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Selected Papers from the 1st International Electronic Conference on Entomology

Edited by
Nickolas G. Kavallieratos

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Editor

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Contents

About the Editor

Nickolas G. Kavallieratos

Nickolas G. Kavallieratos is an Associate Professor of Agricultural Entomology and Acarology at the Agricultural University of Athens (AUA). During 2002-2014, he was a researcher at Benaki Phytopathological Institute (BPI) serving in all scientific ranks and administration positions (Head of the Laboratory of Agricultural Entomology, Head of the Department of Entomology and Agricultural Zoology, and Deputy Director) at BPI. He has also worked at several universities and institutes in Greece, Europe and Africa. Since 2009, he has been a frequent visiting scientist at the United States Department of Agriculture (USDA), Center for Grain and Animal Health Research, in Manhattan KS. Since 2001, he has also been a frequent visiting scientist at the Faculty of Biology, University of Belgrade. He studies morphology, morphometry, systematics, zoogeography, phylogeny, genetics and behavior of the Aphidiinae (Braconidae) parasitoids of aphids, providing descriptions of new species in science, redescriptions of species and genera, revisions of genera and reviews around the world (Europe, Asia, Africa, and North and South America) since 1994. The overall outcome of his research deals with the generation of tools that enable entomologists to identify Aphidiinae parasitoids with ease in different types of habitats, host plants and host aphids. He has also been working on the biology, ecology, demography, mating behavior and management of stored-product pests (insects, mites) with the use of natural (diatomaceous earths and other inert dusts), biological (entomopathogenic fungi, entomopathogenic nematodes and bacteria), chemical insecticides, and novel chemical or natural compounds (essential oils, nanoemulsions and microemulsion) that exhibit insecticidal properties. Prof. Kavallieratos has published >300 papers in peer-reviewed journals.

Preface to "Selected Papers from the 1st International Electronic Conference on Entomology"

This Special Issue was compiled in cooperation with the 1st International Electronic Conference on Entomology (IECE), organized by *Insects* from 1 to 15 July 2021 on the MDPI Sciforum platform. This Special Issue of *Insects* retains the topical subdivisions from the conference in the following fields: Systematics and Morphology, Genetics and Genomics, Biology, Behavior and Physiology, Biodiversity, Ecology and Evolution, Pest Management, Forest and Urban Entomology, Medical and Veterinary Entomology, Apiculture and Pollinators.

Nickolas G. Kavallieratos
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Editorial

Special Issue: Selected Papers from the 1st International Electronic Conference on Entomology

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The 1st International Electronic Conference on Entomology (1IECE) was held between 1 and 15 July 2021 on the MDPI Sciforum platform organized and funded by the international journal *Insects*. This event provided an opportunity for scientists from around the globe to communicate their most recent research findings in entomology. In the last decade, there has been tremendous development of entomological research, leading to the publication of thousands of important studies. 1IECE aimed to rapidly spread worldwide advances in insect science to the entire scientific community through the publication of proceedings of selected papers in a Special Issue (SI). This SI employed the same eight fields used for topical subdivisions during 1IECE: Systematics and Morphology, Genetics and Genomics, Biology, Behavior and Physiology, Biodiversity, Ecology and Evolution, Pest Management, Forest and Urban Entomology, Medical and Veterinary Entomology, and Apiculture and Pollinators. In total, 21 1IECE presentations have been published in the journal *Insects* through the traditional peer review process. The contributors to this Proceedings SI represent 23 countries from across the globe: Togo, Senegal, Italy, China, Pakistan, Saudi Arabia, Greece, Serbia, Hungary, Poland, Portugal, Australia, Switzerland, Japan, Russia, The Netherlands, Cyprus, USA, Spain, South Africa, France, United Kingdom, and Argentina.

The studies in this SI deal with various interesting aspects of research, i.e., identification of termite species in West Africa [1], characterization of the microbial symbionts of *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae) populations [2], the contribution of bacterial symbionts to the thermal tolerance of two aphid species *Rhopalosiphum padi* (L.) and *Sitobion avenae* (F.) (Hemiptera: Aphididae) [3], recording the nematode fauna in Greek forests [4], investigation of genetic variability in *Apis mellifera* L. (Hymenoptera: Apidae) from Serbia through microsatellite loci [5], comparison of damage caused to tomato plants by the biocontrol agents *Nesidiocoris tenuis* (Reuter) (Hemiptera: Miridae) and *Dicyphus cerastii* Wagner (Hemiptera: Miridae) [6], evaluation of numerous essential oil-based microemulsions as grain protectants for management of two major stored-product insects, *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae) and *Trogoderma granarium* Everts (Coleoptera: Dermestidae) [7], investigation of several aspects of the life history of the biological control agent *Neoleucopis kartliana* (Tanasijtshuk) (Diptera: Chamaemyiidae) in Greece [8], validation of theoretical models explaining the persistence of mtDNA variation within populations of *Drosophila obscura* Fallén (Diptera: Drosophilidae) [9], description of how thermal conditions, sex, and population origin may affect stress resistance in *Drosophila subobscura* Collin (Diptera: Drosophilidae) [10], the genetic structure of *Corythucha ciliata* (Say) (Hemiptera: Tingidae) based on mitochondrial DNA analysis [11], utilization of ecological/geographical models to evaluate changes in the distribution of *Oedaleus decorus* (Germar) (Orthoptera: Acrididae) [12], investigation of exposure to heavy metals and population origin on the diversity of microbiota and fitness in *Drosophila melanogaster* Meigen (Diptera: Drosophilidae) and *D. subobscura* [13], a database and checklist of alien insects in Greece [14], evaluation of direct and delayed mortality caused by the anthranilic diamide chlorantraniliprole to adults and larvae of *T. castaneum*, adults of *Rhyzopertha dominica* (F.)

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(Coleoptera: Bostrychidae), adults of *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae) and adults and nymphs of *Acarus siro* L. (Sarcoptiformes: Acaridae) [15], identification of *Pseudococcus jackbeardsleyi* Gimpel and Miller (Hemiptera: Pseudococcidae) and *Maconellicoccus hirsutus* Green (Hemiptera: Pseudococcidae) as potential vectors of cacao mild mosaic virus (CaMMV) [16], defining relationships between coccinellids and aphids on alfalfa in Spain [17], exploration of the diversity and phylogeny of Tingidae species occurring on olive trees in South Africa using morphology and mitogenome sequence [18], and examination of the interaction between *T. castaneum* and *Aspergilus flavus* Link (Eurotiales: Trichocomaceae) in maize flour [19]. Furthermore, this SI includes two review papers addressing the current (2010–2021) global knowledge on taxonomy of Aphidiinae (Braconidae) [20] and adaptation of Echinophthiriidae (Anoplura) surviving in places unfavorable to all other insects [21].

Finally, I would like to thank all authors for their fine contributions, the Academic Editors of Insects Bessem Chouaia, Brian T. Forschler (Editor-in-Chief of *Insects*), Natsumi Kanzak, Silvio Erler, Thomas W. Phillips, Tibor Magura, Marco Salvemini, and David Schlipalius for their critical decisions on certain manuscripts, the reviewers for the time they spent to carefully examine the manuscripts and make valuable suggestions, and the editorial team of *Insects* who processed manuscripts for the SI. My special thanks go to Barbara Wang, Assistant Editor of *Insects*, who exhaustively worked for several months with me to make this important SI come to fruition.

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Article

Record of New Termite (Blattodea, Termitidae) Species in Togo West Africa †

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† This published work and the nomenclatural acts it contains have been registered in ZooBank, the online registration system for the ICZN (International Code of Zoological Nomenclature). The LSID (Life Science Identifier) for this publication is: urn:lsid:zoobank.org:pub:3C2C4571-129C-4BD5-8A95-98F7FC17C5EB.

Simple Summary: In the sub-Saharan regions of Africa, there are many termite species, of which very few have been correctly identified and described. The large majority of these species is either wrongly identified or waiting to be found and described because of the lack of identification keys and the errors within the existing keys. One way to overcome this problem is the use of reference works that contain illustrated parts of the body of termites along with accurate measurements of the features involved in termite identification. The purpose of this study is to provide pictures of the heads of soldiers (commonly used in termite identification) along with measurements of parts of the head and leg. A total of 12 termite species were examined. Seven of these species were already described, while the other five appear to have not been described before. Ten out of the twelve species are new records for the country.

Abstract: In Africa, despite their economic and ecological importance, termites are still relatively unknown. Their systematic remains uncertain, the approximate number of species for many biogeographic areas is underestimated, and there is still confusion in the identification of the species for many genera. This study combined morphological traits with morphometric measurements to determine several species collected in Togo and provided head illustrations of soldiers. Termites were sampled within the frame of transects laid in several landscapes inside three different parks including: Fosse aux Lions, Galangashie, and Fazao Malfakassa. Samples were grouped by morphospecies and measurements of part of the body (length and/or width of head, mandible, pronotum, gula, and hind tibia) were conducted. Twelve termite species including *Foraminitermes corniferus*, *Lepidotermes* sp., *Noditermes cristifrons*, *Noditermes* sp. 1 and *Noditermes* sp. 2, *Promirotermes holmgren infera*, *Promirotermes* sp., *Unguitermes* sp., *Amitermes evuncifer*, *A. guineensis*, *A. truncatus*, and *A. spinifer* were separated and pictured. Ten new species were added to the check list of the country, including five unidentified ones. Further studies such as biomolecular analysis should be carried out in order to clarify the status of these unknown species.

Keywords: termite systematic; morphological traits; morphometric measurements

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1. Introduction

Termites species and generic richness (particularly in the central and west part of the continent) is very important [1–3]. Most termite species are found in tropical forest, undisturbed savanna, and protected parks [4–7]. Although several studies have been conducted (and are still ongoing) on African termites, the diversity and the taxonomy of these termites remain poorly documented. Many species are waiting to be identified, while

others have been incorrectly identified [8–11]. These taxonomical problems were pointed out by a group of researchers working on African termites [12].

In fact, the identification of African termites is based not only on the comparison of samples with reference species (which most of the time have not been correctly identified) but also with reference works by famous taxonomists [13–19]. These reference works combine morphological traits (shape, color of different parts of the body) and morphometric measurements (length, width, and depth) of certain parts of the body of the soldier caste. The worker caste of termites is also used for identification, and the same features as soldiers are examined or measured [20]. In addition to the external morphological traits, gut anatomy is often used to identify termites [21]. All these descriptions are sometimes illustrated by hand drawing of the whole or part of the body of the termites instead of color images. This weakness is understandable as most of these reference works were carried out and published a century ago with tools and means that did not allow color images. However, since then, very few revisions have been conducted on African termite species. Among the useful reference works, several are also written in languages such as German [15], Italian [13,14] and French [8,9,17,18], which unlike English are not easily accessible to many researchers. Fortunately, recent research on termite taxonomy, in addition to using English, also uses images of the whole or part of the body of termites [22,23]. The research on termites in Togo (a West African country) has been hampered by the above-mentioned issues. The check list of termite species of the country needs to be established. Although some recent studies have been carried out on the systematization of termite species, many areas of the country have still not been prospected, and their respective species remain unknown. The purpose of this study is to contribute to the knowledge of termites species in the country and to share color images along with the morphological features and morphometric measurements of several emblematic species from the central and northern parts of Togo.

2. Materials and Methods

2.1. Study Areas

Termites were collected from three different parks (Figure 1) including: Fosse aux lions (10°46′–10°49′ N and 0° 11′–0°14′ E), Galangashie (10°19′–20°28′ N and 0°14′–0°27′ E), and Fazao-Malfakassa (8°20′–9°35′ N and 0°35′–1°02′ E).

Fosse aux lions and Galangashi, both located in the northern part of Togo, are characterized by a Sudanian tropical climate with a long dry season (November to May) and a long rainy season (June to October). In these two parks, the mean temperatures range from 29 ± 2 °C during the rainy season to 30 ± 3 °C during the dry season. The annual rainfall is 986 mm, and the landscape is shrubby savanna. Fazao-Malfakassa, located in the center of the country, is characterized by a semi-humid tropical climate with a rainy season from April to October and a dry season from November to March. The mean temperatures range from 27.5 ± 1.5 °C during the dry season to 27 ± 2 °C during the rainy season. The annual rainfall is 120 mm, and the landscape is composed of dry forests, gallery forests, shrubby savanna, and fallows.

2.2. Termites Sampling

A total of 27 sampling sites were prospected with three transects per sampling site (81 transects for the whole study). The standard protocol [24] adapted to the savanna ecosystem [4,25] was used. Each transect of 100 × 5 m was divided into 20 sampling units of 5 × 2 m, which were sampled for 15 min [26,27]. Termites were searched within the frame of each sampling unit inside mounds, litter, wood, and grasses on trees by two well-trained collectors. After this searching on the surface, termites were also searched throughout eight soil scraps.

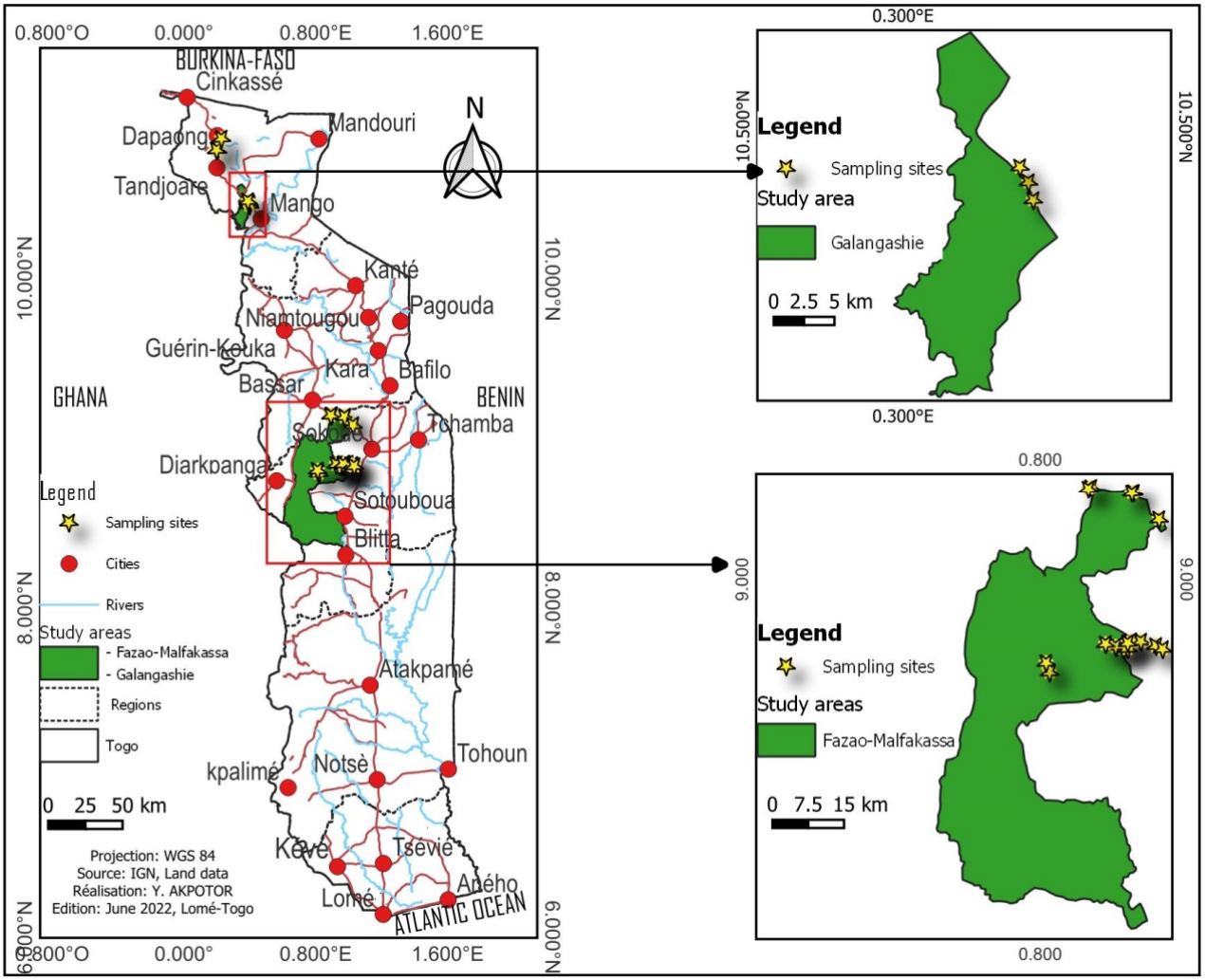

Figure 1. Map of Togo with prospected parks.

2.3. Termites Identification

Morphological traits (shape of the mandibles and the position of the mandible tooth) of the soldier and the number of antennal segments were used to separate species. Measurements of head width and length, left mandible length, pronotum width, and hind tibia length were made with a stereomicroscope (Leica EZ4) equipped with an integrated camera connected to a computer. Voucher specimens are conserved in the "Laboratoire d'Entomologie" of the University of Lomé (Lomé, Togo).

2.4. Statistical Analysis

A total of 5 individual soldiers (when possible) were used for morphometric measurements. Thus, the morphometric data are presented as the mean of the measurements of each of chosen morphological feature from 5 individual soldiers.

Factorial discriminant analysis (using morphometric data) was used to separate species with close measurements within the same genus. XLSTAT (version 6.1.9. 2003 Addinsoft, Inc., Broklyn, NY, USA) software was used for the factorial discriminant analysis.

3. Results

Twelve termite species belonging to seven genera and three subfamilies (Table 1) were examined in this study. All these species belonged to the Termitidae family. Except for *A. evuncifer* and *A. guineensis*, the other 10 species were recorded for the first time in Togo.

Table 1. Termite species examined.

Subfamily	Species	Distribution
Foraminitermitinae Holmgren, 1912	*Foraminitermes corniferus* (Sjöstedt, 1905)	Fazao-Malfakassa
Cubitermitinae Weidner, 1956	*Lepidotermes* sp.	Fazao-Malfakassa
	Noditermes cristifrons (Wasmann, 1911)	Fazao-Malfakassa, Galangashi
	Noditermes sp. 1	Fazao-Malfakassa, Galangashi
	Noditermes sp. 2	Fazao-Malfakassa, Galangashi
	Unguitermes sp.	Fazao-Malfakassa
Termitinae Latreille, 1802	*Amitermes evuncifer* (Silvestri,1912)	Fazao-Malfakassa, Galangashi, Fosse aux lions
	Amitermes guinensis (Sands, 1992)	Fazao-Malfakassa, Galangashi, Fosse aux lions
	Amitermes spinifer (Silvestri, 1914)	Galangashi, Fosse aux lions
	Amitermes truncatidens (Sands, 1959)	Fazao-Malfakassa, Galangashi, Fosse aux lions
	Promirotermes holmgreni infera Silvestri, 1914	Fazao-Malfakassa
	Promirotermes sp.	Fazao-Malfakassa

3.1. Foraminitermes Species

The head of the soldier was yellow-brown and sub-rectangular in the dorsal view (Figure 2). The labrum with a whitish tip was a bit shorter than the mandibles, which were shorter than the head capsule. The mandibles were brown at the base but darker at the top. The antennae had 15 articles. The morphometric measurements of this species are given in Table 2.

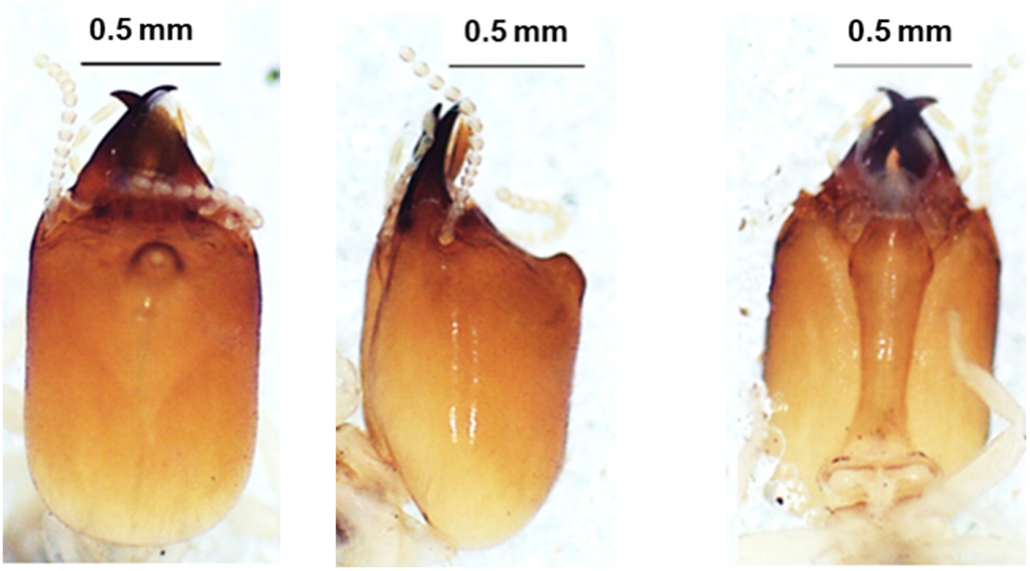

Figure 2. The head of *Foraminitermes corniferus* soldier in dorsal view (**left**), lateral view (**middle**), and ventral view (**right**).

Table 2. The measurements (mm) of the soldiers of *Foraminitermes corniferus*.

Measured Characters	Measurements (mm)
Head length	1.28
Head width	0.853
Left mandible length	0.555
Pronotum width	0.562
Gula width	0.817
Hind tibia length	0.7

3.2. Lepidotermes Species

The head in the dorsal view (Figure 3) was almost square. The mandibles were wider at the base but tapered at the top. Each mandible had a basal tooth. There were 14 antennal articles. The morphometric measurements of this species are presented in Table 3.

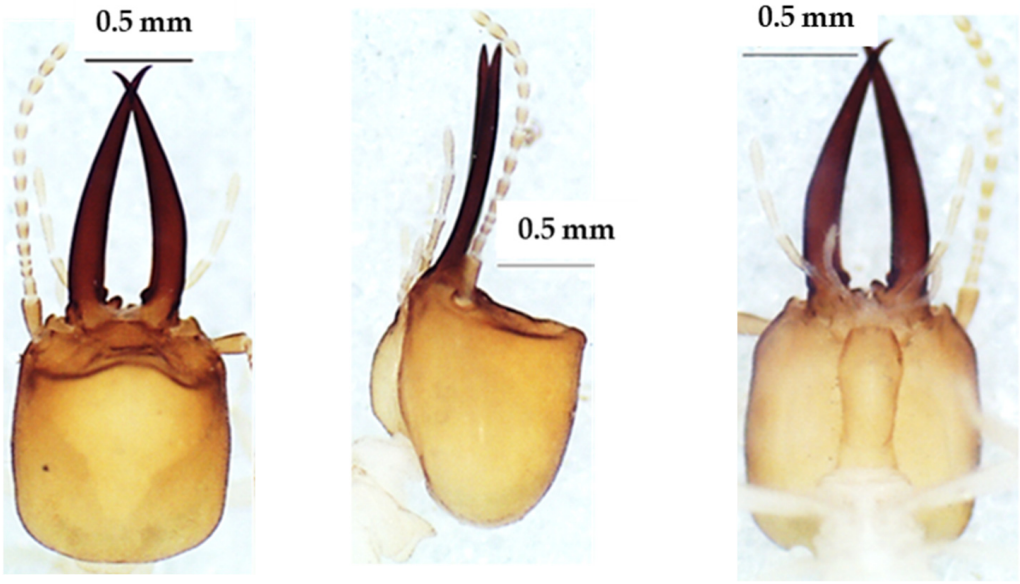

Figure 3. The head of *Lepidotermes* sp. soldier in dorsal view (**left**), lateral view (**middle**), and ventral view (**right**).

Table 3. The measurements (mm) of the soldiers of *Lepidotermes* sp.

Measured Characters	Measurements (mm)
Head length	1.14
Head width	1.05
Left mandible length	1.29
Pronotum width	0.482
Gula width	0.638
Hind tibia length	0.741

3.3. Noditermes Species

The heads of these three species in dorsal view (Figures 4–6) were almost rectangular and orange. Their respective labrum was bifurcate at the top. There were 14 antennal segments. However, they were distinct species, and their differences are highlighted in Tables 4–6. The head capsule of *Noditermes* sp. 1 was larger (1.626 ± 0.027 mm) and wider (1.15 ± 0.046 mm) than the other two *Noditermes*. Similarly, the mandibles of *Noditermes* sp. 1 were also longer (1.35 ± 0.026 mm) than those of the two other species. *N. cristifrons* had the smaller pronotum (0.508 ± 0.008 mm). The factorial differential analysis (Figure 7) shows clearly that these three *Noditermes* species were distinct.

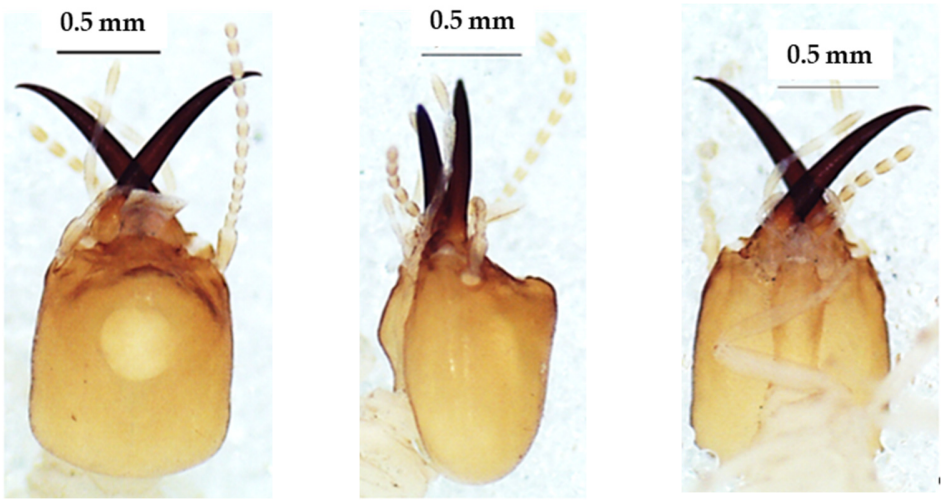

Figure 4. The head of *Noditermes cristifrons* soldier in dorsal view (**left**), lateral view (**middle**), and ventral view (**right**).

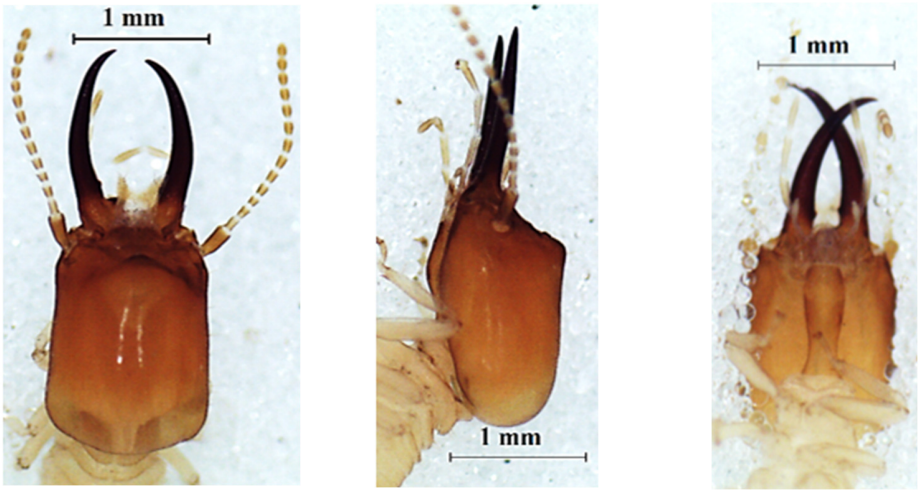

Figure 5. The head of *Noditermes* sp. 1 soldier in dorsal view (**left**), lateral view (**middle**), and ventral view (**right**).

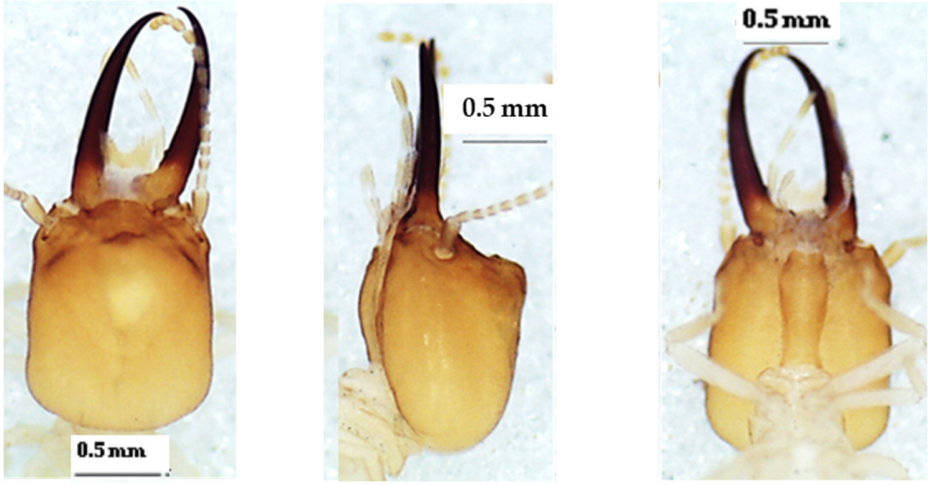

Figure 6. The head of *Noditermes* sp. 2 soldier in dorsal view (**left**), lateral view (**middle**), and ventral view (**right**).

Table 4. The measurements (mm) of the soldiers of *Noditermes cristifrons*.

Measured Characters	Range (mm)	Mean ± SD
Head length	1.22–1.34	1.268 ± 0.038
Head width	0.91–1.09	1.002 ± 0.059
Left mandible length	1.17–1.38	1.237 ± 0.082
Pronotum width	0.5–0.516	0.508 ± 0.008
Gula width	0.239–0.31	0.279 ± 0.025
Hind tibia length	0.754–0.878	0.791 ± 0.044

Table 5. The measurements (mm) of the soldiers of *Noditermes* sp. 1.

Measured Characters	Range (mm)	Mean ± SD
Head length	1.6–1.67	1.626 ± 0.027
Head width	1.07–1.19	1.15 ± 0.046
Left mandible length	1.31–1.38	1.35 ± 0.026
Pronotum width	0.609–0.62	0.616 ± 0.004
Gula width	0.263–0.301	0.284 ± 0.015
Hind tibia length	0.734–0.936	0.886 ± 0.086

Table 6. The measurements (mm) of the soldiers of *Noditermes* sp. 2.

Measured Characters	Range (mm)	Mean ± SD
Head length	1.36–1.39	1.372 ± 0.012
Head width	1.09–1.12	1.105 ± 0.013
Left mandible length	1.26–1.33	1.285 ± 0.031
Pronotum width	0.563–0.597	0.579 ± 0.013
Gula width	0.265–0.288	0.275 ± 0.006
Hind tibia length	0.808–0.832	0.814 ± 0.012

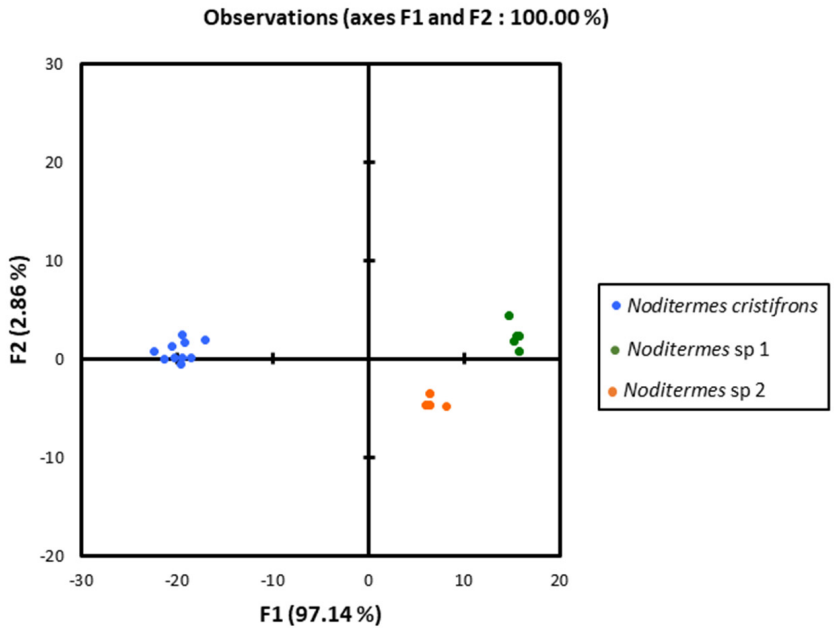

Figure 7. *Noditermes* species discrimination by factorial discriminant analysis.

3.4. Unguitermes Species

The head of the soldier in the dorsal view (Figure 8) was almost square and yellow-orange. The top of the labrum was rectilinear and wider than the base. The mandibles were longer than the head capsule (Table 7). There were 14 antennal articles.

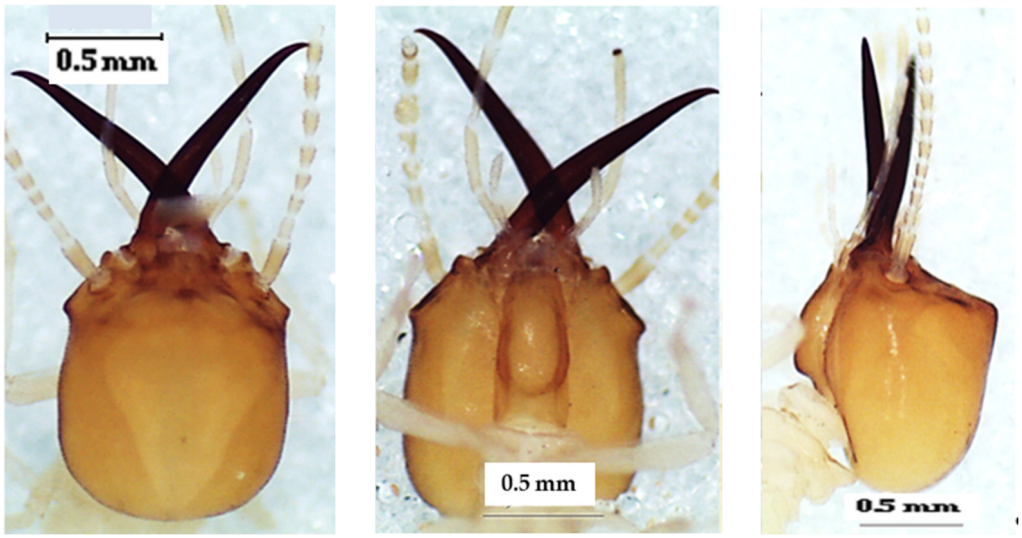

Figure 8. The head of *Unguitermes* sp. soldier in dorsal view (**left**), lateral view (**middle**), and ventral view (**right**).

Table 7. The measurements (mm) of the soldiers of *Unguitermes* sp.

Measured Characters	Range (mm)	Mean ± SD
Head length	1.16–1.2	1.18 ± 0.028
Head width	0.986–1.02	1.003 ± 0.024
Left mandible length	1.21–1.28	1.245 ± 0.045
Pronotum width	0.54–0.6	0.57 ± 0.04
Gula width	0.301–0.32	0.31 ± 0.013
Hind tibia length	0.772–0.793	0.782 ± 0.014

3.5. Amitermes Species

The four species of *Amitermes* including *A. evuncifer* (Table 8, Figure 9), *A. guineensis* (Table 9, Figure 10), *A. spinifer* (Table 10, Figure 11), and *A. truncatidens* (Table 11, Figure 12) were unambiguously identified. Apart from *A. spinifer* (with 13 antennal segments), the soldiers of the other three species had 14 antennal segments. All four species had mandibles strongly curved at the top, and each mandible had a tooth in its inner side. The tips of the mandibular teeth of *A. evuncifer*, *A. truncatidens*, and *A. guineensis* were horizontal, whereas in *A. spinifer* the tips of the mandibular teeth were pointing down. *A. guineensis* differed from the other species by the rectangular shape of the head capsule and especially by the average length, which was greater (1.225 ± 0.031 mm) than those of the other species. The species with a shorter head capsule was *A. spinifer* (0.933 ± 0.018 mm). The left mandible of *A. guineensis* was the longest (0.722 ± 0.058 mm), while *A. truncatidens* had the shortest left mandible (0.547 ± 0.023 mm). The ranges and measurements of head length, head width, left mandible length, pronotum width, gula width, and hind tibia length for each species are presented in Tables 8–11, respectively. Although the *A. evuncifer* and *A. truncatidens* measurements were close (Tables 8 and 11), the factorial discrimant analysis showed that they were separate species (Figure 13), as well as the other two species (*A. guineensis* and *A. spinifer*).

Table 8. The measurements (mm) of the soldiers of *Amitermes evuncifer*.

Measured Characters	Range (mm)	Mean ± SD
Head length	1.06–1.19	1.134 ± 0.049
Head width	0.93–0.975	0.954 ± 0.014
Left mandible length	0.594–0.709	0.660 ± 0.038

Table 8. *Cont.*

Measured Characters	Range (mm)	Mean ± SD
Pronotum width	0.587–0.615	0.600 ± 0.011
Gula width	0.252–0.3	0.279 ± 0.016
Hind tibia length	0.828–1.2	0.970 ± 0.126

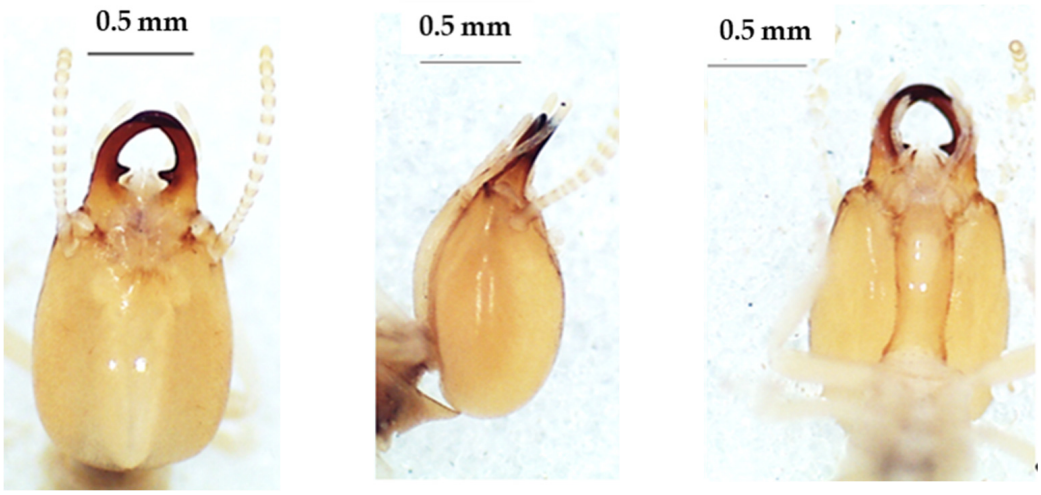

Figure 9. The head of *Amitermes evuncifer* soldier in dorsal view (**left**), lateral view (**middle**), and ventral view (**right**).

Table 9. The measurements (mm) of the soldiers of *Amitermes guineensis*.

Measured Characters	Range (mm)	Mean ± SD
Head length	1.17–1.27	1.225 ± 0.031
Head width	0.677–0.979	0.871 ± 0.008
Left mandible length	0.611–0.795	0.722 ± 0.058
Pronotum width	0.139–0.287	0.438 ± 0.013
Gula width	0.139–0.287	0.213 ± 0.041
Hind tibia length	0.503–0.519	0.509 ± 0.005

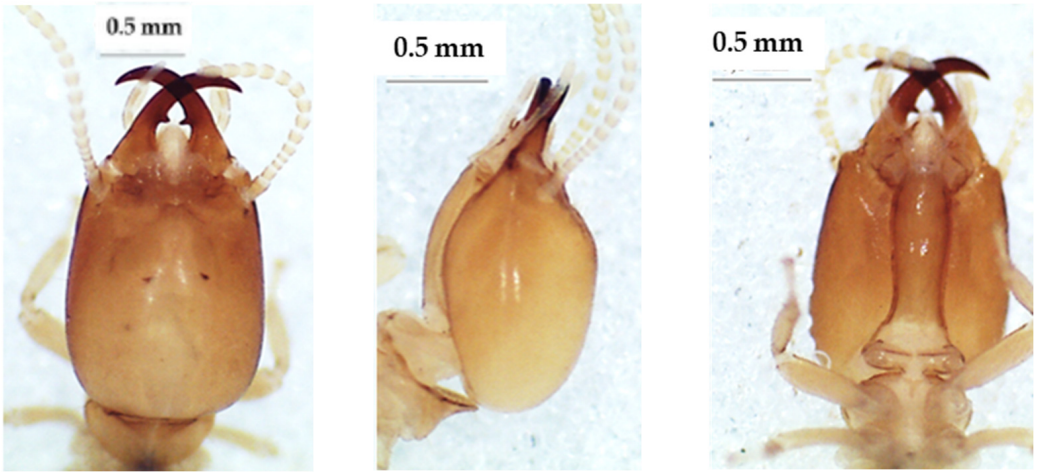

Figure 10. The head of *Amitermes guineensis* soldier in dorsal view (**left**), lateral view (**middle**), and ventral view (**right**).

Table 10. The measurements (mm) of the soldiers of *Amitermes spinifer*.

Measured Characters	Range (mm)	Mean ± SD
Head length	0.865–0.954	0.933 ± 0.018
Head width	0.681–0.742	0.726 ± 0.027
Left mandible length	0.585–0.624	0.613 ± 0.017
Pronotum width	0.472–0.492	0.486 ± 0.009
Gula width	0.257–0.26	0.288 ± 0.020
Hind tibia length	0.642–0.65	0.643 ± 0.017

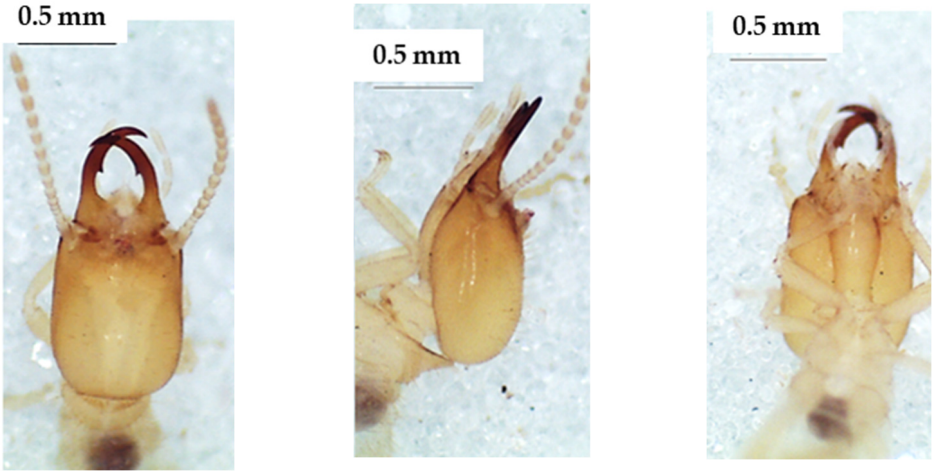

Figure 11. The head of *Amitermes spinifer* soldier in dorsal view (**left**), lateral view (**middle**), and ventral view (**right**).

Table 11. The measurements (mm) of the soldiers of *Amitermes truncatidens*.

Measured Characters	Range (mm)	Mean ± SD
Head length	1.01–1.13	1.064 ± 0.037
Head width	0.943–0.992	0.966 ± 0.016
Left mandible length	0.522–0.577	0.547 ± 0.023
Pronotum width	0.564–0.576	0.575 ± 0.007
Gula width	0.27–0.314	0.284 ± 0.021
Hind tibia length	0.756–0.816	0.785 ± 0.025

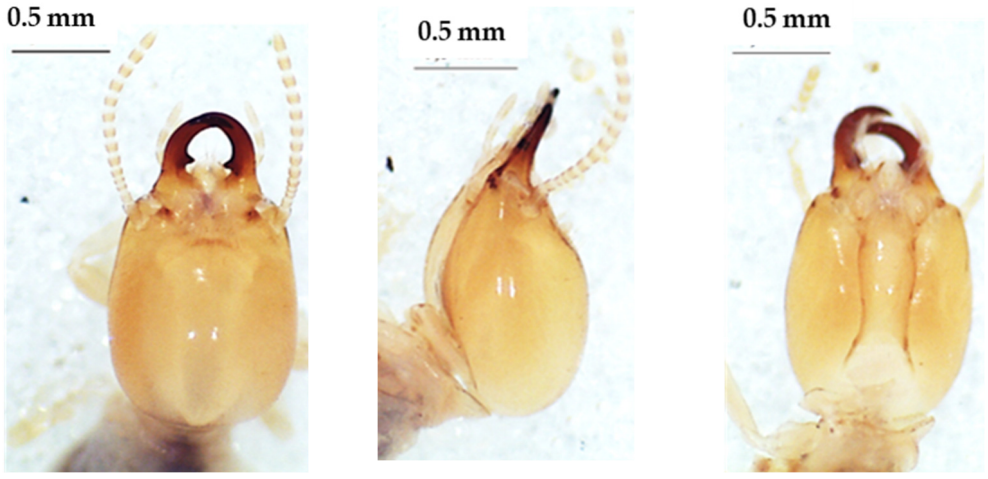

Figure 12. The head of *Amitermes truncatidens* soldier in dorsal view (**left**), lateral view (**middle**), and ventral view (**right**).

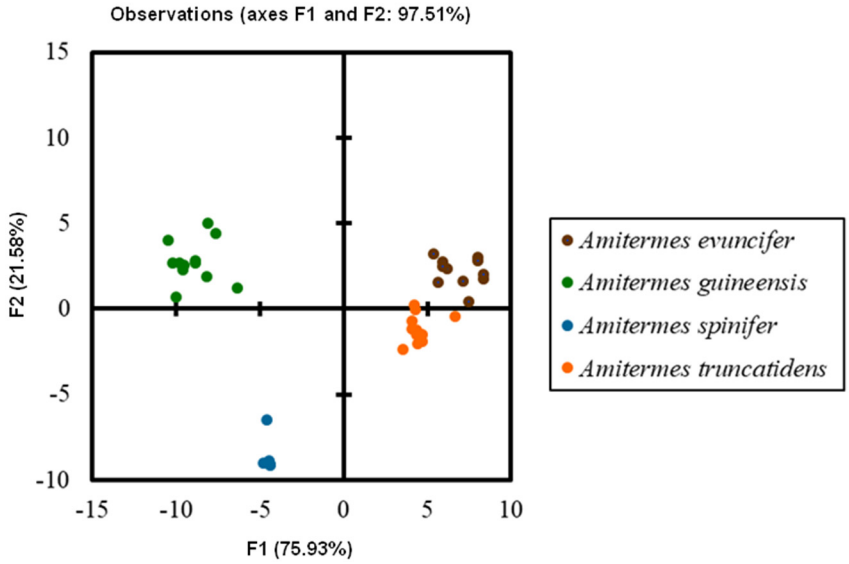

Figure 13. *Amitermes* species discrimination by factorial discriminant analysis.

3.6. Promirotermes Species

For the two species of this genus, *P. holmgreni infera*, (Table 12, Figure 14) and *Promirotermes* sp. (Table 13, Figure 15) the hind part of the head was wider than the front part. The maxillary palps of the two species were as long as the mandibles, which were tapered at the top. Their respective labra were bifurcate and wider at the top.

Table 12. The measurements (mm) of the soldiers of *Promirotermes holmgreni infera*.

Measured Characters	Range (mm)	Mean ± SD
Head length	1.27–1.31	1.29 ± 0.014
Head width	0.994–1.08	1.040 ± 0.034
Left mandible length	1.57–1.74	1.674 ± 0.063
Pronotum width	0.787–0.826	0.802 ± 0.02
Gula width	0.27–0.36	0.31 ± 0.037
Hind tibia length	1.1–1.23	1.136 ± 0.05

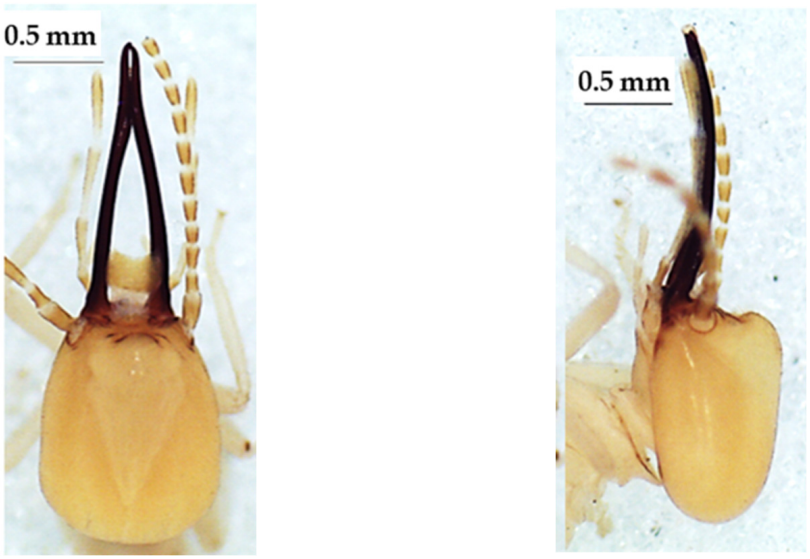

Figure 14. The head of *Promirotermes holmgreni infera* soldier in dorsal view (**left**) and ventral view (**right**).

Table 13. The measurements (mm) of the soldiers of *Promirotermes* sp.

Measured Characters	Range (mm)	Mean ± SD
Head length	0.877–0.879	0.878 ± 0.014
Head width	0.692–0.995	0.693 ± 0.021
Left mandible length	1.11–1.13	1.12 ± 0.015
Pronotum width	0.75–0.77	0.76 ± 0.014
Gula width	0.33–0.344	0.337 ± 0.009
Hind tibia length	0.667–0.67	0.668 ± 0.0016

Figure 15. The head of *Promirotermes* sp. soldier in dorsal view (**left**) and ventral view (**right**).

