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Edited by

Salvatore Moricca and Tiziana Panzavolta

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About the Editors

Salvatore Moricca

Salvatore Moricca, PhD, is Professor of Plant Pathology at the Department of Agriculture, Food, Environment, and Forestry of the University of Florence. He was formerly a research scientist at the CNR (National Research Council) of Italy. The main emphasis of his research is on the biology and epidemiology of fungal pathogens in forests, nurseries, and agricultural systems. His research also explores the ecology and role of alien fungal and oomycete pathogens in natural ecosystems. He uses conventional and molecular methods for plant disease diagnosis as well as to address central aspects of pathogens' life-history strategies. He is an editorial board member of *Plant Pathology* (for 15 years now), F*rontiers in Microbiology* and *Italian Journal of Mycology*. He is a member of many scientific societies including the American Phytopathological Society and the British Society for Plant Pathology. He is currently the coordinator of the IUFRO Unit 7.02.05—Rusts of Forest Trees. He was an expert evaluator for research proposals submitted to the EU and other international funding organizations and agencies. He authored more than 200 scientific publications, six textbooks for Plant Pathology and Forest Pathology classes, and 15 international book chapters. He attended many international congresses, also acting as principal organizer or chairperson. He was a visiting scientist at several international research Institutions and a visiting professor at the San Paulo State University, Brazil.

Tiziana Panzavolta

Tiziana Panzavolta is Associated Professor at the Department of Agriculture, Food, Environment and Forestry of the University of Florence, with teaching experience in alien pest management and forest zoology and entomology. Her research interests involve: 1) invasive alien pests—monitoring of the western conifer seed bug; biology and damage of the oriental chestnut gall wasp; monitoring of *Pityophthorus juglandis*, vector of *Geosmithia morbida*, causal agent of the thousand cankers disease of Juglans; 2) native forest pests—biology and monitoring of bark beetles; 3) ornamental tree pests—biology and damages of the horse chestnut leaf miner and of the oak leaf *Phylloxera*; 3) multitrophic interactions—role of pine terpenes in host selection and oviposition of the pine processionary moth and its egg parasitoids; effects of pine monoterpenes and insect pheromones on predators of saproxylic insects; interactions between phytophagous insects and plant pathogenic fungi; 4) insect pest management with eco-friendly pest control methods—use of pine monoterpenes and insect pheromones in capture traps, or on logs in bark beetles control programs; use of entomopathogenic nematodes and sexual disorientation against chestnut pests. She has published more than 90 scientific papers and has attended, also as a speaker, several national and international conferences.

Preface to "Forest Pathology and Entomology"

Forest ecosystems are vital to life on Earth since they provide a multitude of benefits in terms of water and air purification, nutrient recycling, soil erosion prevention, and carbon storage, as well as habitat, food, and fibers to human populations and wildlife. These irreplaceable products and services are today in increasing danger due to multiple natural and anthropogenic disturbances. Global climate change and trade in particular, besides destabilizing forest ecosystems, offer new chances of survival, reproduction, and spread to both native and introduced forest pathogens and pests. Outbreaks of pathogens and pests have in fact dramatically increased in number and impact in the last few decades, causing loss of significant forest areas worldwide. In this context, studies on forest health have become increasingly prominent and topical, making forest pathology and entomology forefront disciplines to tackle this emerging global threat. A special research effort is aimed at tackling the problem of invasive alien species. The timely identification of new introductions and follow up monitoring of non-native pathogens and pests is of critical importance so that prompt eradication and other control measures can be implemented. At the same time, native pathogens and pests need to be more deeply investigated, since climate change and the consequent environmental stress are causing a resurgence of their attacks in endemic areas and their range expansion in previously free areas. Effective disease and pest management requires reliable information on the life strategies of harmful parasites, from their biology to their ecology and distribution, including the impact on forest ecosystems and possible control strategies.

The 22 papers that make up this Special Issue deal with different aspects of the above subjects, from the diagnosis to the surveillance of causative agents, from the study of parasites' biological, epidemiological and ecological traits to their correct taxonomic positioning and classification, and from disease and pest monitoring to sustainable control strategies. All the authors have invested significant time and energy in writing their papers. The editors of this Special Issue are grateful to all of them for their dedication. The editors also hope that the readers will find this book a useful point of reference and they can gain insights for their own research on the same or related topics.

Salvatore Moricca, Tiziana Panzavolta
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Editorial

Recent Advances in the Monitoring, Assessment and Management of Forest Pathogens and Pests

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Abstract: Tree pathogens and pests are fundamental components of forest ecosystems. By killing and decomposing susceptible trees, they regulate the cycle of nutrients and energy flow, thus shaping the structure and composition of forest stands. However, ecosystems can be seriously disrupted when the population density of these parasites increases beyond their tolerance level. Ascertaining the origin of pathogen and pest outbreaks, recognizing their causal agents in a precise and unequivocal way, while understanding their reproductive and dispersive dynamics are all crucial for the implementation of effective control measures. The studies collected in this special issue cover a wide range of topics in the field of forest pathology and entomology. Investigations range from molecular diagnosis of pathogens and pests to their monitoring and quantification in the field, from measurements of their proliferation rate to the analysis of their genetic variability, from the assessment of the role of plant diversity and ecosystem heterogeneity on pathogen and pest impacts to disease and pest management. Specific case studies show how applied research conducted with innovative methods is key to solving taxonomic issues that were, until now, controversial. The variety of experimental approaches and the range of scientific issues addressed document the trends and topicality of modern forest health protection science.

Keywords: fungi; insects; invasive species; diagnosis; surveillance; disease and pest management

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This special issue was launched in view of the United Nations' International Year of Plant Health in 2020, with the purpose of demonstrating how forest health protection from pathogens and pests must necessarily go through certain fundamental and unavoidable steps: recognizing the "enemy"; measuring/quantifying it; developing suitable strategies to combat it. These stages are stepping stones: better understanding of the parasite's life-history strategies and impacts helps us to progress towards the final goal of disease and pest management.

A first, unavoidable issue, especially when dealing with alien parasites which, as such, are also little known, is to identify them accurately and unambiguously [1]. Accurate and timely diagnosis is furthermore of paramount importance for the subsequent implementation of effective disease and pest management, since it allows us to have a better understanding of the parasite's potential impact on woodland ecosystems. Several studies address this issue with both traditional and molecular approaches. For example, useful guidelines were provided by Mercado et al. [2] for the accurate determination of *Dendroctonus* species attacking *Pinus contorta* Douglas ex Loudon in subalpine forest in the southern Rocky Mountains. Simplified insect identifications based on morphological characters, their attack pattern, and signs have improved classification of these beetles which are normally difficult to distinguish from congeneric species.

Advances in molecular-based diagnostics of infectious diseases are greatly enhancing our ability to identify and characterize damaging agents, allowing a more accurate testing for pathogen identification [3]. Current significant research efforts are aimed at identifying new markers for highly specific diagnosis. In a study targeting the destructive and highly

polyphagous pathogen *Phytophthora cinnamomi*, for instance, a novel biomarker unique to this oomycete, the RxLR effector gene PHYCI_587572, was identified through a comparative genomics approach. Furthermore, a recombinase polymerase amplification-lateral flow dipstick assay targeting the PHYCI_587572 biomarker has proven able to detect, with high specificity, *P. cinnamomi* isolates of diverse sources and different geographic origins, with no cross reactions from 37 other oomycete and fungal species, among which, the sister taxon *P. parvispora* [4].

An additional recent breakthrough is the loop-mediated isothermal amplification (LAMP) technique, initially developed to diagnose animal/human diseases [5] but which is also being applied to phytosanitary issues and to solving taxonomic uncertainties. A LAMP assay was developed for unambiguously identifying *Dothistroma septosporum* (G. Dorog.) M. Morelet, one of the agents of *Dothistroma* needle blight (DNB), an emergent pathogen harmful to both natural and plantation pine forests. The assay proved highly sensitive, detecting as little as 1 pg of fungus DNA; it was also highly specific, discriminating this pathogen from the related *Dothistroma pini* Hulbary and *Lecanosticta acicola* (Thüm.) Syd. [6]. The LAMP method was also employed to solve the question of the identity of the pathogen responsible for the first outbreak of DNB in Italy [7]. This first outbreak of DNB there, about half a century ago (mid-1970s), had been ascribed to *D. pini* on the basis of micromorphological identification alone [8]. The re-checking of this old, never-confirmed report by using pathogen testing with the LAMP method, revealed the causal agent of the early outbreak of the last century to have been, instead, *D. septosporum*, probably introduced into the area by plantations of exotic *Pinus radiata* D. Don.

Once a damaging agent has been accurately identified, the next step is to broaden the knowledge about this species as much as possible [9]. In fact, pest control efforts need to be based on extensive scientific knowledge of this organism's key traits and its population dynamics. The citrus long-horned beetle *Anoplophora chinensis* (Forster) and the gypsy moth *Lymantria dispar* L., for example, are both invasive, destructive pests which can damage many tree species in orchard, urban, and forested habitats. Hence, information on temperature requirements of the citrus long-horned beetle, and on the key traits affecting the gypsy moth's dispersal capability, are provided in order to develop phenological models for management or eradication efforts [10,11]. Specifically, Keena et al. [10] evaluated adult survival, reproduction, and the egg hatching of *A. chinensis* at eight constant temperatures under laboratory conditions; these can be used to predict the timing of stages. Srivastava [11], in addition, provided a review of the current literature on the variations in flight capability and flight distance of gypsy moth populations for each subspecies, as well as shifts in other traits of concern. Rapid tools for assessing key traits in non-native pest populations, as well as knowledge about population dynamics in post-invasion sites, furnish further fundamental information to adequately deal with new introductions into novel habitats. For example, Robinett et al. [12], demonstrated that white ash trees are persisting in several forested areas in the heart of the *Agrilus planipennis* Fairmaire invasion site in Michigan, more than a decade after invasion, indicating that these beetle populations remain below the sites' carrying capacity.

Research efforts in recent years, greatly boosted by modern molecular and bioinformatic methods, have set their sights on surveying the fungal and pseudofungal biodiversity in protected areas. Protected areas are reservoirs of biodiversity that are an essential tool for its conservation, thus they demand particular attention. These areas, being relatively undisturbed ecosystems, are especially useful for studying the ecology and dynamics of populations of plant pathogens among diverse vegetation types. A survey on the diversity and distribution of *Phytophthora* species in aquatic, riparian and terrestrial habitats in a protected area in Eastern Sicily (Italy), for instance, has revealed the presence of 11 species of *Phytophthora* and, interestingly, a correlation between the occurrence of some oomycete taxa and certain vegetation type groups [13]. Another study investigated the impact and spread of the emergent charcoal canker agent *Biscogniauxia mediterranea* (De Not.) Kuntze in a protected, lowland residual forest (Castelfidardo Forest, central Italy) [14]. The impact of

the charcoal canker pathogen in this sub-Mediterranean deciduous forest with mesophilic traits, dominated by oak species, ended up devastating the stand in recent decades. Moreover, it turned out to be related to environmental stresses, such as precipitation deficit and extended drought; further factors predisposing oaks to decay were an absence of silvicultural management, a high competition among physiologically mature trees, and the geographic isolation of this residual forest.

Understanding the factors governing the spread and virulence of pathogens that cause substantial ecological and economic damage continues to remain central to forest pathology studies. Variation in the landscape, in land use (e.g., forest lands vs. former agricultural lands) and, of course, in the main ecological parameters, strongly influences tree susceptibility and the dispersal patterns of forest pathogens [15]. In this regard, Cheng et al. [16] found that in Japanese red pine (*Pinus densiflora* Sieb. et Zucc.) forests, the impact of Diplodia tip blight caused by *Sphaeropsis sapinea* (Fr.) Dyko and B. Sutton, a serious pathogen for several conifer species from the *Abies*, *Cedrus*, *Juniperus*, *Larix*, *Picea*, *Pinus*, *Pseudotsuga* and *Thuja* genera, varied according to stand types and vertical structure layers. Diplodia tip blight disease, for example, was higher in *P. densiflora*–conifer mixed forest than in the *P. densiflora*–hardwood mixed forest or in the purely *P. densiflora* forest. Furthermore, a higher plant diversity in the understory resulted in a more intense competition between young Japanese red pines and shrub–herb plants, resulting in poor resistance of this pine species to Diplodia tip blight [16].

The paper by Klavina et al. [17] provided evidence that Norway spruce (*Picea abies* (L.) Karst.) plantations established in former non-forest lands become highly susceptible to the root rot pathogen *Heterobasidion parviporum* Niemelä and Korhonen after thinning, and that in plantations growing in former pastures and meadows, the pathogen, probably favored by the scarcity of a competing soil microbial community, is able to expand with large territorial clones. For this reason, stump treatment with biological or chemical control agents during thinning operations is fundamental to preventing the transfer of *Heterobasidion* infection to the next generation of trees through root contacts. Stumps are, on the other hand, a primary source of infection for *Heterobasidion annosum* s.l., since they permit pathogen spread to healthy trees by vegetative mycelium via root anastomoses. Airborne *Heterobasidion* sp. spore deposits on the stumps of susceptible species can be estimated by means of wood discs [18]. Brūna et al. [19] have utilized wood discs to investigate the susceptibility of seven conifer species (*Larix sibirica* Ledeb., *P. abies*, *Picea sitchensis* (Bong.), *P. contorta*, *Pinus strobus* L., *Pinus sylvestris* L. and *Pseudotsuga menziesii* (Mirb.)) to artificial inoculation with conidia of *H. annosum* sensu stricto (s.s.) and *H. parviporum* under controlled conditions or to natural airborne infection. These authors observed that the rate of infection of wood discs sprayed with conidial suspensions under controlled conditions was comparable to that obtained from exposing the same species' discs to natural airborne inoculum in spruce stands infested either by *H. annosum* or *H. parviporum*. The importance of such local spore sources in the range of dispersal of the two forest pathogens was confirmed by the fact that the infection rate of wood discs decreased as the distance from the inoculum sources (fruiting bodies) increased.

Proper management of forest pathogens and pests must take into consideration not only the pest itself, but also the other living components of the ecosystem, as well as their multitrophic interactions. The role of insects as vectors of pathogenic fungi is well established [20,21], nonetheless, many aspects of insect/fungus interactions still need to be clarified. A great quantity of *Heterobasidion* spp. fruit bodies, for example, develop on large, infected *P. abies* logs when they are left in stands after forest operations. These high-spore-density fruiting bodies are a considerable risk; therefore, several studies recommend removing large, infected logs from stands. All the same, these logs are important as a habitat for many fungi and invertebrates, including rare and endangered species. Legzdina et al. [22], for instance, found a rich community of invertebrates in *Heterobasidion* fruit bodies, decayed logs and adjacent soil; however, as these authors stated, the nature of such associations requires further investigation. Moreover, Vissa et al. [23] examined

differences in the diversity of symbiotic mite communities associated with *Dendroctonus ponderosae* Hopkins, an economically damaging North American bark beetle. Specifically, they observed that symbiotic mite biodiversity varied geographically according to environmental differences; they suggest that this information may be useful for further steps towards identifying key species interactions in forest ecosystems.

Eco-sustainable control strategies must be favored and improved to minimize the impact of pest control measures on forest ecosystems. The use of these strategies requires more expertise, being more difficult to apply in comparison to traditional measures based on pesticides. Additional studies are needed to better understand how to improve their efficacy, while reducing undesirable effects. Bracalini et al. [24], for example, have investigated the negative effects of *Ips sexdentatus* Böerner mass trapping, especially non-target catches among the target's natural enemies. They tested slot traps modified with mesh screens and escape windows to improve their selectiveness, demonstrating how trap modifications may mitigate the problem, especially for beetles larger than the target. This expedient should be adopted together with precise mass-trapping scheduling, in order to avoid seasons in which the target adults are less active than the main predator adults. Since biodiversity conservation is an integral component of sustainable forest management, considering the impact of all management strategies on the entire biological community is essential. In this regard, Hartshorn [25] assessed the impact of different types of forest management (e.g., clear cutting, burning, and chemical control of invasive species) on leaf litter arthropods, which are some of the most abundant and diverse communities in forests, and which provide several ecosystem services, from decomposition and mineralization to pollination and predation.

Microbial insecticides may be employed in forest ecosystems, where the use of pesticides is not an option. These biological agents are safe for both their users and woodland dwellers, due to their high selectivity. Among these environmentally friendly insecticides, *Bacillus thuringiensis*-based products are the most used all over the world [26]. *Bacillus thuringiensis* var. *kurstaki* (Btk), combined with mating disruption, are reported to be effective in controlling *Thaumetopoea pityocampa* Denis et Schiffermüller [27], a harmful insect for forest, urban and peri-urban pines, that needs to be kept under control because its larvae also cause public health problems for humans and pets. Finally, the use of Btk can also be integrated with other biological products, as proposed by Ruiu et al. [28]. These authors suggested applying Btk in combination with the agent of natural gypsy moth epizootics, the species-specific multicapsid nucleopolyhedrovirus (LdMNPV), to contain the lepidopteran defoliator *L. dispar* in multi-year integrated programs. In other instances, various fungal species have shown a marked antagonism towards important phytopathogenic fungi. The fungus *Phlebiopsis gigantea* (Fr.) Jülich, for example, is a biocontrol agent that has been very successful in the biocontrol of the root rot of conifer trees caused by *Heterobasidion annosum* (Fr.) Bref. Wit et al. [29] utilized elongation factor 1-alpha (EF1α) partial DNA sequencing and in silico data to infer the relationships between representative isolates of *P. gigantea* and selected Basidiomycota species. The results obtained indicated that the EF1α region is a valid marker for selecting the most competitive isolates of *P. gigantea* to be possibly employed for limiting damage by forest tree root pathogens.

This special issue is furthermore enhanced by two reviews that deal with two important themes: the emerging problem of the invasion of forest ecosystems by alien parasites and phytoplasma diseases of forest trees. The former is topical and very popular; the latter, little investigated, if not totally disregarded. The first review, by Panzavolta et al. [30], analyzed the main entry pathways of non-native pathogens and pests, as well as the factors affecting their establishment and spread into forest ecosystems and uncontaminated territories. It spotlights the importance of early diagnosis, monitoring and eradicating harmful organisms, noting the role of research and the valuable contribution of volunteers in early detection (citizen science). It also highlights how government policies must be enacted to effectively counter this international problem. In the second article, Marcone et al. [31] reviewed the major phytoplasma taxa that cause diseases to forest tree species. These dam-

aging, wall-less, obligate bacteria, which harm thousands of plant species worldwide, have been poorly investigated compared to both phytoplasmas infecting fruit trees and other groups of plant pathogens. Deeper research into the vectors, plant host range, strain virulence, pathogenicity and host tolerance/resistance would all be crucial for appropriately managing phytoplasma diseases.

The topics covered in the 21 papers that make up this special issue indicate the current trends in research on forest health protection. The variety of techniques used, spatial scales, methodological approaches, and topics covered are a measure of the versatility and importance of today's forest pathology and entomology research.

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Improved Identification and New Records of *Dendroctonus* Bark Beetles Attacking *Pinus contorta* in the Subalpine Forest of the Southern Rocky Mountains

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Abstract: Research Highlights: Atypical and poorly understood attacks by *Dendroctonus rufipennis* (Kirby) to *Pinus contorta* Doug. ex Loudon were detected in the southern Rocky Mountains (SRM). The phenomenon is confirmed across all examined area. Its reproduction is described for the first time as well as the first attacks of *D. adjunctus* Blandf. in that host. Improved detection and diagnostics of *D. rufipennis* will allow a simpler, and efficient identification of the species. It will improve the detection capacity by pest detection specialists and entomologists, which will increase our understanding of the phenomena within and beyond the known range. Background and Objectives: In addition to *D. ponderosae* Hopk. other *Dendroctonus* species, sometimes together, attacked *P. contorta* that grew intermixed with *Picea engelmannii* in the SRM' subalpine forest. The identification of these beetles was difficult. The goal was to improve the detection and identification of the species from similar *Dendroctonus* spp. attacking that host and to uncover biological facts about the phenomena. Materials and Methods: *Dendroctonus* attacking *P. contorta* were collected along the entire SRM, their attack signs and behavior were recorded. These characteristics were revised from those in the literature and new characters were introduced and tested. Results: The identification of *Dendroctonus* bark beetles attacking *P. contorta* in the SRM was improved using revised and new characters including attack signs, attack behavior, and adult beetle characters. An improved identification key couplet is presented to effectively distinguish *D. murrayanae* from *D. rufipennis*. Conclusions: Simplified insect identifications that are both accessible to users with different levels of expertise and are based on insect characters, their attack pattern, and signs, like the present, improve detection of insects of interest. Efficient insect detections allow a better understanding of the capabilities they have and the impact they cause to the woodland ecosystems we study, protect, and manage around the globe.

Keywords: non-host attack; post-epidemic; facilitation; endemic population strategies

1. Introduction

In the southern Rocky Mountains (SRM) of Colorado and southern Wyoming, *Pinus contorta* Douglas (lodgepole pine) grows from 2400 to 3200 m, intermixing with *Picea engelmannii* Parry ex Engelm (Engelmann spruce) in the subalpine zone, at elevations above 2800 m [1]. In this temperate mountain forest, native *Dendroctonus* bark beetles kill trees altering forest structure and species composition. Two *Dendroctonus* bark beetles attack *P. contorta* natively, *D. ponderosae* Hopk. (mountain pine beetle) and *D. murrayanae* Hopk. (lodgepole pine beetle). The magnitude of the impact on their host is different. For instance, during the most recent epidemic, *D. ponderosae* killed approximately 60% of the mature *P. contorta* within the SRMs [2,3], whereas *D. murrayanae* was reported affecting only small patches of trees [4]. The disparity of the beetles' impact reflects their different biologies. While *D. ponderosae* attacks all *Pinus* species in the SRM, *D. murrayanae* only attacks *P. contorta*. Moreover,

D. ponderosae occurs throughout the SRMs, while *D. murrayanae* only occurs above latitude 39.4°, as it appears to be limited to cooler, northern regions [5]. In addition, *P. engelmannii* has a native *Dendroctonus* enemy, *Dendroctonus rufipennis* (Kirby) (North American spruce beetle), with *D. ponderosae* attacking *Picea* spp. on rare occasions. With reddish elytra that contrasts with a dark brown head and prothorax, adult *D. rufipennis* and *D. murrayanae* closely resemble each other in color, but also in size, shape, and other external characters. The two *Dendroctonus* are also difficult to distinguish behaviorally since they have similar egg galleries, egg placing patterns, and brood aggregation patterns [6]. Therefore, the distinction between the beetle species is usually based on the infested host [5,7]. *Dendroctonus rufipennis* populations have been irruptive during the last 20 years, killing large numbers of suitable *P. engelmannii* in the SRM.

In the SRM, subalpine forest coincides with elevations at which *D. ponderosae* activity can regularly become attenuated by low temperatures [8]. Consequently, *D. ponderosae* attacks to *P. contorta* are seldom documented above 3000 m of elevation [8–10]. However, during the last epidemic, *D. ponderosae* attacks were observed above 3000 m providing the opportunity to study their activity in areas where they occur irregularly. While studying this in the Roosevelt National Forest in northern SRM in Colorado, red-elytra *Dendroctonus* beetles resembling *D. murrayanae* and *D. rufipennis* were attracted to baited (Exobrevicomin-Myrcene-Transverbenol, Synergy Semiochemicals) funnel traps (12-funnel Lindgren) monitoring *D. ponderosae* activity. Due to their resemblance and the response to *D. ponderosae* lures, that included one *P. contorta* tree component, these beetles were difficult to identify to species. Attacks by similar bark beetles were also documented in southern parts of the SRM in Colorado, where *D. murrayanae* has not been documented. On all occasions, these bark beetles were found in mixed stands of *P. contorta* with *P. engelmannii*. Thus, the question of whether it was *D. murrayanae* or *D. rufipennis* the species responsible for some of the attacks to *P. contorta* in the region's subalpine forests became a topic of relevance.

Dendroctonus rufipennis has been reported attacking *P. contorta* in the SRMs before, from 1944–1949 [11] and in 1957 [12] (Figure 1); however, a taxonomic authority [5,7] contradicted those determinations. The phenomena of *D. rufipennis* attacks to *P. contorta* in the SRM may be poorly understood due to the difficulty of separating it from *D. murrayanae* attacking that host in subalpine forests. In this study, *Dendroctonus* beetles attacking *P. contorta* in SRM' subalpine forests of Wyoming and Colorado were examined. The objectives of this study were to (1) improve the identification of adult *Dendroctonus* and the detection of their attack signs and patterns, (2) confirm the identity of *Dendroctonus* spp. with red-elytra attacking *P. contorta* in subalpine forest, and (3) discover new biological information about these *Dendroctonus* species.

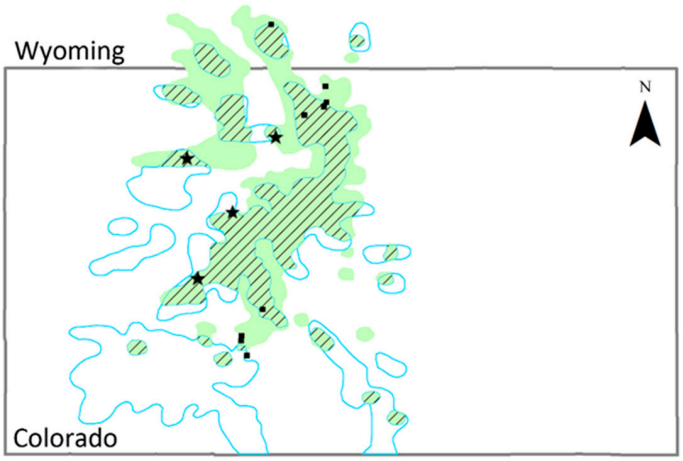

Figure 1. Southern Rocky Mountains sites were *D. rufipennis* was identified attacking *P. contorta*. Records from the 1940s and 1950s are indicated by stars, and squares indicate records made during this study. *P. engelmannii* (green) and *P. contorta* (blue) layers were modified from [13], trees intermix in the dashed areas.

2. Materials and Methods

Sites in this study were in the SRMs, from southern Wyoming to southern Colorado (Figure 1). Yellowing trees in the spring or red ones in the fall with pitch tubes were used as evidence of trees infested by *Dendroctonus* beetles. In these areas, both live and dead adult beetles were collected. Live specimens were collected from live *P. contorta* as these attacked, while dead specimens were found trapped beneath the tree's bark and on the duff around the tree collar. Specimens were placed individually in 1.5 mL microcentrifuge tubes. In the laboratory, specimens were cleaned in an ultrasonic cleaner (100005, Sper Scientific) using warm water and mounted for examination. Previously determined diagnostic characters [5–7,14–17] were used to revise and determine the best diagnostic characters of collected specimens. Voucher specimens from the Hopkins Collection, Rocky Mountain Station, Fort Collins, CO, and collected from *P. contorta* included: (n = 5) Rout N. F. (VII-48, Coll. Unk., No. 34220-L-6; (n = 5) Yeoman Campground, White River N. F. (IX-1947-Coll. Wygant, No. 34220-L-7; (n = 4) Trappers Lake, White River N. F. (VII-1946, Coll. C.J. Hay, No 31409-E; (n = 2) Jumping Creek Campg., Helena, MT (Helena-Lewis and Clark N. F.) (VI-1989, Coll. J.B. Johnson, ID by Furniss), and collected from *Pinus albicaulis* (Whitebark pine) (Engelm.) in Wyoming: (n = 10) Bridger-Teton N. F. (labeled Dubois) (VII-1936, Coll. J. A. Beal, No. 17700-M-1).

A subsample of red-elytra *Dendroctonus* from Colorado and Wyoming were selected to examine male genitalia. In these, sex was determined by examining a secondary sexual character, i.e., the granules in the interstriae declivity, which are greatly reduced (nearly absent) in males in contrast to being abundant in females [7,14,15]. Before dissecting, male beetles were softened in warm water for five minutes, after which the abdomen was removed ventrally by pulling it using a pair of forceps posteriorly to the metacoxae to access to the aedeagus. Removed aedeagi were cleared in a solution of 10% KOH heated in a warm bath (55 °C) for 15 min to clear the sclerotized aedeagal capsule eliminating the need of removing the delicate endophallus [18] for its examination. Structures were examined with a Leica M16 stereomicroscope (Leica Microsystems, Castle Rock, CO, USA).

3. Results

3.1. New Records of Dendroctonus spp. Attacking P. contorta in the Southern Rocky Mountains

Previous reports of *D. rufipennis* attacking *P. contorta* in the SRM where from Routt N. F., Grand Mesa N. F., and White River N. F. limiting records to North-Central Colorado west of the Continental Divide. *Dendroctonus rufipennis* was here detected attacking numerous *P. contorta* in subalpine forests from Medicine Bow N. F. in southern Wyoming and from Roosevelt N. F., Gunnison N. F., and Rio Grande N. F. in Colorado encompassing the entire SRM's latitudinal range (Figure 1) and the east side of the mountain range. *Dendroctonus rufipennis* is evidently affecting a larger number of trees than in 1940 and 1957, where it was reported to affect 250 and 140 trees, respectively. These findings make the occurrence of this event more widespread and potentially more impactful than previously reported. *Dendroctonus adjunctus* Blandf. has never been reported before attacking *P. contorta*. Attacks of this species were detected on a single site in the Rio Grande N. F. where it was found attacking three *P. contorta* together with *D. rufipennis*; therefore, treatment of this species is limited in this manuscript.

3.2. Diagnosing Dendroctonus Adult Beetles Attacking P. contorta in Subalpine Forest

The external color of the three *Dendroctonus* is a simple starting point to separate *D. ponderosae* from the two red-elytra *Dendroctonus*. Adult *D. ponderosae* has an even thorax and elytra color, whereas most adult *D. murrayanae* and *D. rufipennis* have a darker thorax contrasting with their reddish elytra (Figure 2). Although *D. adjunctus* is also even colored, this species is unique among the four species in having a vertical impression on its mid-upper frons. However, these color differences alone are not useful to diagnose a fraction of older *D. rufipennis* and *D. murrayanae* that have black elytra matching their head and thorax. Color should be used in conjunction with the length of dorsal setae, particularly of that in the elytral declivity where the two red-elytra beetles have longer setae than *D. ponderosae*

(Figure 2), these two characters are evident to the naked eye on clean specimens. Low magnifications (< 7X) allow distinguishing the dull surface of the elytral declivity and the clearly impressed (flattened) second and third interstrial space that curve strongly towards the suture in *D. ponderosae.* This character distinguishes *D. ponderosae* from *D. murrayanae* and *D. rufipennis* and, as previously described [14,15], were referred to as striae II and III [7,17].

Figure 2. Three *Dendroctonus* bark beetles attacking *P. contorta* in the southern Rocky Mountains (SRM): (**a**) *D. ponderosae* distinguished from the other two by having equally colored thorax and elytra and shorter elytral declivity setae, (**b**) *D. rufipennis* examined in the SRM had duller exoskeletal surfaces than (**c**) *D. murrayanae*. Pictures by J. Mercado.

Whereas distinguishing *D. ponderosae* from the other two species was relatively easy, red-elytra species (i.e., *D. murrayanae* and *D. rufipennis*) are difficult to distinguish from each other. An early diagnostic character, the presence of punctures on the posterior half of the pre-episternal (synonyms: preëpisternal, proepisternal) area in *D. murrayanae* described as absent in *D. rufipennis* [14] (Figure 3) was examined. This morphological character proved to be confusing since (1) it was poorly defined, (2) its study requires the use of magnifications of 40X, and (3) punctures were present, but were more difficult to see in some *D. rufipennis*. Another character examined described by Wood [7], was the granules and punctures in the frons. He mentioned that "the relative number of punctures and granules offer the only reliable method of separating *D. murrayanae* from *D. rufipennis*."

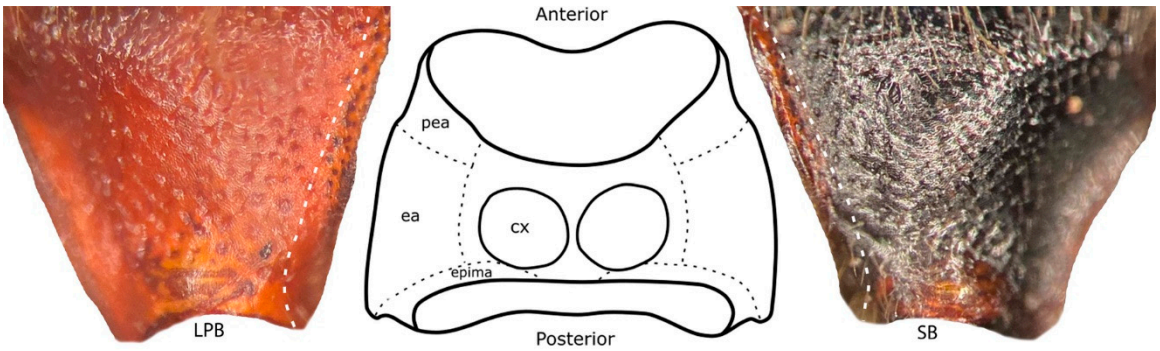

Figure 3. From anterior to posterior, the sides of a *Dendroctonus* thorax is divided into three areas [14]: (**1**), pre-episternal (pea), (**2**) episternal (ea), and (**3**) epimeral (epima). The character of the relative number of punctures at the posterior half of (pea) near white dashed lines, to distinguish *D. murrayanae* from *D. rufipennis* is difficult to use. Pictures by J. Mercado, thorax line drawing adapted from Hopkins [14].

The exoskeletal surfaces of *Dendroctonus* beetles, including the frons, are covered by semi-circular impressions called punctures, and by blunt cusps called granules, the number of these is never quantified, but puncture closeness and granule location and their spread are described between similar species. In both *D. murrayanae* and *D. rufipennis*, punctures are described as being very close; however, in the center of the frons (between the compound eyes), these are described as "distinct" in *D. murrayanae* and "largely obliterated" in *D. rufipennis* [5,7].

Wood [5,7] described that granules in the frons interpuncture space are more clearly separated or "isolated" in *D. murrayanae* than in *D. rufipennis* "coarsely granulate," but in this study granules were found isolated and dispersed in *D. rufipennis* as well. This study found that interpuncture space surfaces in *D. rufipennis* are usually rougher, making granules and punctures hard to discern, whereas these are usually smoother on *D. murrayanae*, making granules and punctures more apparent, especially in the middle of the frons. This character was found to be useful separating most *D. rufipennis* and *D. murrayanae* specimens, but some *D. rufipennis* had distinct mid-frons punctures; therefore, its use is recommended in addition to other characters. The smoother interspaces also helped to distinguish most *D. murrayanae* from *D. rufipennis* when examining other exoskeletal surfaces dorsally and laterally, such as elytra (Figure 2), from similar angles and at similar magnification. At low magnification (7.1X), examined *D. murrayanae* specimens in collection boxes appear glossier than those of *D. rufipennis* (Figure 2). It is recommended that the state of rough vs. smooth to describe the frons interpuncture space surface in the frons center is examined to help separate these species. The accuracy of this character should be studied further for specimens outside the SRM. A new diagnostic character found in this study was simple to use and works with the examined female *D. rufipennis* and *D. murrayanae* specimens. There is a sharp deflection of striae III away from the suture near striae IV and then towards the suture near striae VI in *D. rufipennis*. This deflection is present in *D. murrayanae* but it is smooth, making it less distinct (Figure 4). As described, the character was consistently present on all examined female specimens but not easily seen in some males, and its use in other regions merits further study. In males, or when the above characters are obscure or missing on a specimen in poor condition, the character to examine is the distinctly different endophallus [7] of *D. rufipennis* and *D. murrayanae* (Figure 5).

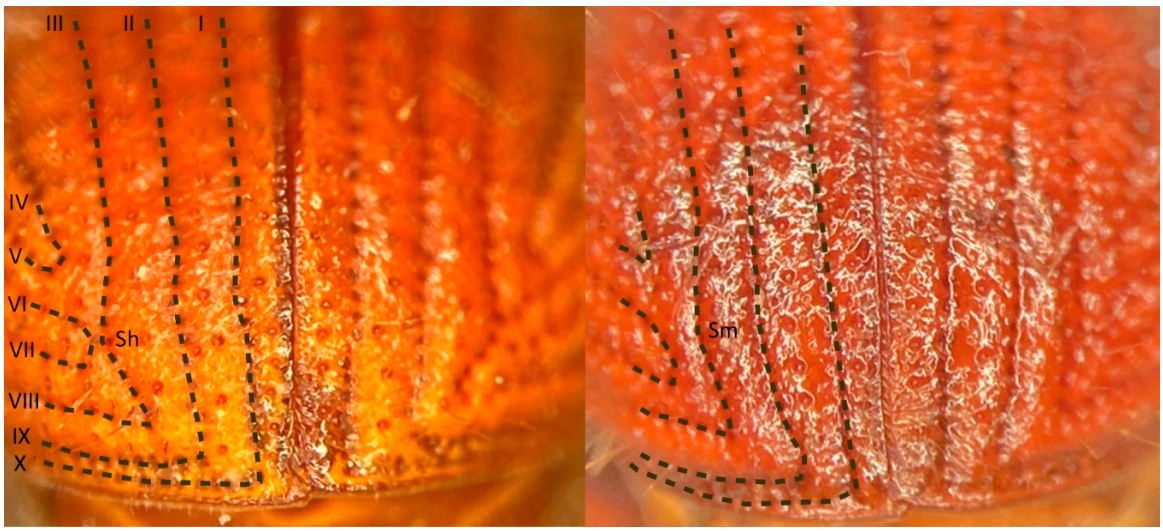

Figure 4. The striae (dashes) and interstriae in the declivity of *D. rufipennis* (**left**) and *D. murrayanae* (**right**). Striae III curves sharply (Sh) towards the suture opposite to striae VI in *D. rufipennis*, but smoothly (Sm) in *D. murrayanae*. Photos by J. Mercado.

Figure 5. The male genital capsule of *D. rufipennis* (**a**) and *D. murrayanae* (**b**). An internal structure in the genital capsule, the endophallus, is seen through the median lobe (also inserts lower right corners). The endophallus is diagnostic for the two species when other means have been exhausted. Photos by J. Mercado. Endophallus line drawings adapted from Wood [7].

To improve diagnosing adult beetles of the similar red-elytra *D. murrayanae and D. rufipennis* attacking *P. contorta* in the SRM, the use of the simplified and improved couplet below is recommended.

Mid-frons punctures distinct; frons and elytra interpuncture spaces smooth; (females, difficult on males) declivity striae III turning sharply (angled) towards suture, opposite to striae VI; (males) genitalia distinctive. *D. murrayanae* Hopk.

Mid-frons punctures usually obscure; frons and elytra interpuncture spaces usually rough (rugose, granulate); (females, difficult on males) declivity striae III turning smoothly (arched) towards suture, opposite to striae VI; (males) genitalia distinctive. *D. rufipennis* (Kirby).

3.3. Diagnosing Dendroctonus Attacks Characteristics to Subalpine P. contorta

Separating *D. rufipennis* from *D. murrayanae* using only morphology is difficult; therefore, to complement adult insect determinations, available behavioral characteristics were reviewed, and a new helpful alternative is presented. These are also helpful in the absence of adult insects. The period and height from the duff of tree attack by the three *Dendroctonus* species are useful to identify the species. Reddish elytra *Dendroctonus* (i.e., *D. murrayanae* and *D. rufipennis*) attack earlier (June), whereas *D. ponderosae* attacks later (July and August). In the fall, *D. rufipennis* move to the lower 60 cm of the tree to overwinter [5], this is the area were *D. murrayanae* develops, making male genitalia examination needed for accurate species determination at that time. In June, attacks of *D. rufipennis* and *D. murrayanae* can be determined by the height at which they attack the tree. Attacks above 60 cm are made solely by *D. rufipennis*, while the attacks of *D. murrayanae* concentrate below that, but especially below 20 cm [6]. In July and August, both *D. ponderosae* and *D. rufipennis* attack above 60 cm, this requires examining other traits in late August to confirm the attacking insect.

Although Wood [7] suggested pitch tubes could be used to identify the attack of *Dendroctonus* species, he did not describe them in his monograph of the genus *Dendroctonus*. The examination of the pitch tubes of beetles attacking above 60 cm made in July and August is recommended here. Pitch tubes that sometimes form a slide on the lower outer border are characteristic of *D. rufipennis* (Figure 6). A few days after the attack, these become bulkier than those of *D. ponderosae* attacking the same tree, which differ as they resemble a perpendicular chimney (Figure 6). The characteristic sign was tested by predicting whether *D. ponderosae* or *D. rufipennis* was the attacking beetle. Out of 26 specimens collected with a chimney type pitch tube present, 58% were *D. ponderosae* (Table 1). In specimens collected with a slide/bulge-type pitch tube, all were *D. rufipennis*. The low accuracy predicting *D. ponderosae* did not occur due to confusion with *D. rufipennis* but with *D. adjunctus*, performing undocumented attacks in *P. contorta* in southern Colorado. It is important to note that soft, sappy pitch tubes that somewhat

resemble a slide can be made by *D. ponderosae* attacking *P. contorta* at lower elevation forests. This occurs when irruptive populations attack healthy trees with a strong quantitative oleoresin response, but a clear slide is not made by *D. ponderosae*. This should be used as the first step before verifying the insect and egg gallery and only in subalpine *P. contorta*/*P. engelmannii* forest type.

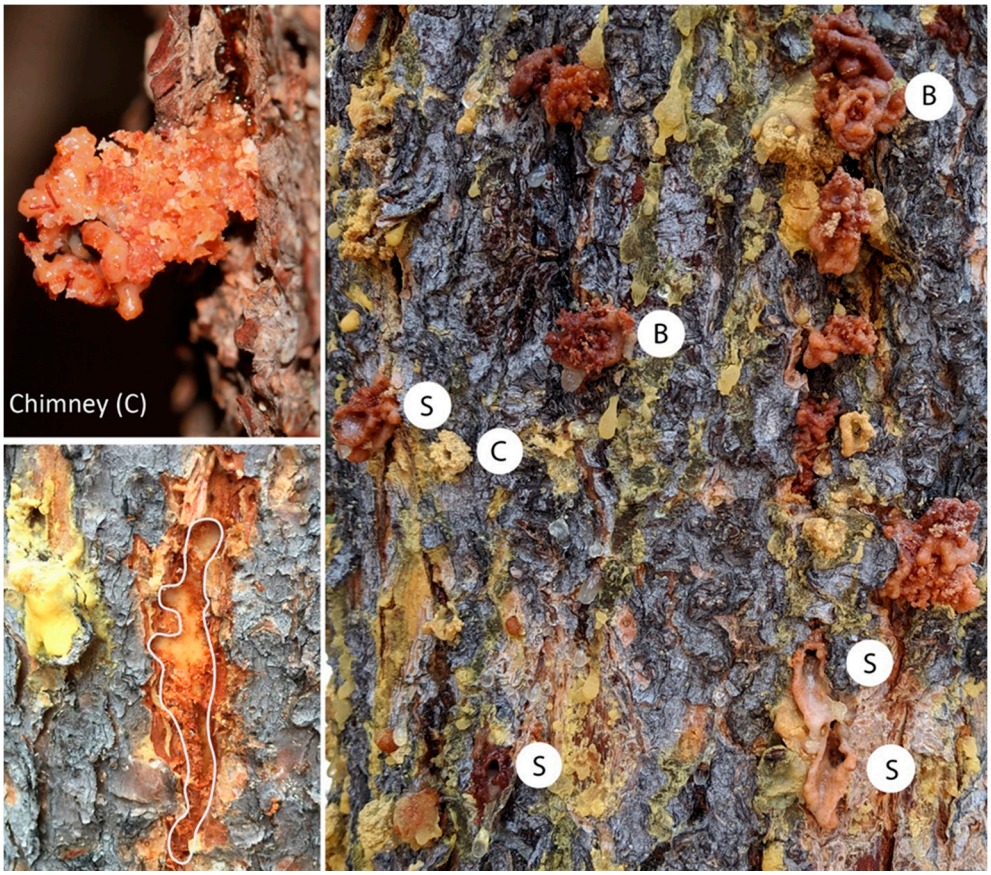

Figure 6. Distinguishing characteristics of *Dendroctonus* attacks to *P. contorta* in the southern Rocky Mountain subalpine forest include the shape of their pitch tubes and the length of their galleries. Clockwise from top left: (**1**) the chimney-like pitch tube of *D. ponderosae* is perpendicular to the tree with a near round outer border, (**2**) a mix of different pitch tube shapes with bulky (**B**) and slide-forming ones (**S**) by *D. rufipennis* and a chimney-shaped one (**C**) made by *D. ponderosae*, and (**3**) the turning notches in the short *D. rufipennis* egg gallery. Photos by J. Mercado.

Table 1. Quantified characters of *Dendroctonus* spp. bark beetles attacking subalpine *P. contorta* in the SRM during this study. Note: *D. murrayanae* Hopk. adults or their attacks were not found during this study.

	Attack Height		**Pitch Tube Type**		**Egg Gallery Length**		
***Dendroctonus* sp.**	<20 cm	60–150 cm	chimney	slide/bulge	<13 cm	>23 cm	Total
D. adjunctus Blandf.	0 of 11	11 of 11	11 of 11	0 of 11	0 of 11	11 of 11	11
D. ponderosae Hopk.	0 of 16	16 of 16	15 of 16	1 of 16	0 of 16	16 of 16	16
D. rufipennis (Kirby)	0 of 75	75 of 75	0 of 75	75 of 75	78 of 75	0 of 75	75

Second, the identification accuracy of beetle attacks made above 60 cm increased by examining the length of egg galleries (n = 102). *Dendroctonus rufipennis* egg galleries averaged 13 cm, whereas those of *D. ponderosae* were longer than 23 cm, as previously described [5]. In the sampled egg galleries, 59.2% of these measuring over 16 cm in length corresponded to *D. ponderosae* (n = 16), and no *D. rufipennis* egg gallery measured over that. Moreover, 11 of the 27 long galleries studied were made by *D. adjunctus*, making this character useful for separating these two species from *D. rufipennis* in

southern Colorado, although not from *D. ponderosae*. *Dendroctonus rufipennis* galleries also have short spurs on the sides along their short length that are used as turning sites for the adult beetle, which are absent in *D. ponderosae* egg galleries (Figure 6).

3.4. Documentation of D. rufipennis Reproduction in Live P. contorta and Verification of Museum Specimens from the 1940's Report of D. rufipennis Attacking That Host

The complete development of *D. rufipennis* adults in *P. contorta* was first suspected in 2014 from the collections of teneral adults from under the bark in Gunnison N. F. in southern Colorado, and in Medicine Bow N. F. in southern Wyoming where these were again collected from the same tree in July 2015 when a dead pupa was found. In mid-July of 2016, standing, live *P. contorta* in northern Colorado contained 12 *D. rufipennis* adults initiating galleries—three of which had laid eggs, which confirmed reproduction initiation attempts by the species. In about the same date, adults *D. rufipennis* were seen emerging a *P. contorta* in the Medicine Bow N. F. and simultaneously a *P. contorta* located 10 m from it was being attacked by *D. rufipennis*. Since there were no other trees with activity within a 30 m radius from these two trees, re-attack to *P. contorta* by *D. rufipennis* in that forest is suspected. As eggs, pupae, and teneral adults have been found under the bark of *P. contorta* reproduction in this host is considered confirmed from the SRM *P. contorta*/*P. engelmannii* subalpine forest.

Red-elytra *Dendroctonus* specimens collected from the 1940's *D. rufipennis* epidemic in Colorado (Wygant, White River N. F., 1947), were examined. These beetle specimens resembled *D. murrayanae* in their color pattern, but the examination of the male genitalia confirmed specimens represented *D. rufipennis*, confirming their prior attack to *P. contorta* in SRM.

4. Discussion

Although, previous reports of *D. rufipennis* attacking *P. contorta* in the SRM exist, there remained uncertainty about their veracity. As discussed in his review of the genus *Dendroctonus*, Wood [7] examined some of the red-elytra *Dendroctonus* specimens reported attacking *P. contorta* in the 1940s. On all suspected cases of *D. rufipennis* attacks to *P. contorta*, he determined that the insect involved was *D. murrayanae* [5,7]. However, it is unclear whether the specimens he studied were the same that Massey and Wygant [11] had determined to be *D. rufipennis* [19]. An issue with Massey and Wygant [11] is that they only report using the characters described by Hopkins [14] to diagnose *D. rufipennis* from *D. murrayanae*. These characters were considered insufficient to separate these species by Wood [7] without studying the male genitalia (endophallus). This insufficiency was confirmed in this study as well, as all red-elytra beetle examined from 2010 to 2016 were incorrectly identified as *D. murrayanae* using the key in [14].

Although it is possible Massey and Wygant [11] knew of an effective way to separate the two red-elytra *Dendroctonus*, this was not effectively expressed in their report. Therefore, characters and keys referred by them should only be used by expert taxonomists of this group of beetles and even then, with great care and understanding of their limitations. Consistently with Wood [7], it is recommended that careful examination of the male' endophallus is used as the best approach to separate these two species when using adult morphology alone, especially when the newly described elytral striae number three character is confusing or missing. However, apart from the endophallus only we found that Wood's [5,7] key couplet describing the clarity of the punctures on the frons center offered diagnostic utility.

There are still questions regarding the activity of *Dendroctonus* bark beetles in subalpine *P. contorta*/*P. engelmannii* forest across their distribution. First, why is *D. rufipennis* attacking this nonhost in the SRMs? Schmid and Frye [19] suggested that under outbreak conditions, *Dendroctonus* species can attack nontypical hosts in the absence of suitable primary hosts. In other forests where *P. contorta* and *P. engelmannii* intermix, *D. ponderosae* has opportunistically attacked and developed in *P. engelmannii* [20] or the hybrid *Picea engelmannii* × *glauca* [21]. Attacks by single *D. ponderosae* pairs to *P. engelmannii* were also observed near one of this study' sites in northern Colorado. However, there were suitable

hosts in the vicinity of both *Dendroctonus* species attacking nonhosts in Colorado. The nonhost attack in subalpine *P. contorta*/*P. engelmannii* forest type by *D. rufipennis* may be possible due to the following factors: (1) irruptive populations of both species can occur simultaneously in areas were their hosts grow intermixed, confusing the insects' olfactory response, (2) a similar tree volatile is produced by stressed trees of both species, i.e., α-pinene, and/or (3) even in trees previously attacked by *D. ponderosae*, *D. rufipennis* is not rejected by any of the semiochemicals released by that species.

Also questioned is whether has the activity of *D. rufipennis* in subalpine *P. contorta* been overlooked or confused? This is difficult to answer, but the author's experience and discrepancies in previous reports suggest [5,7,11] this is possible. Here, guidelines are provided to help elucidate this, and ongoing research by the author is measuring the impacts of this activity in the SRM. Among others, is there a biological function of non-host attacks by bark beetles? One possible function that merits further investigation is the potential of reciprocal facilitation of multiple *Dendroctonus* species attacks to both species. It has been suggested that "primary bark beetles", those that attack primarily live and apparent healthy trees, survive in their endemic state by becoming "secondary bark beetles"—that is, attacking trees that have been previously attacked by other bark beetles or are otherwise compromised [22]. This has been suggested for *D. ponderosae* attacking high latitude/elevation *P. contorta* that had been previously attacked by *Ips mexicanus* bark beetles in Canada [23]. Thus, the study of facilitation for these two co-attacking species is merited. An outcome of facilitation may be the reproduction of a nonhost attacker that, at the same time, benefits the reproductive success of the native host attacker when occurring in its endemic state. This needs to be studied in the SRM with *D. ponderosae* and *D. rufipennis*.

The successful reproduction of *D. rufipennis* has been found in laboratory experiments in BC, Canada, where no developmental differences were noted for *D. rufipennis* raised in *P. contorta* or in white-*P. engelmannii* hybrid bolts of these species [24]. Live *P. contorta*, however, may be more able to defend against *D. rufipennis* attacks, as suggested by hundreds of *D. rufipennis* found dead around the base of attacked trees compared to only a handful *D. ponderosae* throughout the surveyed years. However, the cumulative *D. rufipennis* attacks attempts and the pheromones they release combined with the defensive tree compounds released in their response may elicit an olfactory signal attracting *D. ponderosae*. This may facilitate the attack of that species first, and potentially that of *D. rufipennis* later by reducing the defense capacity of trees attacked by its primary insect enemy. This may represent an ecological adaptation supporting endemic populations of both species in this high-elevation forest. Here, eggs and teneral (immature) adults of *D. rufipennis* in *P. contorta* are documented in trees containing a few *D. ponderosae* attacks, arguably providing a starting point towards establishing this hypothesis. However, there is a need to better measure reproductive success in this host as well as the tree conditions permitting its attack.

5. Conclusions

Useful guidelines for the accurate determination of *Dendroctonus* species attacking *P. contorta* in subalpine forest in the SRM are presented. The approach uses behavioral and morphological characters to provide an undemanding method for determining even the poorly documented attack to *P. contorta* by *D. rufipennis*. All red-elytra *Dendroctonus* bark beetles found attacking *P. contorta* in this study were *D. rufipennis*. This does not mean that *D. murrayanae* is absent from the SRM, but it reflects our sampling from 50 cm to 2 m above the collar and perhaps the rarity of the species in the SRMs. The attacks to *P. contorta* in subalpine forest by nontypical *Dendroctonus* are either poorly reported due to difficulties identifying these *Dendroctonus* and/or on the rise in response to current climate variations. Simplifying the diagnostics of these species is a necessary step and provides the tool for improving our knowledge about bark beetle disturbances in subalpine ecosystems. In the absence of adult male beetles, characteristics of their attack in combination with careful examination of the morphological characters as summarized here can help elucidate the attacking species.

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PHYCI_587572: An RxLR Effector Gene and New Biomarker in A Recombinase Polymerase Amplification Assay for Rapid Detection of *Phytophthora cinnamomi*

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Abstract: *Phytophthora cinnamomi* is a devastating pathogen causing root and crown rot and dieback diseases of nearly 5000 plant species. Accurate and rapid detection of *P. cinnamomi* plays a fundamental role within the current disease prevention and management programs. In this study, a novel effector gene *PHYCI_587572* was found as unique to *P. cinnamomi* based on a comparative genomic analysis of 12 *Phytophthora* species. Its avirulence homolog protein 87 (Avh87) is characterized by the Arg-Xaa-Leu-Arg (RxLR) motif. Avh87 suppressed the pro-apoptotic protein BAX- and elicitin protein INF1-mediated cell death of *Nicotiana benthamiana*. Furthermore, a recombinase polymerase amplification-lateral flow dipstick detection assay targeting this *P. cinnamomi*-specific biomarker was developed. While successfully detected 19 *P. cinnamomi* isolates of a global distribution, this assay lacked detection of 37 other oomycete and fungal species, including *P. parvispora*, a sister taxon of *P. cinnamomi*. In addition, it detected *P. cinnamomi* from artificially inoculated leaves of *Cedrus deodara*. Moreover, the RPA-LFD assay was found to be more sensitive than a conventional PCR assay, by detecting as low as 2 pg of genomic DNA in a 50-µL reaction. It detected *P. cinnamomi* in 13 infested soil samples, while the detection rate was 46.2% using PCR. Results in this study indicated that *PHYCI_587572* is a unique biomarker for detecting *P. cinnamomi*. Although *PHYCI_587572* was identified as an effector gene based on the RxLR motif of Avh87 and the avirulence activity on *Nicotiana*, its exact genetic background and biological function on the natural hosts of *P. cinnamomi* warrant further investigations.

Keywords: plant destroyers; disease diagnosis; RxLR-dEER; soil-borne pathogen; exclusivity; inclusivity

1. Introduction

Phytophthora cinnamomi is a destructive pathogen of nearly 5000 plant species including many economically important crops [1–3]. For example, root rot caused by *P. cinnamomi* is a widely spread and most severe disease of avocado with an estimated annual loss of $40 million in California, USA

alone [4]. *Phytophthora cinnamomi* also causes root and crown rot, canker and dieback diseases on a wide range of woody ornamental crops such as azalea, boxwood, camellia, cedar, and rhododendron [5]. In addition to its impact on agriculture and horticulture, *P. cinnamomi* is a notorious invasive pathogen in natural plantations worldwide. In concert with chestnut blight, *P. cinnamomi* was responsible for the near extinction of wild American chestnut trees in the eastern United States [6,7]. In the forests of south-west Western Australia, *P. cinnamomi* has permanently altered the composition of plant communities and caused drastic decline of biodiversity by killing over 3500 susceptible endemic plant species and threatening macrofungal and bird species [3,8–10]. In China, *P. cinnamomi* has been found in many provinces and municipalities such as Fujian [11–13], Hainan [14], Jiangsu [13], Shaanxi [15], Shanghai [16], and Zhejiang [13], threatening the production of avocado, blueberry, chestnut, kiwifruit, ornamental trees, and the health of natural forests.

Phytophthora species secrete a wide range of effector proteins that overcome host immunity and facilitate the infection of plants [3,17] such as elicitins [18], small cysteine-rich toxins, transglutaminases, and RxLR effectors [3]. RxLR effectors, characterized by an Arg-Xaa-Leu-Arg (RxLR) motif, N-terminal signal peptide, and often Glu-Glu-Arg (EER) motif, are responsible for the translocation of effector proteins from plant apoplast into cytoplasm [19–21]. Although the exact number and pathogenesis roles of *P. cinnamomi* RxLR effectors remain to be determined, a comparative genomic analysis of *P. infestans* and *P. cinnamomi* has identified the presence of at least 171 RxLR effector genes in the *P. cinnamomi* genome [3]. Furthermore, only three out of 10 RxLR effector homologues that have known functional characters are present in the *P. cinnamomi* genome [3], implying the presence of *P. cinnamomi*-unique RxLR effector proteins and genes.

Early detection is the first and arguably most effective step within the hierarchy of plant disease control strategies. Although chemical application and soil sanitation could reduce disease severity and inoculum level [5], regular treatments could be costly and ineffective once *P. cinnamomi* has already widely spread in agricultural settings, and conceivably even more challenging in natural forests due to the scale of the problem. A different approach of disease management is early detection followed by the eradication of the pathogen. It is relatively inexpensive, sustainable, and effective if the pathogen is removed at an early stage. Nevertheless, its success relies on the accuracy and speed of the detection assay.

Detection assays of *P. cinnamomi* have advanced with molecular techniques, while their accuracy has been challenged by emerging new pathogens. Previous molecular detection assays of *P. cinnamomi* were developed based on conventional PCR [22–26], real-time PCR [27–29], and isothermal amplification assays [28,30]. However, many of these assays have failed in differentiating *P. cinnamomi* from *P. parvispora* [31], the species most closely related to *P. cinnamomi* within the genus *Phytophthora* [32–34].

Recombinase polymerase amplification (RPA) is an emerging technique that has been applied in detection assays of *Phytophthora* species [28,35–38]. The RPA process employs three core factors, including recombinase, DNA-binding proteins and polymerase. Recombinases perform to pair oligonucleotide primers with homologous sequence in duplex DNA. Simultaneously, DNA-binding proteins bind to displaced strands of DNA and polymerase begins to synthesize complete double-stranded DNA where the primer has bound to the target DNA. The enzyme mixture conducts exponential amplification of the target region within the template. Compared to PCR and loop-mediated isothermal amplification (LAMP) assays, RPA assays require shorter time span and less complex procedures and equipment for amplifying DNA templates of *Phytophthora* species [36–40]. In addition, RPA amplified products can be detected by lateral flow strips and the results can be easily read without any specialized equipment, suggesting that combination of RPA with lateral flow dipsticks (RPA-LFD) is suitable for developing a simple, rapid, and specific method for the detection of plant pathogens in resource-limited settings. The aims of this study were to identify a RxLR effector gene *PHYCI_587572* using a comparative genomic approach and develop an RPA-LFD assay targeting this *P. cinnamomi*-specific biomarker.

2. Materials and Methods

2.1. Isolate Selection and DNA Extraction

Phytophthora cinnamomi isolates were obtained from diseased roots of *Pinus sp.*, *Rhododendron simsii*, and *Camellia oleifera* from various provinces in China. The roots were surface disinfected, dried, and placed on Phytophthora selective agar PARP-V8 agar [5]. Surface disinfestation of the diseased roots were accomplished incubation in 70% ethanol. When using 70% ethanol, infected roots were given single dips and then dipped in three vials containing autoclaved distilled water for 5–10 min per vial. Following surface disinfestation, roots were surface dried by placing them on filter paper. A total of 76 isolates were used in this study including 19 of *P. cinnamomi*, 40 samples representing 21 *Phytophthora* species, one of *Globisporangium ultimum*, and 16 other fungal species (Table 1). For DNA extraction, each isolate was cultured in 10% clarified V_8 juice (oomycetes) or potato dextrose broth (fungi) at 20–25 °C in the dark for 4–5 days. Genomic DNA (gDNA) was extracted from the harvested mycelia using a E.Z.N.A.® HP Fungal DNA Kit (Omega Bio-tek, Inc., Norcross, GA, USA) following the manufacturer's instructions. Concentrations of gDNA extractions were measured using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) then adjusted as needed. Extractions were stored at −20 °C until use.

Table 1. List of oomycete and fungal isolates used in this study and their detection results in the recombinase polymerase amplification-lateral flow dipstick (RPA-LFD) assay targeting the PHYCI_587572 biomarker.

(Sub) Clade	Species	Isolate	Host or Substrate	Location [a]	RPA-LFD [b]
7c	*Phytophthora cinnamomi*	Pci1	*Pinus* sp.	AH	+
		Pci2	*Rhododendron simsii*	JS	+
		Pci3	*Cedrus deodara*	JS	+
		Pci4	*Camellia oleifera*	JS	+
		Pci5	*Pinus* sp.	JS	+
		Pci6	*Rhododendron simsii*	AH	+
		Pci7	*Rhododendron simsii*	SD	+
		Pci8	*Cedrus deodara*	SD	+
		Pci9	*Cedrus deodara*	AH	+
		Pci10	*Pinus* sp.	SD	+
		JP-07-0035	*Pieris* sp.	Oregon, USA	+
		PCN-18-001	*Castanopsis* sp.	Taiwan	+
		PCN-18-078	*soil*	Taiwan	+
		JP-08-313	*Pieris* sp.	Oregon, USA	+
		JP-07-0376	N/A	Oregon, USA	+
		JP-09-325	N/A	Oregon, USA	+
		ATCC 15400	*Camellia japonica*	South Carolina, USA	+
		ATCC 15401	*Persea americana*	Puerto Rico	+
		CBS 144.22	*Cinnamomum burmannii*	Indonesia	+
7c	*P. parvispora*	CBS132771	*Arbutus unedo*	Italy	−
		CBS132772	*Arbutus unedo*	Italy	−
7a	*P. cambivora*	CBS 248.60	*Castanea sativa*	USA	−
	P. fragariae	CBS 209.46	*Fragaria* × *ananassa*	England, UK	−
	P. rubi	CBS 967.95	*Rubus idaeus*	Scotland, UK	−
7b	*P. melonis*	PMNJHG1	*Cucumis sativus*	JS	−
		PMNJHG2	*Cucumis sativus*	JS	−
		PMNJHG3	*Cucumis sativus*	JS	−
		PMNJDG1	*Benincasa hispida*	JS	−
		PMNJDG2	*Benincasa hispida*	JS	−
		PMNJDG3	*Benincasa hispida*	JS	−
		PMFJHL1	*Lagenaria siceraria*	FJ	−
		IMI 325917	*Cucumis* sp.	FJ	−
	P. sojae	P6497	*Glycine max*	Mississippi, USA	−
		Peng-R3	*Glycine max*	FJ	−
1	*P. cactorum*	Pcac1	*Malus pumila*	JS	−
		Pcac2	*Malus pumila*	JS	−
		Pcac3	*Rosa chinensis*	JS	−
	P. infestans	Pin1	*Solanum tuberosum*	FJ	−
		Pin2	*Solanum tuberosum*	YN	−
	P. nicotianae	Pni1	*Nicotiana tabacum*	YN	−

Table 1. *Cont.*

(Sub) Clade	Species	Isolate	Host or Substrate	Location [a]	RPA-LFD [b]
		Pni2	*Lycopersicum* sp.	JS	–
		Pni3	*Sophora sinensis*	JS	–
		Pni4	*Citrus* sp.	JS	–
2	*P. capsici*	Pcap1	*Capsicum annuum*	JS	–
3	*P. ilicis*	CBS 114348	*Ilex aquifolium*	The Netherlands	–
4	*P. palmivora*	Ppa1	Iridaceae	YN	–
4	*P. quercetorum*	15C7	Soil	South Carolina, USA	–
5	*P. castaneae*	CBS 587.85	Soil	Taiwan	–
6	*P. megasperma*	CBS 305.36	*Matthiola incana*	California, USA	–
	P. mississippiae	57J3	Irrigation water	Mississippi, USA	–
8	*P. drechsleri*	CBS 292.35	*Beta vulgaris* var. *altissima*	California, USA	–
		ATCC 56353	*Citrus sinensis*	Australia	–
	P. hibernalis	CBS 270.31	*Cirrus sinensis*	Setúbal, Portugal	–
	P. syringae	ATCC 34002	*Citrus* sp.	California, USA	–
	P. ramorum	ATCC MYA-2949	*Quercus agrifolia*	California, USA	–
10	*P. boehmeriae*	Pbo1	*Boehmeria nivea*	JS	–
		Pbo2	*Gossypium* sp.	JS	–
		Pbo3	*Boehmeria nivea*	JS	–
		Pbo4	*Gossypium* sp.	JS	–
Oomycete	*Globisporangium ultimum*	Gu1	Irrigation water	JS	–
Fungi	*Alternaria alternata*	LH1401	*Cucumis melo*	JS	–
	Aspergillus oryzae	Ao1	*Glycine max*	JS	–
	Cercospora kikuchii	Ck1	*Glycine max*	JS	–
	Colletotrichum gloeosporioides	Cg1	*Glycine max*	JS	–
	C. truncatum	Ct1	*Glycine max*	JS	–
	Diaporthe phaseolorum var. *caulivora*	DPC	*Glycine max*	JS	–
	Fusarium oxysporum	Fo1	*Pinus* sp.	JS	–
	Fusarium solani	Fs1	*Gossypium* sp.	JS	–
		Fs2	*Glycine max*	JS	–
	Fusarium equiseti	Fe1	*Pinus* sp.	JS	–
	Magnaporthe grisea	Guy11	*Oryza sativa*	French Guiana	–
	Nigrospora sphaerica	Ns1	*Glycine max*	JS	–
	Phakopsora pachyrhizi	Pa1	*Glycine max*	JS	–
	Phomopsis asparagi	Pas1	*Asparagus officinalis*	JS	–
	Rhizoctonia solani	Rs1	*Gossypium* sp.	JS	–
	Verticillium dahliae	Vda1	*Gossypium* sp.	JS	–

[a] Abbreviations of provinces in China: AH, Anhui province; JS, Jiangsu province; SD, Shandong; FJ, Fujian province; YN, Yunnan province; [b] Positive (+) or negative (−) reaction result in the RPA-LFD assay for detecting *P. cinnamomi*.

2.2. Genomic Sequences

Genomic sequences and gene models of *P. capsici*, *P. cinnamomi*, *P. infestans*, *P. ramorum*, and *P. sojae* were retrieved from the MycoCosm portal [41]. Those of *P. asiatica*, *P. cactorum*, *P. cambivora*, *P. hibernalis*, *P. parvispora*, *P. syringae*, and *P. vignae* were also included in the subsequent comparative genomic analysis.

2.3. Identification of A P. cinnamomi-Unique RxLR Effector Gene

To identify RxLR effectors, the genome sequence of *P. cinnamomi* was translated to open reading frames (ORFs). ORFs encoding < 50 amino acids were excluded. SignalP v3.0 was used to identify the N-terminal signal peptide [42] within remaining ORFs. Proteins with Hidden

Markov Model (HMM) scores > 0.9 and without predicted transmembrane domain were predicted as secreted proteins. A regular expressions (REGEX) method was performed by script for identifying the (^\w{1,40}\w{1,96}R\wLR\w{1,40} [ED][ED][KR]) motif [43,44] within the predicted secreted proteins. The RxLR-EER domains of the candidates were aligned and then used to construct a HMM profile to search within the predicted secreted proteins using HMMER. Matches with a bit score of >0 were manually validated.

2.4. Construction of Binary Potato Virus X (PVX) Vectors

As no introns were predicted within *PHYCI_587572*, this gene was directly amplified using the gDNA of isolate Pci1 (Table 1). Each 25-µL PCR reaction include 1 µL of gDNA (100 ng per µL), 1 µL (10 µM) of each of primers PHYCI_587572-PVX-HA(infusion)-F and PHYCI_587572–PVX-HA(infusion)-R (Table 2), 1 µL of PrimeSTAR HS DNA Polymerase (Takara Bio, Kusatsu, Shiga, Japan), 4 µL of dNTP Mixure (2.5 mM), 10 µL of 5× PrimeSTAR Buffer, and 32 µL of nuclease-free water (nfH_2O). PCR was carried out using a Veriti 96-Well Thermal Cycler (Applied Biosystems, Singapore) following the program: an initial denaturation step at 95 °C for 3 min, 35 cycles of 95 °C for 15 s, 56 °C for 15 s, and 72 °C for 15 s, plus a final extension at 72 °C for 5 min. Each set of PCR reactions included a NTC. PCR products were examined in 1% agarose gel electrophoresis at 120 V for approximately 25 min. Agarose gel was stained by ethidium bromide and visualized on a transilluminator. The PCR assay was carried out three times. PCR products were digested with the *Sma*I restriction enzyme (NEB, R0141S) and cloned into the PVX vector pGR107 [45] to form pGR107:*PHYCI_587572*. The constructs were confirmed by sequencing at Genscript (Nanjing, China) using the primers LBA and LBB (Table 2). Recombinant binary plasmids were maintained and propagated in *Escherichia coli* strain JM109 grown in Luria-Bertani (LB) media amended with kanamycin (50 mg per mL) and tetracycline (12.5 mg per mL).

Table 2. Primers used in the construction of binary potato virus X (PVX) vectors, infiltration of *Agrobacterium tumefaciens*, and the recombinase polymerase amplification-lateral flow dipstick (RPA-LFD) assay targeting the *PHYCI_587572* biomarker.

Assay	Name	Sequence (5′–3′)
PVX construction	PHYCI_587572-PVX-HA(infusion)-F	CTAGCATCGATTCCCGGGATGCTCTCGATGACCACAGCCTCC
	PHYCI_587572-PVX-HA(infusion)-R	CTCTAGAGGATCCCCGGGGAAATTCTCCTCGCGCGTG
Agrobacterium infiltration	LBa	CAATCACAGTGTTGGCTTGC
	LBb	GACCCTATGGGCTGTGTTG
RPA	PciRL587572F	GCGAGGCCCTCTCGATGACCACAGCCTCCAACCA
	PciRL587572R	[Biotin]TTGCTGCAGATATGTGCTGCTTGCCTGGACCATC
	PciRL587572P	[FAM]GAGGCAGTCGACGATGATGA[THF]TCCTCCGAAGATTCC[C3-spacer]

2.5. Agrobacterium Tumefaciens Infiltration

Constructs were introduced into *A. tumefaciens* strain GV3101 by electroporation [46,47]. Recombinants (pGR107:*PHYCI_587572*) were selected with tetracycline (12.5 mg per mL) and kanamycin (50 mg per mL). Individual colonies were verified in PCR using the primers LBa and LBb (Table 2).

Nicotiana benthamiana plants were grown in a greenhouse at a day/night temperature of 25/16 °C and a 16 h photoperiod for 4–6 weeks. Recombinant GV3101 cells were grown in LB media amended with kanamycin (50 mg per mL) for 48 h, harvested, washed with 10 mM $MgCl_2$ three times, resuspended in 10 mM $MgCl_2$ to a final OD_{600} of 0.4, then incubated at room temperature for 1 to 3 h prior to infiltration. For pressure infiltration, 100 mL of recombinant GV3101 cell suspension was infiltrated though a small nick into each of three infiltration sites per adaxial side of a *N. benthamiana* leaf using a syringe. After 0, 12, or 24 h, *Agrobacterium tumefaciens* cells carrying the *Bax* gene (pGR107:*Bax*) or *inf1* gene (pGR107:*inf1*) were infiltrated into each of the three filtration sites. *Agrobacterium tumefaciens* cells carrying the enhanced green fluorescent protein (eGFP) gene in place of pGR107:*Bax* or pGR107:*inf1* were infiltrated into three parallelly designated filtration sites of the same leaf after 0, 12, or 24 h as controls. Symptom development was monitored daily. Photographs were taken on 5 and 6 days

after filtration of pGR107:*Bax* and pGR107:*inf1*, respectively. This experiment was conducted twice with three replicate leaves in each repeat.

2.6. RPA-LFD Assay

A set of RPA primers and probe targeting the *P. cinnamomi*-specific biomarker *PHYCI_587572*, including a forward primer PciRL587572F, a 5′-biotin-labeled reverse primer PciRL587572R, and a probe PciRL587572P labeled by fluorescein amidite (FAM), tetrahydrofuran (THF), and C3 spacer (Table 2), was designed according to the instruction for the nfo kit in the TwistAmp® DNA Amplification Kits Assay Design Manual. Primers and probe were synthesized by Sangon Biotech (Shanghai, China).

The RPA-LFD assay was performed following the procedures in the TwistAmp® nfo Kit Quick Guide (TwistDx Ltd., Cambridge, UK). Each RPA reaction in a total volume of 50 µL contained 1 µL of DNA template, 29.5 µL of rehydration buffer, 2.1 µL of each of primers PciRL587572F and PciRL587572R (10 µM), 0.6 µL of probe PciRL587572P (10 µM), 12.2 µL of nfH_2O, and 2.5 µL of magnesium acetate (280 mM). These reagents were added into reaction microtubes and mixed using a pipette. Freeze-dried pellet magnesium acetate was pre-added to the cap of the microtubes. After brief vortex and centrifugation, the reaction microtubes were incubated in a heating block set at 39 °C for 20 min. To visualize amplicons, 10 µL of RPA product was mixed with 90 µL of phosphate buffered saline with Tween 20 (PBST) running buffer. Then 5 µL of the RPA product per each reaction was added to the sample pad of a Milenia Genline HybriDetect 1 LFD (Milenia Biotec GmbH, Giessen, Germany). The LFD was then vertically immersed into 100 µL of PBST (Milenia Biotec GmbH, Giessen, Germany) in a sterile centrifuge tube for up to 5 min at room temperature (aver. 22 °C) until a clear control line was observed. The dipsticks yielded visible control lines, indicating valid tests. Test lines were visible on all dipsticks correlating to positive RPA reactions.A negative detection or NTC in contrast should not result in any signal or color at the position of the test line.

2.7. Evaluation of RPA Specificity and Sensitivity

The specificity of the RPA-LFD assay was evaluated by testing against all isolates listed in Table 1. One µL of gDNA (10 ng per µL) of each isolate was included in each 50 µL reactions. Each isolate was tested in triplicate.

To evaluate the sensitivity of the RPA-LFD assay, 10-fold serial dilution of gDNA of *P. cinnamomi* isolate Pci1 ranging from 100 ng to 100 fg (0.0001 ng) per µL were used as templates. Each concentration was evaluated in triplicate. Each set of reactions in the specificity and sensitivity evaluations included an NTC (nfH_2O in place of DNA template).

2.8. Detecting P. cinnamomi in Artificially Inoculated Pine Needles Using RPA-LFD

Pine needles were collected from a healthy deodar cedar tree (*Cedrus deodara*) at a landscape site of Nanjing Forestry University (Nanjing, China). They were artificially inoculated by *P. cinnamomi*. A 2 × 2 mm mycelial plug of *P. cinnamomi* isolate Pci1 was mixed with approximately 100 g of pine needles and incubated in each of triplicate sterile 250 mL flasks at 22 °C, 100% relative humidity, and a 12-h photoperiod for 5 days. Sterile agar plugs were mixed with pine needles to produce three non-inoculated control samples. Total DNAs were extracted from *P. cinnamomi*-colonized and non-inoculated samples using an NaOH lysis method. Briefly, 20 mg of plant tissues collected from the wound site of each Pine needle were placed into a 1.5-mL microtube containing 200 µL of NaOH (0.5 N). They were grinded for approximately 1 min until no large pieces of plant tissues were visible using a sterile tissue grinder pestle. Then, 5 µL of grinded tissues in NaOH were transferred to a new microtube containing 495 µL of Tris buffer (100 mM, pH 8.0). Two µL of the mixture were used as used as templates in the RPA-LFD assay. Purified gDNA (10 ng per µL) of isolate Pci1 and nfH_2O were used as a positive control and NTC, respectively, in two repeats of the experiment.

2.9. Detecting P. cinnamomi in Soil Samples Using the RPA-LFD Assay

A total of 13 soil samples were collected from five provinces in China (Table 3). A previous study found the presence of *P. cinnamomi* in all 13 samples [30]. Each soil sample was mixed well and divided into two equal subsamples. DNAs were directly extracted from the first set of 13 soil subsamples using a Plant DNA Mini Kit (Omega Biotek, Norcross, GA, USA). For the second set, a baiting method [5] using *C. deodara* leaves was deployed. After 5 days, DNA was extracted from leaf baits of each subsample using the NaOH lysis method. Both RPA-LFD and conventional PCR assays were performed using DNA extractions of both soil samples and leaf baits. Each DNA extraction was tested three times using each assay. Purified gDNA (10 ng) of isolate Pci1 was used in positive control reactions.

3. Results

3.1. Identification of A P. cinnamomi-Unique RxLR Effector Gene PHYCI_587572

A protein (Avh87), translated from the *PHYCI_587572* gene located at scaffold_77:29462-29848, was found containing a signal peptide cleavage site at residue 20, an RLLR motif at 44–47, followed by an SEER motif at 63–66 (Figure 1). Sequences of the *PHYCI_587572* gene and *Avh87* were used to blast against the genome sequences and predicted ORFs of *P. asiatica*, *P. cactorum*, *P. cambivora*, *P. capsici*, *P. hibernalis*, *P. infestans*, *P. parvispora*, *P. ramorum*, *P. sojae*, *P. syringae*, and *P. vignae*, respectively. No homologs of *PHYCI_587572* gene or Avh87 were found within these non-target species.

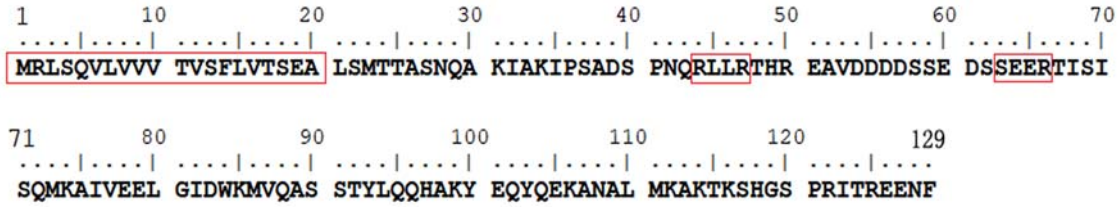

Figure 1. Amino acid sequences of avirulence homolog protein 87 (Avh87) derived from the *PHYCI_587572* gene open reading frame of *Phytophthora cinnamomi*. Boxed residues 1–20: predicted signal peptide; 44–47: an RxLR (RLLR) motif; 63–66: a dEER (SEER) motif.

3.2. Suppression of Programmed Cell Death by Avh87

Avh87 encoded by the *PHYCI_587572* gene consistently suppressed BAX- and INF1-mediated cell death among three replications between two repeats of the *A. tumefaciens* infiltration experiment. BAX (pGR107:*Bax*) could triggered apoptosis of *N. benthamiana* leaves when simultaneously infiltrated along with Avh87 (Figure 2A). However, infiltration leaves with pGR107:*PHYCI_587572* 12 or 24 h prior to that with pGR107:*Bax* inhibited the BAX-mediated cell death (Figure 2A). The inhibition of Avh87 appeared as more effective against INF1. As shown in Figure 2B, INF1-mediated cell death was suppressed when pGR107:*Inf1* was infiltrated promptly following that with pGR107:*PHYCI_587572*. The eGFP protein in place of BAX or INF1 expressed as negative controls consistently induced minimum cell death (Figure 2) in both sets of experiments.

3.3. Specificity and Sensitivity of the RPA-LFD Assay

In the evaluation of specificity, identical results were obtained among three repeats of all isolates. All LFDs had a visible control line, indicating valid tests. Test lines were visible only on LFDs with genomic DNAs (gDNAs) of 19 *P. cinnamomi* isolates (Table 1). No test lines were observed on those of 22 other oomycete and 15 fungal species (Figure 3).

In the sensitivity evaluation, all LFDs had a visible control line. Solid test lines were observed on LFDs of RPA reactions containing 0.1–100 ng of *P. cinnamomi* gDNA (Figure 4). They were also visible on LFDs of 0.01 to 0.001 ng of gDNA (Figure 3). No test lines were observed on those of 0.0001 ng

(100 fg) of gDNA or no template control (NTC). These results were consistent across three repeats of the evaluation. In the comparison PCR assay, ≥1 ng of gDNA was required for consistent positive results using primers PciRL587572F and PciRL587572F (Supplementary Figure S1). The result indicates that RPA-LFD assay was more sensitive than PCR by using LFD strips.

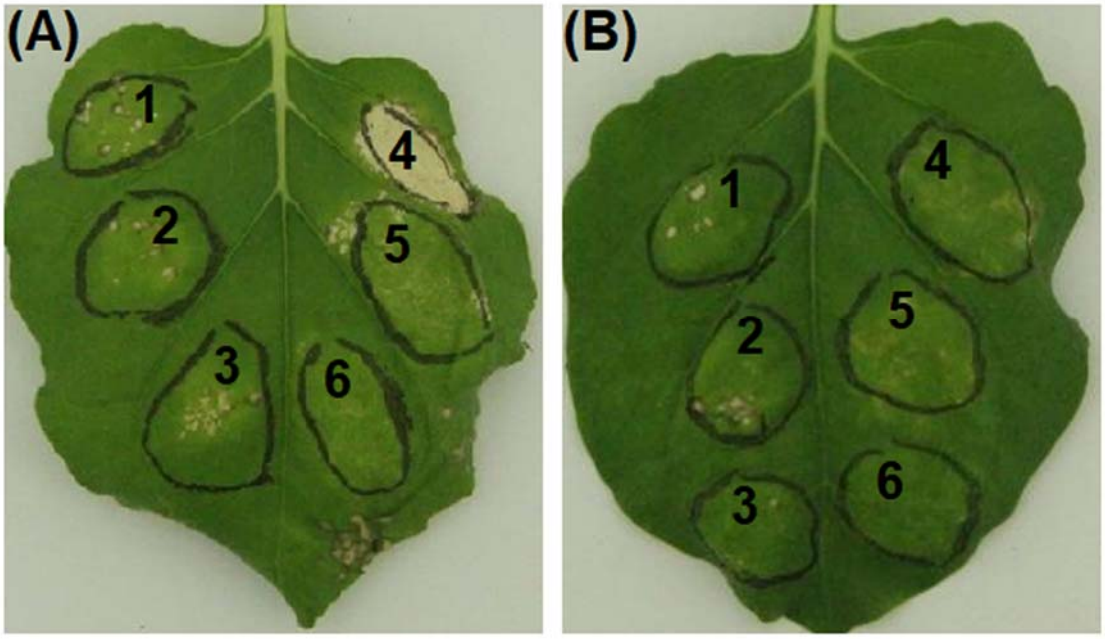

Figure 2. Suppression of BAX- and elicitin protein INF1-mediated cell death of *Nicotiana benthamiana* leaves infiltrated with *Agrobacterium tumefaciens* strains containing a PVX vector carry the *PHYCI_587572* gene (Avh87) or a control gene (eGFP). (**A**): 1, Avh87→eGFP; 2, Avh87→12h→eGFP; 3, Avh87→24h→eGFP; 4, Avh87→BAX; 5, Avh87→12h→BAX; 6, Avh87→24h→BAX. (**B**): 1, Avh87→eGFP; 2, Avh87→12h→eGFP; 3, Avh87→24h→eGFP; 4, Avh87→INF1; 5, Avh87→12h→INF1; 6, Avh87→24h→INF1.

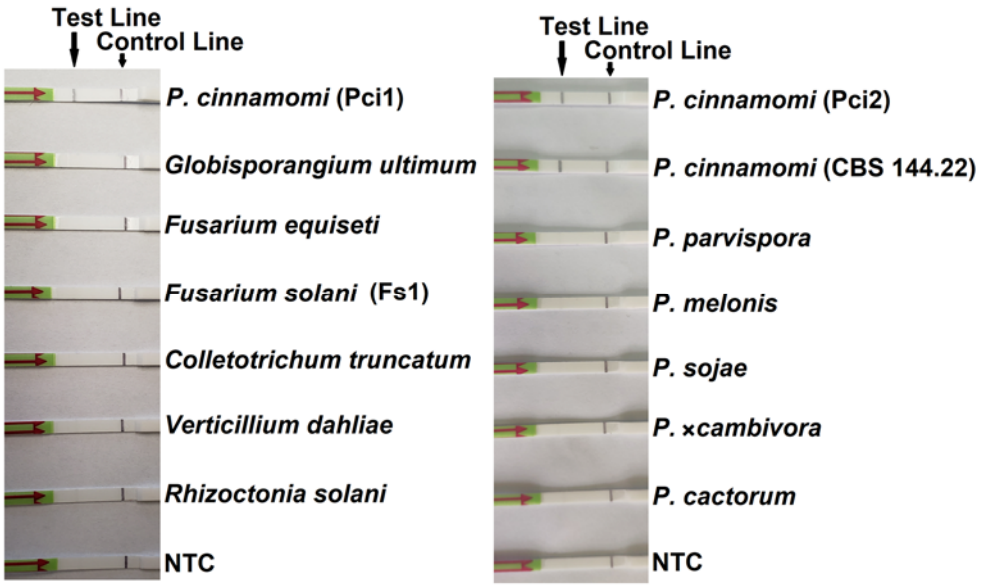

Figure 3. Detection results of the recombinase polymerase amplification-lateral flow dipstick assay targeting the *PHYCI_587572* biomarker. Genomic DNAs (10 ng per 50-µL reaction) of *Phytophthora cinnamomi* isolates and other oomycete and fungal species. Nuclease-free water was used in place of DNA templates in no template controls (NTC).

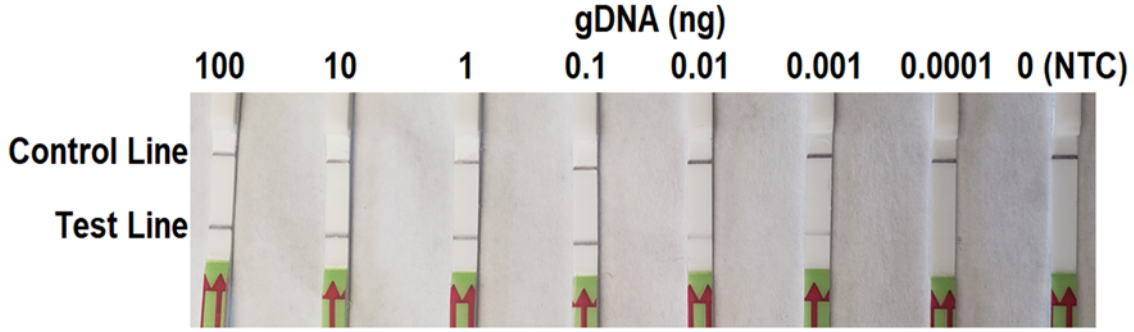

Figure 4. Evaluation results of sensitivity of the recombinase polymerase amplification-lateral flow dipstick assay targeting the *PHYCI_587572* biomarker using a 10-fold serial dilutions of genomic DNA (gDNA) of *Phytophthora cinnamomi* isolate Pci1. Nuclease-free water was used in place of DNA templates in no template controls (NTC).

3.4. Detecting P. cinnamomi in Artificial Inoculated Pine Needles

All LFDs had a visible control line indicating valid tests. *Phytophthora cinnamomi* was detected in the total DNAs extracted from three *P. cinnamomi*-infested pine needle samples and the positive control, whereas not detectable in three non-inoculated samples or NTC (Figure 5). Identical results were recorded in both repeats of the experiment.

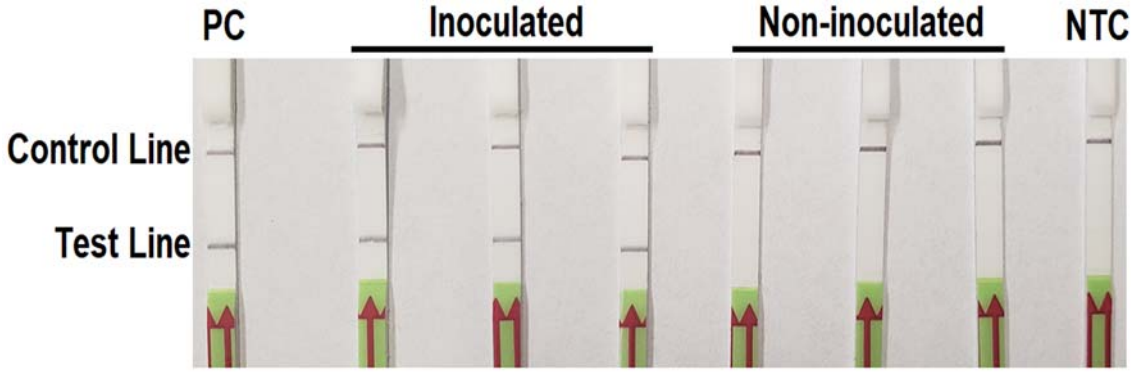

Figure 5. Detection of *Phytophthora cinnamomi* in artificially inoculated pine needles of *Cedrus deodara* using the recombinase polymerase amplification-lateral flow dipstick assay. Genomic DNA (10 ng per μL) of *P. cinnamomi* isolate Pci1 was used as the positive control (PC). Nuclease-free water was used in place of DNA templates in the no template control (NTC). Dipsticks of the first repeat are shown. Identical detection results were observed in the second repeat of the experiment.

3.5. Detection of P. cinnamomi in Infested Soil Samples

The RPA-LFD assay was applied to 13 *P. cinnamomi*-infested soil samples. Detection results were consistent among three repeats of the experiment. Using DNA samples directly extracted from soil samples, *P. cinnamomi* was detected in all 13 samples using the RPA-LFD assay, while it was detected in only six samples using PCR (Table 3). Using DNAs extracted from *C. deodara* leaf baits, both RPA-LFD and PCR assays detected *P. cinnamomi* in all 13 samples.

Table 3. Detection results of *Phytophthora cinnamomi* in 13 infested soil samples using the recombinase polymerase amplification-lateral flow dipstick (RPA-LFD) and PCR assays.

Sample No.	Location [a]	Vegetation	Year	Detection of Pcin			
				DNAs from Soil		DNAs from Leaf Baits	
				RPA-LFD	PCR	RPA-LFD	PCR
1	Hefei, AH	*Pinus* sp.	2014	+	+	+	+
2	Huaibei, AH	*Rhododendron simsii*	2014	+	+	+	+
3	Liuan, AH	*Cedrus deodara*	2016	+	-	+	+
4	Suzhou, AH	*Camellia oleifera*	2016	+	-	+	+
5	Nanjing, JS	*Pinus* sp.	2015	+	+	+	+
6	Yancheng, JS	*Rhododendron simsii*	2015	+	-	+	+
7	Xuzhou, JS	*Cedrus deodara*	2016	+	-	+	+
8	Tianan, SD	*Pinus* sp.	2016	+	+	+	+
9	Jinan, SD	*Rhododendron simsii*	2017	+	-	+	+
10	Xiamen, FJ	*Pinus* sp.	2016	+	+	+	+
11	Zhangzhou, FJ	*Rhododendron simsii*	2016	+	-	+	+
12	Kunming, YN	*Pinus* sp.	2015	+	+	+	+
13	Anning, YN	*Rhododendron simsii*	2015	+	-	+	+

[a] Province abbreviations: AH, Anhui; JS, Jiangsu; SD, Shandong; FJ, Fujian; YN, Yunnan.

4. Discussion

Phytophthora cinnamomi is one of the most invasive plant pathogens with a global impact [3,6]. Early and accurate detection of *P. cinnamomi* is paramount for implementing disease management promptly and avoiding unnecessary costs due to misdiagnosis. The present study describes *PHYCI_587572*, a potentially *P. cinnamomi*-specific RxLR effector gene identified using a comparative genomics approach, and a rapid and equipment-free RPA-LFD detection assay for *P. cinnamomi* that targets this novel biomarker. The findings in this study have important implications on the *P. cinnamomi*-hosts interactions and the management of this aggressive pathogen.

Despite the potential importance of RxLR proteins in the infection of plants by *P. cinnamomi*, to date, the molecular function of only a few RxLR effectors and genes has been determined [3]. Identification of oomycete pathogenicity effector genes has been facilitated by the increasing availability of genome sequences and the recognition of the RxLR-dEER motif. In this study, the *PHYCI_587572* effector gene encoding Avh87 was identified from the genome sequence of *P. cinnamomi*. During this process, we also identified 265 other RxLR candidate genes. Efforts to identify their molecular functions are underway. Additionally, a comparative analysis of 12 *Phytophthora* genomes indicated the specificity of *PHYCI_587572* to *P. cinnamomi* genome. Furthermore, the inhibition of Avh87 on BAX- and INF1-mediated cell death indicated the potentially fundamental role of Avh87 in the pathogenicity of *P. cinnamomi*. Further investigations are warranted on the biological function of *PHYCI_587572* (Avh87) on the natural hosts of *P. cinnamomi*.

Given the important role of effector genes in oomycete pathogenicity, they are unique candidates for developing novel biomarkers. The RxLR effector gene *PHYCI_587572* identified in this study was found lack of homolog in other reference *Phytophthora* species. This implies its unique function in the pathogenicity of *P. cinnamomi*. However, because many effector genes have been found rapidly evolving [21,48], thus have shown a higher degree of intra-specific polymorphism than universal housekeeping genes [49], utilizing a novel effector gene as a diagnostic biomarker may bear the risk of false-negative detections against mutated isolates of the target *Phytophthora* species. In this study, the novel RPA-LFD assay targeting the RxLR effector gene *PHYCI_587572* accurately detected all 19 *P. cinnamomi* isolates recovered from at least eight host species in three provinces of China, two U.S. states, Puerto Rico, and Indonesia, including the ex-type isolate CBS 144.22 (Table 1). This finding indicates that the novel RPA-LFD assay has a high degree of inclusivity within *P. cinnamomi* isolates. Nevertheless, additional isolates should be tested in the future to confirm its inclusivity.

Rapid pathogen detection is extremely important to the management of aggressive diseases. The key advantage of RPA-based assays is their short reaction time span and requirement for less complex instrumentation. The RPA-LFD assay in this study could be finished within 30 min, which is only half of the time taken by a LAMP assay [30] and a quarter of PCR reactions. Also, it does not require a thermal cycler or gel electrophoresis and imaging system, as its results could be visualized on LFDs. Compared to the LAMP assay for *P. cinnamomi* [30], the RPA-LFD assay also needed fewer primers and lower incubation temperature. All these advantages make the RPA-LFD assay an ideal method for detecting *P. cinnamomi* under field conditions and in diagnostic laboratories.

The development of this novel RPA-LFD assay has further improved the sensitivity of isothermal detection assays for *P. cinnamomi*. As determined in this study, this RPA-LFD assay detected 0.001 ng of *P. cinnamomi* gDNA (~12 copies of nuclei) in a 50-µL reaction system. This is at least 200 times more sensitive than our previously developed LAMP assay and 2000 times more sensitive than a PCR assay using primers F3 and B3 [30]. However, this sensitivity appears lower than a previously reported qPCR-based assay [29]. Although the RPA-LFD assay has the advantages of short time span and less instrumentation requirement against qPCR, future studies are warranted to further improve the sensitivity of isothermal assays for *P. cinnamomi*.

5. Conclusions

A novel RxLR effector *PHYCI_587572* was bioinformatically identified from the genome sequence of *P. cinnamomi*. A comparative analysis of genome sequences of 12 *Phytophthora* species indicated that this effector gene is unique to *P. cinnamomi*, thus an ideal biomarker for developing diagnostic assays. This assay was specific to *P. cinnamomi*, while it lacked detection against 37 other oomycete and fungal species, including *P. parvispora*, a sister taxon of *P. cinnamomi*. It was also found to be 200 times more sensitive than a previously developed LAMP assay. In addition, it detected *P. cinnamomi* in artificially inoculated leaves of *Cedrus deodara*. Results in this study indicated that the RPA-LFD assay is an ideal method for detecting *P. cinnamomi* under field conditions and in diagnostic laboratories, while understanding the precise molecular and biological functions of *PHYCI_587572* and its avirulence homolog protein Avh87 requires further investigation.

Supplementary Materials: The following are available online at http://www.mdpi.com/1999-4907/11/3/306/s1.

Author Contributions: T.D., A.W., X.Y., X.Y., and W.T. conceived and designed the experiments. T.D. and A.W. contributed the reagents and materials. T.D., A.W., X.Y., Y.X., and T.H. performed the experiments. T.D., A.W., X.Y., and W.T. analyzed the data. T.D., A.W., and X.Y. contributed the analysis tools and wrote the first draft of the manuscript. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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Development of a Rapid Loop-Mediated Isothermal Amplification Assay for the Detection of *Dothistroma septosporum*

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Abstract: A Loop-Mediated Isothermal Amplification (LAMP) assay was developed for the detection of the pine pathogen *Dothistroma septosporum* (G. Dorog.) M. Morelet. The specificity of the LAMP assay was tested using a selection of pine needle fungi, including *Dothistroma pini* Hulbary, and *Lecanosticta acicola* (Thüm.) Syd.; only *D. septosporum* DNA was amplified by the test. In terms of sensitivity, the assay was able to detect as little as 1 pg of total *D. septosporum* DNA. This assay enables DNA extracted from diseased host needles to be rapidly tested for the presence of *D. septosporum* using relatively simple to operate equipment away from a fully equipped molecular biology laboratory.

Keywords: DNA-based diagnostics; LAMP; *Dothistroma* needle blight

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1. Introduction

Dothistroma needle blight, a disease caused by the pathogens *Dothistroma septosporum* and *Dothistroma pini*, results in significant damage to pines in both natural and plantation settings globally [1]. In British Columbia, the increasing losses due to *D. septosporum* have been linked to climate change [2]. During 2012 and 2013, *D. septosporum* was found on *Pinus contorta* Dougl. var. *latifolia* Engelm., *Pinus banksiana* Lamb., and their hybrid, in northern Alberta, Canada [3]. The identification of *D. septosporum* in Alberta and the potential for eastward spread through the boreal forest have led to research into population genomics [4], host range [3], and, as we describe here, rapid diagnostics for *D. septosporum*, the pathogen associated with the disease in western Canada.

Polymerase chain reaction (PCR)-based diagnostics for detecting *D. septosporum* DNA have been described by Ioos et al. [5], Langrell [6], and Schneider et al. [7]. These diagnostics are sensitive and able to detect the pathogen within infected tissue although they must be performed within a well-equipped laboratory. Loop-Mediated Isothermal Amplification (LAMP) is an alternative to traditional PCR-based methods. LAMP assays use four to six oligonucleotide primers and a DNA polymerase to drive strand displacement DNA synthesis at a constant temperature without thermalcycling [8]. LAMP assays have been developed for point-of-care diagnostics in medicine [9], agriculture [10], and important forest pathogens [11]. Portable instruments such as the Genie® III developed by OptiGene (Horsham, West Sussex, UK) or the BioRanger instrument developed by Diagenetix Inc. (Honolulu, HI, USA) have been developed to allow LAMP assays to be conducted under field conditions.

Here we report the development of a LAMP-based assay to identify *D. septosporum* in culture and to rapidly and accurately detect *D. septosporum* within infected pine needles.

2. Materials and Methods

2.1. Culturing of D. Septosporum

Pure cultures of *D. septosporum* were obtained from infected pine needles by excising a single acervulus and rolling it in a straight line across the surface of a 2% Malt Extract Agar (MEA) plate. Once spore germination was visible under a dissecting microscope (2–3 days at room temperature), a single germinated conidia was transferred to a fresh 2% MEA plate and incubated at room temperature. Alternatively, *D. septosporum* was isolated by surface sterilizing an infected needle in 10% bleach for 5 min to reduce surface contamination and then placing the infected needle on 2% water agar at room temperature to induce sporulation. An erupting conidial mass from an acervulus was then placed on fresh 2% water agar and a single hyphal tip was subcultured onto fresh 1% to 3% MEA [3]. Prior to DNA extraction, liquid cultures were prepared by inoculating 50 mL clarified V8 medium with mycelial plugs from actively growing cultures, then incubating these at 20 °C with occasional shaking for 6–8 weeks. V8 juice amended with 1.42 g $CaCO_3$ per 100 mL was clarified first by centrifugation for 15 min at 2500 rpm and then filtering the remaining supernatant 3 times through Whatman® qualitative filter paper, grade 4. To obtain the clarified V8 growth medium, clarified V8 juice was mixed with dH_2O in a 1:4 ratio.

Pure cultures of D. septosporum, D. pini, and L. acicola were obtained from infected P. contorta var. latifolia, P. banksiana, P. contorta var. latifolia × P. banksiana, Pinus ponderosa P. Laws. ex C. Laws., Pinus monticola Dougl. ex D. Don, Pinus nigra Arnold, Pinus mugo Turra, Pinus attenuata Lemmon × Pinus radiata D. Don, and P. radiata, collected from Alberta, British Columbia, Ontario, Alaska, Oregon, California, Montana, North Dakota, South Dakota, and New Zealand. Cultures of Ceuthospora sp., Cyclaneusma minus (Butin) DiCosmo, Peredo and Minter "simile", C. minus "verum", D. pini, D. septosporum, Lophodermium conigenum (Brunaud) Hilitzer, Pestalotiopsis sp., Phytophthora pluvialis Reeser, Sutton and E. Hanson, and Strasseria geniculata (Berk. and Broome) Höhn. were also used (Table S1).

2.2. Extraction of DNA from Cultures

In Canada, two DNA extraction methods were used to extract DNA from fungal cultures. The Qiagen DNeasy PowerPlant Pro kit (Qiagen, Valencia, CA, USA) was used to isolate DNA from mycelium harvested from liquid V8 cultures. Freeze dried mycelium was first ground with one 3 mm stainless steel bead in a SPEX® Mixer/Mill (SamplePrep, Metuchen, NJ, USA) for 30 s at setting 1/30. DNA was then extracted from the ground mycelium following the manufacturer's protocol with the following modifications: the optional phenolic separation solution was added at the fist extraction step and sample disruption by bead beating was replaced by a 10 min incubation at 65 °C. Alternatively, a simple Tris-EDTA extraction method was used to extract DNA from cultures growing on MEA. A single plug (5 mm diameter) was placed in microcentrifuge tubes with 400 µL of Tris-EDTA (10 mM Tris-1 mM EDTA, pH 8.0) solution, one 4 mm and three 2.8 mm stainless steel beads, and placed in a SPEX® 1600 MiniG® (SamplePrep, Metuchen, NJ, USA) bead beater for 3 min at 1500 rpm to disrupt the mycelium. After disruption the sample was centrifuged at 16,000× *g* for 3 min and the supernatant transferred to a new tube. DNA extraction method for all specimens is specified in Table S1. In either case, DNA stock solutions were diluted 100-fold in sterile PCR-grade water to serve as a template for DNA amplification.

In New Zealand, DNA from cultures of *Ceuthospora* sp., *C. minus* "simile", *C. minus* "verum", *D. pini*, *D. septosporum*, *L. conigenum*, *Pestalotiopsis* sp., *P. pluvialis*, and *S. geniculata* was extracted using the FastDNA kit (MP Biomedicals, Solon, OH, USA) following the manufacturer's protocol. DNA of other *Phytophthora* spp. was provided by collaborators and the extraction method is unknown (Table S1).

