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Forest Insects and Pathogens in a Changing Environment

Ecology, Monitoring & Genetics (IUFRO Joint Meeting of WP7.03.05 & 7.03.10)

Edited by
Dimitrios N. Avtzis and Rudolf Wegensteiner

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Special Issue Editors

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About the Special Issue Editors

Dimitrios N. Avtzis was born in Thessaloniki (Greece) and studied Forestry (B.Sc.—M.Sc. equivalent) at the Faculty of Forestry and Natural Environment (Aristotle University of Thessaloniki—AUTH). In 2003, he enrolled to the PhD program at the Institute of Forest Entomology, Forest Pathology and Forest Protection (IFFF) at the University of Natural Resources and Life Science (BOKU—Vienna, Austria) under the supervision of Professor Dr. Christian Stauffer. From 2006 to 2010, he participated in numerous research projects, whereas in 2008, he received the excellence award (Research Commission of AUTH) that supported a post-doc study at the Faculty of Forestry and Natural Environment (Aristotle University of Thessaloniki—AUTH). Since 2011, he has worked as a researcher in the field of Forest Entomology at the Forest Research Institute (Hellenic Agricultural Organization Demeter) in Thessaloniki (Greece). Dr. Dimitrios N. Avtzis has published more than 45 articles in SCI journals and presented his work both in national and international conferences. Since 2018, he has been the Deputy of IUFRO 7.03.05 (Ecology and Management of Bark and Wood Boring Insects).

Rudolf Wegensteiner was born in Vienna (Austria), PhD at University of Vienna (Zoology and Botany), Habilitation at BOKU University Vienna (Entomology and Invertebrate Pathology), worked at BOKU University from 1986 to 2017 and served as a Co-chair of IUFRO WP 7.03.05 (Ecology and Management of Bark and Wood Boring Insects) from 2005 to 2014.

Preface to "Forest Insects and Pathogens in a Changing Environment: Ecology, Monitoring & Genetics (IUFRO Joint Meeting of WP7.03.05 & 7.03.10)"

The natural environment is constantly changing due to anthropogenic activities, and their impact on forest pests and pathogens cannot be neglected. Frequent insect population outbreaks, invasion of exotic pathogens, and the unpredictable damages they cause all point to the importance of collaborations not only at regional but, more importantly, at international scale. Only this exchange of knowledge can serve as a protective net against the challenging and changing environment. To that end, the current Special Issue of *Forests* includes some of the most up-to-date studies on existing and emerging pests and pathogens worldwide. These studies range from assessing the effect of forest management practices on insect populations (Gallardo et al. 2019, Moon et al. 2019) to comprehending the impact of habitat features on the potential of a forest insect, either endemic (Hut et al. 2019) or invasive (Galko et al. 2019). In the same direction, there are investigations that imprint the current expansion (Olenici et al. 2019) or attempt to decipher the historical processes that shaped the current distribution of abundant insect species (Avtzis et al. 2019) but also examine insects' infection level by microsporidia in native and invaded areas (Zimová et al. 2019). In addition to insects, however, this Special Issue covers some very interesting topics on forest pathogens, either as a review describing the current status of important forest diseases (Diamandis 2018) or as novel approaches to control emerging diseases (Keča et al. 2019). Further to that, new data are presented on the identification of resistant forest tree genotypes (Shestibratov et al. 2019) but also on the implications that might emerge during the restoration of susceptible tree species due to a pathogen (Sena et al. 2019). Given that these studies regard forests globally, we believe and hope that this Special Issue further promotes knowledge that will fortify the forest ecosystems for the future to come.

Dimitrios N. Avtzis, Rudolf Wegensteiner
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Article

Shallow Genetic Structure among the European Populations of the Six-Toothed Bark Beetle *Ips sexdentatus* (Coleoptera, Curculionidae, Scolytinae)

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Abstract: The six-toothed bark beetle, *Ips sexdentatus*, is one of the most abundant scolytid species of the central and southern European countries. It mostly feeds on *Pinus* sp., whereas during population outbreaks it can also attack *Picea* sp. In spite of its broad distribution, its phylogeography has never been studied before. To do that, we employed an mtDNA marker on 489 individuals that covered most of its native range in Europe. Geographic distribution of the 86 haplotypes showed that at least three glacial refugia have played a significant role in shaping the currently observed pattern of genetic divergence in Europe, without excluding the contribution of minor refugial areas that acted in a similar manner. The revealed shallow structure can be considered an artifact of factors that reduced intraspecific diversity, at the same time favoring gene flow. As such, biological traits of the species itself (flying ability and host preference) and even human-mediated transport of wood seem to be the most prevailing and probable reasons that gave rise to the observed pattern.

Keywords: *Ips sexdentatus*; Scolytinae; mtDNA; phylogeography; flying ability; human-mediated transport

1. Introduction

Comparative phylogeographic studies allow the indication of the common refugial areas that species used during ice ages and the migration routes they followed in the postglacial recolonization [1,2]. For Europe, glacial refugia were located in the southeastern parts of Europe where climatic conditions were milder but recently cryptic glacial refugia within the northern parts of Europe were also revealed [3–5]. Consequently, the contemporary patterns of diversity observed for many organisms emerged through the amalgamation of lineages that diverged both in major and cryptic refugia [6–9]. Forest insects are of particular interest as phylogeographic analyses can be used to understand how climatic changes affected their genetic structure and migration behavior in the past. After doing that,

we can then assess their behavior within the context of climate change and even under the influence of human-mediated migration, as both these factors shape species distribution today [10].

The Curculionidae family includes the subfamily Scolytinae, including some of the most notorious forest pests affecting the environment in many ways [11–13]. Among those, *Dendroctonus ponderosae* H. for North America and *Ips typographus* L. for Europe are responsible for enormous economic loss and tremendous ecological damage [14–16]. Numerous studies have attempted to decipher the biology, behavior and distribution of these species, even employing phylogeographic approaches [17,18]. For example, the studies on *I. typographus* [17,19] facilitated the accurate delimitation of the postglacial colonization routes and possible glacial refugial areas in Europe, knowledge that was later further enhanced through investigations on other Scolytinae species [20–23]. However, a species that is relatively less investigated despite its broad distribution is the six-toothed bark beetle, *Ips sexdentatus* B. This bark beetle feeds predominantly on pine trees (*Pinus* spp.) but during outbreaks may attack even spruce (*Picea* spp.) [24,25], which can be found in most of the Palearctic regions [26], although in Europe is spread mostly in central and southern countries [27]. Even though *I. sexdentatus* is generally not considered an aggressive species, this scolytid may cause strong damage in weakened trees particularly after fire or in association with other more aggressive species such as *Tomicus* spp. or other *Ips* spp. [28–30]; at high population levels it can even attack and kill healthy trees [31,32].

As previous studies on European bark beetles have shown, glacial refugia have left a detectable imprint on their genetic diversity pattern, either in a subtler [19,23] or more emphatic manner [33]. The rate of influence is largely determined by the biological traits in concert with the duration of evolutionary history of the species themselves [19]. Here we present the phylogeography of *I. sexdentatus* of European populations based on an mtDNA marker, with the aim to assess the influence of glacial refugia in the observed pattern of intraspecific diversity within its native range.

2. Materials and Methods

2.1. Sampling—DNA Analysis

From 2011 to 2015, *Ips sexdentatus* adults were collected by pheromone traps and shipped to the Forest Research Institute (Hellenic Agricultural Organization Demeter, Thessaloniki, Greece). In total 50 localities from 17 countries were sampled, covering almost the whole European distribution of the species (Supplementary Table S1). Specimens were sent in absolute ethanol and stored at −20 °C in the laboratory. DNA of 489 individuals was extracted using the PureLink® Genomic DNA kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions, with the only modification that the initial tissue grinding was performed using stainless steel grinding balls. Polymerase Chain Reaction (PCR) was run in 25 μL volumes, with primers 2183 and 3041 targeting an 850 bp-fragment of mtDNA's cytochrome oxidase 1 gene [34]. Each reaction contained 8 μL of the extracted DNA, 10.6 μL of double distilled water, 5 μL of Red Bioline buffer (provided with the Taq), 0.5 μL of each primer and 0.4 μL of MyTaq (Red Bioline), adding up to a total of 25 μL. PCR procedure was as follows: 3 min at 94 °C, followed by 40 cycles of 30 s at 94 °C (denaturation), 30 s at 45 °C (annealing) and 1.5 min at 72 °C (extension). The final extension period was carried out at 72 °C over the course of 7 min. Purification of PCR products was performed using the PureLink® PCR Purification Kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. Sequencing took place at CEMIA SA (Larissa, Greece) using an ABI 3730XL.

2.2. Data Analysis

Chromas Lite® was used to visualize the sequences which then were aligned and checked using Clustal X (version 2) [35]. Haplotypes that were represented by a single individual were verified by additional sequencing of an independent amplicon, in order to exclude cases of base misincorporation as a result of a PCR error [36]. After this double check, haplotypes were described and deposited in National Center for Biotechnology Information (NCBI) GenBank.

Patterns of molecular diversity were assessed by estimating haplotype (Hd), nucleotide diversity (p) [37] and the average number of nucleotide differences (k) [38] for every population using MEGA v.6 [39]. The ratio of haplotypes/individuals was then compared with previous mtDNA studies on bark beetles [40]. Bayesian phylogeny was constructed with MrBayes version 3.1.1 [41] using the nucleotide substitution model that best fits the data under the hierarchical likelihood–ratio test (hLRT) and Akaike criteria determined by MrModeltest version 2.1 [42]. The most appropriate model was found to be Hasegawa-Kishino-Yano with invariable sites and gamma distributed heterogeneity (HKY + I + G) [43], subsequently integrated in the Bayesian analysis. The initial run lasted 2×10^6 generations, with a sampling frequency of 100 generations in order to determine the number of trees to be discarded in the final run. Calculation of standard deviation showed that after 5×10^5 generations a plateau has been reached, thus the first 500 trees were discarded.

Population dynamics were assessed using Tajima's *D* [44], Fu and Li's *F* [45] statistics and a mismatch distribution analysis which were calculated by MEGA 6 [39], whereas the statistical parsimony network was estimated using Automated Nested Clade Analysis (ANECA) [46] that relies on TCS [47] and GeoDis [48] to infer the demographic processes that produced the observed pattern of divergence.

Clustering of populations was initially assessed with Spatial Analysis of Molecular Variance (SAMOVA 1.0) [49], to unveil the geographic clustering pattern and the minimum number of groups that produces F_{CT} (among-groups variation) values higher than F_{ST} (among-populations/within groups variation), both with and without taking into account the coordinates of populations. This estimation would allow identification of the point at which variation is mostly shaped by geographic groups rather than single populations. As the impact of isolation by distance [50] is very often heavily imprinted on intraspecific diversity [51], the relationship between genetic and geographic distances was assessed by the Mantel test [52] as this is implemented in Alleles in Space (AIS) [53] with 1000 permutations to assess the statistical significance of the coefficient. AIS was also used to visualize the spatial pattern of genetic diversity by running a Landscape Shape Interpolation (LSI) analysis (distance weighting parameter a = 1/50 × 50 grid). The 3-d surface plot produced exhibited the geographic coordinates (*X*- and *Y*-axis) and the genetic distances (*Z*-axis), with peaks representing areas of high genetic distance among individuals (restricted gene flow) and troughs point out areas of low genetic distance (unimpeded gene flow). Visualization of species diversity distribution was also performed using the Genetic Landscape Toolbox [54] under ArcMap 9.3 (ESRI, Redlands, CA, USA) according to author's instructions. In both these approaches to visualize the distribution of genetic divergence, populations with few individuals (less than 3) were excluded.

3. Results

Analysis of the 674-long region of the mitochondrial Cytochrome Oxidase One (COI) gene of 489 *I. sexdentatus* individuals yielded 86 haplotypes. The mean number of substitutions was 1.9 nucleotides (=0.28%) whereas the maximum was 15 nucleotides (=2.2%). Among those 86 haplotypes, seven had more than ten individuals while 48 haplotypes contained a single individual. No amino acid change could be found translating the DNA of the haplotypes (Supplementary Table S1). Previous genetic studies on bark beetles [40] showed that between the number of individuals analyzed and the respective haplotypes retrieved there is a positive correlation that is statistically significant (Pearson's correlation coefficient $r = 0.6181$, $p < 0.05$). Integrating the number of haplotypes of the current investigation produced a similar coefficient and statistical significance ($r = 0.6276$, $p < 0.05$) (Supplementary Figure S1).

A Bayesian tree was calculated (Figure 1) and only three clusters supported by high (>85%) posterior probabilities indicated by colors could be identified. These clusters were mapped onto a European map (Figure 2). The green clade with six haplotypes only included individuals from Italy and the blue clade with two haplotypes that included only individuals from Greece. The red clade encompassed samples from several central–north eastern countries (Germany, Austria, Slovenia, Hungary and Ukraine)

(Figure 2). All other haplotypes were marked with pale red and thus represented the biggest parts of the pie charts. HT25 was the widest distributed haplotype, occurring in the majority of populations. HT20 was found in populations occurring in the southeastern/central parts of Europe, with its mutationally related haplotypes (HT17, HT18, HT19, HT21, HT51, HT52 and HT53) being geographically limited in southeast Europe. Something similar was observed with HT02, which was also found in southeastern/central populations, whereas the closest related haplotypes are found in the Balkans (Supplementary Figure S2).

In terms of population dynamics, both neutrality indices were negative ($D = -2.30$, $p < 0.01$/$F = -5.09$, $p < 0.02$), showing an excess of rare alleles that can be observed in a population expanding after a recent bottleneck event. This was also verified by the unimodal distribution shown by the mismatched distribution (Supplementary Figure S2). Automated Nested Clade Analysis (ANECA) did not reach a conclusion for the total cladogram, likely a consequence of the shallow structure among populations; for the higher level clusters that corresponded to the identified clades (2-3, 3-1, 4-1), the conclusion was restricted gene flow with isolation-by-distance, suggesting that certain areas became isolated, favoring the genetic divergence of individuals therein (Supplementary Figure S3).

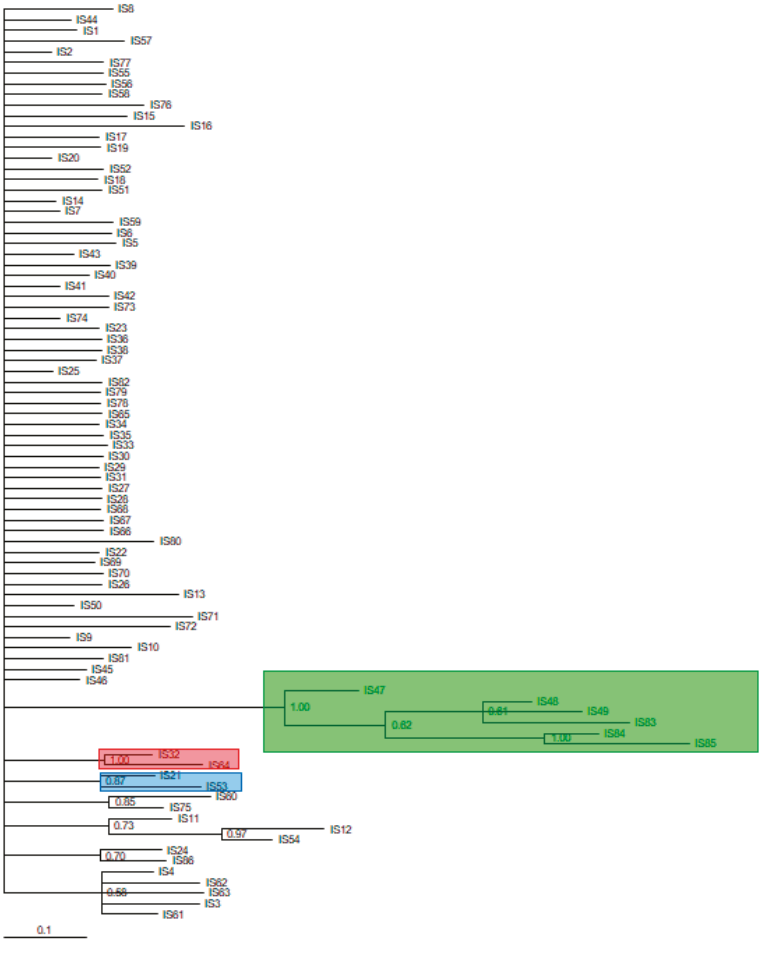

Figure 1. Phylogenetic tree of the 86 haplotypes created with MrBayes version 3.1.1 and inferred using the nucleotide substitution model (HKY + I + G) suggested by MrModeltest version 2.1.

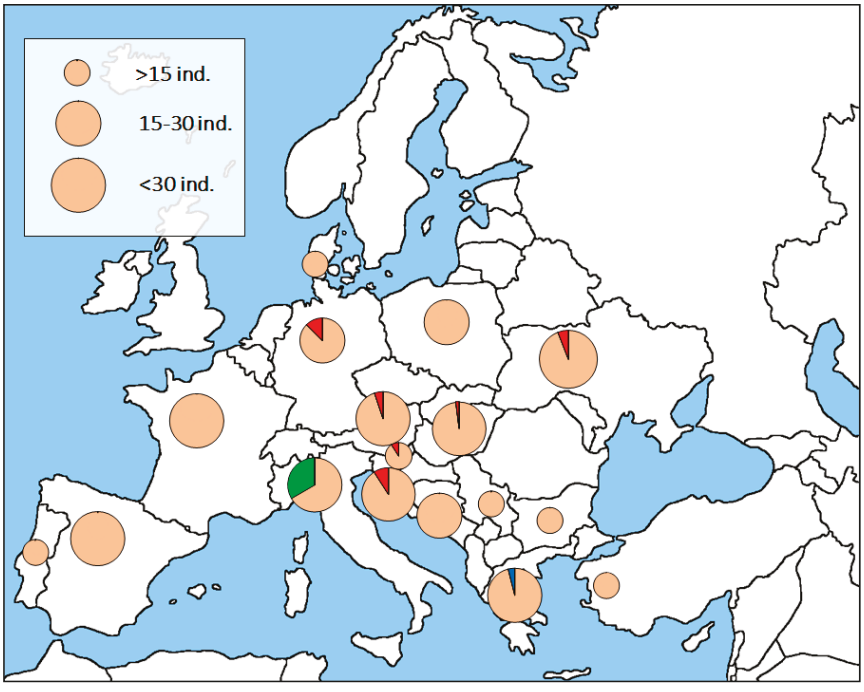

Figure 2. Distribution of mtDNA clades. Colors are similar to Figure 1. Pie charts are proportional to the number of individuals analyzed.

For both options with and without coordinates, the number of clusters that produced an F_{CT} value (=0.39473) higher than F_{ST} (=0.38497) is achieved only when they were separated into many groups (N = 15); however, 2/3 of these groups contained a single population, mainly located in southeastern Europe (data not shown). A similar image was concluded by Alleles in Space that allows the spatial visualization of the areas that exhibit high (peaks) and low (troughs) diversity. Southern populations exhibited overall higher diversity than central and northern ones, with numerous peaks located in the southern longitudes (Supplementary Figure S4). ArcMap 9.1 visualized these data where red color (points of high haplotype diversity) was mostly found in Italy, the northern parts of the Iberian Peninsula and the Balkans, indicating the effect of multiple areas that presumably might have acted as sources of high diversity (Supplementary Figure S5).

4. Discussion

The first genetic analysis of European *I. sexdentatus* populations identified 86 haplotypes analyzing 489 individuals from 17 countries, that covered most of the natural range of this species in Europe. This haplotype diversity falls within the range of previous phylogenetic investigations on bark beetle species worldwide [40] (Supplementary Figure S1). Based on the statistically supported clades observed in the topology of the phylogenetic tree, it can be deduced that at least three refugial areas have shaped the genetic structure of *I. sexdentatus*: one located on the Italian Peninsula, one located in the southern part of the Balkans and one likely in the Dinaric region. The distinct clustering and separation of haplotypes originating from these regions suggest that a fraction of diversity was maintained there at least during the latest glacial periods, a pattern similar to the ones retrieved for other conifer-feeding bark beetles in Europe like *Tomicus piniperda* L. [22,23], *Ips typographus* [8,55] and *Pityogenes chalcographus* L. [8,33,56]. However, the occurrence of additional haplotype clusters with common geographic origins (clusters

2-2 and 2-3; Supplementary Figure S3) argues for a possible impact of additional minor refugia beside the above mentioned major refugial areas on the genetic divergence among the European *I. sexdentatus* populations. This finding is congruent with recent investigations [6] that support the concept of "refugia within refugia" [57]. Southeastern parts of Europe in particular seem to have been such a region, with the diverse landscape favoring the emergence of intraspecific divergence [58], something that was visualized by AIS and ArcMap (Supplementary Figures S4 and S5, respectively). Both approaches demonstrated not only that southern populations outperformed central and northern ones in terms of diversity (Supplementary Figure S4), but also that multiple areas acted as diversity hot spots for *I. sexdentatus* populations (Supplementary Figure S5).

However, this pattern seems to be blurred by factors that acted upon the initial differentiation. Such a shallow genetic structure may arise due to recent lineage divergence [59] or the life history traits of the species itself [60]. The latter particularly seems to have played an important role for *I. sexdentatus*; as it has been recently observed, the flight ability of beetles largely determines their diversification rate, with flightless species being in favor of allopatric speciation not only by retaining higher genetic differentiation among distant populations but also demonstrating a higher number of genetically distinct lineages than flight-capable species [61]. With the flight capability of *I. sexdentatus* ranging between 5 km (98% of the samples) and 45 km (10%) [62], gene flow among populations cannot be overlooked. It thus seems possible that gene flow effectively reduced most of the divergence that could derive from different refugial areas resulting in this shallow pattern of intraspecific differentiation [2,63]. Moreover, in line with the hypothesis expressed previously [64], host specialization of the bark beetle species strongly determines the degree of intraspecific genetic diversity. Specialist species being more prone to higher divergence than generalist species as host selection cannot exert any kind of selection that could facilitate the emergence of differentiation for the latter. As a generalist, *I. sexdentatus* can attack spruce (*Picea abies* (L.) Karst) beside its main host (*Pinus* sp.), particularly during mass outbreaks, and thus the observed low intraspecific divergence could be attributed to this trait. To them, another factor should also be added: The unimpeded trade of wood and wood packaging material among the European countries. Recent studies [65] have shown that *I. sexdentatus* is the second most common scolytid species in international ports and in the associated wood waste landfills. As expected, human-mediated movement of individuals over long distances smoothened the genetic landscape, conforming populations to panmixia as has been already demonstrated [66,67].

5. Conclusions

This study verified the impact of at least three glacial refugia on the European populations of *I. sexdentatus*, with indications of additional minor refugial areas that contributed to the currently observed pattern of diversity. Nevertheless, the shallow structure evinced the effect of biotic (biological traits of the species) and abiotic (human-mediated transport) factors that smoothened divergence. Taking this into account, future studies should aim at a refugia-oriented sampling coupled with a comprehensive set of genome-wide molecular markers using ddRADSeq [33], something that could considerably increase the resolution capacity of the approach, allowing the conclusion even of subtle structuring, as is the case for *I. sexdentatus*.

Supplementary Materials: The following are available online at http://www.mdpi.com/1999-4907/10/2/136/s1, Supplementary Figure S1. Correlation of haplotypes retrieved against the number of individuals analyzed in bark beetle species phylogenetic studies. Supplementary Figure S2. Mismatch distribution diagram inferred from the mitochondrial DNA sequences of *I. sexdentatus*. Supplementary Figure S3. Cladogram with respective conclusions inferred with ANECA. Colored clusters represent statistically supported (>85% posterior probability) clades of the phylogenetic tree (colors identical with Figure 1). Supplementary Figure S4. Landscape of genetic diversity created with Alleles in Space; peaks represent areas of high genetic distance among individuals and troughs point out areas of low genetic distance. Supplementary Figure S5. Distribution of haplotype diversity (based on Tamura-Nei distance) over the populations sampled. Red color indicates points of high and blue color shows areas of low diversity. Supplementary Table S1. Individuals analyzed per population, and their haplotype assignment.

Author Contributions: D.N.A., M.F., C.S. and F.L. conceived the idea; D.N.A. conducted the experiments; R.W., M.P., D.G., F.L. and M.F. organized the thorough sampling; D.N.A., F.L. and C.S. analyzed the data. All authors contributed to the writing.

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

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Review

Management of Chestnut Blight in Greece Using Hypovirulence and Silvicultural Interventions

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Abstract: Sweet chestnut (*Castanea sativa* Mill.) is an important tree for Greece. The invasive fungus *Cryphonectria parasitica*, which causes chestnut blight, was first found in Central Greece in 1963. It has since spread all over the country, significantly reducing the national annual nut production. The increasing decline of forests and orchards due to the disease led to a project in 1995, which aimed at studying the feasibility of applying biological control. A prerequisite study of the existing vegetative compatibility types of the pathogen showed only four, and their distribution was mapped. A pilot project (1998–2000) that consisted of clear cutting heavily infected coppice stands and introducing hypovirulence to the remainder was implemented on Mt. Athos on a 7000 ha sweet chestnut forest. Two evaluations (in 2003 and 2011) revealed that hypovirulence was established in the sweet chestnut forests and spread more or less homogeneously. A nationwide project introducing hypovirulence to 29 counties was implemented in two, 3-yr-periods 2007–2009 (17 counties) and 2014–2016 (12 counties). The new evaluations showed that hypovirulence spread profoundly and forests and orchards started recovering. The appearance of natural hypovirulence cannot be predicted. Introduced hypovirulence and silvicultural interventions can be used to manage the disease. It is the responsibility of the forest/orchard manager to decide whether to wait for appearance of natural hypovirulence, or to introduce it for a faster decline in disease.

Keywords: disease management; biological control; chestnut blight; *Cryphonectria parasitica*; hypovirulence; silvicultural interventions

1. Introduction

Sweet chestnut (*Castanea sativa* Mill.) is an important tree for the European countries in the Mediterranean region. The cultivation of sweet chestnut is particularly important for Greece, as it is a key crop for the economy of mountain areas. It is cultivated in orchards for its edible nuts and in coppice forests for its highly valued timber.

Pollen analysis has shown that Northern Greece had chestnut trees as early as the 10th century B.C. [1], while more recent evidence has shown that sweet chestnut was cultivated in North-Eastern Greece from 2100–2050 B.C. [2]. The migration of human populations, particularly the Greeks and Romans, appears to have played a fundamental role in the spread and cultivation of sweet chestnut, both for its fruit and wood. Sweet chestnut was carried west, initially to the Greek colonies in Italy [3] and later all over the Mediterranean coast.

In Greece, sweet chestnut is found all over the country; from the northernmost border down to the island of Crete. A decline in sweet chestnut cultivation was experienced in the second half of the 20th century in Greece, mainly because of:

- The migration of the mountain people to cities, and consequently, the abandonment of the orchards;

- The rapid spread of chestnut blight, which devastated orchards and coppice forests alike;
- The lack of policies that would encourage and support chestnut growers.

Chestnut blight caused by *Cryphonectria parasitica* (Murr.) Barr has spread all over Greece since 1963, when it was first recorded on Mt. Pelion [4,5]. The disease was quite destructive in many areas, hastening the migration of people who could no longer make their living by chestnut cultivation [6]. As a result, the national, annual chestnut production was reduced from 18,000 tons in the 1960s to 11,000 tons in 2005.

Analyzing the American and European experiences, biological control of *C. parasitica* using its mycoviruses was considered the most promising method to remedy the problem and restore the chestnut orchards and forests [7–11]. Biological control projects have been previously implemented in France and Italy [12,13] with encouraging results.

Knowledge of the vegetative compatibility types (vc types) of the pathogen and their geographic distribution in a country are crucial for the successful application of biological control [14,15]. Thus, before any planning of the biological control of chestnut blight, the identification of the existing vc types, their distribution and mapping is absolutely essential [16,17].

Therefore, the first goal of an attempt to save the sweet chestnut in Greece was to examine the feasibility of applying biological control by using hypovirulence on a national scale. A second goal would be to organize the actual application of biological control, in case it was shown that such a project was feasible and effective.

2. Preparatory Tasks before the Application of Biological Control

Under the pressure of an increasing decline in chestnut production due to chestnut blight, a project aiming to study the vc types of *C. parasitica* and their distribution in the country was set out in 1995. Sampling was carried out in all 29 counties where chestnut grows, either naturally as old growth and coppice forests, or in orchards. In areas where both natural coppice forests and orchards were present, samples were taken from each. Cankered trees were selected more or less evenly throughout the area of each population [6].

The isolation of the fungus from bark samples, identification of vc types and conversion to hypovirulence using the CHV-1 mycovirus (Italian subtype) were conducted following standard techniques [6,7,9]. CHV-1 had occurred spontaneously in isolated spots on Mt. Pelion in Central Greece, where the disease was first detected in 1963 [18].

In 1988, a major threat to the chestnut coppice forests of Mt. Athos, a UNESCO protected site, triggered the need for fast intervention. A study was conducted aiming to apply biological control against chestnut blight. Extensive sampling in the 7000 ha forest revealed that there was only one vc type of the fungus *C. parasitica*, that of EU-12. A work plan was elaborated, consisting of the clear cutting of severely infected compartments and field inoculations. All felling operations were performed in 1998 during the first year of the three-year project. A compatible hypovirulent inoculum was produced using the CHV-1 mycovirus isolated from Mt. Pelion. The field inoculations were conducted in 1998, 1999 and 2000 by trained personnel. Approximately 20,000 accessible cankers were treated, which were more or less evenly distributed throughout the chestnut forest. This project worked as a pilot trial for the next, nationwide assignment.

When chestnut blight has spread all over a country, circumstantial and locally applied measures are ineffective. Action should be taken simultaneously on a national scale if there is to be any hope for fast combat of the disease and the rehabilitation of the chestnut forests and orchards. After the collected information showed that hypovirulence could be used to manage the disease, the decision was made to introduce hypovirulence on the national scale through artificial inoculations in two, 3-yr-periods 2007–2009 (17 counties) and 2014–2016 (12 counties). The spontaneously expressed hypovirulent (hv) strain with the CHV-1 subtype I (Italian subtype) virus from Mt. Pelion was used to prepare the hv inoculum for the tree inoculations, and thus spread hypovirulence all over the chestnut growing areas.

The transmission of viruses by mycelial anastomosis from hypovirulent to virulent strains is feasible only if the two strains fall within the same type of vegetative compatibility (vc type) [12,15,19]. Therefore, the inoculum was specific for each county and compatible with the local vc types of the fungus *C. parasitica* according to a vc type distribution map that had been previously elaborated [6]. All the inoculum was prepared at the Forest Research Institute on a commercial level, but with laboratory care [20]. In counties where more than one vc type was recorded, the Institute supplied a blend of inoculum of the corresponding vc types at the proportion found.

Such a large-scale operation involving chestnut growers, foresters, laboratory technicians, research scientists, laboratory work and field application, and which depends on time restrictions, requires flawless organization and timing. Before the application of biological control in the field, certain prerequisites are fundamental; the most important being a national survey in order to identify all the existing vc types of the pathogen. After this first step, a good deal of other work has to be organized, making the preparation of an "Integrated Biological Control Plan" aimed towards the management of the disease essential [21].

Integrated Biological Control Plan

An Integrated Biological Control Plan aims to give the manager all the necessary information in order to successfully organize the introduction of hypovirulence in chestnut areas where it has not already appeared naturally. Furthermore, it aims to recommend sylvicultural/horticultural interventions in order to reduce the disease occurrence potential, slow down the disease spread and give time for the introduced hypovirulence to establish, spread and dominate the virulent strain/s in the field.

When the Integrated Biological Control Plan refers to a national scale project, then it should comprise a number of Biological Control Plans that refer to smaller areas such as regions, counties, municipalities or individual forests [21].

The objectives of an Integrated Biological Control Plan are:

- To assess the health condition of the forest stands or orchards and estimate the magnitude of the damage;
- To map the exact position of the disease centers for easy access and fast inoculation by the field teams;
- To estimate the approximate quantity of inoculum needed for each particular area;
- To estimate the cost;
- To deal with any other organizational details.

The health condition of the forest stands and orchards was calculated after a statistically adequate sample of trees along transects was determined. Three health categories were established:

- *Stable* when the disease severity was found to be 0%–10%;
- *Unstable* when the disease severity was found to be 11%–40%;
- *Critical* when the disease severity was found to be >50%.

A disease severity between 40% and 50% was handled by considering other criteria such as the age of the trees, location, effects of clear cutting (if any), etc. For example, infected stands on steep slopes that protected the soil from erosion could be assigned a 40% level instead of 50% so that they remain, instead of being clear cut. Similarly, stands near sylvicultural maturity could be kept instead of being clear cut.

3. Results

The field sampling and the bench work revealed that only four vc types existed in Greece: EU-1, EU-2, EU-10, and the dominant EU-12, which accounts for 88% [6]. The perfect (sexual) stage of the fungus was never found. As (a) it was previously established that the fewer vc types in a wide area,

the more effective the control may be; (b) localized, natural hypovirulence had previously occurred in Greece; and (c) the pilot project in Mt. Athos produced positive results and valuable experience, the decision was taken to apply biological control on a nationwide scale using Greek mycoviruses. An Integrated Biological Control Plan was compiled.

Wherever possible, forest stands or orchards in *Critical* condition were clear cut. The timber produced was removed from the forest as soon as possible. As the rotation in sweet chestnut coppice forests in Greece is 20–25 years, most of the produced wood products were fence or vineyard poles, rather than construction timber. All timber was absorbed into local markets without any particular disturbance in prices. No intervention was suggested in *Stable* stands or orchards, but their health condition was closely observed. Tree canker inoculations were applied in *Unstable* stands and orchards to trees aged from 5 to 18 years. The logic behind selecting young trees was that chestnut bark becomes rough and cracks longitudinally after the age of about 20 years. During canker inoculations the holes are made in the periphery of the canker at a distance of 3–4 cm away from the canker edge. The hypovirulent strains can establish successfully only on healthy bark and the edge of the cankers can be seen only in young trees with smooth bark.

Approximately 170,000 accessible cankers were inoculated for three consecutive years, in each of the two periods dating from 2007–2009 and 2014–2016. All field inoculations were carried out by trained foresters and forest workers under the supervision of the Forest Research Institute and Forest Service regional foresters [6]. The entire project was financed by the Forest Service, which meant that the chestnut growers did not have to cover any of the cost.

Several evaluations after the end of the inoculations showed that the introduced hypovirulent strains had established on the inoculated trees, healing the particular cankers, and that they had also started to disseminate to and heal non-inoculated cankers as well [20].

The effect of the management type (orchard vs. coppice forest) was significant for the dissemination of hypovirulence. The spread of hypovirulence was faster in coppice forests than in orchards [20]. In clear cut stands, a rather heavy infection was observed in the first 2–3 years in injuries along the densely sprouting shoots because of crowding. The initial feeling of disappointment was followed by relief, however, when the fatal cankers gradually converted to healing cankers by the already-established hypovirulence.

Forest managers and individual chestnut growers, as well as the official government of Mt. Athos, happily state today that chestnut blight in Greece is now history, and that they have returned to cultivation as before the occurrence of the disease.

4. Discussion

Chestnut is a multipurpose tree cultivated in commercial plantations for nuts and in coppice forests for timber. It combines rural economic importance with important ecological benefits, such as protection against fire and erosion, an excellent habitat for biodiversity and positive effects on climate and recreation. When a disease like chestnut blight enters a country, it is certain that sooner or later it will spread, causing unbearable loss. In several European countries, spontaneous hypovirulence appeared approximately 30 years after the initial entrance of the disease. It is a hard decision for administrators whether to wait for natural hypovirulence to occur and naturally spread, or to implement a costly biological control project after specialists recommend its feasibility.

The project implemented in Greece showed that even though spontaneous (but localized) hypovirulence may start occurring, its spread may be slow and dramatically different from area to area. Impatient chestnut growers would use any means to seek a fast resolution.

Crucial reasons such as (a) the decline of the Greek national chestnut production due to the disease; (b) the socio-economic impact due to property loss and finally the immigration of chestnut growers from their mountain villages, resulting in the abandonment of their orchards; (c) the devastation of sweet chestnut orchards and coppice forests; and (d) the existence of only four vc types of the pathogen,

contributed to the consideration of managing the disease by applying biological control along with silvicultural/horticultural interventions.

The design of a successful inoculation project on a nationwide scale and the preparation of the hypovirulent paste for diverse counties with different vc types of the pathogen is difficult, painstaking and requires a good deal of scientific experience and long preparatory work [6].

The Integrated Biological Control Plan proved highly useful. The financial support of the Forest Service encouraged chestnut forest and orchard owners to participate in the project. As a consequence, all interventions were planned and implemented at the same time and at the right time in all chestnut-producing counties. Cankers extend fast in spring and early summer. The inoculations started on 1 May and lasted until 15 July, so that the virulent strains of the extending cankers hit the hypovirulent strains that were inoculated around the cankers within the growing season. The conversion of the virulent strains to hypovirulent strains and the subsequent production of pycnidia and spores resulted in the dissemination of hypovirulence.

During the evaluation of the project, it was found that there was a remarkable difference in the speed of disease decline from area to area. The age of the trees and horticultural practices may significantly affect the establishment and dissemination of hypovirulence. The spread of hypovirulence was faster in coppice stands than in orchards. As observed in Italy [22], we found that in orchards the results were affected by the horticultural techniques applied by the farmers and the care involved. Interventions in orchards such as grafting, pruning and wounding the trees with tools and machinery hampered the control process. In Mt. Athos we saw cases where silvicultural salvage operations in coppice stands aimed at reducing the disease potential by removing infected trees where no sign of natural hypovirulence had been detected, actually resulted in a sharp increase in the disease and high tree mortality. This was mainly because of tree wounding during the felling and extraction. However, when there is natural or introduced hypovirulence, such wounded trees and their initially lethal cankers gradually convert to healing ones [20].

The dissemination of hypovirulence is a dynamic process that evolves over time. As hypovirulence gradually increases, virulence is expected to be reduced. Consequently, the results of the biological control of chestnut blight are not seen for the first few years after the inoculations. In fact, they cannot be seen by the chestnut growers even several years later, because the growers cannot easily distinguish lethal cankers from healing or even healed ones. After the inoculations and the establishment of hypovirulence, the cankers cannot be visually identified as lethal or healed. There are cankers in the process of conversion which cannot be distinguished, even by an experienced eye. The main criteria are that the branch above a large lethal canker will soon die, and there will be a few adventitious shoots growing on the lower side of the canker. In healing and healed cankers, the adventitious shoots will start dying and the branch will show signs of recovery. Sweet chestnut growers ignore the overall health status of their trees and tend to remove the limbs of the trees every time they observe cankers. As a result, they end up with poorly shaped trees. In addition, by removing the superficial cankers they actually reduce the production of pycnidiospores carrying the viruses, which would eventually remedy the disease. It is very important to educate chestnut growers in all the treated counties about what to expect, how to recognize the identity of the cankers and what to do. For example, significant arguments erupted in several counties between chestnut growers who owned orchards with old trees and the field crews. When the latter refused to inoculate their trees, the chestnut growers responded fiercely and considered the act as discriminatory against them. It is crucial therefore to teach growers well in advance how biological control works and encourage them to have confidence in the science, the scientists applying it, and the importance of patience.

5. Conclusions

We reached the conclusion that when biological control against chestnut blight is implemented in such a way that all involved parameters (such as the identification and mapping of vc types, the preparation of hv inoculum, using trained personnel for field work, and the continuous monitoring

and education of chestnut growers) are thoroughly considered, then the results should be expected to be satisfactory.

Systematic inoculation in a dense network of cankered coppice sprouts or orchard trees can contribute to the establishment of hypovirulence and greatly enhance its spread in comparison with natural, locally-expressed hypovirulence. It is the responsibility of the forest manager/orchard owners to decide whether to wait for natural hypovirulence to occur and spread spontaneously or to introduce hypovirulence for faster results [20].

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Long-Term Assessment of Selective Pruning of *Quercus* Species for Controlling Populations of *Coraebus florentinus* (Coleoptera: Buprestidae) in Mediterranean Forests

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Abstract: *Coraebus florentinus* (Herbst) is one of the most important wood borer pests damaging oak species in Mediterranean forests. Recently, the effect of temperature on the pre-imaginal development of this insect was established, and predictive models of survival and emergence in relation to temperature were performed, which allow scheduled management techniques to be fit in accordance with the biological timing of this species. In this study, the effect of selective pruning of damaged branches of *Quercus* species for controlling the population of this insect is assessed. The study was carried out in three plots located in the Sierra Morena Mountains (southern Iberian Peninsula). In each plot, forest features were typified, and the parameters "infestation level" and "population intensity" were quantified. The assessment was performed one year and five years after applying selective pruning. The most effective time to prune was established according to the predictive model mentioned above. After one year, the results indicated that selective pruning just before adult emergence was effective in reducing the population size and controlling damage. After five years, this effect was still significantly manifested. In addition, the results show that selective pruning is effective even in areas with lower initial rates of infestation.

Keywords: Buprestidae; *Coraebus florentinus*; Mediterranean forests; oak; *Quercus*; selective pruning; wood borer insects

1. Introduction

Coraebus florentinus (Herbts, 1801) (Coleoptera: Buprestidae), commonly known as black-banded oak borer, is a wood borer beetle causing drying and subsequent death of branches of mostabundant *Quercus* species growing in the Mediterranean Region [1]. Therefore, it is not a very aggressive pest and, originally, it had restricted distribution to Mediterranean forests, where cork and evergreen oaks predominate [2].

Damage is the result of the larval feeding activity, which, at the beginning of its development, bores longitudinal galleries beneath the bark of young and healthy branches. At the end of the larval stage, the insect changes its boring direction, turning in the gallery and interrupting the sap flow. This causes the death of the branch upon completion of metamorphosis [3,4].

Because larval development is entirely endophytic and the tree does not show initial signs of infestation, control treatments with phytosanitary products are impractical. In fact, there are no specific insecticides for the black-banded oak borer registered in Spain. Moreover, insecticides would contaminate the cork, risking human health. This is why current research aims to obtain attractants for the capture of adults (chromatic traps, pheromones, or aggregation compounds) and to develop an environmentally friendly approach to control this pest related to the chemical ecology of

C. florentinus [4]. Studies have revealed that odours released by males and females are qualitatively and quantitatively similar, but behavioural assays have demonstrated that the antennae of males are more sensitive to beetle-produced volatiles than those of females [4]. Even if these control procedures were fully developed, it would be difficult to accurately apply them due to the extent of their mature period, along with the shortage of the adult life [5]. In addition, biological control techniques have been explored, i.e., populations of *C. florentinus* could be slightly controlled with natural enemies (*Picusviridis* L.; *Xylophoruscoraebi* Thomson; *Atanycolus sculpturatus* Thomson; *Cerceris bupresticida* Thomson) [6].

Nevertheless, the most effective method to control this pest to date consists of pruning and subsequent removal of damaged branches [1,5,7,8]. This procedure is valid, provided that the branch is cut when the insect has reached the pupa stage, which coincides with the symptomatic manifestation of damage in the branch (first pallor and then redness and dryness of leaves) [5], and is prior to the emergence, dispersion, and reproduction of the adult.

Recent studies dealing with the influence of temperature on the development of this borer have performed predictive models based on the establishment of temperature intervals that induce the optimal progression from pupae to adult. These models allow scheduled management techniques to be fitted with the biological timing of this species, according to the inter-annual thermic variability [1,9]. Previously, other authors recommended pruning as a control method in areas with high levels of infestation (≈90%) [5], but they did not fit the pruning time to the lifecycle of *C. florentinus*. Prior to applying selective pruning, it is necessary to know the effectiveness of the procedure on the course of the insect populations as well as the impact on the next generations.

Taking into account the abovementioned information, the present study was proposed with the objective of designing, executing, and assessing the long-term effectiveness of a management plan consisting of selective pruning of affected branches and the subsequent quantification of *C. florentinus* populations.

2. Materials and Methods

2.1. The Area

Sampling was performed in the Hornachuelos Natural Park, Sierra Morena Mountains (southern Iberian Peninsula) (Figure 1), where the landscape is dominated by Mediterranean mixed sclerophyllous forests. The most representative woodland species are *Q. ilex* subsp. *Ballota* L.; *Q. suber* L.; *Q. faginea*, Lam.; *P. istacialentiscus* L.; *Asparagus albus* L.; *Erica australis* L.; and different species of *Cistus* (*C. ladanifer* L., *C. crispus* L., *C. monspeliensis* L., *C. salviifolius* L., and *C. albidus* L.) in the shrubland [10,11].