4. Discussion

Among the recorded species, several had already been described, while others seemed to be ambiguous, because their measurements did not fall within the range of known species from West Africa. Foraminitermitinae include three genera: *Foraminitermes*, *Labritermes*, Holmgren 1914 and *Pseudomicrotermes*, Holmgren 1912 [28,29]. *Foraminitermes* species have been revised by Krishna [30]. Among the six described species of *Foraminitermes*, two species including *F. tubifrons* Holmgren 1912 and *F. valens* Silvestri 1914 [13], were both recorded in West Africa (Guinea, Ivory Coast, and Nigeria) and neighboring countries (Cameroon and Congo). *F. corniferus* is close to *F. valens* for some morphometric values in comparison to other species. However its head is larger than that of *F. valens*. As the other five *Foraminitermes* species, *F. corniferus* is endemic to the Ethiopian zoogeographical region and was recorded in Congo (Mukimbungu) [30]. The occurrence of *F. corniferus* in our study area indicates that the distribution area of this species, hitherto known from the Congo (Mukimnungu), extends to Togo. The genus *Lepidotermes* contains nine described species [29]. All these species are found principally in southern Africa [30–34]. Among these species, *Lepidotermes* sp. is morphologically close but smaller than the *Lepidotermes lounsburyi* and *Lepidotermes planifacies* described respectively by Silvestri [14] and Williams [35]. *N. cristifrons*, previously described as *Cubitermes cristifrons* [36], seemed to be the sole species of the seven described of the *Noditermes* genus [29] to occur in West Africa. It was recorded in Gambia in a forest ecosystem. The other two undetermined *Noditermes* (*Noditermes* sp. 1 and sp. 2) were all larger than *N. cristifrons* and appeared to not yet be described. This appears to be the same for *Unguitermes* sp. which was smaller than *Unguitermes acutifrons* [14] and *Unguitermes magnus*, Ruelle 1973 [37]. All the representative castes (imago, soldiers, and workers) of the four *Amitermes* species were already described and are all found in the Ethiopian zoogeographical region [19]. In this study, the ranges and means of the measurements of the soldiers fell within the ranges and means of respective species. *Amitermes spinifer* had the shorter and smaller head of all, while *A. guineensis* had the longer and the larger one. Compared to *Promirotermes holmgren holmgren*, *P. holmgren infera*,

and *P. holmgren redundans*, the known species from West Africa, the *Promirotermes* sp. presented was clearly smaller and different by the shape of its head.

5. Conclusions

Twelve termite species were partially (head) illustrated in our study. Seven of these species including the four species of *Amitermes* genus (*A. evuncifer*, *A. guineensis*, *A. spinifer,* and *A. truncatus*), *Foraminitermes corniferus*, *Noditermes cristifrons*, and *Promirotermes holmgren inferea* were already described. The other five (*Lepidotermes* sp., *Noditermes* sp. 1, *Noditermes* sp. 2, *Unguitermes* sp., and *Promirotermes* sp.) were different by their measurements from the known species of the respective genus. This study was the first in Togo to present termite species with measurements and illustrations. It can be used as reference work for future taxonomic research.

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Bacterial Symbionts in *Ceratitis capitata*

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Simple Summary: The Mediterranean fly (Medfly), *Ceratitis capitata*, is considered one of the world's most destructive fruit pests, as it can attack commercially important fruit, thus causing considerable economic damages, estimated to be more than 2 billion dollars annually. The yield reductions are mainly due to the damage incurred when larvae feed directly on the pulp, inducing the premature fruit drop. Additionally, oviposition holes facilitate secondary fungal and bacterial infections, further reducing the yields. Integrated pest management (IPM) strategies for medfly control are highly dependent on the use of insecticides, which, however, pose environmental concerns. Alternative strategies include the Sterile Insect Technique (SIT), which aims to eliminate or suppress pest insects without using pesticides. Lately, the medfly microbiota has been explored to develop new control strategies for insect pests and insect vectors. Here, we report the characterization of the microbial communities associated with selected organs of three different populations of *C. capitata* to identify possible candidates for a Symbiotic Control approach. Our findings provide new knowledge about the microbiota associated with *C. capitata* and stress the characterization of microbial symbionts as possible tools for a Symbiotic Control approach to implementing the pest management programs.

Abstract: *Ceratitis capitata* (Diptera: Tephritidae) is responsible for extensive damage in agriculture with important economic losses. Several strategies have been proposed to control this insect pest including insecticides and the Sterile Insect Technique. Traditional control methods should be implemented by innovative tools, among which those based on insect symbionts seem very promising. Our study aimed to investigate, through the 16S Miseq analysis, the microbial communities associated with selected organs in three different medfly populations to identify possible candidates to develop symbiont-based control approaches. Our results confirm that *Klebsiella* and *Providencia* are the dominant bacteria in guts, while a more diversified microbial community has been detected in reproductive organs. Concertedly, we revealed for the first time the presence of *Chroococcidiopsis* and *Propionibacterium* as stable components of the medfly's microbiota. Additionally, in the reproductive organs, we detected *Asaia*, a bacterium already proposed as a tool in the Symbiotic Control of Vector-Borne Diseases. A strain of *Asaia*, genetically modified to produce a green fluorescent protein, was used to ascertain the ability of *Asaia* to colonize specific organs of *C. capitata*. Our study lays the foundation for the development of control methods for *C. capitata* based on the use of symbiont bacteria.

Keywords: *Ceratitis capitata*; *Asaia*; Symbiotic Control

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1. Introduction

Ceratitis capitata (Diptera: Tephritidae) is one of the main destructive pests of fruit production worldwide [1] because of its significant physical damage to fruits and vegetables and its economic impact on agriculture and forestry. Due to its ability to tolerate and adapt

to a wide range of climates and its capability to attack a broad spectrum of plant species, *C. capitata* has a large distribution. This is the case in Mediterranean countries as well, considering that medfly damage (linked to the population density) is more consistent in intensive cultures such as in Latin America than in Mediterranean agriculture [2]. Several strategies have been proposed to control the medfly distribution. Among them, methods such as the insecticide bait spray and the Sterile Insect Technique (SIT) have been demonstrated to be effective [3,4]. Although the chemical method works efficiently in medfly control, it has some disadvantages related to its toxicity to humans and animals and makes the fruits or plants polluted by leaving residues on them. Despite the fact that SIT has been successful in several countries, releasing X-rays sterile medflies aimed to reduce the wild insect population and helping to reduce the broad spectrum of chemical treatments, new control methods that are easily applicable are urgently needed.

Indeed, the study of microbiota could open new perspectives in medfly control to integrate other control methods. In fact, in several entomological systems (both insect pests and insect vectors), the use of symbiotic microbes to control harmful insects is already common practice in some cases; in others, it may become so in the near future [5–7]. A striking case in this context is represented by mosquitos' vectors of pathogens that are dangerous for humans and animals. In particular, some selected bacteria have been enrolled in mosquito control. One very effective method is the biocontrol of mosquito borne diseases through mosquitoes transinfected with the endosymbiotic bacteria *Wolbachia*. Others refer to the bacterium *Asaia*, which has been isolated from many different mosquito species. *Asaia* is a natural effector for mosquito immune priming and is often detected at a very high prevalence, and this is attributed to several aspects of its biology. It has also been proposed for paratransgenic control applications [8,9].

Nevertheless, up to now, studies on the microbiota composition of *C. capitata* are already limited [10–14] and mostly related to the SIT and Incompatible Insect Technique (IIT). It has been noted that these procedures affect the medfly gut bacterial community, and sterile males are less competent in attracting and mating with wild females and are affected by the genetic background of the medfly population [15–17]. Post-irradiation dietary supplementation with *Klebsiella oxytoca* improved the mating latency of infertile males, suggesting the potential use of this bacterium to improve the SIT success [15]. Additionally, selected bacteria can act as probiotics in the diet of larval and adult stages, improving *C. capitata* mass rearing and enhancing the SIT application [18]. Similar to *C. capitata*, the intestinal probiotic *K. oxytoca* restored the ecological fitness of *Bactrocera dorsalis*, another destructive, polyphagous and invasive insect pest of fruits and vegetables, following a decline post-irradiation [19]. In *B. dorsalis* bacteria belonging to Enterobacteriaceae, *Enterobacter cloacae*, *K. oxytoca*, *Morganella* sp., *Providencia rettgerii* and *C. freundii* were identified as predominant in the gut, suggesting their involvement in promoting host fitness under stressful conditions and host immune system response [20–22]. *K. michiganensis* was implicated in promoting insect resistance to long-term, low-temperature stress [23]. Moreover, the gut symbiont *Citrobacter* sp. seemed to be involved in increasing the host resistance against an organophosphate insecticide [20].

Here, we present the results of a study aimed to characterize the microbial communities in different anatomical districts (guts and reproductive organs) of three different populations of *C. capitata* to determine whether selected symbionts could be translated into potential tools for the Symbiotic Control (SC) of medfly, which could be integrated with more traditional approaches. Considering the presence of *Asaia* in all three medfly populations, we investigate the possibility of using *Asaia* in the SC of *C. capitata.*

2. Materials and Methods

2.1. Ceratitis capitata Rearing

The strains of *C. capitata* used in this work were: (i) the Guatemala strain, established in 1989 from wild pupae collected in Antigua (Guatemala); (ii) the La Réunion strain, established in 1994 from wild pupae collected near St. Denis (La Reunion, France); (iii) the

ISPRA strain, established in 1968 at the European Community Joint Research Centre (Ispra, Italy) with wild flies from Sicily and Greece and maintained in Pavia since 1979. These strains originated from the Department of Biology & Biotechnology, University of Pavia, where they are maintained under standard rearing conditions [24], and, since 2018, they have been maintained in the insectary at the School of Biosciences and Veterinary Medicine, University of Camerino. The insects were kept in cages (25 × 25 × 25 cm) made of a steel frame covered with nettings and maintained at the standard lighting conditions of 12 h light and 12 h dark, 26 ± 1 °C temperature and 75 ± 5% humidity and in aseptic conditions during both the developmental and adult stages.

2.2. DNA Extraction

Before DNA extraction, the insect surface was sterilized in 70% ethanol and rinsed three times in sterile PBS. The samples were homogenized with sterile 0.5-mm wide glass beads (Bertin Instrument, Montigny Le-Bretonneux, France) for 30 s at 6800 rpm by an automatic tissue homogenizer (Precellys 24 Bertin Instrument, Montigny Le-Bretonneux, France). Genomic DNA was extracted using a Jet Flex Genomic DNA Purification kit (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's instructions.

2.3. 16S rRNA Profiling

The 16S Miseq analysis was conducted on the male and female organs (gut and reproductive organs) of three different *C. capitata* populations. Single pools of 20 organs for each group were obtained from cohorts of 10-day-old insects dissecting in sterile conditions. DNA extraction was performed as described above. A negative control consisting of a blank sample was included for each batch of extraction to control for the contamination of bacteria possibly introduced during the DNA extraction. They were not further processed since no quantifiable extract was produced from each negative control.

16S rRNA profiling was conducted by LGC Genomics (Berlin, Germany). Libraries preparation was performed by covering the hypervariable region V3–V4 of 16S ribosomal RNA using 341F and 785R oligonucleotides [25]. The data were pre-processed using the Illumina bcl2fastq 2.17.1.14 software, and the reads were sorted by amplicon inline barcodes. Sequencing adapter remnants were clipped from all reads. 16S pre-processing and OTU picking from the amplicons were analyzed using Mothur 1.35.1 [26]. The sequence alignments were performed against the 16S Mothur-Silva SEED r119 reference alignment. OTU diversity was analyzed with QIIME 1.9.0 [27], and annotations of the putative species level of OTUs were obtained with NCBI BLAST+ 2.2.29 [28]. The raw data were submitted as BioProject accession number PRJNA682004 to the NCBI database.

2.4. Molecular Detection of Asaia

The presence of *Asaia* was investigated in 20 female and 20 male adults from the three *C. capitata* populations. *Asaia* detection was performed by PCR using specific oligonucleotides targeting the 270-bps fragment of the 16S rRNA gene described in Favia et al. [8]. A total of 50 ng of genomic DNA were used in PCR reaction containing 1X Buffer, 0.25 mM dNTPs, 0.9 U DreamTaq Polymerase (Thermo Scientific, Waltham, MA, USA) and 200 nM of Asafor (5′-GCGCGTAGGCGGTTTACAC-3′) and Asarev (5′-AGCGTCAGTAATGAGCCAGGTT-3′) oligonucleotides. The amplification protocol included: initial denaturation at 95 °C for 3 min, followed by 30 cycles consisting of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 30 s and concluding with a final extension step of 10 min at 72 °C. The PCR products were electrophoresed on a 1% agarose gel to determine the presence and size of the amplified DNA. The amplicons were purified and sequenced by the mean of the Sanger method (Eurofins Genomics, Ebersberg, Germany). The 16S RNA sequences were analyzed by BLASTN (http://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 1 May 2022).

2.5. Asaia Isolation from C. capitata Adults

Bacteria isolation was performed from *C. capitata* adults. Before the isolation, the insect surface was sterilized in 70% ethanol and rinsed three times in sterile PBS. *Asaia* isolation was performed as described in Favia et al. [8] using a liquid enrichment medium at pH 3.5 followed by plating in a carbonate-containing solid medium. The enrichment medium allowed for the elimination of those microorganisms not tolerating low pH, while the following plating on $CaCO_3$-containing medium permitted the isolation of acid-producing bacteria capable of creating $CaCO_3$ dissolution haloes around the colonies. To confirm the isolation, pink-pigmented, shiny and smooth colonies were analyzed though *Asaia*-specific PCR.

2.6. Asaia sp. *Transformation*

Asaia strains isolated from the three populations of *C. capitata* were transformed with plasmids pHM2 containing a GFP gene cassette to express a green phenotype, as described in Favia et al. [8]. *Asaia* cells were grown overnight on GLY agar (yeast extract 1%, glycerol 2.5%, agar 2%, pH 5) for 48 h at 30 °C. The culture was diluted 1:20 into 25 mL of GLY and incubated with aeration until the cells reached the early log phase (optical density at 600 nm, 0.5–0.8). After an incubation of 15 min on ice, the cells were harvested (2700× *g*, 10 min, 4 °C) and washed twice with 10 mL of cold 1 mM HEPES (pH 7.0). The pellet was resuspended in 5 mL of cold 10% (*v*/*v*) glycerol, centrifuged again and finally resuspended in 0.5 mL of cold 10% (*v*/*v*) glycerol. Then, 65 microliters of competent cells ($\approx 9.2 \times 10^9$ CFU/mL) were mixed with 0.2 µg of plasmid DNA, transferred to a cold 0.1 cm-diameter cuvette and pulsed at 1800 V in the Electroporator apparatus (Biorad, Hercules, CA, USA). The cells were immediately added with 1 mL of GLY medium, transferred to a tube and incubated at 30 °C for 3 h. Transformant cells were selected by plating on GLY agar medium added with 100 µg/mL kanamycin, 40 µg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (XGal) 24 and 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for testing the Lac+ phenotype. Successful transformants were confirmed by fluorescence microscopy analysis and specific *Asaia*-PCR. A Gfp-tagged transformant from the ISPRA population was hence selected for mosquito recolonization experiments.

2.7. Medfly Recolonization by Asaia Expressing Green Fluorescent Protein (Gfp)

The ISPRA population was fed with modified *Asaia* to evaluate the ability of the bacterium to colonize the insect host. The *Asaia*^{Gfp} strain from the ISPRA medfly was grown for 24 h at 30 °C in GLY medium enriched with 100 µg/mL kanamycin. The cells were harvested by centrifugation, washed three times in 0.9% NaCl, adjusted to a concentration of 10^8 cells/mL and resuspended in sugar solution 25%. For monitoring the long-term colonization of *C. capitata*, the suspension was supplemented with 100 µg/mL of kanamycin to avoid the loss of plasmid from the bacterial cells. After 3, 5, 10 and 15 days of exposure to the feeding solutions containing *Asaia*^{Gfp}, the insects were dissected to monitor the presence of green bacteria in the organs. At each timepoint, the guts and reproductive organs of 7 males and 7 females were dissected in sterile PBS, fixed with 4% paraformaldehyde for 10 min at 4 °C and mounted in glycerol–PBS for analysis with a Nikon C2+ Laser Scanning Confocal Microscope (Nikon, Minato, Tokyo, Japan).

2.8. Isolation of Bacteria from the ISPRA Population

Bacteria from the ISPRA population medfly were isolated, processing a single pool of 10 gut and 10 reproductive organs from males and females. Each pool was homogenized as previously described in 100 µL of sterile 1X PBS, and serial dilution in 1X PBS was prepared from 1:10 to 1:1000. One hundred microliters of each dilution were spread on the surface of Blood Agar plates (ThermoFisher Scientific, Waltham, MA, USA) and incubated at 35 °C for 48 h. The isolated colonies were picked and transferred on Luria Bertani (LB) agar plates (1% NaCl, 1% Tryptone, 0.5% Yeast extract, 2% Agar, pH 7). To isolate gram-negative bacteria, clones were plated on MacConkey agar (ThermoFisher Scientific, Waltham, MA,

USA) and incubated at 35 °C for 48 h. The identification of gram-negative oxidase-negative isolates was performed using the API20E system (bioMérieux, Inc, Marcy-l'Étoile, France).

The other isolates were molecularly characterized using universal oligonucleotides targeting the 16S RNA ribosomal bacterial (805 Rev 5′-TCGACATCGTTTACGGCGTG-3′ and 27 for 5′-AGAGTTTGATCCTGGCTCAG-3′). A total of 50 ng of genomic DNA were used in PCR reaction containing 1× Buffer, 0.25 mM dNTPs, 0.9 U DreamTaq Polymerase (Thermo Scientific, Waltham, MA, USA), 200 nM of 27F and 805R oligonucleotides. The amplification protocol included: initial denaturation at 95 °C for 3 min, followed by 30 cycles consisting of denaturation at 95 °C for 30 s, annealing at 56 °C for 30 s and extension at 72 °C for 30 s and concluding with a final extension step of 10 min at 72 °C. The PCR products were electrophoresed on a 1% agarose gel to determine the presence and size of the amplified DNA. The amplicons were purified and sequenced by the mean of the Sanger method (Eurofins Genomics, Germany). The 16S RNA sequences were analyzed by BLASTN (http://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 1 May 2022).

2.9. Antagonistic Assay

Antagonistic activity of the *Klebsiella oxytoca* strain was evaluated by an Agar diffusion assay against the *Asaia* strain isolated from the ISPRA population medfly as described in Ciolfi and Marri [29], with some modifications. Briefly, overnight cultures of *K. oxitoca* and *Asaia* in a GLY medium were used for the test. A lawn was prepared by mixing 5 mL of soft-agar (0.7% agar) with 59 µL of *Asaia* culture (OD600, 0.1) and poured over the GLY agar medium in plates. Three microliters of *K. oxytoca* (OD600, 0.3) were then spotted on the lawn. The plates were incubated for 4 days at 26 °C and examined daily for zones of inhibition.

3. Results

3.1. 16S rRNA Profiling

The microbiome sequencing of male and female organs of three different populations of *C. capitata* generated a total of 4.2 M reads, varying among samples (minimum = 64.760, maximum = 701.290), with an average of 352.944 reads. Analysis of the rarefaction curves indicated an adequate sampling quality, suggesting a coherent number of sequence reads per sample. The Principal Coordinates Analysis (PCoA) plots show the high similarity of microbial composition among all of the guts analyzed, while a more specific microbial community in the ISPRA reproductive organs was represented. No substantial difference in microbial composition is observed between males and females in any strain (Figure S1). At the phylum level, the Proteobacteria results are the most prevalent in all groups, particularly in guts (males and females 99%). The phyla Actinobacteria and Firmicutes were detected in reproductive organs. Bacteroides phylum was revealed in the reproductive organs of La Réunion (males: 2.7%) and ISPRA strains (male: 6% and females: 2.3%). Additionally, Cyanobacteria phylum was present in ISPRA population reproductive organs (males: 15% and females: 7.8%) (Figure 1 and Table S1).

At the genus level, among the Proteobacteria phylum, *Klebsiella*, belonging to the class of Gammaproteobacteria, was the most abundant bacteria, with a range around 85–98% in the guts and 33–80% in the male and female reproductive organs of all three populations, except in the La Rèunion females, where *Providencia* is highly represented (95.7%), while *Klebsiella* is at 1.8%. *Providencia* was detected with a different percentage range (6–95%) in all samples—although in the male guts of the La Réunion strain and the female guts of the ISPRA strain, the percentage was lower than 1% (0.3% and 0.5%, respectively) (Figures 2 and S2).

Other interesting bacteria belonging to the phylum Proteobacteria (class: Alphaproteobacteria) such as *Asaia* and *Gluconobacter* were also detected, which were present in all samples, albeit with variable percentages (see Supplementary Materials Table S2). Considering the 1% cut-off for sample analysis, *Asaia* was present in the male reproductive organs (1.1%) and female guts (1.4%) in the Guatemala strain, the male reproductive organs (8%) of the La Réunion strain and the male guts (1%) and male (7.4%) and female (8%) reproductive organs of the ISPRA strain.

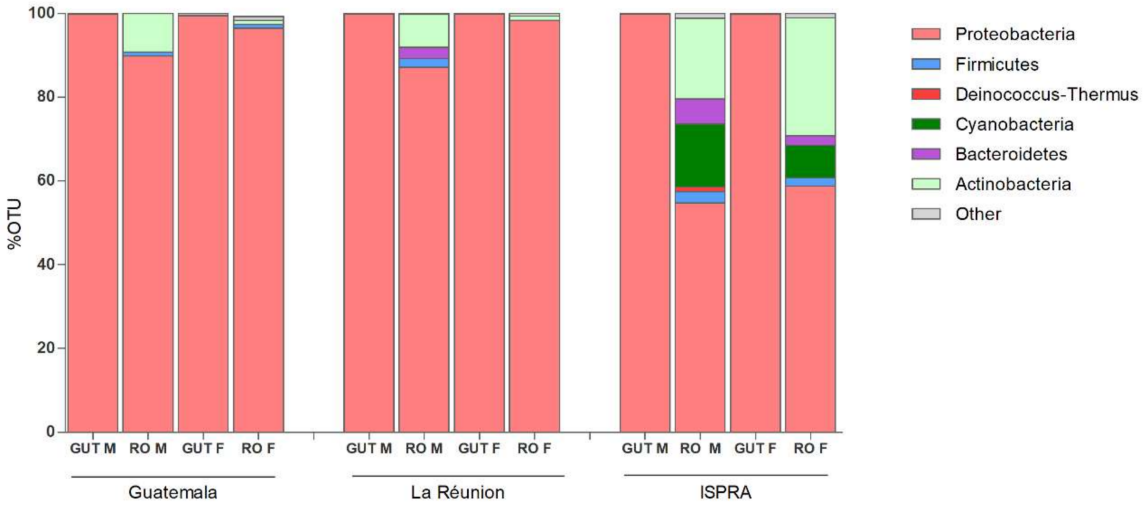

Figure 1. Phylum level composition (% of OTUs) in different organs of three different populations of *C. capitata.* Only OTUs representing >1% of the total reads are represented. RO: reproductive organs; F: females; M: males.

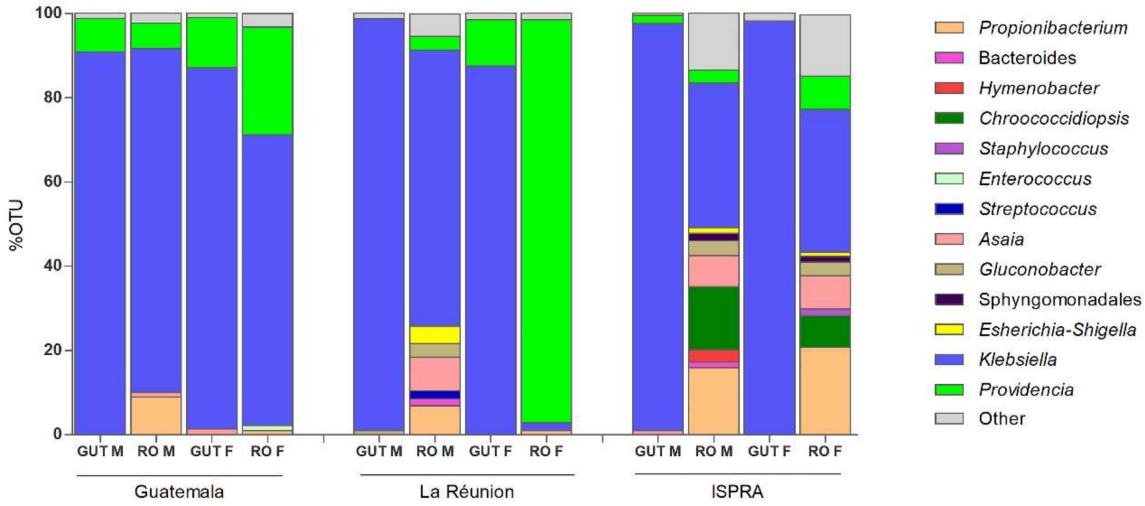

Figure 2. Genus level composition (% of OTUs) in different organs of *C. capitata.* Only OTUs representing >1% of the total reads are represented. RO: reproductive organs; M: male; F: female.

Gluconobacter was mostly detected in the males (guts: 1% and reproductive organs: 3.2%) of the La Réunion population and in the male (3.6%) and female (3.2%) reproductive organs of the ISPRA strain.

Just in the ISPRA population, the bacterium *Chroococcidiopsis*, belonging to the phylum Cyanobacteria, reached a much higher density, being detected in male (14.9%) and female (7.6%) reproductive organs.

In all three populations, *Propionibacterium* was detected only in male and female reproductive organs, with a range of 1–20%.

As already reported in several studies, no *Wolbachia* was detected in any samples [30].

3.2. Detection, Isolation and Transformation of Asaia from C. capitata Populations

Considering the presence of *Asaia* shown in the NGS analysis, we investigated its presence in the new generation of medfly in depth. We tested for *Asaia* presence in 40 adults (20 males and 20 females) of each population by specific PCR. *Asaia* was detected in 100% of samples (a total of 120 individuals). Moreover, *Asaia* was isolated from *C. capitata* adults using a selected medium followed by plating in a carbonate-rich medium. The resulting

single, pink colonies capable of dissolving carbonate in the medium and generating dissolution haloes were confirmed to be *Asaia* by 16S rRNA gene sequencing, with 99.8% nucleotide identity with *A. bogorensis* (Figure 3A–C). Additionally, *Asaia* was genetically modified to express a GFP protein to confirm its versability (Figure 3D–F).

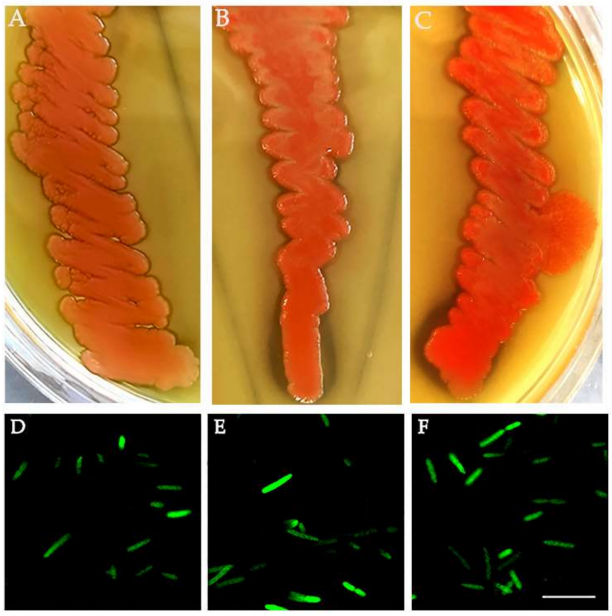

Figure 3. *Asaia* strains from *C. capitata*. *Asaia* isolates from the (**A**) Guatemala strain, (**B**) La réunion strain and (**C**) ISPRA strain. *Asaia* strains genetically modified to express a green fluorescent protein (GFP): (**D**) Guatemala strain, (**E**) La Réunion strain and (**F**) ISPRA strain. Bar = 10 µm.

3.3. Medfly Recolonization Using Asaia Strain Expressing Green Fluorescent Protein (Gfp)

To investigate the possibility of using *Asaia* as a Symbiotic Control tool for medfly, we tested its ability to colonize the insect's organs in the ISPRA population, where *Asaia* was most abundant in the reproductive organs with respect to the other populations. *Asaia*-GFP was observed in the crop, an initial portion of the alimentary tract, of all samples (males: 7/7 and females: 7/7) only at the first time point (3 days after *Asaia* administration) (Figure 4). At the following time points, no individual tested positive for fluorescence. Likely, the presence of strong dominant bacteria, such as *Klebsiella* and *Providencia*, and the unfavorable sugary diet may have prevented *Asaia* from colonizing medfly organs in the long term.

3.4. Bacteria Isolation from the ISPRA Population and Activity Test

Considering the competition phenomena of symbionts to colonize specific niches in insects [31,32], we proceeded with the isolation of other bacteria from male and female organs (guts and reproductive organs) to select microorganisms showing potential features to interfere with the presence of *Asaia*. From each pool of guts plated on MacConkey Agar, *Klebsiella oxytoca* (Enterobacteriaceae family) and *Serratia marcescens* (Yersiniaceae) were isolated. The same species were found in the reproductive organs of both males and females while, *Aeromonas* spp. (Aeromonadaceae) was detected only in female reproductive organs. Moreover, the sequencing of a longer fragment of the16S rRNA gene (780 bps) of other isolates allowed for the identification of the bacterium *Enterococcus* sp. (Enterococcaceae), with an identity of 99% (MK764705.1) in the same organs.

Since *K. oxytoca* has been described to have a killer phenotype [29], we hypothesized that the strong presence of this bacterium in the gut could interfere with *Asaia* stabilization in this anatomical district. We tested the killer activity of *K. oxytoca* against *Asaia*, plating the tester bacterium in soft agar and adding a drop of *K. oxytoca* in the center. After 4 days

at 26 °C, we observed a homogeneous layer of *Asaia* grown around the spot of *Klebsiella*, suggesting that *Asaia* was not sensible to the *K. oxytoca* activity (Figure S2).

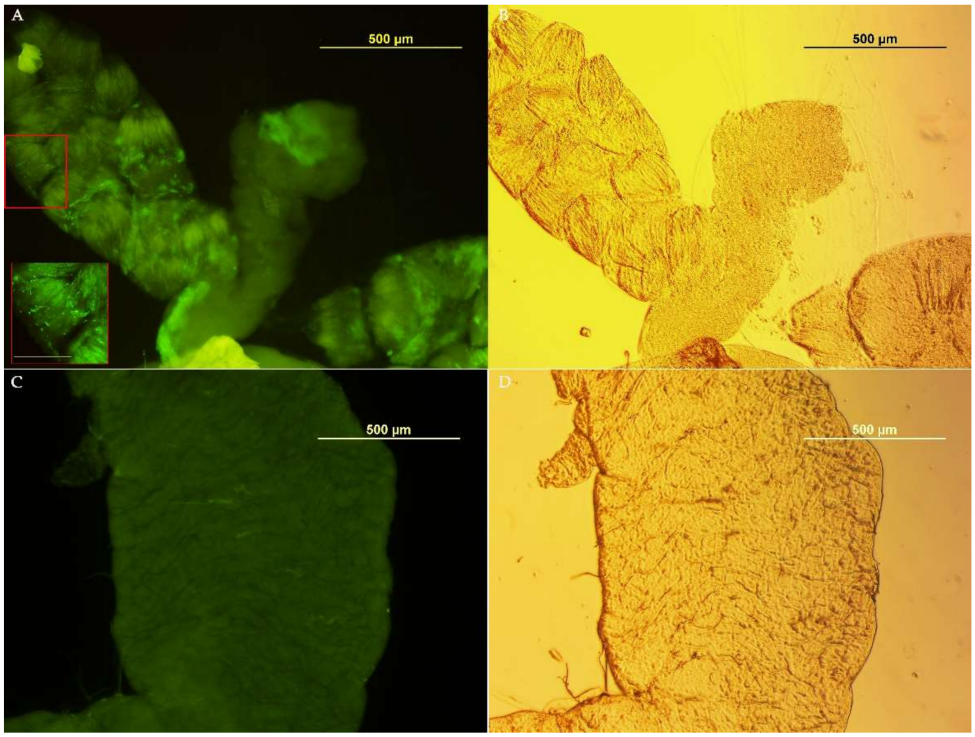

Figure 4. *Asaia*-GFP detection in *C. capitata* organs. (**A**) Gut colonized with *Asaia*-GFP; in the red box, a magnification of a gut portion with green bacteria (bar 50µm). (**B**) Phase-contrast image, (**C**) Gut from *C. capitata* fed on a standard diet (without *Asaia*). (**D**) Phase-contrast image.

4. Discussion

The microbiota composition of three different populations of *C. capitata*, which were reared under the same conditions, showed a homogeneous microbial community in the guts, which is dominated by *Klebsiella* and *Providencia*, as reported in previous studies [29,33–35], while highly different microbial communities were detected in the reproductive organs. These data support the need to investigate bacterial communities associated with different anatomical districts to identify potentially useful microorganisms in the development of medfly Symbiotic Control approaches. In this context, the first detection of *Chroococcidiopsis* and *Propionibacterium* as stable components of the medfly's microbiota may open interesting perspectives. *Propionibacterium* has been already described as part of the microbiota of other insects, such as mosquitos [36], likely accomplishing a possible nutritional role, while *Chroococcidiopsis* has been never detected in insects, and its role in the reproductive organs is still unknown.

Among other interesting bacteria detected in our studies, we focused on *Klebsiella oxytoca* and *Enterococcus* sp, which were isolated from guts and reproductive organs. *Enterococcus* was previously described as part of the microbiota of *Bactrocera dorsalis*, where it seemed involved, together with *K. oxytoca*, in the reproduction and survival of the insect pest [37]. *Klebsiella* sp. has been previously described as a major component of wild medfly's intestine [38], likely involved in nitrogen fixation [38,39] and associated with male mating success [40]. Additionally, *K. oxytoca* strains isolated from the alimentary tract of wild medflies showed an antagonistic activity in vitro against other bacteria such as *Escherichia coli*, *Enterobacter cloacae* and *Salmonella typhimurium* [29].

This is particularly interesting in the light of the fact that we detected *Asaia* in all of the samples but located only in the reproductive organs. In fact, *Asaia*, an insect symbiont proposed as a tool for the control of mosquito-borne diseases by direct paratransgenic

applications and indirectly through the upregulation of the host immune response [9], has been described as strictly associated with several insects, such as mosquitoes, in association with salivary glands, guts and reproductive organs [8]. In some mosquito strains, *Asaia* competes with *Wolbachia* in the reproductive organs [41,42].

Thus, a possible competition between *Klebsiella* and *Asaia* could have been hypothesized. However, the strain of *Asaia* that we have isolated from *C. capitata* did not show susceptibility to *K. oxytoca* in the antagonistic assay. Although *Asaia* is resistant to antagonistic activity, we can hypothesize that interspecies competition among other bacterial populations for nutritional sources could explain the inability of *Asaia* to colonize the gut of medfly, but further investigations are needed to better understand these competitive phenomena.

5. Conclusions

Despite the growing interest in the role of the microbiota in the biology of many insects and in the possible use of microbiota components for the development of innovative control methods, only a few studies have been published to date on the microbiota of *Ceratitis capitata*. Our study, although still intended as a preliminary, has identified some bacteria that could potentially be used for the development of control methods based on symbionts and which, at the same time, offer the possibility of better understanding some aspects of microbial competition between the symbionts of insects.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/insects13050474/s1, Figure S1: Principal Coordinates Analysis (PCoA) plots of samples colored according to different organs; Figure S2: Antagonistic test of *Klebsiella oxytoca* against *Asaia* using the Agar diffusion assay; Table S1: % OTU Phyla in *C. capitata* strains; Table S2: % OTU Genera in *C. capitata* strains.

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Bacterial Symbionts Confer Thermal Tolerance to Cereal Aphids *Rhopalosiphum padi* and *Sitobion avenae*

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Simple Summary: This study assesses the putative association between the chronic and acute thermal tolerance of cereal aphids *Rhopalosiphum padi* (L.) and *Sitobion avenae* (F.) and the abundance of their bacterial symbionts. Thermal tolerance indices were determined for 5-day-old apterous aphid individuals and were associated with the aphid-specific and total bacterial symbionts' gene abundance (copy numbers). The results show a significantly higher bacterial symbionts' gene abundance in temperature-tolerant aphid individuals than the susceptible ones for both aphid species. Moreover, the gene abundance of total (16S rRNA) bacteria and most of the aphid-specific bacterial symbionts for both cereal aphid species were significantly and positively associated with their critical thermal maxima values. Overall, the findings of the study suggest the potential role of the bacterial symbionts of aphids in conferring thermal tolerance to their hosts.

Abstract: High-temperature events are evidenced to exert significant influence on the population performance and thermal biology of insects, such as aphids. However, it is not yet clear whether the bacterial symbionts of insects mediate the thermal tolerance traits of their hosts. This study is intended to assess the putative association among the chronic and acute thermal tolerance of two cereal aphid species, *Rhopalosiphum padi* (L.) and *Sitobion avenae* (F.), and the abundance of their bacterial symbionts. The clones of aphids were collected randomly from different fields of wheat crops and were maintained under laboratory conditions. Basal and acclimated CTmax and chronic thermal tolerance indices were measured for 5-day-old apterous aphid individuals and the abundance (gene copy numbers) of aphid-specific and total (16S rRNA) bacterial symbionts were determined using real-time RT-qPCR. The results reveal that *R. padi* individuals were more temperature tolerant under chronic exposure to 31 °C and also exhibited about 1.0 °C higher acclimated and basal CTmax values than those of *S. avenae*. Moreover, a significantly higher bacterial symbionts' gene abundance was recorded in temperature-tolerant aphid individuals than the susceptible ones for both aphid species. Although total bacterial (16S rRNA) abundance per aphid was higher in *S. avenae* than *R. padi*, the gene abundance of aphid-specific bacterial symbionts was nearly alike for both of the aphid species. Nevertheless, basal and acclimated CTmax values were positively and significantly associated with the gene abundance of total symbiont density, *Buchnera aphidicola*, *Serratia symbiotica*, *Hamilton defensa*, *Regiella insecticola* and *Spiroplasma* spp. for *R. padi*, and with the total symbiont density, total bacteria (16S rRNA) and with all aphid-specific bacterial symbionts (except *Spiroplasma* spp.) for *S. avenae*. The overall study results corroborate the potential role of the bacterial symbionts of aphids in conferring thermal tolerance to their hosts.

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1. Introduction

Global warming is primarily manifested not only by a gradual rise in the Earth's average temperature, but also by the occurrence of frequent extreme high temperature events. Such high temperature events can influence population performances and the demographic parameters of insects, both in temporal and spatial scales [1–3].

Inferring the influence of extreme climate events on insects necessitates a better comprehension of their thermal tolerance limits and the mechanisms behind it [4–6]. Previous works have shown the significant impact of extreme high temperatures, both under acclimated and chronic exposures, on the physiology and thermal biology of invertebrates, including aphids [1,7–13]. Some studies, for instance, have demonstrated that different aphid species respond differently to chronic and acclimated temperature exposures [3,9–13]. However, the underlying mechanisms for such thermal impacts on aphids' biology and ecology are largely unknown.

Aphids have been model systems to study insect–microbial symbiont interactions. They harbor many symbiotic bacteria within their bodies, including primary or obligate (*Buchnera aphidicola*) and secondary or facultative (*Serratia symbiotica*, *Hamiltonella defensa*, *Regiella insecticola*, *Rickettsia* spp. and *Spiroplasma* spp.) symbionts [14–18]. These aphid-specific symbiotic bacteria perform various functions, such as obligatory *B. aphidicola* provides nutritional supplementation and other facultative endosymbionts confer their hosts resistance to natural enemies and environmental extremities [19–24]. Previous studies have demonstrated the potential mediation of heat tolerance in aphids by their obligate and facultative bacterial symbionts [25–28].

In this paper, we address the putative effects of high temperature exposure on the thermal tolerance of aphids and their bacterial symbionts' abundance. We intend to understand if short-term heat acclimation and chronic exposure to high temperatures would mediate any effect on the thermal tolerance, survival and abundance of symbiotic bacterial of aphids and if the thermal tolerance traits of aphids correlate to the abundance of their respective bacterial symbionts. To this end, individuals of two cereal aphids, i.e., bird cherry-oat aphid, *Rhopalosiphum padi* (L.), and English grain aphid, *Sitobion avenae* (F.), were chronically exposed to 31 °C (until death) and were acclimated to 34 °C for 3 h, before subjecting them to basal and acclimated critical thermal maxima (CTmax) determination. The abundance of aphid-specific bacterial endosymbionts was assessed using qPCR and their correlation with host thermal traits was worked out.

2. Materials and Methods

2.1. Collection and Rearing of Aphids

In this study, cereal aphids *R. padi* and *S. avenae* were studied as model species because of their differential performance under extreme temperature regimes [1,3]. About 100 wild clones of *R. padi* and *S. avenae* were randomly collected from winter wheat (*Triticum aestivum* L.) fields near the Henan (35°59′26.0″ N 114°31′37.9″ E) and Hebei (39°30′36.4″ N 115°55′58.2″ E) provinces of China. These aphid clones were transferred separately under cool conditions in plastic tubes. These clones were reared separately on wheat seedlings. Rearing was conducted up to F_3 generations under standard conditions, i.e., at 65 ± 5% relative humidity, 22 ± 1 °C temperature and under 16 h:8 h light:dark photoperiod.

2.2. Experiment of Chronic Thermal Exposure

In order to determine the association between chronic heat tolerance and the gene abundance of aphid-specific bacterial symbionts, we collected three batches of aphids from the three generations reared in the laboratory. There were 33 apterous (5 days old) active

and healthy aphid individuals in each batch. The tested aphids were reared individually on wheat leaves plugged in a moist sponge fixed in plastic tubes (30 mm diameter and 100 mm length) and the leaves were changed on alternate days. The three batches of aphids were exposed until death to 31 °C in a climate chamber (RXZ-280B; Jiangnan Ltd., Ningbo, China) set at 55–70% relative humidity and under 16 h: 8 h light:dark photoperiod. The observations were made at regular intervals of 3–6 h until the end of experiment and dead aphids were transferred immediately in vials containing 95% ethanol and were preserved at −20 °C in a freezer for the extraction of DNA. For the comparison of aphid-specific and total bacterial symbiont communities, dead aphid individuals were categorized into four mortality time periods as per their tolerance to chronic temperature (31 °C). For *R. padi*, mortality time period 1–4 refer to the individuals that died within 6–24, 24–48, 48–72 and 72–96 h, respectively, while for *S. avenae*, mortality time period 1–4 refer to the individuals that died within 6–18, 18–36, 3–54 and 54–66 h, respectively (Figure 1).

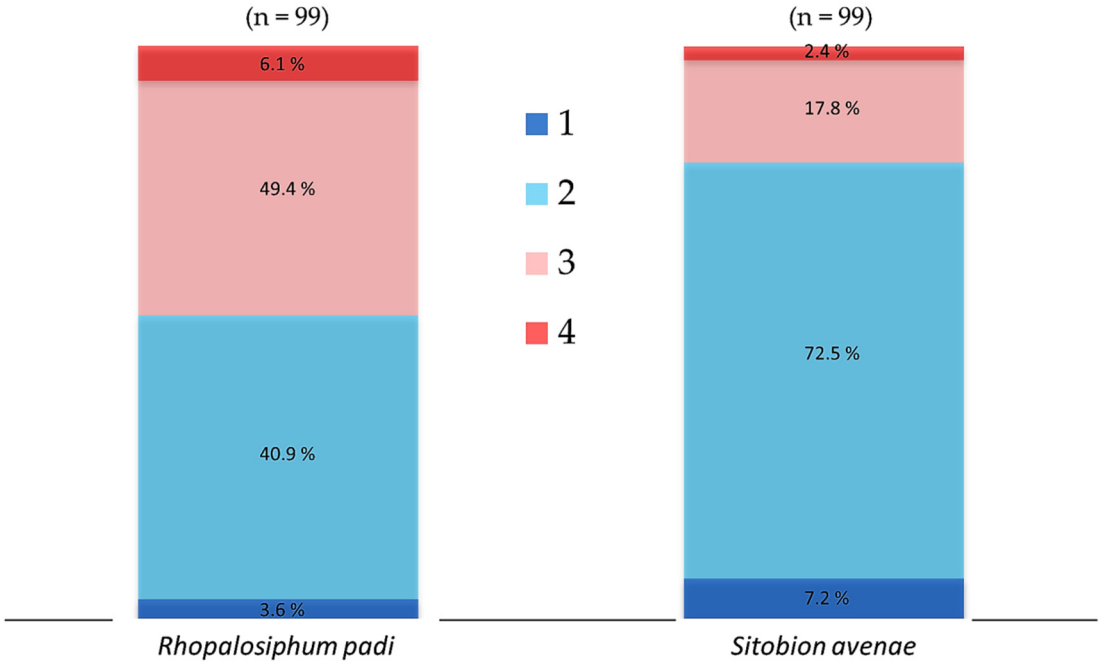

Figure 1. Cumulative percent mortality of cereal aphids *Rhopalosiphum padi* and *Sitobion avenae* exposed chronically to 31 °C. For each species, 33 aphid individuals were exposed from each of the laboratory-reared F_1, F_2 and F_3 generations. Dead aphid individuals were divided into four mortality time periods, i.e., 1–4, representing susceptible-to-tolerant thermal threshold levels. For *R. padi*, the mortality time periods 1–4 refer to the individuals that died within 6–24, 24–48, 48–72 and 72–96 h, respectively, while for *S. avenae*, mortality time periods 1–4 refer to the individuals died within 6–18, 18–36, 3–54 and 54–66 h, respectively.

2.3. Basal and Acclimated Critical Thermal Maxima Determination

To determine basal critical thermal maxima (CTmax), we collected 3 batches of aphids (each batch with 33 individuals) from the 3 generations of aphids reared in laboratory. The tested aphids were placed individually in a multi-well transparent plastic arena, which was then hanged in a vertical position in the middle of a double-layered glass container (20 × 30 cm) of a programed glycol bath with an accuracy of ±0.01 °C (Ministat 230-cc-NR; Huber Ltd., Berching, Germany). The temperature in the container was first maintained at 21 °C for 20 min and then was augmented gradually by 0.1 °C min^{-1} until the death of all test aphid individuals. Panasonic HDC-HS700 HD Camcorder (Panasonic, Osaka, Japan) was employed for recording the entire behavior of aphids in the arena plate during whole heating process. The temperature at which an aphid individual showed body spasms and lost its ability to move was noted as its CTmax value [29]. At the end of experiments, dead

aphids were transferred immediately in vials containing 95% ethanol and were preserved at −20 °C in a freezer for the extraction of DNA.

To determine acclimated CTmax, we collected 3 batches of aphids (each batch with 33 individuals) from the 3 generations of aphids reared in laboratory. The tested aphids were first acclimated at 34 °C for 3 h (this acclimation duration was selected as aphids did not lose their fitness up to 3 h in a pilot test; see Figure S1), and then were subjected to acclimated CTmax determination using same protocol as mentioned above for basal CTmax.

2.4. Quantification of Aphid Bacterial Symbionts

After surface sterilization with 1.0% sodium hypochlorite, the total genomic DNA was extracted from the preserved aphid individuals using TIANamp® genomic DNA Kit (Tiangen Biotech, Beijing, China), according to the protocol provided by the manufacturer. Purified DNA was eluted from each sample using 200 µL of Tris-EDTA buffer provided in the DNA extraction kit, and after quantification by NanoDrop™ spectrophotometer (ND-1000, Thermo Fisher Scientific, Waltham, MA, U.S.A.), it was preserved at −20 °C in a freezer until downstream molecular analysis. Diagnostic PCR tests were carried out with MyCycler™ thermal cycler (Bio-Rad Laboratories Inc., Hercules, CA, U.S.A.) for the detection and subsequent optimization of annealing temperatures of primers. Primer pairs along with their sequences used for the diagnostic PCR amplifications of obligate and facultative bacterial symbionts of aphid individuals are detailed in Table 1.

Table 1. Primer sequences used for the PCR amplification of total and aphid-specific bacterial symbionts of cereal aphids.

Symbiont Category	Type	Taxonomic Name	Bacterial GROUP	Gene Length (kb)	Primer Code	Real Time Quantitative PCR Primer Sequence (5′ to 3′)
Total Bacterial Community (16S rRNA)	Eubacteria			1.47	16S rRNAF	CCTACGGGAGGCAGCAG
					16S rRNAR	ATTCCGCGGCTGGCA
Primary/Obligate Symbiont	P-type	*Buchnera aphidicola*	Gammaproteobacterim	1.5	BaF	TGAGAGGATAACCAGCCACAC
					BaR	ATTTTTTTCCTCCCCGCTGA
Secondary/ Facultative Symbionts	R-type/PASS	*Serratia symbiotica*	Gammaproteobacterim	1.46	SsF	CCCTGGACAAAGACTGACGC
					SsR	CGACATCGTTTACAGCGTGGA
	T-type/PABS	*Hamiltonella defensa*	Gammaproteobacterim	1.3	HdF	CCTCCTAAACGAATACTGACGC
					HdR	CACTTCTCTTGGAAACAACCTC
	U-type/PAUS	*Regiella insecticola*	Gammaproteobacterim	1.3	RiF	AGCCACACTGGAACTGAGAAAC
					RiR	TCCTCCCCGCTGAAAGTGCT
	S-type/PAR	*Rickettsia* spp.	Alphaprotobacterium	1.46	RspF	AGTGGCGAAGGCTGTCATCT
					RspR	GCTGCGAAACTGAAAGAAAATC
		Spiroplasma spp.	Mollicutes	1.4	SspF	GCGGTAATACATAGGTGGCAAGC
					SspR	AAGGCGGTTAGGGGTTGAGC
					BaR	ATTTTTTTCCTCCCCGCTGA

For RT-qPCR amplifications, linearized recombinant plasmids (pGEM-T Easy Vector; Promega) were prepared using pGM-T Cloning Kit (Tiangen Biotech, Beijing, China) having a standard sequence inserts of target genes. Standard curves were created using serial dilutions (10-fold) of purified linearized plasmids containing 10^1 to 10^9 copies of the targeted bacterial genes. Each RT-qPCR reaction mixture of 20 µL was constituted of 10 µL 2× SuperReal PreMix Plus (Tiangen, Beijing, China), 0.6 µL of each of the 10µM forward and reverse primers, 8.4 µL of ddH_2O and 1 µL of 10 ng μL^{-1} of DNA template. The thermal protocol used for RT-qPCR amplifications included an enzyme activation step of 94 °C for 600 s, 40 cycles with denaturation, annealing and extension steps, respectively

at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 60 s. For each sample, three independent biological and technical replicates were run.