2.3. ITS-Based Identification of Cultures

The nuclear ribosomal internal transcribed spacer (ITS) was PCR amplified using the total DNA extracted from putative isolates of *D. septosporum* and other fungi isolated from pine needles using the primers ITS-1F [12] and ITS-4 [13] following the protocol described in Feau et al. [3]. The PCR product was purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA, USA), and sequenced by Macrogen (Seoul, Korea) or CHU de Québec—Université Laval Research Centre (Quebec, QC, Canada). To identify fungal cultures, ITS sequences were queried against the NCBI nr database using the BLASTn algorithm [14].

2.4. Extraction of DNA from Needles

DNA was extracted from pine needles using DNAzol® Direct (Molecular Research Centre, Inc., Cincinnati, OH, USA). Needles putatively infected by *D. septosporum*, as indicated by microscopic examination of conidia produced on needles that had been placed at room temperature in a sealed Petri plate with a moist paper towel to induce sporulation, as well as visually healthy needles, were selected for DNA extraction. Needles were either fresh and stored at 4 °C or dried and stored at −20 °C. When fresh, one to two infected needles, cut into 5 mm sections, were placed in a 1.5 or 2.0 mL centrifuge tube, with 100 µL of DNAzol® Direct. The needles were then ground with a SPEX® 1600 MiniG® (SamplePrep, Metuchen, NJ, USA) bead beater using one 4 mm and three 2.8 mm stainless steel balls at 1500 rpm for 3 min. When the needles were dry, one or two needles were ground using the bead beater prior to adding DNAzol® Direct. The DNAzol® and ground needle mixtures were incubated at room temperature for 15 min with occasional inversion. The tubes were then centrifuged at 16,000× *g* for 3 min to pellet debris, the DNA-containing supernatant was transferred to a fresh tube and a 100-fold dilution in sterile PCR-grade water was prepared to serve as template for DNA amplification. The same procedure was used to extract DNA from needles of *P. contorta* var. *latifolia* that were infected with *Elytroderma deformans* (Weir) Darker, *Davisomycella ampla* (Davis) Darker, *Lophodermella concolor* (Dearn.) Darker, *Lophodermium seditiosum* Minter, Staley and Millar, and *Lecanosticta acicola* (Table S2).

2.5. Development of LAMP Primers

The β-tubulin 2 gene was selected as a target for our LAMP assay as it has previously been shown to distinguish *D. septosporum* from close relatives [5,7]. A consensus of partial *D. septosporum* β-tubulin 2 gene sequences was generated from an alignment of GenBank accessions AY808205–AY808230 [15], assembled by Barnes et al. [15], that is broadly representative of *D. septosporum* diversity. The consensus sequence was then compared to the corresponding portions of the β-tubulin sequence data from *L. acicola* (AY808237, AY808238) [15] and *D. pini* (AY808231–AY808236) [15] to identify regions specific to *D. septosporum* and would therefore make suitable targets for the LAMP primers. LAMP primers for the *D. septosporum*-specific regions were developed using default parameters in the LAMP Designer 1.4 software package (PREMIER Biosoft, OptiGene, Horsham, West Sussex, UK). The top ranked primer set, as determined by the software, that distinguished *D. septosporum* and *D. pini* was selected (Table 1; Figure 1). The specificity of the F3/B3 primer pair and the component sequences of the FIP and BIP primers (F1c and F2, and B1c and B2, respectively (Figure 1)) were assessed using Primer-BLAST [16], with default parameters and the nr database for fungi.

Table 1. LAMP primers specific to the β-tubulin 2 gene of *Dothistroma septosporum*.

Primer Name	Sequence 5′-3′
F3	CAGGACAGTATGTGGAATCC
B3	TCGGTGCTTGCCTAGATA
FIP	GACACTCAGTCGCTCTCGCGCGTCATGCAGATTCGTA
BIP	AGGTAGGTGCTCCTCTCCGCAGGATGACGATGTGCTG
LoopF	TGCAGTGCCTTCGTATCTG
LoopB	CCAGTGCTTCAACACTATGC

Figure 1. Position of LAMP primers based on consensus sequence of *D. septosporum* β-tubulin 2 gene sequences retrieved from GenBank (AY808205–AY808230). Primer FIP is a combination of F1c and F2, while primer BIP is a combination of B1c and B2. Differences from *D. pini* are noted by * symbol.

2.6. Preparation of the LAMP Reaction

Primers were first hydrated to a stock concentration of 500 µM using Tris-EDA diluted 10-fold with sterile PCR-grade water. A working stock of primer mix was then prepared that contained 0.8333 µM F3, 0.8333 µM B3, 1.666 µM LoopF, 1.666 µM LoopB, 3.333 µM FIP, and 3.333 µM BIP in PCR-grade water. Primers and template DNA were then combined in Optigene Isothermal master mix (ISO-001) (OptiGene, Horsham, West Sussex, UK) in the proportions of 0.6 master mix, 0.24 primer mix and 0.16 template DNA (i.e., for a 15 µL reaction, use 9 µL isothermal master mix, 3.6 µL of primer mix, and 2.4 µL of template DNA). The final primer concentrations for the LAMP reaction were 0.2 µM F3, 0.2 µM B3, 0.4 µM LoopF, 0.4 µM LoopB, 0.8 µM FIP, and 0.8 µM BIP. Template DNA from cultures or needles consisted of stock DNA diluted 100-fold in sterile PCR-grade water.

Primers, master mix and template DNA were combined with a final volume of 15 µL. The Genie® III instrument (OptiGene, Horsham, West Sussex, UK) was run at 65 °C for up to 60 min, and then annealing ramped from 98 to 80 °C at a rate of −0.05 °C/s for fragment identification. Both positive (pine DNA spiked with 0.1 to 0.01 ng of DNA from *D. septosporum* per 15 µL reaction) and a blank (sterile PCR-grade water) were included in each run. In New Zealand, the Smart-DART (Digenetix Inc., Honolulu, HI, USA) or BioRanger LAMP devices (Digenetix Inc., Honolulu, HI, USA) were used with the same reaction components as were utilized in Canada although the reaction was overlaid with mineral oil as the Smart-DART and BioRanger do not have heated lids.

2.7. Sensitivity of the LAMP Assay

The sensitivity of the LAMP assay was determined using serial dilutions of total *D. septosporum* DNA extracted from three different isolates, all of which were collected from the north Okanagan region of British Columbia from *P. contorta* var. *latifolia*. Total DNA

concentration was first quantified using a Qubit™ 4 Fluorometer (Invitrogen, Waltham, MA, USA) and the Qubit™ dsDNA HS assay kit (Invitrogen, Waltham, MA, USA) and then standardized to 4.17 ng/µL. Serial dilutions were made from 4.17 ng/µL to 4.17 × 10^{-6} ng/µL and 2.4 µL of template DNA was added to each 15 µL LAMP reaction, providing a dilution series from 1 ng to 1.0 × 10^{-6} ng of template DNA in each reaction. The LAMP reaction was run for 60 min.

2.8. Specificity of the LAMP Assay

The specificity of the LAMP assay was assessed using DNA from cultured isolates of *D. septosporum*, *D. pini*, and *Lecanosticta acicola* (Table S1), and using pine needles infected with *L. acicola*, *Lophodermella concolor*, *Lophodermium seditiosum*, and *Elytroderma deformans* (Table S2). In New Zealand, the assay was tested against *Phytophthora* spp. and fungi associated with the foliage of *Pinus radiata* (Table S1). Confirmation of the ability of the LAMP assay to detect *D. septosporum* within infected needles was conducted by using DNA extracted from a subsample of needles from which *D. septosporum* was previously isolated into pure culture and identified by ITS sequencing. In cases where the LAMP reaction was negative, the quality of the DNA was checked by PCR amplifying the ITS region to confirm that the DNA could be amplified.

2.9. Statistical Comparison of the DNA Extraction Methods

The annealing temperature and amplification time for *D. septosporum*-positive DNA samples extracted using the Qiagen DNeasy PowerPlant Pro kit and the simple Tris-EDTA methods were compared using the nonparametric Kruskal–Wallis test as the assumptions of equal variance and normal distribution were not met. The sample from New Zealand was excluded from the analysis as the annealing temperature and amplification time were not recorded. Raw data are provided in Table S1.

3. Results

Our *D. septosporum* LAMP assay has high specificity. DNA extracted from all isolates of *D. septosporum* included in the screening population produced positive amplification while no amplification occurred when the DNA of other fungi or *Phytophthora* spp. that were included in the screening population were used as template. (Table 2). Further, in silico analysis of the LAMP primers using Primer-BLAST suggested that all six primers were specific for *D. septosporum*. Detection times were significantly shorter for DNA samples extracted using the Qiagen method compared to the TE method (H = 44.5345; p = 2.499 × 10^{-11}). In contrast, there was no significant difference between extraction methods in terms of annealing temperatures (H = 1.7184; p = 0.1904) (Table 2). Detection of *D. septosporum* from DNA extracted from infected needles took longer relative to DNA from cultures regardless of method; average detection time was 28.50 (SD ± 9.42) min although detection took as long as 54 min (Table S2). Examples of positive and negative LAMP reactions are presented in Figure 2.

Table 2. Summary LAMP results based on amplification of template DNA extracted from pure cultures and needles. Cultures—Qiagen indicates results for DNA extracted using the Qiagen method and Cultures—TE indicates the results for DNA extracted using the TE method.

Origin and DNA Extraction Method	No. of Positive Amplifications/No. of True Positive Samples	No. of no Amplifications/No. of True Negative Samples	Average Annealing T °C (±SD)	Time (min) (±SD)
Cultures—Qiagen	40/40	55/55	88.57 (±0.13)	15.82 (±1.15)
Cultures—TE	25/25	3/3	88.62 (±0.06)	22.46 (±3.54)
Needles—DNAzol	27/27	5/5	88.55 (±0.14)	28.50 (±9.42)
All	92/92	63/63	88.58 (±0.12)	21.42 (±7.79)

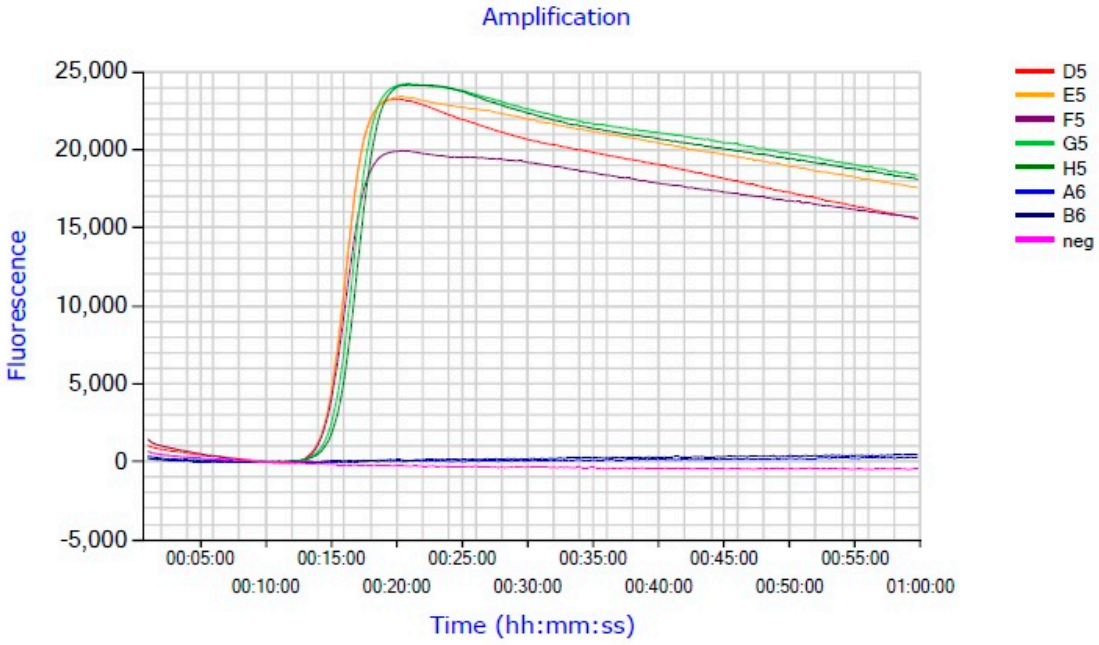

Figure 2. Output from LAMP assay to assess specificity of *D. septosporum* lamp assay. D5 = *D. septosporum* (DSEP-FLNRO-7M-Ne2-TAIGA-591); E5 = *D. septosporum* (DSEP-Whitecourt-Mountain-4077-2-Ne2); F5 = *D. septosporum* (DSEP-EM-Mt-14-Ne1-TAIGA-469); G5 = *D. septosporum* (DSEP-ABTIC-9-Ne1-TAIGA-590); H5 = *D. septosporum* (DSEP-CLG-2-Ne1-TAIGA-614); A6 = *D. pini* (DPIN-Brewer-Lake-Needle1); B6 = *D. pini* (DPIN-Brewer-Lake-Needle2); neg = sterile distilled water.

In sensitivity testing, *D. septosporum* was consistently detected in less than 30 min when at least 2 pg of template DNA was included in the LAMP reaction. The detection limit of our LAMP assay was approximately 1 pg of template DNA (Table 3). Generally, as the concentration of template DNA decreased, the time to detection increased; for example, for 2 pg of template DNA detection took 18–26 min, whereas for 1 ng, detection took 14–16 min (Table 3, Figure 3). The LAMP assay detected the presence of *D. septosporum* within fresh and dried pine needles; however, if needles were damp chambered to induce pathogen sporulation for microscopic examination the LAMP reaction failed.

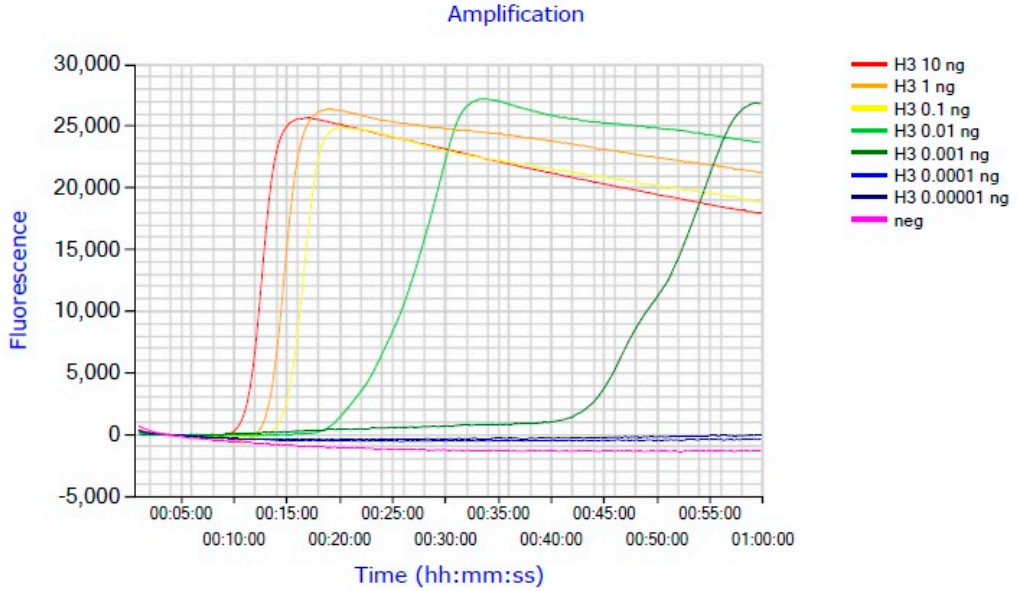

Figure 3. Determining the sensitivity of the LAMP reaction. Isolate H3 = *D. septosporum* (DSEP-Kingfisher-Ne1). Positive results for concentrations from 10 to 0.001 ng/µL. Negative results for 0.0001 ng/µL, 0.00001 ng/µL, and negative control (sterile distilled water).

Table 3. Sensitivity of LAMP assay determined by using serial dilutions of *D. septosporum* DNA.

Sample	ng Template DNA in 15 µL Reaction	Annealing Temp (°C)	Amplification Time (min)
Cherryville Site 2 Tree 2 Needle 2	10	88.67	13.30
	1	88.66	15.45
	0.1	88.73	18.00
	0.01	88.66	25.00
	0.009	88.59	18.00
	0.008	88.54	21.00
	0.007	88.64	21.45
	0.006	88.59	33.15
	0.005	88.57	20.30
	0.004	88.53	23.45
	0.003	88.53	23.45
	0.002	88.54	23.00
	0.001	-*	-
	0.0001	-	-
	0.00001	-	-
Mabel Lake Tree 2 Needle 1	10	88.62	13.30
	1	88.62	15.45
	0.1	88.74	16.15
	0.01	88.71	19.30
	0.009	88.53	20.35
	0.008	88.54	25.29
	0.007	88.57	22.55
	0.006	88.68	23.56
	0.005	88.62	26.31
	0.004	88.64	20.19
	0.003	88.58	20.19
	0.002	88.55	25.13
	0.001	-	-
	0.0001	-	-
	0.00001	-	-
Kingfisher Needle 1	10	88.65	12.30
	1	88.67	14.30
	0.1	88.66	16.15
	0.01	88.68	28.00
	0.009	88.52	22.15
	0.008	88.57	21.30
	0.007	88.59	20.30
	0.006	88.62	24.30
	0.005	88.57	20.45
	0.004	88.57	21.30
	0.003	88.60	22.15
	0.002	88.61	18.30
	0.001	88.46	46.30
	0.0001	-	-
	0.00001	-	-

"-*" indicates no amplification.

4. Discussion

Dothistroma septosporum, one of the causal agents of *Dothistroma* needle blight, is a pathogen of global importance within both natural and plantation pine stands. This pathogen is morphologically similar to the closely related species *D. pini* [15] and DNA-based assays have been developed to differentiate between these two pathogens [5,7]. As historical distribution records in Canada are based on morphology, it will be necessary to use DNA-based methods to fully understand the geographic distribution of these

pathogens. Development of a LAMP diagnostic assay for *D. septosporum* was prompted by the finding that this species was causing *Dothistroma* needle blight in Alberta [3,4].

The LAMP assay reported here is based on the β-tubulin 2 gene, a locus that has previously been shown to distinguish *D. septosporum* from close relatives [5,7,15]. The results of this study further confirm the suitability of this locus as a diagnostic for *D. septosporum*. The sample population of *D. septosporum* assembled for this study was geographically diverse, with samples representing the different geographic subgroups identified by Capron et al. [4], as well as samples from Alaska, California, Oregon, and New Zealand, suggesting that the assay has wide applicability.

Assessment of a dilution series indicated that the LAMP assay is highly sensitive and able to detect as little as 1 pg of pathogen DNA. The LAMP assay is not as sensitive as Langrell's [6] nested PCR assay that targets the ITS region. Schneider et al. [7] also found their ITS-based assay to be more sensitive than the qPCR assay of Ioos et al. [5] that targets β-tubulin 2. These authors suggested that this difference reflects target copy number; β-tubulin 2 is a single-copy gene whereas ITS has a high copy number [7]. The LAMP assay was as sensitive as the duplex-scorpion qPCR assay of Ioos et al. [5], but not as sensitive as their dual-labelled probe qPCR assay [5], despite both targeting β-tubulin 2. Advantages of the LAMP assay include the relatively easy to use equipment and speed of the assay; however, the assays of Ioos et al. [5] and Schneider et al. [7] can simultaneously detect *D. pini* and *D. septosporum* while the LAMP assay only detects *D. septosporum*.

The two methods used to purify DNA from cultures of *D. septosporum* resulted in significantly different time to detection by the LAMP assay; DNA extracted using the Qiagen method amplified more quickly than DNA extracted directly from mycelial plugs using the Tris–EDTA method. The DNA extracted using the Qiagen method is likely to have been associated with fewer impurities as therefore was a better template for amplification. The final concentration of DNA extracted by the Tris-EDTA method was also likely lower than DNA extracted using the Qiagen method. In contrast, there was no significant difference between the two extraction methods in terms of annealing temperature, indicating that extraction method did not influence specificity. Although the assay worked using DNA extracted with both methods, and is very sensitive, these results highlight the importance of using high-quality DNA, especially in situations where the presence of *D. septosporum* may represent a new distribution record.

The time to pathogen detection was shorter when using DNA extracted from cultures than DNA extracted from infected needles. This is likely due to the higher relative concentration of *D. septosporum* DNA in extracts from cultures; DNA from *D. septosporum* makes up all of the DNA extracted from cultures but only a small proportion of the total DNA when extracted from infected needles. The assay was used successfully to assess the presence of *D. septosporum* if needles were fresh or stored dried at room temperature, at 4 °C, or at −20 °C; however, if needles were damp chambered at room temperature to induce pathogen sporulation, the assay became unreliable. In this case, increases in levels of inhibitory compounds, or a reduction in the relative concentration of *D. septosporum* DNA due to the growth of other fungi might explain this result. Regardless, optimal results were obtained with DNA extracted from fresh or properly stored material.

The host range of *Dothistroma* spp. includes 109 taxa within *Pinus*, *Abies*, *Cedrus*, *Larix*, *Picea* and *Pseudotsuga*. Using molecular methods, *D. septosporum* has been confirmed to infect 42 *Pinus* spp., 11 of which have been shown to also be hosts for *D. pini*. Within the 109 identified host taxa, susceptibility to *D. septosporum* ranges from slightly susceptible to highly susceptible [1]. In addition, it has been shown that on *P. radiata*, *D. septosporum* has a hemi-biotrophic life style and is able to infect and colonize host tissue asymptomatically during a biotrophic phase before switching to a necrotrophic phase [17]. If signs and symptoms of *D. septosporum* are identified in a highly susceptible species and in an area where there is an established history of disease, it may not be necessary to confirm *D. septosporum* infection using a genetic assay. However, in slightly susceptible species where morphological identification is difficult, or during a hemi-biotrophic phase prior to

symptom development, or in geographic areas without a history of the disease, the LAMP assay can rapidly provide confirmation of infection. Incorporating a LAMP diagnostic into surveillance could improve disease control as management activities and could be initiated before the pathogen has spread widely.

An advantage of the LAMP approach is that the amplification temperature is constant, making the technology suitable for point-of-care applications. Portable equipment, such as the Genie® III, allows these DNA-based diagnostics to be performed outside of a laboratory setting. Compatible with a point-of-care diagnostic, our assay returns a positive reaction in 30 to 60 min. We are currently optimizing methods for sample preparation in the field to enable tests to be conducted on site.

5. Conclusions

This LAMP assay provides an additional diagnostic assay for the detection of *D. septosporum*. Advantages of this assay include, speed, sensitivity and the potential for field deployment. This pathogen is a research and management focus for many countries [1] and as the number of diagnostic tools increases, it is hoped that management efficacy will also increase, leading to a decline in the effects of this pathogen in stands where pathogen management is necessary to achieve stand management objectives.

Supplementary Materials: The following are available online at https://www.mdpi.com/1999-4907/12/3/362/s1, Table S1. Collection locations and LAMP results for DNA extracted from cultures. Table S2. LAMP results obtained from DNA extracted from needles.

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Molecular-Based Reappraisal of a Historical Record of Dothistroma Needle Blight in the Centre of the Mediterranean Region

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Abstract: In this work, we rechecked, using species-specific Loop mediated isothermal AMPlification (LAMP) diagnostic assays followed by sequencing of fungal isolates at the beta-2-tubulin (tub2) gene region, a historical and never confirmed report of Dothistroma needle blight (DNB) in the introduced Monterey pine (*Pinus radiata* D. Don) in the mountains in the extreme tip of southern Italy. The report dates back to the mid-1970s, and predates the molecular-based taxonomic revision of the genus *Dothistroma* that defined the species accepted today. In the fall of 2019, symptomatic needles of Monterey pine and Corsican pine (*Pinus nigra* subsp. *laricio* (Poir.) Palib. ex Maire) were sampled in the area of the first finding. The applied diagnostic methods revealed the presence of *Dothistroma septosporum* (Dorogin) M. Morelet on both pine species. In this way, we: (i) confirmed the presence of the disease; (ii) clarified the taxonomic identity of the causal agent now occurring at that site; (iii) validated the species-specific LAMP diagnostic protocol we recently developed for *Dothistroma* for use on a portable field instrument, and (iv) showed that the pathogen now also attacks the native *P. nigra* subsp. *laricio*, a species particularly susceptible to the disease, indigenous to the mountains of Calabria, which is one of the very few areas where the species' genetic resources are conserved. Comparative genetic analysis of the rare populations of *D. septosporum* found in the central Mediterranean region and in the native range of *P. nigra* subsp. *laricio* could help to clarify the history of the spread of the pathogen in southern Europe and better evaluate the risk it poses to the conservation of native pine species.

Keywords: *Dothistroma septosporum*; *Mycosphaerella pini*; loop-mediated isothermal amplification; molecular diagnostics; field-portable diagnostics; *Pinus nigra* subsp. *laricio*; forest health protection; forest conservation

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1. Introduction

Dothistroma needle blight (DNB) is a serious foliar disease of pines with a worldwide distribution [1,2]. Causal agents of the disease are two closely related ascomycete fungi, *Dothistroma septosporum* (Dorog.) Morelet (syn. Scirrhia pini Funk & Parker, teleomorph: *Mycosphaerella pini* Rostr.), and *Dothistroma pini* Hullbary (teleomorph:unknown) [3]. These pathogens induce quite similar symptoms consisting initially of water-soaked lesions that later develop into small brown-to-reddish spots. Within these spots, black conidiomata differentiate under favorable conditions. Spots later expand to the whole needle circumference, resulting in transverse bands, hence the alternative name "red band needle blight". Infected needles gradually necrotize and fall [3,4].

Moisture, rainfall and temperature are primary factors in DNB incidence and severity, both in plantations and in nurseries [5]. Stagnation of air and humidity are the most significant factors contributing to the disease at the population scale. The impact of the disease is higher on lower pine branches, especially on those that are shaded and closer to the ground. Pines growing in dense stands are more susceptible than pines growing in more open stands; young pines in open stands, where canopy conditions promote the development of thick understory vegetation, are particularly susceptible; and pines growing inside the stands are always more susceptible than those growing on the margin [6]. On a single tree, infection spreads from the lower branches to the upper parts of the canopy. Under favorable climatic conditions (the optimum is high relative humidity and temperatures around 16–18 °C), the disease can be severe and cause a reduction in photosynthesis, extensive defoliation, stunted growth and mortality. The wide distribution of the disease in large areas of the northern and the southern hemispheres, under varied climatic and ecological conditions, proves the high adaptability of DNB pathogens. This is confirmed by climate models forecasting for the agents of DNB the ability to persist under a range of climates, from sub-arctic to temperate, continental, subtropical and dry tropical regions [7].

Until the 1970s, the disease was known primarily for the severe damage it caused to Monterey pine (*Pinus radiata* D. Don), a fast-growing species that was widely used in southern hemisphere plantations, especially in Tanzania and Kenya, and the causal agent was described as *D. pini* Hulbary [4]. The continued increase in demand for woody material that has driven the growth of *P. radiata* plantations worldwide led to a rapid epidemic and sometimes devastating spread of DNB, which appeared in Chile, New Zealand, and central and southern Africa. At the time, the disease, although present in the northern hemisphere, for instance in British Columbia, California and Oregon, had not had particularly significant epidemic manifestations [4]. However, DNB also appeared in Central France and Southern Europe (Spain, ex-Yugoslavia, Romania), where *Dothistroma* was reported in *P. radiata* and black pine (*Pinus nigra* J.F.Arnold) plantations [8–10].

Additionally, in Italy, in the post-war period, there was an increase in the number of plantations of fast-growing species for wood production, and in particular of *P. radiata* plantations in the central-southern regions. Magnani [11], supported by the opinion of the mycologists de Ana Magan in Spain and Morelet in France, reported the presence of *D. pini* Hulbary in the south of the peninsula. The report was again from an artificial plantation of the non-native *P. radiata*.

This first record of DNB in Italy was in the center of the Mediterranean region, in the southern end of the Apennine mountains (Calabria, southern Italy). The outbreak site, San Pietro di Caridà, is close to the mountain ridge, in an area with a microclimate characterized by high moisture and high annual rainfall [12]. Here, the temperate and humid air masses rising from the Ionian sea and the Tyrrhenian sea, the two seas separated by the small strip of land that is southern Calabria, meet on the ridge, giving rise to turbulence and frequent precipitation.

Because the taxonomy of the genus *Dothistroma* has been decisively revised since the disease was first reported in southern Italy more than forty years ago, and because DNB is now an important foliar disease of pines considered to be emerging globally, in the present study, a survey was carried out in this historical outbreak site in order to: (1) ascertain if DNB was still occurring in the area; and, if this was the case, (2) identify unequivocally and conclusively the causal agent; and finally (3) assess the current host species of the pathogen. A proper identification of the causal agent had both taxonomic and epidemiological relevance, since the disease was originally ascribed to *D. pini* on the basis of micromorphological examination alone [11]. However, later research demonstrated an accurate identification of the agent(s) of DNB to be possible only by means of molecular methods [13,14]. Recently, the differential diagnosis of the pine pathogens *D. pini*, *D. septosporum*, and *Lecanosticta acicola* can be performed more quickly and yet sensitively using specific assays based on loop-mediated isothermal amplification (LAMP) [15]. Considering

the widespread presence of *D. septosporum* in Calabria on *Pinus nigra* subsp. *laricio* in the Sila National Park, located approximately 150 km to the north [16,17], the last objective of the study was related to the concern that, also in this area, the pathogen might have made a host jump from the introduced *P. radiata* to other pine species. In fact, the area falls within the native range of Corsican pine (*Pinus nigra* subsp. *laricio* (Poir.) Maire), which is represented by a few populations distributed between Corsica and Southern Italy [18], in which the genetic resources of the species are conserved [19,20]. *P. nigra* subsp. *laricio* has been found to be particularly susceptible to DNB both in the Sila National Park [17] and in various other localities outside its native range of distribution, for instance in continental France, in the United Kingdom, and in New Zealand, where the species is grown in artificial plantations [21–26].

2. Materials and Methods

2.1. Study Area and Plant Material

Sampling was performed in the fall of 2019 in the Prateria district, in the Municipality of San Pietro di Caridà, Province of Reggio Calabria (Calabria, southern Italy), in which the samples observed by Magnani [11] had also been collected in 1977. The Prateria district, in the Calabrian Apennine, extends between 800 and 1100 m above sea level, in a mountainous area of transition between the southern side of the Serre mountains and the northern side of the Aspromonte massif. Monitoring in search of the old DNB outbreak site focused on the south-facing slopes that drain into the Metramo lake (38°28′ N, 16°13′ E, about 900 m above sea level), an artificial water reserve built at the beginning of this century for irrigation purposes (Figure 1). The vegetation, compared to the descriptions from the 1970s, when there were in the area extensive plantations of *P. radiata* and *P. nigra* subsp. *laricio* cultivated since the middle of the twentieth century for the production of wood, is now very different. Broad-leaved trees prevail, mainly beech (*Fagus sylvatica* L.), which grows in extensive pure high forests and to a lesser extent in coppice forests, covering all the mountains around. There are, however, scattered in the beech forest, nuclei of *P. nigra* subsp. *laricio* and still some remnants of the old plantations of *P. radiata* that have been cut down over time.

Needle samples were collected from adult plants of *P. radiata* and *P. nigra* subsp. *laricio* that showed typical DNB symptoms such as defoliation, reduced growth, and characteristic red bands on the needles, with or without the presence of fruiting bodies attributable to *Dothistroma* on the basis of simple visual inspection with the naked eye. Samples were taken from six plant groups (4 groups of *P. nigra* subsp. *laricio* and 2 groups of *P. radiata*) at least 250 m apart in an area of approximately 4 square kilometers (Figure 1c). Needle samples, bagged in sealed envelopes (two separate envelopes for each pine group and each containing several dozen needles from different pine plants of the same species), were transported to the laboratory and kept refrigerated until further handling.

2.2. Isolation of Fungi

In the laboratory, symptomatic needles that clearly showed deep red bands, orange-red-brown needle apices, and green bases were examined under the stereomicroscope (Optica Lab, Geass, Torino, Italy). In order for fungal fruiting bodies to mature, needles were incubated at room temperature for 5 days in Petri dishes containing moist blotting paper. Conidia were mounted in lactophenol cotton blue and examined under a light microscope (Axiophot, Carl Zeiss) at up to ×40 magnification.

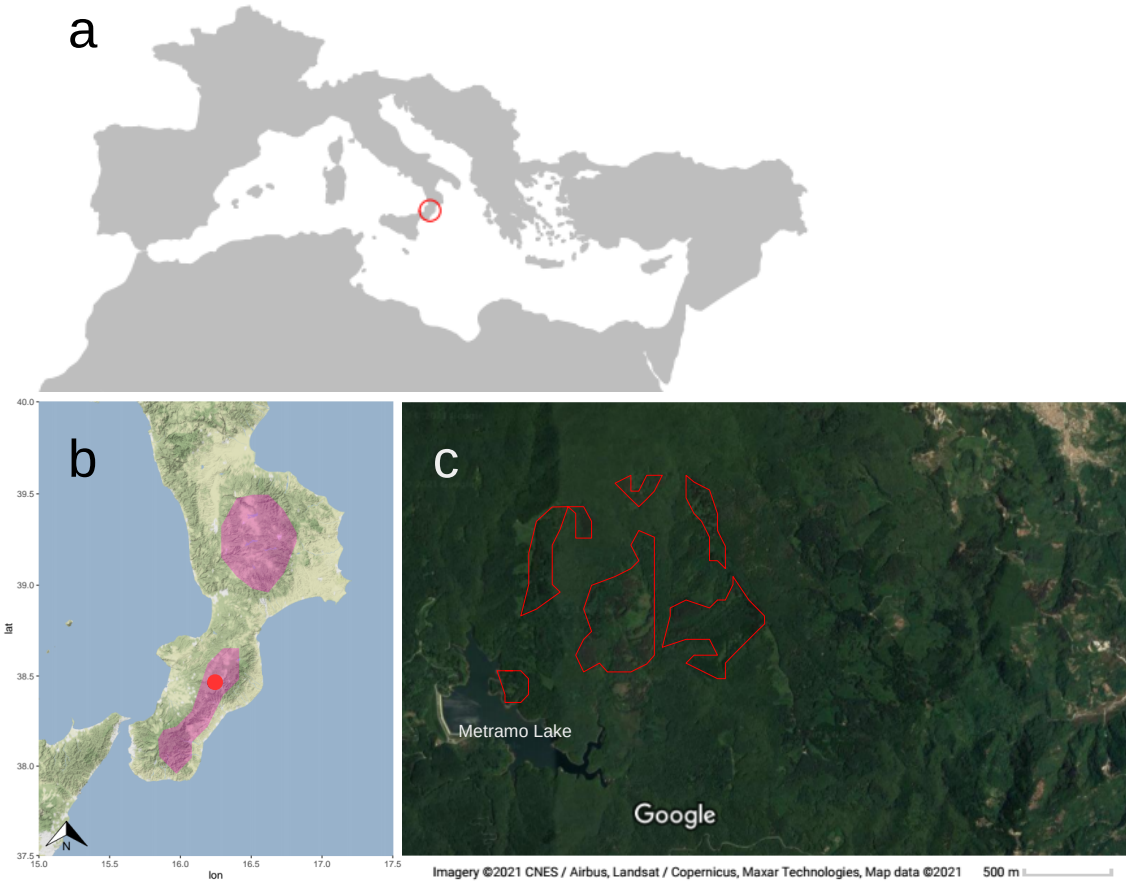

Figure 1. Map position of the study area of Prateria (red symbols) in the center of the Mediterranean region (**a**,**b**) on the south-facing slope of the Serre mountains towards the Metramo Lake (San Pietro di Caridà, Reggio Calabria, Italy) (**c**). The approximate local distribution range of *Pinus nigra* subsp. *laricio* (adapted from the European Forest Genetic Resources Programme, EUFORGEN http://www.euforgen.org/species/pinus-nigra/ (accessed on 3 July 2021)) is shown in magenta coloring (**b**). Maps were drawn using the sf [27] and ggplot2 [28] packages in R [29].

In a laminar flow cabinet, the needles were surface-sterilized by gently wiping with 70% ethanol, and conidiomata were excised from tissues with a fine-pointed scalpel under the stereomicroscope (×10 magnification) and crushed into a drop of sterile distilled water. The dispersion (10 µL) was taken with a pipette and streaked onto a 90 mm Petri dish containing 1.5% Malt Extract Agar (MEA) plus streptomycin (0.050 gL^{-1}). Three streaks (30 µL) were made on each dish. Dishes were incubated in the dark at 20 °C according to species requirements [30].

2.3. DNA Extraction from Mycelia and Pine Needles

The mycelium of axenic cultures was grown on 1.5% MEA plus cellophane into 90 mm Petri dishes and maintained in the dark at 20 °C. After 7–15 days, ca. 70 mg (fresh weight) of mycelium was scraped from the cellophane surface and put in a 2 mL Eppendorf tube. Similarly, fragments (2–5 mm long) of symptomatic *P. radiata* and *P. nigra* subsp. *laricio* pine needles (ca. 70 mg fresh weight obtained by pooling together pieces of different symptomatic needles from the envelope corresponding to a sample) were transferred into 2 mL microcentrifuge tubes (12 samples for DNA extraction in total). Needle samples were frozen at −20 °C overnight and ground for 1 min at 30 Hz with two 3 mm sterile steel beads in a MM400 mixer mill (Retsch GmbH, Haan, Germany).

DNA was extracted from fungal mycelia following the CTAB (cetyl trimethylammonium bromide) protocol described in [31], while DNA was extracted from pine needles using the Nucleospin Plant II kit (Macherey-Nagel, Düren, Germany) following the man-

ufacturer's instructions. Total DNA concentrations were estimated using a NanoDrop® ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Eluted DNA samples were kept at −20 °C until further processing.

2.4. LAMP on DNA Extracts from Needles

Each DNA extract from pine needles was tested by LAMP reaction using the species-specific assays previously optimized for *D. septosporum*, *D. pini* and *L. acicola* [15]. Each run was performed separately on a portable Genie III instrument (OptiGene Limited, Horsham, UK) according to conditions described in [15]. Each sample was tested in duplicate, including in each test, positive controls (containing external target DNA extracts from *D. septosporum*, *D. pini* or *L. acicola* cultures) and two no-template controls (NTC, in which 5 µL of pure water were used instead of DNA), to ensure effective LAMP reaction conditions and verify the absence of contamination.

2.5. Conventional PCR and Sequencing on Mycelial DNA Extracts

DNA from mycelia of four isolates was amplified at the beta-2-tubulin (tub2) gene region using the Btub2Fd/Btub4Rd primer pairs [32]. The expected PCR product size was ca. 431 bp. Each PCR reaction was performed in a final volume of 25 µL using a MB series thermocycler (Cheimika, Italy). Each sample was amplified in duplicate, including in each run no template controls (NTC), in which DNA was substituted by dd-water and positive controls composed by *D. septosporum* DNA. PCR reaction cycle was as follows: a first step of 3 min at 95 °C, 40 cycles composed of (i) 30 s at 95 °C, (ii) 30 s at 54 °C, (iii) 30 s at 72 °C, a final step of 7 min at 72 °C. Each reaction mixture contained 19.25 µL of dd-water, 2.5 µL of 1× DreamTaq Green Buffer (Thermo Fisher Scientific, Waltham, USA), 0.5 µL PCR primer mixture (each at a final concentration of 50 µM), 0.5 µL of 10 mM dNTPs, 0.25 µL of 5 U* μL^{-1} DreamTaq polymerase (Thermo Fisher Scientific, Waltham, USA) and 2 µL of template DNA. For each sample, 5 µL of post-amplification products were visualized by electrophoresis runs in 1% agarose gel (Sigma-Aldrich, St. Louis, MO, USA) using 1× Tris-acetate-EDTA (TAE) as a buffer and ethidium bromide (0.5 µg mL^{-1}) for staining. DNA fragments were purified from the PCR products by using NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel, Düren, Germany), and were sent for Sanger sequencing to StarSEQ® GmbH (Mainz, Germany). Chromatograms were checked and nucleotide sequences trimmed using Unipro EUGENE [33]. Sequences were aligned using T-coffee [34] and alignments visualized with Mview [35] through the EMBL-EBI Job Dispatcher Web Services [36]. Identity searches of consensus sequences were performed with the Blastn tool in the NCBI GenBank database (http://www.ncbi.nlm.nih.gov, accessed on 7 May 2021).

Phylogenetic analyses were performed using a qiime2 version 2021.4 pipeline [37]. De novo multiple sequence alignment was performed with MAFFT version 7 [38] with default settings. Sequences for the outgroup were downloaded from the National Center for Biotechnology Information (NCBI: https://www.ncbi.nlm.nih.gov, accessed on 3 July 2021). Three strains of *Lecanosticta* were included in the analyses (*L. variabilis*, *L. longispora* and *L. acicola*). In order to reduce alignment errors that may introduce noise and confound phylogenetic inference, alignments were masked (i.e., filtered) before phylogenetic analysis, eliminating alignment columns that were phylogenetically uninformative or misleading [39]. Max gap frequency value was set equal to 1, retaining all columns regardless of gap character frequency. The minimum conservation value was set equal to 0.4, retaining a column only if it contains at least one character that is present in at least 40% of the sequences. Phylogenetic analyses were conducted using RAxLM (randomized accelerated maximum likelihood) version 8 [40] with the addition of rapid bootstrapping support values under the gamma model [41]. The tree with maximum likelihood score was selected, setting a seed for the parsimony starting tree equal to 1993, and a seed for rapid bootstrapping equal to 2756. Alignment had 164 distinct alignment patterns, and the proportion of gaps and completely undetermined characters in this alignment corresponded to 20.00%. RAxML rapid bootstrapping and a subsequent maximum likelihood

(ML) search were performed using 1 distinct models/data partitions with joint branch length optimization, executing 1000 rapid bootstrap inferences and thereafter a thorough ML search.

3. Results

3.1. Symptoms, Signs and Conidial Micromorphology

Typical DNB symptoms were present on all needle samples taken in six different groups of pines, two of *P. radiata* and four of *P. nigra* subsp. *laricio*, on the south-facing slopes towards Lake Metramo at elevations ranging from about 900 to 1100 m a.s.l. Trees of both species were defoliated, with transparent crowns and typical lion-tailed branches. These sampling points represent, in the first case, remains of old plantations of the introduced *P. radiata*, and in the second case, naturally regenerated trees of the native *P. nigra* subsp. *laricio*.

Under the stereoscope, necrotic brown spots and black stroma were visible beneath the needle epidermis and typical black conidiomata in the red bands and discolored areas appeared after a few days of incubation in high humidity conditions. Conidia had morphological characteristics consistent with attribution of the species to *D. septosporum* or *D. pini* [13], i.e., they were thin-walled, hyaline, smooth, fusiform to short-clavate, straight or more frequently curved, 2–3(5) septate, and measured 12–40 × 2–3 µm.

3.2. LAMP Assays

DNA extracts from pine needles with typical DNB symptoms, with or without fruiting bodies, all tested positive for *D. septosporum* and negative for both *D. pini* and *L. acicola* by applying LAMP optimized assays. Each sample that tested positive for *D. septosporum* on the portable instrument was amplified in about 20 min with amplification times (tamp), quality of amplification curves and fluorescence (F) (tamp range: 22–23 min, F range: 15,000–30,000) comparable to those obtained on the real time PCR instrument used during the optimization of the assay [15]. No amplification curves were obtained from negative samples. All positive controls were amplified (*D. septosporum* tamp 20–22 min, F 10,000–30,000; *D pini* tamp 15–22 min, F 20,000–25,000; *L. acicola* tamp 21–23 min, F 15,000–25,000), confirming the effectiveness of each run. An example of the reaction kinetics is given in Figure 2.

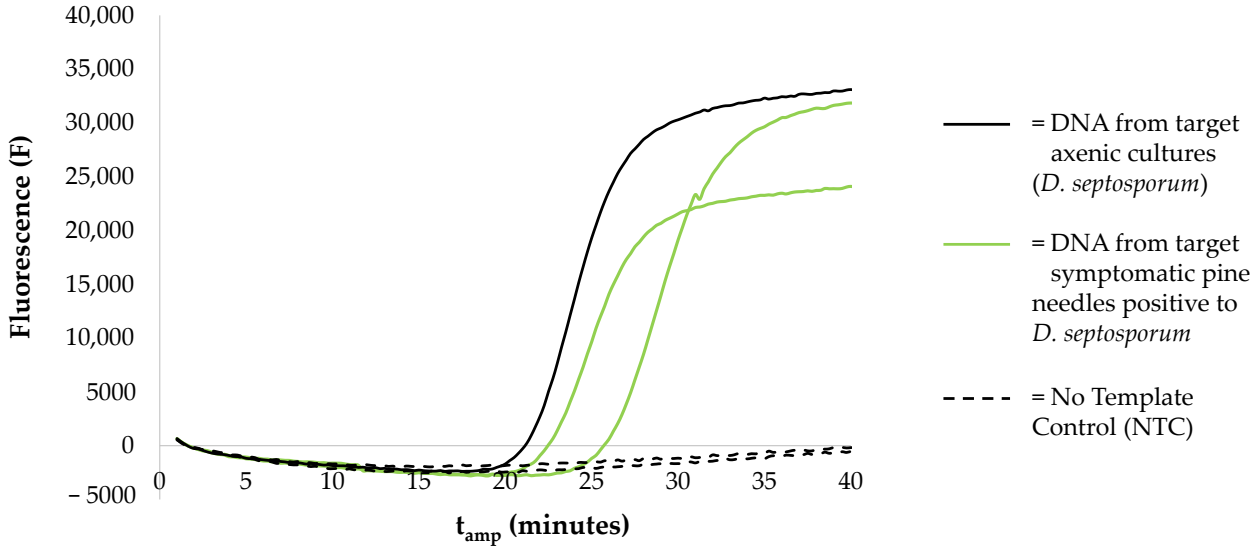

Figure 2. Selection of kinetics showing amplification results of the LAMP assay for the detection of *D. septosporum* [15] on a portable instrument (Genie III OptiGene, Limited, Horsham, UK).

3.3. Isolation of Fungi and Identification by Sequencing

Numerous dark brown or blackish colonies resembling those of *Dothistroma* species were obtained from conidia from all the groups of sampled pines. Colonies grew slowly, as is typical of *Dothistroma*, and stained the substrate red for dothistromin production after 1–2 weeks of growth. Mycelial plugs from 15 axenic colonies were cut from the agar and placed into microeppendorf tubes (1.5 mL), covered with sterile distilled water and stored at 4 °C.

Partial sequences of the tub2 gene region were obtained from four isolates from *Pinus nigra* subsp. *laricio*. Amplicon size was ca. 431 bp as expected. After quality check, trimming and alignment, sequences comprised 297–372 base pairs due to poor quality sequences requiring more trimming in some cases. Blast alignment in the GeneBank database showed that all four fungal isolates could be classified as *D. septosporum* (99.35%–100% sequence identity, best hit *D. septosporum* strain CMW44656). Sequences were deposited in GenBank under accession numbers (MZ594908-MZ594911).

Dothistroma spp. included in phylogenetic analyses formed a cluster independent from *Lecanosticta* spp., which were selected as an outgroup (Figure 3). Sequences from the study site (RCDS001, RCDS003, RCDS007, RCDS009) were placed inside the *Dothistroma septosporum* clade, supporting the conclusion that these isolates belong to this species.

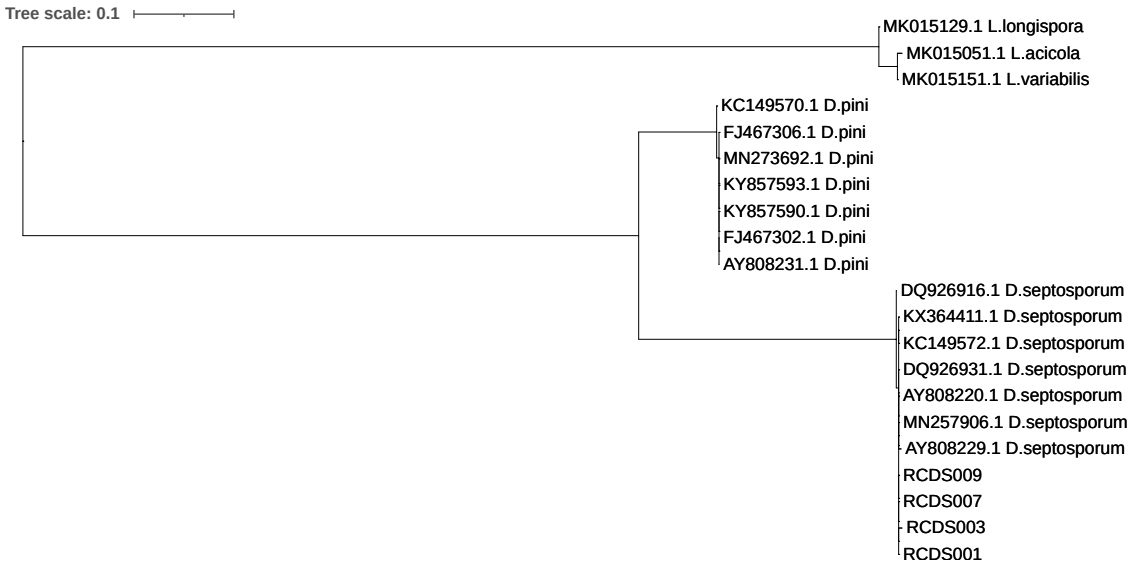

Figure 3. Phylogenetic tree with maximum likelihood score (branch support computed out of 1000 rapid bootstrapped trees) based on partial tub2 gene sequences of isolates RCDSS001, RCDS003, RCDS007, RCDS009 from *Pinus nigra* subsp. *laricio* in Prateria (San Pietro di Caridà, Reggio Calabria, Italy) and of *Dothistroma* species isolates retrieved from GenBank.

4. Discussion

Since the first report of DNB in the mountains of southern Calabria [11], the cultivation of *P. radiata* has been almost abandoned in southern Italy [42], and today, forty-four years later, in the area of that first report, only rather small nuclei of pine, especially the native *P. nigra* subsp. *laricio*, are found scattered in vast pure beech forests. Now that molecular techniques enable us to discriminate among morphologically indistinguishable species and also to recognize cryptic pathogens, we were able to test the authenticity of the record of Magnani [11], conducting our investigations at exactly the same site, and thus confirm that his observations, never verified later, were correct. In fact, the major revision of the genus *Dothistroma*, the name of the species of which changed [13], came long after the publication of Magnani's report. In this study, we can now ascertain that, about half a century after the establishment of *P. radiata* plantations, *Dothistroma* is still present in the area, although trees with clear symptoms of the disease are scattered or in small, isolated groups physically separated from each other by non-host species.

In confirming the persistence of the pathogen in the area, we demonstrated that the recently published LAMP diagnostic assays discriminating the infections by DNB agents and *L. acicola* in pine needles [15], are efficiently applicable on the portable instrument (Genie III Optigene, UK). This result encourages their prospective use directly in the field, after optimization of a suitable crude extraction protocol that is under development. Application of LAMP diagnostics and sequencing in the tub2 gene region confirmed the pathogen found today in the study area to be the species currently identified as *D. septosporum*. Given the peculiar climatic characteristics of the area due to the orography, the position between two seas and also the additional presence of the artificial lake Metramo, which create conditions of higher atmospheric humidity than in the surrounding areas and favor the survival and spread of the pathogen, it is reasonable to assume that the population detected today likely descends from the one found in 1977, although the data do not allow us to confirm this assertion with certainty.

Due to changes in the politics of afforestation as well as in forest management strategies over the past 50 years, the silvicultural importance of *P. radiata* in the area seems today to be greatly reduced compared to the past, but the danger posed by *D. septosporum* to native pines remains serious. In fact, in the area included in this work, as in the Sila Massif [15] (which is also located in Calabria about 150 km north of the present finding), the pathogen was found on the native species *P. nigra* subsp. *laricio*. The few natural populations that still exist of this native species of pine are preserved today in Calabria and Sicily, in addition to the few present on the French island of Corsica. The presence of the pathogen therefore represents a threat to the genetic resources of the species.

Outside the Italian territory, reports based on molecular diagnosis of *D. septosporum* in the central southern Mediterranean area (i.e., Spain, Greece, Turkey) are isolated and sporadic and generally from pine plantations [2,43], with the exception of findings of the fungus in natural forests in Montenegro and southwestern Turkey [44–48]. The scientific debate on the geographic origin of *D. septosporum* is long and not without controversy [13,49–52], partly due to the lack of sufficient sampling of the fungus in some regions of its current range. Recently, a large body of work using microsatellite markers for genotyping an unprecedented number of samples collected from across the worldwide range of *D. septosporum* has indicated Eastern Europe (i.e., the Baltics and Western Russia) and Western Asia as the possible area of origin of the fungus, and hypothesized a possible derivation of Turkish populations from ancestral populations through an ancient separation dating prior to the colonization of Central Europe [53]. In the study by Mullet and colleagues [53], not enough isolates of the fungus from the outbreak in the Sila Massif were included to attempt reconstructing the evolutionary history of that population, while the finding of *D. septosporum* in the areas of the present study was yet to be confirmed. Whether or not *D. septosporum* is indigenous to these areas is currently unknown.

The high susceptibility of *P. nigra* subsp. *laricio* at all sites where it was planted outside its native range and came into contact with *D. septosporum* suggests a lack of coevolution with the pathogen. Similarly, the severe damage observed on this pine in its native range in the Sila Massif [17] also points to a recent introduction, although other possible explanations, e.g., an environment made more conducive to disease by climate change, cannot be excluded [54]. However, it is reasonable to assume that the populations of *D. septosporum* recently found on this pine species in its native range in Southern Italy might be recent introductions. In Corsica, to the best of our knowledge, there is only one published record of DNB, i.e., a single isolate of *D. septosporum* from *P. nigra* subsp. *laricio* included in [53], which has not been transposed yet into the EPPO database (https://gd.eppo.int/taxon/SCIRPI/distribution, accessed on 19 July 2021). According to EPPO, the pathogen was absent from Corsica at least until 2011, when a monitoring campaign was conducted, and no subsequent updates have been reported. Therefore, the finding in Corsica could also follow a recent introduction. No records exist on the occurrence of the pathogen in Sicily, where natural populations of *P. nigra* subsp. *laricio* are also present. As a prospect for a future study, a comparative genetic analysis of the

Calabrian populations of the fungus could help to define in more detail the history of the pathogen's spread in Southern Europe.

5. Conclusions

DNA-based approaches are increasingly used to support and extend more classical taxonomic relationships, as well as to unravel complicated taxonomic histories and circumscript species. Molecular identification as *D. septosporum* of the fungus initially identified as *D. pini* on the basis of micromorphological characterization alone in the first reported Italian outbreak of the disease is a vivid example. The development of a rapid and highly discriminating diagnostic tool such as the LAMP technique will allow us to identify the agents of DNB in the Mediterranean area more accurately and more specifically. Further genetic analysis could also provide a more complete picture of the current distribution of the causal agents of red band needle blight in the region and insights into the origin of the disease.

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Data Availability Statement: The sequence data presented in this study were deposited in the NCBI GenBank data repository.

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Effects of Temperature on *Anoplophora chinensis* (Coleoptera: Cerambycidae) Adult Survival, Reproduction, and Egg Hatch

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Abstract: *Anoplophora chinensis* (Forster) is an invasive species that can damage many tree species in orchard, urban, and forested habitats. Adult survival, reproduction, and egg hatch of *A. chinensis* from Italy and China are evaluated at eight constant temperatures (5, 10, 15, 20, 25, 30, 35, and 40 °C) under laboratory conditions. The estimated T_{max} for longevity was 42 and 33 °C for females and 42 and 39 °C for males from China and Italy, respectively. The estimated T_{max}, T_{min}, and optimum temperature for fecundity were 35, 9, and 29 °C, respectively. Females laid eggs at 15–30 °C and eggs hatched at 15–35 °C. Days to first oviposition increased exponentially from 13 days at 30 °C to >300 days near 10 °C. The estimated T_{min} for egg hatch was 13 °C, the T_{max} at 38 °C, and the optimum 29 °C. Percentage hatch was estimated to be highest at 26 °C and have a T_{max} of 31 °C and T_{min} of 10 °C. These results indicate that summer temperatures over a wide range of latitudes should support beetle survival and reproduction, but at temperatures ≥35 °C, oviposition ceases, and adult survivorship declines. In addition, females may survive into the fall, but lay fewer eggs that may not hatch. These responses of *A. chinensis* to temperature can be used for developing phenological models to predict the timing of stages for management or eradication efforts.

Keywords: *Anoplophora chinensis*; temperature; survival; reproduction; fecundity

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1. Introduction

Temperature is an important abiotic factor that influences insect life-history processes, such as survival, development, and reproduction. Understanding how insects respond to temperature makes it possible to estimate potential geographic ranges and develop phenology models to predict the timing of stages that are critical to deploying survey and management options. It is particularly important to develop these predictive tools for species like *Anoplophora chinensis* (Forster) (Coleoptera: Cerambycidae) that become invasive after being transported, either in live plants or solid wood packaging materials, through international trade routes. The native range of *A. chinensis* largely overlaps that of its congeneric, *Anoplophora glabripennis* (Motschulsky), but it has been recorded from warmer areas of southern Asia and is not found in some of the more northern parts of China and far east Russia [1]. *Anoplophora chinensis* is widely distributed in China, Japan, and Korea, and occasionally found in Vietnam, Malaysia, Indonesia, and the Philippines [2,3]. It is a polyphagous xylophage that attacks 73 different deciduous tree genera (more than *A. glabripennis*) and also is reported to attack some coniferous trees in the genera *Cryptomena* and *Pinus* [4]. The beetle is the most important wood infesting citrus pest in Japan and China, and also causes major damage to *Casuarina equisetifolia* L. shelterbelts in China [5–7]. In its invaded range, *A. chinensis* prefers trees in the genera *Acer*, *Betula*, and *Corylus* but has completed development on 24 genera and partial development on another five genera of trees [3,8]. Females lay their eggs in sites they chew in the bark of the lower bole or exposed roots of the tree, and the early instars feed under the bark, while later instars feed in the xylem [3]. The larvae cause damage to the vascular system of the tree and create structural

weaknesses that can lead to the death of the tree. Adults are present from the late spring through the summer and into the early fall, depending on the local climatic conditions.

A recent revision of *Anoplophora* considered *Anoplophora malasiaca* (Thomson) to be a synonym of *A. chinensis* based on shared morphological traits of the reproductive system [2]. There is, however, still controversy over this synonymy, since they can be differentiated by whether or not two white spots are present on the pronotum and by their mitochondrial COI region haplotypes [9,10]. The first invasive population of *A. chinensis* was found in the Lombardy region of Italy in 2000, but in the last 20 years, additional infestations have been found in Italy, France, the Netherlands, Croatia, and Turkey [11]. The infestations in the Netherlands and Croatia have been traced to live *Acer palmatum* Thunberg imported from China, while the one in France started with beetles that emerged from bonsais imported from Asia [11,12]. A find outside a greenhouse in Tukwila, Washington, United States in 2001 also came from imported bonsais [13]. Using the mitochondrial DNA haplotypes, the Italian infestations from the Lazio and Lombardy regions were found to be similar to Japanese populations, while those from Tuscany were closer to Chinese populations [14]. Therefore, the invasive populations may originate from areas where the two original species are found and could be different biologically in their responses to temperature and other traits. Since all invasive infestations or potential ones (Washington State, US) have resulted in the establishment of eradication efforts, it is important to know if there are differences between these original species. For simplicity, *A. malasiaca* will be referred to as "*A. chinensis* from Japan".

There is some information on the temperature effects on the life-history of *A. chinensis* adults and eggs. At natural temperatures on citrus (twigs for food and bolts for oviposition) adults from a Japanese population of *A. chinensis* survived 77.6 $\pm$ 20.3 days, maturation fed for 9.8 $\pm$ 1.4 days, and females laid 193.8 $\pm$ 65.2 eggs (peaked at 30 days post eclosion and 90% of the eggs hatched) [7]. Another study using Japanese adults found differences in maturation feeding (feeding required to reach sexual maturity), longevity, and fecundity at 24 °C when different hosts were used [15]. Fecundity ranged from 60–140 eggs when another group of Japanese females was offered *Acer*, *Citrus*, and *Platanus* bolts to lay on in outdoor cages [10]. When Chinese *A. chinensis* females were fed on *Melia azedarach* L., on average, they laid 176 eggs and lived 92 days (males 103 days), but they only laid 56 eggs and lived 62 days (males 63 days) when fed *Casuarina* sp. [1]. Finally, it was estimated that a total of 184 degree-days is required for 50% of Japanese *A. chinensis* eggs to hatch when using the estimated T_{min} of 6.7 °C [7].

In this study, we determined the response of *A. chinensis* adults and eggs to a range of constant temperatures. Then the mathematical relationships between temperature and adult survival, reproduction, and egg hatch were developed for *A. chinensis* to facilitate their use in developing phenology models and to compare them to what has been previously reported for *A. glabripennis*. Finally, the implications for management and eradication programs are discussed.

2. Materials and Methods

2.1. Populations and Temperatures

The populations used in this study were established from larvae that were shipped on 26 February 2018, from the USDA Agricultural Research Service (ARS), European Biological Control Laboratory in Montferrier-sur-Lez, France, under permit to the USDA Forest Service quarantine facility in Ansonia, CT. Fifty larvae from the fifteenth ARS laboratory generation of an invasive *A. chinensis* population from Lombardy, Italy (41.58° N and 87.42° W) were used to start the USDA Forest Service (FS) colony. The Italian colony was initiated by ARS in 2002 and augmented each year with freshly collected adults. The Italian colony was determined to have originated from Japan based on molecular tests. Individuals used in this study were from the sixteenth or seventeenth Italian laboratory generations.

Fifty larvae from the fifth ARS generation of a native *A. chinensis* population from Yancun, Guangdong Province, China colony (40.45° N and 73.45° W) were used to start

the FS colony. The Chinese population was started by ARS in 2012 with 180 larvae from China. Individuals used in this study were from the sixth or seventh laboratory generations. Voucher specimens of both populations were deposited at the Entomology Division, Yale Peabody Museum of Natural History, New Haven, CT.

Eight constant temperatures were used for these studies: 5, 10, 15, 20, 25, 30, 35, and 40 °C. Temperature fluctuations in the environmental chambers rarely exceeded 1 degree above or below the set value, and a photoperiod of 16:8 (L:D) h was used. Humidity was passively maintained by placing open reservoirs of water ($32 \times 28 \times 14$ cm^3) in the bottom of the chambers or by humidifiers that were part of the chamber (15, 20 and 25 °C). The humidity in the chambers averaged 80 ± 10%, 85 ± 15%, 70 ± 10%, 80 ± 5%, 60 ± 5%, 45 ± 5%, 68 ± 4%, and 55 ± 10% RH at 5, 10, 15, 20, 25, 30, 35, and 40 °C, respectively. Actual humidity within the rearing containers was likely higher than in the chamber, due to the presence of fresh host material.

2.2. Mating Adults and Obtaining Eggs

Adults used in these studies came from larvae reared on an artificial diet, either the *A. glabripennis* diet designated "AG2" [16] or a drier *A. chinensis* diet that was a modification of a host-free diet developed for *A. glabripennis* [17,18]. Pre-pupae were placed in 50 mL plastic centrifuge tubes (with two pinholes in the lid and a piece of damp paper towel) and held in the dark at 25 °C until pupae formed, and the adults emerged. Callow adults were held for four more days under the same conditions as the pre-pupae/pupae to allow the cuticle to fully sclerotize before they were sexed, weighed, and assigned to a temperature regime. Chinese adults (15 females and 16–18 males) were initially assigned evenly to the 15, 20, 25, and 30 °C temperature regimes, and then ten of each sex were assigned evenly to the 10, 35, and 40 °C temperature regimes. Virgin females in the 15–35 °C temperature regimes were held individually in 3.8-L glass jars with *Acer rubrum* L. (red maple) twigs (3–7 mm diameter with leaves removed) as a food source and *A. rubrum* bolts (3–7 cm diameter and 22 cm long) with both ends waxed as an oviposition substrate. A virgin male was added to the female's jar 0–11 days later (i.e., as soon as a female became available at the same temperature). Males that were waiting for a mate were held at their assigned temperatures in 950 mL glass jars with fresh twigs as a food source. All adults held at 10 and 40 °C were held individually in 950 mL glass jars with fresh twigs as a food source. After twelve weeks at 10 °C (presumably enough time for the females to maturation feed), the three males that were still alive were placed with females in the larger glass jars to see if the females would chew pits or lay eggs. Twigs and bolts were cut twice a month and were stored at 4 °C and ≥80% RH until used in the weekly food and bolt changes. Folded paper towels were placed in the bottoms of the jars to collect frass and excess moisture. Two holes (1–2 mm diameter) in the plastic jar lids allowed airflow.

The oviposition bolts were replaced twice a week (30–35 °C), weekly (15–25 °C), or monthly (10 °C) until a female died. Oviposition bolts held at 15–35 °C were checked daily for oviposition sites. The date of each new oviposition pit chewed in the bark was recorded, and the pit marked to distinguish the day it appeared. When the oviposition bolts were removed from the jars, all pits chewed in the bark, including both pits that obviously contained eggs and those that did not, were counted, and the bolt diameter was measured.

The bark was stripped off many of the daily marked bolts the day they were removed from the jar to obtain the eggs for the egg hatch part of the study. After the bark was stripped off, the eggs were carefully removed from the bolt by lifting a thin strip of wood with the egg on it to prevent breaking the eggshell. Eggs attached to the small pieces of wood were placed individually in wells of a 24-well tissue culture plate that was held in a water box at the temperature at which the egg was laid or to which the egg was moved. Ten eggs maximum, or half of the eggs at minimum, that were laid by females at 15 °C were kept at that temperature. For females held at other temperatures, a minimum of 10 eggs was held at the oviposition temperature, and the others were moved as needed.

To minimize temperature shifts for eggs that had already begun developing, one-day-old eggs were only moved to temperatures that were ±5 °C of the laying temperature, but eggs laid the same day were moved to other temperatures as needed. Eggs laid more than one day before the bolt was removed from the mating jar were held at the oviposition temperature. Eggs laid by Chinese females held at 25 or 30 °C were moved to any temperature, while eggs laid at 15 °C were only moved to 20–30 °C and eggs laid at 20 °C were only moved to 10–35 °C. This was because there were fewer eggs laid at the cooler temperatures. Two to ten eggs from a Chinese female were held at each temperature 15–30 °C, and 1–3 eggs were held at each temperature 5–10 °C and 35–40 °C.

Once enough eggs were obtained from each mating pair for the egg hatch part of the study, the rest of the bolts from each female were held at 25 or 30 °C for 7–10 days to allow the eggs to hatch before stripping the bark off the bolt. This provided a better measure of the percentage of the eggs that were fertile, since removing the eggs could damage or kill them. Eggs were checked daily for 4, 4, 4, 6, 9, 12, 30, and 30 weeks at 40, 35, 30, 25, 20, 15, 10, and 5 °C, respectively. These time periods were chosen to ensure that all eggs that could hatch at a given temperature did. Eggs that had not hatched after the given time period at 5–20 °C were moved to 25 °C and observed for an additional four weeks.

Adults from the invasive Italian population were more limited in numbers, so only 8–10 females and 11–14 males were assigned on a rotating basis to the 15, 20, 25, and 30 °C temperature regimes. All the same feeding and holding methods were used as detailed above for the Chinese population adults and eggs. Since Italian females also laid fewer eggs, their eggs were only held at 15–30 °C. Eggs laid at these temperatures were moved using the same scheme as detailed for the Chinese eggs.

2.3. Adult Survival and Reproduction

Mating pairs and single adults were checked daily for mortality, with the only exception that single adults at 10 °C were checked three times a week. When the female in the pair died, the male was moved to a 950 mL glass jar and maintained until death. When the male in the pair died, the female was maintained in the mating jar until death. For each temperature at which females laid eggs, the mean number of eggs laid per week per live female during that week was calculated. When the female died, she was dissected to determine if she had any fully formed eggs in her ovarioles that she had not oviposited. The following dependent variables were analyzed in PROC GLIMMIX [19]: Female and male weight at four days, female and male longevity, days to the first egg, fecundity, maximum weekly oviposition, a week at which maximum oviposition occurred, mean weekly oviposition pits per female, mature unlaid eggs at female death, and percentage hatch on bolts held at 25–30 °C. A gamma distribution with a log link was used for all the variables except the percentage hatch for which a beta distribution with a logit link was used and the unlaid eggs in the female at death for which a Poisson distribution with a log link was used. The two populations were analyzed separately for each variable. The model used temperature, population (Italy or China), and the interaction between the two as fixed effects. Differences among means were determined using the Tukey–Kramer test with $\alpha = 0.05$ [19].

The relationship between longevity and temperature was separately fit for each sex and population combination to a linear model ($y = bx + a$), and the upper threshold (T_{max}) was calculated to be the point at which the line crossed the x-axis. The relationship between fecundity and temperature for the Chinese population was fit using the following:

$$y = \begin{cases} at(t - Tmax)(Tmin - t)^{\frac{1}{b}} \text{ for } Tmax \leq t \leq Tmin \\ \text{otherwise } 0 \end{cases} \quad (1)$$

where y = fecundity, t = temperature °C are the known values, and the others are estimated. The a is an empirical constant, b modulates the slopes, and T_{max} and T_{min} are the upper and lower temperature thresholds, respectively [20]. Nonlinear convergence for the model

was based on the Marquardt method (PROC NLIN [19]). The optimum temperature for Equation (1) was calculated by equating the first derivative to zero and solving for t.

The relationship between temperature (t) and a number of days from female emergence to first oviposition was fit using the following:

$$y = at^b \quad (2)$$

where a and b are empirical constants, and nonlinear convergence was based on the Marquardt method (PROC NLIN [19]). The percentage of females that oviposited and produced progeny was also calculated for each temperature and population combination.

2.4. Egg Hatch

Two dependent variables were analyzed using PROC GLIMMIX [19]: Days to hatch and percent hatch on bolts held at 25–30 °C. The model treated temperature, population, and temperature by population as fixed effects, whereas maternal family was treated as a random effect. Differences among means were determined using the Tukey–Kramer test with $\alpha = 0.05$ [19]. A gamma distribution with a log link was used for days to hatch, and a beta distribution with a logit link was used for the percentage hatch on bolts. The percentage hatch of eggs laid at or moved to various temperatures after being removed from under the bark was also calculated.

To describe the relationship between temperature and rate of hatch for eggs from the combined populations, Equation (1) was altered by changing $y = 1/\text{days to hatch}$, and the curve was fit using the Marquardt convergence method (PROC NLIN [19]). The cumulative distributions of time to egg hatch at 15, 20, 25, and 30 °C and degree days to egg hatch for all eggs were calculated using the estimated T_{min} from Equation (1). The cumulative proportion of eggs hatching (P) over accumulated degree-days (DD) was described using a Gompertz function:

$$P = \exp(-\exp(-bDD + a)) \quad (3)$$

in which a and b are the lag and the rate of increase, respectively ([21], PROC NLIN and Marquardt convergence method, [19]).

3. Results

3.1. Adult Survival and Reproduction

At the initiation of the temperature treatments, adult females from China (186.5 ± 28.9 mg) were significantly heavier than females from Italy (145.3 ± 25.0 mg) ($F = 39.96$, df = 1, 93, $p < 0.0001$). There was also a significant difference ($F = 40.21$, df = 1, 139, $p < 0.0001$) in the average mass of males from the Chinese population (127.5 ± 22.8 mg) and the Italian population (105.1 ± 21.0 mg). The mass of the beetles held at the different temperatures for either sex did not differ (females: $F = 0.46$, df = 6, 93, $p = 0.8351$; males: $F = 1.17$, df = 6, 139, $p = 0.3274$).

Both male and female longevity increased with decreasing temperature and Italian individuals of both sexes tended to survive a shorter time than Chinese individuals (Table 1). Temperature and population both had significant effects on both female (temperature: $F = 126.6$, df = 6, 113, $p < 0.0001$; population: $F = 39.3$, df = 1, 113, $p < 0.0001$) and male longevity (temperature: $F = 127.8$, df = 6, 139, $p < 0.0001$; population: $F = 60.2$, df = 1, 139, $p < 0.0001$), but the interaction between the two did not (Table 1). Female and male longevity fit a linear model, but variation increased at the lower temperatures so that the confidence of the fit (adjusted R^2) was lower, especially for the Italian population (Figure 1, Table 2). At 10 °C the Chinese population appeared to have two biotypes, one that was dominated by males that survived about 100 days and another that was dominated by females that survived about 375 days. The estimated T_{min} for each sex and population combination for longevity and the parameters for the fitted models are given in Table 2.

Table 1. Effects of temperature on *Anoplophora chinensis* adult survival and reproduction ((mean ± SE (n) or percentage (n)). Means were compared between each temperature and population combination for each parameter.

		Temperature (°C)							
Parameter	**Population**	**10**	**15**	**20**	**25**	**30**	**35**	**40**	**Statistics Population * Temperature**
Female Longevity (d)	China	272.7 ± 35.6a (10)	205.9 ± 21.9ab (15)	116.1 ± 12.4c (15)	83.0 ± 8.8cd (15)	66.0 ± 7.0de (15)	21.6 ± 2.8f (10)	5.1 ± 0.7g (10)	F = 0.3681, df = 3, 113 p = 0.4928
	Italy	*NA*	141.5 ± 20.6bc (8)	55.0 ± 8.0de (8)	44.6 ± 5.8e (10)	40.6 ± 5.9de (8)	*NA*	*NA*	
Male Longevity (d)	China	101.1 ± 12.2bc (10)	201.1 ± 19.2a (16)	109 ± 10.1b (17)	102.6 ± 9.2b (18)	62.8 ± 5.7cd (18)	21.3 ± 2.6e (10)	5.1 ± 0.6f (10)	F = 0.91, df = 3, 139 p = 0.4358
	Italy	*NA*	97.7 ± 10.4bc (13)	68.0 ± 7.8bcd (11)	57.2 ± 6.1d (13)	41.4 ± 4.2d (14)	*NA*	*NA*	
Fecundity	China	0.0 (10)	40.1 ± 8.6abcd (15)	76 ± 16.3cd (15)	92.2 ± 19.7abc (15)	118.0 ± 30.9a (15)	0.0 (10)	0.0 (10)	F = 0.49, df = 3, 69 p = 0.6895
	Italy	*NA*	15.8 ± 5.4d (8)	29.8 ± 11cd (8)	24.0 ± 8.9bcd (10)	26.0 ± 8.8ab (8)	*NA*	*NA*	
Max Oviposition/Wk	China	*NA*	6.8 ± 1c (15)	13.2 ± 1.9ab (15)	18.7 ± 2.6ab (15)	26.3 ± 4.6a (15)	*NA*	*NA*	F = 2.01, df = 3, 69 p = 0.1210
	Italy	*NA*	5.3 ± 1.2c (8)	8.2 ± 2bc (8)	7.8 ± 1.9bc (10)	8.7 ± 1.9bc (8)	*NA*	*NA*	
Week Max Oviposition Occurred	China	*NA*	10.1 ± 0.8a (15)	5.8 ± 0.4bc (15)	4.8 ± 0.4c (15)	4.2 ± 0.4cd (15)	*NA*	*NA*	F = 2.04, df = 3, 69 p = 0.1164
	Italy	*NA*	8 ± 0.9ab (8)	2.8 ± 0.4d (8)	2.8 ± 0.4d (10)	2.7 ± 0.3d (8)	*NA*	*NA*	
Time First Egg Laid (d)	China	*NA*	54.3 ± 3.3a (15)	23.9 ± 1.4b (15)	14.7 ± 0.9cd (15)	14.2 ± 1.1cd (15)	*NA*	*NA*	F = 5.26, df = 3, 69 p = 0.0025
	Italy	*NA*	39.2 ± 3.7a (8)	20.0 ± 2.1bc (8)	20.0 ± 2.1bc (10)	12.8 ± 1.2d (8)	*NA*	*NA*	
Mature Eggs in Female at Death	China	0.00 ± 0.00ab (10)	0.93 ± 0.25a (15)	1.73 ± 0.33ab (15)	1.67 ± 0.33ab (15)	2.67 ± 0.41ab (15)	0.10 ± 0.10b (10)	0.00 ± 0.00ab (10)	F = 3.81, df = 3, 113 p = 0.0121
	Italy	*NA*	2.25 ± 0.57a (8)	2.38 ± 0.55a (8)	2.40 ± 0.50a (10)	1.25 ± 0.38a (8)	NA	*NA*	
Weekly Oviposition Pits Chewed/Female	China	*NA*	13.9 ± 1.5bc (15)	27.7 ± 3a (15)	38.8 ± 4.2a (15)	46.4 ± 6.2a (15)	24.1 ± 4.5 ab (10)	*NA*	F = 2.42, df = 3, 80 p = 0.0718
	Italy	*NA*	8.4 ± 1.3c (8)	12.4 ± 2bc (8)	15.3 ± 2.1bc (10)	36.6 ± 6.3a (8)	*NA*	*NA*	
% Hatch on Bolts Held at 25–30 °C	China	*NA*	24.4 ± 7.1b (15)	59.6 ± 8.1ab (15)	77 ± 6.8a (15)	40.4 ± 11.8ab (15)	*NA*	*NA*	F = 2.28, df = 3, 46 p = 0.0921
	Italy	*NA*	12 ± 7b (7)	88 ± 11.9ab (5)	36 ± 14.8ab (6)	50 ± 23ab (7)	*NA*	*NA*	

Table 1. *Cont.*

Parameter	Population	Temperature (°C)							Statistics Population * Temperature
		10	15	20	25	30	35	40	
% Females Laying Eggs	China	0 (10)	100 (15)	100 (15)	100 (15)	100 (15)	0 (10)	0 (10)	$F = 0.49$, df = 3, 69 $p = 0.6905$
	Italy	*NA*	87.5 (8)	62.5 (8)	60 (10)	87.5 (8)	*NA*	*NA*	
% Females with Progeny	China	0 (10)	60 (15)	100 (15)	100 (15)	100 (15)	0 (10)	0 (10)	$F = 0.49$, df = 3, 69 $p = 0.6907$
	Italy	*NA*	25 (8)	50 (8)	50 (10)	37.5 (8)	*NA*	*NA*	

With rows, means followed by the same letter are not significantly different based on the Tukey–Kramer test with $\alpha = 0.05$. *NA* = parameter not applicable either because no females were held, or no eggs were laid at these temperatures.

Table 2. Parameter values for linear and non-linear models were used to describe the relationships between temperature and selected life-history values for *Anoplophora chinensis*.

Response Variable	n	T_{Max}	T_{Min}	a	b	F	df	p	Adjusted R^2	Temp. Range (°C)	Optimum Temp. (°C)
China Female Longevity (d) [a]	90	41.18	—	326.9 ± 18.7	−8.77 ± 0.71	150.21	1, 89	<0.0001	0.626	10–40	—
Italy Female Longevity (d) [a]	34	33.57	—	211.8 ± 38.1	−6.31 ± 1.63	14.89	1, 32	0.0005	0.296	10–40	—
China Male Longevity (d) [a]	98	41.89	—	226.1 ± 14.8	−5.40 ± 0.57	90.34	1, 97	<0.0001	0.477	15–30	—
Italy Male Longevity (d) [a]	53	39.15	—	158.8 ± 16.8	−4.06 ± 0.72	31.65	1, 52	<0.0001	0.366	15–30	—
China Mean Fecundity [b]	6	35.00 ± 0.00	9.21 ± 2.40	0.07661 ± 0.02420	1.87 ± 0.64	58.52	2, 4	0.0169	0.979	10–35	29.4
China Mean % Egg Hatch on bolts [b]	5	30.77 ± 0.42	10.22 ± 0.55	0.00079 ± 0.00020	1.79 ± 0.38	416	1, 4	0.0368	0.992	10–30	25.5
Mean Time to First Oviposition	8	—	—	10805.4 ± 10369.1	−2.02 ± 0.34	105.8	2, 6	<0.0001	0.880	15–30	—
Hatch Rate (1/d) [b]	1031	38.19 ± 1.29	12.7 ± 0.51	0.000036 ± 0.000015	1.18 ± 0.19	7201	4, 1027	<0.0001	0.529	15–35	29.2

Parameter values are means followed by standard errors. In the table and the models, T_{Max} = upper temperature threshold and T_{Min} = lower temperature threshold. The temperature range used did not always include all the data points, since some were above or below the thresholds based on preliminary plots. [a] model $y = ax + b$. [b] model $y = \begin{cases} \text{at}(t - T_{max})(T_{min} - t)^{\frac{1}{b}} \text{ for } T_{max} \leq t \leq T_{min} \\ \text{otherwise } 0 \end{cases}$, where t is the temperature (°C). [c] model $y = at^b$.

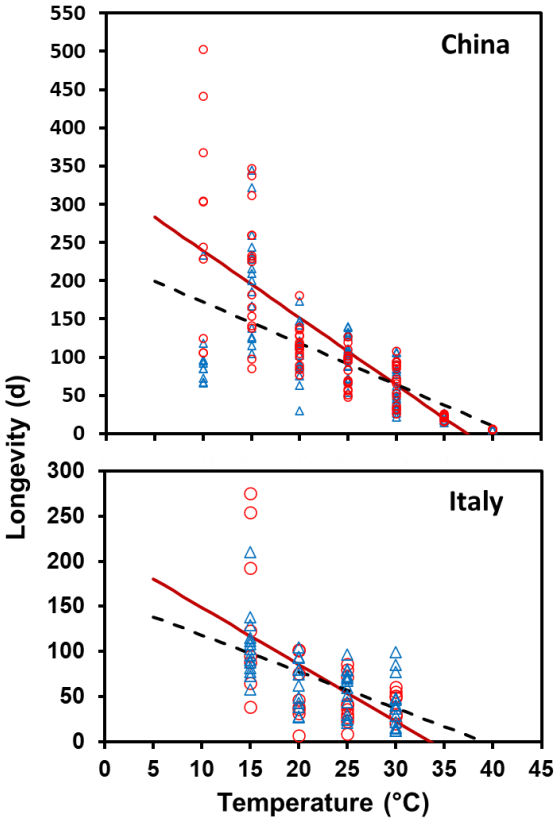

Figure 1. Relationship between temperature and longevity for male and female *A. chinensis* from China and Italy. Observed male data are open triangles, observed female data are open circles, predicted male dashed line, and predicted female solid line.

Once Chinese females started laying eggs, they generally continued laying eggs until about five weeks before they died, while Italian females stopped laying at about two weeks before death at 30 °C. The time to first oviposition increased with decreasing temperature with the maximum being 12 weeks at 15 °C (Figure 2). The fecundity of *A. chinensis* from China peaked at 30 °C and declined with both increasing and decreasing temperatures, while the fecundity of females from Italy did not vary much over the 15–30 °C temperature range (Table 1, Figure 2). This effect of temperature on fecundity was significant ($F = 2.93$, df = 3, 69, $p = 0.0394$) and so was the effect of population ($F = 31.47$, df = 1, 69, $p < 0.0001$), but the interaction between the two was not (Table 1). Both the percentage of Italian females that laid and produced progeny were also lower than that for Chinese females, further reducing their total reproduction over the 15–30 °C temperature range (Table 1). Equation (1) described the relationship between the mean fecundity of Chinese females and temperature reasonably well based on the adjusted R^2 value (Table 2, Figure 3A). No curve was fit for the Italian population because fecundity varied little over the range of temperatures where oviposition occurred.

There were significant effects of temperature ($F = 8.33$, df = 3, 69, $p < 0.0001$) and population ($F = 23.6$, df = 1, 69, $p < 0.0001$) on mean maximum weekly oviposition, peaking at 26 eggs per week at 30 °C and declining below that temperature for Chinese females and remaining constant at about eight eggs per week for Italian females (Table 1). The maximum weekly oviposition recorded for any female was 12, 22, 26, and 35 eggs for Chinese females and 12, 15, 17, and 19 eggs for Italian females at 15, 20, 25, and 30 °C, respectively. The week that the maximum weekly oviposition occurred was significantly affected by temperature ($F = 40.32$, df = 3, 69, $p < 0.0001$), but not population ($F = 43.77$, df = 1, 69, $p < 0.0001$); oviposition peaked sooner at higher temperatures than at lower ones (Table 1). The number of days to first oviposition was significantly affected by the interaction between temperature and population ($F = 5.26$, df = 3, 69, $p = 0.0025$), generally

declining with increasing temperature (Table 1). Equation (2) described the relationship between temperature and number of days to first oviposition well with an adjusted R^2 of 0.88 (Figure 3B). The average number of oviposition pits chewed weekly per female was significantly affected by both temperature (F = 23.03, df = 4, 80, $p < 0.0001$) and population (F = 40.09, df = 1, 80, $p < 0.0001$), peaking at 25 °C and declining both above (Chinese population) and below those temperatures (Table 1).

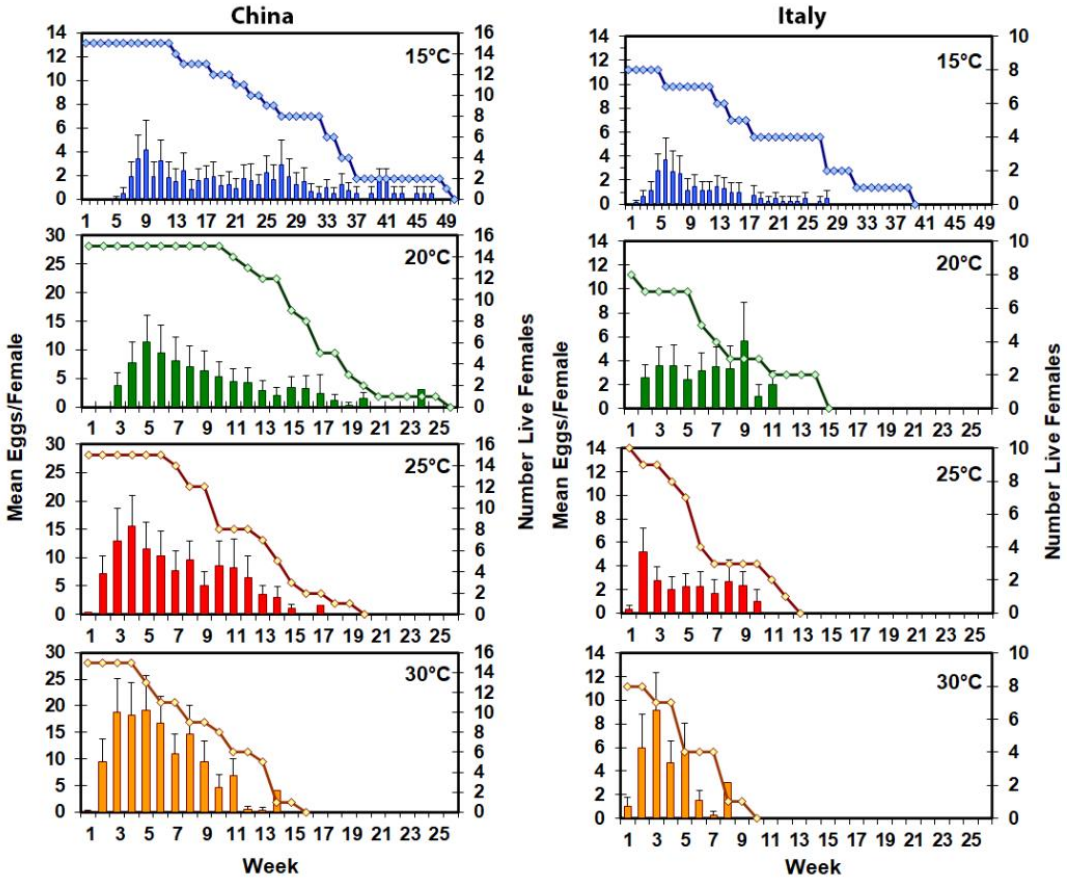

Figure 2. Mean (±SE) weekly oviposition and female survival for female *A. chinensis* from China (Left) and Italy (Right) held at four constant temperatures.

The interaction between temperature and the population the female came from significantly (F = 3.81, df = 3, 113, p = 0.0121) affected the number of mature unlaid eggs found in the female at death. At temperatures between 15 and 30 °C there was no difference in the number of unlaid eggs for Italian females (Table 1). Chinese females had no unlaid eggs at 10 and 40 °C, and only one female had a single matured egg in her ovarioles at death at 35 °C. Chinese females at 15 °C had fewer unlaid eggs than those at 30 °C. The maximum number of unlaid mature eggs found in females from both populations was eight eggs.

3.2. Egg Hatch

Temperature had a significant effect (F = 392.34, df = 4, 897, $p < 0.0001$) on number of days to egg hatch, but population did not (F = 0.13, df = 1, 897, p = 0.7202). The number of days to egg hatch for the populations combined was lowest at 30 °C and increased both above and below that temperature (Table 3). The relationship between hatch rate (1/d) and temperature was fit to Equation (1) with good confidence (R^2 = 0.529, Table 2, Figure 4B). The T_{min} and T_{max} for hatch rate appear to be good estimates, since no hatch occurred at 5, 10, or 40 °C, although only eggs from the Chinese population were used at these temperatures (Table 3).

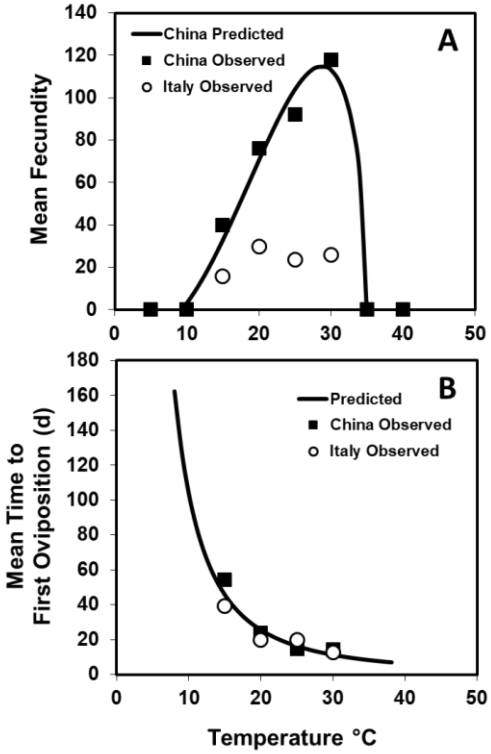

Figure 3. Relationships between temperature and mean fecundity (**A**) and time to oviposition (**B**) for *A. chinensis* from China (solid squares) and Italy (open circles). The solid line in graph A is for only the Chinese population, and in graph B it is for the two populations combined.

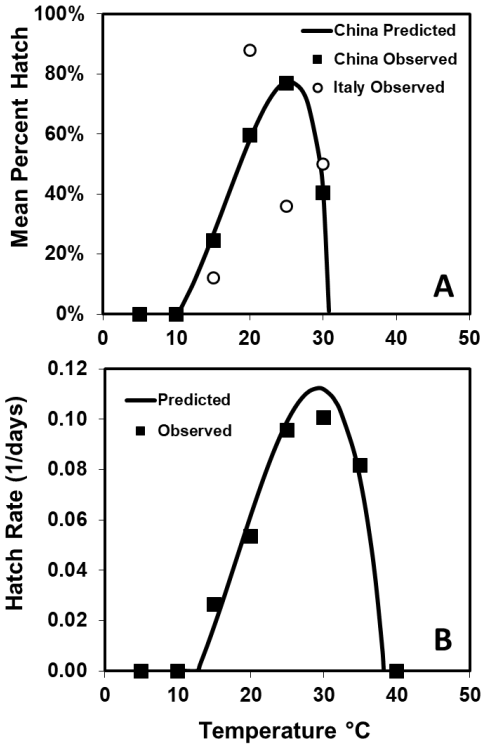

Figure 4. Relationships between temperature and mean percentage egg hatch (**A**) and mean hatch rate (1/d) (**B**) for *A. chinensis*. In graph A, observed points are solid squares (Chinese) and open circles (Italian), and the predicted line is only for the Chinese population. Results are pooled across populations in graph B (solid squares are mean observed points).

Table 3. Effects of temperature on *Anoplophora chinensis* egg hatch (mean ± SE (n)).

		Temperature							
Parameter	**Population**	**5**	**10**	**15**	**20**	**25**	**30**	**35**	**40**
Time to Egg Hatch (d)	Both	NA	NA	38.0 ± 1.2a (34)	18.7 ± 0.4b (125)	10.5 ± 0.1cd (770)	9.9 ± 0.2d (96)	12.3 ± 0.9c (6)	
% Hatch of Eggs Removed from Under the Bark	China	0.0 (51)	0.0 (90)	11.7 (291)	30.9 (369)	60.5 (1138)	27.3 (333)	5.5 (109)	0.0 (51)
	Italy	NA	NA	0.0 (57)	12.1 (91)	52.9 (155)	7.4 (68)	NA	NA

Time to egg hatch values followed by the same letter are not significantly different (F = 392.34, df = 4, 897, $p < 0.0001$) based on the Tukey–Kramer test with $\alpha = 0.05$ [19]. The data for the Chinese and Italian populations were pooled for time to hatch. NA = parameter not applicable, since no eggs were held or hatched at these temperatures. Some eggs laid the day the bolt was removed, or the previous day, were moved to other temperatures and held for hatch. Other eggs were held at the oviposition temperature.

The cumulative proportions of the number of days to hatch at 15 to 35 °C (populations pooled) is shown in Figure 5A. The first hatch occurred at 31, 9, 5, 5, and 11 days and the last at 50, 30, 19, 16, and 15 days for eggs held at 15, 20, 25, 30, and 35 °C, respectively. The cumulative proportion versus accumulated degree-days was fit to Equation (3) with high confidence (R^2 = 0.994; Figure 5B). Degree-day accumulations of 125.5 (95% CI 125.3–125.6) and 165.6 (95% CI 164.4–167.0), are predicted for 50% and 90% of the eggs to hatch, respectively (Figure 5B, 0.5 and 0.9 values).

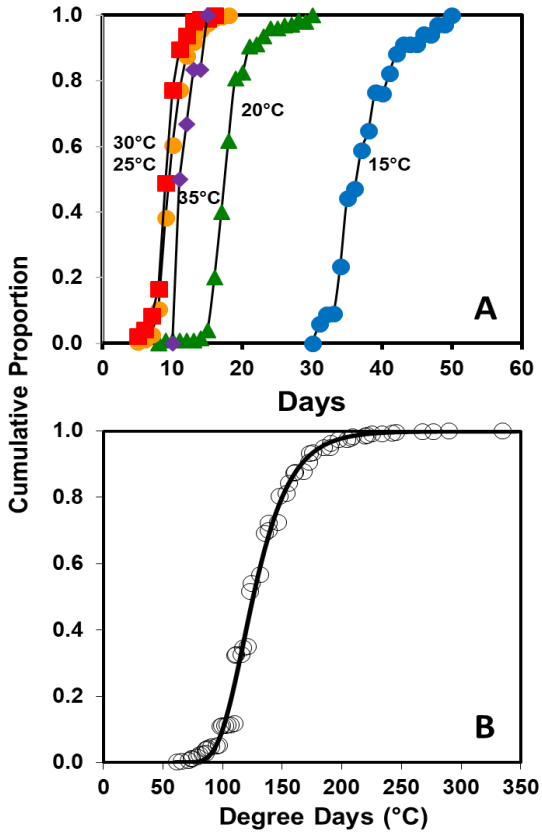

Figure 5. Observed cumulative frequency distributions for *A. chinensis* egg hatch times (d) at five constant temperatures (°C) (**A**). Cumulative proportion of *A. chinensis* eggs hatching over accumulated degree-days (**B**). The solid line in the bottom graph represents the fit to the equation: Predicted proportion hatching = exp (–exp (0.0469 (±0.00139) X accumulated degree-days + 5.5186 (±0.1683))); adjusted R^2 = 0.994.

The percentage hatch of eggs laid at or moved to temperatures for each population is given in Table 3. Because the percentage hatch for eggs removed from under the bark

seemed low, the percentage hatch for eggs laid at different temperatures then held on bolts at 25–30 °C was used for developing the relationship between percentage hatch (as a measure of fertility) and temperature. Percentage hatch on bolts was significantly affected by temperature (F = 5.85, df = 3, 63, p = 0.0014), but not population or the intersection between the two (Table 1). Percentage hatch was highest at 25 °C for the Chinese population and 20 °C for the Italian population and declined as temperature increased or decreased (Table 1, Figure 4A). The relationship between temperature and mean percentage hatch of Chinese eggs on bolts was fit with high confidence (R^2 = 0.992) by Equation (1), but no curve was fit for the Italian egg data, because the maximum likelihood estimator did not converge (Figure 4A, Table 2).

4. Discussion

The observed adult activity over the range of temperatures assessed was comparable to what was seen for *A. glabripennis* [22,23]. Adults held at 15–25 °C exhibited the full range of normal behaviors (resting, feeding, mating, and ovipositing), just more slowly at cooler temperatures. At 10 °C Chinese adults moved very slowly, fed little, and did not chew oviposition sites. At 30–35 °C adult behavior increased, and they would attempt to walk or fly away when their twigs and bolts were changed. At 35 °C, Chinese females did not oviposit even though they chewed some oviposition pits. Chinese adults held at 40 °C were more lethargic, fed little, and did not live long. The attempts to move away from the higher temperatures fit with the observations that *A. glabripennis* seeks shaded areas to rest when the temperature is >29 °C in China [24].

Adult longevity declined with the increase in activity at higher temperatures. This is likely due to increased metabolism at higher temperatures which has been found to reduce the life span in insects [25]. The longevity at 20–25 °C on *A. rubrum* for both sexes from the Chinese population is comparable to that recorded on *Melia azedarach* (91.8 days female and 103.0 days male) and higher than recorded on *Casuarina* sp. (61.6 days female and 63.0 days male) at room temperature in China [1]. Conversely, both sexes of the Italian population on *A. rubrum* at 25 °C lived for a shorter time than did individuals from a Japanese population at 24 °C on either *Citrus unshiu* (Yu.Tanaka ex Swingle) or *Salix schwerinii* E. Wolf [15]. This highlights the effects of host plants on longevity and suggests that caution should be used when applying predictions solely based on a single host in the laboratory. Female *A. chinensis* from both populations lived longer than *A. glabripennis* females at temperatures ≤15 °C, but comparable lengths of time at higher temperatures [23]. However, the male longevity of individuals from the Chinese population was comparable to that of *A. glabripennis* individuals over the 10–35 °C range [23]. The longer survival of female *A. chinensis* than *A. glabripennis* at cooler temperatures is surprising, since the native range of *A. glabripennis* extends further north than that of *A. chinensis*, but fits with the fact that *A. glabripennis* has a higher metabolic rate than *A. chinensis* at cold temperatures [17].

The timing of first oviposition (13–17 days to first egg at 24 °C depending on the host) and the shape of the weekly oviposition curve (bell-shaped with a long right tail) are comparable to the findings of this study for *A. chinensis* [1,7]. The mean fecundity (92 for Chinese and 24 for Italian at 25 °C), however, was lower than that of either Chinese females on *M. azedarach* (176 eggs) or field-reared Japanese females on citrus (193.8 eggs) [1,7]. This is most likely due to the host used in the studies, since the host has been shown to affect fecundity in *Anoplophora* spp. [15,26]. The increasing number of oviposition sites chewed per week, maximum oviposition per week, and fecundity for females from the Chinese population with increasing temperatures 15–30 °C was to be expected because of increased efficiencies of assimilation and conversion of ingested food at higher temperatures [27]. What was not expected was a lack of temperature response for reproductive output for the Italian population, and the causes for this are unknown. Compared to *A. glabripennis*, the *A. chinensis* Chinese females laid more eggs across the entire temperature range of 15–30 °C, and fecundity peaked at a higher temperature [23]. The higher fecundity could have been because *A. chinensis* were, on average, heavier than *A. glabripennis* females, and

fecundity has been correlated with size in both species [7,28]. The ability of *A. chinensis* to handle higher temperatures better than *A. glabripennis* is also suggested by its native range extending further south, even though much of their ranges overlap [2]. At the more extreme temperatures, the pattern of reproductive arrest seen for *A. chinensis* is comparable to that found for *Hylotrupes bajulus* L. [29] and *A. glabripennis* [28]. At 10 °C and ≥35 °C, females laid no eggs, and there were no fully developed eggs in their ovarioles at death. This was either due to a lack of egg maturation or eggs being reabsorbed within these females to use as an energy source.

Eggs of most insect species will hatch over a 15 °C range of constant temperatures, and under varying temperatures like occur in nature, egg hatch may occur 3–4 °C below that range [30]. As temperatures approach (within 2–3 °C), the T_{max} and T_{min}, percentage hatch declines dramatically [30]. *Anoplophora chinensis* eggs from the Chinese population appear to be an exception, as eggs hatched over a broader range of constant temperatures (15–35 °C), although the predicted T_{min} and T_{max} were only 3 °C below the temperatures at which hatch percentage declined sharply. However, the cold tolerance of *A. chinensis* eggs seems to be less than that of *A. glabripennis*, since none of the *A. chinensis* eggs that were held at 5–10 °C for 200 days and then were moved to 25 °C were able to hatch, while eggs of *A. glabripennis* did hatch under similar conditions. This would suggest that *A. chinensis* eggs would not be able to overwinter, unless the more sheltered tree bole and roots location they use provides more protection from colder temperatures than is afforded *A. glabripennis* eggs laid under the bark up higher on the tree. However, care should be taken in making this assumption, since removing the eggs from the bark can damage *A. chinensis* eggs, and we did not confirm this by holding eggs under the bark at the low temperatures.

The percentage of Italian eggs laid at 20 °C and hatched at 25 °C was similar to that for Japanese *A. chinensis* eggs laid and hatched at 20 °C in *Citrus* sp. Bolts (90%) [7]. The lower hatch percentages seen for eggs removed from bolts was probably due to damage sustained when removing them, since there were reductions for all temperatures, and the eggshells of *A. chinensis* broke more easily than those of *A. glabripennis*. The percentage hatch of the Chinese population eggs kept on bolts was comparable to *A. glabripennis* egg hatch when removed from bolts at 15–30 °C. However, unlike *A. glabripennis*, some *A. chinensis* eggs that were removed from bolts were able to hatch at 35 °C, possibly another indication that *A. chinensis* may be better adapted for warm climates than *A. glabripennis.*

The predicted number of degree-days to 50% hatch of *A. chinensis* eggs was 166 *DD* which is comparable to previous estimates for Japanese *A. chinensis* (184 *DD*) [7], but considerably less than the 239 *DD* required by *A. glabripennis* [23]. The T_{min} of 12.7 °C is higher than the 6.7 °C estimated for Japanese *A. chinensis* and slightly higher than the 9.7 °C estimated for *A. glabripennis* [7,23]. In addition, the T_{max} is quite a bit higher for *A. chinensis* than *A. glabripennis.* The lack of hatch for *A. chinensis* eggs held at 10 °C substantiates the T_{min} estimated in this study and suggests that either the previous estimate was low or that some Japanese populations of *A. chinensis* may have a lower threshold.