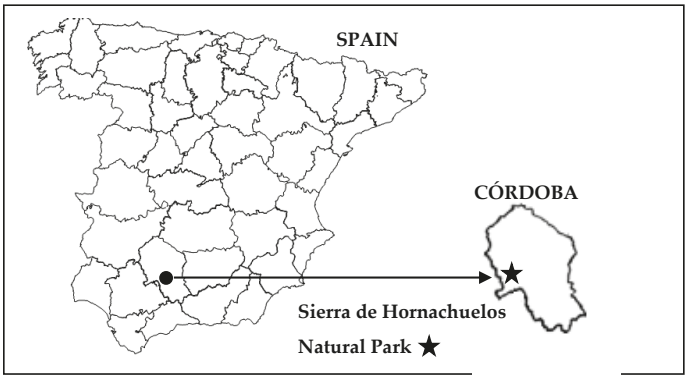

Figure 1. Location of the research area [10].

2.2. Field Work

Field work was carried out in three plots named "Los Lagares", "Mezquitillas" and "El Patriarca" (P1, P2 and P3, respectively), where previously different levels of damage caused by *C. florentinus* had been quantified [9,12]. In each plot, two areas of approximately 1.5 ha and similar environmental features and damage level by *C. florentinus* were delimited and selected for sampling. The main environmental features of each plot (altitude, orientation, orography, surface, vegetal composition and coverage, tree density, and woodland age) and the level of damage are summarised in Appendix (Tables A1–A3). In each area, 100 trees (mainly *Q. ilex*, but also *Q. suber* and *Q. faginea* in minor proportions) were selected and geo-referenced. In one of the areas (selective pruning area), besides assessing damage, all the branches damaged by the buprestid were pruned and removed. In the other area (control area), infestation was evaluated, but no pruning treatment was done. In addition, in both the selective pruning area and control area, a ring around the perimeter of about 35 m wide (34.53 m ± 3.88 S.D.) was considered for assessing damage by *C. florentinus* (areas of influence), because its infestation could be decisive for possible re-invasions of the insect (Figures 2 and 3). The amplitude of this area was established while taking into account the tree density and that *C. florentinus* is a slow-flying beetle, with almost vertical trajectory and very short distances in flight movements [5]. In each area of influence, 100 trees were also selected to evaluate damage.

The amplitude of the perimeter in each area of influence varied depending on the orography and the terrain constraints. To discard significant differences on the extent of the 12 areas of influence, 10 measurements were taken for each, and data were analysed by one-way ANOVA statistic. Results ($P = 0.268$; $F = 1.368$) indicated no significant differences, validating the experimental design.

Based on the temperature/emergence predictive model of Cárdenas & Gallardo [1], pruning was carried out after mid-April 2012, coinciding with the pupation phase and the time when the environmental temperature reached the optimum threshold for the development of this stage (Table A4). Data on environmental temperatures were obtained from the website of the Agriculture and Fisheries Council, of the Junta de Andalucía, Spain [13].

Once the branches were diagnosed by visual surveying, they were sawed (with 4.5 m pole scissors) just below the pupation chamber, which is easily recognisable by the superficial thickening surrounding it [14]. Diagnosis was made by observing the branches that showed clear symptoms of suffering a recent attack (yellowish leaves still in the treetop) [4], well differentiable from those infected in previous years (few, obscure, and dry leaves still hanging on the branch, or the branches are totally defoliated, acquiring a singular aspect, recognizable on the canopy [15,16]).

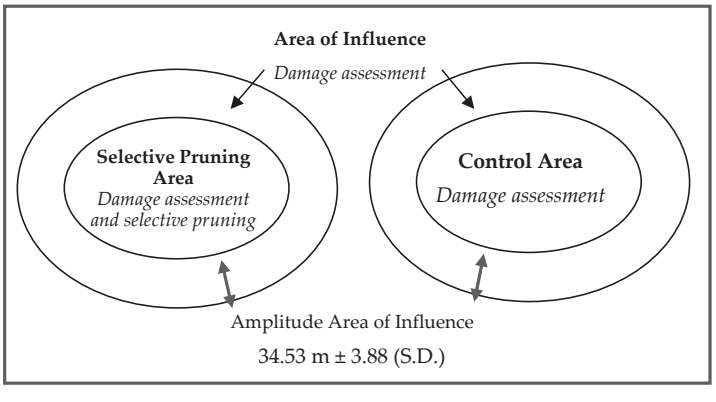

Figure 2. Schematic representation of the different sampling areas and study plots and their respective tasks completed in 2012.

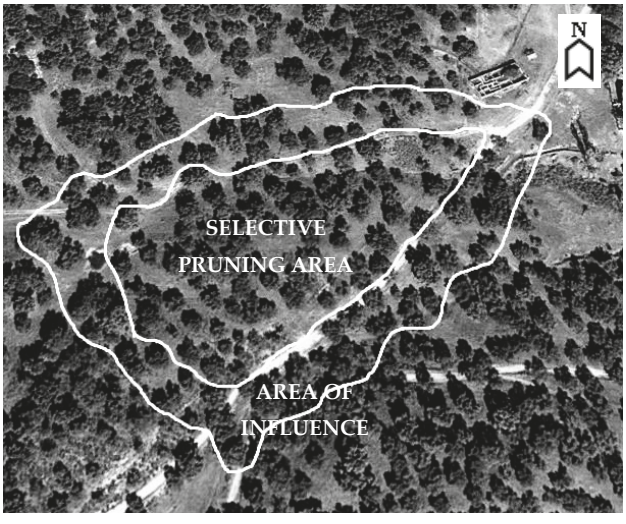

Figure 3. Orthophoto showing one of the Selective Pruning Areas and its Area of Influence.

During the months of May to July 2012, the infestation by the buprestid was evaluated in all control zones and areas of influence (Table 1). In the spring of 2013 and 2017, systematic tree prospections of all the areas were carried out to evaluate again the damages caused by *C. florentinus* and to assess the efficacy of selective branch pruning in the short and long term (Table 1).

Table 1. Schedule of activities carried out in each of the zones established for each plot during the three years of sampling.

Years of Sampling	Dates of Sampling					
	Selective Pruning Area			Area of Influence		
	P1	P2	P3	P1	P2	P3
2012	16 April *	26 April *	20 April *	10 July	10 July	10 July
2013	27 May	27 May	14 May	11 June	10 June	14 May
2017	9 June	15 June	23 May	9 June	15 June	23 May
Years of Sampling	**Control Area**			**Area of Influence**		
	P1	**P2**	**P3**	**P1**	**P2**	**P3**
2012	12 June	19 May	9 May	10 July	12 June	9 May
2013	20 June	28 June	23 May	28 June	12 July	23 May
2017	9 June	15 June	30 May	9 June	15 June	30 May

The asterisk (*) indicates the selective pruning dates.

2.3. Data Analysis

To evaluate damage by *C. florentinus*, the following parameters were estimated: Infestation level (IL), defined as the proportion of trees damaged from the total sampled; and Population intensity (PI), or average number of dry branches per damaged tree.

The independent sample *T*-test was used to check differences in PIs of each area over different monitoring years. If the normality assumptions were not satisfied, after checking by the Shapiro-Wilk test, the equivalent non-parametric Mann-Whitney U/Wilcoxon Ranked Sum test was performed [17].

Analysis of variance (one-way ANOVA) was used to test for differences in PIs among different plots and also among the areas in each plot. The assumptions of normality and homoscedasticity were checked with the Shapiro-Wilk test and Levene test, respectively [17]. If the data did not satisfy the normality and homoscedasticity criteria, the non-parametric Kruskal-Wallis test was applied instead.

To explore relationships between IL and PI in different plots, areas, and years of sampling, a Generalized Lineal Mixed Model (GLMM) was performed using the information criteria to check for GLMM inference. This information-theoretic method quantifies the magnitude of difference between models in expected predictive power [18]. Both the Akaike and the Bayesian information criteria have been considered for interpreting the results. The statistical tests were performed using SPSS (20.0, International Business Machines Corporation, NY, USA) [19].

3. Results

3.1. Initial Incidence of Coraebus florentinus: Analysis of the Starting Situation

Data on ILs and PIs obtained in 2012 in the four sampling areas (pruning area, control area, and their respective areas of influence) established at each sampling plot are recorded in Table 2. It was observed that ILs in the areas selected in plots P1 and P2 were quite similar, with percentages of damaged trees ranging between 10% and 18%. Nevertheless, in P3 the ILs were much higher than those of the other plots, with a percentage between 24% and 34%. Regarding the PI, a statistical comparison did not find significant differences in any of the cases considered, neither among the sampling plots ($P = 0.789$; $\chi^2 = 0.346$; $\alpha \leq 0.05$) nor among the different areas within each plot (P1: $P = 0.146$, $x^2 = 5.379$; P2: $P = 0.607$, $x^2 = 1.835$; P3: $P = 0.863$, $x^2 = 0.745$).

Table 2. Values of Infestation Level (IL) in %, and Population Intensity (PI) in average number of dry branches/tree, corresponding to each sampling area established inside each sampling plot (P1, P2, and P3) in 2012.

Sampling Plots 2012	P1		P2		P3	
	IL	PI	IL	PI	IL	PI
Pruning Area	12	1.25	14	1.64	34	1.26
Area of Influence of Pruning Area	18	1.23	10	1.20	24	1.29
Control Area	17	1.41	15	1.20	32	1.41
Area of Influence of Control Area	11	1.72	11	1.36	27	1.34

Related to the relationships between IL and IP, the values provided by the GLMM analysis for the verisimilitude log are lower than those of the information criteria (Table A5). Thus, no good fit can be inferred, and no relation could be supported from it.

3.2. Incidence of Coraebus florentinus One Year after Selective Pruning: Short-Term Infestation

To determine the effect of selective pruning one year after treatment, we again assessed the parameters in consideration for this study (ILs and PIs) and then made a comparison with respect to the previous year. In 2013, the values of ILs corresponding to the pruning areas were approximately half of those in the control areas. Ostensive differences and lower values were also observed with respect to their areas of influence. This result is generalizable for the three sampling plots (Table 3). On the contrary, the PIs were quite alike. In fact, the statistical tests performed to contrast the appraisal results were not significant among the sampling plots ($P = 0.098$; $x^2 = 4.646$; $\alpha \leq 0.05$) or the different areas within each plot (P1: $P = 0.701$, $x^2 = 1.421$; P2: $P = 0.301$, $x^2 = 3.655$; P3: $P = 0.918$, $x^2 = 0.504$).

Comparing the ILs recorded in 2012 and 2013, a noticeable reduction was perceived, close to 50%, in the three pruning areas, while values were similar in the control areas. In addition, a downward trend was found in the LIs registered in the areas of influence linked to the pruning areas. However, these levels remained similar or slightly higher in the control areas and their perimeters of influence.

The analysis of variations in the distribution of *C. florentinus* populations resulting from the comparison of inter-annual data of PIs revealed no significant changes in the established probabilistic framework (Table 4).

Table 3. Values of Infestation Level (IL) in %, and Population Intensity (PI) in average number of dry branches/tree, corresponding to each sampling area established inside each sampling plot (P1, P2, and P3) in 2013.

Sampling Plots 2013	P1		P2		P3	
	IL	PI	IL	PI	IL	PI
Pruning Area	7	1.14	8	1.75	18	1.45
Area of Influence of Pruning Area	15	1.27	11	1.36	21	1.33
Control Area	15	1.14	17	1.53	30	1.54
Area of Influence of Control Area	18	1.28	20	1.20	27	1.48

Table 4. Statistical comparison of the Population Intensity recorded in 2012 with respect to those of 2013 in the whole of the areas sampled at each sampling plot (P1, P2, and P3). Z = Value of the statistic for the Mann-Whitney *U* test; P = Probability ($\alpha \leq 0.05$).

Sampling Plots Comparative 2012–2013	P1		P2		P3	
	P	Z	*P*	Z	*P*	Z
Pruning Area	0.842	−0.200	0.671	−0.424	0.192	−1.303
Area of Influence of Pruning Area	0.559	−0.585	0.636	−0.474	0.407	−0.828
Control Area	0.239	−1.179	0.163	−1.395	0.386	−0.867
Area of Influence of Control Area	0.098	−1.655	0.570	−0.568	0.375	−0.888

The comparison of the values of the verisimilitude log and the information criteria (Table A5) did not reveal a good fit again, so no relationship between IL and IP could be stated.

3.3. Incidence of Coraebus florentinus Five Years after Selective Pruning: Long-Term Infestation

In the spring of 2017, five years after pruning, the IL and the PI were again evaluated (Table 5). Comparing the ILs in the pruning areas with respect to the control areas, the values continued to be substantially lower in the pruned areas (almost 50% fewer damaged trees). This trend was evident in the three sampling plots and also manifested in the respective areas of influence.

Table 5. Values of Infestation Level (IL) in %, and Population Intensity (PI) in average number of dry branches/tree, corresponding to each sampling area established inside each sampling plot (P1, P2, and P3) in 2017.

Sampling Plots 2017	P1		P2		P3	
	IL	PI	IL	PI	IL	PI
Pruning Area	5	1	8	1	15	1.07
Area of Influence of Pruning Area	7	1.29	10	1.40	14	1.21
Control Area	11	1.09	13	1.46	28	1.46
Area of Influence of Control Area	13	1.31	15	1.07	25	1.48

Regarding the PIs, as had happened in previous comparisons, there was no statistical significance in any of the cases, neither among sampling plots ($P = 0.254$; $x^2 = 2.740$; $\alpha \leq 0.05$) nor among areas within each sampling plot (P1: $P = 0.477$, $x^2 = 2.490$; P2: $P = 0.129$, $x^2 = 5.663$; P3: $P = 0.123$, $x^2 = 5.777$). Five years after treatment, comparison of the initial situation (2012) with respect to the current one (2017) revealed noticeable differences in ILs between the pruning areas and their respective areas of

influence. In the control areas and their perimeters, the ILs were generally maintained, with some slightly oscillations (Tables 2 and 5). The statistical comparison of the PIs in the different areas and sampling plots did not reveal significant differences attributable to the pruning treatment (Table 6).

Finally, as the information criteria are greater than the value of the verisimilitude log (Table A5), no relation between the population indices (IL and IP) could be asserted.

Table 6. Statistical comparison of the Population Intensity recorded in 2012 with respect to those of 2017 in the whole of the areas sampled at each sampling plot (P1, P2, and P3). Z = Value of the statistic for the Mann-Whitney *U* test; P = Probability ($\alpha \leq 0.05$).

Sampling Plots Comparative 2012–2017	P1		P2		P3	
	P	Z	*P*	Z	*P*	Z
Pruning Area	0.347	−0.941	*0.063*	−1.861	0.218	−1.233
Area of Influence of Pruning Area	0.572	−0.565	0.549	−0.600	0.983	−0.021
Control Area	0.189	−1.314	0.411	−0.822	0.375	−0.887
Area of Influence of Control Area	0.130	−1.516	0.147	−1.449	0.652	−0.452

4. Discussion

After the first assessment performed in 2012, the level of damage from *C. florentinus* was determined to be low to medium in the research area. This is consistent with its description as a primary pest of medium importance given its ability to cause growth loss affecting tree canopy shape and not resulting in tree death [8]. Nevertheless, in recent decades the geographical range and damage records of *C. florentinus* have expanded northwardly as a result of global warming [20], and of the heliophilia and thermophilia of the insect [2,21]. Warming conditions are linked to higher reproduction rates and quicker development [1]. In this way, rising temperatures are causing immigration of oak borers toward Central Europe, so much so that *C. florentinus* has recently been included among the wood borer species involved in oak declines in Europe [22].

The spread of the insect, future climate scenarios, and the reduction of oak fitness by water stress can increase damage in forests [20], particularly if reproduction of the beetle occurs in mass [23]. A similar effect has been described for other species of buprestids, such as *Agrilus biguttatus*, and the implication of this species for acute oak (mainly *Quercus robur* and *Q. petraea*) decline has been recently evidenced [24].

Returning to the species under consideration, and particularising for the study area, historical series of data referring to the levels of damage by *C. florentinus* in the Hornachuelos Natural Park are available, which rated the level of infestation between 3% and 8% [5,25], while the data obtained in our initial evaluation ranged between 10% and 34%. This increase confirms the foreseeable upward trend in the population size of this wood borer beetle.

Regardless of the damage caused by *C. florentinus*, strategies for controlling its populations are scarce or non-existent (see the introduction section), and silvicultural management by pruning damaged branches is the main option employed [8,26]. In fact, the same authors proposed monitoring the biology of the insect in the southern Iberian Peninsula, studying also the economic costs of mechanical control by pruning in a "dehesa" with 87% infestation. A year after pruning, the authors indicated that the reduction in the number of affected trees was near 50% (agreeing with our results), thus showing that the effects perpetuated in the residual population responsible for subsequent invasion or re-infestation [5]. The authors carried out the pruning at the beginning of July and proposed applying the technique again, at the same time, in the following two years. However, they did not follow the results on a long-term basis.

Keeping in mind all the information available, the starting point of our research was that pruning is the most suitable procedure to control the populations of *C. florentinus*, but we tried to improve the procedure by introducing the innovation of "selective pruning". Selective pruning is the process of selecting individual branches to be pruned. It provides different benefits for the tree, making

it a recommendable management technique, because it stimulates plant growth, removes diseased structures, can make the tree less attractive to pests, and changes the plant's resistance to pests [27], in addition to destroying weed hosts and serving as an effective control tool [28]. In relation to this last concern, we assessed selective pruning as a method for controlling damage by *C. florentinus*, but we applied the concept of "selection" with a double meaning: first, selection of branches to be pruned (exclusively the branches that show clear symptoms of suffering a recent attack), and second, selection of the optimal time to prune.

Our results indicate that selective pruning is an effective method to reduce the populations of *C. florentinus*. In relation to the course of damage after five years of treatment, comparison of the initial situation with respect to the current one revealed remarkable reduction in the level of infestation of the pruning areas and their respective areas of influence, while in the control areas and their perimeters, the infestation was similar to the initial infestation, with some slightly increased and some slightly reduced. In short, our data support the idea that the pruning procedure is also effective at least five years after applying the treatment. Moreover, the effectiveness of this control measure seems to be independent of the degree of attack. In fact, the ratio of affected branches was reduced by half in the three studied plots, even though the plots had different initial levels of damage. A similar reduction was observed in areas with a great number of damaged trees [5]. At first, it would be expected that the greater the infestation, the greater the achieved reduction after pruning. This did not occur when comparing our data with those of a study by Fernández de Cordova and Cabezuelo [5], which can be explained in terms of the effectiveness of pruning; adequate planning of the pruning time optimises the result, even at low levels of infestation.

When indiscriminate pruning is carried out, dry branches resulting from the attacks of previous years are also eliminated. These branches, from which adults have already emerged, do not represent any additional risk; contrarily, the dry branches become a main trophic resource for many saproxylic organisms [16,29–31]. These organisms play a key ecological role in forest ecosystems, contributing to the maintenance of trophic chains [32], favouring decomposition and recycling of plant matter, and contributing to plant pollination [33], which is of particular relevance for maintaining the biodiversity of Mediterranean woodlands [32,34–36].

Thus, it makes no sense to prune the branches after adults have emerged, because no action is being taken against infestation but forest resources are being removed. Hence the need to adjust field tasks to the particular meteorological conditions of each year, which can be done according to the predictive model developed in previous research [1].

Aside from selecting the branches and the pruning time appropriately, we propose cutting and eliminating the portion of the branch that contains the pupation chamber and leaving the rest in the place of origin in order to conserve forest resources. Considering that the duration of the larval development of *C. florentinus* can extend for two or three years, it would be advisable to repeat the selective pruning treatment for at least two consecutive years until the populations of the insect are maintained at lower levels.

5. Conclusions

- Selective pruning of branches affected by *C. florentinus* is an effective method for controlling the populations of this insect, reducing the IL by up to 50%.
- To be most effective, pruning must be scheduled to take place before the emergence of adults, following the predictive models of emergence depending on inter-annual temperature variations.
- This method of mechanical control is effective in both the short and long term, and the population reduction is appreciable not only in the pruned zone but also in the adjacent area.
- The effectiveness of this control measure is independent of the IL by *C. florentinus* in the managed area.

- The proven effectiveness of selective pruning to control this species warrants its inclusion among the management activities in Mediterranean oak forests as a preventive measure to avoid the foreseeable demographic explosion due to climate change.

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Appendix A

Table A1. Environmental features of each of the selected areas in P1 with specific regard to land (surface, orography, orientation, and altitude) and vegetation (vegetal composition and coverage, tree density, woodland age, and average level of damage by *C. florentinus*) characteristics.

	P1	
	Selective Pruning Area	**Area of Influence**
	Land	
Surface	1.82 ha	1.54 ha
Orography	Hillside with medium slope	Hillside with variable slope
Orientation	South	South
Altitude	426 m above sea level	426 m above sea level
	Vegetation	
Shrubland composition	*Cistus* sp. L., *Phlomis purpurea* L., *Lavandula stoechas* L., *Rubus ulmifolius* Schott, *Daphne gnidium* L.	*Cistus* sp. L., *P. purpurea* L., *L. stoechas*, *R. ulmifolius*, *D. gnidium*, *Genista hirsuta* Vahl., *Pistacia lentiscus* L.
Canopy Cover Fraction	5–25%	50–75%
Woodland composition	*Quercus ilex* L. (80%), *Q. suber* L. (20%)	*Q. ilex* (87%), *Q. suber* (13%)
Average tree density	76 trees/ha	57 trees/ha
Average tree age	Mature trees (average ø = 36.1 ± 14.5 cm)	Mature trees (average ø = 36.04 ± 1.52 cm)
Average level of damage	14.6%	14.6%
	Control Area	**Area of Influence**
	Land	
Surface	2.05 ha	1.96 ha
Orography	Valley/hillside with variable slope	Valley/hillside with variable slope
Orientation	North/no dominant orientation	South/no dominant orientation
Altitude	451 m above sea level	451 m above sea level
	Vegetation	
Shrubland composition	*Cistus* sp., *P. purpurea*, *R. ulmifolius*, *D. gnidium*, *P. lentiscus*, *Crataegus monogyna* Jacq.	*Cistus* sp., *L. stoechas*, *P. purpurea*, *R. ulmifolius*, *G. hirsuta*, *P. lentiscus*, *C. monogyna*
Canopy Cover Fraction	5–25%	5–25%
Woodland composition	*Q. ilex* (72%), *Q. suber* (28%)	*Q. ilex* (80%), *Q. suber* (20%)
Average tree density	55 tress/ha	59 trees/ha
Average tree age	Mature trees (average ø = 44.24 ± 20.20 cm)	Mature trees (average ø = 42.46 ± 17.26 cm)
Average level of damage	14.6%	14.6%

Table A2. Environmental features of each selected areas in P2 with specific regard to land (surface, orography, orientation, and altitude) and vegetation (vegetal composition and coverage, tree density, woodland age, and average level of damage by *C. florentinus*) characteristics.

	P2	
	Selective Pruning Area	**Area of Influence**
	Land	
Surface	1.14 ha	1.59 ha
Orography	Hillside with low/medium slope	Hillside with medium slope
Orientation	South	South
Altitude	541 m above sea level	541 m above sea level
	Vegetation	
Shrub composition	*Cistus* sp., *P. purpurea*, *R. ulmifolius*, *D. gnidium*, *G. hirsuta*, *P. lentiscus*, *Hedera helix* L.	*Cistus* sp., *P. purpurea*, *L. stoechas*, *R. ulmifolius*, *D. gnidium*, *G. hirsuta*, *P. lentiscus*, *Smilax aspera* L., *Rosmarinus officinalis* L., *Nerium oleander* L.
Canopy Cover Fraction	5–25%	25–50%
Woodland composition	*Q. ilex* (95%), *Q. suber* (3%), *Q. faginea* Lam. (2%)	*Q. ilex* (72%), *Q. suber* (25%), *Q. faginea* (3%)
Average tree density	71 trees/ha	120 trees/ha
Average tree age	Young trees (average ø = 25.74 ± 11.30 cm)	Young trees (average ø = 23.89 ± 12.02 cm)
Average level of damage	17.6%	17.6%
	Control Area	**Area of Influence**
	Land	
Surface	1.23 ha	1.32 ha
Orography	Valley with low slope	Hillside with low slope
Orientation	No dominant orientation	No dominant orientation
Altitude	511 m above sea level	511 m above sea level
	Vegetation	
Shrubland composition	*Cistus* sp., *P. purpurea*, *L. stoechas*, *G. hirsuta*, *Scirpus holoschoenus* L.	*Cistus* sp., *L. stoechas*, *P. purpurea*, *G. hirsuta*, *D. gnidium*, *S. holoschoenus*
Canopy Cover Fraction	25–50%	5–25%
Woodland composition	*Q. ilex* (70%), *Q. suber* (30%)	*Q. ilex* (65%), *Q. suber* (35%)
Average tree density	51 tress/ha	49 trees/ha
Average tree age	Mature trees (average ø = 35.99 ± 14.57 cm)	Mature trees (average ø = 41.84 ± 18.25 cm)
Average level of damage	17.6%	17.6%

Table A3. Environmental features of each selected areas in P3 with specific regardto land (surface, orography, orientation, and altitude) and vegetation (vegetal composition and coverage, tree density, woodland age, and average level of damage by *C. florentinus*) characteristics.

	P3	
	Selective Pruning Area	**Area of Influence**
	Land	
Surface	1.05 ha	1.25 ha
Orography	Valley	Valley
Orientation	No dominant orientation	No dominant orientation
Altitude	206 m above sea level	206 m above sea level

Table A3. *Cont.*

P3		
	Selective Pruning Area	**Area of Influence**
	Vegetation	
Shrubland composition	*Cistus* sp., *P. purpurea*, *D. gnidium*, *G. hirsuta*, *G. cinerea* Vill., *Retama sphaerocarpa* L., *P. lentiscus*, *P. terebinthus* L., *Asparagus* sp. L.	*Cistus* sp., *P. purpurea*, *D. gnidium*, *G. hirsuta*, *G. cinerea*, *R. sphaerocarpa*, *P. lentiscus*, *P. terebinthus*, *Asparagus* sp., *Phillyrea angustifolia* L.
Canopy Cover Fraction	75–100%	75–100%
Woodland composition	*Q. ilex* (100%)	*Q. ilex* (100%)
Average tree density	86 trees/ha	89 trees/ha
Average tree age	Mature trees (average ø = 34.74 ± 7.69 cm)	Mature trees (average ø = 31.62 ± 9.15 cm)
Average level of damage	33.5%	33.5%
	Control Area	**Area of Influence**
	Land	
Surface	1.24 ha	1.36 ha
Orography	Valley	Valley
Orientation	No dominant orientation	No dominant orientation
Altitude	197 m above sea level	197 m above sea level
	Vegetation	
Shrubland composition	*Cistus* sp., *P. purpurea*, *Asparagus* sp., *G. hirsuta*, *P. lentiscus*, *P. terebinthus*, *P. angustifolia*	*Cistus* sp., *P. purpurea*, *Asparagus* sp., *G. hirsuta*, *D. gnidium*, *R. sphaerocarpa*, *P. lentiscus*, *P. terebinthus*, *P. angustifolia*
Canopy Cover Fraction	75–100%	75–100%
Woodland composition	*Q. ilex* (100%)	*Q. ilex* (100%)
Average tree density	79 trees/ha	82 trees/ha
Average tree age	Young trees (average ø = 26.03 ± 8.02 cm)	Young trees (average ø = 25.72 ± 7.86 cm)
Average level ofdamage	33.5%	33.5%

Table A4. Maximum, minimum, and average environmental temperatures (°C) recorded in the study area during the selective pruning period in 2012.

Date (DD-MM-YYYY)	Maximum Temperature	Minimum Temperature	Average Temperature
01-04-2012	21.7	10.7	15.3
02-04-2012	19.0	12.1	14.6
03-04-2012	18.9	11.8	13.9
04-04-2012	16.5	11.5	13.7
05-04-2012	18.8	7.5	12.6
06-04-2012	14.7	4.9	9.3
07-04-2012	18.9	6.0	12.1
08-04-2012	23.3	5.3	14.5
09-04-2012	28.7	8.4	18.6
10-04-2012	21.7	10.6	16.4
11-04-2012	21.3	10.2	15.8
12-04-2012	19.3	10.4	14.1
13-04-2012	19.9	9.1	14.5
14-04-2012	18.9	10.0	13.2
15-04-2012	17.8	7.5	12.0
16-04-2012	19.5	7.2	13.6
17-04-2012	24.8	6.0	15.3
18-04-2012	20.3	10.6	15.0
19-04-2012	20.3	11.3	15.3
20-04-2012	21.1	11.1	16.3

Table A4. *Cont.*

Date (DD-MM-YYYY)	Maximum Temperature	Minimum Temperature	Average Temperature
21-04-2012	24.3	9.7	17.1
22-04-2012	24.9	12.1	18.2
23-04-2012	25.2	8.6	16.7
24-04-2012	24.3	11.0	17.1
25-04-2012	22.7	7.9	15.6
26-04-2012	23.2	13.7	17.5
27-04-2012	21.6	14.0	17.4
28-04-2012	17.8	9.1	12.9
29-04-2012	14.1	8.7	10.6
30-04-2012	17.7	6.7	12.1

Table A5. Verisimilitude log values and Akaike (AIC) and Bayesian (BIC) information criteria obtained in the Generalized Lineal Mixed Model (GLMM) analysis.

Years	**2012**			**2013**			**2017**		
Plots	**P1**	**P2**	**P3**	**P1**	**P2**	**P3**	**P1**	**P2**	**P3**
Verisimilitude Log	17.44	15.10	19.02	19.20	20.10	20.10	17.67	16.71	21.62
AIC	23.44	21.10	25.02	25.21	26.10	26.10	23.67	22.71	27.62
BIC	18.54	16.20	20.12	20.31	21.20	21.20	18.77	17.81	22.72

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Review

Distribution, Habitat Preference, and Management of the Invasive Ambrosia Beetle *Xylosandrus germanus* (Coleoptera: Curculionidae, Scolytinae) in European Forests with an Emphasis on the West Carpathians

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Abstract: The black timber bark beetle *Xylosandrus germanus* (Blandford) is an invasive ambrosia beetle that originates from Southeast Asia and has become successfully established within Europe and North America. Herein, we provide a review of the spread and distribution of this tree and timber pest species across Europe, before and after 2000, along with a review of its habitat preferences. Since the spread of *X. germanus* across Europe has accelerated rapidly post-2000, emphasis is placed on this period. *X. germanus* was first recorded in Germany in 1951 and since then in 21 other European countries along with Russia. Ethanol-baited traps were deployed in oak, beech, and spruce forest ecosystems in the Western Carpathians, Central Europe, Slovakia, to characterize the distribution and habitat preferences of this non-native ambrosia beetle. Captures of *X. germanus* within Slovakia have been rising rapidly since its first record in 2010, and now this species dominates captures of ambrosia beetles. *X. germanus* has spread throughout Slovakia from south-southwest to north-northeast over a period of 5–10 years, and has also spread vertically into higher altitudes within the country. While living but weakened trees in Europe and North America are attacked by *X. germanus*, the greatest negative impact within Slovakia is attacks on recently felled logs of oak, beech and spruce trees, which provide high quality timber/lumber. We suggest that the recent rapid spread of *X. germanus* in Central Europe is being facilitated by environmental changes, specifically global warming, and the increasing frequency of timber trade. Recommendations for the management of *X. germanus* in forest ecosystems are proposed and discussed, including early detection, monitoring, sanitary measures, etc.

Keywords: black timber bark beetle; biological invasion; Xyleborini; ambrosia beetle; spread; occurrence; ethanol; forest management

1. Introduction

Invasive ambrosia beetles (Coleoptera: Curculionidae, Scolytinae) can cause severe damage in forest systems [1,2]. In particular, ambrosia beetles in the tribe Xyleborini are among the most successful insect invaders worldwide [2–6]. Key traits likely contribute to their successful establishment and proliferation, including a cryptic lifestyle, fungivory, a broad range of host tree, haplodiploid reproduction, and sibling mating [6,7]. Notably, ambrosia beetles are among the true fungus farming insects, whereby the adults and larvae live within wood in symbiosis with their ambrosia fungi [8]. Consuming the fungal symbiont is the sole source of nourishment for the adults and larvae, and is required for proper development [9–11].

Xylosandrus germanus (Blandford), also known as the black stem borer or black timber bark beetle [12], is a highly successful xyleborine invader and destructive wood-boring pest. Adult female *X. germanus* tunnel into the heartwood of trees and logs, whereby they cultivate fungal gardens of *Ambrosiella grosmanniae* [13]. A variety of secondary microorganisms have also been isolated from galleries of *X. germanus*, including bacteria, yeasts, and filamentous fungi [14,15]. Ranger et al. [16] reviewed additional important aspects related to the biology and ecology of *X. germanus* in detail.

X. germanus is native to Southeast Asia [4,12], but has become established in Europe and North America [6,12,17]. In North America, *X. germanus* was first recorded in New York in 1932 [18], and is now established in 28 US states and three Canadian provinces [6]. The first record of *X. germanus* in Europe was in Germany in 1951 [19,20]. As described in greater detail below, populations are now established in many parts of the European Union, and it has been detected in 21 European countries, along with Russia [12,21] (Figure 1). In most of these countries *X. germanus* is considered a pest species and is expected to spread into other suitable sites, but it could go undetected for many years due to its cryptic behavior [12,21]. The main pathways of spread are human assisted movement via infested wood and wood products, along with natural dispersal [21].

A Rapid Pest Risk Analysis was prepared for *X. germanus* in the United Kingdom [22] and Sweden [21] in 2017, both of which concluded a high likelihood of entry, establishment, and spread. However, the species is not listed in the EU Annexes and is not on the EPPO Alert or Action lists due to its wide distribution in Europe [22]. Since the spread of *X. germanus* across Europe has accelerated rapidly post-2000, a comprehensive review of its distribution, habitat and host preferences within the European regions is needed. We hypothesize that the accelerated spread of *X. germanus* is being facilitated by climate change in combination with increased trade in timber and wooden packaging material. This review discusses the spread and distribution of this pest species within Europe, including a detailed account of its spread in Slovakia. We also include an analysis of habitat preference and suggest measures to be taken in managing *X. germanus* with respect to the production of forest products.

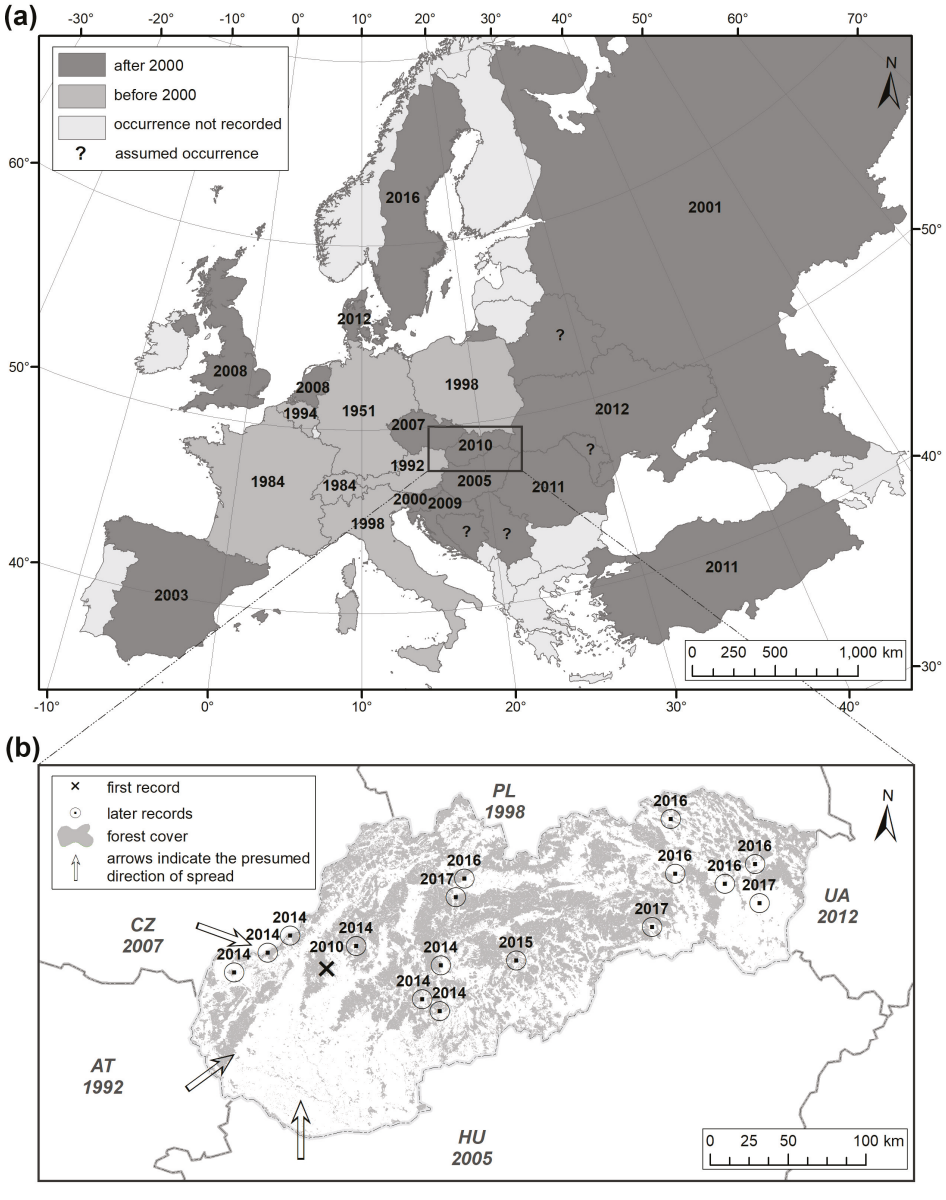

Figure 1. The occurrence and spread of *Xylosandrus germanus* across (**a**) Europe and (**b**) within Slovakia. Records are indicated by years (AT—Austria, CZ—Czech Republic, PL—Poland, UA—Ukraine, HU—Hungary).

2. Spread of *Xylosandrus germanus* across Europe

Here we provide the first records of *X. germanus* within Europe (Figure 1a) with an emphasis on two separate periods: before 2000 and after 2000.

2.1. Before 2000

2.1.1. Germany

Within Europe, *X. germanus* was first recorded in Germany near Darmstadt on oak and beech [19], the oldest record of a single female near Darmstadt (Kranichsteiner Wald) being dated 27 October 1951 [20,23]. Further records followed over the next several years, mostly from regions with a mild climate. Wichmann [24] suggested that *X. germanus* probably arrived in Germany through the importation of oak lumber from Japan, mainly before and after World War I during the periods 1907–1914 and 1919–1929. The unintentional introductions of *X. germanus* from North America after World War II could have also contributed to its spread due to multiple deliveries of timber and wood products to the western part of Germany [20,24].

2.1.2. Switzerland

Maksymov [25] reported the first occurrence of *X. germanus* in Switzerland (near Basel) on a beech trunk in 1984. Germany could have been the source of invading specimens, since the distance between Darmstadt, Germany and Basel, Switzerland is circa 300 km. The first mass attack was recorded two years later on beech, oak, and spruce in June.

2.1.3. France

In France, *X. germanus* was first reported in 1984 [26,27] and additional records followed later [28].

2.1.4. Austria

Holzschuh [29] reported *X. germanus* in Austria in 1992, at two sites in the western part of the country. In 1994 the beetle was found at two more sites near Salzburg [30]. Holzinger et al. [31] describe this species as the most abundant ambrosia beetle collected in 2012.

2.1.5. Belgium

X. germanus was incidentally recorded in Belgium, near Brussels, in 1994 [32]. Later studies describe this species as the most abundant scolytine [17]. Henin and Versteirt [33] reported it from 29 additional sites in Belgium.

2.1.6. Poland

The first record of *X. germanus* from Poland was made in 1998, followed by many other records after 2005 [34,35].

2.1.7. Italy

In Italy, *X. germanus* was first recorded in a walnut plantation in 1998 [36,37]. Later, in northeastern Italy, Rassati et al. [1] collected *X. germanus* at 24 out of 25 sites. *X. germanus* has two generations per year in Italy [1,38], compared with one generation in areas at higher latitudes.

2.2. After 2000

2.2.1. Slovenia

X. germanus was first recorded in Slovenia, near Nova Gorica, on *Castanea sativa* (Mill.) in 2000 [39]. Since then, it has been found at many other sites in various forest ecosystems, mainly in southeastern and central Slovenia [40,41]. The increased number of sites where *X. germanus* has been found recently, and the increased number of beetles caught in traps, suggests that the beetle has become established in Slovenia [39].

2.2.2. Russia

The first record of *X. germanus* in Russia was from Krasnodarsk Krai, in the southwestern region of Russia, in 2001 [42]. The occurrence of *X. germanus* in the Russian far east, near Vladivostok, was later reported by Sweeney et al. [43]. Empirical data support that *X. germanus* is likely established in the Russian far east, but additional collection efforts are required from Krasnodarsk Krai in the southwestern region of Russia.

2.2.3. Spain

X. germanus was first recorded in northern Spain in 2003 [44]; its spread continues, with additional records in the northern part of the country [45].

2.2.4. Hungary

Four specimens of *X. germanus* were recorded in Hungary in 2005. They were collected from felled *Quercus* sp. and *Tilia* sp. logs [46]. According to CABI [12], *X. germanus* is established in Hungary.

2.2.5. Czech Republic

A single *X. germanus* female was first recorded in Moravia (Czech Republic) in 2007 [47]. This record was from a mixed forest. *X. germanus* continues to spread across the country [48]. In 2017 and 2018, this species was obtained through ethanol-trapping in oak forests near Brno and Straznice na Morave, Moravia [49].

2.2.6. Britain

X. germanus was first recorded in Britain during a saproxylic beetle survey in 2008 [50]. Further records of this species came from a mixed pine forest in North Hampshire in 2012 and 2013 [22], and at a site in southern Suffolk (East Anglia, UK) in 2017. While *X. germanus* is established in Britain, it seems to be restricted to the southern region [51].

2.2.7. The Netherlands

In the Netherlands, *X. germanus* was first recorded in 2008 and has been observed at 10 sites over a large part of the country [52].

2.2.8. Croatia

Franjević et al. [53] stated that *X. germanus* has been collected in oak stands in Croatia since 2009. In 2011, it was the second most abundant scolytid species caught in pheromone traps. [54].

2.2.9. Slovakia

X. germanus was first recorded in Slovakia in 2010 [55,56]. For more information, see "Spread and occurrence of *Xylosandrus germanus* in Slovakia".

2.2.10. Romania

The first record of *X. germanus* in Romania was made in 2011, in an old beech forest in the northern part of this country in the East Carpathians, at altitudes between 760 and 900 m a.s.l. The spread of this species continues so that additional areas in Romania have already been colonized or are likely to be colonized soon [57,58].

2.2.11. Turkey

The first record of *X. germanus* in Turkey is from 2011 and was obtained by ethanol trapping in kiwi orchards [59,60]. A later study [61] confirmed the occurrence of this species in hazelnut orchards.

2.2.12. Ukraine

In 2012, *X. germanus* (a single female) was first recorded in the Transcarpathian Region, Uzhgorod District, Ukraine [62], bordering eastern Slovakia. Since *X. germanus* occurs in Hungary, Slovakia and Poland, its establishment in the Ukraine is likely.

2.2.13. Denmark

X. germanus was first recorded in Denmark (a female crawling on an ash tree trunk on Lolland Island) in 2012. More records were gathered in 2013 [63].

2.2.14. Sweden

In 1996, one specimen of *X. germanus* was caught in a window trap in Nybro, inside the flooring manufacturer Kährs, where oak timber from Germany was stored [64]. The second record of one individual in a baited trap was from the Kalmar harbor in 2016 [21]. There were no records of *X. germanus* in 2017 [65]. The likelihood that the beetle could spread to Sweden has increased since its recent establishment in Denmark [21]. According to Björklund and Boberg [21], empirical data do not support the establishment of *X. germanus* or its wide distribution in Sweden, and additional trapping efforts are required to determine if this species is established in the country.

Between 1951 and 1998 *X. germanus* established in 7 of 44 European countries. Since 2000, the beetle has rapidly spread across Europe, where it has been detected in another 13 European countries. As of 2018, *X. germanus* has become established in at least 21 European countries.

3. Spread and Occurrence of *Xylosandrus germanus* in Slovakia

X. germanus was first recorded in Slovakia in 2010 (Figure 1b), in an oak stand in Považský Inovec Mountains, Forest District Duchonka, western Slovakia, where a total of 19 females were obtained through ethanol-baited traps utilized for monitoring purposes [55,56]. Since all of the individuals have been collected deep in a close-canopy forest distant from main traffic routes, the spread of *X. germanus* several years prior to its first detection is highly likely.