2.5. Statistical Analysis

Data were statistically analyzed using Statistix V. 8.1® (Tallahassee, FL, USA) analytical software. For each aphid species, the comparison of basal and acclimated CTmax indices was conducted by Student's paired *t*-test at $p \leq 0.05$. Similarly, Student's paired *t*-test was employed to compare the gene abundance of aphid-specific bacterial symbionts between different critical thermal maxima (CTmax) treatments or between both aphid species. Spearman's rank correlation analysis was worked out to explore the potential association among the gene abundance of aphid-specific endosymbionts and thermal indices of aphids.

3. Results

3.1. Symbionts Gene Abundance and Chronic Thermal Tolerance

When *R. padi* were exposed to 31 °C, mortality was less than 5% within 36 h, then increased rapidly and reached a high level of 25% between 36 to 66 h, and then maintained a low level of less than 10% between 66–96 h (Figure S2). The proportion of tested *R. padi* individuals that died within the mortality time periods 1–4 when exposed chronically to temperature of 31 °C was 3.6, 49.4, 40.9 and 6.1%, respectively (Figure 1). For *S. avenae*, when the tested aphids were exposed to 31 °C, mortality was less than 10% within 18 h, then it increased dramatically and reached a peak of ca. 40% between 18 to 36 h, and then decreased gradually from 15% to very low levels between 36–66 h (Figure S2). The tested *S. avenae* individuals that died within the mortality time periods 1–4 when exposed chronically to temperature of 31 °C were 7.2, 72.5, 17.8 and 2.4%, respectively (Figure 1).

Furthermore, qPCR determinations showed that tolerant *R. padi* individuals (of mortality time periods 3 and 4) harbored significantly higher gene copy numbers of total (16S rRNA) bacterial symbionts and of all aphid-specific bacterial symbionts, i.e., *B. aphidicola*, *S. symbiotica*, *H. defensa*, *R. insecticola* and *Rickettsia* spp. (Figure 2). For *S. avenae*, the difference was significant only for 16S rRNA, *S. symbiotica* and *Rickettsia* spp. (Figure 3).

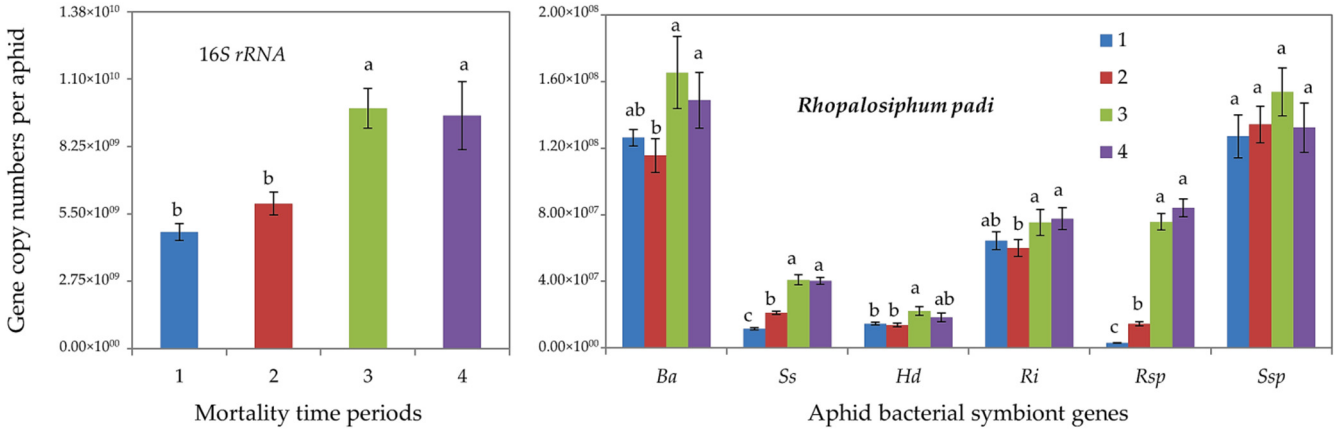

Figure 2. Abundance (mean gene copy numbers ± SD; n = 20) of bacterial symbionts of aphids (*Rhopalosiphum padi*) under chronic exposure to 31 °C. Dead aphid individuals were divided into four mortality time periods, i.e., 1–4, representing susceptible-to-tolerant thermal threshold levels. 16S rRNA = total eubacterial rDNA gene; Ba = *Buchnera aphidicola*; Ss = *Serratia symbiotica*; Hd = *Hamiltonella defensa*; Ri = *Regiella insecticola*; Rsp = *Rickettsia* spp.; Ssp = *Spiroplasma* spp. Different letters at the tops of the treatment bar show significant differences between treatments (one-way ANOVA; $p \leq 0.05$).

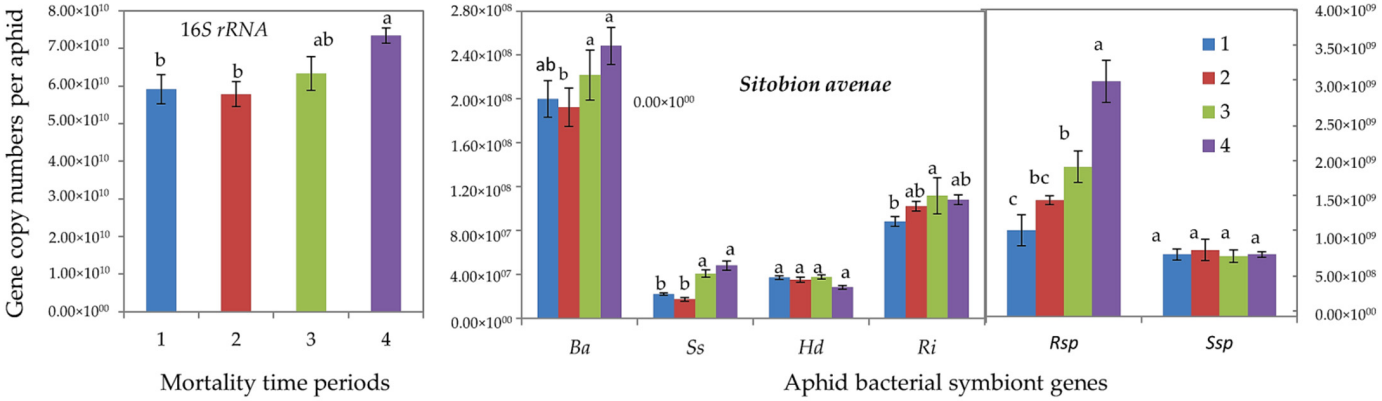

Figure 3. Abundance (mean gene copy numbers ± SD; n = 20) of bacterial symbionts of aphids (*Sitobion avenae*) under chronic exposure to 31 °C. Dead aphid individuals were divided into four mortality time periods, i.e., 1–4, representing susceptible-to-tolerant thermal threshold levels. 16S rRNA = total eubacterial rDNA gene; Ba = *Buchnera aphidicola*; Ss = *Serratia symbiotica*; Hd = *Hamiltonella defensa*; Ri = *Regiella insecticola*; Rsp = *Rickettsia* spp.; Ssp = *Spiroplasma* spp. Different letters at the tops of the treatment bar show significant differences between treatments (one-way ANOVA; $p \leq 0.05$).

3.2. Symbionts Gene Abundance and Acute Thermal Tolerance

Mean acclimated and basal CTmax values were 38.82 ± 0.44 °C and 37.41 ± 0.48 °C for *R. padi* and 37.53 ± 0.51 °C and 36.79 ± 0.46 °C for *S. avenae*(Figure 4). Acclimated individuals of both aphid species exhibited significantly higher CTmax values than the non-acclimated aphids (basal CTmax) (Figure 4). On average, CTmax values of *S. avenae* were about 1.0 °C less than those of *R. padi*.

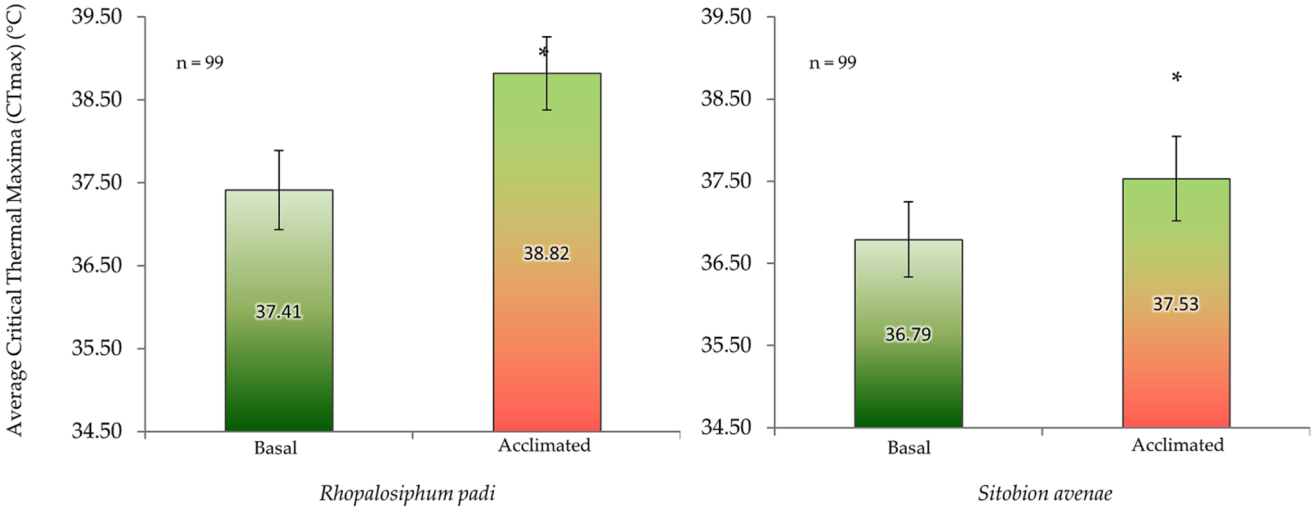

Figure 4. Basal and acclimated critical thermal maxima (CTmax) (mean ± SD; n = 99) of cereal aphids *Rhopalosiphum padi* and *Sitobion avenae*. Asterisks indicate significant difference among acclimated and basal CTmax for each aphid species (Student's paired t-test; $p \leq 0.05$).

Both aphid species had higher absolute gene copy numbers of total bacteria (16S rRNA) in non-acclimated (basal) aphids than the acclimated ones, but it did not reach the significant level. However, the gene copy numbers of *B. aphidicola*, *S. symbiotica*, *H. defensa* and *R. insecticola* in *R. padi* were significantly higher in the acclimated than in the non-acclimated (basal) aphid individuals (Figure 5). Likewise, in *S. avenae*, the gene abundance of *B. aphidicola*, *S. symbiotica* and *H. defensa* were moderately, but significantly, higher in the acclimated than non-acclimated (basal) aphid individuals (Figure 6). The mean gene abundance of total bacterial (16S rRNA) and aphid-specific bacterial symbionts was higher

in *S. avenae* than *R. padi*, except the abundances of B. aphidicola and R. insecticola gene copy numbers, which were similar in both species (Figure S3).

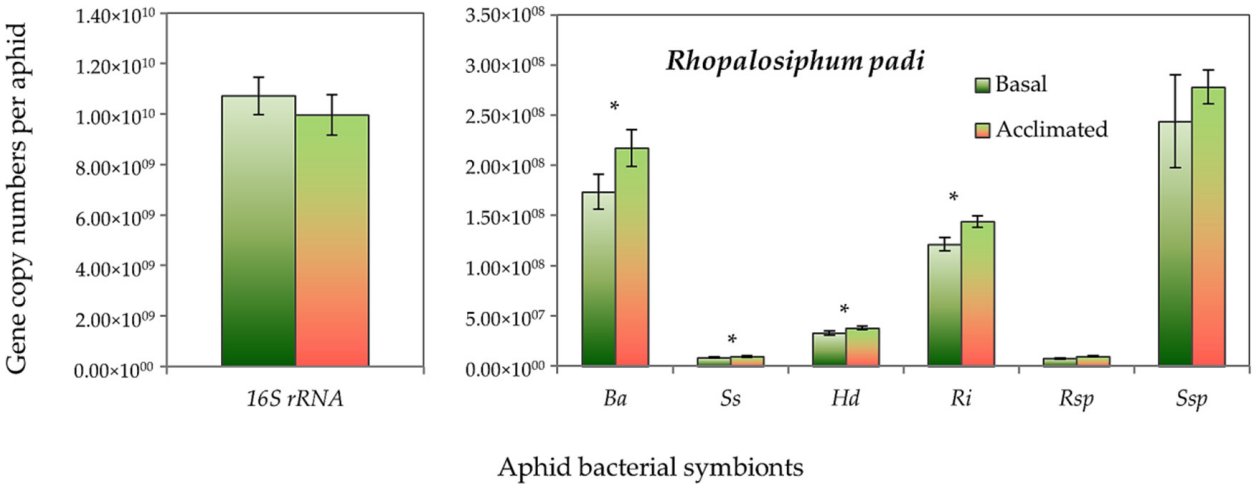

Figure 5. Abundance (mean gene copy numbers ± SD; n = 99) of bacterial symbionts of *Rhopalosiphum padi* aphids in acclimated and basal treatments. 16S rRNA = total eubacterial *r*DNA gene; Ba = *Buchnera aphidicola*; Ss = *Serratia symbiotica*; Hd = *Hamiltonella defensa*; Ri = *Regiella insecticola*; Rsp = *Rickettsia* spp.; Ssp = *Spiroplasma* spp. Asterisks signify significant difference between acclimated and basal treatments (Student's paired *t*-test; $p \leq 0.05$).

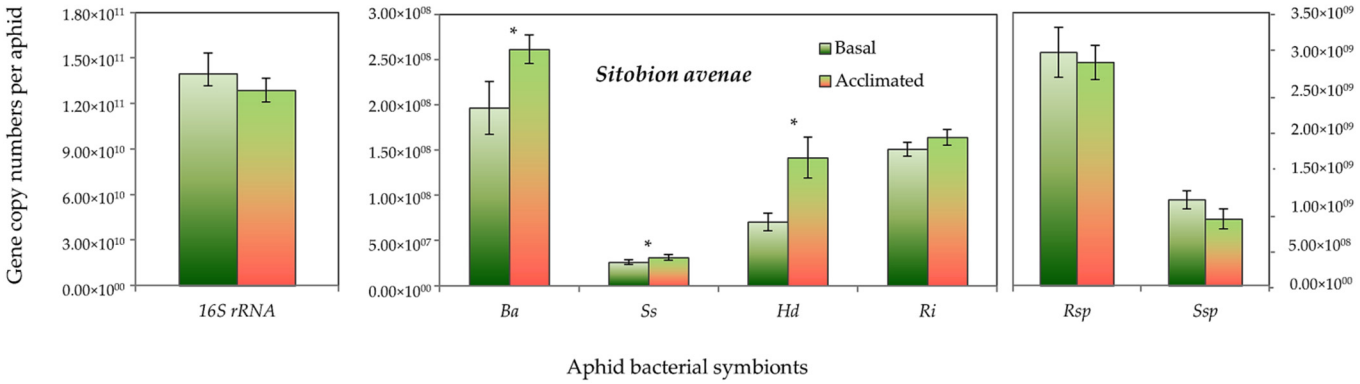

Figure 6. Abundance (mean gene copy numbers ± SD; n = 99) of bacterial symbionts of *Sitobion avenae* aphids in acclimated and basal treatments. 16S rRNA = total eubacterial *r*DNA gene; Ba = *Buchnera aphidicola*; Ss = *Serratia symbiotica*; Hd = *Hamiltonella defensa*; Ri = *Regiella insecticola*; Rsp = *Rickettsia* spp.; Ssp = *Spiroplasma* spp. Asterisks signify significant difference between acclimated and basal treatments (Student's paired *t*-test; $p \leq 0.05$).

Nevertheless, a significant rank correlation was recorded between the thermal tolerance indices and the abundance (gene copy numbers) of total symbiont density, *B. aphidicola*, *S. symbiotica*, *H. defensa*, *R. insecticola* and *Spiroplasma* spp. for *R. pad*i, and with the total symbiont density, total bacteria (16S rRNA) and with all aphid-specific bacterial symbionts (except *Spiroplasma* spp.) for *S. avenae* (Table 2).

Table 2. Correlation among the critical thermal maxima (CTmax) of cereal aphids with their bacterial symbiont gene abundance.

Bacterial Symbionts	*Rhopalosiphum padi*	*Sitobion avenae*
	(*n* = 99)	(*n* = 99)
Total eubacterial community	0.298	0.670 **
Buchnera aphidicola	0.529 **	0.939 **
Serratia symbiotica	0.481 **	0.850 **
Hamiltonella defensa	0.433 *	0.658 **
Regiella insecticola	0.414 *	0.618 **
Rickettsia spp.	0.248	0.390 *
Spiroplasma spp.	0.473 **	0.048
Total symbionts density	0.514 **	0.350 *

Spearman's Rank Correlation Coefficients (rho); * = correlation is significant at the 0.05 level (2-tailed); ** = correlation is significant at the 0.01 level (2-tailed).

4. Discussion

Extreme high temperature events exert significant effects on the thermal tolerance of aphids as simulated through chronic and acclimated exposures to high temperatures [7,9–13]. However, do the bacterial symbionts of aphids mediate the chronic and acute thermal tolerance of their hosts? This question remains to be cleared. This study was performed to determine the association between the chronic and acute thermal tolerance of *R. padi* and *S. avenae* aphids, and the gene abundance of their total (16S rRNA), secondary or facultative (*S. symbiotica*, *H. defensa, R. insecticola*, *Rickettsia* spp. and *Spiroplasma* spp.) and primary or obligate (*B. aphidicola*) endosymbiotic bacteria [17,18].

The findings of this study corroborated the fact that the *R. padi* species is more heat tolerant and exhibits higher evolutionary potential to high temperature events as compared to *S. avenae* [10,13,30]. Moreover, the acclimation-induced enhanced thermal threshold observed in both aphid species is in line with previous studies demonstrating the greater thermal plasticity induced by the acclimation to elevated temperatures and by heat-hardening in *Trichogramma* wasps [31], mites [32] and in other organisms, such as in the aquatic hydra/algae holobiont system [33].

Furthermore, a significantly higher gene abundance of bacterial symbionts, particularly of *B. aphidicola*, *S. symbiotic*, *R. insecticola* and *Rickettsia* spp., were recorded in the cohorts of temperature-tolerant aphid individuals (i.e., of mortality time periods 3 and 4) as compared to susceptible ones (i.e., of mortality time periods 1 and 2) for both aphid species. Likewise, the gene abundance of *B. aphidicola*, *S. symbiotica* and *H. defensa* were significantly higher in the acclimated than non-acclimated (basal) aphid individuals of both aphid species and for *R. insecticola* for *R. padi*. More interestingly, a significant and positive correlation was found among the thermal tolerance indices and gene abundance of total symbionts density, *B. aphidicola*, *S. symbiotica*, *H. defensa*, *R. insecticola* and *Spiroplasma* spp. for *R. padi*, and with the total symbionts density, total bacteria (16S rRNA) and with all aphid-specific bacterial symbionts (except *Spiroplasma* spp.) for *S. avenae*.

These results validate the potential role of aphid symbiotic bacteria in conferring ecological fitness and thermal tolerance to their host aphids [24–26,28,34,35]. Insect–microbial symbiont interactions play a vital role in the evolutionary adaptation of host insects to ecological stresses, such as extreme thermal exposures [36]. Many previous studies have demonstrated the significance of endosymbionts *S. symbiotica* and *H. defensa* in improving aphid tolerance to extreme temperature exposures [26,34–38]. Russell and Moran [35], Montllor et al. [34] and Dunbar et al. [39] demonstrated that bacterial the symbionts *S. symbiotica, H. defensa* and *B. aphidicola* ameliorate the thermal tolerance of their hosts and confer tolerance to high temperature exposures. However, how short-term acclimation boosted the gene abundance in the individuals of both species compared to basal (non-acclimated) needs further investigation. In-large, the results of this study corroborate that

the interactions of aphids and their bacterial symbionts may play an important role in aphids' thermal adaptation to high temperature exposures or events.

5. Conclusions

In short, a significantly higher abundance of bacterial symbionts was harbored by the individuals of the *R. padi* and *S. avenae* species tolerant to chronic thermal exposures than the susceptible ones. Short-term acclimation to 34 °C considerably enhanced the CTmax (thermal tolerance) for both aphid species. Furthermore, interestingly, the critical thermal maxima values of both species were positively associated with the gene abundance of *B. aphidicola*, *S. symbiotica*, *H. defensa* and *R. insecticola* signifying their putative role in conferring thermal tolerance to their host aphids. In future studies, the diversity and community structure of bacterial symbionts associated with these aphids should be conducted by Illumina deep sequencing of 16S rRNA.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/insects13030231/s1, Figure S1: Effect of different acclimation times on critical thermal maxima (CTmax) of 5-day-old apterous adults of cereal aphid *Sitobion avenae*; Figure S2: Cumulative percent mortality of cereal aphids *Rhopalosiphum padi* and *Sitobion avenae* under 31 °C for different exposure times; Figure S3: Gene copy numbers (mean ± SD) of total (16S rRNA) and aphid-specific bacterial symbionts in basal and acclimated 5-day-old apterous adults of cereal aphids *Rhopalosiphum padi* (RP) and *Sitobion avenae* (SA).

Author Contributions: Conceptualization, C.-S.M. Data curation, M.Z.M. and A.R.; Formal analysis, M.Z.M.; Funding acquisition, C.-S.M. and S.S.; Investigation, M.Z.M. and A.R.; Methodology, M.Z.M., A.R. and Z.B.; Project administration, C.-S.M.; Resources, C.-S.M.; Software, M.Z.M. and S.S.; Supervision, C.-S.M. and Z.B.; Validation, M.Z.M. and C.-S.M.; Writing—original draft, M.Z.M.; Writing—review and editing, C.-S.M., Z.B. and S.S.; All authors have read and agreed to the published version of the manuscript.

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

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Nematodes in the Pine Forests of Northern and Central Greece

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Simple Summary: Pine wood nematode *Bursaphelenchus xylophilus* is the agent of pine wilt disease and one of the most important forest tree pathogens worldwide, transmitted through beetles of the *Monochamus* genus. As an invasive species, it has spread beyond its natural range by human activity mainly wood trade. The devastating impact it has on pine forests has led to severe environmental and economic damages in its introduced countries. The wide distribution of *Monochamus* spp. beetles in many parts of the world along with favourable climatic conditions, which are both important factors for the establishment of pine wilt disease, have raised awareness over its continuous expansion. Therefore, in an attempt to control and even inhibit its further spread and consequently its severely adverse impacts, appropriate measures have already been taken and implemented from countries across the globe.

Abstract: In the context of plants or plant products protection by harmful organisms, measures have been taken by EU countries in order to prevent their introduction and establishment into the EU, and also limit their expansion in case they do enter. Such a case is *Bursaphelenchus xylophilus* (Parasitaphelenchidae, Nematoda), already recorded in Portugal and Spain. So, Member States should take all the appropriate steps in order to monitor and confine if necessary susceptible plants and/or plant products. Such measures include annual surveys even in countries where pine wilt disease does not occur yet. Therefore, national survey programs are widely established, sampling and examining samples from pine trees showing suspicious symptoms that could potentially be attributed to *B. xylophilus*. In this direction, such a network has also been established in Greece collecting and examining wood samples nationwide. In total, 123 wood samples were collected from conifer trees of Northern and Central Greece. Though *B. xylophilus* was absent from all samples examined, four other *Bursaphelenchus* species were identified. In addition, other nematode taxa were also recorded, including several phytophagous, microbivorous as well as predatory nematode species. This highlights the fact that besides preventing the introduction of *B. xylophilus* in Greece, national survey programs can significantly contribute to and enhance our knowledge of the indigenous nematode species.

Keywords: *Bursaphelenchus* spp.; national survey program; nematodes; conifers; Greece

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1. Introduction

The pine wood nematode (PWN) *Bursaphelenchus xylophilus* Steiner & Buhrer 1934 is one of the most important pathogens worldwide [1] that causes pine wilt disease (PWD), and it is currently included in the EPPO A2 list of pests that are recommended for regulation as quarantine pests [2]. *B. xylophilus* natural pathway of transport between hosts is by the adult stages of the longhorn beetle of the genus *Monochamus* (Coleoptera, Cerambycidae). *B. xylophilus* is transmitted either during maturation feeding on healthy trees

(primary transmission) or during oviposition on weakened and susceptible trees (secondary transmission) [3]. PWN dispersal juveniles ("dauer" larvae) are carried mainly within the respiratory system (tracheae) of *Monochamus* spp. beetles. During maturation feeding (phytophagous phase), PWN is transmitted on healthy pine trees where it spreads in the vascular system of the tree and resin canals. There it feeds on epithelial cells and living parenchyma causing a rapid reduction in the complete cessation of the resin flow. Cell destruction leads to embolism of the tracheids, blocking water conductance (cavitation) tree's death, or dead trees attract female insects for oviposition and nematodes enter the tree by oviposition slits in the bark (mycophagous phase). *Monochamus* spp. larvae burrow into the wood where nematodes surround the pupal chambers and enter into the insect's body through openings such as the spiracles. The transmission cycle continues through the maturation feeding of the young immature adult *Monochamus* insects. [3–5]. However, the high risk of introduction of the pine wood nematode into other countries, revealed in the relevant Pest Risk Analysis [6], is significantly magnified by human-mediated activities, following the routes and pathways employed by international wood trade either as a commodity or as wood packaging material (WPM) [7].

The pine wood nematode is indigenous in North America (the US and Canada) [8] where native pine species are relatively tolerant to its infestation. That was not the case in Japan, however, the first country where *B. xylophilus* was accidentally introduced at the beginning of the 20th century [9–13]. Japanese pine species were far more susceptible, and *B. xylophilus* expanded rapidly, resulting in extensive damages with reports of an annual loss rate in timber of 1.0 million m^3 in the 70′s that peaked at 2.4 million m^3 in 1979 [14,15]. Soon afterwards, *B. xylophilus* had spread to other neighboring Asian countries [16,17] that were confronted with similarly devastating impacts. In China, more than 1.7 million hectares had been affected by PWD until 2008, with more than 53 million trees dying out within a single year [18,19], whereas, in Korea, *B. xylophilus* is estimated to be causing annual losses of about 8 million US dollars [20] besides any additional costs for pest control and management [21].

In Europe, the pine wood nematode was first recorded in continental Portugal [22], and despite the containment measurements taken immediately [7], it subsequently expanded to Spain [23] and Madeira Island [24]. The estimated mortality risk of pine trees in southern Europe due to PWN is higher than 50%, something that could possibly have devastating effects on the European forests that occupy about 82 million hectares. The risk of *B. xylophilus* further spreading in Europe is even higher in areas where its insect vectors are present [25]. By 2030 the cumulative wood loss in the EU has been estimated at €22 billion representing 3.2% of the total value of PWN sensitive conifer trees [26].

Given the fact that high temperature and low humidity positively affect the spread and establishment of the PWD [10,27–29], coupled with the increase in international trade and movement of goods, the future impact of PWD is expected to increase [21,26], with Southern EPPO regions, in particular, exhibiting very high risk [30]. Situated at the eastern part of the Mediterranean basin, Greece can be readily included among the countries threatened most by a possible introduction of *B. xylophilus*, particularly as pine forests occupy a large proportion of Greek mainland and islands [31,32], its vector *M. galloprovincialis* Olivier 1795 is present [3] and Greece's suitable climate conditions [33,34] favour the progress of the disease [35]. In this direction, numerous samples from all over the country are annually examined in the framework of the Greek national survey program against forest quarantine pests, in the attempt to promptly detect the pine wood nematode and inhibit its unimpeded expansion in forest ecosystems. Nevertheless, survey programs can simultaneously increase our basic knowledge on indigenous nematode species occurring in Greek forests as well, something very important given the limited number of studies on indigenous species [36]. Therefore, the purpose of this study is to document the indigenous nematode community that inhabits Greek conifer forests parallel to the Greek national survey program against quarantine pests, in this case, *B. xylophilus*.

2. Materials and Methods

In the framework of the National Survey Program regarding *B. xylophilus*, wood disc samples were collected from areas situated in Northern and Central Greece as well as the Northern Aegean islands. Samples were collected from fourteen regional units, namely eleven from Northern Greece (Halkidiki, Drama, Evros, Florina, Pella, Pieria, Rodopi, Serres, Thesprotia, Thessaloniki and Xanthi) two from Central Greece (Aitoloakarnania and Karditsa) and one from Northern Aegean islands (Lesvos). Samples were collected from phytosanitary inspectors during their regular inspections on permanent sites or emergency inspections at sites with weakened and dead trees. Wood disc samples were collected from the trunk at breast height and/or the branches, while at the same the location and the coordinates of each site were recorded. Finally, wood disc samples were sent and examined at the Forest Research Institute in Thessaloniki. In total, one hundred and twenty-three wood samples were collected from dying or diseased conifer trees. Samples were processed immediately after their arrival at the laboratory.

Nematodes were extracted using a modified Baermann funnel technique [37], and each sample contained about 10 gr of wood, cut into small to medium-sized pieces. Wood chips were wrapped in fine mesh and placed inside glass funnels of 100 mm in diameter. At the end of each funnel, a piece of soft silicone tube was attached to the stem. The tube was closed with a squeezer clip, and the funnel was then filled with water until it entirely covered the wood chips. Funnels were placed on a wooden custom-made stand appropriately designed for the extraction of multiple samples. Wood chips were soaked in water for at least 48 h at room temperature. The presence of nematodes was detected with the use of a binocular stereoscope (Zeiss SV8, 2× magnification zoom). Isolated nematodes were picked with a micropipette and mounted on a glass slide for further identification under a microscope (Zeiss Axio Imager A1, 10×–100× magnification).

Nematode identification was based on their morphological characteristics such as the stomodeum, reproductive organs, and tail morphology [8,38–41]. Nematodes were also assigned to trophic groups according to Yeates et al. [42], Scholze & Sudhaus [43], and Ferris [44]. Nematodes were identified at species level for the genus *Bursaphelenchus*, and at genus or family level for the rest of the nematodes recovered, while in some cases where deeper taxonomic identification was not possible, they were only classified according to their trophic group. Only nematode occurrence (presence/absence) in each sample was documented.

In order to detect any differences in pine nematode communities between regional units, Cluster analysis was performed based on the identified nematode taxa (species, genera, and families). Nematodes classified only into trophic groups were excluded from the analysis. Unweighted pair-group average (UPGMA) was used as a hierarchical clustering algorithm while distances were estimated using the Dice similarity index. Analysis was performed using PAST 3.0 [45].

Finally, sample distribution was depicted using QGIS Desktop 3.10.12 A Coruña, and the coordinates were projected using the Greek coordinate reference GGRS87. Greek coastline and regional units' shapefiles were obtained from GEODATA.gov.gr (accessed on 10 February 2022) [46].

3. Results

Out of the 123 wood disc samples examined (Figure 1), nematodes were detected in 60 samples, i.e., 49% of the samples. *B. xylophilus* was not detected in any of the 60 samples, although other *Bursaphelenchus* spp. were detected in 35% of them (21 samples). Among those samples, 17 samples contained only one *Bursaphelenchus* species (81% of the samples), while the rest of the samples contained two *Bursaphelenchus* species. At the same time, the majority of wood disc samples with nematodes (95% of the samples) contained other nematode taxa together with *Bursaphelenchus* species.

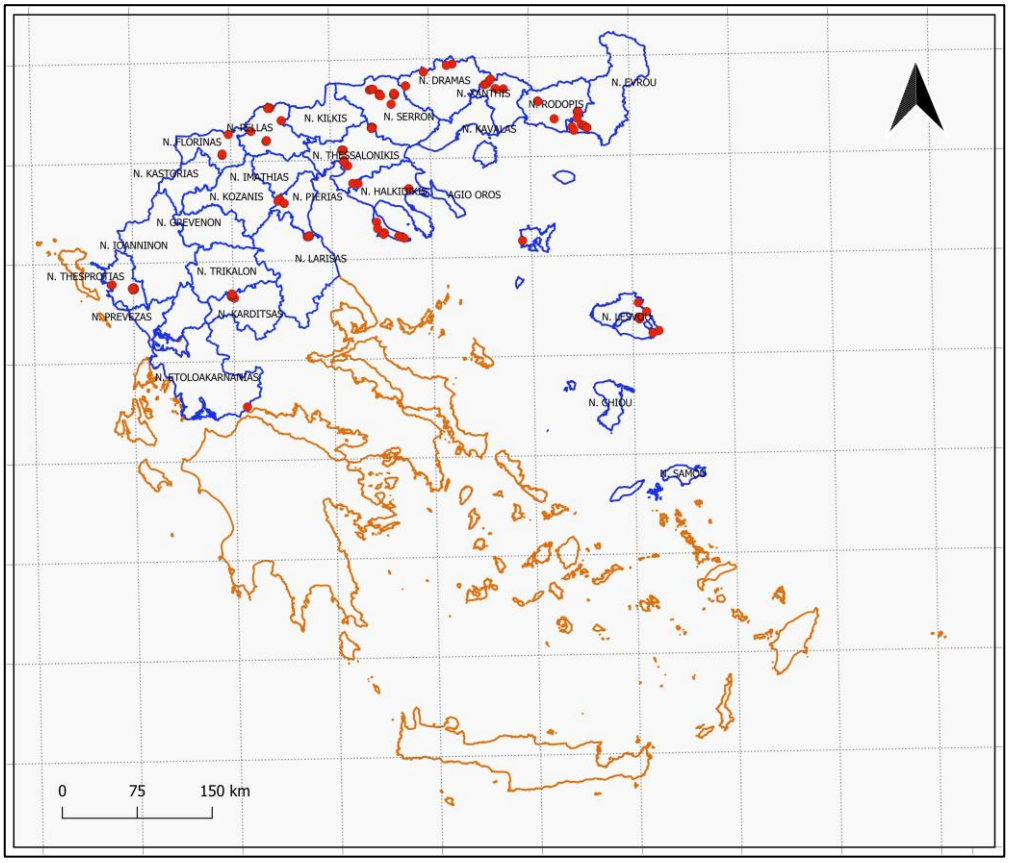

Figure 1. Sample distribution across the sampled areas.

In particular, four *Bursaphelenchus* species were identified: *Bursaphelenchus hellenicus* Skarmoutsos, Braasch, Michalopoulou 1998, *B. leoni* Baujard 1980, *B. mucronatus* Mamiya and Enda 1979 and *B. sexdentati* Rühm 1960. In addition to them, there were also some *Bursaphelenchus* spp. individuals that could not be assigned to a specific species (Table 1) due to either their premature stage and/or the condition of their body. *B. hellenicus* was the most abundant species followed by *B. mucronatus* and *B. sexdentati* with equal frequency, *B. leoni* was the least encountered species.

Regarding the other nematode taxa detected in the wood disc samples (Table 1), they can also be categorized into trophic groups as follows: *Aphelenchoides* sp., *Aphelenchus* sp., *Tylencholaimellus* sp. (fungivores), *Diplenteron* sp., *Eucephalobus* sp., *Panagrobelus* sp., *Panagrolaimus* sp., *Plectus* sp., *Pristionchus* sp., *Rhabditis* sp., *Rhodolaimus* sp. (bacterivores), *Devibursaphelenchus* sp., *Ektaphelenchus* sp., *Clarkus* sp. (predators), *Thonus* sp. (predator/omnivore) and *Parasitorhabditis* sp. (entomophilic). *Laimaphelenchus* sp. is classified in more than one feeding group as it includes non-parasitic plant feeding, fungivorous as well as predatory nematodes. Similarly, although *Devibursaphelenchus* sp. is classified as fungivorous by Ferris [44], it has also been reported predating on other nematodes [47,48]. Some individuals were identified to family level e.g., Anguinidae (fungivores/plant feeders), Tylenchidae (non-parasitic plant feeders), Dolichodoridae (plant parasitic), and Rhabditidae (bacterivores), while some others were separated only after their feeding group based on the structures of the mouthparts.

Nematodes were detected in the wood of the following conifer species: *Abies borisii-regis* Mattfeld, *P. brutia* Tenore, *P. halepensis* Miller, *P. maritima* Aito, *P. nigra* Arnold, and *P. sylvestris* Linnaeus, as well as unspecified *Pinus* species (Table 1).

In total, nematodes were detected in overall thirteen out of fourteen regional units, namely Halkidiki, Drama, Evros, Florina, Karditsa, Lesvos, Pella, Pieria, Rodopi, Serres, Thesprotia, Thessaloniki, and Xanthi (Figures 1 and 2). Among them, Thessaloniki and

Evros were the two regions with the highest number of wood disc samples with nematode presence, and at the same time, these areas exhibited also the highest number of nematode taxa, followed by Halkidiki and Drama (Figure 2). Except for Florina, Pieria, and Serres where no *Bursaphelenchus* spp. were detected at all, wood disc samples from every other area contained both *Bursaphelenchus* species and other nematode taxa (Table 1).

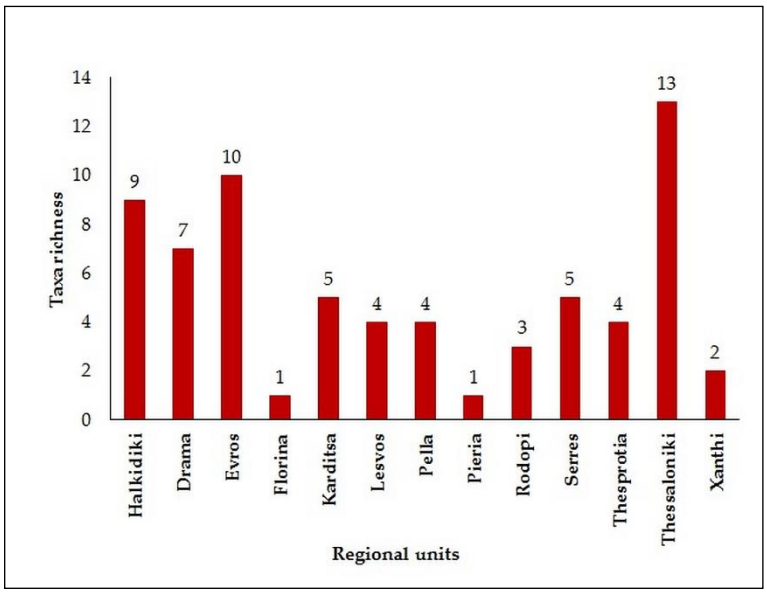

Figure 2. Nematode taxa richness per regional unit.

Cluster analysis based on the occurrence of nematode taxa (Figure 3) resulted in relatively heterogenous clusters with the exception of the marked cluster that includes Drama, Thessaloniki, and Halkidiki. Moreover, Pieria is distinctly separated from all other regions.

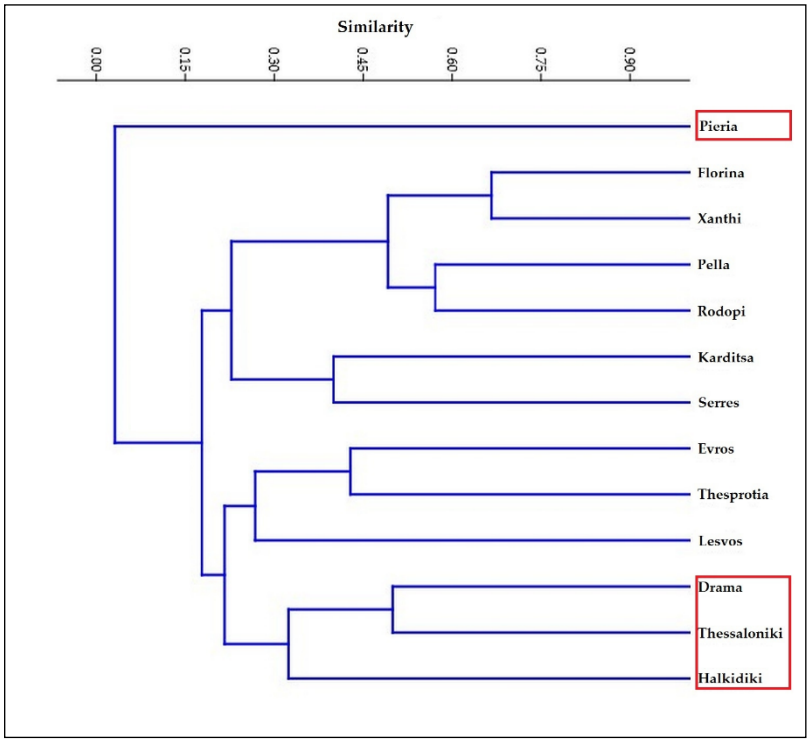

Figure 3. Cluster analysis on nematode taxa.

Table 1. Tree species, *Bursaphelechus* spp. and other nematode taxa detected per regional unit and locality.

Regional Unit	Locality	Tree Species	*Bursaphelenchus* spp.	Other Nematode spp.
Halkidiki	Kassandra	*Pinus halepensis*	*B. leoni*	*Aphelenchus* sp.
		Pinus halepensis	*B. mucronatus*	*Deviburs aphelenchus* sp.
		Pinus halepensis		*Diplenteron* sp.
		Pinus halepensis		*Parasitorhabditis* sp.
		Pinus halepensis		*Panagrolaimus* sp.
		Pinus halepensis		*Pristionchus* sp.
		Pinus halepensis		*Thonus* sp.
Drama	Drama	*Pinus* sp.		*Parasitorhabditis* sp.
		Pinus sp.		Tylenchidae
	Neurokopi	*Pinus* sp.	*B. mucronatus*	Bacterivore
		Pinus sylvestris	*B. hellenicus*	*Aphelenchoides* sp.
		Pinus sylvestris	*B. leoni*	Anguinidae
Evros	Alexandroupoli	*Pinus brutia*		*Ektaphelenchus* sp.
		Pinus brutia		*Panagrolaimus* sp.
		Pinus brutia		*Parasitorhabditis* sp.
		Pinus brutia		*Rhabditis* sp.
		Pinus brutia		Anguinidae
		Pinus sp.	*B. hellenicus*	*Clarkus* sp.
		Pinus sp.	*Bursaphelenchus* sp.	*Eucehpalobus* sp.
		Pinus sp.		*Laimaphelenchus* sp.
		Pinus sp.		*Parasitorhabditis* sp.
		Pinus sp.		Plant parasitic
Florina	Florina	*Pinus nigra*		*Laimaphelenchus* sp.
Karditsa	Mouzaki	*Pinus brutia*	*Bursaphelenchus* sp.	*Aphelenchoides* sp.
		Pinus brutia		*Rhabditis sp.*
		Pinus brutia		Anguinidae
		Pinus brutia		Dolicodoridae
Lesvos	Lesvos	*Pinus brutia*	*B. hellenicus*	*Eucehpalobus* sp.
		Pinus brutia	*B. sexdentati*	*Plectus* sp.
Pella	Aridaia	*Abies borisii-regis*	*B. mucronatus*	
		Pinus sp.	*Bursaphelenchus* sp.	*Aphelenchoides* sp.
		Pinus sp.		*Laimaphelenchus* sp.
	Pella	*Pinus sylvestris*		*Laimaphelenchus* sp.
Pieria	Pieria	*Pinus nigra*		*Panagrolaimus* sp.
Rodopi	Rodopi	*Pinus maritima*	*B. hellenicus*	*Laimaphelenchus* sp.
		Pinus maritima	*B. mucronatus*	Bacterivore
Serres	Sidirokastro	*Pinus brutia*		*Laimaphelenchus* sp.
		Pinus brutia		*Merlinius* sp.
		Pinus brutia		Anguinidae
		Pinus brutia		Dolichodoridae
		Pinus brutia		Rhabditidae
Thesprotia	Thesprotia	*Pinus* sp.	*B. hellenicus*	*Clarkus* sp.
		Pinus sp.	*Bursaphelenchus* sp.	*Tylencholaimellus* sp.

Table 1. *Cont.*

Regional Unit	Locality	Tree Species	*Bursaphelenchus* spp.	Other Nematode spp.
Thessaloniki	Lagkadas	*Pinus* sp.	*Bursaphelenchus* sp.	*Laimaphelenchus* sp.
		Pinus sp.		*Parasitorhabditis* sp.
		Pinus sp.		*Rhodolaimus* sp.
	Thessaloniki	*Pinus maritima*	*B. sexdentati*	*Aphelenchus* sp.
		Pinus maritima		*Laimaphelenchus* sp.
		Pinus maritima		*Panagrobelus* sp.
		Pinus maritima		*Plectus* sp.
		Pinus sp.	*B. hellenicus*	*Aphelenchoides* sp.
		Pinus sp.	*B. leoni*	*Merlinius* sp.
		Pinus sp.	*B. sexdentati*	*Parasitorhabditis* sp.
		Pinus sp.	*Bursaphelenchus* sp.	Anguinidae
Xanthi	Xanthi	*Pinus sylvestris*	*Bursaphelenchus* sp.	*Laimaphelenchus* sp.
		Pinus sylvestris		Bacterivore

4. Discussion

In the current study, nematodes and their communities in pine forests were systematically examined and recorded for the first time in Greece, enhancing significantly our basic knowledge of the indigenous nematode fauna. The study was conducted alongside the annual survey programme against harmful organisms, in this case, *B. xylophilus*. *B. xylophilus* was not detected in any of the wood samples examined. In general, the introduction of PWN in Greece through natural dispersal is not very likely since *Monochamus* spp. beetles, PWN insect vector, cover relatively short distances [3,49]. This fact, however, does not significantly reduce the risk of PWN invading Greece, as international trade and transport of wood products is considered to be the main pathway of *B. xylophilus* invasion and expansion [50,51], especially when *B. xylophilus* and its vector are introduced together [51]. In spite of the attempts to ensure proper treatment or monitoring of wood products, materials infested with *B. xylophilus* and/or its insect vector are being regularly recorded worldwide at points of entry, such as ports [52], even from countries known to be PWN-free [4,53]. For example, in Portugal, *B. xylophilus* presence is consistently recorded in areas around ports that are associated with the trade of goods [4]. Greece's ports as possible entry points for *B. xylophilus* are among the ones' that require high priority surveillance in order to prevent a rapid invasion of *B. xylophilus* and pine wilt disease across Europe [54].

Greece, like many other EPPO countries, is considered a risk area for the introduction and establishment of *B. xylophilus*, given the abundance of its host trees coupled with the occurrence of its insect vector [6]. Out of the seven indigenous *Pinus* spp. in Greece [31] four are susceptible to PWD: *P. halepensis*, *P. nigra*, *P. pinea* Linnaeus, *P. sylvestris*. In fact, *B. xylophilus* can be found in almost any conifer species (except *Thuja* and *Taxus* spp.) weakened enough to allow *Monochamus* species to oviposit and transmit the nematode in addition to pine species that express pine wilt disease [55].

Additionally, climatic conditions in Greece further favour a possible establishment of *B. xylophilus*. Average summer temperatures in the Mediterranean regions are high enough to support pine wilt disease in susceptible trees [56]. In Greece, the lowest minimum summer temperature is 20 °C [57], ideal for the development of both *B. xylophilus* and *Monochamus* spp. and consequently the expression of pine wilt disease. Both the nematode and its insect vector strongly depend on temperature. In fungal cultures of *Botrytis cinerea* Persoon (1794), the postembryonic development of *B. xylophilus* requires 12, 6, 4–5, and 3 days at 0 °C, 15 °C, 21 °C, 26 °C, and 30 °C, respectively [58], while it reproduces in 12 days at 15 °C, 6 days at 20 °C and 3 days at 30 °C [56]. *M. galloprovincialis* larval development is also dependent on temperature. There is a linear relationship between temperature and development duration in days between 15 °C and 30 °C [59]. However, the developmental rate seems to decrease above 30 °C for both PWN and pine sawyer

beetles [56,59], although areas with climatic conditions that do not favour the expression of the disease could possibly act as reservoirs. [6,50].

The natural dispersal of *B. xylophilus* between host trees occurs primarily during the maturation feeding of *Monochamus* (Coleoptera, Cerambycidae) species. Even though the main vector of PWN in Europe is *M. galloprovincialis* [60], and this species occurs widely yet in low population levels in Greece [61], there is always the risk of accidentally introducing non-native sawyer beetles [62,63]. Besides *M. galloprovinciallis* in Europe and *M. carolinensis* Olivier 1792 in North America or *M. alternatus* Hope 1842 in East Asia, many other *Monochamus* species have been reported capable of carrying *B. xylophilus* [3,49,64]. The remarkable biological similarities among *Monochamus* species globally, render many of these species putative vectors of *B. xylophilus*, particularly in the presence of their host trees [49]. Even though it is still not clear whether *Monochamus* species can directly cause tree mortality, infestation by the pine sawyer beetle is definitely weakening tree physiology, making it more susceptible to other secondary pests and diseases that ultimately lead to significant timber degradation and economic losses [3,51,65,66].

Moreover, besides *Monochamus* species as vectors of *B. xylophilus*, PWN has been found in association with other Coleoptera species such as *Acanthocinus griseus* Fabricius 1793, *A. gundaiensis* Kano 1933, *Amniscus sexguttatus* Dillon 1956, *Arhopalus rusticus* Linnaeus 1758, *Aromia bungii* Faldermann 1835, *Asemum striatum* Linnaeus 1758, *Corymbia succedanea* Hua 2002, *Neacanthocinus obsoletus* Olivier 1795, *N. pusilus* Kirby 1837, *Spondylis buprestoides* Linnaeus, 1758, *Uraecha bimaculata* Thomson 1864, *Xylotrechus sagittatus* Germar 1821, *Hylobius pales* Herbst 1797, *Odontotermes formosanus* Shiraki 1909, *Pissodes approximates* Hopkins 1911, *Tomicus piniperda* Linnaeus 1758, as well as other genera (e.g., *Acalolepta* sp., *Chrysobothris* sp., *Rhagium* sp.). However, there is still no evidence that any of these species can act as vectors of the nematode in nature [3,56,67].