Diurnal and seasonal temperature variations in the lower bole and exposed roots of trees where *A. chinensis* eggs are laid could be substantially different from ambient or from tree trunks and branches where *A. glabripennis* eggs are laid. Previous studies have shown that phloem temperatures in the upper bole and branches can be much higher than ambient (averaging 2–10 °C higher and maximum 21 °C higher), especially when the tree section is exposed to direct sunlight [31,32]. However, other factors can also alter within-tree temperatures, such as reflected and thermal radiation, wind chills, and soil temperature around the roots. In fact, the deeper in the trunk or under soil, the more insulated the wood is, and the slower the temperatures may respond to ambient changes [32]. When using the estimated *DD* and T_{min} for predicting when *A. chinensis* eggs will hatch, ambient air temperatures will likely not provide accurate estimates. Further work to determine exactly how air temperature differs and temperature changes diurnally and seasonally in the root zone of trees is needed to accurately predict egg hatch timing and potential effects on percentage hatch for *A. chinensis.*

The impact of temperatures on the adults can be seen if the temperatures from the most northern and southern invasive *A. chinensis* populations in Europe are compared. Monthly mean temperatures in Rome, Italy are 17, 21, 23, 24, 21, and 17 °C during May, June, July, August, September, and October, respectively, while the corresponding temperatures in Rotterdam, Netherlands were 12, 15, 17, 17, 14, and 11 °C, respectively (https://weatherbase.com, accessed 2 April 2021). For Rome, Italy, during the same time period, the maximum temperatures averaged 22, 26, 29, 29, 26, and 22 °C, the maximum recorded temperature was 37 °C for July-September, and there are, on average, four days a year that the temperature exceeds 32 °C. Thus, Rome experiences temperatures at which the beetles can survive and reproduce well from June to August, and summer temperatures rarely reach >32 °C, so it should not reduce adult survival or suspend oviposition. Average temperatures in Rotterdam, however, barely reach levels at which beetles will oviposit June–August, and even high averages only reach ≥20 °C in July and August—so although the beetles may survive a long time, they will lay few eggs. This may partially explain the total of 25 exit holes seen in the Westland, Netherlands, infestation and the relative ease of eradicating that infestation, while the infestation in Rome is larger and more difficult to eradicate [11]. Average summer temperatures in major citrus-growing areas in California like Visalia would be near-optimal, but average highs and maximum recorded temperatures could suspend oviposition and reduce adult longevity part of the time. Citrus-growing areas in Florida (e.g., Bartow) would be near-optimum for beetles, and even the maximum recorded temperatures would rarely reach the T_{max} for adult survival or result in the suspension of oviposition. These data on temperature effects on reproduction and survival of adults and eggs of *A. chinensis*, if combined with temperature response data for larvae, could be used to develop maps of the potential geographical range for this species and stage-based phenological models to predict the timing of life stages. Such information is critical for informing management and eradication programs, both in the native and introduced ranges.

5. Conclusions

In the current study, the temperature had significant impacts on all the life-history traits of the Chinese population and most of the Italian population traits, with the exceptions of fecundity and mean maximum oviposition per week. Effects of temperature on the longevity of both sexes differed between the populations; Chinese individuals lived longer than Italian individuals. Also, adults from the Chinese population were heavier than those from the Italian population. Temperature effects on longevity also differed between the sexes. The percentage of females that oviposited and produced progeny was lower for females from the Italian than the Chinese population. There were no differences between the populations in the time to first oviposition or egg hatch rate over the temperature range that was evaluated. The mathematical relationships between temperature and the life-history parameters provided estimates of the lower and/or upper temperature thresholds and optimum temperatures for many of the traits evaluated. These relationships will facilitate the development of phenology models and improve predictions of potential geographic ranges to aid managers in deploying control and eradication measures.

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Conflicts of Interest: The authors declare no conflict of interest.

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Communication

Potential Differences and Methods of Determining Gypsy Moth Female Flight Capabilities: Implications for the Establishment and Spread in Novel Habitats

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Abstract: The introduction of the Asian gypsy moth into novel environments continues with frequent interceptions in North America. There is a concern that these subspecies will pose a greater threat to the forests and urban environments of North America than the established gypsy moths (*Lymantria dispar dispar* L.), due to their greater capacity for female flight. Asian gypsy moth populations vary in many key traits, including female flight capabilities. The potential impacts of female flight, in combination with the other key traits, on the ecology and spread of this insect are first discussed in this communication. This also provides the first review of most of the current literature on the variations in flight capability and flight distance of gypsy moth populations, as well as variation in other traits of concern and the potential methods of identification, with special attention paid to the Asian subspecies *Lymantria dispar japonica* Motschulsky and *Lymantria dispar asiatica* Vinkovskij. There are currently good tools for identifying the general origin of introduced gypsy moth populations, but these do not provide enough information to effectively manage introductions. Gypsy moth key traits differ among populations, even within each subspecies of the gypsy moth, so introduction of gypsy moths from other world areas into locations where the gypsy moth is already present could result in unwanted changes in gypsy moth biology. It also appears that the introduction of flight-capable females could enhance a population's dispersal capability and require modifications to management protocols used for flightless females. Therefore, rapid tools to assess key traits in introduced populations are needed to adequately plan for, or deal with, new introductions into novel habitats.

Keywords: Asian gypsy moth; *Lymantria dispar*; invasive species; forest pests

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1. Introduction

The gypsy moth, *Lymantria dispar* (Lepidoptera: Lymantriidae), is a forest insect capable of causing severe harm to ecosystems and individual trees through repeated defoliation [1,2] and is responsible for substantial economic losses caused by subsequent damage [2]. Liebhold et al. [3] estimated that caterpillars of gypsy moth populations currently present in North America can feed on over 300 (~100 preferred species) shrubs and tree species. Furthermore, Keena and Richards [4] found that gypsy moths of European and Asian origin were able to establish on North American tree types even without prior exposure to the host species, thus making them a threat to temperate forests around the globe.

Three subspecies of gypsy moth have been described: European gypsy moth (*Lymantria dispar dispar*), Asian gypsy moth (*Lymantria dispar asiatica*), and Japanese gypsy

moth (*Lymantria dispar japonica*) [5,6]. The European gypsy moth (EGM) is native to Eurasia, where it is found from Portugal, in the west, to the Ural Mountains, in the east [7]. This subspecies was introduced into North America in 1869 and is now established in many forests of the Eastern United States and Canada [8,9]. The native range of the Asian gypsy moth (AGM) extends east from the Ural Mountains through Russia, China, Korea, and into portions of Japan, while the Japanese gypsy moth (JGM) is limited to the main islands of Japan and the Kuril Islands of Russia [5].

The European gypsy moth (EGM) was first introduced into the northeastern state of Massachusetts, in the United States, in 1869, by a French mathematician, in order to conduct breeding experiments with a native silkworm. During this time, gypsy moth larvae escaped and became established in the surrounding area. Twenty years after this initial establishment, a large outbreak occurred and attracted public attention. Over the next 100 years, the gypsy moth continued to expand into the forests of the Eastern United States and Canada, despite numerous eradication efforts led by local and federal agencies [10]. Within the United States, the rate of spread of EGM averaged 9.45 km/year between 1900 and 1915, 2.82 km/year between 1916 and 1965, and 20.78 km/year between 1966 and 1990 [11]. McManus and Csoka [10] believe that the increased rate of spread that occurred in the second half of the 19th century could be due to the following: (1) a decrease in the use of DDT and other chemical pesticides; (2) the fact that newly infested areas contained contiguous forests dominated by oak species (*Quercus* spp.), the EGM's preferred host; and (3) severe outbreaks that occurred in the 1970s and early 1980s that increased the spread rates of the insect. After finding that stratified dispersal was important in the spread of EGM [11], a barrier-zone management program called "Slow the Spread" was instituted that has reduced the annual rate of spread to <4 km per year [12]. Moreover, outbreaks in established areas are now better regulated in a density-dependent form by two host-specific pathogens, (1) the fungus *Entomophaga maimaiga* Humber, Shimazu, and Soper (Zygomycetes: Entomophthorales) and (2) a nucleopolyhedrosis virus, *LdNPV* [13,14]. In addition, aerial application of *Bacillus thuringiensis* Berliner serovar *kurstaki* (Btk) is currently the most effective control method to suppress gypsy moth outbreaks in Europe [15]. In areas with established gypsy moth populations, outbreaks generally occur every five to ten years [16] and last between one and three years [17,18]. However, the longest outbreak periods were recorded to be 12.5 and 13.0 years in Poland and the Czech Republic, respectively [19]. The outbreak period decreases from the northwest to the southeast regions of Europe. For example, in Central Europe, the period is approximately 12 years, while in Mediterranean and Balkan regions, it is seven to eight years [20]. A study by Hlasny et al. [21] found outbreak periods to be strongly related to geographical gradients and revealed substantial spatial and temporal variability in the outbreak patterns in the regions surrounding the Carpathian Mountains.

There are ecological costs to the EGM invasion. In general, the EGM displays a preference for certain tree species during outbreaks [10], and these tree species are defoliated at higher rates than species that are consumed by only some larval stages or species rarely, if ever, consumed by any larval stage [22]. Once defoliated, the weakened trees are far more susceptible to other agents, such as boring insects and parasitic fungi, which cause eventual tree mortality [10]. Consequently, EGM has caused a decrease in the dominance of their preferred host species, specifically oaks, in Eastern North American forests and could result in a long-term shift in species composition [23].

Substantial economic costs are also associated with the EGM invasion of North America. These costs generally come from the following sources: (1) timber value loss due to tree mortality or a reduction in growth; (2) residential-property value loss caused from dead or dying trees; (3) household expenditures for tree care; (4) local government expenditures for tree removal and replacement, or treatment; and (5) federal government expenditures for research, regulation, management, and outreach. Aukema et al. [24] estimated that the EGM was responsible for $254 million in damages on an annual basis, with a considerable proportion of this ($120 million) coming from residential property value loss.

The AGM was not introduced into North America until much later. The first recorded introduction occurred in 1991, near the port of Vancouver, in British Columbia, Canada [25]. Shortly after this, individual AGMs were discovered in the United States, in Washington and Oregon, in addition to other parts of British Columbia. It is thought that ships from Eastern Russia transported AGM larvae to ports along the Pacific Northwest Coast. These infestations were eradicated quickly through treatment and trapping. However, since this initial introduction, AGM has been detected and eradicated on at least 20 separate occasions throughout the United States [25]. In 2020, an eradication program occurred in Western Washington State for recently discovered introductions of EGM with AGM genetic traits, as well as the Hokkaido gypsy moth (*Lymantria umbrosa* Butler) [26].

If AGMs were to establish in North America, there is a high probability that the ensuing economic and ecological damage would exceed that of the EGM for several reasons (Figure 1). Although males of both AGM and EGM can fly, AGM females are capable of flight, while EGM females are not [7]. Studies have also found that Asian and Japanese female gypsy moths display an attraction to light sources, which has commonly led to egg deposits on ships and vehicles [27,28]. In addition, AGMs have a wide geographic range that encompasses various forest ecosystems. If introductions were to occur with AGMs from multiple origins, they might produce offspring with a wider host range than the original populations [4]. Gypsy moth populations also vary in both the larval and egg responses to temperature. The amount of chill an egg mass requires before hatching varies between populations across all subspecies. Hence, if genotypes that require less chill were introduced into already infested areas, variation in egg hatch within the population could increase, and a non-diapausing strain could develop in uninfested areas with mild climates [29]. Some AGM larvae take a longer time to develop and grow to larger sizes, and this could cause greater defoliation at lower numbers than the EGM larvae [30]. However, with longer time to grow and develop, there is increased opportunity for natural enemies and other factors to influence population dynamics.

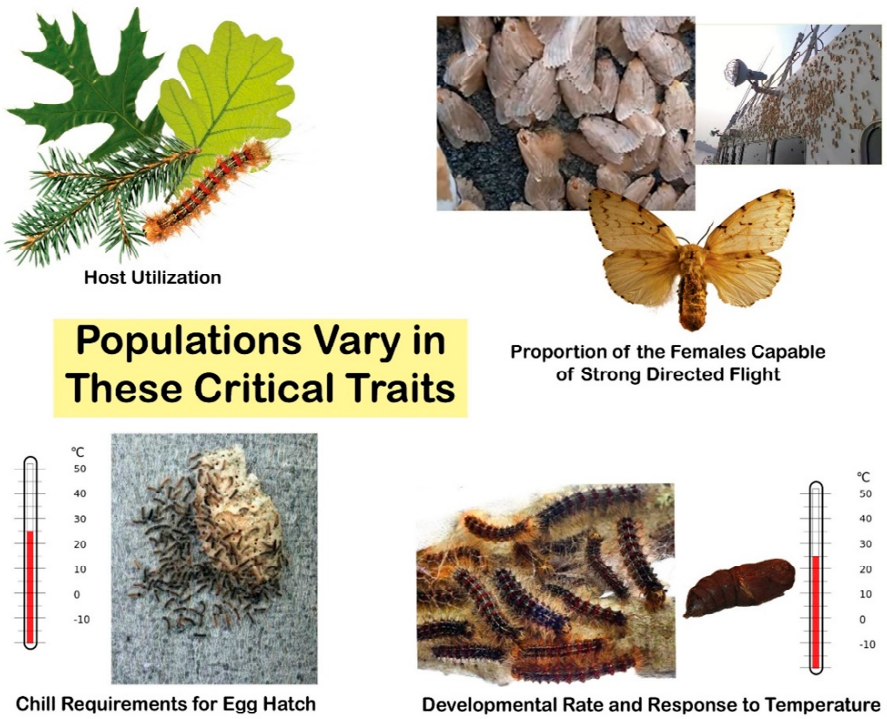

Figure 1. Gypsy moth populations vary in several key traits that can alter the dispersal capabilities or their potential to cause damage in novel habitats.

One of the most important variables used in estimating the spread of gypsy moths is flight ability and flight distance of female moths. While current spread and distribution

modelling approaches have used general flight distance estimates [31], more specific information would help to improve projections of future potential distribution and spread. However, there does not appear to be a distinct line separating the flight capacity of each gypsy moth subspecies. While dividing gypsy moths into three subspecies can be useful for descriptive purposes, traits can be passed between subspecies through hybridization [32]. Because of this, it is especially important to examine flight capability on a distinct population basis instead of limiting the inquiry to a subspecies level. The overall objective of this communication is to document what is currently known regarding the flight capability, and potential flight distances, of females from different gypsy moth populations. We also place this information on flight capability within the context of other factors important to a program targeted at preventing the establishment and spread of flight-capable females in novel habitats. This includes assessing the current ability to distinguish populations with flight-capable females and evaluating the effects of female flight on the population dynamics (establishment and spread) in novel habitats.

2. Gypsy Moth Population Dynamics (Establishment and Spread) and Potential Impacts of Female Flight

Gypsy moths represent one of the most studied and best-documented biological invasion systems in North America [33]. Novel introductions and establishments are regularly monitored through extensive surveys, and this information is used to understand population dynamics and other factors affecting establishment, such as dispersal ability, environmental tolerance, and host preferences [9]. Gypsy moth population dynamics are complex due to the fact that there are multiple natural enemies that regulate population density or reduce outbreak populations [34]. Gypsy moth populations immediately after outbreaks are mostly regulated by two host-specific entomopathogens, *Entomophaga maimaiga* and the nucleopolyhedrosis virus *LdMNPV* [35]. Outbreaks have been recorded in areas that have at least 20% of the moths' preferred host species [36]. However, there are multiple other key factors involved in influencing the population dynamics of the species. One of these factors is land use. A study by Lentini et al. [37] found that outbreaks are generally more frequent in overexploited forests and woodlands (<25% canopy cover rate) than natural ones. Moreover, gypsy moth population dynamics may be further subjected to changes depending on control measures [15]. A study by Mannu et al. [15] found a forward shift in abundance peak with early *Btk* applications during the population growth, leading to postponed outbreak peak by two to three years, whereas *Btk* applications during the culmination phases were found to be more effective in suppressing gypsy moth populations.

A recent finding showed that gypsy moth establishment and spread dynamics are very different along the species' leading edge [33,38]. Gray et al. [34] studied the relationship among gypsy moths, forest composition, stand architecture, and natural enemies. They found that habitat structure did not influence natural enemy populations and that the pre-eruptive phase was different from the pre-establishment phase due to high mating success. Moreover, they found high mating success and adult female survival in the pre-eruptive phase across all of the sites and habitat structures. This hints at the inability to find mates at very low densities (pre-establishment), resulting in the Allee effect, a positive relationship between individual fitness and population density [38] that may be a limiting factor in the establishment of newly founded gypsy moth populations [39]. In this scenario, an individual of a species under the influence of an Allee effect will experience a decrease in its fitness level when the population density is low. In the case of invasive species, low density at initial introduction provides an opportunity for Allee effects to modify the invasion dynamics and could cause longer lag times, slower spread, and reduced establishment probability [40].

Mate-finding failures, as a source of Allee effect, are also an important factor determining the spread and establishment of gypsy moths in flightless female populations of North America [41,42]. It has been found that both Allee effects and stratified diffusion into new areas play roles in gypsy moth dispersal, so tactics based on these findings have been used

along the leading edge to slow the spread. Isolated colonies of gypsy moths with flightless females are prone to Allee effects and extinction given their low abundance. In this case, mating success seems to be a prominent density-dependent factor affecting sexually active sporadic populations [43]. Sharov et al. [44] found a significantly higher likelihood of mating in higher-density populations. Gypsy moth fecundity decreases rapidly in adults greater than two days old, which means there is limited time for mating before death [45]. Consequently, female movement may or may not be helpful in low-density populations.

The role of the Allee effect may be diminished when flight-capable females are present. Flight-capable females fly to lights (the moon or manmade light sources) or light-colored surfaces to lay their eggs. Virgin females fly less frequently than mated females, as they call for a mate first [46] (Figure 2). However, virgins that are not mated quickly will eventually fly to another location, to call for a mate. Since both sexes are attracted to lights, virgin females may be increasing their chances of finding a mate by flying to nearby light sources, where males will also congregate [27]. Once mated, females fly and congregate near lights, to lay their eggs, effectively creating local concentrations of flight-capable individuals. These concentrations could be within an already infested area with mixed-flight individuals, or out ahead of the generally infested area, if moving into new habitats. These local concentrations could then coalesce and increase the rate of expansion just as the small populations ahead of the leading edge in flightless female populations increase the speed of invasion into new areas. With flighted females, however, the number of these small populations outside the generally infested area could be higher, and they could be further from the leading edge of the infestation. There has been evidence of this already occurring when it was observed that female flight capability was maintained at higher levels (even up to 70%) than random mating within European populations where flighted types and non-flighted types were sympatric [7]. When individuals from populations with flight-capable females are mated with individuals from populations where females were flightless, the first-generation females are only capable of a descending glide, and only about 15% of the female progeny of matings between the hybrids are capable of strong directed flight [32].

In addition to the Allee effect, climate suitability is also an important factor in determining the establishment probability of gypsy moths when suitable hosts are present [31]. In the past, various authors have used phenology (Gypsy Moth Life Stage model) and climate matching models (MaxEnt) to assess the establishment probability of gypsy moths [31,47]. These studies show that the potential southern margin of gypsy moth spread in the USA will be limited by insufficient chilling that terminates diapause, while exposure to extreme cold temperatures will result in egg mortality in the northern regions of North America [48]. Moreover, cold winter temperatures were found to be associated with reduced summer trap captures of EGMs [49]. Interestingly, May et al. [50] found no evidence of metabolic adaptation to colder environments based on their comparisons between northern and southern populations of North America gypsy moths. Moreover, Tobin et al. [51] found that the average development rates of North American gypsy moths were faster in areas experiencing higher temperatures despite larvae being exposed to repeated supraoptimal temperatures. Here, Tobin et al. [51] provide evidence of ongoing gypsy moth southern range margin retraction and suggest higher optimal temperatures during larval and pupal development period to be associated with gypsy moth range margin retraction. The authors further suggest that higher temperatures could exceed the positive effects on gypsy moth population growth. Moreover, recent works by Faske et al. [52] and Banahene et al. [53] provide evidence of local physiological adaptation under warmer climates and occurrence of strong temperature-dependent selective pressures in the southern populations of gypsy moth, respectively.

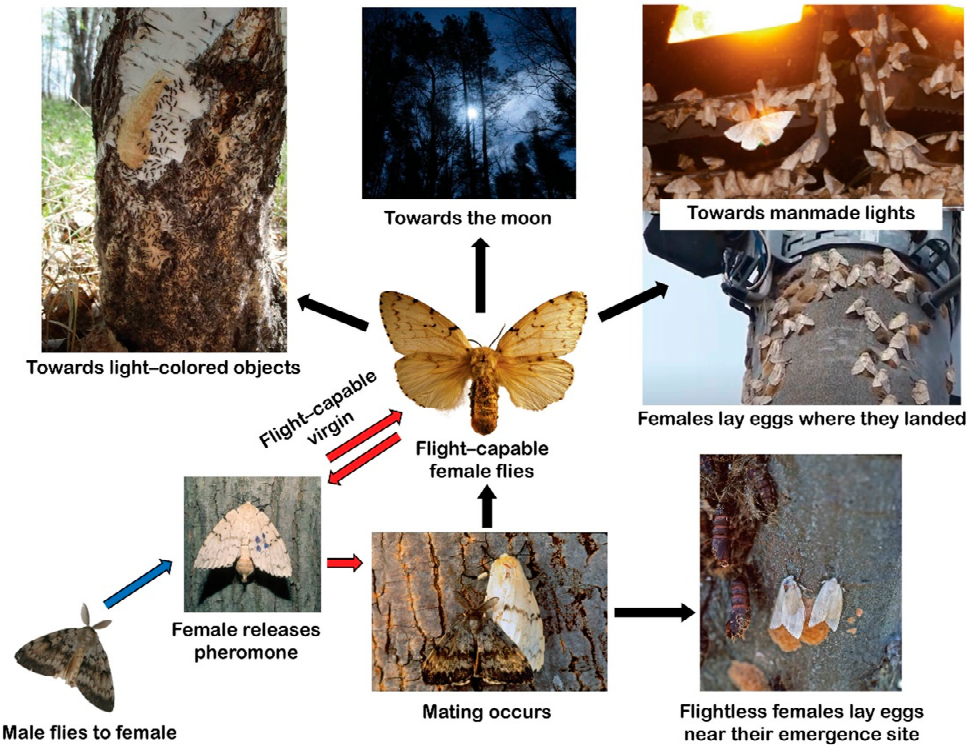

Figure 2. Schematic showing the sequence of mate finding, mating, female flight, and oviposition for populations with flight-capable or flightless females. Note that flight-capable virgin females will fly after calling for a few hours, when no mating occurs, but they will resume calling in the new location. Strong directed female flight to lights or light-colored objects occurs after mating, and females will congregate, in order to oviposit.

Just like their EGM counterparts, the larvae and pupae of gypsy moth from Asian origins with flight-capable females may struggle to survive and develop in regions that experience extended periods of temperatures $\geq$ 30 °C [30]. The developmental rates and responses to temperature of many AGM populations are very similar to those of EGM populations, with the exception of the most slowly developing ones from the *L. dispar japonica* subspecies [30]. In addition, the variation between AGM populations in both host utilization and egg chill requirements is already present between EGM populations [4,29], so establishment potential will depend in part on the traits of the source AGM population and the climate and hosts present in the area where it is introduced. AGM populations should be able to establish in most cases where EGM can establish, based on modeling that has been done [54].

Environmental cues may also impact flight behavior in gypsy moths. Temperature and light intensity may play important roles in determining flight propensity [55]. Iwaizumi and Arakawa [56] collected egg masses from four different localities in Japan: Chiba, Kobe, Hachinohe, and Tomakomai. From the reared larvae, female flight duration under yellow and black light sources was examined. The authors observed yellow fluorescent light that cut out ultraviolet radiation had a suppressive effect on female flight. The relatively high light intensity of 10 lux most likely suppressed the nocturnal flight behavior of the tested females. Iwaizumi and Arakawa [56] found that females from three of the populations flew an average of 200 m, while females from the Tomakomai population had a mean flight distance of roughly 600 m.

Chen et al. [57] measured the flight propensity of female AGM from seven different populations under three combinations of light intensities. Three of the populations originated in China, two from Russia, and one each from Japan and South Korea. Seventy-two percent of all females tested had the ability of directed flight. The authors also found that the range of light intensities used within the study (0.05, 0.10, and 0.40 lux) did not have a

significant effect on flight propensity. This agrees with research by Charlton et al. [55] that found female gypsy moth flight to begin when light intensity falls below 2 lux.

3. Variation in Female Flight Capability and Flight Distances

3.1. Female Flight Capability

Keena et al. [46] investigated the propensity and capability of flight in female gypsy moths from populations established in Russia and North America, as well as the flight capability of hybrids bred from these two populations. While the majority of female moths from the Russian populations were able to fly, females gathered from the population established in the United States were not flight capable. To further analyze how hybridization could affect flight capability, the authors bred four new populations and assessed flight abilities. Parents used during breeding, as well as the percentage of offspring capable of sustained flight (FL) and those capable of gliding flight (GF), are shown below in Table 1.

Table 1. Origin and flight-capacity percentages of North American, Russian, and hybrid gypsy moths [18].

Origin		Sustained Flight	Gliding Flight
Female	**Male**		
Russia	Russia	88%	11%
Russia	North America	0%	51%
North America	Russia	2%	65%
North America	North America	0%	0%

Gypsy moths with both parents from North America were not capable of sustained or gliding flight. However, over 50% of the crosses of North American and Russian populations were able to glide. This suggests that if individuals from flight-capable populations become established in North America and mate with the currently established gypsy moth populations, it is likely that the first-generation offspring would have substantially improved gliding abilities. The possibility of sustained flight amongst hybrids is less established. While only 2% of the Russian–North American crosses were able to fly within this study, a separate investigation found that 8% of captive hybrid females were able to attain sustained flight if they were fed foliage, while no hybrids were flight-capable when reared on an artificial diet [58]. This suggests that female flight capacity of gypsy moths of all strains may be underestimated in studies if an artificial diet is used.

Female gypsy moth flight capacity has been further explored in a study that cross-bred gypsy moths that have established in North Carolina, USA, that are incapable of flight with individuals from Mineralni, Russia, that have a high capacity for ascending flight [32]. The authors compared the parents, reciprocal F1 hybrids, double reciprocal F2 hybrids, and all potential backcrosses. Heritability of female flight was found to be at least 0.60, while relative wing size had a heritability of 0.70. However, overall, flight capacity is determined by a combination of behavior, flight musculature, and wing size, which is controlled by multiple genes. The authors go on to suggest the following:

> "*In a freely hybridizing population, the amount of flight capability maintained would depend on several factors: initial ratio of flight capable to flightless females, costs versus fitness of flight in the particular environment, propensity of different hybrids to mate, etc. Should females with full flight capability be introduced into North America in an area where the flightless females are already established, the populations would hybridize, and the ability of L. dispar to spread could be increased*".

Conversely, if a population of gypsy moths with flight-capable females were to become established in an area of North America where gypsy moth populations were not present, flight-capable females could remain in the population for many generations [32]. Introduction of AGM strains into North America in locations with established EGMs would most likely create hybrid populations. The probability of hybridization will be highest when both

introduced and native populations have similar developmental responses to temperature and numbers of instars, since these traits can have an impact on the seasonal dynamics of the gypsy moth. These hybrid populations would have reduced flight capability, as compared to their Asian parentals [46], and may also incorporate other undesirable traits from the invading population, depending on the genetics of the trait. For example, the phenotype that requires less chill before eggs are able to hatch is a dominant trait that, once introduced, will increase in the population [29]. Subsequent introductions of other AGM strains could further increase the EGM population's flight capacities and introduce other traits that could alter the population's biology and behavior further.

3.2. Female Flight Distance

The nocturnal flight activities of female gypsy moths from Yokohama City and Chiba City, Japan, were assessed by Iwaizumi et al. [56]. Female moth behavior was monitored in a cage under different photoperiodic conditions. Mean flight distances of 226 and 269 m were observed for virgin females and mated females, respectively. However, the maximum recorded value of 746 m for virgin females is closer in agreement with Liebhold et al. [59], who estimated that female gypsy moths in Kanazawa, Japan, had a maximum flight distance per night of around 1 km.

Yang et al. [60] evaluated the effect of mating status and age on the flight activity of female gypsy moths from seven populations within China. Using a computer-linked flight mill, the authors found flight activity peaked when females were one day old and decreased thereafter. However, no observable difference was seen between mated and unmated females. Total flight difference varied among the populations. One-day-old females from Guizhou, China, had a mean flight distance of 7.5 km, with one individual even reaching 10.67 km, while one-day-old females from Inner Mongolia flew an average of 3.95 km. However, the authors caution that these distances may not represent entirely realistic conditions, as the tethering used within the flight mill experiment most likely increases flight distances. It is also important to consider the life cycle of gypsy moths in estimating their spread. The time females spend in the moth stage is, in part, dependent on how soon they mate, as individuals die after depositing their eggs. Gypsy moth adults live no more than three days, and generally only one day [61].

To accurately predict the likely rate of spread of an AGM outbreak in North America, information on potential flight distances is needed. Table 2 summarizes all the literature on flight distances for studies conducted with gypsy moth populations from China, Japan, and Eastern Russia. By using data collected at ports in the Republic of Korea, Japan, and Russia [62,63], minimal potential flight distances can be estimated. Average distances between general egg collection areas and forest edge were 1.7 km in the Republic of Korea, 3.1 km at Japanese ports, and 0.5 km in Russia. Flight distances for all ports are shown in Table 3. These numbers could provide a rough estimate for flight distances for these populations, but because the actual distances between the forests and the ports differ between countries, care should be taken in using this data.

Table 2. Flight distances of gypsy moth populations in China, Japan, and Eastern Russia.

Authors	Year	Population	Distance	Notes
Rozkhov and Vasilyeva	1982	Asia (unspecified)	100 km	
Baranchikov	1986	Russia Far East	3–5 km	
Savotikov et al.	1995	Asia (unspecified)	20–40 km	
Liebhold et al.	2008	Japan (Kanazawa)	$\leq$1 km	max distance, one night
Iwaizumi et al.	2010	Japan (Yokahoma City, Chiba City)	746 m	max distance, one night, virgin female
			511 m	max distance, one night, mated female
			226 m	mean distance, one night, virgin female
			269 m	mean distance, one night, mated female

Table 2. *Cont.*

Authors	Year	Population	Distance	Notes
Iwaizumi and Arakawa	2010	Japan (Tomakomai)	659 ± 335 m	mean distance, one night
		Japan (Hachinohe)	188 ± 202 m	mean distance, one night
		Japan (Kobe)	356 ± 351 m	mean distance, one night
		Japan (Chiba)	255 ± 116 m	mean distance, one night
Yang et al.	2017	China (Jining, Inner Mongolia)	3.95 ± 0.29 km	mean distance, one night; one-day-old females; measured with flight mill
		China (Sandeli, Liaoning)	6.63 ± 1.40 km	mean distance, one night; one-day-old females; measured with flight mill
		China (Yanzikou, Beijing)	5.56 ± 1.16 km	mean distance, one night; one-day-old females; measured with flight mill
		China (Longhua, Hebei)	4.03 ± 0.99 km	mean distance, one night; one-day-old females; measured with flight mill
		China (Lianyungang, Jiangsu)	5.79 ± 1.44 km	mean distance, one night; one-day-old females; measured with flight mill
		China (Liuan, Anhui)	6.54 ± 1.12 km	mean distance, one night; one-day-old females; measured with flight mill
		China (Xifeng, Guizhou)	7.50 ± 2.28 km	mean distance, one night; one-day-old females; measured with flight mill

Table 3. Potential flight distance of gypsy moth populations from the Republic of Korea, Japan, and Russia.

Country	City Port	Distance from Port to Forest Edge (km)	Notes
Republic of Korea	Donghae	2	
Republic of Korea	Okgye	1	
Republic of Korea	Incheon	2	
Republic of Korea	Pyongtaek	2–5	Depends on size of forest needed
Republic of Korea	Busan	2	
Republic of Korea	Pohang	2	
Republic of Korea	Ulsan	3	
Republic of Korea	Gunsan	1–3	Depends on size of forest needed
Republic of Korea	Mokpo	1–2.5	Depends on size of forest needed
Republic of Korea	Gwangyang	1.5	
Republic of Korea	Yeongilman	1.5	
Republic of Korea	Onsan	2–4.5	Depends on size of forest needed
Republic of Korea	Daesan	1	
Japan	Kokura	3–5	Depends on size of forest needed
Japan	Ube	4	
Japan	Oita	3–8	Depends on size of forest needed
Japan	Hirosihima	3–4	Depends on size of forest needed
Japan	Matsunaga	2	
Japan	Tsuruga	1.5	
Japan	Kanazawa	1–7	Depends on size of forest needed
Japan	Chiba	5–8	Depends on size of forest needed
Japan	Fushiki	7	
Japan	Toyama-shinko	6–14	Depends on size of forest needed
Japan	Sakata	0	Port directly next to forest
Japan	Hachinohe	5	
Japan	Nagahama	4.5	
Japan	Aomori	4	
Japan	Hakodate	0.5–4	Depends on port location used
Japan	Tomakomai	3	
Japan	Otaru	1	

Table 3. *Cont.*

Country	City Port	Distance from Port to Forest Edge (km)	Notes
Russia	Vladivostok	3.5–9	9 k to closest trap; 3.5 k to closest forest edge
Russia	Nakhodka	0–3	3 k to closest trap; 0 k to closest forest edge
Russia	Vostochny	0.5	
Russia	Olga	0	Port directly next to forest
Russia	Slavyanka	1	
Russia	Zarubino	0	Port directly next to forest
Russia	Posyet	0	Port directly next to forest
Russia	Plastun	0	Port directly next to forest
Russia	Vanino	0.2	
Russia	Kozmino	0	Port directly next to forest
Russia	Korsakov	0.5	

All of these factors combined suggest that it may be appropriate to use a range of techniques when estimating flight capacity and flight distances of AGMs. Further research is required to classify all populations, using genetic markers and flight distance to improve predicted rate of spread accuracy associated with introduced gypsy moths in novel environments.

4. Identification of Gypsy Moth Subspecies and Specific Traits within Populations

4.1. Genetic and Genomic Analyses of Subspecies and Populations

Understanding the underlying mechanisms of flight capacity is an important goal. Various approaches have been used, from physiological inquiries to genomics. The ability to identify subspecies is of vital importance in stopping the spread of flight-capable AGMs. However, easily observed morphological differences between subspecies are minimal [64]. Genetic methods of identification have been developed which help to differentiate between subspecies and populations [65–67] and therefore aid in determining flight capability and potential flight distance.

Gypsy moth genetic variation and distribution of female flight has been described by Keena et al. [7]. Gypsy moths were obtained from 46 locations around the world, representing the current established range of the species. Female flight capability, pre-flight behaviors, female muscle strength, and female wing size were evaluated for all 46 strains. For 31 of these strains, the authors determined the mitochondrial DNA (mtDNA) haplotype, the nuclear DNA genotype at one locus, and the microsatellite genotype at four loci. Female gypsy moths that were capable of directed flight came from Northeastern Europe, Siberia, and Asia. Wing musculature and size were found to be useful in predicting female flight capacity within populations. An analysis of all DNA haplotypes and genotypes was able to place 94% of individuals into their general geographic origin: Asian, Siberian, European, or North American. However, a clear genetic distinction was not found between European strains that differed in flight-capable females.

By analyzing microsatellite loci and mitochondrial DNA sequences, Wu et al. [68] detected four genetic clusters of *Lymantria dispar*, which correspond to the general place of origin for the named gypsy moth subspecies—Western Europe, the Asian continent, and the islands of Japan. The fourth cluster was found for gypsy moth populations established in North America and was most likely caused by a population genetic bottleneck after initial establishment on the continent. Wu et al. [68] also found evidence of continuing hybridization between subspecies, with high levels of genetic variation in East Asian gypsy moth populations. Analysis of genome-wide single nucleotide polymorphisms (SNPs) is a powerful approach that also enables clear distinction of subspecies and geographic variants, while revealing introgression near the geographic boundaries between subspecies [69]. These polymorphisms could be translated into genomic bio-surveillance tools [70].

Receptors in the olfactory systems of insects have been shown to be important in flight orientation [71]. Thus, it could be possible to differentiate between populations

containing flight-capable and flightless gypsy moth females by examining olfactory genes. McCormick et al. [72] studied the differences between olfaction-related genes in gypsy moth females from one flightless and two flight-capable populations. The flightless population, considered to comprise EGMs, came from New Jersey, USA, while the flight-capable populations had origins in Northern Japan and Far Eastern Russia and were considered to comprise JGMs and AGMs, respectively. By using next-generation sequencing, they identified 115 chemosensory genes and conducted a principal component analysis to determine gene-expression patterns. The olfactory-related genes of JGMs appeared more divergent, whereas the AGM and EGM populations shared many commonly expressed genes. This suggests that these differences are not related to flight capability [72].

Lymantria genomes have been sequenced [73,74], and analyses revealed that the genomes of *Lymantria* are among the largest in the lepidoptera [73]. Genetic comparisons of AGM and EGM genomes found differences in metabolic pathways which suggest strong signatures of energy-related pathways in both subspecies, dominated by metabolic functions related to thermogenesis [73]. Divergent proteins associated with muscle contraction could possibly help to explain differences in wing musculature and, therefore, flight [74]. Further functional studies are needed to confirm these predictions.

4.2. Physical Differences among Subspecies and Populations

Physical characteristics of insects play a large role in determining flight capacity. In order for insects to fly, total flight muscle mass must make up a minimum of 12% to 16% of total body mass [75]. Several studies have sought to examine the physical characteristics of female gypsy moths between flight-capable and flightless populations.

Keena et al. [32] evaluated the muscle strength and wing size of female gypsy moths from North American and Russian populations, as well as various crosses between the two strains. Muscle strength was tested by inverting females onto their backs and evaluating the ease with which they were able to right themselves by beating their wings. Over 90% of the females from the Russian population were able to right themselves within one or two quick wingbeats, while the majority of North American females were unable to do so. More than 50% of the hybrid females were able to right themselves, although many individuals were significantly challenged with the movement. Female wing measurements varied considerably among the populations and crosses. The wings of Russian females were greater in size than those of hybrids, which in turn were greater than those of the individuals from North America.

To further explore the relationship between wing characteristics and female flight capability within gypsy moth populations, Shi et al. [76] conducted a morphometric analysis of 821 gypsy moths from eight different strains. Individuals of both sexes were sourced from one site in the United States, two sites in Europe, two sites in Russia, two sites in China, and one site in Japan. Body mass, length, width of fore- and hindwings, wing area, wing aspects, and wing loads were measured for all individuals. Average wing loading was significantly lower, while average wing area was larger, for females from flight-capable populations. The authors of the study created a model, using female wing load and forewing length, that correctly predicted female flight capability of the populations 97% of the time. This information could be especially useful if used in conjunction with reliable molecular identification methods in determining the flight capability of invasive gypsy moth populations.

4.3. Identification of Flight-Capable Populations

No single tried and true technique exists to identify flight-capable AGM populations and individuals. However, the two methods described above show the most promise. The model developed by Shi et al. [76] could be used to predict flight capability of a population (native or introduced) by measuring female forewing length and wing loading. A disadvantage of using this method is that it is necessary to capture an adult female gypsy moth. While male gypsy moths can be captured with the use of traps baited with

a synthetic sex pheromone called "disparlure" [77], female gypsy moths remain elusive. Black-light traps can capture both male and female AGMs but are generally not considered specific enough for detection purposes [25]. Another method of capturing female gypsy moths involves the collection of egg masses, followed by the rearing of juveniles into adulthood [46]. However, the additional time this would take could hamper detection and eradication efforts.

An alternative method of identifying flight-capable gypsy moth populations was employed by Picq et al. [69]. They used single nucleotide polymorphism (SNP) markers to identify the geographic origins of gypsy moth samples. With this method, genetic material could be taken from both female and male moths, to determine geographic origin. This information could then be compared to known flight-capability information for gypsy moth populations. The disadvantage of this is that it does not directly predict flight capacity. A combination of this approach with genetic markers associated with flight by genome-wide association studies, or by functional analyses, could provide solutions in the future [78].

5. Conclusions: Current Challenges and Future Directions

Gypsy moths from many different populations have been and continue to be introduced into novel environments where they have the potential to establish and spread. There is a suite of traits that are of concern because of how they impact the establishment and dispersal potential of gypsy moth populations, including female flight capability, host utilization, and egg chill requirements. It has become clear that these traits differ among populations, even within each subspecies of gypsy moth, so introduction of gypsy moths from other world areas into locations where the species is already present could result in unwanted changes in gypsy moth biology. It also appears that introduction of flight-capable females, even from Europe, could enhance dispersal capability and require modifications to management protocols. Therefore, information on not only the origin of the population but also its biological traits is needed to determine risks associated with new introductions of gypsy moths into a novel habitat. Having a tool to rapidly identify the female flight capability of newly introduced gypsy moths is a critical component of any system instituted to evaluate the risks associated with the introduction but we currently do not have such a tool. Other rapid tools to assess larval host utilization (particularly conifers) and egg chill requirements would also be useful but are not available. Although much is known about gypsy moths from several world areas, we still do not have enough knowledge to adequately plan for or deal with new introductions into novel habitats.

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Article

Captures of Emerald Ash Borer (*Agrilus planipennis*) Adults in Post-Invasion White Ash Sites with Varying Amounts of Live Phloem

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Abstract: Emerald ash borer (EAB), (*Agrilus planipennis* Fairmaire), first identified in 2002 in southeast Michigan, has caused catastrophic ash (*Fraxinus* spp.) mortality in forests within the core of the invasion and has spread to 35 states and five Canadian provinces. Little is known about persistence and densities of EAB populations in post-invasion sites after most ash trees have died. We monitored EAB populations from 2014 to 2016 using double decker (DD) traps set in the midst of white ash (*F. americana*) trees in 30 post-invasion sites in southeast and south-central Michigan. Two DD traps were deployed at each site. One trap had a dark green upper prism and light purple lower prism, both baited with *cis*-3-hexenol lures. The other had two dark purple prisms baited with *cis*-3-hexenol on the upper prism and Manuka oil on the lower prism. In 2014 and 2016, size and condition of ash trees were recorded and area of live white ash phloem was estimated in an 18-m-radius plot centered around each of the DD traps. Area of live white ash phloem per site ranged from approximately 24 to 421 m^2 in 2014 and from 24 to 411 m^2 in 2016. Canopy condition of live white ash trees generally improved; 65% and 89% of the trees had healthy canopies (<20% dieback) in 2014 and 2016, respectively. Traps in 28, 29 and 30 of the sites captured a total of 580, 585, and 932 EAB adults in 2014–2016, respectively. Area of live ash phloem explained relatively little of the variation in total EAB captures in all three years. Low trap catches, along with relatively stable canopy conditions and continued abundance of live white ash, indicate that EAB populations remain below the carrying capacity of the sites, and ash phloem availability is not a limiting factor for EAB abundance. Further monitoring to track both EAB dynamics and tree condition is needed to determine the long-term outlook for white ash in these sites.

Keywords: Emerald ash borer; *Agrilus planipennis*; post-invasion conditions; insect traps; prism trap; *Fraxinus americana*

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1. Introduction

Emerald ash borer (EAB) (*Agrilus planipennis* Fairmaire) (Coleoptera: Buprestidae), a phloem-feeding beetle native to Asia, was first identified in 2002 in North America when adult beetles were collected from dying ash (*Fraxinus* spp.) landscape trees in the Detroit metropolitan area in southeast Michigan, USA [1,2]. An extensive dendrochronological study spanning 1.5 million ha showed that EAB had killed ash trees in southeast Michigan by 1998, indicating it likely became established in this area by the early 1990s [3]. Infestations expanded as EAB adults dispersed and as satellite populations, resulting from inadvertent transport of infested ash nursery trees, logs, firewood and related materials, coalesced. By 2003, EAB-caused ash mortality extended across much of a five-county area in southeastern Michigan [3]. As of February 2021, EAB populations had been found in at least 35 U.S. states and five Canadian provinces [4]. Hundreds of millions of ash in forests,

rural and urban areas have been killed by EAB, which has become the most destructive and costly invasive forest insect in North America [5–7]. In plots established in the original core of the EAB invasion in southeast Michigan, 80 to 99% of green ash (*F. pennsylvanica* Marsh.), black ash (*F. nigra* Marsh.), and white ash (*F. americana* L.) trees in forest overstories had died by the early- to mid-2000s [8–10].

Adult beetles feed on ash leaves throughout their life span and mature females lay eggs under bark flakes or in bark crevices [1,11]. Larvae feed on phloem and cambium in serpentine galleries that typically score the outer sapwood, disrupting translocation of nutrients and water [1,6]. As larval densities build, injury to the vascular system increases, leading to canopy thinning, dieback, and eventual mortality of the tree [1,2,6].

Interspecific differences among North American ash species in EAB host preference and/or resistance can affect ash mortality rates, potentially influencing local EAB persistence or dynamics, and eventual forest composition. Several studies have shown that green ash and black ash are consistently highly preferred and vulnerable EAB hosts, and blue ash (*F. quadrangulata* Michx.) is much less preferred and/or relatively resistant, while white ash appears to be an intermediate host for EAB [7,12,13]. Although nearly all white ash have died in some sites in southeast Michigan [9,10,14], a high proportion of white ash in other forested sites in the same region remains relatively healthy despite more than 12 years of EAB presence. For example, in a recent survey of 28 forested areas in southeast and south-central Michigan, white ash mortality ranged from more than 90% to less than 10% of the trees and basal area per site [15]. In a plantation with 21 randomized blocks consisting of five ash species, all green ash and black ash were heavily colonized, with more than 200 EAB larvae per m^2 of phloem area, most of which died from intraspecific competition for phloem. Larval densities on blue ash and Manchurian ash (*F. mandshurica*), an Asian species, were $\leq$2.0 per m^2, and most trees remained uninfested [13]. White ash trees averaged 41.0 $\pm$ 12 larvae per m^2, and, while a few trees were heavily infested, seven of the 21 white ash had zero to ten larvae per m^2 [13].

Potential density of EAB in a given tree or within an area of interest depends on the availability of live ash phloem to support larval development [16,17]. McCullough and Siegert [18] related ash tree diameter at breast height (DBH) to phloem area and EAB production. Using measurements from 148 green ash and white ash trees ranging from 5.2 to 65.0 cm DBH, they determined that on average, 89 $\pm$ 4.6 EAB adults could potentially develop per m^2 of phloem. Similarly, empirical data collected from debarked trees across multiple sites showed individual EAB larvae each require approximately 10 cm^2 of phloem for development [16–18]. Hence, the number of EAB adults that could potentially be produced in a specific area depends on abundance and size of ash trees.

The carrying capacity for EAB can be orders of magnitude lower in post-invasion areas, i.e., areas where peak EAB infestations occurred several years earlier and most overstory ash have been killed, than in pre-invasion conditions [19–21]. Ash regeneration, including recruits (e.g., young trees 2.5 to 10 cm DBH), saplings, or stump sprouts can be abundant in some, but not necessarily all, post-invasion stands [8,15,22–24]. Although trees as small as 2.5 cm in diameter are occasionally colonized and killed by EAB [1,2,25], they produce relatively few EAB adults [17,18,26], and EAB larvae developing in small trees may be more likely to be killed by parasitoids than larvae in larger trees with thicker bark [27,28]. Whether small ash trees in post-invasion sites provide adequate phloem to sustain an EAB population over time at densities capable of killing trees is not yet clear.

We hypothesized that EAB population levels in a given site would vary with the availability of live ash phloem, which depends on the number and size of live white ash. Various trap designs and lures have been developed and evaluated for detecting and monitoring EAB populations (see reviews in References [29–32]). Green or purple double-decker traps, consisting of upper and lower sticky prisms attached to a 3-m tall PVC pipe and baited with ash volatiles, consistently capture relatively high numbers of EAB and have high detection rates at field sites with very low to high EAB infestation levels [31,33–36]. In a previous study conducted in 24 green ash sites in 2010 and 2011, EAB populations

were monitored with dark purple double-decker traps baited with *cis*-3-hexenol on the top prism and Manuka oil on the bottom prism and with sticky bands on girdled and ungirdled trap trees [19]. Adult EAB captures reflected the east-west temporal gradient of EAB invasion in southern Michigan and were significantly correlated with larval attack densities. In 2010, for example, 13% of EAB adults were captured in post-invasion Core sites in the southeast where most overstory ash were dead, while 12% of adults were captured in recently infested Cusp sites in the southwest. The remaining 75% of EAB adults were captured in south central Crest sites where EAB densities were near peak levels, and a mix of healthy, declining and dying ash were present [19]. Similarly, Poland and McCullough [36] captured more beetles (>100 per trap) on baited DD traps at heavily infested sites with >35% dieback than at sites with moderate infestations and <35% dieback (60–100 beetles per trap), or sites with low EAB population densities and no visible signs of tree infestation (<25 beetles per trap). At a newly infested site with ultra-low EAB infestations levels, only 1–4 beetles were captured on baited green or purple DD traps [35].

We hypothesized that live white ash phloem should represent a critical but limited resource for EAB populations in post-invasion areas. Our objectives were to (1) determine the area of live white ash phloem available for EAB colonization across a range of post-invasion sites that experienced high EAB infestation and overstory mortality at least 10 years previously; (2) assess EAB persistence and relative abundance at the sites using baited DD traps; and (3) determine if EAB trap catches were correlated with white ash phloem availability.

2. Methods

2.1. Site Selection

We used a Michigan county atlas in April 2014 to identify 70 large, forested areas on state, county, or municipal property in southeast and south central Lower Michigan where EAB infestations had peaked at least 8 to 10 years earlier, and nearly all overstory green ash had died. We queried managers of each area to assess white ash presence and abundance. We visited 40 post-invasion sites where managers confirmed the presence of white ash trees and identified 30 areas where white ash trees, whether dead or alive, appeared to be relatively abundant (Figure 1). We established a center point and recorded GPS coordinates in the midst of the white ash trees within each site. Stand conditions, including species composition of overstory trees and regeneration, are presented in detail in Robinett and McCullough [15]. Briefly, white ash trees ranged from 10.0 to 44.0 cm DBH (diameter at breast height), and survival varied substantially among sites, ranging from 0 to 100%. Across all sites, however, 75% of white ash trees, and 66% of the white ash basal area, ranging from 0.3 to 3.5 $m^2 \cdot ha^{-1}$, were alive. Nearly all live white ash had signs of previous EAB colonization, but 83% of trees in 2016 had healthy canopies ($\leq$10% canopy dieback). Green ash trees were recorded in 27 sites, but 92% had been killed by EAB in previous years.

2.2. Fixed Radius Plots

We centered a circular plot with an 18 m radius around each of the two DD traps in each site and recorded DBH of live and dead white ash trees ($\geq$6 cm DBH) during the summer in 2014 and again in 2016. We visually estimated canopy dieback in increments of 10%, where <10% indicated a full, healthy canopy, and $\geq$80% indicated severe decline [37]. Ash trees were grouped by dieback class (0–20%, 21–40%, 41–60%, 61–80%, 81–100%) for some analyses. Each ash tree was examined for signs of EAB infestation, including holes left by woodpeckers preying on EAB larvae, D-shaped adult EAB emergence holes, and bark cracks over old larval galleries. Trees were re-surveyed from early July to mid-August in 2016.

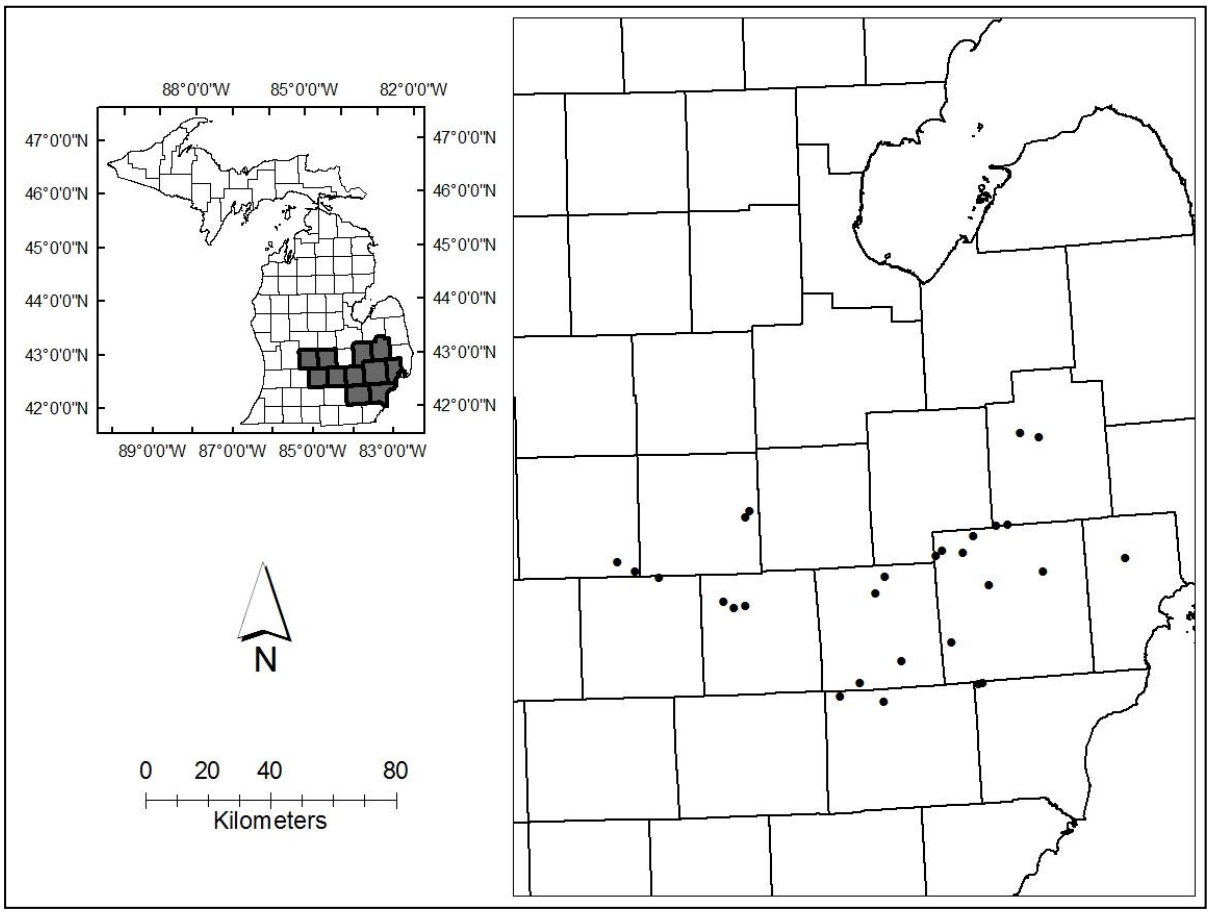

Figure 1. Distribution of 30 white ash sites in southeast and south central Lower Michigan.

Density of white ash stems and white ash basal area ($m^2 \cdot ha^{-1}$) were calculated for each plot and summed for each site in 2014 and 2016. White ash trees were assigned to four DBH classes (6.0–13.0, 13.1–25.0, 25.1–42.0, and 42.1 to 60.0 cm). Area of phloem (m^2) in live and dead white ash trees was calculated using DBH (cm) from trees recorded in 2014 and 2016, following methods of McCullough and Siegert [18]. We also adjusted area of live phloem in individual trees to account for dead portions of the canopy. When dieback estimates were >10%, the estimated live phloem area was reduced by the proportion of the canopy that was dead. Previous studies have shown correlations between canopy decline and larval densities or the portion of phloem covered by larval galleries [12,13,38,39]. We also estimated live phloem area by DBH and canopy dieback classes.

2.3. Trapping

Presence and relative abundance of EAB was assessed at each of the 30 sites over a period of 3 years based on trap captures. We set up two double decker (DD) traps, 36 to 50 m apart, between 24 May and 12 June 2014 in each site. We recorded GPS coordinates at each trap, allowing us to install traps in the same locations between mid-May and mid-June in 2015 and 2016. Traps remained in place through mid-September each year. Each trap was placed in full sunlight, 5–10 m from the edge of wooded areas, or in open areas among scattered ash trees. A DD trap consisted of two coroplast prisms (36 × 60 cm on each side; Great Lakes IPM, Vestaburg, MI, USA) fastened to a 3-m-tall PVC pipe (10 cm diam at 3.0 m and 1.8 m above-ground and coated with clear Pestick™ (Hummert International, Earth City, MO, USA). The PVC pipe was supported by sliding it over a T-post embedded in the ground (34). One DD trap was comprised of a dark green upper prism (Sabic green, λ = 530 nm, 48% relative reflectance) and a light purple lower prism (Sabic purple,

λ = 420 nm 21.7% reflectance; 670 nm 13.6% reflectance [40,41]. Two *cis*-3-hexenol bubble caps (release rate of 3.7 mg/day per bubble cap for a combined release rate of 7.4 mg/day; Contech Enterprises Inc., Delta, BC, Canada) were attached to the lower edge of both prisms. The second DD trap was comprised of two dark purple prisms (λ = 430 nm 20% reflectance, 600 nm 6% reflectance; 670 nm 13.5% reflectance (56). The top prism was baited with two *cis*-3-hexenol bubble caps, while the lower prism was baited with a Manuka oil pouch (release rate of 50 mg/day, Synergy Semiochemicals, Corp., Delta, BC, Canada). Lures were replaced after 6 weeks to ensure consistent volatile release, at which time Pestick™ was also re-applied. The same trap designs were placed in the same locations in all three years.

Captured EAB adult beetles were collected at two- to three-week intervals beginning on 10 June and continuing through mid-September each year. Beetles were typically collected from multiple sites on a given day, but it required approximately a week to check traps at all 30 sites. For each collection period, sites were visited in the same order as when they were set up to ensure uniform time between collection periods. Beetles on each prism of the two traps were bagged separately and returned to the Michigan State University (MSU) Forest Entomology Laboratory, East Lansing, MI USA. Beetles were soaked in Histoclear II (National Diagnostics, Atlanta, GA, USA) to remove Pestick™ and then examined under a microscope to confirm species and sex. We acquired cumulative growing degree days (GDD) (base 10 °C; beginning 1 January annually) corresponding to each collection period from the nearest MSU Enviro-weather station (Enviroweather.msu.edu) at each site. Euclidean distances between each site and the nearest weather station were determined using ArcMap 10.3 and ranged from 5 to 43 km.

2.4. Statistical Analysis

Adult male and female beetles captured per trap (total on both prisms) and per prism were tallied for each collection period and summed by year. Differences in EAB captures were compared among years and between the two trap designs, and among prism colors or lures within trap types, using generalized linear mixed models (PROC GLIMMIX). Main effects of trap type, year, prism color, position (upper or lower), and lure were tested as fixed effects in separate models. Site was a random effect in all models. To account for collinearity among years, an autoregressive order one covariance structure was used for all models, with the Kenward-Rogers modification of the denominator degrees of freedom [42]. Because the response variable was count data, a negative binomial distribution was used with the log link function [43]. Differences among years, trap types, prism color within trap types, and lure within trap types for the separate models were tested with the Tukey-Kramer means comparison procedure. Residuals were tested for homogeneity of variance assumption using Levene's test, and models were adjusted if needed.

Simple linear regression was used to assess whether the area of live white ash phloem (m^2) in the 18 m radius around each trap significantly predicted EAB adult captures within sites (PROC REG). Captures of EAB in 2014, 2015, and 2016 were compared to total area of live phloem measured in 2014, and EAB captures in 2016 were regressed on live phloem area measured in 2016. All analyses were conducted using the SAS 9.4 for Windows statistical package [44] with an α-level of 0.05.

3. Results

3.1. Captures of EAB on Traps

Overall, 580, 585, and 932 EAB adults were captured on the 60 DD traps in 2014, 2015, and 2016, respectively. In 2014, no EAB adults were captured in two sites, while one to 61 beetles were captured in the other 28 sites, with an average of 19 $\pm$ 2.8 beetles captured per site (Figure 2). In 2015, EAB adults were captured in 29 sites; the site with no EAB captures was one of the sites with no EAB captures the previous year. Total beetles captured per site in 2015 ranged from one to 64, with an average of 19 $\pm$ 3.0 per site (Figure 2). Between 2014 and 2015, EAB captures increased by $\geq$50% in 12 sites, remained consistent in four

sites, and decreased by ≥50% in five sites. In 2016, EAB adults were captured in all 30 sites. Total captures ranged from one to 127 EAB adults per site and averaged 31 ± 5.3 beetles per site (Figure 2). Between 2014 and 2016, the number of captured beetles increased by approximately 60% in 14 of the 30 sites.

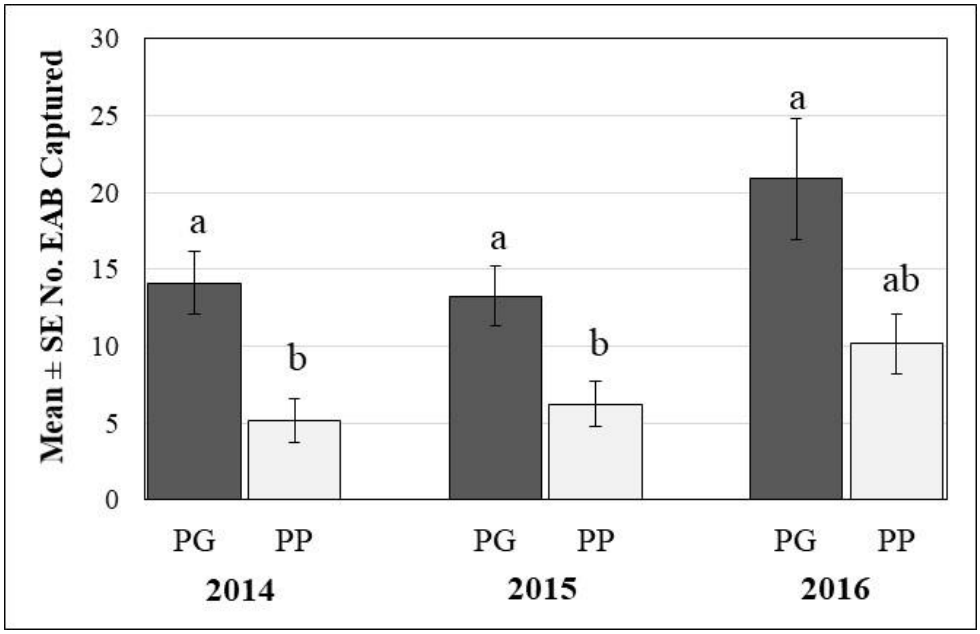

Figure 2. Mean (+SE) number of emerald ash borer adults captured per trap in 2014, 2015, and 2016 on green-purple (GP) double decker (DD) traps and purple-purple (PP) DD traps. Bars topped by the same letter are not significantly different; Tukey-Kramer means comparison procedure, $p > 0.05$.

Cumulative growing degree days (GDD) (base 10 °C/50 °F) associated with the first trap checks in June in each site ranged from a minimum of 347 to a maximum of 538 $GDD_{10\,°C}$ in 2014–2016. Peak EAB activity occurred in late June to early July each year, corresponding to a minimum of 521 to a maximum of 789 $GDD_{10\,°C}$ in 2014–2016, when 54%, 52%, and 62% of the total EAB adults were captured, respectively. Following this peak, EAB captures decreased by 48% and 39% in 2014 and 2015, respectively, and by 85% in 2016. Traps captured ≤21 EAB total during our final checks, which occurred between 17 and 23 September each year.

Cumulative number of EAB adults captured throughout the season differed between the two trap designs ($F = 8.86$; degrees of freedom [df] = 1.138; $p < 0.003$) and among years ($F = 6.08$; $df = 2.42$; $p = 0.005$), but the interaction was not significant ($F = 0.46$; $df = 2.42$; $p = 0.6$). Overall, for all three years combined, traps with green upper and light purple lower prisms (GP) caught significantly more EAB adults (mean = 15.75 ± 3.0) than traps with two dark purple prisms (PP) (mean = 6.85 ± 1.4), accounting for 73%, 68%, and 67% of total captures in 2014, 2015, and 2016, respectively. Significantly more EAB adults were captured in 2016 for both trap types combined (mean = 14.54 ± 2.4) than in 2014 (mean = 8.5 ± 1.4) or in 2015 (mean = 9.06 ± 1.5) (Figure 2).

Prism color and lures contributed to differences in trap captures between the two DD trap designs; however, prism color, position, and lures were confounded, making it difficult to fully determine their relative importance for EAB attraction. Overall, green or light purple prisms baited with *cis*-3-hexenol lures captured more EAB adults than dark purple prisms baited with either *cis*-3-hexenol or Manuka oil ($F = 15.50$; $df = 3.87$; $p < 0.001$). Prism position (upper or lower) did not have a significant effect on trap captures ($F = 3.34$; $df = 1.87$; $p = 07$).

Male beetles dominated captures on both trap designs in all three years. Overall, 70%, 69%, and 77% of the EAB adults captured in 2014, 2015, and 2016, respectively, were males. Across all years, green prisms caught significantly more EAB males than either upper or lower dark purple prisms, while captures on light purple prisms were intermediate ($F = 14.4$; $df = 3.48$; $p < 0.0001$). Light purple prisms on the GP traps caught significantly more EAB females than either of the dark purple prisms on the PP traps or the upper green prisms on the GP traps ($F = 13.3$; $df = 3.348$; $p < 0.0001$). Significantly more EAB males ($F = 7.50$; $df = 2.348$; $p = 0.0006$) and females ($F = 6.06$; $df = 3.348$; $p = 0.003$) were captured in 2016 than in 2015 or 2014 (Table 1).

Table 1. Mean (± SE) number of emerald ash borer (EAB) adult males and females captured per prism on double-decker (DD) traps with green upper and light purple lower prisms (GP) or dark purple upper and lower prisms (PP) in 2014, 2015, and 2016 in 30 sites located in southeast and south central Lower Michigan ($N = 30$).

		Double Decker (GP) Trap		Double Decker (PP) Trap		*F; df; p*
		Green Upper	**Light Purple Lower**	**Dark Purple Upper**	**Dark Purple Lower**	
2014	Males	7.1 ± 1.2a [1]	4.5 ± 0.9ab	2.2 ± 0.5b	1.6 ± 0.6b	6.21; 3.116; 0.0006
	Females	0.7 ± 0.2ab	1.9 ± 0.4a	0.6 ± 0.2b	0.7 ± 0.3b	8.73; 3.116; <0.0001
	Total	7.8 ± 1.3a	6.4 ± 1.1a	2.9 ± 0.7b	2.3 ± 0.9b	8.76; 3.116; <0.0001
2015	Males	5.5 ± 1.1a	4.1 ± 0.8a	2.3 ± 0.6ab	1.3 ± 0.4b	4.72; 3.116; 0.004
	Females	1.4 ± 0.3ab	2.3 ±o.4a	1.4 ±0.3ab	1.1 ± 0.3b	2.56; 3.116; 0.05
	Total	6.9 ± 1.3a	6.4 ± 1.1ab	3.8 ± 0.9ab	2.4 ± 0.6b	4.40; 3.116; 0.006
2016	Males	8.7 ± 2.0a	7.9 ± 1.6a	4.6 ± 0.9ab	2.8 ± 0.8b	4.11; 3.116; 0.008
	Females	1.0 ± 0.2b	3.3 ±0.7a	3.9 ± 0.8ab	1.0 ± 0.3b	5.81; 3.116; 0.001
	Total	9.8 ± 2.1a	11.2 ± 2.2a	6.3 ± 1.2ab	3.9 ± 0.9b	4.49; 3.116; 0.005
All years	Males	7.1 ± 0.8a	5.5 ± 0.7ab	3.1 ± 0.4b	1.9 ± 0.3b	14.4; 3.348; <0.0001
	Females	1.0 ± 0.1b	2.5 ± 0.3a	1.2 ± 0.2b	0.9 ± 0.3b	13.3; 3.348; <0.0001
	Total	8.1 ± 0.9a	7.8 ± 0.9a	4.3 ± 0.6b	2.9 ± 0.5b	16.3; 3.348; <0.0001

[1] Means within the same year and sex followed by the same letter are not statistically different; Tukey-Kramer means comparison procedure ($p > 0.05$).

3.2. White Ash Phloem

In 2014, we recorded a total of 1109 white ash trees (≥6 cm DBH) across the 30 sites, including 736 live and 373 dead trees. Most (87%) of the live white ash had <50% canopy dieback and 477 trees had <20% dieback (Table 2). Conversely, 44 trees were severely declining with ≥80% dieback (Table 2). External signs of EAB infestation were apparent on 58% of the live white ash, including 78% of trees with <50% dieback and 43 of the 44 trees with ≥80% dieback. Nearly all (98%) of the dead trees had clearly been killed by EAB, as evidenced by abundant galleries and sloughing bark.

Overall, white ash trees across the 30 sites represented an estimated 3627 m^2 of phloem area, which included 1794 m^2 (49.5% of the total) in the live trees tallied in 2014. Within sites, phloem area in live white ash trees averaged (± SE) 120.9 ± 17.51 m^2 and ranged from 24.4 to 421.0 m^2. Small ash (6.0 to 13.0 cm DBH) contributed approximately 685 m^2 of phloem. Live trees in the two largest size classes (25.1 to 42.0 and 42.1 to 60.0 cm DBH) contributed 279 m^2 of phloem, while pole-sized trees (13.1 to 25.0 cm DBH) contributed the remainder. On average, an estimated 10,761 ± 1558 EAB adult beetles per site could have potentially developed if all the phloem available in 2014 was colonized, ranging from a minimum of 1673 beetles at the site with the least phloem to a maximum of 37,466 beetles at the site with the highest amount of white ash phloem. When we adjusted phloem area to account for dead portions of the canopy, total live phloem decreased to 1427 m^2. This included 557 m^2 of live phloem within trees in the smallest DBH class (6.0 to 13.0 cm), 675 m^{2i}n pole-sized trees (13.1 to 25.0 cm), and 195 m^2 of live phloem in trees in the two largest DBH classes (25.1 to 42.0 and 42.1 to 60.0 cm).

Table 2. Number of live and dead white ash (*F. americana*) trees and estimated area of phloem (m^2) recorded for trees in fixed radius plots (18-m radius) in 30 sites in Michigan in 2014 and 2016. Phloem area was calculated following methods of McCullough and Siegert [18] and adjusted to account for the proportion of the canopy that was dead.

Percent Canopy Dieback Class	No. Trees	Total Phloem (m^2)	Adjusted Live Phloem (m^2)
2014			
<10.0%	353	829	829
10.0 to 19.9%	124	248	233
20.0 to 39.9%	131	307	232
40.0 to 59.9%	49	151	80
60.0 to 79.9%	35	106	37
80.0 to 99.0%	44	154	20
Dead	373	1833	0
2016			
<10.0%	608	1300	1300
10.1 to 19.9%	54	138	124
20.0 to 39.9%	50	119	90
40.0 to 59.9%	14	72	39
60.0 to 79.9%	17	48	16
80.0 to 99.9%	14	58	8
Dead	430	1837	0

In 2016, we again established plots (18 m radius) and measured DBH of white ash trees surrounding the traps. A total of 1187 white ash trees were tallied across the 30 sites, including 743 live and 430 dead trees (Table 2). The slight increase in number of trees between 2014 and 2016 reflected growth of young trees that were > 6 cm DBH in 2016 but not in 2014, as well as minor differences in plot boundaries between the two years. Overall, 96% of the live trees had < 50% canopy dieback and 662 trees, including most of the small trees, had $\leq$ 10% dieback (Table 2). Fourteen trees were heavily infested and severely declining with $\geq$ 80% canopy dieback (Table 2). External signs of previous EAB infestation were apparent on 71% of the live white ash and 99% of the dead trees. All of the severely declining trees were or had been heavily infested and 94% of the 118 trees with 10 to 50% dieback had external signs of past EAB infestation.

In 2016, white ash trees (live and dead) represented a total of 3596 m^2 of phloem area, including 1759 m^2 (48.5%) of phloem in the live trees. Phloem area of live white ash within sites averaged ($\pm$SE) 119.0 $\pm$ 16.44 m^2 and ranged from 24.4 to 410.9 m^2. Live trees in the smallest DBH class (6.0 to 13.0 cm) accounted for 729 m^2 of the phloem recorded, and trees in the two largest DBH classes (25.1 to 60.0 cm) accounted for 259 m^2 of phloem, while pole-sized trees (13.1 to 25.0 cm DBH) provided the remainder. Live phloem area decreased between 2014 and 2016 in 13 sites, increased in 11 sites, and differed by < 1.5 m^2 in six sites. On average, an estimated 10,881 $\pm$ 1434.9 EAB adults could have developed if all phloem available in 2016 were fully colonized, ranging from a minimum of 2095 at the site with the lowest amount of white ash phloem to a maximum of 36,567 beetles at the site with the most phloem available. After adjusting phloem to account for dead portions of the canopy, total live phloem decreased to 1457 m^2. Trees in the smallest DBH class (6.0 to 13.0 cm) provided 636 m^2 of live phloem, and pole-sized trees (13.1 to 25.0 cm) contributed another 620 m^2 of live phloem, while trees in the two largest DBH classes (25.1 to 42.0 and 42.1 to 60.0 cm) contributed 201 m^2 of live phloem.

Levels of EAB infestation and tree condition ranged from sites in which nearly all trees were alive and appeared relatively healthy to sites where $\geq$90% of the white ash were dead [15]. Linear regressions between live phloem area, adjusted for dieback, and numbers of EAB captured on traps were run after excluding one site with an unusually high amount of live phloem. Linear relationships between area of live white ash phloem derived from

the 2014 surveys and the number of EAB adults captured on traps were significant in 2014 (F = 4.86; p = 0.0361) and 2015 (F = 5.83; p = 0.0229) but explained less than 20% of the variation in EAB captures (Figure 3). Captures of EAB adults increased substantially in 2016 but were not significantly related to live phloem area measured in 2014 (F = 4.10; p = 0.0529) nor measured in 2016 (F = 2.90; p = 0.1001) (Figure 3).

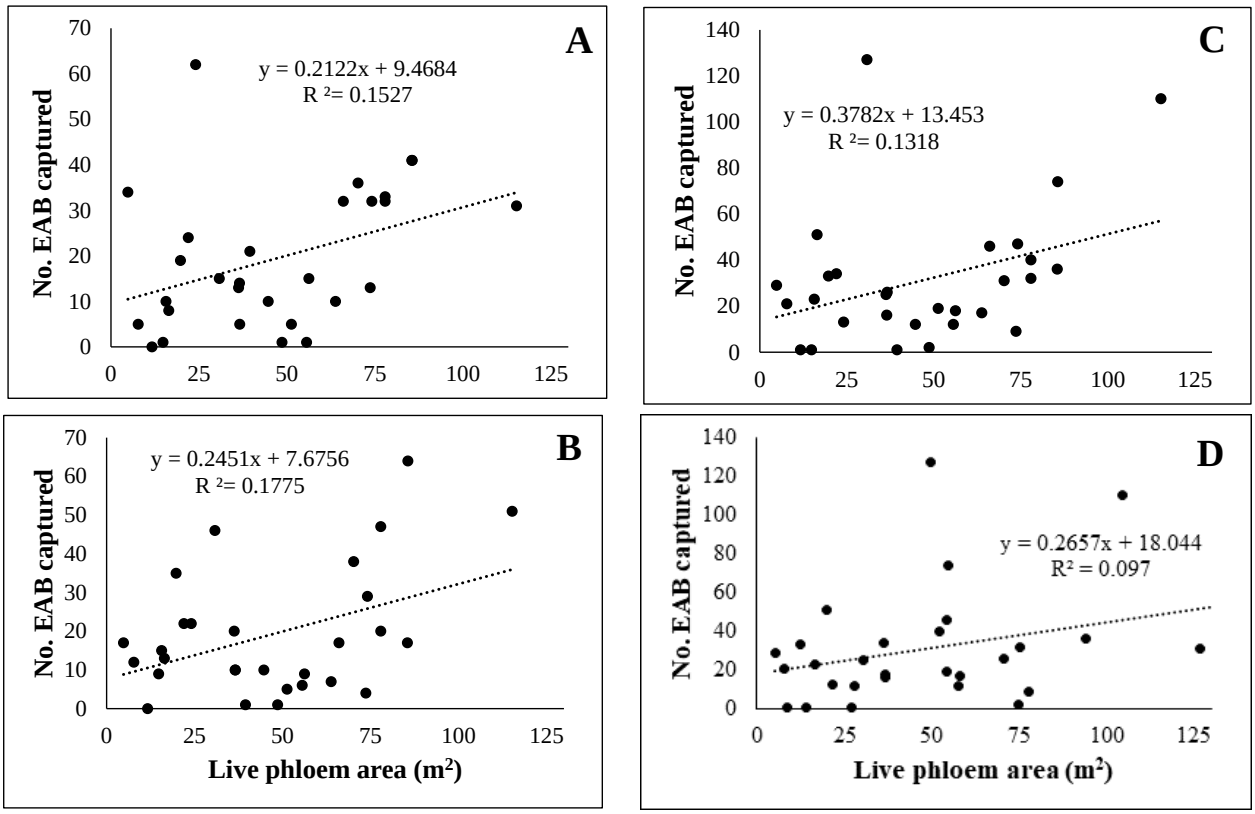

Figure 3. Linear relationship between emerald ash borer (EAB) adult captures in (**A**) 2014, (**B**) 2015, and (**C**) 2016 and adjusted area of live white ash phloem (m^2) surrounding traps (18 m radius) measured in 2014 and between EAB adult captures in 2016 (**D**) and adjusted area of live white ash phloem (m^2) measured in 2016. Phloem area (m^2) of tallied trees was estimated following methods of McCullough and Siegert [18]; then, it was reduced by the proportion of the canopy that was dead.

4. Discussion

Populations of both EAB and white ash trees continue to co-exist in southeast and south central Lower Michigan more than a decade after these forests were invaded. Across the 30 sites, the level of EAB infestation and condition of white ash trees varied from locations where nearly all trees were alive and in relatively good condition, to areas where ≥90% of the white ash were dead [19]. Even in areas where ash mortality was high, however, a substantial amount of live phloem remained available for EAB colonization; estimated area of live phloem per site ranged from 24.4 to 421.0 m^2 in 2014 and from 24.4 to 410.9 m^2 in 2016. Between 2014 and 2016, 57 trees that were seriously declining in 2014 had died, but, overall, live phloem area was only 3.5% lower in 2016 than in 2014. Moreover, across sites, canopy condition of live trees generally improved. For example, 65% and 89% of the live trees had healthy canopies with <20% dieback in 2014 and 2016, respectively.

We originally hypothesized that if availability of live phloem was a limiting resource for larval development, fewer EAB adults would be captured on traps in sites where ash mortality was high than in sites where live ash phloem was abundant. Relationships between EAB captures and live phloem area, adjusted to account for canopy dieback, were significant, but live phloem area explained less than 20% of the variation in captures in any

year. Overall, results indicate that phloem availability was not a limiting factor driving EAB population levels and trap captures at our sites. Similarly, McCullough et al. [35] found that abundance of ash phloem near traps had no discernable effect on EAB captured on traps at sites with low population densities. They reported that position of traps and sun exposure had only weak effects, while trap type had a significant effect on the number of EAB captured. Additionally, Mercader et al. [45] found little relationship between EAB captures on baited purple prism traps suspended from branches of ash trees and local EAB densities across a large, recently infested area with low EAB populations.

While availability of live phloem was nearly the same in our sites in 2014 and 2016, captures of EAB adults increased significantly in 2016 compared to the previous two years. More dead trees were tallied in 2016 than in 2014, reflecting mortality of the 57 trees that were seriously declining in 2014. However, canopy condition of live trees generally improved; the number of trees tallied in each dieback class (>10% to >80% dieback) decreased between 2014 and 2016. Therefore, increased trap catches in 2016 did not appear to be related to live phloem available or increased levels of canopy dieback within the sites. It is possible that some portion of the EAB adults captured on our traps developed on trees beyond our plots and immigrated into our sites. While most EAB colonize ash trees within 100 to 200 m of their point of emergence when phloem is abundant [46,47], mature adult females are physiologically capable of dispersing several kilometers [48,49]. Ash trees up to 800 m from the emergence point of adults have been colonized [46–48,50], and adults have been captured on traps at least 300 m away from the nearest ash trees [33].

An alternative explanation is that larvae from eggs laid by beetles in 2014 required two years to complete development on the relatively healthy trees in our plots and were captured as adults in 2016. Previous studies have shown that, on recently infested, healthy trees where larval densities remain low, a high proportion of larvae overwinter as early instars the first year, feed and overwinter as prepupae the second winter, and then emerge as adults the following summer [1,48,51]. If most beetles captured in 2016 developed on trees within the plots, EAB densities may have been building, although signs of infestation and canopy decline did not increase between 2014 and 2016.

Despite the increase in EAB adult captures in 2016, the number of beetles captured per trap was still relatively low and indicative of very low population levels. For example, McCullough et al. [35] captured an average of approximately 1 to 4 EAB on baited green or purple DD traps in a newly infested forest where the EAB population was ultra-low, trees had no visible signs of EAB infestation, and mean larval densities were approximately 2 larvae per m^2 and 12 larvae per m^2 in non-girdled and girdled trees, respectively. Similarly, <25 beetles were captured on baited DD traps at sites with low EAB population densities and no visible signs of tree infestation, while approximately 60–100 beetles were captured at sites with moderate infestations and <35% canopy dieback, and >100 EAB were captured at heavily infested sites with >35% dieback [36]. In heavily infested sites where canopy dieback ranged from 35–50%, green or purple prism traps hung 1.5 m or 13 m above ground captured >60 and >100 beetles per trap, respectively [52,53]. In our sites, trap catches averaging <20 beetles per trap indicate population levels were very low, and few visible signs of infestation and little canopy decline would be expected.

Estimating the area of phloem available for EAB development provides a means to estimate potential EAB production for a specific location and to assess pre- and post-invasion dynamics of a local EAB population. McCullough and Siegert [18] found that, on average, roughly 89 EAB could successfully develop and emerge per m^2 of phloem surface area, and that EAB production averaged 68.8 $\pm$ 5.9 beetles per m^2 on small trees (2.5–13 cm DBH) and 105.3 $\pm$ 5.7 beetles per m^2 on larger trees (DBH > 13 cm). We estimated that approximately 10,761 $\pm$ 1558.4 and 10,881 $\pm$ 1434.9 EAB adults could have potentially emerged across the sites based on the trees recorded and phloem area calculated in 2014 and 2016, respectively. However, low trap catches even in 2016, along with relatively stable canopy conditions and continued abundance of live white ash, indicate that EAB populations have not come close to achieving projected levels in most of the sites.

Persistence of both EAB populations and live white ash in the post-invasion sites we monitored may reflect multiple factors, including host preference and oviposition behavior of adult females. Previous research with ash trees in common gardens or plantations, landscapes, and forests has consistently shown that, when green ash and white ash tree co-occur, EAB females will preferentially oviposit on green ash trees [7,12,13,38]. In the large forested parks and game areas where our study was conducted, most overstory green ash were killed by EAB several years ago, but green ash regeneration, including recruits, saplings, and stump sprouts, remains abundant in many areas [8,10,22–24]. Some portion of female beetles that developed on white ash trees in our plots may have dispersed and oviposited on young green ash. Additionally, while DBH of live white ash trees in our sites ranged from 6.0 to 40.6 cm, many trees were small, with a DBH of $\leq$ 13 cm, and some were likely too small to have been colonized when local EAB populations were peaking in the early and mid-2000s [3,8,10,19]. When young white ash trees are colonized by EAB, females typically lay most eggs on the trunk or around branch junctions where rough-textured bark is present, avoiding the relatively smooth bark on branches and upper portions of the trunk [54]. If larvae feed predominantly on the trunk of small trees, mortality typically occurs rapidly compared to the gradual decline and dieback characteristic of larger trees where feeding begins on canopy branches. However, with the exception of 57 severely declining trees that died, most white ash trees tallied in our sites appeared healthy in 2014 and remained healthy in 2016 [15]. Further, more than half of the white ash trees we tallied, including many small trees, had holes left by woodpeckers preying on late instar EAB larvae, exit holes left by EAB adults, or old larval galleries, indicating these trees were suitable hosts.

Natural enemies of EAB larvae, including woodpeckers [55–57], native parasitoids [54,57], and introduced parasitoids native to Asia [58,59], could be playing a role in keeping EAB populations below carrying capacity within these sites. In study sites in southern Michigan where two introduced parasitoids, *T. planipennisi* and *O. agrili*, have been established since 2008, healthy ash saplings (400–1600 per ha) and young trees (200–900 per ha) are persisting despite formerly high EAB populations that killed most overstory ash [28]. Life table analyses of EAB population dynamics at these sites indicate *T. planipennisi* killed 36%–85% of the late instar larvae in saplings, contributing to net population growth rate of EAB that was near or below replacement levels. Another study in southeast Michigan indicated ash mortality was lower, while ash regeneration and diameter growth were generally higher in post-invasion sites with parasitoid releases compared to control sites [60]. Based on the increasing trend in EAB trap catches between 2014/2015 and 2016, it is difficult to predict whether populations will continue to build in our study sites. Additional years of monitoring to track both EAB dynamics and tree condition in these sites would be helpful to project the long term outlook for white ash in these sites.

The two double-decker (DD) trap designs we used to assess EAB presence and relative infestation levels in our sites also provided useful information on beetle attraction to the green and purple prisms. Overall, the GP (green-light purple) DD traps baited with *cis*-3-hexenol on both prisms captured significantly more EAB beetles than the PP (dark purple-dark purple) traps baited with *cis*-3-hexenol on the upper prism and Manuka oil on the lower prism. Males were captured in significantly higher numbers on the upper green panels, while the lower light purple panels captured significantly more females. In another study with DD traps, Poland et al. [31] similarly reported that GP traps captured significantly more females, males, and total EAB than did PP traps, but differences were minimal between traps with *cis*-3-hexenol lures on both prisms compared to traps with *cis*-3-hexenol on the upper prism and Manuka oil on the lower prism. Previous studies have shown that males were more attracted than females to green prisms, while females were more attracted than males to purple prisms [31,41,53,61–64]. Males, which tend to hover around the canopies of ash trees and then land on leaves or approach females feeding in the canopy [65,66], are presumably attracted to colors and volatiles associated with ash leaves. In contrast, purple wavelengths, which are similar to the spectra associated with

tree bark, attract females who likely use visual cues to locate suitable oviposition sites on branches and trunks of ash trees [66].

Multiple studies across dozens of sites have shown detection rates (e.g., capture of ≥one EAB adult) or total EAB adult captures (standardized by trapping surface area) are higher on DD traps baited with *cis*-3-hexenol or a combination of *cis*-3-hexenol and Manuka oil than on baited purple or green prisms or multiple funnel traps hung from ash branches [33–36,40,67]. Furthermore, DD traps tend to have lower variation in trap catches within sites and among sites with different levels of EAB infestation [31,40]. In contrast, the number of EAB captured in traps hung in the canopy of ash trees is substantially more variable and may reflect the infestation level of the individual trap-bearing trees or nearby trees [31,36,45].

Emergence of EAB adults typically begins in mid-May in southern Michigan when accumulated growing degree days (GDD) (base 10 °C) reach approximately $250_{10\,^{\circ}C}$ [68]. As in previous studies, EAB adult captures peaked in late June to early July in all three years [33,68,69], but, in 2016, the proportion of EAB adults (approximately 29%) captured during our first trap check beginning June 10 was twice as high as in the previous two years (approximately 13%). We suspect this pattern was driven by early spring weather. For example, on 1 June, a total of 215, 254, and 248 growing degree days (base 10 °C) had accumulated in Commerce Township in southeast Michigan in 2014, 2015, and 2016, respectively. On 1 April, however, at this same location, a total of 1.9 and 4.6 growing degree days (base 10 °C) had accumulated in 2014 and 2015, respectively, compared to 29.7 degree days in 2016. A period of unusually warm weather in March 2016 likely enabled many larvae to begin pupating, complete development, and emerge as adults relatively early in the summer.

Our results highlight the need for long-term monitoring to track persistence and dynamics of native ash species and EAB populations in post-invasion sites in North America. While nearly complete mortality of overstory white ash trees has certainly been documented in areas of southeast Michigan [9,10,70], in many sites we evaluated, EAB populations appear to be persisting at densities below carrying capacity, and white ash are growing and presumably reproducing. Whether this will continue over time remains to be seen, especially if trees become stressed by severe drought or other unfavorable conditions that would increase their attractiveness and vulnerability to EAB [2,68–71].

If EAB populations build and white ash dieback and mortality begin to increase, releasing parasitoids for EAB biological control could perhaps help protect remaining trees and regeneration. Research on individual "lingering" green ash trees that survived longer than surrounding trees or resistance mechanisms of Asian ash species is ongoing [72], but there has been comparatively little effort to address white ash resistance to EAB. Furthermore, practical aspects of establishing resistant ash in forested settings, such as the need for herbicide applications to reduce competing vegetation, protection from deer browse, and probably irrigation, may limit the viability of this approach in many areas. A comprehensive, area-wide integrated strategy that could include periodically treating a portion of trees with a highly effective systemic insecticide, biological control releases, and perhaps use of girdled ash "trap trees" would reduce EAB density, population growth, and ash mortality [73], preserving white ash as a functional component in these forests [7].

5. Conclusions

Our results demonstrate that white ash trees are persisting in several forested sites in the core of the EAB invasion in southeast and south central Michigan more than a decade after invasion. Approximately 60% to 70% of the live white ash had evidence of past EAB attacks and some trees with severe canopy dieback in 2014 were dead by 2016. Nevertheless, most trees had relatively healthy canopies (<20% dieback), and canopy condition across the 30 sites generally improved between 2014 and 2016. Low EAB trap catches along with the abundance of live white ash and relatively stable canopy conditions indicate that EAB populations remain below the carrying capacity of the sites, and ash

phloem availability is not a limiting factor for EAB abundance. Further monitoring to track both EAB dynamics and tree condition is needed to determine the long-term outlook for white ash in these sites.

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Diversity of *Phytophthora* Communities across Different Types of Mediterranean Vegetation in a Nature Reserve Area

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Abstract: Research Highlights: Protected natural areas are a reservoir of *Phytophthora* species and represent the most suitable sites to study their ecology, being less disturbed by human activities than other environments. Background and Objectives: The specific objective of this study was to correlate the diversity and distribution of *Phytophthora* species with the vegetation in aquatic, riparian and terrestrial habitats within a protected area in Eastern Sicily, Southern Italy. Materials and Methods: Environmental samples (water and soil) were sourced from two streams running through the reserve and six different types of vegetation, including *Platano-Salicetum pedicellatae*, the *Sarcopoterium spinosum* community, *Myrto communis-Pistacietum lentisci*, *Pistacio-Quercetum ilicis*, *Oleo-Quercetum virgilianae* and a gallery forest dominated by *Nerium oleander* (Natura 2000 classification of habitats). *Phytophthora* species were recovered from samples using leaf baiting and were classified on the basis of morphological characteristics and sequencing of internal transcribed spacer (ITS) regions of ribosomal DNA (rDNA). Results: As many as 11 *Phytophthora* species, within five different ITS clades, were identified, including *P. asparagi*, *P. bilorbang*, *P. cryptogea*, *P. gonapodyides*, *P. lacustris*, *P. multivora*, *P. nicotianae*, *P. oleae*, *P. parvispora*, *P. plurivora* and *P. syringae*. No *Phytophthora* species were found in the *Sarcopoterium spinosum* comm. *Phytophthora asparagi*, *P. lacustris* and *P. plurivora* were the prevalent species in the other five plant communities, but only *P. plurivora* was present in all of them. Overall aquatic species from clade 6 (100 out of 228 isolates) were the most common; they were recovered from all five types of vegetation, streams and riparian habitats. *Phytophthora* populations found in the *Platano-Salicetum pedicellatae* and *Oleo-Quercetum virgilianae* show the highest diversity, while no correlation was found with the physicochemical characteristics of the soil. *Conclusions:* The vegetation type and the aquatic or terrestrial habitat were identified as major environmental factors correlated with the diversity of *Phytophthora* communities in this reserve.

Keywords: leaf baiting; rDNA ITS regions; soil; water; ITS clades; Mediterranean vegetation; ecology; soil inhabitants; aquatic species

1. Introduction

The genus *Phytophthora* (Pythiaceae, Peronosporales, Oomycota, Chromista) comprises to date over 180 described species while, according to a conservative estimate, the actual number of species in this genus is at least double, if not triple [1,2]. Many *Phytophthora* species, such as *Phytophthora infestans*, *P. cinnamomi* and *P. ramorum*, are destructive plant pathogens causing severe crop losses and tree decline worldwide [3–10]. Most plant pathogenic *Phytophthora* species are polyphagous, with a host range encompassing plants of different families [11–15], and are typically soil inhabitants, although a number of them that produce deciduous sporangia have partially or temporarily adapted to an aerial lifestyle [16]. A more restricted number of species in the phylogenetic ITS clade 6 recovered from water courses, lakes and irrigation basins are functionally more adapted to aquatic habitats [17–21]. In general, these aquatic *Phytophthora* species are weakly aggressive as plant pathogens and, consequently, it has been assumed they behave as saprotrophs in plant debris in water and as opportunistic pathogens in riparian habitats. However, their ecological role in ecosystems is not fully understood.

Human-mediated transport, mainly the trade of nursery plants, has been identified as a major pathway for the introduction of non-native *Phytophthora* species into new areas [22–26]. It was demonstrated, e.g., that there is a causal link between the ornamental plant industry and the introduction of the destructive oak pathogen *P. ramorum* in the wildland in North America [27]. The use of nursery plants for forest restoration and afforestation is a way to introduce and spread exotic *Phytophthora* species in natural habitats and forests [28–30]. In a survey of protected natural areas in Sicily, 13 out of 20 *Phytophthora* recovered species were putatively exotic and only seven could be considered endemic to Europe [31]. In many cases, *Phytophthora* species found in forests and natural or naturalized ecosystems included aggressive plant pathogens of cultivated plants, suggesting that these ecosystems may act, in turn, as potential sources and reservoirs of *Phytophthora* inoculum for agricultural crops [32]. As a consequence, monitoring of forest and natural ecosystems should be included in *Phytophthora* species surveillance and biosecurity schemes. The establishment of alien invasive *Phytophthora* species in natural ecosystems has destabilizing effects as it affects the ecosystem homeostasis and resilience. The invasion of natural and semi-natural ecosystems by these pathogens may endanger native and rare plant species and are a threat to the diversity of plant communities [27,32–34]. Hence, the knowledge of resident *Phytophthora* populations should be a prerequisite for a rational management strategy of protected natural areas (PNAs).

The refinement of baiting and sampling methods, together with rapid advances in molecular diagnostics and DNA-sequencing technology [35–54], facilitated the detection of *Phytophthora* in environmental samples and stimulated the study of *Phytophthora* communities in forest and natural ecosystems all over the world, including watercourses and still unexplored areas [55–67]. These surveys revealed the richness of *Phytophthora* diversity in these ecosystems and led to the discovery of an impressive number of cryptic new species in this genus. They contributed to a better understanding of the global diversity of *Phytophthora,* the geographic radiation pathways of single *Phytophthora* species and clades from their centers of origin, their lifestyle and, in particular, their reproductive behavior and adaptation to different environments [2]. Despite numerous surveys, the environmental factors shaping the *Phytophthora* populations and conditioning their compositional changes in the wild have been poorly investigated. However, PNAs, being less disturbed by human activities, are the most suitable context for studying the ecology of *Phytophthora* species. The main objective of this study was to investigate whether the diversity of *Phytophthora* species and their frequency and spatial distribution across a small nature reserve in Sicily (Southern Italy) are correlated with the type of vegetation and the preferential habitat.

2. Materials and Methods

2.1. Sampling Area

Sampling activities were carried out in the "Complesso Speleologico Villasmundo—S. Alfio" regional nature reserve (strict nature reserve), part of the Special Areas of Conservation (SAC) (ITA090024) "Cozzo Ogliastri" in the municipality of Melilli (Sicily, Italy) (Figure 1). The reserve is managed by the CUTGANA (University of Catania) and was established in 1998 in order to protect one of the most important karst systems of the Hyblean area. The reserve covers a surface of 72 hectares in the north-eastern sector of the Climiti Mountains, between the "Belluzza" and "Cugno di Rio" streams. Along the river "Cugno di Rio", there are cave entrances (A zone, the core zone of the reserve). The buffer zone of the reserve (B zone) hosts several natural ecosystems of great importance.

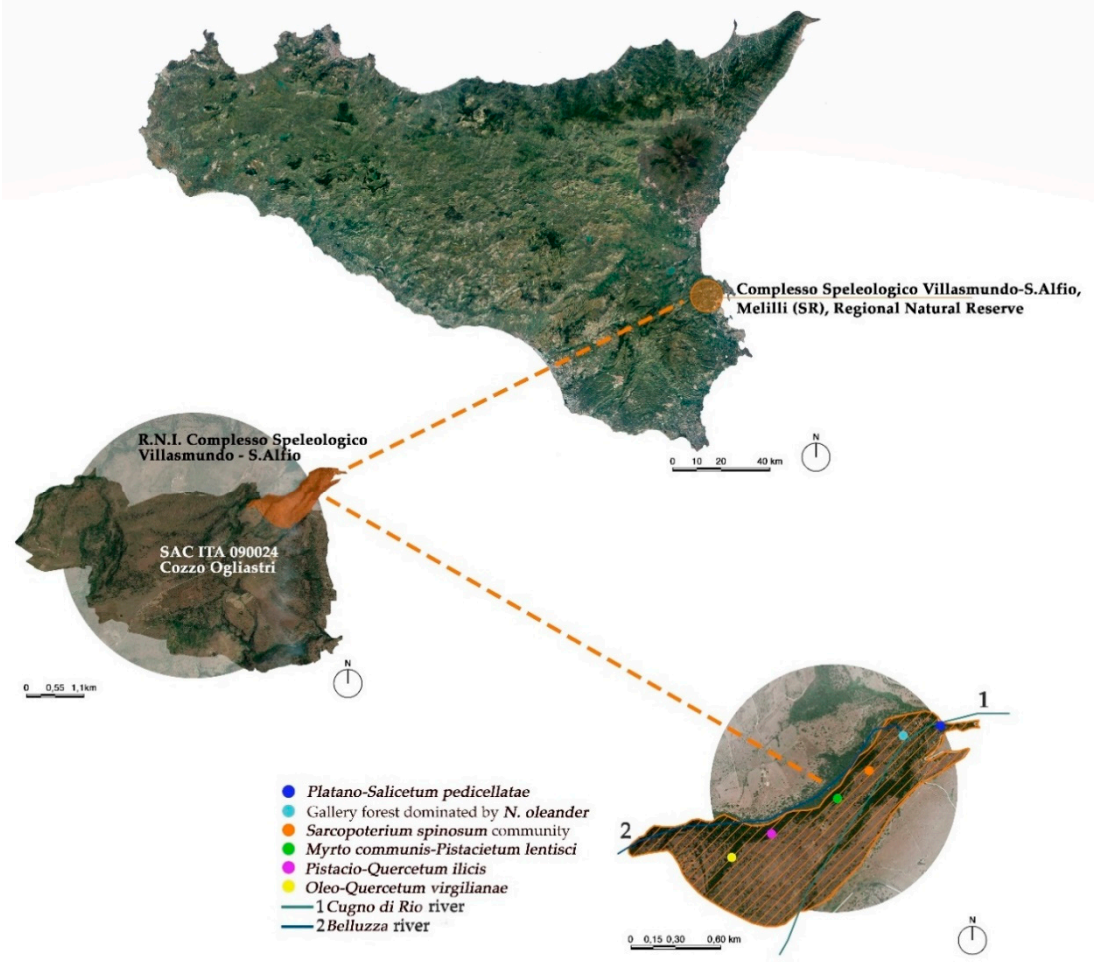


Figure 1. Geographical location of "Complesso Speleologico Villasmundo—S. Alfio" regional nature reserve (RNR) and plant communities within the reserve.

The plant communities and their respective habitats (according to the Habitats of Directive 92/43/EEC) where samples were collected are located in the B zone of the reserve and are described herein. (i) *Platano-Salicetum pedicellatae* Barbagallo, Brullo and Fagotto (Nature CODE 92C0 I): forests and woods, prevalently riparian, dominated by oriental plane (*Platanus orientalis* L.) and willow (*Salix pedicellata* Desf.), probably a relic of a more extended plane tree wood. (ii) *Sarcopoterium spinosum* community (Nature CODE 5420): low, thorny formations of hemispherical shrubs of the coastal thermo-Mediterranean zone of Aegean islands, of mainland Greece and the Ionian islands, of coastal Anatolia, much more widespread and diverse than the Western Mediterranean formations. (iii) *Myrto*

communis-Pistacietum lentisci (Molinier) Rivas-Martínez (Nature CODE 9320): thermo-Mediterranean woodland dominated by arborescent mastic (*Pistacia lentiscus* L.) and myrtle (*Myrtus communis* L.). (iv) *Pistacio-Quercetum ilicis* Brullo and Marcenò (Nature CODE 9340): Mediterranean oak stand characterized by holm oak (*Quercus ilex* L.). (v) *Oleo-Quercetum virgilianae* Brullo (Nature CODE: 91AA): mature climax community typified by southern live oak *(Quercus virgiliana* (Ten.) Ten.) in association with cork oak (*Q. suber* L.), holm oak (*Q. ilex* L.), carob (*Ceratonia siliqua* L.), wild olive (*Olea europaea* L. subsp. *sylvestris* (Mill.) Hegi)) and mastic (*P. lentiscus* L.). (vi) Gallery forest dominated by oleander (*Nerium oleander* L.) (Nature CODE 92CO I): thermo-Mediterranean community dominated by oleander (*N. oleander* L.) in association with willows (*Salix* spp.) and poplars (*Populus* spp.). Sampling activities were carried out during the autumn of 2015/2016 and 2017/2018. Plant nomenclature follows Pignatti [68], while the syntaxa classification follows Biondi et al. [69]. For the correlation between plant communities and habitat types, we referred to the Italian Interpretation Manual for the Habitats of Directive 92/43/EEC [70].

2.2. Sampling and Phytophthora Isolation

Twenty rhizosphere soil samples, including fine roots, were collected randomly from 20 mature trees and shrubs growing in all six plant communities (Table S1).

Soil sampling and isolation were performed in accordance with Jung et al. [31]: four soil cores were collected under each tree or shrub, 50–150 cm away from the stem base, and rhizosphere soil from all four cores was bulked together (about 1 L).

For each sample, subsamples of 400 mL were used for baiting tests that were performed in a walk-in growth chamber with 12 h natural daylight at 20 °C. Young leaves of *C. siliqua* and *Quercus* spp. floated over flooded soil were used as baits. After 24–48 h incubation, necrotic segments (2 × 2 mm) from symptomatic leaves were plated in Petri dishes onto selective PARPNH agar medium which consisted of 100 mL V8 juice (Campbell Grocery Products Ltd., Ashford, UK), 15 g agar, 3 g CaCO3, 200 mg ampicillin, 10 mg rifampicin, 25 mg pentachloronitrobenzene (PCNB), 50 mg nystatin, 50 mg hymexazol, and 1 L of deionised water [71]. Petri dishes were incubated at 20 °C in the dark. Outgrowing *Phytophthora* hyphae were transferred onto V8 juice agar (V8A) under the stereomicroscope. All the *Phytophthora* isolates were maintained on V8 agar in the dark at a temperature of 6 °C.

Additional isolations were performed directly from river water by using an in situ baiting technique. To this end, 10 non-wounded young leaves of *C. siliqua* and *Quercus* spp. were arranged in a mesh-bag styrofoam raft (25 × 30 cm) fixed to float on the water surface (Figure 2). In total, five mesh-bag-styrofoam rafts were placed: two on the surface of the Belluzza stream and three on the Cugno di Rio river, the two water courses crossing the reserve. The rafts were collected after 3 days. All obtained isolates were maintained on V8A and stored at 6 °C in the dark.

Figure 2. Mesh-bag styrofoam raft: (**A**) raft placed in Cugno di Rio river; (**B**) raft floating on the water surface of the Belluzza river.

2.3. Morphological Characterization of Isolates

Cultures of seven days, grown on V8A at 20 °C in the dark, were used to group all isolates into morphotypes on the basis of their colony growth patterns. For each host plant and plant community, the different morphological types have been labeled with progressive numbering (Roman numbering); then, isolates belonging to the same sampling hosts have been tagged with the relative type number.

Moreover, morphological features of chlamydospores, sporangia, oogonia, antheridia and hyphal swellings were carefully analyzed and compared with species descriptions in the literature [17,72].

2.4. Molecular Identification of Isolates

The DNA of the pure cultures of isolates obtained from soil and rafts was extracted by using PowerPlant® Pro DNA isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA), following the manufacturer's protocol. The DNA was preserved at −20 °C. The identification of *Phytophthora* species was performed by the analysis of internal transcribed spacer (ITS) regions of ribosomal DNA (rDNA). DNA was amplified using forward primers ITS6 (5′-GAAGGTGAAGTCGTAACAAGG-3′) [73] and reverse primer ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) [74]. The PCR amplification mix and thermocycler conditions were in accordance with Cooke et al. [73]. All PCRs were carried out in a 25 µL reaction mix containing PCR Buffer (1×), dNTP mix (0.2 mM), $MgCl_2$ (1.5 mM), forward and reverse primers (0.5 mM each), Taq DNA Polymerase (1 U) and 100 ng of DNA. The thermocycler conditions were as follows: 94 °C for 3 min; followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s and then 72 °C for 10 min.