The number of catches of *X. germanus* have been rising rapidly since 2010. For example, monitoring traps set in the same oak stand and the same site repeatedly over time yielded a total of 40 specimens in 2011, 77 specimens in 2012 [55], 322 specimens in 2013, and over 1000 specimens per year in subsequent years [66]. *X. germanus* has rapidly become dominant over other species of ambrosia beetle in Slovakia. A similar pattern has been observed in other European countries [1,17,27,31,33,67,68]. Hence, *X. germanus* may substantially alter the diversity of scolytine assemblages in forests [21,27,33]. It could be assumed that native competitors do not substantially limit *X. germanus* in nature [33].

In Slovakia, *X. germanus* was spreading from the south/southwest to the north/northeast (Figure 1b). It has spread throughout the whole country (its length is approximately 400 km) over 5−10 years. The rate of active spread of *X. germanus* has been described as tens of kilometers per year in Western Europe [33].

Not much is known about the vertical spread of *X. germanus* in European forests. Several works state that everywhere the species has established, it has permanent populations only at relatively low elevations [29,32,33]. Henin and Verstein [33] concluded that in Western Europe *X. germanus* does not appear to be able to settle and establish permanent populations above approximately 350 m. Similarly, Bruge [32] noted that *X. germanus* had not been observed above 500 m within Europe. Moreover, 578–600 m is the highest elevation previously reported for a population of *X. germanus* [69,70]. However, Olenici et al. [57] stated that in the Voievodeasa Forest in the East Carpathians, Romania, a permanent population was discovered at an altitude of 760–900 m on a slope with a southeasterly aspect. These accounts are considerably higher than the maximum altitudes described by others in Western Europe, and herein we provide support for them from the West Carpathians.

The prevailing forest systems in the West Carpathians in Central Europe are composed of oak (*Quercus* spp.), European beech (*Fagus sylvatica*) and Norway spruce (*Picea abies*). These systems and their associated fauna show distinct zonation along altitudinal gradients [71]. Systematic ethanol-trapping, carried out in the Kremnické vrchy Mountains, West Carpathians, Central Slovakia, yielded many *X. germanus* females (N = 37,760) during their flight dispersal between May and August 2016. Analysis of these count data provided the first insights into the occurrence of *X. germanus* in the prevailing forest systems on southern slopes of volcanic hills with the variation of altitudes between 259 and 882 m, and with Norway spruce planted within this range of altitudes. A high measure of association between forest type (levels: oak, beech and spruce) and altitude (ANOVA, log-transformed altitudes, eta = 0.76) allowed separate analyses of the effects of these variables on the catches of *X. germanus* at randomly selected collecting sites or forest stands, respectively (n = 36).

Although approximately 51% of the collected beetles were obtained from beech stands, the factor forest type alone (irrespective of elevation) did not influence the abundance of the beetle within the given range of altitudes (Kruskal-Wallis test, χ^2 = 3.9204, df = 2, $n_1 = n_2 = n_3$ = 12, p = 0.141) (Figure 2a). In North America, the abundance of *X. germanus* collected in ethanol-baited traps did not differ between hardwood and coniferous habitats [72]. These results confirm broad ecological plasticity of this habitat generalist [9].

The beetles were found along the entire altitudinal gradient. The significant effect of altitude on the abundance of *X. germanus* (negative binomial GAM, edf = 3.524, χ^2 = 24.38, $p < 0.0001$) suggests the species optimum conditions are in the beech forest between approximately 500 and 700 m a.s.l. (Figure 2b). The moist climate found in this forest type is supposed to retain more stable microhabitats for *X. germanus* and its fungal symbiotic associates compared with the warm and dry climate found in oak forests at lower altitudes. Similarly, cool sub-montane forests at altitudes from approximately 800–900 m and above could also be less suitable for the beetle, as indicated by the smoother (Figure 2b). Although *X. germanus* in Slovakia was observed to attack felled spruce logs as high as 1020 m a.s.l. [73], it has not yet been found in cool mountain forests above 1100 m a.s.l. [74].

The model (Figure 2b) explains 38% of the deviance in the data. It could be extended by using more observations from the same and/or broader ranges of altitudes (depending on local topographies), slope aspects and canopy openness. The extent to which canopy openness could affect the occurrence of *X. germanus* in the prevailing forest systems in the West Carpathians has not yet been analyzed; however, the preference of this species for shaded habitats or microhabitats in regions with both an oceanic [67] and continental climate [75] within Europe is evident. In the western part of Germany (Königsforst, near Cologne), for example, *X. germanus* females produced approximately five times more entrance holes in spruce logs shaded by trees than in unshaded logs [67]. In the studied Carpathian forest, the canopies of all stands were closed, and the breeding substrates attractive to the beetle were not scarce across all forest stands.

Minimum winter temperature has been proposed as a key parameter in limiting the survival of *X. germanus* [29,32,33]. The period 2010–2016 in Slovakia included successive mild winters, of which the winters of 2013/2014 and 2014/2015 were among the mildest since 1950 (data source: Slovak Hydrometeorological Institute). This could explain in part the rapid spread and establishment of *X. germanus* in the country. The expected effects of low freezing temperatures (under −20 °C) over several days in January 2017 on the occurrence of *X. germanus* in Slovakia have not been analyzed, but were probably not the principal factor inhibiting the survival and spread of this species. It is highly probable that the vertical spread of *X. germanus* is connected with a warming climate [69,70].

On the basis of this, we can assume that the spread of *X. germanus* in Europe does not occur only horizontally in individual countries, but also vertically, into higher altitudes where local topographies can play an important role. Yet, Ito et al. [77] hypothesized that *X. germanus* probably lacks sufficient dispersal ability to cross large geographical barriers such as oceans, high mountains, and grassland ecosystems, due to an absence of woody hosts. Their study found that the Tsugaru Strait, between Honshu and Hokkaido islands, acts as a geographic barrier to *X. germanus*. It is unknown whether

the Carpathian mountain range will affect the natural dispersal of *X. germanus*, especially in Slovakia, Ukraine, and Romania. Judged from the known altitudinal distribution of *X. germanus* and the topography of the West Carpathians in Slovakia, this species would be able to cross this mountain range in many areas below 1000 m a.s.l. with ease.

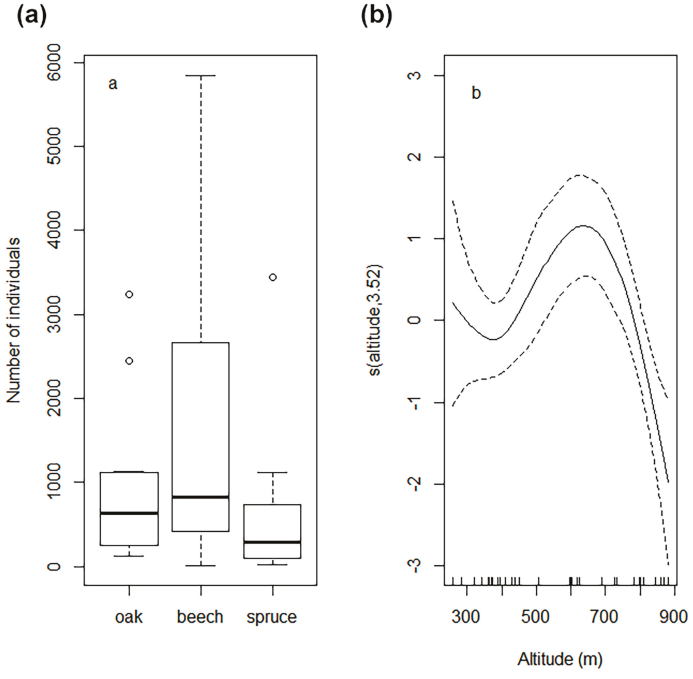

Figure 2. The number of dispersing *X. germanus* females caught in ethanol-baited flight interception traps (n = 36) set in the Kremnické vrchy Mountains, West Carpathians, Slovakia, during the whole dispersal period of the beetle in 2016. Plastic bottle traps were baited with 100 mL of 85% absolute ethanol and replaced at one-week intervals. (**a**) Boxplots (median, IQR, minimum and maximum, outlying values as open circles) of the number of *X. germanus* for oak, beech and spruce forest stands as categorical explanatory variables. (**b**) Estimated smoothing curves for the NB GAM model containing altitude as a solo explanatory variable; the solid line is the smoother, dotted lines are the 95% point-wise confidence intervals, 3.52 is the effective degrees of freedom, and the vertical lines along the x-axis are the altitude values of the observations. Statistical data analyses and graphics were performed in *R* [76].

4. Host Selection and Preference

The known host spectrum of *X. germanus* currently comprises over 200 shrub and tree species in 51 families [78], which includes trees growing in woodlands, plantations, ornamental nurseries, fruit tree orchards, and forested stands, along with recently felled logs, stored timber, and stumps [16,17,33,46,67,79–87]. Thin-barked deciduous trees in ornamental nurseries are more commonly attacked than coniferous trees [16], but *X. germanus* is known to attack stored timber from both deciduous and coniferous trees [25,46,88].

Despite a broad host range, host quality plays an important role during host selection by *X. germanus*, with weakened, dying, or stressed trees being preferentially attacked [16]. Healthy, non-stressed trees are not perceived as hosts. Ethanol is an important kairomone used during host location and selection by *X. germanus* that acts as a chemical indicator of suitable trees or host logs [89,90]. Ethanol also increases the colonization success of ambrosia beetles by promoting the

growth of their fungal symbiont and suppressing the growth of fungal garden competitors [91]. A variety of stressors can induce the emission of ethanol from living but weakened trees, including flood and drought stress, freeze stress, girdling, impaired root function, root and crown disturbance, pollutants, and pathogens [92–96]. In particular, flood stress and freeze stress have been demonstrated to induce the emission of ethanol and predisposes trees to attack by ambrosia beetles [85,96–99]. The emission of ethanol from aging logs also attracts and induces attacks by *X. germanus* and other ambrosia beetles [46,67,100–103].

In North America, *X. germanus* is mainly a pest on a number of species of deciduous trees, particularly in ornamental nurseries [16,80,83,104] and fruit tree orchards [86,87]. For instance, *X. germanus* is the most abundant and problematic ambrosia beetle attacking trees in ornamental nurseries in Ohio [16,82,83]. Attacks have also been documented on logs in North America, especially of deciduous species rather than conifers [105,106]. However, *X. germanus* is not currently considered an important pest of logs or lumber in the USA. Yet, new records of *X. germanus* were obtained during trapping surveys of high-risk sites in Oregon, for instance, businesses importing untreated solid wood packing material, raw wood products, port and industrial areas, and urban forests [107]. The intracontinental movement of untreated solid wood and raw wood products were proposed to be the basis for the introduction of *X. germanus* into Oregon [107].

In Europe, *X. germanus* is considered a secondary pest that attacks mostly felled tree logs [46] (Figure 3). In North America *X. germanus* is most problematic in tree nurseries and orchards, while in Europe most published records are from forests (see "Spread of Xylosandrus germanus within Europe"), with the exception of an attack within a walnut (*Juglans regia*) plantation in Italy [37,108] and in kiwi and hazelnut orchards in Turkey [59,61]. Host reports from Europe include beech (*Fagus sylvatica*), oak (*Quercus* spp.) (Figure 3), spruce (*Picea abies*), pine (*Pinus sylvestris*), fir (*Abies alba*), elm (*Ulmus* spp.), lime (*Tilia* spp.), sweet chestnut (*Castanea sativa*), and hornbeam (*Carpinus betulus*), especially on felled logs and stumps [1,22,25,32,46,50,84,88,109]. It is clear that, especially in the last 20 years, damage caused by *X. germanus* was reported on many tree species which are thus susceptible to be attacked by this beetle [33].

Previous studies from Europe indicate that *X. germanus* prefers to colonize logs that were debarked during logging and transport [25,66,67] (Figure 3). Beginning in 2014, *X. germanus* was first recognized as a pest since it started attacking freshly cut beech and oak logs (Figure 3) in many parts of western and even central Slovakia [84]. To our knowledge, attacks by ambrosia beetles to freshly cut logs had not been observed in Slovakia before. In 2016, we also recorded the first damage of oak and beech lumber by *X. germanus* in eastern Slovakia [110].

There have not yet been any reports of *X. germanus* attacking trees in plantations, orchards, or ornamental nurseries in Slovakia. Colonization of living trees was observed in Slovakia, mostly on beech that were physiologically stressed by other adverse factors (wood-decay fungi, drought, bark burn, frost injury) [110]. Colonization of live, albeit stressed, beech trees was also observed in Western Europe [17,33,97].

While *X. germanus* shows an apparent preference for thin-barked trees [16], it does not appear to discriminate on the thickness (diameter) of host material [25]. Logging residues, as well as thick and highly valuable lumber, are attacked [84]. For instance, Henin and Verstein [33] state that *X. germanus* was found in a 4000 ha beech stand on all types of substrate: stumps, small branches, limbs, and logs. Presumably *X. germanus* can attack any sort of woody material from any species of woody plant, as long as key factors are met—the presence of ethanol in host tissues and sufficient humidity for the development of mutualistic ambrosia fungi [12,91].

Taking into account its broad host range, preference for weakened hosts, and capability to attack standing trees, recently felled logs, and lumber, we assume that many economically important species of woody plants, logs, and lumber could act as potential hosts and be vulnerable to attack.

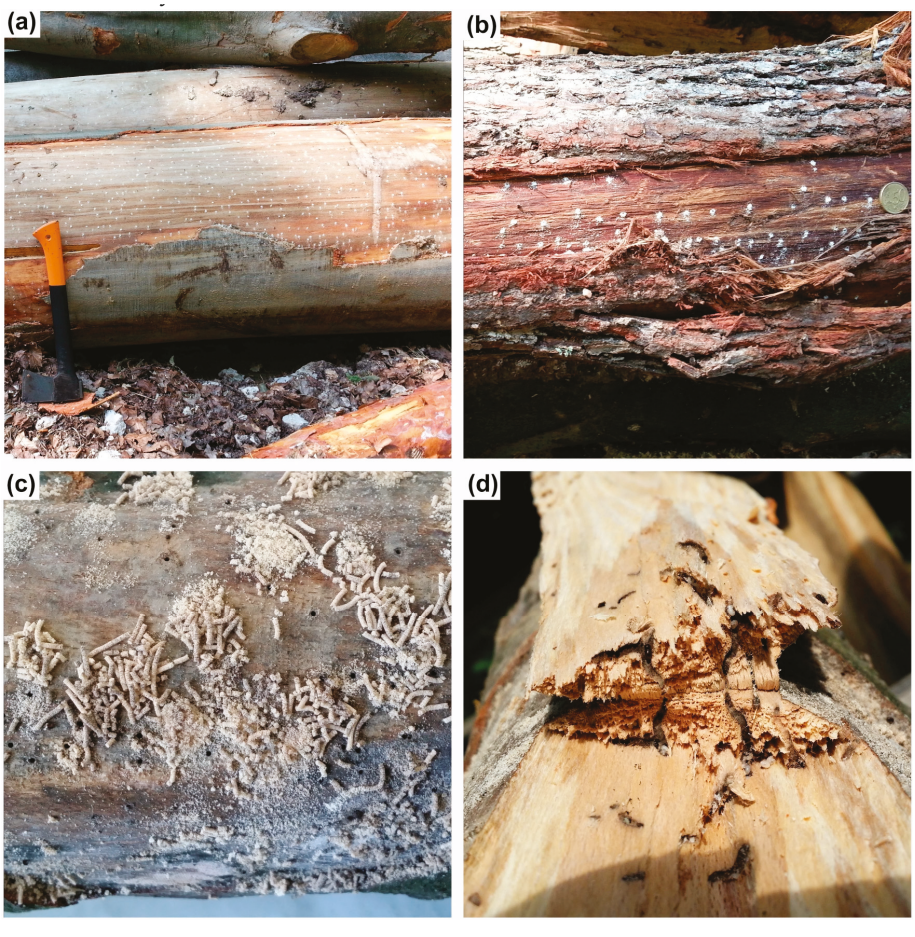

Figure 3. Fresh attacks (white sawdust) of *Xylosandrus germanus* on beech timber (**a**) and oak timber (**b**). After several weeks females expelled noodles of sawdust (**c**) from the gallery (**d**). Photo by Juraj Galko.

5. Forest Management and Recommendations

Due to its extreme inbreeding, haplodiploidy, fungiculture, and broad host range, *X. germanus* has become a very efficient invasive species [6,7]. It is probable that further spread of *X. germanus* will be difficult to prevent, considering that a single fertilized female can found a population in a new region without any negative effects stemming from inbreeding depression [11]. However, it is possible to avoid considerable damage to timber using suitable management measures, as described below in greater detail.

For early detection of attacks, entrance holes with a 1 mm diameter and whitish, light colored sawdust (Figure 3) are typical signs of this species' presence [16,86]. At later stages, when sawdust is expelled from galleries in typical cylindrical formations (noodles) [16,23] (Figure 3), chemical treatment is ineffective. We assume that an effective preventive measure is early treatment of high quality lumber with an authorized insecticide. If the material is already attacked, a chemical treatment with a concentration at upper recommended limits may be used, but would only be effective during initial attack.

As previously noted, *X. germanus* mostly causes damage to felled lumber in Europe [25], which can greatly reduce the value of timber products. The damage caused by attacking high quality oak timber can be especially costly because strict European standards do not allow for any timber infestation [53]. For instance, according to current pricing [111], the monetary value of high-end quality oak timber goes from about 500 EUR/m^3 up to 1000 EUR/m^3 (depending on properties of the timber, this price can be up to 30% higher). After attacking such timber, its price drops significantly to under 200 EUR/m^3. The price of top quality beech timber is up to 350 EUR/m^3, but when attacked it falls below 100 EUR/m^3. The resulting loss exaction between the procurer and purchaser may be very complicated, especially when damages manifest later in the purchaser's storage [112]. Thus, the greatest losses result from attacks on high quality lumber. For instance, in 1995 in Switzerland, *X. germanus* caused major mechanical damage on spruce and fir, amounting to a loss of 1 million CHF [108].

Generally, it can be said that the purchaser can choose to purchase infested wood [112]. Even though *X. germanus* does not drill deep into suitable material (only about 2–3 cm [25]), the purchaser may not buy such infested lumber, fearing the presence of other species (psychological effect on the purchaser) with similar infestation symptoms (such as holes and whitish sawdust) which drill much further into wood and thus cause greater damage, such as *Gnathotrichus materiarius* (Fitch) (Coleoptera: Curculionidae, Scolytinae) [67].

We recommend the following management tactics to preserve the monetary value of quality lumber and minimize economic losses:

1. Logging, transport, storage, and processing of lumber should be carried out at periods without an increased abundance of technical pests. For instance, Franjević et al. [53] recommended the main felling period should be from October through March, harvesting should be prohibited during April and May, and thinning should take place from June through September.
2. We recommend that valuable, top quality lumber, resulting from spring and winter logging stored at vulnerable sites from March to August, is preventively treated with an authorized insecticide (chemical treatment) [25].
3. An alternative to chemical treatment is covering valuable lumber with protective nets infused with insecticides (Storanet®/Woodnet®, BASF®) [53,54]. According to Franjević et al. [53] and our personal observations, the netting system provided excellent control against bark and wood-boring insects attacking fresh cut logs. The Forest Stewardship Council (FSC) and World Health Organization (WHO) have approved the use of these chemically treated reusable fabrics [53].
4. Auctions of high quality products should not take place at sites during periods when wood-boring insects occur there.
5. Inspection of attacked wood material should be carried out visually (entrance holes, white piles of sawdust) (Figure 3). White sawdust is a typical sign of infestation.
6. It is essential that personnel working with wood at vulnerable sites are aware of this species' symptoms, since chemical treatments are only effective during the initial stages of attack.
7. For monitoring the presence of *X. germanus* it is possible to use different types of traps [16,113] baited with ethanol. Traps provide information on the place, time, and abundance at which the monitored pest occurs [113]. However, mass trapping of *X. germanus* using ethanol-baited traps is not currently an effective management tactic [17,53].
8. Heavily infested material should also be chipped or burned to avoid population build up [2].
9. Wood products being imported into countries or regions where *X. germanus* has not yet reached should also be closely inspected and monitored.

There are other modern, albeit costly methods of protecting lumber, such as treatment with heat; microwave [114] or other radiation; and special, direct injecting of insecticides into damaged spots [115]. Biocontrol measures such as breeding and introduction of natural insect enemies

(e.g., hymenopteran parasitoids), utilizing entomopathogenic fungi, or parasitic nematodes, still require additional research [115]. Promising results have been obtained using entomopathogenic fungi to control *X. germanus* and other ambrosia beetles [116–118], but the low threshold for attacks on ornamental and horticultural trees, logs, and lumber could hamper implementation.

6. Conclusions

In conclusion, we have summarized the known information on *X. germanus* and its significance in European forest ecosystems. Based on the aforementioned literature, we conclude that *X. germanus* is established in 20 European countries and Russia. It will be virtually impossible to stop further spread, since a single female can found an entire new population. However, preventing human-assisted movement of infested material will help to slow the spread. The spread of *X. germanus* in Europe has accelerated since 2000, and the species became established throughout Slovakia within 5–10 years. Our analyses indicate that *X. germanus* is also spreading vertically into higher altitudes. Climate change within Europe [119,120], and specifically mild winters, could be assisting the spread of *X. germanus*. Similarly, freeze stress events following mild winters could also increase the availability of suitable host material and lead to an increased incidence of attacks [97,98]. Heavy precipitation and flood stress can also predispose trees to attack. The capability of *X. germanus* to attack a broad range of deciduous and coniferous trees, along with logs and lumber, also poses a challenge to preventing its spread.

In Europe, and especially Slovakia, *X. germanus* mainly causes damage to felled lumber and forested systems, and much less so in tree plantations, orchards, and ornamental nurseries. In the future it may shift host preferences and become an important pest in European orchards, plantations, nurseries and vineyards, as it is currently in North America. Notably, trees growing under controlled production systems that are weakened and emitting stress-induced ethanol are highly vulnerable to attack by *X. germanus*.

We recommend that forest management measures should primarily focus on preventive treatment of high quality lumber. Insecticide-treated netting shows considerable promise for protecting logs and lumber. While ethanol-baited traps are very effective and important for detecting and monitoring *X. germanus*, a mass trapping strategy is not currently available. Like other Xyleborini ambrosia beetles, *X. germanus* is an excellent example of the rapid spread by an alien species into new regions; therefore, its future movements and population changes should be watched carefully [17].

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Stand Characteristics and Soil Properties Affecting the Occurrence of Kunyushan Web-Spinning Sawfly (*Cephalcia kunyushanica* Xiao) in Japanese Red Pine (*Pinus densiflora*) Pure Forests in the Kunyushan Mountains, China

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Abstract: The Kunyushan web-spinning sawfly (*Cephalcia kunyushanica*) is a major pest in the Japanese red pine (JRP, *Pinus densiflora*) pure forests in the Kunyushan Mountains of China. In this study, four stand types (ST1–4) were identified in plots of JRP pure forests, based on the pest severity index (PSI; ranging from 0–100). The order of infestation ratio in the four type stands was as follows: ST4 > ST3 > ST2 > ST1. We investigated the correlation of *C. kunyushanica* occurrence with stand characteristics and soil physicochemical properties in the four stand types. The results showed that all stand characteristics were different among the four stand types. Compared with infested plots, healthy (ST1) plots had a higher soil bulk density, and the differences among the groups were significant. Differences in soil water content, non-capillary porosity, and total porosity were significant among the four ST groups. The average organic matter content, total nitrogen (N), and available N were lower in ST1 plots, whereas total potassium (K) was higher compared with other ST groups. In addition, a redundancy analysis suggested that seven (total N, diameter at breast height (DBH), soil water content, bulk density, available K, zinc ion (Zn^{2+}), and stem density) of 24 environmental variables were significantly correlated with the ordinations of *C. kunyushanica* occurrence. The results provide theoretical guidance for the ecological control of *C. kunyushanica*, and are also useful for the management of forests in areas where *C. kunyushanica* is a major pest and where site and stand conditions are similar.

Keywords: Japanese red pine pure forests; *Cephalcia kunyushanica*; stand type; stand characteristics; soil properties

1. Introduction

Rotation times of forests are usually estimated in decades and trees can be infected with a variety of different insect pests and diseases during the juvenile and mature stages of growth [1]. Therefore, suitable forest management practices are needed. Forest managers have traditionally used chemical pesticides rather than non-chemical methods for forest management, which has caused several negative consequences such as the development of pesticide resistance, resurgence of pest populations, emergence of secondary pests, and environmental pollution [2]. Compared with traditional forest management practices, integrated pest management (IPM) is a more effective and environmentally sustainable approach to pest management [3]. In a review of pest management, O'Neil [4] highlighted

that the practice of IPM is often inconsistent with the underlying philosophy. This has resulted in a new concept of ecological control of forest pests [5], which mainly includes biological control, cultivation of insect/pathogen resistant species, site preparation, and the dynamic monitoring of pests. These measures are effective for restricting the growth and development of pests and maintaining balance in the ecosystem [6].

Stand and soil characteristics in a particular site impact the herbivore population at that site. For example, Marchisio et al. [7] reported that severe water stress occurs in the years preceding an outbreak of *Cephalcia arvensis*. The density of pine false webworm (*Acantholyda erythrocephala*) is positively correlated with the relative dominance of host plants and negatively correlated with tree diversity [8]. Site factors (elevation and stand density) and the microclimate of the Japanese red pine (JRP; *Pinus densiflora* Sieb. et Zucc.) forest ecosystem affect the survival, resource selection, and distribution of Kuyushan web-spinning sawfly (*C. kunyushanica*) by affecting the growth potential of host plants or the forest population dynamics [9]. Therefore, studying the effects of stand and soil characteristics on diseases and insect pests is useful for forest managers and is an important component of the ecological control of forest pests.

JRP forests are one of the most economical coniferous forest species in the Kunyushan Mountains of China, where JRP pure forests account for approximately 70% of the vegetation [1,10]. The health and stability of JRP forests are closely related to the development and safety of the local forest ecosystem. Kunyushan web-spinning sawfly (*Cephalcia kunyushanica* Xiao) is a specialized phytophagous insect species [11], which exists only in the Kunyushan Mountains. It was first reported in the Kunyushan Mountains in 1983, and was identified as a new species in 1990 [12]. *C. kunyushanica* can be found on pine stands of all ages, but it prefers the newest needles when the resources are rich. Although it feeds mainly on JRP, it occasionally also feeds on Armand pine (*P. armandi* Franch.), Korean pine (*P. koraiensis* Sieb. et Zucc.), and lodgepole pine (*P. thunbergii* Parl.). *C. kunyushanica* parasitizes pine trees of all ages, with irregular but severe outbreaks in the Kunyushan Mountains. To date, only two studies have been conducted on the biological performance of *C. kunyushanica* [13,14]. Each year, adults of *C. kunyushanica* emerge in May or June and lay eggs in groups on pine needles. Once hatched, larvae spin silk nests; generally, two to four larvae live in one nest [11]. At the base of the nest or in a feces-covered silk tube, the larvae cut and eat needles, and do not disperse actively in the larval stage. The larvae then drill a 5–12 cm deep hole into the tree and prepare to overwinter. Pupation occurs in April of the next year, and adults emerge approximately after one month [13].

In recent years, many studies have been conducted to understand the effect of stand types, site conditions, forest spatial structure, and species connectivity on population of *C. kunyushanica*. However, the influence of soil properties on sawflies has rarely been investigated, the scope of previous research in this area is relatively small, and therefore it does not accurately reflect the influence of JRP stand characteristics on sawfly populations. Further research is needed to advance our understanding of the relationship between stand characteristics and pest severity. This is the first study of the relationship between soil properties and *C. kunyushanica*, and is part of a more comprehensive investigation of pine-insect interactions. The objective of the study was to understand the differences in stand characteristics and soil properties between healthy and infested stands of JRP pure forests. We also inferred the feasibility of ecological approaches to control *C. kunyushanica* densties, based on the effect of environmental factors on the occurrence of *C. kunyushanica*.

2. Materials and Methods

2.1. Study Area

The Kunyushan Mountains are located in the Jiaodong Peninsula in Shandong Province in eastern China (121°41′34″–121°48′04″ E; 37°11′50″–37°17′22″ N). The climate of this region is moderate due to warm temperate monsoons, with a mean annual temperature of 12.3 °C. The frost-free period ranges from 200–220 days, with a mean annual precipitation of 800–1200 mm and mean annual relative

humidity of 62.6%. The soil is mostly brown sandy loam. JRP trees are the main indigenous conifers in this region, and they are also found in Northeast China, Japan, Korea, and Russia. JRP forests are adapted to a wide range of environments and are naturally distributed from the piedmont to the peak of the mountain (800 m above sea level).

2.2. Site and Stand Characteristics Survey

The survey work followed the "Observation Methodology for Long-term Forest Ecosystem Research" of the national standards of the People's Republic of China (GB/T 33027-2016). A total of 121 temporary plots (30 × 30 m) were established systematically in JRP pure forests (from May to August 2017), sharing a similar soil type. The approximate distance between plots was between 30–60 m. The pines were natural secondary forests, covering an area of 15,416 ha in the Kunyushan Mountains. More than 90% of the JRP ages were 32–36 a. The soil had not been treated, such as with tilling or fertilizing. Eight trees were tagged per plot and surrounded by red ropes to serve as temporary markers. Four trees were tagged in the four corners, and another four trees were tagged in the middle of each plot. Trees with a diameter at breast height (DBH) $\geq$2 cm were identified in each plot, and the height, crown width, and DBH of these trees were recorded. The stem density was expressed as the number of trees per hectare. The basal area at breast height and stand volume in sample plots were calculated according to the following equations [15]:

$$G_{1.3} = \pi D^2/4 \quad (1)$$

$$M = G_{1.3} \times (h + 3)\, fa \quad (2)$$

Here, $G_{1.3}$ is the basal area at breast height ($m^2 \cdot ha^{-1}$), D represents the DBH (cm), M is the stand volume ($m^3 \cdot ha^{-1}$), h is the tree height (m), and fa is the experimental form factor (0.42).

2.3. Determination of the Larval Density of C. kunyushanica

Web spinning and nesting in branches of JRP trees were used as evidence of infestation by the larvae of *C. kunyushanica*. The infestation ratio (IR) was defined as the proportion of trees in a plot with insect nests (Equation (3)). The status of insect attacks in JRP pure forests in each plot was described by the pest severity index (PSI), which was measured by the five-spot method. Briefly, two trees were selected from each corner and the center of each plot. Insect nests in these ten trees were observed using binoculars or the naked eye. Because each nest usually contained three larvae on average, the larval density per investigated tree was calculated by trebling the nest numbers. The PSI was calculated according to Equation (4), and the pest classification standard is shown in Table 1.

Table 1. The pest classification standard of the *C. Kunyushanica* per tree.

Pest Classification	Value of Corresponding Grade (CGV)	Basis of Classification (larvae·tree^{-1})
I	0	0
II	1	1–10
III	2	11–30
IV	3	31–50
V	4	>50

The IR and PSI were calculated according to the following equations:

$$\mathrm{IR}\ (\%) = \frac{\sum \mathrm{NTN}}{\sum \mathrm{NT}} \times 100 \quad (3)$$

$$\mathrm{PSI} = \frac{\sum_{i=1}^{10} (\mathrm{NIT} \times \mathrm{CGV})}{(\sum \mathrm{NT}) \times \mathrm{MV}} \times 100 \quad (4)$$

Here, NTN is the number of trees with nests in each plot, NT is the number of trees in each plot, i is the number of investigated trees, i.e., 10 per plot, NIT is the number of infested trees, CGV is the value of the corresponding grade, MV is the maximal value of CGV, which was always 4 (Table 1).

2.4. Collection and Processing of Soil Samples

To analyze soil physical properties, composite soil samples were collected from each plot at a depth of 0–10 cm using a cutting ring (100 cm^3), according to the five-spot sampling method. Soil bulk density (BD, $g{\cdot}cm^3$) were obtained by using a cutting ring (100 cm^3) and calculated as the ratio of the oven-dry soil mass to the cutting ring volume. The soil water content (%) was calculated from the mass loss after oven drying the samples at 105 °C to a constant weight. The natural state of the soils, along with the cutting rings, was weighed (m_1, g) after soaking for 12 h in water to estimate the maximum moisture capacity (MMC, %). The cutting rings were then placed on dry sand for 2 h, allowing the non-pore water to be completely drained, then weighted (m_2, g). Finally, the soil was sampled from the cutting rings and dried in an aluminum box to a constant weight (m_0, g). The equations of maximum moisture capacity, capillary porosity (CP, %), non-capillary porosity (NP, %), and total porosity (TP, %) were as follows [16]:

$$\mathrm{MMC} = \frac{m_1 - m_0}{m_0} \times 100\% \quad (5)$$

$$\mathrm{CP} = \frac{m_2 - m_0}{m_0} \times 100\% \times \mathrm{BD} \quad (6)$$

$$\mathrm{NP} = (\mathrm{MMC} - \frac{m_2 - m_0}{m_0} \times 100\%) \times \mathrm{BD} \quad (7)$$

$$\mathrm{TP} = \mathrm{CP} + \mathrm{NP} \quad (8)$$

To analyze soil chemical properties, soil samples were collected from a depth of 0–20 cm, also according to the five-spot sampling method, and thoroughly mixed. Impurities in the soil samples, such as stones, animal and plant residues, and other litter, were removed manually. The cleaned soil samples were air-dried and then ground to a final particle size of <2 mm. Soil organic matter (%) was analyzed using the potassium dichromate oxidation-external heating method [17]. The soil pH level was measured in a 1:5 mixture of soil. Total nitrogen (N) was determined using KD310-A distillation and titration unit (OPSIS, Furulund, Sweden) [18]. Total phosphorus (P), total potassium (K), ferric ion (Fe^{3+}), cupric ion (Cu^{2+}), zinc ion (Zn^{2+}), and manganese ion (Mn^{2+}) were measured by using microwave digestion system (CEM, Matthews, NC, USA) and plasma emission spectrometer (Thermo, Waltham, MA, USA) [19]. Available nitrogen was monitored by alkaline solution-diffusion method [20]. Available phosphorus was extracted with hydrochloric acid and sulfuric acid solution, while available potassium used ammonium acetate solution for extraction. Then they were monitored with a plasma emission spectrometer (Thermo, Waltham, MA, USA) [21].

2.5. Data Analysis

To compare the differences in stand characteristics and soil properties among 121 plots, we divided these plots into four stand types (ST1–4), according to the PSI of each plot: ST1 plots were uninfected (PSI = 0), whereas ST2 ($0 < \mathrm{PSI} \leq 20$), ST3 ($20 < \mathrm{PSI} \leq 40$), and ST4 (PSI > 40) plots were infested with *C. Kunyushanica*.

The homogeneity of variance of all indicators were tested before the one-way analysis of variance (ANOVA). The results show that the basal area at breast height, stand volume, bulk density, soil water content, maximum moisture capacity, Cu^{2+}, and available K didn't correspond to normal distribution. So, for basal area at breast height, stand volume, and available K, data were transformed by logarithm; for soil water content and maximum moisture capacity, data were dealt with by sine transformation; for Cu^{2+}, data was transformed by $1/x$, where x was Cu^{2+} content in each plot; for bulk density, data was transformed by i^6, where i was the bulk density in each plot. Then a one-way ANOVA was conducted

to test the difference among four stand types, using Tukey's multiple comparisons. Differences were considered significant at the 5% level of significance. Those were done in SPSS v22.0 (IBM, New York, NY, USA).

The ordination of the occurrence of web-spinning sawflies among the four stand types was determined using CANOCO v5.0 (Microcomputer Power, Ithaca, NY, USA), according to [22]. A detrended correspondence analysis (DCA) showed that the largest gradient length was 1.79 (<3), and therefore redundancy analysis (RDA) was considered an appropriate analytical method [18]. RDA is a multivariate direct gradient analysis method used for a multiple regression analysis of many environmental variables [22]. In this study, the effect of plot averages of 24 environmental variables (including stand characteristics and soil properties) on pest occurrence was investigated in each plot. On the basis of a Monte Carlo permutation test with 499 iterations, the forward selection procedure was used to select environmental variables with p-values < 0.05 for the ordination of stand characteristics, and the selected variables were used in final analyses [23,24].

3. Results

3.1. The Distribution of PSI in Four Stand Types

Table 2 shows the distribution of PSI in four stand types. The number of sawfly-non-infested plots is 34, accounting for 28.10% of the total investigated plots. Among the sawfly-infested plots, the mean values of ST2-ST4 are 11.89, 29.37 and 58.95, respectively. In addition, the table shows the minimum value, maximum value and stand deviation in four stand type.

Table 2. The distribution of PSI in four stand types.

	Stand Type	Plot Number	Minimum Value	Maximum Value	Mean Value	Standard Deviation
PSI	ST1	34	0.00	0.00	0.00	0.00
	ST2	32	3.13	20.00	11.89	5.55
	ST3	31	20.83	40.00	29.37	5.56
	ST4	24	43.75	91.67	58.95	13.67

PSI is the abbrevation of pest severity index; ST1-4 refer to the four stand types classified according to the pest severity index.

3.2. Differences in Stand Characteristics

Table 3 summarizes the stand characteristics in the four stand types of JRP pure forests. The stem density in infested plots was significantly different ($p < 0.01$) form that in healthy plots (ST1), and differences among the four stand types were highly significant. The stem densities in ST3 and ST4 plots were much lower than in ST1 plots. Other growth parameters of trees showed an increasing trend with an increase in PSI values, and the differences in these characteristics among groups were all significant ($p < 0.01$). However, the differences among infested plots (ST2, ST3, and ST4) were non-significant ($p > 0.05$) for tree height, DBH, basal area at breast height, and stand volume. The value of crown width was much higher in ST4 plots than in ST1, ST2, and ST3 plots.

Table 3. Stand characteristics of Japanese red pine (JRP) pure forests in different stand types.

Stand Type	Stem Density (trees·ha^{-1})	Tree Height (m)	Crown Width (m)	DBH (cm)	Basal Area at Breast Height (m^2·ha^{-1})	Stand Volume (m^3·ha^{-1})
ST1	2319 ± 84 a	5.0 ± 0.3 b	2.3 ± 0.2 c	9.8 ± 0.4 b	8.86 ± 0.76 b	32.39 ± 4.16 b
ST2	1991 ± 106 ab	6.3 ± 0.3 a	2.9 ± 0.1 bc	13.6 ± 0.6 a	16.52 ± 1.31 a	68.30 ± 6.17 a
ST3	1910 ± 109 b	6.5 ± 0.3 a	3.0 ± 0.1 b	13.3 ± 0.6 a	16.90 ± 1.54 a	69.12 ± 7.52 a
ST4	1621 ± 88 b	6.8 ± 0.4 a	3.7 ± 0.4 a	13.7 ± 0.7 a	17.39 ± 1.70 a	73.47 ± 8.02 a
F-value	7.487 **	6.605 **	11.483 **	11.286 **	11.145 **	8.371 *

ST1-4 refer to the four stand types classified according to the pest severity index. * $p < 0.05$, ** $p < 0.01$. *F*-value is for comparing across stands. The number after ± is standard error. The different letters indicate significant difference between the stand types, and vice versa.

3.3. Differences in Soil Physicochemical Properties

The differences in soil physical and chemical properties among JRP pure forests with different infestation grades are summarized in Tables 3 and 4, respectively. Soil bulk density and soil water content in healthy plots were significantly different ($p < 0.01$) from those in infested plots. Differences in non-capillary porosity and total capillary porosity among the four stand types were significant ($p < 0.01$) and showed an upward trend with the severity of *C. kunyushanica*. No significant differences were detected in non-capillary porosity and total capillary porosity among the infested plots (ST2, ST3, and ST4). No remarkable differences in maximum moisture capacity and capillary porosity were observed among infested plots (Table 4).

Table 4. Water-related physical properties of soil in four stand types of JRP pure forests.

Stand Type	Bulk Density (g·cm^{-3})	Soil Water Content (%)	Maximum Moisture Capacity (%)	Capillary Porosity (%)	Non-Capillary Porosity (%)	Total Porosity (%)
ST1	1.22 ± 0.01 a	10.38 ± 0.41 a	19.76 ± 0.38 a	13.35 ± 0.61 a	6.46 ± 0.32 b	19.81 ± 0.82 b
ST2	1.14 ± 0.02 bc	9.04 ± 0.63 ab	20.35 ± 0.71 a	15.22 ± 0.58 a	7.67 ± 0.48 ab	22.89 ± 0.72 a
ST3	1.19 ± 0.02 ab	8.29 ± 0.46 b	20.53 ± 0.52 a	14.87 ± 0.47 a	8.45 ± 0.32 a	23.33 ± 0.46 a
ST4	1.10 ± 0.01 c	7.44 ± 0.34 b	19.76 ± 0.83 a	15.20 ± 0.84 a	8.08 ± 0.36 a	23.82 ± 0.99 a
F-value	8.124 **	4.538 **	0.997	2.196	5.817 **	6.730 **

ST1-4 refer to the four stand types classified according to the pest severity index. ** $p < 0.01$. *F*-value is for comparing across stands. The number after ± is standard error. The different letters indicate significant difference between the stand types, and vice versa.

An analysis of soil chemical properties revealed strongly significant differences ($p < 0.01$) in the organic matter content, total N, and available K among the four stand types, and significant differences ($p < 0.05$) in available N. There was an increasing trend in the organic matter content, total N, and available N with the level of infestation, with the values of these variables being lowest in ST1 plots and highest in ST4 plots. In contrast, the amount of available K decreased significantly with an increase in PSI values. No significant differences were observed among the four ST groups for total P, total K, available P, various ions (Cu^{2+}, Zn^{2+}, Fe^{3+} and Mn^{2+}), and pH ($p > 0.05$) (Table 5).

Table 5. Chemical properties of soil in different stand types of JRP pure forests.

Stand Type	Organic Matter Content ($g \cdot kg^{-1}$)	Total N ($g \cdot kg^{-1}$)	Total P ($g \cdot kg^{-1}$)	Total K ($g \cdot kg^{-1}$)	Available N ($mg \cdot kg^{-1}$)	Available P ($mg \cdot kg^{-1}$)	Available K ($mg \cdot kg^{-1}$)	Cupric Ion ($mg \cdot kg^{-1}$)	Zinc Ion ($g \cdot kg^{-1}$)	Ferric Ion ($g \cdot kg^{-1}$)	Manganese Ion ($g \cdot kg^{-1}$)	pH
ST1	32.37 ± 3.14 b	0.96 ± 0.07 b	0.17 ± 0.02 a	22.84 ± 0.71 a	74.57 ± 6.06 b	0.96 ± 0.10 a	73.89 ± 3.68 a	3.45 ± 0.29 a	0.081 ± 0.003 a	21.61 ± 1.04 a	0.430 ± 0.023 a	4.47 ± 0.04 a
ST2	49.26 ± 3.94 a	1.51 ± 0.12 a	0.22 ± 0.03 a	22.89 ± 0.85 a	101.71 ± 10.38 ab	1.20 ± 0.13 a	67.24 ± 4.06 a	5.42 ± 1.06 a	0.087 ± 0.003 a	24.51 ± 0.97 a	0.436 ± 0.024 a	4.39 ± 0.06 a
ST3	45.52 ± 3.87 ab	1.66 ± 0.010 a	0.25 ± 0.02 a	22.40 ± 0.74 a	101.84 ± 7.98 ab	1.20 ± 0.11 a	66.52 ± 2.56 ab	6.27 ± 0.95 a	0.081 ± 0.003 a	24.52 ± 0.87 a	0.434 ± 0.020 a	4.35 ± 0.04 a
ST4	59.14 ± 4.96 a	1.72 ± 0.114 a	0.23 ± 0.02 a	21.30 ± 0.76 a	107.18 ± 9.18 a	1.32 ± 0.20 a	54.48 ± 2.81 b	5.78 ± 0.89 a	0.086 ± 0.002 a	24.62 ± 0.77 a	0.465 ± 0.025 a	4.30 ± 0.04 a
F-value	7.701 **	11.435 **	1.996	0.812	3.322 *	1.260	5.074 **	0.717	0.930	2.380	0.420	1.832

ST1-4 refer to the four stand types classified according to the pest severity index. * $p < 0.05$, ** $p < 0.01$. *F*-value is for comparing across stands. The number after ± is standard error. The different letters indicate significant difference between the stand types, and vice versa.