Even though *B. xylophilus* was not identified among the nematode species retrieved from the wood disc samples, four *Bursaphelenchus* spp., *B. hellenicus*, *B. leoni*, *B. mucronatus,* and *B. sexdentati*, were detected in about half of the samples with nematode presence indicating a strong occurrence of this genus in pines. *B. hellenicus*, *B. mucronatus*, *B. leoni,* and *B. sexdentati,* as well as *B. eggersi* Rühm 1956 and *B. vallesianus* Braasch, Schönfeld, Polomski, Burgermeister 2004 have already been documented in Greece [32,68,69]. However, *B. eggersi* [32] a member of the *eggersi* group [38,40,41,70] and *B. vallesianus* [69], a member of the *sexdentati* group [38,40,41,70], were not detected in the present study. In general, *B. mucronatus* and *B. sexdentati* are acknowledged as the most abundant species in Europe, with the latter being more frequent in the southern European regions [70,71]. In contrast, *B. leoni* is recognized as a typical Mediterranean species, based on their dispersal and frequency, although they have also been occasionally found in Central Europe [68]. Finally, *B. hellenicus* exhibits the most limited natural range, which contains only two other countries, namely Italy [72] and Turkey [73] besides Greece [32,74]. Additionally, in terms of pathogenicity, *B. mucronatus*, *B. vallesianus,* and *B. sexdentati* have been characterized to be highly pathogenic [36,75,76] although such findings have not been confirmed under natural forest stand conditions [77], and the expression of virulence could also be dependent on host susceptibility as shown by Carropo et al. [78]. *B. leoni* was found to be less pathogenic whereas, *B. helleniccus* is considered to be non-pathogenic [36,68].

Most of the aforementioned *Bursaphelenchus* species have also been documented in Greece's neighboring and surrounding countries (Table 2). For example, in addition to *B. leoni* and *B. sexdentati, B. idius* Rühm 1956 have also been recovered from weakened trees in Cyprus [79,80]. Similarly, *B. anamurius* Akbulut, Braasch, Baysal, Brandstetter, Burgermeister 2007, *B. pinophilus* Brzeski, and Baujard 1997 and *B. vallesianus* are already known to occur in Turkey, besides *B. hellenicus*, *B. mucronatus,* and *B. sexdentati* [73,81–84]. On the other hand, species richness of *Bursaphelenchus* spp. in Italy is considerably higher, with numerous other *Bursaphelenchus* species (e.g., *B. abietinus* Braasch and Schmutzenhofer 2000, *B. andrassyi* Dayi, Calin, Akbulut, Gu, Schröder, Vieira, Braasch 2014, *B. eremus* Rühm 1956, *B. fraudulentus* Rühm 1956, *B. fungivorous* Franklin and Hooper 1962, *B. minutus*

Walia, Negi, Bajaj, Kalia 2003 and *B. tusciae* Ambrogioni and Palmisano 1998) having been identified [72,85–88] besides the ones already known in Greece [72,89], something that needs particular attention given the strong commercial relationships.

Table 2. *Bursaphelenchus* spp. records in Greece and neighboring countries (•) indicates presence [1].

***Bursaphelenhus* spp.**	**Cyprus**	**Greece**	**Italy**	**Turkey**
B. abietinus			•	
B. anamurius				•
B. andrassyi			•	
B. eremus			•	
B. fraudulentus			•	
B. fungivorous			•	
B. hellenicus		•	•	•
B. idius	•			
B. leoni	•	•	•	
B. minutus			•	
B. mucronatus		•	•	
B. pinophilus				•
B. sexdentati	•	•	•	•
B. tusciae			•	
B. vallesianus		•		•

[1] For references see text.

As more than one *Bursaphelenchus* species were found in almost 20% of the wood disc samples inhabited by nematodes, it can be easily deduced that a single tree can host more than one species at the same time. This is something that has also been reported in the past, with up to four different *Bursaphelenchus* species co-existing in one tree [70,89]. Furthermore, Penas et al. [90] have verified that one insect vector could possibly carry several *Bursaphelenchus* species, while one *Bursaphelenchus* species can have different insect vectors [8,70], suggesting a non-specific relationship between insect vectors and *Bursaphelenchus* spp. [90]. As a consequence, both these mechanisms could explain and maintain the co-existence of different *Bursaphelenchus* spp. in a single tree. Several insect species can carry *Bursaphelenchus* nematodes acting as vectors, mainly longhorn beetles (Cerambycidae), bark beetles (Curculionidae-Scolytinae), and jewel beetles (Buprestidae) [8,70,91,92]. For example, *B. mucronatus* was found to be associated with *Ips sexdentatus* Börner 1776, while *B. sexdentati* was associated with *Orthotomicus erosus* Wollaston 1857, *Acanthocinus aedilis* Linnaeus 1758, and *Arhopalus rusticus* Linnaeus 1758 [93]. On the other hand, insect species are capable of vectoring more than one *Bursaphelenchus* species, e.g., *O. erosus* carried three different *Bursaphelenchus* spp., *Hylurgus ligniperda* Fabricius 1787 two *Bursaphelenchus* species and both *Tomicus piniperda* and *I. sexdentatus* one *Bursaphelenchus* species each. [94].

To elucidate further the behavior and occurrence of *Bursaphelenchus* spp. within a host tree, sampling effort should aim at screening different parts of the same tree. Even though *Bursaphelenchus* species have been recovered from all parts of the tree, occurrence frequencies can differ [95]. Specifically, *Bursaphelenchus* spp. have been detected both in the stem and the branches, with numbers greater in the lower part of the stem compared to branches, whereas they have been detected even in the roots [75,96]. Similar findings have also been reported for *B. xylophilus* on several occasions. For instance, it has been shown that *B. xylophilus* nematodes migrate within infected trees soon after the initial infection or inoculation [97,98]. Trunk samples had significantly higher nematode density levels than the branches, as *B. xylophilus* nematodes moved from the infected branches to the stem after infestation [99–101].

Apart from *Bursaphelenchus* spp., other nematode taxa were also recovered in many wood disc samples. The retrieved taxa belong to different trophic groups, ranging from bacterivores and fungivores, to phytophagous and predatory nematodes, most of which have never been recovered from wood disc samples in Greece before. Many of the genera

recorded, apart from *Bursaphelenchus* spp., belong to families that are typical of environments with nutrient availability (Rhabditidae, Panagrolaimidae) or to families adapted to stress with a wide ecological range (Cephalobidae, Aphelenchidae, Aphelenchoididae, Anguinidae). On the other hand, Mononchidae and Quadsianematidae are more sensitive to disturbance and are commonly present in more stable environments [102]. Nevertheless, the presence of many different groups of nematodes appears feasible given the great variety of available resources as it is suggested by Moll et al. [103].

Many of the free-living nematode taxa recovered in the present study have also been reported to be associated with insects, in addition to their initial trophic group assignment [42,104–106] such as members of the families Aphelenchoididae, Rhabditidae, Neodiplogasteridae, and Panagrolaimidae [42,95,107]. *O. erosus, H. ligniperda*, *T. piniperda,* and *I. sexdentatus*, as well as *Hylastes linearis* Erichson 1836 and *Pissodes castaneus* De Geer 1775, have all been found to carry nematodes belonging to different genera of the Aphelenchoididae family or other taxonomic groups. As already mentioned, *O. erosus, H. ligniperda, T. piniperda,* and *I. sexdentatus* also carried members of the genus *Bursaphelenchus* [94]. Therefore, it is not uncommon for many nematode species to co-exist in a single host, as has been demonstrated in previous studies [75,80,83,89,108]. For example, Caroppo et al. [89] recorded the co-occurrence of Rhabditida, Aphelenchida, and Tylenchida nematodes, while Đođ et al. [109] found that saprophytic nematodes such as Rhabditidae, Diplogasteridae, and Cephalobidae were found to be dominant but also co-existing with low density populations of *Bursaphelenchus* spp. Similarly, numerous other nematode genera were found together with the *Bursaphelenchus* species recovered from *Pinus pinaster* Aiton trees in Portugal [100].

In order to investigate whether nematode communities from different areas differ from each other, a Cluster analysis was performed. The analysis was based on all reported taxa i.e., both on *Bursaphelenchus* spp. and the other documented nematode taxa (genera and families), and resulted in the formation of rather heterogenous groups. One would expect that areas with close proximity to each other would group together as in the case of Drama, Thessaloniki, and Halkidiki (Figure 3), which could mean that there is a great possibility that these areas share similar nematode communities, although this could not be verified at the present time. At present, it seems that the different areas examined are classified based on nematode taxa richness rather than community composition.

Many factors affect both nematode presence and community structure such as tree species, environmental variables, as well as the time of the year that sampling took place. For example, Moll et al. [103] who studied nematode communities from deadwood of 13 different tree species came to the conclusion that nematode composition was strongly related to tree species as well as the presence of other co-occurring biota such as fungi and prokaryotes. As already mentioned, environmental variables play an important role in nematode community composition. For instance, soil nematode communities appear to differ across different latitudes [110] while climate variables such as temperature and precipitation are strongly related to nematode community structure and composition [111,112]. Finally, even the time of sampling during the year could also be an important factor influencing nematode community studies since nematode community composition tends to differ among seasons [113].

Environmental traits such as temperature and humidity, as already mentioned, are factors of essential importance influencing the manifestation of PWD. Nematode infection of a healthy pine tree occurs from early June to late July, coinciding with the period of maturation feeding of adult pine sawyers when high temperature and low humidity promote the progress of PWD [10]. As Ichihara et al. [28] have shown, temperature affects migration patterns of *B. xylophilus* in the tissues of *Pinus thunbergii* Parlatore and the expression of PWD. Estimated optimal temperatures that PWD progresses have been reported to range from 25 °C to 30 °C [27,29].

Relative to climate, climate change is a great concern regarding *B. xylophilus* expansion into other countries and continents. As climate changes the distribution of PWN is expected

to expand along with the expression of the disease. Different global scenarios predict the expansion of PWN risk areas globally even in areas that are currently not suitable for the expression of the disease [114]. For instance, future climatic scenarios predict that by 2030 there will be a significant increase in the distribution of PWD across Europe ranging from 8% up to 34% of its total area, or even up to 55% under even more extreme scenarios [54]. As a result, the predicted changes in habitat suitability for the potential host trees (e.g., *Pinus sylvestris*) would additionally impair the physiology of these trees, rendering them more susceptible to pests and pathogens. This could ultimately alter the current PWD risk areas into high-risk areas in the near future [114]. In general, it can be easily deduced that as climate change progresses, both the intensity and the expansion of PWD is expected to increase, leading to even greater economic damages [21].

5. Conclusions

In summary, it can be easily deduced that in the framework of the national survey programs focusing on *B. xylophilus*, significant knowledge can be gained and accumulated regarding other *Bursaphelenchus* and nematode species as well. One-year observations and screening of samples from the northern and central parts of Greece have already resulted in the record of four different indigenous *Bursaphelenchus* species, coupled with the identification of 24 additional nematode taxa, enhancing significantly our knowledge of the poorly studied nematode species inhabiting pine forests in Greece.

Further future investigation, covering greater parts of Greece, even the whole Greek domain, together with the employment of molecular techniques will provide significant and more complete and accurate information regarding indigenous *Bursaphelenchus* spp. as well as the rest of the local coniferous nematode fauna.

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Further Evidence of Population Admixture in the Serbian Honey Bee Population

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Simple Summary: The western honey bee is one of the most ecologically and economically important pollinator species. Due to human interference, it faces serious challenges, not only in number decline and habitat loss, but also in natural subspecies diversity and distribution. The conservation of genetic diversity and perseverance of locally adapted populations and subspecies becomes a crucial task in the face of rapid environmental changes. In order to further assess present genetic variability in Serbian honey bee populations, we analyzed 14 microsatellite loci and then compared nine of them with previously published data. Our results suggest that Serbia now harbors a distinct, relatively homogenous honey bee population, although some local differences are still preserved.

Abstract: Socioeconomic interests and beekeeper preferences have often taken precedence over the conservation of locally native honey bee subspecies, leading to the predominance of admixture populations in human-dominated areas. To assess the genetic diversity of contemporary managed Serbian honey bee colonies, we used 14 microsatellite loci and analyzed 237 worker bees from 46 apiaries in eight localities of northern and southern Serbia. Furthermore, we compared data for nine microsatellite loci with 338 individuals from Italy, Hungary, Poland, and Spain. The standard parameters of genetic diversity in Serbian honey bee populations were in line with other analyses, although somewhat smaller. STRUCTURE analysis showed the existence of two equally distributed genetic clusters and Analysis of molecular variances could not confirm the presence of a geographically discrete population but showed local differences. Discriminant analysis of principal components showed overlapping of worker bees from different parts of Serbia. Clear genetic differentiation can be observed when comparing all populations between geographical regions and their corresponding subspecies. The absence of the *A. m. macedonica* subspecies from its historical distribution range in southern Serbia as well as the lack of distinctive geographical groups suggest that selective breeding, queen import, and migratory beekeeping practices strongly influence the genetic structure and diversity of honey bees, leading to the genetic uniformization and creation of the admixture population.

Keywords: honey bee; microsatellite; population genetics; genetic diversity

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1. Introduction

The western honey bee (*Apis mellifera* Linnaeus, 1758) is one of the species that is a subject of constant human interference. Although its domestication began more than 10,000 years ago, this species has never become truly domesticated despite all efforts, mainly due to its complex mating behavior [1–3]. The species' native range of distribution in western Asia, Africa, and Europe was expanded to all other continents, except Antarctica, to regions marked by highly distinct ecological and climate conditions. In addition to large-scale, transcontinental movements and expansion of the natural range, beekeeping practices and preferences for perceptively more suitable subspecies significantly changed distribution and variability in the historical range of distribution. In recent years, a substantial body of evidence has confirmed that, deliberately or not, humans shape the current diversity of honey bees worldwide [4].

Since the classical Ruttner categorization of the *A. mellifera* subspecies [5], there has been an ongoing debate about its taxonomy, number of subspecies, distribution range, and origin due to the specificity of population structure, features of biology, and resolutions of honey bee subspecies discrimination methods. Based on the results of genomic analysis [6–9], it was proposed that this species originated in northern Africa or the Middle East [10,11], but the most recent work [12] showed adaptive radiation of subspecies from Asia. From there, it colonized its native geographic range, and followed by multiple colonization waves and glacial events, it diverged into 33 existing subspecies [13] and divided into five evolutionary lineages (A, C, M, O, and Y). However, it still remains unclear how accurate this number of subspecies is, since many subspecies, due to high phenotypic plasticity, have ecotypes previously defined as subspecies [4,14–16]. The main differences between subspecies, often referred to as geographic races, are most likely the result of both local adaptations to distinct environments and geographical isolation. However, reproductive isolation does not often exist, and subspecies readily interbreed when they come into contact, although partial reproductive isolation is observed [17]. When subspecies come into contact, naturally or by human interference, it is inevitable that an admixture population will be established and that introgression of foreign genetic variants can be detected in native populations. This situation is especially prominent in Europe, which is the natural area of distribution of mitochondrial lineages A, M, and C. Beekeepers prefer subspecies *A. m. carnica,* and *A. m. ligustica*, both classified as C lineage, and their Buckfast hybrid has been imported to almost all parts of the continent [18–21]. It resulted in a considerable degree of genetic admixture among subspecies, even though in some areas the specific genetic footprint of autochthonous subspecies is still preserved [22–26]. For example, *A. m. mellifera* populations (belonging to the M lineage) have been hybridized in varying degrees in most of their native areas and in some parts, such as Germany, have been almost replaced by *A. m. carnica* because local beekeepers preferred this subspecies [27]. The loss of native subspecies and specific genetic diversity they harbor made conservationists and lawmakers in several countries establish protected areas for their native subspecies (Denmark and Great Britain) [19] as well as make laws that prohibit the breeding of nonnative bees (for example, Serbia and Croatia) [28].

Due to the spread of Varroa mites in the last decades of the twentieth century, it was believed that wild honey bee colonies became extinct in Europe, but now new evidence is emerging that some areas have thriving feral or even possibly wild honey bee colonies [29–32]. Therefore, it is not surprising that most research on the genetics of honey bees is conducted in managed colonies. Large-scale genetic comparisons show that genetic structure in any given area is heavily dependent on several factors, the most prominent of all being the level of importation of foreign queens and the presence and the type of breeding strategy implemented by beekeepers and their organizations. Extensive management by beekeepers promotes population admixture [33], which is expected when humans facilitate the movement and interbreeding of previously structured populations [34]. The general conclusion is that many lines used for contemporary beekeeping in Europe consist of a mixture of different source populations [35]. The bees from areas with frequent queen

importation show a high level of admixture and are hardly assignable to distinct subspecies anymore, but for those in areas where breeding lines were selected and maintained at their geographical origin, genetic identity was preserved and they resemble their native source populations [28,31,36–39].

It is noted that in areas that are natural contact zones of different *A. mellifera* subspecies, natural hybridization occurs [40,41] and the hybridization is inevitable in regions where human interference due to beekeeper preferences is high, which occurred in the C lineage native area of distribution [24–26,42,43]. Serbia, located in the center of the Balkan Peninsula, is geographically in the middle of the distribution range of the C lineage. Previous analysis showed that of four C lineage subspecies, Serbia harbors two (*A. m. macedonica* and *A. m. carnica*) which are clinaly distributed from the northwest (*carnica*) to the southeast (*macedonica*) with a hybrid zone between them [23,26,44,45]. Furthermore, nine described mtDNA haplotypes for *tRNA^{leu}-cox2* of which two are novel [46] and three distinct ecotypes belonging to specific geographical regions [47] reflect significant genetic variability of *A. mellifera* in this region. In the past 30 years, the variability of Serbian honey bees has been extensively described on morphological [26,48], etiological [49,50], and genetic levels [26,44,45,47,51–53]. Microsatellite analysis of Serbian honey bees from the first decade of the 21st century showed that although substantial admixture between *A. m. carnica* and *A. m. macedonica* in the central part of Serbia can be detected, populations from the northwest and southeast retain a distinctive subspecies genetic footprint [26]. The results of microsatellite and tRNA^{leu}-cox2 mtDNA variability [23] confirmed this clinal distribution of subspecies and their hybrids, with worker bees from northern Serbia forming a distinct genetic cluster characterized as *carnica-2* ecotype, and those from southern Serbia forming a different distinct genetic cluster characterized as the *macedonica*-1 ecotype.

However, beekeeping practices in Serbia have changed dramatically in the last decade. First, the number of managed beehives has doubled since 2009 [54], partly due to the government's financial support, and now Serbia has the largest number of beehives per capita worldwide (one beehive per six inhabitants). According to our field data, the traditional way of beekeeping is lost and the number of stationary apiaries is dwindling. The production of beekeepers who prefer *A. m. carnica* queens intensified, and the number of queen breeding institutions focused on its desired traits is growing. Serbia is one of the countries that has embedded in its legislation the preservation of autochthonous species, subspecies, and races, and as per the Law on Animal Breeding from 2009, breeding and keeping of only *A. m. carnica* are allowed in its territory [55]. Recent work based on the variability of the tRNA^{leu}-cox2 mtDNA region [46] suggests that the composition and distribution of honey bee populations in Serbia has changed over the past decade, invoking the need for further examination of genetic variability on various levels.

Biparental inherited microsatellite loci proved to be an excellent genetic marker for inferring overall population genetic variability, deciphering the distribution of different *A. mellifera* subspecies [19,56], detecting population admixture [7,26], and determining the presence of distinct locally adapted populations [20,57]. Large-scale analysis of microsatellite loci enables a better understanding of large and fine-scale population differentiation. To better understand the genetic variability of contemporary Serbian honey bee populations, we analyzed 14 microsatellite loci in 237 worker bees from the northern and southern parts of Serbia. In addition, we compared our data for nine microsatellite loci with previously published data [58] for 338 individuals from Hungary, Poland, Spain, and Italy belonging to *A. m. mellifera, A. m. carnica*, *A. m. iberiensis*, *A. m. ligustica*, and the Buckfast hybrid.

2. Materials and Methods

2.1. Sampling

A total of 237 worker bees were collected from 46 stationary apiaries during late August and early September in 2020 and stored in 95% ethanol at −20 °C for further analysis. Eight localities from southern and northern parts of Serbia were chosen, four in the south (Leskovac (L), Vlasina (V), Stara Planina (SP), and Tromeđa (T)) and four in the

north (Subotica (S), Vršac (Vr), Deliblatska pesščara (DS), and Fruška Gora (FG) (Figure 1). Approximately five worker bees from the apiary were chosen for genetic analysis, each representing one beehive. The detailed specification of sampling sites can be found in [46]. Furthermore, the DNA of 338 individuals from Hungary, Poland, Spain, and Italy belonging to *A. m. mellifera*, *A. m. carnica*, *A. m. iberiensis*, *A. m. ligustica*, and Buckfast hybrid from Péntek-Zakar 2015 were obtained for comparison.

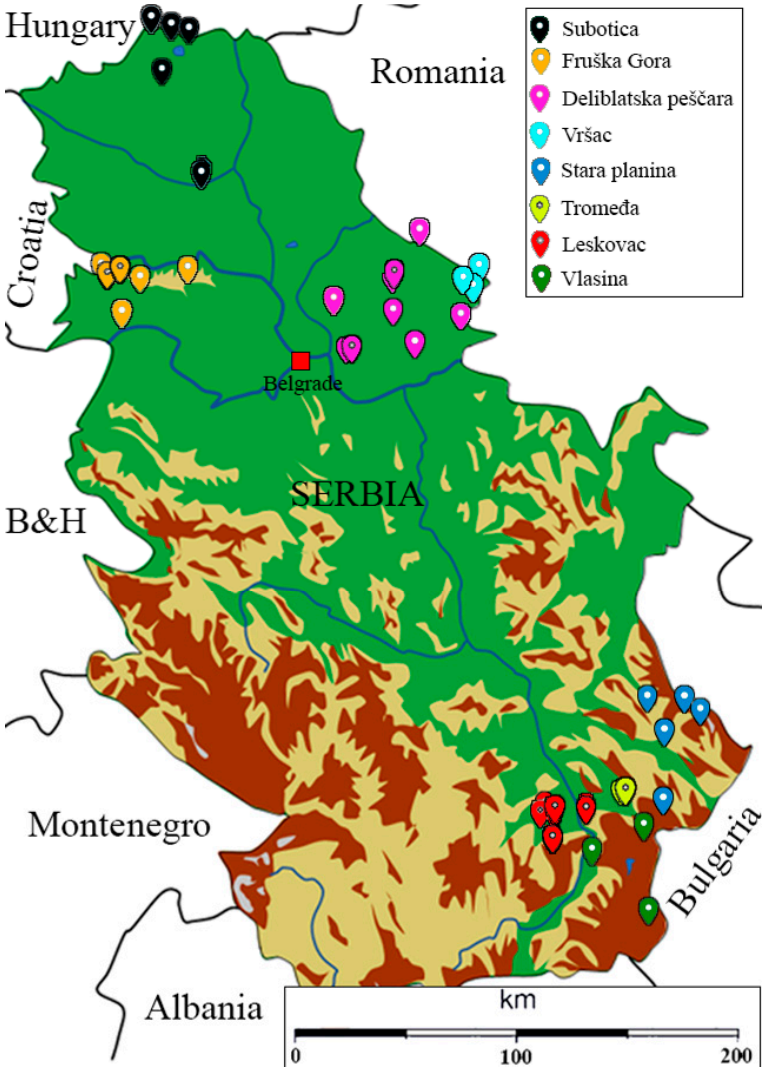


Figure 1. Sampling localities (from [46]).

2.2. DNA Extraction and PCR-RFLP Analysis

Whole-genomic DNA was extracted using the protocol described in [59]. The concentration of the extracted DNA and its quality were checked both with a spectrophotometer (NanoPhotometer, IMPLEN, Germany) and an agarose gelelectrophoresis.

The PCR-RFLP method described by [42] was used to distinguish *A. m. carnica* from *A. m. macedonica*. For the amplification of mtDNA COI fragment, the following primers were used: 5′-GATTACTTCCTCCCTCATTA-3′ [60] and 5′-AATCTGGATAGTCTGAATAA-3′ [53]. The PCR amplification of the COI fragment and subsequent digestion with *Nco*I and *Sty*I restriction enzymes were performed according to the protocol described in [46,61].

2.3. Microsatellite Analysis

For comparison of different honey bee colonies on the autosomal level, we have 14 microsatellite loci described in [61]. The choice of loci used in the analysis was made

according to the most frequent microsatellite loci used in a number of different studies dealing with the variability of these genetic markers. The selected loci, primer pairs used for amplification, and the corresponding annealing temperatures are presented in Table S1. The microsatellite loci were amplified in PCR reactions in which forward primers were labeled with a fluorescent dye (Table S1). PCR was performed in four reactions that differed in annealing temperature (Table S1) using the following program: one cycle of initial denaturation at 94 °C for 5 min, after which there were 30 cycles of 35 s at 94 °C, 35 s at annealing temperature (Table S1) and 35 s at 72 °C. The final elongation step was performed at 72 °C for one hour. Loci were amplified in a MiniAmp Plus Thermal Cycler (Applied Biosystems, ThermoFisher Scientific) in four multiplex reactions. The amplification was carried out in a volume of 20 µL with the following final concentrations of reaction components: 1 × Taq Buffer with $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 0.8 mM dNTP mix, 1 U of reverse Taq polymerase (all components were produced by Thermo Fisher Scientific, EU), and 5 pmol of each forward and reverse primer. For the amplification of the microsatellite loci, 1.5–1.9 ng of DNA was used. To verify the reliability of the data, 10% of samples were reamplified for the second time.

To use data from the study [58] we performed calibration by reanalyzing 10 samples and 9 microsatellite loci from this data set. The DNA of the same worker honey bees used in [58] was processed in the same way as the samples from Serbia.

2.4. Fragment Analysis

For fragment analysis, the first and second multiplex reactions were multipooled, and the third and fourth multiplex reactions were multipooled. All reactions were mixed in equal volumes and plated as one reaction in a volume of 1 µL. Each amplification mix contained seven different loci. GeneScan 600 LIZ size standard was used to score alleles (Applied Biosystems, Warrington, UK). Fragment analysis was performed on the 3130 Genetic Analyzer (Applied Biosystems, UK). Data were analyzed using Gene Mapper Software (Life Technologies, Foster City, CA, USA).

2.5. Statistical Analyses

The standard parameters of genetic diversity for microsatellite loci (number of alleles, allelic size range, average gene diversity over loci, number of alleles based on a minimal sample size (obtained by rarefaction), number of private alleles based on a minimal sample size (obtained by rarefaction), observed (H_O) and expected (H_E) heterozygosity, random match probability (RMP), and the mean number of pairwise differences (MPD)) were calculated using Arlequin ver. 3.5.2.2 software [62] and HP-Rare 1.1 [63]. The RMP parameter is used to express the probability that two randomly sampled individuals from a population have a matching genotype and is calculated as the sum of square frequencies [64]. MPD is a parameter that represents the measure of differences between all pairs of haplotypes in the sample. Arlequin ver. 3.5.2.2 software was also used to assess genetic differentiation among populations by analysis of molecular variances (AMOVA) and to estimate the pairwise population and overall F_{ST} and F_{IS} values. The statistical significance of all performed tests was assessed with 10,000 permutations. The matrix of pairwise population F_{ST} values was visualized using a multidimensional scaling method (nonmetric MDS) implemented in the PAST 3.25 software [65], and the R functions connected with Arlequin ver. 3.5.2.2 software. The Hardy–Weinberg equilibrium was tested using Arlequin ver. 3.5.2.2 software with 1,000,000 steps in MC and 100,000 dememorization steps. To correct the probabilities when multiple tests were performed simultaneously, we performed a sequential Bonferroni test for the Hardy–Weinberg equilibrium. The linkage disequilibrium between the pairs of loci was estimated using the likelihood ratio test in Arlequin ver. 3.5.2.2 software with 10,000 steps in MC and 10,000 dememorization steps.

The number of genetic clusters represented in the sample was estimated with STRUCTURE v 2.3.4 software [66–68]. For the analysis, the admixture model was used with a burn length of 10,000 and a Markov chain Monte Carlo (MCMC) of 100,000 randomizations.

The range of the possible number of clusters (K) was from 1 to 10, with a series of 10 runs for each K. The results obtained by STRUCTURE were analyzed by the STRUCTURE harvester [69]. To detect the number of K groups that best fit the data set, this software used results generated by the STRUCTURE software to create a plot of the mean likelihood value per K value and calculated the highest value of the second-order rate of change (Δ K) using the of Evanno method [70]. The model choice criterion, LnP (D), implemented in the STRUCTURE which detects the true K as an estimate of the posterior probability of the data for a given K, was evaluated as well. The most likely scenario was chosen and used to graphically plot both the individuals and populations analyzed.

The observed distances among samples are presented using discriminant analysis of principal components (DAPC) [71]. This method consists of performing the linear discriminant analysis (LDA) on the principal components analysis' (PCA) transformed matrix. In the case of samples from Serbia, only LDA was performed on the first 32 PCs and in the case of all analyzed populations on the first 55 PCs which cumulatively conserve 98.9% of the total variance. The number of retained PCs was estimated using randomly repeated cross-validation (100 iterations), which consisted of performing DAPC on 90% randomly sampled training set observations (stratified sampling was used so the training set consisted of 90% of the observations from each population) after retaining 10–183 PCs and using the obtained model to predict the groups (populations) in the remaining 10% of samples (test set). Average prediction success per group was used as a metric. Additionally, the PCA transformed matrix (all 183 PCs) was used to find the optimal number of clusters using Ward's method [72]. We tested 2–50 clusters, and the optimal number of clusters was chosen using BIC statistics using the "diffNgroup" method. This method uses Ward's clustering method to split the differences between successive values of the BIC summary statistic into two groups to differentiate sharp decreases from mild decreases or increases. The retained K was the one before the first group switch. Thus, estimated clusters of samples were compared with the a priori defined populations.

3. Results

3.1. PCR-RFLP

The size of the PCR-amplified COI segment for the RFLP analysis was 1029 bp. Digestion with both *Nco*I and *Sty*I did not show a restriction pattern characteristic for mtDNA lineage found in *A. m. macedonica*. Since no restriction sites were observed after RFLP analysis, we presume that all individuals in our sample belong to *A. m. carnica* [46,61].

3.2. Genetic Diversity Analysis

3.2.1. Genetic Diversity Analysis for 14 Microsatellite Loci in the Serbian Sample

The standard diversity parameters for the sampled localities in Serbia for all 14 analyzed microsatellite loci are presented in Table 1 and Table S2. The average numbers of alleles' observed heterozygosity and average gene diversity over loci were the highest in L, the lowest values for these parameters were found in T, Vr, and FG, respectively. Considering the mean number of private alleles based on minimal sample size, the highest number was observed in L, but values in other analyzed localities were in the same range. The observed heterozygosity was generally lower than expected and F_{IS} values varied between −0.04 in T and 0.19 in Vr (Table S3). It is interesting to note that the departure from the Hardy–Weinberg equilibrium coincided with significant heterozygote deficiencies, especially for locus A43 in all localities except FG. Furthermore, in all localities except L, observed heterozygosity before Bonferroni corrections for selectively adaptive locus Ap249 was significantly lower than expected (Table S2), and after correction it remained significant for T, FG, S, and DP. Linkage disequilibrium was also observed for some pairs of loci, mostly prominent for two selectively adaptive loci (Ap249 and B124) in three northern and one southern locality (Table S4).

Table 1. Standard diversity parameters for sampled localities in Serbia for 14 microsatellite loci.

Locality	N	Na	Agd	Ho	He	Ar	RMP	MPD	G-W	Ar8	Apr
Leskovac (L)	51	9	13.571	0.5984	0.5617	0.5958	0.00980	7.7786	0.6638	3.44	0.37
Vlasina (V)	14	5.7140	8.429	0.5584	0.5192	0.5888	0.03570	6.7011	0.6350	3.41	0.33
Tromedja (T)	15	5.1430	7.929	0.5184	0.4698	0.5397	0.03330	6.2207	0.6605	3.05	0.21
Stara Planina (SP)	25	6.7860	8.643	0.5559	0.5291	0.5785	0.02000	5.5592	0.6890	3.38	0.35
Fruska Gora (FG)	29	7.0710	10.714	0.5063	0.4662	0.5444	0.01780	5.5693	0.6163	3.2	0.26
Subotica	37	7.2140	10	0.5510	0.5054	0.5552	0.01350	7.7138	0.6667	3.19	0.22
Deliblatska Pescara (DP)	50	8.2140	13.071	0.5121	0.5098	0.5734	0.01020	4.0968	0.6096	3.3	0.34
Vrsac (Vr)	16	5.7860	8.500	0.5783	0.4311	0.5674	0.03120	6.9395	0.6516	3.28	0.35

N—number of genotyped individuals, Na—the average number of alleles, Ar—allelic size range, Agd—average gene diversity over loci, Ho—observed heterozygosity, He—expected heterozygosity, RMP—random match probability, MPD—mean number of pairwise differences, G–W—Garza–Williamson index, Ar8—number of alleles based on a minimal sample size of 8 diploid individuals, and Apr—number of private alleles based on a sample of 8 diploid individuals. Part of the results are published in Proceedings of the The 1st International Electronic Conference on Entomology session Apiculture and Pollinators, 1–15 July 2021, MDPI: Basel, Switzerland, doi: 10.3390/IECE-10720.

3.2.2. Population Genetic Analysis for Nine Microsatellite Loci in All Sampled Localities

The standard diversity parameters for all sampled localities for nine analyzed microsatellite loci are presented in Tables 2 and S5. The average number of alleles was the highest in Hungary and the lowest in the Polish sampled site, Wroclaw. The average gene diversity over loci was the highest in Poland and Spain and the lowest in Serbian populations. Heterozygosity excess was observed in all populations except those in Serbia, both for all analyzed loci and individual loci per population as well as for F_{IS} values (Tables S5 and S6). Linkage disequilibrium analysis was also performed for this set of data and the results are presented in Table S7.

Table 2. Standard diversity parameters for all analyzed localities for 9 microsatellite loci.

Locality	N	Na	Agd	Ho	He	Ar	RMP	MPD	G-W	Ar10	Apr
Hungary (*A. m. carnica*)	237	14	0.63694	0.89613	0.65753	35	0.0025	5.7325	0.5158	3.85	0.27
Spain (*A. m. iberiensis*)	10	5.3	0.73895	0.81687	0.64477	9.375	0.0500	3.6947	0.62704	4.18	1.22
Poland (*A. m. mellifera*)	45	9.3	0.75006	0.86408	0.75869	12.44	0.0111	6.7506	0.70839	4.62	0.36
Poland Aug forest	15	6.4	0.71239	0.88148	0.71239	7.667	0.0333	6.4115	0.74374	4.23	0.23
Poland Bialowieza	15	7.4	0.74738	0.82222	0.74738	11.667	0.0333	6.7264	0.63242	4.66	0.21
Poland Siedlice	15	6.1	0.75603	0.88889	0.75648	7.556	0.0333	6.0483	0.75483	4.35	0.06
Poland (*A. m. carnica*)	21	7.6	0.72887	0.90476	0.74671	9.000	0.0249	6.5598	0.78886	4.49	0.37
Poland Krakow	15	6.9	0.74253	0.9037	0.73498	8.667	0.0333	5.9402	0.76265	4.47	0.19
Poland Wroclaw	6	3.8	0.70076	0.90741	0.70932	5.222	0.0972	5.6061	0.65743	3.63	0.03
Buckfast (Hungary)	10	4.3	0.63158	0.84321	0.64419	7.556	0.05	5.0526	0.55482	3.57	0.13
Italy (*A. m. ligustica*)	15	5.3	0.60977	0.91111	0.6349	6.778	0.0333	4.8782	0.69998	3.58	0.08
Southern Serbia (*A. m. carnica*)	105	10.2	0.51083	0.46459	0.52209	14.67	0.0051	4.0866	0.69461	3.47	0.48
Serbia Leskovac	51	8.6	0.54021	0.50215	0.53994	12.444	0.0106	4.3217	0.68713	3.52	0.21
Serbia Vlasina	14	5.3	0.50970	0.44445	0.50970	8.222	0.0357	4.5873	0.64462	3.38	0.13
Serbia Tromedja	15	4.8	0.42644	0.41235	0.44805	7.889	0.0333	3.4115	0.63851	2.98	0.08
Serbia Stara Planina	25	6.7	0.46898	0.42468	0.51138	9.222	0.0208	2.8139	0.62132	3.44	0.20
Northern Serbia (*A. m. carnica*)	131	10.4	0.46634	0.40063	0.47838	13.333	0.0044	3.7307	0.72894	3.23	0.36
Serbia Fruska Gora	29	6	0.38355	0.38641	0.438	9.111	0.0196	2.6848	0.5726	3.02	0.08
Serbia Subotica	36	6.7	0.47344	0.44136	0.47344	9.556	0.0143	4.261	0.6193	3.11	0.06
Serbia Deliblatska Pescara	50	7.9	0.3918	0.40292	0.49063	12.889	0.0104	1.959	0.6065	3.28	0.13
Serbia Vrsac	16	5.2	0.49568	0.33029	0.49716	8.667	0.0312	3.4698	0.59755	3.23	0.26

N—number of genotyped individuals, Na—the average number of alleles, Ar—allelic size range, Agd—average gene diversity over loci, Ho—observed heterozygosity, He—expected heterozygosity, MPD—mean number of pairwise differences, RMP—random match probability, G–W—Garza–Williamson index, Ar10—number of alleles based on a minimal sample size of 10 diploid individuals, and Apr—number of private alleles based on a sample of 10 diploid individuals.

3.3. Population Structure

3.3.1. Population Structure Based on 14 Microsatellite Loci in the Serbian Sample

The average number of pairwise differences between and within Serbian localities together with *Nei's* distances is visualized in Figure 2a and pairwise F_{ST} is visualized in Figure 2b.

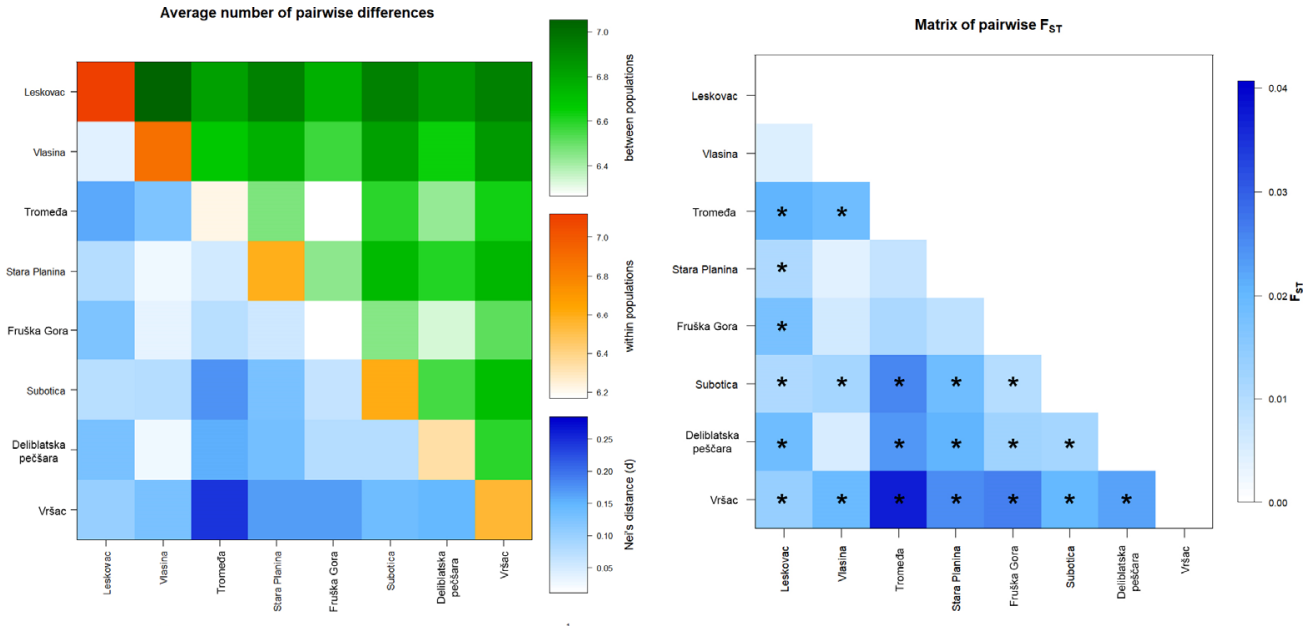


Figure 2. Matrixes of the average number of pairwise *Nei's* (**a**) and F_{ST} (**b**) distances based on the analysis of 14 microsatellite loci for localities in Serbia. (**a**) The average number of pairwise differences between populations is presented above diagonally, the average number of pairwise differences within the population is presented diagonally, and *Nei's* distances are presented below diagonally. (**b**) Statistically significant F_{ST} values are marked with an asterisk (*). Part of the results are published in Proceedings of the The 1st International Electronic Conference on Entomology session Apiculture and Pollinators, 1–15 July 2021, MDPI: Basel, Switzerland, doi: 10.3390/IECE-10720.

Differences between some pairs of localities were consistent in all analyses with the Vr locality showing statistically significant pairwise differences with all analyzed localities. Statistically significant differences between localities were observed for pairs of the south (L-SP and L-T), all north and south/north (L-FG, L-DP, L-S, V-S, SP-S, and SP-DP) comparisons (Figure 1, Table S8). Overall, the north showed greater population differences than the southern regions, and there is no clear pattern of differentiation between the geographical regions.

The AMOVA performed across all 14 loci showed a low but significant value of genetic variance between localities (0.047), with 1.42% of the genetic variance being attributed to the variation among localities (Table 3). When localities were grouped according to their geographical region, the percentage of variation was higher within regions than among them. Additionally, when localities were grouped according to their region, the percentage of variation among localities within regions remained statistically significant, while differentiation between geographical regions could not be observed (Table S9).

The results of the analysis performed by the DAPC method are shown in Figure 3, Supplementary Figure S1, and the visualization by the MDS plot shows the positioning of populations in two dimensions (Figure 4). Although individuals from T, SP, L, and DP tend to cluster separately from others, DAPC analysis showed that individuals from geographically remote localities are grouped in cluster overlaps, indicating similarity between them. Moreover, assignment to the previous predesigned group was relatively

low, with p ranging 0.2–0.3, indicating admixture. The MDS plot placed localities separately from each other, which is in correlation with AMOVA, suggesting the presence of distinct genetic variability in all analyzed localities. However, there is no clear grouping of localities according to their geographical region, which is also in concordance with AMOVA.

Table 3. AMOVA results when all localities in Serbia were analyzed without grouping.

Source of Variation	d.f.	SS	Variance Components	Percentage of Variation
Among localities	7	43.424	0.047	1.42 (**p = 0.005**)
Among individuals within localities	229	797.685	0.184	5.49 (**p = 0.0001**)
Within individuals	237	738.685	3.116	93.1 (**p = 0.000**)
Total	473	1579.61	3.347	

d.f.—degrees of freedom, SS—the sum of squares, and p-statistical significance (statistically significant values are in bold).

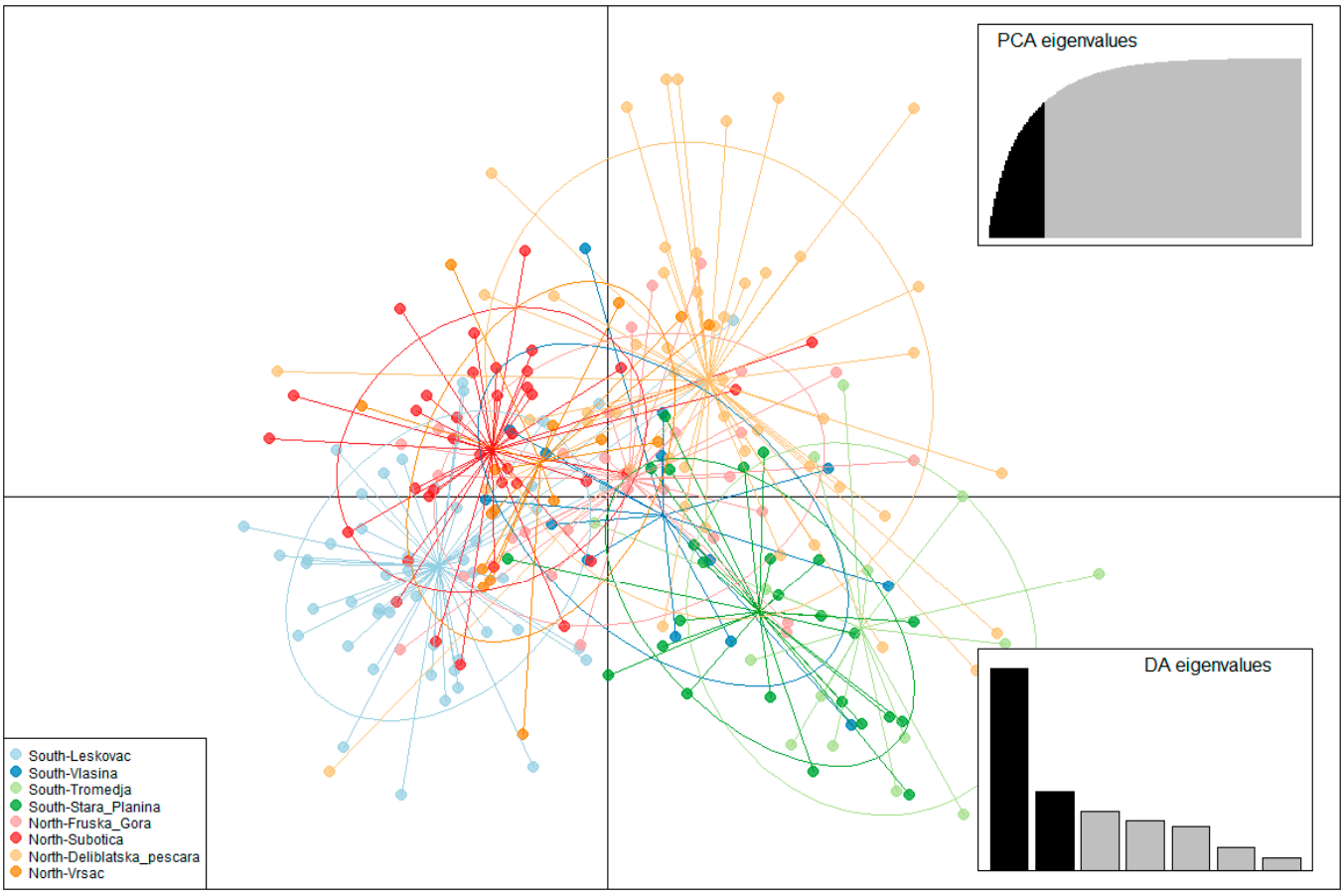


Figure 3. Discriminant analysis of principal components. The first and second linear discriminants are presented in the plot.

The STRUCTURE Harvester showed that K = 2 is the most likely scenario (Supplementary Figure S2). The same number of clusters was inferred by LnP (D) analysis (Supplementary Figure S3). Both clusters inferred by STRUCTURE are equally distributed in all sampled localities. Additionally, the number of clusters inferred with the DAPC method was 8, with mixed distribution across localities (Supplementary Figure S4).

3.3.2. Population Structure Based on Nine Microsatellite Loci in All Sampled Localities

The average number of pairwise differences between and within localities grouped according to their geographical region and subspecies together with *Nei's* distances is visualized in Figure 5a, and pairwise F_{ST} is visualized in Figure 5b. As expected, individuals

from Spain were shown to be the most separated from others, but the separation between region and subspecies can also be observed since statistically significant F_{ST} values were obtained (Table S10). The same conclusion can be inferred when all sampled localities were compared separately (Supplementary Figures S5 and S6, and Table S11). It is interesting to note that some differences between Serbian localities disappear but that there is a clear distinction between Serbian localities and other analyzed localities and subspecies. Moreover, very low but statistically significant F_{ST} value was detected between southern and northern Serbia.

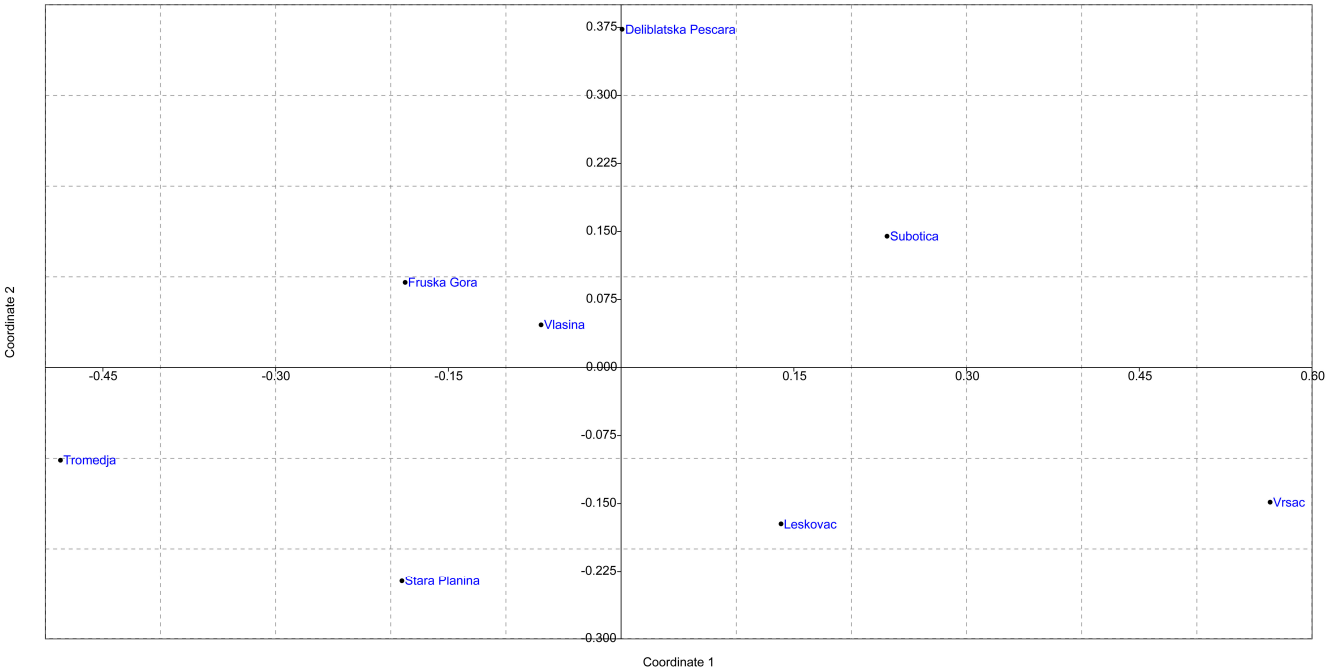
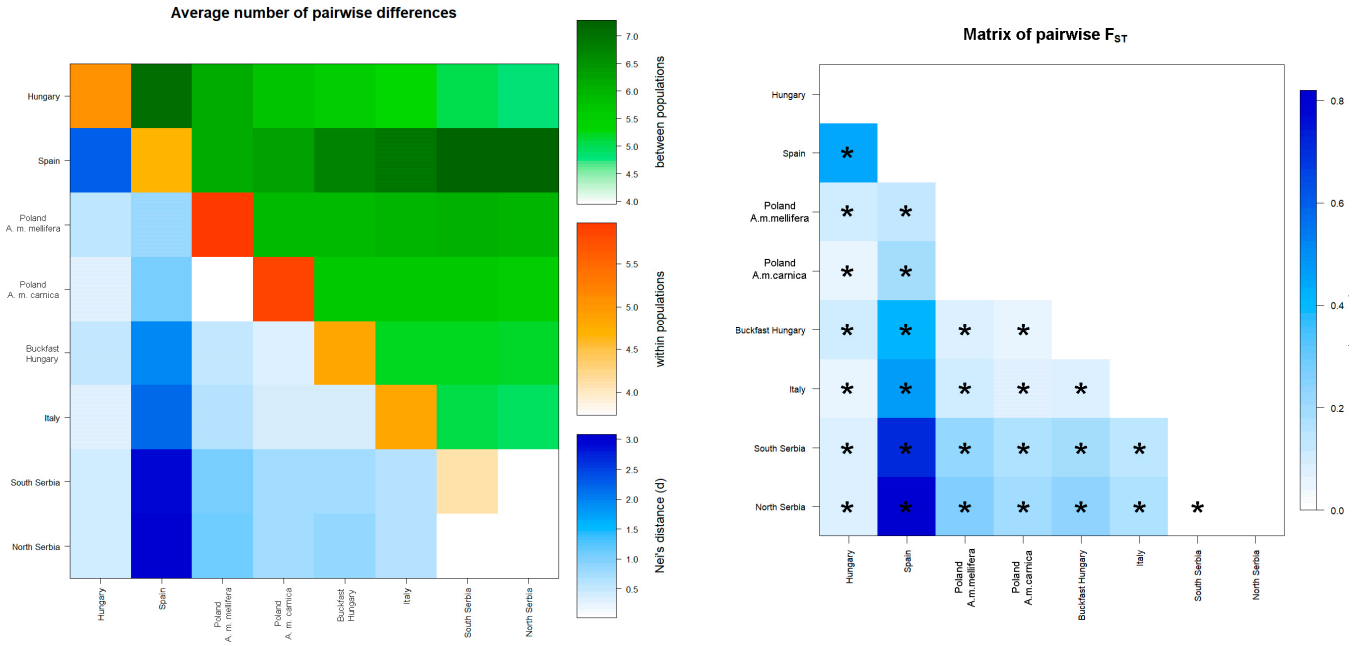


Figure 4. Non-metric multidimensional scaling plot of F_{ST} distances between localities in Serbia. The goodness of fit is expressed with the stress value which is 0.1519 for this data set. Population pairwise F_{ST} values are presented in Table S8.