Amplicons were detected in 1% agarose gel and sequenced in both directions by an external service (Amsterdam, The Netherlands). Derived sequences were analyzed using FinchTV v.1.4.0 (Geospiza Inc., Seattle, WA, United States) [75]. For species identification, blast searches [76] in the *Phytophthora* Database [77], GenBank [78] and in a local database containing sequences of ex-type or key isolates from published studies were performed. Isolates were assigned to a species when their sequences were at least 99–100% identical to a reference isolate.

2.5. Analysis of Phytophthora Diversity

The *Phytophthora* diversity of soil samples sourced from the six plant communities was assessed by using the Shannon diversity (H = −Σpiln(pi)), the Pielou evenness (J = H/lnS) and the Simpson dominance (λ = 1/Σpi2) indices, where pi represents the frequency of each species and S the number of different species per plant community. Since the assumption of normal distribution was violated (the Shapiro–Wilk test was applied), the statistical differences in the diversity among sampling areas were assessed by the chi-square non-parametric test of Kruskal–Wallis followed by Dunn's multiple comparison post-hoc test (the R software [79] was used).

2.6. Soil Analysis and USDA Classification

An additional twenty rhizosphere soil samples were collected from plants within the selected sampling areas. The soil analysis was performed by a private laboratory (Progetto Ambiente & C. s.a.s., Catania, Italy) following the "official method of soil chemical analysis", in accordance with standard protocols defined by D.M. 13/09/1999, G.U. n°248, 21/10/99 and D.M. 25/03/2002, G.U. n°84, 10/04/2002. The following characteristics of the soil were determined: pH-H_2O, electrical conductivity at 25 °C, active limestone, organic matter content, nitrates and soil texture.

To define the soil texture of each sample, the USDA classification method [80] was used. The percentage of each soil component (sand, clay and silt) has been used in order to assign each sample to a textural class.

3. Results

Molecular analyses were performed on 228 isolates, of which there were 45 from rivers and 183 from the soil of the reserve. These isolates represented all morphotypes recovered in soil samples and baiting rafts. Morphological and ITS sequence analyses revealed the occurrence of multiple *Phytophthora* species in each type of plant community with the only exception of the *Sarcopoterium spinosum* vegetation, where no *Phytophthora* species was detected. ITS sequence analyses showed that all 228 isolates (65.9%) matched with 99–100% identity reference sequences of 11 known *Phytophthora* species belonging to five different ITS clades. The ITS sequences of isolates of this study were deposited at GenBank, and, since isolates of the same species were all the same, only some sequences were deposited in GenBank. The *Phytophthora* species recovered in the reserve, the host species and the accession numbers are given in Table S2.

Among the isolates, 100 belonged to species in ITS clade 6 (i.e., *P. bilorbang*, *P. asparagi*, *P. lacustris* and *P. gonapodydes*), 72 to species in ITS clade 2 (i.e., *P. multivora*, *P. oleae* and *P. plurivora*), 20 to species in ITS clade 8 (i.e., *P. cryptogea* and *P. syringae*) and 36 to *P. parvispora* and *P. nicotianae*, in ITS clades 7 and 1, respectively. The distribution of each *Phytophthora* species in each plant community type is shown in Table 1.

Table 1. Diagrammatic representation of the diversity and distribution of *Phytophthora* species recovered from six plant communities in the "Complesso Speleologico Villasmundo—S. Alfio" RNR. The proportion of isolates of each *Phytophthora* species recovered from each plant community is reported on the same row. The color intensity indicates the frequency of each species in each plant community (see legend).

Plant Community	*Phytophthora* Species Recovered in the Reserve										
	Clade 1		Clade 2			Clade 6			Clade 7	Clade 8	
	NIC	MUL	OLE	PLU	ASP	LAC	GON	BIL	PAR	CRY	SYR
Platano-Salicetum pedicellatae	16.7%	6.7%		3.3%		36.7%	3.3%	3.3%	6.7%	23.3%	
Sarcopoterium spinosum comm.											
Myrto communis-Pistacietum lentisci				34.4%	59.4%						6.25%
Pistacio-Quercetum ilicis				81.8%	4.5%	13.6%					
Oleo-Quercetum virgilianae	48%		8.7%	4.3%	35%					4.3%	
Gallery forest dominated by *N. oleander*				14.3%		71.4%		14.3%			

ASP = *P. asparagi*; **BIL** = *P. bilorbang*; **CRY** = *P. cryptogea*; GON = *P. gonapodyides*; LAC = *P. lacustris*; **MUL** = *P. multivora*; **NIC** = *P. nicotianae*; **OLE** = *P. oleae*; **PAR** = *P. parvispora*; **PLU** = *P. plurivora*; SYR = *P. syringae*. Underlined, the species that are considered native to Europe; in bold, species regarded as exotic [20,25,31]; the origin of *P. oleae* has not been established yet. % isolated species in each plant community: <25% 25–50% 50–75% >75%.

3.1. Phytophthora Diversity and Distribution in Different Plant Communities

With the only exception of the *Sarcopoterium spinosum* community, *Phytophthora* species from ITS clade 6 were isolated from all the plant communities (Figure 3). The most common *Phytophthora* species were aquatic species from ITS clade 6, such as *P. lacustris*, which were recovered from all river and riparian systems. *P. gonapodyides* and *P. bilorbang* were isolated from rhizosphere soil of willow while *P. lacustris* was recovered from river water and rhizosphere soil of mastic. With regard to species from clade 2, *P. oleae*, *P. multivora* and *P. plurivora*, they were detected in five out of six surveyed plant communities. In particular, *P. oleae* was recovered from rhizosphere soil of southern live oak, *P. multivora* from river water and *P. plurivora* from river water and rhizosphere soil of mastic, cork oak, southern live oak and holm oak. The clade 8 species *P. cryptogea* and *P. syringae* were found in three out of six plant communities. *P. cryptogea* was recovered from rhizosphere soil of willow, plane tree and southern live oak, while *P. syringae* was recovered only from rhizosphere soil of mastic. *Phytophtora parvispora* (ITS clade 7) was isolated exclusively from rhizosphere soil of mature willow trees in the *Platano-Salicetum pedicellatae* plant community. Finally, the ITS clade 1 species *P. nicotianae* was found in the *Platano-Salicetum pedicellatae* and *Oleo-Quercetum virigilianae* plant communities and was isolated from rhizosphere soil of plane, willow, southern live oak and cork oak (Table 1). *Phytophthora plurivora*, *P. asparagi*, *P. lacustris* and *P. nicotianae* were the prevalent species, accounting for 28%, 25%, 17% and 14% of all the isolates, respectively. Conversely, *P. syringae*, *P. gonapodyides*, *P. bilorbang*, *P. multivora*, *P. oleae* and *P. parvispora* were represented by less than 3% of isolates.

The structure of the community *of Phytophthora* species in soil samples differed significantly between the vegetation types. Among 11 *Phytophthora* species detected, only *P. plurivora* was found in all five plant communities. By contrast, *P. oleae* was detected exclusively in the *Oleo-Quercetum virgilianae*, *P. syringae* in the *Myrto Communis-Pistacietum lentisci*; *P. gonapodyides*, *P. bilorbang* and *P. parvispora* in the *Platanum-Salicetum pedicellatae.*

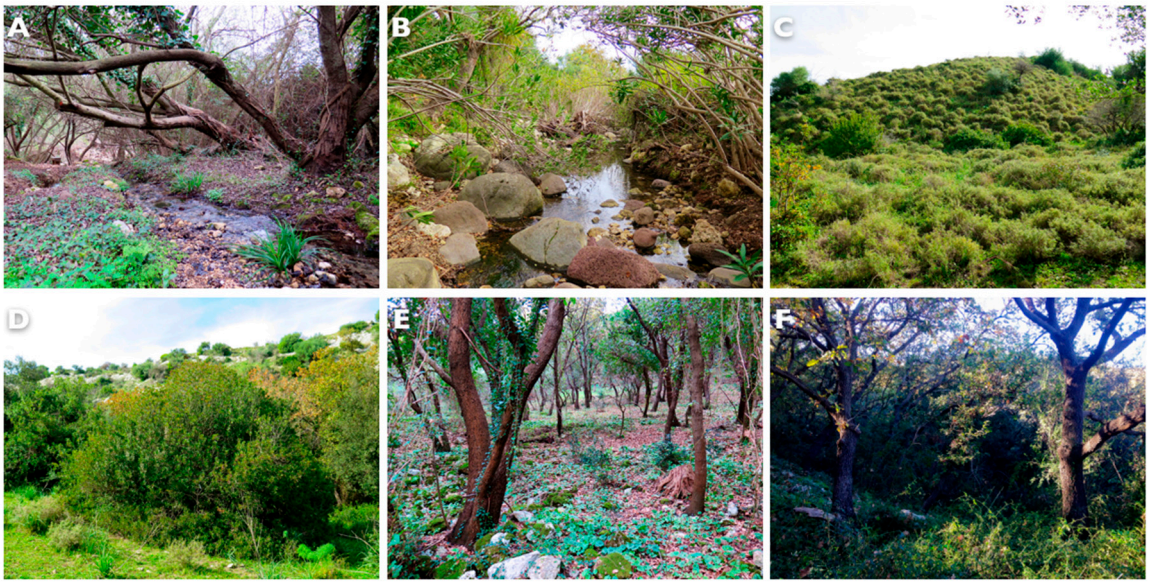
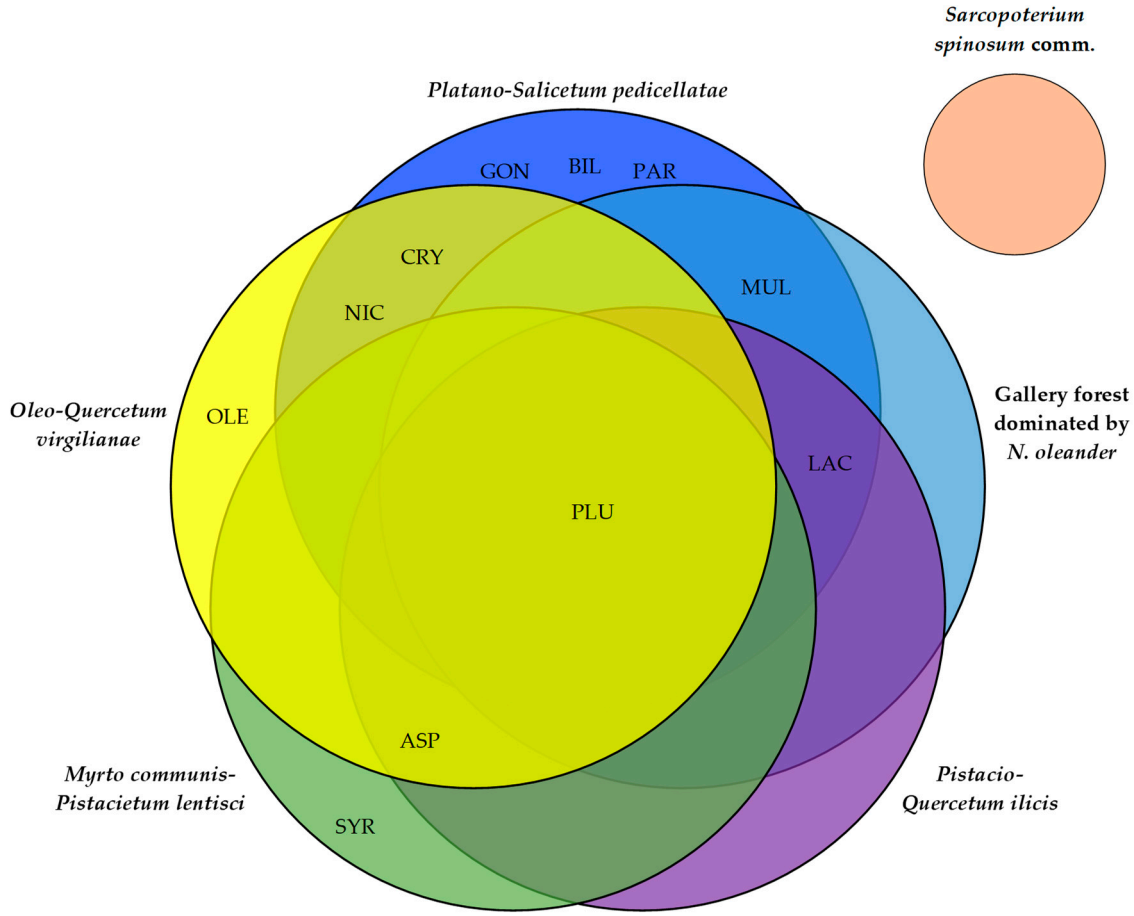


Figure 3. Plant communities: (**A**) *Platano-Salicetum pedicellatae*; (**B**) gallery forest dominated by *Nerium oleander*; (**C**) *Sarcopoterium spinosum* community; (**D**) *Myrto communis-Pistacietum lentisci*; (**E**) *Pistacio-Quercetum ilicis*; (**F**) *Oleo-Quercetum virgilianae*.

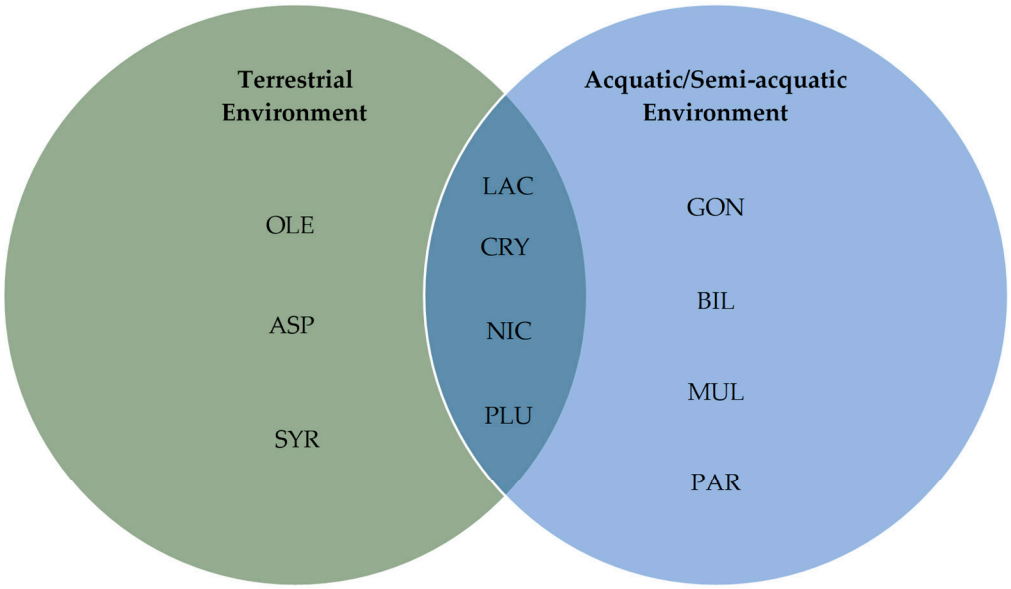
Overall, the sets of *Phytophthora* species from different plant communities strongly overlapped (Figure 4).



ASP= *P. asparagi*; BIL= *P. bilorbang*; CRY= *P. cryptogea*; GON= *P. gonapodyides*; LAC= *P. lacustris*; MUL= *P. multivora*; NIC= *P. nicotianae*; OLE= *P. oleae*; PAR= P. *parvispora*; PLU= *P. plurivora*; SYR= *P. syringae*

Figure 4. Venn diagram showing the distribution of *Phytophthora* species in six different plant communities in the "Complesso Speleologico Villasmundo—S. Alfio" RNR.

Comparing the different environments from which the species have been isolated, it can be observed that *P. cryptogea*, *P. lacustris*, *P. nicotianae* and *P. plurivora* were recovered from both terrestrial and aquatic or semi-aquatic environments, while *P. asparagi*, *P. oleae* and *P. syringae* were found exclusively in terrestrial environments and *P. bilorbang*, *P. gonapodyides*, *P. multivora* and *P. parvispora* only in aquatic or semi-aquatic environments. Overall, the sets of species from terrestrial habitats and from aquatic or semi-aquatic (riparian) habitats were distinct (Figure 5).



ASP= *P. asparagi*; BIL= *P. bilorbang*; CRY= *P. cryptogea*; GON= *P. gonapodyides*; LAC= *P. lacustris*; MUL= *P. multivora*; NIC= *P. nicotianae*; OLE= *P. oleae*; PAR= P. *parvispora*; PLU= *P. plurivora*; SYR= *P. syringae*

Figure 5. Venn diagram showing the distribution of *Phytophthora* species in terrestrial and aquatic or semi-aquatic environments in the "Complesso Speleologico Villasmundo—S. Alfio" RNR.

3.2. Analysis of Soil

Results of soil analyses are schematically summarized in Table 2. Values of soil pH from all plant communities were above 7.5 and not significantly different from each other. Concerning the electrical conductivity at 25 °C, moderately high values were found in soil samples from the *Platano-Salicetum pedicellatae* (1100 ± 48 µS/cm), *Sarcopoterium spinosum* comm. (973 ± 45 µS/cm) and *Myrto communis-Pistacietum lentisci* (894 ± 43 µS/cm) plant communities, high values in soil of the *Pistacio-Quercetum ilicis* (1414 ± 63 µS/cm) and significantly lower values in soil from the *Oleo-Quercetum virgilianae* (439 ± 36 µS/cm) plant community. As far as the active limestone is concerned, a high value was found in soil of the *Oleo-Quercetum virgilianae* (127 ± 5 g/Kg), while a relatively low value was recorded in soil from *Pistacio-Quercetum ilicis* (31 ± 2 g/Kg). The amount of nitrates was relatively high in soil from *Pistacio-Quercetum ilicis* and *Oleo-Quercetum virgilianae* plant communities (12.8 ± 1 and 11.59 ± 1 mg/Kg, respectively) and significantly lower in soil taken from *Platano-Salicetum pedicellatae*, *Sarcopoterium spinosum* comm. and *Myrto communis-Pistacietum lentisci*. Soils from all plant communities were rich in organic matter. The highest content of organic matter was found in soil from *Pistacio-Quercetum ilicis* (15%).

According to the USDA soil textural classification, soils from *Platano-Salicetum pedicellatae*, *Sarcopoterium spinosum* comm. and *Oleo-Quercetum virgilianae* were sandy clay loam; soil from *Pistacio-Quercetum ilicis* was clay loam and soil from the *Myrto communis-Pistacietum lentisci* was sandy clay. No obvious correlation was found between soil characteristics and *Phytophthora* species diversity.

Table 2. Soil characteristics in each plant community type of the "Complesso Speleologico Villasmundo—S. Alfio" RNR.

Soil Properties	Plant Community [a]				
	Platano-Salicetum pedicellatae	*Sarcopoterium spinosum* Comm.	*Myrto communis-Pistacietum lentisci*	*Pistacio-Quercetum ilicis*	*Oleo-Quercetum virgilianae*
pH	7.5 ± 0.1	7.4 ± 0.1	7.3 ± 0.1	7.4 ± 0.1	7.6 ± 0.1
Electrical conductivity at 25 °C (µS/cm)	1.100 ± 48	973 ± 45	894 ± 43	1.414 ± 63	439 ± 36
Active limestone (g/Kg)	69 ± 3	86 ± 4	81 ± 4	31 ± 2	127 ± 5
Soil texture	Sandy clay loam	Sandy clay loam	Sandy clay	Clay loam	Sandy clay loam
Nitrates (mg/Kg)	6.7 ± 0.7	6.8 ± 0.6	4 ± 0.5	12.8 ± 1	11.59 ± 1
Organic matter (%)	5 ± 0.3	6 ± 0.5	8.2 ± 0.7	15 ± 2	6.1 ± 0.5

[a] Only rafts were placed in the gallery forest dominated by Nerium oleander and no soil sample was collected from this vegetation community.

3.3. Analysis of Phytophthora Diversity

The analysis of the diversity of *Phytophthora* populations from different plant communities showed a high variability of evenness (Table 3). Significantly higher values of Shannon and Pielou evenness diversity indices were observed in the *Phytophthora* populations from the *Platano-Salicetum pedicellatae* and *Oleo-Quercetum virgilianae* plant communities, while the evenness was moderate to low in *Phytophthora* populations from *Myrto communis-Pistacietum lentisci*, *Pistacio-Quercetum ilici* and the gallery forest dominated by *N. oleander*. In contrast, values of the Simpson dominance index were significantly higher in *Phytophthora* populations from the gallery forest dominated by *N. oleander* and *Pistacio-Quercetum ilicis*, intermediate in the *Phytophthora* population from *Myrto communis-Pistacietum lentisci* and significantly lower in the *Phytophthora* populations from *Platano-Salicetum pedicellatae* and *Oleo-Quercetum virgilianae* plant communities.

Table 3. Values of the diversity indices, Shannon diversity, evenness and Simpson dominance of *Phytophthora* populations from six different plant communities in the "Complesso Speleologico Villasmundo—S. Alfio" RNR. Data were analyzed with the Kruskal–Wallis test. Different letters indicate significant differences according to Dunn's multiple comparison test ($p \leq 0.01$).

Plant Communities	Diversity Indexes					
	Shannon Index		Pielou Evenness		Simpson Dominance	
Platano-Salicetum pedicellatae	1.707	a	0.821	a	0.229	c
Sarcopoterium spinosum comm.	-		-		-	
Myrto communis- Pistacietum lentisci	0.760	b	0.692	b	0.563	b
Pistacio-Quercetum ilicis	0.652	bc	0.593	c	0.649	a
Oleo-Quercetum virgilianae	1.205	a	0.749	ab	0.361	c
Gallery forest dominated by *N. oleander*	0.451	c	0.650	b	0.722	a

4. Discussion

As many as 11 *Phytophthora* species, including putatively endemic and exotic species as well as pathogens associated prevalently to agriculture, such as *P. nicotianae* [14,81], were found in the "Complesso Speleologico Villasmundo—S. Alfio" RNR, a relatively high number compared to the limited extension of the reserve (0.72 km^2). This can be explained with the quite recent establishment of the reserve (D. ARTA n° 616 04/11/1998), which, until 22 years ago, also comprised tree crops whose relics still survive and a high variability of plant communities included within the boundaries of the reserve, each occupying a different ecological niche and constituting a distinct ecosystem. The variability of ecosystems within the reserve is further increased by the presence of two streams, which favor the settlement of species with aquatic or semi-aquatic habitats along the banks and in the rhizosphere soil of the riparian vegetation.

Phytophthora plurivora, *P. asparagi* and *P. lacustris* were the most widespread species in the "Complesso Speleologico Villasmundo—S. Alfio" RNR, whereas the other species had a scattered distribution. Among the species recovered from the RNR, some, such as *P. cryptogea*, *P. gonapodyides, P. plurivora* and *P. syringae*, are common in natural and forest ecosystems throughout Europe [35,60,61,82], while others, like *P. bilorbang*, *P. oleae* and *P. parvispora,* occur more sporadically. Both *P. plurivora* and *P. gonapodyides* were reported in a previous survey of protected natural areas in Sicily [31]. In this study, *P. oleae* was isolated only from the rhizosphere soil of southern live oak *(Q. virgilianae* (Ten.) Ten.) in the *Oleo-Quecetum virgilianae* plant community. This was the first time that this recently described species was reported from a host plant other than olive (*O. europaea* L.) [83,84]. *Phytophthora bilorbang* is a prevalently aquatic species, but it has been occasionally reported as an opportunistic, aggressive plant pathogen [85]. *Phytophthora parvispora* (formerly *P. cinnamomi* var. *parvispora*), a species in clade 7a, is an aggressive plant pathogen with a prevalently terrestrial habitat. In this survey, it was recovered from the rhizosphere of willow (*S. pedicellata* Desf.) in a riparian semi-aquatic environment. In a previous study, it had been reported as a pathogen of strawberry tree (*Arbutus unedo*) in Sardinia (central Italy)

and described as a new species distinct from *P. cinnamomi* [86]. The first record of this species in Italy dates back to 2010 and was from ornamental plants in nursery [87].

Phytophthora gonapodyides is a species with a prevalently aquatic lifestyle but may be an aggressive opportunistic plant pathogen [88]. It inhibits seed germination and causes root rot and stem lesions in *Quercus robur* L. and *Q. ilex* L.; also, in association with other species of *Phytophthora*, it was recovered from declining oak stands even in xerophytic environments [71,89,90]. According to Erwin and Ribeiro [91], the damage caused by this species is underestimated and is overshadowed by that of other *Phytophthora* species, since it is traditionally regarded a minor pathogen. Presumably, the role of this and other *Phytophthora* species with a prevalently aquatic lifestyle in natural ecosystems is more complex than that of mere plant pathogens and deserves to be further investigated.

Phytophthora plurivora is a very polyphagous pathogen whose host range encompasses more than 80 woody host species including oaks (*Quercus* spp.), willows (*Salix* spp.), oleander (*N. oleander*) and oriental plane (*P. orientalis* L.) [31,92]. In the "Complesso Speleologico Villasmundo—S. Alfio" RNR, it was the species with the widest distribution and was isolated from the rhizosphere soil of mastic (*P. lentiscus* L.), southern live oak (*Q. virgilianae* (Ten.) Ten.), holm oak (*Q. ilex* L.) and cork oak (*Q. suber* L.), as well as from river water of *Platano-Salicetum pedicellatae*. Its polyphagia and the ability to produce resting spores, such as thick-walled oospores, might explain the cosmopolitan attitude of this species and its widespread occurrence in Mediterranean natural ecosystems. Interestingly, both *P. plurivora* and the other three species found exclusively in terrestrial habitats, i.e., *P. asaparagi*, *P. oleae* and *P. syringae*, are homothallic and produce oospores with thick walls. This could be a common adaptive strategy of these species to cope with adverse environmental conditions typical of many ecosystems in Mediterranean climate, such as long periods of drought alternating with intense rainfall, mild wet winters, high temperatures in summer, wide excursion of daily temperature and rapid fluctuation of air and soil humidity. In general, the ability to produce resting structures increases the competitiveness of *Phytophthora* spp. in terrestrial environments [93]. All the species isolated from the rhizosphere soil samples collected from diverse plant communities and streams in the "Complesso Speleologico Villasmundo—S. Alfio" RNR, with the only exception of *P. lacustris*, produce at least one kind of resting structure. However, only *P. asparagi, P. bilorbang*, *P. multivora*, *P. plurivora* and *P. syringae* are homothallic. In addition, *P. nicotianae, P. oleae, P. parvispora* and possibly *P. syringae* are able to produce chlamydospores.

Phytophthora lacustris is a ubiquitous species in riparian ecosystems, such as reed belts and riparian alder stands, throughout Europe and North America [20,94]. Like other species in clade 6, this species tolerates high temperatures, is sexually sterile and produces a great amount of zoospores [20]. For the clade as a whole, this combination of characters has been interpreted as an adaptation to riparian environments [17,95]. However, although *P. lacustris* is significantly less aggressive than other *Phytophthora* spp. with a terrestrial habitat, it can seriously infect fine roots of trees stressed by episodes of flooding or drought [20]. Accordingly, in the present study, *P. lacustris* was isolated prevalently from the stream crossing the gallery forest plant community, which is dominated by oleander (*N. oleander*), and from the rhizosphere soil of willow (*S. pedicellata*) trees in the *Platano-Salicetum pedicellate* riparian plant community, but *P. lacustris* was recovered even from the rhizosphere soil of southern live oak (*Q. virgilianae*) in the xerophytic *Pistacio-Quercetum ilicis* plant community.

Phytophthora asparagi, another species included in clade 6 but in a separate subclade [96], was recovered exclusively from terrestrial environments and was common in different plant communities, including *Myrto communis-Pistacietum lentisci*, *Pistacio-Quercetum ilicis* and *Oleo-Quercetum virgilianae*. In a previous survey of the National Park of La Maddalena archipelago (Sardinia), this species was isolated frequently from the rhizosphere soil of typical plants of the Mediterranean maquis vegetation, showing symptoms of decline, including white asparagus (*Asparagus albus* L.), *Phoenician juniper* (*Juniperus turbinata* Guss.) and mastic (*P. lentiscus* L.) [65]. In the "Complesso Speleologico Villasmundo—S. Alfio" RNR, *P. asparagi* was recovered from the rhizosphere soil of mastic as well as from the rhizosphere soil of evergreen oaks, including southern

live oak (*Q. virginiana* (Ten.) Ten.), holm oak (*Q. ilex* L.) and cork oak (*Q. suber* L.). As a result, the list of known host plants with which this species is associated has expanded.

In the present survey, two other species from clade 6, *P. gonapodiydes* and *P. bilorbang*, were detected. They were found exclusively in the riparian ecosystem and rivers, confirming a prevalently aquatic lifestyle [20,65,95,97]. Very probably, the high number of isolates of clade 6 species obtained in this study and their widespread occurrence is related to the high adaptability of these species to different environmental conditions and their ability to produce numerous zoospores, which in natural ecosystems are easily transported and spread by water courses.

The diversity and species richness of *Phytophthora* populations associated with different plant communities—as measured using three distinct indexes, Shannon diversity, Pielou evenness and Simpson dominance—were significantly higher in *Platano-Salicetum pedicellatae* and *Oleo-Quercetum virgilianae*, very probably reflecting the richness of host plant species and the complexity of these termo-mesophilous communities. Moreover, the higher richness and diversity of species in the *Platano-Salicetum pedicellatae* might be explained by the presence of both aquatic and terrestrial environments within this plant community. The Pielou evenness index value also suggests that the *Phytophthora* community in the *Platano-Salicetum pedicellatae* was more balanced than in the *Oleo-Quercetum virgilianae*, due to the dominance of *P. nicotianae*, an aggressive and polyphagous plant pathogen, in the latter community. Considering the proportions of isolates recovered from each of the abovementioned plant communities regarded together as an ecological succession, it can be supposed that the progressive decrease in complexity causes both the reduction in the diversity of *Phytophthora* communities and the progressive unbalance in their composition, resulting in the dominance of the most aggressive *Phytophthora* species. In accordance with this hypothesis, the very low complexity of the garrigue of *Sarcopoterium spinosum* (L.) Spach is the extreme ecological limit for the establishment of *Phytophthora* communities.

Nevertheless, the sets of *Phytophthora* species in each plant community, as represented by the Venn diagram, showed a tendency to cluster together, probably due to both the dominant presence of two invasive species (i.e., *P. plurivora* and *P. asparagi*) and the proximity of different ecosystems. The strong overlapping of *Phytophthora* species sets also indicates that environmental conditions are conducive to these oomycetes in all types of plant communities examined in this study. In particular, no obvious correlation was observed between the diversity of distribution of *Phytophthora* populations and some physicochemical soil properties that may influence the ecology of *Phytophthora* species, their aggressiveness and ability to survive, such as pH, salinity, textural class, active limestone, nitrates and organic matter content [98–104]. In all plant community types, the content of organic matter was relatively high, but this was particularly true for the *Pistacio-Quercetum ilicis*. This might explain the occurrence of *P. lacustris* in the rhizosphere soil of trees in this vegetation community. *Phytophthora* species, in fact, including several aggressive plant pathogens, have the ability to either survive or complete their lifecycle as saprobes, despite their poor ability to compete with other saprophytic organisms. This saprophytic attitude is more pronounced in clade 6 species, like *P. lacustris* [20]. Therefore, higher levels of organic matter favor the establishment and survival of *Phytophthora* spp., in general, and of clade 6 species, in particular.

Moreover, other soil parameters, with the only exception of the high level of active limestone in the *Oleo-Quercetum virgilianae*, which could be a limiting factor, can be considered within the optimum range in all plant community types, including the *Sarcopoterium spinosum* comm., where no *Phytophthora* species was found. This indicates that the aforementioned physicochemical soil properties were not a limiting factor for the settlement and survival of *Phytophthora* spp. in this xerophytic plant community type. Other major environmental factors conditioning the ecology of soilborne *Phytophthora* species are soil moisture and temperature [91]. The effects of soil water status, generally expressed in terms of matric potential, on the ability of *Phytophthora* species to sporulate and cause disease have been extensively investigated in agricultural systems [91,105]. The geographical range of many soilborne *Phytophthora* species or their ability to thrive and survive at high altitudes depend on their

extreme and optimum temperatures for growth [7,57,106] and many aquatic *Phytophthora* species are thermophilic [19,20,107], suggesting that this is an adaptive functional trait of this group of species. However, the values of these two soil parameters, matric potential and temperature, are not constant and, in the Mediterranean climate, they vary considerably and suddenly across the seasons or even the same day, so they were not considered in this study.

Overall, comparing *Myrto communis- Pistacietum lentisci*, *Pistacio-Quercetum ilicis* and *Sarcopoterium spinosum* comm., with *Platano-Salicetum pedicellatae* and *Oleo-Quercetum virgilianae*, the *Phytophthora* diversity shows a trend that could be related to the degree of maturity of plant communities. Results of this study are in agreement with those of a similar study carried out in forests of South Africa [108]. Conversely, the geographic distribution of *P. cinnamomi* in the Iberian Peninsula turned out to be influenced primarily by abiotic factors, including soil texture and climate, followed by land use and lastly by the presence of main host plant species [109]. However, comparisons with the results of this last study are impaired by the scale of the survey, the heterogeneity of environments investigated and the fact that only one Phytophthora species was involved.

When the *Phytophthora* species found in the "Complesso Speleologico Villasmundo—S. Alfio" RNR were grouped on the basis of the type of environment from which they were recovered, the Venn diagram clearly separated them into two distinct sets, including species with a prevalent or exclusive terrestrial habitat, such as *P. asparagi*, *P. oleae* and *P. syringae,* and species with a prevalent or exclusive aquatic or semi-aquatic habitat, such as *P. bilorbang* and *P. gonapodyides*, respectively. The first group was characterized by homothallic species, able to produce thick-walled oospores allowing them to survive adverse soil conditions; the second group comprised species that have adapted to thrive in aquatic and semi-aquatic environments. The presence of prevalently aquatic species in the first set and the presence of typically terrestrial species in the second set may be explained by the proximity of vegetation to water streams, flooding events and runoff of rain water. Consistently with our results, in a very recent study a correlation was found between the *Phytophthora* community and both the type of vegetation and environment in the French Guiana rainforest, which, like other neotropical forests, is considered a major plant diversity hotspot [110]. However, unexpectedly, the *Phytophthora* community in this forest showed a low diversity compared to the richness of species recovered from the "Complesso Speleologico Villasmundo—S. Alfio" RNR.

5. Conclusions

This study showed a correlation between the vegetation type and the diversity and distribution of *Phytophthora* species in a small natural reserve where different types of plant communities typical of the Mediterranean macroregion were represented. Moreover, it confirmed that *Phytophthora* species may be grouped according to an ecological criterion on the basis of their prevalently terrestrial or aquatic lifestyle. The ecology of these two distinct groups is conditioned by their aggressiveness as well as by some other biological characteristics, such as high temperature requirement, the ability to produce resting structures, the prevalence of asexual reproduction and the amount of zoospores that they produce. The presence of different ecosystems in a restricted area, i.e., in comparable environmental and climatic conditions, was a unique opportunity to highlight this correlation between the type of vegetation and the diversity of *Phytophthora* communities. However, only a more extensive survey of the same plant community's types in distant geographic areas would definitely demonstrate that the type of vegetation is a major driving factor shaping the *Phytophthora* communities in natural ecosystems. Other ecological and epidemiological aspects that deserve further attention are the seasonal pattern of *Phytophthora* populations in these ecosystems, the selective pressure exerted by invasive *Phytophthora* species on natural vegetation and their impact on spontaneous regeneration.

Supplementary Materials: The following are available online at http://www.mdpi.com/1999-4907/11/8/853/s1, Table S1: Vegetation, geographic coordinates, type of samples and soil texture of the 20 sites sampled in the "Complesso Speleologico Villasmundo—S. Alfio" regional nature reserve. Table S2: Isolates of the Phytophthora species characterized in this study, plant hosts of the "Complesso Speleologico Villasmundo—S. Alfio" regional Nature reserve and the GenBank accession numbers of their ITS sequences.

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Status of Charcoal Canker on Oak Trees at a Site of Community Importance: Case Study of the Relict Castelfidardo Forest (SIC Area IT520008, Castelfidardo, AN, Italy)

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Abstract: Oaks are dominant and key tree species in Mediterranean forest ecosystems. However, in recent decades, oak forests have been heavily impacted by oak decline, a worldwide phenomenon exacerbated by climate change. The charcoal disease agent *Biscogniauxia mediterranea* is involved in the decline of Mediterranean oak formations in a variety of contexts. Here, we investigated the impact and role of *B. mediterranea* in the decline of oaks in Castelfidardo Forest, a relict wood of the late Holocene and a Site of Community Importance. We established five plots within which we recorded tree positions, any symptoms and signs of decline, association of *B. mediterranea* to declining trees, and deadwood and associated mycota. Of 471 oaks inspected, 7.0% showed brownish exudates on the stems, 46.9% showed epicormic shoots along the main trunk, and 24.4% showed black carbonaceous stromata on diseased branches and trunks. The decline was most severe for *Quercus cerris*, which comprised plots #4 and #5, at 50.0% (81/162 trees) and 29.0% (33/114), respectively; then for *Quercus robur* for plot #3, at 40.0% (38/95); and finally for *Quercus pubescens* for plots #1 and #2, at 13.7% (7/51) and 12.3% (6/49), respectively. Bark tissues were collected from trees with charcoal cankers and taken to the laboratory for microscopic examination and identification by mycological and molecular methods. This investigation revealed a close association between oaks with pronounced reduction of vitality and incidence of *B. mediterranea*. Deadwood was equally distributed among the five plots, and was heavily colonized by *Basidiomycota*. The high incidence of the charcoal canker pathogen *B. mediterranea* appeared to be related to environmental stresses. However, the absence of silvicultural management, high competition among physiologically mature trees, and the geographic isolation of this residual forest may have predisposed oaks to decline.

Keywords: *Biscogniauxia mediterranea*; oak decline; dieback; Site of Community Importance (S.I.C.); tree competition; warming conditions

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1. Introduction

Forest decline may be considered the most worrying and complex syndrome that affects wide forested areas across a range of ecosystems throughout the world [1–5]. When dealing with forest decline in the Mediterranean area, special attention should be focused on oak forests, due to the importance and widespread presence of oaks in Mediterranean forest ecosystems and the severity and extent of the decline in oak formations [6].

Oak decline involves various members of the genus *Quercus*, and it is seen as a general and progressive decline that is accompanied by a range of symptoms. The most common of these are: leaf chlorosis and yellowing; microphyllia; premature leaf drop and crown transparency; branch dieback; emission of dark exudates through the trunk; and production

of epicormic shoots along the main branches and stems [7,8]. Indeed, oak decline has long been recognized as a complex, multifactorial disease that involves a variety of predisposing factors, inciting factors, and contributing factors [1,9].

The most common predisposing factors are those that reduce the natural host defenses, which can induce chronic metabolic disorders that debilitate trees and make them more prone to long-term effects of damaging agents. These include factors related to climate, site, and anthropogenic interference, such as water stress from extended drought, poor soil conditions, waterlogging, silvicultural mismanagement and land-use disturbance. Inciting factors can be physical or biological, and they can act episodically and over short durations, such as hail, frost, acute water shortage, and defoliating insects. These factors induce acute, short-term damage, so these symptoms of the decline can regress, and the plants can recover. However, if the damage is prolonged and repeated over several years, this can irreversibly weaken the trees, and potentially cause their death. Contributing factors are those factors that further debilitate trees, and when these act on physiologically impaired trees, they can become lethal. Among these, there are secondary biotic agents, such as weakening pathogens, boring insects, and bark beetles [1,10–15].

All of these damaging factors can be implicated singly or cumulatively in the decline of oaks, with the variously focused actions showing different levels of intensity and, as a consequence, resulting in varying damage severity. Cumulative or alternative involvement of these stressors can give rise to a number of syndromes that are variable from site to site. This has resulted in the generation of diverse and sometimes conflicting hypotheses on the etiology of these phenomena, which has resulted in the definition of oak decline as a "complex of different diseases" [1,10].

In recent years, changes in temperature and rainfall patterns have begun to have increasingly important and decisive roles in oak decline [16–19]. The climate of the Mediterranean basin has shown a general tendency to become warmer and drier [20,21]. Here, the persistent stressful conditions caused by prolonged water deficit and drought stress can weaken the trees and make them more vulnerable to infection by secondary, opportunistic pathogens [22,23].

Members of the genus *Quercus* are widespread on the Italian peninsula, and thus they characterize its national and local landscape [24,25]. Castelfidardo Forest (*Selva di Castelfidardo*) is an example of a forest with high landscaping and ecological value in which *Quercus* spp. are the dominant tree component. This forest is recognized as a 'Site of Community Importance, and has been included in the European "Natura 2000" network, an international system of protected areas that is designed to safeguard European habitats and biodiversity. As Castelfidardo Forest is a protected area of natural heritage, and as many oaks within this forest have recently shown increasing symptoms of decline and mortality, we investigated the causes of this general, progressive decline and dieback.

Castelfidardo Forest is a sub-Mediterranean deciduous forest with mesophilic traits that today covers an area of only 36 ha, although at the beginning of the seventeenth century it was known to have extended to the sea, with an estimated area of ~350 hectares. The original area has therefore undergone significant contraction over time, to the point that Castelfidardo Forest can be considered today as a lowland residual forest within a widely cultivated hilly landscape [26].

A temperate bioclimate is predominant in this area, with rainfall patterns that provide between 300 mm and 600 mm per year of rainfall that is mainly concentrated over the autumn and winter months, resulting in prolonged, dry summers. The northern exposure, the geological characteristics of the site, and the relatively humid soil due to the superficial aquifer (especially in the lower portion of the forest) have created a microclimate that differs from the surrounding hills and from the coastal areas, such that the vegetation expresses sub-Mediterranean phytoclimatic conditions [27].

The composition of Castelfidardo Forest has been shaped by the nature of the geological substrate, the morphological structure of the area, and anthropogenic interference. Its core areas mainly include deciduous *Quercus* species (i.e., *Quercus cerris*, *Quercus robur*,

Quercus pubescens), whereas parts of the edges of the forest have been reforested with *Pinus* spp. and *Cupressus* spp. (*Ministerial Decree*, 30 March 2009) (Figure 1). However, Castelfidardo Forest has been undergoing diffuse deterioration, with loss of biodiversity and general decline. This is due to the monoplane structure of Castelfidardo Forest, which has a dominant layer of roughly even-aged trees, and lack of silvicultural management, high stand density, strong competition among individuals, and the pedoclimatic characteristics of the area. Furthermore, it represents a small, isolated remnant of a late Holocene forest that has long been isolated from continuous forests.

Figure 1. Map of Castelfidardo Forest (*Selva di Castelfidardo*; Site of Community Importance; Castelfidardo, AN, Italy) instituted by *Ministerial Decree* 30 March 2009.

The aims of this study were to: (i) determine the health conditions of the oak trees in the Castelfidardo Forest; (ii) describe the incidence of the charcoal canker pathogen *Biscogniauxia mediterranea* on the declining oaks; (iii) determine the amounts of deadwood and take a census of the associated Mycota; and (iv) determine whether there is a cause-effect relationship between climatic conditions and the decline of the oak species.

2. Materials and Methods

2.1. Study Site, Plot Design, and Tree Health Assessment

An examination of the phytosanitary status of the oak trees was carried out in the Castelfidardo Forest (43°27′59″ N, 13°35′30″ E) from May to July 2015. For this purpose, five plots were located in the upper, middle, and lower areas that were representative of the soil types and vegetation that characterize the forest (Table 1). Each plot was circular, with an area ranging from 0.6 ha to 0.9 ha. The plots were of variable sizes and were designed to include all of the heterogeneity conditions in terms of soil type, vegetation, and intensity of decline.

Table 1. Main characteristics of plots studied in Castelfidardo Forest (Castelfidardo, AN, Italy).

Plots	Area (m^2)	Tree Species	Stand Density (Trees/100 m^2)	Trees Monitored (n)
#1	6480.4	*Quercus pubescens* + *Fraxinus ornus*	11	51
#2	8569.6	*Quercus pubescens* + *Fraxinus ornus*	9	49
#3	6870	*Quercus robur* + *Carpinus betulus*	17	95
#4	7700	*Quercus cerris* + *Carpinus orientalis*	15	162
#5	8890	*Quercus cerris* + *Carpinus orientalis*	19	114

In the upper layer, plots #1 and #2 were characterized by the arenaceous substrate where *Q. pubescens* was predominant, and was associated with sporadic presence of *Fraxinus ornus* trees. In plot #3, in the middle layer, the dominant species was *Q. robur*, with rare *Carpinus betulus* found. In the lower layer, where plots #4 and #5 were located, the arenaceous component definitively disappeared, and the dominant forest vegetation was *Q. cerris*, with *Carpinus orientalis* occurring sporadically. In all of these plots, *Ruscus aculeatus* was the predominant understory species, and this covered large proportions of the plots, strongly limiting the natural regeneration of the oaks.

As summarized in Table 1, the main parameters recorded in each plot were: surface area; tree species; tree densities; and total numbers of trees inspected. A number of trees were selected according to the expression of symptoms (i.e., production of tarry exudates, epicormic shoot formation, and eruption of charcoal cankers through the bark), from 49 to 162 trees in each plot. For each tree selected, the diameter at chest height (i.e., 1.3 m above the ground) and its sanitary status were assessed and recorded, noting in particular the presence and frequency of: early symptoms of decline (e.g., emission of exudates, production of epicormic branches); advanced symptoms and/or signs of decline (e.g., dieback, black carbonaceous stromata indicative of charcoal cankers); and tree mortality. The position of each tree was recorded using the Global Positioning System (GPS; e-Trex 30, Garmin), and metallic labels with progressive identification numbers were affixed to the trunks. The data obtained relating to individual trees were managed through the Quantum Geographic Information System (GIS) open source software, and displayed using the Google Earth software.

The occurrence of saprotrophic and facultative necrotrophic fungi (e.g., *Armillaria* spp.; able to decompose deadwood) was also determined. Fruit bodies of these fungi were photographed, collected, and later identified through conventional morphological keys [28–30].

2.2. Sample Collection, Mycological Analysis, and Morphological Identification

Bark tissue with charcoal canker (mean dimension, 4 × 4 cm) was collected from 20 *Q. pubescens* trees in plots #1 and #2, from 10 *Q. robur* trees in plot #3, and from 20 *Q. cerris* trees in plots #4 and #5. These samples were incubated in a humidity chamber with five layers of tissue paper that had previously been soaked in sterile distilled water, and placed in glass Petri dishes (diameter, 14 cm), at 25 °C, in the dark, for 10 days. The samples were then observed under a stereo microscope (MZ125; Leica) to document the sporulation. Various samples were photographed using a digital camera (FinePix S1 Pro; Fujifilm).

The fungal agents were isolated from the bark after the incubation in the humidity chamber, from differentiated fruiting structures. Under aseptic conditions, portions of the mycelia were transferred onto Petri dishes with potato dextrose agar (PDA) with added antibiotics (150 mg/L streptomycin, 150 mg/L ampicillin; to prevent bacterial contamination). Five original mycelial samples were put into each plate, and five replicates were prepared from each sample. Each plate was labeled, sealed with parafilm, and incubated at 25 °C in the dark. After 5 days, the developed fungal colonies were subcultured onto fresh PDA plates, then transferred into glass test tubes, and finally stored at 4 °C. For 50 isolates (10 for each plot), identification was carried out by recording the characteristics of 50–100 units of spores for each isolate, by light microscopy (Eclipse E600; Nikon).

2.3. Molecular Identification of Fungal Colonies

Mycelia were collected from 25 representative fungal colonies grown on PDA, transferred into 1.5 mL microcentrifuge tubes and pulverized, with the addition of 600 µL extraction buffer (20 mM EDTA, 0.1 M Tris-HCl, pH 8.0, 1.4 M NaCl, 2% cetyltrimethylammonium bromide, 4% polyvinylpyrrolidone, 0.1% sodium metabisulfite [added just before use]). The extraction of total DNA was carried out using the CTAB protocol [31]. The quality and quantity of the extracted DNA were determined directly on 1% agarose gels, with evaluation using a biophotometer (Eppendorf, Hamburg, Germany). The DNA was

finally diluted to 10 ng/μL for further amplification. Nucleic acids were stored at −20 °C, and later analyzed by polymerase chain reaction (PCR). Amplification of the 5.8S ribosomal DNA (rDNA) and flanking internal transcribed spacer (ITS) region was performed in 20 μL PCR reactions that contained 2 μL genomic DNA (at ~20 ng/μL) from the fungal isolate, 10 μL EmeraldAmp GT PCR Master mix 2× (Takara, Madison, WI, USA), and 0.5 μL of each primer (10 μM), in a thermal cycler (MyCycler; Bio-Rad Laboratories, Hercules, CA, USA). MED1/MED2 primers were used, which are specific for *B. mediterranea* [32]. The PCR products (9 μL per sample) were separated by electrophoresis in 1.5% agarose gels stained with Red Gel (Biotium, Hayward, CA, USA), then visualized and captured using an imagining system (Gel Doc XR; BioRad, Hercules, CA, USA). The same ITS region was also amplified from fungal isolates with universal primers ITS1/ITS4 [33]. Amplicons from three isolates (WP101, WP20, WP376), were purified using Wizard SV gels and PCR clean-up kits (Promega Corporation, Madison, WI, USA), and quantified using the biophotometer (Eppendorf, Hamburg, Germany). Sequencing was carried out by Genewiz UK. The Bioedit software, v. 7.0.0 (http://www.mbio.ncsu.edu/Bioedit/bioedit.html, accessed on 12 April 2021) was used to cut off 20 bp to 30 bp of the terminal end sequence. All of the sequences were used as the query sequences in BLAST searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 26 April 2021), to determine the nucleotide identities available in GeneBank NCBI. The nucleotide sequences were deposited in the NCBI database (MZ407593-MZ407595).

2.4. Deadwood and Associated Lignicolous Macrofungi

As deadwood and deadwood-dwelling macrofungi are fundamental for the maintenance of the biodiversity of forest ecosystems, the quantity and quality of the deadwood served as a proxy indicator for the level of biodiversity of Castelfidardo Forest. In particular, the deadwood was classified into standing dead trees (snags) and lying dead woody debris (logs). The quantities were expressed as percentages, considering the snags and logs with respect to the total number of oak trees in each plot.

The associated lignicolous macrofungal assemblage was analyzed concurrently. The macrofungi were collected gently and taken to the laboratory for detailed mycological examination.

2.5. Climatic Data

Data for the minimum, maximum, and average temperatures, and for the average annual and seasonal rainfall for the period 1951-2015 were obtained from the Regional Informative System located at Ancona Torrette 2944 (RT 701) (13°26′, 43°36′).

3. Results

3.1. Phytosanitary Monitoring

A total of 471 oak trees were assessed from May to July 2015 for their health conditions in the five plots (Table 1). Many of the trees showed an advanced decline. The patterns of symptom appearance showed a succession of symptoms of increasing severity. Oaks in the initial stages of decline showed some nonspecific symptoms, such as leaf yellowing, early leaf browning, and thinning of canopy cover. Those in advanced states of decline showed foliar rolling and leaf wilting, production of epicormic shoots on the main branches and stems, and loss of branches, which eventually lead to tree mortality.

The emission of dark-brownish mucilaginous exudates through the bark (Figure 2A,B) occurred mainly during the growing season, and this was recorded for 7.2% (34/471) of the trees. Debarking the trees at the points of emission of exudates showed clearly visible necrotic areas of the underbark tissue. Furthermore, the trees with these symptoms often showed marked transparency of the canopy. Trees with production of exudates through the bark were more frequently recorded for plots #5 and #2, at 9.7% (11/114 trees) and 8.2% (4/49), respectively (Figure 3A). Epicormic shoot formation along the main trunk was recorded for 42.7% (201/471) of the trees (Figure 2C,D), and it occurred equally in all

of the five plots (Figure 3B). Charcoal cankers erupted through the bark in about 27.6% (130/471) of the trees (Figure 2E). In cross-sections of the trunks, necrosis of the xylem and wood decay were observed in correspondence with the black carbonaceous stromata that had developed externally (Figure 2F). The charcoal-black cankers were more frequently recorded for plot #3, at 40.0% (38/95), followed by plots #4 and #5, at 30.3% (49/162) and 25.4% (29/114), respectively; in plots #1 and #2, occurrence of the typical charcoal-black surface was lower (Figure 3C). Declining trees generally died in the growing season following the appearance of these erumpent, coriaceous black stromata. In plot #4, there were sporadic fallen trees that showed root rot and thick, black, string-like structures, which were usually seen under the bark or in the soil near the roots, as typical structures of *Armillaria* sp. (Figure 2G,H).

Figure 2. Representative photographs of main symptoms observed on the stems of declining oak trees in Castelfidardo Forest (Castelfidardo, AN, Italy). (**A**,**B**) Dark-brownish exudates. (**C**,**D**) Epicormic shoot proliferation. (**E**) Charcoal cankers erupting through the bark. (**F**) Cross-section of a tree with discoloration and decay of the outermost portions of the woody cylinder in correspondence with areas of external development of *B. mediterranea* carbonaceous stromata; (**G**) Lying tree with clear symptoms of root rot. (**H**) Dark rhizomorphs of *Armillaria* spp. under the oak bark.

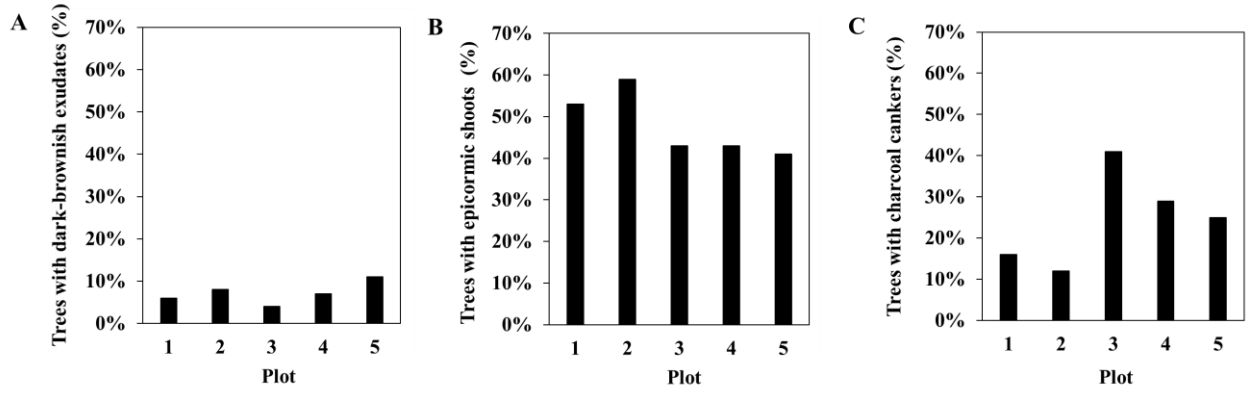

Figure 3. Quantification of the symptoms of oak decline recorded for the five experimental plots (#1–#5) in Castelfidardo Forest. (**A**) Dark-brownish exudates. (**B**) Epicormic shoot proliferation. (**C**) Charcoal cankers.

3.2. Mycological and Molecular Identification

We sporadically isolated from the bark tissue of oaks some fungi belonging to the genera *Botryosphaeria*, *Diplodia*, *Neofusicoccum*, *Cytospora*, *Discula* (*Apiognomonia*). However, we focused our attention on the agent of charcoal canker *B. mediterranea* as the incidence of this fungus was, in terms of isolation frequencies, extraordinarily higher.

Stereoscopic observations revealed carbonaceous, perithecial stromata in all of the 50 samples. The stromata appeared slightly convex, ellipsoid, and elongated, 7.2 to 20.5 × 3.5 to 4.2 cm. Perithecia were ovoid to tubular, 0.74 to 0.80 × 0.12 to 0.15 mm. A total of 152 fungal colonies were obtained in purity, most of which (94.1%; 143) were identified as *B. mediterranea* by mycological examination of their distinguishing micromorphological traits (Figure 4). The colonies completely filled the agar plates (diameter, 90 mm) after 7 days of incubation at 25 °C. The colonies were white-grey in color and velvety (viewed from the top). Short-stipitate, amyloid asci that were dark brown were observed; these produced ellipsoid ascospores, 14 µm to 19 × 7 to 9 µm, which perfectly matched the description by Mirabolfathy et al. [34].

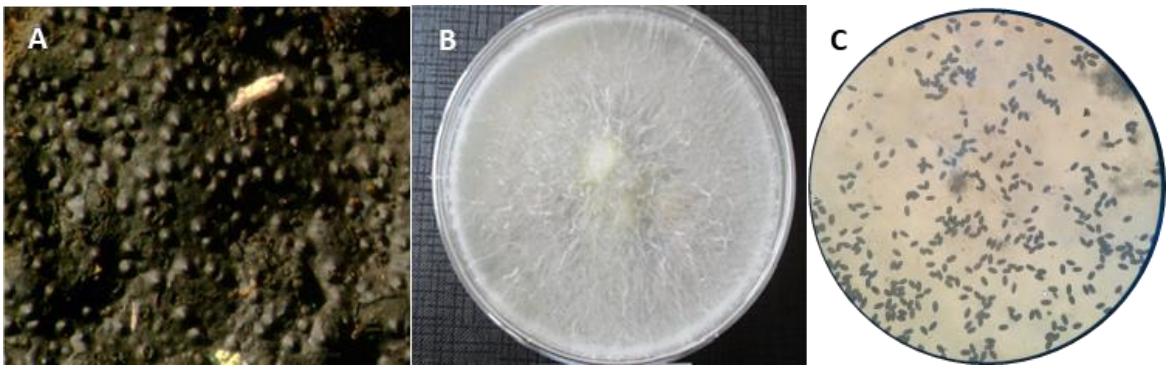

Figure 4. Representative images of carbonaceous stromata of *B. mediterranea* harboring perithecia, with a clearly visible layer of ostioles on its surface. (**A**) As observed under the stereo microscope. (**B**) A colony of *B. mediterranea* after 5 days on a PDA plate at 25 °C. (**C**) Ascospores of *B. mediterranea* as observed under the microscope.

No reproductive structures were observed in cultures that could be used to identify the microorganisms on a micromorphological basis. The reverse of the colonies were darker, tending to dark grey-black. Colonies of *Trichoderma* sp. were also sporadically isolated from the black carbonaceous stroma (four isolates).

The morphological identification of the fungi was supported by molecular identification. The DNA extraction protocol was very efficient, and provided ~210 ng/µL, with high quality (A260/280 > 1.87; A260/230 > 1.92). Only a few of the extracted samples (n = 6)

showed low quality DNA values (A260/280 < 1.80; A260/230 < 1.80), and these were not processed further.

Amplification with the specific MED1/MED2 primers gave fragments of the expected length (377 bp) in all 25 of the samples analyzed. No ambiguous bands were generated, and the lack of bands in the water control test confirmed the absence of contamination. A portion of the ITS region (approximately 580 bp) was sequenced for additional confirmation of the identity of the fungus. Three nucleotide sequences selected revealed 100% identity with reference sequence MT819849 in the NCBI database, as *B. mediterranea* isolated from *Quercus suber* in Portugal.

3.3. Deadwood and Deadwood-Dwelling Mycobiota

The ground within Castelfidardo Forest was characterized by high amounts of deadwood, for all of the plots investigated. Plot #2 showed the lowest abundance of snags (12.2%, versus total number of oak trees) and logs (4.0%), while the highest abundance was for plot #5 (39.5%, 18.4%, respectively) (Figure 5).

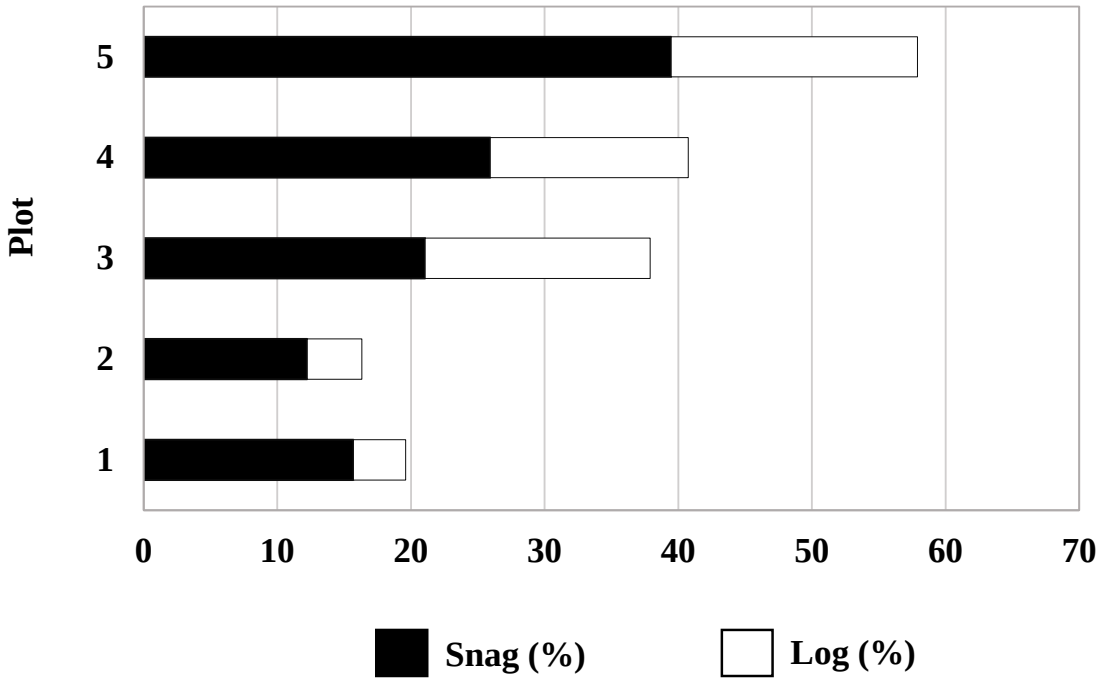

Figure 5. Abundance and type of deadwood (% of snags and logs) recorded in the five plots in Castelfidardo Forest (Castelfidardo, AN, Italy).

The deadwood was colonized by various fungi and other saprophytic microorganisms that thrive on dead and/or decaying trees. Most of these fungi belonged to *Basidiomycetes*. The most frequently found in plots #4 and #5 were *Armillaria mellea*, *Macrolepiota* spp., *Trametes* spp., and *Artomyces* spp., while in plots #1, #2, and #3 they were mainly *Pluteus* spp., *Hypholoma* spp., *Macrolepiota* spp., *Armillaria mellea* and *Armillaria ostoyae* (Figure 6).

Figure 6. Representative photographs of the main macrofungi associated with the deadwood in Castelfidardo Forest (Castelfidardo, AN, Italy): (**A**) *Trametes* spp. (**B**) *Cyathus* spp. (**C**) *Pluteus* spp. (**D**) *Postia* spp. (**E**) *Hypholoma* spp. (**F**) *Macrolepiota* spp. (**G**) *Armillaria ostoyae*. (**H**) *Armillaria mellea*. (**I**) *Clathrus* spp. (**J**) *Tapinella* spp. (**K**) *Macrolepiota* spp. (**L**) *Mycena* spp. (**M**) *Micromphale* spp. (**N**) *Coprinus* spp. (**O**) *Artomyces* spp.

3.4. Climatic Data

Analysis of climatic data related to Castelfidardo Forest revealed that the average annual temperature (17 °C) recorded over the period from 2001 to 2015 has increased by 2 °C with respect to the historical series (1957–2000) (Figure 7). For rainfall, there were no evident differences, with two negative peaks recorded in 1983 (376 mm) and 2014 (488 mm). A more detailed analysis of the climatic data relating to the growing seasons from 2001 to 2015 revealed that in the spring and summer periods for that time interval, the temperatures increased by 2 °C and 2.5 °C respectively, compared to the former period of 1957 to 2000.

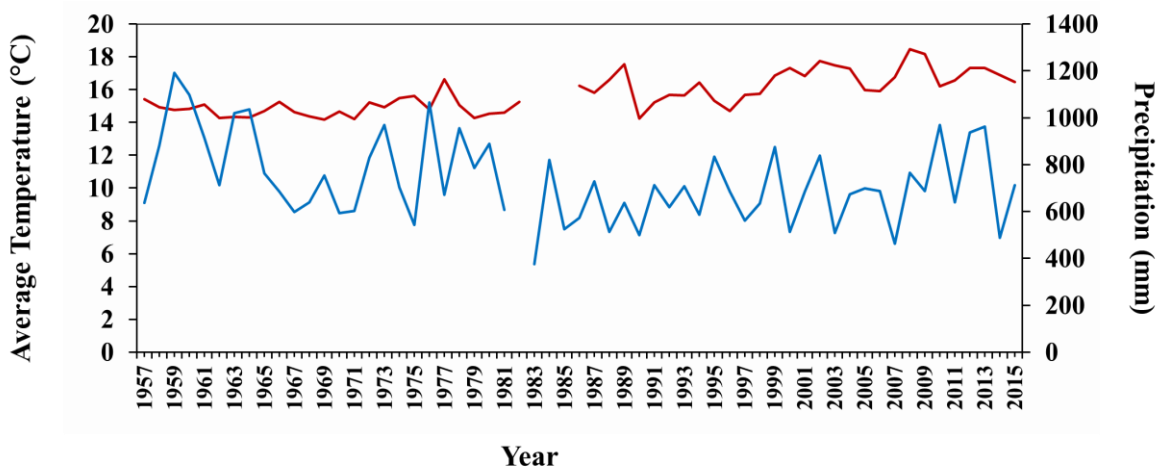

Figure 7. Graph of mean annual temperature (red lines) and rainfall (blue lines) cycles recorded from 1957 to 2015 at the Ancona Torrette 2944 weather station (RT 701) (13°26′, 43°36′) (Regional Informative System).

4. Discussion

Oak forest ecosystems are of paramount importance in the Mediterranean basin due to the multiplicity of functions and services they provide in this region. Indeed, this region is at risk of desertification due to climatic and socio-economic driving forces, including rainfall variability, droughts, changes in land-use patterns, overgrazing, and demographic saturation of rural areas [35]. Oak formations have crucial roles here for the prevention of land degradation and for climate mitigation, carbon storage, and the safeguarding of the environment; they also generate economic, esthetic and landscaping benefits [25].

Unfortunately, Mediterranean oak forests have been plagued by oak decline for decades now, which is a phenomenon that appears strongly related to climate anomalies, as mainly heat stress, but also water scarcity and droughts [18,36]. Indeed, the Mediterranean region is a hotspot for climate change, as it is characterized by a negative water balance and pronounced warming [37,38]. This region is currently experiencing high water stress conditions and prolonged droughts, and these are worsening the effects of climate change, with a strongly negative impact on plant biomass production. Our analysis of climatic data showed increased temperatures during spring and summer for the period from 2001 to 2015 in the Castelfidardo area, which confirms the general warming trend that is constraining the whole Mediterranean region.

The stressful conditions determined by the water deficit, high temperatures, and droughts have created conditions conducive to the pervasive spread of the thermophilic fungus *B. mediterranea* in oak forests [39]. Several fungal species have also been isolated from trunk and branch cankers as well as from twigs and foliage of declining oaks, including *Botryosphaeria dothidea*, *Diplodia corticola*, *Diplodia seriata*, *Neofusicoccum parvum*, *Cytospora* spp., *Discula quercina*, and *Neocucurbitaria cava* [22,23,40,41]. The occurrence of these fungi was relatively erratic in the plots investigated, with lower isolation frequency compared to *B. mediterranea*. However, in particular here, we focused our attention on trees with evident symptoms of decline that were accompanied by the specific presence of *B. mediterranea* stromata. The relationship between oak decline, climate change, and the proliferation of *B. mediterranea* has been well documented in Mediterranean countries [12,42–46]. Indeed, recent outbreaks of *B. mediterranea* in countries where it had not been reported to cause harm, like in Turkey, Iran, Slovenia [47,48], and Croatia [49], confirm that this fungus is expanding its range and is accentuating its role as a contributing factor to the decline of oak formations.

Members of the Xylariaceae fungi are widely known for surviving across a range of lifestyles. They live as saprotrophs, as they can decompose many natural substrates, and they can also be pathogenic, promoting disease on living hosts, and especially those that are water-stressed. The Xylariaceae fungi can persist within trees in a latent stage, due

to their endophytic aptitude [50]. *B. mediterranea* is a weakness pathogen that can live as an endophyte inside healthy plant tissues, thus causing extensive, although symptomless, infections. Such a cryptic existence can last for undefined periods, as long as the host does not become weakened by adverse environmental conditions. When stressful conditions that alter the carbohydrate physiology in trees occur, *B. mediterranea* switches to a pathogenic behavior to extensively colonize its debilitated host. This pathogenic endophyte then moves externally to sporulate profusely over the plant surfaces, a mechanism used to escape from a dying tree and colonize other trees that are already severely weakened, and possibly injured [11,23].

The aggressive colonization of oaks by *B. mediterranea* in the Castelfidardo Forest must thus be mainly ascribed to the chronic debilitation of these trees by hot temperatures, recurrent water deficit and droughts. Exposure to these environmental adversities will have triggered tree infection by this opportunistic fungus whereby its pathogenic action will also be facilitated by the intrinsic, low resilience of this residual forest. As a relict population, Castelfidardo Forest is more prone to any disturbance. Indeed, the extent of oak decline in Castelfidardo Forest appears greater than for other oak stands of the Italian Peninsula. Millennia of anthropogenic pressure, which was mainly represented by agricultural encroachment and overgrazing, led to the isolation of this residual portion of primeval forest. Such forest fragmentation can result in long-lasting and complex changes in biodiversity that can go beyond the loss of species, and thus include alterations to the functional diversity of the remaining communities, with isolated forests being more fragile and vulnerable [51].

The saprophytic stage is of fundamental importance in the epidemiology of *B. mediterranea* in Mediterranean oak stands. Through its extensive production of stromatal charcoal cankers, this xylariaceous fungus can introduce massive amounts of spores into the oak forest ecosystem, enormously increasing its biomass. The number of individuals introduced at a given location is a primary determinant of the successful establishment of an invasive organism into new environments [52]. On the other hand, the importance of propagule pressure in the spread of fungal pathogens and in the colonization of new hosts in oak stands has already been established [53].

Recent studies have shown how some functional traits can exacerbate the negative effects of drought, such as stand density and intraspecific and interspecific competition, which can then predispose trees to decline [54,55]. The Castelfidardo Forest was subjected to intense cutting in 1970, which has not been followed by any other interventions to date. This lack of management for half a century has had repercussions on the tree physiology and vigor; in particular, the high density of the stands causes excessive competition among the trees for the already limited resources (i.e., water, nutrients, light). This has led to depletion of the plant carbon reserves, and therefore to less resistance to colonization by the weakness pathogen *B. mediterranea*. Indeed, higher incidence of charcoal canker and higher tree mortality were recorded for *Q. robur* in plot #3 and for *Q. cerris* in plots #4 and #5. In these plots, tree densities were much higher than in plots #1 and #2. Differential susceptibility to decline across oak species was shown in previous studies. In particular, in France, *Q. robur* was more susceptible than *Quercus petraea* [56]. In North America, species of the red oak group were more susceptible than those of the white oak group [57]. In central and southern Italy, *Q. cerris* is more susceptible to decline than *Q. pubescens* [58].

In the present study, the disease caused by *B. mediterranea* was also tree-density dependent, and it represented only the terminal stage of infection. In this context, the fungus would function as an ecological factor that regulates the structure and composition of forest stands, to make them better suited to changing environmental conditions [59].

We recorded an abundance of deadwood that was equally distributed across all of the plots. The role of deadwood in forest ecosystems is linked to various aspects, which include improvement of natural diversity [60], storage and slow release of carbon and nutrients [61,62], maintenance of soil fertility, creation of pedoclimatic conditions conducive to natural regeneration of the forest, conservation of the soil, and improvement

of the stability of slopes from hydrogeological risk [63]. The deadwood richness of the Castelfidardo Forest suggests that the carbon reserves in the stand are substantial and can therefore be used by the trees. However, it must be considered that when trees are chronically impaired by abiotic (i.e., environmental) and biotic (mainly anthropogenic) stressors beyond a threshold of tolerance, their energies become so depleted that they can no longer benefit from the availability of substrate. This happens especially to physiologically overmature and veteran trees, like the majority of oaks in Castelfidardo Forest [64,65]. *Armillaria mellea* and *A. ostoyae* were also associated with the deadwood. These two Agaricales deserve special attention because they play a central role in the dynamics of numerous woody ecosystems [66]. They are ubiquitous species, more commonly found in mature stands with an abundance of old trees, where they play a valuable ecological role in the recycling of dead wood. However, *A. ostoyae* and *A. mellea* are also polyphagous, facultative parasites with parasitic ability, causing white rot to a number of host species [67]. In Castelfidardo Forest these two fungi live preferentially as saprotrophs on dead wood and only sporadically are they found to attack pathogenically live but visibly weakened trees [65].

5. Conclusions

The present study focused on the decline of oaks in Castelfidardo Forest, which is a forest of great ecological importance. The attacks of *B. mediterranea* on oaks in this forest have increased dramatically in recent years and must be related to hot temperatures and recurrent droughts which chronically weaken trees (Eugenio Paoloni, personal communication). The life history strategy of *B. mediterranea* has been investigated in other oak woods of central Italy where it emerged, as in this study, that the agent of charcoal canker takes advantage of environmental stresses to aggressively colonize physiologically impaired oaks [68]. Since we cannot interfere with climatic conditions, to mitigate the impact of the decline, it would be advisable to reduce the source of inoculum and tree density. This opportunistic fungus sporulates abundantly on the trunks and branches of dying or standing dead trees, as well as on deadwood on the ground. Therefore, among the possible control strategies, it would be appropriate to: (i) eliminate all dead trees with sporulating cankers; (ii) carry out thinning, to reduce tree densities, particularly in plots #3, #4, and #5; and (iii) explore the possibility of using antagonistic microorganisms (e.g., *Trichoderma* spp.) for repressing this stress-mediated disease [69].

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Conflicts of Interest: The authors declare that they have no conflict of interest.

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Effect of Plant Diversity on the Occurrence of Diplodia Tip Blight in Natural Secondary Japanese Red Pine Forests

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Abstract: Diplodia tip blight caused by *Sphaeropsis sapinea* (Fr.) Dyko and B. Sutton is a serious threat to the health of natural secondary Japanese red pine (*Pinus densiflora* Sieb. et Zucc.) forests. To explore the effect of plant diversity on Diplodia tip blight disease occurrence, the correlation between Diplodia tip blight in *P. densiflora* and plant diversity in various stand types and vertical structure layers were analyzed by Pearson correlation coefficients and generalized linear model while keeping environmental factors relatively consistent. Disease index of Diplodia tip blight in *P. densiflora* was positively correlated with the plant diversity indices in the tree layers of *P. densiflora*–conifer mixed forest. In contrast, it was negatively correlated with the plant diversity indices in the tree layers of *P. densiflora*–hardwood mixed forest and *P. densiflora* pure forest. In shrub–herb layers of all stands except the *P. densiflora* pure forest herb layer, the disease index was positively correlated with the plant diversity indices. The prediction models for the disease index of Diplodia tip blight in the *P. densiflora*–hardwood mixed forest, *P. densiflora*–conifer mixed forest, and *P. densiflora* pure forest were also established. The foregoing results suggest that in the Kunyu Mountains of China, the differences in plant diversity among various stand types and vertical structure layers have different effects on the occurrence of Diplodia tip blight in *P. densiflora*. Plant diversity largely reflects the occurrence of Diplodia tip blight disease. However, if the effect of plant diversity on Diplodia tip blight is fully reflected, the plant species characteristics, especially tree genetic relationships, should be considered. In this way, plant species diversity structure in natural secondary Japanese red pine forests may be modified through forest management to lower the incidence of Diplodia tip blight.

Keywords: Diplodia tip blight; *Pinus densiflora*; plant diversity; *Sphaeropsis sapinea*; stand type; vertical structure layer

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1. Introduction

The fungus *Sphaeropsis sapinea* (Fr.) Dyko and B. Sutton is a critical pathogen for almost 60 conifer species that belong to *Abies*, *Cedrus*, *Juniperus*, *Larix*, *Picea*, *Pinus*, *Pseudotsuga*, and *Thuja* [1] (p. 186). It causes tip blight, stem canker, rhizome decay, and sapstain and has serious threats to forest health in nearly 40 countries such as New Zealand, Australia, South Africa, the United States of America, Finland, Germany, and China [2–8] (pp. 224–227). As an endophyte, it may occur as a latent pathogen and produces no visible symptoms in the hosts [9]. *Sphaeropsis sapinea* infesting coniferous trees of any age can induce disease when the hosts are subjected to physiological stress or physical damage [10]. A high incidence of the disease results in extensive forest blight, and adversely affects the integrity and stability of forest ecosystems [11–14].

The natural secondary Japanese red pine (*Pinus densiflora* Sieb. et Zucc.) forests of the Kunyu Mountains are the original habitats and distribution centers of Japanese red pine in China and even Northeast Asia. They have abundant species, genetic, and ecosystem

diversity. Their germplasms are considered to have high scientific research and practical value [15,16]. However, Diplodia tip blight caused by *S. sapinea* is a major disease affecting the natural secondary Japanese red pine forests of the Kunyu Mountains. It threatens the healthy and stable development of forests. Nevertheless, it is unknown why this pathogen has induced an epidemic in this region.

Studies on the relationships between Diplodia tip blight in pine and environmental factors showed that *S. sapinea*, as an endophyte, can become pathogenic, when its hosts have been weakened by abiotic stresses such as precipitation deficits, elevated temperatures, drought, and hailstorms [6,7,17–19].

Theoretical studies revealed that tree species diversity has a significant impact on disease prevalence. For example, the risk of *Picea abies* (L.) H. Karsten root rot caused by *Heterobasidion annosum* (Fr.) Bref. is lower in the *P. abies* mixed forest than it is in the *P. abies* pure forest [20]. Hantsch et al. [21] found a negative correlation between the disease index of oak powdery mildew (*Erysiphe alphitoides* (Griffon and Maubl.) U. Braun and S. Takam. and *Erysiphe hypophylla* (Nevod.) U. Braun and Cunningt.) and tree species diversity. By contrast, Menges and Loucks [22] suggested that the mortality of certain host tree species, such as *Quercus velutina* Lam., *Quercus borealis* Michx., and *Quercus ellipsoidalis* E. J. Hill infested with *Ceratocystis fagacearum* (Bretz.) Hunt) rise with the increase of *Quercus* species diversity. Furthermore, the relationship between plant diversity and disease is highly complex in multi-host forest ecosystems [23]. For instance, most plants in forest ecosystems are hosts of *Phytophthora ramorum* Werres, De Cock and Man in't Veld [24,25]. However, substitute hosts can impede disease spread in highly susceptible hosts and reduce the impact of the pathogen on host plants [26]. Xie and Liang [27] identified that the disease index of moso bamboo (*Phyllostachys edulis* (Carriere) J. Houzeau) shoot blight (*Ceratosphaeria phyllostachydis* Zhang) is highest in the moso bamboo–conifer mixed forest, moderate in the moso bamboo–hardwood mixed forest, and lowest in the moso bamboo pure forest. Meanwhile, the shrub–herb plants are important components of the forest community. Their diversity may alter humidity, soil, light, and other factors inside the forest, and further affect tree growth and the forest environment, which act on the occurrence and development of diseases [28,29]. Plant diversity in the shrub and herb layers of different moso bamboo stands have different effects on the development of moso bamboo shoot blight [27]. While the species richness and Shannon–Wiener index increased and Simpson index decreased in the shrub–herb layers of *Dalbergia odorifera* T. Chen mixed forest, the disease index of black scurf (*Phyllachora dalbergiicola* Henn.) in *D. odorifera* sharply decreased [30]. These studies demonstrate that there are explicit relationships between disease and environmental factors. Nevertheless, the relationship between plant diversity and disease is complex. The differences in plant diversity among different stand types and vertical structure layers have different influences on disease occurrence.

In the present study, we focused on whether the plant diversity has an impact on the occurrence of Diplodia tip blight in *P. densiflora*. The effect between plant diversity and Diplodia tip blight in *P. densiflora* in various stand types and vertical structure layers of natural secondary Japanese red pine forests in the Kunyu Mountains in China was expounded. In this analysis, environmental factors were kept relatively consistent. The assumption was that plant diversity has significantly different effects on the disease occurrence in different forest types. It could infer the feasibility of a method for adjusting plant diversity structure to control the prevalence of Diplodia tip blight in *P. densiflora*, based on this effect.

2. Materials and Methods

2.1. Study Area and Plot Layout

The Kunyu Mountains (121°41′34″–121°48′04″ E; 37°11′50″–37°17′22″ N) are located on the Jiaodong Peninsula of Eastern China. The region has a warm temperate monsoon climate with mean annual temperature of 12.3 °C, mean annual precipitation of 800–1200 mm, mean annual relative humidity of 62.6%, and frost-free period of 200–220 d. The forest soil is mainly brown sandy loam [31,32]. *Pinus densiflora* is the main constructive

species. Between June and October 2020, a total of 120 temporary sample plots, each with an area of 20 m × 20 m, were set up in natural secondary Japanese red pine forests of the Kunyu Mountains. All stand plots distributed evenly and had similar conditions of altitude 200 ± 25 m, middle slope position, slope 30° ± 3°, sunny slope aspect, brown sandy loam, and forest age 40 ± 4 years. There were 40 plots of *P. densiflora*–hardwood mixed forest, 40 plots of *P. densiflora*–conifer mixed forest and 40 plots of *P. densiflora* pure forest. Plots were considered to contain pure forest if their ratios of cross-sectional area at breast height of *P. densiflora* to the cross-sectional area at breast height of all tree species were ≥70%. Otherwise, the plots were deemed mixed forests. Shrub plots of 5 m × 5 m were established by the five-point method at each 20 m × 20 m tree plot. In this method, the center and four corners of each plots were selected. Herb plots of 2 m × 2 m were established in each shrub plot.

2.2. Plant Diversity Analysis

Plant species and quantity were counted in the tree, shrub, and herb layers in the plots of *P. densiflora*–hardwood mixed forest, *P. densiflora*–conifer mixed forest and *P. densiflora* pure forest. For the tree layers, the diameter at breast height (DBH) of living trees was ≥ 5 cm. Plant species richness, and the Shannon–Wiener, Simpson, and Pielou indices were calculated. Each plant diversity index has its own unique meaning. Plant species richness refers to the total number of species in plant communities. All plant species are treated equally and only the presence of the plant species is considered, and relative species abundance is not assessed here. The Pielou index measures the distribution of individuals of plant species in plant communities. The Shannon–Wiener index predicts that plant individuals randomly selected within plant communities belong to the uncertainty of a certain plant species. It considers the abundance and evenness of plant species. The Simpson index indicates the probability that two plant species selected randomly within the plant community are the same plant species [33] (pp. 7–10), [34,35]. It also considers both plant species abundance and evenness. However, the Simpson index is more strongly affected by evenness than the Shannon–Wiener index. The formulae for the above four indices are as follows [35–37]:

$$\text{Species richness } (S) = \text{Total plant species per plot} \tag{1}$$

$$\text{Shannon} - \text{Wiener index } (H') = -\sum_{i=1}^{s} \frac{n_i}{N} \ln\left(\frac{n_i}{N}\right) \tag{2}$$

$$\text{Simpson index } (D) = 1 - \sum_{i=1}^{s} \left(\frac{n_i}{N}\right)^2 \tag{3}$$

$$\text{Pielou index } (J) = -\sum_{i=1}^{s} \frac{n_i}{N} \ln\left(\frac{n_i}{N}\right) / \ln S \tag{4}$$

where n_i is the total abundance of a single plant species per plot and N is the total abundance of all plant species per plot.

2.3. Disease Index

The disease index of Diplodia tip blight in *P. dentiflora* was measured by the five-point method, in that six trees were taken from each of the four corners and the center of each plot. These 30 *P. densiflora* were divided into upper, middle, and lower layers, for which one branch from each direction (east, south, west, and north) were selected [34]. The needles are treated as cylinders with equal radii. Hence, the ratios of the lesion areas on the needles with tip blight symptoms (Figure 1) to the total needle areas could be transformed into the length ratios. Total needle lesion lengths and total needle lengths for all sampled branches per tree were measured with a steel tape. The ratios of the former to the latter were calculated and then the representative values per tree were determined according to the plant disease classification standard in Table 1. The representative values and the

corresponding total numbers of *P. densiflora* were substituted into the formula to calculate the sample plot disease index. The formulae are as follows [38,39]:

Table 1. Plant disease classification standard of Diplodia tip blight in *Pinus dentiflora*.

Disease Grade	Representative Value	Plant Disease Classification Standard
I	0	No disease symptoms
II	1	The lesion area is less than 25%
III	2	The lesion area is 25–50%
IV	3	The lesion area is 50–75%
V	4	The lesion area is more than 75%

$$DITP = \frac{\sum NDPd \times RV}{TPd \times MRV} \times 100 \quad (5)$$

$$DISP = \frac{TDISP}{TNSP} \quad (6)$$

where *DITP* is the Diplodia tip blight disease index in each temporary sample plot, *NDPd* is the number of diseased *P. densiflora* at a certain disease grade, *TPd* is the total number of *P. densiflora* (30), *RV* is the representative value of the corresponding grade, and *MRV* is the maximum representative value of the corresponding grade (4). *DISP* is the disease index of a certain stand type, *TDISP* is the sum of all *DITP* values in a single stand type, and *TNSP* is the sum of temporary sample plots in a single stand type (40).

Figure 1. Symptoms of local (**A**) and overall (**B**) tip blight in *Pinus densiflora* caused by *Sphaeropsis sapinea*.

2.4. Data Analysis

The variables of disease indices and plant diversity indices of each *P. densiflora* stand conformed to the normal distribution according to quantile–quantile (Q–Q) plot tests. Brown-Forsythe variance analysis followed by Tamhane's T2 test at $p < 0.05$ level [40] (pp. 114–115) was used to compare the means of the Diplodia tip blight disease indices in all plots of the three Japanese red pine stand types and the plant diversity indices in all plots of various Japanese red pine stand types and vertical structure layers. The scatter plots had linear trends between the plant diversity indices and the disease indices of Diplodia tip blight in *P. densiflora*, thus Pearson correlation coefficients based on these two normally distributed sets of variables were calculated to determine the correlations [41,42]. Generalized linear model (GLM) analysis for all test factors (namely plant species richness and the Shannon–Wiener, Simpson, and Pielou indices among various vertical structure

layers and the same stand type) was performed to examine plant diversity indices that had significant impacts on the disease indices of Diplodia tip blight in *P. densiflora*. The optimal model for each stand was selected by Akaike information criterion (AIC) and Bayesian information criterion (BIC). Plant species richness and the Shannon–Wiener, Simpson, and Pielou indices for each stand were replaced with x_1–x_4 for the tree layer, x_5–x_8 for the shrub layer, and x_9–x_{12} for the herb layer, respectively. In this manner, the prediction models for the Diplodia tip blight disease indices in each stand were respectively acquired. Data processing and statistical analyses were performed by R v4.0.2 (R Core Team, Vienna, Austria), Microsoft Excel 2016 (Microsoft Corp., Redmond, WA, USA), and SPSS v23.0 (IBM Corp., Armonk, NY, USA).

3. Results

3.1. Disease Indices in Three Different Japanese Red Pine Stand Types

The disease indices of Diplodia tip blight in *P. densiflora* in three Japanese red pine stand types significantly differed ($F(2, 117) = 415.197$; $p < 0.01$) as follows: *P. densiflora*–conifer mixed forest (44.215 ± 2.288), *P. densiflora* pure forest (36.202 ± 2.444), and *P. densiflora*–*hardwood* mixed forest (29.875 ± 1.930).

3.2. Plant Species Composition and Diversity

3.2.1. Plant Species Composition

The sum of the tree species in the tree layers of the three Japanese red pine stands was in the range of 13–19 (Table 2). In the *P. densiflora*–hardwood mixed forest, a total of 19 tree species in 15 genera and 11 families were identified. The highest relative abundance was of *P. densiflora* (47.85%), followed by *Quercus variabilis* (22.30%), *Q. acutissima* (15.96%), etc. In the *P. densiflora*–conifer mixed forest, one tree variety and 16 tree species in 12 genera and eight families were found, the highest relative abundance was of *P. densiflora* (52.42%), followed by *P. thunbergii* (29.94%), etc. In the *P. densiflora* pure forest, a total of 13 tree species in nine genera and six families were detected. The highest relative abundance was of *P. densiflora* (71.04%), followed by *Q. acutissima* (14.52%), etc. (Table 2).

The shrub layers of the three Japanese red pine stands consisted of 29–35 plant species (Table 3). In the *P. densiflora*–hardwood mixed forest, one shrub variety and 34 shrub species in 31 genera and 20 families were found. The highest relative abundance was of *Rhus chinensis* (29.89%), followed by *Indigofera kirilowii* (29.38%), etc. In the *P. densiflora*–conifer mixed forest, one shrub variety and 34 shrub species in 31 genera and 20 families were identified. The highest relative abundance was of *I. kirilowii* (33.87%), followed by *R. chinensis* (26.71%), *Lespedeza bicolor* (12.09%), etc. In the *P. densiflora* pure forest, one shrub variety and 28 shrub species in 25 genera and 17 families were detected. The highest relative abundance was of *I. kirilowii* (39.41%), followed by *R. chinensis* (30.02%), *L. bicolor* (11.12%), etc. (Table 3).

The herb layers of the three Japanese red pine stands comprised of 49–53 plant species (Table 4). In the *P. densiflora*–hardwood mixed forest, one herb subspecies, two herb varieties, and 49 herb species in 45 genera and 24 families were found. The highest relative abundance was of *Carex lanceolata* (23.90%), followed by *Sanguisorba applanata* (12.81%), *Pteridium aquilinum* var. *latiusculum* (10.76%), *Commelina communis* (10.39%), etc. In the *P. densiflora*–conifer mixed forest, two herb subspecies, two herb varieties, and 49 herb species in 47 genera and 21 families were identified. The highest relative abundance was of *Imperata cylindrica* (18.25%), followed by *C. lanceolata* (16.28%), *S. applanata* (12.04%), etc. In the *P. densiflora* pure forest, one herb subspecies, two herb varieties, and 46 herb species in 44 genera and 23 families were detected. The highest relative abundance was of *C. lanceolata* (20.37%), followed by *S. applanata* (18.49%), *I. cylindrica* (14.50%), *Miscanthus sinensis* (10.77%), etc. (Table 4).

Table 2. Relative abundance of tree species in the three stands.

Tree Species	Relative Abundance (%)		
	PHF	PCF	PPF
Rhus chinensis Mill.	1.41	0.40	0.33
Catalpa bungei C. A. Mey	0.31	0.07	—
Cryptomeria japonica var. *sinensis* Miquel	—	0.26	—
Cunninghamia lanceolata (Lamb.) Hook.	0.38	0.70	0.41
Metasequoia glyptostroboides Hu & W. C. Cheng	—	—	0.04
Albizia julibrissin Durazz.	—	—	0.07
Albizia kalkora (Roxb.) Prain	2.13	0.40	0.96
Robinia pseudoacacia L.	1.13	1.46	1.15
Castanea mollissima Bl.	—	0.18	—
Quercus acutissima carr.	15.96	5.56	14.52
Quercus serrata Murray	0.34	0.51	—
Quercus variabilis Bl.	22.30	1.87	5.32
Larix kaempferi (Lamb.) Carr.	0.09	0.33	0.07
Pinus armandii Franch.	0.66	4.39	0.55
Pinus densiflora Sieb. et Zucc.	47.85	52.42	71.04
Pinus taeda L.	—	1.32	—
Pinus thunbergii Parl.	6.77	29.94	5.5
Rhamnella franguloides (Maxim.) Weberb.	0.09	—	—
Cerasus serrulata (Lindl.) G. Don ex London	0.06	—	—
Pyrus calleryana Dcne.	0.03	—	—
Sorbus alnifolia (Sieb. et Zucc.) K. Koch	0.19	0.04	—
Ailanthus altissima (Mill.) Swingle	0.22	0.15	0.04
Symplocos paniculata (Thunb.) Miq.	0.03	—	—
Ulmus pumila L.	0.03	—	—

Note: *Pinus densiflora*–hardwood mixed forest, *P. densiflora*–conifer mixed forest, and *P. densiflora* pure forest are denoted as PHF, PCF, and PPF, respectively.

Table 3. Relative abundance of shrub species in the three stands.

Shrub Species	Relative Abundance (%)		
	PHF	PCF	PPF
Rhus chinensis Mill.	29.89	26.71	30.02
Eleutherococcus senticosus (Rupr. Maxim.) Maxim.	—	0.03	—
Blumea balsamifera (L.) DC.	0.03	—	—
Lonicera japonica Thunb.	0.14	0.30	—
Viburnum erosum Thunb.	0.20	0.28	1.45
Celastrus orbiculatus Thunb.	0.61	2.81	0.27
Euonymus oxyphyllus Miq.	0.06	0.03	0.04
Rhododendron simsii Planch.	0.06	—	—
Albizia kalkora (Roxb.) Prain	6.94	3.26	2.97
Amorpha fruticosa L.	0.25	1.87	2.07
Campylotropis macrocarpa (Bge.) Rehd.	—	0.51	0.20
Cercis chinensis Bunge	—	—	0.04
Indigofera kirilowii Maxim. ex Palibin	29.38	33.87	39.41
Lespedeza bicolor Turcz.	5.94	12.09	11.12
Pueraria montana (Lour.) Merr.	0.03	0.05	—
Deutzia grandiflora Bunge	5.69	2.73	3.56
Callicarpa bodinieri Levl.	—	0.13	—
Lindera glauca (Sieb. et Zucc.) Bl.	0.08	0.15	0.39
Lindera obtusiloba Bl.	0.98	0.61	0.39
Yulania denudata (Desr.) D. L. Fu	—	0.13	0.08
Grewia biloba var. *parviflora* (Bunge) Hand.-Mazz.	3.29	3.31	3.48
Broussonetia papyrifera (Linn.) L'Her.r ex Vent.	—	—	0.08
Morus alba L.	—	—	0.04
Morus mongolica (Bur.) Schneid.	—	0.05	0.04

Table 3. *Cont.*

Shrub Species	Relative Abundance (%)		
	PHF	PCF	PPF
Jasminum nudiflorum Lindl.	—	0.03	—
Ligustrum lucidum Ait.	0.11	0.86	—
Flueggea suffruticosa (Pall.) Baill.	0.11	—	0.12
Rhamnella franguloides (Maxim.) Weberb.	0.53	—	—
Rhamnus davurica Pall.	0.06	0.18	0.16
Armeniaca sibirica (L.) Lam.	0.06	0.03	—
Cerasus japonica (Thunb.) Lois.	5.52	2.61	1.84
Cerasus tomentosa (Thunb.) Wall.	0.11	0.03	0.12
Rosa multiflora Thunb.	0.56	0.18	0.08
Rubus parvifolius L.	0.70	0.81	0.12
Rubus phoenicolasius Maxim.	0.45	0.91	—
Zanthoxylum schinifolium Sieb. et Zucc.	2.65	1.67	0.74
Koelreuteria paniculata Laxm.	0.03	—	—
Smilax china L.	0.98	0.76	0.23
Smilax sieboldii Miq.	1.14	2.02	0.35
Symplocos paniculata (Thunb.) Miq.	2.45	0.30	0.31
Ulmus macrocarpa Hance	0.03	0.03	—
Boehmeria japonica (Linnaeus f.) Miq.	0.11	0.53	—
Ampelopsis humulifolia Bge.	0.70	0.15	0.27
Vitis amurensis Rupr.	0.17	—	—

Note: The meanings of PHF, PCF, and PPF are the same as those in Table 2.

Table 4. Relative abundance of herb species in the three stands.

Herb Species	Relative Abundance (%)		
	PHF	PCF	PPF
Amaranthus tricolor L.	—	0.04	—
Bupleurum chinense DC.	0.10	—	—
Saposhnikovia divaricata (Turcz.) Schischk.	0.03	—	—
Cynanchum chinense R. Br.	0.20	0.04	—
Cynanchum paniculatum (Bunge) Kitag.	0.79	0.31	0.13
Metaplexis japonica (Thunb.) Makino	—	0.10	0.10
Anaphalis sinica Hance	0.13	—	—
Artemisia argyi Lévl. et Van.	0.03	0.16	0.03
Artemisia japonica Thunb.	—	0.24	0.10
Artemisia stechmanniana Bess.	0.13	0.53	—
Aster hispidus Thunb.	—	0.06	—
Aster indicus L.	0.07	—	—
Atractylodes Lancea (Thunb.) DC.	0.10	0.10	—
Bidens pilosa L.	1.39	0.01	0.06
Carpesium cernuum L.	—	—	0.03
Chrysanthemum chanetii H. Lév.	—	0.27	0.19
Chrysanthemum indicum L.	0.50	3.24	0.77
Cirsium arvense var. *integrifolium* C. Wimm. et Grabowski	—	0.07	—
Crepidiastrum denticulatum (Hout.) Pak & Kawano	0.13	0.01	0.22
Echinops grijsii Hance	—	0.01	—
Elephantopus scaber L.	0.03	—	0.10
Erigeron bonariensis L.	0.03	—	—
Erigeron canadensis L.	—	0.09	0.86
Leibnitzia anandria (L.) Turcz.	0.07	—	—
Senecio scandens Buch.-Ham. ex D. Don	—	0.03	—
Sonchus wightianus DC.	—	0.24	0.48
Taraxacum mongolicum Hand.-Mazz.	—	0.03	—
Brassica juncea (L.) Czern.	0.03	—	—
Adenophora petiolata subsp. *Hunanensis* (Nannfeldt) D. Y. Hong & S. Ge	2.52	1.18	2.04

Table 4. *Cont.*

Herb Species	Relative Abundance (%)		
	PHF	PCF	PPF
Adenophora stricta Miq.	0.26	0.10	0.10
Codonopsis lanceolata (Sieb. et Zucc.) Trautv.	—	0.19	—
Platycodon grandiflorus (Jacq.) A. DC.	0.60	0.74	0.54
Dianthus chinensis L.	0.07	0.26	0.03
Gypsophila oldhamiana Miq.	—	0.13	0.41
Pseudostellaria heterophylla (Miq.) Pax	0.26	—	—
Silene conoidea L.	—	0.03	—
Commelina communis L.	10.39	6.16	0.26
Hylotelephium erythrostictum (Miq.) H. Ohba	—	0.03	0.10
Orostachys fimbriata (Turcz.) A. Berger	—	0.23	0.67
Phedimus aizoon (L.) 't Hart	0.66	0.01	0.03
Trichosanthes kirilowii Maxim.	—	—	0.03
Carex lanceolata Boott	23.90	16.28	20.37
Discorea nipponica Makino	—	—	0.10
Swertia bimaculata (Sieb. et Zucc.) Hook. f. et Thoms. ex C. B. Clark	0.03	—	—
Belamcanda chinensis (L.) Redouté	—	0.33	0.19
Juncus effusus L.	9.63	—	5.71
Agastache rugosa (Fisch. et Mey.) O. Ktze.	0.10	—	—
Isodon amethystoides (Benth.) H. Hara	0.50	—	—
Asparagus cochinchinensis (Lour.) Merr.	0.07	0.01	—
Convallaria majalis L.	—	—	0.29
Lilium concolor var. *pulchellum* (Fisch.) Regel	0.03	—	0.10
Ophiopogon japonicus (L. f.) Ker-Gawl.	0.60	0.23	0.29
Polygonatum odoratum (Mill.) Druce	—	0.01	0.06
Cocculus orbiculatus (L.) DC.	0.53	2.33	1.47
Phryma leptostachya subsp. *Asiatica* (Hara) Kitamura	—	0.09	—
Phytolacca acinosa Roxb.	0.10	—	—
Arthraxon prionodes (Steud.) Dandy	0.03	—	—
Arundinella hirta (Thunb.) Tanaka	1.36	—	0.41
Deyeuxia pyramidalis (Host) Veldkamp	0.33	5.15	1.88
Imperata cylindrica (L.) Beauv.	7.08	18.25	14.50
Miscanthus sacchariflorus (Maxim.) Hackel	—	—	0.19
Miscanthus sinensis Anderss.	7.85	6.65	10.77
Oplismenus undulatifolius (Ard.) Beauv.	0.86	3.92	1.79
Themeda triandra Forsk.	—	0.51	0.92
Fallopia multiflora (Thunb.) Harald.	0.10	—	—
Polygonum divaricatum L.	3.21	0.67	1.34
Androsace umbellata (Lour.) Merr.	—	0.03	—
Lysimachia barystachys Bunge	0.13	—	0.10
Lysimachia fortunei Maxim.	0.03	—	—
Lysimachia pentapetala Bunge	0.10	2.85	1.31
Pteridium aquilinum var. *latiusculum* (Desv.) Underw. ex Heller	10.76	9.19	9.56
Clematis florida Thunb.	0.03	0.17	0.03
Fragaria vesca L.	—	0.03	—
Sanguisorba applanata Yü et Li	12.81	12.04	18.49
Galium odoratum (L.) Scop.	0.07	—	—
Rubia cordifolia L.	—	0.37	0.03
Rubia yunnanensis Diels	0.17	—	—
Ostericum sieboldii (Miq.) Nakai	0.07	—	—
Patrinia scabiosifolia Link	0.03	5.57	1.66
Viola arcuata Bl.	0.96	0.19	0.51
Viola philippica Cav.	—	0.47	0.22
Alpinia japonica (Thunb.) Miq.	—	—	0.45

Note: The meanings of PHF, PCF, and PPF are the same as those in Table 2.

3.2.2. Comparison of Plant Diversity Indices

Plant Diversity Indices among Various Stand Types and the Same Vertical Structure Layer

Comparison of the diversity indices among various stand types (Table 5) showed that the cumulative values for the species richness, the Shannon–Wiener, and Simpson indices in the tree–shrub–herb layers were, in descending order, *P. densiflora*–conifer mixed forest > *P. densiflora*–hardwood mixed forest > *P. densiflora* pure forest. The differences among groups in terms of the species richness ($F(2, 117) = 90.606$; $p < 0.01$) and the Shannon–Wiener ($F(2, 117) = 59.715$; $p < 0.01$) and Simpson ($F(2, 117) = 62.622$; $p < 0.01$) indices were all significant. The cumulative values of the Pielou index in the tree–shrub–herb layers were highest for the *P. densiflora*–hardwood mixed forest, moderate for the *P. densiflora*–conifer mixed forest, and lowest for the *P. densiflora* pure forest. There were no significant differences among groups in terms of this index ($F(2, 117) = 2.651$; $p > 0.05$).

In the tree layers of the three stands, the differences in the species richness ($F(2, 117) = 17.361$; $p < 0.01$) and the Shannon–Wiener ($F(2, 117)' = 70.503$; $p < 0.01$), Simpson ($F(2, 117) = 127.614$; $p < 0.01$), and Pielou ($F(2, 117) = 36.521$; $p < 0.01$) indices among groups were all significant. Multiple comparisons showed that the species richness and the Simpson and Pielou indices of the tree layers in the *P. densiflora*–hardwood mixed forest and the *P. densiflora*–conifer mixed forest were significantly different from those of the tree layers in the *P. densiflora* pure forest ($p < 0.01$) (Table 6).

In the shrub layer of the three stands, the differences in the species richness ($F(2, 117) = 26.891$; $p < 0.01$;) and the Pielou index ($F(2, 117) = 3.904$; $p < 0.05$) among groups were significant. Multiple comparisons revealed that the species richness of the *P. densiflora* pure mixed forest significantly differed from those of the other two stands ($p < 0.01$). The Pielou indices significantly differed between the *P. densiflora* pure forest and the *P. densiflora*–conifer mixed forest ($p < 0.05$). The differences among stands in terms of the Shannon–Wiener ($F(2, 117) = 1.964$; $p > 0.05$) and Simpson ($F(2, 117) = 2.408$; $p > 0.05$) indices were not significant (Table 6).