3.4. Ordination of the Occurrence of C. kunyushanica

The infestation ratio (IR) of *C. kunyushanica* raged from 0–90%. The mean values of IR in ST1-ST4 were 0%, 37.68%, 42.93%, and 67.33%, respectively. IR and PSI were taken as the species variables in the redundancy analysis. Among the 24 environmental variables, seven (total N, DBH, soil water content, bulk density, available K, Zn^{2+} concentration, and stand density) were significantly related ($p < 0.05$) to the occurrence of *C. kunyushanica*. Their effect on the occurrence of *C. kunyushanica* followed the order of total N > DBH > soil water content > bulk density > available K > Zn^{2+} concentration > and stand density (Table 6, Figure 1). The first two components of the RDA axes explained 54.75% of the variance in the relationship between the occurrence of *C. kunyushanica* and the seven selected environmental factors. In addition, the *F*-ratio was 38.45 and the *p*-value was 0.002 (Figure 1), indicating that the linear correlation between the sorting axis and environmental factors reflected the relationship of *C. kunyushanica* with stand and soil factors, and the result of the sequencing was reliable.

Table 6. Forward selection of seven significant environmental variables by Monte Carlo permutation test in RDA.

Variables	Contribution %	*F*-Ratio	*p*-Value (5%)
Total N	39.9	38.4	0.002
DBH	25.5	30.7	0.002
Soil water content	6.4	8.2	0.004
Bulk density	7.9	10.9	0.002
Available K	4.1	5.9	0.02
Zn^{2+}	3.5	5.3	0.02
Stem density	2.1	3.2	0.046

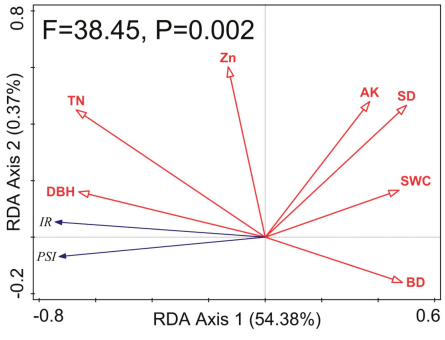

Figure 1. First two canonical axes of redundancy analysis (RDA) ordination biplot between the occurrence of web-spinning sawflies and significant environmental variables. SD, stem density; TN, total nitrogen; AK, available potassium; SWC, soil water content; DBH, diameter at breast height; BD, bulk density; Zn, Zinc ion; IR, infestation ratio; PSI, pest severity index.

Forward selection of the seven significant environmental factors in the RDA ordinations showed that the occurrence of *C. kunyushanica* was primarily influenced by total N, DBH, soil water content, bulk density, available K, Zn^{2+}, and stem density, and their total contribution to *C. kunyushanica* occurrence was 89.4% (Table 6). The RDA ordination biplot revealed specific associations between the occurrence of *C. kunyushanica* and environmental factors; DBH, total N, and Zn^{2+} were positively associated with IR and PSI, whereas available K, stem density, soil water content, and soil bulk density had a negative association with IR and PSI (Figure 1).

4. Discussion

To determine the relationship between stand characteristics, soil properties, and the occurrence of *C. kunyushanica* in the Kunyushan Mountains of China, we examined the differences of these variables in healthy (sawfly-non-infested) and infested stands of JRP pure forests. Stand characteristics of non-sawfly-infested stands of JRP pure forests were different from those of sawfly-infested JRP stands. Stem density was lower, while tree height, crown width, DBH, basal area at breast height, and stand volume were higher in stands infected by *C. kunyushanica*. Soil bulk densities and soil water content were higher in non-sawfly-infested stands than those in sawfly-infested stands. Among the soil chemical properties, the mean values of organic matter, total N, and available N were lowest in healthy plots, while available K was lowest in infected plots.

Although several studies have analyzed the relationship between pest density and stand characteristics, the results have always been ambiguous. In a previous study, Japanese larch (*Larix leptolepis* Gordon) density, tree height, DBH, and the proportion of larch stems were not strongly correlated with the prepupal densities of larch web-spinning sawflies (*C. koebelei* Rohwer) when larch stands had closed canopies [25]. McMillin et al. [26] reported inconsistent effects of stem density on three species of sawflies. In this study, we observed that the PSI increased as the stem density decreased, and *C. kunyushanica* prefers to live in better growing (with higher tree height, DBH, crown width, and so on) JRP forests. Differences in stem densities may have been derived from differences in pest species; for example, the density of *Neodiprion autumnalis* Smith was highest in areas with low tree density and stem density had no influence on *N. xiangyunicus* Xiao and Huang [26]. However, Sun et al. [1] suggested that "*C. kunyushanica* prefers to occur in dense Japanese red pine stands", which is inconsistent with our results. The main reason for this discrepancy may be that Sun et al. used only seven plots, which was not sufficient for the accurate analysis of *C. kunyushanica* in the Kunyushan Mountains. Differences in the size, thickness, and vigor of trees are reflected in the DBH, height, and canopy of trees [27], and these stand characteristics are positively correlated with each other [28]. Therefore, the densities of sawflies were higher in trees with a greater height, canopy width, and DBH than in trees with shorter height, smaller crown width, and DBH. This phenomenon is explained by the plant vigor hypothesis [29]. We showed that *C. kunyushanica* was most likely to occur in large tree stands, which is consistent with the results of De Somviele et al. [30], who reported that variations in outbreak intensity, as measured by defoliation intensity, are positively correlated with mean stand volume, mean stand height, and basal area, but are negatively correlated with stand density. This may be because host plants contain reserves to restore their foliage, food does not become scarce, and thus, do not drive the sawflies out to less preferable plots.

Among the water-related physical properties of soil, our results showed that soil water content in ST4 plots was significantly lower than that in ST1 plots. It is possible that the highest PSI value of ST4 plots was caused by water stress [7]. The association between herbivore outbreaks and sites under water stress may be caused by stress-induced physiological changes in the host, leading to more attractive or acceptable foliage with lower levels of defense compounds. Additionally, these changes enable insects to escape regulation by their natural enemies [31]. Soil bulk density is a key soil parameter that is directly related to many soil properties and processes, including porosity, soil moisture, and erodibility [32]. Soil bulk density can be estimated from the organic matter content of soil, because these variables are negatively correlated [33]. Many abiotic factors affect mortality of pupae in the soil, for examples, teneral *Dacus oleae* suffered a higher mortality in hard soil [34]. The mature larvae of *C. kunyushanica* also overwinter in soil from August to April of the next year [14]. Soil porosity strongly affects the movement of air and water through the soil layers, as well as the soil hardness [35–37], which affects how the mature larvae or pupae survive. The overwintering larvae populations of the previous year are the basis of the adults populations this year. Thus, ST4 plots had the highest PSI, with higher non-capillary porosity and total porosity than other plots.

The ST4 plots had the highest total N and available N, indicating a positive correlation between PSI and these soil variables. It is possible that N enrichment indirectly affects herbivores by changing

the internal microclimate, structure, and quality of host plants, thus changing the behavior and physiological and chemical defense mechanisms of herbivores [38,39]. Increases in the available N content of soil increase the leaf N content, photosynthetic rate, yield, and seed protein content, which enrich the nutrient resources and consequently increase the herbivores populations. This phenomenon is explained by the resource concentration hypothesis [40–43]. Changes in N content also affect host plant allelopathy, which further affects the oviposition and feeding sites of herbivores, and alters their ability to escape predators [44,45]. A positive correlation has been reported between leaf N concentration and insect survival, development, growth, and reproduction [46]. Cheng et al. [47] reported that the total N content is positively correlated with the organic matter content, which improves the availability of soil nutrients. So, similarly to the nitrogen nutrients, soil organic matter enriches the nutrient resources and consequently increases the herbivores populations. Besides, Nemer et al. [48] showed that the mortality of *C. tannourinensis* prepupae is 100% in sandy soils, which lack organic matter. This also suggests that soil organic matter restricts the development and survival of the pupae of Pamphiliidae insects. The PSI of *C. kunyushanica* increased as the level of available K decreased, which can be explained by the plant stress hypothesis [49]. This suggests that the number of herbivores increases with increased translocation of nutrients in host plants due to environmental stress [50]. In our study, it was possible that the deficient potassium nutrition of the JRP could have enhanced nitrogen nutrition available to *C. kunyushanica*.

The RDA ordination graph indicated that different variables either positively or negatively influenced stand characteristics. Seven significant environmental factors affected the occurrence of *C. kunyushanica* by affecting the growth of JRP trees. The RDA was useful because it not only simplified the number of variables effectively but also determined the independent contribution rate of each variable to the environment. Moreover, RDA describes the explanatory ability of specific indicators and enables reliable quantitative ranking [22,51]. In this study, we used RDA to explore the relationship between environmental factors and *C. kunyushanica* and intuitively explained the interaction among multiple variables.

The ecological control of forest pests can make use of various ecological factors, including pest themselves, to control the structure and function of ecosystem, and reach the goal of pest control-sustainable forestry development [6]. From a practical standpoint, our results strongly suggest that control of *C. kunyushanica* through ecological approaches can be achieved by increasing JRP density appropriately. Meanwhile, the overwintering larvae or pupae populations of the previous year are the basis of the adults populations this year. Thus, reducing the number of larvae or pupae may be a potential approach for preventing sawfly outbreaks. Our study shows *C. kunyushanica* usually occurs in soils with higher levels of organic matter and nitrogen. Thus, in these places, we can destroy the soil chamber constructed by the mature larvae so as to prevent them from completing pupation.

5. Conclusions

This study revealed differences in the stand characteristics and soil properties among healthy JRP pure forests and those infested with *C. kunyushanica*. The results may be useful for the management of forests in areas where *C. kunyushanica* is a major pest and where site and stand conditions are similar. Among the 24 factors, stem densities, DBH, total N, Zn^{2+}, available K, soil water content, and bulk density are the most significant factors affecting *C. kunyushanica* density. Therefore, the seven factors should be considered for controlling the *C. kunyushanica* population in the future, such as adjusting the stem density. In addition, this study was conducted at the stand scale. We propose that the further research is needed to explore the relationship between stand factors and the occurrence of *C. kunyushanica* at tree scale, thus using roadside sampling methods to cover large areas in a cost-effective approach.

Author Contributions: R.H. and J.L. conceived and designed the experiments; R.H. and X.X. performed the experiments; Y.Z. and X.Z. conducted the data analysis.

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Survival of European Ash Seedlings Treated with Phosphite after Infection with the *Hymenoscyphus fraxineus* and *Phytophthora* Species

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Abstract: The European *Fraxinus* species are threatened by the alien invasive pathogen *Hymenoscyphus fraxineus*, which was introduced into Poland in the 1990s and has spread throughout the European continent, causing a large-scale decline of ash. There are no effective treatments to protect ash trees against ash dieback, which is caused by this pathogen, showing high variations in susceptibility at the individual level. Earlier studies have shown that the application of phosphites could improve the health of treated seedlings after artificial inoculation with *H. fraxineus*. Three-year-old *F. excelsior* seedlings were inoculated with the following pathogens: a *H. fraxineus*, *Phytophthora* species mixture (*P. plurivora*, *P. megasperma,* and *P.* taxon *hungarica*), in combination with two pathogens and mock-inoculated as the control, and then either watered or treated with ammonium phosphite (Actifos). Results showed significant differences in the survival of seedlings and symptoms of disease development among the treatments. Chlorophyll-*a* fluorescence parameters indicated a decrease in photosynthetic efficiency in infected plants, suggesting that they were under strong biotic stress, but none of the parameters could be used as a reliable bioindicator for ash decline disease. The application of Actifos enhanced the production of triterpenes (ursolic and oleanolic acid), and decreased the production of phenols (tyrosol) and sterols (β-sitosterol) in seedlings infected with *H. fraxineus*. Treatment with Actifos caused seedlings to enhance their response to pathogen(s) attack and increase their survival probability.

Keywords: *Fraxinus excelsior*; invasive pathogens; ash dieback; chlorophyll-*a* fluorescence; phenols; triterpenes; sterols; ammonium phosphite

1. Introduction

European ash (*Fraxinus excelsior* L.) is a valuable component of forest ecosystems where small pure or mixed stands are created with other broadleaved species in deep humid soil [1,2]. Ash as a species was for a long time considered to be free from substantial pests and disease that could threaten

the cultivation of a high-quality wood for a wide range of uses by the forestry industry. Due to fast growth and usable wood, ash for many years was the tree of choice for foresters [3].

Suddenly, about 25 years ago, reports of the decline of ash stands appeared in Poland [4]. At that time, there was not much attention directed to ash, because Europe was already facing a decline in oak [5], beech [6], spruce, and fir [7,8], which were both ecologically and economically more significant species. Approximately a decade later, the problem of ash dieback started becoming apparent, with reports not only from Poland, but also from neighboring Baltic countries, indicating that the problem with ash health was present in the wider region of the northeastern part of continental Europe [9]. Thorough research in the affected parts of Poland indicated a new fungal pathogen, which was named *Chalara fraxinea* by Kowalski [10], as a potential cause of the chronic decline of ash [11]. The species was later associated with its teleomorph [12], and it was subsequently named *Hymenoscyphus fraxineus* (Baral, Queloz, and Hosoya) [11].

Molecular studies have demonstrated that the pathogen was imported from eastern Asia, where it occurs on Manchurian ash (*Fraxinus mandshurica* Rupr.) [13]. After introduction into Europe, the pathogen was quickly established because of the abundance of a susceptible host population, and therefore spread epidemically across the European continent [14]. By 2012, the alien invasive pathogen was reported in the continental part of Europe, the Scandinavian Peninsula, the eastern part of Russia, and also in the British Isles and Ireland [15]. Currently, only countries belonging to the Mediterranean basin (Spain, Greece, Turkey, the southern part of Italy) have not yet reported the presence of the ash decline (EPPO, 2018).

In the affected regions, damage caused by the fungus *H. fraxineus* was higher in younger stands than in older ones, ranging from ≥60% in Germany [16], 57% up to 80% in young stands in Norway [17], or 3% to 35% in older and younger stands in France, respectively [18], but even in the devastated areas, some trees showed good crown conditions and a high survival rate during an attack [19], indicating the presence of genetic resistance existing within the populations [15,20]. Individual resistance between resilient populations of Manchurian and susceptible European ash individua was ascribed to the presence of a chemical compound such as iridoid glycoside in the leaves of the more resistant trees [21,22].

Changes in the health condition of single trees were observed yearly [23], indicating that survival is strongly influenced by environmental conditions and the genetic potential [20] of individuals to initiate the production of secondary metabolites such as iridoid glycoside, which induces plant resistance against the pathogen [15,21,24].

The complex action of phosphites (phosphonates) and their priming in plant-pathogen interactions is well known [25,26] and applied in the induction of resistance against *Phytophthora* root pathogens on beech [27] and *Eucalyptus* [28]. Phosphites are widely used in plant and nursery production as plant growth stimulators, and are available on the market under different brands, i.e., Kalex (K_3HPO_3) or Actifos ($(NH_4)_2HPO_3$) [29–31]. A study performed in a laboratory has shown that phosphites can alter the development of *H. fraxineus* colonies in laboratory conditions [32], but also plays a role in the survival of artificially inoculated seedlings [30,33].

Based on previous knowledge, we hypothesized that there would be clear differences in the tested parameters between three-year-old *F. excelsior* seedlings that were treated and not treated with ammonium phosphite (Actifos) after infection with three types of inocula (*H. fraxineus*, *Phytophthora* species mixed, or *H. fraxineus*–*Phytophthora* mixed combination). We assumed that seedlings infected with *H. fraxineus* would have the highest mortality rate, and those with the combination of pathogens would have the worst health status, according to the measured physiological parameters.

The chlorophyll fluorescence method is often used for the selection of species or varieties that are resistant to stress factors that also act on the photosynthetic apparatus as well as on the overall condition/performance of plants and their yielding. Studies based on the chlorophyll fluorescence method concern both crop plants and trees. Much research has shown that this method helps to predict the hidden changes caused by abiotic and biotic stresses in trees. Moreover, it has been proven that

measuring chlorophyll fluorescence parameters allows the non-invasive estimation of external stressor effects [34–36].

Trees have evolved an array of diverse chemical defenses to cope with pathogen attack. Among these, terpenes and phenolic metabolites are among the most studied components in the resistance of trees to pathogens [37,38]. Phenolic such as pinosylvin, pinosylvin monomethyl ether, stilbenes, and flavonoids are connected with increased resistance to *Ceratocystis polonica*, *Heterobasidion annosum* s.l., *Gremmeniella abietina*, etc. [39]. The different phenolics extracted from aspen showed antifungal activity against *Phellinus tremulae*, and catechol and salicin were found to be inhibitory to *Hypoxylon mammatum* [40]. There is a lack of information about the composition of the chemical compounds involved in resistance to pathogens in angiosperm trees and ash species. In order to obtain information about biochemical processes occurring in infected host tissues, an exploratory analysis of chemical compounds produced in inoculated seedlings versus control plants was conducted with the use of gas chromatography followed by mass spectrophotometry (GC-MS) [41,42]. Both methods should allow an insight into the physiological processes reflecting the interaction between the *H. fraxineus* and *Phytophthora* species playing a part in the dieback phenomenon of European ash.

This paper aims to present the differences in: (i) the survival of *F. excelsior* seedlings over a period of two years (2016/2017); (ii) growth parameters including root development, (iii) re-isolation and PCR-based confirmation of the pathogens from dead and living seedlings; (iv) an exploratory analysis of triterpenes, phenols, and sterols in the cortical tissue samples; and (v) the chlorophyll-*a* fluorescence (ChlF) of leaves as an indicator of the plant metabolism and health status of the seedlings [43].

2. Materials and Methods

2.1. Plant Material

One hundred and sixty three-year-old European ash seedlings were grown in a greenhouse at the Forest Research Institute (IBL, Sękocin Stary, Poland) and planted in 1-L pots filled with a 1:1 (v:v) peat:perlite mixture at the beginning of the vegetation period in May 2016. The temperature range in the greenhouse was between −5 °C and 30 °C, and the photoperiod was the same as in nature with the dormant period from December till March. Fertilization was done at the beginning of the experiment with N:P:K fertilizer with 20 g per plant. At the beginning of the experiment, *F. excelsior* seedlings had a mean stem height of 127.7 ± 2.52 mm and mean stem diameter (at soil level) of 4.14 ± 0.06 mm.

Eighty seedlings were used for treatment with water/control and Actifos ($(NH_4)_2HPO_3$—Agropak, Poland), as shown in Table 1. In the treatment variant with Actifos, seedlings were treated with a 0.6% Actifos water solution on 26 July 2016. Plants were regularly watered up to field capacity, every two to three days or daily during the summer months.

Table 1. Experimental design of three-year-old European ash seedlings.

	Mock Inoculation and Control	***Hymenoscyphus fraxineus* (Hf)**	***Phytophthora* Mix ***	***H. fraxineus* + *Phytophthora* Mix**	**Total**
Water	20	20	20	20	80
Actifos	20	20	20	20	80
Inoculation time	No inoculation	26 September 2016	10 July 2016	10 July 2016 26 September 2016	

* *Phytophthora* mix—*P. plurivora*, *P. hungarica*, *P. megasperma*.

The seedlings were measured for root collar diameter (mm—at the soil level) and stem height (mm) at the beginning of the experiment, in November 2016 and at the end of the experiment in September 2017. The plants' shoots and roots were dried in an oven at 65 °C for 48 h to obtain a total dry weight measured with 10^{-3} g accuracy. Twenty seedlings per treatment were analyzed.

At the end of the experiment, the ash seedlings were removed from the soil and thoroughly washed before scanning with an Epson Perfection V700 Photo Scanner. Subsequently, root morphology

was assessed with WinRhizo Pro (Regent Instruments Inc., Quebec City, QC, Canada). After assessment, the roots were separated into fine roots (<2 mm) per length of mother roots (2–5 mm) and oven-dried and weighed [44].

2.2. Fungal Inoculum

Seedlings in each of the treatments were separated into four groups of 20 plants, which were inoculated with (i) *Hymenoscyphus fraxineus* (Hf); (ii) *Phytophthora* mix (Phy) (mixture of three species *P. plurivora* T. Jung and T.I. Burgess, *P. megasperma* Drechsler, *P.* taxon *hungarica*—which were isolated from ash stands in Poland [9]; (iii) *H. fraxineus* + *Phytophthora* mix (Hf + Phy) and (iv) mock inoculation as control (Mi) (Table 1).

Pathogenicity tests with *Phytophthora* species were performed using a soil infestation test according to Jung et al. [45], and inoculum consisted of four to six-week-old cultures of mix isolates of *P. plurivora*, *P. megasperma*, and *P. hungarica* grown at 20 °C in 500-mL Erlenmeyer flasks on an autoclaved mixture of 250 cm^3 of fine vermiculite (Agra vermiculite® RHP, Rhenen, The Netherlands) and 20 cm^3 of whole millet seeds thoroughly moistened with 175 mL of vegetable juice broth (200 mL 1-1 of vegetable juice (Fortuna®, Agros-Novasoki, Warsaw, Poland), 800 mL 1-1 distilled water amended with 3 g 1-1 $CaCO_3$). After this period, the inoculum was put into the soil at a ratio of 20 cm^3 to 25 cm^3 of inoculum per 1000 cm^3 of the soil mixture. Control groups of plants were inoculated only with rinsed sterile vermiculite-vegetable juice mixture at the same ratio. Each box with pots was flooded immediately after inoculation for 72 h. The inoculation with *Phytophthora* mix was performed on 12 July 2016, which was two months before the *H. fraxineus* inoculation.

A three-week-old culture of *H. fraxineus* (strain KY613994, Sękocin Stary, Poland), growing on 2% malt extract agar (MEA) (Merck, Darmstadt, Germany) in Petri dishes, was used for the stem inoculation in a small cut in the bark made by a scalpel sterilized in 95% ethanol. A plug of bark was removed, and a 3-mm disk of mycelium from the margin of a colony was placed in the wound. All of the wounding control was inoculated with sterile agar plugs. After inoculation, the stems were sealed with Parafilm (Sigma-Aldrich, Taufkirchen, Germany). Inoculation with *H. fraxineus* was carried out on 26 September 2016.

Stem lesion lengths were measured upward and downward from the inoculation point successively after the death of the seedlings.

2.3. Re-Isolation and Confirmation of H. fraxineus and Phytophthora spp.

Re-isolation of the pathogen was carried out by taking small fragments (2 mm × 2 mm) of wood surface sterilization in a solution of 1% NaOCl and placing them in 90-mm Petri plates containing 2% MEA. The dishes were incubated at 4 °C for 20 days and checked regularly.

The fragments of wood and bark (approx. 50 mg) were taken from four inoculation points at the stem as marked from one to four (Figure 1) and ground to powder in liquid nitrogen. The sampling point (1) was at the place of artificial inoculation, while points two and three were usually at the margin or outside of the cankers. Point four was 1 cm above point three, and outside the range of the cankers. DNA was extracted using the Genomic Mini AX Plant kit and cleaned with Anti-Inhibitor Kit (A&A Biotechnology, Gdynia, Poland) according to the manufacturer's instructions. *H. fraxineus* was confirmed with species-specific primers HFrax-F—5′CTTTAGCAGGTCGCCCTCT 3′ and HFrax-R—5′TGCTGGCAAGACACCGCAA 3′ to amplify a 389-bp fragment of the ribosomal DNA [45].

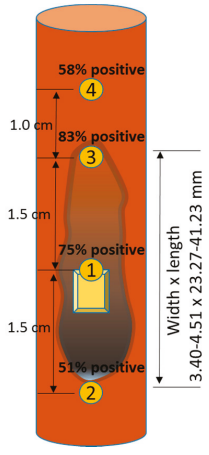

Figure 1. Sampling points (1–4) for PCR confirmation of *H. fraxineus* pathogen in the seedlings. Point 1 refers to the inoculation place; points 2 and 3 were 1.5 cm below and above sampling point 1, respectively; point 4 was 1 cm and 2.5 cm above points 3 and 1, respectively.

The 25-µL PCR mixture consisted of 2.5 µL of 10× Buffer (GenoPlats, Rokocin, Poland), 2 µL of $MgCl_2$ (25 mM), 0.5 µL of dNTP's, 0.25 µL Taq (5 U/µL)(GenoPlats, Rokocin, Poland), 1.0 µL of each primer (40 nM final conc.), 1 µL of DNA (ca. 100 nM), and up to 25 µL of Milli-Q water. Cycling conditions were slightly changed from Drenkhan et al. (2016) with initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 68 °C for 30 s, elongation at 72 °C for 1 min, and a final extension at 72 °C for 10 min. PCR products were electrophoresed on 1.5% agarose gels and visualized under UV light using a GelDoc XR + gel documenting system (BioRad, Hercules, CA, USA).

To confirm the presence of Phytophthora species in the root tissue, five samples (around 2 mm in length) were collected from rotten parts of the roots. Tissue samples were surface-sterilized with 1% sodium hypochlorite. Afterwards, small fragments (approximately 3 mm × 3 mm) were cut out with a scalpel and placed on PARP (Pimaricin, Ampicillin, Ryfampicin and PCNB) selective media. Additionally, the tissue fragments extracted from the uninfected plants were placed on a medium. Petri dishes were incubated at 22 °C for one week. The growth of mycelium was monitored each day [46].

2.4. Chlorophyll-a Fluorescence Measurements

Chlorophyll-*a* fluorescence (ChlF) measurements of leaves were performed using a Handy PEA fluorimeter (Hansatech Instruments, King's Lynn, Norfolk, UK). Measurements were performed after 20 min of dark adaptation of leaves using leaf clips [36,41,43]. An excitation red light (emitted from three diodes with a wavelength peak of 650 nm and intensity of 3500 µmol m^{-1} s^{-1}) was used for the induction of chlorophyll fluorescence, and 1 s of transient fluorescence was measured [43,47]. ChlF transients were used for calculation of an OJIP test (major phases of fluorescence rise from O to P with two intermediate steps J and I) and basic parameters. One measurement per plant was taken in each seedling on 9 September 2016 and 27 July 2017.

2.5. Analyses of Chemical Composition of Seedlings

Forty-two seedlings from eight treatments were randomly selected from the total of 160 seedlings at the end of the experiment in September 2017. In the treatment with *H. fraxineus* infection, only dead plants were the subject of analyses, while in other treatments, living plants were analyzed.

Diethyl ether and pyridine were purchased from POCH S.A. (Gliwice, Poland), whereas *N*,*O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) and a standard mixture of *n*-alkanes (C_{10}–C_{40}) were purchased from Sigma-Aldrich (Poznań, Poland).

The chemical composition of 42 ash seedlings was analyzed according to the method of Stocki et al. [48]. Ash shoots up to 5 mm in thickness were selected, milled into a 0.5-mm fraction, and dried for 48 h at 50 °C. Raw materials (1 g) were extracted three times with 25 mL of diethyl ether. Extracts were filtered through paper filters. The solvent was removed using a rotor evaporator (Büchi, Switzerland) [49,50]. Dry residues of diethyl ether extracts (10 mg) were dissolved with 1 mL of pyridine, and 100 µL of BSTFA was added. Mixtures were heated for 30 min at 60 °*C* and analyzed by gas chromatography-mass spectrometry (GC-MS) using an Agilent 7890A gas chromatograph (USA) equipped with an Agilent 5975C mass selective detector (USA). An injection of a 1-µL sample was performed using an Agilent 7693A autosampler (USA). The separation was performed on an HP-5MS (30 m × 0.25 mm × 0.25 µm film thickness) fused silica column at a helium flow rate of 1 mL/min. The injector worked in a split (1:50) mode at an injector temperature of 300 °C. The initial column temperature was 50 °C, rising to 320 °C, at 3 °C/min; the final temperature was held for 10 min. The ion source and quadrupole temperatures were 230 °C and 150 °C, respectively. Electron ionization mass spectral (EIMS) was obtained at an ionization energy of 70 eV. The detection was performed in a full scan mode from 41 a.m.u. to 600 a.m.u. After integration, the percentage of each component in the total ion current (TIC) was calculated. Mass spectral data and calculated retention indices were used to identify compounds. Mass spectrometric identification was carried out with an automatic system of GC-MS data processing supplied by the National Institute of Standards and Technology (NIST) and a library of mass spectra [51]. Retention indices of analyses were determined, taking into account C_{10}–C_{40} *n*-alkanes retention times and comparing them with the NIST and databases [51].

2.6. Statistical Analysis

Differences in mean parameters measured for the seedlings were detected by analysis of variance with one-way ANOVA followed by Tukey tests. Homogeneity of variance was confirmed by Levene's test.

Wood chemical component, ChlF parameters, and root morphology data were not normally distributed (checked by means of the Kolmogorov–Smirnov test), so they were processed with the Kruskal–Wallis test, followed by the Mann–Whitney U test for multiple comparisons, as the data were not normally distributed after the log transformation.

The effects of treatments on chlorophyll-*a* fluorescence parameters were calculated using one-way ANOVA, with statistically significant differences being determined by the LSD Fisher post-hoc test with significance $p < 0.05$.

The Spearman rank correlation was used to measure the strength of association and the direction of the relationship between selected parameters of chlorophyll fluorescence, survival of seedlings, growth parameters, and chemical component analyses.

Statistical analyses were performed using SPSS ver. 20 (IBM Corp., Armonk, NY, USA).

3. Results

At the control, in April 2017, and six months after inoculation, 100% of the seedlings inoculated with *H. fraxineus* (Hf) and 55% of those inoculated with *H. fraxineus* + *Phytophthora* mix were dead in water treatment (Figure 2). In Actifos treatments, all of the *H. fraxineus* infected plants were alive, and in the joint infection with *Phytophthora* (Phy), only three seedlings died (Figure 2).

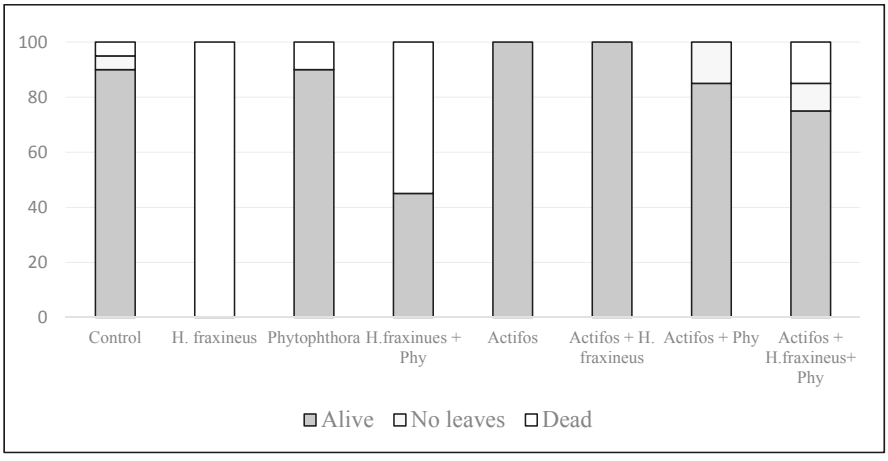

Figure 2. Survival of ash seedlings vs. mortality rate at the end of experiment (September 2017).

Dead seedlings were examined for extension of necrotic lesions and wood discoloration. The lesion length and width values were 23.27–41.23 mm and 3.40–4.51 mm, respectively, without significant differences among treatments ($p > 0.05$).

During re-isolation of *H. fraxineus* from the tissues of dead seedlings, the species was identified in only 2.4% of all of the cultures. Among the obtained isolates, *Fusarium* spp. dominated (38.8%), followed by *Alternaria* spp. (28.7%) and *Phomopsis* spp. (17.7%) (data not presented), suggesting the succession of fungal communities on dead ash tissues.

Molecular identification of the pathogen from necrotic tissues at four sampling points (Figure 1) was successful in 66% of the samples. *H. fraxineus* DNA was isolated and amplified most often from the point that was 1.5 cm above the inoculation place (point 3) in 83% of samples, followed by 75% at inoculation point (1), and 58% and 51% at the upper and lower outermost points of sampling, respectively (Figure 1). In order to confirm the presence of the pathogen's DNA in living plants, from the treatment with Actifos (Act + Hf, Act + Hf + Phy) samples of tissues from the margin of the callus were taken, but the PCR product was obtained in only 8% (3/37) of the reactions.

One year after the start of the experiment, small but significant variations in the diameter and height of the seedlings were observed between eight treatments (Table 2). According to the analyses, these differences resulted from both the individual variability of the seedlings and the type of treatment applied.

Table 2. *Fraxinus excelsior* seedling growth characteristics (mean ± SE) for eight treatments at the end of the experiment (September 2017).

	Diameter (D)	Diff in D	Height (H)	Diff in H	Shoot/Root Dry Weight Ratio	Leaf Area
		10^{-3} m			g	cm^2
Water	±	0.41 ± 0.06 b	114.50 ± 7.01 a	22.70 ± 5.93 a	0.29 ± 0.26 a	106.47 ± 12.04 a
Hf	6.07 ± 0.17 **b	0.08 ± 0.01 d	174.04 ± 6.69 c	0.71 ± 0.07 d	0.55 ± 0.47 b	0.00 ± 0.00 c
Phy	6.26 ± 0.23 a	0.57 ± 0.14 a	151.36 ± 6.93 c	11.79 ± 2.38 b	0.35 ± 0.19 ab	65.91 ± 8.91 b
Hf + Phy	6.25 ± 0.21 a	0.16 ± 0.08 c	171.54 ± 7.98 c	9.29 ± 4.45 c	0.53 ± 0.67 b	101.09 ± 27.41 b
Actifos	5.38 ± 0.19 c	0.19 ± 0.05 c	134.39 ± 7.46 b	23.11 ± 7.56 a	0.34 ± 0.17 ab	111.31 ± 17.28 a
Act + Hf	6.06 ± 0.16 b	0.39 ± 0.07 b	183.20 ± 8.84 d	14.05 ± 3.17 b	0.36 ± 0.29 ab	91.22 ± 24.24 a
Act + Phy	6.08 ± 0.20 b	0.26 ± 0.04 b	136.89 ± 5.65 b	11.97 ± 1.82 b	0.29 ± 0.21 a	40.35 ± 9.48 b
Act + Hf + Phy	5.93 ± 0.10 b	0.40 ± 0.15 b	150.13 ± 8.75 c	8.65 ± 3.43 c	0.29 ± 0.31 a	62.76 ± 17.21 b

* mean ± SE. ** Different letters behind values indicate significant differences obtained by Tukey's post hoc tests ($p < 0.05$).

The smallest values for the diameter (D) were observed for treatment with Actifos, while the highest were measured for *Phytophthora* and combined *Phytophthora* and *H. fraxineus* treatments, respectively (Table 2). Differences in the diameter between the 2016 and 2017 measurements (annual plant radial increment) (diff. in D) were significant between the water and Actifos treatments, but not for the other treatments with Actifos. Reduction of the height increment (diff. in H) was significant for all of the infestation treatments, while the values for water and Actifos treatments were highest, and did not differ from each other. The leaf area at the end of the experiment was reduced for all of the *Phytophthora* treatments (Table 2).

The nine tested root parameters showed a significant difference between treatments and allowed division into three groups (Table 3). If we compare with the water (control) treatment, treatment, total root length (total and fine root) and surface area were significantly different only for Hf + Phy and Act + Phy treatments. In the Actifos treatments, there were fewer root tips—both in the total number of tips (NoT) and fine root tips (FRT)—compared to the water treatment (Table 3). Seedlings in the Actifos treatments produced fewer fine roots—as measured by fine root length (FRL) versus mother root length (MRL) and fine root tips (FRT/MRL) in relation to the length of mother roots compared to the water treatment (Table 3).

During 2016, the photosynthetic efficiency (based on chlorophyll fluorescence signals analysis) of the studied ash seedlings showed that the combinations of Actifos + *Phytophthora* mix and *H. fraxineus* + *Phytophthora* mix were the most similar to the control treatment (water). This was expressed by showing less loss of absorbed light energy as heat dissipation (DI parameters), a higher level of reaction center reduction in photosystem II (PSII) (Phi and Psi parameters), better electron transport (ET parameters), and reduction of the first acceptors of photosystem I (PSI) (RE parameters) (Figure 3). The total positive response of the photosynthetic machinery for these two combinations can also be seen by the values of the performance index parameters (PI). On the other hand, worse functioning of the photosynthesis apparatus was noted in the case of *H. fraxineus* (Hf) alone (Figure 3). During the following year (2017), we were not able to measure chlorophyll fluorescence signals for the latter due the loss of leaves. Plant treatment with Actifos showed a very positive response of the photosynthetic efficiency of the tested tree seedlings that were not affected by *H. fraxineus*. Moreover, some enhancements at the level of PSII functioning (performance index, or PI) that were expressed as an increase in the absorbed and trapped light energy and accelerated electron transport rate were observed (Figure 3). A similar response was observed when the trees were treated with an Actifos + *Phytophthora* mix. The application of the *Phytophthora* mix, whether alone or with Actifos, maximally reduced the effect of *H. fraxineus* (Hf) on the trees' photosynthetic efficiency. The application of Actifos by itself on the trees affected by *H. fraxineus* did not help improve this efficiency (Actifos + Hf). In both years of the study, the maximal photosynthetic efficiency of the PSII (Fv/Fm) parameter did not show significant changes (Figure 3).

In the water treatments, there was no significant difference in the content of triterpenes between the infected and control seedlings. The total amount of sterols was significantly lower in the Hf inoculation type, while the highest value was held by the combined Hf + Phy inoculation. *H. fraxineus*-infected plants had a significantly higher content of phenolic compounds compared to other water treatments (Figure 4).

The Actifos treatments showed differences in the chemical composition of seedlings, but only the reduced amount of total triterpenes in the Hf + Phy inoculation type was significant. The measured amounts of sterols and phenols in Actifos treatments were not significantly different; however, the content of phenols in Hf + Phy-infected seedlings was four times higher compared to other Actifos variants (Figure 4).

Table 3. Mean values and results of Tukey test for eight root morphology parameters of common ash seedlings observed in eight treatments.

	Water	Hf	Phy	Hf + Phy	Actifos	Act + Hf	Act + Phy	Act + Hf + Phy
TRL (cm)	1014.83 ± 76.44 a*	846.93 ± 71.98 a	1065.22 ± 61.93 a	1541.93 ± 90.66 b	1061.89 ± 60.16 a	1346.74 ± 58.42 b	941.83 ± 36.91 a	889.81 ± 64.57 a
FRL (cm)	978.85 ± 75.05 a	793.48 ± 69.36 a	1012.55 ± 59.24 a	1474.27 ± 92.32 b	1012.31 ± 58.89 a	1268.28 ± 54.86 b	893.9 ± 34.87 a	842.42 ± 62.38 a
SA (cm^2)	173.07 ± 12.83 a	208.34 ± 18.21 a	217.88 ± 12.69 a	347.37 ± 28.9 b	207.8 ± 10.26 a	320.72 ± 17.44 b	194.8 ± 11.14 a	187.12 ± 13.91 a
FRSA (cm^2)	97.99 ± 7.9 a	97.49 ± 9.74 a	113.09 ± 7.24 a	174.16 ± 9.87 b	115.71 ± 6.83 a	161.98 ± 8.21 b	105.21 ± 4.5 a	101.32 ± 8.32 a
NoT (n)	5387.95 ± 612.69 a	3500 ± 480.15 abc	5093.2 ± 637.52 ab	7340.44 ± 2510.38 a	2084.7 ± 305.99 c	5178 ± 628.92 ab	4016.3 ± 662.69 abc	2494.05 ± 361.36 bc
FRT (n)	5384.7 ± 612.65 a	3496.29 ± 479.88 abc	5087.6 ± 637.19 ab	7334.22 ± 2510.51 a	2081.55 ± 305.97 c	5172.75 ± 628.81 ab	4012.25 ± 662.24 abc	2489.5 ± 361.08 bc
FRL/MRL	43.1 ± 4.27 b	30.56 ± 5.46 ab	33.84 ± 2.39 ab	32.69 ± 4.78 ab	29.32 ± 2.53 ab	24.73 ± 1.79 a	24.19 ± 1.68 a	30.3 ± 6.91 ab
FRT/MRL	273.09 ± 55.7 b	158.2 ± 39.07 ab	165.8 ± 23.42 ab	196.08 ± 75.8 ab	57.08 ± 7.74 a	105.44 ± 18.16 a	100.4 ± 15.59 a	84.81 ± 13.91 a

TRL—Total Root Length; **SA**—Surface Area; **NoT**—Number of Tips; **FRL**—Fine Root Length; **MRL**—Mother Root Length; **FRL/MRL**—Fine Root Length/Mother Root Length; **FRT/MRL**—Fine Root Tips/Mother Root Length; **FRSA**—Fine Root Surface Area; **FRT**—Fine Root Tips; * different letters in the row indicate significant difference according to Tukey test ($p < 0.05$).

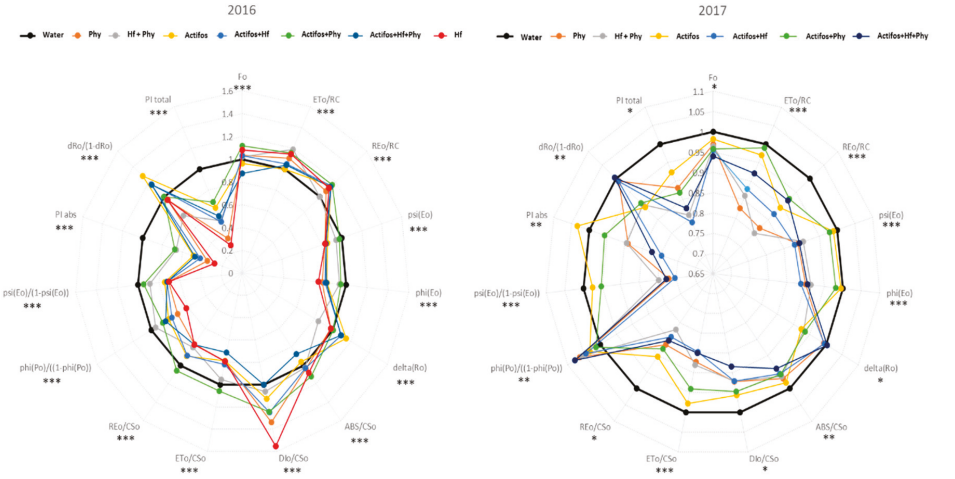

Figure 3. Comparison of JIP parameters for selected treatments. All of the elements are normalized to the control treatment. Asterisks (*, **, ***) denote significant differences between control and treated plants according to the Fisher LSD test at $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$, respectively.

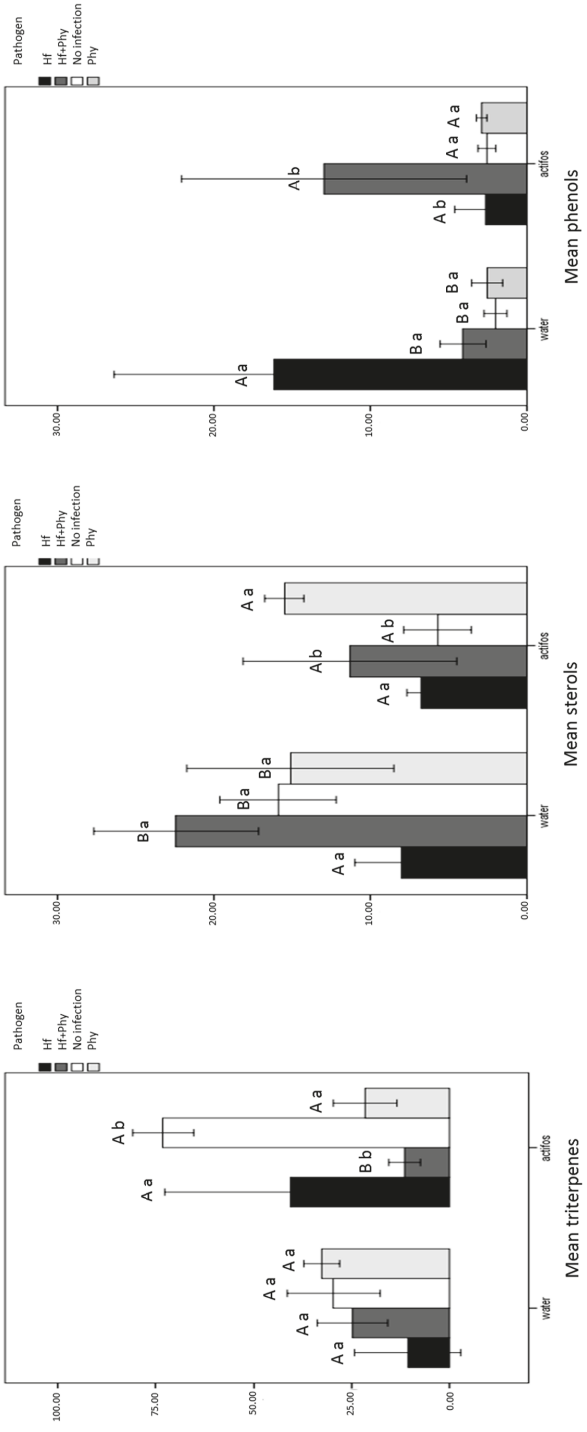

Figure 4. Content (%) of triterpenes, sterols, and phenols of cortical tissue samples of *F. excelsior* seedlings subjected to ammonium phosphite treatment (water and Actifos) after control, Hf, Phy, Hf + Phy infection. Uppercase letters indicate significant differences ($p < 0.05$ Kruskal–Wallis and Mann–Whitney *U*-test) between mock-inoculated and fungal inoculated plants within the same treatment (water and Actifos). Lowercase letters indicate significant differences ($p < 0.05$ Mann–Whitney *U*-test) between plants subjected to different treatments and the same inoculation type.