The AMOVA performed across nine loci showed a high and significant value of genetic variance among localities (0.28) with 10.79% of the genetic variance being attributed to the variation among the localities (Table 4). A negative value of differences among individuals within localities indicates that individuals in any given sampled population are mostly uniform and closely related to each other. Additionally, when AMOVA was performed with a different grouping of localities and subspecies, differences among geographical regions and subspecies remained significant, indicating regional differentiation that reflects subspecies and geographical distribution (Table S12).

The results of AMOVA were further corroborated by DAPC analysis (Figure 6 and Supplementary Figure S7) and the positioning of the populations in two dimensions in the MDS plot (Figure 7). When localities were grouped according to geographical region and subspecies, clear differentiation could be observed. As expected, Spain's population is the most separated from the others. Buckfast individuals from Hungary are closer to Italian individuals than Hungarian ones, and the Hungarian population is relatively homogeneous as previously reported. Serbian localities were in a cluster overlap and separated from other analyzed populations. The alternative grouping of localities and subspecies does not change the relative relations among the analyzed localities; localities from the same geographical region and subspecies were always clustered together and separated from others (Supplementary Figures S5–S7).



(**a**) (**b**)

Figure 5. Matrixes of the average number of pairwise *Nei's* (**a**) and F_{ST} (**b**) distances based on the analysis of 9 microsatellite loci when all localities were grouped according to geographical region and subspecies. (**a**) The average number of pairwise differences between populations is presented above diagonally, the average number of pairwise differences within the population is presented diagonally, and *Nei's* distances are presented below diagonally. (**b**) Statistically significant F_{ST} values are marked with an asterisk (*).

Table 4. AMOVA results when all localities were analyzed without grouping.

Source of Variation	d.f.	SS	Variance Components	Percentage of Variation
Among localities	16	286.781	0.2801	**10.79 (p = 0.00)**
Among individuals within localities	557	1034.257	−0.4601	−17.72
Within individuals	574	1594.000	2.7770	106.93
Total	1147	2915.037	2.5970	

d.f.—degrees of freedom, SS—the sum of squares, and p-statistical significance (statistically significant values are in bold).

The STRUCTURE Harvester showed that K = 8 is the most likely scenario (Figure 8), since the LnP (D) showed that K = 8 best fits the data even though Δ K suggested K = 2 has the highest probability (Figure S8). In our data, K = 8 gives the most plausible distribution of inferred genetic clusters, which were specifically distributed among the individuals in the populations originating from different geographical regions or subspecies. Furthermore, the number of clusters inferred with the DAPC method was four, with specific distribution of clusters across sampled geographical regions (Supplementary Figure S9).

Based on all analyses it can be concluded that strong geographical differentiation exists between analyzed geographical regions and their corresponding subspecies.

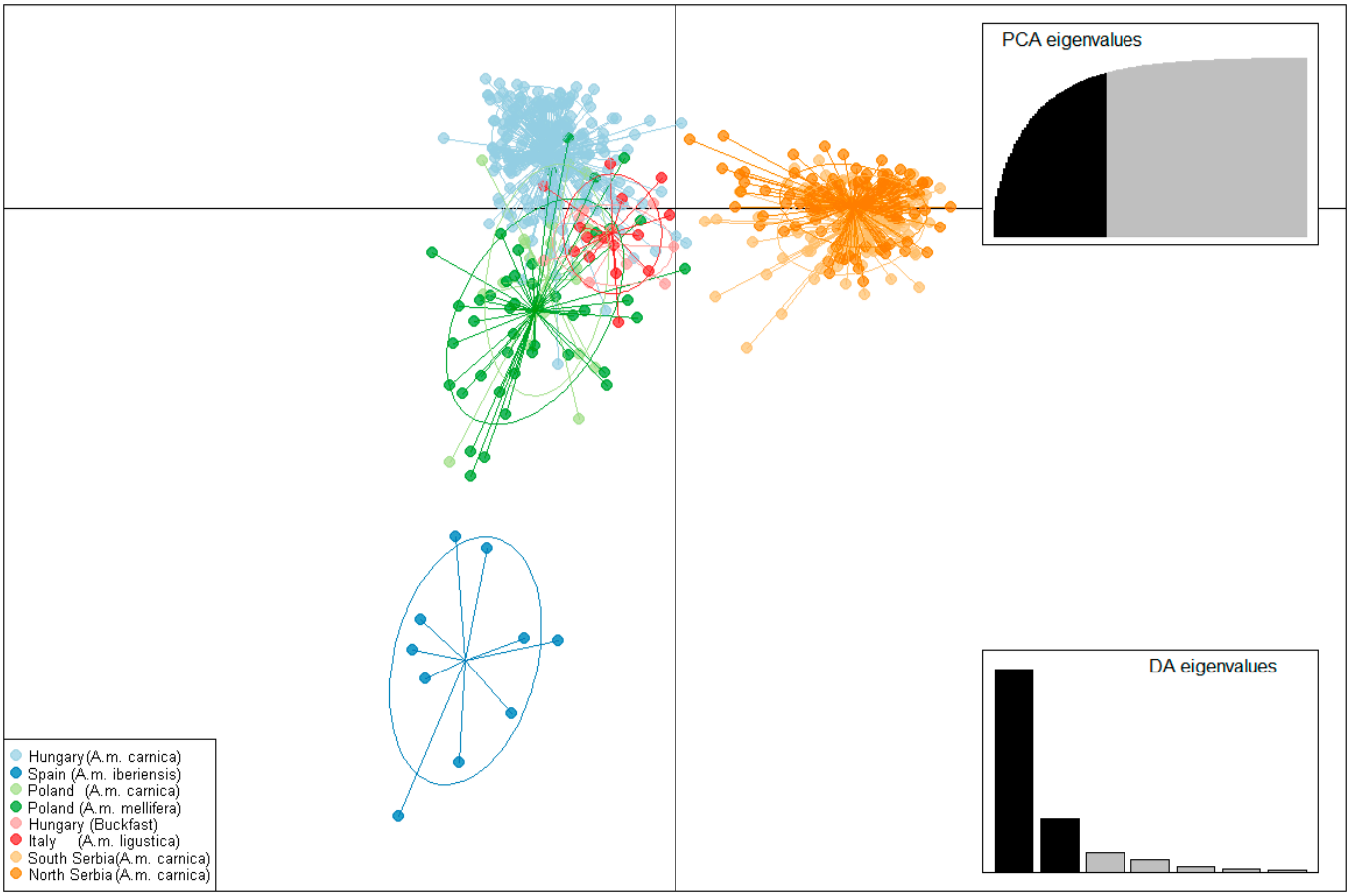


Figure 6. Discriminant analysis of principal components when all localities were grouped according to their geographical region and subspecies. The first and second linear discriminants are presented in the plot.

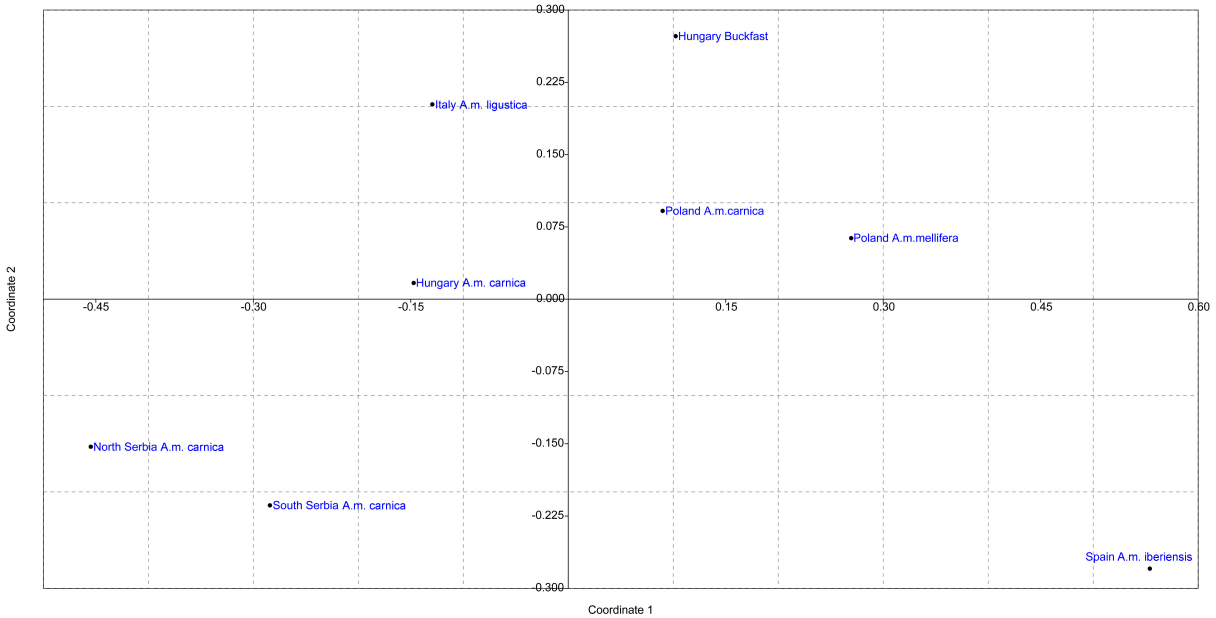


Figure 7. Non-metric multidimensional scaling plot of F_{ST} distances between analyzed geographical regions and subspecies. The goodness of fit is expressed with the stress value which is 0.0426 for this data set. Population pairwise F_{ST} values are presented in Table S10.

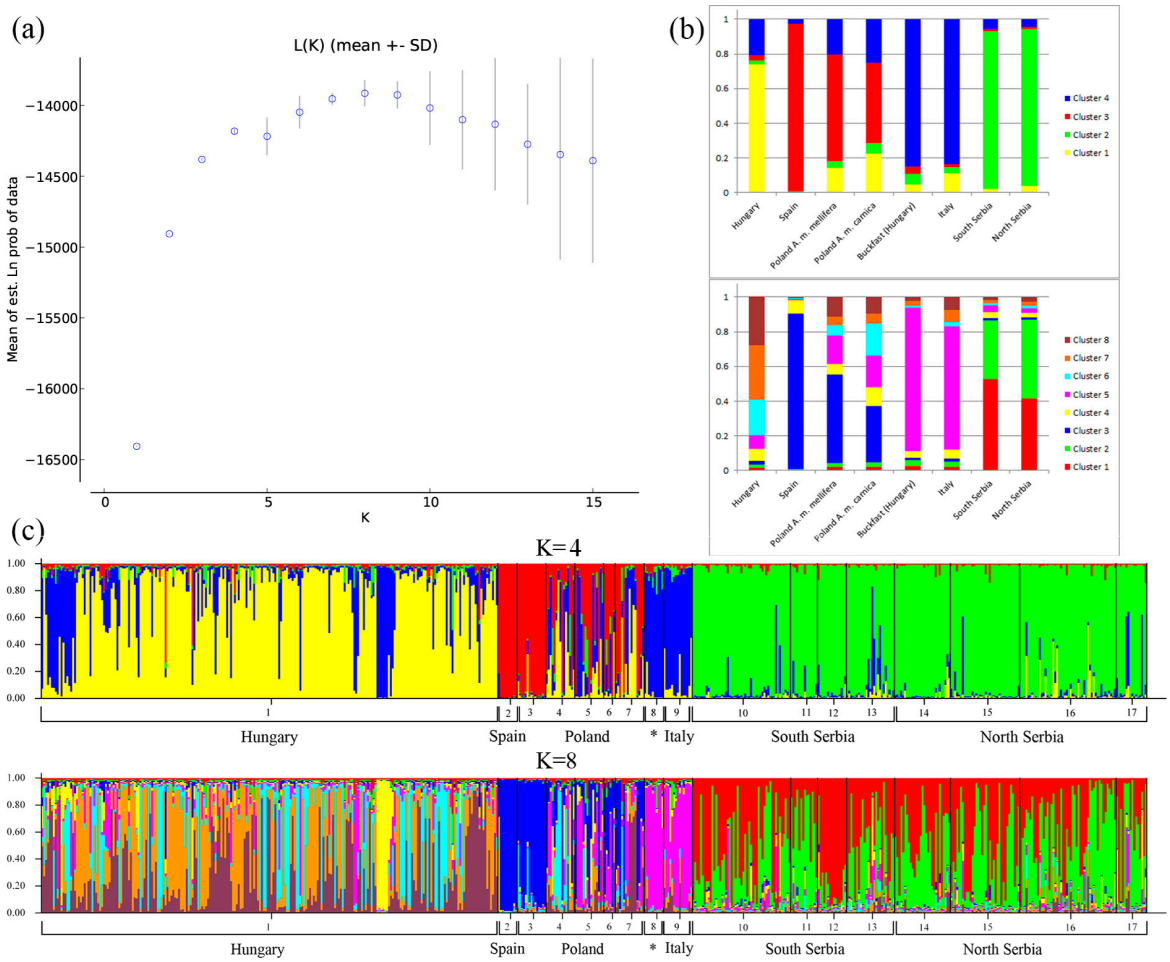


Figure 8. (**a**) L(K) mean for the assumed number of genetic clusters. (**b**) Proportions of inferred STRUCTURE clusters (K = 4 and K = 8). (**c**) Proportions of the inferred STRUCTURE clusters (K = 4 and K = 8) from the individuals. 1—Hungary, 2—Spain, 3–7—Poland (3—August forest, 4—Krakow, 5—Bialowieza, 6—Wrocław, and 7—Siedlice), * 8—Buckfast lineage from Hungary, 9—Italy, 10–13—Southern Serbia (10—Leskovac, 11—Vlasina, 12—Tromeđa, and 13—Stara Planina), and 14–17—Northern Serbia (14—Fruška Gora, 15—Subotica, 16—Deliblatska peščara, and 17—Vršac).

4. Discussion

The modernization of beekeeping practices and rapidly growing numbers of beehives in Serbia have invoked the need to re-study previously described genetic variability in the Serbian honey bee population [23,24,26,44,45,53]. Most of the previous genetic studies on the Serbian honey bee, even the most recently published ones, are based on samples from the first decade of the 21st century. Since then, significant changes in beekeeping practices together with stricter implementation of Serbian legislation and an increased number of beehives have led to changes in genetic variability in Serbian honey bee populations, as suggested in [46,61]. Therefore, we examined 14 microsatellite loci in Serbian worker bees from eight different localities to further shed light on the current status of the genetic diversity of Serbian honey bees. Furthermore, we compared nine microsatellite loci in our sample with previously published samples from Hungary, Poland, Spain, and Italy [58] to infer broader genetic relations between different *A. mellifera* populations and subspecies. Our results suggest that the Serbian honey bee population is relatively homogenous with preserved local differences and separated from the other populations analyzed.

The parameters of genetic diversity in Serbian localities are relatively high but lower than in other analyzed localities. Moreover, reference localities showed significant heterozygosity excess, while in Serbian localities Ho was in line with He, and for some loci, heterozygote deficiency may have been observed. These results, together with the G–W

index, indicate that the Serbian honey bee population did not experience recent bottleneck events. For some loci, departures from Hardy–Weinberg and linkage disequilibrium were observed, which may be an indication of recent gene flow from other subspecies or populations [73].

Although significant F_{ST} values were obtained between some pairs of localities, there is no clear pattern that indicates a south/north geographical distribution of microsatellite loci in the Serbian honey bee population. The results of AMOVA analysis suggest that grouping according to region may indicate some geographical distribution since the percentage of variation among localities within groups slightly decreases, but the value is not statistically significant (0.32, $p = 0.119$). Together with the equal distribution of two clusters inferred by STRUCTURE analysis in all localities and cluster overlapping inferred by DAPC analysis, the presented results indicate population admixture and a relatively homogenous population. However, local differences are still preserved since significant F_{ST} values can be observed between pairs of localities, and some differentiation may be observed according to the position of population in DAPC and MDS landscapes.

Our results could not confirm the presence of *A. m. macedonica* and north/south differences between individuals from different parts of the country previously reported for the Serbian honey bee population [23,26,53] but are in concordance with recently published work on the genetic diversity of the mtDNA $tRNA^{leu}$-cox2 for the same sample [46]. Both uniparental and biparental markers showed that although local specific genetic variants and weak regional differences can be observed, previously reported regional differences indicative of subspecies distribution could not be confirmed. The formation of an admixture population may be one of the reasons behind the presented results. Extensive hybridization between *A. m. carnica* and *A. m. macedonica* subspecies in the central part of Serbian territory was previously described [23,26], and it is possible that the hybridization zone expanded reflecting recent changes in beekeeping practices as was shown by mtDNA data. The absence of north/south regional differentiation may also be partially attributed to the intensification of migratory beekeeping, since apiaries from the south are transported to the north during the flowering season of agricultural plants. As there is no human control of mating between individuals from different apiaries, when migratory apiaries return to the region where the stationary apiaries sampled in this study are located, admixture may be propelled. The Serbian leading beekeeper organization strongly encourages strict implementation of Serbian legislation that only *A. m. carnica* subspecies can be present in apiaries which, with a growing number of *A. m. carnica* queen manufacturers, may also contribute to the observed loss of *A. m. macedonica* and the admixture of the Serbian honey bee population.

Population structure analysis showed that each geographical region and each corresponding subspecies are separated. *A. m. iberica* from Spain is represented by its own cluster in STRUCTURE analysis as well as clearly separated in MDS and DAPC plots. The Italian *A. m. ligustica* is represented by its own structure cluster which is present in the Hungarian Buckfast sample as expected. The same result was obtained from DAPC and MDS analysis. The Polish populations of *A. m. carnica* and *A. m. mellifera* were located close to each other but still separated. The Hungarian population is well separated from the other populations studied, and although relatively homogeneous, some *A. m. ligustica* introgression may be observed, as previously reported by [58]. Serbian populations are well separated from others with significant overlap between individuals from the south and north, although very low but significant F_{ST} value can be observed. Structure analysis showed weak but still detectable introgression of *A. m. ligustica* alleles, which is in concordance with our field data that some illegal importation of Italian bees occurred in the past, since this subspecies has been one of beekeepers' favorites. Two distinct clusters can be observed in the Serbian honey bee population, and they are almost equally distributed among localities, suggesting population admixture.

Our results suggest that, as already shown in many studies [74], geographical distance together with environmental factors maintain the specific genetic diversity of *A. mellifera*

subspecies within any given geographical region. However, this genetic diversity is under constant anthropogenic influence due to the modernization of beekeeping practices, such as migratory beekeeping, importation of foreign queens, and even legal practices [4,33,35,37]. Serbia is the natural area of contact between warmer climates preferring *A. m. macedonica* and colder ones preferring *A. m. carnica* and, although relief and ecological differences exist between these two parts of the country, climate conditions are favorable for both subspecies, and the main reason between their distinct distribution may lie in isolation by distance. The distances between the southern and northern parts of Serbia may be too great for bees, but for beekeepers they are rather small and they readily travel 500 km in the flowering season for different plants. Together with a vast increase in the number of beehives and beekeepers in the past decade [54] and legislation that specifically allows breeding of a single subspecies, it is not surprising that the previous composition of the diversity of honey bees in Serbia has changed. However, specific local genetic variability may still be retained since differences between analyzed localities can be observed.

Unfortunately, socioeconomic interests and beekeeper preferences for more productive and gentler individuals have often taken precedence over the conservation of locally native subspecies [27,37,39], leading to the predominance of admixture populations in human-dominated areas [4]. Our results suggest that this scenario happened in Serbian honey bee populations and that for the above-mentioned reasons Serbia now harbors a distinct hybrid honey bee population. Further analysis that will include honey bee populations from eastern and western parts of Serbia are needed in order to better understand the pattern of genetic variability of managed honey bees in Serbia, so that the best managing strategies, with the goal of preserving the existing genetic diversity, can be implemented.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/insects13020180/s1, Figure S1: Discriminant analysis of principal components. The first three linear discriminants are presented in the plot; Figure S2: (a) Delta *K* values for the assumed number of genetic clusters. (b) Proportions of inferred STRUCTURE clusters ($K = 2$). (c) Proportions of the inferred STRUCTURE clusters ($K = 2$) from the individual worker bees; Figure S3: Ln values of probability for the assumed number of genetic clusters; Figure S4: Distribution of clusters according to the DAPC method and inffered number of 8 clusters; Figure S5: Non-metric multidimensional scaling plot of F_{ST} distances between 8 localities in Serbia (*A. m. carnica*) and other analysed *A. mellifera* populations from Hungary (*A. m. carnica* and Buckfast), Poland (Krakow and Wroclaw–*A. m. carnica*; August forest, Bialowieza and Siedlice–*A. m. mellifera*) Spain (*A. m. iberica*) and Italy (*A. m. ligustica*); Figure S6: Non-metric multidimensional scaling plot of F_{ST} distances between 8 localities in Serbia (*A. m. carnica*) and other analysed *A. mellifera* populations from Hungary (*A. m. carnica* and Buckfast), Poland (Krakow and Wroclaw–*A. m. carnica*; August forest, Bialowieza and Siedlice–*A. m. mellifera*) Spain (*A. m. iberica*) and Italy (*A. m. ligustica*); Figure S7: Discriminant analysis of principal components. The first three linear discriminants are presented in the plot; Figure S8: Delta *K* values for the assumed number of genetic clusters; Figure S9: Distribution of clusters according to the DAPC method and inffered number of 4 clusters; Table S1: List of loci used in genetic analyses, primers used for their amplification, fluorescent dyes used for tagging the forward primers, annealing temperature (Tm) used in each reaction for the amplification of specific microsatellite loci with the combination of loci amplified together in multiplex reactions I, II, III and IV; Table S2: Parameters of genetic diversity calculated per locus per populations including Garza Williamson index, Hardy Weinberg equilibrium (1,001,000 steps done)–Serbian localities; Table S3: Population specific Fis indices for 10,100 permutations (Serbian localities); Table S4: Tables of significant linkage disequilibrium (Serbian localities); Table S5: Parameters of genetic diversity calculated per locus per populations including Garza Williamson index, Hardy Weinberg equilibrium (1,001,000 steps done) (all populations); Table S6: Population specific Fis indices for 10,100 permutations (all populations); Table S7: Tables of significant linkage disequilibrium (all populations); Table S8. Pairwise population Fst (below diagonal) and Fst *p* values (above diagonal) between the populations based on the variability of 14 microsatellite loci found in 8 different localities of *Apis mellifera carnica* in Serbia; Table S9: Outcomes of AMOVA analysis based on the variability of 14 microsatelite loci when population sample from Serbia was grouped according to the geographical region: North vs South; Table S10: Pairwise population Fst (below diagonal) and Fst *p* values (above

diagonal) between the populations based on the variability of 9 microsatellite loci when Serbian localities were grouped according to their geographical region and other *A. mellifera* populations from: Hungary (*A. m. carnica* and Buckfast), Poland (*A. m. carnica* and *A. m. mellifera*), Italy (*A. m. ligustica*) and Spain (*A. m. iberica*); Table S11: Pairwise population Fst (below diagonal) and Fst *p* values (above diagonal) between the populations based on the variability of 9 microsatellite loci found in 8 different localities of *Apis mellifera carnica* from Serbia and other *A. mellifera* populations from: Hungary (*A. m. carnica* and Buckfast), Poland (*A. m. carnica* and *A. m. mellifera*), Italy (*A. m. ligustica*) and Spain (*A. m. iberica*); Table S12: Outcomes of AMOVA analysis based on the variability of 9 microsatelite loci when population sample from Serbia was compared with other analysed populations.

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Half Friend, Half Enemy? Comparative Phytophagy between Two Dicyphini Species (Hemiptera: Miridae)

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Simple Summary: Omnivorous predators, such as some mirids, are important biological control agents in several vegetable crops since they are generalists and can survive in the crop in the absence of prey. *Nesidiocoris tenuis* is a mirid used worldwide and its phytophagy is well known, which is not the case for the Palearctic *Dicyphus cerastii.* To use the latter in biological control it is crucial to evaluate the damage it causes to plants. We compared these two mirid species, under laboratory and semi-field conditions, regarding the damage they caused to plants and fruit, and their location on the plant versus on the fruit. Both species produced plant damage (scar punctures on leaves and necrotic patches on petioles) and caused flower abortion, at a similar level, however, only *N. tenuis* produced necrotic rings. Overall, *N. tenuis* females produced more damage to tomato fruit than *D. cerastii*. There was an increased frequency of *D. cerastii* females found on the plants over time, which did not happen with *N. tenuis*. Our results suggested that although *D. cerastii* caused less damage to tomato fruit than *N. tenuis*, it did feed on the fruit and could cause floral abortion, which requires field evaluation and caution in its use.

Abstract: Despite their importance as biological control agents, zoophytophagous dicyphine mirids can produce economically important damage. We evaluated the phytophagy and potential impact on tomato plants of *Dicyphus cerastii* and *Nesidiocoris tenuis*. We developed a study in three parts: (i) a semi-field trial to characterize the type of plant damage produced by these species on caged tomato plants; (ii) a laboratory experiment to assess the effect of fruit ripeness, mirid age, and prey availability on feeding injuries on fruit; and (iii) a laboratory assay to compare the position of both species on either fruit or plants, over time. Both species produced plant damage, however, although both species produced scar punctures on leaves and necrotic patches on petioles, only *N. tenuis* produced necrotic rings. Both species caused flower abortion at a similar level. Overall, *N. tenuis* females produced more damage to tomato fruit than *D. cerastii.* There was an increased frequency of *D. cerastii* females found on the plants over time, which did not happen with *N. tenuis*. Our results suggested that, although *D. cerastii* caused less damage to fruit than *N. tenuis*, it still fed on them and could cause floral abortion, which requires field evaluation and caution in its use in biological control strategies.

Keywords: omnivorous predator; *Nesidiocoris tenuis*; *Dicyphus cerastii*; plant damage; zoophytophagy; fruit injury; protected crops; biological control; tomato

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1. Introduction

Zoophytophagous mirid species (Hemiptera: Miridae) are important biological control agents in several crops. Dicyphine (Miridae: Bryocorinae: Dicyphini) species, such

as *Nesidiocoris tenuis* (Reuter), and several species of the genera *Macrolophus* Fieber and *Dicyphus* Fieber, are used worldwide as generalist predators [1] on several vegetable crops, both in conservation and augmentative biological control strategies.

Dicyphus cerastii Wagner is a Palearctic mirid, reported in the Mediterranean Basin, which spontaneously colonizes Portuguese greenhouses [2]. It is currently being evaluated as a candidate biological control agent (BCA), since it can feed on several horticultural pests [3]. *Nesidiocoris tenuis* is currently commercialized and released to control whiteflies and *Tuta absoluta* (Meyrick) in Mediterranean greenhouses [1,4,5]

Dicyphine mirids may resort to phytophagy in periods of prey scarcity [6,7], and to obtain water [8] and nutrients [9] from plants. Despite being advantageous as a feeding strategy, phytophagy can have negative effects in an agronomical context. Plant feeding may lead to a decrease in predation activity [9,10], and, more importantly, phytophagy can cause damage of economic importance, such as necrotic rings in stems and leaf petioles, as well as flower or fruit abortion, and punctures in the fruit [6,11–14]. This is particularly evident with *N. tenuis*, which is often the target of pesticide sprays that are used to control its populations when there is a risk of plant damage occurring, a practice that negatively impacts other natural enemies present on crops.

The increasing demand for food products without pesticide residues, combined with the need to control pests, highlights the urgency for sustainable alternatives that reduce negative effects on both the consumer and the environment's health. The use of predatory mirids in biological control has been very successful in protected tomato crops (e.g., [5,15,16]), despite the damage produced by some species. Therefore, to enhance biological control in tomato crops, research should focus on less phytophagous yet efficient dicyphine predators.

Plant damage can greatly vary with host plant species [6], mirid species [6,17,18], and even among populations of the same species [19,20]. Therefore, understanding the risk that phytophagy represents to crops is a key point in the evaluation of a new candidate BCA [13], such as *D. cerastii*, that will help decision making while choosing the best suited mirid species. Although damage caused by *N. tenuis* has been studied (e.g., [6,11,21,22]), little is known about those induced by *D. cerastii.* The latter has been observed producing chlorotic punctures on leaves [4] and also necrotic damage on tomato stems and leaf petioles, as well as feeding punctures on fruit (our pers. obs.). However, the influence of its damage on plant development and possible economic impacts has never been evaluated or compared with other mirid species.

We hypothesized that *D. cerastii* and *N. tenuis* display different phytophagous behavior. Within this context, the aim of this study was to compare the phytophagy and potential impact on tomato production of these two Dicyphini species. For this, we characterized the type of plant damage produced by these species on tomato plants. Then, we assessed the effect of fruit ripeness, mirid age, and prey availability on feeding injuries on tomato fruit. Finally, we compared the location of both species on either tomato fruit or plants.

2. Materials and Methods

2.1. Rearing of Mirid Predators

Stock colonies of both species (*D. cerastii* and *N. tenuis*) are maintained at the Instituto Superior de Agronomia (ISA), Lisbon, Portugal, on tobacco plants (*Nicotiana tabacum* L.). The colony of *D. cerastii* was started with individuals from different geographical sites in Portugal (Fataca, Ferreira do Zêzere, Lisbon, and Póvoa de Varzim) and is frequently refreshed with individuals, mostly from the Oeste region (Mafra and Silveira). The colony of *N. tenuis* was started with individuals from the Oeste region (Silveira) and from Koppert Biological Systems (The Netherlands). For rearing details, see [3]. Young adult females (between 1 and 7 days old), for all three bioassays, were obtained from the regular collection of large nymphs from breeding cages that were transferred to separate cages, where they could reach adulthood. For nymph experiments (see Section 2.3, Fruit Damage), 4th/5th instar nymphs were collected from immature rearing cages.

2.2. Phytophagy in Semi-Field Conditions

Phytophagy was observed for *N. tenuis* and *D. cerastii* in semi-field conditions in a greenhouse at ISA's *campus*. For this, mesh cages (1.5 m high and 1.0 m wide) were used. In each cage, there were two tomato plants (cv. Vayana), each one in a 15 L pot. Plants were fertilized and watered as needed, using an organic fertilizer solution (Húmus Líquido Horta®, SIRO, Mira, Portugal). When plants had 4 to 5 developed leaves (ca. 30–40 cm high), six couples of *D. cerastii* or *N. tenuis* were released. Control cages were set without any insects. Each treatment (12 *D. cerastii*, 12 *N. tenuis* and a control) had five replicates. To simulate the natural presence of prey, a teaspoon (ca. 4 g) of a mix of *Artemia* spp. (Anostraca: Artemiidae) and *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) eggs (Entofood®, Koppert Biological Systems) was sprinkled evenly on the plants at the time of insect release, and every two weeks after that. Six weeks after release, all insects in the cages were captured into vials containing 96% ethanol and counted. Plant damage, expressed as the total number of necrotic rings or necrotic patches, was recorded for every cage. Flower abortion was counted as the proportion of missing flowers/total number of flowers in the flower rachis, for every cage. When trusses already had small fruit, these were counted as flowers. The mirids were released on 28 April 2021, and the assay ended on 11 June 2021.

2.3. Fruit Damage

The following factors were considered to compare the puncture level on fruit: mirid species (*D. cerastii* and *N. tenuis*), mirid age (nymph and adult female), tomato ripeness (unripe and fully ripe), and availability of food and/or water. For each species, three adult females or three nymphs were placed in plastic cups (8 cm high and 6 cm diameter) with one tomato fruit that was approximately 4–5 cm long. The lid of the cups had a hole (3 cm ϕ) covered with fabric to allow ventilation. Four treatments were considered (15 replications), for each species, mirid age, and tomato ripeness: (a) fruit only (N); (b) fruit with water (W) (supplied through an Eppendorf vial with moist cotton wool); (c) fruit with water and alternative food (FW) (*Ephestia kuehniella* eggs and *Artemia* spp. cysts Entofood®, Koppert Biological Systems, Berkel en Rodenrijs, The Netherlands), supplied on a sticky paper strip (2.0 cm $\times$ 0.8 cm); (d) fruit with alternative food (F) (Entofood®) but no water. Tomato fruit (Figure 1, cv. Suntasty) were obtained from an organic commercial tomato greenhouse. Before the experiment, the fruit were washed with abundant water and individually inspected for any marks or possible feeding punctures. Fruit with any defect were discarded. The unripe condition was considered as fully grown green fruit and fully ripe was considered as fully red fruit. For each bioassay, a control (i.e., only fruit) was made to ensure that punctures were caused by mirids. Insects were allowed to feed for 24 h, after which punctures (injuries resulting from fruit feeding) were counted under a stereomicroscope with a magnification of 50$\times$. Injury was considered as a puncture surrounded by a small whitish or yellowish halo [12,23]. Replicates in which death occurred, or nymphs molted into adults, were discarded.

2.4. Location on Tomato Plant versus Fruit

The tomato fruit were the same cultivar as those in the fruit-damage bioassay (see Section 2.3, above). Females of both species were placed individually in 600 mL transparent plastic cups covered with fabric to allow ventilation. The cups contained a young tomato plant (cv. San Pedro) held in water in a 10 mL glass bottle, and an unripe (green) tomato fruit. The unripe tomato was elected after analyzing the data from the tomato-damage bioassay, in which the most injured tomato was unripe. Females were placed individually for 24 h before the experiment in empty test tubes (starved). After release into the plastic cups, the position of the females was recorded at 1 h, 2 h, 6 h, and 24 h as being on the young plant, on the fruit, or elsewhere in the cup (as proposed by McGregor et al. [24]). Observations were conducted in a controlled chamber (Fitoclima CP500, Aralab Lda., Albarraque, Sintra, Portugal) at a temperature of 25 °C and photoperiod of 14 h. At the

end, fruit were inspected for feeding punctures. Females were used only once. In total, 20 replicates were made for each species.

Figure 1. Plant damage by *Nesidiocoris tenuis* on tomato plants: (**a**) necrotic rings on leaves and shoots; (**b**) shoot wilting; (**c**,**d**) detail of necrotic rings on leaves.

2.5. Data Analysis

Phytophagy in semi-field conditions. Both *D. cerastii* and *N. tenuis* populations at the end of the experiment and plant damage (necrotic rings or necrotic patches) were compared between species using one-way ANOVA with species as an independent variable. Differences in flower abortion among treatments were compared with Pearson's χ^2 tests. These statistical analyses were performed with IBM SPSS statistics v.26 (IBM, Armonk, NY, USA).

Fruit damage bioassay. Classification tree methods were used to understand the relative importance of the variables used (i.e., the absence or presence of water and/or food, developmental stage *of* the mirid, tomato ripeness, and species) on the number of feeding punctures. Statistical analyses were performed using R software version 4.1.0 implemented in RStudio version 1.4.1106. Conditional inference trees were made using the "*ctree*" function (R package party, http://cran.r-project.org/web/packages/party/index.html, accessed on 9 June 2021), which bases node splitting on statistical tests, providing a *p*-value for the significance of splitting [25]. The importance of the variables was measured using the random forest algorithm [26] and computations were performed in the randomForest package with 1001 trees (ntree = 1001). The random forest algorithm combines many classification trees to produce more accurate classifications and has measures of variable importance and measures of similarity of data points as by-products of its calculations [27]. Data were analysed together (i.e., considering counts for both species) and separately for each species. All preliminary analysis considered the modalities in two groups: (i) without food (N and W); and (ii) with food (F and FW). We grouped them into absence (A) and presence (P) of food and/or water, respectively, in the presented output.

Position on tomato plant versus fruit. The position of insects was compared between species for each observation time using the Fisher's exact test and z-test with Bonferroni correction method. For the comparison among locations within each species and each observation time, the non-parametric χ^2 test for one sample was used. These statistical analyses were performed with IBM SPSS statistics v.26 (IBM, Armonk, NY, USA).

3. Results

3.1. Phytophagy in Semi-Field Conditions

The average temperature during the assay was 24.3 °C, with a minimum of 10.4 °C and a maximum of 45.4 °C. The relative humidity was ca. 50%. At the end of the experimental period, *N. tenuis* had a larger average population (137.4 ± 30.3 individuals/cage) than *D. cerastii* (68.4 ± 14.4 individuals/cage); this difference, however, was not significant (F = 4.182; df = 1; $p = 0.075$).

Nesidiocoris tenuis produced both necrotic patches (ca. 10% of total damage) on leaves and stems and necrotic rings (ca. 90% of total damage) (Figure 1), whereas *D. cerastii* only produced necrotic patches (Figure 2). Necrotic rings caused by *N. tenuis* occasionally led to withering of young shoots or leaves (Figure 1b), while this was not observed with *D. cerastii* patches. Plant damage numbers were significantly different between species (F = 17.114; df = 1; $p = 0.003$), and *N. tenuis* produced more necrotic injuries on the plants than *D. cerastii* (Figure 3).

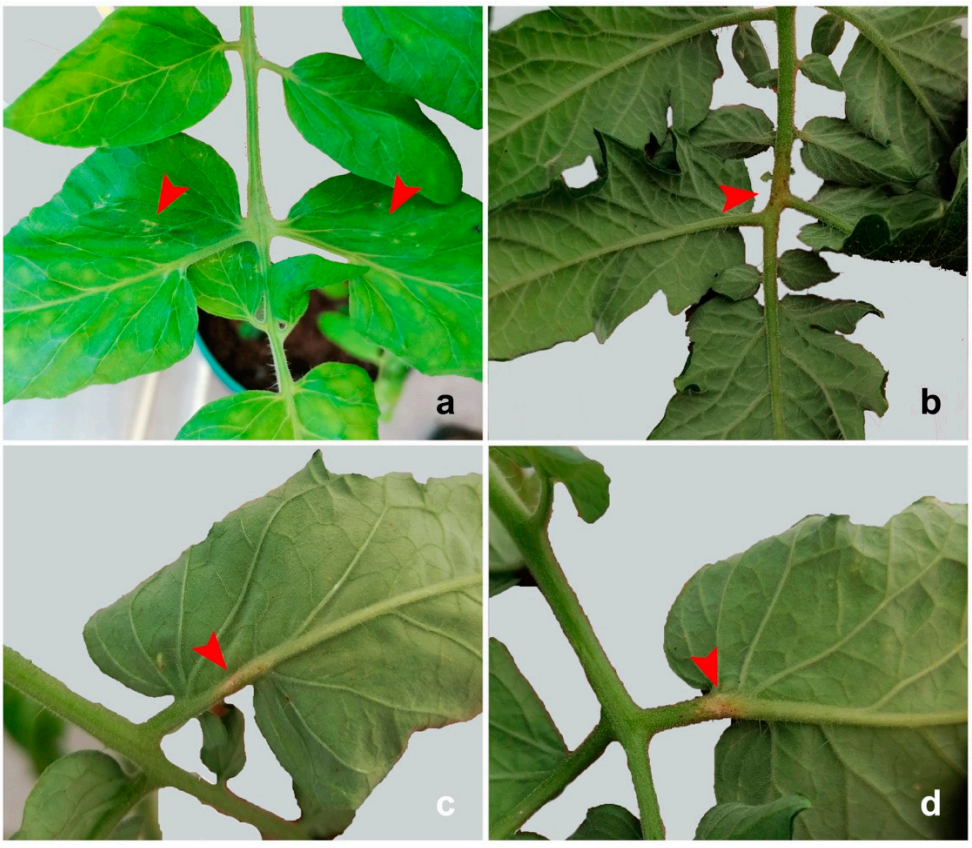

Figure 2. Plant damage by *Dicyphus cerastii* on tomato plants: (**a**) puncture scars on expanded leaves, (**b**–**d**) detail of necrotic patches on leaves.

Both species also produced puncture scars on leaves as a result of feeding on young stem/leaf tissues (Figure 2a).

Control cages had significantly lower flower abortion than both *D. cerastii* ($\chi^2 = 12.047$; df = 1; $p = 0.001$) and *N. tenuis* cages ($\chi^2 = 16.395$; df = 1; $p < 0.001$), whereas the two mirid species displayed similar levels of flower abortion ($\chi^2 = 0.670$; df = 1; $p = 0.413$) (Figure 4).

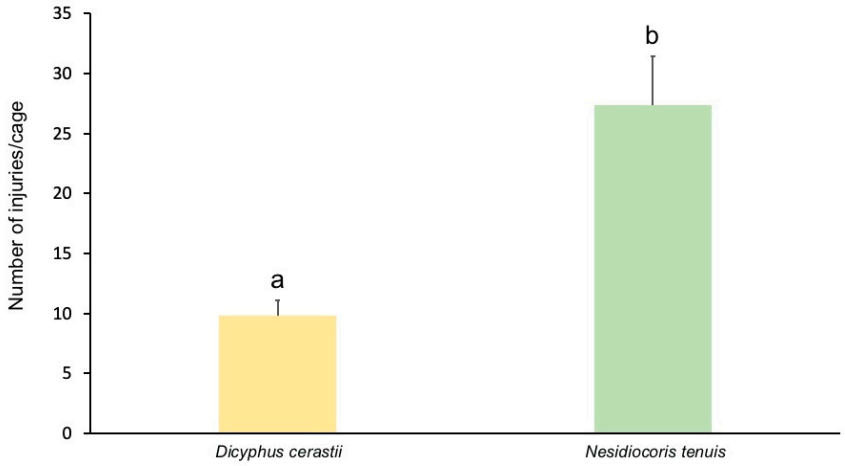

Figure 3. Number of plant injuries (necrotic rings or patches) per cage, by *Dicyphus cerastii* and *Nesidiocoris tenuis* on tomato plants (two plants/cage). Bars topped by different letters represent means that are significantly different (ANOVA, $p < 0.05$).

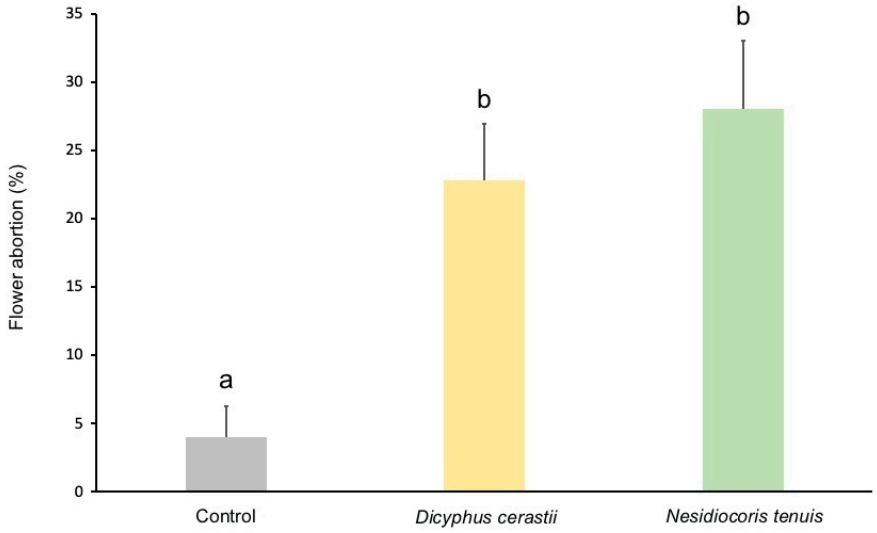

Figure 4. Percentage (+SE) of flower abortion (missing flowers/total number of flowers × 100) on control, *Dicyphus cerastii*, and *Nesidiocoris tenuis* tomato plants. Bars topped by different letters represent significantly different percentages (χ^2, $p < 0.05$).

3.2. Fruit Damage

Both species fed on tomato fruit and produced punctures that appeared as damaged epicarp/mesocarp cells. It was often possible to observe that the damaged area extended beyond the puncture point following stylet movement inside the fruit. Punctures were structurally similar (Figure 5), but the pattern of each species was different. *Dicyphus cerastii* punctures tended to be aggregated, forming clearly visible patches in cases of highly damaged fruit. *Nesidiocoris tenuis* punctures appeared less aggregated compared to *D. cerastii*. Punctures on fruit did not heal, as punctures on green fruit did not disappear even when fruit changed color during maturation. Punctures produced by females and nymphs appeared similar, for both species. We observed that, occasionally, females of both species laid eggs on fruit. It is possible that an amount of the punctures may have been egg laying attempts or the result of probing.

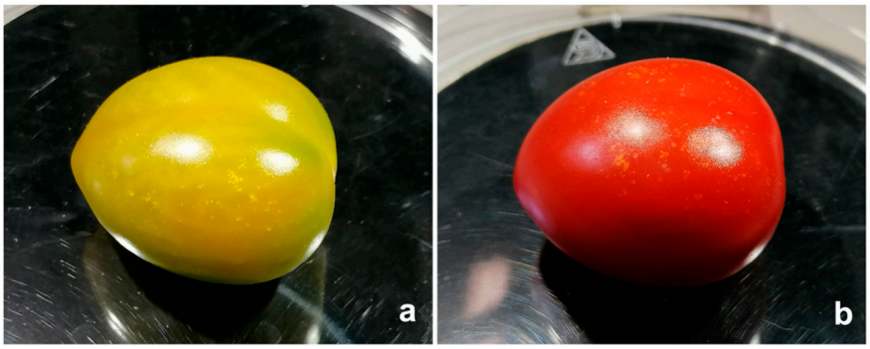

Figure 5. Feeding punctures in tomato fruit: (**a**) unripe fruit; (**b**) ripe fruit.

A high variability was observed in the number of punctures inflicted in all treatments and in both species, with low numbers or even no punctures to high numbers of punctures in the same treatment, which generated great variance in the data for both species studied. When analyzing the dataset considering both species or for each species separately, there was no difference between the two treatments without food (N and W) or between the two treatments with food (F and FW). Considering both species, the most important variable was the tomato ripening stage (tomato_age), with the unripe (green) tomato being the one with the highest number of feeding punctures, followed by the presence/absence of food. Food presence (F, FW) or absence (N, W) was only significant in the case of green fruit, and species was only significant for females in the presence of food in the case of green fruit, and for females in the ripe fruit. While the most important factor for *D. cerastii* was also the tomato ripeness, for *N. tenuis* the most important factor was the individual's stage of development (i.e., whether it is a nymph or an adult). Food was the second factor for *D. cerastii* but only the third, and more distant, for *N. tenuis* (Figures 6 and 7).

3.3. Location on Tomato Plant vs. Fruit

The locations of *N. tenuis* and *D. cerastii* were only significantly different at the first observation (1 h) (Fisher's exact test value = 6.423, $p = 0.033$), with the former more present on the young plant than the latter and the inverse regarding the cup ($\alpha = 0.05$) (Figure 8). Both *N. tenuis* and *D. cerastii* were mainly found on the young plant. However, in the case of *D. cerastii*, differences among locations were only verified at 2 h, 6 h, and 24 h, with females more present on the young plant (or on the young plant or on the cup walls) than on the fruit. In the case of *N. tenuis*, the females were more often found on the young plant than on fruit or cup walls, except at 24 h; at this time there were no differences between the young plant and fruit and no female was observed on cup walls.

In this bioassay, damage on plants was not quantified since, in some treatments, they would not be identified, especially with *D. cerastii*. Feeding punctures on fruit were found (although they were not counted) in all cases when the females of both species were sighted on the fruit. Furthermore, in both species, at least one fruit with feeding punctures was found, although the female was never observed in that same fruit during the bioassay (one case in *N. tenuis* and two cases in *D. cerastii*).