In the herb layers of the three stands, the differences in the species richness ($F(2, 117) = 127.444$; $p < 0.01$) and the Shannon–Wiener ($F(2, 117) = 93.937$; $p < 0.01$) and Simpson ($F(2, 117) = 53.329$; $p < 0.01$) indices between groups were significant. Multiple comparisons showed that these indices significantly differed between the *P. densiflora*–conifer mixed forest and the other two stands ($p < 0.01$). The differences among the herb layers of the three stands in terms of the Pielou indices ($F(2, 117) = 2.910$; $p > 0.05$) were not significant (Table 6).

Plant Diversity Indices among Various Vertical Structure Layers and the Same Stand Type

Overall, the species richness and the Shannon–Wiener, Simpson, and Pielou indices were higher for the herb layers than the shrub and tree layers of natural secondary Japanese red pine forests. In the *P. densiflora*–hardwood mixed forest, the differences in the species richness ($F(2, 117) = 26.001$; $p < 0.01$) and the Shannon–Wiener ($F(2, 117) = 18.011$; $p < 0.01$), Simpson ($F(2, 117) = 15.598$; $p < 0.01$), and Pielou ($F(2, 117) = 11.601$; $p < 0.01$) indices among groups were all significant. Multiple comparisons revealed that the latter three indices of the herb layer significantly differed from those of the shrub and tree layers ($p < 0.01$). The species richness significantly differed between the tree layer and the other two layers ($p < 0.01$). In the *P. densiflora*–conifer mixed forest, the differences in the species richness ($F(2, 117) = 107.719$; $p < 0.01$) and Shannon–Wiener ($F(2, 117) = 177.587$; $p < 0.01$), Simpson ($F(2, 117) = 139.297$; $p < 0.01$), and Pielou ($F(2, 117) = 20.258$; $p < 0.01$) indices among groups were all significant. Multiple comparisons revealed that the latter three indices of herb layer were significantly differed from those of the shrub and tree layers ($p < 0.01$). In the *P. densiflora* pure mixed forest, the differences in the species richness ($F(2, 117) = 87.678$; $p < 0.01$) and the Shannon–Wiener ($F(2, 117) = 153.491$; $p < 0.01$), Simpson ($F(2, 117) = 166.290$; $p < 0.01$), and Pielou ($F(2, 117) = 59.575$; $p < 0.01$) indices among groups were all significant (Table 6).

Table 5. The total of each plant's diversity indices for the three stands.

Stand Type	*S*	*H′*	*D*	*J*
PHF	18.917 ± 1.682 ^{b}	3.792 ± 0.205 ^{b}	1.924 ± 0.070 ^{a}	2.221 ± 0.137
PCF	22.797 ± 3.205 ^{a}	4.083 ± 0.404 ^{a}	1.975 ± 0.119 ^{a}	2.171 ± 0.109
PPF	16.066 ± 1.419 ^{c}	3.379 ± 0.216 ^{c}	1.743 ± 0.097 ^{b}	2.152 ± 0.164

Note: The species richness and the Shannon–Wiener, Simpson, and Pielou indices are denoted as *S*, *H′*, *D*, and *J*, respectively. The meanings of PHF, PCF, and PPF are the same as those in Table 2. The lowercase letters (a, b, c) show the significant difference between various stand types and the same vertical structure layer ($p < 0.05$). For non-significant differences among groups, no letters are appended to the values. All values are means ± standard deviation.

Table 6. Plant diversity indices among various stand types and vertical structure layers.

Stand Type	Layer	*S*	*H′*	*D*	*J*
PHF	Tree	5.388 ± 0.881 ^{a,B}	1.202 ± 0.099 ^{a,B}	0.624 ± 0.037 ^{a,B}	0.719 ± 0.055 ^{a,B}
	Shrub	6.964 ± 1.182 ^{a,A}	1.229 ± 0.131 ^{B}	0.626 ± 0.048 ^{B}	0.719 ± 0.070 ^{a,b,B}
	Herb	6.566 ± 0.964 ^{b,A}	1.360 ± 0.144 ^{b,A}	0.675 ± 0.051 ^{b,A}	0.783 ± 0.079 ^{A}
PCF	Tree	5.153 ± 1.424 ^{a,C}	1.105 ± 0.218 ^{b,B}	0.598 ± 0.071 ^{a,B}	0.701 ± 0.071 ^{a,B}
	Shrub	7.366 ± 1.680 ^{a,B}	1.185 ± 0.141 ^{B}	0.596 ± 0.059 ^{B}	0.693 ± 0.075 ^{b,B}
	Herb	10.278 ± 1.584 ^{a,A}	1.794 ± 0.169 ^{a,A}	0.781 ± 0.035 ^{a,A}	0.777 ± 0.047 ^{A}
PPF	Tree	4.075 ± 0.764 ^{b,C}	0.820 ± 0.099 ^{c,C}	0.436 ± 0.057 ^{b,C}	0.592 ± 0.086 ^{b,C}
	Shrub	5.347 ± 0.936 ^{b,B}	1.163 ± 0.176 ^{B}	0.614 ± 0.076 ^{B}	0.745 ± 0.100 ^{a,B}
	Herb	6.644 ± 0.893 ^{b,A}	1.395 ± 0.158 ^{b,A}	0.693 ± 0.059 ^{b,A}	0.815 ± 0.094 ^{A}

Note: The meanings of *S*, *H′*, *D*, and *J* are the same as those in Table 5. The meanings of PHF, PCF, and PPF are the same as those in Table 2. The lowercase letters (a, b, c) show the significant difference between various stand types and the same vertical structure layer ($p < 0.05$). The uppercase letters (A, B, C) show the significant difference between various vertical structure layers and the same stand type ($p < 0.05$). For non-significant differences among groups, no letters are appended to the values. All values are means ± standard deviation (SD).

3.3. Correlation between Plant Diversity Indices and Disease Index of Diplodia Tip Blight in P. densiflora

3.3.1. Tree Layer

In the tree layer of the *P. densiflora*–hardwood mixed forest, the disease index of Diplodia tip blight in *P. densiflora* was significantly negatively correlated with the species richness and the Shannon–Wiener, Simpson, and Pielou indices ($p < 0.05$). In the tree layer of the *P. densiflora*–conifer mixed forest, the disease index of Diplodia tip blight in *P. densiflora* was significantly positively correlated with the Shannon–Wiener, Simpson, and Pielou indices ($p < 0.05$). However, the disease index of Diplodia tip blight in *P. densiflora* was not significantly correlated with the species richness. In the tree layer of the *P. densiflora* pure mixed forest, the disease index of Diplodia tip blight in *P. densiflora* was significantly negatively correlated with the Shannon–Wiener and Simpson indices ($p < 0.05$). However, there was no significant correlation between the disease index of Diplodia tip blight in *P. densiflora* and the species richness or the Pielou index (Table 7).

3.3.2. Shrub Layer

In the shrub layers of all three stands, the disease index of Diplodia tip blight in *P. densiflora* was significantly positively correlated with the Shannon–Wiener and Simpson indices ($p < 0.01$). However, the disease index of Diplodia tip blight in *P. densiflora* was not significantly correlated with the species richness or the Pielou index (Table 7).

3.3.3. Herb Layer

In the herb layer, the disease index of Diplodia tip blight in *P. densiflora* was significantly positively correlated with the Shannon–Wiener and Simpson indices ($p < 0.05$) of the *P. densiflora*–hardwood mixed forest and the Shannon–Wiener, Simpson, and Pielou indices of the *P. densiflora*–conifer mixed forest. However, the disease index of Diplodia tip blight in *P. densiflora* was not significantly correlated with the species richness and the Pielou index of the *P. densiflora*–hardwood mixed forest, the species richness of the *P. densiflora*–conifer mixed forest, or the species richness, and the Shannon–Wiener, Simpson, and Pielou indices of the *P. densiflora* pure forest (Table 7).

Table 7. The correlation coefficients between plant diversity indices and disease index of Diplodia tip blight in *Pinus densiflora*.

Layer	Stand Type	*S*-DI	*H*′-DI	*D*-DI	*J*-DI
Tree	PHF	−0.430 **	−0.883 **	−0.796 **	−0.367 *
	PCF	0.218	0.667 **	0.672 **	0.385 *
	PPF	−0.221	−0.471 **	−0.392 *	−0.139
Shrub	PHF	−0.024	0.640 **	0.655 **	0.259
	PCF	0.114	0.471 **	0.463 **	0.196
	PPF	−0.122	0.522 **	0.444 **	0.228
Herb	PHF	0.118	0.431 **	0.397 *	−0.062
	PCF	0.204	0.637 **	0.660 **	0.433 **
	PPF	−0.059	−0.006	0.023	0.086

Note: * means $p < 0.05$, and ** means $p < 0.01$. '-' before the correlation coefficient means negative correlation, and no '-' means positive correlation. *S*-DI, *H*′-DI, *D*-DI and *J*-DI represent the correlation coefficients between the species richness, the Shannon–Wiener index, the Simpson index, and the Pielou index and Diplodia tip blight in *Pinus densiflora*, respectively.

3.4. Generalized Linear Model Analysis between Plant Diversity Indices and Disease Index of Diplodia Tip Blight in P. densiflora in Three Japanese Red Pine Stand Types

In the *P. densiflora*–hardwood mixed forest, the Simpson index of the tree layer was the most significant influence on the disease index of Diplodia tip blight in *P. densiflora* ($p < 0.05$), followed by the Simpson index of the shrub layer ($p < 0.01$) and the Shannon–Wiener index of the tree layer ($p < 0.01$). The values of AIC and BIC were 94.214 and 102.659, respectively. The optimal disease index prediction model in this stand was:

$$y = 43.538 - 11.165x_2 - 11.616x_3 + 11.195x_7 \ (p < 0.01) \quad (7)$$

In the *P. densiflora*–conifer mixed forest, the Simpson index of the herb layer was the most significant influence on the disease index of Diplodia tip blight in *P. densiflora* ($p < 0.01$), followed by the Simpson indices of the tree and shrub layers ($p < 0.01$). The values of AIC and BIC were 138.477 and 146.921, respectively. The optimal disease index prediction model in this stand was:

$$y = 7.467 + 14.639x_3 + 10.985x_7 + 27.454x_{11} \ (p < 0.01) \quad (8)$$

In the *P. densiflora* pure forest, the Shannon–Wiener index of the tree layer was the most significant influence on the disease index of Diplodia tip blight in *P. densiflora* ($p < 0.01$), followed by the Shannon–Wiener index of the shrub layer ($p < 0.01$). The value of AIC and BIC were 172.441 and 179.197, respectively. The optimal disease index prediction model in this stand was:

$$y = 36.495 - 8.693x_2 + 5.874x_6 \ (p < 0.01) \quad (9)$$

4. Discussion

In the present study, the disease index of Diplodia tip blight in the *P. densiflora*–conifer mixed forest was larger than the disease indices of Diplodia tip blight in the *P. densiflora*–hardwood mixed forest and the *P. densiflora*–pure mixed forest. Moreover, the disease index was positively correlated with the tree diversity indices. Similarly, Gerlach et al. [43] reported that the extent of damage caused by root rot (*Armillaria* sp.) rises with the increase of the proportion of coniferous species in a mixed forest. Our result was in good agreement with the associational susceptibility hypothesis that the disease severity intensifies when multiple host plants are combined [44–47]. In the *P. densiflora*–conifer mixed forest, there are high relative abundances of both native *P. densiflora* and introduced *P. thunbergii*. These species have a close genetic relationship and are hosts of the Diplodia tip blight pathogen. They have similar morphological, physiological, and biochemical characteristics, such as similar nutrients, which could provide enough

resources to support pathogen proliferation and increase the risks of host parasitization and Diplodia tip blight epidemics in *P. densiflora* [48].

The present study also showed that the disease index of Diplodia tip blight in *P. densiflora* was lowest in the *P. densiflora*–hardwood mixed forest and negatively correlated with the tree diversity indices. It is in line with Karlman et al.'s [49] conclusion that certain fungal diseases in conifer are negatively correlated with the hardwood richness. This phenomenon upholds the associational resistance hypothesis that the disease severity gradually decreases with increasing tree diversity in mixed forest consisting of both host trees and non-host trees [44,46]. In the *P. densiflora*–hardwood mixed forest, the host tree species of *S. sapinea*, *P. densiflora*, and its non-host tree species, *Q. acutissima* and *Q. variabilis*, all have high relative abundances. Non-host trees may create form physical or chemical barriers that impede the extensive localization, spread, and colonization of the pathogen [50]. In this way, non-host trees could lower the incidence of Diplodia tip blight in *P. densiflora*. In addition, the increase of the relative abundance in hardwood in the *P. densiflora*–hardwood mixed forest diminishes the amount of resources available to the pathogen, thereby hindering its growth and population establishment [47,50,51]. For example, as there are fewer *P. abies* in the *P. abies-Pinus sylvestris* L. mixed forest than there are in the *P. abies* pure forest, the range and quantity of *H. annosum* infections on adjacent trees are reduced in the former case [20].

The disease index of Diplodia tip blight in the *P. densiflora* pure forest is higher than that in the *P. densiflora*–hardwood mixed forest and lower than that in the *P. densiflora*–conifer mixed forest. Furthermore, the differences among stand types were significant. Meanwhile, the disease index was negatively correlated with the tree diversity indices. As the *P. densiflora* pure forest has a large proportion of host-*P. densiflora*, the disease index there is significantly larger than that in the *P. densiflora*–hardwood mixed forest. However, the other tree species with ratios of cross-sectional area at breast height <30% are mainly non-host hardwoods. Therefore, the disease index of Diplodia tip blight is lower in the *P. densiflora* pure forest than it is in the *P. densiflora*–conifer mixed forest.

Based on the foregoing findings, it is speculated that there are some promotion or inhibition effects between different tree species and the disease of Diplodia tip blight in *P. densiflora* that need to be confirmed. Pan [16] found that *Cephalcia Kunyushanica* Xiao have significant positive effects with host *P. densiflora* and *P. thunbergii*, and have significant negative effects with *Populus ussuriensis* Kom. and *Hovenia acerba* Lindl. The purpose of alleviating the harm of *C. Kunyushanica* could be achieved by properly matching *P. ussuriensis* and *H. acerba* among hosts. Therefore, taking some forest management measures to appropriately increase non-host broad-leaved tree species could mitigate the Diplodia tip blight disease.

There were significant positive correlations between the disease index of Diplodia tip blight in *P. densiflora* and the plant diversity indices in the shrub–herb layers of all three stands except the understory herb layer of *P. densiflora* pure forest. Clearly, there may be possible explanations that because of forest resource limitations, competition between trees and understory plants might dramatically increase with shrub–herb diversity. These factors influence the host growth regulation and reduce the host resistance to the pathogen of Diplodia tip blight [30]. Another reason may be that as plant diversity increases in the shrub–herb layers, high-humidity microclimates rapidly develop in the understory environments, which favor the proliferation and dispersal of pathogen mycelia and conidia in diseased needles, shoots, and stems. [1,52]. In this manner, the infection risk of Diplodia tip blight in *P. densiflora* increases with decreasing host tree vigor. To control the occurrence and dispersal of Diplodia tip blight in *P. densiflora*, the diversity structure of the understory vegetation must be modified in natural secondary Japanese red pine forests.

Researchers in China reported that excess precipitation causes root waterlogging and poor ventilation in host trees of *S. sapinea*, which results in weakening of the host trees and further aggravates the occurrence of Diplodia tip blight [1,17,53]. By contrast, the occurrence of Diplodia tip blight caused by *S. sapinea* is different in countries such as Germany, Italy, the United States of America, and South Africa, where asymptomatic endophytic

fungi become pathogen since host resistance is weakened by abiotic stresses such as precipitation deficits, elevated temperatures, drought, and hailstorms [8] (pp. 224–227), [6,54]. The above results inferred that extreme abiotic stresses increase disease susceptibility of host trees, and then lead to the prevalence of the disease. Therefore, to avoid the influence of the related abiotic factors, the effect of plant diversity on the occurrence of Diplodia tip blight in *P. densiflora* was analyzed in the case of relatively consistent environmental factors in the research plots.

Generalized linear model analysis revealed that the three prediction models respectively established with the Simpson indices of the tree and shrub layers and the Shannon–Wiener index of the tree layer as independent variables in the *P. densiflora*–hardwood mixed forest, the Simpson indices of the herb, tree, and shrub layers as independent variables in the *P. densiflora*–conifer mixed forest, and the Shannon–Wiener indices of the tree and shrub layers as independent variables in the *P. densiflora* pure mixed forest could better explain the variation of Diplodia tip blight in *P. densiflora* disease index in three stands. Both the Shannon–Wiener index and Simpson index contain the information of plant species richness and evenness and can reflect characteristics of plant diversity. In the practical application, the models obtained in the three stands were used to predict the disease index of Diplodia tip blight in *P. densiflora*, which could provide a reference for prevention and control of this disease.

5. Conclusions

Differences in the plant diversity among various stand types and vertical structure layers have different effects on the occurrence of Diplodia tip blight in *P. densiflora* in the Kunyu Mountains of China. In the tree layers, the occurrence of Diplodia tip blight in the *P. densiflora*–hardwood mixed forest and the *P. densiflora* pure forest upheld the associational resistance hypothesis, and that in the *P. densiflora*–conifer mixed forest supported the associational susceptibility hypothesis. The higher the plant diversity under the forest, the more intense competition between the pathogen host *P. densiflora* and shrub–herb plants, resulting in poor host disease resistance. Moreover, humid microclimates can develop and establish under the forest and facilitate the spread of the pathogen borne by diseased tissues. Plant diversity largely reflects the occurrence of Diplodia tip blight disease. However, if the effect of plant diversity on the disease is fully reflected, the plant species characteristics, especially tree genetic relationships, should be considered. Then, the plant species diversity structure may be modified through the forest management measures to lower the incidence of this disease.

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Infection and Spread of Root Rot Caused by *Heterobasidion parviporum* in *Picea abies* Stands after Thinning: Case Studies on Former Pasture and Meadow Lands

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Abstract: Afforestation of former agricultural lands is a common practice in several countries. This is beneficial for avoiding diseases carry-over from previous forest generations and to expand forest areas. However, several biotic and abiotic risks have been reported in such stands, including a higher risk of *Heterobasidion* root rot after thinning. Therefore, this study investigates the spread of *Heterobasidion* root rot in three *Picea abies* (L.) Karst. plantations in Latvia established on former pasture and meadow lands and subjected to forest management practices. Initially, to determine average infection rate, we sampled all standing trees (157 in total) along transects within sampling areas. On the transects, the proportion of trees suffering from *Heterobasidion* root rot varied from 16 to 33% among stands. Based on those data and observed dieback over all the stands, we established circular sample plots in disease centres where all trees and stumps were analysed. The average infection rate in the circular plots varied from 34 to 41%. Obtained *Heterobasidion* isolates were analysed with species-specific primers and were all determined to be *Heterobasidion parviporum* Niemelä & Korhonen. Isolates were paired to detect the number and size of genotypes. Of 141 genets examined, 99 were isolated from only one tree or stump, while 42 formed genets including two or more trees or stumps indicating spread of infection through root contacts. The total number of *Heterobasidion* genets per hectare in studied stands varied from 72 to 484. The following conclusions were reached: (i) stands on former non-forest lands (pastures and meadows) can be highly susceptible to *Heterobasidion* infection after thinning, (ii) the pathogen may form expanding territorial clones in both former pasture and meadows and (iii) stump treatment with biological or chemical control agents is recommended to prevent *Heterobasidion* infections.

Keywords: Norway spruce; *Heterobasidion* root rot; primary infection; secondary infection; first rotation forest; afforestation

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1. Introduction

Changes in land use from pastures, meadows, or agricultural fields to forest plantations is a common practice in many countries. For instance, in the European Union, 12.9 million hectares of abandoned lands were afforested in 1995–2015 [1]. Agricultural lands differ from lands with previous forest coverage, and thus forest management strategies should consider the most appropriate tree species and potential risks [2–4]. Therefore, several studies have compared stand growth rate and growth conditions in forest lands vs. former agricultural lands [5,6].

One of the most commonly planted conifer tree species in former agricultural lands in Latvia and other Baltic and Nordic countries is Norway spruce (*Picea abies* (L.) Karst.) [7].

Norway spruce is highly susceptible to root rot caused by *Heterobasidion* spp. [8–13]. In Latvia, the country-scale inventory revealed that about 22% of spruce trees contained heart rot, mostly caused by *Heterobasidion parviporum* Niemelä & Korhonen, which results in severe economic losses [14,15]. More than half of the territory of Latvia is covered by forest and the forest sector contributes almost 20% to total exports from Latvia [16]. Consequently, reduction of economic losses caused by root rot is crucial.

Infected previous-generation stumps are important for transfer of *Heterobasidion* infection to subsequent tree generation through root contacts [10,11,17–21]. Therefore, forest planting in infection-free areas and afforestation on former agricultural lands or pastures are important strategies to decrease infection potential [22].

Nevertheless, several studies have reported similar butt rot incidence rate in former fields or grazing land in comparison with former forestlands [23] or even severe damage in former agricultural lands [24–27]. Modelling based on stump infection rate with *Heterobasidion* in spruce stands revealed that former pastures and arable lands are more susceptible to root rot than forest stands [28]. A more recent study form Czech Republic indicated differences in infection rate among different previous land uses, indicating higher infection in former arable lands than in former pastures [29].

Higher infection rate on former agricultural lands has been related to different factors: (i) soil chemical parameters such as high carbonate content, high nitrogen content or overall unbalanced nutrient status [4,30–32], (ii) soil compaction that stimulates the superficial root system of Norway spruce [26], (iii) biotic factors such as lack of fungi antagonistic to *Heterobasidion* [4,26,31,33–36], and (iv) increased percentage of grass cover [30].

Forest management practices, especially cuttings, are significant for the establishment of *Heterobasidion* infections in stands on former agricultural lands where primary spore infection of freshly cut conifer stumps is the main route for disease spread. In contrast, in former forest lands, mycelial infections originating from the old stumps or roots can be a more significant source of disease spread. Therefore, several studies on former agricultural land have focused on root rot development after first thinning [37–39]. Although the incidence of *Heterobasidion* root rot is highly dependent on the degree of stump infection, the possibility of direct root infection cannot be completely omitted. For instance, Rönnberg et al. [40] showed high incidence of *Heterobasidion* root rot in unthinned spruce stands planted on pastureland. Thor and Stenlid [41] found 0.5–8.1% of trees infected in unthinned spruce stands established on former agricultural land and even up to 16.9% infected in an unthinned stand in former pastureland.

Secondary infections occur when hyphae of the pathogens spread to root systems of adjacent trees via root contacts, which can produce expanding disease centres [42,43]. To obtain detailed knowledge on secondary infection, Swedjemark and Stenlid [44] analysed the population structures of *Heterobasidion annosum* s.l. in spruce stands on former arable lands and found that seven years after thinning, 63% of trees have decay symptoms and were infected by mycelial spread from stumps. In another Swedish study, comparing the growth rate of artificially inoculated *Heterobasidion* mycelia in the stem and roots of Norway spruce growing on forest land and arable land, concluded that on average, *Heterobasidion* growth rate in both tree and stump roots did not differ significantly between former arable and forest sites [19].

However, the previously mentioned studies were carried out on former agricultural lands in Sweden and Scotland and the results are not necessarily applicable to other soil and forest types as well as different management practices (both agricultural and forestry). Comparable studies have not been performed in Norway spruce stands in Latvia or neighboring countries, indicating the need for such studies. In a recent Latvian study dealing with *Heterobasidion* root rot in Scots pine stands, Zaļuma et al. [45] reported a lower expansion rate of *Heterobasidion* root rot in pine plantations established on former agricultural land in comparison with former forestland. Consequently, in addition to the previously mentioned studies, it is very important for practical forestry in Latvia to obtain

detailed, more specific information about the spread of *Heterobasidion* root rot in Norway spruce stands on sites with non-forest history.

The aim of this work was to investigate the distribution patterns (primary and secondary infection) of *Heterobasidion* spp. in managed Norway spruce plantations established on former meadows and pasturelands in central part of Latvia.

2. Materials and Methods

2.1. Study Sites and Experimental Design

The study was established in three Norway spruce stands in Central Latvia, in the municipalities of Babite, Auce, and Olaine (Figure 1). In this region, average annual temperature ranges from 6.8 to 7.9 °C and annual precipitation rate is 574 to 636 mm. For Central Latvia, dry pine and spruce forests on sandy or loamy soils, water-saturated areas (peat bogs, wet forest types, flood plain meadows, etc.) with peat soils or drained forests and a large proportion of former agricultural fields on loamy soils are characteristic. The first-rotation spruce plantations in the study represent this variety: a drained forest within a region of peat bogs (Stand 1), a spruce plantation within a region dominated by agricultural fields (Stand 2), and a dry-type (*Oxalidosa*) stand (Stand 3). Characteristics of the investigated areas are presented in Table 1.

Table 1. Description of Norway spruce stands selected for the study: general information, forest characteristics, and details of sampling area and soil parameters.

	Stand 1	**Stand 2**	**Stand 3**
General information			
Location	56°84′ N, 23°77′ E	56°38′ N, 23°12′ E	56°82′ N, 24°07′ E
Municipality	Babite	Auce	Olaine
Altitude, m	17	85	13
Annual rainfall, mm	600–650	550–600	650–700
Forest data			
Total area, ha	8.7	2.75	1.54
Age, years	73	82	52
Average height, m	27.1	28.8	23.5
DBH, cm	35	30	24
Yield per ha	342	387	268
Forest type	*Vacciniosa mel.*	*Myrtillosa mel.*	*Oxalidosa*
Thinning years *	2007, 2015	1991, 2001	1987, 2017
Previous land use	Pastureland	Wet meadow	Meadow
Other tree species (<5% admixture)	*Betula pendula*	*Populus tremula*	*Pinus sylvestris*
Sampling area			
Length of transect, m	500	400	250
Area of transect, m^2	1000	800	500
Total No. of circle sample plots	11	6	5
Total area of circles, m^2	3454	1884	1570
Soil			
pH (KCl)	4.1	5.9	4.6
C, (g/kg)	146	103	106
N, (g/kg)	7.6	8.5	7.5
P, (g/kg)	0.03	0.06	0.03
K, (g/kg)	0.02	0.05	0.02
Ca, (g/kg)	4.4	8.9	6.9
Mg, (g/kg)	0.16	0.88	0.25

* All stands were thinned twice. No data of the exact thinning period and season is available.

All stands had selective or sanitary thinning (Table 1); since after thinning, stumps were not treated with any control agents to prevent infection of root rot, at least potentially, stumps were exposed to primary infection of *Heterobasidion*.

Soil samples for chemical analysis were collected at 0–20 cm depth along transects every 50 m. Chemical analyses of soil were conducted using established standard methods (International Organization for Standardization (ISO) standard) at LSFRI Silava, Laboratory of Forest Environment, Salaspils, Latvia. Samples were prepared for analysis according to ISO 11464:1994. The soil pH was determined in 1 M KCl (soil—extractant mixture 1:2.5). Total carbon content was determined using an elemental analyser ELTRA CS530 (Eltra GmbH, Haan, Germany) according to LVS ISO 10694. The concentration of total N was determined using the Kjeldahl method (ISO 11261:1995) and P was assayed by colourimetry using the ammonium molybdate spectrometric method in 1 M HCl solution (soil/extractant mixture 1:5). Concentration of K, Ca, and Mg was determined by atomic absorption spectrophotometer with an acetylene-air flame in 1 M HCl extract.

Figure 1. Location of the study sites in the central part of Latvia.

To determine average infection rate, we sampled all standing trees within a 2-m-wide transect parallel to the longest edge of each stand with starting points in the middle of the shortest edge. Health status was assigned to each tree in the transect: (i) visually healthy crowns and (ii) trees with severe dieback symptoms, dead and windthrown trees [9]. To detect the pathogen under laboratory conditions, wood samples were collected from all trees on the transect with the aid of an increment borer (one sample per tree at root collar height). Additionally, to characterise stand growth rate, we measured the diameter at breast height and, for living trees, the tree height (Table 1).

Based on the transect data about the distribution of *Heterobasidion* infected trees and visual observation of potential infection centers, we established from five to eleven circular sample plots per stand (10 m radius, 314 m^2) where all trees and stumps were sampled and examined for *Heterobasidion* fruit bodies. In addition, stumps were classified as either stumps from the 1st or 2nd thinning. Broken dead tree trunks of different heights were categorized as stumps. Wood samples from trees were collected with an increment borer (one sample per tree at root collar height) and from stumps—either with the same method or with the aid of an axe if they were heavily decomposed. Wood samples from each tree were placed into individual plastic tubes and stump samples into individual plastic bags. All sampled trees and stumps (both in transects and circular plots) were numbered and their location mapped. Field work was carried out from March 2019 to April 2020.

2.2. Isolation and Identification of Heterobasidion Genotypes

In the laboratory, all wood samples were surface sterilized by flaming and placed on petri dishes containing malt extract agar (MEA) media (for tree samples) or Hagem agar media (for stump samples). After a 5 to 7-day incubation, the plates were examined under a Leica stereomicroscope M205C (20–30× magnification) to detect the presence of

Heterobasidion spp., observing its characteristic asexual sporulation (conidiophores and conidia) on wood samples [10]. If *Heterobasidion* was observed, colonies were sampled with sterilised surgical forceps and placed on new MEA media. Outgrowing *Heterobasidion* mycelia were subcultured to obtain pure fungal cultures.

Isolates originating from the same circular sample plot were subjected to pairwise somatic compatibility tests by confronting their mycelia on Hagem agar media in 9 cm Petri dishes in all combinations. The genets were identified either by recording the line of demarcation in the contact zone of confrontation (demonstrating that the genotypes are different) or by observing free fusion of the mycelia (implying that the genotypes were identical) [46].

The results of this test provided an information about the total number and size of *Heterobasidion* genets: number of genets encompassing only one tree or stump and number of genets encompassing two or more trees and (or) stumps. Based on these data, we estimated the number of trees/stumps infected by *Heterobasidion* genets per hectare and per disease centre. To estimate the area and maximum width of each genet including two or more trees/stumps, we used the map of each site with all sampled trees and stumps mapped. We used species specific primers (*Heterobasidion parviporum* specific primers KJ-F and KJ-R and *Heterobasidion annosum* specific primers MJ-F and MJ-R) to determine the *Heterobasidion* species of each genotype [47]. DNA was extracted from fungal cultures representing each genotype using a CTAB method [48]. Two PCR reactions were run with each sample—one with *H.parviporum* and other with *H.annosum* species specific primers listed above. PCR conditions, were a described by Hantula and Vainio [47]. DNA from *H. parviporum* and *H. annosum* was used as a positive control.

2.3. Data Analysis

A chi-square (χ^2) test of independence from the actual number of observations of infected or decaying trees and stumps was used to compare abundance patterns between sites and sample plots [49].

Using the geospatial tool QGIS 3.2.0-Bonn (bc43194) (https://qgis.org/api/3.2/), tree spatial location was visualised and the width and the area of genets including two or more trees/stumps was measured (tool "MeasureLine" and "MeasureArea"). The maximum expansion rates were calculated as a reference taking years from the first thinning for each stand (2007, 1991 and 1987, respectively, Stand 1 to 3) or from the second thinning (2015, 2010 and 2017, respectively, Stand 1 to 3).

To assess which factors have an impact on the number of trees per genet, we used generalized linear mixed models (GLMM) assuming a Poisson error distribution. The full model included number of trees per genet as a response variable and as fixed effects we tested site, stand age class (older stands (Stand 1 and 2) vs. younger stand (Stand 3), disease centre infection indicators (classes of infection (<40% vs. >40%), stump and dead tree proportion) and as a random factor in all models we included disease centre. Models were calculated using the program package lme4 [50].

Area and width of genets including two or more trees/stumps as well as maximum expansion rates (area and width) were tested for normality using the Shapiro and Wilk normality test at significance level $\alpha = 0.001$ [51]. If normality was assessed, one-way analysis of variance (ANOVA) with Fisher LSD post-hoc test was calculated [52]. If normality was not assessed, nonparametric Wilcoxon test (unpaired samples) was used to compare parameters. For calculations, R version 4.0.2 was used [53].

3. Results

3.1. Incidence of Dieback and Heterobasidion Infection

In total, in all three study sites, 851 wood samples were collected for *Heterobasidion* isolation (397 from living trees, 118 from dead trees and 336 from stumps). The number of sampled trees and stumps are given in Table 2. In all stands, stumps from both 1st and

2nd thinning were present and in addition there were some trees broken close to their base (mainly in Stand 2) (Table 2).

Table 2. Characteristics and dieback rate in Norway spruce stands on former pastures (Stand 1) and meadows (Stands 2 and 3). (T—transect; C—disease centres (circular sample plots).

	Stand 1		Stand 2		Stand 3	
	T	C	T	C	T	C
Living trees						
Total number *	72	95	25	73	34	153
Number per hectare	720	275	313	387	680	975
Dead trees						
Total number *	8	41	10	37	8	29
Number per hectare	80	119	125	196	160	185
Stumps						
Total number	n.d.	142	n.d.	96	n.d.	98
No. of 1st thinning stumps	n.d.	65	n.d.	45	n.d.	29
No. of 2nd thinning stumps	n.d.	75	n.d.	39	n.d.	69
No. of other stumps **	n.d.	2	n.d.	12	n.d.	-
Number per hectare	n.d.	411	n.d.	510	n.d.	624

* Several trees were present in both transect and disease centre. ** Category "other stumps" includes trees broken at different heights.

In Stand 2 and 3, chi-square test of independence showed that transect and disease center data have similar tendencies regarding the number of living and dead trees ($p > 0.05$). In contrast, chi-square test for Stand 1 data indicated significant differences in living and dead tree abundance in disease centers and the transect ($\chi^2 = 11.65$; df = 1; $n = 216$) (there were less dead trees in the transect ($\chi^2 = 5.67$) than expected).

Additionally, a chi-square test of independence was performed to examine the numbers of living trees and stumps among sites. The relation between these variables was significant, $\chi^2 = 24.17$ (df = 2, $n = 657$), $p < 0.05$. The highest chi-square values were observed for both living trees and stumps in Stand 3: more living trees ($\chi^2 = 7.52$) and less stumps ($\chi^2 = 7.18$) than predicted.

The transects of both Stand 2 and 3 had several openings, which explains the lower number of trees per hectare in comparison with disease centres (Table 2). In Stand 3, these openings were related to stand planting design (there were several gaps left to facilitate thinning). Stand 3 had higher planting density that reflects in the number of trees per hectare (Table 2).

In addition, fruit bodies were recorded as an indicator of disease of trees and stumps. There were more records of fruit bodies in Stand 1 (two dead trees and 10 stumps with fruit bodies) compared to other stands (there were two fruit bodies recorded in Stand 2 and four fruit bodies in Stand 3, found exclusively on stumps in both stands).

After incubation of 851 collected wood samples, we detected pathogen conidiophores with conidia in 302 samples (35%); infection rate varied among stands (30% in Stand 1 and 39% in Stands 2 and 3) (Table 3).

Chi-square test indicated that in transects and disease centres, the incidence of *Heterobasidion* infection was similar among sites ($p > 0.05$). In addition, the infection incidence of both living and dead trees in disease centres have a similar pattern among sites ($p > 0.05$). In total, we confirmed the presence of the pathogen in 31.8 and 48.3% of all living and dead tree samples, respectively. Infection incidences of living trees among sites was 24.5, 38.6 and 35.2% (Stand 1 to 3, respectively) and of dead trees 58.1, 35.7 and 51.5% (Stand 1 to 3, respectively). In total, we confirmed the presence of the pathogen in 34.2% of all stump samples. Infection incidences varied among stump samples at different sites: 26.1, 39.6 and 40.8% (Stand 1 to 3, respectively). However, the differences were not significant.

Table 3. Frequency (% *) of Norway spruce trees or stumps infected by *Heterobasidion parviporum* in managed forests on former pastures and meadows.

	Stand 1	Stand 2	Stand 3
Transect; %	**16.3**	**28.6**	**33.3**
Living trees; %	12.5	25.7	23.8
Dead trees; %	3.8	2.9	9.5
Disease Centres; %	**33.5**	**41.3**	**38.6**
Living trees; %	11.5	15.5	18.9
Dead trees; %	8.6	7.3	5.4
Stumps; %	13.3	18.4	14.3
Total; %	**29.6**	**38.5**	**38.9**

* Percentage expresses the proportion of trees/stumps where *Heterobasidion parviporum* was detected of all trees/stumps sampled.

3.2. Analysis of Heterobasidion Genets

Pure *Heterobasidion* sp. cultures were successfully isolated from 90.5% of a total of 286 wood samples producing *Heterobasidion* conidiophores with conidia collected from disease centers (76.3, 98.8, and 96.3% from Stands 1 to 3, respectively) (Table 3). Identification with species specific primers revealed that all obtained isolates belonged to species *Heterobasidion parviporum*.

In total, 141 *Heterobasidion* genets were detected resulting in 72 to 484 *Heterobasidion* genets per hectare among study sites (Table 4). Less genets per hectare were detected in Stand 1 (2.9 times and 6.7 times less in comparison with Stand 2 and Stand 3, respectively).

Table 4. Distribution pattern and characteristics of *Heterobasidion parviporum* genets in disease centres of managed Norway spruce stands on former pasture and meadows.

	Stand 1	Stand 2	Stand 3
Genets including one tree/stump			
Infection per hectare	38	143	376
• Trees infected	35	64	274
• Stumps infected	3	79	102
Frequency (%)	21%	25%	56%
Genets including two or more trees/stumps			
Number of trees and stumps per genet (min.-max.)	4.8 (2–12)	4.4 (2–11)	2.6 (2–7)
Infection per hectare	170	302	293
• Trees infected	120	185	191
• Stumps infected	50	117	102
Distance among trees and (or) stumps, m			
• Mean ± SE	9.6 ± 1.7	6.9 ±1.0	3.1 ± 0.5
• Min.-max.	1.8–18.0	2.1–14.6	1.0–6.9
Area, m^2			
• Mean ± SE	56.4 ± 19.7	12.4 ± 4.9	5.9 ± 2.9
• Min.-max.	1.2–137.4	0.3–52.2	1.3–17.3
Frequency (%)	79%	75%	44%
Total infection per hectare	208	445	669

The mean number of trees/stumps per genet was 2.8, 2.1 and 1.4 in Stand 1 to 3, respectively. Genets including one tree/stump were the most abundant in all sites: 13, 27, 59 (i.e., 52, 68 and 78% of all genets per stand), in Stand 1 to 3, respectively. Expanded

genets including two or more trees/stumps were less frequent 12, 13 and 17 (Stand 1 to 3 respectively). Chi-square test indicated differences among abundance of genets including one tree/stump and genets including two or more trees/stumps per sites ($\chi^2 = 6.1$, df = 2, $n = 141$; $p > 0.05$).

Frequency of trees or stumps infected by genets including one tree/stump of all infected trees and stumps was 21, 25 and 56% (Stand 1 to 3, respectively). Chi-square test indicated differences among abundance of trees and stumps infected by genets including one tree/stump per sites ($\chi^2 = 26.3$, df = 2, $n = 259$; $p > 0.05$), in particular abundance of trees and stumps of genets including one tree/stump in Stand 1 was lower than predicted ($\chi^2 = 7.4$) and in Stand 3 higher than predicted ($\chi^2 = 8.4$).

In comparison with Stand 1 and 2, Stand 3 had more isolates and consequently more genets (in total and both genets including one tree/stump and genets including two or more trees/stumps). Stand 3 had a lower number of trees per genet and less expanded genets including two or more trees/stumps (lower distance among trees and area) (Table 4). Modeling the number of trees per genet and including disease centres as a random factor revealed that grouping Stand 1 and 2 together (older stands) vs. Stand 3 identified a significant difference ($p < 0.05$).

The maximum number of trees infected by genets including two or more trees/stumps are presented in Table 4. The largest genets included both living and dead trees and stumps. The longest mean distance between trees and (or) stumps with shared genets was about 7–10 m (maximum 15–18 m) (Stands 1 and 2) compared with values of Stand 3 (mean longest distance 3 m; maximum 7 m) (Table 4). Analysis of variance showed significant differences and post-hoc tests indicated that the genets including two or more trees/stumps in Stand 3 were significantly ($p < 0.05$) smaller in size than genets in Stand 1 (3.3 times smaller) and in Stand 2 (2.3 times smaller).

The maximum area of genets including two or more trees/stumps in Stand 1 was 2.6 to 8 times greater than in Stand 2 and 3 (Table 4). The mean area of genets including two or more trees/stumps was also 5 to 9 time larger in Stand 1. Analysis of variance showed significant differences and post-hoc tests indicated that the genets including two or more trees/stumps in Stand 1 had significantly ($p < 0.05$) larger area than both Stand 2 and 3 ($p < 0.05$).

The maximum expansion length per year calculated from the first thinning was 1.5, 0.5 and 0.2 m (Stand 1 to 3, respectively) and maximum expansion area per year: 11.5, 1.9, and 0.5 m^2 (Stand 1 to 3, respectively). Wilcoxon test showed that also mean expansion indicators (distance and area per year) were higher in Stand 1 in comparison with Stand 2 and Stand 3 ($p < 0.05$).

4. Discussion

4.1. Dieback and Observed Heterobasidion Infection of Norway Spruce on Former Pasture and Meadow Lands

Visual observation of stand health status revealed expansive spread of root rot in the studied Norway spruce stands on former non-forest lands (pastures and meadows) in Latvia. The greater total number of trees and stumps per hectare in Stand 3 is mainly related with greater planting density. The number of stumps indicates intensity of thinning and, therefore, potential infection risk of stumps by *Heterobasidion* spores. Greater abundance of stumps might also favor the mycelial spread of *Heterobasidion* since its growth rate in stump roots is significantly faster than in living trees [19,54]. Moreover, in densely planted stands, where the trees or stumps are closer to each other, the possibility of root contacts is higher, thus allowing greater probability of infection transmission [4].

In Stand 2, there were several trees broken at different heights (Table 2), which indirectly indicates reduced tree vitality. In addition, gaps and a low number of trees per transect might indicate not only a reduced number of trees due to the stand age but also poor current and previous pathological conditions within the stand.

Fruit bodies were found in all study sites, which indicates that environmental conditions were suitable for fruiting of *H. parviporum*. Consequently, the risk of primary infection

by *H. parviporum* spores should be taken into account when planning future thinning in these stands or other similar sites.

The detected *H. parviporum* infection rate (30% to 39%) was on average in line with data from other studies from both former agricultural and forest lands. It indicates that even though no inoculum is present in former non-forest lands, infection can establish and expand as successfully as in forest lands or even more successfully. For instance, in a Swedish study carried out on a spruce stand on former agricultural land, seven years after thinning, 63% of the trees had decay symptoms [45]. In Sweden, modelling of spruce stands including different former land use indicated 2–90% of rotted trees in final cuttings [55]. In Denmark, on average, 23% (max. 94%) of Norway spruce individuals growing on former agricultural or grazing fields were found to suffer from butt rot [23].

The success rate of *H. parviporum* isolation from dead spruce individuals was 35.7 to 58.1%. However, since the pathogen was not detected in all dead spruce individuals, it cannot be excluded that either the infection has not yet spread higher in the stem or that causes other than *Heterobasidion* root rot were responsible for a proportion of declining or dead trees. Other fungal agents as *Armillaria* sp. can cause root rot in Norway spruce stands [56]. In addition, spruce dieback not related to root rot has been observed in several regions in Latvia [57].

The frequency of stumps colonized by *H. parviporum* (34.2% on average) was relatively high in spruce stands planted in former pastures and meadows in Latvia. Viable *Heterobasidion* mycelium can survive in stumps for decades [58]. However, with time, it develops more intensively in deeper layers of stumps [59] and isolation can be difficult due to wood decomposition and presence of other stump colonizing fungi. Since the stumps in the studied stands were not recently cut, total stump infection may be even higher. Infected spruce stumps left after thinning transfer root rot to residual trees and serve as a source of infection [21,54]. Therefore, winter thinning and use of biological control agents containing spores of *Phlebiopsis gigantea* or other preventive substances such as urea is recommended to limit the infection of conifer stumps by basidiospores of *Heterobasidion* spp. [43,60–62], especially in stands with no previous rot incidences such as in stands established on former agricultural lands [4,63,64].

4.2. Heterobasidion Genets

All obtained *Heterobasidion* isolates were identified as *Heterobasidion parviporum*, which is the most common *Heterobasidion* species causing damage in spruce stands in Northern Europe and Latvia [15,65]. *H. parviporum* is adapted to spruce as the host species [66,67] and is therefore more often observed in spruce stands, being predominant or exclusive in some cases [9,68–70], and is able to spread more frequently from stumps to neighbouring trees than *H. annosum* [71]. Korhonen et al. [72] reported that *Heterobasidion* infections in spruce stands on former agricultural lands were mainly caused by *H. parviporum* and concluded that in such first rotation spruce stands without any history of pine growth and woody inoculum, spores of *H. parviporum* are the principal infection source. Moreover, Vasiliauskas and Stenlid [18] showed that *H. parviporum* is better adapted to spruce wood, and spreads more extensively in spruce stems compared to *H. annosum*.

In our study, the number of trees/stumps infected by *H. parviporum* per hectare was 208, 445 and 669 (Stand 1 to 3, respectively) (Table 4). In addition, the total number of *Heterobasidion* genets per hectare in the present study was high: 72, 212, and 484 (Stand 1 to 3, respectively). In comparison, in a Finnish study on *Heterobasidion* infections on clear-felled forests on old forest sites, the number of fungal genets per hectare varied from 2 to 158 (mean 28) [73]. The high infection rate and high number of *Heterobasidion* genets in the present study may indicate a higher susceptibility of Norway spruce to *Heterobasidion* root rot on former agricultural lands compared to old forest lands [2].

The number of genets including only one tree or stump was lower in older stands (Stand 1 and 2) than in the youngest one (Stand 3). Time from the first thinning did, however, not differ significantly between the stands (Table 1). However, even in final cuttings on

old forest sites, genets including one tree/stump and therefore primary infection can be dominant [73]. In general, a lower number of trees infected by the same *Heterobasidion* genet and occupying a smaller stand area indicate that these clones are relatively younger than those comprised of more trees and covering a larger area [44]. However, the age of the genets of *Heterobasidion* may affect the pathogenicity of the fungus, with younger genets being more pathogenic [39].

Development of territorial clones following primary infection of *Heterobasidion* takes at least 7–8 years [42,44,74]. In our study, 22 to 48% of detected genets formed expanding territorial clones, which included 44 to 79% of infected trees and (or) stumps. This indicates that infection in all sites is well established and expanding. Due to well-developed clonality, in Stand 1 and 2, it is most probable that infection was initiated due to the first thinning (12, 28 years ago), and not by the more recent ones (4, 18 years ago) (Stand 1 and 2, respectively). In Stand 3, infection history seems to be much more recent than in other stands and probably more expansive after the last thinning (two-years before sampling) than after the first one (32-years before sampling). In addition, greater planting density in Stand 3 could favour development of territorial clones in a shorter period as indicated previously [35,42,44].

Comparing the maximum size of *Heterobasidion* genets with other studies, we found genets of similar size also reported from stands with a much longer infection history. For instance, Vasiliauskas and Stenlid [18] reported territorial clones of *H. annosum* s.l. up to 20 m in diameter in a 60 year-old spruce stand planted in a previously infected pine site, while in the oldest stands of our study (Stand 1 and 2), territorial clones reached up to 14–17 m. In addition, the largest distance between isolates from the same genet in Finnish study [69] in 70 to 100 year-old spruce stands was 13 m, which is less than in our study on former non-forest lands. Previous studies indicate that *H. parviporum* can form large territorial clones. Stenlid [46] studied a 120-year-old and thinned *P. abies* stand and detected *H. parviporum* territorial clones reaching up to 30 m in diameter. Similarly, up to 40 m long territorial clones of *H. parviporum* were observed in an intensively thinned *P. abies* stands in Serbia [74]. In related studies from Finland, the maximal length of territorial clones varied between approximately 10 and 30 m [9,66] or reached up to 55 m [73]. Our study shows that even 12 to 32 years after thinnings in stands on former pastures and meadows, *H. parviporum* can form large territorial clones.

The maximum number of trees per genet in our study was 12 and this is lower in comparison to stands on former forest lands (i.e., a Finnish study [67] reported 16 trees per genet) and also to stands on former pasturelands, where a greater number of trees (up to 25) forming territorial clones have been reported [40]. However, the real number of infected trees and stumps in our study could be greater than detected due to various reasons: (i) stump decomposition and poor isolation success from some samples, (ii) sampling height of trees (root collar) excluding infection in lower zones, (iii) sample number (one sample per tree) not covering all stem inner surface where decay column can be located, and (iv) limited sampling area (territory of circular sample plot). In addition, to estimate the exact borders and size of *Heterobasidion* genets, a thorough analyses of excavated root systems would be required. Consequently, the size of the genets may actually be bigger.

All the largest *H. parviporum* genets of our study contained three to nine dead trees or stumps. Stumps colonized by *Heterobasidion* provide a substrate from which the fungus can spread and cause infection in standing trees [21]. A study by Capretti and Mugnai [26] indicated that dead wood (buried wood in their study) might be an important factor for extensive spread of the pathogen especially in former pasture soil. Recent studies show that root fragments are important for *H. parviporum* infection transfer [75]. Therefore, both abundance of dead wood and roots could be related with the spread of the pathogen in Stand 1 (former pastureland). The average expansion rate of territorial clones of *H. parviporum* on Stand 2 and 3 (former meadows) (0.25 and 0.10 m·year^{-1}) corresponds to results obtained by Bendz-Hellgren et al. [19], where the average *H. annosum* s.l. growth rate on former agricultural lands was 0.25 m·year^{-1} in spruce stump roots and 0.09 m·year^{-1} in

living tree roots. The average *H. parviporum* growth rate in the Stand 1 (former pasture-land) was high, 0.8 m·year^{-1}, in comparison with results on both forest and arable lands reported by Bendz-Hellgren et al. [19]. This might indicate an erroneous assumption of infection year (i.e., infection had occurred before first thinning via wounding). Since no wounds were observed on trunks, current primary infections probably occurred via root injuries. Spores of *Heterobasidion* are present in soil, where they can maintain their vitality at least for a year and infect injured tree roots [32]. Rönnberg et al. [40] reported a high incidence of *Heterobasidion* root rot in unthinned spruce stands planted on pastureland. In addition, Vollbrecht and Agestam [28] and Capretti and Mugnai [26] indicated that former pastures were highly susceptible to root rot. Capretti and Mugnai [26] also concluded that *Heterobasidion* growth rate in dead wood was particularly high in pasture lands. The increased capacity of *H. parviporum* to colonise dead wood and form fruitbodies in Stand 1 of our study is a probable scenario, explaining the greater expansion of genets.

In research sites, stumps were not treated against *Heterobasidion* root rot, which in a short period resulted in severe damage as reported above. Therefore, we suggest that stump treatment should be considered as a general practice on former pastures and meadows to prevent both primary and subsequent infections, as suggested by other authors [41,43,62,64]. This is particularly relevant for private forest owners who are often managing forests on former agricultural lands, but often do not utilise preventive stump treatments.

Further studies should focus on the presence of *Heterobasidion* spores in soils of former agricultural lands in relation to the soil microorganism community and root injuries.

5. Conclusions

This study generated new data on the ecology and patterns of spread of *Heterobasidion parviporum* in Norway spruce stands on former pastures and meadows. From these novel data, we concluded the following: (i) on average, primary infection via spores in such stands is dominant, but secondary infection increases with stand age, (ii) *H. parviporum* can form large (up to 137 m^2) territorial clones in forests on former pasture and meadow lands causing extensive tree dieback and mortality. Therefore, to reduce economic losses in spruce plantations on former pastures or meadows, stump treatment with biological or chemical control agents that prevent *Heterobasidion* infections should be considered in thinning operations. It is important to facilitate the implementation of stump treatment for private forest owners, who in many cases are owners of forests on former agricultural lands.

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Article

Inferences on the Susceptibility of Wood of Different Tree Species to *Heterobasidion annosum* Sensu Lato Primary Infections and on the Range of Pathogen Spores Dispersal

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Abstract: Stumps play a pivotal role in the epidemiology of the fungal forest pathogens *Heterobasidion* spp. because they are the main courts of primary airborne infections. The aims of this study were (i) to determine the susceptibility of seven tree species (i.e., *Larix sibirica*, *Picea abies*, *Picea sitchensis*, *Pinus contorta*, *Pinus strobus*, *Pinus sylvestris* and *Pseudotsuga menziesii*) to primary infection by *H. annosum* and *H. parviporum* through comparative inoculation experiments of conidia on wood discs in controlled conditions; (ii) to compare the susceptibility of wood discs of the same tree species to natural airborne infections in two Latvian Norway spruce forest stands infested either by *H. annosum* or *H. parviporum*; (iii) to explore the rates of infection of wood discs at increasing distances from spore sources in these two forests to make inferences on the range of spores dispersal. Results obtained by spraying wood discs with conidial suspensions in controlled conditions are in agreement with those obtained by exposing wood discs to the natural airborne inoculum in the forests, as clearly supported by the significant correlation ($r = 0.79$; $p < 0.05$) between the two sets of data. Susceptibility was highest in *Pinus* species, followed by *P. abies* and *P. sitchensis*. Susceptibility was lowest for *L. sibirica* and *P. menziesii*. The area colonized by *Heterobasidion* spp. in the sapwood of wood discs was much greater than that colonized in the heartwood. A sharp decrease in the rate of infection of wood discs with distance from spore sources (i.e., fruiting bodies) was observed, further confirming the importance of local spore sources in the epidemiology of *Heterobasidion* spp. Taken together, these findings could help designing tactics to manage these fungal forest pathogens.

Keywords: basidiospores; conidia; *Heterobasidion* spp.; spore dispersal; susceptibility; wood discs

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1. Introduction

Heterobasidion is a genus of basidiomycetes in the family of Bondarzewiaceae, including several species, two of which, i.e., *H. annosum* (Fr.) Bref. and *H. insulare* (Murrill) Ryvarden, have been reported to be species complexes [1]. While most *Heterobasidion* are saprotrophs, those included in the *H. annosum* species complex, hereafter referred to as *H. annosum* sensu lato (s.l.), are pathogenic and may be particularly damaging, especially in managed coniferous stands [1,2]. Within *H. annosum* s.l., three native species occur in Europe: *H. abietinum*, *H. annosum* sensu stricto (s.s.), hereafter referred to as *H. annosum*, and *H. parviporum*. While the former is found mainly in central and southern Europe in association with its main host, silver fir (*Abies alba* Mill.), *H. annosum* and *H. parviporum* are more widespread throughout Europe, including the Baltic States [2–4]. *Heterobasidion annosum* and *H. parviporum* display different host preferences: *H. parviporum* mainly infects Norway spruce (*Picea abies* (L.) H. Karst.), whereas *H. annosum* is commonly associated

with pines (*Pinus* spp.), especially Scots pine (*Pinus sylvestris* L.), although it can also infect Norway spruce and some deciduous trees [5–7]. In addition, the North American invasive *H. irregulare* has been reported in Italy on Italian stone pine (*Pinus pinea* L.) [8–10].

Heterobasidion annosum s.l. spreads by means of spores infecting either freshly cut stump surfaces or trees through tree wounds [1]. Once the fungus has colonized the root systems of stumps or trees, it can spread to neighboring healthy trees by the propagation of the mycelium, provided that root contacts are present [1]. Infection through spores and by the propagation of the mycelium are known as primary and secondary infections, respectively. Primary infection is mainly caused by basidiospores (sexual spores), which are produced in large amounts by fruiting bodies. The fungus also produces conidia (asexual spores), although the role of conidia in the infection process in nature seems to be negligible [11].

The susceptibility of different tree species to species of *H. annosum* s.l. has been extensively analyzed by determining the disease incidence in secondarily infected trees in naturally infested stands [12–17]. On the contrary, the susceptibility of stumps of different species to primary infection by *H. annosum* s.l. has received much less attention, and only a few studies have been conducted on this topic. In the Alps, Norway spruce was reported as significantly more susceptible to airborne stump infection by *H. annosum* s.l. than European larch (*Larix decidua* Mill.), silver fir and Scots pine, in that order [18]. Studies carried out in Latvia also showed that Norway spruce stumps are more susceptible to *H. annosum* s.l. airborne infection compared to Scots pine stumps [19].

As previously postulated [18], the comparison of levels of susceptibility of stumps of different tree species to primary infection by *H. annosum* s.l. is complicated by the fact that such analyses would require stumps of different species to co-occur in the same forests, which is not always the case. However, referring to *H. annosum* s.l., epidemiological traits related to spore release and deposition have been successfully revealed by exposing in the forest wood discs of conifers simulating stumps [17,18,20–26]. For those purposes, wood discs of Norway spruce, less often Scots pine, silver fir and larch have been employed. In addition, wood discs of Norway spruce, Italian stone pine and silver fir have been recently and successfully used to test the efficacy of biological and chemical treatments against species of *H. annosum* s.l. [27]. Only a few studies explored the susceptibility to artificial or natural spore infection by *H. annosum* s.l. of wood discs of different tree species at the same time. Redfern [28] analyzed the germination of *H. annosum* basidiospores inoculated on stem sections of Scots pine, lodgepole pine (*Pinus contorta* Douglas) and Sitka spruce (*Picea sitchensis* (Bong.) Carr.), whereas Thomsen and Jacobsen [29] investigated the growth of formerly inoculated *H. annosum* mycelium in discs of Norway spruce, Scots pine, hybrid larch (*Larix* × *eurolepis* Henry), Sitka spruce, and Douglas fir (*Pseudotsuga menziesii* (Mirbel) Franco). Dimitri et al. [30] determined the number of *H. annosum* s.l. spores deposited on *P. abies* and *P. sylvestris* wood discs exposed in Norway spruce stands, while Wang et al. [26] made the same by exposing wood discs of *Larix* × *eurolepis*, *P. abies* and *P. sylvestris* in hybrid larch stands. To date, no studies have compared the levels of susceptibility of wood discs of different tree species to the infection by spores or conidia of *H. annosum* s.l. using wood discs originating from the same trees.

All the analyses on the susceptibility of stumps to airborne infections by *H. annosum* s.l. have been made by considering the species complex level; therefore, there is little knowledge on whether the susceptibility of stumps of different tree species may differ depending on the *Heterobasidion* species involved, as previously postulated [18]. The assessment of the risk of primary infection by *H. annosum* s.l. would be particularly important to predict the likelihood of establishment of the pathogen but also to modulate the intensity of thinnings on a given tree species in order to minimize the risk of outbreaks.

In Latvia, forests occupy 52% of the country's territory, and the area covered by Norway spruce and Scots pine is 46% of the total forest area [31], which underlines the importance of these dominant tree species in Latvian forestry. However, in the context of climate change, the proportion of other non-native conifer tree species may increase [32], re-

quiring investigation of their susceptibility to native *H. annosum* s.l. species, i.e., *H. annosum* and *H. parviporum*.

Primary infection by *H. annosum* s.l. on stumps depends on the levels of spore loads, which have been reported to vary depending on the season and on the distance from spore sources (i.e., fruiting bodies) [1]. Studies conducted in Sweden showed that spore deposition rate and the risk of stump infection reach a maximum in May to August [33], while in the Alps, spore deposition rate and risk of stump infection peak at the end of summer and autumn [18,24]. Most of *H. annosum* s.l. spores land within a few meters from fruiting bodies, and only a minority can travel 100 m and further [34,35]. Other studies indicate that a considerable amount of spores can spread 100 m from spore sources [21,36]. Most of the information on the range of spore dispersal of *H. annosum* s.l. refers to Norway spruce stands of Northern Europe [21,25] or to mixed oak–Italian stone pine stands of the Mediterranean Region [36]. There is a complete lack of knowledge on the levels of spore loads and on the range of spore dispersal in Norway spruce stands of the Baltic Region.

The aims of this work were (i) to determine the susceptibility of seven tree species to primary infection by *H. annosum* and *H. parviporum* through comparative inoculation experiments of conidia on wood discs in controlled conditions; (ii) to compare the susceptibility of wood discs of the same tree species to natural airborne infection in two Latvian Norway spruce forest stands infested either by *H. annosum* or *H. parviporum*; (iii) to explore the rates of infection of wood discs at increasing distances from spore sources in these two forests to make inferences on the range of spore dispersal.

2. Materials and Methods

2.1. Comparative Susceptibility of Wood Discs of Seven Coniferous Tree Species to Inoculation with H. annosum and H. parviporum Conidial Suspensions in Controlled Conditions

Two healthy looking trees with no signs of root rot or other diseases were selected in forest stands of central and eastern Latvia for each of the following tree species: Siberian larch (*Larix sibirica* Ledeb.), *Picea abies*, *Picea sitchensis*, *Pinus contorta*, Weymouth pine (*Pinus strobus* L.), *Pinus sylvestris* and *Pseudotsuga menziesii* [32]. All selected trees were without visible decay at stump level. Moreover, as *Heterobasidion* mycelium can be found 30–60 cm in advance of the discoloration [37,38], all discs used in the experiment were cut at least 1 m upward the stem basis. The age of trees was between 23 and 44 years. Wood discs were prepared in an area distant from forests by cutting the basal portion of stems (up to 2 m height from the root collar) into slices of 2–3 cm thick. Wood discs, 10.7–16.5 cm in diameter, were kept at -5 °C until the day prior to the experiment when they were thawed at room temperature.

Conidial suspensions for the experiment were obtained by mixing conidia of three isolates, each of *H. annosum* (No. 93186, No. 98040, No. 03058, Finland) and *H. parviporum* (No. 98036, No. 03129, No. 05029, Finland). All isolates were cultured in Petri dishes on malt extract agar medium for 3 weeks at 20 °C. Conidial suspensions for each *Heterobasidion* species were prepared in 0.5 L of tap water by washing the conidia several times from three Petri dishes. The number of conidia in the suspension was calculated by counting conidia within 30 sight fields per Petri dish in Hagem agar medium in two repetitions under a microscope (magnification 100×) [39]. Conidial concentration in the suspensions was 42.16×10^3 conidia/mL of water for *H. annosum* and 45.83×10^3 conidia/mL of water for *H. parviporum*.

The experiment was performed on 26 August 2008. The surface of each wood disc was divided into four sectors. Sectors were numbered clockwise (No. 1–4), and sector No. 1 and sector No. 3 (opposite sectors) were treated with conidial suspensions of either *H. annosum* or *H. parviporum* (Figure 1) by using a sprayer. The other sectors were covered with a sheet of paper during treatment. Three wood discs for each tree species were treated with conidial suspensions of both fungal pathogens.

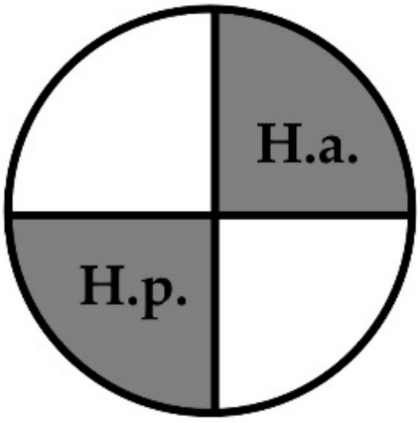

Figure 1. Spraying scheme of wood discs. Abbreviations: H.a.—area treated with *Heterobasidion annosum* conidial suspension; H.p.—area treated with *H. parviporum* conidial suspension.

After treatments, each disc was placed individually in a loosely closed plastic bag and incubated for 7 days in the dark at room temperature. After incubation, discs were inspected for the presence of typical conidiophores of *Heterobasidion* spp. by using a dissecting microscope with 20–30 magnification, as previously described [24]. The area colonized by *Heterobasidion* spp. was marked. Afterwards, discs were scanned, and areas were measured using a planimeter (PLANIX 10S 'Marble', Tamaya, Japan). Borders between the sapwood and heartwood in each disc were visually determined by the same person.

2.2. Comparative Susceptibility of Wood Discs of Seven Coniferous Tree Species to Heterobasidion spp. Natural Airborne Infections and Rates of Infection at Increasing Distance from Spore Sources

2.2.1. Study Areas and Characterization of *Heterobasidion annosum* s.l. Disease Centres

Experiments were established in two sample plots: Kalsnava and Tireli. The Kalsnava plot was located in the eastern part of Latvia (56°41′9″ N, 25°55′40″ E). It represented a 76-year-old Norway spruce stand on drained peat soil (forest type *Oxalidosa* turf. mel.) with an admixture of Scots pine, silver birch (*Betula pendula* Roth) and black alder (*Alnus glutinosa* (L.) Gaertn.). The Tireli sample plot (56°50′35″ N, 23°47′51″ E) was located in a 30-year-old Norway spruce stand with an admixture of *P. sylvestris* on drained mineral soil (forest type *Oxalidosa*).

The Kalsnava sample plot was heavily infested by *H. annosum* s.l. At the center of the sample plot, in a radius of four meters, there was a windthrown Norway spruce tree with *H. annosum* s.l. fruiting bodies with a total area of hymenophores of 1431 cm^2, two infected Norway spruce trees with fruiting bodies on the upper roots and a partly uplifted birch stump with *H. annosum* s.l. fruiting bodies under the roots. Some fruiting bodies were found at a distance of 20–40 m from the infection center. The total area of hymenophores of *H. annosum* s.l. fruiting bodies found in the Kalsnava sample plot was 2048 cm^2. To identify the fungus at the species level, wood samples were taken with a sterilized increment borer or an axe from the windthrown Norway spruce, birch stump and from the roots of the infected Norway spruce.

In the Tireli sample plot, the *H. annosum* s.l. infection center was a gap of 8 m radius containing six Scots pine stumps. At the margin of the gap, there were five Scots pines with symptoms of *H. annosum* s.l. infection (thin crown). At the center of the gap, in a 3 m radius, four Scots pine stumps were located, two of them partly uplifted, with *H. annosum* s.l. fruiting bodies on the roots showing hymenophores with a total area of 485 cm^2. Wood samples were collected from these two Scots pine stumps according to the method described above for the Kalsnava sample plot.

Isolations from wood samples were made according to Arhipova et al. [40]. Isolates were identified at the species level by assessing their ability to heterokaryotize the homokaryotic tester strains No. 05247 (*H. annosum*) and 05146 (*H. parviporum*) [41], kindly provided by Kari Korhonen. All isolates obtained from Tireli belonged to *H. annosum*, and all those obtained from Kalsnava belonged to *H. parviporum*.

2.2.2. Samplings to Determine the Susceptibility of Wood Discs to *Heterobasidion annosum* s.l. Natural Airborne Infections and the Rates of Spore Deposition at Increasing Distance from Spore Sources

In the Kalsnava sample plot, *H. annosum* s.l. spore deposition on wood discs was assessed along two transects in the four directions: north (N), south (S), east (E) and west (W) (Figure 2). The cross point of transects was established one meter from the largest *Heterobasidion* fruiting body group. The Tireli sample plot was located 20 m from a forest road, and therefore, spore deposition on wood discs was assessed along a single transect oriented south-east (SE) and north-west (NW) (Figure 2).

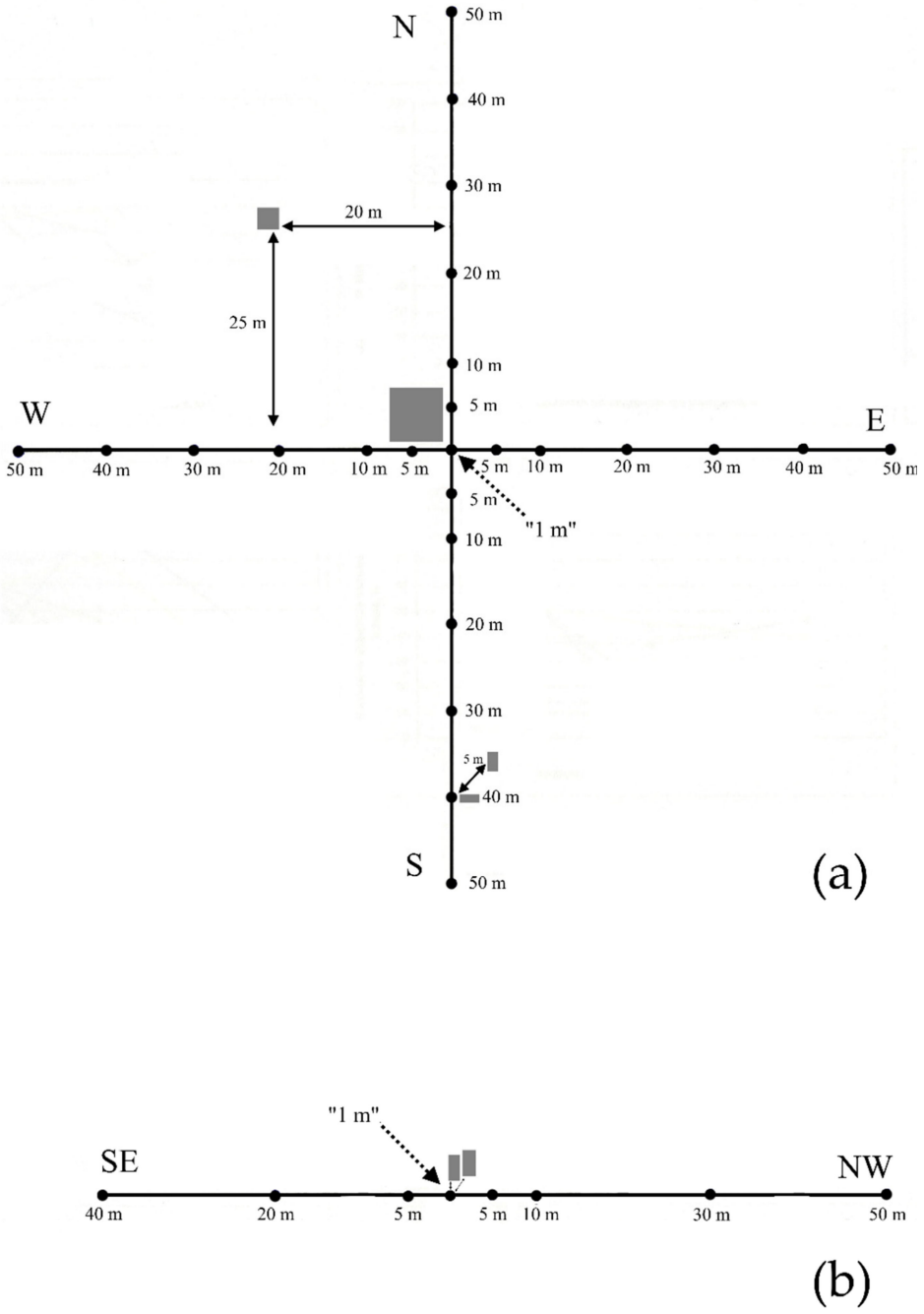

Figure 2. Location of sampling points and groups of *Heterobasidion annosum* s.l. fruiting bodies: (**a**) the Kalsnava sample plot and (**b**) the Tireli sample plot). Relative area of *H. annosum* s.l. fruiting body hymenophores; dashed line with arrow shows the cross point of transects located 1 m from the largest group of *H. annosum* s.l. fruiting bodies.

Four and two sampling campaigns were conducted at different time periods in Kalsnava and Tireli sample plots, respectively (Table 1). Ten wood discs prepared as described above were placed in each sampling period at each sampling point (two discs of *P. sitchensis*, two discs of *P. menziesii*, one disc of *P. abies*, one disc of *P. sylvestris*, two discs of *P. contorta*, one disc of *P. strobus* and one disc of *L. sibirica*) as summarized in Figure 2. Two discs of some species instead of one were used because of their smaller diameter. In total, 520 discs were exposed in the Kalsnava sample plot and 159 discs in the Tireli sample plot. At all sampling points, discs were placed on transparent plastic sheets during exposition to avoid any contamination originating from the ground. In all experiments, wood discs were exposed for approximately 17 h (4 p.m.–9 a.m.) (Table 1).

Table 1. Main features and experimental conditions of sampling campaigns of airborne *Heterobasidion annosum* s.l. spores in Kalsnava and Tireli.

Sample Plot	Date of Experiment in 2008	Analyzed Directions	Distance from the Group of Fruit Bodies, m	Air Temperature, °C	Air Humidity, %	Speed (m/s) and Direction of Wind
Kalsnava	15.07–16.07	N, S, E, W	1 *, 5, 10, 20, 30, 40, 50	16	80	1–3, SW
	03.08–04.08	N, S, E, W	1, 10, 30	18	80	1–4, SW, W
	11.08–12.08	N, S, E, W	1, 10, 30	17	89	1–3, S, SW
	21.08–22.08	N, S, E, W	1, 20, 40	15	89	3–4, SW
Tireli	30.08–31.08	NW SE	1, 5, 10, 30 10, 20, 40	12	86, heavy rain	1–4, NW
	11.09–12.09	NW SE	1, 5, 10, 30 10, 20, 40	8	72	3–4, NE

*: the transect cross point at the distance of 1 m from the largest *H. annosum* s.l. fruiting body group.

After exposure, each disc was placed individually in a plastic bag and brought to the laboratory. Discs were incubated for 7 days in loosely closed plastic bags at room temperature. Discs were inspected by using a dissecting microscope for the presence of *H. annosum* s.l. as described above.

Additionally, in order to explore the levels of spore deposition at increasing distance from spore sources in a way unbiased by the woody substrate, in the Kalsnava sample plot, a Petri dish filled with malt extract agar was placed directly under the largest *H. annosum* s.l. fruiting body so that the margin of the Petri dish gently touched its hymenophore. Additional Petri dishes were placed on the ground at 1 m distance from the largest *H. annosum* s.l. fruiting body group and in four directions (N, S, E, W) at a distance of 10 m and 30 m. Petri dishes were exposed during the daytime on 7 and 14 October 2008 for 1, 2, 4 and 8 min. Air temperature during exposure in these two days was 8 °C and 10 °C, respectively. The following day, spore counts were performed using light microscopy in 30 random sight fields in the Petri dish on Hagem agar medium.

2.3. Calculations and Statistics

In the experiment carried out under controlled conditions, the area colonized by either *H. annosum* or *H. parviporum* on the total surface of discs sprayed with conidial suspensions of either species (%) was determined. The sapwood area was calculated by subtraction of the heartwood area from the total area of the disc. Areas colonized by *Heterobasidion* spp. in sapwood and heartwood of wood discs (treated sectors) of different tree species were compared using non-parametric Mann–Whitney tests.

In the experiments carried out in the field, infection frequency expressed as the percentage of infected discs on the total number of discs exposed was compared between tree species in each sample plot using a chi-square test. The mean area occupied by *H. annosum* s.l. (percentage of the total area of the disc) in discs of each tree species, hereafter referred to as infection rate, was calculated for all discs in each sample plot (Kalsnava and Tireli). The correlation between the mean area occupied by *Heterobasidion* in

different tree species and distances from the largest *H. annosum* s.l. fruiting body group was explored using Spearman's rank-order correlation test.

The correlation between the mean proportion of area occupied by *H. annosum* s.l. on wood discs of different tree species after treating discs with conidial suspensions (data from *H. annosum* and *H. parviporum* pooled together) and by exposing discs in the field (data from Kalsnava and Tireli pooled together) was determined by using Spearman's rank-order correlation test.

H. annosum s.l. basidiospores deposition in Petri dishes was calculated taking into account the number of spores, the area of sight field, the area of the Petri dish and the exposure time. Spore deposition was expressed per m^2 in one hour (h). Data analyses were performed by using R 3.6.1 [42].

3. Results

3.1. Comparative Susceptibility of Wood Discs of Seven Coniferous Tree Species to H. annosum and H. parviporum Conidial Suspensions in Controlled Conditions

Heterobasidion annosum colonized a larger area than *H. parviporum* on wood discs of all the tested tree species except *P. contorta*, although differences were significant only for wood discs of *P. abies* ($p < 0.05$). The area occupied by *H. parviporum* was significantly larger in *P. contorta* compared to *P. sitchensis* and *L. sibirica* ($p < 0.05$) (Figure 3). The area occupied by *H. parviporum* in *P. menziesii* was significantly smaller compared to *P. contorta*, *P. sylvestris*, *P. abies* and *P. strobus* ($p < 0.05$).

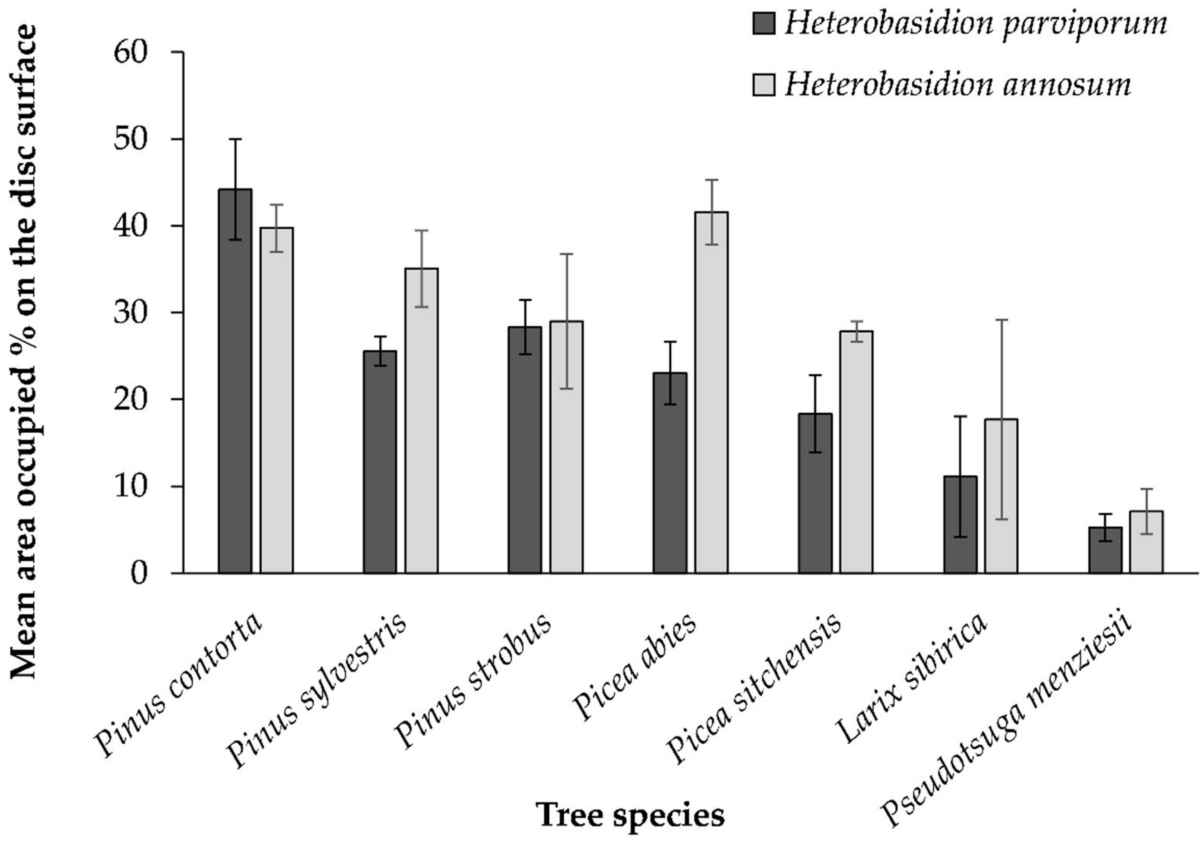

Figure 3. The mean area occupied by *Heterobasidion annosum* and *H. parviporum* on wood discs of seven coniferous tree species inoculated with conidial suspensions.

The area occupied by *H. annosum* in *P. sitchensis* was significantly smaller compared to that in *P. contorta* ($p < 0.05$). The area occupied by *H. annosum* in *P. menziesii* was significantly smaller than that in *P. contorta*, *P. sylvestris*, *P. abies* and *P. sitchensis* ($p < 0.05$).

The overall areas colonized by either *H. annosum* or *H. parviporum* on wood discs of different tree species were correlated ($r = 0.75$, $p = 0.052$). In the discs of all the tree species and for both *Heterobasidion* species, the area colonized by the fungi in sapwood was much greater than that colonized in heartwood (Table 2).

Table 2. The percentage of the area occupied by *Heterobasidion annosum* and *H. parviporum* in the sapwood and heartwood of the discs of seven tree species.

		Mean Area (%) Occupied by *Heterobasidion* spp.						
		Larix sibirica	***Picea abies***	***Picea sitchensis***	***Pinus contorta***	***Pinus strobus***	***Pinus sylvestris***	***Pseudotsuga menziesii***
H. parviporum	Sapwood	24.9	43.4	25.8	48.4	71.5	67.8	11.0
	Heartwood	0.1	2.1	4.7	0.0	0.2	0.0	0.1
H. annosum	Sapwood	40.8	71.4	40.6	44.4	68.0	80.6	18.0
	Heartwood	0.5	6.3	3.0	0.5	1.8	0.01	0.1

3.2. Comparative Susceptibility of Wood Discs of Seven Coniferous Tree Species to H. annosum s.l. Natural Airborne Infections

At Kalsnava, more discs were infected in the first and second experiment compared to the third and fourth experiment: 97% and 98% compared to 77% and 84%, respectively. Most frequently, *P. strobus*, *P. sylvestris* and *P. abies* discs were infected: 100%, 96% and 89%, respectively. The proportion of infected *L. sibirica*, *P. contorta*, *P. menziesii* and *P. sitchensis* discs was comprised between 83% and 87% (Table 3).

Table 3. The *Heterobasidion annosum* s.l. infection frequency (%) of wood discs of different tree species in the Kalsnava and Tireli study sites.

Tree Species	**Kalsnava**					**Tireli**		
	1 *	**2**	**3**	**4**	**Average**	**1**	**2**	**Average**
Larix sibirica	96	100	67	67	83	88	50	69
Picea abies	100	100	67	89	89	88	88	88
Picea sitchensis	98	100	72	78	87	100	69	85
Pinus contorta	92	100	78	72	86	94	69	82
Pinus strobus	100	100	100	100	100	75	100	88
Pinus sylvestris	96	100	89	100	96	100	100	100
Pseudotsuga menziesii	100	89	67	83	85	69	50	60
Average	97	98	77	84	89	88	75	82

*: Experiment number 1 to 4.

At Tireli, the average infection frequency of wood discs was 88% and 75% in the first and second experiment, respectively. The wood discs of *P. sylvestris* were the most frequently infected (100%), whereas only 60% of wood discs of *P. menziesii* and 69% of those of *L. sibirica* were infected by *H. annosum* s.l. The frequency of infected *P. abies*, *P. sitchensis*, *P. contorta* and *P. strobus* wood discs was comprised between 82% and 88% (Table 3).

The area occupied by *H. annosum* s.l. on discs exposed in the Kalsnava sample plot was significantly greater ($p < 0.05$) than that on discs exposed in the Tireli sample plot for all tree species (Figure 4). In both Kalsnava and Tireli, the mean area occupied by *H. annosum* s.l. on wood discs was greater for *P. contorta* and *P. sylvestris* and lower for *P. menziesii*.

At Kalsnava, the area occupied by *H. annosum* s.l. in wood discs of *P. sylvestris* was significantly larger compared to that occupied in wood discs of *P. abies*, *L. sibirica* and *P. menziesii* ($p < 0.05$). The area occupied by *H. annosum* s.l. in wood discs of *P. menziesii* was significantly smaller compared to that in wood discs of *P. strobus* and *P. contorta* ($p < 0.05$). The area occupied by *H. annosum* s.l. in heartwood was small—the average for the analyzed tree species ranged from 0.01% to 0.55%, and there were no significant differences between tree species ($p > 0.05$).

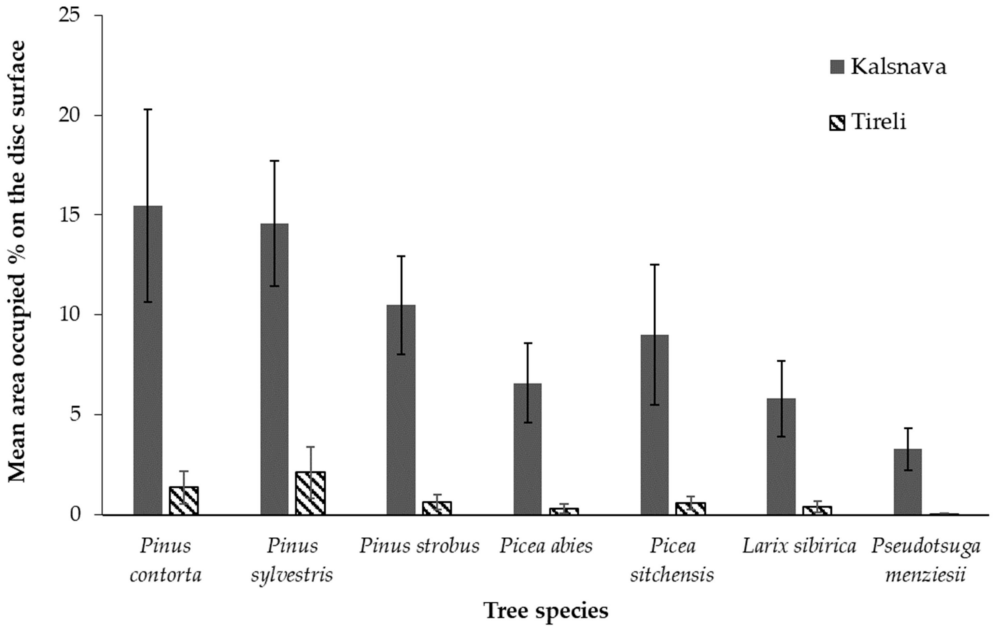

Figure 4. The mean area occupied by *Heterobasidion annosum* s.l. on wood discs of different tree species exposed in the forest plots of Kalsnava and Tireli.

At Tireli, the largest area occupied by *H. annosum* s.l. was in discs of *P. sylvestris* and *P. contorta*: 2.12% and 1.38% of the total surface of discs, respectively. The smallest occupied area was for *P. menziesii*—0.05%. The mean area occupied by *H. annosum* s.l. in discs of the other tree species ranged from 0.31% to 0.65%. At Tireli, no *H. annosum* s.l. infection was detected in the heartwood of any wood disc.

A positive and significant correlation was found between areas colonized by *H. annosum* s.l. on wood discs of different tree species in Kalsnava and Tireli sample plots ($r = 0.90$, $p < 0.05$). A positive and significant correlation was also found between the mean area occupied by *H. annosum* s.l. on discs treated with conidial suspensions in controlled conditions and on discs exposed in the forest plots of Kalsnava and Tireli ($r = 0.79$; $p < 0.05$).

3.3. Rates of Infection at Increasing Distance from Spore Sources in the Field

By analyzing the rates of infection of wood discs at different distances from the largest *H. annosum* s.l. fruiting body group at Kalsnava, a rapid decrease in the rate of infection of wood discs with distance was observed for most of the analyzed tree species and as an average (Figure 5). In the W direction, *P. sylvestris* and *P. contorta* showed higher rates of infection at a distance of 20 m compared to the other tree species. At 40 m distance in the S direction, a peak in rates of infection was observed for all tree species.

In Tireli, the highest infection rate in the first experiment was observed in the NW direction 5 m far from *H. annosum* s.l. fruiting bodies. In the second experiment, the highest infection rate was also observed in the NW direction at a distance of 1 m from *H. annosum* s.l. fruit bodies, and at a distance of 5 m. The infection rate decreased rapidly with distance from *H. annosum* s.l. fruiting bodies.

The levels of spore deposition under fruiting bodies of *H. parviporum* in Kalsnava determined by using Petri dishes was 87.17×10^6 spores per m^2h^{-1} on 7 October and 12.85×10^6 spores per m^2h^{-1} on 14 October (average 50.01×10^6 spores m^2h^{-1}). On 7 October, spore deposition attained more than one million spores per m^2h^{-1} at a distance of 1 m from spore sources, and 82,250 and 61,688 spores m^2h^{-1} at distances of 10 and 30 m from spore sources, respectively. On 14 October, spore deposition was more than 400,000 spores m^2h^{-1} at a 1-m distance from spore sources and varied between 4113 and 41,125 spores m^2h^{-1} at a distance of 10 m from the spore sources. At a distance of 30 m from spore sources, no spores were detected in any direction on 14 October.

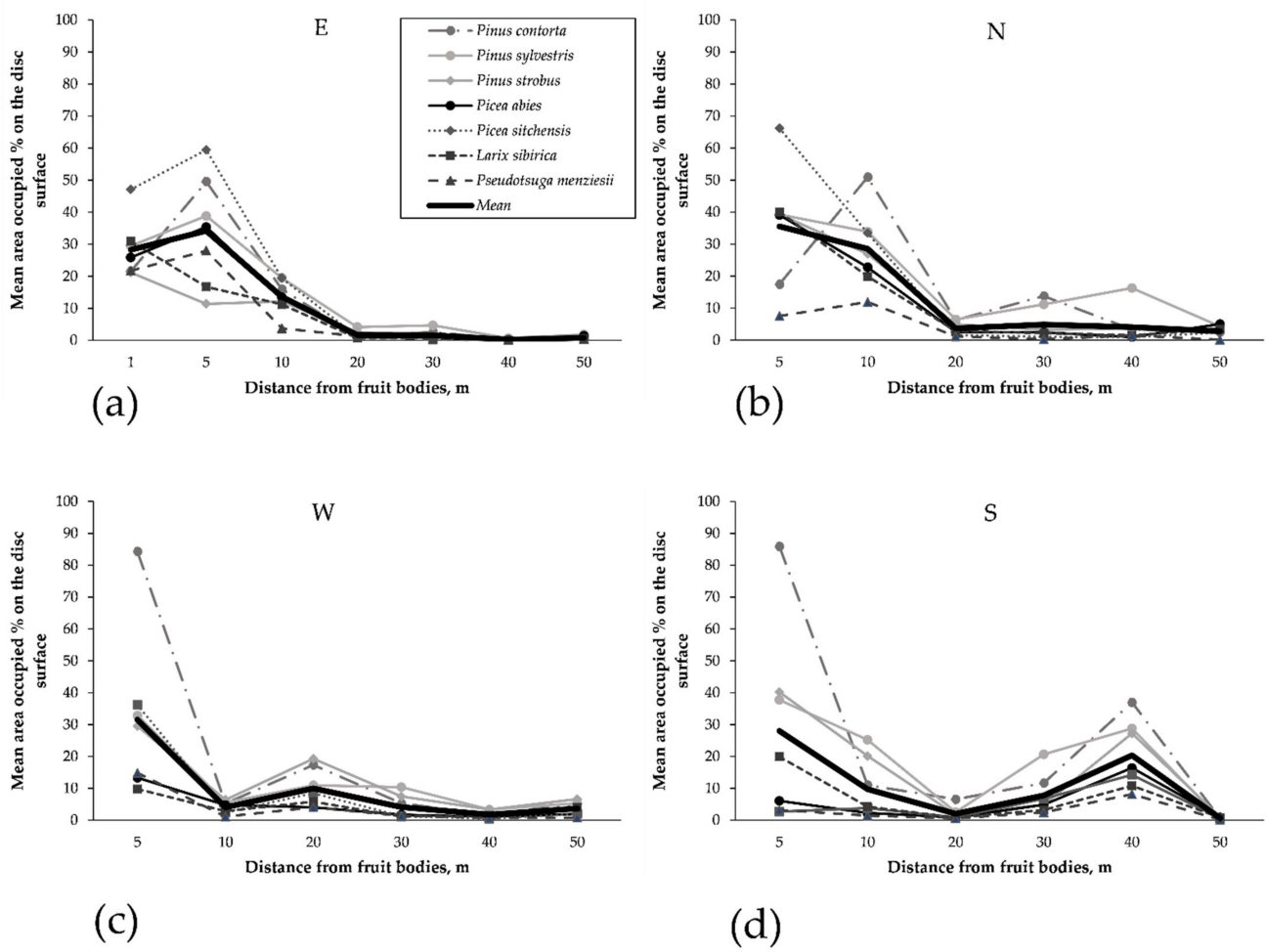

Figure 5. The rates of infection of wood discs of seven coniferous tree species by *Heterobasidion annosum* s.l. in the Kalsnava sample plot at increasing distances from spore sources. Disc exposition in different directions—(**a**) east, (**b**) north, (**c**) west and (**d**) south; only in figure (**a**) the area occupied by *Heterobasidion annosum* s.l. at a distance "1 m" from the largest group of fruiting bodies is shown; the same numbers refer to other directions.

4. Discussion

Our data shows variation in susceptibility of wood discs of different tree species to primary infection by *H. annosum* s.l., and, in this regard, results obtained by exposing discs to airborne infections in the forest are fully consistent with those of artificial inoculations in controlled conditions using conidial suspensions.

In comparative inoculation experiments on wood discs in controlled conditions using conidial suspensions, larger surfaces were colonized by *H. annosum* s.l. on wood discs of *P. sylvestris*, *P. strobus* and *P. abies*, and smaller in wood discs of *P. menziesii*. These findings support previous reports by Thomsen and Jacobsen [29], who observed higher growth rate of the fungus following artificial inoculation on wood discs of Scots pine and Norway spruce, followed by Douglas fir and hybrid larch. Nevertheless, in that study, the slowest growth rate was observed on Sitka spruce. Interestingly, on wood discs of Norway spruce, a significantly larger area was occupied by *H. annosum* compared to *H. parviporum*, despite many studies suggested that *H. parviporum* is better adapted to Norway spruce wood than *H. annosum* [43–46]. However, it should be noted that the development of *Heterobasidion* spp. on wood may vary depending on whether trees are alive or dead. In an experiment conducted in Latvia by inoculating Norway spruce billets with the same two *Heterobasidion* species [32], a slower growth rate was found for *H. parviporum* compared to *H. annosum*. In addition, a higher rate of isolation of *H. annosum* compared to *H. parviporum*

was observed following primary infection on Norway spruce stumps in subalpine stands heavily infested by *H. parviporum* [47], supporting the hypothesis that *H. annosum* may be a better saprobe than *H. parviporum*, as previously documented on the wood blocks in laboratory conditions [48].

When exposing wood discs in the forest at Kalsnava and Tireli to *H. annosum* s.l. natural airborne infections, the fungus colonized the largest areas on wood discs of pine species, followed by *P. sitchensis*, *P. abies* and *L. sibirica*. Again, the smallest areas colonized by the fungus were found on wood discs of *P. menziesii*. Thus, our findings are in agreement with data obtained by Dimitri et al. [30] and Wang et al. [26], who reported higher susceptibility to *H. annosum* s.l. airborne infections of pine discs compared to Norway spruce discs. However, this is not consistent with the results of studies conducted in the Alps reporting higher infection frequencies in Norway spruce stumps compared to pine stumps [18]. More infected Norway spruce stumps compared to pine stumps have also been found in mixed spruce–pine stands in Latvia [19], as well as in small diameter stumps after pre-commercial thinning [49,50]. The above inconsistencies could be related to the substrate that was analyzed—discs vs. stumps. Discs are more prone to drying, and this may have accounted for the inconsistencies. In addition, the discs analyzed in this study had different diameters, and therefore, they could have been subjected to different degrees of drying.

In our study, the area occupied by *H. annosum* s.l. on wood discs of *P. contorta* was larger compared to that occupied by the fungus on wood discs of *P. sitchensis*, which is consistent with data provided by Redfern [28] using stumps as a substrate. At the same time, our results pointing to a lower susceptibility of wood discs of Douglas fir to primary infections by *H. annosum* s.l. corroborate previous observations by Morrison and Johnson [51], who found less infection of Douglas fir stumps compared to Sitka spruce stumps after precommercial thinning.

Rönnberg et al. [52] concluded that in pine stumps *H. annosum* s.l. spore infection was mainly found in sapwood, whereas Oliva et al. [53] indicated that on Norway spruce stumps, the fungus develops both in sapwood and heartwood. In our study, wood discs of all coniferous tree species analyzed showed very little heartwood infection. Referring to *P. sylvestris* and *P. abies*, our observations are in agreement with those of other studies showing that *H. annosum* s.l. spore infection mainly occurs in sapwood [52,54–58]. Conversely, other studies documented higher infection of heartwood compared to sapwood in Sitka spruce [59,60]. This might be due to the different methodology we used, particularly the storage of the discs at a temperature below 0 °C. Although this methodology (even using wood discs 6–9 cm in diameter) had already been used in other studies [21,25], it is possible that the conditions during storage could have modified the properties of heartwood in some tree species.

The results obtained by spraying wood discs with conidial suspensions in controlled conditions are in good agreement with those obtained by exposing wood discs to the natural airborne inoculum in the forests, as clearly supported by the significant correlations between the two sets of data. In both cases, these data suggest higher susceptibility to primary infections by *H. annosum* s.l. for *P. contorta* and other *Pinus* and *Picea* species, followed by *L. sibirica*, and lower susceptibility for *P. menziesii*.

The results obtained in this study, both through the comparative inoculation experiments of conidia on wood discs in controlled conditions and by exposing wood discs to *H. annosum* s.l. natural airborne infections in the two Norway spruce stands are in agreement with the results obtained by Zaluma et al. [32], who investigated the development of *H. annosum* s.l. mycelia in the wood of different tree species. In this latter study, a relevant *H. annosum* s.l. mycelium development deep in the wood was found with *P. sylvestris*, *P. strobus* and *P. abies*, an intermediate with *P. contorta* and *P. sitchensis*, and a limited development with *L. sibirica* and *P. menziensii*. It should be noted that the similarity of results between our study and the study by Zaluma et al. [32], who also used conidial suspensions of both *H. annosum* and *H. parviporum*, was more pronounced for the

inoculation experiment with conidia of *H. parviporum* in controlled conditions and for discs exposed in the Kalsnava plot where, incidentally, all fruiting bodies sampled were identified as *H. parviporum*. Taken together, these findings suggest that it could be desirable to test the susceptibility of different *Larix* species to *H. annosum* s.l. in Latvian conditions. *Larix sibirica* is of particular interest because of a relatively limited development of *H. annosum* s.l. mycelia were observed in the wood of this tree species but, in contrast to *P. menziesii*, the development of *Phlebiopsis gigantea*—a well-known and effective antagonistic fungus against *H. annosum* s.l.—has been reported to occur in the wood of *L. sibirica* [32]. Other studies have also reported *Larix* spp. to be less susceptible to *H. annosum* s.l. compared to Norway spruce [17,18,61].

In our study, the highest rate of infection of most wood discs was identified at a distance of 1 m from the infection center, but at 5 and 10 m, the wood discs' infection rates significantly decreased. This finding supports the results of investigations conducted in Fennoscandia [34,35], indicating that the maximum number of spores is released up to 10 m from the fruiting bodies. The exception to the above gradient in our study was the southern direction, where a significant increase in the infection rate was observed at a distance of 40 m. However, it should be noted that after additional surveys, initially undetected *H. annosum* s.l. fruiting bodies were found on rotten Norway spruce root fragments under the moss close to the points where discs were exposed. This confirms the importance of local spore sources in the prevalence of *H. annosum* s.l. infection in forest stands, as pointed out by several studies [20,23,35,36,62–64].

In this study, the spore load of *H. annosum* s.l. in a heavily infested Norway spruce stand was also analyzed using Petri dishes with agar medium. The number of spores observed in our study under the *H. parviporum* fruiting body was lower than that reported by Möykkynen et al. [21]. However, it should be noted that in this latter study conducted in Finland, the number of spores below the fruiting body was analyzed in June/August, at the peak of *H. annosum* s.l. sporulation in northern Europe [33], while in our study, samplings were conducted in October/November when sporulation is expected to be lower. In our study, the number of spores detected per m^2 in different directions at a distance of 10–30 m from the largest group of fruiting bodies varied widely from 0 to 131600 spores. Other studies have also reported a relatively large variation in spores' deposition levels in Norway spruce stands: 0–447 spores m^2 h^{-1} [25] and 40–1500 spores m^2 h^{-1} [30].

In conclusion, this study provides evidence that woods of different tree species differ in their susceptibility to primary infection by both *H. annosum* and *H. parviporum*. This study also supports the notion of a limited dispersal range of *H. annosum* s.l. basidiospores, pointing to the importance of local spore sources in the epidemiology of these pathogens.

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Invertebrates in Fruitbodies of *Heterobasidion* spp., Infected *Picea abies* Logs and Adjacent Soil

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Abstract: *Heterobasidion* spp. pathogenic fungi produce conspicuous fruitbodies on infected wood, which may represent a habitat for a range of organisms, including invertebrates. The aim was to: (i) assess and compare invertebrate diversity in *Heterobasidion* spp. fruitbodies, infected *Picea abies* wood and adjacent soil; (ii) test methods for collecting invertebrates from *Heterobasidion* spp. fruitbodies. A total of 69 *Heterobasidion* fruitbody samples, 46 wood samples and 19 soil samples were collected at two forest sites in Latvia. In total, 7311 invertebrate individuals were collected representing 62 different taxa, among which 55 were in fruitbodies, 47 in wood and 36 in soil. The dominant invertebrates in fruitbodies were Acari (82.4%), Collembola (6.2%), Coleoptera (4.7%); in wood were Acari (78.6%), Collembola (9.6%), Diplopoda (3.1%); in soil were Acari (90.6%), Collembola (3.4%) and Coleoptera (1.4%). Among all taxa, 17.7% were found exclusively in fruitbodies, 3.2% in wood, 4.8% in soil, while 48.4% were shared among different substrates. Although many invertebrate taxa were shared, their relative abundance differed substantially among different substrates. The sampling methods used provided valuable information on invertebrate communities. Further development of these methods may be needed to obtain more detailed and specific information about invertebrates inhabiting different habitats.

Keywords: *Heterobasidion*; carpophores; fauna; Norway spruce; Tullgren funnels

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1. Introduction

Heterobasidion spp. pathogenic fungi cause considerable economic losses to forestry due to the infection of living trees and decay of wood. The impact of the pathogen is more severe in Norway spruce (*Picea abies*) stands as the fungal mycelia from the root system can expand several meters up in the stem, thereby causing rot in the most valuable part of the stem [1]. In Latvia, ca. 22% of *P. abies* stands are infected by *Heterobasidion* spp. and, on average, the economic loss at a final cutting exceeds EUR 4000/ha [2]. *Heterobasidion* spreads by basidiospores (primary infections) and by vegetative mycelia through root contacts (secondary infections) [3]. The abundance of fruitbodies of the pathogen in a particular stand has an impact on the primary infection by basidiospores. It is known that these fruitbodies mainly affect the rate of infection at the stand level but not in adjacent stands [4]. Both *Heterobasidion parviporum* Niemelä & Korhonen and *Heterobasidion annosum* (Fr.) Bref. are found in Latvia, and both of these species form fruitbodies on infected *P. abies* wood at a similar rate [5]. Studies conducted in Germany and Finland [6,7] have indicated that a large number of fruitbodies are formed on 3- to 4-year-old large dimension *P. abies* logs that are left in stands after forest operations. In Latvia, the area covered by fruitbodies on 3- to 4-year-old *P. abies* logs is on average 3759 cm^2/m^3, and can exceed 5000 cm^2/m^3 on logs in stands growing on drained peat soils [5].