Differences between the treatments (water and Actifos) within the same inoculation type were significant for the total triterpene, sterol, and phenol amounts. The content of the triterpenes and sterols was significantly varied for uninfected as well as Hf + Phy-infected seedlings. The amount of sterols was significantly lower in Actifos-treated seedlings infected with Hf + Phy, while the amounts of phenols within the same combination of treatment/infection type showed an opposite trend (Figure 4).

In the water treatments, there was no significant difference in the content of main triterpenes, sterols, and phenols between the infected and control seedlings. However, *H. fraxineus*-infected plants had a higher amount of tyrosol and lower content of ursolic acid and β-sitosterol compared to other water treatments (Figure 5).

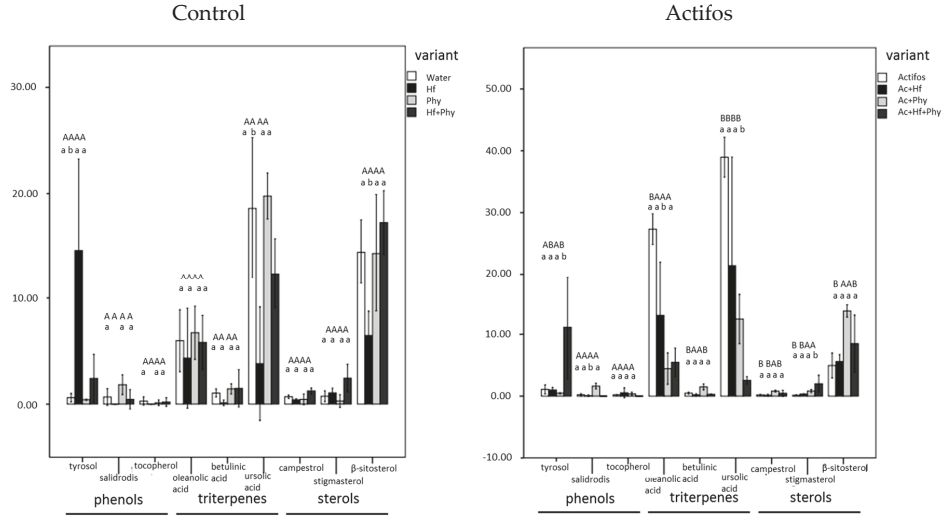

Figure 5. Content (%) of selected compounds in extract from the shoots of *F. excelsior* subjected to ammonium phosphite treatment (water and Actifos) after control for Hf, Phy, and Hf + Phy infection. Lowercase letters indicate significant differences ($p < 0.05$ Kruskal–Wallis and Mann–Whitney *U*-test) between mock-inoculated and fungal inoculated plants within the same treatment (water and Actifos). Uppercase letters indicate significant differences ($p < 0.05$ Mann–Whitney *U*-test) between plants subjected to different treatments and the same inoculation type.

The Actifos treatment showed significant differences in the amount of all sterols, as well as tyrosol, oleanolic, and betulinic acids (Figure 5).

The profiles of the main triterpenes, sterols, and phenols showed a significant difference between treatments (water and Actifos) and infection type. The amount of tyrosol was significantly decreased for seedlings infected with Hf, and increased for those infected with Hf + Phy. The content of ursolic acid within the same combination of treatment/infection type showed opposite trends. Some infected seedlings treated using Actifos had a significantly lower content of salidroside, oleanolic acid, stigmasterol, and β-sitosterol (Figure 5).

A strong correlation that was both positive and negative, ranging from 0.46 to 0.81, was observed for the tested chlorophyll fluorescence (Chlf) parameters (Table 4). The negative correlation was for both performance indexes and energy dissipation (DI/CSo). There were no statistically significant differences in correlations between the survival of seedlings and Chlf parameters. A positive correlation was observed between the survival and height and diameter of seedlings. The diameter of the tested plants strongly correlated with the height of the tested seedlings. Phenols were negatively correlated to triterpenes and sterols.

Table 4. Spearman rank-based correlation matrix for the interaction of the 11 selected parameters.

	Parameters	Mean	s.d.	1	2	3	4	5	6	7	8	9	10
Chlorophyll fluorescence (159)	(1) PIabs	0.743	0.065	—									
	(2) PItot	1.230	0.893	**0.60** **	—								
	(3) DI/CSo	67.476	21.529	**−0.81** **	**−0.46** **	—							
	(4) ET/CSo	89.500	24.877	**0.53** **	**0.68** **	−0.05	—						
No. of living plants (8)	(5) Survival	14.875	7.060	−0.39	**−0.53**	0.02	−0.53	—					
Growth parameters (187)	(6) Height	141.30	38.94	−0.03	**−0.16** *	−0.04	−0.09	0.58	—				
	(7) Diameter	5.79	1.044	−0.12	**−0.21** **	0.10	−0.09	0.65	**0.57** **	—			
	(8) Leaf area	88.52	82.303	−0.02	**−0.04**	0.07	0.08	0.34	0.11	**0.19** *	—		
Chemical analysis (24)	(9) Phenols	5.714	6.400	**−0.50** *	−0.11	**0.43** *	**−0.23**	**0.39**	0.18	0.16	−0.09	—	
	(10) Triterpenes	30.615	21.612	0.24	0.04	−0.25	−0.01	−0.05	0.05	0.13	0.31	**−0.50** *	—
	(11) Sterols	12.590	6.260	0.14	−0.12	−0.04	0.05	−0.32	−0.16	−0.12	−0.03	−0.32	−0.28

* $p < 0.05$ level (2-tailed); ** $p < 0.01$ level (2-tailed).

4. Discussion

The mortality of seedlings in ash stands infected with *H. fraxineus* constitutes major damage and a threat to the existence of the species on the sites [16,52]. The regeneration of ash on heavily infested sites is endangered both by infestation and a lower production of seed [53], and by a high infection rate of seedlings [54]. The survival rate of young trees is very low because of the small crown volume and girdling of the main stem [18,55], while in older trees, this process is slower and takes time to be observed while the disease progressively develops on the shoots [56–58].

The high pathogenicity of *H. fraxineus* was demonstrated in this study. The mortality rate in the water treatment after the first experimental period on three-year-old seedlings was 100%. None of the infected seedlings survived the winter 2016/17. This confirms earlier findings that the disease progresses during the winter months, and that ash trees with later flushing and longer dormancy suffer from greater damage [59]. Molecular confirmation of the pathogen spread in the bark tissues, and wood showed the development of lesions above and below the point of inoculation (Figure 1) [10,12,60]. The pathogen preferentially followed the acropetal mode of transport in the plant, progressing faster and further up to 2.5 cm in the direction of the movement of water and mineral material rather than in the opposite direction, which is typical for vascular pathogens [61–63]. All of the plants treated with ammonium phosphite—Actifos before infection with *H. fraxineus*—survived until the end of the experiments. Even though some authors speculate that this pathogen can behave as an endophyte in vigorous plants, it was not possible to prove the presence of fungal DNA in the transient zones surrounding the callus tissues [64].

Although we hypothesized that the seedlings infected with *Phytophthora* mix through the soil and *H. fraxineus* in the stem would have the highest mortality rate, the two-year experiment showed that nine and 17 of 20 seedlings survived in the control and Actifos treatment groups, respectively. The efficiency of phosphites against the *Phytophthora* species is well documented [27,28,65], and it seems that the application of Actifos prevented the development of *Phytophthora* and reduced damage in roots [30]. Also, there is a possibility that the *Phytophthora* species in the mix for soil infestation contained less pathogenic species, but we cannot neglect that the survival of seedlings infected with *H. fraxineus* in these variants was much higher than in the water/control groups infected with the fungus. The results presented allow us to hypothesize that two months earlier, the soil infection with the *Phytophthora* mixture triggered still unknown resistance mechanisms, and ensured the higher survival of seedlings infected with *H. fraxineus.*

Seedlings showed differences in development, especially in the second year of the experiment. Reduction in height increment was significant in the infections with *H. fraxineus,* suggesting a decrease in the vitality of seedlings infected with *Phytophthora* species [30]. A similar conclusion could be drawn for the leaf area of plants infected with the *Phytophthora* mixture, which had obviously smaller leaves. This situation is well known for root system infections with pathogens such as *Phytophthora* spp., *Armillaria* spp., and *Phellinus weirii* [66–69]. Regarding Actifos-treated seedlings infected with *H. fraxineus,* there were no differences compared with both the control and Actifos, while differences in height growth were significantly smaller from the control, but still higher than in all of the other inoculation variants (Table 2).

Root analyses were not correlated with the seedlings' above-ground development. No differences existed among variants and treatments for total root length (TRL) and surface area (SA), suggesting that *Phytophthora* did not cause a notable destruction of the root system. The number of root tips decreased in the treatments with phosphites [28,70], and a similar situation was noted with number of tips (NoT) and fine root tips (FRT). The toxicity of phosphites to roots probably triggers a resistance mechanism of the plants against pathogens. The differences observed in the seedlings' above and below-ground development point to the importance of the individual vulnerability of ash trees to both pathogens [15,71].

Chlorophyll fluorescence enables a fast and non-invasive assessment of the photosynthetic apparatus function of any photosynthesizing organisms, so it has recently become a very popular

method for the detection of any plant stressors [42,72,73]. It also allows changes in the tested material (mostly leaves) to be predicted before any visible changes can be seen [36,74,75]. However, studies on trees and specialized host–pathogen interactions are still scarce [76]. The analyzed chlorophyll fluorescence parameters showed a clear difference between the control variants, Actifos-treated plants, and inoculated seedlings before any visible symptoms could be detected by the naked eye (at the end of the first study period). The worst photosynthetic performance was observed for infections by *H. fraxineus*. As a result, in the conditions of the experiment, none of the seedlings survived until spring. Also, autumn 2016 measurements showed that seedlings sprayed with Actifos suffered from the treatments, which is common for trees under stress [77]. This was not the case in the second year, where Actifos-treated plants showed chlorophyll fluorescence parameters that were quite similar to the control plants.

Plants infected with *H. fraxineus* showed the lowest level of photosynthetic performance and the highest "cost" of photosynthetic machinery survival, which was expressed as a loss of the absorbed light as heat energy. This is a standard response when plants deal with any biotic or abiotic stress [36].

Treatment of healthy seedlings with Actifos had a slightly positive effect on the photosynthetic efficiency, maintaining a similar level as in the control, but the application of Actifos did not help improve this efficiency when the plants were infected by *H. fraxineus*, suggesting that some other host–pathogen interactions occur in asymptomatic plants [78]. The photosynthetic efficiency of seedlings was reduced when plants were treated with the *Phytophthora* mix alone. However, its application helped the tree seedlings infected by *H. fraxineus* to maintain photosynthetic performance at an adequate level. The application of the *Phytophthora* mix in combination with Actifos did not have a significant effect on the photosynthetic apparatus status in seedlings both infected and uninfected by *H. fraxineus*. This result suggests that resistance reactions are not connected with the changes in biochemical energy consumption (boost of photosynthesis) to protect plants [44], but with the production chemical compounds (phenols, tannins, etc.) that can kill or delay fungal development in the infected tissues [79].

The very well-known and most used chlorophyll fluorescence (ChlF) parameter (Fv/Fm or Phi_{po}) related to the maximal efficiency of the PS-II photosynthesis system (data not shown) did show significant changes, which means that it cannot be recommended as a reliable bioindicator [36].

Based on the correlation analysis, it seems that there is a strong and significantly different correlation between ChlF parameters. The survival of the seedlings seems to be negatively correlated with the tested performance indexes (PI_{abs}, PI_{tot}) and electron transport (EI/CSo). The significant correlation between PIabs and DI/CSo suggests their relatedness to phenol production, but it is not possible to make further conclusions based on the knowledge presented.

Stronger plants had an improved chance of surviving either an *H. fraxineus* or *Phytophthora* attack (Table 3), which correlates with studies showing that damage to developed trees is less frequent [16,18]. A positive correlation was observed between the height and diameter of seedlings and their survival, and also the height/diameter interaction was strong and significant.

Trees have developed a wide range of defense mechanisms that can help them survive interactions with forest pathogens and insects [79]. Resistance to pests can be constitutive or induced, and has the potential to physically or chemically inhibit or stop pathogens or insects [80,81]. After physical barriers have been bypassed, multiple mechanisms are induced to produce chemical compounds such as phenols, terpenes, PR proteins, and secondary resins in localized or systemic induced resistance [79,82]. Induced resistance can be activated by biotic (pathogens, endophytes) [83] or abiotic compounds such as phosphites [28].

In the variant where plants were infected with Hf (only watered), the disease developed and seedlings died, while the content of phenolic compounds amounted to almost 800% compared to the control (=100%). The application of Actifos increased the amount of phenolics by 27%, which was the same as in the variant when the seedlings were sprayed with Actifos and infected with Hf (~32%). This level prevented lesion development and activated callus formation. When the infection

combined Hf and *Phytophthora*, the ash trees defended themselves by increasing their content of phenolic compounds by over six times (>650%) after treatment with Actifos, and in this case, some of them did not die. Changes in terpene and sterol amounts were notable, too. The seedlings treated with Actifos showed a notable increase in terpenes both in the control and Hf treatments, while a decrease was observed for infections with the *Phytophthora* mix. The total amounts of sterols were suppressed in the Actifos treatments. A few compounds originating from the different chemical groups that were suppressed by the application of Actifos may be of interest for further analyses. Nevertheless, the observed differences in total amounts indicate great individual variation between plants. The interaction between phenols and triterpenes was negative (Table 3), suggesting that an increase of phenols induces a significant reduction in triterpene production, but the role of these and other components involved in host–pathogen interaction and the survival of ash seedlings/trees under *H. fraxineus* attack needs further clarification.

5. Conclusions

F. excelsior seedlings inoculated with *H. fraxineus* showed significant differences in survival, lesion development, the production of phenols, terpenes, and sterols, and ChlF responses in plants sprayed with ammonium nitrate in the Actifos compared to water (control) treatment. Seedlings treated with Actifos prior to inoculation with *H. fraxineus* managed to survive the pathogen attack and prevent the development of the disease. It is not possible to be certain whether the pathogen is eradicated from the plant, as it was not possible to confirm this with species-specific primers, or whether it was merely suppressed in a latent phase of development, which is in line with the parameters obtained for the ChlF response. However, several attempts to re-isolate the pathogen on artificial media failed.

The combination of the possibility to manipulate plant vigor and individual genetically conditioned resistance, which was earlier reported, could be a method to ensure the improved survival of seedlings in the first years after their establishment until they move to higher social status/DBH (Diameter at breast height) classes, where they will have a greater chance of avoiding lethal damage from pathogens.

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Effects of Forest Management Practices on Moth Communities in a Japanese Larch (*Larix kaempferi* (Lamb.) Carrière) Plantation

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Abstract: Biodiversity in forests is strongly affected by forest management practices, such as clearcutting and aggregated retention. Therefore, the assessment of the effects of forest management on biodiversity is a major concern in forest ecology. In the present study, we aimed to characterize the effects of forest management practices, after one year, on the abundance, species richness, community composition, and functional groups of moths in forests. The moths were sampled in four different forest stands: three stands (clearcutting, aggregated retention, and no cutting) in a planted Japanese larch forest and one stand in a natural Mongolian oak forest. The results revealed that the moth communities changed in response to the changes in vegetation after the implementation of forest management practices, and clearcutting increased the abundance and species richness of herbivorous and warm-adapted species. The structure and function of moth communities were affected by the forest management practices such as clearcutting and aggregated retention, which were reflected by a decrease in community indices and change in moth community composition with changes in vegetation.

Keywords: aggregated retention; clearcutting; coniferous forest; deciduous forest; functional group; Lepidoptera; multivariate analysis

1. Introduction

Forest management practices abruptly change the environmental condition of forests, affecting their ecological structures, including biodiversity. Therefore, the assessment of the effects of forest management on biodiversity is a major concern in forest ecology [1], and it is important to evaluate the changes in local flora and fauna caused by the forest management practices [2]. Clearcutting as a timber harvest technique is widely used in forest management practice because it is a cheapest and the most efficient [3,4]. However, many concerns regarding this technique are related to its detrimental effects on the forest ecosystems [5]. To minimize the negative ecological effects of clearcutting, recently the aggregated retention is widely promoted as a way to conserve forest biodiversity [6]. Clearcutting affects the environmental condition of forests, including an increase in sunlight exposure, and fluctuations in temperature, humidity in the ground layers, soil bulk density, and soil hardness [7–9]. This results in changes in the vegetation due to changes in the canopy and stem density of trees in

the forest [10]. Furthermore, the light-demanding plant species become more abundant than the shade-tolerant species [11]. The growth of some vascular plant species, such as herbaceous forest species (*Epilobietea angustifolii* Tüxen. and *Carex pilulifera* L.), is favored by clearcutting [2,12], whereas, several higher plants, bryophytes, and lichens are negatively affected by clearcutting [2].

In South Korea, the retention harvest experiment was initiated in Mongolian oak (*Quercus mongolica* var. liatungensis (Koidz.) Nakai) forest in 2010 with two patterns (aggregated and dispersed) and two levels of retention (15% and 50%) in 1 ha area [13]. For the maintenance of ecologically healthy forest ecosystems and sustainable forest ecosystem management, Korea Forest Service established a standard in detail for environmentally friendly logging in "Creation and Management of Forest Resources Act" in 2010. After the establishment of the Act, the aggregated retention method is popularly conducted by the local government. However, there are limited studies on the effects of the aggregated retention practices on the ecosystem health and biodiversity [13–18]. Among these studies, Kim [13], Ming [14], and Jeong et al. [16] reported the effects of aggregated retention on vegetation and environmental factors. The aggregated retention area showed lower daily change in micro-environment (transmitted light, temperature, humidity) compared to harvested area due to the remaining canopy, showing fewer effects on plant species diversity and ecosystem stability that the dispersal retention [13,14]. Meanwhile, Jeon et al. [15] studied the response of insect fauna in designated regeneration forests for a baseline of comparative analysis of insect diversity, and Roh et al. [17] and Kim et al. [18] studied the changes of coleopteran insect communities in a green-tree retention forest.

The changes in vegetation caused by forest management practices affect the distribution and abundance of animals including insects, which use the forest as a habitat and food resource. Several studies have reported the negative effects of clearcutting on the distribution of insects, such as saproxylic beetles, carabids, lepidopterans, isopods, and Opiliones [7,11,19–22]. However, clearcutting can increase the abundance of some species adapted to fragmented environments [23].

Among insects, moths are frequently used as bioindicators to evaluate terrestrial ecosystem conditions [24,25]. Most moths can be identified easily, and they live in diverse environments. Some moth species are used as an indicator of environmental changes. Summerville [26] selected the members of Arctiidae and Notodontidae as biodiversity indicators to evaluate habitat disturbance and fragmented landscapes. The abundance of some subfamily members of Arctiidae increased after harvest in Australian rainforest [24]. The abundance of geometrid moths increased during later successional stages, which are characterized by dense vegetation and canopy [27]. Summerville and Crist [28] reviewed that the moth communities respond predictably to forest management practices, outcomes of post-management response are largely driven by the changes in plant community, and significant reductions in moth species richness and changes in community composition correlate with clearcutting.

Moth assemblages have strong temporal occurrence patterns influenced by meteorological factors, host plants, natural enemies, etc. Temporal occurrence patterns of moth assemblages are one of major concerns to forest managers and ecologists because they build high species diversity and larvae of most moths are herbivores taking a role as a pest on forests. Seasonal patterns of moth assemblages have been studied in both larvae and adult stages [29,30]. There are limited studies on the seasonal occurrence of adult moth assemblages [31–33], although there are more studies on the occurrence of larvae moth assemblages including the relation to the weather conditions, quality of foliage, and natural enemies [29,34–38]. Sanyal [30] analyzed the temporal variation in diversity and species composition of diverse ensembles of moths along an altitudinal gradient in a Western Himalayan landscape, and Sayama et al. [29] showed that the high species turnover of adult moths during the active season was due to the short occurrence period of each species in northern Japan. These seasonal patterns would be influenced by weather condition and available food resources, resulting in the changes of various biological traits of moths such as species-specific host plant and adaptability to temperature [29,39].

Recently, functional traits are increasingly used to understand the mechanisms of biodiversity responses to environmental changes [40,41]. These traits are characteristics of organisms, such as body size and food resources, which are expressed in the phenotype of individual organisms, including morphology, physiology, structure, phenology, and behavior [40,42,43]. The analysis of functional traits can elucidate the response of communities to the changes in environment. Through the evaluation of the effects of current near-to-nature management strategies on the functional diversity of saproxylic beetles in forests, Gossner [44] reported that forest-stand variables do not have a statistically significant effect on the overall functional diversity, but the beetles were significantly affected by the diversity of single functional traits. Choi et al. [45] reported that the seasonal changes in coleopteran functional groups shifted from regulators of primary production to regulators of decomposition, reflecting their available food resource in pine forests. Schmidt and Roland [46] studied the diversity of moths in a fragmented habitat focusing on the importance of functional groups and landscape scale in a boreal forest and reported that there was no change in the overall moth diversity between low and moderately fragmented stands. However, the changes in diversity pattern within functional groups showed that the total diversity measures might mask the changes in community structure.

In the present study, we aimed to characterize the effects of forest management practice on the abundance, species richness, community composition, and functional groups of moths in forests. We tested two hypotheses: (1) moth communities will change in response to the changes in vegetation after the implementation of forest management practices and (2) clearcutting and aggregated retention will increase the abundance and species richness of herbivorous and warm-adapted species.

2. Materials and Methods

2.1. Field Sampling

The present study was conducted in four different forest stands with different forest management practices in Japanese larch and Mongolian oak forests in Mount Nambyeong (1150 m altitude, 37°25′ N, 128°27′ E) in South Korea (Figure 1), which is adjacent to Mount Gariwang (1560 m altitude). This area is a national forest, and public access and usage are controlled by regional forest service. The forests in the mountains consist of natural broad-leaved tree stands dominated by *Quercus mongolica* and coniferous stands with *Pinus densiflora* Siebold & Zucc., *Larix kaempferi*, *Abies nephrolepis* (Trautv. ex Maxim.) Maxim., *Abies holophylla* Maxim., and *Taxus cuspidate* var. caespitosa (Nakai) Q.L. Wang [47]. Various forest management practices, including clearcutting and tree planting, have been conducted in Nambyeong and Gariwang mountains.

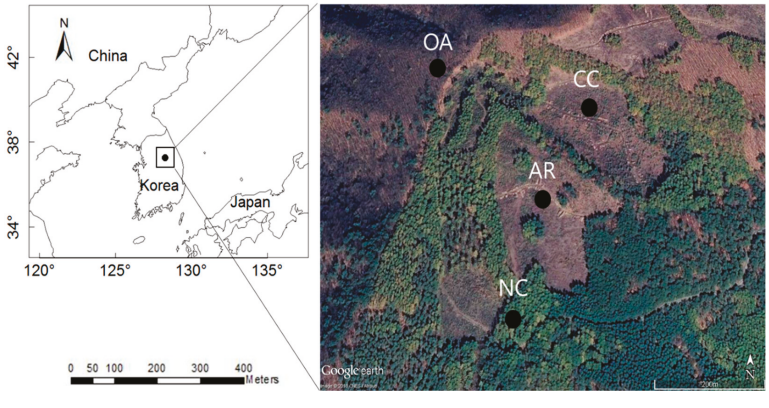

Figure 1. Location of the study sites in South Korea. CC: clearcutting area; AR: aggregated retention area; NC: no cutting area; OA: Mongolian oak area.

Japanese larches were planted in the study area (excluding the area of Mongolian oak stand) in the 1970s. Clearcutting and aggregated retention as forest management practices were carried out in two different Japanese larch stands (5 ha each) in the study area in 2015 (Figure 1) to investigate the effects of forest management practices on the biodiversity of forests. Aggregated retention was conducted by leaving three patches of diameter 40 m (0.13 ha) with 60 m between patches in an area of 5 ha. Only herbs and small shrubs including Nepalese smartweed (*Persicaria nepalensis*) and silvervine (*Actinidia polygama*) developed as vegetation in these stands after clearcutting and aggregated retention (Table 1).

Table 1. Characteristics of environmental factors at each study site.

Study Site (Stand)	Acronym	Altitude (m)	Area (ha)	Dominant Vegetation
Clearcutting	CC	980	5	*Actinidia polygama*, *Persicaria nepalensis*
Aggregated retention	AR	950	5	*Magnolia sieboldii* K. Koch, *Actinidia polygama*, *Persicaria nepalensis*,
Japanese larch (no cutting)	NC	910	5	*Larix kaempferi*, *Acer pseudosieboldianum* var. ambiguum Nakai, *Commelina communis* L., *Actinidia polygama*
Mongolian oak (no cutting)	OA	950	5	*Quercus dentate* Thunb., *Actinidia polygama*, *Ainsliaea acerifolia* var. *subapoda* Nakai

Moths were collected in four different forest stands: three stands (clearcutting: CC, aggregated retention: AR, and no cutting: NC) in a planted Japanese larch forest and one stand in a natural Mongolian oak forest (OA). There was no forest management practice in OA area. The study area is located 910–980 m above sea level (Table 1, Figure 1). NC was used as a control treatment against CC and AR treatments. We collected the moths every month from May to October in 2016 using bucket light traps with black light powered by a battery (12 V, 8 W) in each study stand. The traps were installed for 3 h after sunset, and the function of the light traps was automatically controlled using a timer. Most moth specimens were identified to the species level by a moth expert, whereas micro-moths (microlepidopterans) were identified to higher taxa level, because of the difficulty in identification due to their small body size.

2.2. Functional Groups

We characterized the differences in functional groups among the four study stands and seasons. The functional traits were divided in two categories: the type of host plant as food resource and type of distribution region [48,49]. With respect to the type of host plants, the moths were assigned to one of the plant types (i.e., herbs, shrubs, trees, and lichens) based on the host plant of the larval stage. The distribution region included three groups (i.e., north, south, and others) based on the geographical distribution of moth species in East Asia including Korea and Japan. Northern species inhabit relatively cold northern areas, whereas, southern species inhabit relatively warm southern areas. Others group includes species habiting both southern and northern areas.

2.3. Data Analyses

We compared the community indices including species richness, abundance, and Shannon diversity index (natural logarithm transformed) among four different stands. The species rank abundance curve was plotted to present the relative species abundance to overcome the shortcomings of biodiversity indices that cannot display the relative roles of different variables [50].

We used the hierarchical cluster analysis [51] to classify the samples based on the similarities in moth communities among the study stands by Ward's linkage method with Bray-Curtis distance measure [52]. Ward's linkage method reflects the similarities between two clusters based on the increase in the error sum of squares (ESS) when the two clusters are combined. It is robust to noise and

outliers and is more appropriate for quantitative variables than for binary variables [53]. Bray-Curtis distance is a widely used for distance measure, as it is easy to handle ecological data with a large proportion of zeroes [54]. The abundance of each species was transformed with natural logarithm to reduce variations among species. One was added to the abundance value to avoid log 0 prior to log-transformation. The data obtained in October were used only to evaluate community indices, and they were excluded in the multivariate analysis because only limited species were collected in October. The same dataset was used for non-metric multidimensional scaling (NMDS) to characterize the effects of forest management practice on moth assemblages. NMDS reduces the number of dimensions to two or three dimensions that can be easily visualized and interpreted. Unlike other ordination techniques that rely on distances for ordination, NMDS is a flexible technique that enables the handling of a variety of data types [55]. The proportion of both abundance and species richness of functional groups is presented as biplot in the NMDS ordination of moth assemblages. The cluster analysis and NMDS were carried out using the package vegan [56] in R (https://cran.r-project.org). The biplot in the NMDS with functional groups was fitted using the function envfit in the vegan package.

3. Results

3.1. Differences in Community Composition

We collected 2358 individuals representing 301 moth species belonging to 13 families from four different stands. Three community indices (species richness, abundance, and Shannon diversity index) were higher in the no cutting areas (the NC and OA stands) than in the forest management practice areas (the CC and AR stands), and they presented the highest value in July (Table 2). Among the four treatment stands, the OA stand exhibited the highest species richness and abundance with 201 species and 1444 individuals, respectively, followed by the NC stand with 108 species and 383 individuals, whereas the CC and AR stands presented low species richness and abundance (Table 2, Table S1). Furthermore, Shannon diversity index showed a pattern similar to that of species richness and abundance.

Table 2. Community indices of the moths in four different treatment stands at each sampling month.

Community Index	Treatment	May	June	July	August	September	October	Total
Species richness	CC [a]	8	20	32	21	24	0	86
	AR	2	16	28	17	22	0	67
	NC	9	36	33	24	30	2	108
	OA	13	57	87	54	44	4	201
	Overall	24	84	122	85	74	6	301
Abundance	CC	8	40	96	71	86	0	301
	AR	3	52	67	52	56	0	230
	NC	24	135	91	74	57	2	383
	OA	36	418	576	171	232	11	1444
	Overall	71	645	830	368	431	13	2358
Shannon diversity	CC	2.08	2.46	2.57	2.01	2.53	0.00	3.19
	AR	0.64	2.14	2.55	1.76	2.33	0.00	2.95
	NC	1.90	2.89	2.96	2.23	3.05	0.69	3.69
	OA	1.93	2.64	3.1	2.87	2.83	1.16	3.65
	Overall	2.56	2.95	3.24	2.86	3.20	1.52	3.84

[a] CC: Clearcutting area; AR: Aggregated retention area; NC: No cutting area; OA: Mongolian oak area.

The species rank abundance curve elucidated the effects of forest management practices on moth communities (Figure 2). The curve of the OA stand was on the right with gentle slopes, whereas those of the CC and AR stands were on the left with steep slopes. The curve of the NC stand was in the middle of the OA and CC stands, with a gentle slope.

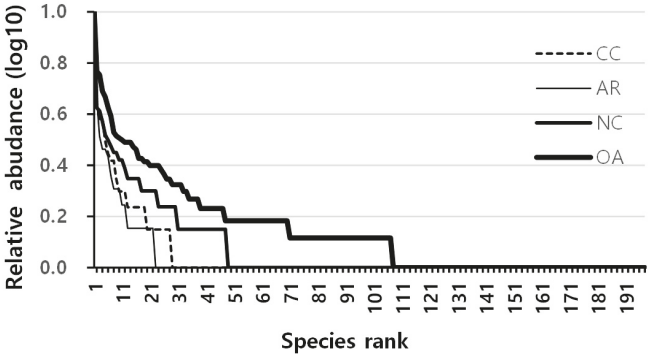

Figure 2. Species rank abundance curve of moths collected from four different treatment stands. CC: clearcutting area; AR: aggregated retention area; NC: no cutting area; OA: Mongolian oak area.

The composition of moth assemblages was different among the four stands. The most abundant family was Noctuidae in the CC stand, Geometridae in the AR and NC stands, and Erebidae and Geometridae in the OA stand (Figure 3). The abundance of Noctuidae gradually increased in all the four stands from May to September, and decreased in October, with the highest value in September. The members of Erebidae were abundant in July, whereas, the geometrids were abundant in June.

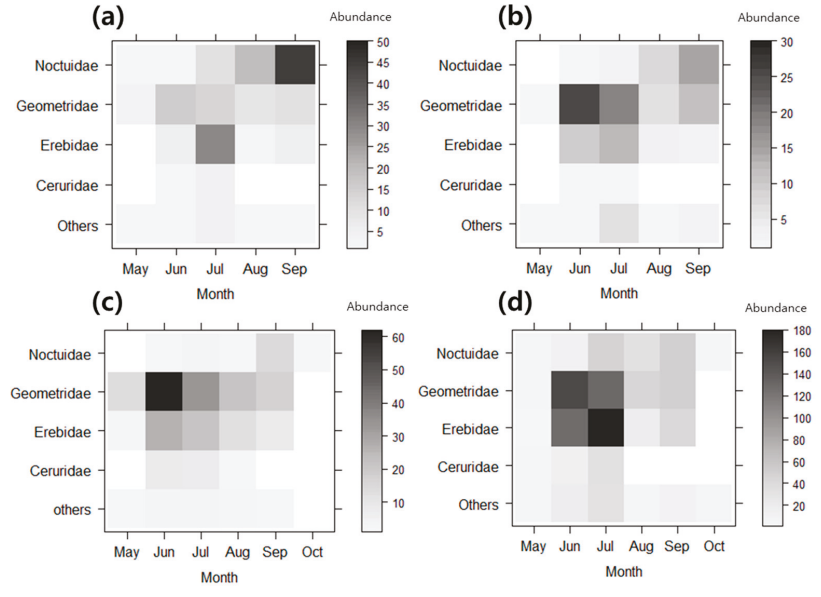

Figure 3. Abundance of the dominant family of species in the four treatment stands at different sampling months. (**a**) Clearcutting area, (**b**) aggregated retention area, (**c**) no cutting area, and (**d**) Mongolian oak area.

The occurrence of dominant species was different among the four stands. The species *Xestia fuscostigma* (Bremer), *Athetis gluteosa* (Treitschke), and *Sineugraphe exusta* (Butler) of the family Noctuidae accounted for 20.6% of the total abundance in the CC stand (Table 3). *Xestia fuscostigma* and *A. gluteosa*, which exhibit host plant preference, such as herbs for food resource, were collected only in the CC and AR stands. *Sineugraphe exusta* was collected mostly in the CC stand. The species

Idaea biselata (Hüfnagel) and *Eustroma melancholicum* (Butler) of the family Geometridae were collected only in the AR and OA stands, respectively. *Alcis angulifera* (Butler), which has a wide range of host plant preference, was abundant in the NC stand. *Hydrillodes pacifica* Owada, which belongs to the family Erebidae, was the dominant species in the OA stand. *Zanclognatha griselda* (Butler) was collected only in the NC stand, and *Duliophyle agitata* (Butler) was relatively abundant in the NC stand. *Paracolax tristalis* (Fabricius) was the dominant species in the CC stand, although it was abundant in all the stands.

Table 3. Biological trait of dominant moth species in four different treatment stands.

Family	Species	Dominant Species [a]				Biological Trait	
		CC [b]	AR	NC	OA	Host Plant [c]	Distribution Pattern [d]
Noctuidae	*Xestia fuscostigma*	2	5			HR	M
	Athetis gluteosa	3				HR	N
	Sineugraphe exusta	4				Unknown	N
Geometridae	*Idaea biselata*	5	1		3	HR/TS	M
	Eustroma melancholicum		2			SR	M
	Alcis angulifera		4	1	4	HR/SR/TS/LC	M
	Duliophyle agitata			4		SR/TS	M
Erebidae	*Paracolax tristalis*	1	3	5	2	SR/TS	N
	Hydrillodes pacifica			2	1	Unknown	S
	Zanclognatha Griselda			3		TS	N
	Zanclognatha lunalis (Scopoil)				5	SR/TS	N

[a] Dominant species ranking from first to fifth: 1 stands for the most dominant and 5 for the least one. [b] CC: Clearcutting area; AR: Aggregated retention area; NC: No cutting area; OA: Mongolian oak area. [c] HR = herbs SR = shrubs TS = trees LC = lichens. [d] M = miscellaneous, N = northern, S = southern.

3.2. Functional Groups

The composition of functional groups was different among the four stands (Figures 4 and 5). Both species richness and abundance of groups feeding on herbs were high in the CC and AR stands, whereas those of the groups feeding on trees were high in the NC and OA stands (Figure 4). In addition, they were higher in the AR stand than in the CC stand. The moth species feeding on lichens were also higher in NC areas than in the forest management areas, although their overall proportion was low. The proportion of species richness inhabiting the northern region was the highest in the OA stand, whereas, that of species inhabiting the southern region was high in the CC and AR stands (Figure 5).

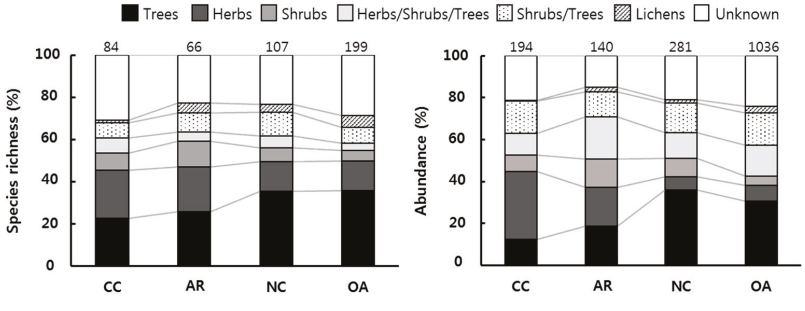

Figure 4. Proportion of species richness and abundance of the functional groups based on the type of host plants. The numbers on the bar indicate species richness (number of species) and abundance (number of individuals) in the corresponding treatment. CC: clearcutting area; AR: aggregated retention area; NC: no cutting area; OA: Mongolian oak area.

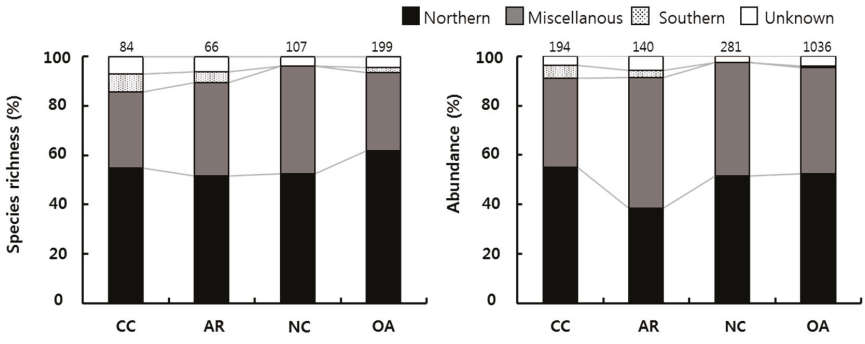

Figure 5. Proportion of species richness and abundance of the functional groups based on the distribution region. The numbers on the bar indicate species richness (number of species) and abundance (number of individuals) in the corresponding treatment. CC: clearcutting area; AR: aggregated retention area; NC: no cutting area; OA: Mongolian oak area.

3.3. Forest Management Practices and Functional Groups

The cluster analysis revealed the differences in moth communities in the four stands (Figure 6a), reflecting the effects of forest management practice. The CC and AR stands were grouped together, whereas the OA and NC stands formed another group. This was also reflected in the NMDS ordination (Figure 6b,c). The CC and AR stands located on the left side in the ordination map, whereas the OA and NC stands were on the right side. The CC stand was characterized by groups feeding on herbs, the AR stand was characterized by groups feeding on herbs, shrubs, and trees, the OA stand was characterized by groups feeding on shrubs, trees and lichens, and the NC stand was characterized by groups feeding on trees (Figure 6b). Meanwhile, the species inhabiting the southern region were prevalent in the CC stand, whereas, species inhabiting the northern region were prevent in the OA stand. This relationship was more pronounced in richness within the distribution types (Figure 6c).

Additionally, the monthly samples were classified into four clusters (1–4) based on similarities in moth assemblages, reflecting mainly the seasonality of moth communities (Figure 7a). Cluster 1 represented samples for May, cluster 2 for June, cluster 3 for July, and cluster 4 for August and September. However, the effects of forest management practices were reflected in each cluster. For example, in cluster 4, the samples from the OA and NC stands were grouped together, whereas, the samples from the CC and AR stands were grouped together. Similar patterns were observed in other clusters.

The NMDS ordination also elucidated similar patterns in the cluster analysis, presenting the seasonality of moth assemblages and the effects of forest management practices (Figure 7b,c). Cluster 1 was on the left side in the NMDS ordination, whereas cluster 3 and 4 were on the right side. Clusters 2 was in the upper middle area. The abundance of functional groups of species feeding on shrubs and trees were represented in cluster 1 and their richness in cluster 2. The assemblages associated with groups feeding on trees, in particular, the samples from the OA stand, were mainly in cluster 2, and the assemblages associated with groups feeding on herbs were in cluster 4 consisting of the samples from the CC and AR stands (Figure S1). The functional groups based on the distribution region reflected the temperature preference of species. Clusters 1 representing samples collected in May (early season) were characterized by the species inhabiting the northern regions, whereas, cluster 4 representing samples collected in August and September, in particular, the samples from the CC and AR stands, were characterized by the species inhabiting the southern regions.

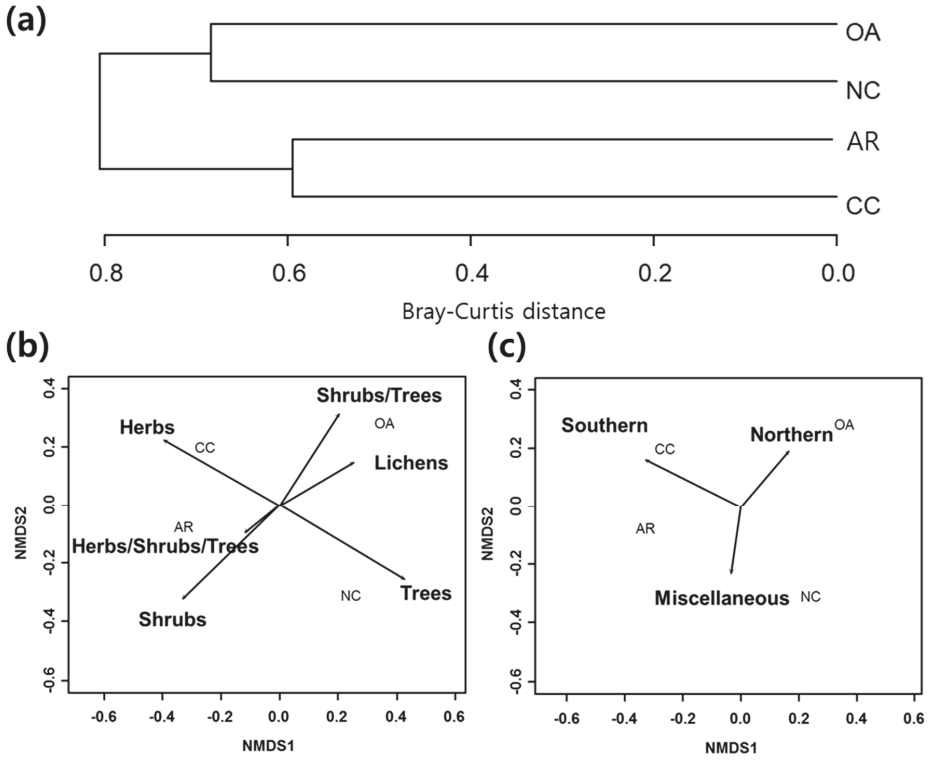

Figure 6. (**a**) Cluster analysis of the four treatment stands based on the abundance of moth communities using Ward's linkage methods with Bray-Curtis distance measure. (**b**,**c**) Ordination of non-metric multidimensional scaling (NMDS) using the same data used in the cluster analysis (stress value for the first two axes = 0.01). The abundance proportion of each functional group for host plants (**b**) and distribution patterns (**c**) is presented in the NMDS. CC: clearcutting area; AR: aggregated retention area; NC: no cutting area; OA: Mongolian oak area.

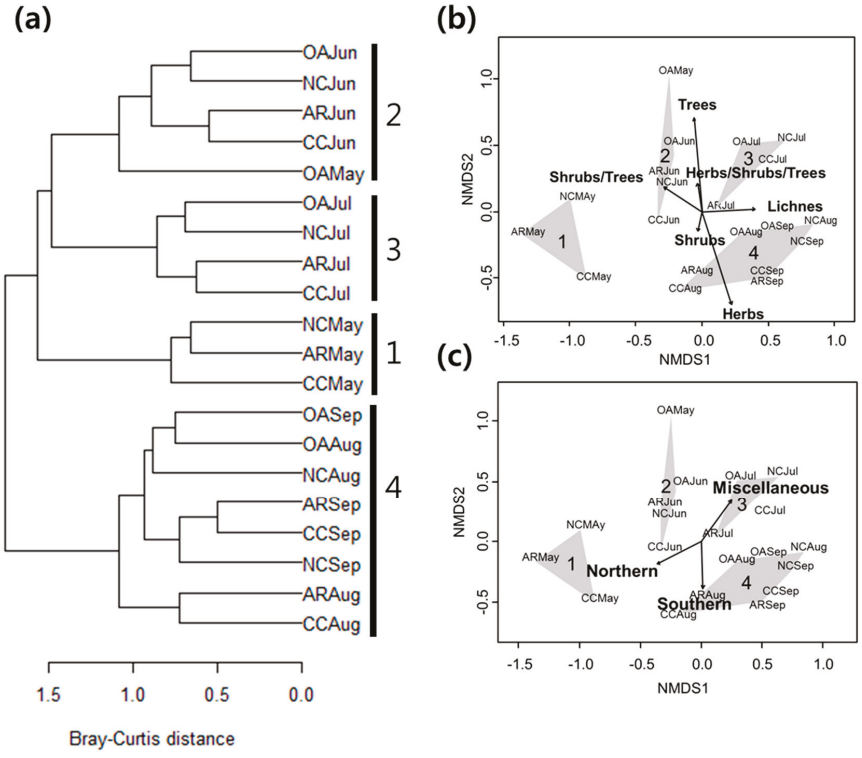

Figure 7. (**a**) Cluster analysis of species abundance of moths in the monthly samples using Bray-Curtis distance and Ward's linkage method. (**b**,**c**) Ordination of NMDS using the same data used in the cluster analysis (stress value for the first two axes = 0.15). The abundance proportion of each functional group for host plants (**b**) and distribution patterns (**c**) is presented in the NMDS. The first two characters in sample names indicate study stands, and the last three characters are sampling months.