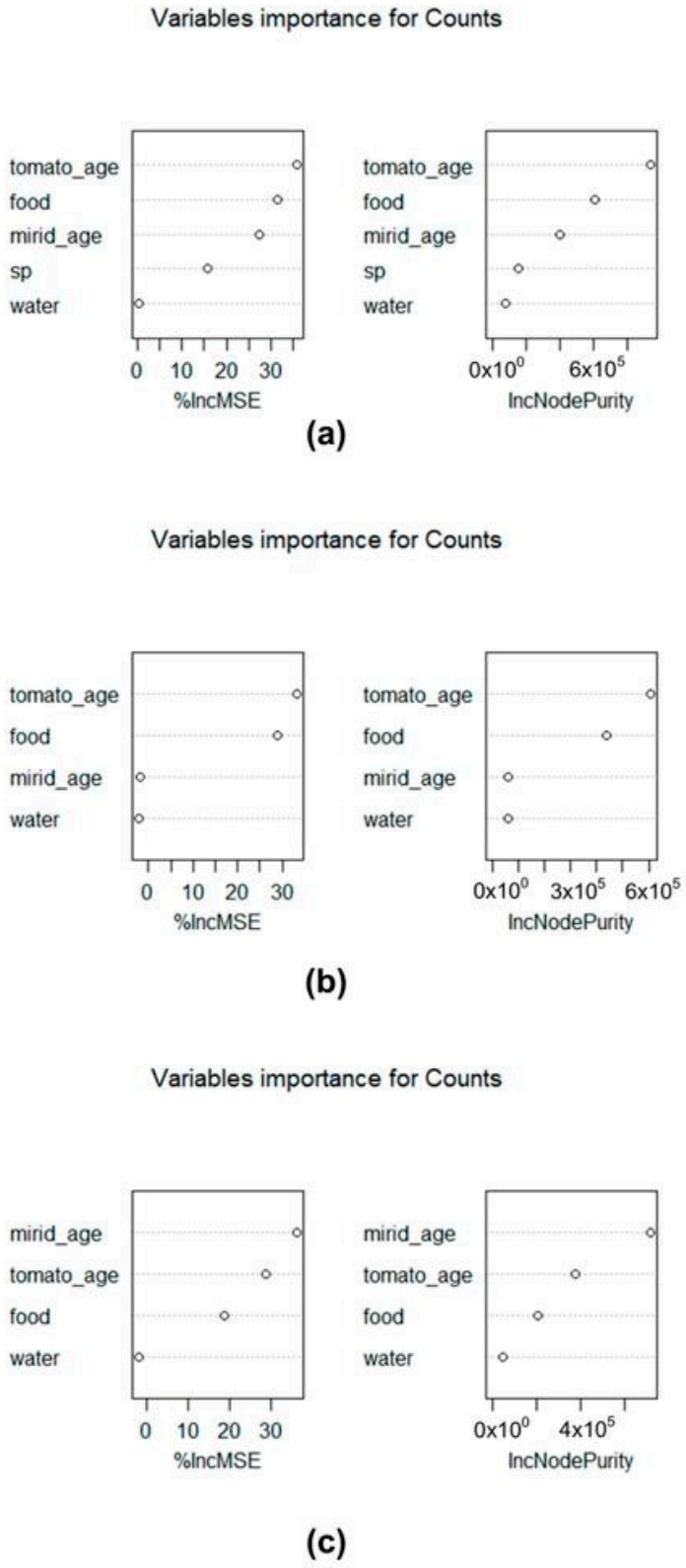

Figure 6. Variable importance plot from the random forest model (randomForest). The variables are ordered top-to-bottom as most-to-least important for an increase in feeding punctures (counts) on tomato fruit. (**a**) Using all datasets with data from both species, *Dicyphus cerastii* and *Nesidiocoris tenuis*; (**b**) database containing data collected only for *D. cerastii*; (**c**) database containing data collected only for *N. tenuis*. %incMSE: increase in mean square error of predictions as a result of the variable being permuted; inNodePurity: importance of each predictor variable.

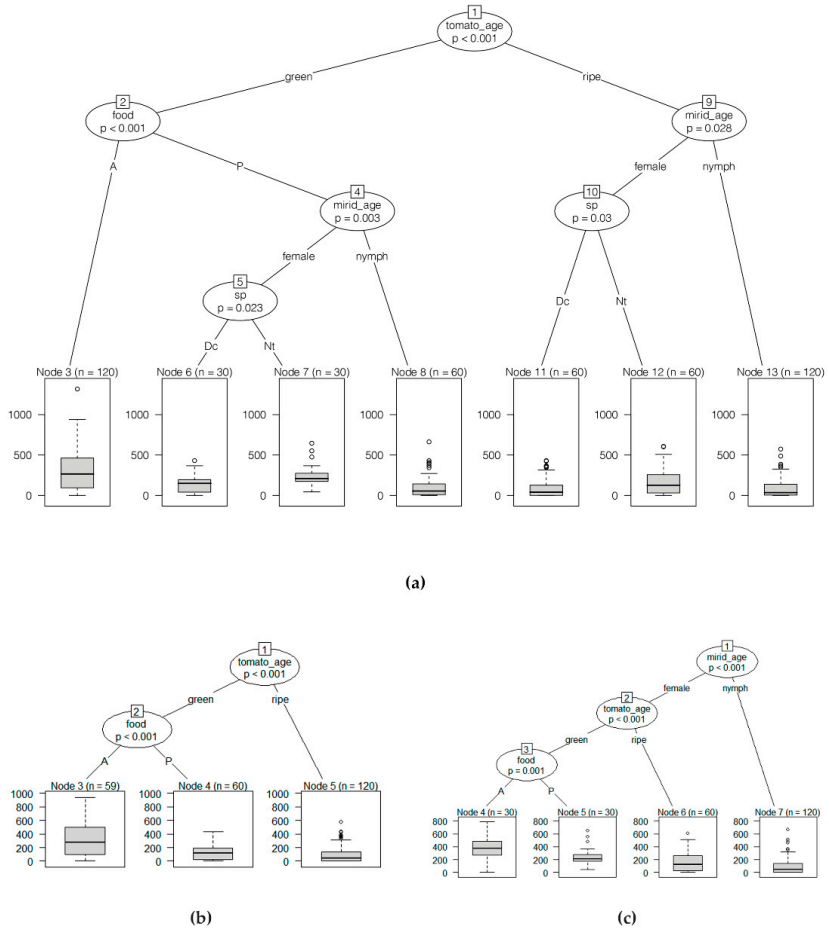

Figure 7. Classification trees from the conditional inference trees (ctree) model. For each internal node, input variable and P values are provided, the boxplot of the number of feed punctures is displayed for each end node. Numbers in boxes above the variable indicate the node number. Number above boxes (n) indicates number of fruit. (**a**) Using all datasets with data from both species, *Dicyphus cerastii* and *Nesidiocoris tenuis*; (**b**) database containing data collected only for *D. cerastii*; (**c**) database containing data collected only for *N. tenuis*. Dc: *Dicyphus cerastii*; Nt: *Nesidiocoris tenuis*; A: absence of food; P: presence of food.

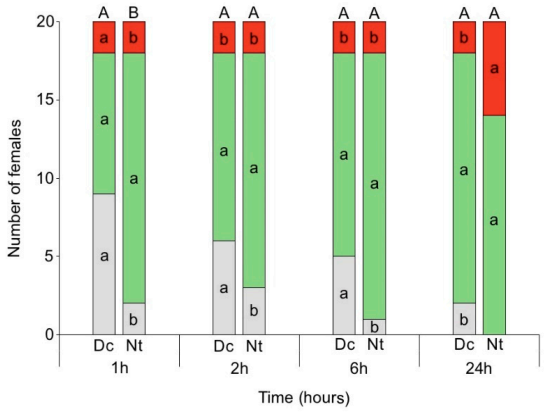

Figure 8. Number of *Dicyphus cerastii* (Dc) and *Nesidiocoris tenuis* (Nt) females on the young tomato plant (green), tomato fruit (red), or cup wall (grey) after 1 h, 2 h, 6 h, and 24 h. Bars topped by different letters for each observation time represent significant differences between species (Fisher's exact test, $p < 0.05$); different letters within the same column indicate differences among location for each species (χ^2, $p < 0.05$).

4. Discussion

The occurrence of plant damage production by zoophytophagous mirids is influenced by factors such as prey scarcity (e.g., [11,21,28]), water stress (e.g., [8]), and also host plant and mirid species (e.g., [6]).

Phytophagous behaviour in *N. tenuis* is regarded as more severe than in other dicyphine mirids [6,17,18] since this species has the particularity to feed on vascular tissues and to aggregate on feeding sites [29]. Our study corroborates this, as plants with *N. tenuis* suffered more damage than those with *D. cerastii*. Moreover, the higher number of necrotic rings produced by *N. tenuis* were also more severe for the plant compared to the necrotic patches observed for *D. cerastii*. We observed that, in some *N. tenuis* infested plants, the apical shoots or leaflets withered because of necrotic rings in stems, whereas this was not observed in *D. cerastii* plants.

Besides the damage to vegetative parts of the plant, dicyphines are also reported to damage reproductive organs, such as flowers and fruit [6]. *N. tenuis* is also recognized for causing flower and fruit abortion on tomato plants [30], and in a study by Sanchez et al. [31] the percentage of flower abortion by *N. tenuis* reached up to 50% during population peaks. Flower and fruit abortion on tomato plants has also been reported for *M. pygmaeus* [32]. However, to our knowledge, this type of damage has not been previously described for *Dicyphus* spp. In our experimental conditions, we found that the percentage of flower abortion was not different between both mirid species. As flower abortion is particularly important on cluster tomato cultivars, *D. cerastii* may have a similar impact to *N. tenuis* on such cultivars, despite the lower damage to vegetative tissues produced by *D. cerastii*.

In this study, the presence of water did not influence fruit puncture level. Therefore, both mirid species and both development stages could obtain the water they needed from green or ripe tomato fruit, at least when water was not provided. Water provision has been reported as one reason for phytophagy on heteropteran predators (e.g., [9]). However, as puncture numbers did not differ when water was supplied for both *N. tenuis* and *D. cerastii*, these mirids may have looked for other resources when they fed on the fruit.

Among the nutrients obtained from phytophagy, carbohydrates may have a particular ecological function since they have been reported to influence both predation and reproduction in dicyphines. This was demonstrated for *N. tenuis*, which was able to reduce the amount of prey feeding needed to establish itself on tomato plants [5], and increased its progeny [33], in the presence of sucrose dispensers. In another study, *N. tenuis* reduced its phytophagy when provisioned with sucrose dispensers [34].

In our study, when considering both species combined, tomato ripeness was the most important factor, with green fruit suffering more punctures than mature ones. This difference may be due to distinct nutritional profiles between unripe and ripe fruit. Sugar concentration, among other nutrients, may be higher in ripe tomato fruit [35,36], so it is possible that mirids may obtain more nutritional value per feeding puncture on ripe fruit than on green ones. On green fruit, the main effect was the presence of prey, which reduced fruit damage. There were differences between the species as *N. tenuis* females produced more damage than those of *D. cerastii*. On ripe fruit the most important factor was mirid age, with females producing most damage and, in these fruit, food did not significantly reduce damage. However, and once again, females of *N. tenuis* produced more damage than those of *D. cerastii*.

Considering *N. tenuis*, the most important factor on fruit damage was age, with females damaging more fruit than nymphs. Differently, in studies with whole plants, *N. tenuis* nymphs showed higher carbohydrate content [34] and spent more time feeding on the apical part of the plant [20], compared to adults. A similar trend was found for nymphs of the neotropical mirids, such as *Macrolophus basicornis* (Stål), *Engytatus varians* (Distant) and *Campyloneuropsis infumatus* (Carvalho), that also produced fruit punctures, whereas females did not [23]. Even though we observed less fruit damage by *N. tenuis* nymphs than females, our results indicated that nymphs were less influenced by factors such as fruit ripeness or presence of prey, suggesting that *N. tenuis* nymphs may be less prone to change

their phytophagous behavior than adults. Following mirid age, fruit ripeness was the next important factor for *N. tenuis*, with green fruit sustaining more damage. The presence of prey reduced the amount of damage on green fruit, whereas on ripe fruit it did not produce differences. This further suggests that green fruit may be a less valuable nutritional source for *N. tenuis*.

Tomato ripeness was the most relevant factor to explain fruit punctures by *D. cerastii*. The presence of prey was also important for fruit damage reduction in green fruit. Differently to *N. tenuis*, mirid age was not important in this species, which may suggest that *D. cerastii* may not be as dissimilar in phytophagy between adults and nymphs as *N. tenuis*.

Plant damage by zoophytophagous mirids has been associated with prey scarcity [11,21]. However, in our study the presence of food did not affect puncture level on ripe or green fruit with *N. tenuis* nymphs. Similarly, McGregor et al. [24] reported that the presence of food did not influence the level of fruit feeding by *Dicyphus hesperus* on mature tomato fruit, and Lucas and Alomar [37] reported that in whole caged plants the presence of *E. kuehniella* eggs did not prevent fruit injury by *D. tamaninii*.

Plant damage by zoophytophagous mirids may be determined by a complex combination of factors, besides prey abundance. Different species may have distinct preference or behavior that produce different types and levels of damage. Under the same conditions *M. caliginosus* (in fact, *M. pygmaeus*, C. Castañé, pers. comm.) did not produce fruit damage, whereas *D. tamaninii* did [37]. A different dicyphine, the nearctic *D. hesperus* preferred to feed on tomato leaves producing negligible damage on fruit [24]. Host plant and cultivar may also determine phytophagy, as was demonstrated for *N. tenuis*, which varied its phytophagy among different tomato cultivars [38]. The health of the host plant may also shape phytophagy by dicyphines. *Macrolophus pygmaeus* was reported to increase the number and produce more evident fruit damage on tomato plants infected with Pepino mosaic virus (PepMV) [12], but the same did not occur with *N. tenuis* [14]. Defence-activated plants may also be less susceptible to mirid phytophagous behavior. This was demonstrated for *N. tenuis*, which produced less plant damage on tomato plants inoculated with the endophytic *Fusarium solani* K strain, a fungal isolate that confers tomato resistance to foliar and root fungal pathogens [39].

Other factors may explain differences in phytophagy, such as genetic variation within species [19,20]. In fact, for the same treatments, we observed high variability in puncture numbers inflicted on fruit. As the large majority of the individuals used to initiate, and all the ones used to refresh the rearings, came from nearby locations (less than 45 km of linear distance), it is likely that the geographic origin was not a key determining factor in the high variability in feeding puncture number. This suggests that other factors, other than those considered in our study, may be driving fruit feeding in both *N. tenuis* and *D. cerastii*, and genetically determined behaviors should probably be considered in future research.

The fact that most *N. tenuis* and *D. cerastii* females were found on the young plants rather than elsewhere in the cup, and that there was an increased frequency of *D. cerastii* females found on young plant over time, may be related to the search for a better oviposition site [24]. Despite this, we could observe a slight increase over time of *N. tenuis* females occurring on fruit, which became the same as that for young plants at 24 h, suggesting a potential risk to fruit by this species. Furthermore, although few females of both species were observed on fruit compared to plant parts, feeding punctures were observed on fruit where females were not seen throughout the observations, showing that the female was at some moment on the fruit and fed on it. Finally, as *D. cerastii* preferred to be on young tomato plants than tomato fruit for plant feeding over time, the potential for damage to tomato fruit by this zoophytophagous mirid may be lower when compared to *N. tenuis.*

In the field, in commercial, protected tomato crops, necrotic rings, shoot, and flower cluster withering, and also punctures on fruit, are common when *N. tenuis* is present at high densities. This has repercussions on tomato production, leading growers to use a tolerance threshold and apply control measures. In the case of *D. cerastii*, necrotic tissues and punctures on fruit have been observed in the field by our team in commercial greenhouses

when this species is present in high population densities. In order to fully assess how *D. cerastii* may affect tomato production (both in quantity and quality), further research is needed in semi-field conditions and commercial greenhouses, to establish safe population density thresholds. Since the damage caused by *D. cerastii* was apparently different from the necrotic rings of *N. tenuis*, histological studies are needed to characterize the necrotic patch damage reported here. Furthermore, it is important to understand if the feeding behaviour of *D. cerastii* induced the production of volatile defence compounds in the damaged plant, as reported for *N. tenuis*, *M. pygmaeus* and *Dicyphus maroccanus*, Wagner (syn. *D. bolivari* Lindberg) [40,41], with the consequent attraction of other biological control agents [41].

5. Conclusions

Overall *D. cerastii* damage was less severe than *N. tenuis*, as it did not cause necrotic rings and was more likely to seek out parts of the plant than the fruit. Despite this, it fed on fruit and caused flower abortion. Therefore, as was already known for *N. tenuis* and *M pygmaeus*, *D. cerastii* has the potential to cause an economic impact on tomato fruit production, particularly for cluster tomato cultivars, since its damage is related to the parts of the plant responsible for fruit production. We suggest that decision making regarding its use as a biological control agent should be made through field evaluation considering different cultivars.

We also found that fruit damage was highly variable within treatments, indicating that there may be differences in phytophagy on both species and individual levels. Therefore, in the future, selection of less phytophagous populations/strains combined with adequate management strategies may also benefit from the predatory behavior of dicyphine mirids with lower negative impact on tomato production.

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Review

Sizing the Knowledge Gap in Taxonomy: The Last Dozen Years of Aphidiinae Research

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Simple Summary: Taxonomy is a biological discipline with the task to identify, name, and describe organisms, and as such, it provides necessary data for all other biological disciplines. The biodiversity crisis through which we are living draws attention to the crucial role of taxonomy in biology today. At the same time, the scientific community, as well as society in general, has become more aware of the difficulties associated with taxonomy, such as gaps in taxonomic knowledge, a lack of taxonomic infrastructure, and an insufficient number of taxonomic experts ("taxonomic impediment"). With this study, we tried to size this knowledge gap by analyzing the taxonomical studies on Aphidiinae (Hymenoptera: Braconidae) conducted from 2010 to 2021. Aphidiinae are endoparasitoids of aphids; a single specimen completes its development inside the living aphid host, which are used in biological control programs. Here, we summarize the knowledge gathered over the last dozen years and discuss it in a general context.

Abstract: Taxonomic impediment is one of the main roadblocks to managing the current biodiversity crisis. Insect taxonomy is the biggest contributor to the taxonomic impediment, both in terms of the knowledge gap and the lack of experts. With this study, we tried to size the knowledge gap by analyzing taxonomical studies on the subfamily Aphidiinae (Hymenoptera: Braconidae) conducted from 2010 to 2021. All available taxonomic knowledge gathered in this period is critically summarized: newly described species, detection of alien species, published identification keys, etc. All findings are discussed relative to the current state of general taxonomy. Future prospects for taxonomy are also discussed.

Keywords: taxonomy; taxonomic impediment; Aphidiinae; parasitoids

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1. Prologue

The biodiversity crisis has been known about for decades, but has just recently started drawing the public attention it deserves [1]. There is an urgent need to mitigate the crisis by exploring, managing, and conserving biodiversity. The first step is to realize that the most important unfinished job in biology is discovering and describing biodiversity [2,3]. This brings us to the vital role of taxonomy in today's biology. As a biological discipline with the tasks of identifying, naming, and describing organisms [4], taxonomy represents a bridge between two basic biological disciplines, morphology and systematics, and provides necessary data for all other biological disciplines (Figure 1). The strongest link between taxonomy and systematics/morphology is in phylogenetic taxonomy, which uses data about common ancestry from systematic (and phylogenetic) studies (based mainly on morphology and/or molecules) and combines it with morphological data in species descriptions. To the untrained eye, taxonomy might look like an easy job, but it must collate information from various scientific fields (e.g., morphology, anatomy, ecology, molecules, geography, etc.), which makes it the most integrative biological discipline [5,6] (Figure 1). Unfortunately, at the moment, taxonomy is not the highway bridge that we need. It is like an old, decrepit bridge that is still standing on a good foundation, but is full of gaps, holes,

and obstacles. The difficulties associated with taxonomy are gaps in taxonomic knowledge, a lack of taxonomic infrastructure, and an insufficient number of experts; together these are called the "taxonomic impediment". With more than 1 million described species, insects comprise more than half of all known species [7], and with an estimate of 5.5 million living species [8], they represent the biggest proportion of the taxonomic impediment.

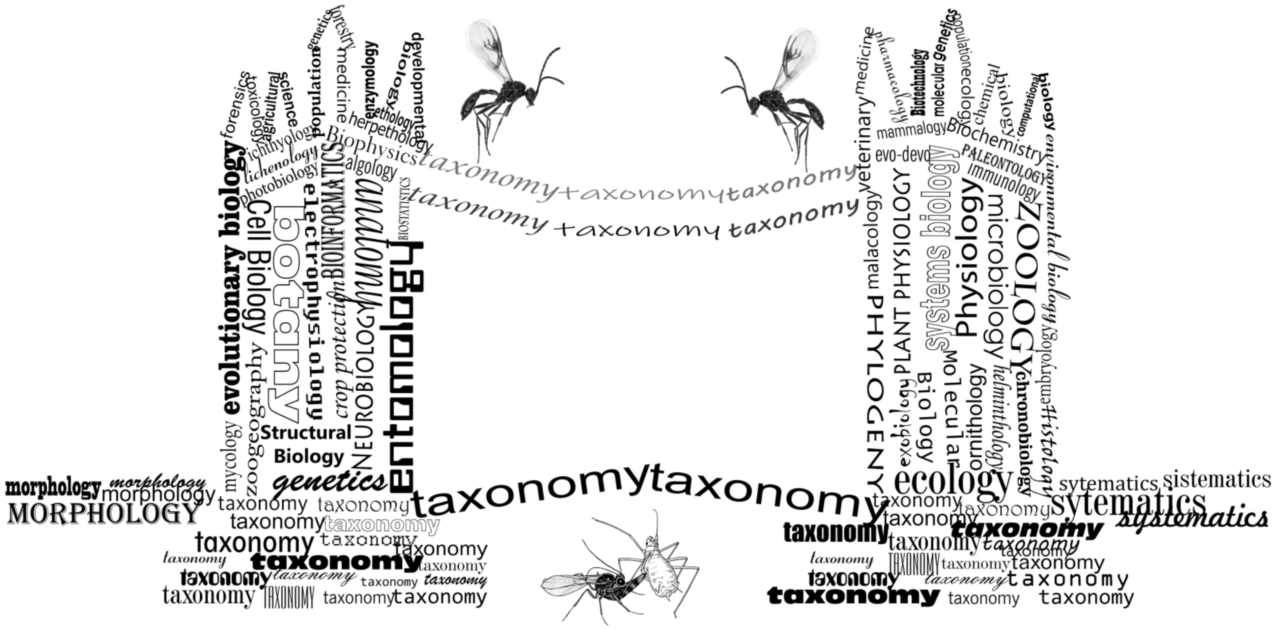

Figure 1. Position of taxonomy in biology today (inspired by *words are bridge* by mariateresa.dapolito).

Knowledge gaps in taxonomy are present for all insect groups, differing only in size. Generally, the size of the gap is negatively correlated with body size and positively correlated with the number of species in the group. Families of small Diptera and parasitoid wasps (Hymenoptera) are recognized as groups with the highest proportions of dark taxa (little-known and unknown species) [9–11].

In order to present the size of the knowledge gap in taxonomy, the subfamily Aphidiinae (Hymenoptera: Braconidae) is chosen as an example.

All species belonging to the subfamily Aphidiinae are solitary koinobiont endoparasitoids of aphids and, as such, a single specimen completes its development inside the living aphid host, which continues to feed and grow [12]. Aphidiinae are highly specialized to attack only aphids and several species are widely used as biocontrol agents. Primarily because of their economic importance, Aphidiinae are one of the best studied parasitoid groups. The taxonomy and systematics of Aphidiinae are especially well studied in Europe, with over 70 years of continuous research [13]. The history of Aphidiinae taxonomy dates back to the dawn of nomenclature and taxonomy, with the first species described in the 10th edition of *Systema Naturae* (*Aphidius rosae* Haliday, 1834 described as *Ichneumon aphidum* L. 1758) [14]. In the last 263 years, many notable entomologists have contributed to Aphidiinae taxonomy (e.g., Christian Gottfried Daniel Nees von Esenbeck, Alexander Henry Haliday, Thomas Ansell Marshall, William Harris Ashmead, and many others), but the real pioneers are our contemporaries: Professor Manfred Mackauer (Simon Fraser University, Burnaby, Canada) and Dr. Petr Starý (Biology Centre of the Czech Academy of Sciences, České Budějovice, Czech Republic). More than six decades ago, they started their research on Aphidiinae, and have published numerous papers concerning taxonomy, systematics, and all other aspects of Aphidiinae biology. Dr. Petr Starý, with more than 500 published papers dealing with Aphidiinae, can undoubtedly be labeled as a man who made a difference. One may ask why there is a knowledge gap when one man has performed so much research. The answer would be that taxonomy is a never-ending story;

so, here we present taxonomical studies on Aphidiinae conducted from 2010 to 2021, as a proxy of the knowledge gap size.

2. What Has Been Accomplished in the Last Dozen Years?

The first problem that emerges when one starts reading about the subfamily Aphidiinae is the number of described species and genera. Those numbers differ significantly in various sources, starting with 400 species in 50 genera in Boivin et al. [12], then 505 species in 38 genera according to Žikić et al. [15], up to more than 600 species in 65 genera according to Tian et al. [16], and 700 species according to Mackauer and Finlayson [17]. There are several reasons for this discrepancy, such as the use of outdated references (the last comprehensive world checklists of Aphidiinae were published in the late 1960s [13,18]); uncritical use of available databases such as Taxapad World Ichneumonidae database [19]; counting both recent and fossil species, etc. The determination of the exact number of Aphidiinae species is beyond the subject of this paper, but according to the available data (critical use of data from Taxapad World Ichneumonidae [19] and Fauna Europea [20], combined with Starý 2006 [21] and references therein, and references from this study), our best estimate is that there were about 500 living species classified in 52 genera prior to 2010.

2.1. Bookworm on the World Wide Web

The subfamily Aphidiinae is an excellent model to emphasize the "taxonomic impediment." It is a group of insects that are frequently used for different studies. The aim of the literature survey was to identify as many studies as possible on Aphidiinae, including articles, books, book chapters, conference proceedings, Master's and PhD theses, research reports, etc., and to identify all new Aphidiinae taxa described in 2010–2021. Literature surveys face specific problems in these times of a constantly growing volume of research. Choosing the right tool for the survey is essential, and there are numerous studies that assess the usefulness of different search systems ([22–24] and references therein). In most studies, search engines (such as Google Scholar, Microsoft Academia, etc.) are not recommended to be used solely for literature surveys [22,23], but there are some studies that showed that Google Scholar (GS) can at least be used (with some limitations and extra labor) as a data source for research assessment [24].

In order to determine how many studies dealing with Aphidiinae were published in the last 12 years (2010–2021) a bibliographic search was performed using Web of Science Core Collection (WoS), Scopus, and GS. In all three search systems, the following words and word combinations were used as descriptors for the search: Aphidiinae, Aphidiidae, and aphid parasitoids in 2010–2021. All the descriptors were searched throughout all fields (the whole article without references). Considering that the relevance-based sorting algorithm of GS provides only 1000 results per query (biased towards highly cited documents) [22,24], independent searches were performed for every pair of consecutive years (2010–2011, 2012–2013, . . . 2020–2021). In this way, all search queries had fewer than 1000 results and bias was eliminated. In all three search systems, the following methodology was used for article selection:

(1) All search results were inspected by eye and those studies with at least one of the descriptors appearing in the title, keywords, or abstract were treated as relevant. (2) All studies that did not meet the first criterion were further inspected by checking the Materials and Methods and Results sections of research articles, and the full text of all other types of studies. Studies in which Aphidiinae were identified as an object of the study were treated as relevant. (3) Studies that did not meet the previous two criteria were excluded. (4) Studies written in languages unfamiliar to the author were automatically translated with Google Translate (although the translation is not always perfectly accurate, it can be easily used for all detected languages if the one who is using it is familiar with the subject). (5) Obtained sets of studies were then checked and all duplicates were excluded (only for GS results; Scopus and WoS searches resulted in no duplicate results). (6) Results that matched the previous selection criteria were exported as .csv (excel) files. In order to

export results from GS, every individual result was manually added to a library and then exported. (7) Obtained datasets had different structures, and it was not possible to compare them automatically (mainly because of the unusual structure of the .csv file exported from GS), so it was completed manually. Every study title obtained in Scopus and WoS searches (which both resulted in a significantly smaller number of results) was searched for again in GS, and if it had already been added to the library, it was treated as a duplicate; those that were not in the library were treated as unique results and were added to the final dataset.

Search results differed significantly between WoS, Scopus, and GS (Table 1), which is as expected based on the differences in their coverage. GS, as the most comprehensive academic search engine (with over 300 million records) [25], provided the highest number of both initial and relevant results.

Table 1. Results of literature surveys for Aphidiinae studies using Google Scholar (GS), Scopus, and Web of Science (WoS).

Search System	Total Number of Results	Number of Relevant Results	Number of Unique Results Included in Analysis
GS	3570	1753	1752
Scopus	874	803	125
1-4 **WoS**	1082	654	25
Total	5526	3209	**1902**

After a final comparison of datasets obtained from different search systems, we had a total set of 1902 studies published on Aphidiinae in 2010–2021. Interestingly, in the GS search, 1752 records were acquired, so only 150 of the total records were omitted. An additional GS search (a search of individual articles) resulted in finding all 150 previously unrecorded articles (from Scopus and WoS). Although it remains unknown why those results were omitted in the initial search, it can be concluded that GS has 100% coverage of Aphidiinae studies from the analyzed time period.

Among the 1902 analyzed results, the biggest proportion (86%) were papers published in scientific journals, but there was also a significant number (14%) of conference papers, books, book chapters, Master's and PhD theses, etc. (almost all recorded just by GS search). Besides the difference in non-journal-article records, the GS, Scopus, and WoS search results differed significantly in terms of the number of records published in languages other than English. There is a small number of non-English-language journals indexed in both Scopus and WoS (all with abstracts in English), and thus a GS search recovered 292 results in 19 languages other than English, while Scopus detected 22 results in six languages, and WoS detected only 17 in five languages.

Based on the Aphidiinae literature survey results obtained with GS, Scopus, and WoS, it is obvious that the sole use of curated databases, such as WoS and Scopus, is inadequate for reviews of taxonomical and faunistic research. Although GS receives (mainly deserved) criticism for not being suitable as a primary tool for systematic reviews (because of inadequate recall, precision, transparency, and reproducibility) [22], in this particular case (a survey of Aphidiinae studies), it has been shown that a specifically performed search (as described above) makes GS suitable for a survey of taxonomy literature (with 100% coverage in this study). This search was performed only for the subfamily Aphidiinae, but it will most likely provide similar results for the majority of insect groups because of the specific nature of taxonomic publications. Insect taxonomists often publish their studies in journals that are not indexed in databases. Some of the journals are local, while some are well-respected journals with a long tradition. For example, oo ooooo oa (Proceedings of the Russian Entomological Society, published since 1861), The Entomologist's Monthly Magazine (published since 1864), Entomologisk tidskrift (Entomological Journal, published since 1880), Entomofauna (published since 1980), Insecta Mundi (published since 1985), and many more are not indexed in either Scopus or WoS. This "old-fashioned" method of

literature searching (checking every article by eye, which resembles a search in the library) might appear time-consuming and labor-intensive, but provides the most comprehensive data.

2.2. New Taxa in the Old World and All Other Worlds

Within the analyzed search results, a vast majority of studies have been conducted on various applied aspects of Aphidiinae biology (life history, demography, functional response, host preference, foraging behavior) and, to a lesser extent, on local fauna, all with the aim of using those parasitoids in biological control. There were fewer than 60 papers focusing on Aphidiinae taxonomy and systematics, and only 42 in which new Aphidiinae taxa are described [16,17,26–65] (Table S1 and Figure 2). The obvious discrepancy between applicative and taxonomical studies can be treated as a proxy of the "taxonomic impediment" in Aphidiinae taxonomy. Within those 42 papers, four new genera (*Choreopraon* Mackauer, 2012; *Sergeyoxys* Davidian, 2016; *Astigmapraon* Tian et Chen, 2017, *Ishtarella* Martens, 2021) [16,17,43,65] and 64 new species were described around the world (Table S1).

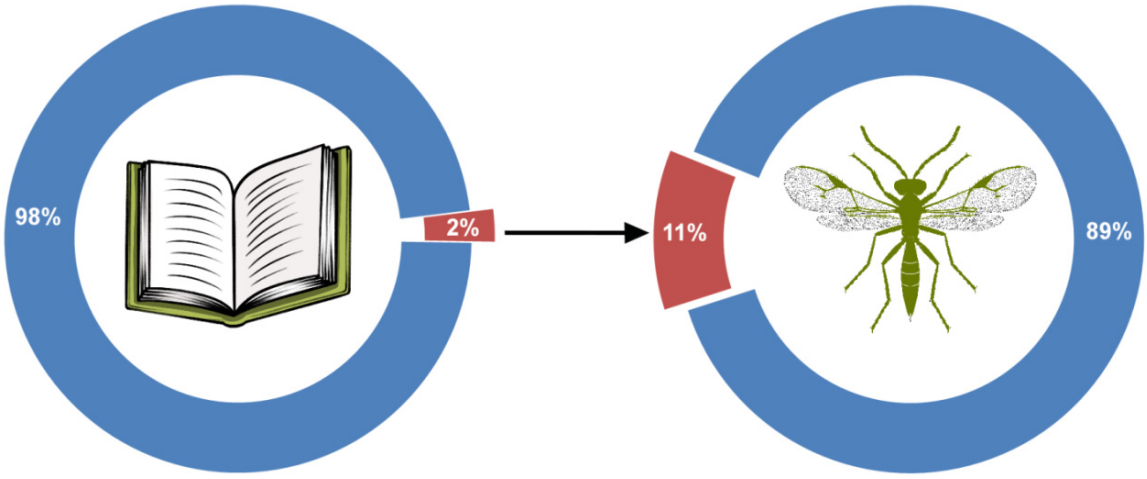

Figure 2. Share of taxonomic papers (red) in Aphidiinae studies conducted in the period 2010–2021 (**left**) and share of new Aphidiinae species described in the same period (red) compared to the total number of Aphidiinae species (**right**).

Newly described taxa represent 7% and 11% of all known Aphidiinae genera and species, respectively (Figure 2).

The fact that such a high percentage of all known Aphidiinae species have been described just in the last 12 years indicates that there are potentially many more species waiting to be found, and thus the knowledge gap in Aphidiinae taxonomy is still large. The assumption can be rightly criticized that the only way to determine the knowledge gap is to estimate the total number of species, and determine the ratio of described to undescribed. Estimating the species number of any insect group, as well as of insects in general, is challenging for many reasons. There are numerous datasets and methodologies that can be used [8]. There are also numerous assumptions that must be made and thus, the obtained results can vary significantly. So, let us try to justify the previous assumption with the simplest and probably most conservative estimation of the number of Aphidiinae species. For this purpose, the parasitoid–host (P:H) ratio can be used, because Aphidiinae are obligatory aphid parasitoids. The main idea is to calculate P:H for some representative areas or territories that are characterized by well-researched aphid and Aphidiinae fauna, and then calculate the number of Aphidiinae species based on the total number of aphid species. Based on the fact that the majority of aphid species are known from the Palaearctic region [66], it is logical to look for representative areas in this realm. The only two areas with up-to-date and relatively well researched faunas of both aphids and parasitoids are the Czech Republic and Serbia. The Czech Republic is a Central European country with

755 recorded aphid species and 135 (between 130–140) Aphidiinae species [21]. Serbia is a South European country with 385 recorded aphid species [67,68] and 121 Aphidiinae species [69]. The calculated parasitoid–host ratio for the Czech Republic is P:H = 0.18 and for Serbia it is P:H = 0.31. Taking the mean P:H ratio (0.245) and multiplying it by the number of currently known aphid species, which is around 5000 [70], gives us a rough, conservative estimate of 1225 Aphidiinae species. Taking into account that the assumptions that are made for this estimation (the current number of aphid species is treated as the final number, two representative areas are perfectly explored, etc.) are far from the truth, there is a high probability that the real number of Aphidiinae species is several times higher than the obtained result.

Recently, Engel et al. [6] emphasized that the shortage of brains and hands involved in taxonomy is one of the main reasons for such a large knowledge gap in taxonomy. Further analysis of the data about Aphidiinae taxa gathered since 2010 provides us with exact evidence for their claim. Two out of four genera were described from the Palaearctic (*Sergeyoxys*—Russia, *Astigmapraon*—China) while *Choreopraon* and *Ishtarella* were discovered in New Zealand (Australasia) and Thailand (Indomalayan realm), respectively. The predominance of discoveries in the Palaearctic is much more obvious at a species level, with 70% of species being described from this region. At the same time, none of the species were discovered in Afrotropical and Neotropical regions (Figure 3). In general, Aphidiinae species richness depends on habitat richness and, most importantly, aphid species richness, and is a product of the evolutionary history of the group. As obligatory parasitoids, Aphidiinae follow their hosts in distribution, and consequently the majority of species are found in temperate regions of the Northern Hemisphere [17]. However, such a large difference in species discoveries between Palaearctic and the rest of the world can be largely attributed to the insufficient number of taxonomic experts and their uneven distribution around the world.

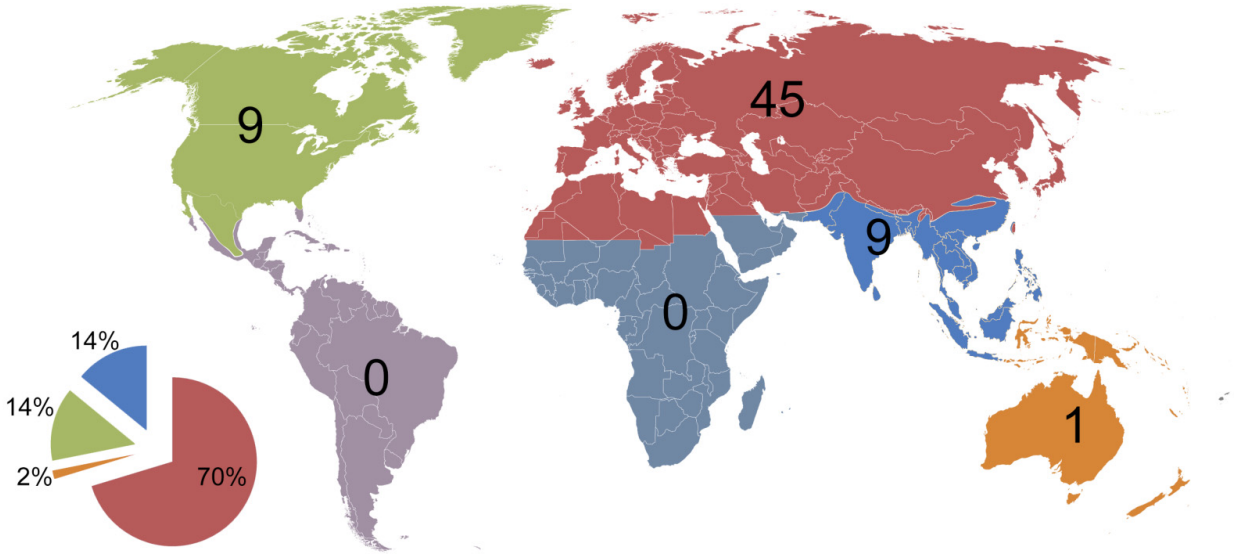

Figure 3. Number and percentage (pie chart) of Aphidiinae species described in different biogeographic realms in 2010–2021.

The uneven distribution of experts was even more obvious when species authors were analyzed. The 64 Aphidiinae species described between 2010 and 2021 were named by 22 researchers (up to three authors per species), from which five were from the USA (Nearctic), one from India (Indomalayan), and 16 from the Palaearctic (two from China; one each from Japan, Iran, and South Korea; and 11 from Europe). Only nine species were described by authors not from Europe.

Although the number of species per area/country cannot be used for a biodiversity assessment per se (as explained above), in this particular case it could be very informative

as a proxy of uneven distribution of Aphidiinae taxonomy experts (Table 2). Areas or countries are chosen as representatives of a specific continent or region (the largest or most extensively researched). A closer look at those simple data reveals strange patterns in species richness. The only biologically (and biogeographically) logical fact is that the highest number of Aphidiinae species was recorded from Russia (which occupies 30% of the Palaearctic). For example, some of the biggest areas and countries of the world, such as North America, India, and China, all have a similar number of species to two relatively small, landlocked European countries: Czech Republic and Serbia. This discrepancy can be explained only by the lack of Aphidiinae taxonomy experts, because the number of aphid species is much higher, especially in North America [71], where around 1500 aphid species are recorded. Although taxonomists examine specimens from all over the world, most of their work is related to their place of residence, which means those areas are investigated in much more detail.

Table 2. Number of Aphidiinae species recorded in some countries/areas, with references in square brackets.

Country/Area	Number of Species	References	Area (km^2)
America north of Mexico (Nearctic)	~130	[47,57,58,72,73]	19,782,990
Mexico (North and Central America)	33	[74]	1,972,550
Costa Rica (Central America)	10	[75]	51,100
Chile (Neotropics)	23	[76]	756,096.3
Brazil (Neotropics)	19	[77]	8,515,767
Australia (Australasia)	23	[78]	7,692,024
New Zealand (Australasia)	15	[79]	268,021
Subsaharan Africa (Afrotropics)	22	[80–84]	23,290,000
Madagascar	7	[84]	592,800
Russia (Palaearctic)	198	[54,61,85]	17,098,246
Middle East and North Africa	108	[86]	11,695,164
China (Asia)	130	[16]	9,596,961
India (South Asia)	127	[29,34]	3,287,263
Japan (Far East)	≈80	[42,55,64,87]	377,975
Kyrgyzstan (Central Asia)	35	[60]	199,951
Norway (Northern Europe)	26	[58,88,89]	385,207
Czech Republic (Central Europe)	≈135	[21]	78,871
Germany (Central and Western Europe)	109	[88,90]	357,022
Great Britain and Ireland (British Isles)	96	[88,91]	293,752
Serbia (Southern Europe)	121	[69]	88,361

2.3. Aliens in Europe

Species descriptions and rate of species description are often used as the sole indicator of taxonomic activity, but taxonomy is much more than just naming a species, and various indicators can and should be used to assess the current state of taxonomy and systematics [92]. One such indicator, which is widely underestimated, is the crucial role of taxonomists in the identification of alien species. In European Aphidiinae, only five species were marked as alien (*Aphidius colemani* Viereck, 1912; *Aphidius smithi* Sharma and Subba Rao, 1959; *Lysiphlebus testaceipes* (Cresson, 1880); *Pauesia cedrobii* Starý and Leclant, 1977 and *Pauesia unilachni* (Gahan, 1927)) before 2010, and all were intentionally introduced as biocontrol agents [93,94]. *Lysiphlebus testaceipes* deserves special attention. As a promising biocontrol agent of citrus aphids (*Toxoptera aurantii* (Boyer de Fonscolombe, 1841) and *Aphis spiraecola* Patch, 1914), it was introduced in Europe in 1973 (in the Mediterranean part of France), and very quickly became invasive and widespread over the Mediterranean area [95]. In the last decade, *L. testaceipes* made a breakthrough in the cooler territories of the continental part of Europe [95,96]. On the other hand, the same species was introduced

in South Africa (in 1969), where it apparently failed to establish colonies, and also in Kenya, but the fate of the released parasitoids is unknown [80]. Recently, *L. testaceipes* was recorded in high numbers in both Western (Benin) [97] and Eastern Africa (Malawi) [98]. Although its origin is unknown in both countries, it most likely spread naturally from South Africa or Kenya.

Three more alien species, namely *Lysiphlebus orientalis* Starý and Rakhshani, 2010, *Aphidius ericaphidis* Pike and Starý, 2011 and *Trioxys liui* Chou and Chou, 1993, were detected in Europe in the last decade; all were accidentally introduced [99–101]. Interestingly, two species were detected in Europe soon after their descriptions. *Lysiphlebus orientalis* was described in 2010 from China and detected in samples from Serbia collected in 2010 and 2011 [99], while *Aphidius ericaphidis* was described in 2011 from the USA and was detected in samples from Serbia and Scotland collected in 2014 and 2015, respectively [100]. Additional revision of collections indicated that both species were present in Europe long before their formal descriptions (*L. orientalis* in 1995, and *A. ericaphidis* in 1965). *Trioxys liui* was first collected in Spain in 2017 [101]. Furthermore, *Trioxys sunnysidensis* Fulbright and Pike, 2007 was described as a parasitoid of bird cherry-oat aphid (*Rhopalosiphum padi* (Linnaeus 1758)) in Washington State [102]. In 2019, Čkrkić et al. determined that this species is widely distributed in North America and Europe, and also present in New Zealand [88]. Although *T. sunnysidensis* is most likely a cereal aphid parasitoid, it remains undetected because of its rarity. They emphasized that some rare and even new or alien species can be easily overlooked in large-scale ecological studies [88].

Considering that Aphidiinae are reducing populations of aphids (which could be treated as pests), some economically oriented (and environmentally unenlightened) policymakers/politicians may conclude that the introduction of alien parasitoids is a good thing. Yet, this could not be further from the truth. Every alien species undoubtedly has negative effects on a new environment. The majority of effects were recently summarized in "Scientists' warning on invasive alien species" [103]. In this particular case, alien Aphidiinae species can affect the richness and abundance of native species and modify trophic networks, which may lead to changes in ecosystem functioning and the delivery of ecosystem services [103]. For example, alien species can outcompete some native species, resulting in native species' extinction from occupied areas, or in host shift by native species, which will then trigger a whole cascade of events. At the same time, alien species could have unusual characteristics that reduce their capacity to control aphid populations, such as in the case of the relationship between *L. orientalis* and its host, which is characterized by transgenerational fecundity compensation [104]. Transgenerational fecundity compensation is a phenomenon in which the offspring of parasitized aphids produce more progeny than the offspring of nonparasitized aphids [104]. On the other hand, climate change will inevitably affect local fauna, with local extinctions of native species and/or the appearance of new exotic species. Even relatively small climatic changes can affect aphid–parasitoid communities and result in changes over a relatively short period of time [105].

2.4. Shrinking the Gap by Revising the Knowledge

A single description of a species is of inestimable importance, but sometimes alpha-taxonomy [106] can be in discrepancy with beta-taxonomy [106] because of the "superficial description taxonomic impediment" (older descriptions often can be too superficial by today's standards) [3]. In such cases, a revision of a whole group of organisms (species group, genus, tribe, etc.) is necessary in order to reduce the knowledge gap.

The majority of Aphidiinae tribes, subtribes, and genera were revised 30–50 years ago and those revisions are mainly outdated. In the last decade, a lot of effort has gone into the improvement of Aphidiinae taxonomy and systematics and the clarification of species status. Revision of the world Monoctonina Mackauer, 1961, is the only revision on the subtribe level, and included all available species [53,57]. Several genera with fewer species were also reviewed: *Monoctonia* Starý, 1962 [42], *Lipolexis* Foerster, 1862 [55], *Areopraon* Mackauer, 1959 [49], *Pseudopraon* Starý, 1975 [49], *Paralipsis* Foerster, 1862 [44,51],

Acanthocaudus Smith, 1944 [47], *Euaphidius* Mackauer, 1961 [107], and *Remaudierea* Starý, 1973 [107]. Most of those revisions resulted in a higher number of species within the genus, while the genera *Euaphidius* and *Remaudierea* were determined as junior synonyms of *Aphidius* [107]. European species of the genera *Adialytus* Foerster, 1862, [108] and *Lysiphlebus* Foerster, 1862, [50] were also revised, and a new subgeneric classification of European *Ephedrus* Haliday, 1833, species was proposed [52]. *Lysiphlebus* revision [50] can serve as a classical example of the importance of revisions in taxonomy, and consequently in biodiversity research. Prior to revision, there were 15 *Lysiphlebus* species known in Europe. With this study, the number of European *Lysiphlebus* species was reduced to 13; four species were synonymized and two new species were described. Although this quantitative taxonomic information is very important, even more important is the quality of the taxonomic information [92]. Within this study, based on molecular markers and morphology, Tomanović et al. determined that only two descriptions of *Lysiphlebus* species match today's standards in Aphidiinae taxonomy, and redescribed all other species [50]. In addition, remarks about species biology and distribution were provided.

There are several published studies dealing with the taxonomic status of Aphidiinae species groups [37,40,41,94,109–112], among which the most important are those about the taxonomy of biocontrol agents belonging to the *Aphidius colemani* and *A. eadyi* species groups [94,111]. It was determined that the *Aphidius colemani* species group consisted of three species: *A. colemani*, *A. transcaspicus* Telenga, 1958, and the almost forgotten *A. platensis* Brethes, 1913 [111]. At the time when this study was conducted, parasitoids within globally commercially distributed materials were a mixture of all three species [111], and most likely, the situation is the same now. Interestingly, similar results were obtained within the *A. eadyi* species group [94]. Three species of biocontrol agents against *Acyrthosiphon pisum* (Harris) were identified (*A. smithi*, *A. eadyi* Stary, Gonzalez and Hall, 1980, and *A. banksae* Kittel, 2016). *Aphidius banksae*, which was previously known only from Israel and Turkey, was identified as a widely distributed species with a range that covers most of the western Palaearctic [94]. There were also a few studies conducted to test taxonomic status and relationships among three closely related biocontrol agents of cereal aphids, *Aphidius uzbekistanicus* Luzhetzki, 1960, *Aphidius rhopalosiphi* De Stefani Perez, 1902, and *Aphidius avenaphis* (Fitch, 1861) [109,113]. Unlike the previous two studies [94,111], the results of those studies were not straightforward (which is quite common) considering taxonomy. Using molecular and morphological data, the authors determined the incongruence between those two datasets and discovered the possible existence of cryptic species, which could not be morphologically identified and described because of a small number of samples [109,113]. *Aphidius rhopalosiphi* is one of the most studied Aphidiinae species from various aspects of biology, which was also confirmed with this literature survey. High genetic diversity was discovered within *A. rhopalosiphi*, which may imply that it is a species complex, or at least a group of separate evolutionary lineages (which can also differ in biology, ecology, etc.) [109], which calls into question the results of all these studies. Such huge knowledge gaps in the taxonomy of economically very important biocontrol agents raise other questions, especially about the taxonomic status of many other less explored Aphidiinae species.

2.5. Keys to Unlock an Easier Scientific Existence

So-called "bad taxonomy" can have a significant effect on our knowledge of nature [114]. As a bridge that connects and supports many (if not all) biological disciplines, taxonomy needs to be very precise. A single taxonomic error (incorrect identification) may be incorporated in numerous ecological and biological studies, and later even in some environmental programs. With every new step, an error multiplies its impact and could have different negative consequences [114]. In order to reduce the potential for error, taxonomists must provide usable and reliable identification keys. For nontaxonomists, this may seem trivial and easy, but from a taxonomist's perspective, an identification key is one of the most challenging publications to create. In the last dozen years, there were 40 papers published

in which the authors provided identification keys for Aphidiinae genera and species. The studies can be divided according to subject into several groups: (1) identification keys for Aphidiinae from specific (local) areas: from the Middle East and North Africa [86], Iran [33,86 and references therein], Malta [115], Argentina [116], Pakistan [117,118], Australia [78], Costa Rica [119], Serbia [69], and China [120]; (2) identification keys for species within tribes [120], subtribes [53], genera [16,42,44,47,49–52,55,108,112,121], and species complexes [111]; and (3) identification keys for Aphidiinae species related to specific aphids [30], plants [32,36,122–127], and habitats [128].