Heterobasidion fruitbodies can produce between 35,000 and 151,000 basidiospores per cm^2 of the fruitbody in one hour [8], and, in *P. abies* stands, the number of spores can exceed 1000 per m^2 per hour [9]. This spore density is a high risk to freshly cut *P. abies* stumps during tree felling as fresh stumps are the primary substrate for colonisation by *Heterobasidion* spores. Therefore, to reduce the risk of infection, several studies have recommended the removal of large dimension *P. abies* logs that are infected by *Heterobasidion* spp. from forest stands [5–7]. However, other studies have indicated that large dimension *P. abies* logs are important as a habitat to many fungal and invertebrate species [10,11]. Fungal fruitbodies are known to be inhabited by a number of invertebrate species [12,13]. Fruitbodies provide not only a food source for invertebrates but also shelter and a place for reproduction, thus influencing the density of invertebrates in a given substrate [11]. Invertebrate fauna have been investigated in the fruitbodies of *Fomitopsis pinicola* (Fr.) Karst, a fungal species commonly found in conifer forests [14–17]. However, in most studies on invertebrate fauna inhabiting fungal fruitbodies, multiple fungal species were analysed, without differentiation. The focus has often been on macro arthropods that feed on fungi, typically Coleoptera and Diptera [17–19]. However, fungal fruitbodies are also known to be commonly inhabited by Acari and Collembola [20,21]. Some studies have also investigated *Heterobasidion* spp. fruitbodies, however, the majority of these have examined a small number of fruitbodies, from which only adult Coleoptera were collected as these are relatively easy to identify. In a study that included several fungal species, adult Coleoptera were found in only two *H. parviporum* fruitbodies from a total of 44 collected [22], while in another similar study, only half of examined *H. parviporum* fruitbodies were inhabited by Coleoptera [23]. *Heterobasidion annosum* was shown to be associated with several Coleoptera species from the family Ciidae [24], and reproduction in fruitbodies was confirmed for *Cis lineatocribatus*, with both adults and larvae identified [18]. The possible role of insect vectors for dissemination of *Heterobasidion* spp. has previously been highlighted by Stenlid [25] and Piri et al. [26]. Indeed, spores of *Heterobasidion* spp. are known to be disseminated by the large pine weevil (*Hylobius abietis*) [27,28], but the role of other insects is largely unknown. To determine the effect of the removal of infected *P. abies* wood from forest stands on invertebrate diversity, quantitative and qualitative assessment of invertebrate fauna in *Heterobasidion* spp. fruitbodies is required, particularly focussing on rare and endangered species. The aim of this study was to: (i) assess and compare invertebrate diversity in *Heterobasidion* spp. fruitbodies, infected *P. abies* wood and adjacent soil; (ii) develop and test methods for collecting invertebrate samples from *Heterobasidion* spp. fruitbodies.

2. Materials and Methods

2.1. Sample Plots

Samples were collected from two sample plots in the autumn of 2016: (i) a 70-year-old forest stand consisting of 80% *P. abies* with an admixture of *Betula pendula* and *Pinus sylvestris* on peatland (forest type Oxalidosa turf.mel. [29]) near the Forest Research Station in the Kalsnava forest region (56°41′40.4″ N, 25°54′21.8″ E), further referred to as Kalsnava; (ii) a 50-year-old forest stand consisting of 90% *P. abies* with an admixture of *B. pendula* on mineral soil (forest type Oxalidosa [29]) in JSC Latvia's State ForestsCentral Daugava region, Ogre division (56°50′50.8″ N, 24°48′17.8″ E), further referred to as Ogre. Sample plots contained infected *P. abies* logs of different sizes and decomposition stages, where *Heterobasidion* spp. fruitbodies of varying sizes and ages were previously identified.

2.2. Field Methods

To sample fruitbodies, 46 infected *P. abies* logs with large *Heterobasidion* spp. fruitbodies (Figure 1) were randomly selected in each plot. For each log, the root end diameter and length were measured. The length of logs ranged between 107 and 487 cm (average 274 cm); diameters ranged between 15 and 42 cm (average 25 cm). Fruitbody samples (100 cm^2) were collected by cutting a 10 × 10 cm piece from the fruitbody closest to the root end

of the log. During the collection of fruitbody samples, it was noted if the fruitbody was in contact with the soil and if any damage was visible. A similarly sized (100 cm^2) wood sample was collected 20 cm from the fruitbody in the direction away from the root end of the log. One wood sample and at least one fruitbody sample was collected from each log. If multiple and well-separated fruitbodies were found on one log, two to three fruitbody samples were collected. Where the log was in contact with the soil, a 5 cm deep, 100 cm^2 soil sample was collected. If multiple logs were found less than 10 m from each other (in log piles), only one soil sample was collected. After collection, all samples were placed into plastic bags, and kept at +4 °C until processing. A total of 69 *Heterobasidion* spp. fruitbodies, 46 *P. abies* wood samples and 19 soil samples were collected. In Kalsnava, 46 fruitbodies, 30 *P. abies* wood samples and 15 soil samples were collected, while in Ogre, 23 fruitbodies, 16 *P. abies* wood samples and 4 soil samples were collected.

Figure 1. Large *Heterobasidion* spp. fruitbody.

2.3. Laboratory Methods

In the laboratory, each sample was weighed using a Kern EMB 200-2 balance (Balingen, Germany) before and after placing samples in Tullgren funnels (Figure 2), and macrofauna were collected (if present) using forceps. By weighing the samples before and after placing them in Tullgren funnels, the relative humidity of samples was calculated. The relative humidity of fruitbodies ranged from 40.6% to 85.6% with an average of 70.1%. In Ogre, the relative humidity of fruitbodies was on average 69.4%, but in Kalsnava it was on average 71.6%. The average weight of a fruitbody sample before drying was 68.7 g, maximal and minimal values being 151.0 g and 14.2 g, respectively. After drying, the weight varied between 5.1 g and 42.3 g with an average of 19.9 g.

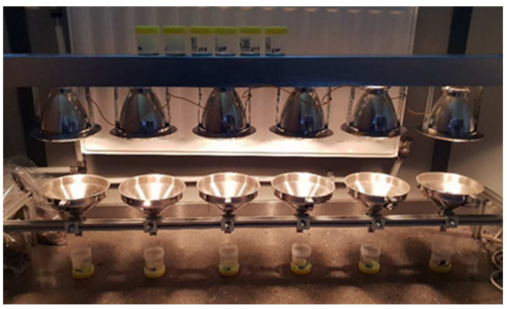

Figure 2. Tullgren funnels utilized for the collection of invertebrates.

The thickness of fruitbodies was measured with a ruler. Most of the collected fruitbodies were between 0.4 and 2.5 cm thick, but some fruitbodies that were collected close to the root collar were up to 5.0 cm thick. The majority of fruitbodies collected were at least 3 years old with some being between 6 and 7 years old.

After measurements, samples were placed in Tullgren funnels for at least 72 h. Tullgren funnels (Figure 2) are commonly utilized to obtain quantitative data about arthropods in soil [30], but this method is also effective to obtain data on invertebrates in fruitbodies and

wood [14,31,32]. A 1:3 mixture of glycerine and 70% ethanol was placed in the collection vials to preserve the collected invertebrate individuals.

The collected invertebrates were examined using Leica NBO MSV266 (Wetzlar, Germany) and Optika B-383PH (Ponteranica, Italy) microscopes. Invertebrates were classified to the lowest possible taxonomic level, most to family level. Springtails (Collembola) were classified according to W. Dunger [33], mites (Acari) according to unpublished material by Dr U. Kagainis on the classification of oribatid mites (Oribatida) morphogroups in Latvia. Beetles (Coleoptera) were classified to the family level using the key by D. M. Unwin [34]. Taking into account the available information on Coleoptera of the Ciidae family inhabiting *Heterobasidion* spp. fruitbodies, these were classified to species level using the publicly available key by Lompes [35], which was developed from the results of studies by Reibnitz [24].

2.4. Statistical Analyses

To analyse invertebrate diversity in each substrate, the invertebrate data (actual observations) were used to construct accumulation curves [36], which was conducted in R [37] using the *vegan* package and *specaccum* function [38]. Differences in invertebrate taxa richness in different substrates (study sites as well as adults and larvae combined) were compared by nonparametric chi-square tests, taking into account the Bonferroni correction [39]. Shannon index values [40] were calculated for each substrate to determine the diversity in invertebrate communities. Values were calculated using invertebrate frequencies by taxa, i.e., the subclass or family taxonomic level. The invertebrate community structure in different substrates (fruitbodies, wood and soil) was analysed using principal component analysis in Canoco v.5.02 (Microcomputer Power, Ithaca, NY, USA).

3. Results

A total of 7311 invertebrate individuals were collected from all substrates and from both sample plots, including 7151 adults and 160 larvae. The number of invertebrates collected per individual fruitbody was between 1 and 396 (average 70). In wood and soil samples, invertebrate numbers ranged from 0 to 316 (average 35) and 0 to 192 (average 41), respectively. There were 55 different invertebrate taxa in 69 fruitbody samples, 47 taxa in 46 wood samples and 36 taxa in 19 soil samples. Consequently, the chi-square test showed that the absolute richness of invertebrate taxa differed significantly among all substrates, being highest in the soil, then in the wood and lowest in fungal fruitbodies ($p < 0.004$). A possible reason for the observed differences may be an uneven number of samples collected of different substrates. Indeed, when the same number of samples was taken from each substrate following the rarefaction analysis, the richness of invertebrate taxa did not differ significantly among different substrates ($p > 0.05$).

The accumulation curves for different substrates did not reach the asymptote (Figure 3).

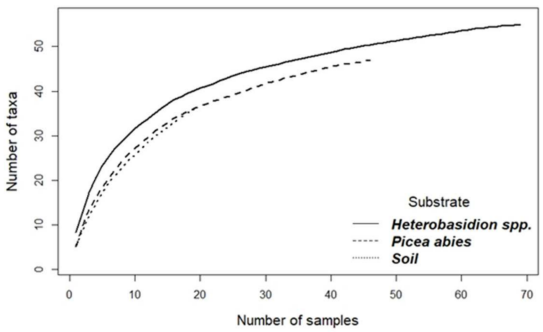

Figure 3. Accumulation curves showing the relationship between the cumulative number of invertebrate taxa and the number of collected samples.

The largest number of invertebrates was collected from *Heterobasidion* spp. fruitbodies —4886 adults and 101 larvae (Table 1). The dominant invertebrate taxa were Acari (82.4%),

with the other invertebrates being Collembola (6.2%), Coleoptera (4.7%), Diplopoda (2.5%) and Diptera (1.8%). In addition, Haplotaxida (0.8%), Thysanoptera (0.7%), Hymenoptera (0.3%), Aranea (0.2%), Gastropoda (0.1%) and Chilopoda (0.1%) were found in fruitbodies. Several invertebrate groups were represented by very few individuals in fruitbodies—Lepidoptera, Isopoda and Hemiptera (0.04% each) and Protura and Opiliones (0.02% each).

Table 1. Occurrence and relative abundance (%) of invertebrate taxa identified in *Heterobasidion* fruitbodies, *Picea abies* wood and soil samples. Absolute numbers for higher taxonomic groups are shown in parentheses.

Invertebrate Taxa	***Heterobasidion***		***Picea abies* Wood**		**Soil**	
	Fruitbodies					
Subclass/Family	**Adults**	**Larvae**	**Adults**	**Larvae**	**Adults**	**Larvae**
Acari	**(4111)**		**(1277)**		**(634)**	
Hypochthoniidae	0.6	-	2.6	-	1.4	-
Pthiracaridae	0.9	-	4.8	-	2.2	-
Euphthiracaridae	0.2	-	5.9	-	0.3	-
Crotonioidae	0.7	-	0.1	-	1.4	-
Nanhermanniidae	0.1	-	0.3	-	0.4	-
Damaeidae	0.2	-	0.1	-	0.7	-
Eremaeidae	0.1	-	0.1	-	-	-
Tenuialidae	0.04	-	0.1	-	0.3	-
Xenillidae	0.02	-	0.2	-	-	-
Carabodidae	65.9	-	12.4	-	11.4	-
Oppiidae	7.3	-	39.2	-	32.7	-
Scheloribatidae	-	-	-	-	0.6	-
Ceratozetidae	0.8	-	5.3	-	34.9	-
Galumnidae	0.04	-	0.1	-	0.6	-
Other [1]	7.1	-	10.3	-	4.3	-
Collembola	**(309)**		**(156)**		**(24)**	
Hypogastruridae	0.2	-	0.2	-	0.1	-
Neanuridae	4.2	-	6.4	-	2.3	-
Onychiuridae	0.8	-	1.5	-	0.4	-
Entomobryidae	0.1	-	1.2	-	0.3	-
Isotomidae	0.7	-	0.1	-	0.3	-
Tomoceridae	0.2	-	0.4	-	-	-
Sminthuridae	0.02	-	0.1	-	-	-
Coleoptera	**(170)**	**(62)**	**(12)**	**(29)**	**(7)**	**(3)**
Anobiidae	0.02	-	-	-	-	-
Cantharidae	-	17.8	-	16.7	-	40.0
Carabidae	0.1	-	-	-	-	-
Ciidae	3.0	11.9	-	1.9	-	-
Cryptophagidae	-	-	-	-	0.1	-
Elateridae	0.02	23.8	-	33.3	-	-
Pselaphidae	-	-	0.1	-	0.1	-
Ptilidae	0.1	-	0.5	-	-	-
Silphidae	0.1	-	0.1	-	-	-
Staphylinidae	0.2	7.9	0.1	1.9	0.7	20.0
Diplopoda	**(126)**		**(50)**		**(1)**	
Blaniulidae	2.3	-	2.9	-	0.1	-
Julidae	0.02	-	-	-	-	-
Polydesmidae	0.3	-	0.3	-	-	-
Diptera	**(50)**	**(39)**	**(10)**	**(22)**	**(3)**	**(1)**
Bibionidae	0.02	0.9	-	-	-	-
Brachycera	0.1	15.8	-	7.4	-	20.0
Cecidomyiidae	0.04	2.9	-	-	-	-
Ceratopogonidae	0.1	-	-	3.7	-	-
Chiromidae	0.4	14.8	0.3	25.9	-	-

Table 1. *Cont.*

Invertebrate Taxa	*Heterobasidion*		*Picea abies* Wood		Soil	
	Fruitbodies					
Subclass/Family	**Adults**	**Larvae**	**Adults**	**Larvae**	**Adults**	**Larvae**
Drosophilidae	-	-	0.1	-	-	-
Limoniidae	-	-	-	3.7	-	-
Psychodidae	0.02	-	-	-	-	-
Rhagionidae	-	-	-	-	0.1	-
Sciaridae	0.3	0.9	0.3	-	0.3	-
Stratiomyidae	0.02	2.9	-	-	-	-
Haplotaxida	**(41)**		**(18)**		**(8)**	
Lumbricidae	0.4	-	0.6	-	0.7	-
Enchytraeidae	0.4	-	0.5	-	0.4	-
Thysanoptera	0.7 **(35)**	-	0.6 **(10)**	-	0.1 **(1)**	-
Hymenoptera	**(15)**		**(7)**		**(8)**	
Parasitica	0.3	-	0.4	-	1.2	-
Formicidae	0.02	-	-	-	-	-
Aranea	0.2 **(10)**	-	0.5 **(8)**	-	0.4 **(3)**	-
Gastropoda	**(6)**		**(6)**		**(1)**	
Arionidae	0.02	-	-	-	-	-
Clausiliidae	0.02	-	-	-	0.1	-
Zonitiidae	0.1	-	0.4	-	-	-
Chilopoda	0.1 **(5)**	-	0.2 **(3)**	-	0.1 **(1)**	-
Hemiptera	0.04 **(2)**	-	0.1 **(1)**	-	0.3 **(2)**	-
Lepidoptera	**(2)**			**(3)**		**(1)**
Tineidae	0.04	-	-	5.5	-	20.0
Isopoda	**(2)**		**(2)**			
Trichoniscidae	0.04	-	0.1	-	-	-
Opiliones	0.02 **(1)**	-	-		-	-
Protura	0.02 **(1)**	-	-	-	-	-
Pseudoscorpionida	-	-	0.6 **(10)**	-	0.3 **(2)**	-

[1] Acari individuals that were not identified to family level.

Coleoptera of the Ciidae family were most prevalent in fruitbodies (86.8% of all beetles collected): *Cis nitidus* (27.6% of all beetles collected), *C. castaneus* (19.4%), *C. lineatocribatus* (18.8%), *C. jacquemartii* (14.7%) and *C. glabratus* (5.8%). Adult *Phosphuga atrata, Daliopius marginatus* and *Oxypselaphus obscurus* were observed, as well as larvae of several beetle families, i.e., Elateridae, Cantharidae, Ciidae and Staphylinidae. Larvae of these taxa were also found in wood samples; however, they were in a smaller number.

Three Diplopoda species were found in fruitbodies. The most prevalent species was *Proteroiulus fuscus*, which was twice as prevalent than in wood samples. One *Cylindroiulus caeruleocinctus* individual was found only in fruitbodies, as well as thirteen *Polydesmus denticalatus* individuals. Three earthworm species *Lumbricus castaneus, L. rubellus* and *L. terrestris* were found in fruitbodies. These species were also found in wood samples; however, they were in a smaller number.

The second largest number of invertebrates was collected from wood samples, i.e., 1570 adults and 54 larvae (Table 1). The dominant invertebrate taxa were Acari (78.6%), with the other invertebrates being Collembola (9.6%), Diplopoda (3.1%), Coleoptera (2.5%), Diptera (1.9%) and Haplotaxida (1.1%). Several invertebrate groups were represented by a few individuals in wood samples, which were Pseudoscorpionida and Thysanoptera (0.6% each), Aranea (0.5%), Hymenoptera (0.4%), Gastropoda (0.4%) Lepidoptera larvae (0.2%), Chilopoda (0.2%), Isopoda (0.1%) and Hemiptera (0.1%).

The smallest number of samples were collected from soil samples, i.e., 695 adults and five larvae (Table 1). As in the fruitbody and wood samples, Acari were the dominant invertebrate taxon (90.6%). Other invertebrates were present in small numbers: Collembola (3.4%), Coleoptera (1.4%), Haplotaxida (1.1%) and Hymenoptera (1.1%). Several

invertebrate groups were represented by very few individuals in soil samples: Diptera (0.6%), Aranea (0.4%), Pseudoscorpionida (0.3%) and Hemiptera (0.3%), and Chilopoda, Diplopoda, Thysanoptera, Lepidoptera larvae and Gastropoda (0.14% each).

Shannon index values were 2.37 in wood samples, 1.63 in fruitbodies and 1.95 in soil samples.

Dominance of one invertebrate taxonomic group was pronounced with Acari dominating in all substrates, and Collembola was the second most common group (Table 1). The third most prevalent taxonomic group varied between substrates, i.e., in *Heterobasidion* spp. samples it was Coleoptera, in wood it was Diplopoda and in soil it was Coleoptera and Haplotaxida. Among the 62 invertebrate taxa found in the present study, eleven (17.7%) were exclusively found in fruitbodies, two (3.2%) in the wood and three (4.8%) in the soil, while thirty (48.4%) were common to all substrates (Figure 4). There were 43 shared invertebrate taxa between fruitbodies and wood, 31 between fruitbodies and soil and 32 between wood and soil (Figure 4).

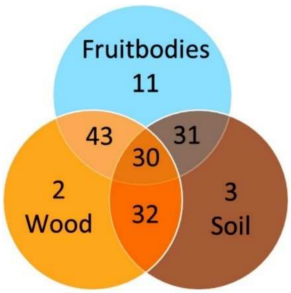

Figure 4. Venn diagram showing the diversity and overlap of invertebrate taxa in different substrates (sites as well as adults and larvae are combined).

In principal component analysis (PCA) of invertebrate communities, axis 1 explained 81.7% of variation and axis 2 explained 18.3%. PCA showed that invertebrate communities from different substrates were separated from each other on axis 1, i.e., on the axis, which explained most of the variation (Figure 5).

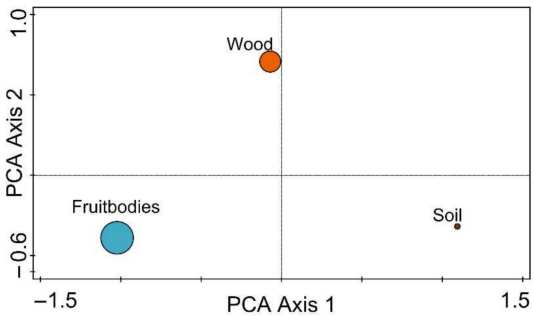

Figure 5. Ordination diagram based on principal component analysis of invertebrate communities from *Heterobasidion* spp. fruitbodies, *Picea abies* wood and soil samples. The size of each point reflects the relative richness of invertebrate taxa.

4. Discussion

The taxonomic composition of invertebrates found in *Heterobasidion* spp. samples reflects the biology of fruitbodies. Fruitbodies develop on the part of the infected substrate that are in contact with the soil [7]. If the wood substrate is embedded in the soil, the fruitbody develops in a narrow line at the point of contact between the soil and the wood [5]. Therefore, the dominant invertebrate taxa in fruitbodies were also present in wood and soil samples (Table 1, Figure 4). Although the majority of invertebrate taxa were shared among different substrates, the PCA showed that invertebrate communities in different substrates were separated from each other, which was likely due to differences in the abundance data

of different taxa (Table 1), thereby demonstrating the specificity and substrate preferences of different invertebrates. Our data show that Acari were the predominant invertebrate taxa found in *Heterobasidion* spp. fruitbodies (Table 1). This is consistent with other reports about fauna of invertebrates found in *Heterobasidion* spp. [32] and *F. pinicola* [14] fruitbodies. Some of the Acari that were not identified to family level belong to the order Mesostigmata, which was previously identified in fruitbodies from other species, such as *F. pinicola* [41]. Acari of the genus *Carabodes* were the most prevalent in fruitbodies. The presence of this genus in fruitbodies has been studied extensively in coniferous forests in Norway [14,42], and many taxa inhabiting soil also occupy fruitbodies as an additional food resource. This was also observed for Acari collected from *Heterobasidion* spp. [32].

Several studies have noted that *Heterobasidion* spp. fruitbody development is promoted by shade and moisture [5,43,44]. In this study, invertebrate groups that prefer moist environments, such as Haplotaxida, Diplopoda and Chilopoda, were also frequently detected in fruitbodies. However, these invertebrates are probably incidental and temporary inhabitants of fruitbodies even though their numbers in the analysed samples was relatively high (Table 1). Diplopoda and Chilopoda are prone to dehydration, and therefore seek moist places to forage [45]. The relative moisture content of the fruitbodies analysed in this study was 70%, and water drained from them when compressed. A portion of the Haplotaxida was collected from the oldest peripheries of the fruitbodies, where other invertebrates had already formed tunnels.

Heterobasidion spp. form perennial fruitbodies with a layered, cork-like structure [3]. Several Diptera adults and larvae were identified in fruitbodies, however the number of individuals collected was relatively small in comparison with the total number of invertebrates collected, which could be due the structure of fruitbodies. Diptera most often inhabit softer, fruitbodies of annual fungi that decay faster [46]. However, despite the structural differences of various fungal fruitbodies, our study is in agreement with the results from other studies [47,48], i.e., showing the low level of specialization between Diptera. Therefore, fungal fruitbodies can be colonized by various groups of Diptera. The number of amassed Diptera could be affected by the time of sampling as these insects are mostly inactive during the dormancy period.

One of the aims of this study was to compare invertebrate fauna inhabiting fruitbodies, rotting wood and soil. While these substrates are in close contact with each other in nature, differences in the invertebrate fauna inhabiting them were observed (Table 1, Figures 4 and 5). These differences may be due to the season when samples were collected (October). Assessment of invertebrate fauna during the vegetation period may be more representative. Each substrate had a characteristic invertebrate fauna profile (Figure 5), and the number of individuals and taxa collected varied between different substrates. For example, Acari of the genus *Carabodes* were most prevalent in fruitbodies but were less common in wood and soil samples (Table 1). By contrast, some other Acari were most often found in wood and soil samples (Oppiidae and Ceratozetidae, respectively) but were less commonly found in fruitbodies. In total, 90% of invertebrate individuals collected from soil samples were Acari, while in fruitbody and wood samples the proportion of Acari was lower (82% and 78%, respectively). Although the majority of invertebrate taxa were found in all substrates, some characteristic taxa were only found in fruitbodies (e.g., Ciidae), but not in wood or soil samples (Figure 4).

In studies of polypore inhabitants, usually the entire fruitbody is collected [17,32]. Studies in Latvia on the development of *Heterobasidion* spp. fruitbodies on decaying *P. abies* wood indicate that the size of fruitbodies can vary widely, and older fruitbodies coalesce with each other [5]. Therefore, in this study, only partial fragments of fruitbodies were collected. Although *Heterobasidion* spp. form large fruitbodies on windthrown trees and large dimension logging debris in favourable conditions [3,5], collection of a large number of 10 cm × 10 cm fragments of *Heterobasidion* spp. fruitbodies is difficult. Although the area of collected fruitbodies was uniform (100 cm^2), the volume of fruitbodies varied. This was due to differing thicknesses of the collected fruitbodies, which is influenced by the age

of fruitbodies. In addition, the dry weight of collected fruitbodies varied between 5.1 g and 47.3 g, which has also been reported to influence invertebrate numbers and diversity in *H. annosum* fruitbodies [32]. The obtained data indicate that in further research, a larger number of samples of smaller size could be collected.

The Shannon diversity index values indicated that invertebrate diversity in fruitbodies was moderate. As mentioned before, the results of the study were influenced by the season when samples were collected. *Heterobasidion* spp. sporulate throughout the year, however, the maximum rate is between June and August [49,50]. Collection of fruitbodies at this time may identify invertebrate taxa that feed on spores at some developmental stage or taxa that are inactive during colder periods of the year. Analysis of invertebrates present in fruitbodies during peak sporulation times may identify potential vectors that can influence the primary infection pathways of this pathogen [51]. Previous studies on the role of different invertebrates as vectors of *Heterobasidion* have mainly focused on fungal conidia [3,52], but further studies are required to better understand the nature of such associations.

In this study, samples were placed in Tullgren funnels to collect invertebrates for three days. In total, 95% of the individuals collected were collected within the first 24 h. Some reports [45,53] indicate that in order to identify certain invertebrates, samples need to be held in Tullgren funnels for a longer period, for example, to allow development of larvae. Some Diptera and Coleoptera larvae were collected in this study, however, the proportion of larvae of the total number of individuals collected was low (2% in fruitbodies). To obtain a more comprehensive assessment of invertebrate fauna inhabiting *Heterobasidion* spp., methods that allow the development of larvae should be considered, by not preserving collected individuals in glycerine and placing collected larvae in rearing chambers, or by placing fruitbodies in vials and collecting emerged insects. In addition, Tullgren funnels are only partially effective for the collection of mobile invertebrates such as Collembola and should be used in conjunction with other collection methods [54], for example, by covering funnels. In this study, Collembola were observed on the surface of the samples during both sample sorting and collection in Tullgren funnels.

The stage of wood decay also influences invertebrate numbers and diversity, similarly to the decay stage of fruitbodies. The majority of organisms associated with polypores utilise fruitbodies in the later succession stages [11]. During the sterile phase, when the fungal hymenium layer is developing, perennial polypore species produce chemicals that protect them against invertebrate damage, which ceases when the fruitbody starts to decay [14]. Invertebrates may only start to inhabit fruitbodies after several years. In addition, fruitbodies on a single log do not start to decay simultaneously, ensuring suitable conditions for invertebrates that colonise decaying fruitbodies, even over several decades. Studies in Latvia have identified *Heterobasidion* spp. fruitbodies in various stages of decay on more than ten-year-old rotting spruce wood [5].

Ciidae was the most prevalent Coleoptera family collected from fruitbodies. This is a functionally important group that inhabits fruitbodies during both larval and adult stages, and several successive generations are often found in a single fruitbody. The diversity of Ciidae species is significantly influenced by host organism incidence within a territory, as well as fruitbody developmental stages [17,55]. *Cis bidentatus* was the only Coleoptera species collected from *H. parviporum* fruitbodies in Finland, and that colonised decaying fruitbodies or decaying parts of still living fruitbodies [23]. However, this species was not identified in this study.

While a comparatively small number of *Cis glabratus* individuals were identified in this study, this species has been reported to be prevalent in *H. annosum* [24] and *F. pinicola* fruitbodies [15,17]. In addition to species collected in this study (*C. lineatocribatus*, *C. nitidus*, *C. glabratus* and *C. castaneus*), another study also reported the presence of *C. bidentatus* and *Ennearthron cornutum* on *Heterobasidion* spp. [24].

According to the European Union Council directive 92/43/EEK (Protecting Europe's biodiversity (Natura 2000)), no rare or protected species of invertebrates were identified in this study.

Further research is needed to reveal the effect of the decay stage of wood and fruitbodies, as well as the effect of wood dimensions on the invertebrate fauna found in *Heterobasidion* spp. fruitbodies. Differences in the invertebrate fauna inhabiting decaying wood in areas of mineral and peat soils could be assessed by collecting samples from forest stands of different forest types.

5. Conclusions

The results demonstrated the presence of a taxa-rich community of invertebrates inhabiting *Heterobasidion* fruitbodies, decayed *P. abies* logs and adjacent soil. Although a large number of invertebrates were shared among different substrates, their relative abundance varied substantially showing certain specificity and substrate preferences of particular taxa.

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forests

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Article

Patterns of Diversity in the Symbiotic Mite Assemblage of the Mountain Pine Beetle, *Dendroctonus Ponderosae* Hopkins

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Abstract: The mountain pine beetle, *Dendroctonus ponderosae* (Coleoptera: Scolytinae), is an economically important bark beetle species with a wide geographic range spanning from the southwestern United States into northern Canada. This beetle causes extensive tree mortality to 13 pine species. Mites (Acari) are common and abundant symbionts of mountain beetles that may influence their fitness through positive and negative interactions. We present a unique assessment of the mite associates of mountain pine beetles using measures of alpha and beta diversity. We sampled phoretic mites from five beetle populations: Arizona, Colorado, South Dakota, Utah (USA), and Alberta (Canada) that varied in host tree species, local climate, and beetle population level. We collected 4848 mites from 8 genera and 12 species. Fifty to seventy percent of beetles carried mites in flight with the highest mite loads occurring in middle and southern populations; decreasing in northern populations. Mite assemblages (i.e., both richness and composition) varied along a south to north latitudinal gradient and were driven by species turnover (i.e., species replacement). Differences in mite composition increased with distance between populations. We discuss climatic variation, environmental filtering, and host tree differences as factors that could affect differences in mite composition between beetle populations and discuss implications for functional shifts. Our results could represent a model for estimating diversity patterns of mite symbionts associated with other major insect pests in coniferous forest systems.

Keywords: biodiversity; bark beetles; symbionts; species assemblage; beta diversity; forest ecosystems

1. Introduction

Globally, forests face increased pressure from insect pests, such as bark beetles (Coleoptera: Scolytinae) [1–3]. Bark beetle impacts can be severe as they actively damage tree phloem tissue and vector phytopathogens, resulting in large events of tree mortality [4,5]. The mountain pine

beetle, *Dendroctonus ponderosae* Hopkins, has been responsible for tree mortality across more than 30 million hectares of forest in western North America in the last decade [4,5]. Previously constrained by temperature, a warming climate has allowed the mountain pine beetle to expand its habitable range to higher elevations and its geographic distribution northward [6–8]. The mountain pine beetle colonizes 13 pine species of which it most commonly attacks lodgepole pine (*Pinus contorta* Doug.), white pine (*Pinus strobiformis* Englm.), limber pine (*Pinus flexilis* E. James), ponderosa pine (*Pinus ponderosa* Doug. ex C. Lawson) [9], and more recently, jack pine (*Pinus banksiana* Lamb.) [7,10].

Most of the mountain pine beetle's life is spent within the phloem layer of its host tree where beetles tunnel, deposit blue-staining fungi, lay eggs, and complete development from larval to adult [11–13]. The microhabitats created by beetle activity are used by numerous organisms hereby referred to as 'symbiota' or 'symbionts' [14]. The beetle's symbiota can also influence its fitness via ecological interactions (e.g., alimentary mutualists and fitness antagonists) [15,16]. For example, fungal symbiota associated with the mountain pine beetle can positively influence beetle reproductive success by providing nutritional resources [13,17,18] or negatively via parasitism and competition [19,20]. Mites (Acari) are important associates of bark beetles [16,21] and perform various direct and indirect functional roles in bark-beetle galleries such as vectoring fungi, predating each-other and beetle young, etc. [14,22,23]. They may specialize on a single beetle host, act as generalists, and form complexes of cryptic species with varying degrees of host specificity [24,25]. As in other closely related bark beetles, mite functions influence a beetle's success both directly and indirectly [26–29]. Mites can indirectly affect tree death by facilitating the transmission of plant pathogens into a new tree [29,30]. For example, mites associated with the bark beetle, *Scolytus multistriatus* (Marsham), contribute to the transmission of the destructive vascular wilt disease agent, *Ophiostoma novo-ulmi* Brasier in *Ulmus* spp.; interestingly, this fungus can significantly reduce beetle fitness [31].

Mites associate with bark beetles and other forest insects via phoresy [32], i.e., by using the beetles as a means of passive transport between trees and under the bark within a tree. Very high loads of phoretic mites can impair host mobility and negatively affect the host or 'carrier' beetle [27,33]. In carrion beetles (Coleoptera: Silphidae) mites at abnormally large densities negatively affected their host beetle fitness [34]. Several factors are known to influence phoretic mite loads across beetle populations, including forest tree composition [16,35], temperature [36–38], geographic distance and dispersal (reviewed in [39]), and beetle abundance [29,40]. Since the mountain pine beetle shows varying preferences for certain pine species [12], with the abundance of preferred host trees, and local climate varying among forests [4–6], it is expected that mite assemblages differ across beetle populations, but this is rarely tested.

Disregarding mites in the study of bark beetles not only underestimates the total biodiversity associated with bark beetles, but also reduces our ability to interpret variation in beetle population dynamics [16,21,41]. Additionally, previous studies of mountain pine beetle mites have largely been restricted to single populations, lacking comparisons of different populations [5,21,42]; thereby limiting our understanding of how specific mite assemblages relate to their beetle host success or population stage.

Here we examine, describe, and analyze the phoretic mite assemblages associated with mountain pine beetles in five different beetle populations spread across a latitudinal gradient and varying in host tree species, local climate, and beetle population stage. We use measures of alpha and beta diversity to describe these compositional differences and discuss mechanisms that might be driving these differences; specifically host tree variability, climatic variability, beetle abundance, and mite functional group. We predict that increased latitudinal separation correlates with differences in mite assemblages, and that differences may be caused by climate and environmental factors. Our aim is to set the stage for future work that would examine broad scale patterns of mite assemblages associated with mountain pine beetles and other economically and ecologically important forest insect species.

2. Materials and Methods

2.1. Mite Collection

Between 2009 to 2017, 2225 mountain pine beetle specimens were collected in Arizona, Colorado, Utah, South Dakota (United States), and Alberta (Canada) during the beetle's main emergence event, which usually occurs in the late summer months between July–August (Figure 1; Table 1). Beetles were captured using a combination of baited flight traps (Lindgren funnel traps; ref. [43]) and infested logs placed inside emergence chambers [44] (Table 1). Sampling size of beetles varied due to differences in funding and resources, and the sampling strategies of each author/collaborator. Host tree species, sampling years, and beetle population phase also varied with location (Table 1). This broad dataset, therefore, represents nearly all stages and climate types of mountain pine beetle populations across its North American distribution. Differences in sample size of beetles and associated mite loads were accounted for through the use of multi-variate non-parametric statistical analyses. Between 161 and 910 beetles from each population were examined for mites. Mites were removed from each beetle, identified by clearing and mounting on glass slides under a dissecting microscope, and stored using mite collection protocols and identification resources as described in Vissa, et al. [35] for Arizona and Utah populations, Mori, et al. [42] for Alberta, Reboletti [45] for South Dakota, and Mercado, et al. [21] for Colorado. These vary only subtly in mite storage (particularly location of specimen), and otherwise converge on the same collection, clearing and mounting protocols used for processing bark-beetle mites.

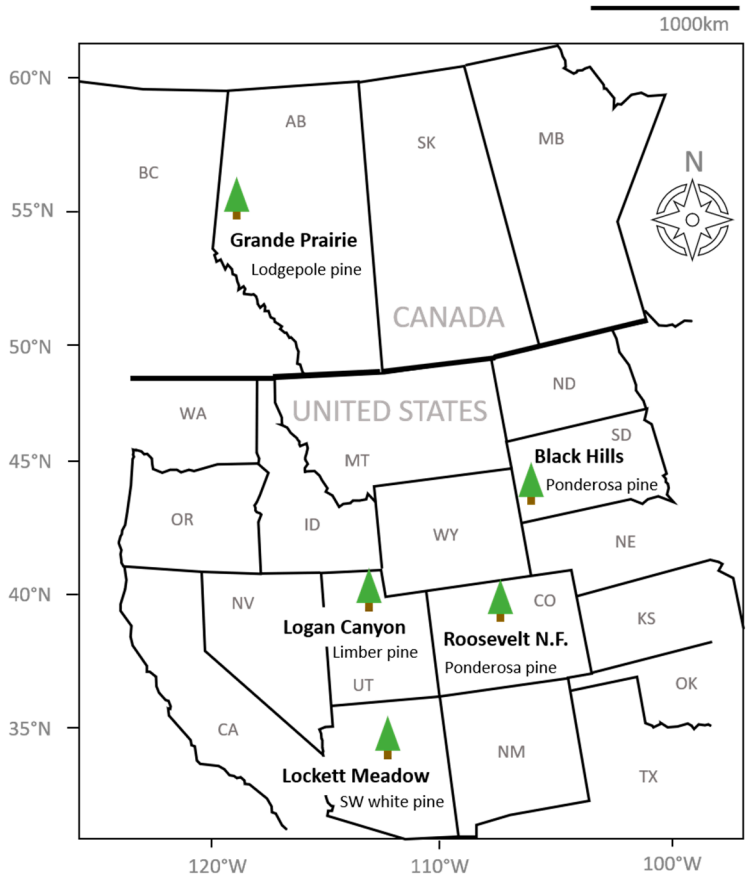

Figure 1. Map of sampling locations for the five sampled beetle populations.

Table 1. Mountain Pine Beetle (MPB) population, sampling, and site information. (#) refers to the "total number of" beetles and/or mites.

Population	Sampling Year(s)	Lat (N) N to S	Descriptive Latitude	Elevation (m)	MPB Attacked Host Tree	Year	MPB Population Phase	Descriptive Precipitation	# MPB	# mites	Average Mites per Beetle	Total No. of Mite Species
Grande Prairie, Alberta, Canada (CAN)	2009	54.69	northern	~750	*Pinus contorta*	2009	Epidemic	most dry	910	801	0.88	3
Black Hills, South Dakota (USA)	2007	43.5	middle	~2200	*Pinus ponderosa*	2011	Epidemic	dry	350	1106	3.16	7
Logan Canyon, Utah (USA)	2016/2017	41.93	middle	~2300	*Pinus flexilis*	2016–2017	Endemic	wet	161	886	5.50	5
Roosevelt N.F., Colorado (USA)	2012/2013	40.4	middle	~2250	*Pinus ponderosa*	2012–2013	Epidemic to Endemic	dry	565	1460	2.58	5
Lockett Meadow, Arizona (USA)	2016/2017	35.36	southern	~2600	*Pinus strobiformis*	2016–2017	Endemic	wettest	239	595	2.49	5

2.2. Assessing Climatic Conditions

To assess sampling year and population location factors, we compared sampling year average and 30-year annual averages of precipitation and temperature extracted from the PRISM (Parameter-elevation Regressions on Independent Slopes Model) Climate Group, Oregon State University database [46] using field coordinates listed in Table 1. The same data for Alberta, Canada was retrieved from the Canadian Climate Normals database (Environment and Climate Change Canada, 2019; ref. [47]).

Alberta, the northern-most population, had the lowest minimum and maximum temperatures overall; and had particularly low average minimums in the sampling year (Figure 2). South Dakota had the highest average maximum and average minimum temperatures overall. Extracted climate data showed low precipitation in both regions. Colorado's climatic conditions were similar to that of South Dakota during the 2012 sampling year but experienced a marked increase in total precipitation during the 2013 sampling year. Utah experienced similar average maximums as those seen in Colorado, however, lower average minimum temperatures were observed. Arizona, the southern-most population, had higher maximum and minimum temperatures compared to Utah, although Arizona received less total precipitation compared to Utah (Figure 2).

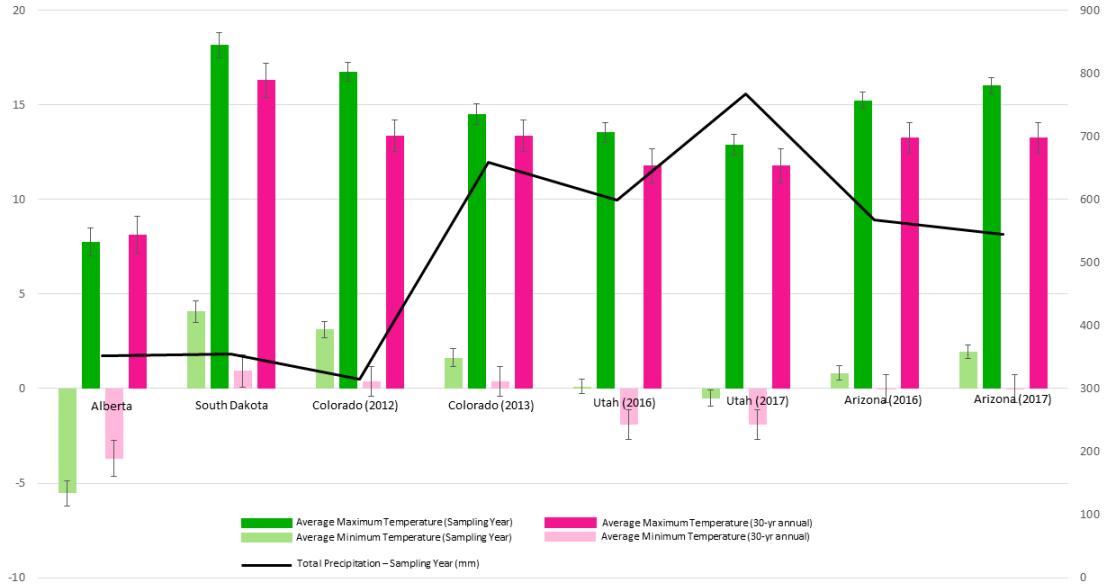

Figure 2. Basic climate information for sampling locations and years compared to 30-year annual averages. Obtained from the PRISM Climate Group database [46], Oregon and the Canadian Climate Normals database [47].

2.3. Mite Data Analysis

Analyses were carried out in R. ver. 3.6.2 (R Core Team, 2019) using raw mite abundance data. Species accumulation curves for each population and the overall total sample pool were estimated using the *specaccum* function in the *vegan* package [48]. To test for differences in mite loads across populations, we used generalized linear models (GLM) using the *glm* function in the *MASS* package [49]. To test for pairwise differences in the assemblage of species across sampling locations based on the presence and absence of species and their abundances, a negative binomial GLM with pairwise comparisons using the *manyglm* function in the *mvabund* package [50] was used. The use of the negative binomial distribution was determined by model selection using the Akaike information criterion (AIC) [51] where Poisson, zero-inflated binomial and negative binomial distribution models were compared.

The multivariate species data were fit and analyzed with the *mvabund* package. The principle model fitting function, *manyglm* fits a GLM via resampling for each sampled species using species abundances. It allows for multiple species testing and uses a likelihood ratio test (LRT) and resampled

p-values to detect significance where the null-hypothesis (H_0) considers beetle population to have no effect on mite species composition. To account for correlation in testing 1000 resampling iterations were used in our analysis via 'pit.trap' resampling in testing [50].

Beta diversity metrics for each population were calculated with the *betapart* package [52]. This package uses multiple-site dissimilarity measures to assess the spatial patterns of beta diversity. It also accounts for compositional heterogeneity across sites, thereby accounting for multivariate structure of dissimilarity in species composition [53]. Beta diversity calculations were conducted using both the Sorenson and Jaccard dissimilarity indices with no differences in results observed between the two. The Sorenson dissimilarity is used in all visualizations where: β_{sor} is the overall Sorenson dissimilarity index, β_{sne} is dissimilarity explained by nestedness, and β_{sim} is dissimilarity explained by turnover. β_{sor}, β_{sne}, and β_{sim} are measured on a 0–1 scale where $\beta_{sor} = 0$ indicates all species are shared and $\beta_{sor} = 1$ indicates no species are shared. β_{sor} may also be expressed as ($\beta_{sne} + \beta_{sim}$). Degrees of nestedness were visually represented in a cluster dendrogram made within the *betapart* package. Finally, indicator species associated with each sampling location were assessed with the *indicspecies* package [54]. The indicator species analysis [55] highlights the occurrence of species that determine the community assemblage or its diversity within the given sampling area.

3. Results

3.1. Mite Abundance, Taxa Found, and Species Richness

A total of 4848 mites were collected across all sampled mountain pine beetle populations (Table 1). Mites represented 8 genera and 12 species (Table 2). No species were endemic to any population, while two species (*Tarsonemus endophloeus* Lindquist and *Proctolaelaps subcorticalis* Lindquist) were found in all populations. Mites from over 100 beetles were sampled from each population with species accumulation curves beginning to approach or fully approaching asymptote in all populations (Figure 3). The number of mites per beetle varied significantly (GLM analysis; $p < 0.01$) in all but three comparisons across populations (Table 3). Mite loads were highest in middle to southern populations; and decreased in northern populations. Alberta, the northern-most population, had significantly lower mite loads than all other populations. Percentage of phoretic mites per beetle was lowest in beetle populations in Alberta, Canada. Arizona and Utah had the greatest percentage of beetles carrying mites, followed by South Dakota where half the beetles carried mites (Figure 4).

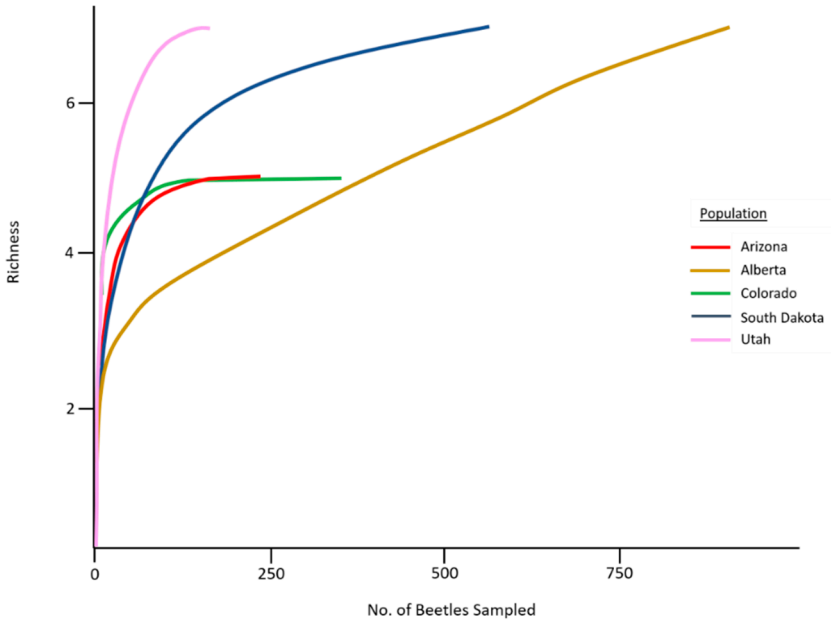

Figure 3. Mite species accumulation curves for our five sampled mountain pine beetle populations.

Table 2. Average abundance per beetle (±standard error), functional and taxonomic information for mite species captured. Indicator species ($p < 0.05$) for each population are indicated with an asterisk (*). Species with average abundance <0.1 are considered rare species. Significant difference between abundances listed below across populations were confirmed (see generalized linear models (GLM) analysis; Table 3).

Taxa Information		**Average Abundance Per Beetle (±S.E.)**				
Mite Species	**Functional Group**	**Alberta**	**South Dakota**	**Utah**	**Colorado**	**Arizona**
Tarsonemus ips Lindq.	Fungivore	0.34 (0.03)	3.26 (0.14)	3.82 (0.46)	1.19 (0.15)	0.79 (0.1) *
Tarsonemus endophloeus Lindq.	Fungivore		0.32 (0.01)	0.04 (0.02)	0.27 (0.05) *	
Proctolaelaps subcorticalis Lindq	Predator	0.5 (0.07) *	1.73 (0.07) *	0.03 (0.03)	0.39 (0.07) *	0.01 (0.01)
Dendrolaelaps quadrisetus Berl.	Predator			0.22 (0.05)		0.08 (0.02)
Trichouropoda sp. (*T. utahensis* Wis. and Hirch. in Utah, Arizona; and *T. utahensis* + *T. maeandralis* Hirsch. in Colorado)	Omnivore			1.39 (0.22) *	2.3 (0.35) *	1.32 (0.16)
Nanacarus sp.	Omnivore		0.01 (0.00)			
Histiogaster sp.	Detritivore		0.43 (0.02)			0.28 (0.19) *
Histiogaster arborsignis Woodr.	Detritivore	0.04 (0.01) *			0.02 (0.01)	
Schweibea sp.	Unknown		0.18 (0.01) *			
Parawinterschmidtia sp.	Unknown		0.04 (0.00)			
Overall mites/beetle		0.9	3.2	5.5	2.6	2.49
Total Species Richness		3	7	5	5	5

Table 3. GLM test statistics for total mite abundance per beetle across five sampled populations. Asterisk (*) shows significane at $p \leq 0.05$.

Analysis of Deviance Table		
Population	*t*-value	*p*-value
Intercept (Arizona)	7.079	<0.01 *
Alberta	−6.8	<0.01 *
South Dakota	−1.542	0.1
Colorado	3.154	<0.01 *
Utah	4.091	<0.01 *
Pairwise Comparisons		
Population Pair	*z*-value	*p*-value
Arizona-Canada	6.8	<0.01 *
Arizona-Colorado	−3.154	0.01 *
Arizona-South Dakota	1.542	0.53
Arizona-Utah	−4.091	<0.01 *
Canada-Colorado	−12.016	<.01
Canada-South Dakota	−6.685	<.01
Canada-Utah	−11.037	<0.01 *
Colorado-South Dakota	5.687	<0.01 *
Colorado-Utah	−1.574	0.51
South Dakota-Utah	−6.122	<0.01 *

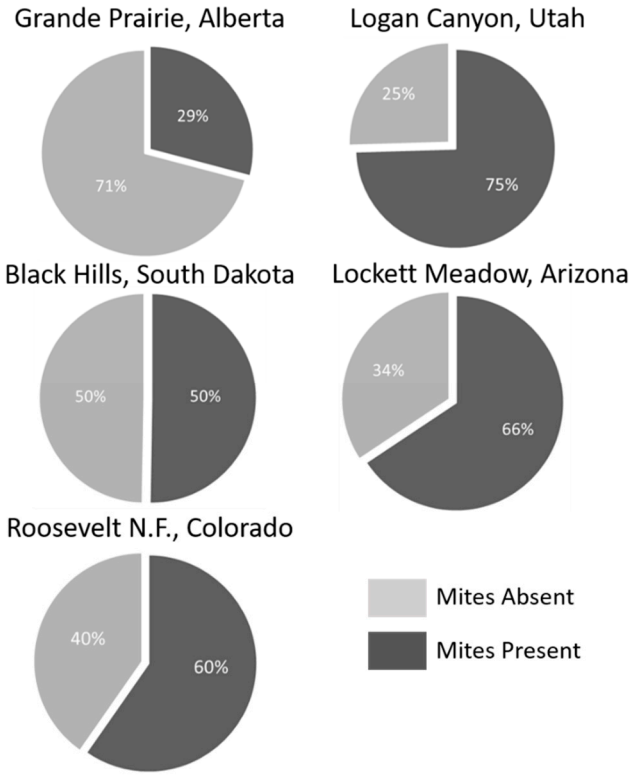

Figure 4. Percent beetles carrying mites in each sampled population.

A pairwise comparison of the total mite species richness per beetle showed no difference between populations ($p > 0.05$; Table 4). Specific indicator species in each population varied, with some overlap occurring across Alberta, South Dakota, and Colorado. There was no overlap in indicator species between the southern-most and northern-most populations (Table 2).

Table 4. Pairwise population comparisons of total mite species richness per beetle.

Population Pair	|*t*-Value|	Std. Error	*p*-Value
Arizona-Alberta	0.164	0.01	0.99
Arizona-Colorado	0.797	0.01	0.93
Arizona-South Dakota	0.032	0.012	1
Arizona-Utah	1.412	0.01	0.62
Alberta-Colorado	1.089	0.01	0.81
Alberta-South Dakota	0.157	0.012	0.99
Alberta-Utah	1.706	0.01	0.43
Colorado-South Dakota	0.961	0.01	0.87
Colorado-Utah	0.762	0.012	0.94
South Dakota-Utah	1.604	0.01	0.5

3.2. Partitioning Compositional Differences Using Measures of Beta Diversity

The mite assemblages associated with the five bark beetle populations differed geographically based on the presence and absence of species and their relative abundances (Table 5; LRT = 7159; $p = 0.001$). *Tarsonemus endophloeus* was present in mid-latitude populations (Utah, Colorado, and South Dakota), with highest abundances in South Dakota and Colorado. *Tarsonemus endophloeus* was rare in Utah and absent from the southernmost (Arizona) and northernmost (Alberta) populations in our study

(Figure 5; Table 2). *Dendrolaelaps quadrisetus* Berlese was found only in Arizona and Utah; however, these were captured in 2016 and not in 2017. *Trichouropoda utahensis* Hirschmann and Wisniewski, in particular, was unique to middle and southern mountain pine beetle populations from Utah, Arizona, and in Colorado (where *T. maeandralis* Hirschmann also was found). It is also indicative of Utah where it was most abundant (Figure 5; Table 2). Mites in the genus *Trichouropoda* are not reported in Alberta in our study location; however, they have been observed on mountain pine beetles from the southwest of the province near Banff National Park [56]. Some rare species, which include *Histiogaster arborsignis* Woodring, *Nanacarus* sp., and *Parawinterschmidtia* sp., were captured in very low numbers.

Table 5. (**A**) Multivariate test statistic showing likelihood ratio test (LRT) values for population effect on mite composition based on the presence/absence of species and abundances where D_f = degrees of freedom; (**B**) Wald test statistics for population differences shown in (**A**); (**C**) LRT for differences in mite species across all populations. Asterisk (*) shows significance at $p \leq 0.05$.

(A) Multivariate Test—Analysis of Deviance Table				
	D_f	D_f Difference	LRT	*p*-value
(Intercept)	2030	4	7159	0.01 *
Population	2026			
(B) Wald Test Statistics by Population				
Population	Wald Value			*p*-Value
Intercept (Arizona)	6.311			<0.01 *
Alberta	6.646			<0.01 *
South Dakota	4.937			<0.01 *
Colorado	3.922			<0.01 *
Utah	5.31			<0.01 *
(C) Univariate Comparisons of Species Abundances Across All Populations				
Mite Species	LRT			*p*-value
Tarsonemus ips Lindq.	192.849			<0.01 *
Tarsonemus endophloeus Lindq.	116.192			<0.01 *
Trichouropoda utahensis Wis. and Hirsch.	223.699			<0.01 *
Trichouropoda sp. complex (*T. maeandralis* Hirsch. + *T. utahensis)*	459.59			<0.01 *
Proctolaelaps subcorticalis Lindq.	65.354			<0.01 *
Histiogaster arborsignis Woodr.	27.042			<0.01 *
Histiogaster sp.	24.011			0
Schweibea sp.	18.026			<0.01 *
Other/Rare spp.	9.689			0.04

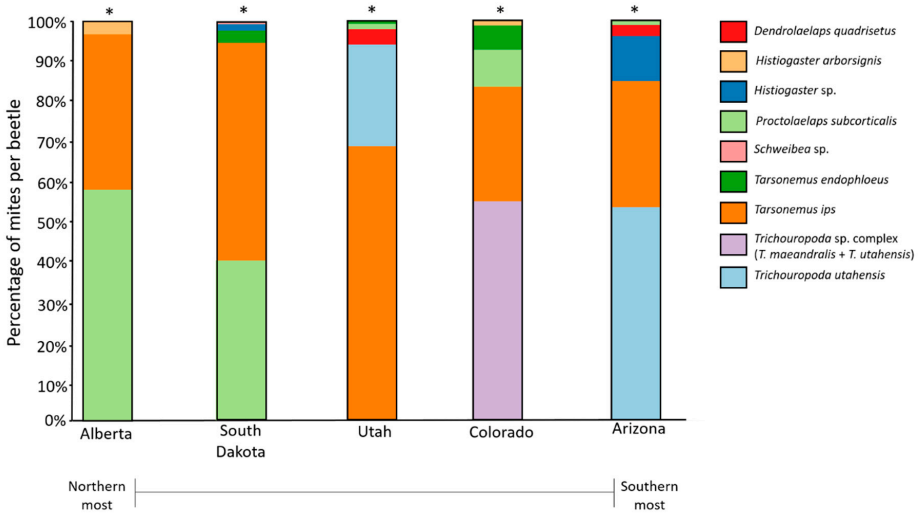

Figure 5. Mite assemblages shown as percentage of each mite species (excluding extremely rare species) sampled across five populations. Asterisk (*) indicates significantly different assemblages between populations (for full statistics, see Table 5).

Differences in mite composition between populations can be further explained by measures of nestedness and turnover (species replacement). On a scale of 0–1 where 1 = complete dissimilarity, the total dissimilarity in mite composition (β_{sor}) across populations was 0.54. The majority of dissimilarity resulted from turnover (β_{sim}) which explained 0.43 of the total dissimilarity, while nestedness (β_{sne}) accounted for 0.114 of the total dissimilarity.

The highest rates of turnover occurred between the southernmost beetle populations (Arizona and Utah) and the other populations, particularly Alberta (Figure 6a). High rates of turnover were also seen between southern-most population (Arizona) and each of the mid-latitude populations (Colorado and South Dakota) as well as between the northernmost population (Alberta) and the mid-latitude populations (Figure 6a). The mite assemblages of Arizona and Utah were completely nested, i.e., the mites found in Utah were a subset of those found in Arizona. Alberta mite assemblages were a subset of those found in South Dakota and other middle populations (Figure 6a,b).

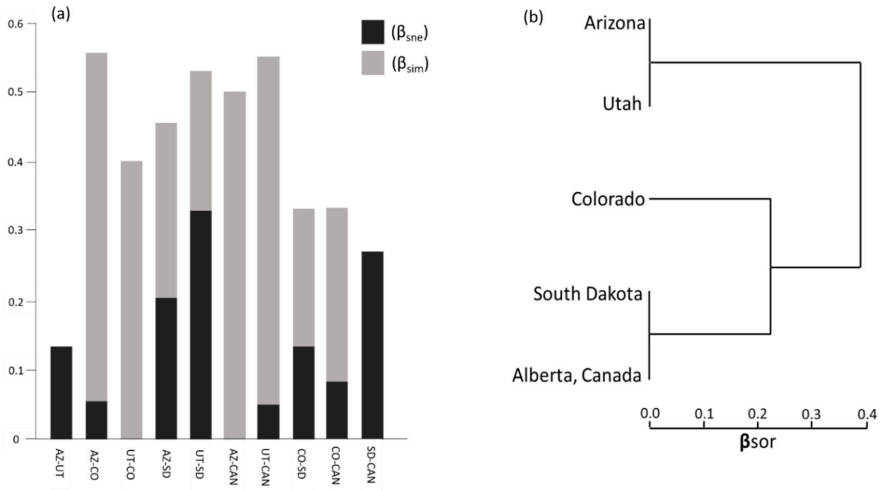

Figure 6. (**a**) Sorenson dissimilarity indices of Beta (β) diversity for mite communities partitioned into nestedness (β_{sne}) and turnover (β_{sim}) from southern-most (Arizona [AZ]) to northern-most (Alberta, Canada [CAN]) sampled populations; (**b**) cluster dendrogram showing degree of nestedness based on overall dissimilarity (β_{sor}).

4. Discussion

Geographically distant mountain pine beetle populations have different symbiotic mite assemblages despite some commonalities reflected in species overlap. Sorenson's dissimilarity values for beta diversity indicate that the mite assemblage of beetle populations geographically closer to one another are more similar than populations that are geographically more distant. This was most evident between Alberta and all other populations. Turnover and nestedness patterns varied although species richness did not show any significant variability across populations.

4.1. Differences in Species Richness and Abundance

No significant differences were detected in mite species richness, presumably because richness per beetle was generally very low for all populations. Species accumulation curves approached asymptote in all populations (although not at the same rates), indicating that our sampling efforts adequately captured the overall mite richness associated with these populations. However, the disparities in species accumulation curves between populations may also be due to the possible presence of cryptic species that could not be uncovered. The number of mites per beetle (mite load) did not vary on a latitudinal gradient (as predicted), suggesting that other factors may better explain these differences. We observed that post-outbreak and 'endemic' stage populations had higher mite abundances, and observably higher total richness (Table 2) suggesting that mite loads may be indicative of beetle population phase. Hofstetter, et al. [57] show that higher mite loads dominated by *Tarsonemus* mites correlated with decreased beetle progeny densities because of mite introduction of antagonistic blue-stain fungi. While our sampling efforts could not test particular hypotheses, our observed mite load trends may provide insight on population specific ecological dynamics in mountain pine beetles. High mite loads can affect beetle flight [27,33] or influence their reproductive success within trees [58]. The specific effects of different mite levels on mountain pine beetles has not been tested. Our analysis of mite community patterns provides a foundational step towards understanding these effects.

The finding that *Tarsonemus ips* and *Proctolaelaps subcorticalis* occurred in all populations suggests that these mites may not be affected by latitude, pine tree host, climate, or beetle population stage. *Trichourpoda utahensis* was only found in Arizona and Utah; and although also found in Colorado, occurred alongside a second *Trichouropoda* species, *T. maeandralis* (which was not the case for Arizona and Utah). *Trichouropoda* mites carry fungi that differ from the mutualistic fungi carried by the mountain pine beetles, and its relationship with collapsing beetle population has been suggested [16].

4.2. Beta Diversity Patterns

As expected, populations relatively closer together shared more species between them than those geographically distant. High nestedness at these sites may indicate a low overall phoretic mite biodiversity [59]. Utah and Arizona (and likewise South Dakota and Alberta) showed nested patterns where mite species were likely removed rather than replaced. This could be due to the large latitudinal and subsequent climate shift between these populations resulting in loss of species with changes in local climate.

High rates of species turnover are explained by environmental differences causing some species to be replaced by others more suited to the new environment [60]. Although not specifically tested in this study, we hypothesize that environmental filtering, specifically temperature, serves as a likely explanation for differences in mite community assemblages across latitude. Beta diversity patterns driven by turnover are associated with temperature seasonality, which may be experienced across a latitudinal gradient [61]. Other complementary abiotic factors may contribute to high levels of turnover, particularly dispersal ability. Hill, et al. [62], showed that spatial patterns and environmental heterogeneity play a large role in explaining high levels of dissimilarity [in community assemblage] among sites in passively transported invertebrates and are better explained by increased turnover.

However, dispersal limitations are often defined for arthropods by temperature [63], which may be the ultimate driver of the latitudinal patterns.

Differences in host tree species may also help explain mite composition patterns across bark beetle populations. Our data analysis could not accommodate testing for differences in mite composition by host tree because host tree and location are confounding factors except for South Dakota and Colorado which shared the same host tree species (Table 1), and beetles collected via flight traps may come from a variety of tree species. Fungal symbiota associated with the mountain pine beetle induce different secondary chemical responses in different host tree species which may contribute to the tree's attractiveness as a host environment for the beetle and its associates [64,65]. In our study, although Colorado and South Dakota populations shared the same host (*Pinus ponderosa*), the overall mite compositions associated with these populations differed significantly. However, it should be noted that sampling years varied. However, these two populations did differ in their beetle population stage—Colorado was a collapsing population from an epidemic while the South Dakota population was increasing to epidemic status. Vissa et al. [35] reported vastly different mite loads associated with beetles attacking two different tree species within a single region in Portugal, suggesting that dominant host tree type can also affect beetle-associated mite abundances. Here we found that the population from limber pine (Utah) had beetles with the greatest mite loads. Tree genotype has also been shown to drive different defense production, insect preference, and long-term population dynamics [66,67].

Temperature affects patterns in species diversity across latitudinal gradients [68]. Our colder population of Alberta had the lowest mite diversity while the others, similarly warmer populations, had contrasting higher mite diversities. Evans, et al. [69] showed that a *Trichouropoda* sp. associated with the southern pine beetle had the highest reproductive success in warmer temperatures. This was reflected in our results where populations with higher average minimum and maximum temperatures also had more mites per beetle. Species of *Trichouropoda* were also absent in Alberta but prevalent in Colorado, Utah, and Arizona, where both temperature and precipitation were higher during development years; however, this may be confounded with the population stages in these populations.

4.3. Implications for Shifts in Functional Groups of Mites

The concept of environmental filtering in symbiotic mite communities associated with bark beetle systems is not well explored. Swenson et al. [70] hypothesized that abiotic (i.e., environmental) filtering not only alters species assemblages in plants, but also alters the functional diversity. Thus, in climatically 'extreme' regions, not only are there fewer species [70], but the functional groups can be altered as well [71]. While we were unable to identify specific shifts in the functional groups of mites, the observed compositional differences may contain vital information on the resulting functional shifts.

High taxonomic turnover does not directly translate to a significant functional turnover [72]. Fish faunas with high taxonomic beta diversity were associated with low functional beta diversity, suggesting that although species assemblages varied, they maintained shared functional attributes [72]. Our methods were unable to tease apart the functional diversity patterns associated with symbiotic mite populations or allow us to specifically identify which mite species are being replaced and by whom; however, we did observe some uniformity in the abundant functional groups across all populations. Fungivores (particularly *Tarsonemus* mites) were abundant in each epidemic population, followed by a generalist predator (*P. subcorticalis*), suggesting that these species likely affect beetle population dynamics (either by promoting the spread of fungi or by direct predation).

5. Conclusions

To our knowledge, this is the first study to examine and partition the differences in the beta diversity of symbiotic mite communities associated with an economically important North American bark beetle (the mountain pine beetle) across a wide range of its distribution; and may serve as a model system for estimating diversity patterns of symbiota associated with other major insect pests in pine forest systems. As anticipated, the composition of mite biodiversity associated with mountain pine

beetles varied geographically although no mite assemblage was entirely unique, and environmental differences driven by geography can best explain these differences. Our assessment of mite species composition patterns provides foundational information for further steps to identifying key species interactions shaping forest insect systems.

Author Contributions: Corresponding author S.V., R.W.H. and D.A.U. contributed significantly to the conceptualization, mite collection, sample processing and editorial process of this manuscript. D.A.U. additionally provided significant assistance in statistical analyses. Authors D.M., J.E.M., and B.A.M. conducted extensive sampling and mite data processing for South Dakota, Colorado, and Alberta respectively; and also actively contributed to the written materials in this manuscript. Author W.K. served as the primary identification resource for mite data in this manuscript and also contributed as a manuscript editor. Finally, author M.L.E. conceptualized experimental design of the Canadian component, provided some editorial help, and facilitated the Canadian research work by obtaining the necessary funding. All authors have reviewed this manuscript in its entirety and approve of its submission. All authors have read and agreed to the published version of the manuscript.

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Ips sexdentatus Mass-Trapping: Mitigation of Its Negative Effects on Saproxylic Beetles Larger Than the Target

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Abstract: Research Highlights: We investigated the negative effects of bark beetle mass trapping, especially non-target catches among the target's natural enemies. Slot traps modified with mesh screen and escape windows were tested to improve their selectiveness. Background and Objectives: Two of the main natural enemies of bark beetles, *Thanasimus formicarius* (L.) and *Temnoscheila caerulea* (Olivier), are frequently trapped in high numbers in *Ips sexdentatus* (Böerner) pheromone traps, along with other saproxylic insects; this may lead to much larger pest populations in the successive 4–20 beetle generations. From 2016–2019, during *I. sexdentatus* mass-trapping in a pine forest of Tuscany (Italy), non-target catches were tallied. Trap modifications were evaluated to mitigate non-target catches, especially those concerning bark beetles' natural enemies. Materials and Methods: A total of 25 bark-beetle slot traps were placed about 75 m apart in a pine stand infested by *I. sexdentatus*. Traps were baited with *I. sexdentatus* aggregation pheromone, whose main components are ipsenol, ipsdienol, and 2-methyl-3-buten-2-ol. Catches were collected every 10 days from March to December. In 2019, 13 traps were modified by applying a 6-mm mesh screen on top of the collection container and by providing three 60 mm × 8 mm escape windows immediately above the screen. These "modified traps" and their captures were considered separately from the 12 remaining "standard traps." All bark beetle species were recorded, as well as all beetle species > 8 mm. Results: Overall, target catches amounted for <10% of the total beetle catches. The most-collected species was the bark beetle *Orthotomicus erosus* Wollaston. Trap modification allowed the escape of larger species, resulting in the reduction of the average size of caught specimens. Even though non-target catches among predators were still high, the proportion of major predators (*T. formicarius* and *T. caerulea*) to bark beetles showed a statistically significant reduction of predator catches in modified traps, an encouraging outcome. Conclusions: Trap modifications may mitigate the problem of non-target catches during mass trapping, especially reducing catches of beetle species larger than the target. However, the key is to schedule mass trapping only during those seasons when the target adults are more active than the main predator adults, thus limiting their catches and, consequently, the negative effects on pest management and biodiversity.

Keywords: bark beetles; natural enemies; aggregation pheromones; pest management; Mediterranean pine forests

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1. Introduction

Due to favorable conditions, bark beetle outbreaks are alarmingly increasing, with a severe impact on European conifer forests [1–3]. In recent years, climate change has caused a higher frequency of extreme weather events, including windstorms and severe drought [1]. These disturbances increase the suitable breeding materials for bark beetles, such as stressed or uprooted trees [1]. Therefore, bark beetle population densities may

rise and, during epidemic outbreaks, aggressive bark beetle species are able to move from stressed and weakened trees to healthy ones; this behavior was confirmed in the six-toothed pine bark beetle, *Ips sexdentatus* (Böerner) (Coleoptera: Curculionidae: Scolytinae) [4].

I. sexdentatus occurs in pine forests throughout the Eurasian continent. It is generally considered a secondary pest [5] and less aggressive than the congeneric *I. typographus* (Linnaeus) [6], but sometimes it can become a more serious pest. For example, in Italy this bark beetle has recently been favored by the exotic scale *Matsucoccus feytaudi* Ducasse, which causes the decline of the maritime pine (*Pinus pinaster* Aiton) forests. In the suitable climatic conditions of Southern Europe, with large availability of suitable hosts (the stressed maritime pines), *I. sexdentatus* populations have rapidly increased. Under these conditions, their attacks also involve healthy trees of other pine species [7–9]. Coastal pine forests are particularly important in Italy, as they provide numerous goods and services, especially for recreational tourism [10]; thus, protecting these forests is essential to maintaining their social, environmental, and economic functions.

Only a few direct control measures are available to contain bark beetle populations: prompt cutting of infested trees, trap-trees, trap-logs, and pheromone-baited mass-trapping [11]. Though it is generally carried out in combination with the prompt cutting of infested trees, mass-trapping is essential, particularly when phytosanitary felling cannot be swiftly carried out [12]. Several studies report the efficacy of mass-trapping in bark beetle control, alone or in combination with other measures [13–17]. However, its effectiveness is still controversial, as it is not easy to prove in terms of real tree mortality reduction, particularly since other biotic and abiotic factors can affect the results [16,17].

Mass-trapping, however, may lead to significant negative effects. Indeed, many studies show its negative impact on non-target insect species, which may also be lured by the pheromone baits [18–20]. Among non-target catches, predators are of particular concern [18,19]. Bark beetle natural enemies track their prey using aggregation pheromones as kairomones; thus, they too may be attracted by pheromone traps [21,22]. Two of the main natural enemies of bark beetles, *Thanasimus formicarius* (L.) (Coleoptera: Cleridae) and *Temnoscheila caerulea* (Olivier) (Coleoptera: Trogossitidae), are frequently captured in high numbers in *I. sexdentatus* pheromone traps [9,19,20,23,24]. Furthermore, other saproxylic insects that exploit the same pheromones to locate suitable host plants (typically weakened by the target bark beetle species) may also be caught during mass-trapping programs [19]. Thus, non-target catches may negatively affect the natural regulation of bark beetle populations, as well as the conservation of other saproxylic species [19].

Several studies focus on the development of more selective trapping techniques to mitigate these negative effects [8,9,20]. For example, mass-trapping selectiveness could be improved by finding the best component blends. Although bark beetles' natural enemies often use their prey's pheromones to track them [22], prey and predators may have different preferences in terms of optical isomers or other specific pheromone components [25–27] that can be exploited to reduce predator catches [18]. In regards to *I. sexdentatus*, Etxebeste et al. [8] determined that the composition of non-target catches associated with this species varied greatly with different pheromone blends. A reduction in mass-trapping negative effects can also be achieved by choosing the right type of trap. Martin et al. [9], for instance, found that slot traps are more selective than multi-funnel traps, showing fewer predator catches, particularly favoring the escape of *T. formicarius*. Finally, traps can be modified to prevent non-target beetles from entering, especially those bark beetle predators that are larger than their prey. Martin et al. [9] and Ross and Daterman [28] assessed how modifying multiple-funnel traps and slot traps by adding a 6-mm mesh screen and by opening escape windows may help to limit predator catches. In this study, non-target beetle catches were recorded during a mass-trapping program in a pine forest in Tuscany (Italy); trap modifications similar to those used by Martín et al. [9] were evaluated.

2. Materials and Methods

2.1. Study Area and Sampling Design

The present study was carried out during 2016–2019 in a Tuscan coastal pine stand, approximately 70 hectares, between Marina di Grosseto and Principina a Mare, in the Province of Grosseto (Italy) (42°42′17.22″ N, 10°59′47.59″ E). This private forest plot was mainly composed of *P. pinaster*, with a small amount of stone pine (*Pinus pinea* L.); here, since 2006, the former species has been gradually infested by *M. feytaudi*, which has caused serious damages and has stressed the trees. Since 2014, the huge availability of weakened pines caused a high increase in the population density of *I. sexdentatus*. Another aggressive bark beetle species, *Tomicus destruens* (Wollaston), was also present in this stand and contributed to pine mortality. In addition, the bark beetle *Orthotomicus erosus* Wollaston was abundant, although it was mostly found on pines already infested by *I. sexdentatus* or *T. destruens*, and/or highly stressed by abiotic factors. This area has a high touristic value due to its many campsites; therefore, to reduce the population density of *I. sexdentatus* and the number of attacked pines, prompt cutting of infested trees, as well as mass-trapping were carried out. On 28 April 2016, a total of 25 Super Forest bark beetle slot traps (Serbios s.r.l., Badia Polesine, Rovigo, Italy) were placed in the pine stand along the south-western borders of the forest plot, spacing each trap by about 75 m (Figure 1). This pattern addressed the higher *I. sexdentatus* infestation in the pine stand closer to the coastline—and outside our study area—where no control measures were carried out. Super Forest traps are a variant of the common Theysohn design, with fewer, longer slots, but with the same amount of slot rows (seven) (Figure 2A). Traps were baited with the commercial blend of the *I. sexdentatus* aggregation pheromone manufactured by Serbios s.r.l., whose main compounds are ipsenol, ipsdienol, and 2-methyl-3-buten-2-ol. Catches were collected every 10 days from March (except in 2016 when traps were installed in April) to December. Bait was renewed every 30–50 days, according to the dispensers' lifespan (as indicated by the manufacturer) and to the weather conditions in the study area, since warmer climates of Tuscany deplete these dispensers considerably faster during summer months [29]. Thus, sticking to manufacturer instructions would lead to errors in interpreting flight patterns. In order to ensure a continuous presence of adequately baited traps in the field, a baiting rotation system based on alternating pairs was set up: group A consisted of six pairs plus the 25th trap; group B included the remaining six pairs (Figure 1). The two groups were baited 20 days apart, always following the same renewal schedule based on the dispensers' lifespan and on weather conditions.

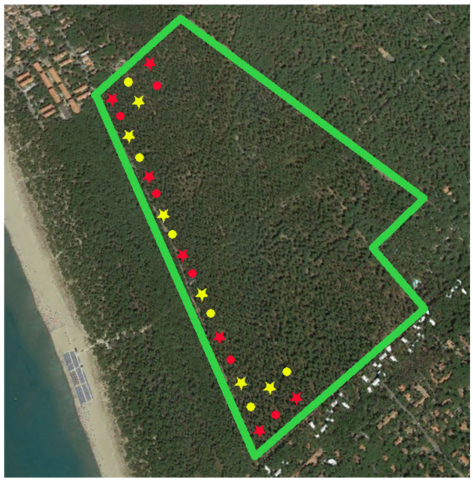

Figure 1. Study area with trap pattern as applied in 2019. Part of the coastal pine stands in the Municipality of Grosseto (Tuscany, Italy) is shown; the green line delimits the study area's borders. Bait rotation alternated groups A (in red) and B (in yellow; symbols indicate modified (stars) and standard (circles) traps.

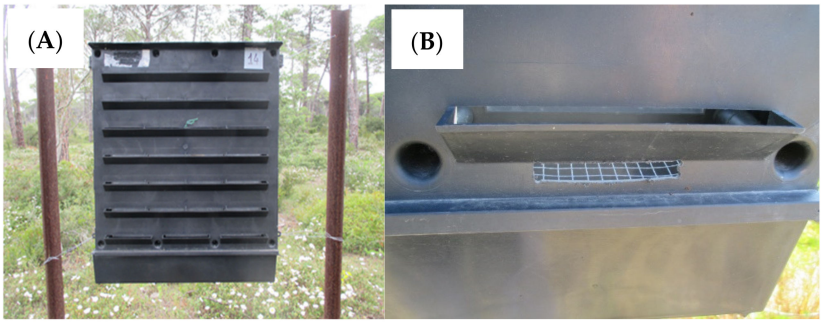

Figure 2. Standard trap without modifications (**A**) and detail of a modified trap (**B**): through one of the three escape windows the 6mm mesh screen can be seen inside.

Besides the target beetle, in the first three years of the study (2016–2018), special attention was reserved for some of the other beetles trapped: the major non-target bark beetles and the two major bark beetle predators, *T. formicarius* and *T. caerulea*. In 2019, additional non-target beetles were considered, we grouped them into two categories according to their mean length: (1) all bark beetles; and (2) all beetles on average > 8 mm, among which *T. caerulea* and *T. formicarius* were grouped. In this year, 13 traps were modified by applying a 6-mm mesh screen on top of the collection container as in Martìn et al. [9], and by providing three additional 60 mm × 8 mm escape windows immediately above the mesh screen (one central window on one side and two lateral windows on the other side) (Figure 2B). These traps were called "modified traps" and their captures were considered separately than those from the "standard traps," which were not modified. "Modified traps" were evenly distributed in the bait renewal rotation by alternating them with "standard traps." Finally, a random sub-sample of 30 specimens per each species (when possible) per each kind of trap (modified or standard) was measured, recording their body length.

2.2. Statistical Analysis

The analysis of variance (ANOVA) for repeated measures (catches in the same trap on different dates were considered repeated measures) was used to compare catches in standard traps and modified traps of (1) *I. sexdentatus;* (2) non-target bark beetles (all species together); (3) all bark beetles together; (4) beetles > 8 mm; and finally (5) species of this last category, with enough catches (more than 30), were analyzed separately. All the data analyzed with ANOVA were log transformed. Simpson's index was used to compare species diversity in the two kinds of traps employed in 2019. As regards the two main predators, *T. caerulea* and *T. formicarius,* the 2019 mean catches, separated according to the kind of trap, were then compared with ANOVA with those of previous years when the traps had not yet been modified but were in the same positions. Pearson correlation was applied to assess the correlation between the main predators and bark beetle catches. In addition, the percentages of these two predators were compared using the chi square test, taking into account the total bark beetles and predators caught by standard and modified traps. The size of beetles > 8 mm trapped in 2019 in modified traps were compared with the size of those from standard traps with ANOVA; the same test was separately carried out for those species with enough catches (more than 30) in each kind of trap.

3. Results

3.1. Catches in I. sexdentatus Pheromone Traps

During the first three years of the study (2016–2018), 42,823 *I. sexdentatus* adults were caught in pheromone traps (Table 1). However, these were not the most numerous catches; in fact, *I. sexdentatus* was less than a tenth compared to the non-target beetle species. The most-trapped beetles (469,545 specimens) were non-target bark beetles (Table 1): *O. erosus*, *Hylurgus ligniperda* (Fabr.), and *T. destruens*. While only few *T. destruens* specimens were caught (62 in total in the three years), the other two species were trapped frequently and in great numbers. More specifically, *O. erosus* was the most-caught species in the first

three-year period, with 452,756 individuals, which represented almost 95% of all beetles snared. Among the non-target beetles were two of the bark beetles' major predators: *T. caerulea* and *T. formicarius*; each year more than 2000 specimens were caught, with a total of 7500 in the three years. The predator/prey (*I. sexdentatus*) ratios for *T. formicarius* were 0.13, 0.12 and 0.08 in 2016, 2017, and 2018 respectively, while for *T. caerulea* they were 0.09, 0.05, 0.07. Finally, other beetles, larger than bark beetles, were also captured, but these were neither identified nor counted in the first three years of the study.

Table 1. Total number of beetles trapped with the *Ips sexdentatus* aggregation pheromone during the 2016–2018 period in the study site (Municipality of Grosseto, Tuscany, Italy).

Year	*I. sexdentatus*	Non-Target Beetles				Predator/Prey Ratios	
		Other Bark Beetles	*T. formicarius*	*T. caerulea*	Total	*T. formicarius*	*T. caerulea*
2016	11,042	231,284	1447	1013	233,744	0.13	0.09
2017	12,774	120,574	1587	621	122,782	0.12	0.05
2018	19,007	117,687	1450	1382	120,519	0.08	0.07
Total	42,823	469,545	4484	3016	477,045		

During 2019, the last year of the study, 182,641 specimens were caught across both types of traps; this tally takes into account only the species that had been counted during the first three-year period (*I. sexdentatus*, *T. formicarius, T. caerulea*, and other bark beetles). Again, the great majority were non-target bark beetles (170,219) (Table 2): about 98% of these trapped beetles were *O. erosus*, which was the most captured species, with 167,773 individuals; as regards the other bark beetles, 1855 adults of *H. ligniperda* and one *T. destruens* were recorded. Besides these main species, other minor bark beetles were recorded with a total of 590 specimens: *Carphoborus pini* Eichhoff (33), *Coccotrypes dactyliperda* Fabricius (8), *Crypturgus mediterraneus* Eichhoff (31), *Crypturgus pusillus* Gyllenhal (143), *Hylurgus micklitzi* Wachtl (97), *Pityogenes bidentatus* Herbst (251), *Pityophthorus pubescens* Marsham (21), and *Xylocleptes biuncus* Reitter (6). Again, the two most important predator species, *T. caerulea* (2409) and *T. formicarius* (925), were trapped. The predator/prey ratios calculated on standard traps were 0.13 for *T. formicarius* and 0.33 for *T. caerulea.* In 2019, however, other large non-target beetles were also taken into account, for a total of 1,662 recorded specimens. This group included specimens from five different families: Melolonthidae, with one species, *Amadotrogus grassii* (Mainardi) (112); Elateridae, with two species, *Lacon punctatus* (Herbst) (191) and *Melanotus (Melanotus) crassicollis* (Erichson) (342); Buprestidae, with two species, *Buprestis (Buprestis) novemmaculata* Linnaeus (92), and *Chalcophora detrita* (Klug) (5); Cerambycidae, with three species *Monochamus galloprovincialis* (Olivier) (8), *Acanthocinus griseus* (Fabricius) (838), *Oxypleurus nodieri* Mulsant (3), and finally Curculionidae not in the sub-family of Scolytinae, with one species, *Brachyderes (Brachyderes) incanus* (Linnaeus) (71).

Table 2. Number of beetles trapped with the *Ips sexdentatus* aggregation pheromone during 2019 in the study site (Municipality of Grosseto, Tuscany, Italy) with standard and modified slot traps.

Type of Trap	N. of Traps	*I. sexdentatus*	Non-Target Beetles				
			Other Bark Beetles	Larger Beetles			Total
				T. formicarius	*T. caerulea*	Others	
Standard	12	3982	53,622	505	1333	1392	56,852
Modified	13	5106	116,597	420	1076	270	118,362
Total	25	9088	170,219	925	2409	1662	175,214

Catches of target and non-target beetles were differently distributed during the year. *I. sexdentatus* adults in 2016, 2017, and 2018 had three peaks in activity: spring (March/April) (in 2016 this first peak was not noted because traps were deployed later),

summer (July/August), and particularly autumn (October/November). On the contrary, in 2019 the autumnal catch showed a remarkable drop (Figure 3). The two main *I. sexdentatus* predators (*T. caerulea* and *T. formicarius*), instead, were more active in spring, particularly in April, and summer, while in autumn only a very low number of adults were trapped. In fact, in the spring/summer period during the years 2016/2018, the mean predator/prey ratio was 0.43, while during the autumn it was 0.03. This activity distribution was also true for all other non-target beetles trapped. In fact, during the study period non-target bark beetles were caught particularly during the spring/summer period, with a non-target/target bark beetle ratio of 21, while during autumn these non-target catches were much lower, plummeting to a ratio of 0.51. Accordingly, beetles > 8 mm and non-target bark beetles were caught mainly during the 2019 spring/summer period, while very low catches occurred in the autumn (Figure 4).

3.2. Standard Traps Versus Modified Traps

The mean number of *I. sexdentatus* catches in standard traps was not different when compared to the average catches of *I. sexdentatus* in modified traps (ANOVA, $df = 1$, $F = 0.528$, $p = 0.475$). The mean number of non-target bark beetle catches did not differ between the two types of traps (ANOVA, $df = 1$, $F = 2.325$, $p = 0.141$). On the contrary, the mean number of all non-target beetles > 8 mm was lower in the modified traps (ANOVA, $df = 1$, $F = 27.744$, $p < 0.001$). In fact, the percentage of all non-target beetles > 8 mm trapped by the standard traps was 44.79% of the total (7,212 specimens, not including the non-target bark beetles) (Figure 5A), while in the modified ones this percentage dropped to 25.70% of the total (6871 specimens) (Figure 5B). In addition, the modified traps had a lower species diversity (Simpson's index = 0.56) compared to the standard ones (Simpson's index = 0.75). For 10 out of the 11 species included in the "non-target beetles > 8 mm", a decrease in catches was recorded with the modified traps, although this decrease was statistically significant only for seven species: *L. punctatus* (ANOVA, $df = 1$, $F = 34.181$, $p < 0.001$), *M. crassicollis* (ANOVA, $df = 1$, $F = 30.968$, $p < 0.001$), *B. novemmaculata* (ANOVA, $df = 1$, $F = 14.493$, $p < 0.01$), *A. griseus* (ANOVA, $df = 1$, $F = 18.985$, $p < 0.001$), and *B. incanus* (ANOVA, $df = 1$, $F = 6.163$, $p < 0.05$); both *C. detrita* and *M. galloprovincialis* were caught only in standard traps.

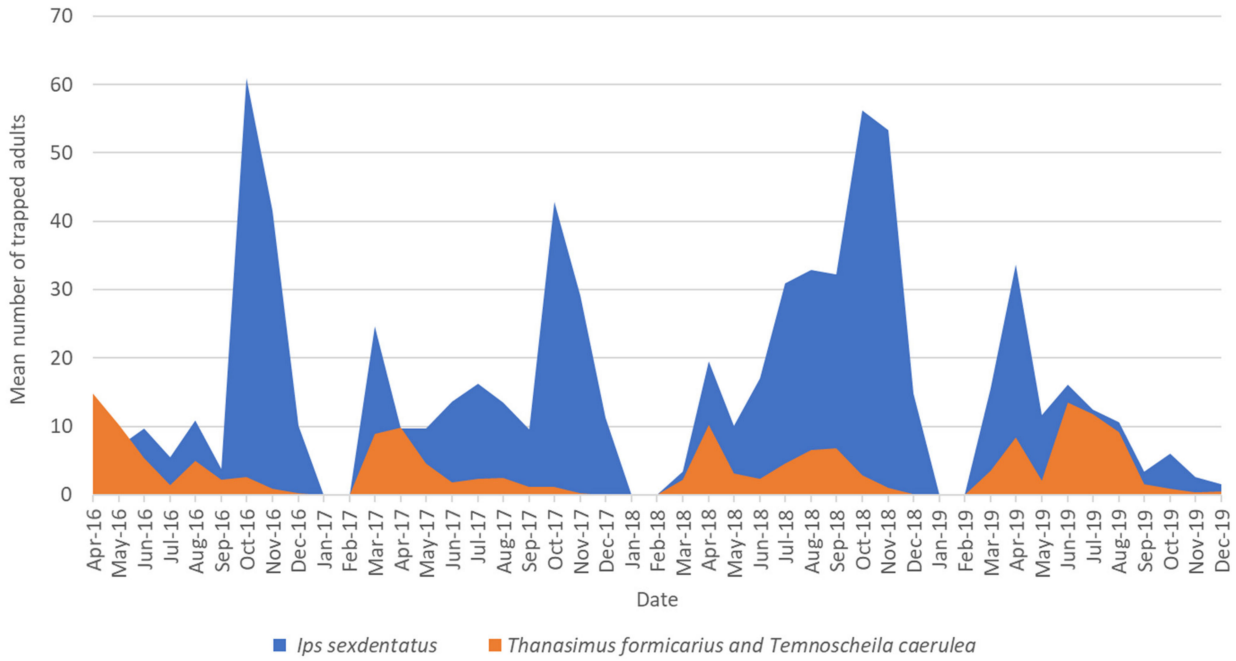

Figure 3. Mean number of trapped adults of *Ips sexdentatus* and its main predators (*Thanasimus formicarius* and *Temnoscheila caerulea*) in the study site (Municipality of Grosseto, Tuscany, Italy) using standard slot traps, from 2016 to 2019.

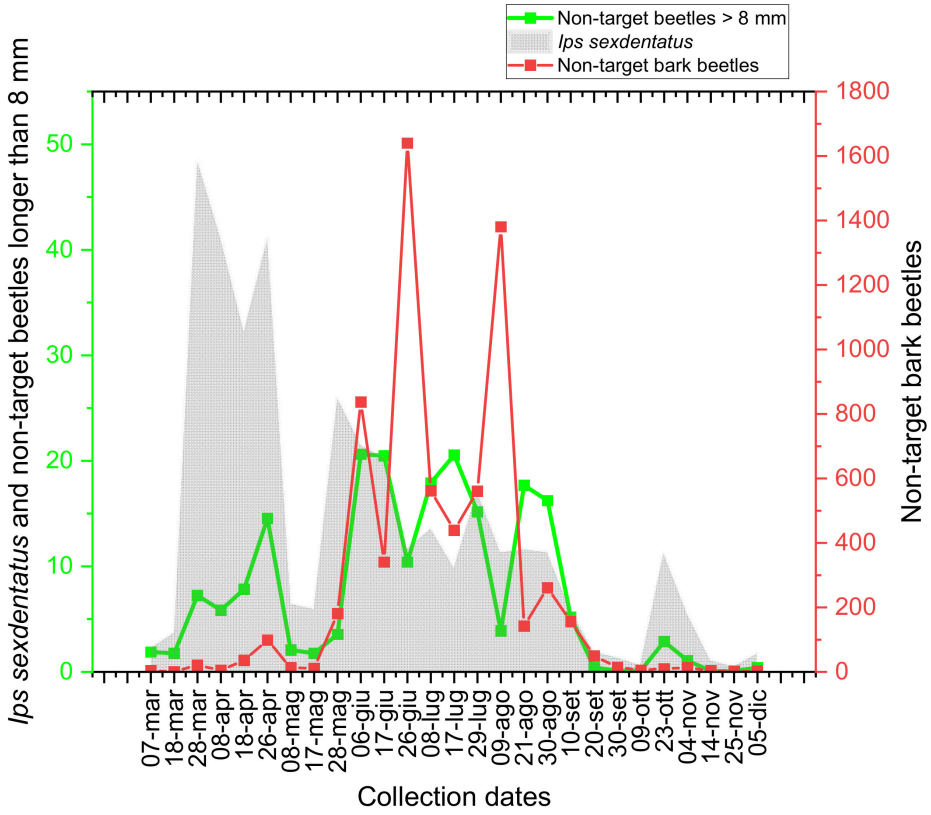

Figure 4. Mean number (standard and modified traps together) of trapped adults of *Ips sexdentatus*, non-target bark beetles, and non-target beetles > 8 mm in the study site (Municipality of Grosseto, Tuscany, Italy) during 2019.

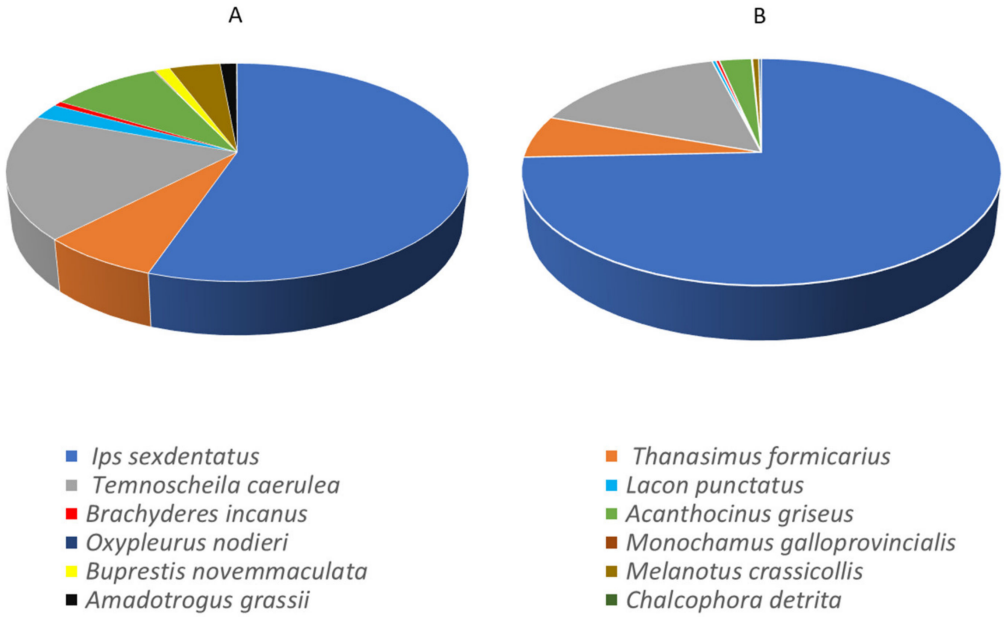

Figure 5. Percentages of *I. sexdentatus* and non-target beetles > 8 mm caught in standard traps (**A**) and modified traps (**B**) baited with the *I. sexdentatus* aggregation pheromone in the study site (Municipality of Grosseto, Tuscany, Italy) during 2019.

The two main bark beetle predators were the most-caught species in this category (non-target beetles > 8 mm), though the efficacy of modified traps was less evident. Indeed, catches in 2019 in the two types of traps were not statistically different (ANOVA, df = 1,

$F = 3.882$, $p = 0.061$). Furthermore, the predator/prey ratios for *T. formicarius* in standard and modified traps were 0.13 and 0.08, respectively, while for *T. caerulea* the predator/prey ratios were 0.33 and 0.21. Predator (*T. formicarius* and *T. caerulea*) catches were positively correlated to bark beetle (*I. sexdentatus* and non-target bark beetles) catches (Pearson correlation, $r = 0.404$, $p < 0.01$), while no differences in all bark beetle captures emerged between standard and modified traps (ANOVA, $df = 1$, $F = 2.314$, $p = 0.142$). However, the percentage of predators in modified traps (1.22% of the total bark beetles and predators) was significantly lower than that in standard traps (3.10%) (Chi square test, $\chi^2 = 790.909$, $p < 0.001$). In addition, considering standard traps throughout the whole study period, the mean number of catches for 2019 were significantly higher than the previous three years' (GLM, Wald Chi-Square = 46,295, $p < 0.001$). In modified traps (in 2016–2018 the same traps, located in the same sites, but not yet modified, were used) there was not a statistically significant difference (Figure 6).

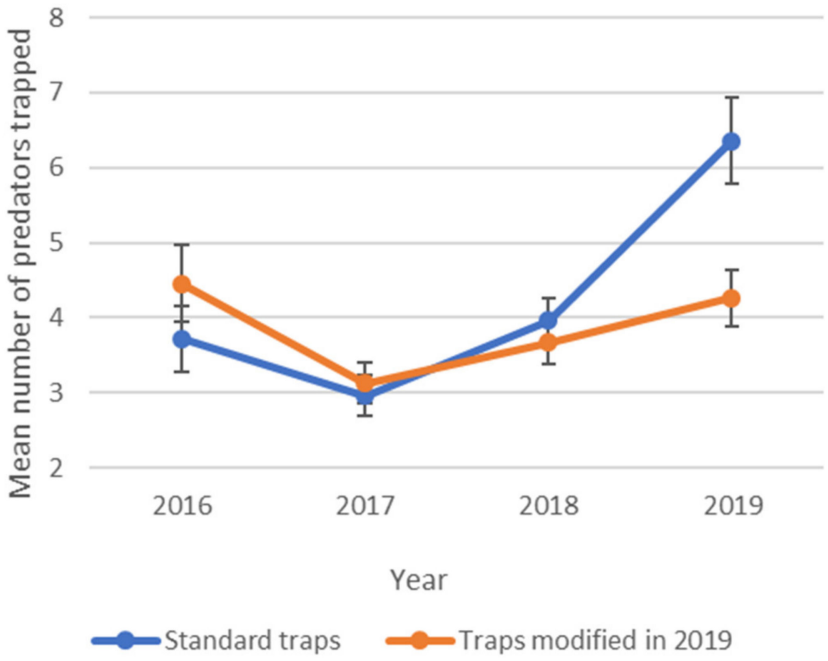

Figure 6. Mean catches per trap per control date of *T. caerulea* and *T. formicarius* in standard and modified traps in the study site (Municipality of Grosseto, Tuscany, Italy). Trap modifications were not present until 2019; however, trap position never changed during the four-year study. Bars indicate standard error.

Trap modification favored the larger species (Figure 7); in fact, beetles captured in standard traps had a higher mean size (14.22 mm) compared to those in the modified traps (12.14 mm) (ANOVA, $df = 1$, $F = 15.014$, $p < 0.001$). However, *T. caerulea* did not benefit from trap modification despite its size (Figure 7). Comparing the size of trapped specimens of each species in standard and modified traps, a general significant difference was observed: for each species, specimens captured by modified traps were smaller in size (ANOVA, $df = 7$, $F = 14.972$, $p < 0.05$). However, only in the case of *A. griseus* did this difference end up being statistically significant (Wald Chi square = 8.878, $p < 0.01$).

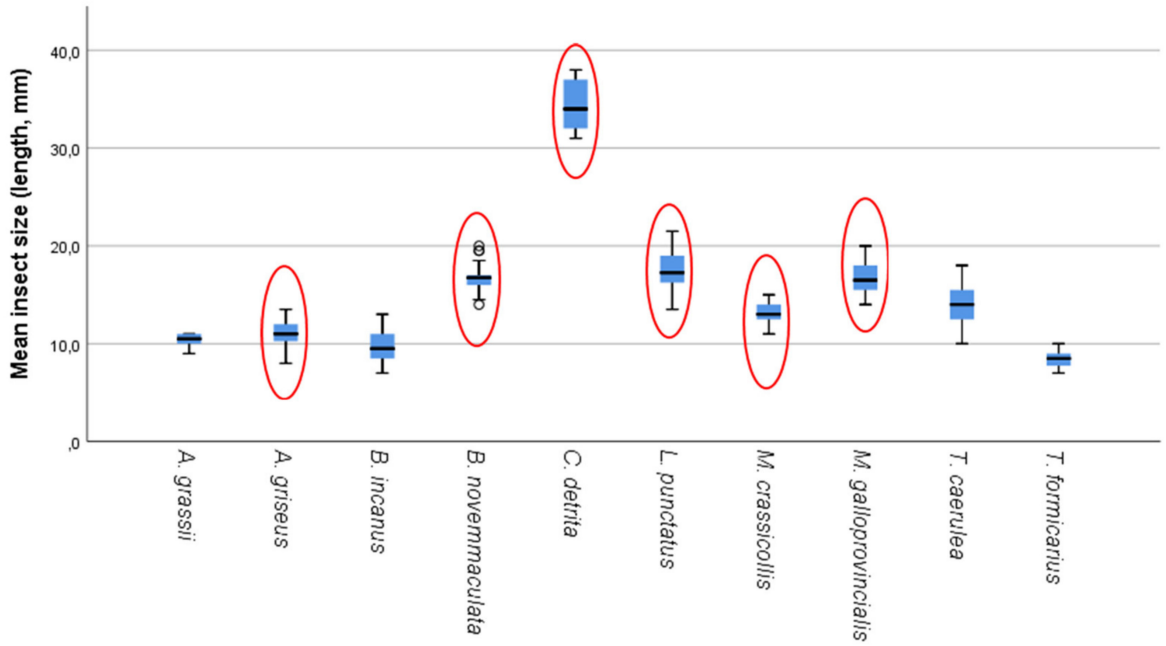

Figure 7. Body length (mm) of the trapped insect species. Red ovals represent species with a subsequent significant catch reduction in modified traps. Bars indicate standard error.

4. Discussion

As already assessed in other studies [19,20] *I. sexdentatus* aggregation pheromone attracts many other saproxylic beetles, among which are several bark beetles receptive to the same pheromonal components [30]. In our study, the most-trapped beetles were *O. erosus* and *H. ligniperda*, both of which had been shown to be captured when the *I. sexdentatus* pheromone was used as bait [8,9,20]. Furthermore, Serez [31] demonstrated how commercially available pheromones for *I. sexdentatus* can be used to attract *O. erosus*. The numbers of specimens trapped in our studies were in agreement with the observations of Serez [31] in Turkish areas infested by *O. erosus*; moreover, *O. erosus* has been recently confirmed as an increasingly dangerous pest in Mediterranean pine forests [32]. However, in our study area, *O. erosus* seems less aggressive. In fact, despite the abundant catches and the fact that *O. erosus* is able to kill whole trees, in our study only small pine branches were attacked, while no pines showed large areas of the trunk or of the main branches infested by this species. Interestingly, *T. destruens*, the other bark beetle which causes serious damage in the study area's pine forest, was trapped only in low numbers (63 specimens total over the four years).

Other saproxylic beetles trapped in *I. sexdentatus* traps were bark beetles' predators, which can be kairomonally attracted by the bait intended for their prey, as stated by other studies [8,19]. In fact, these species can use aggregation pheromones, together with volatiles, to locate their bark beetle prey [18]. In our study, four predators, *T. formicarius*, *T. caerulea*, *L. punctatus*, and *M. crassicollis* were trapped. The first three species had already been collected in traps baited with pheromone blends of *I. sexdentatus* in other studies [8,19,20,33]. *T. formicarius* and *T. caerulea* are important bark beetle natural enemies that contribute to their population control by preying on their larvae under the bark [9]; thus, catching these predators during mass-trapping constitutes a problem for bark beetle management. Bark beetle control methods must be sustainable, impacting only (or almost only) the target species. In this study, the predator/prey ratios were considerably high compared to other studies [34]. This could cause a boomerang effect, since it was estimated that a reduction of predator densities could lead, within 4–20 bark-beetle generations, to a doubling of the pest population, prolonging bark beetle outbreaks [35].

Yet another important consideration is that other saproxylic beetles, including vulnerable species, also locate their plant hosts by exploiting bark beetle pheromones [36]. In our study's catches, almost all beetles > 8 mm were saproxylic. Among these, we found several vulnerable species: Melolonthidae *A. grassii* is considered rare; one species of Buprestidae, *C. detrita*, is categorized as "endangered" in the European red list of the Italian saproxylic beetles; and one, Cerambycidae *O. nodieri*, which is "near threatened" and is also considered a facultative predator of bark beetles [37]; all other species were of lesser concern [38]. Many of the captured species had already been caught in similar studies with traps baited with conifer monoterpenes and/or bark beetle pheromones containing ipsenol and 2-methyl-3-buten-2-ol, or ipsdienol [8,9,19,20,36]. Therefore, besides the problem of predator decline, mass-trapping might reduce the overall biodiversity of saproxylic insects, with even higher impacts on endangered species.