4. Discussion

4.1. Changes in Community Structure and Function Due to the Forest Management Practices

The results of the present study revealed that the species richness, abundance, and Shannon diversity index of moth communities changed due to the forest management practices. The community indices decreased in the forest management areas compared with NC areas. This is consistent with the findings of earlier studies, that is, moths exhibit high diversity in unmanaged forests than in managed forests [57,58]. In the present study, the NC area presented a complex diversity in plant layers. Willott [58] reported that the composition of species in the understory and canopy of a primary forest was different, with high diversity in the understory. High plant species diversity reflects the heterogeneity of habitat [59,60], providing various habitat conditions for feeding, drying of wings, resting, and breeding of lepidopterans [26,61]. Meanwhile, the forest management practices create a homogeneous habitat, inducing changes in the abiotic factors, such as sunlight exposure, humidity, and temperature [7], and the stand returns to the initial stage of succession. Therefore, it is covered with herbaceous plants [62], which increases species feeding on herbaceous plants. In the present study, the functional groups of moths revealed the effects of forest management practices. The percent of moths feeding on herbs was high in the forest management areas (CC and AR), whereas the percent of moths feeding on lichens and trees was high in the no cutting areas (NC and OA) (Figure 4).

Furthermore, the abundance of warm-adapted species (i.e., the species distributed in the southern area) was higher in the forest management areas, with higher light exposure, than in the NC areas (Figure 5).

Furthermore, the functional groups of moths exhibited temporal variability. The functional groups of species feeding on herbs, shrubs, and trees were abundant in June, July, and August, respectively (Figure 7), suggesting that the functional group of moths, which are known to prefer young leaves, is closely related to the leafing period [34,63]. Murakami et al. [64] reported that species diversity in the lepidopteran larval communities that feed on trees was higher during summer than during spring. Choi et al. [45] presented that the herbivores among functional groups of Coleoptera in the pine forest were high in July. The results of the present study supported these findings, suggesting that the phenology of dominant host plants coincided with the abundance of moths. For example, *Actinidia polygama* (a shrub) leaves in June and *Persicaria nepalensis* (an herb), leaves and blooms in August. The dominant trees were the members of Pinaceae, including *Larix kaempferi* that buds in mid-May to early June and grows up to July. Therefore, July is a suitable period for moths feeding on these trees.

4.2. Aggregated Retention and Biodiversity

The green-tree retention is the recolonization sources of the postharvest forests, and the aggregated retention provides continuity in structure, function, and composition of ecosystems between forest generations [65]. Therefore, the recovery of biodiversity is faster in the aggregated retention areas than in the clearcutting areas [66]. This concept in term of biodiversity is related to the metapopulation and metacommunity [65]. In our study, overall species richness decreased in the clearcutting and aggregated retention areas compared to NC areas, and it was higher in clearcutting area than in the aggregated retention area. Halaj et al. [67] reported similar results that overall species richness was lower in high-level of retention (40%) than in low-level of retention (15%). This was caused by an increase of species feeding herbs due to the changes in vegetation.

Meanwhile, our study presented that the differences in the moth community reflected the effects of retained trees in the aggregated retention stand. The proportion of tree feeding group in both species richness and abundance was higher in the AR stand than in the CC stand although the difference was small (Figure 4). The CC stand was characterized by species feeding herb, whereas AR was by the species feeding on herbs, shrubs, trees, and shrubs (Figure 6), supporting the idea that the green-tree retention is the recolonization sources of the postharvest forests.

Richness of forest species, such as species feeding on trees, has a positive relationship with the proportion of retained trees [66]. Forest species richness was higher in the no treatment areas (NC and OA) than in the treatment areas (CC and AR), and it was higher in the AR stand than in the CC stand. The proportion of retention area was 7.5% in this study. Therefore, we could expect more species richness with an increase of the level of retention, enhancing the recolonization of the postharvest forest. However, a threshold of the proportion of retained trees to satisfy all groups of species was not clearly identified [66,67] because different species have different habitat preference and tolerance ranges.

The patterns of retention also influence the regeneration of postharvest forest [66], although it was not considered in this study. In addition, we did not have replication for each treatment, and it might have affected on our results. However, our results are not strongly different from literature, showing the decrease of species diversity and changes of community composition in clearcutting and aggregated retention stands. There are limited studies on the effects of the forest management practices in particular aggregated retention on biodiversity and forest ecosystems in Asian countries including Korea. Our research is the first study on the effects of aggregated retention on the moth communities, especially in Korea, although there are some limitations in this study. In the further study, therefore, integrated studies are expected to be conducted by considering the levels of retention, the patterns of retention, vegetation, and multitaxa with several replications.

4.3. Long-Term Effects of Forest Management Practice

We evaluated the effects of forest management practices, which act on forest ecosystems as an intervention. After the implementation of forest management practices, it takes a long time for the forest to return to its natural condition, following the recovery successional process. Axmacher et al. [68] showed the differences in moth communities according to environmental conditions, such as food resources, elevation, biogeographical and historical condition, and forest types. The results of the present study showed the short-term effects of forest management practice on moth communities after cutting the trees. However, we did not evaluate the long-term responses of moth communities according to the successional stages. The community composition of moths might be dependent on the vegetation, which acts as food resource for moths.

After cutting the trees in the study areas, the vegetation might have changed from grasses and shrubs to trees. Guariguata and Ostertag [69] reported that grasses and shrubs dominated the first decade of forest succession after intervention. Subsequently, long-lived, tall-statured species and tree species were dominant with the characteristics of old-growth forest. We expect that the vegetation in the forest management areas might be similar to that of the OA stand, which represents the natural condition, through the secondary forest successional process. These changes in vegetation can induce changes in the community composition of moths, which inhabit the study areas. Therefore, long-term studies can elucidate the effects of forest management practices on the structure and function of moth communities by comparing the differences in the responses of moths in the successional process of forests.

5. Conclusions

The results revealed that the moth communities changed in response to the changes in vegetation after the implementation of forest management practices. Clearcutting and aggregated retention decreased overall species diversity of moths, but increased diversity of herbivorous and warm-adapted species. The results supported both the hypotheses tested in the present study. The structure and function of moth communities were affected by the forest management practices, reflected by a decrease in community indices and changes in moth community composition. Clearcutting increased the diversity of herbivorous species and warm-adapted species.

Supplementary Materials: The following are available online at http://www.mdpi.com/1999-4907/9/9/574/s1, Table S1: A list of species and their abundance at each study site. Figure S1: Percent of species richness and abundance of each functional group based on the host plants at each site. The first two characters in sample names indicate study stands, and the last three characters are sampling months. Aggregated retention was differentiated from the clearcutting in the composition of functional groups in the moth communities, reflecting the differences of forest management practice techniques.

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Widespread Distribution of *Trypodendron laeve* in the Carpathian Mountains (Romania)

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Abstract: *Trypodendron laeve* Eggers, 1939 is a species of ambrosia beetle much less known than the other three *Trypodendron* species occurring in Europe. Its status (native or alien) in Central Europe has been a subject of debate over the past two decades. In Romania, the species was discovered in 2008 and the aim of the research presented in this paper was to investigate its distribution in the Carpathians, mainly at high altitudes (>800 m), in tree stands with Norway spruce (*Picea abies* [L.] H. Karst). Panel intercept traps baited with synthetic pheromone for *Trypodendron lineatum* (Olivier, 1795) were used in the spring of 2015, at 31 locations. Adults of *T. laeve* were caught in 20 of them. Additional observations were made within some studies using similar baits and *T. laeve* specimens were caught in eight locations. *T. laeve* was always trapped together with *T. lineatum*, and at some locations also together with *T. domesticum* (Linnaeus, 1758) and *T. signatum* (Fabricius, 1787). In all traps, fewer specimens of *T. laeve* were caught compared to *T. lineatum*. The species has a widespread distribution in the mountain regions, within forests composed of native tree species and generally located far away from commercial routes. There, it occurs together with other native species of the same taxonomic genus. It seems to be more abundant at high altitudes, but overall its populations are less abundant than those of *T. lineatum*.

Keywords: *Trypodendron laeve*; Carpathian Mountains; Romania; distribution

1. Introduction

The species of the genus *Trypodendron* Stephens, 1830 are ambrosia beetles, which make breeding galleries in wood, but feed on symbiotic fungi [1]. They infest the sapwood of weak, dying, and newly dead trees, logs, and stumps [2]. The beetles locate the suitable hosts using the ethanol released from wood undergoing anaerobic fermentation as a guiding cue [3]. However, they are able to distinguish broadleaved species from conifers. *Trypodendron* species living on conifers use the monoterpenes, mainly α-pinene, to recognize their hosts, the ethanol and α-pinene acting synergistically in attracting the beetles [4–6]. Then, the beetles aggregate by means of aggregation pheromones [7,8]. In fact, conifer living species use lineatin along with the two host volatiles as a very effective aggregation

signal [9,10], while the adults of *T. domesticum* appear to be repelled by terpenes [5,11]. The tunnels excavated by insects and fungal staining can cause important economic loss through the degradation of the infested logs. In addition, some ambrosia beetle species attack apparently healthy trees [12] and consequently are important economic pests [13].

According to the latest reference work on bark beetles, the Holarctic region contains 13 species of *Trypodendron* [14], with four in Europe including *T. domesticum* (Linnaeus, 1758), *T. signatum* (Fabricius, 1787), *T. lineatum* (Olivier, 1795), and *T. laeve* Eggers, 1939 [15]. The first two species occur on broadleaved trees, with the second one seeming to prefer the oaks (*Quercus* spp.) [2]. In the Palaearctic region, *T. lineatum* was found on many species of *Picea* A. Dietr., 1824, *Pinus* L. 1753, *Abies* Mill. 1754, *Larix* Mill. 1754, and *Cedrus* Trew 1757 [16], while in North America, it is also occasionally found attacking species of *Alnus* Mill., *Betula* L. 1753, *Acer* L. 1753, and *Malus* Mill. 1754 [13,17]. The host tree species of *T. laeve* are less known. It was found in logs or dead trees of *Picea abies* (L.) H. Karst. 1881 [18–20] and *Pinus sylvestris* L. 1753 [20,21]. *Picea obovata* Ledeb. 1833 and *P. jezoensis* (Siebold & Zucc.) Carr. are also between its hosts [16].

While *T. lineatum* and the European species associated with broadleaved trees are quite well studied, *T. laeve* is less known, because its taxonomic status was only recently clarified. It was firstly described in Japan [22] and secondly (a few years later) in Norway, under the name *Trypodendron piceum* A. Strand, 1946 [18]. However, during the next four decades, the species was not reported in other parts of Europe. The standard taxonomic literature and identification keys used during that time, such as Balachowsky [23], Stark [24], Nunberg [25], and Pfeffer [26], did not include *T. laeve* or *T. piceum*. Consequently, *T. laeve* was "forgotten" or overlooked, particularly by applied entomologists. Later, Grüne [27] did not refer to *T. laeve* or *T. piceum* at all in the illustrated key of the European scolytids, and Schedl [28] regarded both names as synonyms for *T. lineatum*. Even Annila et al. [29] did not distinguish between *T. piceum* and *T. lineatum* [30], although it was known that the species was present in the Nordic countries.

Eventually, Holzschuh [19], comparing some specimens captured in 1982–1983 that looked different from those of *T. lineatum*, with specimens from the Schedl's collection of scolytids, identified those specimens as belonging to *T. laeve* and drew attention to the fact that they differ from *T. lineatum* both in the conformation of male genitalia and in the femur colour, suggesting the treatment of *T. laeve* as a distinct species. Pfeffer [31] mistakenly synonymised *T. piceum* Strand with *T. proximum* (Niijima, 1909), but it was corrected in Pfeffer [16,32], where he presents *T. laeve* as a separate species and the name *T. piceum* as a synonym for *T. laeve*, as Wood [33] suggested. Due to the original mistake in Pfeffer [31], *T. laeve* (=*T. piceum*) was mentioned by Martikainen [20] under the name *T. proximum*, but Mandelshtam and Popovichev [34] demonstrated that they are different species. This clarifies the taxonomic status of the *T. laeve* species in Europe, but not its origin.

In the 1980s, in Austria, various species of Siberian insects were reported, and Holzschuh [19] assumed that *T. laeve* is also an alien species that came to Europe with imported wood from Russia. The idea was reiterated in later works [35–37] and some authors mentioned this species as an invasive one in Europe [38,39]. However, the information gathered until the year 2000 about the distribution of the species in Fennoscandia led Martikainen [20] to reconsider the status of the species. Consequently, Kenis [38] regarded the species to be native to Scandinavia but alien to central Europe, where its distribution was less known. Apart from the above-mentioned reports from Austria, there were few reports from Germany [40] and the Czech Republic [41,42].

Currently, the species is also reported as being present in Estonia, Finland, Latvia, Norway, Poland, Slovakia, Sweden, Switzerland, the Central European Territory and North European Territory of Russia, China, and Japan [15], as well as from Romania [43] and the Russian Far East [44]. However, while the species distribution in the Fennoscandia is well documented [20,21,30,45–51], in Central and Eastern Europe, the knowledge is quite poor, although some studies have been done [43,52,53]. Consequently, the objective of the research presented in this paper was to determine the species distribution in Romania.

2. Materials and Methods

2.1. Field Research

To determine the species distribution of *T. laeve* in Romania, in the spring of 2015, with the help of field forestry personnel, 78 pheromone traps were placed in 31 locations situated along the Carpathian Mountains chain (Table 1).

Table 1. Location of study sites and tree stand characteristics where pheromone traps were set up.

No.	Location, County	Forest District, Production Unit, Compartment	Coordinates N/E	Elevation (m)/Aspect	Forest Composition (%)	Forest Age (Years)
Trap locations for detection of *Trypodendon laeve* in 2015						
1.	Vișeul de Sus, Maramureș	Vișeu, VI Mira, 9C	47.7227333 24.7672555	1520 NW	100 Pa	160
2.	Paltinu, Suceava	Vama, II Paltinu, 87A	47.6236667 25.4564722	1180–1250 E	100 Pa	80
3.	Cacica, Suceava	Solca, II Cacica, 9B	47.6322889 25.9207638	460 E	40 Aa 40 Pa 20 Fs	120
4.	Cârlibaba, Suceava	Cârlibaba, VI Cârlibaba, 129A	47.5716028 25.1482083	1100 NW	100 Pa	80
5.	Lunca Ilvei, Bistrița-Năsăud	Lunca Ilvei, I Lunca Ilvei, 150	47.3729028 25.0417055	1070 SW	60 Pa 30 Fs 10 Aa	120
6.	Almaș, Neamț	Gârcina, III Almaș, 28B	47.0176806 26.3233083	650 NE	40 Fs 20 Aa 20 Pa 20 Ap	55
7.	Stânceni, Mureș	Lunca Bradului, Trupul Gudea, 132B	46.864583 25.245783	1144 W	80 Pa 10 Aa 10 Fs	115
8.	Brateș_3, Neamț	Tarcău, VII Ața, 54A	46.7954444 26.0342027	983 NW	60 Pa 30 Aa 10 Fs	150
9.	Brateș_1, Neamț	Tarcău, V Bolovăniș, 179A	46.7748000 26.1507416	580 NE	60 Pa 30 Aa 10 Dt	80
10.	Brateș_2, Neamț	Tarcău, VI Brateș, 179A	46.7554583 26.0735944	818 NW	70 Pa 30 Aa	110
11.	Dărmănești, Bacău	Dărmănești, III Dărmănești, 79B	46.3614333 26.3454722	1020 E	50 Fs 30 Pa 20 Dt	100
12.	Sălătruc, Bacău	Lignum, UBII Lapos, 101, 102	46.351275 26.398533	820 E	50 Fs 30 Aa 20 Pa	100
13.	Poiana Uzul, Bacău	Dărmănești, III Bârzăuța, 54A	46.2393833 26.3076777	1160 N	90 Pa 10 Dr	100
14.	Soveja, Vrancea	Soveja, II Soveja, 64C	45.9973083 26.6106000	810 SE	80 Pa 20 Aa	75
15.	Covasna, Covasna	Comandău, IV Obârșia Bâscii, 99B,C	45.8171028 26.3339861	1340 N	100 Pa	30–130
16.	Brădăcești, Vrancea	Zăbala-Nereju, I Bârsești, 124	45.7158333 26.6588888	1060	60 Fs 40 Pa	110
17.	Predeal, Brașov	RPLP Kronstadt, III Postăvaru, 133A	45.5023472 25.6298277	1345 N	100 Pa	110
18.	Poiana Brașov, Brașov	RPLP Kronstadt, VI Tâmpa, 38A	45.5970556 25.5668472	1096 NW	40 Pa 30 Aa 30 Ld	110
19.	Moroeni, Dâmbovița	A.O.S. Carpathia, III Raciu, 56C	45.2785944 25.3500000	1200 NE	100 Pa	80
20.	Bădeni, Argeș	A.O.S. Carpathia, VII Bădeanca, 100A	45.3062000 25.2803972	1420 SW	90 Pa 10 Fs	90
21.	Căpățâneni, Argeș	Vidraru, VI Limpedea, 42B, 43B	45.3556778 24.6877555	1520 S/SW	100 Pa	125
22.	Cârțișoara, Sibiu	Arpaș, V Bâlea, 14H	45.6500000 24.6119444	1503 W	100 Pa	100
23.	Paltin, Sibiu	Sibiu, I Dealul Paltinului, 34G	45.5313889 24.1694444	1494 NE	100 Pa	120
24.	Bistra, Sibiu	Miercurea Sibiului, III Miercurea Sibiului, 164B	45.6716667 23.6638888	1490 N	100 Pa	100

Table 1. *Cont.*

No.	Location, County	Forest District, Production Unit, Compartment	Coordinates N/E	Elevation (m)/Aspect	Forest Composition (%)	Forest Age (Years)
25.	Jieț, Hunedoara	Petroșani, V Jieț, 62A	45.4090278 23.5594361	1205 SE	60 Fs 40 Pa	150
26.	Cugir, Alba	Cugir, Valea Bosorog-Parva, 130A,B	45.6228889 23.4930194	1400 E/SE	100 Pa	95
27.	Poiana Mărului, Caraș-Severin	Oțelu Roșu, VI Obârșia Bistrei Mărului, 66A/66B/69B	45.3493333 22.6219722	1215 NW/NE/N	90 Pa 10 Aa/100 Pa/100 Pa	90/130/130
28.	Rusca Montană, Caraș-Severin	Rusca Montană, V Rusca Montană, 138A, 139	45.6071111 22.4974722	802 N/N	70 Pa 30 Fs 60 Pa40Fs	110 110
29.	Padiș_2, Bihor	Beliș, II Ponor, 69A	46.6338028 22.7409138	1135 N	100 Pa	115
30.	Doda Pilii, Cluj	Beliș, II Ponor, 143A	46.657875 22.7680111	1280 SE	100 Pa	160
31.	Padiș_1, Bihor	Beliș, II Ponor, 128A	46.650975 22.7502583	1440 NE	70 Pa 30 Fs	110
Trap location for other studies with synthetic pheromone of *Trypodendron lineatum*						
32.	Bobeica, Suceava	Cârlibaba, VII Buhăiescu, 49I	47.7132194 25.0763333	1200 S	100 Pa	115
33.	Putna_1, Suceava	Putna, II Putnișoara, 105A	47.827604 25.607495	650 SE	70 Pa 10 Aa 20 Fs	110
34.	Putna_2, Suceava	Putna, II Putnișoara, 121A	47.799167 25.603056	750 E	40 Pa 20 Aa 30 Fs 10 Ap	90
35.	Putna_3, Suceava	Putna, II Putnișoara, 131A	47.792221 25.611334	850 V	40 Pa 20 Aa 40 Fs	45
36.	Demacușa, Suceava	Tomnatic, I Demacușa, 50G	47.6701575 25.4389286	890–1000 NE	60 Pa 20 Aa 20 Fs	80
37.	Ciumârna, Suceava	Vama, III Dragoșa, 344A	47.6943417 25.5870722	800 SE	60 Pa 20 Aa 20 Fs	110
38.	Iacobeni, Suceava	Iacobeni, U.P. VI Botoș—Orata, 5A	47.4114722 25.3118611	835–1115 W	100 Pa	100
39.	Borca, Neamț	Borca, II Borca, 73A	47.1216389 25.7174388	1010 N	100 Pa	40

Aa—*Abies alba*, Ap—*Acer pseudoplatanus*, Fs—*Fagus sylvatica*, Ld—*Larix decidua*, Pa—*Picea abies*, Dr—Other conifers, Dt—Other broadleaved trees.

Trapping locations were chosen to be—generally—at altitudes above 800 m, within pure Norway spruce (*Picea abies*) stands or mixed stands of spruce and other native species, mainly silver fir (*Abies alba*), European beech (*Fagus sylvatica* L.), sycamore (*Acer pseudoplatanus* L.), and European larch (*Larix decidua*), most of them aged over 75–80 years. The selected sites were tree stands located at 50–100 m from the places where harvested logs were stored in 2014 before transport to a mill or nearby in one-year old clear-cut areas, but far from the main rail or car transport routes and woodworking factories.

At each location, three traps were placed in general, but at some points, there were only one to two traps (Table 2). The pheromone traps were of the Intercept® type. They were set up within the stand, at 10–15 m from the stand edge and at a distance of 50–100 m from each other.

The traps were primed with pheromone lures whose composition was optimized to attract beetles of *T. lineatum*, like in other studies [19,20,35,51,52], because there are no commercial products designed to attract *T. laeve*. The lures contained diluted lineatin in methylbutenol, ethanol, and alpha-pinene. This mixture diffused through a polyethylene film at a rate of 30 mg/day at 20 °C. The pheromone lures and traps were provided by the Research Institute in Chemistry "Raluca Ripan" Cluj-Napoca.

Table 2. Captures of *Trypodendon* species at the 31 sites monitored in 2015.

Site No.	Location	No. of Traps	Monitored Period	Start of Flight	No. Flight Days Lost	$T_{max.aver}$	Total Number of *Trypodendron* Beetles per Study Site				Ratio *T. laeve*/*T. lineatum*
							laeve	*lineatum*	*domesticum*	*signatum*	
1.	Vișeul de Sus	3	21.04–19.05.15	26.04.15	0		43	1832	1	0	1/42.6
2.	Paltinu	3	20.03–16.05.15	12.04.15	0		56	1270	314	0	1/22.5
3.	Cacica	3	03.04–18.05.15	24.03.15	3	15.9	0	63	9	12	
4.	Cârlibaba	3	07.04–25.05.15	26.03.15	1	13.9	217	4464	0	0	1/19.6
5.	Lunca Ilvei	3	26.03–14.05.15	26.03.15	0		3	60	118	0	1/17.0
6.	Almaș	3	04.04–07.05.15	25.03.15	2	14.9	0	474	18	3	
7.	Stânceni	3	15.04–29.05.15	26.03.15	4	14.4	46	4527	13	0	1/89.8
8.	Brateș_3	1	08.04–20.05.15	26.03.15	1	14.6	0	5	6	0	
9.	Brateș_1	1	01.04–12.05.15	24.03.15	4	15.1	0	12	3	2	
10.	Brateș_2	1	01.04–12.05.15	25.03.15	2	14.9	1	130	4	0	1/22.0
11.	Dărmănești	1	30.03–11.05.15	26.03.15	1	13.6	6	51	14	0	1/8.5
12.	Sălătruc	2	30.03–11.05.15	25.03.15	2	14.4	9	181	45	17	1/19.1
13.	Poiana Uzul	3	02.04–07.05.15	11.04.15	0		61	204	41	0	1/3.3
14.	Soveja	3	28.04–02.06.15	26.03.15	13	17.4	0	180	9	29	
15.	Covasna	3	28.04–26.05.15	11.04.15	9	14.2	11	3609	10	0	1/298.3
16.	Brădăcești	3	26.04–31.05.15	11.04.15	8	15.9	0	71	6	0	
17.	Predeal	3	23.05–04.07.15	11.04.15	19	15.5	0	4285	10	0	
18.	Poiana Brașov	3	30.03–11.05.15	26.03.15	1	13.3	178	1387	17	3	1/7.8
19.	Moroeni	3	02.04–21.05.15	11.04.15	0		0	69	5	0	
20.	Bădeni	3	02.04–23.05.15	11.04.15	0		67	722	348	56	1/3.2
21.	Căpățâneni	3	03.05–14.06.15	16.04.15	5	13.8	0	165	5	0	
22.	Cârțișoara	3	02.04–14.05.15	16.04.15	0		68	517	0	0	1/7.4
23.	Paltin	3	02.04–14.05.15	16.04.15	0		6	606	18	0	1/101.0
24.	Bistra	3	01.05–05.06.15	11.04.15	6	13.9	2	365	1	0	1/151.5
25.	Jieț	3	20.04–25.05.15	26.03.15	6	15.7	83	818	67	0	1/7.7
26.	Cugir	3	23.04–08.06.15	11.04.15	4	14.8	1	769	0	0	1/671.0
27.	Poiana Mărului	3	14.04–19.05.15	26.03.15	4	15.9	1	88	12	0	1/68.0
28.	Rusca Montană	3	14.04–19.05.15	25.03.15	7	17.1	0	64	2	0	
29.	Padiș_2	1	07.05–10.06.15	25.03.15	16	16.5	3	262	19	0	1/31.3
30.	Doda Pilii	1	07.05–10.06.15	26.03.15	13	16.1	1	364	12	0	1/364.0
31.	Padiș_1	1	07.05–10.06.15	26.03.15	10	15.6	0	427	22	0	
	Total	78					863	28,041	1149	122	

Since February 2015 and the first ten days of March 2015 were warmer than normal, the installation of the traps in the forest was scheduled for March, so that they would be operational before the maximum daily temperature reached 13 °C, but in the second half of March and the beginning of April, the weather had become very variable, including snowing periods, and in many cases, the installation of traps was postponed for April or even May.

The traps were kept in the field for four to eight weeks and the captures were generally checked weekly, and in some cases every two weeks. The collected insects were preserved in ethanol until their identification.

Additional data on the presence of the *T. laeve* species in other sites was obtained from the processing of biological material captured in eight other studies using the same type of pheromone, and in one case (at Borca, Neamţ county), a flying beetle collected on logs.

2.2. Determining the Date of the Commencement of the Flight and the Number of Missed Flight Days

Given the way in which field traps were installed, for a fair interpretation of the results, it was necessary to indirectly determine whether they were set up before or after the beginning of flight, and the respective number of missed flight-friendly days.

For this purpose, the maximum daily air temperature (T_{max}) values for January–May 2015 have been extracted from the E-OBS data base [54] version 15.0, for the sites where the traps had been installed. The average altitude of the area covered by the grid cell ($0.25° \times 0.25°$) corresponding to each site has been obtained from the same data base.

Then, the maximum daily temperature was corrected for the difference between the elevation of the field location and the mean elevation of the grid cell, taking into account a mean thermal gradient for the maximum daily temperature during the spring of 0.86 °C/100 m for the Eastern Carpathians, 0.92 °C/100 m for the Southern Carpathians, and 0.79 °C/100 m for the Western Carpathians [55].

Daily corrected maximum temperatures ($T_{max.correct}$) were then compared to the thermal threshold (13 °C) at which, according to the data published by Martikainen [20], the flight of *T. laeve* species begins. The first day of the year when this threshold was reached was considered to be the start date of the flight.

If the trap installation was made after that date, the time interval between the start of the flight and the day of the trap installation was considered a delay time, expressed in days of delay. In the period of delay, the maximum daily temperature was often below the thermal threshold and the insects did not fly. Subtracting from the total number of days of delay those in which the maximum corrected temperature was below 13 °C resulted in the number of days actually missed and for those days, the average maximum temperature ($T_{max.aver}$) was computed.

The same procedure was used for *T. lineatum* for which a temperature threshold of 15 °C [20] was taken into account.

2.3. Studies of Collection Material

In order to find out if there are specimens of *T. laeve* in the country collections, the main collections of Scolytinae in the country have been checked, namely those from the Museum of Natural Sciences Suceava—"Ştefan Negru" collection, presented in the catalogue published by Vasiliu et al. [56]; from the Brukenthal Museum in Sibiu—a collection presented by Negru [57]; and from the Faculty of Biology of Babeş-Bolyai University in Cluj-Napoca—the non-catalogued Orest Mark's collection.

Species identification was done using the key published by Pfeffer [16].

2.4. Data Processing

Since our primary goal was to determine whether *T. laeve* is present in the locations where traps were installed, the results are given as the total number of captures per site. However, in order to facilitate the interpretation of data on *T. laeve* captures, taking into account the captures of *T. lineatum*, a species collected at all sites and which uses the same substrate with *T. laeve* [18–20],

the potential correlation between the captures of these species was analysed. For this analysis, only the places with *T. laeve* captures were taken into account. Because the data were not normally distributed (Shapiro-Wilk test), even after their log or square root transformation, Spearman's rank order correlation was run.

To understand if *T. laeve* responds differently than *T. lineatum* to the pheromone we used, a fact that could have affected *T. laeve* captures, the proportion of males in the total catches of the two species was analysed. It has been assumed that the sex ratio in nature for both species is the same, 1:1, as is known for *T. lineatum* [58]. Only locations where at least 30 specimens of each species were captured were considered, and the proportions have been calculated for each place. Testing for the difference between the two proportions was done using the Z-test [59], because theoretically, both sexes have the same chance of being captured.

A significance level of 0.05 was taken into account both for correlation analysis and for the comparison of two proportions. Statistical calculations were made with XLSTAT 2012 version (Addinsoft: Paris, France).

2.5. Maps

The distribution map for each *Trypodendron* species was obtained in ArcMap 10.2.2 software (Esri: Redlands, CA, USA). The projected coordinate system used was GCS WGS 1984.

3. Results

At the 31 sites where traps were installed to detect *T. laeve* in the spring of 2015, a total of 30,175 specimens of *Trypodendron* were captured, of which 863 (2.9%) were *T. laeve*, 28,041 (92.9%) *T. lineatum*, 1149 (3.8%) *T. domesticum*, and 122 (0.4%) *T. signatum* (Table 2).

T. laeve was trapped at 20 places, while *T. lineatum* was captured at 31, *T. domesticum* at 29, and *T. signatum* at seven places (Figures 1–4).

There was a strong positive and statistically significant correlation ($r_s = 0.6225$, $p = 0.0041$) between *T. lineatum* and *T. laeve* captures.

The number of *T. laeve* captures varied greatly from one place to another, being between 0.33 and 72.3 beetles/trap, with most of them in Cârlibaba (25.1%) and Poiana Braşov (20.6%), where the traps were set up in the spring of 2015 sufficiently early in relation to the altitude of the place and the evolution of the weather; in both cases, only one day of flight was lost.

Where the traps were placed before the flight or at most four days favourable to flight were lost, and the maximum daily temperature of those days did not exceed, on average, 15 °C, the ratio between the number of *T. laeve* and *T. lineatum* (caught during the *T. laeve* flight period) was, with only two exceptions, greater than 1:25.0. Where more than four flight-favourable days have been missed, the number of *T. laeve* captures has considerably decreased both in absolute terms and in relation to *T. lineatum* captures. In the places where more than 10 flight-favourable days have been lost, at most one to three beetles have been caught (e.g., at Doda Pilii and Padiş_2).

T. laeve was not captured at Soveja, Padiş_1, and Predeal, where the traps were installed at the latest time.

Some beetles of *T. laeve* were also caught in other locations (Table 3 and Figure 1), in traps baited with synthetic pheromone for *T. lineatum*, but—in the most cases—captures were very low compared to those of *T. lineatum*. However, in Putna, at 850 m above sea level, the captures of *T. laeve* were higher than those in Carlibaba and Poiana Brasov.

The proportion of males was 45.2–81.7% and 32.6–50.7% in *T. lineatum* and *T. laeve* captures, respectively, at the locations monitored in 2015. At all locations, except one (Bădeni), the male proportion in *T. laeve* captures was statistically significantly lower than in *T. lineatum* captures ($z = 2.26–5.56$; $p < 0.05$). In one of the eight additional studies using traps with similar baits (Putna, in 2017), the male proportions were 78.0–84.4% in *T. laeve* captures and 76.8–78.4% in *T. lineatum* captures, and the differences between proportions were not statistically significant ($z = 0.18–1.10$; $p > 0.05$).

No specimens of *T. laeve* were found in the coleopteran collections analysed in the study.

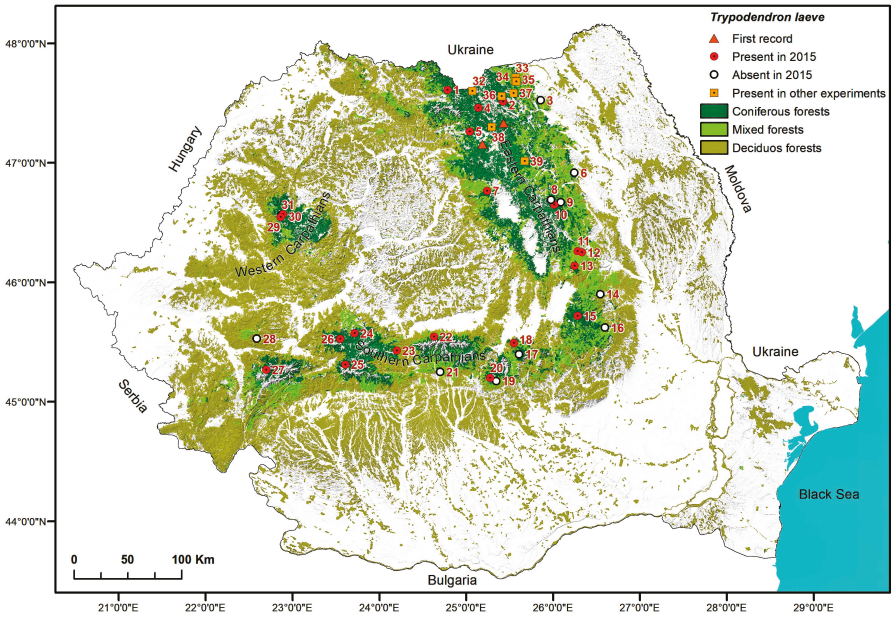

Figure 1. Sampling locations surveyed for the occurrence of *Trypodendron laeve* in 2015 and locations where it was found on other occasions mentioned in this study.

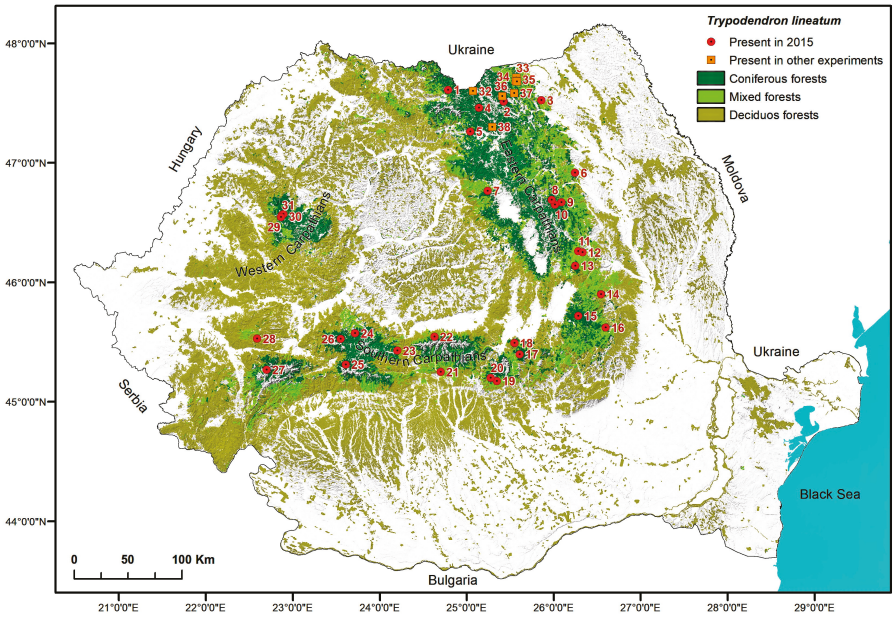

Figure 2. Sampling locations where *Trypodendron lineatum* was found in 2015 or on other occasions mentioned in this study.

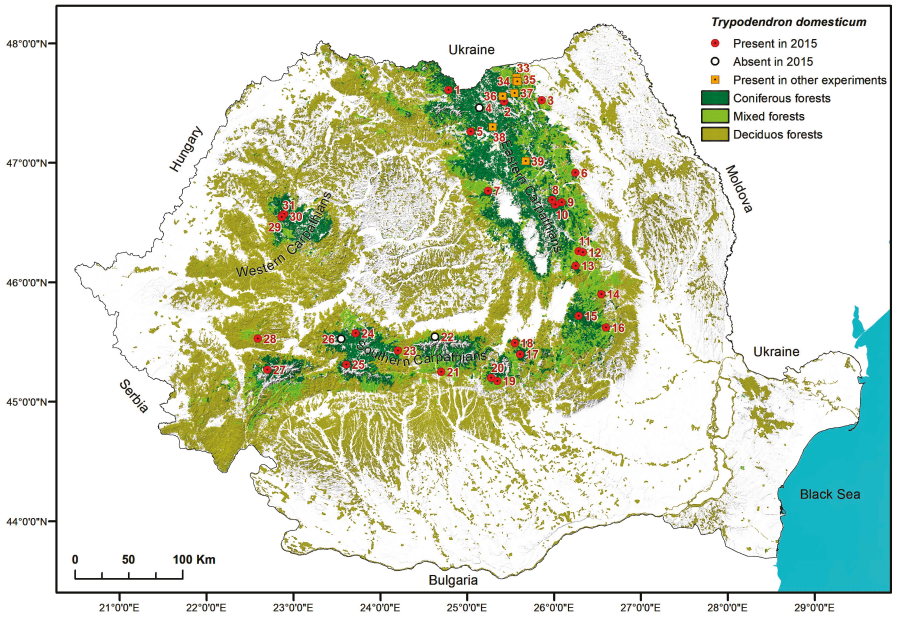

Figure 3. Sampling locations where *Trypodendron domesticum* was found in 2015 or on other occasions mentioned in this study.

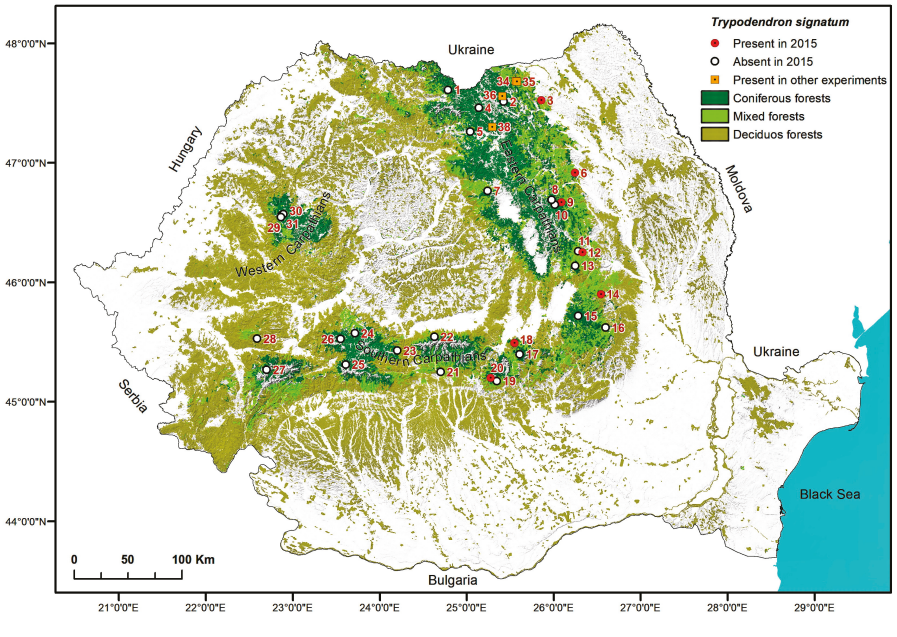

Figure 4. Sampling locations where *Trypodendron signatum* was found in 2015 or on other occasions mentioned in this study.

Table 3. Captures of *Trypodendron* species in other studies that have been conducted with synthetic pheromone of *T. lineatum*.

Site No.	Location	No. of Traps	Monitored Period	Total Number of *Trypodendron* Beetles per Study Site			
				laeve	*lineatum*	*domesticum*	*signatum*
32.	Bobeica	3	19.04–16.09.14	1	37,291	0	0
33.	Putna_1	3	13.03–24.08.17	5	353	19	0
34.	Putna_2	3	13.03–24.08.17	41	686	42	4
35.	Putna_3	3	13.03–24.08.17	237	4125	47	5
36.	Demacuşa	20	12.04–15.06.16	1	19,366	524	1
37.	Ciumârna	20	06.05–09.06.15	1	14,095	57	0
38.	Iacobeni	20	13.04–16.06.16	3	31,906	47	3
39.	Borca	-	23.03.17	1	-	2	-
	Total	72		290	107,822	738	13

4. Discussion

Adults of *T. laeve* were captured along the entire Carpathian Mountains chain in Romania and in nine of the sampling locations the catches were substantial (more than 30 individuals). At some sites, the catches were very low, and in one third of all sampling sites, no specimens of *T. laeve* were trapped.

Considering that *T. laeve* fly very early and the intense flight takes only about three weeks [21,37,51], it is quite normal for the number of specimens to be smaller where the traps have not functioned throughout the flight period (as was the case for 23 of 31 places), or even to capture nothing where the traps have been set up after the end of the flight (e.g., in Predeal).

On the other hand, the abundance of *Trypodendron* individuals in a given location is dependent on the abundance of the ephemeral substrate in which these species develop, varying with it. Where the host resource is reduced, the populations decline, while they increase where the available host habitats increase [13,51,60]. Consequently, relatively small or missing *T. laeve* captures from some places where the installation of the traps was not delayed (Lunca Ilvei, Moroeni) or where only few favourable days for flight were lost (Sălătruc, Dărmăneşti, Paltin, Brateş_1–3, Almaş, Cacica) could be due to the poverty of the suitable breeding material (wind–thrown trees, stumps, coniferous trees killed by bark beetles). This is suggested by *T. lineatum*'s low captures from the same places, with the captures of the two species (*T. laeve* and *T. lineatum*) being closely correlated according to our results and to data from other studies [61], because both species colonize coniferous wood. The results from Putna confirm this hypothesis. While the traps at the elevations of 650 m and 750 m were set up in tree stands with only a few stumps (all older than one year), at an 850 m altitude, the traps were located at about 50 m apart from the logs (about 100 cubic meters) abandoned in the forest from the previous spring (2014) and many more beetles have been captured.

It is known that *T. laeve* hibernates in tree bark and wood [20], while *T. lineatum* hibernates in soil [13], which allows the first species to fly when the soil is still covered with snow. This gives *T. laeve* a competitive advantage over *T. lineatum* in occupying the available substrate [62], but only when the soil is covered by snow or when the warming in the spring does not proceed very quickly, but gradually. If warming is very fast, there is no delay or only a very short one between the dates when the maximum daily temperature reaches 13 °C and 15 °C when the flight of *T. laeve* and *T. lineatum* respectively starts [20]. This happened in 13 of the places where traps were set up in 2015. The almost simultaneous beginning of the flight of the two species was observed in Austria at low altitudes by Krehan and Holzschuh [37] and in southern Sweden in the nemo-boreal zone, where there is little or no snow in winter, by Öhrn et al. [51]. Even in many parts of the very north or high altitude forests, the beginning of the flight is more or less the same for both species (Torstein Kvamme, personal communication). In this context, *T. laeve* is also disadvantaged by the fact that the adults of this species apparently do not produce a sister-brood [20,51], as is the case with *T. lineatum* [13,63].