Although a significant number of Aphidiinae identification keys have been published in the last 12 years, there is still a significant shortage, primarily because of species coverage in existing taxonomic keys. Currently, we lack identification keys even for areas that are relatively well explored. There are several very usable keys that cover European species of a specific subtribe or genus, e.g., [50,51,53,55]. We are still lacking keys for some of the most difficult and problematic genera (*Aphidius*, *Praon*, *Pauesia*, and *Trioxys*). The most comprehensive identification key is one about Aphidiinae in Serbia (covers 121 species) [69]; unfortunately, it is published only in the Serbian language. At the same time, users of taxonomic end-products (such as species descriptions and identification keys) largely avoid using them, or at least do not cite such work. Bortolus [114] found that 62.5% of papers published in top-ranked ecological journals are missing any information about the literature used for the identification of the organisms in the study.

3. Looking in and through the Mirror—Current Situation and Future Prospects

Taxonomy is one of the most undervalued biological disciplines [6]. It is safe to say that the current state of Aphidiinae taxonomy is better than in most parasitoid groups, but it is far from satisfactory. The taxonomy of Aphidiinae is facing the same problems as taxonomy in general. The knowledge gap is still very large, as illustrated in the previous sections. Considering the number of experts, subfamily Aphidiinae is in a more favorable situation than other subfamilies and families of parasitoids. Ten taxonomy experts (Željko Tomanović (Belgrade, Serbia), Elena Davidian (St. Petersburg, Russia), José Manuel Michelena Saval (València, Spain), Nickolas Kavallieratos (Athens, Greece), Ehsan Rakhshani (Zabol, Iran), Keith Pike (Washington State, USA), Jelisaveta Čkrkić (Belgrade, Serbia), Korana Kocić (Belgrade, Serbia), Maryna Kaliuzhna (Kyev, Ukraine), and myself) for a relatively small group of parasitoids looks great, but their distribution is highly inconvenient: 8 out of 10 researchers are from Europe, and half of them are from the same research team (University of Belgrade Faculty of Biology). Fortunately, there are a few young Aphidiinae taxonomists who will replace retired pioneers Petr Starý, Manfred Mackauer, and Ulf Gärdenfors. Thus, although there are numerous cracks in the mirror's surface, the reflection still looks good and promising, and we can say that the current state of Aphidiinae taxonomy is on its way to becoming almost satisfactory.

Now, it is time to look through the mirror. Future prospects for Aphidiine taxonomy are the same as for taxonomy in general. There are ever-increasing demands for scientific names [129], while the rate of naming species is constant or just slightly increasing. In 2010–2021, Aphidiinae species were described at a rate of 5.33 species per year on average. For almost two decades, we have been witnessing different attempts to accelerate the taxonomic process and numerous debates for and against some of the proposals. The two most "revolutionary" proposals are, in fact, technological approaches in which species descriptions should be replaced with DNA barcodes [130,131], while type specimens should be replaced with photographs of species taken in the field [131,132]. From its beginnings, taxonomy has been integrative, but species descriptions are based on a set of characters (with emphasize character state), and, in most cases, illustrated with line drawings and/or (later) photographs. In a broader context, those "revolutionary" approaches are just simplifications of taxonomy in the way of using just one character (DNA barcodes) instead of many [6], and keeping the illustration, but not the voucher specimens. Researchers who advocate those ideas give many different reasons why it is "better" than traditional taxon-

omy. For example, Minteer et al. stated that voucher specimens should be replaced with a "series of good photographs, which can even be used to describe a species, complemented by molecular data and a description of a species' mating call for birds, amphibians, or insects" in order to avoid the extinction that can be caused by collecting [132]. There are at least two questionable aspects of this statement. Firstly, if you are studying small insects (less than 5 mm), and the majority of insects are small, it is highly improbable that one will be able to take a series of high-resolution photos in the field (sometimes you cannot even take one, if you manage to see the insect in the first place). In addition, in most cases, characters for species' identification are very small body parts such as the genitals, tarsal claws, tibial spurs, etc. The equipment for taking good photographs of those characters is too robust and heavy for fieldwork, and the insect needs to be still for photographing. After that, a DNA sample should be taken. Everyone with experience with small insects (e.g., parasitoids) knows that most small insects do not survive such disturbance, and then we will get an unwanted voucher specimen. Secondly, if collecting one or few specimens can threaten species survival, that species will most probably go extinct in the blink of an eye anyway, and then we have lost out on an opportunity to gather knowledge.

We often hear that DNA barcoding is becoming cheaper and cheaper, and thus affordable to all. Those who write those statements are simply unable to see the insects for the pipette tips. If you look beyond your comfort zone, you will realize that at least half of the scientific community can access basic scientific literature only thanks to a modern Robin Hood figure named Alexandra Elbakyan (creator of the website Sci-Hub, which provides free access to research papers without regard for copyright). For example, back in 2007, Godfray wrote that DNA sequencing was becoming cheaper and more affordable [131], but in order to read his article (and find out what was so cheap) you needed to pay $32, which represents a significant proportion of the average monthly salary in some parts of the world.

The idea behind the Barcode of Life Initiative is excellent, but the authors of the initial paper [130] wrote, "When fully developed, a COI identification system will provide a reliable, cost-effective and accessible solution to the current problem of species identification." Currently, we are still far from a fully developed system, and yet there are some studies that exclusively use DNA barcodes for species diagnosis in Braconidae [133]. There is already a wide debate considering the justification of such minimalist revisions [3,6,134,135]. Meier at al. stated that such studies will become the next "superficial description impediment", which is probably the strongest argument against this practice [3]. Indeed, taxonomic diagnoses should be clear, and involve a minimal number of statements which will allow us to distinguish a given specimen from other taxa [135]. Although Sharky et al. revised 11 Braconidae subfamilies, Aphidiinae were not analyzed [133].

DNA barcoding is a widely accepted method in Aphidiinae taxonomy, but only as a part of an integrative taxonomic approach that (in most cases) employs DNA barcodes along with morphological and ecological data. In the last two years, 10 Aphidiinae species have been described thanks to cooperation between taxonomists and the Barcode of Life Initiative [53,55,57], and even more species were described using the DNA Barcode as part of an integrative approach. All three alien species recorded in Europe since 2010, together with *T. sunnysidensis*, were also identified by integrative taxonomy. In this small scientific community, the opinion prevails that using all available types of data is the only way to shrink the knowledge gap in taxonomy.

In the last few years, different researchers started using DNA barcodes for the molecular identification of Aphidiinae [79,98,136]. The identification of Aphidiinae based solely on molecular data (barcodes) is not reliable, because species and genera boundaries, based on barcoding sequences, vary significantly. For example, within the genus *Ephedrus*, there are two groups of species that differ by as much as 20% (the genetic distance between *E. persicae* and *E. plagiator* clade is 20.7%) [52]. On the other hand, within the genus *Aphidius*, genetic distances between species are much lower, in some cases less than 1.5% [137], and within *Lysiphlebus* there are species with an even lower genetic distance. There are several

documented cases where DNA barcodes fail to discriminate species that are morphologically and ecologically different, such as *Aphidius ervi* Haliday, 1834 and *Aphidius microlophii* Pennacchio and Tremblay, 1987, as well as *A. uzbekistanicus* and *A. avenaphis*. Considering the aforementioned, it is almost impossible to use only molecular markers in Aphidiinae species identification. Barcodes can be used as guidance for identification, but it is still obligatory to perform morphological identification to confirm species identity. This brings us to the importance of voucher specimens. In ecological studies, the use of the fastest DNA extraction protocols is still common practice, even if they are destructive and leave no voucher specimens. The aforementioned studies about the *Aphidius colemani* group in Eastern Africa [98,136] and the native Aphidiinae of New Zealand [68] used destructive DNA extraction protocols and lost potentially very valuable information, while those from New Zealand probably also lost several as yet undescribed species. There are a number of nondestructive protocols, e.g., [138–140] that could and should be used for voucher specimens.

If we manage to keep the current core of Aphidiinae taxonomists and engage some more from different parts of the world, there is a relatively bright future for Aphidiinae taxonomy. Another important task would be closer cooperation with other researchers, especially ecologists. This collaboration could bring about many interesting and more precise results that will give us a better understanding of our world.

4. Conclusions

The bridge from the beginning of the story looks a bit different after a decade of research. Some holes have been filled, and some obstacles have been removed. Now, we can see better. We can see some new holes, obstacles, and gaps that need to be fixed.

Even with the tremendous work that has been completed so far, the knowledge gap in Aphidiinae taxonomy is still significant, and all aspects of the taxonomic impediment are obvious.

Although there is no meaningful research in biology without reliable taxonomy [60], its importance in the modern world is far from fully acknowledged [141]. Recently, the Swiss Re Institute report concluded that 55% of global gross domestic product (GDP) is dependent on biodiversity and ecosystem services [142], but we are still lacking large research grants (and even small ones) for taxonomy, which is considered only as a cost [141]. Until institutions, governments, and the world realize the importance of this kind of research, taxonomists need to be cleverer, and utilize data from all available resources such as museum collections, as well as cybertaxonomy [143], and molecular data.

Anyone who intends to take this interesting walk over the bridge called taxonomy should be aware that in Aphidiinae taxonomy, barcodes are not enough, and must be used only as a part of integrative taxonomy. A similar situation is seen for most other taxa, and using just one type of information can make our knowledge gaps even larger.

It seems appropriate to finish with a citation of a song by Jonathan Coulton (https://theportalwiki.com/wiki/Still_Alive_(song) (accessed on 10 June 2021)) adapted for taxonomy:

"We've experiments to run
There is research to be done
On the species who are
Still alive."

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/insects13020170/s1, Table S1: List of the Aphidiinae species described in the period 2010–2021.

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Article

Essential Oil Coating: Mediterranean Culinary Plants as Grain Protectants against Larvae and Adults of *Tribolium castaneum* and *Trogoderma granarium*

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Simple Summary: The protection of stored agricultural products has been established as a global priority serving both food safety and security. Toxicity and residual issues of synthetic insecticides shifted the research focus towards natural pest control agents. In this context, six edible plants were selected for the conduction of a novel bioprospecting effort aiming to identify potential control agents against the red flour beetle, *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae) and the khapra beetle, *Trogoderma granarium* Everts (Coleoptera: Dermestidae). The proposed bioprospecting effort aims to identify the chemodiversity of essential oils (EOs) and exploit the potential of EO-based microemulsion (ME) coating as alternative tools for the management of the tested stored-product insects and the concomitant postharvest losses. Elevated toxicity was recorded against *T. castaneum* larvae and *T. granarium* adults. The fact that these EO-based MEs originate from culinary plants renders them safe for human consumption. The present study pioneers the utilization of EO-based MEs as grain protectants in the form of grain coating.

Abstract: Postharvest agricultural losses constitute a major food security risk. In contrast, postharvest protection is strongly linked with food safety. The present study aims to develop novel postharvest protection tools through a bioprospecting protocol utilizing edible essential oils (EOs) as grain coatings. For this purpose, six Mediterranean culinary plants were selected for evaluation. The EOs of juniper, *Juniperus phoenicea* L. (Pinales: Cupressaceae), marjoram, *Origanum majorana* L. (Lamiales: Lamiaceae), oregano, *Origanum vulgare* ssp. *hirtum* (Link) A.Terracc. (Lamiales: Lamiaceae), bay laurel, *Laurus nobilis* L. (Laurales: Lauraceae) and tarhan, *Echinophora tenuifolia* ssp. *sibthorpiana* (Guss.) Tutin (Apiales: Apiaceae) were retrieved through steam distillation, while lemon, *Citrus limon* (L.) Osbeck (Sapindales: Rutaceae) EO was retrieved through cold press extraction. All EOs were formulated to microemulsions (MEs) and applied uniformly as a coating on wheat against larvae and adults of *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae) and *Trogoderma granarium* Everts (Coleoptera: Dermestidae). All EO-based MEs have been evaluated for the first time as grain coatings. They caused moderate to high mortality to *T. castaneum* larvae (67.8–93.3% 14 days post-exposure) and *T. granarium* adults (70.0–87.8% after 7 days of exposure). *Citrus limon*, *O. majorana* and *E. tenuifolia* ssp. *sibthorpiana* EO-based MEs were the most efficient against *T. castaneum* larvae, by exhibiting 93.3%, 91.1% and 90.0% mortality 14 days post-exposure, respectively. *Origanum majorana*, *L. nobilis* and *J. phoenicea* EO-based MEs were the most efficient against *T. granarium* adults, exhibiting 87.8%, 84.4% and 83.3% mortality after 7 days of exposure, respectively. These results indicate that EO-based ME coating is a potent tool against the tested postharvest pests.

Keywords: micromulsion; postharvest pest; grain coating; essential oil; stored-product pest

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1. Introduction

Food security, under the perspectives of global population increase and shifting consumer habits, is one of the main future challenges for the agricultural sector [1–3]. In the European Union, the main instrument of agricultural development, i.e., the Common Agricultural Policy (CAP), has set as a target, in order to address this challenge, the increase of agricultural production by 20% in 2030 [4]. The Food and Agricultural Organization (FAO) provides an alternative perspective of food security by focusing on the postharvest losses of agricultural production which is estimated to be 10% in developed countries and exceed 20% in developing countries [5,6].

The interconnectivity of these two approaches is well established and has been highlighted as a challenge since the Bronze Age [7–9]. While postharvest pest infection is horizontal across the sum of agricultural products [10], it is elevated as a significant risk for global food security in the case of staple food infections, with cereals and legumes being prominent among them [11]. Numerous efforts towards the eradication of fungal infestations have been summarized by Schmidt et al. [12], concluding the necessity of a combinatorial approach against the microbe contamination of stored grains such as cold atmospheric pressure plasma and electrolyzed water treatments. Similar advances may also be traced for insect and mite postharvest pests, focusing on their judicious management [13]. A prominent position among orders of insects that are related to stored products is reserved for Coleoptera. More than 600 coleopteran species have been identified as pests of food commodities with a cosmopolitan anthropochore distribution and have been established as major factors of stored-grain degradation [14]. Adults and larvae of these holometabolous insects cause serious direct and indirect damages in stored products by biting and chewing with their mandibles. Although some adults of these species do not feed or rarely feed upon stored commodities, they are also important since they are the vehicles of reproduction. Furthermore, due to the fact that several coleopterans are strong fliers, they can be easily distributed within/among storage facilities and between field and storage facilities [5,14].

Despite the fact that chemical insecticides are effective against a wide spectrum of insects, they may negatively affect the environment and health of consumers [15,16]. It is well documented that stored-product insects have developed resistance to major classes of insecticides, such as pyrethroids and organophosphates, due to their continuous exposure to synthetic insecticides [17–20]. Therefore, recent advances in policies but also on the regulation of active substances emphasize the use of non-synthetic plant protection products [21,22]. While novel approaches such as cold plasma [23] and ozone [24] treatments have been proven efficient, they have also presented significant side effects, mostly in relation to the nutritional value and physical and chemical properties of grains. On the other hand, natural products have been demonstrated as a promising source of plant protection tools [22,25–27].

Among natural products, essential oils (EOs) constitute a distinct class, representing complex clusters of plant secondary metabolites, with decreased mammalian toxicity and ecosystem penetrability and a selective mode of action circumnavigating the risk of resistance development [28,29]. Essential oils have been studied in relation to their fumigant toxicity [30–32] and their contact toxicity [33–35], but only recently has there been a focus on novel application methods of EOs [36,37]. This research interest became fruitful by providing a solid methodological approach for the application of volatile compounds as stored grain coatings in the form of nanoemulsions (NE) [38–41]. Microemulsions (ME), on the other hand, are kinetically stable, oily droplets in water, with a Surfactant-to-Oil Ratio (SOR) usually higher than 2 [42]. Previous reports have indicated that MEs are effective against different species of insects [43,44].

The present study builds upon the advances of MEs and aims at ameliorating the knowledge on EOs toxicity against stored-product pests through the introduction of a novel bioprospecting protocol. For this purpose, EO-based MEs have been implemented for first time as grain coating agents. The subjects of investigation were retrieved from the

Greek biodiversity pool with a distinct focus on edible and/or culinary plants [45–50]. This way, the EO-based ME grain coating will be compatible with human consumption. The ME preparation utilized food grade emulsifiers and solvents. As target pests, the red flour beetle, *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae), a highly destructive stored-product insect pest of Indo-Australian origin [51,52], and the khapra beetle, *Trogoderma granarium* Everts (Coleoptera: Dermestidae), a highly destructive pest affecting a wide variety of commodities worldwide of animal and plant origin [52–57] and included in the 100 most important invasive species worldwide [58], were selected.

2. Materials and Methods

2.1. Plant Material

Plant material from six Greek indigenous culinary species was examined in the present study. These species are lemon, *Citrus limon* (L.) Osbeck (Sapindales: Rutaceae), juniper, *Juniperus phoenicea* L. (Pinales: Cupressaceae), bay laurel, *Laurus nobilis* L. (Laurales: Lauraceae), tarhan, *Echinophora tenuifolia* ssp. *sibthorpiana* (Guss.) Tutin (Apiales: Apiaceae), marjoram, *Origanum majorana* L. (Lamiales: Lamiaceae) and oregano, *Origanum vulgare* ssp. *hirtum* (Link) A.Terracc. (Lamiales: Lamiaceae) (Table 1). All authentic samples utilized for the identification of EO compounds were obtained from Sigma-Aldrich (Steinheim, Germany), except for germacrene D and α-thujene, which had been isolated in the context of previous studies. The food grade emulsifier, TWEEN® 20 (97%) (Sigma-Aldrich, Steinheim, Germany), was utilized for the preparation of formulations.

Table 1. Essentials oils, plant origin and stock solution composition.

Taxon	Source	Stock Solution		
		EO	TWEEN® 20	Water
Citrus limon	Industrial byproduct	20%	20%	60%
Juniperus phoenicea	Wild gathered	20%	20%	60%
Laurus nobilis	Cultivated	20%	20%	60%
Echinophora tenuifolia ssp. *sibthorpiana*	Wild gathered	20%	20%	60%
Origanum majorana	Cultivated	20%	20%	60%
Origanum vulgare ssp. *hirtum*	Cultivated	20%	20%	60%

2.2. Commodity

Hard wheat, *Triticum durum* Desf. (var. Claudio), commercially acquired, that was free from pest infestations and pesticides was used in the bioassays. Wheat was sieved to remove the impurities and stored at subzero temperatures for several months. Prior to experimentation, the wheat was warmed under room temperature. The moisture content was 12.2% as determined by a calibrated moisture meter (mini GAC plus, Dickey-John Europe S.A.S., Colombes, France).

2.3. Insect Species

The insect species used in the bioassays were obtained from cultures that are kept at the Laboratory of Agricultural Zoology and Entomology, Agricultural University of Athens. The founding individuals of *T. castaneum* and *T. granarium* have been collected from Greek storage facilities since 2003 and 2014, respectively. The selected insect individuals of both species and developmental stages, as well as the conditions they were cultured in, were adapted from previous studies [40,59].

2.4. Essential Oil Isolation and Analysis

All EOs were obtained by hydro-distillation using a modified Clevenger apparatus, according to previously described procedure [60]. The isolation yields of all EOs are included in Table 1. The chemical composition of EOs was determined on a gas chromatographer (GC) coupled to a mass spectrometer (MS) and Flame Ionization Detector (FID) in accor-

dance with a previously described method [60]. Mass spectra were compared with NIST 11 and Willey 275 databases and authentic samples where available.

2.5. Bioassays

A stock solution of EO and TWEEN® 20 (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) (1:1) was prepared from each plant according to the specifications presented in Table 1. The analogy of the EO and the emulsifier was decided according to previously a described protocol [61] in order to produce an ME upon dilution with water. The EO-based ME stock solutions were tested at the concentration of 1000 ppm, where water (0.05% TWEEN® 20) served as control. The selection of this concentration was based on preliminary tests. The experiments were conducted according to Kavallieratos et al. [62], while the application protocol of the MEs followed the guidelines provided by Golden et al. [38]. In this task, treatments were performed on plates, each one representing a treatment replicate. Quantities of 0.20 kg of wheat were each sprayed with 1 mL of the test solution by using an AG-4 airbrush (Mecafer S.A., Valence, France). Different plates were used per spraying. Between treatments, the airbrush was cleaned with alcohol to avoid cross contamination. The sprayed whole wheat was inserted separately in 1 kg plastic canisters and was shaken for 10 min to achieve the balanced distribution of the EO-based MEs on the whole quantity of grains. Three subsamples of 10 g were obtained and placed in Petri dishes (9 cm diameter, 1.5 cm height) using a different scoop that was inside each canister. The covers of the dishes bore a circular opening (1.5 cm diameter) on their centers that was covered by muslin cloth. Thus, the content of the dishes would be adequately aerated. The upper internal vertical sides of each dish were covered by polytetrafluoroethylen (60 wt % dispersion in water) (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) to prevent the escape of insects from the treated wheat. The samples of 10 g of wheat were weighed with a Precisa XB3200D electronic balance (Alpha Analytical Instruments, Gerakas, Greece) on filter paper. Paper was changed each time weighing was conducted. After this, 10 adults or larvae of each species were transferred. The mortality of larvae of both species and *T. castaneum* adults was determined after 1, 3, 7 and 14 days of exposure, while the mortality of *T. granarium* adults was determined after 1, 3 and 7 days due to the shorter adult longevity of this species [63].

2.6. Data Analysis

Mortality in the control treatments was low (<5%) for both species, therefore no correction was considered necessary for the mortality values (correction is conducted when control mortality ranges between 5% and 20% [64]). In order to normalize the variance, mortality data were log (x + 1) transformed prior to being submitted to ANOVA separately for each tested species and life stage [62,65]. The pairwise comparisons were conducted by using the Fischer LSD test ($\alpha = 0.05$). All the analyses were performed with SigmaPlot 14.0 [66].

3. Results

3.1. Phytochemical Analysis

The results of the EO analysis revealed the presence of 48 phytochemical compounds, which are explicitly presented in Table 2. The main compounds in each plant's EO are presented in Figure 1, while the presence of principal and secondary molecular structures in each EO are included in Table 1. From the EOs included in the present study, *O. vulgare* ssp. *hirtum* and *O. majorana* presented phenol carvacrol as the main compound, while in *O. majorana*, isomer thymol was also present in comparable quantity. *Citrus limon* and *J. phoenicea* EOs contain limonene and α-pinene as major compounds, respectively. *Laurus nobilis* EO was found to contain eucalyptol as a major compound, and *E. tenuifolia* ssp. *sibthorpiana* almost equal amounts of methyl eugenol and α-phellandrene.

Table 2. EOs qualitative and quantitative composition. RI = Retention Index; Identification Method: a = MS, b = RI, c = comparison with authentic standards.

Compounds	RI	*C. limon*	*J. phoenicea*	*L. nobilis*	*E. tenuifolia* ssp. *sibthorpiana*	*O. majorana*	*O. vulgare* ssp. *hirtum*	Identification
α-thujene	930	0.6			0.2	1.5	0.1	a, b, c
α-pinene	939	2.3	73.9	3.9	0.6	0.9		a, b, c
camphene	954		0.5			0.3		a, b, c
sabinene	975		0.3	10.2	0.1			a, b, c
β-pinene	980	10.5	1.9	3.6	0.1	0.3		a, b
1-octen-3-ol	981					0.3		a, b
myrcene	991	2.0	3.3	0.9	0.2	1.5	0.2	a, b, c
α-phellandrene	1003		3.1	0.5	32.5	0.2		a, b
α-terpinene	1017			0.7	0.9	2.0	0.2	a, b
para-cymene	1025				10.3	0.1	1.0	a, b
ortho-Cymene	1027			0.8		8.1		a, b
limonene	1029	37.2						a, b, c
β-phellandrene	1031				6.5			a, b
eucalyptol	1032			45.7		t		a, b
trans-β-ocimene	1051	0.2						a, b
γ-terpinene	1060	10.4	0.2	0.7	0.6	14.1	0.9	a, b, c
α-terpinolene	1089	0.7	0.7		0.5		0.4	a, b
linalool	1098			1.7		0.2		a, b
nonanal	1101	0.2						a, b
camphor	1145		0.5					a, b
citronelal	1153	0.3						a, b
borneol	1168					0.5	0.2	a, b
4-terpineol	1178		0.2	2.4		0.5	0.1	a, b
α-terpineol	1189	0.3	0.4	3.0				a, b
neral	1238	1.2						a, b
carvacrol methyl ether	1245					0.7		a, b
piperitone	1253		0.1					a, b
bornyl acetate	1287			0.9				a, b
lavandulyl acetate	1290	0.9						a, b
thymol	1293				0.1	18.3	0.6	a, b
carvacrol	1299				0.5	43.7	95.3	a, b, c
citral	1320	2.0						a, b
δ-eIemene	1338		0.1					a, b
a-terpinelyl acetate	1351		1.1	14.0				a, b
eugenol	1359			2.5				a, b
neryl acetate	1362	1.2						a, b
β-elemene	1391		0.2					a, b
methyl eugenol	1406			1.2	43.8			a, b
β-caryophyllene	1419	0.6	1.3			1.9	0.4	a, b, c
α-bergamotene	1435		1.0					a, b
γ-elemene	1437		0.2					a, b
α-humulene	1455		0.6					a, b
germacrene D	1485		4.2					a, b, c
valencene	1496	0.2						a, b
bicyclogermacrene	1500	0.1						a, b
β-bisabolene	1506	1.5				0.3	0.2	a, b
δ-cadinene	1523		0.2					a, b
germacrene B	1561		1.2					a, b
Total		72.4	95.3	92.7	96.8	95.4	99.8	

3.2. Insecticidal Activity against T. castaneum

The mean mortality rate of *T. castaneum* larvae was significantly increased 1, 3 and 7 days after application of the EO-based MEs (Table 3). Thereafter, a significant increase in larval mortality was detected only for the application of the *C. limon* EO-based ME. Mean mortality rates of *T. castaneum* larvae 14 days after the application of *C. limon*, *J. phoenicea*, *L. nobilis*, *E. tenuifolia* ssp. *sibthorpiana*, *O. majorana* and *O. vulgare* ssp. *hirtum* EO-based MEs were 93.3%, 67.8%, 77.8%, 90.0%, 91.1% and 87.8%, respectively. However, all the tested EO-based MEs showed low mortality on *T. castaneum* adults. Thus, the observed mean mortality rates were 16.7%, 26.7%, 34.4%, 17.8%, 24.4% and 25.6% on wheat treated with *C. limon*, *J. phoenicea*, *L. nobilis*, *E. tenuifolia* ssp. *sibthorpiana*, *O. majorana* and *O. vulgare* ssp. *hirtum* EO-based MEs, respectively, 14 days post-exposure.

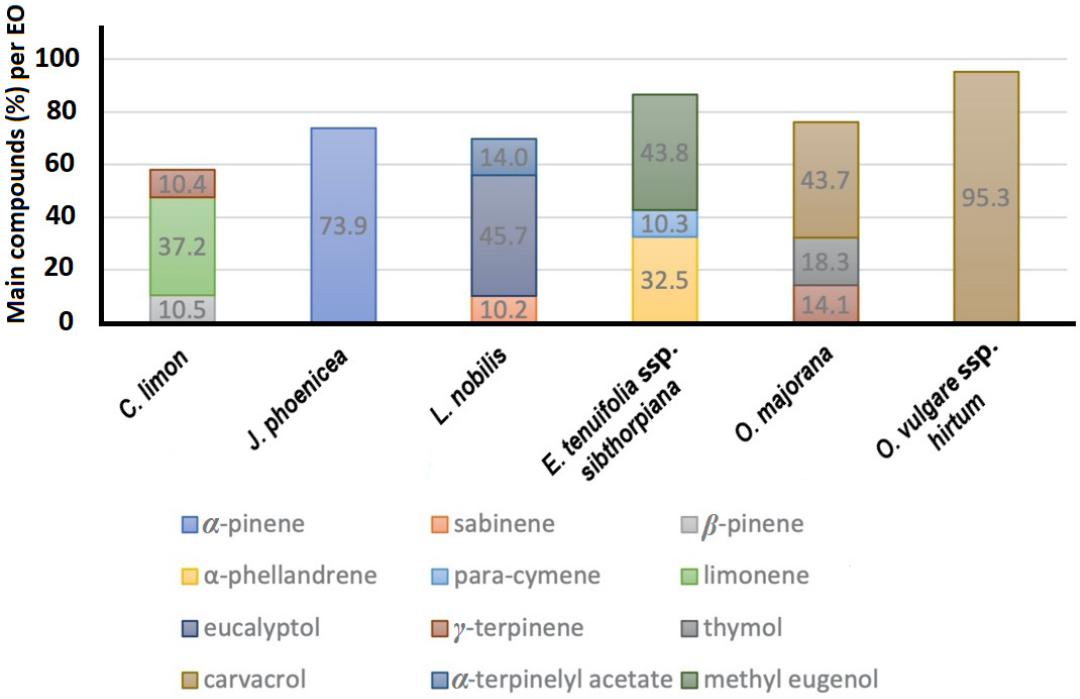

Figure 1. Main compounds of the EOs (>10%).

Table 3. Mean mortality rate (% ± SE) of *T. castaneum* (larvae and adults) 1, 3, 7 and 14 days after application with EO-based MEs. Means in the same row followed by different uppercase letters are significantly different; means in a column followed by different lowercase letters are significantly different (Fisher LSD test, $\alpha = 0.05$).

Plant Species	Developmental Stage	Days after the Treatment				DF	*F*	*p*
		1 Day	3 Days	7 Days	14 Days			
C. limon	Larvae	8.9 ± 2.0 Aa	40.0 ± 7.1 Bab	72.2 ± 6.0 Cad	93.3 ± 2.4 Da	3	59.772	<0.001
	Adults	0.0 ± 0.0 Aa	0.0 ± 0.0 Aa	3.3 ± 2.4 Aa	16.7 ± 6.9 Ba	3	5.095	0.005
J. phoenicea	Larvae	13.3 ± 5.3 Aa	32.2 ± 6.8 Bab	54.4 ± 8.2 Cbc	67.8 ± 8.5 Cb	3	11.801	<0.001
	Adults	2.2 ± 1.5 Aa	7.78 ± 4.0 Aa	13.3 ± 5.3 ABa	26.7 ± 6.5 Ba	3	4.960	0.006
L. nobilis	Larvae	14.4 ± 4.1 Aa	33.3 ± 5.3 Bab	57.8 ± 5.7 Ccd	77.8 ± 8.0 Cbcd	3	22.470	<0.001
	Adults	5.6 ± 4.4 Aa	8.9 ± 5.6 Aa	15.6 ± 5.8 Aa	34.4 ± 6.0 Ba	3	5.555	0.003
E. tenuifolia ssp. *sibthorpiana*	Larvae	13.3 ± 4.4 Aa	40.0 ± 4.1 Bbc	77.8 ± 2.2 Ca	90.0 ± 2.9 Cad	3	83.773	<0.001
	Adults	0.0 ± 0.0 Aa	2.2 ± 1.5 Aa	5.6 ± 1.8 Aa	17.8 ± 3.6 Ba	3	13.814	<0.001
O. majorana	Larvae	15.6 ± 1.8 Aa	57.8 ± 5.5 Bc	80.0 ± 4.7 Ca	91.1 ± 3.5 Cac	3	80.600	<0.001
	Adults	7.8 ± 4.7 Aa	14.4 ± 5.3 Aa	17.8 ± 7.2 Aa	24.4 ± 6.5 Aa	3	1.386	0.265
O. vulgare ssp. *hirtum*	Larvae	11.1 ± 4.2 Aa	44.4 ± 6.5 Bbc	72.2 ± 4.9 Cad	87.8 ± 3.2 Cad	3	50.233	<0.001
	Adults	0.0 ± 0.0 Aa	2.2 ± 1.5 Aa	4.4 ± 1.8 Aa	25.6 ± 4.8 Ba	3	22.016	<0.001
DF	Larvae	5	5	5	5			
	Adults	5	5	5	5			
F	Larvae	0.411	2.409	3.796	3.431			
	Adults	1.605	2.296	1.844	1.232			
p	Larvae	0.839	0.050	0.005	0.010			
	Adults	0.177	0.060	0.122	0.309			

3.3. Insecticidal Activity against T. granarium

Concerning the efficacy on *T. granarium*, the tested EO-based MEs showed low mortality on insects' larvae (Table 4). Depending on plant species, the efficacy of EO-based MEs ranged from 8.9% (*L. nobilis*) to 30.0% (*O. majorana*) 14 days after the exposure. However, *T. granarium* adults showed an increasing mortality rate after 1, 3 and 7 days exposure. Thus, mean mortality rates of insects' adults 7 days after the application of *C. limon*, *J. phoenicea*,

L. nobilis, *E. tenuifolia* ssp. *sibthorpiana*, *O. majorana* and *O. vulgare* ssp. *hirtum* EO-based MEs on wheat were 72.2%, 83.3%, 84.4%, 70.0%, 87.8% and 82.2 %, respectively.

Table 4. Mean mortality rate (% ± SE) of *T. granarium* (larvae and adults) 1, 3, 7 and 14 days after application with EO-based MEs. Means in the same row followed by different uppercase letters are significantly different; means in a column followed by different lowercase letters are significantly different (Fisher LSD test, $\alpha = 0.05$).

Plant Species	Developmental Stage	Days after the Treatment				DF	*F*	*p*
		1 Day	3 Days	7 Days	14 Days			
C. limon	Larvae	3.3 ± 1.7 Aa	4.4 ± 2.4 Aab	10.0 ± 4.1 Aac	13.3 ± 3.7 Aab	3	2.282	0.098
	Adults	8.9 ± 2.6 Aa	26.7 ± 2.4 Ba	72.2 ± 4.0 Ca	N/A	2	111.285	<0.001
J. phoenicea	Larvae	3.3 ± 1.7 Aa	16.7 ± 2.3 Bc	26.7 ± 4.4 BCb	34.4 ± 4.4 Cc	3	16.049	<0.001
	Adults	14.4 ± 2.9 Aa	27.8 ± 2.8 Ba	83.3 ± 5.3 Ca	N/A	2	90.937	<0.001
L. nobilis	Larvae	3.3 ± 1.7 Aa	4.4 ± 1.8 Aab	8.9 ± 2.6 Aac	8.9 ± 2.6 Aa	3	1.719	0.183
	Adults	11.1 ± 2.0 Aa	25.6 ± 2.4 Ba	84.4 ± 2.9 Ca	N/A	2	214.397	<0.001
E. tenuifolia ssp. *sibthorpiana*	Larvae	1.1 ± 1.1 Aa	8.9 ± 2.6 Bbc	16.7 ± 3.7 BCbc	20.0 ± 3.3 Cbd	3	9.122	<0.001
	Adults	8.9 ± 2.6 Aa	17.8 ± 2.8 Aa	70.0 ± 7.5 Ba	N/A	2	52.800	<0.001
O. majorana	Larvae	2.2 ± 1.5 Aa	16.7 ± 4.4 Bc	23.3 ± 6.0 BCbc	30.0 ± 6.0 Ccd	3	6.232	0.002
	Adults	6.7 ± 3.3 Aa	21.1 ± 3.5 Ba	87.8 ± 5.7 Ca	N/A	2	90.808	<0.001
O. vulgare ssp. *hirtum*	Larvae	2.2 ± 1.5 Aa	5.6 ± 2.4 Aab	12.2 ± 1.5 BCac	14.4 ± 1.8 Cab	3	10.171	<0.001
	Adults	12.2 ± 2.2 Aa	23.3 ± 1.7 Ba	82.2 ± 1.5 Ca	N/A	2	310.817	<0.001
DF	Larvae	5	5	5	5			
	Adults	5	5	5	N/A			
F	Larvae	0.356	4.143	3.310	7			
	Adults	1.142	2.040	2.157	N/A			
p	Larvae	0.876	0.003	0.012	<0.001			
	Adults	0.351	0.090	0.075	N/A			

4. Discussion

The composition of *O. majorana* EO is compatible with previous reports that indicate both thymol [67] and carvacrol [68] as main compounds and its significant chemical diversity is recognized. It must be noted that previous analyses of *O. majorana* EO from Greece [69] have also revealed the molecule of cymene as a major compound but not γ-terpinene. The *L. nobilis* EO composition is also consistent with previous reports identifying eucalyptol as the main compound [70], while the major compound α-terpinenyl acetate has also been reported [71]. The composition of *E. tenuifolia* ssp. *sibthorpiana*, *O. vulgare*, *C. limon* and *J. phoenicea* EOs has been presented and extensively discussed in previous studies [60,72,73].

EOs exhibit a significant range of pesticidal activities [32,72,74–76]. They can be produced easily, in a green and low-cost way, i.e., not including organic solvents or complicated methods of extraction [22]. In addition, EOs provide secondary metabolites that can act as modifying agents to resistant organisms, by inhibiting their proteins [77]. *Citrus limon* EO has been previously studied as a fumigant against *T. castaneum* adults with elevated efficacy [78,79]. By testing the contact toxicity and repellency of *O. majorana* EO against *T. castaneum* adults, Teke et al. [80] found potent repellency (97.2%) but not insecticidal activity after 3 days of exposure. Likewise, *O. vulgare* EO exhibited high fumigant and repellent properties against *T. castaneum* adults [81,82]. The evaluation of *L. nobilis* EO against *T. castaneum* in semolina suggested significant insecticidal potentials with simultaneous retention of crucial semolina quality characteristics [83].

Our study clearly shows the effectiveness of the EO-based MEs of the Mediterranean plants *C. limon, J. phoenicea, L nobilis, E. tenuifolia ssp. sibthorpiana, O. majorana* and *O. vulgare* ssp. *hirtum* against the two tested stored-product insect pests. *Citrus limon*, *O. majorana* and *E. tenuifolia* ssp. *sibthorpiana* EO-based MEs were the most effective for the management of *T. castaneum* larvae, by killing 93.3%, 91.1% and 90.0% of the exposed individuals after 14 days of exposure, respectively. *Origanum majorana*, *L. nobilis* and *J. phoenicea*

EO-based MEs killed 87.8%, 84.4% and 83.3% of *T. granarium* adults after 7 days of exposure, respectively. The findings indicate that the evaluated EO-based MEs are effective grain protectants for the management of *T. granarium* adults and *T. castaneum* larvae. So far, limited research has been conducted on EOs as grain protectants. For example, Demirel et al. [84] suggested that the EOs extracted from rosemary, *Rosmarinus officinalis* L. (Lamiales: Lamiaceae), *O. majorana* and thyme, *Thymus vulgaris* L. (Lamiales: Lamiaceae), can be used as a potential source of environment-friendly wheat protectants for the control of the confused flour beetle, *Tribolium confusum* Jacquelin du Val (Coleoptera: Tenebrionidae). Recently, Kavallieratos et al. [85] showed that EOs obtained from horse mint, *Mentha longifolia* (L.) Huds. (Lamiales: Lamiaceae), wormseed, *Dysphania ambrosioides* (L.) Mosyakin & Clemants (Caryophyllales: Chenopodioideae), stemless carline thistle, *Carlina acaulis* L. (Asterales: Compositae), and anise, *Pimpinella anisum* L. (Apiales: Apiaceae) are stored maize and wheat protectants against two stored-product insects pest, the larger grain borer, *Prostephanus truncatus* (Horn) (Coleoptera: Bostrychidae) and *T. granarium*. In addition, Pavela et al. [76] revealed that the essential oils of *Ferula assa-foetida* L. (Apiales: Apiaceae) and *Ferula gummosa* Boiss. (Apiales: Apiaceae) were highly effective against adults of *T. granarium* when applied on stored wheat.

The insect developmental stage is a critical aspect of the efficacy of the EO as grain protectants [62,76]. *Tribolium castaneum* larvae were more susceptible to the EO-based MEs than adults. On the basis of our results, *C. limon*, *J. phoenicea*, *L. nobilis*, *E. tenuifolia* ssp. *sibthorpiana*, *O. majorana* and *O. vulgare* ssp. *hirtum* EO-based MEs provided low adult mortality levels, ranging from 16.7% to 34.4% 14 days post-exposure. In contrast, in the case of larvae, the same EO-based MEs provided moderate to high mortality levels, ranging from 67.8% to 93.3% after 14 days of exposure. Similarly, a 6% (w/w) *Hazomalania voyronii* (Jum.) Capuron (Laurales: Hernandiaceae) EO-based NE caused low mortality to *T. castaneum* adults (i.e., 18.7%) vs. high mortality to larvae (i.e., 97.4%) 7 days post-exposure [40]. However, in the case of *T. granarium*, adults were more vulnerable than larvae. The tested EO-based MEs caused the death of a low percentage of the exposed *T. granarium* larvae (8.9–30.0%) after 14 days of exposure, while they caused elevated mortality (72.2–87.7%) to *T. granarium* adults, 7 days post-exposure. It is well documented that *T. granarium* larvae are more tolerant than adults to EOs [85] and compounds of botanical origin [22,40,62] when applied on wheat. This could be attributed to the long and dense hairs that cover the body of larvae, protecting them from coming in contact with the treated wheat [86]. In contrast, larvae of *Tribolium* spp. are covered by few hairs [87], an issue that increases the likelihood of their contact with the toxicant. The increased tolerance of *T. castaneum* adults in comparison to larvae could be attributed to the different structure of their cuticles [88]. Another hypothesis is that the expression of the *TcCYP6BQ7* gene, which is responsible for the detoxification of plant toxicants, is higher in adults than larvae of *T. castaneum* [89].

In general, pesticide treatment with synthetic insecticides is a common practice against stored-product insect pests [36,62]. However, food safety is generally associated with integrated pest management, aiming to use alternative protectants and/or low-risk pesticides [22,90–92]. Botanicals are low-risk alternative products, linked with reduced regulatory registration procedures [93]. Our results lean towards this direction, as we showed that the EO-based MEs of several plants have the potential to serve as efficient tools against major stored-product insect pests. Developing grain protectants from plants will bring benefits to the food supply chain with simple and cost-effective products of insecticidal activity [94].

5. Conclusions

All EO-based MEs included in the current bioprospecting study exhibit the prevailing phytochemical EO profile for the respective plant taxa. This fact enhances the replicability and upscale of the findings, since the exploited raw materials are widely available in nature [34,45–50,95,96]. In addition, we expect our results to have bearing on the control and the integrated pest management of stored-product insect pests. Further research on

the insecticidal activity of several Mediterranean plants as grain protectants will gather together more information towards efficient, more sustainable management strategies in storage facilities.

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Stress Resistance Traits under Different Thermal Conditions in *Drosophila subobscura* from Two Altitudes

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Simple Summary: The global warming and rapid climate change that we are witnessing is generally influencing all of the living world, so all species must necessarily cope with these changes in order to survive. The ability to withstand environmental stress, especially during the last two decades, has been of great importance for any species' long-term survival. For that purpose, we studied these abilities in the *Drosophila subobscura* species, which is known to be a good model organism for studying adaptations to environmental changes such as in temperature. We chose to investigate thermal stress responses in *D. subobscura* populations from two different altitudes, through four traits linked to stress tolerance: desiccation resistance, heat knock-down resistance, starvation resistance, and chill coma recovery time. Correlations between the populations' origin and these traits were found, as well as the significant influence of the laboratory thermal conditions and sex on these traits showing that males and cold-adapted populations are expected to fare much worse in a fast-changing warming environment.

Abstract: Global warming and climate change are affecting many insect species in numerous ways. These species can develop diverse mechanisms as a response to variable environmental conditions. The rise in mean and extreme temperatures due to global warming and the importance of the population's ability to adapt to temperature stress will further increase. In this study, we investigated thermal stress response, which is considered to be one of the crucial elements of population fitness and survival in fast-changing environments. The dynamics and variation of thermal stress resistance traits in *D. subobscura* flies originating from two natural populations sampled from different altitudes were analysed. Three different temperature regimes (25 °C, 19 °C, and 16 °C) were used for the F1 progeny from both localities to establish six experimental groups and investigate stress resistance traits: desiccation resistance, heat knock-down resistance, starvation resistance, and chill-coma recovery time. We detected that laboratory thermal conditions and population origin may have an effect on the analysed traits, and that sex also significantly influences stress resistance. Individuals from the lower altitude reared at higher temperatures show inferior resistance to thermal shock.

Keywords: *D. subobscura*; desiccation resistance; starvation resistance; chill coma recovery time; heat knock-down resistance; global warming; life history; adaptation; laboratory evolution

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1. Introduction

Global warming and climate change are associated with an increase in average and extreme temperatures. There is a growing mass of evidence that temperature is one of the foremost important factors shaping the distribution and evolution of different

species populations. It is also considered to be a particularly important stressor because variable thermal environments are common and may represent substantial challenges for the survival and reproduction of these populations [1–4]. Average global temperature has risen drastically since the 19th century, and there are predictions that temperature extremes will be 1.5 °C to 4.5 °C higher than pre-industrial levels by the end of this century [5]. This increase in temperature will lead to shifts in the distribution areas of numerous species and changes in species abundance [6–8]. All of the known species on Earth have been affected by these changes and it is of particular interest to better understand thermal adaptation, especially in geographic gradients along which climate strongly varies. Studies of altitudinal changes in phenotype and genotype can complement studies of latitudinal patterns and provide evidence of natural selection in response to climatic factors [2,9,10].

Drosophila species are widely used in studies of adaptation to climate change at different biological levels, with mechanisms of adaptation and response to extreme temperatures of particular interest [9–13]. Their widespread abundance, small body size, short life cycle, ease to manipulate and rear and limited genetic redundancy make them good model organisms for research in biology, ecology, evolution and genetics. *D. subobscura* Collin (Diptera: Drosophilidae), a Palearctic species widespread throughout Europe, may be considered as an ideal model for thermal adaptation research due to its clinally distributed inversion polymorphism that corresponds to the warmer and colder climate and previously described population-level genetic responses to various stressful conditions [14–20]. A recent study [21] on temperature changes in Serbia indicated that temperature is increasing at an accelerated rate and that an increase in maximum temperature, especially during summer, is more pronounced. Previous work has indicated that *D. subobscura* populations from that region respond to thermal changes in habitat with an alteration of chromosome arrangements frequencies and changes in population structure [22]. Moreover, this species allows us short-time multigenerational maintenance, precisely controlled conditions, and a better estimation of the effects under long-term environmental changes. Latitudinal and altitudinal variations at phenotypic and genotypic levels have been thoroughly studied in *Drosophila* sp. [23–28]. For example, selection at low latitudes/altitudes (or at warm temperatures in the lab) may lead to decreased body size in these species; but the decreased size might lead to reduced stress tolerance [29].

Our aim was to try to disentangle the effect of the origin of populations according to altitude from their laboratory thermal adaptations on three fitness-related traits directly linked to thermal stress tolerance—one among which is often referred to as part of the "survival mode" mechanism which helps individuals cope with a stressful environment.

The choice of ecologically relevant traits is of special importance in studies of experimental thermal adaptation. Desiccation resistance (DR), heat knock-down resistance (HKDR), and chill coma recovery time (CCRT) are considered to be reliable indicators of thermal adaptation, all of which are related to thermal stress tolerance [23,30]. Starvation resistance (SR) is also considered to be a reliable indicator of resistance to other forms of environmental stress [31,32]. Temperature plays an important role in the evolution of these characteristics; flies that evolve at lower temperatures are typically larger, have a longer developmental time and larger eggs, unlike the flies that evolve at higher temperatures whose body size is smaller, developmental time is shorter and have smaller eggs [33–40].

In nature, high temperature is correlated with desiccation resistance and there are examples of adaptive patterns in that phenotype [41]. Desiccation is a significant stress for terrestrial animals, and the successful evolution of insects in terrestrial environments can be partly ascribed to their ability to effectively tolerate desiccation [42]. Desiccation stress continues to be a primary factor shaping insect distribution and behaviour, and an inability to respond to desiccation stress may contribute to the negative consequences of climate change for some insect species and populations. Desiccation resistance-related traits are diverse and vary in function of geographic locations and environmental conditions including water availability and environmental temperature [43,44]. The example of *Drosophila birchii* (Diptera: Drosophilidae) that appears to be restricted to the rainforest fragments due to

its inability to survive desiccation indicates that if the population's capacity to respond to selection is low, the drying of that habitat is likely to lead to extinction [35]. Even the insects that inhabit the most arid environments are ecologically successful, at least partly, because of their great capacity to conserve internal water stores [43]. Previous studies of 32 *Drosophila* species also suggested that basal resistance, rather than adult hardening (resistance based on short-term treatments under more severe stress conditions [45,46]) is relatively more important in determining species' differences in desiccation resistance and sensitivity to climate change [44].

High temperature can affect the reproduction, mating success, abundance, and distribution of species [47,48]. Today, when many environments are becoming increasingly arid and temperature extremes are much more frequent, thermotolerance is crucial for insects' survival. Heat knock-down resistance has been suggested as an important heat adaptation trait that correlates with natural adaptation to higher temperature environments [49]. It is suggested that the knock-down trait might be a better indicator of adaptation to natural high temperature environments than the traditionally used heat shock assays [41]. In the knock-down assay, the time taken for flies to be knocked down—become unconscious under the high temperature—is measured, reflecting the environmental conditions which disable the normal functionality of individuals.

Starvation resistance is a phenotypic trait that represents the species' ability to withstand prolonged periods of food deprivation. Starvation is the most frequent environmental stress faced by animals inhabiting environments where food availability fluctuates and is unpredictable [50]. In natural populations, reduction in food resources and their availability are very common—challenges which are faced by animals whose capacity to survive prolonged periods of starvation is linked to their diet and nutritional status. Additionally, starvation resistance is correlated with a relatively long life span, slower development, reduced egg production, and its size, larger body size, etc. [51]. It is expected that starvation resistance increases with altitude, with the feeding resources being more limited in highlands than in lowlands [52,53], but not being coupled with altitude itself but with body size that allows the storage of higher amounts of energy. Flies of the *Drosophila* genus have been widely used to study the ecology, evolution, and genetic basis of starvation resistance, and the nutritional basis of their resistance to starvation was correlated with dietary composition, fat, and glycogen reserves [54–58]. Some data show the apparent association between starvation resistance and longevity in *D. melanogaster* [31]. In this study, we measured the time of death from starvation in *D. subobscura* flies from two different altitudes (populations) under three different temperature regimes as a measure of a more general response to stress conditions.