The hypothesis that low *I. sexdentatus* populations may negatively affect target/non-target ratios in traps was proposed by Panzavolta et al. [20], who, in their two-year study, observed low population densities and low percentages of the target bark beetle in traps (ranging between 2% and 35%). In the present study, higher averages of *I. sexdentatus* specimens were caught by pheromone traps, and more severe damage to the pine forest was observed, both indicative signs of a larger population density. In fact, considering only the study period of Panzavolta et al. [20] (from June to the end of *I. sexdentatus* flight), we trapped a mean of about 492 specimens per trap per year, while in their study only 44.3 and 3.7 mean catches were obtained. However, in the current study, higher *I. sexdentatus* catches corresponded to higher non-target catches. In fact, during the four years, *I. sexdentatus* accounted for only 8% of the total specimens collected in the pheromone traps. Therefore, the low specificity of the pheromone blend used is confirmed, regardless of *I. sexdentatus* population level.

This study confirmed the effectiveness of the trap modifications proposed by Martín et al. [9] if all saproxylic beetles > 8 mm were considered together, although modifications applied are still not enough to negate non-target catches of some species taken individually. More specifically, results were not satisfactory for all natural enemies: only for *L. punctatus* and *M. crassicollis* was the catch reduction in modified traps significant in comparison with standard traps, while the two main predators *T. formicarius* and *T. caerulea* were caught in high numbers in modified traps as well, in agreement with Martin et al. [9]. Traps in our study were also provided with escape windows, not present in the slot traps modified by Martin et al. [9], but this did not lead to fewer catches of this non-target species when compared with their study, as we recorded a ratio of one trapped *T. caerulea* for every five *I. sexdentatus*. However, we found that catches of *T. formicarius* and *T. caerulea* were positively correlated with those of bark beetles (all trapped species). Probably, as suggested by Bakke and Kvamme [39], predators may be also attracted by already caught bark beetles inside the trap, which may release pheromones with their frass. In fact, some bark beetle species are able to produce pheromones after emergence without feeding, as demonstrated for *I. paraconfusus* Lanier and *I. pini* (Say) [40]. In our study, traps with high bark beetle catches resulted uniformly distributed between modified and standard traps (differences in mean catches were not statistically significant). However, if we consider predators in proportion to bark beetles, a statistically significant reduction of predator catches emerged in modified traps, showing an encouraging outcome for trap modifications.

Another important strategy to reduce the impact of mass-trapping on non-target beetle species is to adjust the period of traps set in the field, exploiting the different seasonal abundance of target and non-target beetles. Overall, we observed the lowest non-target catches during autumn, both as regards beetles > 8 mm long and non-target bark beetles (Figure 4). This is in agreement with other studies that confirmed the lowest flight activity of bark beetles' predators during autumn [20,27], or at least their higher activity during spring [18,25]. The highest catches of *I. sexdentatus* in our study area occurred, on the contrary, in autumn, at least in the 2016–2018 period. This was not confirmed in 2019; however, this was likely due to the phytosanitary felling of numerous attacked pines in the

study area which was carried out at the end of 2018. As a result, *I. sexdentatus* population density was reduced, and the study area traits were also modified significantly.

5. Conclusions

The results of our study give emphasis to the low specificity of *I. sexdentatus* aggregation pheromone, as well as its negative effects if used in mass-trapping programs. Slot trap modifications may mitigate the negative effects of mass-trapping on non-target saproxylic beetles, particularly on larger beetles > 8 mm; however, catches of the main bark beetles' predators, *T. formicarius* and *T. caerulea*, still remain too high, a negative effect which could escalate in successive generations, leading to a doubling of bark beetle numbers and prolonging outbreaks. Thus, it would be particularly advantageous to exploit the different seasonal abundance of target and non-target beetles particularly in mild areas where the target species' main flight peak occurs in autumn, when other saproxylic species adults are generally less abundant. Therefore, to mitigate the negative effects of *I. sexdentatus* management on the forest, especially to reduce non-target catches, we recommend choosing slot traps modified with a 6-mm mesh screen and three additional escape windows, as well as concentrating mass-trapping in the autumn.

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A Review of Forest Management Effects on Terrestrial Leaf Litter Inhabiting Arthropods

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Abstract: Leaf litter arthropods are some of the most abundant and diverse communities in forests and provide myriad ecosystem services from decomposition and mineralization to pollination and predation. They are important to forest health and management and, in turn, are affected by how we manage our forests. Various forest management techniques such as clear cutting, burning, and chemical control of invasive species all have differing effects on ground dwelling arthropods and, despite their importance, a review of the literature on these effects does not currently exist. My objective with this paper is to review the effects of different types of forest management on arthropods in leaf litter systems.

Keywords: leaf litter; forest management; arthropods

1. Introduction

Leaf litter-dwelling arthropods (Table 1) are some of the most prominent components of ecosystems in terms of both biomass and diversity [1,2]. They are critical parts of forested ecosystems [3–5] due to the wide breadth of ecosystem services they provide. Namely, litter-inhabiting arthropods act as predators [6–10], decomposers of organic matter [11,12] and serve to mineralize certain nutrients into the soil [13]. Feeding by these arthropods stimulates microorganism growth [14] and also adds fecal matter to the substrate [15]. Some arthropods that inhabit leaf litter, like termites and ants, are even considered "ecosystem engineers" due to their important effects on the ecosystem [16–18].

Table 1. Arthropods commonly encountered in forest leaf litter.

Class	Order	Family
Arachnida	Acari	Ixodidae
		Oribatida
	Araneae	Gnaphosidae
		Lycosidae
		Salticidae
		Theridiidae
	Opiliones	
	Pseudoscorpionida	
Crustacea	Amphipoda	
	Decapoda	
	Isopoda	
Chilopoda		
Diplopoda		
Hexapoda	Coleoptera	Cantharidae
		Carabidae
		Chrysomelidae
		Curculionidae

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Table 1. *Count.*

Class	Order	Family
		Elateridae
		Histerida
		Psephalidae
		Ptilodatilidae
		Scydmaenidae
		Staphylinidae
		Tenebrionidae
	Collembola	
	Dermaptera	
	Diplura	
	Diptera	Muscidae
		Simuliidae
	Orthoptera	Gryllidae
	Hemiptera	Cercopidae
		Cicadellidae
		Pentatomidae
	Hymenoptera	Formicidae
		Microhymenoptera
	Lepidoptera	Geomteridae
		Noctuidae
	Thysanoptera	

Due to their phylogenetic and functional diversity, leaf litter arthropods can have significant impacts on other trophic levels as well [19]. For example, leaf litter composition has been shown to alter the alkaloid profiles of litter-dwelling ants which then impacts the chemical defenses of poison frogs which feed on these ants [20]. Leaf litter-dwelling arthropods have also been developed as bioindicators of ecosystem and soil health [5,21–24]. Some ground dwelling taxa, like beetles (Coleoptera) and spiders (Araneae), are very sensitive to habitat modifications [25,26] and forest management both directly and indirectly impacts these groups more than others.

Forest management impacts on arthropod groups also differs according to place of origin. Most studies on this subject have been done in tropical systems [27–30], however, studies from Europe [23] and North America [31,32] also exist and shed light on the different impacts of forest management in temperate forests. For instance, arthropods in tropical systems tend to decline in relation to forest harvests and altered land use—for example, urbanization—while temperate systems see increases in arthropod diversity in these situations [33]. These effects are taxa dependent, however. Acari, especially oribatid mites, respond positively to a moderate degree of urbanization while Collembola [34] and Diplopoda [35] respond negatively to all levels of urbanization. Responses may also be specific to plant species, as others have found differences in diversity and abundance of Collembola by leaf litter type, regardless of management or land-use practice [36]. High diversity in places like urban forests does not necessarily translate to high diversity in surrounding areas. Man-made barriers like roads and sidewalks form a substantial disruption to the dispersal of arthropods in these systems, so diversity in these scenarios is likely patchy and inconsistent [37].

While forest management type and regionality affects the intensity and directionality of impacts to arthropods, some general trends do exist. For example, contrary to some beliefs, plantation forestry does support leaf litter arthropod biodiversity [38,39], and arthropods are resilient to both site preparation [40] and harvesting practices [41]. However, many of these interactions are taxa dependent, with mobile species being affected differently than sessile species [42]. For example, community composition of winter-active spiders in plantation forests differed based on plantation species and whether it was native to the region [43]. My objectives with this paper are to review the effects of different types

of forest management on leaf litter arthropods and to outline gaps in knowledge with regards to ground-dwelling arthropods in forested systems.

To assess the available literature on forest management practices and their effects on leaf litter-dwelling arthropods, I used Google Scholar and ISI Web of Knowledge to search for "leaf litter", "arthropods", and "forest management" as well as combinations of these terms. Articles were considered acceptable if they quantified leaf litter arthropods and detailed the type of forest management used (e.g., clear-cut, slash management). Articles that investigated the impacts of arthropods on forest productivity, as well as articles evaluating soil arthropods, were not included in this review.

2. Effects of Forest Harvesting

Forest management is frequently done in such a way as to mimic natural disturbances like fire, insect outbreaks, or hurricanes [44]. Natural disturbances may occur frequently and create partial-stand clearings, resulting in a mosaic of stand types and ages. Natural disturbances may also occur as whole-stand replacing events [45]. Forest management affects microclimates of the forest floor through altering light availability, wind, temperature, and moisture, thereby indirectly affecting the arthropods that inhabit the leaf litter [46].

Harvest methods which mimic large disturbances (e.g., clear-cuts, gap cuts) create significant amounts of edge habitats which tend to have more extreme microclimates [47] and support a lower abundance of leaf litter arthropods [48]. Clear cuts also increase the temperature extremes of the forest floor and remove organic matter inputs, both of which negatively impact leaf litter arthropods [49]. Compared to unforested habitats, however, forests may have lower diversity [50] and more clustered phylogenetic relationships of leaf litter communities [32]. Single-tree selection also has significant impacts on some leaf litter dwelling arthropods and has been found to shift carabid communities towards those more associated with grassland habitats, even without substantial tree removal [51].

In addition, soil compaction from logging equipment results in a reduction in arthropod diversity [52]. In intensively managed forests like oil palm plantations, abundance and diversity of leaf litter arthropods was significantly lower compared to untouched forests [53]. Forest harvesting may also have positive effects on leaf litter arthropod communities, however. Ground-dwelling arthropods are more abundant near course woody debris that is left over from harvest operations [31,54]. This increase in course woody debris may explain observed increases in functional diversity of ants, specifically [55].

While some information is known on the impacts of forest management on leaf litter arthropods, little is known on how specific operations (e.g., single-tree selection, gap harvests) affects individual groups or their interactions. There is also little known about how arthropod communities respond to forest harvests over time or how their responses compare to that of natural disturbances.

3. Effects of Forest Restoration

Rainforest restoration presents unique forest management challenges with a variety of techniques available [56,57], and some ground-dwelling groups, like ants, have been developed as biological indicators of the progress of restoration efforts [29,30]. While restoration methods undoubtedly affect leaf litter arthropods, little has been done to examine the effects of these efforts on said communities.

In general, decomposers are less abundant in anthropogenic sites that have been cleared for pasture or other agricultural use [58,59]. However, some authors have found no differences among certain communities, such as ants, among primary forests and forests disturbed for agricultural uses [60]. Also, increased amounts of detritus leads to higher abundance of all arthropod feeding guilds in leaf litter communities, indicating that there are bottom-up limitations in rainforests [19]. These limitations have implications for methods of restoration in degraded rainforests. For example, reforestation efforts that lead to greater structural diversity of plant life likely lead to greater diversity of leaf litter as well. This diversity creates a wide range of microclimates which are beneficial to many ground-dwelling beetles [61].

Compared to other restoration methods, island plantations most closely resembled untouched forests, however, leaf litter arthropods recovered eight years post-harvest even with no restoration [27]. In contrast, methods such as brushwood transposition, wherein woody material is transported to the damaged area to create a heterogenous environment, had low leaf litter arthropod diversity [62]. In North America, second-growth forests support highly diverse populations of leaf-litter inhabiting beetles [63,64] indicating considerable resilience to forest harvesting and conservation efforts.

It is of paramount importance to keep in mind that not all arthropod species are affected in the same ways by these restoration efforts. For example, common species appear to be unaffected while rarer species are more effected by both deforestation and reforestation [65]. Considering the amazing biodiversity of arthropods in rainforests, the effects on rare species are highly concerning. There are significant gaps in knowledge regarding what those rare species are, how they interact with more common species, and how these transition periods of reforestation affect them.

4. Invasive Species Control

Control of forest pests often involves intense management including sanitation harvests and chemical control. In general, control of invasive species negatively impacts leaf litter arthropod communities, although in different ways. As previously mentioned, sanitation harvests, which are often clear cuts, negatively impact leaf litter dwelling arthropod communities through an increase in forest floor temperatures and a reduction in organic matter inputs [49]. Chemical control also impacts arthropods on the forest floor. For example, diflubenzuron, a commonly used chemical control of the invasive gypsy moth, caused reductions in mites, thrips, centipedes and spiders [3,66].

A lack of invasive species control also impacts ground dwelling arthropods in the leaf litter. For example, increased ash wood on the ground from emerald ash borer (EAB) infestation causes increases in leaf litter arthropods [67]. Tree-killing invasive insects, like EAB, may also result in changes to leaf litter-dwelling arthropod communities by temporarily increasing the litterfall input, which is then followed by significant light gaps in the canopy [68]. Invasive plants may represent an initial pulse of novel resources for leaf litter arthropods, thereby showing some benefit early on. However, following decomposition, there is an overall negative effect of invasive plants on leaf litter arthropods [69].

There are several significant gaps in knowledge with regards to invasive species control and leaf litter arthropods. The current literature mainly covers the effects of chemicals or impacts of the invasive species itself on leaf litter arthropod groups. However, there is little known about how leaf litter affects the resiliency of forests to invasive pests or how invasive plants affect these communities.

5. Effects of Fire

Compared to logging operations, fire has a much bigger impact on leaf litter arthropods [70]. Fire has been used for thousands of years by indigenous peoples around the world in agricultural, forest, and wildlife management [71–74]. Decades of fire suppression has led to an increase in fuel load and significant changes to the landscape, such as loss of dominant fire-dependent species, and there have been recent efforts to get fire back on the ground and reincorporated as an essential part of forest management [75]. Fire has complex effects on leaf litter arthropods through the creation of more structurally diverse habitats [76,77] and through changes to the forest floor microclimate [78]. Fire increases temperature and changes soil pH, as well as fluctuations in soil moisture [79]. The exposure to extreme temperatures, increased light, and reduced moisture frequently result in habitat loss [80], especially for those fire-intolerant species that are prone to inhabiting mid- and late-successional forests [81,82]. Changes to plant communities through fire can also modify soil moisture, invariably affecting ground-dwelling arthropods [83].

Fire, like harvesting operations and invasive species control, impacts different taxa in different ways [84–86]. For example, species richness of beetles increased following fire in

the Sierra Nevada mountain range, but the intensity and directionality of impacts were taxon specific with no general patterns [87]. Some general patterns have emerged, however, from the literature. There is typically a significant, immediate reduction in arthropod abundance following fire [28,88,89]. These communities, however, appear to be resilient to fire [86,90–95] and abundance and diversity of leaf litter arthropods actually increases following burning [96,97].

Leaf litter arthropods also have complicated relationships with fungi [98] and fire has been shown to reduce total fungal biomass in forests [99]. Arthropod feeding on senescent hyphal structures of fungi stimulates fungal growth [97], and the presence of leaf litter decomposing arthropods is significantly related to mycorrhizal growth in some tree species like red maple [14].

While it is known that fire has significant effects on leaf litter dwelling arthropods, there is a significant gap in knowledge as to how various arthropod characteristics affect their interactions with fire. How different environmental and landscape characteristics affect leaf litter following a burn and what this means for associated arthropod communities remains unexplored.

6. Conclusions

While we have determined some general trends of effects of forest management on leaf litter arthropods, there is still much to uncover. Arthropods are most definitely affected by forest management activities, but appear to be resilient to these human activities. However, it is unknown how specific types of management affect specific taxa, and this is important from the standpoint of monitoring overall forest health. More work is necessary to understand how various types of forest management affect specific groups like beetles, spiders, ants, and other bioindicators.

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forests

MDPI

Article

Monitoring and Management of the Pine Processionary Moth in the North-Western Italian Alps

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Abstract: The pine processionary moth (PPM), *Thaumetopoea pityocampa* (Denis and Schiffermüller, 1775) (Lepidoptera, Notodontidae), is considered one of the main insect defoliators of conifers in Southern Europe and North Africa. The species is oligophagous on pines and cedars in Mediterranean countries. This 6-year investigation (2014–2019), carried out in Aosta Valley (NW Italy), on *Pinus sylvestris* L. aimed to: (i) Monitor the PPM population with pheromone-baited funnel traps; (ii) assess the infestation index (0–5 classes) according to the degree of defoliation; and (iii) apply control strategies, namely *Bacillus thuringiensis* var. *kurstaki* (*Btk*) and mating disruption (MD). In total, 9618 ha were visually monitored and assigned an infestation index. The percentage of woodland stands that were strongly defoliated by PPM (infestation index ≥ 3) increased progressively between 2015 and 2016, affecting from 8% to 19% of the total area monitored; this area decreased to 16% and 13% in 2017 and 2018, respectively, followed by an abrupt decline to 4% in 2019. Both *Btk* applications and mating disruption significantly reduced the infestation. Where *Btk* was applied, the rate of larval mortality ranged from 79.47% to 98.43%; conversely, in the control plots, the larval mortality was, on average, 1.56%. The mean number of PPM males captured in traps was significantly lower in the plots where MD was performed, ranging from 8.36 ± 2.37 to 13.47 ± 4.68. The mean number of males captured in the control plots was, on average, 119.16 ± 12.68. The total number of nests recorded per tree was significantly lower in MD plots, ranging from 0.41 ± 0.05 to 0.94 ± 0.14. In the control plots, there were, on average, 4.37 ± 0.76 nests per tree. As already documented for several defoliating forestry insect pests, both *Btk* and mating disruption proved effective in controlling PPM infestations, and thus, microbial insecticides should be strongly encouraged and adopted by forest managers.

Keywords: *Thaumetopoea pityocampa*; seasonal flight activity; sexual pheromone traps; *Pinus sylvestris*; forest insect pest; population suppression

1. Introduction

The pine processionary moth, *Thaumetopoea pityocampa* (Denis and Schiffermüller, 1775) (Lepidoptera, Notodontidae) (hereafter referred to as PPM), is one of the most economically important pine defoliators. It is a very ravenous species and is the major phytophagous insect pest in Mediterranean

forests, being distributed in the Mediterranean Basin in southern Europe, the Balkan area, and North Africa [1,2]. The moth's geographic range is constrained by its requirements for sunshine in winter and its susceptibility to both cold winter and high summer temperatures, and large differences in adult flight period may occur depending on the geographical area. In the last 30 years, the PPM has shifted its altitudinal range by about 110–230 m in the Alps [3]. Changes in the distribution of the pest have been recorded, with its range greatly influenced by winter air temperature and annual solar radiation [4]. The PPM caterpillar is herbivorous and is a very severe defoliator, causing significant pine tree health decline [5]. The moth is typically found in the pine forests of the Mediterranean area, but the urban environment is also a suitable habitat. Larvae feed on a wide range of *Pinus* species, especially *Pinus nigra* Arnold and *P. sylvestris* L., but other conifers, such as cedars and even Douglas fir, may also be threatened. A severe reduction in photosynthetic area, annual radial growth, biomass, and major economic losses are the main consequences of infestations in pine plantations [6–10]. Young plantations can be severely compromised by massive attacks [11], thus creating favorable conditions for fungi and/or secondary insect pests, such as scolytids and weevils [10,11]. Furthermore, larvae from the 3rd instar onwards possess urticating hairs that can have a serious social impact, and associated health problems in humans and domestic or farm animals in urban and suburban areas have been reported [12–14].

The PPM exhibits periodic outbreaks, with a roughly 6-year periodicity in several southern European countries. The cycle, however, is not regular and may vary from 3 to 10 years [15]. Traditional control methods to tackle the PPM include the physical removal of winter nests, ballistic methods (shooting nests from the ground), trunk injection with insecticides, bioinsecticide (e.g., *Bacillus thuringiensis* Berliner var. *kurstaki*) [4,16,17], and the use of trunk barriers/traps [12,18]. Understanding the impact of the PPM and precisely when adults will emerge is essential for developing appropriate control strategies. To this end, the sexual female pheromone, (*Z*)-13-hexadecen-11-ynyl acetate, was identified and extracted by Guerrero (1981) [19], and is used in pheromone-baited traps to monitor male flight activity [20] or for mass trapping [4].

In the present paper, a 6-year study (2014–2019) was carried out to investigate the PPM population density on *P. sylvestris* in Aosta Valley (NW Italy) using pheromone-baited traps. Given the lack of data for this area in the literature, the aim was to assess the seasonal flight activity of the PPM, recording male adults, and to determine the infestation index according to the degree of defoliation. Moreover, field studies evaluated the effectiveness of control strategies by comparing treated and untreated areas. The control strategies applied were (i) microbial treatments using commercially available *B. thuringiensis* var. *kurstaki*, and (ii) mating disruption.

2. Materials and Methods

2.1. Study Area

Aosta Valley is a mountainous autonomous region located in the north-western part of Italy. It has a total surface area of about 3262 km^2, 1080 km^2 of which is covered by forests. The territory is mainly mountainous, and the average altitude is 2100 m, ranging from about 295 m to 4810 m a.s.l. The main valley axis is prevalently west-east oriented, presenting a semi-continental climate.

The tree vegetation consists of *Larix decidua* Miller, *Picea abies* L. Karst, mixed stands of Scots pine (*P. sylvestris*), Mountain pine (*P. uncinata* Ram.), and Austrian black pine (*P. nigra*), and *Quercus pubescens* Will., with the sporadic presence of *Populus tremula* L. and *Betula pendula* Roth.

2.2. Seasonal Flight Activity

Over a 5-year period (2015–2019), the PPM population was monitored by highly skilled and trained forestry technicians from the "Corpo Forestale della Valle d'Aosta" (CFVDA). Surveys were carried out in four (2015), and 42 municipalities (2016–2019) of the Aosta Valley, and all details are given in Supplementary Materials Table S1.

Monitoring was performed on the PPM's host, *P. sylvestris*, and commercially available funnel traps were used at the sampling sites. All trap devices (Super Green, Serbios s.r.l., Badia Polesine (RO), Italy) were baited with dispensers containing the sex pheromone component (Z)-13-hexadecen-11-ynyl acetate (loading rate: 1 mg per dispenser), and placed randomly on trunks and branches of *P. sylvestris* stands at an average height of 2 m. Six traps were placed per hectare. All traps were installed during early June and inspected for adult males at weekly intervals until mid-September to detect the flight period of the target species in the study area. Captured males were counted and removed from the traps. Devices were rotated clockwise to minimize the influence of the individual trapping location. The lure in each trap was replaced every six weeks. The first pheromone trap-check date was the date on which the first captures were recorded.

2.3. Infestation Index

The infestation index was recorded in 42 municipalities over a 6-year period (2014–2019) during the winter season (December–February). A 1-ha plot was selected in each municipality. All the plots were characterized by the presence of pure *P. sylvestris* stands, with a density of approximately 300 plants/ha, and an average tree age of about 60 years. Each tree was visually inspected by eye or with binoculars when necessary. The infestation index was recorded in all pine stands by counting the number of nests and the degree of defoliation, according to six classes: 0, zero, no nest, no defoliation; 1, very low ≤ 0.1 nest per plant, < 10% defoliation; 2, low = scattered nests, 0.2–0.5 nests per plant, 10–25% defoliation; 3, medium = 0.6–2 nests per plant, 26–50% defoliation; 4, high = 3–5 nests per plant, 51–75% defoliation; 5, very high ≥ 5 nests per plant, massive defoliation (>75%).

2.4. Application of Control Strategies

The infestation index was used to evaluate tree damage and determine threshold values above which control strategies were applied to control PPM infestations. Treatments with *Btk* were applied in those plots with an infestation index ≥ 3. Conversely, mating disruption was applied at sites characterized by a low PPM population density (infestation index < 2).

Microbial applications were performed at 20 different sites (5 sites per year) over a 4-year period (2016–2019). Each of these sites was at least 1 km away from monitoring traps to avoid compromising male catches, with a preference for areas with pure *P. sylvestris* stands and avoiding areas where the pines were mixed with other species, such as *L. decidua*, other *Pinus* species, and broadleaved trees. At each site, a plot of 100 pine saplings (about 0.30 ha) was sprayed with a commercially available bioinsecticide product based on *B. thuringiensis* var. *kurstaki* (*Btk*) (Kristal 32 WG, HD-1 strain, Serbios s.r.l., Badia Polesine (RO), Italy) at the rate of 32,000 BIU ha^{-1}, in the presence of L1 and L2 larvae. Bioinsecticide was sprayed from the ground, up to a height of about 20 m, between mid-August and mid-September, at about 20 °C (in the twilight hours) and in partly cloudy weather conditions, using atomizer equipment supplied by CFVDA and the phytosanitary service of the Aosta Valley. A control plot, with an area and number of trees comparable to the treated plots, was chosen in the surroundings of each treated area, but far enough away to avoid contamination by the bacterium (at least 1 km). Treatments were not performed if wind speed exceeded 7 km h^{-1} and were repeated in the case of heavy rain. The effectiveness of the microbial treatment was assessed 10 days after treatment evaluating larval mortality. To calculate the percentage of larval mortality, the number of dead larvae was divided by the total number of dead and live larvae recorded on all the saplings for each site.

Four municipalities were chosen for mating disruption over a 2-year period (Saint Nicolas and Sarre in 2016; Arvier and Villeneuve in 2017). In total, 23 and 19 plots were chosen in 2016 and 2017, respectively (11 in Saint Nicolas, 12 in Sarre, 10 in Arvier, and 9 in Villeneuve). At each site, a 1-ha plot of about 300 pine saplings was chosen. Pheromone dispensers (only lures without the traps) containing the specific sex pheromone component (loading rate: 50 mg per dispenser) were placed on branches of *P. sylvestris* stands at an average height of 2 m within tree canopies immediately before adult emergence. Ten and 12 dispensers/ha were used in 2016 and 2017, respectively. For each plot,

a control plot with an area and number of trees comparable to the treated plot was chosen in the surrounding area, but far enough away to avoid the effects of pheromone dispersion (at least 2 km). The effectiveness of the technique was assessed using pheromone traps. The same type of traps as those used for monitoring was placed to collect male adults in the treated and control plots (6 traps/ha; loading rate: 1 mg per dispenser). The traps were placed in a regular pattern throughout each plot to ensure even coverage. All traps were installed immediately before adult emergence and inspected for adult males at weekly intervals until mid-September. Captured males were counted and removed from the traps. The effectiveness of the treatment was assessed during December by recording the mean number (±SEM) of nests/tree in all the treated and control plots.

2.5. Statistical Analysis

All data were first tested for homogeneity of variance (Levene's test), and transformations (log transformation and arcsine square rooted) were employed to stabilize variances and normalize the data. A paired t-test was used to assess differences in larval mortality comparing treated and control plots. Furthermore, a paired *t*-test was used to assess differences in male catches comparing plots where mating disruption was applied and control plots. Statistical significance was set at $p < 0.05$. All analyses were performed using SPSS version 22.0 (SPSS, Chicago, IL, USA).

3. Results

A network of permanent plots intended for monitoring of the PPM was set up during the survey period. In total, 277 georeferenced traps were placed in the same position in each of the five years, except for 2015, when the number of traps was 135 (Figure 1).

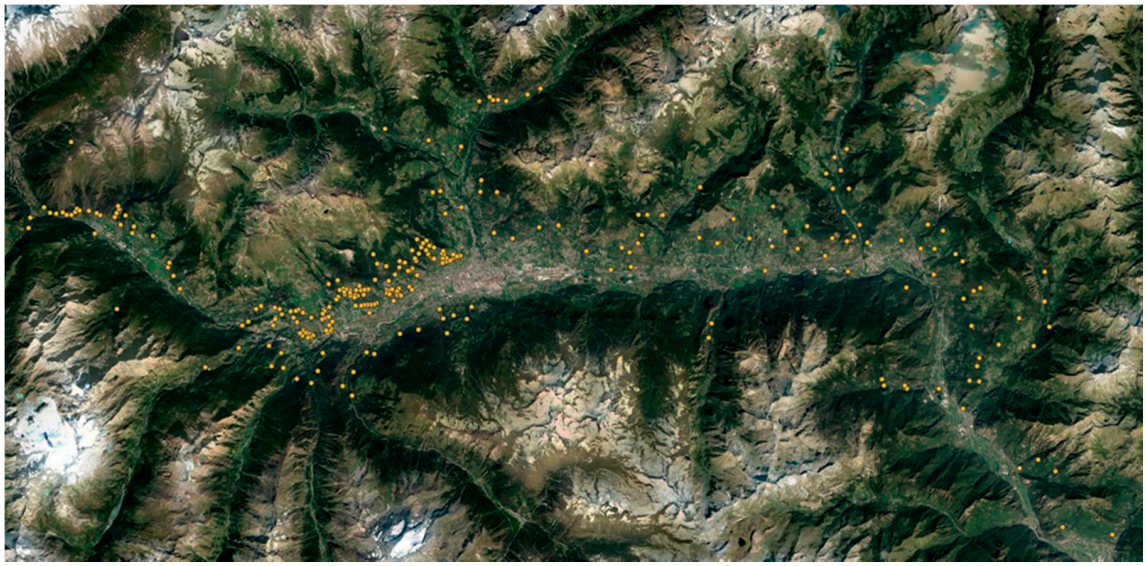

Figure 1. Map of the surveyed area in the Aosta Valley (north-western Italian Alps), showing the georeferenced pheromone baited traps placed in 2015–2019).

The total number of PPM adult males captured with funnel traps was 86,790, 150,652, 157,300, 152,118, and 61,612, in 2015, 2016, 2017, 2018 and 2019, respectively. The mean catches per week per trap ranged from 1.91 in Brusson in 2017 to 138.05 in Morgex in 2016. All data referring to the mean number of adult male PPM captured per week per trap and the total catch per week at all the surveyed sites are presented in Supplementary Materials Table S2.

In all years, the seasonal flight activity started at the beginning of June with the peak of adults on 3rd July (2015, 2016), 10th July (2017), 26th June (2018), and 17th July (2019). Males were found in traps until mid-September (Figure 2).

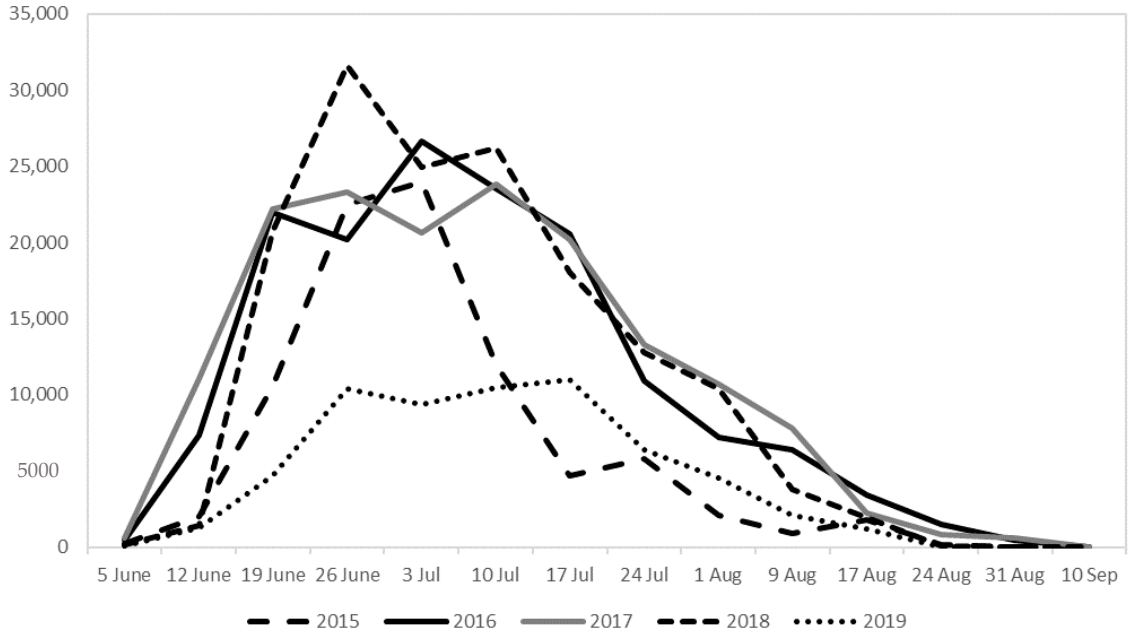

Figure 2. Total number of adults male of *Thaumetopoea pityocampa* collected with the pheromone-baited traps in the five-year period. (Data collected always refers to 277 traps, except for 2015 when the traps were 135).

In total, 9618 ha were visually monitored and assigned an infestation index. The percentage of woodland stands strongly defoliated by PPM (infestation index ≥ 3) increased progressively between 2015 and 2016, increasing from 8% to 19% of the total area monitored. In spring 2016, "very high" defoliation (76–100% loss of leaves) affected an area of 45 hectares, corresponding to 1% of the infested pine forests, while defoliation was "high" (with leaf loss between 51 and 75%) in 10% of the pine forests. The infestation index subsequently decreased to 16% and 13% in 2017 and 2018, and then suffered an abrupt decline to 4% in 2019 (Table 1). The most serious damage occurred in the municipalities of Morgex, Sarre, and Saint-Pierre.

In total, 367, 333, 208, and 253 ha were treated in 2016–2019, respectively. *Btk* treatments involved about 312 km of roads, covering a total area of about 1116 hectares of pine forests.

The mortality rate in the plots treated with *B. thuringiensis* was significantly higher than that recorded in the control plots. Where the bacterium was applied, the rate of larval mortality ranged from 79.47% to 98.43%, with an average of 90.47%. Conversely, in the control plots, the larval mortality ranged from 0.34% to 2.18%, with an average of 1.56% (Figure 3).

Mating disruption was performed in 23 and 19 ha in 2016 and 2017, respectively. The mean number (±SEM) of PPM male adults collected per trap and per week is given in Figure 4. The mean number of PPM males was significantly lower in the plots where MD was performed, ranging from 8.36 ± 2.37 to 13.47 ± 4.68. Conversely, the mean number of males captured in the control plots was, on average, 119.16 ± 12.68.

The total number of nests recorded per tree was significantly lower in MD plots, ranging from 0.41 ± 0.05 to 0.94 ± 0.14. In the control plots, there was an average of 4.37 ± 0.76 nests per tree.

Table 1. Infestation index recorded, and the total area (ha) treated with *Bacillus thuringiensis* var. *kurstaki*, and mating disruption in 2014–2019.

		2014		2015		2016		2017		2018		2019	
		No. Municipality	**ha**	**No. Municipality**	**ha**	**No. Municipality**	**ha**	**No. Municipality**	**ha**	**No. Municipality**	**ha**	**No. Municipality**	**ha**
		42		42		42		42		42		42	
Infestation index	0		7598		7310		5482		4809		4713		4520
	1		1058		1154		1539		2597		2212		4040
	2		481		385		769		673		866		673
	3		385		481		866		673		614		289
	4		96		192		673		577		439		96
	5		0		96		289		289		197		0
Total treated (ha)			-		-		367		333		208		253
***Bacillus thuringiensis* var. kustaki**		-	-	-	-	14	344	9	314	12	208		250
Mating disruption		-	-	-	-	2	23	2	19	-	-		-

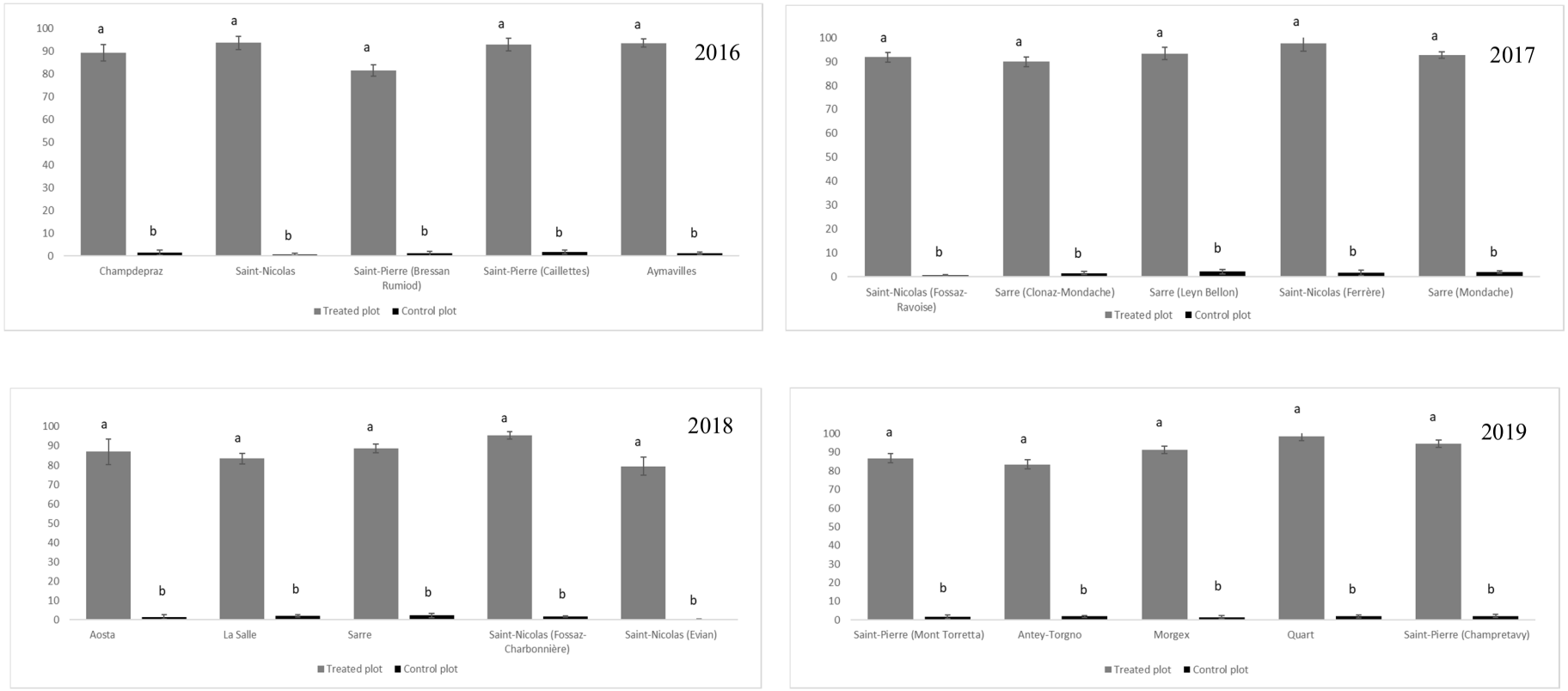

Figure 3. Percentage mortality of PPM larvae (±SEM) recorded inside the nests collected in treated and control plots after applications of *Bacillus thuringiensis* var. *kurstaki* in 2016–2019. Bars with different letters are significantly different, according to the paired *t*-test ($p < 0.05$).

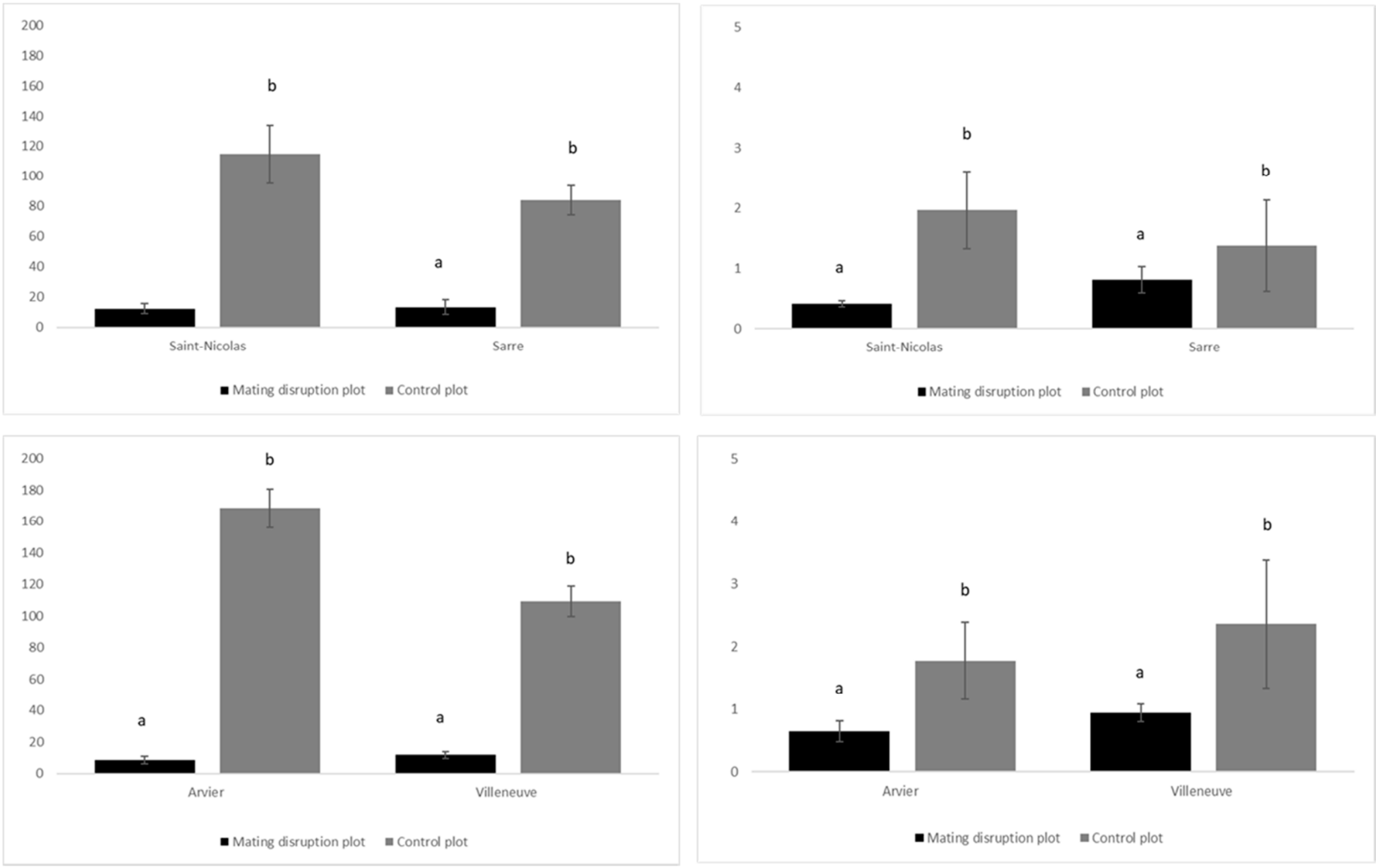

Figure 4. Mean number (±SEM) of male adults/per trap/per week of *Thaumetopoea pityocampa* collected with pheromone-baited traps, and mean number (±SEM) of nests/tree recorded in plots where mating disruption (MD) was applied compared to control plots in 2016 (top) and 2017 (bottom). Bars with different letters are significantly different, according to the paired *t*-test ($p < 0.05$).

4. Discussion

Although *T. pityocampa* is a key pest of pine trees in the Alps, there is little information about its phenology and seasonal occurrence in the north-western Italian Alps. In the Aosta Valley, PPM infestation has been consistently investigated in forest monitoring programmes from the 1960s onwards, but the defoliator moth has only been intensively studied since the 2000s. The Aosta Valley pine forests mainly consist of black pine and Scots pine; defoliation damage ranging from mild to very severe, due to PPM feeding activity, has been observed in about 50% of this area over the years (IR and LD). In 2016, the Department of Agriculture and Natural Resources, therefore, activated a plan to control PPM outbreaks.

Generally, mixed forests are less sensitive to pests and disease than pure stands. Although olfactory cues are commonly used to locate host trees, visual cues may affect the degree of infestation as well. Even in the case of PPM, mixed pine stands have been found to suffer less damage than pure pine stands [21], as non-host trees may act as physical barriers [22,23]. However, over the last century, several species of the genus *Pinus* have been widely used for afforestation [24], and this has clearly facilitated the spread of the PPM in the surveyed area. *P. nigra* is considered the primary native host of PPM, and the planting of this species has almost certainly promoted PPM population growth and outbreaks. Furthermore, PPM is reported to be sensitive to the severity and duration of the cold winter period, since it overwinters at the larval stage. The lethal temperature threshold is between approximately −10 °C and −16 °C [3], and the mean temperature increase over the last few decades has, thus, favored a higher survival rate from year to year, as already reported by Robinet [25]. In Aosta Valley, Mont Avic was used as a study site to monitor the expansion of PPM in the Italian Alps, where both an elevation gain (113.7 ± 23.0 m) and distance gain (232.7 ± 22.4 m) were observed in 2003 [26].

In our survey area, the network of permanent plots established in 2015 revealed that the PPM population progressively increased to reach its highest values in 2017, and then decreased rapidly afterward. The seasonal flight activity started at the beginning of June and continued until mid-September. The trapping of male adults in pheromone-baited traps peaked between the end of June and the beginning of July, with a similar trend in each year that was consistent with the literature [27,28]. Reliable population density data from monitoring and assessment surveys are required for effective suppression of PPM infestations. Although the absolute number of catches per trap may not be entirely representative of the population, being strongly influenced by sex ratio and trap frequency and position [24], monitoring traps may be useful to identify low- or high-density population areas where physical removal of the nests, insecticides, microbial treatments and/or mating disruption can be applied. Since several control methods are available, a practical evaluation of their effectiveness is essential for decision making in current PPM control protocols.

The Aosta Valley is a renowned tourist area, and in the past decades, there has been an increasing need for effective pest management programs, with traditional control methods (e.g., intensive pruning or chemical pesticides) strongly discouraged. The most common environmentally friendly control strategies (e.g., mechanical, biological) have now been adopted in the forestry environment.

Microbial insecticides offer a great deal of promise for pest management, being effective in controlling several defoliating forestry insect pests (e.g., spruce budworm, gypsy moth), without posing the serious environmental hazards associated with conventional pesticides [29,30]. Several microbial antagonists (e.g., bacteria, fungi, and viruses) have been reported for the control of PPM infestations [16], but the only large database based on scientific investigations concerns the use of *Bacillus*-based preparations. Applications of commercially available bioinsecticide products based on *Btk* have increased in the last few decades, targeting PPM [16,31,32], and other forest-defoliating lepidopterans [33–35]. At the surveyed sites, different control strategies were applied with the aim of containing and mitigating PPM outbreaks. *Btk* applications and mating disruption showed promising results in terms of effectiveness in controlling the infestations. Where *Btk* treatment was performed, the PPM mortality was very high. The average larval mortality was 90.47% in the treated plots *versus* 1.56% in the control plots. Similar values, with PPM larval mortality rates up to 90%, have previously

been reported for *Btk* ground application in EU and Mediterranean countries [16,32]. In the literature, the susceptibility of the larvae to *Btk* was found to be directly proportional to the application dose, and some authors reported that a second *Btk* spray within 10–15 days of the first application might be useful [16]. In the current study, only one application was performed targeting L1–L2 larvae, using the dose of 32,000 BIU ha^{-1}, and the average larval mortality rate proved very high at all the surveyed sites and in all years, making a second application unnecessary. Similar to *Btk* applications, the use of pheromones to disrupt the mating of PPM was effective at all the surveyed sites, suggesting that pheromone lures may effectively decrease male captures. The number of males in pheromone-baited traps was significantly lower in all MD plots (on average, 11.47 males/captured/week in all sites) in comparison with the control (on average, 119.16 at all sites) during 2016–2017. The number of nests per tree also confirmed the effectiveness of this technique, as also demonstrated by Trematerra [36]. The values recorded were significantly lower in all MD plots (on average, 0.7 nests/tree) compared to the control plots (on average, 1.87 nests/tree), highlighting the success of this technique. Although some constraints regarding the size of the plot have been reported for agricultural pests because of the risk of adult immigration [37], our investigations were performed in 1-ha plots, and the satisfactory results are consistent with those reported elsewhere [36].

PPM has a typical cyclical pattern, where intermittent outbreaks are interspersed with periods of lower population densities. In the literature, peak defoliations are reported to occur every 6–11 years on average [38–40], while in the Italian Alps, no regular cycles have been detected [41] (although the different statistical methods applied may have influenced the final output). A negative gradation phase characterized by a decline in the PPM population commonly occurs when there is a significant reduction in food availability. In the current study, the PPM population experienced a clear decline in 2019, but given that pine stands were not severely defoliated, it is more likely that this was due to the effectiveness of the set of control strategies applied rather than to the PPM's cyclical pattern. The pest is a strong public-health concern, due to the presence of urticating hairs that are present from the L3 larvae onwards, and which may affect humans, pets, and livestock in urban areas and forestry [13]. *Btk* applications and mating disruption have turned out to be the most appropriate control strategies for forest managers targeting the PPM. The adoption of such control strategies should be strongly encouraged by local authorities and administration policies, especially in areas frequented by people and in pine stands used for biomass production. Repeated annual applications of microbials and/or mating disruption could dramatically reduce population densities to levels below the economic injury level, with populations subsequently maintained at low levels with little management effort [42].

The role played by natural enemies is currently being investigated in Europe [40] and some Italian regions [42,43], and at least 18 parasitoid and 15 predator species have been recorded on the different stages of PPM. To date, the establishment of natural enemies in the Aosta Valley has not been studied, and little is known about their presence and potential impact along latitudinal or longitudinal gradients. Some questions remain to be addressed, and further studies should focus on the occurrence and adaptation of invertebrate natural enemies regarding PPM population dynamics. Since it is not completely clear how natural enemies respond to PPM outbreaks and what their role is in population regulation, specific research is needed to deepen our knowledge about the potential contribution and beneficial role of generalist parasitoids in controlling this defoliator moth in the surveyed area.

Further studies must also be performed regarding climatic conditions. Changes in diel activity and seasonal phenology patterns of both the PPM and oak processionary moth have been associated with global climate change [44], and moth emergence may be strongly affected by weather conditions, as already reported for the congeneric species *T. wilkinsoni* Tams [45]. All these further investigations, in addition to the data already recorded about seasonal occurrence and effectiveness of control strategies, will help provide a current global picture of PPM in the Aosta Valley, and allow for effective and sustainable management strategies to be adopted in the long term.

Supplementary Materials: The following are available online at http://www.mdpi.com/1999-4907/11/12/1253/s1, Table S1: List of the monitored municipalities in Aosta Valley in 2015–2019, Table S2: Total number of male adults of *Thaumetopoea pityocampa* monitored with pheromone-baited traps in Aosta Valley in 2015–2019.

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Gypsy Moth Management with LdMNPV Baculovirus in Cork Oak Forest

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Abstract: (1) Research Highlights: Applications of a species-specific baculovirus is a promising method to control the gypsy moth and regulate its population dynamics in forest ecosystems. (2) Background and Objectives: Cork oak protection against the Lepidopteran defoliator *Lymantria dispar* requires an appropriate forest ecosystem management program, involving the application of eco-sustainable microbial products during population outbreaks. The species-specific multicapsid nucleopolyhedrovirus (LdMNPV), agent of natural epizootics in gypsy moth populations, represents an option that was investigated in a multi-year field study, involving viral applications either from the ground or by aerial treatment. (3) Materials and Methods: Efficacy trials against *L. dispar* populations were conducted in 2018 and 2019 in Sardinia, according to a randomized block design. Each year, two trials were conducted, applying a baculovirus commercial formulation with an atomizer from the ground and assessing the effects of different doses and application timing, respectively. An aerial application trial distributing LdMNPV at ultra-low volumes (2 L/ha) was also conducted in 2019 to assess the virus efficacy at a larger field scale. (4) Results: In both years, a significant increase in larval mortality was detected in plots treated with higher viral occlusion body (OB) doses and with an earlier application targeting younger larvae, in comparison with untreated controls. Due to an observed retrogradation phase of the target pest in 2019, no significant differences in larval density between areas treated from a helicopter and control were detected, but in the few weeks following application, a meaningful vitality decrease in larval samples from treated plots was observed. (5) Conclusions: Based on the results of this study, the use of LdMNPV in forest protection programs against gypsy moth can be worth consideration in multi-year integrated program strategies to modulate population dynamics.

Keywords: biocontrol; bioinsecticide; entomopathogen; microbial; ecosystem

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1. Introduction

Lymantria dispar (L.) (Lepidoptera: Erebidae), also known as gypsy moth, is a univoltine species whose larvae, hatching from overwintering eggs, cause significant damages to cork oak leaves. The combination of their feeding behavior and a high biotic potential are the cause of periodic outbreaks, determining wide forest defoliations [1]. In order to reduce such deleterious effects, the implementation of appropriate biocontainment measures is necessary. Accordingly, the application of bioinsecticides was proven to be a successful approach to counteract this pest action, ensuring limited environmental impact [2]. For this purpose, available formulations based on the entomopathogenic bacterium *Bacillus thuringiensis* exploit the highly specific mode of action of bacterial toxins selectively targeting moth larvae [3]. On the other hand, the risks of possible side-effects on non-target lepidoptera inhabiting the forest ecosystem have sometimes been reported [4]. Another group of entomopathogens is represented by baculoviruses, very specific microorganisms co-evolved with their host [5] and able to cause fatal infections to larvae after the ingestion of viral particles. The bioinsecticidal activity is associated with crystalline occlusion bodies

that, after being ingested by susceptible insects, release occlusion-derived viruses (ODVs) acting on the host midgut epithelial cells. The infection spread in the host body relies on the production of a second type of virions, namely, budded viruses (BVs) [6].

Lymantria dispar multicapsid nucleopolyhedrovirus (LdMNPV) is specifically associated with gypsy moth, being co-evolved with this species [7]. This biocontrol agent represents a natural regulator of the defoliator population, as a result of periodic viral epizootics, especially under high-density conditions [8]. Hence, the baculovirus, reproduced in the laboratory on live larval material and applied in the field, becomes a tool able to significantly affect the population dynamics and therefore be used artificially to counteract gypsy moth outbreaks [9]. While this strategy appears promising, the use of LdMNPV is relegated to specific contexts where commercial products are available (i.e., Canada, USA). Due to the high costs associated with the production of viral material and the lack of available products in the market of several global regions (i.e., Europe) where local *L. dispar* ecotypes represent a constant threat to the different tree forest species, experimental work is needed to implement the use of baculovirus in different environmental conditions.

The present study had the purpose of evaluating the efficacy of a LdMNPV formulation under use in other world areas, against European *L. dispar* in cork oak forests affected by this pest. The study was conducted through two steps in different years, involving either small-scale viral applications from the ground, and larger-scale aerial treatments.

2. Materials and Methods

2.1. Tested Formulations

A suspension concentrate formulation of LdMNPV, commercially authorized in Canada, was provided by Andermatt Biocontrol AG (Switzerland) for experimental applications from the ground or by helicopter. The concentration of the active substance was 2.8×10^{10} OB/L. Foray 76B (Sumitomo Chemical Agro Europe S.A.S), containing 20 billion international units (BIU)/L of *Bacillus thuringiensis kurstaki* (*Btk*) strain ABTS-351, was used as a reference product.

2.2. LdMNPV Applications from the Ground

Two different trials were conducted to evaluate the effects of the baculovirus: (1) time–response and (2) dose–response. Treatments and application details are summarized in Tables 1 and 2, respectively. Time–response and dose–response trials were conducted in different experimental fields in the same year.

Table 1. Treatments in the time–response trial.

Treatment	Description	Application Date		Application Rate
		2018	2019	
Untreated Check	Not treated	-	-	-
LdMNPV Early	Earlier application	9 May	4 May	2 L/ha
LdMNPV Later	Later application	16 May	11 May	2 L/ha
Foray 76B	Reference product	16 May	11 May	2 L/ha

Table 2. Treatments in the dose–response trial.

Treatment [a]	Description	Application Rate
Untreated Check	Not treated	-
LdMNPV Low	1/3 standard rate	0.66 L/ha
LdMNPV Standard	Standard rate	2 L/ha
LdMNPV High	3× standard rate	6 L/ha
Foray 76B	Reference product	2 L/ha

[a] All applications were made on one date (9 May 2018, and 11 May 2019).

The trials were conducted in 2018 and 2019 in forests in north-western Sardinia (Italy) in compliance with Good Experimental Practice (GEP) guidelines established by the European and Mediterranean Plant Protection Organization (EPPO PP 1/210(1), Efficacy evaluation of insecticides—Defoliators of forest trees). The completely randomized experimental design involved four plots (100 m^2) per each treatment. Gypsy moth larval density was recorded before treatments and during the following three weeks (7, 14, and 21 days after LdMNPV application). Assessments were based on counting the number of larvae in eight 30 cm long branches randomly sampled from each plant. Defoliation levels in plots were also evaluated after treatments.

In the time–response trial, early application was conducted one week earlier (9 May 2018, and 4 May 2019), targeting eggs and just-hatched first instar larvae, while standard applications (16 May 2018, and 11 May 2019) targeted first and second instar. In the dose–response trial, applications were made on one date (9 May 2018, and 11 May 2019). Baculovirus applications were carried out with a motorized atomizer (M3 series, Cifarelli SpA, Italy), with a volume of 10 L per plot.

2.3. Aerial Applications

Aerial applications were carried out on 11 May 2019 on a forest area located in the Centre of Sardinia (Abbasanta, Italy). Treatments were performed in ultra-low volumes (ULVs), employing a helicopter (LAMA SA 315/B) equipped with 4 electronic Micronair rotary atomizers (model AU) treating a 20 m wide lane. Treatments were performed early in the morning so that environmental conditions ranged within sub-optimal limits. During product application, a global positioning system (GPS) was employed to trace and record the helicopter route, ensuring accurate and homogeneous distribution. Untreated check plots were compared with plots (around 100 ha each) treated with LdMNPV or *Btk* (Foray 76B). Direct assessments were based on counting the number of larvae on four 30 cm long branches per each of ten plants randomly sampled in each experimental plot. In addition, samples of larvae (n = 100) were collected from each plot and maintained in the laboratory on foliage collected from the same plots after treatment, in order to compare insect survival. The experiment involved three replicates.

2.4. Data Elaboration and Statistical Analysis

Overtime differences in average larval density among treatments in application experiments from the ground were tested using repeated measures ANOVA (PROC MIXED), and means were separated by LSMEANS comparison (adjust = Tukey), using SAS software (version 9.1) [10] with the significance level set at $\alpha = 0.05$. Analysis of variance (ANOVA) followed by least significant difference (LSD) test ($p < 0.05$) was used to compare efficacy data on a specific date and defoliation levels among treatments.

For different datasets in this study, in order to verify assumptions of normality and heteroscedasticity, the Shapiro–Wilk [11] and Levene's tests [12] were performed, respectively. If necessary, data were transformed as the arcsine of the square root of the percentage.

Field treatment efficacy was evaluated in terms of larval density reduction, where percent reduction in treatment x after t days (ΔD_{xt}) was calculated as:

$$\Delta D_{xt} = \frac{D_{x0} - D_{xt}}{D_{x0}} \times 100 \quad (1)$$

where D_{x0} is the initial larval density in treatment x at sampling time 0 (i.e., before application), and D_{xt} is the larval density t days after applications in treatment x. Efficacy differences between treatments were tested separately for each sampling date (i.e., 7, 14 and 21 days after applications) using one-way ANOVA. Tukey's test at a significance level of 0.05 was used for means separation if necessary.

Aerial application trial data obtained from laboratory observations on field-collected larvae were analyzed by a mixed effects Cox proportional hazard model using survival [13], and coxme [14] packages in R software [15]. In each model, treatments were considered

as fixed factors and the larval cage (i.e., replicate) as a random effect factor. Further post hoc analysis was performed using the multcomp package in R [16], applying a Bonferroni correction for multiple testing. Moreover, larval density reduction (%) was corrected for natural mortality to take into account the effect of natural population decreases (C_{xt}) using the Schneider-Orelli formula [17]:

$$C_{xt} = \frac{\Delta D_{xt} - \overline{D}_{Ct}}{100 - \overline{D}_{Ct}} \times 100 \quad (2)$$

where ΔD_{xt} is the larval density reduction (%) in treatment x after t days, and $\overline{D}_{Ct}$ is the average larval density reduction (%) in untreated control t days after applications. After this correction, transformed data were used to evaluate the merely effect of *Btk* or LdMNPV against gypsy moth larvae as assessed in the laboratory. Student's t-test at the 0.05 level of significance was used to test for differences between different treatments 7, 14, and 21 days after application.

3. Results

3.1. LdMNPV Applications from the Ground

Comparing the different plots involved in trials, a homogeneous larval density was observed before insecticidal applications in both years for time–response (2018: $F_{3,15} = 0.31$; $p = 0.82$; 2019: $F_{3,15} = 1.33$; $p = 0.31$) and dose–response (2018: $F_{4,15} = 0.26$; $p = 0.90$; 2019: $F_{4,15} = 0.16$; $p = 0.95$) trials.

In the time–response trials conducted in 2018, no significant changes in larval density were observed one week after the application of LdMNPV in the "LdMNPV early" experimental thesis. On the other hand, a significant larval density reduction was found during the following two weeks in the same plots, in comparison with the untreated check ($F_{9,63} = 15.07$; $p < 0.01$). No significant changes in larval density were instead associated with the "LdMNPV later" thesis (Figure 1). A significant dose-dependent effect was observed in the trial conducted in 2018 ($F_{12,79} = 19.13$; $p < 0.01$). A higher larval density reduction was associated with a higher LdMNPV dose, and this decrease became more significant as time advanced (Figure 2).

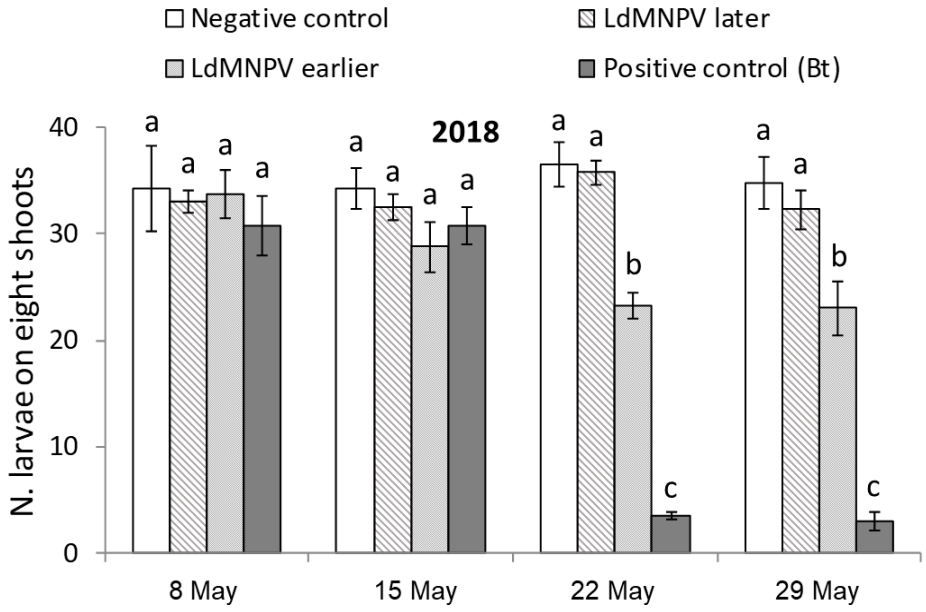

Figure 1. Larval density (mean ± SE) assessed by sampling 8 shoots/plot in the time–response trial with LdMNPV applications from the ground in 2018. Different letters (a, b, c) above bars indicate significant differences among means within each sampling date (ANOVA Mixed Proc., LSMEANS, $p < 0.05$).

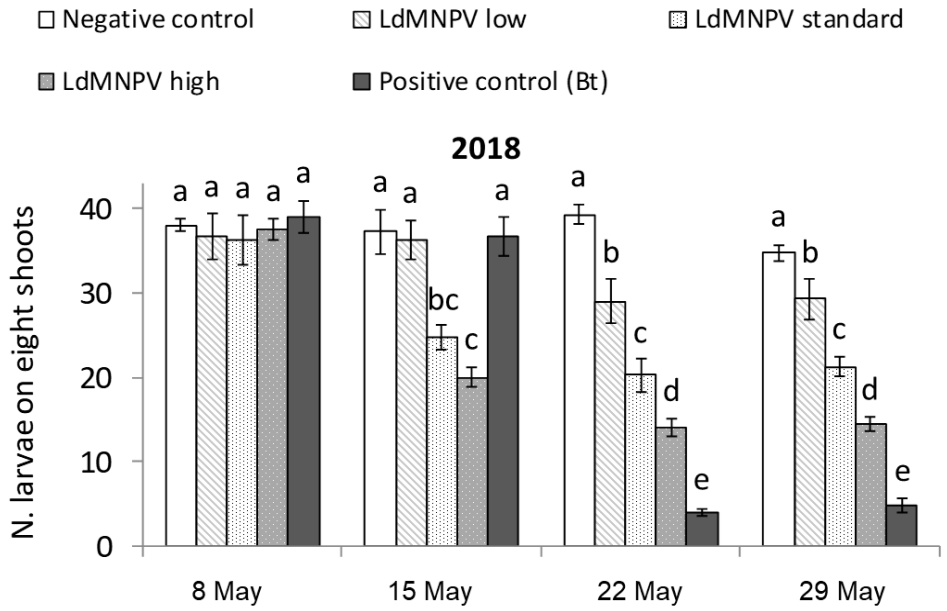

Figure 2. Larval density (mean ± SE) assessed by sampling 8 shoots/plot in the dose–response trial with LdMNPV applications from the ground in 2018. Different letters (a, b, c, d, e) above bars indicate significant differences among means within each sampling date (ANOVA Mixed Proc., LSMEANS, $p < 0.05$).

These results were comparable to the output of trials conducted in 2019. In the case of the time–response trial, a significant larval density decrease was achieved by both early and later applications of the baculovirus ($F_{9,63} = 16.62$; $p < 0.01$), with a higher and faster effect of the earlier treatment in comparison to the untreated check (Figure 3). A good efficacy was also observed in the dose–response trial, in which the LdMNPV doses assayed showed a significant biocontrol action on gypsy moth larvae with a dose-dependent effect, in comparison with the untreated control ($F_{12,79} = 9.43$; $p < 0.01$). A greater protection of trees was associated with the highest doses applied (Figure 4).

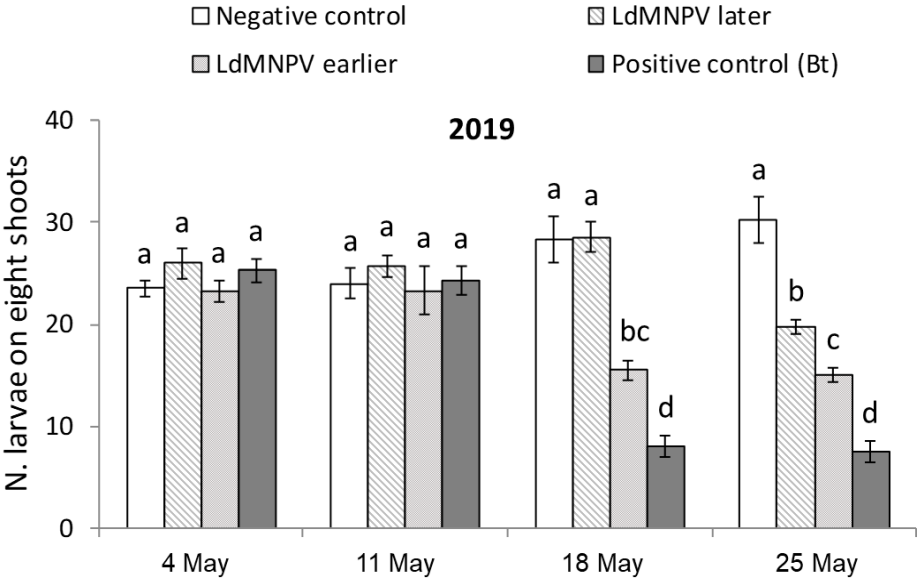

Figure 3. Larval density (mean ± SE) assessed by sampling 8 shoots/plot in the time–response trial with LdMNPV applications from the ground in 2019. Different letters (a, b, c, d) above bars indicate significant differences among means within each sampling date (ANOVA Mixed Proc., LSMEANS, $p < 0.05$).

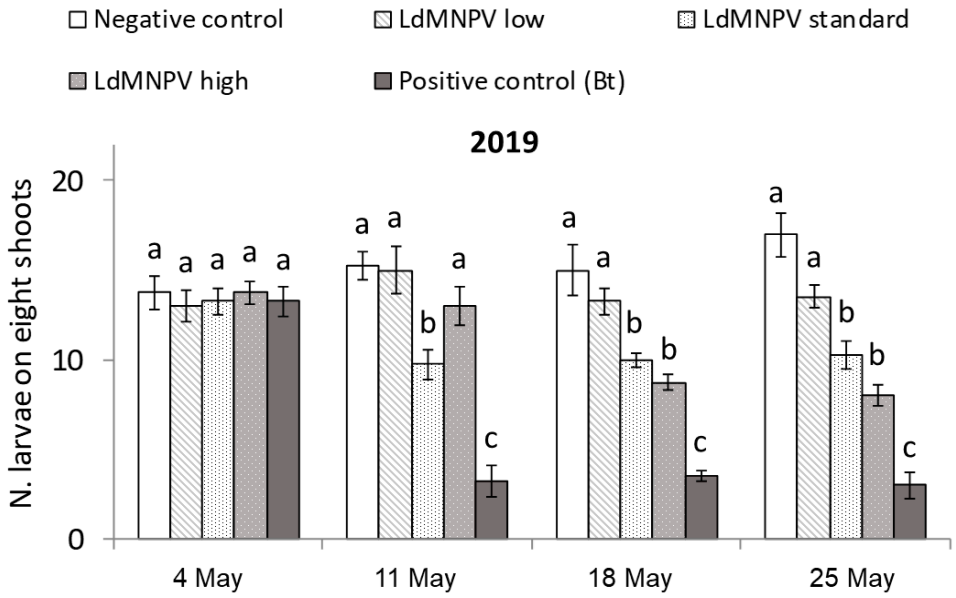

Figure 4. Larval density (mean ± SE) assessed by sampling 8 shoots/plot in the dose–response trial with LdMNPV applications from the ground in 2019. Different letters (a, b, c) above bars indicate significant differences among means within each sampling date (ANOVA Mixed Proc., LSMEANS, $p < 0.05$).

In general, a higher percentage of defoliation was found in the untreated check, while a significant protection was associated with higher LdMNPV doses (2018: $F_{4,19} = 24.50$; $p < 0.001$; 2019: $F_{4,19} = 15.43$; $p < 0.01$) and earlier treatments (2018: $F_{3,15} = 33.08$; $p < 0.01$; 2019: $F_{3,15} = 47.61$; $p < 0.01$) (Figure 5).

In all trials, the decrease in larval density and the protection against defoliation in plots treated with the *Btk* reference product was the best and associated with greater and faster action (Figures 1–5).

3.2. Aerial Applications

LdMNPV formulation applied at a dose of 2 L/ha appeared to be well and homogeneously distributed in the treated plots.

In 2019, a general drop in larval density during the season was observed in the experimental area involved in the aerial application study, outlining a retrogradation phase of gypsy moth population in this forest ecosystem in Sardinia. Accordingly, such a reduction was observed in all plots, with no differences among treatments 7 ($F_{2,8} = 3.52$, $p = 0.13$), 14 ($F_{2,8} = 0.95$, $p = 0.46$), and 21 ($F_{2,8} = 1.85$, $p = 0.27$) days after applications (Table 3).

Table 3. Percentage (mean ± SE) of larval density reduction in the field at different time intervals after bioinsecticidal application, in respect to pre-treatment. Percentage data are corrected using the Schneider-Orelli formula.

Days [a]	Treatment			F	*p*
	Foray 76B	LdMNPV	Untreated Check		
7	64.40 ± 8.82 [b]	31.71 ± 4.00	39.66 ± 17.02	3.52	0.13
14	70.28 ± 8.94	34.34 ± 34.33	46.87 ± 18.57	0.95	0.46
21	75.30 ± 9.11	45.59 ± 5.83	50.58 ± 20.39	1.85	0.27

[a] Days after application. [b] No significant differences among means were observed (ANOVA, $p > 0.05$).

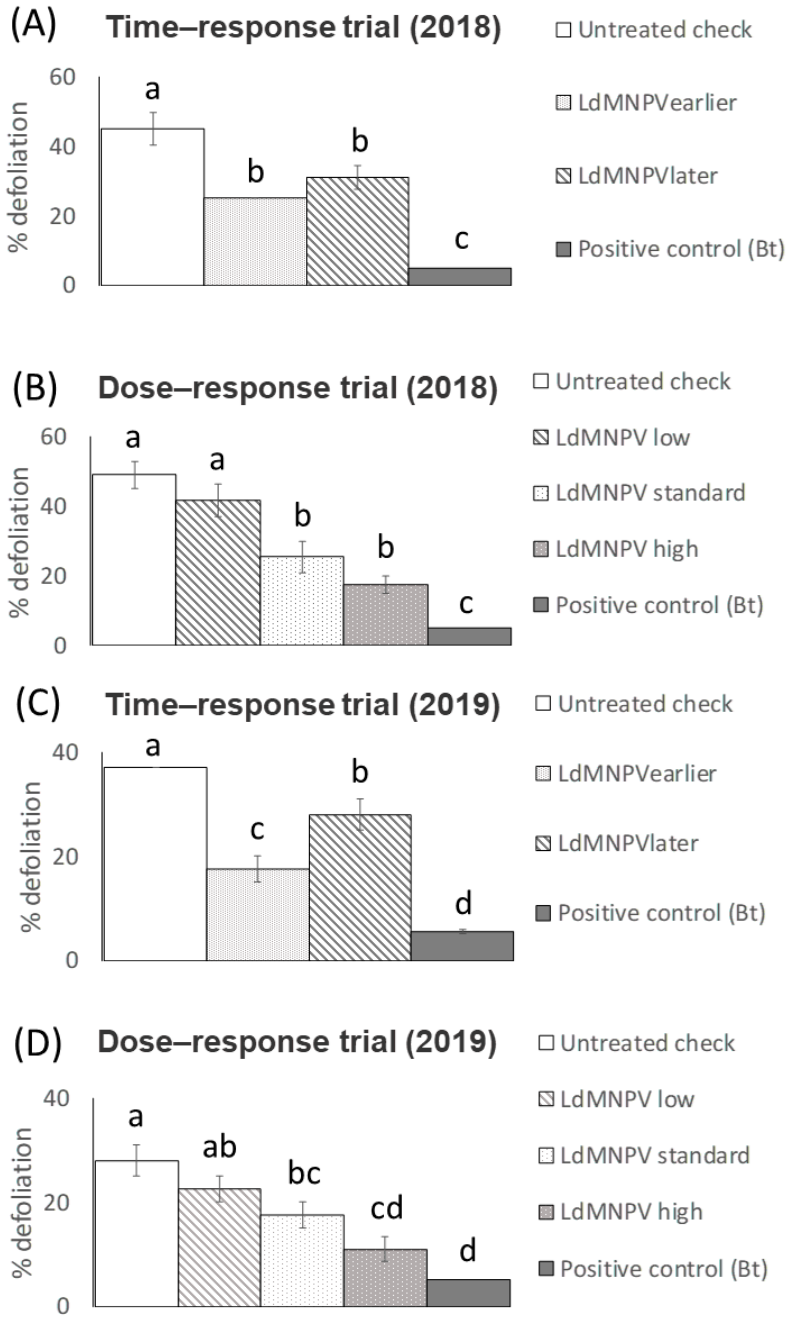

Figure 5. Defoliation percentage (mean ± SE) in different plots treated with LdMNPV from the ground in 2018 (**A**,**B**) and 2019 (**C**,**D**). Different letters (a, b, c, d) above bars indicate significant differences among means (ANOVA, Tukey test, $p < 0.05$).

On the other hand, significant differences in survival rate were observed in the laboratory on the field-collected larvae from different plots ($\chi^2 = 486.79$, $p < 0.01$), with a significant reduction associated with larvae from plots treated with either LdMNPV or *Btk* (Figure 6). In more detail, the survival rate achieved at the end of the observation period was higher for LdMNPV (12%) than *Btk*-treated larvae (0.7%) ($z = -15.73$, $p < 0.01$). The highest survival rate (52%) was instead associated with larvae from untreated plots ($z = -9.08$, $p < 0.01$). The reduction in surviving larvae attributable exclusively to *Btk* and LdMNPV was significantly different between these formulations, either 7 ($t = 7.16$, $p < 0.01$) and 14 ($t = 13.44$, $p < 0.01$) days after applications. Instead, no statistical differences in

corrected larval reduction were found between *Btk* and LdMNPV, 21 days after application (t = 2.75, p = 0.10) (Table 4).

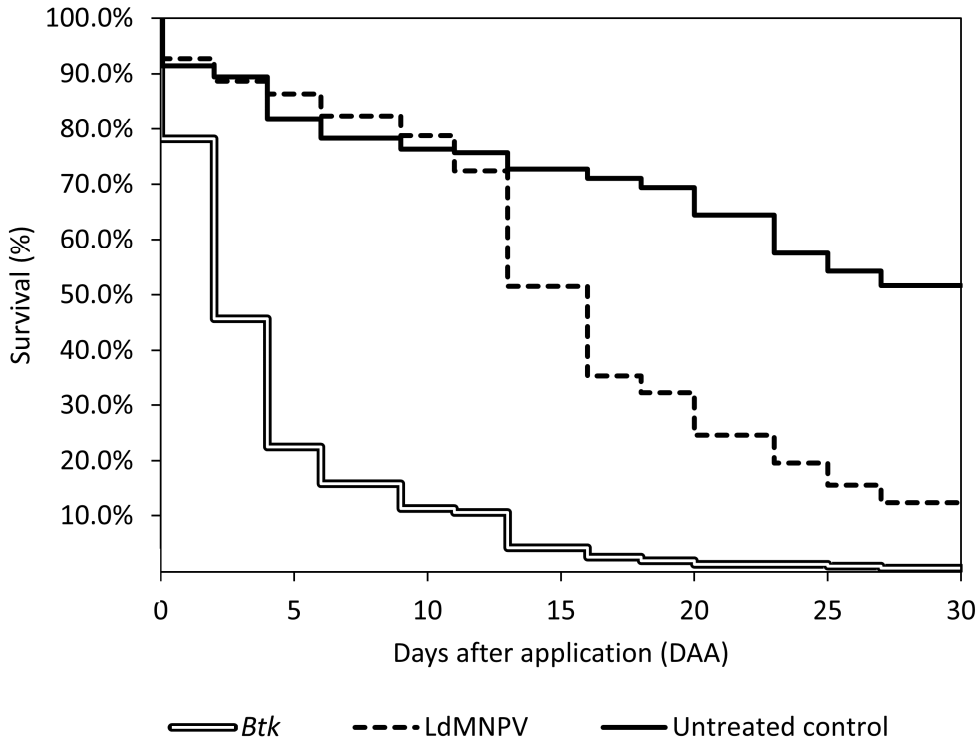

Figure 6. Survival rate of field-collected *Lymantria dispar* larvae from plots treated with Foray76B, LdMNPV formulation, or untreated (control).

Table 4. Reduction percentage (mean ± SE) of surviving larvae in the laboratory attributable exclusively to treatments at different time intervals from bioinsecticidal application.

Days [a]	Treatment [b]		t	p
	Foray 76B	LdMNPV		
7	75.44 ± 4.55 [a]	10.05 ± 2.67 [b]	7.16	0.004
14	86.74 ± 1.80 [a]	21.94 ± 2.12 [b]	13.44	<0.001
21	96.33 ± 0.41 [a]	70.19 ± 5.47 [a]	2.75	0.010

[a] Days after application. [b] Different letters in each line indicate significantly different means (Student's t-test, $p < 0.05$).

4. Discussion

Baculoviruses represent natural and selective bioinsecticides and have successfully been used against several Lepidopteran pests worldwide. However, their use is limited to niche contexts, due to their narrow host range, a delayed insecticidal action in respect to synthetic chemicals, and economical issues related to industrial production technologies still necessarily relying on the use of living insects as substrates for virus replication [18].

Lymantria dispar multicapsid nucleopolyhedrovirus (LdMNPV) formulation used in this study showed good efficacy against gypsy moth larval populations in Sardinian forest areas, where this pest is the cause of important defoliations during its periodic outbreaks [19]. In the experiments conducted with applications from the ground, the lethal effects were dose- and time-dependent, with a higher efficacy achieved with higher doses and earlier treatments. These results align with a pathogenic process that begins with the ingestion of occlusion bodies (OBs) releasing occlusion-derived viruses (ODVs) that act in the midgut, infecting epithelial cells [20]. Accordingly, a stronger and faster effect is

expected as a consequence of the earlier ingestion of a higher number of viral particles [21]. It follows that in order to ensure baculovirus' short-time effectiveness, an early application in the season, possibly against the first instar larvae, is of primary importance.

While a good baculovirus efficacy was achieved in these experiments, larval mortality was significantly lower in comparison with plots treated with *Btk*, which was confirmed to be a powerful bioinsecticide against gypsy moth [22,23].

Higher scale experiments involving larger areas and aerial applications of the bioinsecticidal products employing standard doses (2 L/ha) confirmed a reduced survival rate of baculovirus-treated larvae, in respect to the untreated control. Additionally, in this case, *Btk* treatments generated a higher lethal-effect. This greater knock-down power relates to the mechanism of action of solubilized and activated bacterial crystal toxins (Cry proteins) interacting with and disrupting midgut epithelial cells, which leads to insect paralysis and death [24]. This direct toxicity caused by *Btk* is in antithesis with a slower action of the baculovirus depending on an infectious process involving replication of the virus and its spread within the insect body through the tracheal system [20]. Everything considered, a milder action of the virus compared to *Btk* clearly emerged in field trials. Despite such differences, larval population density in 2019 was affected by a natural reduction associated with all treated and untreated plots and related to gypsy moth population retrogradation in Sardinian forest. Accordingly, a more evident efficacy of baculovirus applications in large areas is expected during population progradation, when the baculovirus can express its full potential as a natural regulator of moth population dynamics [25]. Thus, a higher host density triggers horizontal transmission processes, determining a greater number of infected individuals [26]. While these microparasites can naturally regulate periodic cycles of host abundance, their artificial introduction in the forest ecosystem by early applications in the season would produce a similar effect, under appropriate density dynamic conditions. Such density-dependent containment ability has also been demonstrated in laboratory experiments, in which different degrees of resistance to the baculovirus were associated with diverse larval densities [27].

Besides an action normally contained during the season of application, the virus introduced into the forest environment is expected to produce an additional impact on the following generations as a result of sub-lethal effects and vertical transmission [28]. This expectation supports the use of baculovirus against gypsy moth even if the efficacy in the application season is limited. Following an integrated approach to forest management, baculovirus with a slow action, but a detectable midterm impact on subsequent generations, could be combined with applications of *Btk* that generate a more immediate knockdown effect. However, such an emerging hypothesis needs specific multiyear studies to be appropriately documented.

On the other hand, however, it is important that the application of these microbiological control agents is calibrated on the basis of the actual conditions of population dynamics at a given time, in order to produce the desired pest containment effects and make these low-environmental impact interventions even more economically viable. Thus, gypsy moth baculoviruses are good candidates to be introduced in gypsy moth multi-year management programs aiming at interfering with their natural population dynamics.

5. Conclusions

Based on the obtained results in small-scale trials, *L. dispar* showed a significant susceptibility to the LdMNPV formulation, when applied at higher doses and against younger larvae. The highest dose achieved a good efficacy in protecting the crop, albeit at a lower degree than the *Btk* reference product. Such efficacy was not confirmed in larger-scale trials conducted by aerial applications, partly due to population dynamics affected by a natural retrogradation phase. However, a significant increased mortality of larvae collected in plots treated with the baculovirus was detected. Given a higher susceptibility of younger larvae, earlier applications are recommended.

Everything considered, the use of LdMNPV in forest protection programs against the gypsy moth is worth further consideration under different infestation conditions. Its efficacy in regulating population dynamics during outbreaks is expected to be maximized under progradation [29]. This ecological effect could be exploited in a multi-year integrated program involving the combined use of *Btk* to contain infestations and of the baculovirus to modulate population dynamics.

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Phylogenetic Relationships between *Phlebiopsis gigantea* and Selected *Basidiomycota* Species Inferred from Partial DNA Sequence of Elongation Factor 1-Alpha Gene

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Abstract: *Phlebiopsis gigantea* (Fr.) Jülich has been successfully used as a biological control fungus for *Heterobasidion annosum* (Fr.) Bref., an important pathogen of pine and spruce trees. The *P. gigantea* species has been known for many years, but our understanding of the relationship between various isolates of this fungus has been substantially improved through the application of DNA sequence comparisons. In this study, relationships between *P. gigantea* and selected *Basidiomycota* species was determined, based on elongation factor 1-alpha (*EF1α*) partial DNA sequence and *in silico* data. A total of 12 isolates, representing the most representatives of *P. gigantea*, with diverse geographic distributions and hosts, were included in this study. Phylogenetic trees generated for sequences obtained in this research, grouped the European taxa of *P. gigantea* and partial sequence of the genome deposed in NCBI database, in a strongly supported clade, basal to the rest of the strains included in the study. *P. gigantea* isolates originating from Poland, Finland, Sweden, Great Britain and partial sequence of genome formed a monophyletic group. Within this group, isolates of *P. gigantea* constituted two subclades, showing their partial difference like the two SNPs (single nucleotide polymorphisms) between one and the rest of isolates. The intron and exon relationships among *P. gigantea* isolates were moreover resolved. The results obtained using the *EF1α* region should be useful in the selection of more efficient *P. gigantea* isolates for limiting forest tree root pathogens.

Keywords: *Phlebiopsis gigantea*; *EF1α*; introns; exons; phylogenesis

1. Introduction

Phlebiopsis gigantea (Fr.) Jülich has widely been used as a biological control of the fungus *Heterobasidion annosum* (Fr.) Bref., the causative factor of conifer root rot infections [1–12]. In the literature this species is described with multiple synonymous names, e.g., *Corticium giganteum* (Fr.) Fr., *Peniophora gigantea* (Fr.) Massee, *Peniophora gigantea f. pruinosa* Pilát, *Phanerochaete gigantea* (Fr.) S.S. Rattan, *Phlebia gigantea* (Fr.) Donk. The fungus, after the taxonomic revision made by Jülich (1978) In: Parmasto and Hallenberg [13], was finally placed in the new genus *Phlebiopsis*, which is now commonly accepted. The fungus is a typical saprotroph, regularly colonizing dead parts of coniferous woods, occurring mostly in the Northern Hemisphere, but it has also has a worldwide distribution; for example, few strains have been retrieved from South Africa, New Zealand and Canada [9,14].

According to the Index Fungorum database (www.indexfungorum.org) the taxonomic status of *P. gigantea* is well recognized and there are at least 23 species included in the genus *Phlebiopsis*, based on morphological characteristics. A number of techniques have been employed to identify *P. gigantea* and traditionally, the morphological characters of the spores were used for this purpose. Although the classical methods are reasonably easy and fast to apply [15], molecular techniques confirm identification of this fungus and are very useful for identification of species [16,17].

Various molecular markers used in barcoding, like ITS 1/2, β-tubulin, histone H3 and elongation factor α (syn. *EF1α*), are the most commonly applied in fungal taxonomy. Their application allows determination of fungal genotypes at a species level [18–20]. Comparisons of DNA sequence data are increasingly being used in order to gain knowledge concerning the phylogenetic relationships among *P. gigantea* isolates [7,10,11,21,22]. Many studies have utilised DNA sequence data of the *EF1α* gene for phylogenetic analyses including a wide range species of fungi [23,24].

The objectives of this study were to obtain DNA partial sequences for *EF1α* for *P. gigantea* strains, and to compare them with other some *Basidiomycota* species from the NCBI database. This gene is a highly conserved ubiquitous protein involved in translation that has been suggested to have desirable properties for phylogenetic inference [25]. It has been successfully used in phylogenetic studies as a phylogenetic marker for *Eukaryotes*, *Acomycetes* and *Basidiomycota* [23–25] Additionally, the partial sequences (intron and exon partial regions) obtained in the study of *EF1α* gene isolates of *P. gigantea* from Poland, Finland, Sweden and Great Britain, and partial genome sequence (gi:752829739) deposited in GenBank by Hori et al. [26] were investigated. To date, in studies on the differentiation of chosen *P. gigantea* isolates on the known activity of linear growth and wood decay [3,4,11,27] the *EF1α* gene has not been studied.

Our study provides an additional gene region useful for testing taxonomic groupings and phylogenetic relationships, previously identified based on the other gene regions like ITS [2,3]. Several factors can affect biological activity of the fungus. Grossbard [28] reported, that the presence of some fungi in soil can modify biological traits of co-occurring taxons. Schardl and Craven [29] described that the variation in enzyme and decay activity of fungal isolates in time may suggested risks in lost or change the molecular and biochemical characteristics. The cause is showed in possible hybridisation from the mating of clearly homozygous individuals. Żółciak et al. [3] and Sierota et al. [27] suggested the changes in the activity of different *P. gigantea* isolates with time, its origin, and wood density. For the effective use of competitive fungi used in biopreparations against pathogens in biological control (e.g., *P. gigantea*), there is a need for periodic exchange of strains for more effective ones [4]. Checking the utility of the *EF1α* region can be a valuable clue and can help in making decision regarding the selection of the most effective *P. gigantea* isolates as a competitor of *Heterobasidion* spp.

2. Materials and Methods

2.1. Cultivation of Isolates

Twelve previously identified and tested isolates of *P. gigantea* [11] were used in the experiment: six from Poland (not registered as biocontrol agent) and one from Finland, one from Sweden and four from Great Britain (registered as biocontrol agent) (Table 1). The number of Polish isolates was limited to six due to difficulties in obtaining homogeneous single-spore cultures, while the Finnish and British isolates were accepted as previously tested and approved. Isolates were grown on potato dextrose agar (PDA) medium (Difco™, Sparks, MD, USA) in Petri dishes for ten days at 20 °C according to Kwaśna et al. [30].

Table 1. List of *P. gigantea* isolates used in the study.

Polish Isolate Codes	Location Coordinates/Forest District/Country	Host	Substrate	Collector	Collection (Institution Name, Isolate Code, Country)
PL Pg_1	x:52.09859 y: 20.85479 Chojnów FD, Poland	*Pinus sylvestris* L.	Stump wood	A. Żółciak	Forest Research Institute, 01.06.08.1.5; 02.10.23.1.2; 03.11.04.1.1; 03.11.13.1.3; 99.09.10.1.1; 04.11.19.1.2 Poland
PL Pg_2					
PL Pg_6					
PL Pg_9					
PL Pg_{11} (Accession number: KU886024)	x:51.22000 y: 20.33157 Barycz FD, Poland		Fruitbody on stump	Z. Sierota	
PL Pg_{12}	x:53.42508 y: 20.59593 Nidzica FD, Poland		Stump wood	A. Żółciak	
SE Pg_8	Råberg near Uppsala, Sweden	*Picea abies*(L.) Karst.		K. Korhonen	Lallemand Plant Care - Verdera Oy, VRA 1984 *, Finland
FI Pg_{10}	Loppi, Finland		Log wood		Lallemand Plant Care - Verdera Oy, VRA 1835 *, Finland
GB Pg_{14}	Mull, Great Britain	*Pinus contorta* Dougl. ex Loud.	No data	No data	Forestry Commission, FOC PG 410.3 *, Great Britain
GB Pg_{15}	Roslin, GB	No data			Forestry Commission, FOC PG SP log 5 *, Great Britain
GB Pg_{16} (Accession number: KU886025)	NRS, GB	*Pinus sylvestris* L.			Forestry Commission, FOC PG B 20/5 *, Great Britain
GB Pg_{17}	Buchan, GB				Forestry Commission, FOC PG BU 3 *, Great Britain

* isolates registration in European Union.

2.2. DNA Extraction

Total fungal DNA of *P. gigantea* was extracted from mycelium grown on PDA by using DNeasy Plant Mini Kit (Qiagen, Hilden, Germany), according to the protocol. Quality of the DNA was checked with an Infinite 200 PRO multimode plate reader (Tecan, Group Ltd., Männedorf, Switzerland).

2.3. Primers and PCR Conditions

The PCR reactions were done in 25 µL volumes using a Veriti Thermal Cycler (Applied Biosystems, Foster City, CA, USA). Each reaction contained 1 unit (0.25 µL) of Taq DNA polymerase (recombinant) (Thermo Scientific, Life Technologies Inc., Carlsbad, CA, USA), 2.5 µL of 10 × Taq buffer, 2 mM of each dNTP (0.5 µL), 1.5 mM of $MgCl_2$ (1.5 µL), 12.5 pmol of forward/reverse primers (0.125 µL), and 20 ng (1 µL) of DNA.

The primers and the touchdown PCR reaction conditions were used according to modified procedure of Rehner [18]. Amplicons of the partial region of the *EF1&* gene were generated using two overlapping primer combinations, 526F (5′ GTC GTY GTY ATY GGH CAY GT 3′) × 1567R (5′ AC HGT RCC RAT ACC ACC RAT CTT 3′) and EFdF (5′ AAG GAY GGN CAR ACY CGN GAR CAY GC 3′) × 2218R (5′ AT GAC ACC RAC RGC RAC RGT YTG 3′). For 526F × 1567R primers the touchdown PCR was as follows: 5 min at 94 °C (an initial denaturation), 30 s at 94 °C (denaturation) and 45 s at 60 °C (annealing) in the first cycle, successively reducing the Tm by 1 °C per cycle over the next 9 cycles to a

final Tm 50 °C, which was used in the remaining 36 cycles. An extension step per cycle was 1 min 30 s at 72 °C. The final extension step was 1 cycle of 7 min at 72 °C.

PCR parameters for EFdF × 2218R primers were as follows: 5 min at 94 °C, 45 cycles of (30 s at 94 °C, 30 s at 63 °C, 1 min 30 s at 72 °C) and 7 min at 72 °C. Amplicons were run in 1.5% (w/v) ethidium bromide-stained agarose gels (Sigma-Aldrich, St. Louis, MO, USA) and the bands were visualized under UV illumination. PCR products were sequenced using Sanger's sequencing method at the Polish Academy of Sciences Institute of Biochemistry and Biophysics (Warsaw, Poland).

2.4. DNA Sequence Analysis

Sequencing results were analyzed using the BLAST algorithm on The National Center for Biotechnology Information (NCBI, https://blast.ncbi.nlm.nih.gov/) database and MEGA version 5 [31]. The DNA sequences were aligned using Clustal W version 2.0. [32]. Genetic variability was estimated for the sequences of *P. gigantea* and realigned separately for intron and exon regions using FGENESH 2.6 [33,34].

The obtained sequences were analyzed using Tajima's Neutrality Test. The number of sequences (m), number of segregating sites (S), mean number of segregating sites (Ps), nucleotide diversity (π) and estimates of Theta (Θ) per site as well as the Tajima test statistic (D) were estimated using MEGA5 program [35,36].

EF1α partial gene sequences of *P. gigantea* and selected *Basidiomycota* species from the NCBI database were analyzed using the maximum likelihood approach for phylogeny reconstruction and tested by bootstrapping with 1,000 replicates. Missing and ambiguous characters were excluded from the analysis. Phylogenetic trees were generated based on maximum likelihood method [37]. Two sequences of *P. gigantea* were deposited in GenBank NCBI (Accession numbers: KU886024 and KU886025, www.ncbi.nlm.nih.gov.genbank/).

3. Results

EF1α Partial Gene of P. gigantea Analysis

The region of the *EF1α* gene was successfully amplified for all *P. gigantea* isolates described in this study. All amplifications yielded a single band approximately 1,500 bp long. The final analyzed length partial sequence of the *EF1α* gene was 1,411 bp, the mRNA length was 1,403 bp. The structure of *EF1α* partial gene from the *P. gigantea* (Accession numbers: KU886024 and KU886025) is shown in Figure 1.

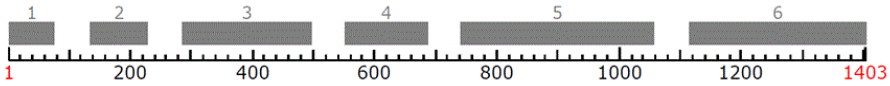

Figure 1. The structure of *EF1α* partial gene from the *Phlebiopsis gigantea*. The positions for exons are presented respectively: exon 1 (1–75), exon 2 (134–228), exon 3 (284–496), exon 4 (550–686), exon 5 (739–1056), exon 6 (1113–1403). (mRNA) 1–6 exon (s): 1–1403. The numbers indicate the positions of the bases identified using FGENESH software.

Genetic diversity was calculated for all obtained sequences including exons and introns of 12 partial sequences of the *EF1α* gene and partial genome sequence (gi:752829739) of *P. gigantea* (Figure 1) and separately for exons and introns (Table 2) The number of segregating sites (*S*) and mean number of segregating sites (*Ps*) were 32 and 0.022679 respectively. The value of the Θ, expressing the total variability was 0.007308 for analyzed 13 partial sequences mentioned above. The nucleotide diversity (π) was 0.005761. The value of Tajima's *D* was below zero (−0.931820).

In the I_1 and I_2 intron region the number of segregating sites was one. The remaining introns I_3, I_4 and I_5 showed three and six segregating sites, respectively. The value of the Θ, expressing the total variability ranged from 0.005556 to 0.034526 for all intron part sequences. The nucleotide diversity (π)

ranged from 0.004863 to 0.025641. The analysis of exons of the coding regions showed no segregating sites for partial E_1, however the number of segregating sites ranged from one for E_2 to seven for E_6. The value of the Θ was zero for partial E_1 and the highest 0.007544 for E_6. Among exons the nucleotide diversity (π) ranged from zero to 0.006176. The value of Tajima's *D* was below zero for all intron and E_2–E_6 exon regions (Table 2).

Table 2. Genetic polymorphism and neutrality tests of *EF1α* partial gene sequences of 12 Polish isolates and partial genome sequence (gi:752829739) of *P. gigantea*, including introns and exons.

Region	Sequence	*bp*	*m*	*s*	P_s	Θ	π	*D*
Intron	I_1	58	13	1	0.017241	0.005556	0.004863	−0.274290
	I_2	55	13	1	0.018182	0.005859	0.005128	−0.274290
	I_3	53	13	3	0.056604	0.018240	0.013546	−0.813698
	I_4	52	13	3	0.057692	0.018591	0.013807	−0.813698
	I_5	56	13	6	0.107143	0.034526	0.025641	−0.950320
Exon	Partial E_1	75	13	0	0.000000	0.00000E+000	0.000000	*n/c*
	E_2	95	13	1	0.010526	0.003392	0.002969	−0.274290
	E_3	213	13	4	0.018779	0.006052	0.004093	−1.099317
	E_4	137	13	3	0.021898	0.007057	0.006176	−0.394391
	E_5	318	13	3	0.009434	0.003040	0.002661	−0.394391
	E_6	299	13	7	0.023411	0.007544	0.006174	−0.688629

Abbreviations: *bp*, base pairs; *m*, number of sequences; *s*, number of segregating sites; $P_s = s/m$, mean number of segregating sites; $\Theta = P_s/a_1$, estimates of Theta per site; π, nucleotide diversity; *D*, Tajima's *D* Tajima test statistic; *n/c*, no changes.

Bootstrap values supported the separation of species into distinct clades (Figure 2). Among partial 12 sequences of *P. gigantea* the closest one to the partial genome sequence of *P. gigantea* (gi: 752829739) was GB Pg 16 (KU886025) from Great Britain. The couple of single mutations differentiated GB Pg 16 sequence from the rest of identical isolates of this fungus.

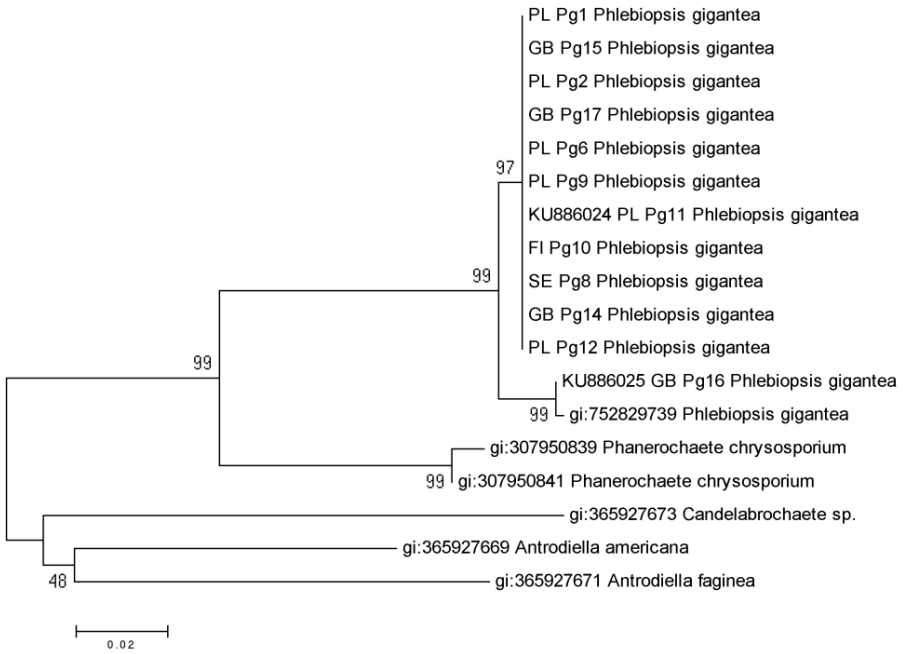

Figure 2. Phylogenetic tree for *Phlebiopsis gigantea* isolates and other species from the *Phanerochaetaceae*, based on *EF1α* gene partial sequences. Branch length values were shown, the tree was reconstructed using the maximum likelihood approach and tested by bootstrapping (1,000 replicates).

For *Phanerochaetaceae* family, two major and well supported groups were identified. One of the group represented all *P. gigantea* and *Phanerochaete chrysosporium* Burds. partial sequences (97–99% bootstrap support). The second group included: *Candelabrochaete* sp., *Antrodiella Americana* Ryvarden & Gilb. and *Antrodiella faginea* Vampola & Pouzar (48% bootstrap support). Phylogenetic analysis of partial sequences of *EF1α* gene for *P. gigantea* and selected *Basidiomycota* species, showed separate clades for following orders: *Polyporales*, *Agaricales*, *Boletales*, *Atheliales*, *Russulales* and *Sebacinales* (Figure 3).