The above-mentioned issues could explain why *T. lineatum* was captured in a much larger number (at least 3.2 times more) than *T. laeve*, even if only the flight time of *T. laeve* is taken into account and only a maximum of one to two days of *T. laeve* flight were missed. However, it is possible that the

number of captures has also been influenced to a certain extent by a possible differential response of the two species to the synthetic pheromone used in the traps, as Öhrn et al. [51] suggested for their study. If the natural sex ratio in the case of the two species is the same, the different proportions of males in the total captures indicate quite a different response of the two species to the synthetic pheromone used in this study. Such differences have also been reported by Krehan and Holzschuh [37], Martikainen [20], and Lukášová and Holuša [61]. The fact that the two species do not respond in the same way to olfactory stimuli was also evidenced by Kvamme [21], who found that *T. lineatum* adults (especially the males) were attracted to alpha-pinene released at a rate of 1.2–1.4 mg/h, while those of *T. laeve* were not.

In the case of a very low population density in some places, the lack of *T. laeve* captures may also be the result of other factors, such as the low sampling effort (up to three traps and in some places only one) or the inappropriate placement of some traps, so that in five places, only one or two of three traps captured adults of this species. Observations made in other studies have highlighted the fact that traps that are not seen due to the foliage of young trees (with branches close to the ground) capture much less insects than those that are not surrounded by obstacles (Olenici, unpublished data). On the other hand, Öhrn et al. [51] noted that both *T. lineatum* and *T. laeve* were captured in larger numbers in traps placed in shade than in the sun-exposed traps. This may be the result of beetles responding better to olfactory stimuli under relatively still conditions [64], which they find inside the forest rather than in open settings [65].

Of the *Trypodendron* species which develop in the wood of broadleaved trees, it is worth noting that, although in many places the traps were located in coniferous stands, *T. domesticum* was found almost everywhere, suggesting that it is a common species in Romania, like *T. lineatum*. On the other hand, *T. signatum* was collected from a much smaller number of places.

It seems that *T. signatum* is a rare species compared to *T. domesticum*, previously being reported in Romania from only a few places (Haţeg, Huluzu in Latoriţa Mountains, Sibiu, Braşov, Şumuleu Ciuc, Mihăileni, Frumoasa—Harghita, Tazlău—Neamţ, and Remeţi—Maramureş) [56,66–68].

The lack of specimens of *T. laeve* in the collections of Scolytinae analysed by us may be due to several reasons. First of all, the scolytids have been much less collected and studied in Romania than in the countries with a rich entomological tradition. Secondly, entomological excursions and insect sampling are not usually done so early, when *T. laeve* is flying. Even nowadays, it seems unusual for many people, including practitioners, to search for insects during the winter months or when snow is still on the ground. Thirdly, until recently, when there were no pheromone lures, collecting bark and ambrosia beetles by an axe and chisel was a more difficult and uncertain task than today, especially for rare species. Eloquent is the fact that almost all the data obtained after 1980 on the presence of *T. laeve* in different places were obtained using traps baited with synthetic attractants [19,21,35,37,44,52,53]. Even in countries with a very rich entomological tradition, there is relatively little historical data. Specimens of *T. laeve* were found as early as the 19th century or the beginning of the 20th century only in Sweden and Finland [30], while in Germany, there is a single historical record which dates back to 1953 [40].

Summarizing the above, one can say that *T. laeve* is a widespread species in the Carpathian Mountains, where it coexists with at least two other indigenous species: *T. lineatum* and *T. domesticum*. It has a continuous range, accompanying the Norway spruce even where it has been extended into the altitudinal belt of beech forests, but appears to be more abundant in the spruce forests at high altitudes.

5. Conclusions

T. laeve has a widespread distribution in the Carpathian Mountains and it seems that the species is more abundant at high altitudes. Overall, its populations are less abundant than those of *T. lineatum*.

Author Contributions: N.O. conceived and designed the study; I.V. prepared the pheromone baits and tested them to establish the release rate; M.-L.D. and G.I. dealt with fieldwork, collected all the data about the places where the traps were installed, and verified the three entomological collections; N.O. identified the beetles and M.K. verified the correctness of the identifications; N.O. wrote the paper and M.-L.D. prepared the maps. All co-authors assisted the lead author in writing and revising the manuscript.

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Phytophthora cinnamomi Colonized Reclaimed Surface Mined Sites in Eastern Kentucky: Implications for the Restoration of Susceptible Species

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Abstract: Appalachian forests are threatened by a number of factors, especially introduced pests and pathogens. Among these is *Phytophthora cinnamomi*, a soil-borne oomycete pathogen known to cause root rot in American chestnut, shortleaf pine, and other native tree species. This study was initiated to characterize the incidence of *P. cinnamomi* on surface mined lands in eastern Kentucky, USA, representing a range of time since reclamation (10, 12, 15, and 20 years since reclamation). Incidence of *P. cinnamomi* was correlated to soil properties including overall soil development, as indicated by a variety of measured soil physical and chemical parameters, especially the accumulation of soil organic carbon. *P. cinnamomi* was detected in only two of the four sites studied, aged 15 and 20 years since reclamation. These sites were generally characterized by higher organic matter accumulation than the younger sites in which *P. cinnamomi* was not detected. These results demonstrate that *P. cinnamomi* is capable of colonizing reclaimed mine sites in Appalachia; additional research is necessary to determine the impact of *P. cinnamomi* on susceptible tree species at these sites.

Keywords: forest health; mine reclamation; Forestry Reclamation Approach; Phytophthora; ink disease; American chestnut

1. Introduction

Appalachian forests are threatened by many stressors, including climate change [1,2], land use change [3–5], and invasive pests and pathogens [6,7]. Forest restoration and management efforts must be informed by a clear understanding of these and other impacts to ensure forest health and resilience in the future [8].

The American chestnut (*Castanea dentata* (Marsh.) Borkh.) story is a well-known example of the dramatic effects of invasive pathogens. American chestnut was once a dominant canopy species throughout the Appalachian region, which includes the states of West Virginia and parts of Alabama, Georgia, Kentucky, Maryland, Mississippi, New York, North Carolina, Ohio, Pennsylvania, South Carolina, Tennessee, and Virginia. Composing 50% or more of the forest canopy over much of its range, American chestnut was functionally eliminated from eastern forests in only a few decades in the early 1900's by the introduced fungal pathogen causing chestnut blight, *Cryphonectria parasitica* (Murr.) Barr [7,9,10]. Thanks to breeding targeted at introducing resistance genes from blight-resistant

Chinese chestnut (*Castanea mollissima* Blume) into American chestnut, American chestnut varieties with reasonable levels of blight resistance are becoming available for use in restoration plantings [11].

Unfortunately, even before the introduction of chestnut blight, *Phytophthora cinnamomi* Rands, a pathogen causing ink disease in American chestnut, had been introduced in the southeastern US and had been slowly causing American chestnut decline in the mid-late 1800s [12,13]. Thought to have originated from Papua New Guinea or Taiwan (but since distributed globally) [14], *P. cinnamomi* is a soil-borne oomycete pathogen with >2000 susceptible species [15], with disease symptoms including black exudate staining infected roots, chlorotic leaves, and thinning crowns [7]. The pathogen continued its slow march northward, overshadowed by the much more dramatic activity of chestnut blight [7]. Unfortunately, genes conferring resistance to chestnut blight do not necessarily also confer resistance to *P. cinnamomi*; thus, some of the early blight-resistant American chestnut varieties were killed by *P. cinnamomi* when planted [16,17]. These early observations have led researchers to focus on identifying genes conferring resistance to *P. cinnamomi* and developing strategies for improving blight-resistant varieties to also be *P. cinnamomi* resistant [18,19]. In addition to the genetic improvement of susceptible hosts, such as American chestnut, a recent review recommended the construction of a more robust dataset on *P. cinnamomi* distribution in eastern US forests [20].

P. cinnamomi is broadly distributed globally. At very small spatial scales (i.e., ~1 m^2), the pathogen appears to be somewhat randomly distributed; although it may be present within a given square meter soil patch, it is likely to be detected in only a fraction of samples collected within that patch [21–23]. Pathogen propagules are also capable of moving vertically within the soil profile, retreating to depth to survive inclement conditions [21,24]. Across broader spatial scales, *P. cinnamomi* is thought to be associated with moist soils at low topographic positions, such as drainages. Conversely, the pathogen is generally thought to be absent from higher and drier soils [22,23,25,26]. However, *P. cinnamomi* has been isolated from dry ridgetop soils in Australia [27] and eastern Kentucky [28], suggesting that environmental conditions limiting *P. cinnamomi* and its distribution are complex. *P. cinnamomi* has been present in the southeastern US for over 150 years [12], and is known to be widely distributed in the Appalachian region [29,30], but topographic and soil factors controlling its distribution at smaller spatial scales (e.g., within a watershed) have not been elucidated in any detail. These patterns must be well-understood before informed decisions about the restoration of susceptible tree species can be made.

In addition to introductions of non-native pests and pathogens, Appalachian forests have been degraded by a legacy of surface mining for coal. An estimated 600,000 ha of formerly forested land in Appalachia have been converted to novel grassland systems, characterized by high soil compaction and vegetative competition, and generally unfavorable for colonization by native trees [31–33]. These grassland patches perpetuate negative impacts of surface mining into the future, increasing forest fragmentation, increasing species invasion opportunity, and inhibiting site productivity and carbon storage [3,33–35].

This situation prompted researchers, regulators, and industry practitioners to develop a set of recommendations for reforestation at surface mined sites, termed the Forestry Reclamation Approach (FRA, [36]). These recommendations encourage minimal spoil compaction and reduced vegetative competition, improving the growth and survival of native trees over traditional reclamation practices [37–39]. These mine spoils, while initially devoid of organic matter, can support rapid tree growth and organic matter accumulation, and are considered soils after reclamation [34]. Yellow poplar (*Liriodendron tulipifera* L.) and white oak (*Quercus alba* L.) planted in FRA plots exhibited growth rates similar to the growth of these species in naturally regenerating clear cuts [40]. A chronosequence study in West Virginia found that soil organic carbon (SOC) accumulation in mine soils could be predicted by a logarithmic model, with 75% of 50-year SOC stock (13.3 Mg ha^{-1}) accumulating in the first ten years [41]. Other studies have found even higher SOC accumulation—stocks of 19.2 Mg ha^{-1} 13 years after reclamation and 38 Mg ha^{-1} 25 years after reclamation at sites in Ohio [34,42], and 16.8 Mg ha^{-1} 16 years after reclamation at sites in Kentucky [34]. Together with SOC accumulation, above-ground biomass and litter accumulation contribute to overall ecosystem C

sequestration, projected at 140.8–162.3 Mg ha^{-1} after 60 years in forest reclamation sites from Kentucky, Ohio, Indiana, and Illinois [34].

Because these mine soils are typically not reclaimed with native topsoil, *P. cinnamomi* and other soil-borne pathogens are generally thought to be initially absent from these sites [43–46]. FRA sites may also be unfavorable for *P. cinnamomi* for some time after reclamation due to high infiltration rates, low moisture retention, and relatively high temperatures associated with low shade, low organic matter, and little to no clay content [47–49]. *P. cinnamomi* was not detected in mine reforestation plots in southeastern Kentucky one and three years after reclamation [50], and was also not detected in a number of sites 3–20 years after reclamation in Ohio [46]. However, a recent chronosequence study in eastern Kentucky suggests that rates of microbial activity (assayed by dehydrogenase activity), microbial biomass C and N, litter decomposition rates, and CO_2 efflux in mined sites eight years after reclamation were similar to native forests 12 years after clear-cutting [51]. It is possible that *P. cinnamomi* can colonize these sites over time, along with other soil microorganisms, as soil development progresses. This study was initiated to evaluate whether *P. cinnamomi* colonizes FRA sites representing a chronosequence of time since reclamation in eastern Kentucky, and relate this incidence to soil development parameters, especially SOC accumulation.

2. Methods and Materials

Eight reclaimed surface mined sites of varying ages (two sites each reclaimed in 1997, 2003, 2005, and 2007) in eastern Kentucky were selected for screening for the presence of *P. cinnamomi* (Figure 1).

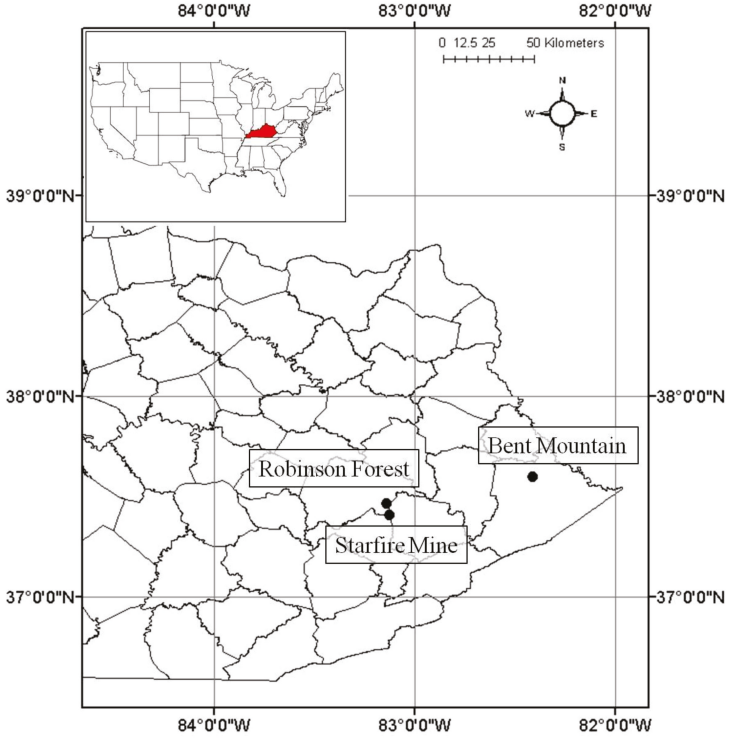

Figure 1. Location of reclaimed mine sites and unmined forest control, Breathitt (Robinson Forest), Perry (Starfire Mine), and Pike (Bent Mountain) Counties, Eastern Kentucky, USA.

Sites reclaimed in 1997 and 2003 were located at Starfire Mine (Breathitt County, Kentucky, 37.40939° N, −83.1229° W), and sites reclaimed in 2005 and 2007 were located at Bent Mountain Mine (Pike County, Kentucky, 37.60023 N, −82.40848 W). Climate in this region is temperate humid continental, with an average temperature of 13.3 °C [52]. Average annual precipitation in Robinson Forest (adjacent to the Starfire Mine site) is 117.5 cm [53], and average annual precipitation in Pike County is 114 cm [47]. Typical temperatures range from 18–30 °C in summer and −5–6 °C in winter at Robinson Forest, and from 18–32 °C in summer and −4–7 °C in winter in Pike County [47]. Sites were constructed using low-compaction spoil placement techniques [36], with overburden sourced from the Breathitt formation, which is dominated by sandstones and shale [54]. For site details, see [40] for the 1997 site, [51] for the 2003 site, [55] for the 2005 site, and [56] for the 2007 site. Similar tree species were planted at each of these sites during reclamation, and white oak was present at all sites at the time of sampling. Because these sites were reclaimed using similar techniques with spoil from the same geologic formation, and are subject to similar weather conditions, we employed a site-for-time substitution (chronosequence approach) to evaluate trends over time [51].

Twenty soil samples were collected underneath white oak at each site in October–November 2017, for a total of 160 samples, and all 160 samples were screened independently. Samples for *P. cinnamomi* screening were collected in 50 mL tubes from the top 5 cm of soil. Samples were screened using a rapid screening approach developed in our laboratory [28]. Briefly, ~40 mL soil samples were flooded with sterile water in 50 mL tubes, and baited with six ~6 mm diameter rhododendron leaf discs for five to seven days. Baits were then removed and frozen in 1.5 mL tubes for subsequent DNA extraction. DNA was extracted from baits using the DNeasy UltraClean Microbial DNA Extraction Kit [Qiagen]. Amplifiable DNA was confirmed for each DNA extraction using universal ITS1–ITS4 primers [57]. DNA was screened for *P. cinnamomi* using a conventional PCR assay with primers Ycin3F and Ycin4R targeting the Ypt gene recommended for *P. cinnamomi*-specificity [58,59]. Samples were screened in duplicate with positive controls, *P. cinnamomi* isolate RF5 (isolated from Robinson Forest, GenBank Accession #MF966152) limit of detection 1.5×10^{-2} ng per PCR, and no template negative controls. Primer annealing temperatures were 55 °C and 58 °C for the ITS and Ypt PCRs, respectively, with PCR amplicons visualized using agarose gel electrophoresis, 1.5% (*m/v*). *P. cinnamomi* incidence was assessed as % of total samples (20 samples screened per site) screened as positive.

In addition to screening for *P. cinnamomi*, a number of soil physical and chemical parameters were assessed at these reclaimed mine sites, as well as a mature white oak stand in Robinson Forest (Breathitt and Perry Counties, Kentucky) selected as an unmined forested control. Three soil samples per site age (sampled to 10 cm using a soil probe) were collected for chemical and physical analyses, and data were averaged by site age (10, 12, 15, and 20 years since reclamation), to permit direct comparison to data reported by previous studies [60]. Particle size was assessed by quantifying the mineral grain size distribution, and sand, silt, and clay fractions as defined by the Wentworth Scale [61]. These samples were dried at ~75 °C for 24–48 h, gently disaggregated, wet-sieved through 2 and 0.5 mm sieves, and treated with dilute H_2O_2 to destroy organic binding agents [62,63]. Samples were then analyzed using a Malvern Mastersizer S-2000, a laser-optical particle size characterization instrument capable of accurately resolving particles over a size range of 0.02 to 2000 µm.

Concentrations of Al, Mn, Fe, Mg, K, Ca, and Na were assessed by inductively coupled plasma mass spectrometry (ICPMS) after samples were completely dissolved using concentrated acids (HF, HCl, and HNO_3) over heat. Bulk density was assessed by the excavation method [64], and SOC was measured using an LECO CHN 2000 analyzer after an acid pretreatment (HCl). Although conventional SOC assessment (using a LECO analyzer) follows an acid pretreatment to remove inorganic carbon (e.g., carbonate minerals), this step has been known to incompletely eliminate carbonates from carbonate-rich mine soils, and can overestimate SOC [60,65]. Thus, soil organic matter (SOM) was evaluated by the thermogravimetric method—this method more accurately differentiates "new organic carbon" contributed by biomass from "old organic carbon" contributed by coal fragments or inorganic carbon contributed by carbonate minerals [60,65]. $\delta^{13}C$ (‰) was measured after HCl

pretreatment (and was thus reflective of organic C only) on a Thermo-Finnigan Delta XP Isotope Ratio Mass Spectrometer.

Soil physical and chemical data (means by site age) were analyzed by regression, together with data from previous studies at these sites where available [40,49–51,55,56,60], with years since reclamation as the main effect (PROC GLM, SAS 9.3). Both linear and quadratic relationships were tested for each variable; data were interpreted using a quadratic regression if the quadratic factor was significant ($p < 0.05$), but were interpreted using a linear regression if the quadratic factor was insignificant ($p > 0.05$). Sand and silt data were available from these sites at times representing 0, 1, 2, 3, and 8 years after reclamation [40,49–51,55,56,60]. SOM, SOC, and $\delta^{13}C$ data were available for these sites at times representing 0, 2, 3, and 8 years since reclamation [51,60]. *P. cinnamomi* incidence was interpreted in light of trends over time in soil physical or chemical parameters to provide insight into *P. cinnamomi* colonization of these sites.

3. Results

Soil particle size distribution was significantly correlated ($p < 0.05$) with time since reclamation for % sand and % silt (Figure 2). Sand decreased from 70% in new mine soils to <40% in mine soils 12–20 years after reclamation. In contrast, silt increased from 20% in new mine soils to 55% in mine soils 12–20 years after reclamation. This shift in particle size distribution toward a dominance of silt may be related to increasing trends in concentrations of some of the metal analytes evaluated in this study (e.g., Al, Fe, Mg, and Ca, Table 1). Decreased soil particle size corresponds to dramatically increased soil surface area and reactivity, which very likely increases the sorption of these and other cations [66,67].

Table 1. Soil physical and chemical characteristics.

	Years Since Reclamation				Robinson Forest
	10	12	15	20	
Al (ppm)	36,800 ± 640	36,800 ± 1200	46,600 ± 7200	48,100 ± 9800	50,800 ± 5000
Mn (ppm)	184 ± 8.5	316 ± 25	321 ± 38	259 ± 39	997 ± 150
Fe (ppm)	9770 ± 140	17,800 ± 960	17,400 ± 2500	19,800 ± 3400	25,600 ± 2000
Mg (ppm)	1420 ± 37	2280 ± 63	2700 ± 550	3300 ± 680	1980 ± 230
K (ppm)	13,500 ± 400	12,400 ± 800	15,400 ± 2500	14,600 ± 2700	12,900 ± 1400
Ca (ppm)	489 ± 36	1040 ± 130	1540 ± 440	1220 ± 62	455 ± 110
Na (ppm)	3880 ± 31	2740 ± 120	2650 ± 220	1800 ± 590	833 ± 57
% Sand	60.6 ± 3.3	37.5 ± 1.9	48.4 ± 5.9	38.1 ± 2.1	27.3 ± 3.7
% Silt	35.9 ± 3.0	56.4 ± 2.2	47.1 ± 5.8	55.1 ± 2.0	62.6 ± 3.6
% Clay	3.45 ± 0.38	6.07 ± 0.48	4.53 ± 0.33	6.80 ± 1.6	10.1 ± 0.28

Total SOC, determined using an LECO analyzer, also demonstrated a significant correlation with time since reclamation, increasing from very low levels in new mine soils (0.1–0.2% in two to three year old soils) to >3.0% in 15–20 year old soils, nearing the SOC levels in unmined forest soils (Figure 3). Similarly, "new organic carbon" represented by SOM measured by the thermogravimetric method increased from 0.03–0.10% in zero to two year old mine soils to 1.5–2.2% in 15–20 year old mine soils, nearing SOM levels in unmined forest soils (Figure 4). SOC concentrations measured by the LECO analyzer tended to be higher than SOM measured by thermogravimetry (Figure 5).

While organic carbon concentrations increased with time since reclamation, $\delta^{13}C$ values decreased with time since reclamation, approximating $\delta^{13}C$ values in unmined forest soils (Figure 6). This relationship between $\delta^{13}C$ and SOM is negative and quadratic, with lower $\delta^{13}C$ values and higher SOM concentrations in the older mine soils, nearing levels in unmined forest soils (Figure 7). Total SOM stocks (Mg C ha^{-1}) exhibited different trends across sites, suggesting some differences in site quality, likely related to differences in regional geology and site construction (Figure 8) [60]. However, at both sites, SOM stocks approached 20 Mg ha^{-1} by eight to 12 years after reclamation.

P. cinnamomi was detected at all four Starfire sites (15 and 20 years after reclamation), but was not detected in any of the Bent Mountain sites (10 and 12 years after reclamation, Figure 9). Of the samples screened at the Starfire sites, 27.5% of samples (range: 5–50%) at the 15-year old sites were positive, and 12.5% (range: 10–15%) of samples at the 20-year old sites were positive. These sites tended to have lower % sand and higher % silt, as well as increased SOC and SOM stocks, compared to the younger Bent Mountain sites.

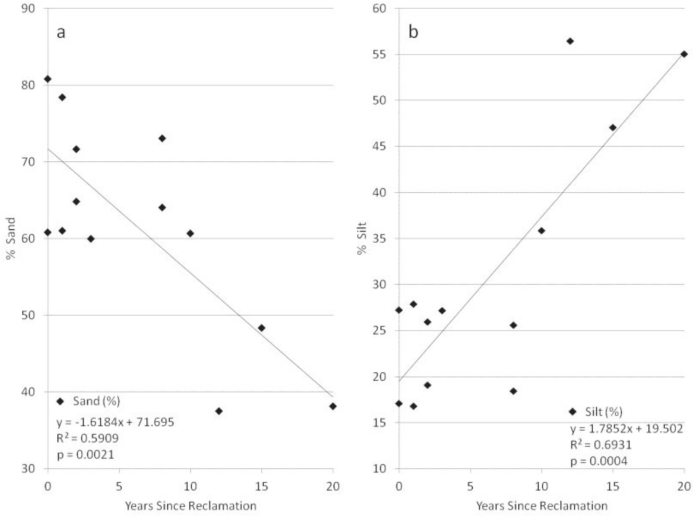

Figure 2. Changes in (**a**) % sand and (**b**) % silt in mine soils over time since reclamation.

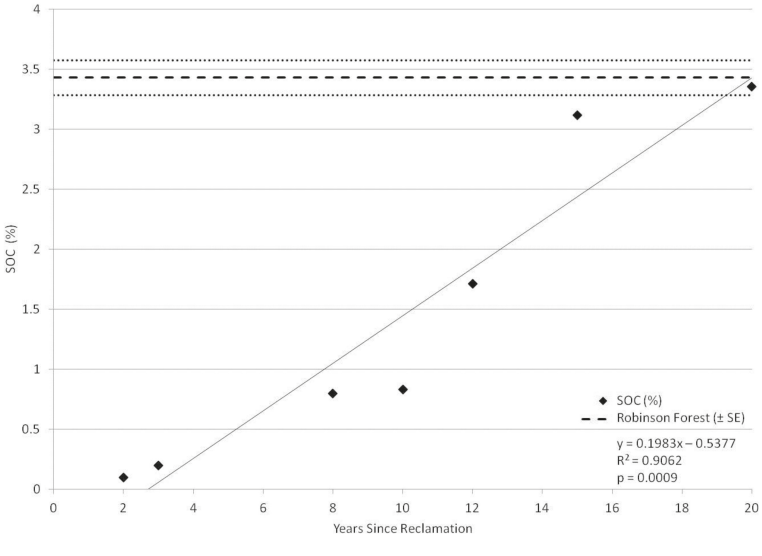

Figure 3. Changes in soil organic carbon (SOC; LECO analysis) in mine soils over time since reclamation, with mean % SOC (±SE) for Robinson Forest plotted for reference.

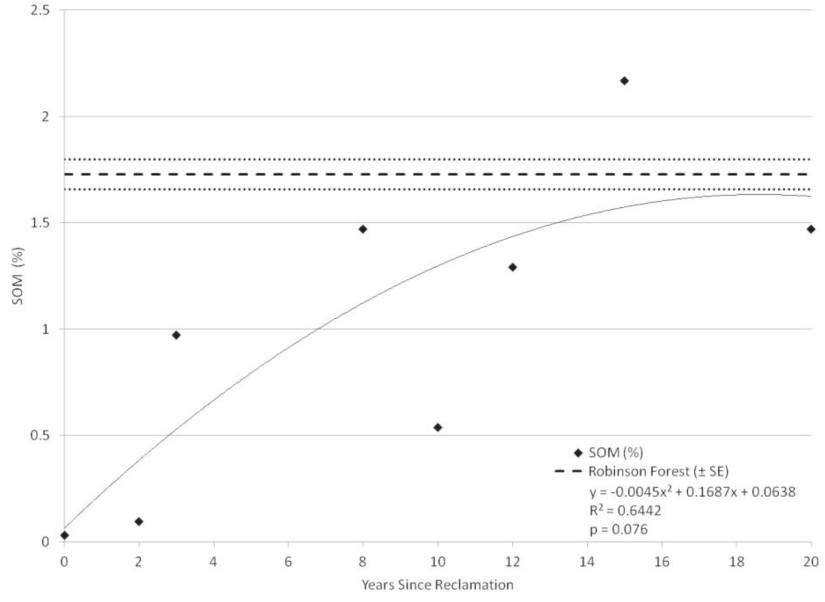

Figure 4. Changes in soil organic matter (SOM; thermogravimetric analysis) in mine soils over time since reclamation, with mean % SOM (±SE) for Robinson Forest plotted for reference.

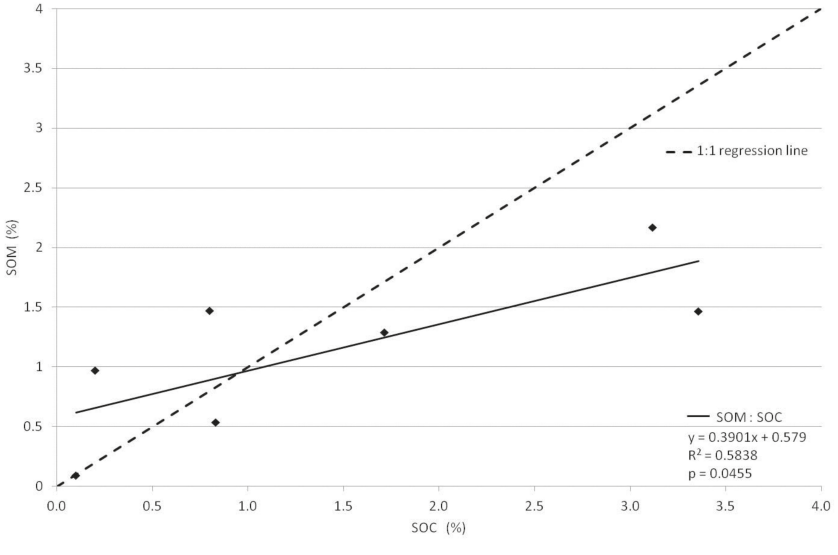

Figure 5. Correlation of SOC (LECO analysis) and SOM (thermogravimetric analysis), compared to a 1:1 reference line.

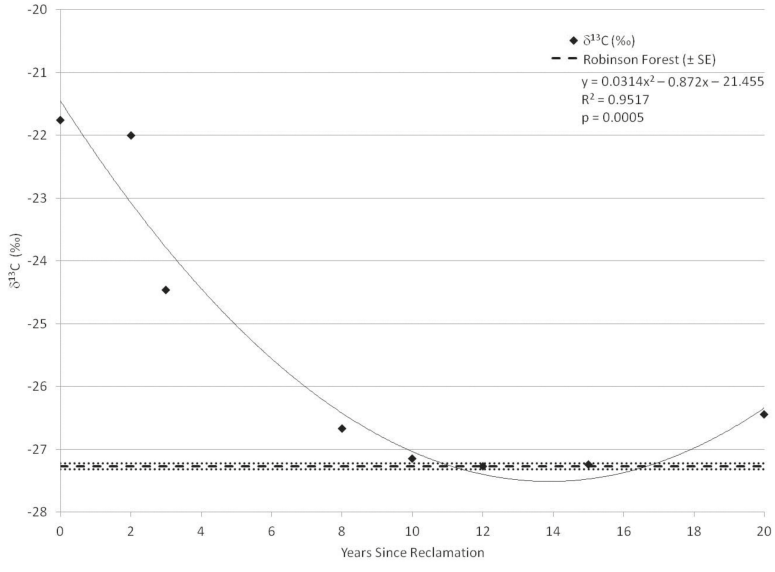

Figure 6. Changes in $\delta^{13}C$ (‰) in mine soils over time since reclamation, with mean $\delta^{13}C$ (±SE) for soil from a mature white oak stand in Robinson Forest plotted for reference.

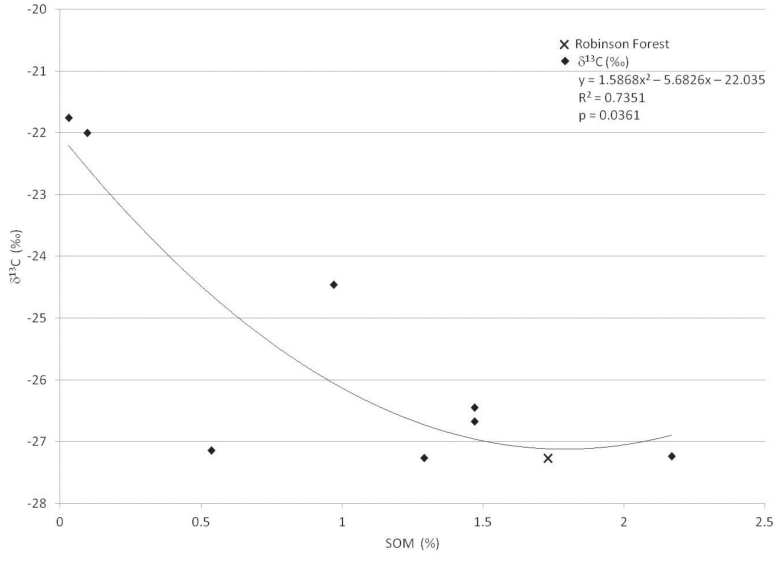

Figure 7. Correlation between % SOM (thermogravimetric analysis) and $\delta^{13}C$ (‰) in mine soils representing a range of time since reclamation, with Robinson Forest plotted for reference.

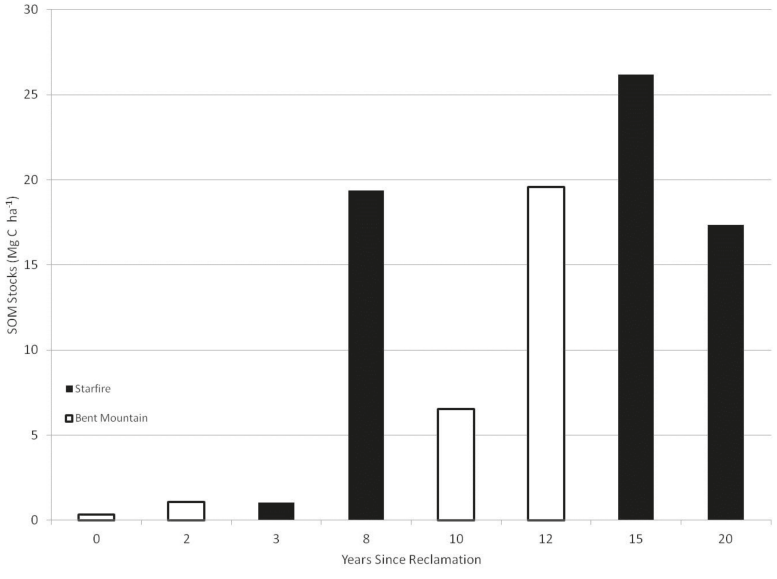

Figure 8. SOM stocks in mine soils over time since reclamation. Rates of accumulation of SOM in Bent Mountain sites appeared to lag slightly behind those of Starfire Mine sites.

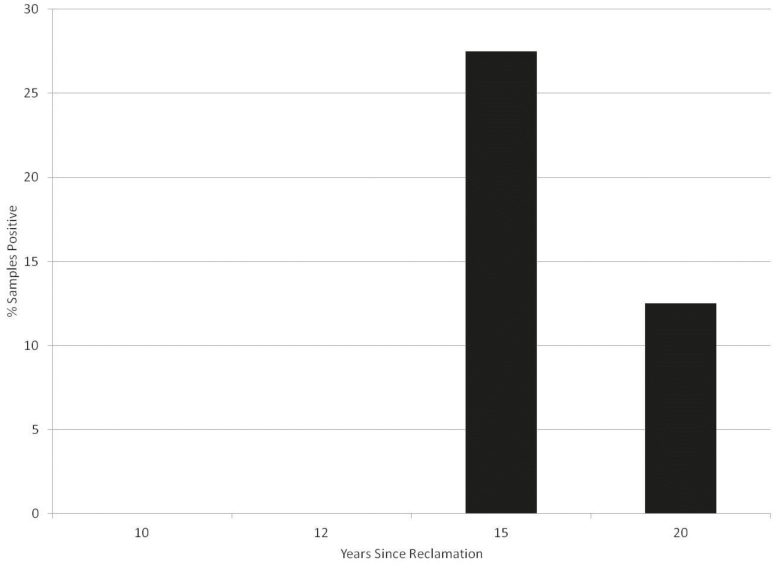

Figure 9. Incidence of *Phytophthora cinnamomi* in mine soils over time since reclamation.

4. Discussion

Soil particle size shifts are consistent with those observed by previous studies at these sites, generally reporting increased fines and decreased coarse fractions with time [39,56]. Over time, this shift in particle size is certain to increase soil reactivity, reflected in the increasing concentrations of metals

seen in this study [66,67]. Increased SOC and SOM concentrations with time since reclamation are also consistent with other studies investigating mine soil development [34,41,60]. Values of SOC (LECO analyzer) tended to be higher than values of SOM (thermogravimetric method) in this study. Acid (HCl) pretreatment followed by measurement of SOC on a LECO analyzer can inadequately remove inorganic carbon by incompletely dissolving carbonate minerals in carbonate-rich mine soils. LECO analysis also does not distinguish "old organic carbon" in coal fragments from "new organic carbon" in biomass, which can also lead to the overestimation of SOC [60,65]. The thermogravimetric method used in this study to assess SOM more accurately differentiates "new organic carbon" in biomass from inorganic carbon (e.g., carbonate minerals) and "old organic carbon" (e.g., coal fragments); thus, values of SOM (thermogravimetric method) are expected to be lower than those of SOC (LECO analyzer) [60,65]. SOM stocks at the sites included in this study (20 Mg ha^{-1} by eight to 12 years after reclamation) were similar to those reported by other studies on reclaimed sites in Appalachia, ranging from 13.3 Mg ha^{-1} over 10 years in West Virginia [41], to 26 Mg ha^{-1} after 10 years in Ohio [34,42].

Phytophthora cinnamomi was only detected at sites 15 and 20 years after reclamation, demonstrating that *P. cinnamomi* is capable of colonizing FRA reclaimed sites over time. The older sites where *P. cinnamomi* was detected were located on Starfire Mine (Perry County), while the younger sites where *P. cinnamomi* was not detected were located at Bent Mountain (Pike County). While some differences in site quality are suggested by observed differences in SOM accumulation, *P. cinnamomi* has been documented in areas adjacent to both mine sites, isolated from soils in Robinson Forest (adjacent to Starfire Mine, Breathitt County) [17,50] and from a dead American chestnut seedling at a nearby reforestation plot on Bent Mountain (Pike County) [68]—suggesting that sources of *P. cinnamomi* are available for colonization at all sites. A parallel study in Robinson Forest detected *P. cinnamomi* in 45% of screened samples [28], suggesting that the incidence of *P. cinnamomi* in unmined forest may be higher than the frequencies reported for reclaimed mined land in this study. Future surveys will clarify whether *P. cinnamomi* becomes more prolific in these sites over time.

Infected seedlings may be an important source of contamination on these sites—a California study documented the introduction of *P. cinnamomi* to previously uninfested sites via seedlings used in restoration plantings [69]. As mentioned above, *P. cinnamomi* was detected on a dead American chestnut in a restoration planting on Bent Mountain; it is unknown whether this seedling was infected before planting (i.e., with propagules from the nursery at which it was grown) or after planting (i.e., with propagules already present at the site) [68]. Nurseries supplying seedlings for the restoration of these surface mined areas should be screened for the presence of *P. cinnamomi* to reduce the risk of contaminating restoration sites.

To our knowledge, this is the first study reporting *P. cinnamomi* incidence at FRA sites. In a previous study, *P. cinnamomi* was not detected at a site in eastern Kentucky (the 10 year old site in this study) during the first season after spoil placement [50]. *P. cinnamomi* was also not detected at a series of reclaimed sites in Ohio ranging from three to 20 years since reclamation [46]. These researchers did not report surveys of adjacent forest soils for *P. cinnamomi*; thus, while *P. cinnamomi* had been previously reported in more southerly regions of Ohio [70], it is unknown whether *P. cinnamomi* is present in unmined forests in their study area.

More generally, a recent study in eastern Kentucky found that some microbial community metrics, such as microbial biomass C and N, and microbial activity (assessed by dehydrogenase activity), in mine soils eight years after reclamation, were similar to regenerating clear cut soils in unmined forests [51]. The current study supports observations that microbial community development occurs over time since reclamation, alongside plant community and soil development, including the recruitment of individual plant pathogens such as *P. cinnamomi*.

In previous studies on FRA-reclaimed sites, such as one of the Bent Mountain sites referenced here, forest development occurred rapidly on favorable mine soils, with planted trees achieving partial canopy closure after only nine growing seasons [39]. Alongside tree growth and canopy closure, these researchers also reported the development of a litter layer and recruitment of shade-tolerant

understory species [39]. Over time, shading provided by canopy closure and moisture storage provided by accumulating litter are expected to regulate soil moisture conditions, increasing site favorability for the recruitment of soil microbes. *P. cinnamomi* is thought to prefer moist soils—development of conditions increasing soil moisture may improve site quality for *P. cinnamomi* [71]. However, the presence of *P. cinnamomi* alone is not sufficient to cause disease in some susceptible hosts. For example, although *P. cinnamomi* was widespread at reclaimed bauxite mine sites in Western Australia, root rot in susceptible jarrah (*Eucalyptus marginata*) was related to high moisture conditions in poorly drained sites with ponding rainwater [72]. In these systems, researchers recommended intentional site preparation (e.g., deep tillage to improve drainage) to reduce ponding and reduce *P. cinnamomi* infection risk [73]. FRA sites are constructed with low-compaction spoil placement techniques, and are characterized by high infiltration and low runoff rates [36,47–49]. Although *P. cinnamomi* was detected on 15- and 20-year old FRA sites, no above ground symptoms of Phytophthora root rot were observed in the chestnuts and white oak growing at these sites; additional studies on roots of these species will be necessary to definitively document infection status at these sites. Although *P. cinnamomi* is present, it is unclear whether or not conditions at these sites are conducive for the development of Phytophthora root rot in susceptible species. Also, follow-up studies will be required to assess whether *P. cinnamomi* will eventually colonize the 10- and 12-year old sites screened in this study in which *P. cinnamomi* was not detected.

5. Conclusions

These data suggest that site quality at FRA-reclaimed mine sites is sufficient by 15 years after reclamation for colonization by *P. cinnamomi*. To our knowledge, this is the first study documenting *P. cinnamomi* colonization of FRA-reclaimed mine sites, and demonstrates that these sites do not remain "Phytophthora-free" over time. Additional research will be necessary to clarify the impact of *P. cinnamomi* on susceptible hosts at these sites. While *P. cinnamomi* was detected at these sites, it is unclear whether or not environmental conditions are conducive to the development of *P. cinnamomi*-related disease in susceptible hosts (such as white oak). Finally, potential routes of invasion of *P. cinnamomi* onto reclaimed mine sites should be assessed—especially distinguishing whether *P. cinnamomi* is more likely to colonize sites via infected seedlings used in plantings, or by the transport of propagules from adjacent infested forest sites.

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Article

Early Detection and Identification of the Main Fungal Pathogens for Resistance Evaluation of New Genotypes of Forest Trees

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Abstract: The growing importance of forest plantations increases the demand for phytopathogen resistant forest trees. This study describes an effective method for early detection and identification of the main fungal phytopathogens in planting material of silver birch (*Betula pendula*) and downy birch (*B. pubescens*), based on the estimation of the size of the internal transcribed spacers (ITS1 and ITS2) in the 18S-5.8S-28S rDNA gene cluster, which are species-specific for most micromycetes. The electrophoretic assay of the ITS1 and ITS2 loci has allowed us to identify predominant phytopathogenic fungal species in downy and silver birch in planta. This new molecular genetic method can be used to screen birch and other forest trees for different fungal pathogens to evaluate disease resistance. This information can be useful in breeding new genotypes of forest trees, including transgenic clones with modified wood composition.

Keywords: *Betula*; birch; fungal phytopathogens; ITS

1. Introduction

Fungal diseases are a serious problem in forestry, and can be the cause of epiphytotics leading to the death of forests: as examples, American chestnut, butternut, and American elm [1]. This is particularly relevant for forest plantations, which have less diversity and stability than natural forests and, therefore, are more susceptible to diseases. Modeling has indicated that short rotation in forest plantations accelerates both the virulence evolution in root-rot pathogenic fungi and the development of epiphytotics [2]. Moreover, global climate change may also promote distribution of forest pathogens. It has been shown that the expected changes in temperature and precipitation will favor the spread of beech bark disease in the forests of North America [3]. Thus, special attention is needed to assess

disease resistance of new genotypes of forest trees, including transgenic lines. Lignin manipulation is one of the main objectives in forest biotechnology. Its content in wood directly correlates with the efficiency of the pulping process, and affects waste management. However, lignin plays an important role in plant defense against pests and other phytopathogens [4]. Thus, a change in the composition and/or content of lignin can reduce plant resistance to phytopathogens. Generally, in addition to the biotechnologically generated desirable traits (intended effects), the appearance of unintended effects that can negatively affect agronomic performance is possible [5]. The detection of such effects can be done by comparing transgenic genotypes with related conventional counterparts [6]. Testing whether transgenic genotypes that have lower lignin content are less resistant to phytopathogens would mean detection of these phytopathogens in transformed and untransformed clones.

Birch species (*Betula* L.) are among the most widespread forest trees, and have great importance in forestry, forest formation, and soil improvement. They also have an important ecological role as pioneer species after clear-cuts and forest fires [7]. They are a fast growing species that provide high quality timber for industrial purposes. Downy birch (*B. pubescens* Ehrh.) and silver birch (*B. pendula* Roth) are commercially important forest species in Europe [8]. Their natural area includes North Africa, Western Asia, and Central Asia, as well as the entire Europe and Northern Eurasia (excluding the Iberian Peninsula). In Northern Europe, these species are the most important deciduous trees in plantation forestry [9]. Intensively managed forest plantations are characterized by a limited number of clones of the same species, which increases the risk of pathogen attacks. In this regard, the methods of diagnosing and identification of various phytopathogens based on DNA analysis have a great potential value [10]. Molecular diagnostic methods have been developed for detection of various pathogens in oak [11], plane trees [12], pines [13], and other forest species, but not in birch. Moreover, these methods were designed to identify pathogens of only one particular species or genus in a single analysis.

The traditional method of phytopathogen detection is based on visual inspection of disease symptoms and is often unreliable, performed in the late stages of the disease, and requires qualified personnel [14], especially for tree species. Molecular diagnostics, based on the detection of pathogen DNA using PCR methods, allows assessing the resistance of a new genotype quickly, with high accuracy, and at the early stages of disease development [15]. For several reasons, the ribosomal DNA (rDNA) loci encoding 5S, 5.8S, 18S, and 28S ribosomal RNAs (rRNAs) are widely used marker regions for the detection and identification of micromycetes [16]. The rDNA loci encoding 5.8S, 18S, and 28S rRNAs form a cluster of the 18S–5.8S–28S loci with two internal transcribed spacers (ITS1 and ITS2) between the 18S–5.8S, and 5.8S–28S loci, respectively. There are at least 50 copies of this cluster per genome, and this multiplicity enhances the sensitivity of the PCR analysis (i.e., the probability of pathogen detection at its low concentration in plant tissue). The ITS loci are relatively conserved within a species [16], but highly divergent between species, which facilitates taxonomic identification of the pathogen causing infection. These loci are well studied and their nucleotide sequences are well-represented in sequence databases, such as NCBI GenBank (https://www.ncbi.nlm.nih.gov), DNA Data Bank of Japan (DDBJ, NIG) (http://www.ddbj.nig.ac.jp), European Molecular Biology Laboratory (EMBL, EBI) (http://www.embl.de), Barcode of Life Data System (BOLD) (http://www.boldsystems.org), and DOE JGI Fungi Portal (https://genome.jgi.doe.gov/programs/fungi/index.jsf), which are very important for pathogen identification. Large scale molecular genetic studies of different fungi have revealed conserved rDNA regions and allowed the development of sets of universal primers for PCR amplification of ribosomal genes and intergenic spacers across different species [17]. DNA-based methods have been proposed for the identification of fungal species based on electrophoretic assay of the PCR amplified marker regions without preliminary sequencing of samples, including the identification of micromycetes [18]. The ITS region has been suggested as a universal marker for DNA barcoding of fungi [18]. However, most of the proposed protocols are not universally applicable, and have limitations in different phytopathological assays. For example, the use of an intergenic spacer (IGS) located between tandemly repeated copies of the rDNA gene clusters as a genetic marker

may be limited for studying pathogenic basidiomycetes, because of the high sequence variation within species in this region, and the challenges of amplifying DNA regions larger than 3 kilobase pairs (Kbp), particularly from decayed tissues [19]. The application of single-strand conformation polymorphism (SSCP) analysis does not directly generate nucleotide sequence data, which reduces their compatibility with nucleotide sequence databases [20]. Moreover, the likelihood of methodological mistakes and artifacts becomes greater when complex procedures are required for sample preparation and electrophoretic mobility analysis [21]. Finally, the sequencing of DNA markers involves a relatively high analytical cost and special laboratory equipment.

In the present study, we have developed the ITS1 and ITS2 genetic markers, which can be used without sequencing. Their species-specific variation in size makes them highly informative and sufficient for identification of the main pathogenic species of birches, using gel electrophoresis following PCR amplification. It is very important that there is almost no intraspecific size variation of the ITS markers in micromycetes that could be similar to the interspecific size variation, which almost excludes false positive results.

Amplicon size analysis was carried out by denaturing polyacrylamide gel electrophoresis, which allowed species identification using both the application of standard DNA samples, and information about the amplicon sizes from nucleotide sequence databases.

2. Materials and Methods

2.1. Plant Material

Samples from silver and downy birch plantings with different infection symptoms were collected during 2017, in the fields and greenhouses at the Korenevskaya Experimental Forest Enterprise of the Forest Research Institute of the National Academy of Sciences of Belarus (Belarus), in the forest enterprises of Gomel Region (Belarus), and in the Moscow Region (Russia).

2.2. Phytopathological Analysis

During the phytopathological assay of the birch planting material, the main diseases that caused the highest losses of yield during commercial cultivation were determined. The determination of disease type was carried out, based on the symptoms defined by the generally accepted system of phytopathological assays (http://www.forestpathology.org/index.html).

2.3. Species-Specific Molecular Genetic Identification of Phytopathogens

Specific phytopathogenic micromycetes were identified using molecular genetic methods for fungal identification in planta [22]. For the pathogen diagnosis, samples of plant tissue were collected at the initial infection stage, which simplified the diagnosis by minimizing the content of saprotrophic microflora. All of the plant samples (e.g., leaf disc cuttings, and stem or root fragments) were fixed in sterile polypropylene tubes with 70% ethanol and stored at −18 °C. During sample preparation, the analyzed fragments of plant material were removed from the tubes, washed thoroughly with running water, and pieces that exhibited a particular infection type were taken for further analysis. They were washed thoroughly with distilled water and cut with a razor blade into 3–8 mm pieces under sterile conditions, so that a junction between healthy and infected tissues was located in the middle of each piece. The samples were then placed in Eppendorf centrifuge tubes for subsequent DNA isolation.

2.4. DNA Isolation and PCR Amplification

The total DNA was extracted from the samples according to a modified cetyltrimethyl ammonium bromide (CTAB) protocol [23]. PCR was carried out using 2× DreamTaq™ Green PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) with combinations of ITS1–ITS2 (amplifying partial 18S rRNA, ITS1, and partial 5.8S rRNA loci) or ITS3–ITS4 (amplifying partial 5.8S rRNA, ITS2, and partial

26S rRNA loci) PCR primer pairs for the amplification of the fungal rDNA species-specific genetic markers [16]. The forward primers were labeled with a fluorescent dye. The primer sequences are shown in Table 1. The amplification reaction mixture (25 µL) contained 1 µL (0.5–50 ng) of DNA template, 12.5 µL of 2× DreamTaq™ Green PCR Master Mix, 1 µL of 5 µM Dye-labelled (e.g., with FAM-dye) forward primer, 1 µL of 5 µM reverse primer, and 9.5 µL nuclease-free water. The DNA reaction mixtures were amplified in a PCR thermocycler (TProfessional Basic Thermocycle) (Biometra GmbH, Göttingen, Germany) by algorithm: 1 cycle at 95 °C for 3 min, followed by 35 cycles of 20 s at 95 °C, 20 s at 60 °C, and 20 s at 72 °C. The reaction was ended with final extension at 72 °C for 4 min before holding the sample at 4 °C for analysis.

Table 1. Primer sequences used for the PCR amplification of the fungal ITS1 and ITS2 loci.

Locus	Primer	Primer Sequence (5′–3′)
ITS1	ITS1	FAM-TCCGTAGGTGAACCTGCGG
	ITS2	GCTGCGTTCTTCATCGATGC
ITS2	ITS3	FAM-GCATCGATGAAGAACGCAGC
	ITS4	TCCTCCGCTTATTGATATGC

2.5. Gel Electrophoresis

For high resolution gel electrophoresis and amplicon fragment analysis the PCR products were diluted to 1 ng/µL in deionized water, and 1 µL of the diluted PCR product was mixed with 18 µL of formamide and 1 µL of GeneScan™ 500 LIZ™ dye Size Standard (Thermo Fisher Scientific, Waltham, MA, USA) used as internal molecular weight markers. The mix was heated to 95 °C for 5 min to denature the products into single DNA strands and then cooled immediately on ice for 2 min. The denatured PCR products were then loaded into an ABI Prism 310 Genetic Analyzer (Thermo Fisher Scientific, Waltham, MA, USA) and electrophoretically separated in POP-4 polymer, according to the manufacturer's manual. The fragment calls and analysis were performed using the GeneMapper v. 4.0 software (Thermo Fisher Scientific, MA, USA). In addition, all alternatively sized amplicon variants were sequenced. Initial species identification based on the amplicon sequences was carried out using an on-line BLAST tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The subsequent species identification was based on the determination of species-specific fragments with a unique size that represented particular fungal species in the ABI-generated electrophoregram, with multiple peaks representing amplicon sequences in the PCR amplified sample (Figure 2).

3. Results and Discussion

The traditional phytopathological assay has determined a list of fungal infections in the birch planting material, such as leaf (leaf spots, powdery mildew, and rust leaves), shoot (necrosis and cancer pathologies), root, and vascular system (rot and wilting) diseases (Figure 1; [24]). Various leaf diseases were most common, with a predominance of powdery mildew (23.2%). In addition, for precise genetic identification of fungal pathogens, we used molecular genetic markers representing the nucleotide sequences of the ITS1 and ITS2 regions. They have indicated that multiple species of micromycetes were present in more than 80% of the infected plant tissue samples, although typically one or several fungal species predominated. Dominant phytopathogenic micromycetes species were detected both alone and in association with other fungal species, suggesting the key role of these fungi in pathogenesis. The presence of other minor micromycetes showed no particular pattern in different plant's samples, either alone or as part of associations with other microbes, and, after species identification, they appeared to represent a group of secondary pathogens and saprophytic fungi. Table 2 presents a list of the main 12 phytopathogenic fungal species, based on the ITS1 and ITS2 markers.

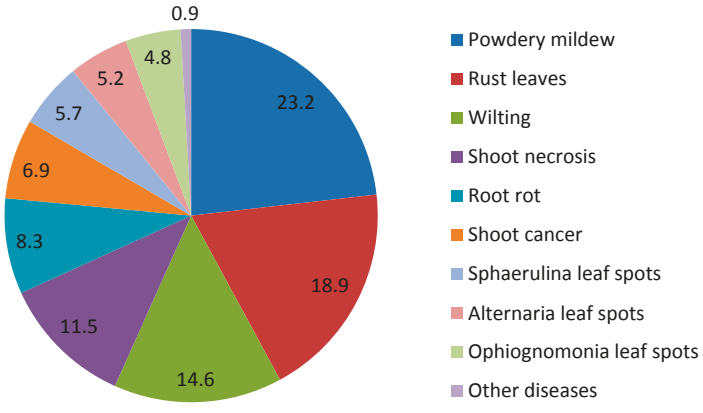

Figure 1. Infectious diseases identified in downy and silver birch based on visual phytopathological inspection (%).

Table 2. The occurrence of the main phytopathogenic fungal species and diseases in silver and downy birch.

Phytopathogen	Disease Type	Occurrence, %
Phyllactinia guttata (Wallr.) Lev.	Powdery mildew	13.2
Erysiphe ornate (U. Braun) U. Braun & S. Takam.	Powdery mildew	10.0
Melampsoridium betulinum (Pers.) Kleb.	Rust leaves	18.9
Fusarium avenaceum (Fr.) Sacc.	Wilting	13.2
Nectria sp. (Fr.) Fr.	Shoot necrosis	4.1
Melanconium bicolor Nees.	Shoot necrosis	3.7
Phytophthora cactorum (Leb. & Cohn) Schroeter	Shoot necrosis	3.5
Pythium sp. Pringsheim	Root rot	7.6
Botryosphaeria dothidea (Moug. & Fr.) Ces. & DeNot.	Shoot cancer	6.2
Ophiognomonia intermedia (Rehm) Sogonov	*Ophiognomonia* leaf spots	4.8
Sphaerulina betulae (Pass.) Quaedvlieg, Verkley & Crous	*Sphaerulina* leaf spots	5.7
Alternaria alternata (Fr.) Keissl.	*Alternaria* leaf spots	4.9
Other species	Other diseases	0.9

The analysis showed that the causative agents of powdery mildew were two pathogens: *Phyllactinia guttata* and *Erysiphe ornate*, mainly the first one. *Melampsoridium betulinum* was the most common pathogen (18.9%). This fungus causes birch rust, which is harmful in nurseries and also decreases seedling growth during the next spring after planting [25]. The disease was most severe in downy birch [26]. There were clear genetic differences in susceptibility to rust among birch clones [27], and an effective diagnostic method for detecting resistance to this pathogen should be useful in breeding. Leaf spots on birch are caused by a number of fungi [28]. In our study, the pathogens were *Ophiognomonia intermedia*, *Sphaerulina betulae,* and *Alternaria alternate*, in approximately equal proportions. The analysis of the amplicon nucleotide sequences amplified by the ITS1 and ITS4 primer pair (which included the rDNA region representing partially 18S rRNA, ITS1, 5.8S rRNA, ITS2, and partially 26S rRNA loci) has showed that all the revealed phytopathogens possessed a species-specific unique nucleotide sequence corresponding to the marker locus. The sizes of diagnostic loci in the same pathogen were identical to the samples from different geographic regions. The amplicon size variation was mainly due to polymorphism in the ITS1 and ITS2 loci (Table 3). The 5.8S rRNA gene and partial sequences of the 18S and 26SrRNA genes varied only in a few cases. In general, the main interspecies differences were due to nucleotide substitutions [29].

Table 3. The list of the main phytopathogenic fungal species identified in silver and downy birch and the sizes of their species-specific diagnostic ITS amplicons obtained using the ITS1–ITS2 and ITS3–ITS4 primer pair combinations.

Phytopathogenic Species	ITS1–ITS2, bp	ITS3–ITS4, bp
Sphaerulina betulae (Pass.) Quaedvlieg, Verkley & Crous	225	231
Ophiognomonia intermedia (Rehm) Sogonov	268	351
Alternaria alternata (Fr.) Keissl.	244	346
Phyllactinia guttata (Wallr.) Lev.	314	364
Botryosphaeria dothidea (Moug. & Fr.) Ces. & DeNot.	259	344
Erysiphe ornate (U. Braun) U. Braun & S. Takam.	298	362
Melampsoridium betulinum (Pers.) Kleb.	328	406
Pythium sp. Pringsheim	298	633
Phytophthora cactorum (Leb. & Cohn) Schroeter	295	602
Fusarium avenaceum (Fr.) Sacc.	233	355
Melanconium bicolor Nees.	270	349
Nectria sp. (Fr.) Fr.	217	348

Typical computer-generated capillary gel electrophoregrams, derived for infected birch samples, are presented in Figure 2. In the absence of fungal infection, with only the genomic DNA of silver or downy birch present as a template, only a single electrophoretic peak corresponding to the amplicon DNA fragment of the host plant should be present, as the birch ITS regions have similar annealing sites for the ITS1, ITS2, ITS3, and ITS4 primers. Thus, it is either a 299 bp long fragment, when the ITS1–ITS2 primer pair is used (Figure 2), or a 411 bp long fragment, when the ITS3–ITS4 primer pair is used. These fragments can be used as an additional internal control of the PCR reaction, and their absence may indicate a PCR or DNA isolation failure (or other technical errors in the protocol). If a single pathogen, or multiple pathogens, are present, DNA fragments of more than one size should be amplified. One of them should correspond to the host DNA, while others would indicate a phytopathogenic or saprophytic infection. Species identification of phytopathogens is based on the amplicon sizes (Table 3). To improve the resolution of the method, the electrophoretic fragment analysis can be performed with PCR products amplified by both primer pairs—ITS1–ITS2 and ITS3–ITS4. It is also possible to multiplex the amplicon analysis using primers labeled by spectrally different fluorescent dyes.

The ITS regions of the rDNA were used for identification of fungal pathogen in forest trees [30], including resistance evaluation [31,32], but only by sequencing DNA from pure microbial cultures. Alternatively, the 16S rRNA terminal restriction fragment length polymorphism (T-RFLP) method was used for profiling bacterial communities [33], but it required restriction enzyme treatment. We combined these two techniques, and developed a method that allows fast and efficient detection and identification of fungal phytopathogens in plant samples without using pure cultures. We confirmed that the nucleotide structure of pathogen diagnostic loci was conservative, regardless of the geographic origin of the samples, and, therefore, the size of the amplified diagnostic loci can be reliably used for fungal species identification. This method allows studying mycobiomes of different plants, by comparing their species compositions. In addition, we plan to use this method for the evaluation of disease resistance of transgenic aspen and birch clones with a modified wood composition [34].

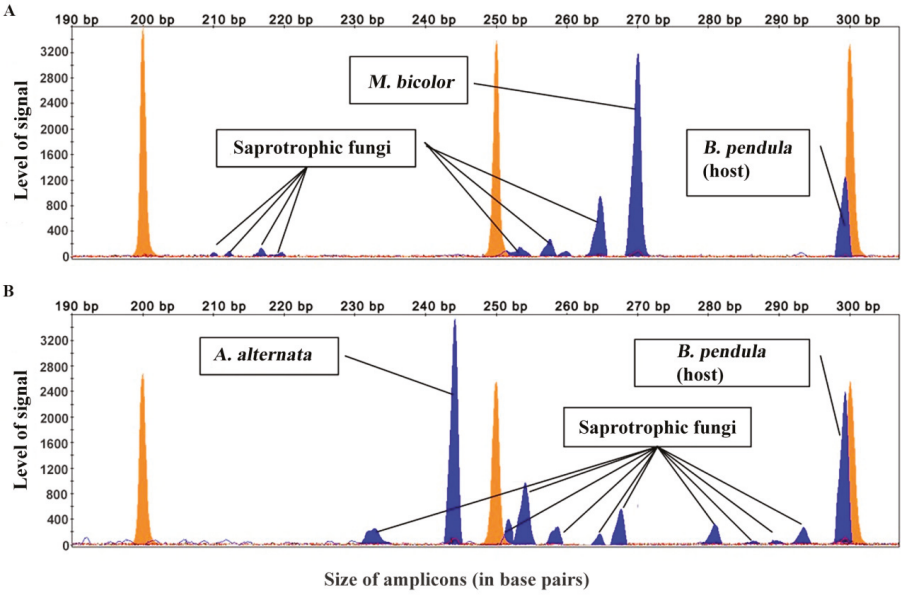

Figure 2. The example of two computer-generated electrophoregrams with multiple peaks representing species-specific fungal and host plant (*B. pendula*) amplicon DNA fragments from the two PCR-amplified samples of DNA isolated from infected silver birch leaf (**A**) and shoot (**B**) tissues, amplified using the ITS1–ITS2 primer pair combination, and separated in the capillary gel electrophoresis using an ABI Prism 310 Genetic Analyzer. Brown peaks represent the DNA fragments of the GeneScan™ 500 LIZ™ dye Size Standard.

4. Conclusions

We proposed a relatively simple method for molecular genetic detection and identification of phytopathogens, and demonstrated its efficiency on the main species of phytopathogenic micromycetes. The method is based on the PCR fragment analysis of the ITS1 and ITS2 loci, which allows for the identification of micromycetes without the need to sequence the amplicons. The proposed molecular genetic method is faster (processing time is approximately 4–5 h), does not require designing species-specific PCR primers, and is less expensive than direct sequencing. The obtained results are more reliable than those based on species-specific PCR, as cross-amplification is not a problem for this method. It is also applicable for early assessment of disease resistance in new genotypes of forest trees developed for short-rotation plantations, including both nontransgenic and transgenic clones. Moreover, this analysis allows detection and identification of not only distinct species, but also their associations, thereby enabling metagenomic analyses. Although it was tested on birch tree species, the developed PCR primers can be used to amplify pathogenic DNA isolated from any other forest tree species.

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Infection Levels of the Microsporidium *Larssoniella duplicati* in Populations of the Invasive Bark Beetle *Ips duplicatus*: From Native to New Outbreak Areas

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Abstract: The microsporidium *Larssoniella duplicati* (Weiser, Holuša, Žižka, 2006) is a specific pathogen of the bark beetle *Ips duplicatus* (C.R. Sahlberg, 1836), which is a serious pest of Norway spruce (*Picea abies* (L.) H. Karst) in Europe. From 2011 to 2016, infection levels of *L. duplicati* and other pathogens in *I. duplicatus* populations were assessed along a gradient, ranging from areas in the north, where the beetle is native, to areas in the south, where the beetle has only recently invaded. The 21 study sites ranged in altitude from 229 to 1009 m a.s.l. We found that pathogen infection levels in *I. duplicatus* populations decreased from the native areas in the north to the new areas of beetle expansion in the south. We also found that pathogen level increased with altitude. The *L. duplicati* infection levels were not associated with the infection levels of other beetle natural enemies. The infection level decreased with the length of time of beetle establishment in an area. The infection level increased with the number of beetles trapped and dissected at a site.

Keywords: *Ips duplicatus*; pathogen; vector; infection level; invasion; latitude

1. Introduction

Changes in climate and land use can increase the spread of organisms [1]. Many of these organisms are non-native to their new area of distribution; some spread to new areas but also increase their population densities in their former areas [2,3]. In some cases, such invasive species begin to damage habitats that are important for humans, like forests with fast-growing tree species [4]. One of the most commercially important tree species in Europe is the Norway spruce (*Picea abies* [5]). This tree is attacked by many species of bark beetles of which *Ips typographus* (Linnaeus, 1758) is the most important in terms of loss of mature trees before final cutting [6].

The double-spined spruce bark beetle *Ips duplicatus* is a native species in Scandinavia, eastern and northern parts of central Europe and northeast Asia, where it occurs on Norway spruce. The beetle is currently spreading to Norway spruce in many parts of Europe. Its high outbreak potential is

supported by climatic change, the physiological weakness of trees, and the attack of such weakened trees by the fungus *Armillaria ostoyae* (Romagn. Herink, 1973) and other pathogens [7,8]. Current studies focusing on wind and bark beetle disturbances suggest increase damages in Europe under climate change [9–11]. The combination of increasing frequency of drought events, Norway spruce planting in non-native habitats and warmer temperatures are considered important predisposing factors triggering the double-spined spruce bark beetle outbreaks. As a result of these factors affected by climate change, the number of *Ips duplicatus* generations is increasing to two to three during one vegetation period in the central European area [12].

From the beginning of the 20th century, the beetle began spreading from its origin in the Palearctic region to the south because spruce monocultures were being increasingly established in the south in Europe [8], unlike most other bark beetle invasions that extend from south to north [13,14]. *I. duplicatus* was first noted in eastern Czech Republic and south Poland in 1960s [15–17]. That area experienced massive *I. duplicatus* outbreaks in the 1990s. During the last 200 years, Norway spruce has been planted in many areas of Europe, mainly out of the natural range of this tree. As the planted trees are growing out of their natural range, they may be stressed [18], and this has increased the spread of *I. duplicatus* to southern Europe [19]; *I. duplicatus* has even been recorded in south Slovakia [15,20] and throughout Romania [21].

The microsporidium *Larssoniella duplicati* appears to be a specific pathogen of *I. duplicatus*; its presence in other spruce bark beetles, such as *I. typographus*, *Pityogenes chalcographus* (Linnaeus, 1761), and *Ips amitinus* (Einchoff, 1871), has not been reported [22–24]. This specificity of *L. duplicati* is not as usual among pathogens of bark beetles; i.e., the same pathogen usually occurs in multiple bark beetle species, but other examples are known [25–29].

L. duplicati was first described in the Czech Republic and Poland [24], where its infection levels in *I. duplicatus* populations are stable and where the disease is probably chronic [22]. This microsporidium infects the midgut muscularis, the ovaries, and the Malpighian tubules of adult beetles. The infection is always in the infected tissue, because infected muscle fibres hold the spores in position [24]. Its infection levels in the native area of beetle (Scandinavia) and the new outbreak area (Romania) have not been studied [23].

The current study had two objectives. The first was to compare the infection levels of *L. duplicati* in the native and new outbreak areas of *I. duplicatus* in Europe. The second objective was to identify variables associated with differences in *L. duplicati* infection levels in *I. duplicatus* populations.

2. Materials and Methods

Pathogens of *I. duplicatus* were studied at 21 sites: four in the Czech Republic, five in Romania, eight in Poland, and four in Sweden. The altitudes of study sites ranged from 229 to 1009 m a.s.l. (Figure 1). During the years of 2011–2016, beetles were collected using Theysohn pheromone traps (Theyson Kunststoff. GmbH, Germany) or Intercept traps (only in Romania) baited with pheromone lures ID Ecolure (FYTOFARM Group s.r.o., Slovakia), Pheagr IDU (Sci-Tech, s.r.o, Czech Republic), Duplodor (Chemipan, Poland), or an experimental lure (Romania) [30] (Table 1). In all used pheromone lures, the main compound is always E-myrcenol—the main aggregation pheromone component for *I. duplicatus* [31]. The pheromone lures were changed after 10 weeks. Each site was sampled in only 1 or 2 years.

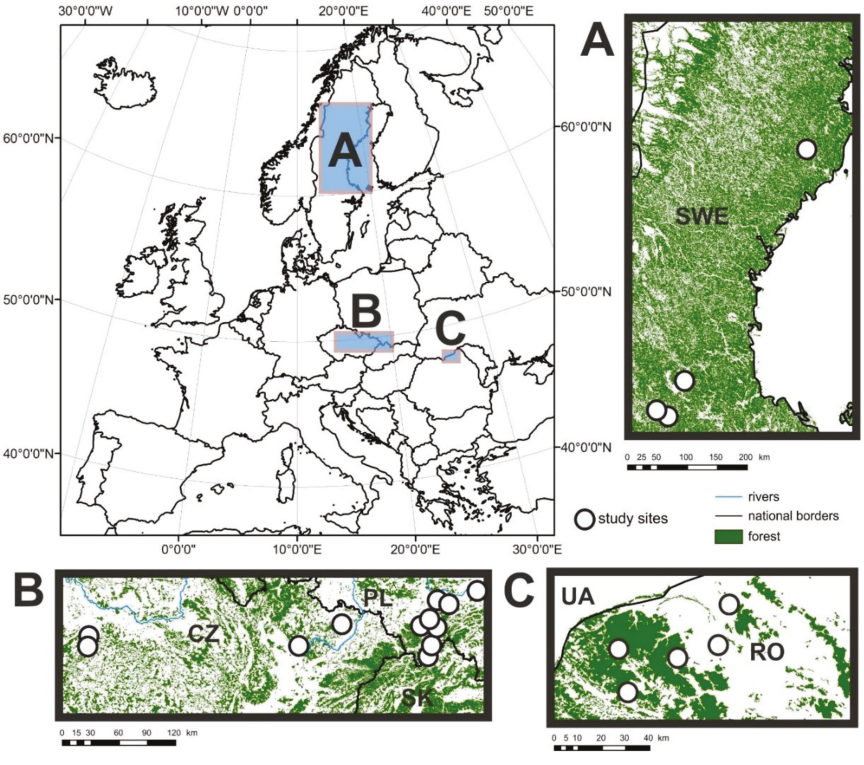

Figure 1. Study sites (circles) in Europe where *Ips duplicatus* was collected during 2011–2015 in forest areas (green).

Beetles were collected from the beginning of May to the end of August. In the study sites, flight barrier traps were placed 1.5 m above the ground and approximately 15–20 m from a standing spruce tree that was more than 30 years old. All forest stands at study sites were composed of a mosaic of trees of all ages, so there was enough suitable material for *Ips duplicatus* infestation.

Trapped beetles were placed in Eppendorf micro-test tubes with a piece of damp gauze to maintain humidity. The tubes were stored frozen until the beetles were dissected.

Each beetle was identified to species [32] and then dissected by removing the gut, Malpighian tubules, ovaries, and the body fat. The dissected tissues were examined with a light microscope (Nikon Eclipse 50 Ni, Nikon Instruments Inc., Melville, NY, USA) at 40 to 400× magnification to determine the presence of *L. duplicati* (oval spores of two sizes, 3–3.5 × 1.5–2 and 2–2.5 × 1.5 in intestinal muscles) and other pathogens and nematodes.

Data concerning the distribution of coniferous forests relative to the study sites were obtained from [32] and were corrected using Corine Land Cover. The program ArcMap 10.0 (ESRI, Redlands, CA, USA) was used to create Figure 1, which shows the distribution of the study sites.

Basic statistical analyses were performed in Statistica 13.1 (Dell software, Austin, TX, USA). We used the Shapiro Wilk test to determine the normality of the data (infection level). The Wilcoxon matched pair test was used to compare infection levels between sexes (percentages of infected males vs. females). Non-parametrical analyses were used as a control for the potential influence of local differences in infection levels at the country level.

Detailed analyses were done in SAM v4.0 [33]; we computed Moran's I to assess the spatial autocorrelation of our dependent variable (infection level of *L. duplicati*) in seven distance classes.

We assessed the relationships between infection level of *L. duplicati* (the percentage of infected individuals at a site, and the dependent variable) and the following independent variables: altitude, latitude (north-south gradient), longitude (east-west gradient), infection level of *Chytridiopsis typographi* parasitism by intestinal nematodes, parasitism by hemolymph nematodes, number of *I. duplicatus* beetles captured and dissected, and year (time of beetle collection). For linear regression of infection level on independent variables, infection level data were arcsine square root transformed to obtain normality. Analyses of the interaction among studied independent variables indicated multicollinearity for longitude (VIF = the variance inflation factor >2), which was the variable that described the east-west gradient in outbreak area of *I. duplicatus*. Thus, longitude was not further analysed. As some independent variables were not significant, we selected variables for inclusion in the final model based on AICc (Akaike information criterion with correction for small sample sizes) as implemented in SAM. In further analyses of *L. duplicati*, we used seven independent variables: latitude (north-south gradient); the infection level of the microsporidium *Chytridiopsis typographi*; the parasitism by intestinal nematodes; the parasitism by nematodes in hemolymph (hereafter termed hemolymph nematodes); the altitude of the study site; the number of *I. duplicatus* beetles trapped and dissected at a site; and the year of beetle collection.

Table 1. Background information on the study sites where *I. duplicatus* specimens were collected and assessed for pathogen infection. Country of origin (Country): Sweden (SWE), Poland (PL), Czech Republic (CZ), Romania (RO). In traps were used different pheromone lures: ID Ecolure, Duplodor, Pheagr IDU and in Romania the experimental lure (exp. lure) [30].

Study Sites	Country	GPS Coordinates		Year of Collection	Pheromone Lure	Altitude (m a.s.l.)
		N	E			
Nås	SWE	60.4677	14.5003	2014	ID Ecolure	232
Siljansfors	SWE	60.9730	15.0578	2014	ID Ecolure	324
Vansbro	SWE	60.5229	14.2389	2014	ID Ecolure	229
Vindeln	SWE	64.2000	19.7833	2014	ID Ecolure	291
Petkówka	PL	49.7333	19.2333	2015; 2016	Duplodor	668
Rajcza	PL	49.7666	19.2333	2015; 2016	Duplodor	646
Romanka Górna I	PL	49.5805	19.2246	2016	Duplodor	829
Romanka Górna II	PL	49.9338	19.3989	2015	ID Ecolure	1009
Sopotnia Dolna	PL	49.9350	19.4664	2015	ID Ecolure	953
Tokarnia	PL	49.9833	19.9833	2015	ID Ecolure	688
Ujsoły	PL	49.7508	19.2009	2015; 2016	Duplodor	859
Złatna	PL	49.4833	19.1666	2015	ID Ecolure	638
Hlubočky	CZ	49.6920	17.4146	2013	ID Ecolure	382
Jílové u Prahy I	CZ	49.8866	14.5055	2016	Pheagr IDU	354
Jílové u Prahy II	CZ	49.9166	14.5071	2016	Pheagr IDU	457
Pustá Polom	CZ	49.8510	18.0242	2014	ID Ecolure	454
Calafindești	RO	47.8513	26.1459	2011	exp. lure	497
Ionu	RO	47.6134	25.4817	2013	exp. lure	1080
Solca	RO	47.7000	25.7963	2013	exp. lure	625
Sucevița	RO	47.7767	25.4817	2013	exp. lure	605
Todirești	RO	47.7127	26.0328	2013	exp. lure	415

3. Results

A total of 1539 adults of *I. duplicatus* from the 21 study sites located throughout the Czech Republic, Romania, Poland, and Sweden were dissected and analyzed.

The *L. duplicati* infection level in *I. duplicatus* populations (i.e., the percentage of specimens at a site with *L. duplicati*) across all countries averaged ± standard error (SE) 16.7% ± 8.4% and ranged from 0% to 39.1%. *L. duplicati* was detected in 20 of the 21 sites (Table 2). *L. duplicati* infection levels did not significantly differ between *I. duplicatus* sexes ($Z = 1.33$, $p > 0.05$). Infection occurred only in the intestinal muscles of *I. duplicatus*.

Table 2. Infection levels of four pathogens in *I. duplicatus*. Infection level refers to the percentage of beetles with the indicated pathogen. The location of the study site (Country): Sweden (SWE), Poland (PL), Czech Republic (CZ), Romania (RO). For each study site there is a number of inspected beetles (N) and infection levels of: *Larssoniella duplicati* (*L.d.*), *Chytridiopsis typographi* (*C.t.*), parasitism by intestinal nematodes (I.n.) and hemolymph nematodes (H.n.).

Study Sites	Country	N	*L.d.* (%)	*C.t.* (%)	I.n. (%)	H.n. (%)
Nås	SWE	46	39.1	-	15.2	-
Siljansfors	SWE	70	21.4	1.43	10.0	4.3
Vansbro	SWE	156	16.7	-	3.2	1.3
Vindeln	SWE	72	23.6	-	11.1	5.6
Petkówka	PL	107	19.6	-	3.8	4.6
Rajcza	PL	103	13.6	-	14.1	5.5
Romanka Górna I	PL	27	7.4	-	14.8	-
Romanka Górna II	PL	192	20.8	-	4.7	7.3
Sopotnia Dolna	PL	35	25.7	-	5.7	2.9
Tokarnia	PL	139	19.4	-	6.5	3.6
Ujsoły	PL	22	9.1	-	13.6	9.1
Złatna	PL	20	10.0	-	10.0	-
Hlubočky	CZ	22	13.6	-	18.2	4.6
Jílové u Prahy I	CZ	18	-	-	5.6	-
Jílové u Prahy II	CZ	43	7.0	2.3	4.7	4.7
Pustá Polom	CZ	237	27.4	0.8	10.1	1.7
Calafindești	RO	20	20.0	-	10.0	-
Ionu	RO	33	18.2	-	12.1	3.0
Solca	RO	80	11.3	-	3.8	3.8
Sucevița	RO	45	8.9	-	13.3	6.7
Todirești	RO	52	1.9	-	5.8	9.6

Average levels of *L. duplicati* infection did not significantly differ among countries ($H = 4.96$; $p > 0.05$). The *L. duplicati* infection level increased from south to north, averaging 12.1% ± 6.5% in Romania, 15.7% ± 6.1% in Poland, 16.1% ± 8.5% in the Czech Republic, and 25.2% ± 8.4% in Sweden (Table 2).

The microsporidium *Chytridiopsis typographi* ((Weiser, 1954) Weiser, 1970) was found at only three study sites, and these were in the Czech Republic and Sweden. Its infection levels were very low (Table 2).

In contrast, nematodes were found in *I. duplicatus* at 21 study sites. The parasitism rate ranged from 3% to 16% for intestinal nematodes and from 0% to 10% for hemolymph nematodes (Table 2). For both kinds of nematodes, average parasitism rate did not significantly differ among countries (intestinal nematodes: $H = 0.08$; $p > 0.05$; nematodes in the hemolymph: $H = 0.81$; $p > 0.05$).

The spatial autocorrelation for *L. duplicati* infection levels was not significant (Table 3). This indicated that infection levels tended to be randomly distributed in space, without a tendency toward clustering or regular spacing. The expected Morans I value was −0.06.

Table 3. Statistics for spatial autocorrelation analysis of *L. duplicati* infection levels in *I. duplicatus* populations at the 21 sites in Europe.

Distance Class	Distance Centre	Moran's I	*p*
1	45.2	0.1	0.6
2	306.6	0.1	0.7
3	650.3	−0.2	0.2
4	877.8	−0.1	0.6
5	1137.8	0.1	0.7
6	1510.1	−0.1	0.9
7	1975.8	−0.4	0.1

In regression analyses, the *L. duplicati* infection level was significantly related to altitude, latitude, year, numbers of dissected beetles, and the infection level of all other pathogens ($F = 6.63$; $p < 0.01$; Table 4). A regression model with all of the variables listed in Table 4 (significant and non-significant) explained a total of 71.2% of the adjusted variance in the *L. duplicati* infection level. The *L. duplicati* infection level was not significantly related with the infection levels of *C. typographi*, parasitism by intestinal nematodes, or hemolymph nematodes. The *L. duplicati* infection level significantly increased with latitude, altitude, and the number of beetles captured and dissected at a site, but significantly decreased with the year of the study (Table 4).

Table 4. Results for a regression model describing the relationship between the *L. duplicati* infection levels in *I. duplicatus* populations and the following variables: latitude (north-south gradient); infection level of *C. typographi*; parasitism by intestinal nematodes; parasitism by hemolymph nematodes (i.e., nematodes detected in the hemolymph); altitude; number of *I. duplicatus* beetles captured and dissected; and year (date of beetle collection). Variance Inflation Factor (VIF), corrected Akaike's Information Criterion (AICc) = −11.93. Significant variables are in bold.

Variable	VIF	*t* Value [a]	*p* Value
Constant		**3.1**	**0.01**
Latitude	**1.1**	**3.5**	**0.01**
C. typographi	1.3	1.4	0.18
Intestinal nematodes	1.4	0.8	0.43
Nematodes in hemolymph	1.1	−0.8	0.46
altitude	**1.4**	**3.8**	**0.01**
number	**1.1**	**2.9**	**0.02**
year	**1.6**	**−3.4**	**0.01**

[a] Positive and negative *t* values indicate positive and negative associations, respectively.

In the next step of the statistical analysis, we deleted non-significant variables from the model; the resulting model explained 70.1% of the adjusted variance in (r^2adj = 0.701; Table 5). We found that only significant variables from the previous regression left in the model and their *p* values were more significant, except of number of dissected beetles.

Table 5. Results of the model that best described (delta AICc <2 based) the relationship between the *L. duplicati* infection level in *I. duplicatus* populations (arcsine square root transformed). The best model included four predictor variables: latitude (north-south gradient); altitude; number of *I. duplicatus* beetles captured and dissected; year (date of beetle collection). Variance inflation factor (VIF). Corrected Akaike's Information Criterion (AICc) = −25.95 (significant variables are in bold).

Variable	VIF	*t* Value	*p* Value
Constant		**3.9**	**0.002**
Latitude	**1.0**	**4.0**	**0.002**
Altitude	**1.2**	**3.7**	**0.002**
Number	**1.0**	**2.8**	**0.020**
Year	**1.1**	**−3.9**	**0.002**

4. Discussion

The current research studied the species-specific pathogen *L. duplicati* associated with the double-spined spruce bark-beetle in areas where *I. duplicatus* is native, as well as in areas where *I. duplicatus* is newly established in Europe. We found two interesting patterns: *L. duplicati* infection levels in *I. duplicatus* populations significantly decreased across the latitudinal gradient from the north to the south and significantly increased with increasing altitude.

In the areas where *I. duplicatus* is native, the *L. duplicati* infection level was as high as 30%; in the areas experiencing new outbreaks of the beetle, infection levels varied around 10% [34]. In the current

study, the highest infection level was 39.1%, and the infection level was higher than 10% at most of the study sites, what is consistent with previous reports [22–24]. The spatial distribution of infection levels was not influenced by the spatial arrangement of the study sites (i.e., sites with high or low infection levels did not tend to cluster in space). This was true even though some of the sites, especially those in Poland and the Czech Republic, were located near areas with spruce forests that have been highly stressed by drought and fungal diseases. Such stressed forests typically support higher population densities of *I. duplicatus* than non-stressed forests [20,35]. Generally, latitude-altitude gradient can be explained by increasing number of individuals in population at northern study sites and in long-term outbreak areas. Study sites with more abundant populations of bark beetles are collected more often and with higher infection levels of pathogens [36].

We also suspect that *L. duplicati* may influence the invasive potential and spread of *I. duplicatus*. This is because *L. duplicati* is likely to reduce the fitness of the infected beetles and infection level is growing more slowly in the newly established outbreak areas. In addition to being infected by *L. duplicati*, *I. typographus* and related bark beetles are also attacked by ectoparasitoids and by the pathogen *Mattesia schwenkei* Purrini, 1977. Infection level of this antagonists of *I. typographus* had lower infection levels in areas with new outbreaks of the beetle than in areas with long-lasting outbreaks (more than 10 years) of the beetle [37]. When bark beetle numbers are low or when contacts between individuals in breeding systems are limited, e.g., as is the case in managed forests, there is a reduced probability of pathogen transmission and therefore a low infection level of some common pathogens [23].

The infection level of *L. duplicati* increased with the number of individuals dissected at a site. Nevertheless, the infection levels do not change with changes in host population density [22–24,34], which suggests that transmission is vertical rather than horizontal [23], as it is for some other microsporidium pathogens [29,38]. Therefore, it is unclear why the infection level should increase with number of analyzed beetles of *I. duplicatus.* In the case of horizontally transmitted pathogens, the infection levels sometimes double or triple during the beetle reproductive period of even one generation [39]. In the current study, the main factor associated with low *L. duplicati* infection levels in *I. duplicatus* was the length of time that the area had been infested with the beetle. This effect of time since beetle establishment is somewhat unclear in the current study, however, the latter factor was confounded with collection date.

I. duplicatus produces only one generation per year in the boreal forests and in northern Poland [8,40] but up to three generations per year in Central Europe [12,15,41]. Although new outbreaks of *I. duplicatus* occur only sporadically at higher altitudes [16,20,42–44], the *L. duplicati* infection level was related to altitude in the current study. This could be explained by the relationship between latitude and altitude, i.e., the more southern sites had both low infection levels and low altitudes.

We also found that the *L. duplicati* infection level did not differ between *I. duplicatus* sexes or among the studied countries, which is consistent with previous reports for *L. duplicati* as well as for other pathogens of bark beetles [39,45].

The only insignificant relationships between *L. duplicati* infection levels and the other variables were with the infection levels of *C. typographi*, parasitism by intestinal nematodes, and hemolymph nematodes. Nematodes and *C. typographi* are the most frequently reported antagonists of *I. duplicatus* [23,26,27,46]. The infection level of *C. typographi* is often very low [23]. In our study, we found *C. typographi* at only three sites, and infection was always less than 2.4%, suggesting that *C. typographi* was probably not affecting *I. duplicatus* population density. Parasitic nematodes are commonly associated with *I. duplicatus*, occurring in more than 70% of the beetle's gallery systems [46–49]. As in the case of *C. typographi*, nematodes did not appear to affect *L. duplicati* infection levels.

5. Conclusions

L. duplicati is probably a chronic pathogen of *I. duplicatus* and might have little or even no negative effect on the beetle—especially out of the native distribution area of its host. This microsporidium may negatively influence the flight capability of pioneer beetles and their ability to successfully invade new host trees, but in time (few years) the infection level of this microsporidium increases in a new population and the differences are minimized. The infection levels of *L. duplicati* in *I. duplicatus* populations decreased with latitude; it was highest in the north (Sweden), where the beetle is native, and was lowest in the south (Romania), where the beetle has only recently invaded. This is most probably connected with colonization aspect of a new sites. Infection levels increased with altitude, but the effect of altitude was confounded with the effect of latitude.

The most important conclusions of our research on an alien pest and its pathogen is that they follow a latitude-altitude gradient. This most probably reflects fact that spread of pathogen is prolonged (e.g., similar to known escape from enemies' hypothesis in bark beetles) [50]. Nevertheless, altitude in coincidence with latitude, indicate some climatic limits of the pathogen—as north sites and high elevations are often more cold and wet than the opposite. This is also important regarding pest management. Even if *L. duplicati* does not have a strong impact on alien bark beetle, its virulence could have some impact on invasive success of the bark beetle.

Author Contributions: S.Z., K.R., and H.V. provided methodology of research and collected field or laboratory data; J.H. (Jakub Horák) and J.T. performed the statistical analyses, and the visualizations; M.K., A.L., and M.-L.D. managed the research and collection of material in their country and took part in finalizing the manuscript; K.R., J.H. (Jakub Horák) and J.H. (Jaroslav Holuša) supervised the research and edited the manuscript. All authors provided feedback on the manuscript and approved the final manuscript.

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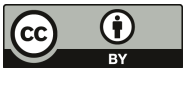

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