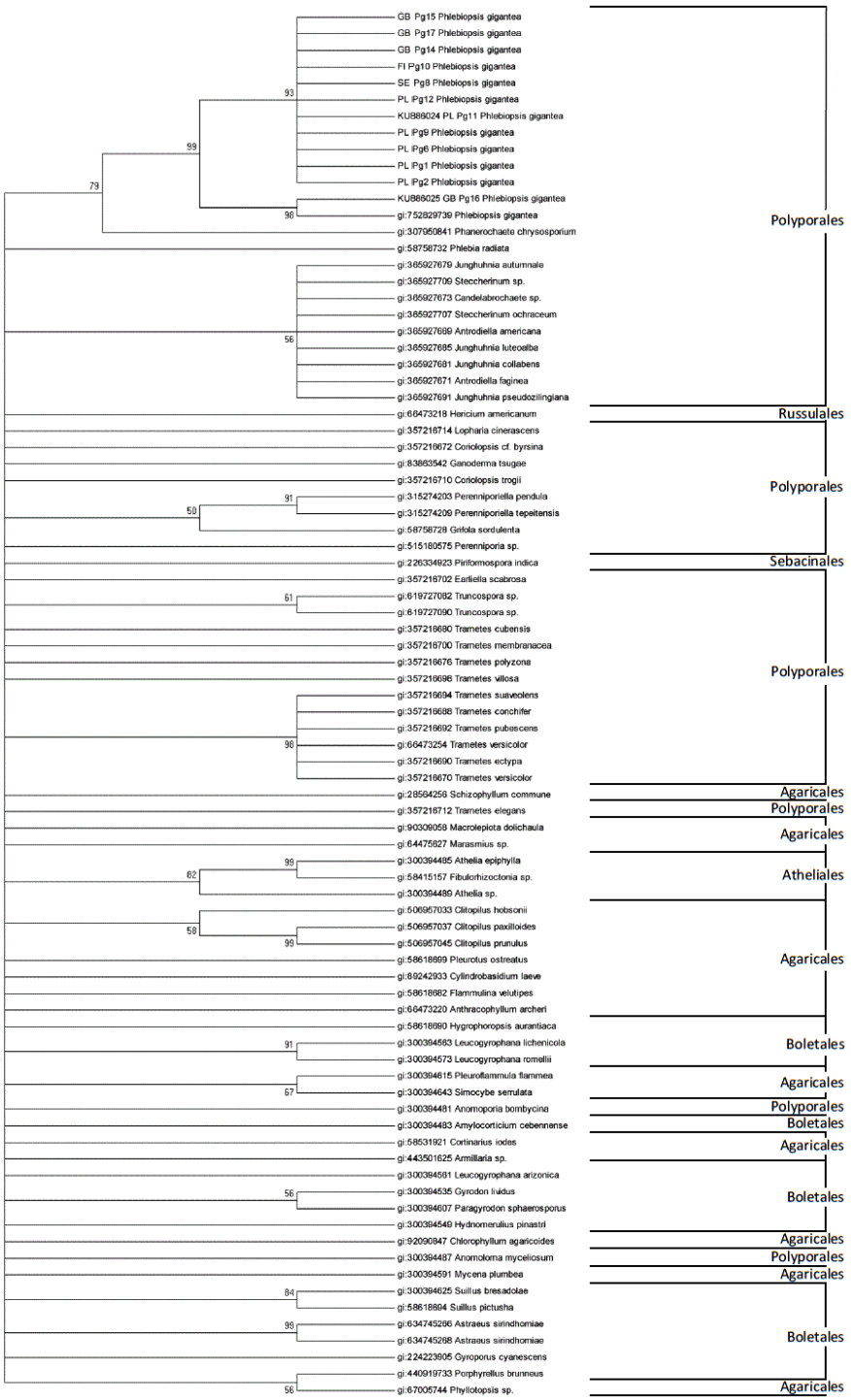

Figure 3. Phylogenetic tree for *Phlebiopsis gigantea* isolates and selected *Basidiomycota*, based on the *EF1α* partial gene sequences. The tree was reconstructed using the maximum likelihood approach and tested by bootstrapping (1000 replicates).

4. Discussion

In this study, DNA partial sequence for the *EF1α* gene of *Phlebiopsis gigantea* was successfully identified and analyzed. All *P. gigantea* isolates yielded PCR products of similar size, indicating that the amplified partial gene region does not include large indels, and therefore is a suitable choice for phylogenetic studies. The aligned sequences showed considerable homology among *P. gigantea* but various species-specific nucleotide substitutions and indels were observed among all *Basidiomycota* species. No sequence variation was observed among all *P. gigantea* except of one isolate (GB Pg 16) from Great Britain and partial sequence of *P. gigantea* genome (gi: 752829739).

The remarkable variation was observed between different strains. This is consistent with previous studies employing ITS region and genetic fingerprinting using random amplified microsatellite (RAMS) markers in taxonomic studies of *P. gigantea* [21].

For the analyzed *P. gigantea EF1α* partial gene the occurrence of exons and introns was identified. Introns represented the so-called 3rd introns group, spliced during the maturation of RNA with the participation of spliceosome [38]. The size of introns ranged from 52 to 58 bp, at the average length of the gene from 0.75 to 1,000 bp of gene.

Among representatives of *Basidiomycota* six introns in gene are observed average [39]. Compared to higher *Eukaryota*, introns occurring in fungi are relatively short [40]. In the case of the analyzed gene, introns size corresponds to the average size of introns identified in representatives of the *Fungi* kingdom (50–200 bp) [41]. The length of introns of *EF1α* gene was slightly smaller than the average for the *Puccinia graminis* (0.65 kb, NCBI accession number: X73529.1) and *Neurospora crassa* gene (0.81 kb) [42].

Despite the randomness and variability of the structure of introns, in the case of I_5 of *EF1α* partial gene, a larger number of segregating sites (six) was identified. In the sequences of other introns this number were from 1 to 3. Furthermore, the characteristic feature of the identified intron sequences was the presence of the sequence GT on the donor side (5′) and the sequence AG at the acceptor side (3′). They are essential in the process of identifying and splicing by spliceosome. Identified GT and AG sequences are considered as the most commonly occurring canonical dinucleotide fragments, respectively starting and ending the introns [43].

Intraspecies analysis of *EF1α* partial gene showed small variability within the coding and non-coding regions. Introns of analyzed *EF1α* partial gene were well conserved among all tested isolates of *P. gigantea.* The evolution of the structure of all introns and E_2–E_6 exon regions of *EF1α* partial gene was neutral. Isolates representing *P. gigantea* from Poland, Great Britain, Finland and Sweden had similar partial sequences of *EF1α* gene and were grouped together. The only British isolate (GB Pg 16 = FOC PG B 20/5) of this fungus showed coupled single mutations like single nucleotide polymorphisms (SNPs) and was very similar to *P. gigantea* (gi: 752829739) with identity 99% and query cover 100%.

Results of the study using DNA-RAMS markers indicated genetic similarity among isolates collected in Finland and Great Britain [10]. Additional studies using the previously mentioned markers showed that Polish isolate (PL 12) of *P. gigantea* was genetically analogous to FC 16 from Great Britain [11]. Vainio and Hantula [21] also showed that European and North American ITS/A alleles of *P. gigantea* were identical, while ITS/C alleles were different. The authors mentioned that the analysis of molecular variation and neighbor joining analysis using 28 RAMS markers revealed a considerable degree of differentiation between Europe and North America [21].

The main advantage of this study is the phylogenetic analysis of *EF1α* partial DNA sequence data for *P. gigantea* in comparing it to species belonging to the same family—*Phanerochaetaceae* and selected *Basidiomycota* species. Phylogenetic trees showed that *P. gigantea* is closely related to *Phanerochaete chrysosporium. EF1α* is also the first protein-coding gene and first single-copy gene used for phylogenetic analysis of *P. gigantea*. The whole genome sequence of *P. gigantea* (gi: 752829739, accession number: AZAG01000080) was published by Hori et al. [26]. The whole genome sequence size is approximately 30 Mbp and number of predicted genes (11,891). Sequence data from the majority of isolates belonging

to the different species showed unique species-specific substitutions, allowing the isolates to be differentiated into clades representing the species.

The results of this study demonstrate that the *EF1α* region is useful for phylogenetic analysis and classification of *Polyporales* species. This is a large and taxonomically difficult order, which include several genera, for example: *Phlebiopsis*, *Phanerochaete*, *Phlebia*, *Junghuhnia*, *Steccherinum*, *Androniella*, *Ganoderma*, *Coriolopsis*, *Perenniporiella* and *Trametes*.

5. Conclusions

Our in silico studies showed a relationship between *P. gigantea* and selected *Basidiomycota* based on elongation factor 1-alpha partial DNA sequence. The partial *EF1α* gene sequence of *P. gigantea* isolates originating from Poland, Finland, Sweden and Great Britain formed a monophyletic group, except for one British isolate GB Pg 16 (FOC PG B 20/5). The results of our study proved that the partial sequence of *EF1α* gene is useful for phylogenetic analysis of the *Phanerochaetaceae* family.

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forests

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Review

Alien Invasive Pathogens and Pests Harming Trees, Forests, and Plantations: Pathways, Global Consequences and Management

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Abstract: Forest health worldwide is impacted by many invasive alien pathogens and pests (IAPPs) that cause significant harm, with severe economic losses and environmental alterations. Destructive tree pathogens and pests have in the past devastated our forests, natural landscapes and cityscapes and still continue to represent a serious threat. The main driver of pathogen and pest invasions is human activities, above all global trade, which allows these invasive species to overstep their natural distribution ranges. While natural transport occurs according to a regular, expected colonization pattern (based on the dispersive capacity of the organism), human-mediated transport takes place on a larger, unpredictable scale. In order for a pathogen or pest species to become invasive in a new territory it must overcome distinct stages (barriers) that strongly affect the outcome of the invasion. Early detection is crucial to enabling successful eradication and containment. Although sophisticated diagnostic techniques are now available for disease and pest surveillance and monitoring, few control and mitigation options are usable in forestry; of these, biological control is one of the most frequently adopted. Since invasion by pathogens and pests is an economic and ecological problem of supranational relevance, governments should endorse all necessary preventive and corrective actions. To this end, establishing and harmonizing measures among countries is essential, both for preventing new introductions and for diminishing the eventual range expansion of IAPPs present at a local scale. Research is fundamental for: (i) developing effective and rapid diagnostic tools; (ii) investigating the epidemiology and ecology of IAPPs in newly introduced areas; and (iii) supporting policymakers in the implementation of quarantine regulations.

Keywords: forest insects; forest diseases; diagnostics; mitigation options; citizen science

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1. Introduction

Forests are of primary importance, being a source of both income and well-being for human populations worldwide; however, biological invasions pose a serious threat to their economic and ecological sustainability globally. The damage caused by biological invasions is estimated to be second only to that resulting from habitat destruction/fragmentation [1]. In fact, every year a substantial part of the world's forests is destroyed or seriously damaged by invasive alien pathogens and pests (IAPPs) [2].

The exponential increase in introduction of IAPPs to new habitats is a complex phenomenon with multiple factors. Currently, the foremost cause is the increase in trade, especially that of plants, on a global scale [3]. The economic and social transformations that have taken place in recent decades have had, and continue to have, a direct effect on the introduction of harmful organisms. In particular, the evolution in transportation vehicles has favoured an ever greater and faster handling of plants, foodstuffs, and plant materials [4]. Another leading factor is the dramatic increase in human mobility, whether for work or tourism; it is not uncommon for alien organisms to be transported from one country to another this way, both inadvertently (for example, through contaminated seeds

or dirt stuck to footwear or clothing) and intentionally (e.g., seeds, bulbs, fruits, souvenirs made with plant materials) [5,6].

Once the IAPPs have reached a new territory, other factors, such as anthropogenic disturbance, can favour their establishment. The alteration of habitats and changes to natural ecosystems—via the construction of roads, dams, power lines, buildings, or due to crop conversions, deforestation, changes in land use, etc.—create favourable conditions for their invasion [7,8]. Specifically, all these modifications: induce a release of resources; influence the interactions between species; alter the substrates; and modify the physical environment [9]. It is no coincidence that the habitats most disturbed by urbanization and tourism, such as those in coastal and island areas, lakes, rivers, and periurban forests, are where most alien species are found [10].

Another factor involved in IAPP invasions is climate change. The current climate trend towards global warming acts directly on IAPPs by creating favourable conditions for their establishment in formerly unsuitable areas [11]. In addition, higher temperatures enhance the fitness of the more thermophilic IAPPs, favouring their reproductive and dispersive biology; furthermore, milder climates increase the number of yearly reproductive cycles and of individuals surviving the winter season [12]. Changes in climate additionally bolster IAPPs indirectly by making plants more susceptible, since adverse climatic factors, such as extended drought and extreme weather events (e.g., rainstorms, windstorms, hail, and severe flooding), impact plant health, reducing plant growth and vigour, altering phenology, impairing physiological processes, and creating wounds that favour IAPP attacks [13,14].

Some of the major impacts of forest IAPPs are: reduction of primary production; alterations of evolutionary processes, both in host populations and in organisms taxonomically related to the invaders; changes in ecological relationships within tree communities (for example, alteration of symbiotic relationships, either mutualistic or antagonistic, between the tree species and their animal or microbial symbionts); decrease or loss of ecosystem services (water filtration and supply, climate mitigation, erosion prevention, nutrient cycling, carbon storage, and habitat provision); and demise of native species (extinction) [12,15].

The governments of various countries are paying great attention to the problem of the introduction of organisms harmful to agriculture and forestry; hence, phytosanitary protection has become an important component of their agricultural and forestry policies [16]. In an attempt to counter this growing phenomenon, many countries have started adopting increasingly stringent measures aimed at ensuring the early detection of banned pathogens and pests, as well as at eradicating harmful quarantine organisms present locally [17]. Furthermore, intergovernmental regional plant protection organizations try to tackle the problem on a regional scale, aiming to ensure adequate levels of plant protection through a coordinated series of phytosanitary actions [18].

2. IAPP Pathways

The movement of IAPPs to novel locations can occur naturally or through human-mediated processes. Natural movement, even over long distances, always occurs along a trajectory that follows a regular, expected colonization pattern [10]. The populations that colonize a new environment normally originate from nearby areas, such as neighbouring islands or the adjacent mainland, and, in any case, they follow a path that can be predicted according to existing knowledge about their biology, the biology of their vectors, the prevailing winds and water currents (in particular for oomycetes), as well as their dispersal capabilities (migratory capacity) [10]. Human-mediated transport, on the other hand, takes place on a much larger scale [19], since a given species can be taken from one point on the planet and released on the opposite side of the globe. Such transportation both breaks up natural biogeographic barriers and disrupts natural evolutionary processes, constituting a concrete threat to the planet's biodiversity [20]. Such anthropogenic conveyance of agricultural and forest pests has increased immensely in terms of frequency, geographical breadth, and number of species involved.

Among the human-dependent causes of the introduction of forest IAPPs, postal traffic, tourist activities, and, of course, global trade play a leading role [6]. The movement of people in general, either international or domestically, influences the amount of interception. Liebhold et al. [21] has quantified this and showed how the number of insect pests intercepted in baggage was positively correlated to the number of travellers entering the U.S. As regards trade-related transport, the main pathways are germplasm (tree seeds, cuttings, and plants for planting) and wood (logs, firewood, processed wood, as well as packaging material, etc.). According to Meurisse et al. [6], live plants and wood packaging material are the main source for movement of forest IAPPs beyond their range limits into new, uncontaminated forest areas, based on the data collected in the ports of entry in the U.S. and New Zealand.

Live plants and wood packaging material have also been confirmed as the main pathways for IAPPs in Europe based on interceptions at ports of entry during the last 5 years (Figure 1). The European Union Notification System for Plant Health Interceptions (Europhyt) (https://ec.europa.eu/food/plants/plant-health-and-biosecurity/europhyt/interceptions_en, accessed on 1 June 2021) records interceptions of new pests and diseases made by EU Member States [22]. Among the main intercepted commodities in this database, there are the following categories: "plants", which includes plants for planting, scions, cuttings, leaves, flowers, branches with foliage; "wood-packaging material", such as wooden crates, wood pallets, wooden packaging material, dunnage; "wood and bark", including roundwood, bark, and processed wood; "produce", such as fruits, vegetables, seeds; "soil", including soil and growing media. "Plants", in particular those coming from Asia and Africa, constitute the most important pathway of forest IAPPs into Europe. The "plants" pathway is followed by the "wood-packaging material", again mainly coming from Asia. Although wood has historically been one of the most common introduction pathways, Europhyt data relating to the 2016–2020 period show that interceptions of harmful organisms on this commodity have reduced significantly, while "wood-packaging material" has moved up to second place. In Europe, there is a third major pathway: "produce", which is an important entry route mainly for agricultural IAPPs (Figure 1). One feature of imports which is overlooked is "soil". The scarce or absent interception of alien organisms in the "soil" (Figure 1) is to be attributed to the very nature of this material, which hampers detection. This is because harmful IAPPs present in the soil remain hidden inside this matrix [5]. Asia was found to be the main source of alien plant insects, followed by Africa and, to a lesser extent, South America (Figure 1). On the other hand, fungal pathogens were intercepted on commodities coming mainly from South America, and secondly, from Asia. However, it should also be considered that EU phytosanitary rules impose a ban on different commodities imported to EU, a restriction that could impact the rate of interceptions on imports from different continents.

A fact that immediately stands out, according to McCullough et al. [23], is that insect interceptions largely prevail over fungal interceptions. This essentially depends on the fact that many fungal pathogens possess a latent phase during which they are not visible on plant surfaces [24,25]. Figure 1 shows how fungal infections become more frequent on fresh plant produce. This is because the herbaceous (fleshy) consistency of fresh plant produce makes this commodity more easily colonizable and, as a consequence, disease symptoms and/or signs become noticeable in a shorter time [23].

The higher frequency of insect interceptions at ports of entry compared to fungal pathogens, corresponds also to a higher number of insects established in new areas (Figure 2). This is because fungi, as microorganisms, are generally not visible (the fungal fruiting bodies are not always present), thus they remain less noticeable and less detected. However, even considering only the species with a high impact, whose effects have been tangible in terms of damage, insects continue to be reported in the highest numbers, as results from the European Alien Species Information Network indicate (EASIN; http://easin.jrc.ec.europa.eu, accessed on 10 June 2021).

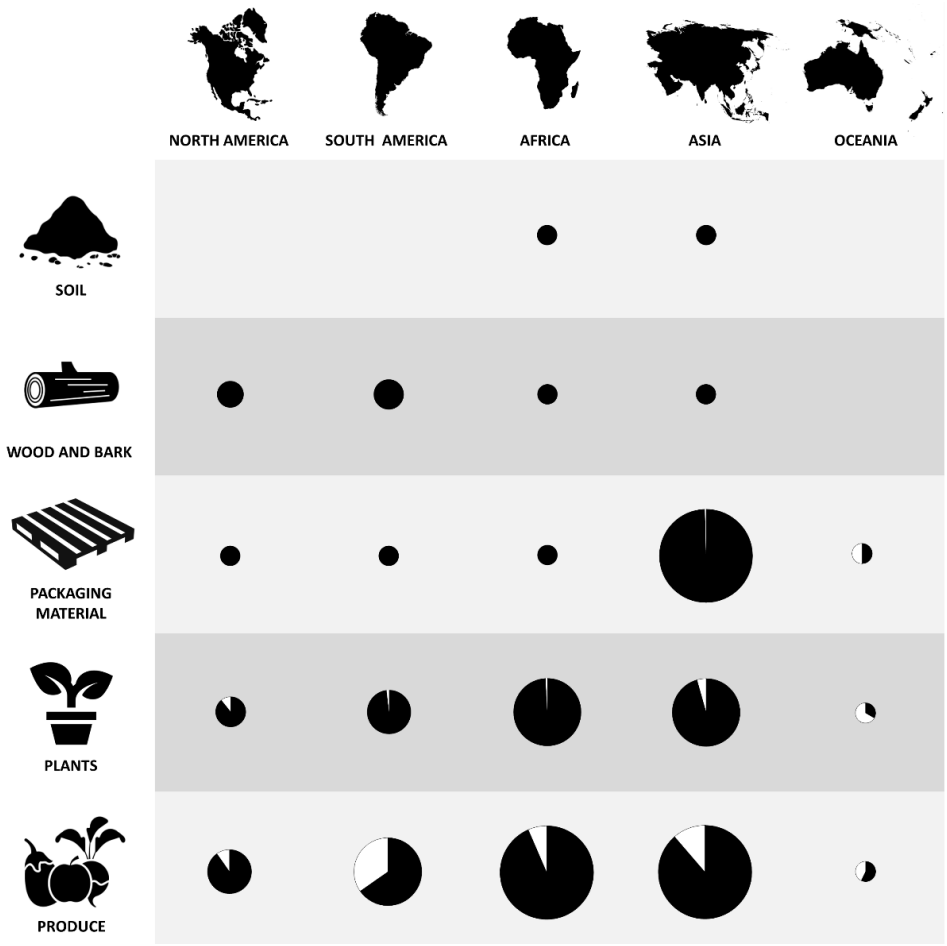

Figure 1. The frequency of fungus and insect interceptions at ports of entry in Europe in the 2016–2020 period (data obtained from reports of Europhyt). Circle sizes indicate the relative amount of interceptions of IAPPs (fungi and insects) within each transport pathway according to their continents of origin. The black sector of the circle denotes the relative frequency of insect interceptions; the white indicates fungi interceptions. Legend: soil = soil and growing media; wood and bark = roundwood, bark, and processed wood; packaging material = wooden crates, wood pallets, wooden packaging material, dunnage; plants = plants for planting, scions, cuttings, leaves, flowers, branches with foliage; produce = fruits, vegetables, seeds.

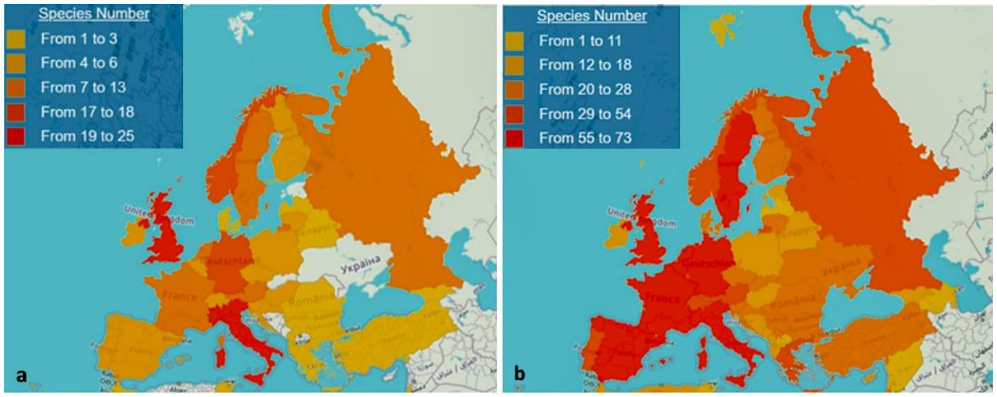

Figure 2. EU geographical distribution of invasive alien species of fungi (**a**) and insects (**b**) introduced into terrestrial and fresh-water environments from 1999 to 2021 (source: EASIN—European Alien Species Information Network). Fresh-water environments were also surveyed to include water moulds (Oomycetes).

The European climate turns out not to be a key factor in determining the abundance of IAPPs introduced. In fact, high numbers of new reported infestations are found in countries from both southern and northern Europe (Figure 2). Instead, other factors, such as trade volume, the presence of roads, and human population density are all correlated with the introduction of alien species to Europe [26].

3. Factors Driving the Invasion Process

The chance of newly introduced harmful organisms becoming invasive in new geographic areas is always uncertain [10]. Indeed, few of these survive either the chronic or occasional forces they encounter in the new environment. Only a very small percentage of them ever become naturalized, and even a smaller fraction ends up becoming invasive [27]. In fact, all non-native species must survive three distinct phases, not always clearly distinguishable, before being able to inflict ecological and economic damage: (1) transport: individuals are taken from their area of origin, transported to a new area, and released into the recipient environment; (2) establishment: the newcomers settle in the non-native environment and implant a reproductive population—among the set of the transported individuals, only a few are capable of this; (3) spread: colonizers grow in abundance and expand their geographical range. These phases are mandatory steps in the invasion process, and the advancement from one to the next one involves overcoming hefty ecological barriers [28]. These hindrances, in fact, allow only a small percentage of the introduced species to advance to the next phase (generally between 5% and 20%, with an average of roughly 10%). Based on this assumption, Williamson [29] introduced the so-called "10 rule", according to which only one out of 10 new introductions would be successful in overcoming any single barrier. This approach has made it possible to analyse the various opportunities IAPPs have to successfully establish into new areas. Invasions, for example, always start with a limited number of individuals being transported and released into a novel location; here, they must be able to survive and give rise to an initial population capable of persisting in this new area. Only when the newcomer species becomes numerically abundant and begins to spread rapidly may "an outbreak" be generated, causing damage to agriculture, the environment, and the local economy, and being perceived as "invasive". If, on the contrary, the population is not able to grow and expand, it remains small in number and is only local (an "endemic occurrence") [30,31]. However, there is an ongoing debate within the scientific community regarding the reliability of the "10 rule", since it can lead to underestimating the impact the introduced organisms can have in new introduction areas [32].

An alien species may have different outcomes in distinct sites of a same region [33]. The first arrivals may survive, reproduce, and spread to become invasive at some locations, while they may succumb at others, even if all individuals of that species share the same traits: e.g., aggressiveness, competitiveness, frugality, polyphagy, tolerance to adverse environmental conditions [34]. In fact, the physical and biological external forces, collectively known as "environmental resistance", which the alien species has to face, vary from site to site, ultimately determining their fate [35]. Among the most common physical forces the alien species has to withstand are: the availability of nutrients, water, oxygen, light, space, shelter, or protection (e.g., the inner tissues of a plant for a fungal endophyte). Biological forces that can effectively hinder the invasion, instead, are: competition, predation, parasitism, and diseases, as well as other interactions with the resident community, including anthropogenic perturbation [36].

Evidence exists that IAPPs perform better than native pathogens and pests in the invaded community, and this can be attributed to various factors. One key reason for their successful infiltration is a higher host susceptibility in the new non-native range, due to the absence of coevolution with the indigenous hosts, the newcomer IAPPs can grow very rapidly and inflict greater damage, in some instances generating overwhelming outbreaks [37]. Another cause is that in the new environment the IAPP may also jump onto new host(s), which may even be taxonomically unrelated to its native hosts [38]. This

is a fairly common phenomenon, whose frequency probably depends on the presence in the introduction area of native plants taxonomically close to the original host plant [39]. Another factor favouring IAPP outbreaks is the scarcity of natural enemies in the new range compared with their native range. According to this "enemy release hypothesis", IAPPs leave behind all those natural enemies that would naturally keep their population under control in the area of origin. In the new environment, moreover, it encounters very few natural enemies able to use it as a trophic resource [40,41]. All these factors enable the IAPPs to exploit their enormous reproductive potential and to spread rapidly on a large scale, even displacing native species [42].

4. Invasive Alien Pathogens and Pests Harming Forests

The harm caused by the introduction of alien forest pathogens and pests is often difficult to fully quantify due to the multiple ecosystem services forests offer, and because each invasion event must be analysed within the specific context (forest, nursery, district, country, region) in which it occurs [43,44]. The damage may not only be significant at the economic, ecological and landscape level, but it might also generate negative repercussions in related sectors, such as in marketing and tourism [45].

4.1. A Retrospective Look

The forest and rural landscapes of various areas of the planet were transformed in the last century by the introduction of dangerous non-native forest pathogens [46]. Between the 1920s and 1940s, the agent behind Dutch elm disease (DED), *Ophiostoma ulmi* (Buisman) Nannf, spread pervasively throughout Europe and North America on native elm species [47]. The invasion by this vascular pathogen, native to Asia, soon turned into a true pandemic [48]. Moreover, the several variants the fungus spawned over time, including the more aggressive subspecies *americana* of *Ophiostoma novo-ulmi* Brasier, were introduced to Europe from North America via infected logs [49]. As clear evidence of the fungus' high evolutionary potential and destructiveness, it eventually led to the almost total disappearance of the European field elm (*Ulmus minor* Mill.) from many countryside and urban districts of Europe [50].

Similarly, iconic tree species of the genus *Castanea*, such as the American chestnut *Castanea dentata* (Marsh.) Borkh. and the sweet chestnut *Castanea sativa* Mill., were, in the last two centuries, wiped out from forest ecosystems and orchards due to the combined, sometimes synchronous, attack of the two fearsome oomycete pathogens, *Phytophthora cambivora* (Petri) Buisman and *Phytophthora cinnamomi* Rands [51], as well as of the ascomycete chestnut blight agent *Cryphonectria parasitica* (Murrill) M.E. Barr [52]. In many European countries, the epidemic spread of chestnut blight led to a severe loss of chestnut cultivation. The disease devastated coppices and orchards, with tragic effects on the already poor economy of many farming communities, for which the edible chestnut fruit was a primary source of food, causing famine and emigration [12].

Around the 1930s, another harmful pathogen, *Ceratocystis platani* (J.M. Walter) Engelbr. and T.C. Harr., the agent behind blue stain canker in plane trees or sycamores (*Platanus occidentalis* L.), spread epidemically first in the suburbs of Philadelphia, and then in many cities in the south-eastern U.S. [53]. Subsequently introduced into the Mediterranean basin either during World War II or soon after, in just a few decades this vascular pathogen gradually killed a multitude of monumental *P. occidentalis* and *Platanus orientalis* L., and their hybrid *Platanus x acerifolia* (Aiton) Willd., trees in city parks, squares, and boulevards [54]. The high genetic uniformity of host populations (most urban trees were clones) and the lack of knowledge about the high infectivity of fungus propagules, which can remain viable at length on pruning tools, favoured the outbreaks of blue stain canker [55].

Seiridium cardinale (W.W. Wagener) B. Sutton and I.A.S. Gibson, the agent behind cypress blight, is an anamorphic fungus native to the west coast of the U.S. (California), where it was first recorded on Monterey cypress (*Cupressus macrocarpa* Hartw. ex Gordon) in 1927 [56]. Despite lacking sexual reproduction, the pathogen was able to spread worldwide

epidemically in a short time [57]. Accidentally introduced into Mediterranean Europe during the mid-1900s [58,59], in a few decades it killed planted and ornamental cypress trees (*Cupressus sempervirens* L.) in stands, windbreaks, parks, villas, cemeteries, and treelines. The destructive cypress blight outbreaks deeply altered the Mediterranean landscape, causing a true ecological disaster [60]. Cypress is, in fact, an iconic part of the Mediterranean countryside, so its disappearance also impacted local economies and tourism [57].

Alien insects can pose a direct danger to plants, but they can also act as vectors for phytopathogenic microorganisms [61]. In addition, native insects sometimes also play an important role in the spread of exotic diseases. For example, the bark beetle, *Phloeosinus aubei* (Perris), native to the Caucasus, Asia Minor, and the Mediterranean area, contributed to the increase in damage caused by the exotic *S. cardinale* by carrying its fungal spores; thus the beetle facilitated the fungus' colonization of host trees. In addition, over the last few decades, this bark beetle has continued to spread into Central Europe, where it is considered an alien pest [62]. Similarly, *Scolytus multistriatus* (Marsham) (Coleoptera: Curculionidae), with a Palearctic native range (from western Europe to Russia and northeast Africa) [63], is the main vector of D.E.D., not only in its native range but also in introduced areas, such as North America, where, before the 1930s, 50%–75% of the elm population in the north-eastern part of the continent were killed by this vascular pathogen [64].

Insects harmful to forest trees are also included among the world's worst 100 IASs [65]; those of forest concern include the gypsy moth, *Lymantria dispar* L. (Lepidoptera, Lymantriidae), and the cypress aphid, *Cinara cupressi* (Buckton) (Hemiptera, Aphididae). The gypsy moth, native to Europe and Asia, was introduced near Boston, Maine, in the late 1860s, and currently is listed as one of the most destructive invasive pests, ranking third among the costliest IASs in the world [66]. Its larvae cause serious defoliation of many tree and shrub species, which may ultimately die or become susceptible to subsequent infestation by secondary pests. From 1924 to 2013, in the north-eastern and mid-western U.S., over 37 million hectares were defoliated [67], with a high impact on timber, costs and losses to urban and suburban forests, as well as damage to the recreational sector [68,69]. In addition, gypsy moth outbreaks have contributed to the decline of oak forests in eastern North America [70]. The cypress aphid is also a highly invasive insect; probably native to North America and Syria, since the 1960s it has become newly established in parts of Europe, Africa, South America, and the Middle East [71]. The cypress aphid, which sucks sap from twigs, causes yellowing to browning of the foliage. Depending on the severity and duration of the infestation, trees may suffer more or less severe damage, or even death in the worst cases [72]. In recent decades, sudden and explosive outbreaks of the cypress aphid have occurred in many countries (some African countries, Italy, Jordan, Yemen, Mauritius, and Colombia). As a consequence, populations of the host trees (*Cupressus*, *Juniperus*, *Widdringtonia,* and other Cupressaceae) have been decimated, both in commercial and ornamental plantings or native stands [73].

4.2. Emerging Invaders

Among the many currently emerging threats to forest ecosystems, oomycete *Phytophthora* species are at the pinnacle. For example, *Phytophthora ramorum* Werres, De Cock et Man in 't Veld, a polyphagous, invasive pathogen originally from the laurosilva forests of eastern Indochina and Japan [74], attacks a multitude of shrub ornamentals and tree species. After causing extensive tree mortality in oak (*Quercus* spp.) and tanoak *Notholithocarpus densiflorus* (Hook. & Arn.) Manos, Cannon and S. H. Oh) forests in California (for this it was called the agent of "sudden oak death") [75], this destructive oomycete has been more recently killing off plantations of Japanese larch [*Larix kaempferi* (Lamb.) Carr.] in the UK, where it was renamed the agent behind "sudden larch death" [76]. Furthermore, the infamous *P. cinnamomi*, the causal agent of ink disease in chestnuts, already mentioned above, continues to destroy woody plants in natural environments, plantations and urban forests, as well as in parks, horticultural nurseries, and urban ornamental plantings

around the world, to the point of earning the nickname of "biological bulldozer" [77]. *P. lateralis* Tucker and Milbrath is considered to be of Asian origin, such as the several *Chamaecyparis* species that, due to the similar native range, exhibit some tolerance or resistance to infection. It is also a harmful pathogen, responsible for serious root and aerial infections [78]. This oomycete shows considerable host specificity, with Port-Orford cedar (*Chamaecyparis lawsoniana* (Murray) Parl.) its main host, but it has also caused epidemic damage to *Taxus brevifolia*, *Thuja,* and other *Chamaecyparis* species growing in proximity to infected *C. lawsoniana* in forests, plantations and in the landscape [79].

Rust fungi also pose a constant threat to many forest ecosystems, mainly because of their high dispersal ability over long distances. It is therefore no coincidence that rust fungi are the largest group of pathogenic fungi in the EPPO (European and Mediterranean Plant Protection Organization) A1 List of Pests Recommended for Regulation as Quarantine Pests (EPPO, https://www.eppo.int/ACTIVITIES/plant_quarantine/A1_list, accessed on 30 June 2021). One invasive rust that has been spreading pervasively throughout Europe in recent years is the fungus *Melampsoridium hiratsukanum* S. Ito ex Hirats. f., of Asian origin [80]. This rust is currently causing heavy defoliation, stunted growth and death to riparian *Alnus incana* (L.) Moench trees in the eastern Alps, threatening the stability of some riverbanks. In this mountain range the fungus has also found *Larix europea* L. to be a suitable alternate host on which to complete its life cycle [81].

Moreover, a highly destructive fungal disease of ash trees (*Fraxinus* spp.) is at present causing significant damage to ash populations throughout the European continent. The causative agent, *Hymenoscyphus fraxineus* (T. Kowalski) Baral, Queloz and Hosoya (anamorph: *Chalara fraxinea* T. Kowalski), kills young and coppiced ash trees in a very short time, whereas older trees can resist its irritative action for some time, until they are exposed to high inoculation pressure [82]. The resultant ash dieback is seriously impacting woodland biodiversity and ecology, reducing landscape aesthetics and gravely damaging hardwood industries, with heavy economic losses [83].

Yet another tree staple, pine stands, are currently being impacted worldwide by the destructive "Pine Pitch canker", caused by *Fusarium circinatum* Nirenberg and O'Donnell, (teleomorph: *Gibberella circinata*) [84,85], as well as by some highly damaging foliar pathogens, such as *Lecanosticta acicola* (Thümen) H. Sydow [86], agent of Lecanosticta needle blight (LNB) and the related fungi, agents of Dothistroma needle blight (DNB) or of "red band needle blight", *Dothistroma septosporum* (Dorog.) Morelet (syn. *Scirrhia pini* Funk and Parker, teleomorph: *Mycosphaerella pini* Rostr.) and *Dothistroma pini* Hulbary (teleomorph: unknown) [87–89]. *F. circinatum*, considered native to Mexico, has spread in the last half century to many pine growing areas, causing severe damage to artificial plantations in the Americas, the Far East (Japan and the Republic of Korea), and in southern Africa [90]. DNB causes premature needle abscission, stunted growth, and, in some cases, tree mortality [91]. The disease represents a serious threat to both natural forest ecosystems and exotic pine plantations on all continents (it occurs everywhere except Antarctica) [92]. The disease has been primarily associated with exotic plantations of Monterey pine (*Pinus radiata* D. Don) but, starting from the 1990s, it has caused serious damage in stands of both planted and native pine species, e.g., in *Pinus contorta* subsp. *latifolia* stands in Canada and in *P. nigra* subsp. *laricio* stands in the Mediterranean region [8,93].

Some invasive pests involve symbiotic relationships between insects and fungi; in this case, the whole complex (the insect and its fungal symbiont) is introduced into the new environment. For example, the complex of *Geosmithia morbida* Kolařik (Ascomycota, Hypocreales, Bionectriaceae) and its vector, the bark beetle *Pityophthorus juglandis* Blackman (Coleoptera, Curculionidae, Scolytinae) is responsible for Thousand Cankers Disease (TCD) in walnut trees. First described in North America, TCD causes progressive crown decline, with the fungus colonizing the copious feeding and reproductive galleries made by its symbiont, the phloem-boring bark beetle. This results in numerous, coalescent cankers that girdle branches and trunks, ultimately leading to the tree's death [94]. This insect–fungus complex has been recorded in Italy, where in northern and central regions several

plantations were found to be being attacked [95,96]. Another example is the polyphagous shot hole borer, *Euwallacea fornicatus* (Eichhoff) (Coleoptera: Curculionidae), an ambrosia beetle native to Asia that was accidentally introduced into Central and North America, Israel, and South Africa. This emerging tree pest carries the spores of three ambrosial fungi within specific body structures (mycangia), which are the only trophic source for insect adults and larvae. One of these fungi, *Neocosmospora* (= *Fusarium*) *euwallaceae* (S. Freeman, Z. Mendel, T. Aoki and O'Donnell) Sand.-Den., L. Lombard and Crous, a moderately virulent pathogen, is responsible for causing dieback in a range of susceptible host trees, or even mortality, depending on the level of infestation. This insect–fungus complex has caused significant damage to orchards and ecosystems around the world, even attacking important forest species [97].

Other insect species of great impact are the Asian long-horned beetle, *Anoplophora glabripennis* (Motschulsky) (Coleoptera: Cerambycidae), and the citrus long-horned beetle, *A. chinensis* Forster (Coleoptera: Cerambycidae). These are xylophagous insects native to Asia, whose big larvae can kill healthy broadleaf trees. The first record of an established population of *A. glabripennis* outside its native range was in 1996 [98], while that of *A. chinensis* was in 1999; both occurred in North America [99]. In Europe, eradication was successful at the sites where *A. chinensis* was discovered shortly after its introduction, proving how early detection is key to prevent the lasting establishment of the pest and to implement effective control measures [100]. Furthermore, the importance of citizen involvement in early detection was highlighted in 2008, when every new outbreak was first reported by members of the public [101].

However, when an IAPP species is not considered a major pest in its native range, it is unlikely to be included on IAS watch lists. Consequently, specific early detection surveys for that pest are not in place. Furthermore, it is difficult to predict the invasiveness of a non-native species in a new environment. Such was the case not only for pathogenic fungi, such as the ash dieback agent *H. fraxineus*, but also for several insect species, such as, for example, the emerald ash borer, *Agrilus planipennis* (Fairmaire) (Coleoptera: Buprestidae), a phloem-feeding beetle native to Asia, where it was only marginally studied as a minor pest. Once accidentally introduced (in 2002) in the United States, however, it became the most destructive forest pest ever recorded in North America [102]. Millions of ash trees have already died because of *A. planipennis*; the infestations have spread over 35 US States and two Canadian Provinces, as of 2020 [103]. The emerald ash borer has also reached European Russia, being first recorded in Moscow in 2003; it is now already present in nine Russian regions [104]. In 2019, it was also confirmed for the first time in Ukraine, revealing how the beetle is continuing to expand its range towards Europe [105]. Because of what already happened in North America, the beetle is being addressed in Europe with more groundwork, trying to develop all the necessary measures to contain the problem [106]. However, it has yet to be determined whether the European ash (*Fraxinus excelsior* L.) is going to be susceptible to severe damage; in fact, most of the infested trees in Russia are in artificial urban settings of *F. pennsylvanica* Marshall [104].

5. Mitigating Threats to Forest Health: Possible Solutions and Action Strategies

In natural forests, the better approach is to actively maintain ecological integrity to ensure forest resilience, whereas in forest plantations the restoration of increasing levels of biodiversity should be favoured as the first line of defence against invasive pests and pathogens [107]. In this last case, the careful choice of appropriate plant material is always fundamental, specifically, avoiding the use of species unsuited to site conditions, as well as limiting the number of exotic plant species [108], since in the last century, non-native tree species, widely used as ornamentals or in afforestation programmes, acted as "Trojan horses" for many harmful diseases and pests [8]. Clear examples are the accidental introductions of *P. ramorum* into North America with nursery plant material [75], of *Teratosphaeria (Mycosphaerella) nubilosa* (Cooke) Crous and U. Braun, a causal agent of Mycosphaerella leaf disease of eucalypt, into South America (Uruguay) with propagation material of this exotic

tree species [109], of *Rhynchophorus ferrugineus* (Olivier), harmful to palm trees widely diffused in South Europe [110], and of *Ophelimus maskelli* (Ashmead) (Hymenoptera: Eulophidae), which damages eucalypt plants in the Mediterranean Basin [111]. Furthermore, in forestry it is always advisable to avoid genetic uniformity, such as clonal plantations, as in poplar cultivation. Mixed forests, instead, are generally less prone to diseases and pests than monotypic stands, with nonhost trees creating effective physical and chemical barriers to the spread of pathogens' inoculum, as well as hindering host searches by pests. In addition, higher biodiversity favours natural enemies and competitors which, interacting with IAPPs, contribute to keeping the invaders' population densities low [107].

Preventing the introduction of dangerous IAPPs into new areas is the first step in their management, so this has become an important priority in many countries. Preventing introductions is carried out through effective surveillance activities, namely phytosanitary inspections at ports of entry (ports, airports, and custom barriers), as well as at vulnerable sites, i.e., those places where the plant material is in transit or processed, such as nurseries, loading and sorting stations, and wood processing companies [112,113]. Considering the enormous volume of commerce at present, and the incessant mobility of humans for work, tourism, etc., many IAPPs elude preventative measures, it being practically impossible to intercept all the species that transit on all commodities [114,115].

Careful prediction of potential invasiveness of harmful pests and diseases can aid interceptions [116]. Important predictive tools are those provided by international organizations such as the CABI (Commonwealth Agricultural Bureaux International) and the EFSA (European Food Safety Authority), as well as various Regional Plant Protection Organizations such as the EPPO, the NAPPO (North American Plant Protection Organization) and the APPPC (Asia and Pacific Plant Protection Commission), which were created to develop international strategies to fight the introduction and spread of harmful pests and diseases and to promote safe and effective pest control methods. In fact, they provide constantly updated lists of organisms considered as threats to agriculture, forestry, and the environment and that are most likely to be introduced into Europe and North America. These agencies' published assessments of invasion risks are important tools since they focus the efforts of inspection services, plant health scientists, and all the stakeholders affected by imported pests and pathogens [117]. Another important early warning system of new and emerging pest and pathogen risks is sentinel plants [118]. These can be: (a) plantings made with young, frequently exported native plants which are used to identify pests and diseases that may be introduced to import countries through the trade in live plants; and (b) plantings made of non-indigenous young or mature plants in import countries whose monitoring could give useful information about the potential damage of non-native pathogens and pests if they were to arrive and become established [119].

A primary obstacle to managing and mitigating alien forest pests is correctly identifying the agents of damage [117]. Traditional identification methods, essentially based on the analysis of macro- and/or micro-morphological characters, have inherent limitations. Considerable experience is necessary to induce the formation of fungal distinguishing structures in vitro and substantial expertise is needed for their microscopic observation and identification; moreover, special growth media (i.e., differential or selective substrates) may not be available for certain, fastidious microorganisms. In any case, these procedures are not applicable for unculturable microbes such as biotrophic fungi [120]. The above difficulties involve the fact that some taxa, for example, are not easily identified through morphological characters, so this entails inspectors with specific expertise [114]. In addition, some insects have juvenile stages that are indistinguishable by classical diagnostic methods. Furthermore, phytosanitary inspectors may face plant material that has been attacked, but which has already been abandoned by its phytophages; therefore, they have to track the pest only on the basis of the traces left behind, which becomes a more difficult task. It is also true, on the other hand, that the reproductive structures of many forest pathogens can be observed on infected plant material, and these are of a great diagnostic value [81]. Furthermore, when the reproductive structures (e.g., fruiting bodies) are not

present on the host surface, they can still be obtained in the laboratory by using a moist chamber; alternatively, the isolation from infected plant material into pure culture can permit the growth and identification of the causal agent.

Many of the difficulties inherent in traditional morphological identification can nowadays be overcome by employing molecular techniques. Furthermore, for an effective inspection system which can cover the ever-increasing volume of import–export material, simple, timely, and repeatable methods have to be developed. Modern molecular diagnostic tools, which detect DNA polymorphisms, ensure high accuracy and diagnostic sensitivity, allowing the detection of even minimal traces of nucleic acids, such as those remaining in the frass of xylophagous insects [113]. Some of these methods have the advantage of being able to process a large number of samples in a short time [121]. For example, loop-mediated isothermal amplification (LAMP) is a nucleic acid amplification method that offers rapid, accurate, and cost-effective diagnosis of diseases and pests [122–124]. The simplicity and the portability of the equipment also make it a tool of choice for carrying out tests in situ, making inspection greatly easier [8]. Molecular methods are therefore of great help when it is necessary to focus attention on clearly defined species, for example for delimiting surveys at vulnerable sites known or suspected to harbour a given pathogen or pest, while conventional (e.g., morphology-based) methods continue to retain their high validity for the control of the territory for the occurrence of unknown IAPPs. In other words, morphological and molecular methods should go hand-in-hand and complement each other, with DNA-based techniques representing versatile tools to support and extend more classical species identification and taxonomy [125].

Novel diagnostic methods are also useful in monitoring the dynamics and ecology of a given IAPP after its introduction. These techniques are in fact also highly effective in: detecting early disease and insect foci; tracing and quantifying pathogens' inoculum (especially airborne inoculum) and insect populations; and locating infection and infestation reservoirs (e.g. storage facilities) that sustain invasion events [126]. National and local inspection services also make use of innovative strategies, including remote sensing technology (GIS and unmanned aerial vehicles like drones) [127,128] and detection dogs, whose keen smell can locate biological targets [129]. This last method was used for the first time for gypsy moths and later for other alien and invasive insect species [130].

The mobilisation of citizens as additional observers and reporters (citizen science) has become increasingly utilized for the early detection of unwanted IAPPs. Active monitoring by citizens, when extensively distributed throughout the territory, has in fact become an important part of biosecurity efforts to prevent IAPPs' establishment and spread [131]. However, to obtain the consent and collaboration of citizens, they must be properly informed about the risks and dangers associated with non-native pathogens and pests for native plants, also because citizens have sometimes opposed management interventions aimed at containing and eradicating harmful alien organisms [132]. Well-informed citizens, besides supporting the accurate monitoring of the territory by inspecting sites and trees, are also a fundamental resource for collecting, categorizing, transcribing, and analysing data [133]. Citizen science is possible today due to a growing number of citizens with ecological awareness and knowledge eager to contribute as volunteers [134]. Some examples of citizen science projects include Observatree (https://www.observatree.org.uk, accessed on 31 July 2021) in the UK and LIFE ARTEMIS (https://www.tujerodne-vrste.info/en/, accessed on 31 July 2021) in Slovenia; in addition there are several citizen science networks, such as the EASIN (https://easin.jrc.ec.europa.eu/easin/CitizenScience/BecomeACitizen, accessed on 31 July 2021), which is an initiative of the European Commission that aims to connect citizens, scientists and policymakers in an effort to manage harmful alien invasive species.

Once an IAPP has been detected within an area of concern, the next move is to try to eradicate invasion foci [135,136]. Quick action while IAPP populations are still small is the most efficient way to prevent ecological or economic harm. With forest IAPPs the only control options are mechanical approaches (such as sanitation cutting) [137], biological

control, and the use of semiochemicals (e.g., mass trapping) [10]. While eradication of alien forest insects has been effective in some cases [138], the eradication of forest pathogens has always been incredibly challenging, with most of the successful interventions taking place in controlled environments (greenhouses and tree nurseries) or in urban green spaces (e.g., parks, trees, and gardens) [139].

After an IAPP population has newly established in a forest, biological control is one of the main measures to achieve self-sustaining long-term control [140]. In some cases, biological control has managed to restore more sustainable pest population dynamics, succeeding in keeping the population density of the invasive species below the damage thresholds [141–143]. For instance, the parasitoid *Torymus sinensis* Kamijo (Hymenoptera: Torymidae) proved to be a good control agent of the Asian chestnut gall wasp *D. kuriphilus* in several areas of the world, significantly reducing the pest populations with a self-sustaining effect [144]. Similarly, in North European conifer forests, the fungus *Phlebiopsis gigantea* (Fr.) Jul. has been successfully employed in the biological control of the root and butt rot pathogen *Heterobasidion annosum* (Fr.) Bref. [145,146]. Commercial formulations based on this biocontrol agent have even since been developed and are commercially available under the names of 'PG suspension™' in the UK, 'Rotstop®' in Finland, and PG IBL in Poland [146,147].

Biological control is not, however, a universal remedy; it is not always effective and several negative effects may occur. This variability in the efficacy of biological control in forest ecosystems is a frequent drawback, mainly due to the complex and multi-partite interactions between the tree, the IAPP, the biocontrol agent, and the other biotic factors [148], all of which are, in turn, impacted by the physical environment and human interference. Biological control in heterogeneous forest ecosystems, therefore, in order to be successful, should take into consideration the multiple biological factors involved: including the environmental conditions (e.g., soil properties, microclimate, etc.), type of forest stand, and silvicultural management [149]. Controlling IAPPs by introducing their natural enemies, many of which are exotic themselves, is a controversial issue whose effects may be viewed as "deliberate ecological invasions". Retrospective analyses of several biological control programs around the globe are now available, thus providing quantitative data on negative effects: parasitism/predation on non-targets; competition with native natural enemies; intraguild predation; vectoring of pathogens [150]; and risk of hybridization with related native species [151]. Due to the increasing awareness of the related environmental and economic risks, many countries have implemented regulations for the release of biological control agents [152].

For the management of IAPP invasions, more stringent rules and policies are needed to protect plant health and to ensure a safer trade [153]. The need for such measures has long been recognised: at a regional level Plant Protection Organizations are trying to enforce phytosanitary policies, regulations and technical recommendations [154]. For example, the defence of the European territory and its plants has been recently tackled with the adoption of new rules (Regulation (EU) 2016/2031), which improve the European phytosanitary regime through more effective measures. A global effort is also required, involving all the countries and policymakers that in various ways may contribute to counteracting the problem. Specifically, international politics should work towards: consolidating phytosanitary coordination among the countries; harmonizing phytosanitary measures; eliminating the gaps between international and national laws; filling legislative gaps by implementing preventive and/or corrective legal actions; promoting more incisive and restrictive actions at national, regional, and international levels; and establishing legal responsibility frameworks to sanction countries that do not respect international regulations [117].

6. Role of Research

The introduction of IAPPs into new territories is a current environmental issue and a dominant theme of scientific research. Phytosanitary protection has become one of the priorities of many nations, as proven by the numerous funded research projects on this

subject [155]. Information about the taxonomy, biology, and ecology of IAPPs during the onset of an invasion process is often lacking, especially as regards their reproduction and dispersal abilities into areas where they have been newly introduced; this knowledge gap may favour their establishment and uncontrolled spread [156]. Plant health scientists, besides being involved in the diagnosis of IAPPs, which, often being new, are sometimes little known, are also concerned with their taxonomic positioning, as well as with the characterization of those subspecific entities that are difficult to determine by traditional approaches [157]. The development of diagnostic methods is especially important for those IAPPs endowed with a latent phase, because latency increases the risk of their spreading undetected. However, research also needs to be performed in other, fundamental aspects of the IAPPs' life history strategies: their demographic history (gene flow, migration); mode of reproduction (i.e., sexual vs. asexual); recombinational events outside the sexual cycle, such as parasexuality and horizontal gene transfer, affecting pathogen virulence [158]; occurrence of hybridization events between related taxa [159]; microbial/pest virulence/aggressiveness versus host resistance. A constant threat is posed to forests by the possible arrival and co-occurrence of new mating types and haplotypes. While the coexistence of different mating types increases the chances for sexual recombination between genetically divergent lineages, continual haplotype introgression can give rise to the emergence of new pathotypes with heightened virulence. These natural events, which have become more frequent as a result of international trade and climate change, are strongly feared in many pest–plant interactions. The evolutionary and pathogenetic potential of *P. ramorum*, *H. fraxineus*, as well as of the walnut twig beetle *P. juglandis*, vector of TCD, to cite but a few examples, strongly depend on these mechanisms [78,137,160]. Research can thus dramatically advance our understanding of the evolution of pathogenesis and aggressiveness of pathogens and pests and of the epidemic risks associated with these phenomena.

This knowledge is crucial for enabling stakeholders (landowners, foresters, phytosanitary inspection services, and policymakers) to implement adequate management strategies. In addition, accurate information from the research community would also permit valid, standardized phytosanitary certification, which could be adopted in partecipating countries and support inspections of feared IAPP introduction, including in the exporting country. On an open market, which allows the free movement of plant material, the certification of propagation material is of primary importance to avoid the spread of diseases and pests into new areas [161].

7. Conclusions

The problem of invasions by forest IAPPs is enormous and complex. It concerns countries, international organizations, and agencies; it affects economic interests, involving a multitude of activities from local to global scales [155,162]. At a local level, the problem must be tackled by concentrating efforts both on preventing new introductions and on eradicating early invasion foci [136]. Since trade-related transport is the main source of forest IAPPs, particularly that involving germplasm and wood-packaging material, this is where immediate action should be taken [6]. Implementing surveillance campaigns on these pathways would therefore help prevent, or at least minimize, the risks of introducing forest IAPPs.

In addition, forest health monitoring has now become of crucial importance. It is, however, challenging, due not only to the vast expanse of many forested areas, but also to the complexity of forest ecosystems, whose various compartments (soil, water, live plants, snags and logs) must be inspected by well-trained personnel [129]. Furthermore, due to the increasing rate of introduction of IAPPs, monitoring should be continual, and articulated across several activities: extensive monitoring of vast areas; intensive monitoring of vulnerable sites (loading stations, nurseries, import–export material checking points) [112]; the use of sentinel plants [118]; early warnings assisted by both conventional

and innovative molecular diagnostic tools [123]; and involving volunteers in early detection (citizen science) [131].

Research has always played a key role in pest management and it continues to do so even more nowadays when dealing with IAPPs. As we have pointed out in this paper, the scientific community is looking into a multitude of issues related to IAPPs, from correct taxonomic positioning [157] to diagnosis [126], from the analysis of recombinational events between non-native and native species [159] to investigations into IAPPs' adaptation to new environments [137]. This scientific support will also be crucial to government agencies' setting up of effective control strategies [100], as well as to lawmakers tasked with enacting laws and regulations [117], who should be routinely notified of scientific advancements in this field.

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Review

On Some Significant Phytoplasma Diseases of Forest Trees: An Update

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Abstract: This paper provides an updating of information of a selected number of major phytoplasma diseases of forest trees, with a focus on the associated phytoplasma taxa. Phytoplasma diseases of forest trees have been less extensively studied than those affecting fruit trees. Research on the role of phytoplasmas as the cause of diseases of forest trees has only in the last few years been intensified, after sensitive and specific detection methods greatly based on PCR technology became available. Various phytoplasma taxa have been identified in naturally infected elm, ash, conifer, sandal, and eucalyptus trees, whereas only one phytoplasma taxon has been recorded in naturally infected alder trees. However, for almost all of the reviewed diseases, there is still sparse information about insect vectors, plant host range, strain virulence, pathogenicity, and host tolerance and resistance. Knowledge of these aspects is the basis for appropriate disease management. In particular, further research is required to clarify the role of phytoplasmas in asymptomatic trees. In addition, the etiological role of various "non-specific" phytoplasma taxa, which have been recorded in forest trees, while no data from pathological studies are available, needs to be further investigated.

Keywords: '*Candidatus* Phytoplasma' species; 16Sr group/subgroups; PCR; yellows diseases; witches' broom; phloem discoloration; die-back; phytoplasma strains; etiology; eucalyptus little-leaf; disease incidence

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1. Introduction

Phytoplasmas are a very large group of wall-less, obligate bacteria associated with diseases of more than a thousand plant species worldwide, including economically and ecologically important forest trees. In the plant host, they colonize the phloem elements and are naturally transmitted by phloem-feeding homopteran insects [1]. Phytoplasmas cause either specific symptoms (virescence, phyllody, witches' brooms) or non-specific symptoms (yellowing, reddening, reduced growth, die-back, and decline). However, symptom expression may be highly variable for a given plant–host combination.

Phytoplasmas are members of the class *Mollicutes*, which are closely related to acholeplasmas, and they are currently assigned to the provisional genus '*Candidatus* Phytoplasma' mainly through 16S rDNA sequence analysis [2,3]. Approximately 34 phytoplasma groups or 16Sr groups (= taxonomic groups) were established by restriction fragment length polymorphism (RFLP) analysis of PCR-amplified rDNA sequences within the mentioned genus [3,4]. In addition, multi-locus sequence typing (MLST) using less-conserved genes proved a useful tool for the identification of genetically closely related but pathologically or epidemiologically distinct strains. Identification of these strains is essential for epidemiological studies [5].

The aim of this paper is to update information of some phytoplasma diseases of forest trees, with a focus on the molecular and taxonomic aspects of the associated phytoplasmas.

2. Phytoplasma Diseases of Forest Trees

2.1. Elm

Various *Ulmus* (elm) species and hybrids are affected by elm yellows (EY), which is a disease common in North America and Europe [6]. This disease was formerly named elm phloem necrosis because the discoloration and death (= necrosis) of phloem tissue are the most characteristic symptoms in highly susceptible hosts, e.g., *Ulmus americana* (American or white elm) [7]. EY was first reported in Ohio in 1938 [7]. However, there is evidence that it was present there as well as in neighboring states long before this time [8,9]. Once established in the midwestern states, EY spread into eastern states and southeastern Ontario [10]. It is known for causing destructive epidemics in North America, which killed thousands of elm trees. Until the 1980s, EY was considered a typical North American disease. Conti et al. [11] first reported the occurrence of this disease in Italy, although it was observed in Italy since at least 1918 (for a review, see [6]). Following this finding, phytoplasma diseases of elm have also been been recorded in several other European countries [6,12,13]. The detection of EY in Europe was first made on the basis of symptoms [11,14]. Later, molecular studies using mainly RFLP and sequence analyses of PCR-amplified rDNA showed that the phytoplasma diseases of elm in Europe and North America are caused by the same organism, the EY agent [15–18]. This pathogen was taxonomically delineated as '*Candidatus* (*Ca*.) Phytoplasma ulmi' [2,19].

Taxonomically, the EY agent '*Ca*. Phytoplasma ulmi' is assigned to the EY phytoplasma group or 16SrV group, subgroup 16SrV-A [19]. Other members of this group are phytoplasmas causing mainly diseases of trees and shrubs in the northern hemisphere [6]. An updated picture of the phylogenetic relationships among phytoplasmas associated with diseases of forest trees reviewed in this paper is shown in Figure 1. In contrast to 16S rRNA gene, genetic diversity within the EY pathogen have been observed in *rplV*, *rpsC*, *secY*, *map*, *groEL*, and *imp* gene sequences [19–24].

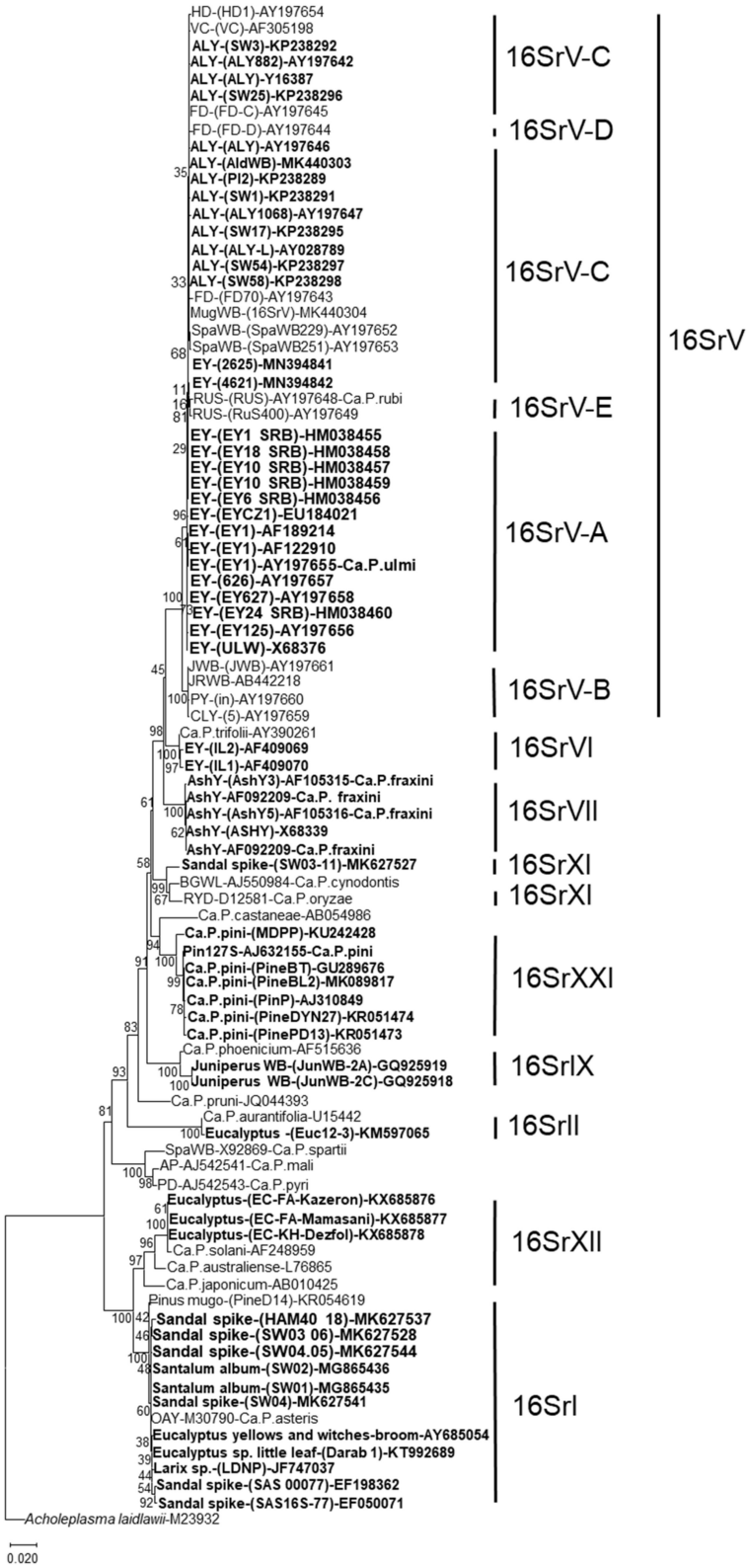

Figure 1. Phylogenetic dendrogram generated by using the neighbor-joining method software MEGA, version X [25] with 16S rDNA sequences from phytoplasmas detected in naturally infected elm, alder, ash, conifer, sandal and eucalyptus trees (in bold type), and a number of reference phytoplasmas. *Acholeplasma laidlawii* was used as the outgroup. Bar represents a phylogenetic distance of 2%. GenBank accession number is given for each phytoplasma. Bootstrap values are shown on branches of the dendrogram.

A phytoplasma of the clover proliferation group (=16SrVI group), subgroup 16SrVI-C, the Illinois elm yellows (ILEY) phytoplasma, was detected in American elm trees showing EY symptoms in Illinois, USA [26]. However, some trees proved to be doubly infected with the ILEY phytoplasma and a phytoplasma of the aster yellows (AY) group (16SrI group) [26]. Diseased elm trees doubly or multiply infected with the EY agent and a phytoplasma of the 16SrI and/or the stolbur group (16SrXII group) were also recorded in Italy [27]. However, in these trees, the EY agent was predominant, while the other phytoplasmas were present at low titer. A few diseased elm trees showing yellowing symptoms, singly infected with 16SrI and 16SrXII-A phytoplasmas, were found in Croatia [23], whereas diseased elm trees in China harbored 16SrV-B and 16SrI-B phytoplasmas [28]. A '*Ca*. Phytoplasma fragariae'-related strain (16SrXII-E subgroup) was detected in five symptomless elm trees in Belgium, whereas 16SrV-C phytoplasmas were detected in two elm trees in Germany with the absence of yellows disease symptoms [13,29].

EY symptoms may differ among elm species. In Northern American species, e.g., *U. americana*, *U. rubra* (red or slippery elm), *U. alata* (winged elm), *U. serotina* (September elm), and *U. crassifolia* (cedar elm), the main symptoms are yellowing, premature casting of the leaves, early bud break, brown discoloration of the phloem tissue, and tree death. Red elm often shows witches' brooms. Discolored phloem tissue of American, winged, September and cedar elms usually have a characteristic odor of oil of wintergreen (methyl salicylate). In *U. minor* (syn.: *U. carpinifolia*, European field elm), witches' brooms are predominant. For this reason, the disease occurring in Europe is often called elm witches' broom. Other symptoms are yellowing, stunting, and small leaves (Figure 2).

Figure 2. Small leaves, yellowing, and witches' broom on European field elm (*Ulmus minor*) shoot affected by elm yellows disease. Left, healthy shoot.

Brooming and stunting are also typical symptoms of EY-affected *U. glabra* (Scots or wych elm) and *U. parvifolia* (Chinese elm) [14,15,30]. Yellowing or reddening, reduced vigor, witches' brooms, and die-back but not phloem discoloration are known to occur in several other European and Asian elm genotypes, including *U. pumila* (Siberian elm), *U. chenmoui* (Chenmou elm), *U. villosa* (cherry bark elm), *U. laevis* (European white elm), *U. wallichiana* (Himalayan elm), *U. wilsoniana* (Wilson elm), *U. japonica* (Japanese elm), and *U.* × *hollandica* (Dutch elm) [11,21,22,27,31,32]. In diseased American elm trees, an abnormal callose deposition on the sieve plates is usually followed by the collapse of sieve tube elements. Thereby, phloem transport is severely compromised. Starch accumulates in the aerial parts of affected trees while roots starve and die. The sieve tubes of affected *U. americana* and *U. rubra* trees are so sensitive to '*Ca*. Phytoplasma ulmi' that they suddenly collapse without allowing the pathogen to reach a high titer [14,33,34]. In contrast, sieve tubes of *U. minor* and *U. parvifolia* trees as well as of other European and Asian elm genotypes being rather tolerant to the EY pathogen are only slightly damaged and allow it to reach a quite high titer [14,17,35,36]. '*Ca*. Phytoplasma ulmi' also causes stomatal closure and elevated diffusive resistance, which is associated with high water potential,

in leaves of *U. americana* and *U. rubra* trees [10]. There are also several reports on the presence of '*Ca*. Phytoplasma ulmi' in non-symptomatic trees belonging to some European and Asian elm genotypes [13,23,27,29–32,37]. In China, elm trees of unspecified species showing yellowing and witches' brooms were found to be infected by 16SrV-B and 16SrI-B phytoplasmas, respectively [28].

EY is common in eastern North American states where several severe epidemics spread at rates of 5 to 8 km per year in some areas and destroyed a large number of native elm tree [30]. In some states, EY epidemics occurred together with the Dutch elm disease and exacerbated the latter by providing additional breeding material for elm bark beetles, which are the insects responsible for the spread of the Dutch elm disease [30]. In Italy, significant EY epidemics have been observed in some experimental fields established during the 1980s to test the adaptability of a number of elm species and various hybrid clones to local environmental conditions [31]. *U. americana*, *U. villosa*, *U. parvifolia*, *U. japonica*, *U. parvifolia* × *U. wallichiana* (= clone P628), (*U. wallichiana* × (*U.* × *hollandica* 'Vegeta' × *U. minor*) (= clone 793), [(*U. wallichiana* × *U. minor*)] × *U. laciniata*) × *U. laciniata* 'Nikkoensis' open-pollinated (= clone 1094), and (*U. wallichiana* × *U. minor*) × *U. laciniata* open-pollinated (= clone 1098) were severely affected [31]. In one of the experimental field, 30% of trees were infected five years after the first evidence of the disease, reaching nearly 80% within fourteen years. EY epidemics observed in southern and northern Italy on European field elm and Siberian elm showed a disease incidence greater than 80% [6,11,31,37]. A detection rate of 75% of EY phytoplasma in European white elm trees has been recorded in Croatia using nested PCR assays. More than half of the infected trees were symptomless [23]. In Croatia, 75% of the European white elm trees that were examined by nested PCR assays proved to be infected by 'Ca. Phytoplasma ulmi'. Most of the examined trees were non-symptomatic [23]. In a recent survey performed in Germany on a nationwide scale by using real-time PCR, '*Ca*. Phytoplasma ulmi' was detected in almost 28% of elm trees examined. Among the elm species examined, the highest detection rate was recorded in the European white elm, followed by the Scots elm and European field elm. EY-specific symptoms were rarely observed only in infected Scots and European field elm trees [13]. In Illinois, a severe EY outbreak associated to ILEY phytoplasma occurred in the 1990s and caused the death of more than one thousand American elms [26]. No epidemics are known to occur in China where single disease occurrences were recorded [28].

The leafhopper *Scaphoideus luteolus* is known to transmit '*Ca*. Phytoplasma ulmi' in North America. However, other vectors are most probably involved in the transmission process since numerous homopteran insects were captured on elm, whereas *S. luteolus* is not present in some EY-infested areas (for references see [6,30]). In New York State, of the various homopteran insects collected in EY-infested areas and examined for their ability to transmit '*Ca*. Phytoplasma ulmi', single transmissions were obtained for the leafhopper *Allygus atomarious* and the spittlebug *Philaenus spumarius* [10]. In addition, '*Ca*. Phytoplasma ulmi' was detected by real-time PCR in several leafhoppers belonging to *Allygus*, *Colladonus*, *Empoasca*, *Erythronneura*, *Graphocephala*, *Homalodisca*, *Orientus*, *Scaphoideus*, and *Typhlocyba*, which were collected in the University Park Campus of Pennsylvania State University, USA [38]. However, it has not been shown whether these leafhoppers transmit the pathogen. Work by Rosa et al. [39] revealed that three out of 30 American elm seedlings exposed to individuals of *Lepyronia quadrangularis*, *P. spumarius*, and *Latalus* sp., captured from an EY-infected red elm tree in the Pennsylvania State University campus, were infected by '*Ca*. Phytoplasma ulmi'. *S. luteolus* is not present in Europe, whereas Carraro et al. [37] provided evidence that *Macropsis mendax* is vectoring '*Ca*. Phytoplasma ulmi' in northern Italy. There are no reports on the role of *M. mendax* in the natural transmission of '*Ca*. Phytoplasma ulmi' in other European countries as well as on its transmission efficiency. In Germany, the finding of naturally infected elm trees at elevations beyond 750 m suggests the presence of a vector different from *M. mendax*, since this insect occurs only to an altitude of 400 m [13]. '*Ca*. Phytoplasma ulmi' was also detected by PCR in individuals of *Hyalesthes luteipes*, and *Iassus scutellaris*, *Allygidius furcatus*, and *Cixus* sp., captured from EY-affected

elm trees in Serbia and France, respectively [40,41]. However, no transmission experiments were carried out in both countries. '*Ca*. Phytoplasma ulmi' seems to be transmitted only among elm trees, because other plants growing near EY-infected elm trees have so far not been found infected by the pathogen. It is also transmitted by natural root grafts. Although in nature this pathogen preferentially infects *Ulmus* plants, it was also detected in *Zelkova serrata* (Japanese zelkova) and *Ugni molinae* (murta) plants showing symptoms of yellowing and witches' brooms in central Italy and Chile, respectively [42,43].

Several elm genotypes that are resistant to the Dutch elm disease have been examined for EY resistance or tolerance by graft-inoculation experiments. The inoculated trees of these genotypes greatly differed in their response to '*Ca*. Phytoplasma ulmi' [44]. Diseased trees of 'Frontier', 'Pathfinder', and 'Patriot' showed foliar yellowing and reddening, witches' brooms, reduced terminal growth, and stunting. Since phloem necrosis and death were not observed, these elms may be rated as tolerant. Trees of 'Pioneer' were considerably more affected; most of them became infected and died. Only two out of 20 inoculated 'Prospector' trees became infected, one of which died, whereas none of the inoculated 'Homestead' trees was infected. The latter trees showed localized phloem necrosis as a defense reaction that prevented spreading of the pathogen, suggesting thus resistance of 'Homestead' [44]. Trees of 'American Liberty' proved to be highly susceptible to '*Ca*. Phytoplasma ulmi', which is a finding that is confirmed also under natural infection conditions [30]. The clonal cultivars Independence, New Harmony, Valley Forge, and the triploid putative hybrid cultivar Jefferson were susceptible as well [45]. Since EY is lethal to North American species, while some Eurasian genotypes are tolerant or resistant, it seems that the disease is of European origin [30]. Tolerance or resistance is usually expected in regions where the pathogen and its natural hosts co-evolved.

Currently, PCR assays are most widely used for the detection of '*Ca*. Phytoplasma ulmi'. Primers differing in specificity have been developed and directed to either ribosomal or non-ribosomal DNA sequences. The sensitivity of detection can be increased by the use of nested PCR. For a review on primer sequences and primer combinations for the detection of '*Ca*. Phytoplasma ulmi' and other phytoplasmas infecting elm, see [6,46,47]. A real-time PCR (qPCR) assay using nonribosomal primers in combination with TaqMan minor-groove-binder (MGB) probe chemistry was developed for the specific detection of '*Ca*. Phytoplasma ulmi'. This assay allowed detection of the pathogen in affected trees and several leafhoppers, but it did not show cross reactivity with the DNA of closely related phytoplasmas belonging to other 16SrV subgroups [38]. In Germany, '*Ca*. Phytoplasma ulmi' was nationwide surveyed by using a universal 16Sr DNA-based qPCR assay and a newly developed '*Ca*. Phytoplasma ulmi'-specific qPCR Taq-Man assay directed to 16S–23S spacer region sequences. Both assays enabled the detection of '*Ca*. Phytoplasma ulmi' in the same number of elm trees (1801 trees out of 6486 trees examined), although the pathogen specific assay showed a slightly lower sensitivity as shown by different cycle threshold (Ct) values. In addition, the universal phytoplasma assay enabled the identification of two elm trees each infected by a different 16SrV-C phytoplasma strain [13]. Scots elm trees showed a higher phytoplasma titer in comparison to the other two elm species examined. However, the phytoplasma titer was not correlated to the presence of EY symptoms [13]. In most trees, a high titer of '*Ca*. Phytoplasma ulmi' was identified, as evidenced by a Ct value $\leq$28, which corresponds to a titer of 10^6 per gram of phloem tissue at least. In addition, all parts of an infected Scots elm tree such as roots, trunk at both ground and aerial levels, branches, leaves, and buds from the top and bottom of the tree were heavily colonized by '*Ca*. Phytoplasma ulmi' throughout the year. The survey also revealed that the pathogen was not homogeneously distributed throughout Germany. Sites with a high number of infected trees occurred in east, south, and central Germany, whereas only a few sites with low infection rates were found in the remaining parts of the country. Infection rates did not vary considerably among sites located at different altitude levels [13]. On the basis of *groEL* and *imp* gene sequences, 29 and 74 genotypes, respectively, have been identified among '*Ca*. Phytoplasma ulmi' strains infecting elms in Germany [24]. In phylogenetic

analysis, genotypes obtained from Scots elm trees clustered together, while those from European white elm and European field elm trees did not. A regional distribution pattern was evident only for a few genotypes [24].

2.2. Alder

Alder yellows (ALY) is a phytoplasma disease affecting several *Alnus* (alder) species. This disease has been described in several European countries such as Germany, Italy, Austria, France, Switzerland, Hungary, Serbia, Slovenia, Lithuania, Serbia, Montenegro, Macedonia, and Poland (for reviews, see [48,49]). ALY has also been recorded in the Washington State (USA) and Ontario, Canada, on *A. rubra* (red alder) [16,50,51]. However, molecular identification and classification of the phytoplasma(s) infecting *A. rubra* in the Washington State have not yet been reported.

Symptoms of ALY are yellowing, premature autumn coloration, sparse foliage, small leaves, die-back, and decline. Shoot proliferation may occur at the base of trunk of affected trees (Figure 3). Latent infections have been observed as well [50,52–54]. Lederer and Seemüller [50] examined 500 *A. glutinosa* (European alder) trees for phytoplasma infections using DAPI (4'-6-diamidino-2-phenylindole) fluorescence methods. All trees older than 5 years tested positive for phytoplasma infections. However, the majority of infected trees were non-symptomatic. Usually, non-symptomatic trees revealed a higher colonization density than symptomatic ones. The colonization density was always higher in leaves and young twigs than in other parts of the trees. The phytoplasma distribution within the affected phloem tissue was uneven, with some sieve tubes filled by phytoplasmas, while others completely empty or containing only a few phytoplasmas. Similar findings were obtained by examining *A. incana* trees [50]. Under natural infection conditions, high detection rates ranging from 85 to 100% of ALY phytoplasma infections in *A. glutinosa* were also recorded by PCR [53,55–58]. No phytoplasma infections have been recorded in *A. viridis* either by DAPI fluorescence methods or PCR assays [50,57]. This lack of infections may be ascribed to the host preference or unfavorable conditions of the insect vector(s).

Figure 3. Pronounced shoot proliferation at the base of trunk of a several-year-old alder (*Alnus glutinosa*) tree affected by alder yellows disease.

The ALY phytoplasma is taxonomically assigned to subgroup 16SrV-C within the EY group [19]. It has a 99.7%–100% 16S rDNA sequence similarity with other members of the mentioned subgroup such as flavescence dorée (FD), Palatinate grapevine yellows (PGY), and spartium witches' broom (SpaWB) agents and phytoplasmas infecting *Clematis vitalba* in Italy and the Balkans, and hemp dogbane (*Apocynum cannabinum*) in New York state, respectively [19,59,60]. ALY phytoplasma strains originating from different parts of Europe

are identical at the 16S rDNA and 16S–23S rDNA spacer region sequence level. However, studies through ribosomal protein gene and nonribosomal loci analyses showed a considerable molecular variability among them. Some strains proved to be identical or near identical to either FD phytoplasma strains or PGY phytoplasma strains [19,20,49,53,55,56,61,62]. Many different strains of the ALY phytoplasma have been identified through *map*-based gene analysis in singly and multiply infected alder trees throughout Europe. These strains, phylogenetically, do not form a homogeneous group but are scattered in various clusters that comprise FD, Palatinate grapevine yellows (PGY), and SpaWB phytoplasma strains. Some ALY phytoplasma strains were more closely related to FD and/or PGY phytoplasma strains than to other ALY phytoplasma strains. In addition, clustering was not linked to the geographic origin of the strains [20,53,55,61]. Phylogenetic analyses of the concatenated *tuf*, *rplV-rpsC*, *rplF-rplR*, *map*, and *uvrB-degV* gene sequences showed that strains infecting alder trees in Canada (two strains) form together with strain HD1 of hemp dogbane (*Apocynum cannabinum*) phytoplasma a monophyletic discrete genetic cluster clearly separated from the cluster comprising ALY phytoplasma strains from Europe [51].

The ALY phytoplasma is naturally spread by the leafhopper *Oncopsis alni* [52], which is very common on alder trees and is characterized by an oligophagous feeding behavior. The distribution of this leafhopper in Europe corresponds to that of ALY [52]. ALY phytoplasma infections were also recorded in individuals of the psyllid *Psylla alni* and leafhoppers *Orientus ishidae*, *Allygus mixtus*, and *Allygus modestus*, which were all collected from ALY-infected alder trees. However, all attempts to transmit the ALY phytoplasma to healthy alder plants using ALY phytoplasma-infested *P. alni* psyllids failed, whereas successful transmission was obtained with ALY phytoplasma-infested *O. ishidae*, *A. mixtus*, and *A. modestus* leafhoppers [52,58,63]. It is well known that ALY phytoplasma strains are also responsible of the Palatinate grapevine yellows (PGY) disease in Germany. Spreading of these strains from alder to grapevine occurs through *O. alni*, which feeds occasionally on grapevine [64–66]. Thus, infected alder trees growing in proximity of vineyards may act as pathogen reservoirs. In addition, due to the close phylogenetic relationship of ALY phytoplasma with the FD agent, diseased alder trees may also function as pathogen reservoirs in areas where FD disease occurs. In these cases, some ALY phytoplasma strains may be transmitted to grapevine by occasional grapevine-feeding vectors leading to FD disease, which is then spread from grapevine to grapevine by *Scaphoideus titanus*, the strictly ampelophagous vector of FD phytoplasma strains. *S. titanus* is not known to occur in the Palatinate [20,55,58,61]. A recent study by Jurga and Zwolińska [49] has shown that diseased common mugwort (*Artemisia vulgaris*) plants showing witches'-broom symptoms, grown next to an ALY-affected alder (*A. glutinosa*) tree in Southern Poland, were infected by a 16SrV-C subgroup phytoplasma that had the same 16S rDNA sequences of the phytoplasma present in ALY-affected alder trees. Therefore, it is possible that common mugwort serves as alternative host of the ALY phytoplasma in eastern Europe [49].

As mentioned above, in nature, a high percentage of alder trees infected by ALY agent do not develop obvious symptoms. The reasons were elucidated by Berges and Seemüller [67] through graft-inoculation of healthy alder plants with icoculum from both differently affected and symptomless alder trees and observation of symptom development for five years. It resulted that the severity of symptoms shown by inoculated plants corresponded to that of plants from which icoculum was taken. Thus, it was concluded that ALY phytoplasma may affect alder by inducing severe symptoms, but avirulent strains occurring within this phytoplasma are responsible for the latent infections that are widespread in Europe. It is also possible that avirulent strains mediate cross-protection and suppress severe strains, since infected alder trees remain symptomless over time in spite of the presence of an abundant and efficient insect vector such as *O. alni*. Differences in strain virulence are also known for other phytoplasmas, including those causing apple proliferation, European stone fruit yellows, ash yellows and aster yellows diseases, whereas antagonistic interactions among different strains of the same pathogen have been observed in many instances [68–70]. On the other hand, it is also possible that symbiotic microorganisms

of alder such as *Frankia alni* may compensate for the detrimental effects of phytoplasma infections [54]. However, it is unknown whether these microorganisms are able to colonize systemically the phloem tissue of alder trees.

2.3. Ash

Ash yellows (AshY) is a prominent phytoplasma disease of *Fraxinus* (ash) species known to occur widely in North America [71]. This disease is caused by the AshY agent '*Ca*. Phytoplasma fraxini', which is a pathogen of the AshY group or 16SrVII group, subgroup 16SrVII-A [72]. Although AshY may have occurred in the northeastern USA states since the 1930s, it was not considered as a major forest disease until the early 1980s. This late recognition was due to the difficulties met in identifying phytoplasma diseases and to the inconsistency with which infected trees show specific symptoms [71]. In North America, AshY occurs in twelve ash species and numerous intraspecific and interspecific ash genotypes [71]. Most of the affected species are native to North America. A 16SrI phytoplasma has been identified in yellows-diseased trees of *F. excelsior* (European ash) in Poland and *F. uhdei* (Urapan ash) in Colombia [73,74]. In the latter country, other studies showed that *F. uhdei* trees showing typical AshY symptoms were infected by '*Ca*. Phytoplasma fraxini' [75,76]. In France, ash trees showing die-back symptoms were infected with '*Ca*. Phytoplasma fragariae' (subgroup 16SrXII-E) [77].

AshY causes reduced growth, loss of apical dominance, suppressed root development, premature flowering, shoot proliferation, and witches' brooms (Figure 4). Light green and deformed leaves are also common. Highly susceptible taxa show mainly die-back, shoot proliferation, loss of apical dominance, and premature death. Short roots and necrosis of rootlets that precedes wilting and death are predominant in diseased *F. americana* (white ash) trees. Infected ash trees are highly sensitive to frost damages, which appear as cracks of the bark at the base of the trunk [10]. Histological symptoms which are more readily observed in the roots than in the aerial parts of affected plants, include autofluorescent sieve tubes and pathological sclerenchyma. Autofluorescent sieve tubes are sieve tubes that fluoresce without the addition of any reagent when exposed to UV radiation. Such sieve tubes are presumed to be non-functional and often collapse. Pathological sclerenchyma is a distinct parenchyma tissue adjacent to infected sieve tubes, which is characterized by prominently thickened and lignified cell walls, which greatly differs from the sclerotic parenchyma that is often seen in healthy phloem. Stomatal closure and high diffusive resistance are also known to occur in the leaves of affected white ash trees [10]. Witches' brooms are regarded as specific symptoms of the disease, whereas other symptoms are non-specific, since they can be induced by several other biotic and abiotic factors. A disease incidence of $\geq$50% has been observed in some *F. americana* stands in northern USA states and in a *F. velutina* (velvet ash) stand in Utah. Incidence ranging from of 3 to 27% was recorded in *F. pennsylvanica* (green ash) in Iowa and Wisconsin (for review, see [71]). '*Ca*. Phytoplasma fraxini' was also detected in non-symptomatic ash trees [71,78]. In Colombia, more than 53,000 *F. uhdei* trees showing typical AshY symptoms were recorded in several areas of the Andes; many of them died. Phytoplasma infections were detected in all symptomatic trees examined (100 trees) either through DAPI fluorescence methods or PCR assays. Phytoplasma infections were strictly related to the presence of symptoms [76]. The phytoplasma identified in diseased *F. uhdei* trees, the AshY agent '*Ca*. Phytoplasma fraxini', was also transmitted to *Catharanthus roseus* (periwinkle) through dodder (*Cuscuta* sp.) [76]. In a study conducted in France to test the presence of the ascomycete fungus *Hymenoscyphus fraxineus* in ash trees displaying symptoms of die-back, a relatively large proportion of symptomatic trees (27%) proved to be not infected by such fungal pathogens [79]. This finding prompted a further study aimed at verifying the presence of phytoplasma infections in affected trees [77]. '*Ca*. Phytoplasma fragariae' was identified in 5% of the examined ash trees (13 out of 260 trees). Four phytoplasma-positive trees were also infected by the fungus *H. fraxineus*. However, further data are necessary to validate the role of phytoplasma infections in the die-back disease affecting ash in France.

Figure 4. Witches' brooms at the root collar of an ash yellows-affected velvet ash (*Fraxinus velutina*) tree (courtesy W.A. Sinclair, Department of Plant Pathology, Cornell University, Ithaca, NY, USA).

Sinclair and Griffiths [68] investigated the virulence of 12 '*Ca*. Phytoplasma fraxini' strains, originating from different areas of North America, by graft-inoculating *F. pennsylvanica* seedlings and periwinkle plants and monitoring symptom development under greenhouse conditions. In graft-inoculated plants, the examined strains caused symptoms that ranged considerably from imperceptible to severe. The same study [68] revealed the occurrence of interference among strains of the same taxon. Strains of '*Ca*. Phytoplasma fraxini' differing in virulence have also been recorded under natural infection conditions [80]. These strains greatly differed in aggressiveness as indicated by the different rates of growth suppression, reduction of foliar greenness, and frequency of witches' brooms that they caused. However, specific interactions between strain and cultivar were not discovered [80]. Several investigations have shown that *F. pennsylvanica* and *F. velutina* are more tolerant than *F. americana* to '*Ca*. Phytoplasma fraxini' and that heritable intraspecific variation in tolerance occurs in *F. pennsylvanica* and is supposed to be present also in other ash species [81,82]. In graft-inoculation trials, the shoot growth of *F. americana* and *F. pennsylvanica* was suppressed earlier than that of *F. velutina*. In addition, the highest growth losses in height, stem diameter, and root volume were recorded for *F. americana* followed in decreasing order by *F. pennsylvanica* and *F. velutina*. The growth of diseased *F. velutina* scions on *F. americana* rootstocks was severely suppressed in comparison with that of diseased own-rooted *F. velutina*. On the other hand, diseased *F. americana* scions grafted on *F. velutina* rootstocks registered a significantly lower growth suppression than diseased own-rooted *F. americana*. When witches' brooms arisen from *F. americana* trees were grafted on *F. velutina* rootstocks, they continued to maintain their original form but did not proliferate new shoots. These findings imply that although tolerant rootstocks may alleviate the impact of '*Ca*. Phytoplasma fraxini' on scions, the management of AshY through tolerant genotypes requires tolerance in both scions and rootstocks [82]. Among the ash cultivars tested by graft inoculation to evaluate their responses to '*Ca*. Phytoplasma fraxini', *F. pennsylvanica* cvs. Bergeson, Dakota Centennial, and Patmore and *F. americana* cv. Autumn Applause were least affected, i.e., they sustained less growth and foliar color depression [80].

There are no reports concerning insect vectors involved in the natural spread of AshY. A previous work showed that field-captured *Paraphlepsius irroratus* and *P. spumarius* individuals transmitted acquired phytoplasmas to ash seedlings [10]. However, the molecular identities of these phytoplasmas were not determined, and further experiments using pro-

genies of these insects failed to transmit '*Ca*. Phytoplasma fraxini'. In the work by Hill and Sinclair [83], 33 taxa of leafhoppers were collected in two locations where AshY incidence was high, in New York State, and tested by PCR assays for phytoplasma infections. The most abundant genus was *Scaphoideus*. '*Ca*. Phytoplasma fraxini' infections were recorded in some individuals of *Scaphoideus* spp. Therefore, these insects should be further examined to prove their transmission ability [83].

2.4. Conifers

After a few findings that were not confirmed by other studies on the presence of phytoplasma infections in various gymnosperms using electron microscope observations, phytoplasmas were detected and molecularly identified in several conifers over the last few years. Schneider et al. [84] reported on the presence of a previously undescribed taxon, '*Ca*. Phytoplasma pini', in *Pinus sylvestris* (Scots pine) and *P. halepensis* (Aleppo pine) trees in Germany and Spain. Affected trees showed symptoms of yellowing, stunting, needle dwarfing, and shoot proliferation. In addition, affected *P. sylvestris* trees were characterized by the presence of ball-like structures in the canopy, resulting in the combination of proliferation of shoots with dwarfed needles. '*Ca*. Phytoplasma pini' was detected in symptomatic and non-symptomatic parts of *P. sylvestris* and *P. halepensis* trees as well as in some neighboring non-symptomatic trees of both species.

Following the finding of Schneider et al. [84], '*Ca*. Phytoplasma pini' and related strains were identified in several other *Pinus* species as well as in *Abies procera* (noble fir), *Tsuga canadensis* (Canadian hemlock), and *Picea pungens* (Colorado blu spruce) in Poland, the Czech Republic, Croatia, Lithuania, and Maryland (USA). In all affected species, the symptoms were similar to those observed in Germany and Spain, whereas ball-like structures were recorded other than in *P. sylvestris* also on *P. mugo* (mountain pine) in Lithuania (Figure 5). Abnormal shoot branching resulting in typical witches' brooms were shown by pine trees in Maryland [85–90]. Furthermore, '*Ca*. Phytoplasma pini' was identified in China [91] in *Taxodium distichum* var. *imbricarium* (pond cypress) showing symptoms of shoot proliferation, little leaf, and leaf necrosis.

Figure 5. Phytoplasma-infected Scots pine (*Pinus sylvestris*) (**left**) and mountain pine (*P. mugo*) (**right**) trees showing ball-like structures.

'*Ca*. Phytoplasma pini' is a member of the pine shoot proliferation group or 16SrXXI group, subgroup 16SrXXI-A. A '*Ca*. Phytoplasma pini'-related strain detected in a witches'-

broom affected pine tree in Maryland, strain MDPP, was assigned to a new subgroup, designated as 16SrXXI-B within the group 16SrXXI, on the basis of virtual RFLP pattern similarity coefficient values [90]. A phytoplasma of the X-disease group (16SrIII group) was detected in *Cupressus* sp. (cypress) trees showing witches'-brooms, stunting, and fasciation in Italy [92] and in *Picea abies* (Norway spruce) and *P. glauca* (white spruce) trees with symptoms similar to those associated with '*Ca*. Phytoplasma pini' in Poland [86]. 16SrI phytoplasmas were identified in diseased *P. pungens* and *Larix* sp. (larch) trees in Poland and Ukraine, respectively [86,93]. The main symptoms observed in diseased larch trees were dwarfing and the proliferation of needles. Therefore, the name "larch dwarfed needle proliferation (LDNP)" was given to the pathogen identified in this species [93]. Valiunas et al. [89] examined 300 *P. sylvestris* and *P. mugo* trees in Lithuania for phytoplasma infections using PCR assays. Of the examined trees, 80% proved to be phytoplasma-positive, of which 98% were infected by *Ca*. Phytoplasma pini-related strains, whereas the remaining trees harbored a subgroup 16SrI-A phytoplasma. A '*Ca*. Phytoplasma phoenicium'-related strain, subgroup 16SrIX-F, was found in diseased *Juniperus occidentalis* (western juniper) plants in Oregon (USA) showing symptoms of shoot proliferation, little leaves, shortened internodes, and ball-like structures. The percentage of affected plants was about 1% [94]. In India, yellows-diseased trees of *Araucaria heterophylla* (Norfolk Island pine) were infected with a '*Ca*. Phytoplasma trifolii'-related strain, which is a member of the clover proliferation group or 16SrVI group. The incidence of the disease was about 15% [95].

Phytoplasma titer in gymnosperms is usually so low that infections could be detected only through nested PCR assays. In addition, the mechanism by which phytoplasmas colonize systemically the affected gymnosperm plants is unknown, since pore sizes in the sieve cells of these plants being too small may hinder phytoplasma movement. Valiunas et al. [96] developed direct PCR methods based on primer pairs directed to 16S rDNA and tuf gene sequences which allowed detecting '*Ca*. Phytoplasma pini'-related strains infecting pine trees in Lithuania with great sensitivity and specificity. The amplified sequences contain molecular markers which are suitable for distinguishing closely related strains of this pathogen. The mentioned PCR methods, which did not allow the amplification of DNA from the pine-infecting phytoplasma strain MDPP recorded in Maryland, are useful tools for detecting and identifying '*Ca*. Phytoplasma pini' on large-scale studies. A draft genome sequence of the '*Ca*. Phytoplasma pini'-related strain MDPP has recently been reported [97]. This sequence, which consists of 474,136 bases, will facilitate comparative genomic studies of phytoplasmas infecting conifer plants.

2.5. Sandal

Sandal spike (SSD) is a serious disease of *Santalum album* (sandal) that is widespread in India, especially in Karnataka, Tamil Nadu, and Kerala states [98–101]. The disease was first described in the late 1890s and was supposed to be of viral origin. Later, further investigations using fluorescence and electron microscopy and symptom remission achieved by tetracycline treatments proved its phytoplasmal etiology (for references, see [48]). SSD has spread gradually during the last decades, devastating large forest areas and threatening the sandal industry of southern India. The most characteristic symptom of SSD is small and extremely narrow leaves, which stand out stiffly from the shoots, giving them a spike-like appearance. Other symptoms include yellowing and decline. Severely affected trees die within a two- or three-year period (Figure 6). Disease incidences reaching up to 33 and 55% were recorded in southern Karnataka [100,102]. SSD is caused by '*Ca*. Phytoplasma asteris', subgroup 16SrI-B [98,103]. In SSD-affected sandal trees in the Marayoor sandalwood reserve in Kerala, a new strain of the 16SrI group, which on the basis of the virtual RFLP pattern similarity coefficient values differed from the phytoplasmas of all 16SrI subgroups, has been identified. This strain represents a new 16SrI subgroup [104]. In addition, a survey conducted in the same reserve has shown the presence in SSD-affected sandal trees of a phytoplasma of the rice yellow dwarf group or 16SrXI group, subgroup 16SrXI-B (Figure 1).

This phytoplasma was recorded either in singly infected trees or in doubly infected trees with '*Ca*. Phytoplasma asteris' [105]. SSD phytoplasma was transmitted to periwinkle, which induced witches' brooms, and from this host back to sandal through dodder (*Cuscuta subinclusa*) [106]. However, attempts to transmit SSD phytoplasma from diseased sandal trees to *Stachytarpheta indica*, a weed common in sandal plantations, through dodder (*C. subinclusa*) failed [100]. SSD is naturally spread by the leafhopper *Coelidia indica*, which was formerly known as *Jassus indicus* [1].

Figure 6. Decline, small and narrow leaves in sandal spike-infected *Santalum album* (sandal). Right, healthy plant.

2.6. Eucalyptus

Eucalyptus little-leaf (ELL) is a phytoplasma disease affecting several eucalyptus (*Eucalyptus* spp.) species that was first observed in India in 1971 and was thought to be caused by the virus [107]. Later, ELL was observed by other Indian researchers, who discovered phytoplasma infections in diseased eucalyptus trees by using electron and light microscope observations [48]. The disease has also been recorded in other countries including Italy, China, Sudan, Iran, and Brazil [48,108–112]. The symptoms described from the various geographic areas and different *Eucalyptus* spp. are similar. They include small leaves, yellowing, and proliferation from axillary buds that confers a broom-like appearance. Diseased trees or their symptomatic parts do not set fruits, are stunted, and die-back. Severely affected trees decline (Figure 7). A 16SrV phytoplasma was identified in ELL-affected trees in Italy [108]. However, in the affected trees, the phytoplasma concentration was so low that infections could be identified only by nested PCR assays. In addition, 16SrI-B and 16SrI-C phytoplasmas were identified in diseased eucalyptus trees in Italy, whereas a 16SrI-B phytoplasma was recorded in diseased eucalyptus (*E. camaldulensis*) trees in Iran [48,110]. In the latter country, phytoplasmas of other taxonomic groups were recorded as well. Azimi et al. [111] reported on the detection of a '*Ca*. Phytoplasma aurantifolia'-related strain, subgroup 16SrII-D in yellows-diseased *E. camaldulensis* trees in Ahvaz, southwestern Iran. The percentage of affected trees was approximately 5%. Baghaee-Ravari et al. [112] reported on the detection of a '*Ca*. Phytoplasma solani'- related strain, subgroup 16SrXII-A in yellows-diseased *E. camaldulensis* trees in Fars and Khozestan provinces of Iran. This strain was detected through nested PCR assays in 14 out of 22 (approximately 64%) symptomatic trees examined. A '*Ca*. Phytoplasma aurantifolia'-related strain, subgroup 16SrII-C, was detected in diseased *E. urophylla* trees in Brazil. Fourteen out of 22 (approximately 64%) symptomatic eucalyptus trees examined using nested PCR assays tested positive [109]. The identity of phytoplasmas infecting eucalyptus in India, China, and Sudan has never been determined by molecular methods.

Figure 7. Small leaves, yellowing, and shortened internodes on eucalyptus (*Eucalyptus* spp.) shoots affected by eucalyptus little-leaf. Healthy shoot on the left of the left panel.

3. Discussion

With few exceptions, phytoplasma diseases of forest trees have been less extensively studied than those affecting fruit trees. One reason may be that until 10–15 years ago, the majority of the research carried out in forest pathology was mainly focused on fungal diseases and insect damages. A second reason is the technical difficulty in detecting and identifying phytoplasmas in forest trees due to the low phytoplasma titer and the presence of non-specific symptoms in affected plants. Research on the role of phytoplasmas as cause of diseases of forest trees has only in the last few years been intensified, after sensitive and specific detection methods, greatly based on PCR technology, became available. By using these methods, phytoplasma infections have been detected for the first time in several forest trees, highlighting that phytoplasma diseases may play in forest trees the same role as in fruit trees. Therefore, the number of phytoplasma diseases of forest trees and associated phytoplasmas has recently been increased considerably [48]. Many phytoplasma diseases of forest trees are each associated with taxonomically distinct phytoplasmas, which induce identical symptoms in a given plant and are present in the same areas or different countries. In addition, a given plant host may be singly or multiply infected with various phytoplasmas. This seems common in woody plants, which have more opportunities to be visited by insect vectors over time. Phytoplasmas belonging to various taxonomic groups and subgroups have been identified in naturally infected elm, ash, conifer, sandal, and eucalyptus trees (Figure 1). In elm, in addition to the specific agent '*Ca*. Phytoplasma ulmi' (16SrV group, subgroup 16SrV-A), phytoplasmas of the 16SrI group, subgroup 16SrI-B, 16SrVI group, subgroup 16SrVI-C, and 16SrXII group, subgroups 16SrXII-A and 16SrXII-E were identified. Subgroups 16SrV-B and 16SrV-C phytoplasmas were also detected in elm. While the pathological role of the EY agent '*Ca*. Phytoplasma ulmi' has been well established by numerous graft-inoculation experiments, the pathogenicity of other phytoplasma taxa present in elm has to be verified. In addition, the current knowledge on the latter phytoplasmas are insufficient to draw conclusions that EY can be considered as a disease induced by various phytoplasmas. Only one phytoplasma taxon, the ALY agent (16SrV group, subgroup 16SrV-C), has been recorded in naturally infected alder trees. Strains of this pathogen are highly homogeneous at the rDNA sequence level, but they greatly differ on the basis of less-conserved gene sequences. In particular, many different strains of the ALY phytoplasma have been identified through *map*-based gene analysis in singly and multiply infected alder trees. These strains phylogenetically do not form a homogeneous group but are scattered among various clusters, which comprise strains of agriculturally important phytoplasmas such as FD and PGY agents. In addition, the presence of avirulent strains occurring within this taxon may be responsible for the latent infections as has been shown by graft-inoculation experiments [67]. AshY-affected ash trees

were found to harbor only '*Ca*. Phytoplasma fraxini' (16SrVII group, subgroup 16SrVII-A), whereas ash trees with symptoms of decline and die-back were infected with phytoplasmas of the 16SrI group and 16SrXII group (subgroup 16SrII-E), respectively. The etiological role of the AshY agent '*Ca*. Phytoplasma fraxini' has been clearly demonstrated by numerous graft-transmission experiments and field observations. However, more data are needed to clearly elucidate the role of 16SrI and 16SrII-E phytoplasmas in the decline and die-back diseases of ash. '*Ca*. Phytoplasma fraxini'-infected ash trees have been recorded only in North America and Colombia. Phytoplasmas belonging to several taxonomic groups such as 16SrXXI (subgroups 16SrXXI-A and 16SrXXI-B), 16SrI (subgroups 16SrI-A and 16SrI-B), 16SrIII, 16SrVI, and 16SrIX (subgroup 16SrIX-F) have been detected in several yellows-diseased conifer species. Among these, the most frequently detected agent is '*Ca*. Phytoplasma pini' (16SrXXI group, subgroup 16SrXXI-A). This pathogen seems particularly widespread in Europe. In sandal trees affected by SSD, in addition to the AY agent '*Ca*. Phytoplasma asteris' (16SrI group, subgroup 16SrI-B), a new strain of the 16SrI group, which on the basis of virtual RFLP pattern similarity coefficient values differed from phytoplasmas of all 16SrI subgroups, as well as a 16SrXI phytoplasma have been identified [104,105]. Taxonomic groups of phytoplasmas infecting eucalyptus trees include 16SrV, 16SrI (subgroups 16SrI-B and 16SrI-C), 16SrII (subgroups 16SrII-C and 16SrII-D), and 16SrXII (subgroup 16SrXII-A).

4. Conclusions

Phytoplasma diseases of forest trees are of considerable economic and ecological significance throughout the world. Over the last few years, several phytoplasma taxa associated with various yellows and decline diseases of forest trees have been identified in singly or doubly or multiply infected trees. However, for almost all of these diseases, including EY and AshY, which are the most studied phytoplasma diseases of forest trees, there is still sparse information about insect vectors, plant host range, strain virulence, pathogenicity, and host tolerance and resistance. Knowledge of these aspects is the basis for appropriate disease management. In particular, further research is required to elucidate the role of phytoplasmas in asymptomatic trees and its relationship to the lack of symptom expression. In addition, the etiological role of various 'non-specific' phytoplasma taxa, which have been recorded in forest trees while no data from pathological studies are available, needs to be further investigated.

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