The chill coma recovery time (CCRT) describes species' thermal adaptation and can reflect the cold tolerance of the species [31,59,60]. Cold resistance in insects has been measured in terms of survival after long-term induced stress, but short essays such as CCRT are being increasingly used. The time that ectotherms need to recover from cold stress is a nonlethal and useful index of cold tolerance [7]. CCRT is relevant for insects' ecology and can explain variation among individuals, species, or populations. For example, in temperate climatic zones, there are daily and seasonal temperature cycles and being cold tolerant is a physical advantage during cold seasons. Freezing conditions induce coma in fruit flies and CCRT is the measure of their cold tolerance. Species such as *D. subobscura* which inhabits colder/temperate zones tend to be more chill tolerant and better cold-adapted, and they recover almost instantaneously after exposure to 0 °C, unlike tropical species [61]. To explore whether geographic populations of *D. subobscura* from different altitudes might differ in terms of their cold tolerance, we exposed them to 0 °C for some time and measured the time taken for them to stand up.

Phenotype is not a sum of different traits; rather it is a complex network of interactions of different traits and the environment surrounding it. All described separately measured fitness characteristics are good indicators of a response to a changing environment, but when analysed together, they may provide a better insight into population response to

different types of thermal stress. To investigate the influence of altitude and different temperature conditions on populations' ability to cope with temperature stress, we analysed four stress resistance traits in *D. subobscura* flies originating from two natural populations sampled from different altitudes and then maintained in three temperature regimes in laboratory conditions. The chosen temperature regimes are in range with the natural thermal experience of *D. subobscura* and are chosen as a non-lethal but stressful environment according to previously published data on thermal limitation for this species (reviewed in [62]). Past studies have indicated that laboratory maintenance at 25 °C may induce male sterility [62], 19 °C is considered to be the optimal temperature for this species [62,63] and previous but unpublished work from our laboratory suggested that 16 °C significantly prolongs development time without decreasing viability. Although limited to two populations, the results of this study add to the body of knowledge on the thermal adaptation of individuals from different latitudes. In order to predict the future abundance and distribution of species [64] and the impact of global warming on biological systems, more studies contributing to a better understanding of the mechanisms behind thermal adaptations are needed [64].

2. Material and Methods

Flies were collected in mid-August 2018, from two different elevations, 1080 m (N 43.395255; E 22.603995) and 1580 m (N 43.374145; E 22.618110), along the Stara planina mountain slope in Serbia. GPS was used to determine the geographical coordinates. The sampling locations were separated by an approximately 1200 m-high mountain ridge. However, as expected due to the difference in altitude, they differed in some vegetation characteristics of the habitat, both representing wild forests in closed canopy woodland. The lower locality is mainly composed beech old forest, while the higher locality is a mixture of beech and spruce. Since specific climate data could not be obtained for the sampling sites, the average temperature and humidity (H%) for both localities were measured during collection. The average temperature/humidity in the lower locality was 19.4 °C/65%, Tmax/Hmax was 28.8 °C/87.5%, and Tmin/Hmin was 13.4 °C/42.7%. In the higher locality, the average temperature/humidity was 18 °C/71%, Tmax/Hmax was 27 °C/91.2%, and Tmin/Hmin 13.5 °C/50%. As can be seen, the lower locality has a higher average and maximal temperature compared to the higher one.

The flies were collected in the evening peak of activity, by net sweeping over fermented fruit traps. Wild-caught females were individually placed into the falcon tubes to establish isofemale lines (IF). Approximately 100 flies per population were used for establishing IF lines. All lines were maintained under constant laboratory conditions: temperature of 19 °C, approximately 60% relative humidity, light of 300 lux, and 12/12 h light/dark cycles. After one generation, three to five pairs of males and females from each IF line were used to establish two synthetic mass populations (H—high altitude and L—lower altitude). Mass populations from both localities were used to establish experimental groups at three different temperature regimes: 25 °C, 19 °C, and 16 °C. All groups were maintained on a discrete generation and reared on standard *Drosophila* medium: 14 g agar, 208 g cornmeal, 188 g sugar, 40 g dry active yeast, 5 g Nipagin diluted in 60 mL of 96% ethanol in 2.2 L distilled water. Mass populations were maintained under described laboratory conditions for 12 generations controlling for larval density by transferring approximately 50 pairs to 12 food vials containing 40 mL of medium and allowing them to lay eggs for three days. After three days, parental generation was transferred to additional 12 fresh food vials for another three days and before being discarded. All the emerged progeny flies were collected, mixed, and then randomly transferred to fresh food vials as parents of the next generation. Therefore, the estimated population census counts approximately 1200 flies per generation.

After 12 generations of maintaining two populations at different temperatures under laboratory conditions, freshly emerged flies (previously kept in the dark to prevent possible mating) were collected. Males and females were separated to prevent mat-

ing and transferred to fresh food vials. All experiments, with minor alterations, followed the protocols of [52,65] and were conducted with five- to six-day-old virgin flies (Supplementary Scheme S1).

2.1. Desiccation Resistance (DR)

Single virgin flies were transferred into empty 3 mL tubes. Approximately 100 flies (50 ♀ and 50 ♂) were used from each group. All groups were tested for DR at three temperatures: 25 °C, 19 °C, and 16 °C. Mortality was scored every hour. Flies that were not able to move were considered dead.

2.2. Heath Knock-Down Resistance (HKDR)

To score HKDR, five virgin flies were placed into empty falcon tubes (50 mL). Fifty flies per group were observed. Falcon tubes were closed with moistened plugs to prevent desiccation. All groups with 50 ♀and 50 ♂flies per group were placed at 37 °C for seven hours, and mortality was checked every 30 min.

2.3. Starvation Resistance (SR)

Five virgin flies of the same sex were transferred into empty falcon tubes (50 mL) with moistened cotton plugs to prevent desiccation. Ten falcon tubes per experimental group (50 ♀ and 50 ♂) were used and placed at three temperatures: 25 °C, 19 °C, and 16 °C. Mortality was scored every three hours. Flies that were not able to move were considered dead.

2.4. Chill Coma Recovery Time (CCRT)

Single virgin flies were put into 3 mL tubes which are placed in a water/ice mixture. Approximately 80 (40 ♀and 40 ♂) flies per group were used. After seven hours on ice, the tubes were moved to room temperature and the recovery time was scored for each fly (in seconds). The flies were considered to have recovered when they were able to stand up.

2.5. Statistics

The results for all traits were analysed using the full factorial general linear model (GLM) procedure and Bonferroni post hoc test in the STATISTICA version 12 (StatSoft. Inc. Tulsa, OK, USA). Desiccation and starvation resistance were analysed with fixed factors: population, rearing temperature, treatment, and sex, and the recovery time and heat knock-down time fixed factors for the chill coma were: population, rearing temperature, and sex.

3. Results

3.1. Desiccation Resistance

The mean values, standard error, and variance for desiccation resistance are shown in Table 1. As expected, flies died faster from desiccation under high temperature treatment conditions in all scored groups. Additionally, the females showed a higher overall desiccation resistance, especially females from a lower altitude reared at 25 °C.

The results of the full factorial GLM analysis with a fixed factors' population, rearing temperature, treatment, and sex are shown in Figure 1. The population, treatment, and sex showed a significant influence on desiccation resistance ($F_{(1)} = 30.76$, $p < 0.001$; $F_{(2)} = 788.14$, $p < 0.001$; $F_{(1)} = 315.50$, $p < 0.001$, respectively (Table S1)). The rearing temperature showed no significant effect on the desiccation resistance ($F_{(2)} = 2.8$, $p = 0.063$ (Table S1)). There is also a significant interaction between almost all combinations of factors, except the interaction between all four factors and the population, rearing temperature, and sex combined.

Under cold temperature conditions (16 °C), both sexes from L reared at 25 °C have higher desiccation resistance than individuals from the H population, as can be seen in Figure 1 (Bonferroni $p < 0.01$ (Table S2)). Under the same conditions, L males reared at 19 °C have a higher desiccation resistance than H males (Bonferroni $p = 0.017$; Figure 1;

Table S2). For females, a different although not statistically significant trend was observed, notably that the females from the H population reared at 19 °C and 16 °C showed a higher desiccation resistance than L. Under the optimal temperature for *D. subobscura* species (treatment 19 °C), L males reared at 19 °C and 25 °C had higher desiccation resistance than H males (Bonferroni $p = 0.017$, $p = 0.035$, respectively; Figure 1; Table S2). On the contrary, under the same conditions, H females had a better but not statistically significant desiccation response. Under high temperature (treatment 25 °C), there is no significant difference between groups, but H females reared at 25 °C have a higher desiccation resistance than L females.

There was no difference between males and females when flies were reared at the optimal temperature (19 °C), irrespective of population origin and the temperature of the treatment.

Table 1. Mean values with standard errors and variance for all groups for a time of death (measured in hours) under desiccation stress. Population L—population originating from lower altitude; population H—population originating from higher altitude; rearing temperature—T at which flies evolve in the laboratory; treatment—T at which flies were tested for desiccation resistance; F—females; M—males; time of death—mean value ± SE (standard error). This Table was published in the *Proceedings of the 1st International Electronic Conference on Entomology*, 1–15 July 2021, doi:10.3390/IECE-10532.

			Population L		**Population H**	
Rearing Temperature	**Treatment**	**Sex**	**Time of Death**	**Variance**	**Time of Death**	**Variance**
16 °C	16 °C	F	70.86 ± 2.06	214.1229	79.85 ± 2.84	394.25
		M	63.28 ± 1.83	167.3486	66.98 ± 2.76	373.8954
	19 °C	F	63.6 ± 2.35	134.8163	73.63 ± 2.35	269.6539
		M	58.67 ± 1.91	178.4328	59.54 ± 1.42	67.84354
	25 °C	F	33.48 ± 1.44	103.6016	32.90 ± 1.18	100.2943
		M	28.2 ± 0.91	41.71429	28.04 ± 0.93	43.26367
19 °C	16 °C	F	82.23 ± 3.16	510.4235	86.64 ± 3.10	481.8678
		M	67.76 ± 2.22	246.9616	52.98 ± 2.33	249.5302
	19 °C	F	70.86 ± 2.37	280.6535	74.28 ± 1.66	138.5322
		M	62.94 ± 2.17	234.5065	46.14 ± 1.42	100.7759
	25 °C	F	44.68 ± 1.19	159.5282	39.44 ± 1.35	90.45551
		M	30.28 ± 1.02	52.36898	26.1 ± 1.03	53.43878
25 °C	16 °C	F	98.14 ± 5.00	1250.735	64.44 ± 4.06	823.1902
		M	72.84 ± 4.21	886.7086	34.46 ± 2.18	237.2739
	19 °C	F	72.64 ± 5.01	1256.235	78.4 ± 4.18	872.0816
		M	63.82 ± 2.67	356.6404	49.62 ± 2.22	245.5873
	25 °C	F	30.56 ± 1.42	100.8229	39.16 ± 1.91	181.6065
		M	28.82 ± 1.58	125.0486	29.14 ± 1.17	68.57184

3.2. Heat Knock-Down Resistance

The mean values, standard errors, and variance for the heat knock-down time are given in Table 2. Individuals reared at 25 °C showed the longest heat knock-down time. The results of the full factorial GLM analysis with fixed factors population, rearing temperature, and sex are shown in Figure 2.

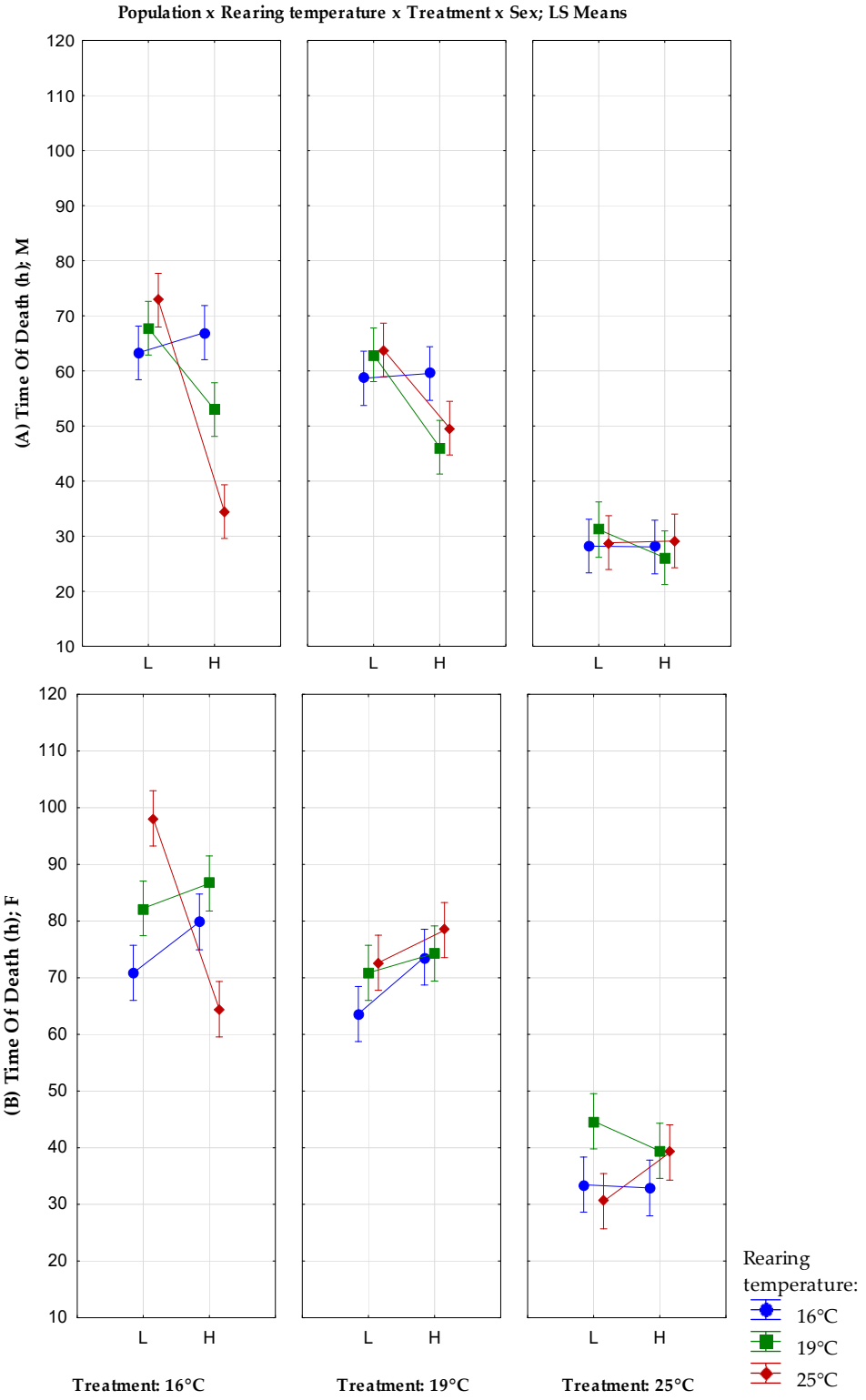

Figure 1. Full factorial GLM analysis of desiccation resistance with a fixed factors population, rearing temperature, treatment temperature, and sex; (**A**) males; and (**B**) females. Vertical bars denote a 0.95 confidence interval. M—males; F—females; L—population originating from lower altitude; H—population originating from the higher altitude. The modified figure was previously published in the Proceedings of the 1st International Electronic Conference on Entomology, 1–15 July 2021, doi:10.3390/IECE-10532.

Table 2. Mean values with standard errors and variance for all groups for the heat knock-down time (measured in minutes). Population L—population originating from lower altitude; population H—population originating from higher altitude; rearing temperature—T at which flies evolve in the laboratory; F—females; M—males; heat knock-down time—time by flies have been knocked down (mean value ± SE (standard error)). This Table was previously published in *Proceedings of the 1st International Electronic Conference on Entomology*, 1–15 July 2021, MDPI: Basel, Switzerland, doi:10.3390/IECE-10532.

		Population L		Population H	
Rearing Temperature	**Sex**	**Heat Knock-Down Time**	**Variance**	**Heat Knock-Down Time**	**Variance**
16 °C	F	115.8 ± 3.93	771.7959	126 ± 3.53	624.4898
	M	105 ± 2.74	376.5306	116.4 ± 3.16	501.0612
19 °C	F	163.8 ± 3.66	668.9388	182.4 ± 5.06	1279.837
	M	162 ± 3.64	661.2245	183.6 ± 4.67	1088.816
25 °C	F	310.2 ± 12.99	8434.653	357.5 ± 14.06	9491.489
	M	349.17 ± 17.77	11,373.57	282.6 ± 14.32	10,252.29

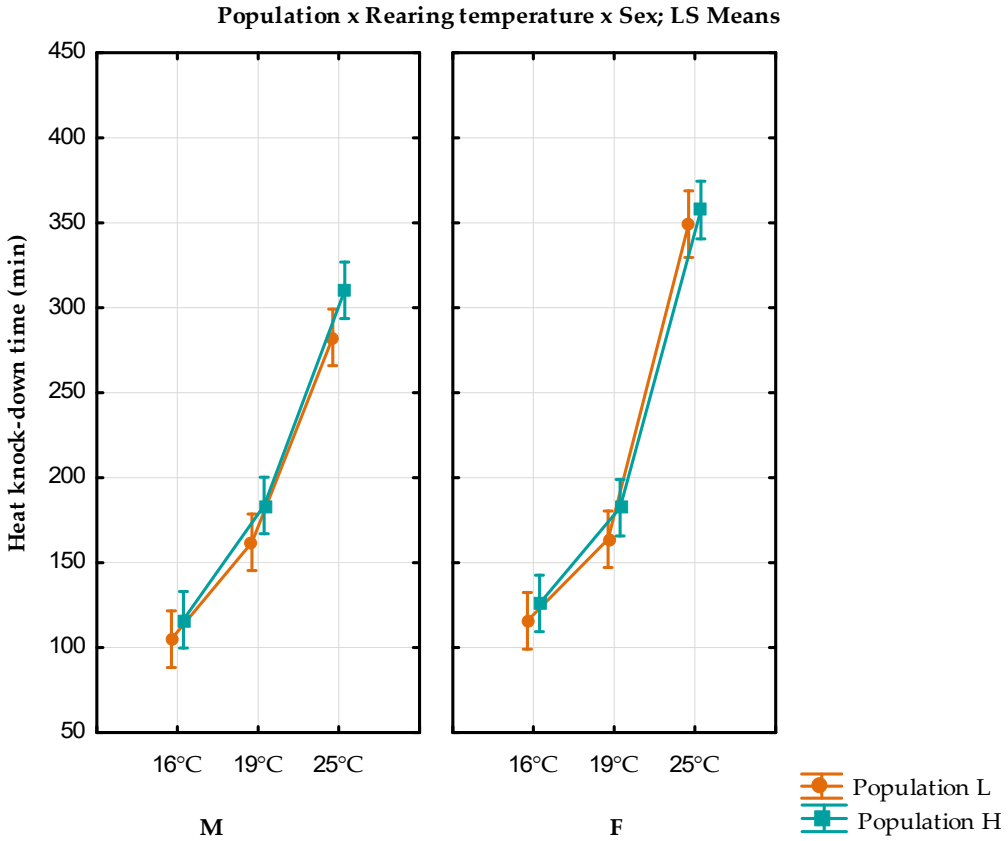

Figure 2. Results of full factorial GLM analysis of heat knock-down resistance with fixed factors population, rearing temperature, and sex. Vertical bars denote 0.95 confidence interval. M—males; F—females; L—population originating from lower altitude; H—population originating from a higher altitude. This Figure was previously published in Proceedings of the 1st International Electronic Conference on Entomology, 1–15 July 2021, MDPI: Basel, Switzerland, doi:10.3390/IECE-10532.

The population, rearing temperature, sex, and rearing temperature x sex interaction showed a statistically significant influence on the heat knock-down time ($F_{(1)} = 10.707$, $p = 0.001$; $F_{(1)} = 20.389$, $p < 0.01$, $F_{(2)} = 610.236$, $p < 0.01$; $F_{(2)} = 11.918$, $p < 0.01$, respectively (Table S3)).

Individuals from the higher altitude showed significantly longer heat knock-down time than individuals from the lower altitude (Bonferroni $p < 0.01$; Table S4). The higher rearing temperature significantly prolongs the heat knock-down time (Bonferroni $p < 0.01$ for all combinations; Table S4). For both populations, females reared at 25 °C have a significantly longer heat knock-down time than males (Bonferroni $p = 0.006$ and $p < 0.01$, respectively (Table S4)).

3.3. Starvation Resistance

The mean values, standard errors, and variance for starvation resistance are shown in Table 3. The results of full factorial GLM analysis with fixed factors origin, rearing temperature, treatment, and sex are shown in Figure 3.

Table 3. Mean values with standard errors and variance for all groups for starvation resistance (measured in hours). Population L—population originating from lower altitude; population H—population originating from higher altitude; rearing temperature—T at which flies evolve in the laboratory; treatment—T at which flies were tested for starvation resistance; F—females; M—males; time of death—mean value ± SE (standard error).

			Population L		**Population H**	
Rearing Temperature	**Treatment**	**Sex**	**Time of Death**	**Variance**	**Time of Death**	**Variance**
16 °C	16 °C	F	67.5 ± 2.26	256.1327	72.42 ± 2.45	300.8608
		M	63.86 ± 1.58	121.875	61.72 ± 2.05	197.1175
	19 °C	F	48.88 ± 2.10	221.2914	54.36 ± 1.71	147.1739
		M	53.58 ± 1.30	85.79755	55.75 ± 1.79	154.2766
	25 °C	F	46.02 ± 0.99	48.71388	38.46 ± 1.35	90.62082
		M	40.92 ± 0.70	24.52408	38.46 ± 0.93	43.23306
19 °C	16 °C	F	103.02 ± 3.42	585.0404	98.64 ± 3.02	455.0106
		M	84.6 ± 1.92	184.0408	81.12 ± 1.81	161.9853
	19 °C	F	87.06 ± 1.90	180.9147	88.44 ± 2.02	204.7004
		M	70.14 ± 2.29	262.6127	73.5 ± 2.18	237.398
	25 °C	F	56.76 ± 1.54	117.8596	55.92 ± 1.48	109.3812
		M	43.86 ± 1.00	50.28612	45.54 ± 0.92	42.86571
25 °C	16 °C	F	123.24 ± 3.48	604.5943	115.4 ± 2.61	341.5922
		M	82.68 ± 2.41	290.6302	78.72 ± 2.80	391.4302
	19 °C	F	119.7 ± 3.19	508.1327	99.84 ± 3.98	790.5453
		M	88.14 ± 2.03	207.1433	72.54 ± 3.44	593.1514
	25 °C	F	87.18 ± 3.20	512.2322	72.18 ± 4.12	851.6608
		M	64.56 ± 1.99	197.7208	48.66 ± 2.82	396.4739

Population, rearing temperature, treatment temperature, sex, population x rearing temperature, and sex x rearing temperature interactions showed a statistically significant influence on starvation resistance ($F_{(1)} = 19.4$, $p < 0.001$; $F_{(2)} = 356.8$, $p < 0.001$, $F_{(2)} = 607.57$, $p < 0.001$; $F_{(1)} = 238.4$, $p < 0.001$; $F_{(2)} = 16.6$, $p < 0.001$; $F_{(2)} = 58.3$, $p < 0.001$, respectively (Table S5)).

Individuals reared at 25 °C showed significantly longer starvation resistance than individuals from the lower rearing temperatures. Lower altitude (L) population reared at 25 °C shows better starvation resistance under all treatments. A higher rearing temperature significantly prolongs starvation resistance, but at 25 °C, the time of death from starvation is much shorter than at lower temperatures. The females reared at 25 °C from both populations are significantly more resistant to starvation than males (Table S6).

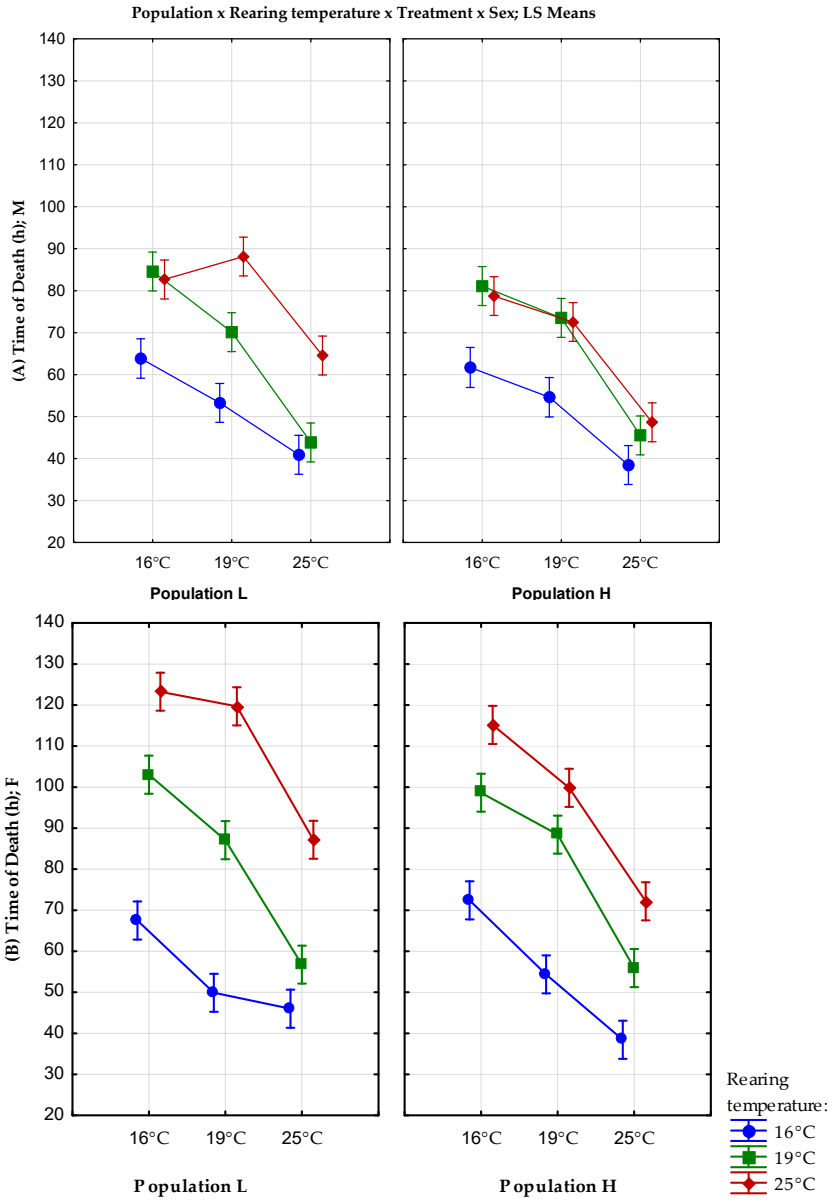

Figure 3. Results of the full factorial GLM analysis of starvation resistance with fixed factors population, rearing temperature, treatment temperature, and sex; (**A**) males; and (**B**) females. Vertical bars denote a 0.95 confidence interval. M—males; F—females; L—population originating from lower altitude; H—population originating from a higher altitude.

3.4. Chill Coma Recovery Time

The mean values, standard errors, and variance for the chill coma recovery time are shown in Table 4. Individuals reared at 25 °C showed the longest chill coma recovery time, with significantly different values between females from both populations.

The rearing temperature and population x rearing temperature x sex interaction showed a statistically significant influence on chill coma recovery time ($F_{(2)}$ = 111.4978, $p < 0.001$; $F_{(2)}$ = 10.0838, $p < 0.001$, respectively; Table S7). Female individuals from a higher altitude (H) showed a significantly longer chill coma recovery time than females from lower (L) altitude (Bonferroni $p = 0.018$; Table S8); but in males, the inverted trend was observed. A higher rearing temperature significantly prolongs chill coma recovery time (Bonferroni $p < 0.01$ for all combinations). For the L population, females reared at 25 °C have a significantly shorter chill coma recovery time than their male counterparts (Bonferroni $p = 0.002$).

The results of the full factorial GLM analysis with fixed factors population, rearing temperature, and sex are shown in Figure 4.

Table 4. Mean values with standard errors and variance for all groups for chill-coma recovery time (measured in minutes). Population L—population originating from lower altitude; population H—population originating from higher altitude; rearing temperature—T at which flies evolve in the laboratory; F—females; M—males; chill coma recovery time—time by flies were recovered from CCRT (mean value ± SE (standard error)).

		Population L		Population H	
Rearing Temperature	**Sex**	**Chill Coma Recovery Time**	**Variance**	**Chill Coma Recovery Time**	**Variance**
16 °C	F	17.61 ± 1.72	143.7924	22.74 ± 1.75	146.3962
	M	17.85 ± 1.77	156.0721	17.26 ± 1.83	167.116
19 °C	F	43.14 ± 5.76	1160.334	35.65 ± 2.53	268.8758
	M	23.72 ± 2.46	206.3637	65.80 ± 12.67	6901.601
25 °C	F	68.04 ± 9.10	3481.358	107.11 ± 14.31	8810.542
	M	115.21 ± 15.60	8751.539	87.09 ± 9.99	4484.564

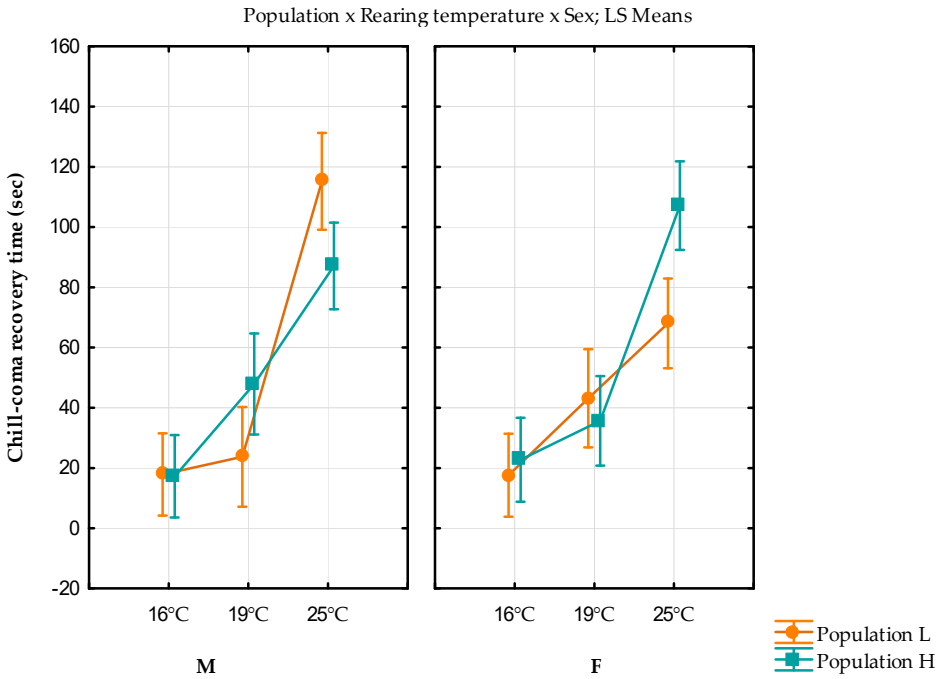

Figure 4. Results of the full factorial GLM analysis for chill coma recovery time with fixed factors population, rearing temperature, and sex. Vertical bars denote 0.95 confidence interval. M—males; F—females; L—population originating from lower altitude; and H—population originating from a higher altitude.

4. Discussion

Organisms in populations of different species are coping with the impacts of climate change and consequently with environmental changes that occur very rapidly, so they must adapt to these changes in order to survive. Extreme temperatures, periods of droughts, or food deficiency which are happening in every environment can wipe out a population unless it is capable of adapt to those conditions or escape in some other way [66]. In any given species, the shape and the speed of adaptation depend on numerous factors, such as the studied trait, population sampled, environmental conditions, and type of response [64]. Temperature is one of the most important selective agents, and studies of laboratory thermal

evolution may provide insight into adaptation mechanisms as the responses to climatic change [35]. The present study analyses the dynamics and variation of four stress resistance traits, namely desiccation resistance, heat knock-down resistance, starvation resistance, and chill coma recovery time in *D. subobscura* flies originating from natural populations from two different altitudes kept under different strictly defined temperature regimes under laboratory conditions.

Our results suggest that population origin has a significant influence on all analysed traits, except CCRT. For desiccation resistance, the lower altitude population generally responds better to desiccation than the high-altitude population, which is expected considering the fact that high temperature is a cause of desiccation in the wild, and temperature generally decreases with increasing altitude [23]. For heat knock-down resistance, we have a different population response, where the high-altitude population showed better resistance to heat knock-down. At higher altitudes, weather conditions are generally correlated with temperature extremes, and population H reared at 25 °C showed the highest resistance to short-term extreme temperature. The results of the starvation resistance assay showed that the L population is more tolerant to food shortages than the H population. The flies reared at 16 °C were the least starvation tolerant, and flies reared at 25 °C were the most resistant to food deprivation. This can be explained by temperature acclimation and the possibility that highland populations do not have the ability to cope with the deficiency of food and very high temperature at the same time. A recovery time from chill coma takes the longest time for flies reared at 25 °C, while the flies reared at 16 °C recovered almost instantly, which could be explained with their preacclimation to a certain temperature. We did not notice a significant difference between the populations' responses to CCRT, but this result is not surprising as differences in this trait were not observed in *Drosophila melanogaster* populations from distinct geographic and climate origins [67]. The chill tolerance traits are thus mediated by different physiological mechanisms and measuring more than one trait is important for evidence of temperature selection pressure. The influence of population origin, detected for three of the four analysed traits, is an indication that altitude may have an influence on shaping the response to temperature stress. However, deciphering the influence of altitude population distribution on thermal stress traits may require the analysis of several independent low- and high-altitude populations to further corroborate these claims. Considering the fact that only two populations from different altitudes are included in our analysis, we cannot exclude some other mechanisms such as drift which play a significant role in shaping population response to thermal stress—as detected in our study. However, even though this species is known for its high mobility and mostly large effective population size, local adaptations at a small spatial scale have been described [68]. Moreover, local adaptation to colder and warmer environments for reproductive performance traits in two *D. subobscura* populations was described [69], indicating that fitness traits depend on both the thermal origin and laboratory conditions of the population and that, if local conditions are different, two populations may be sufficient to support such claims.

Our results suggest that laboratory thermal conditions have an effect on all analysed traits, but as expected, this is more prominent for HKDR and CCRT, which are positively correlated with a high developmental temperature, as previously shown in *D. melanogaster* and *D. buzzati* [31,41,42,67]. As previously shown, adaptation to a colder environment decreases HKDR [70] and our results suggest that with the increased rearing temperature, HKDR and CCRT are prolonged, indicating a trade-off between HKDR and CCRT at a high rearing temperature. Previously published work in *D. subobscura* showed that as few as nine generations of laboratory thermal conditions may lead to the rapid and consistent evolution of wing shape [71], indicating that adaptation to laboratory thermal conditions may take a relatively short time period. Although the lab adaptation process is faster in the first 15–20 generations, it still extends to many more generations after [72]. Our results suggest that the thermal regime that individuals experienced in the laboratory for 12 generations influence the population's ability to endure temperature extremes, indicating

that individuals accustomed to colder environments may face serious challenges in light of global warming. Our results suggest that laboratory thermal conditions and adaptations to imposed thermal conditions influence the population response to desiccation and starvation resistance under short-term cold conditions, but do not have the same influence under higher temperature conditions. Although our analysis detected the influence of laboratory thermal conditions on all analysed traits, we cannot exclude the effect of developmental plasticity as a factor contributing to the observed patterns and shaping individuals response. Our results add to the body of knowledge that temperature extremes, whether high or low, may have a stronger effect on populations preadapted to lower temperatures, and extreme fluctuations may jeopardise their ability to adapt and survive [40,65]. Further studies on more populations of different geographical origin and following an experimental evolution design including replication are needed to better understand the true effect of extreme temperature fluctuation on population ability to survive and adapt to such conditions.

Not surprisingly, all the traits apart from CCRT were strongly influenced by sex. Females, as we previously described, showed better heat resistance performances than males, indicating that males have more difficulties coping with rising temperatures. Females have overall better DR, SR, and HKDR. A previous work showed that an elevated temperature has diverse effects on the different sexes of this species and that, for instance, exposure to 25 °C can even induce male sterility in *D. subobscura* [62]. Additionally, it has been suggested that mating success under heat stress in *D. buzzati* can be a direct target of thermal selection and that thermal sexual selection has a negative effect on cold resistance [49]. We noted that with an elevated rearing temperature, the CCRT for females from the L population needed a shorter time to recover than males, but in the H population, a different trend was observed, possibly indicating preadaptation to harsher environmental conditions.

Drosophila suboscura is considered a cold-adapted species with a thermal range between 6 °C and 26 °C [73] and a thermal optimum of 18 °C [62]. Behavioural assays revealed that this species shows a thermal preference of 16.6 °C when placed in a linear thermal gradient [63]. Desiccation, heat knock-down resistance, and chill coma recovery are considered reliable indicators of thermal adaptation, all of which suggest that these correlate with natural adaptation to high-temperature environments [35]. Although the temperatures used in this experiment are not extreme, in the range experienced by *D. subobscura* developing in nature, our results show that almost all traits are sensitive to rearing temperature and to the origin of the population. However, the patterns of the traits did not respond the same: they show different sensitivities to non-optimal temperatures, indicating different mechanisms responsible for thermal stress adaptation. For instance, for HKDR and CCRT, flies have different responses depending on their rearing temperature, which was expected. The population from a high altitude (H) showed better HKDR and CCRT which can be correlated with a better tolerance to extreme weather conditions in the highlands. The patterns of knock-down resistance and chill coma recovery correlate well with the thermal history of the two populations investigated, where flies reared at 25 °C were shown to be the most heat tolerant via an HKDR assay, but also the less cold tolerant via a CCRT assay, which is indicative of a trade-off association between this two traits regarding rearing temperature. For flies reared at 16 °C, the opposite results were obtained. The lowland L population showed an overall better resistance to desiccation and starvation resistance. Higher rearing temperature prolongs survival for both desiccation and starvation, suggesting that preadaptation to warmer conditions plays an important role in the mechanisms enabling the organism to cope with different environments. Desiccation is significant stress for terrestrial animals, specifically for insects, which are particularly vulnerable to the loss of water due to their relatively small body size [43]. Our results indicate that flies reared at 16 °C and tested for desiccation resistance at 25 °C are the least desiccation tolerant of all groups, especially the highland population, probably because this population is preadapted to cold environment.

There is clear evidence that many habitats are becoming increasingly threatened by stressful climate changes, and anthropogenic factors are altering thermal conditions and are

also mostly responsible for the increased rates of current and expected future extinctions. To understand the effects of climate warming on some species and ecosystems, long-term observations of the occurrence of species and detailed knowledge on their ecology and life-history is crucial, but studies such as this one, despite its limitations, can also make a significant contribution to understanding how species will respond to ongoing climate change. Environmental stress resistance traits are complex quantitative genetic traits that are influenced by the combined effect of genes and environmental conditions. Our results suggest that both long- (rearing temperature) and short-term (HKDR and CCRT temperatures) exposure to a high temperature has a greater detrimental influence on all analysed traits compared to any low temperature exposure. Population history is also an important factor that shapes the individual response to suboptimal and extreme temperature stress, but again, heat stress has a more pronounced effect than cold stress. In light of global warming, our results add to the body of knowledge that cold-adapted species are expected to fare much worse in a fast-changing environment.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/insects13020138/s1, Table S1: Results of GLM for desiccation resistance; Table S2: Bonferroni correction for all pairwise comparisons for desiccation resistance; Table S3: Results of GLM for heat knock-down resistance; Table S4: Bonferroni correction for all pairwise comparisons for heat knock-down resistance; Table S5: Results of GLM for starvation resistance; Table S6: Bonferroni correction for all pairwise comparisons for starvation resistance; Table S7: Results of GLM for chill coma recovery time; Table S8: Bonferroni correction for all pairwise comparisons for chill coma recovery time; Supplementary Scheme S1: Experimental scheme.

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Temperature-Specific and Sex-Specific Fitness Effects of Sympatric Mitochondrial and Mito-Nuclear Variation in *Drosophila obscura*

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Simple Summary: Does variation in the mitochondrial DNA sequence influence the survival and reproduction of an individual? What is the purpose of genetic variation of the mitochondrial DNA between individuals from the same population? As a simple laboratory model, *Drosophila* species can give us the answer to this question. Creating experimental lines with different combinations of mitochondrial and nuclear genomic DNA and testing how successful these lines were in surviving in different experimental set-ups enables us to deduce the effect that both genomes have on fitness. This study on *D. obscura* experimentally validates theoretical models that explain the persistence of mitochondrial DNA variation within populations. Our results shed light on the various mechanisms that maintain this type of variation. Finally, by conducting the experiments on two experimental temperatures, we have shown that environmental variations can support mitochondrial DNA variation within populations.

Abstract: The adaptive significance of sympatric mitochondrial (mtDNA) variation and the role of selective mechanisms that maintain it are debated to this day. Isofemale lines of *Drosophila obscura* collected from four populations were backcrossed within populations to construct experimental lines, with all combinations of mtDNA *Cyt b* haplotypes and nuclear genetic backgrounds (nuDNA). Individuals of both sexes from these lines were then subjected to four fitness assays (desiccation resistance, developmental time, egg-to-adult viability and sex ratio) on two experimental temperatures to examine the role of temperature fluctuations and sex-specific selection, as well as the part that interactions between the two genomes play in shaping mtDNA variation. The results varied across populations and fitness components. In the majority of comparisons, they show that sympatric mitochondrial variants affect fitness. However, their effect should be examined in light of interactions with nuDNA, as mito-nuclear genotype was even more influential on fitness across all components. We found both sex-specific and temperature-specific differences in mitochondrial and mito-nuclear genotype ranks in all fitness components. The effect of temperature-specific selection was found to be more prominent, especially in desiccation resistance. From the results of different components tested, we can also infer that temperature-specific mito-nuclear interactions rather than sex-specific selection on mito-nuclear genotypes have a more substantial role in preserving mtDNA variation in this model species.

Keywords: *D. obscura*; *Cyt b* gene; desiccation resistance; developmental time; viability; sex-ratio; mtDNA; intra-population variation

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1. Introduction

The acquisition of a primordial free-living prokaryote ancestor of mitochondria by early eukaryotes is probably the most important step in the evolution of complex life [1]. The mitochondrial genome codes for only a few genes, but they are immensely important for the metabolism and the high energy efficiency of the eukaryotic cells. Products of the genes encoded by the mitochondrial genome act in conjunction with products of the nuclear genome. These complex interactions include cellular respiration as well as mtDNA replication, transcription and translation, all remarkably important biological processes [2].

The mitochondrial electron transport chain (METC), which is the site of the oxidative phosphorylation pathway (OXPHOS), is tightly orchestrated by the epistasis of (the genes encoded over) the two genomes. Adenosine triphosphate (ATP) is a cell fuel that is produced in the OXPHOS, by five multi-subunit enzyme complexes, four of which are comprised by the subunits encoded by both genomes [3]. METC function is dependent on the synchronised interaction between mtDNA-encoded proteins (and RNAs) and nuclear-encoded proteins that are imported in the mitochondria. These protein subunits originating from two different genomes require high compatibility analogous to a 'lock and key' principle to preserve their configuration and enzymatic activity. Any incompatibilities can compromise their structural and biochemical properties, which in turn can cause electron leakage and consequently oxidative stress.

Apart from the aforementioned direct influence on the OXPHOS, mito-nuclear interactions have an indirect impact on it by being enrolled in the processes of transcription and translation of mitochondrial subunits involved in METC, as well as replication of the mtDNA. Transcription of these mtDNA genes involved in the OXPHOS pathway is completely regulated by the nucleus. All polypeptides involved in the process are nuclear-coded and imported to the mitochondria. This process usually consists of two transcription factors and a single subunit mitochondrial RNA polymerase (POLRMT) which is liable for promoter-binding specificity and strength. This is the basis for transcriptional mito-nuclear interactions because the nuDNA-coded protein needs to be structurally complementary to the control region of the mitochondrial DNA, or regulation of mtDNA genes transcription is compromised [4]. Experiments have shown that when combining factors of the mitochondrial transcription machinery from different taxa, the more distant the taxa, the more deficient the transcription [5–7]. The authors suggest that this is due to coevolution between binding motifs in the POLRMT or splicing peptides and the mtDNA recognition sites in each species [5]. Replication of the mtDNA is also reliant on the activity of the POLRMT, as it is responsible for RNA primer creation after which replication can begin using the mtDNA polymerase complex. This way, mito-nuclear co-adaptions are accountable for mtDNA replication as well [8,9]. Apart from coding for subunits involved in OXPHOS, mtDNA also encodes for transfer RNAs (tRNAs), which cooperate with nuclear-encoded mitochondrial aminoacyl-tRNA synthetases (mt-aaRSs) in the process of translating mtDNA proteins [2,10], giving rise to another level on which mito-nuclear interactions are recognized by selection.

Since mtDNA products play such a pivotal role in the eukaryotic metabolism and normal cell functioning, scientists have long thought that any variation in the mitochondrial genome that impacts fitness would quickly be either purged or fixed by natural selection [11,12], resulting in erosion of genetic variation. This view was encouraged by the attributes of the mitochondrial genome, specifically being haploid and its exclusively maternal inheritance. A further consequence of haploidy in the absence of recombinations and heterozygosis, in which recessive alleles would be masked by dominant ones, is that all alleles are exposed to selection. Scientists thought that the high mutation rates of mtDNA are the reason for the relatively high levels of functional mtDNA variation, but theoretical models have proven that the observed levels of standing genetic variation are much higher than expected under the mutation–selection balance [13].

In the last few decades, this traditional paradigm of rejecting mitochondrial DNAs functional and evolutional relevance has shifted, with the accumulating evidence linking

specific mtDNA sequence polymorphisms to substantial phenotypic effects [14]. First, signs of positive selection acting on the mtDNA in a broad spectrum of phyla have been reported by comparing the distribution of synonymous and nonsynonymous substitutions within and between species [11,15–19]. Second, a correlation between environmental factors and the distribution of mtDNA haplotypes has been observed [18,20,21]. Not long after, experimental evidence started to amass, pointing to fitness consequences ensued by mtDNA sequence variation [22–25].

The presence of adaptive variation in mtDNA between populations is quite easy to explain because distinct populations usually mean independent genetic pools from which the stochastic forces sieve different alleles, but also different selection pressures favouring distinct haplotypes. Even detrimental alleles can become specific for a particular population if they are compatible with arising compensatory mutations in the nuclear genome [26–32]. Therefore, the joint mito-nuclear interactions mentioned above are important in that respect [33].

However, the existence of stable adaptive sympatric variation is much more difficult to explain, given its haploid nature and the importance of mitochondrial genes. Over the years, many ambiguous results have been published on the subject of the role of mito-nuclear interactions in sustaining adaptive sympatric variation. While some authors [25,34] could not prove sympatric variance being maintained with mito-nuclear interactions, others proved it while using the same model species and similar experimental set-ups [26,35]. Current theoretical understanding suggests that conditions required for maintenance of adaptive within-population variance are restrictive [36–38]. Experimental papers on the subject have been scarce throughout the years [1,26]. A recent growing body of theoretical and experimental work has started to shed light on the subject, especially in light of cytonuclear interactions.

Negative frequency-dependent selection (NFDS) was one of the first mechanisms of balancing selection proposed to be maintaining sympatric mitochondrial variation. When it comes to this type of balancing selection, an allele's relative fitness is inversely proportional to its frequency in the population. In a nutshell, NFDS constitutes that rarer alleles are favoured by natural selection over more common ones. The role of negative-frequency dependent selection in maintaining genetic variation in the nuclear genome is well described in the example of chromosomal inversions in *Drosophila ananassae* [39] and maintaining self-incompatibility in plants [40]. The first proposition of the NFDS's role in maintaining mtDNA variation by acting on cytonuclear gene interactions was brought by Gregorius and Ross [38], but it was not until recently that it was hypothesised and experimentally proven that NFDS is responsible for sustaining mtDNA polymorphism in laboratory populations of seed beetle [41,42]. Furthermore, experiments on *Drosophila subobscura* are starting to give weight to NFDS effects on preserving mtDNA variation [43,44].

Sex-specific selection (SSS) is an additional balancing selection mechanism that was proposed to be maintaining standing sympatric mtDNA variation [45,46]. Theory predicts that some mito-nuclear haplotype combinations result in a higher relative fitness in one sex, while different combinations provide better fitness in the other sex. Since mitochondria are maternally transmitted, the selection can act on them only through females. Because of the different reproduction strategies and behavioural patterns and sex-specific life histories in general, males and females have different fitness, as selection only recognizes females, favouring mutations that have a positive impact on females' fitness even though these mutations can have deleterious effects in males. This hypothesis is known as the mother's curse [47,48]. Different mutations in mitochondria importing nuclear-encoded proteins that are favourable to males may arise, pulling corresponding mitochondria-coded proteins to compensatory adapt and adjust to the new biochemical and structural stability and to preserve the high compatibility and efficacy [49]. The idea of SSS maintaining intra-population variation was reinforced in the next couple of years by many experiments on *Drosophila* species [50–52], confirming that SSS acting on these mito-nuclear interactions can maintain intra-population variation.

Rand [35] developed a model of joint transmission of X chromosome and cytoplasm in *Drosophila melanogaster* and showed that maintenance of sympatric mitochondrial variation can be supported if the nuclear component of the interaction is located on the X chromosome.

Another mechanism proposed is the idea that the mitochondrial or mito-nuclear variation is shaped by differences in environmental factors that vary temporally or within habitat [22,50]. In that respect, genotype-by-environment interactions are crucial in upholding sympatric mtDNA variation. Dowling et al. [50] showed that multiple mito-nuclear haplotypes can be maintained by epistatic interactions between mitochondrial and nuclear genes in a random mating population. Their findings show that the adaptive value of specific mito-nuclear combinations are determined by the environment in which they are expressed. Furthermore, Willet et al. [53] have shown the substantial influence of temperature and light regime on selection on interpopulation mito-nuclear crosses of intertidal copepod *Tigriopus californicus*. In this study, it is advocated that differences in protein interactions at varying temperatures are the reason why specific mito-nuclear combinations are favoured at a given temperature range. Oscillating temperature settings over space and time could act to maintain not only mitochondrial but mito-nuclear variation within populations, when this mtDNA × nuDNA × environment epistasis occurs [54].

Thus, different mitochondrial polymorphisms can be auspicious in different extrinsic terms, for example, temperature, or different intrinsic conditions, be it a different nuclear genetic background (if possible, X linked) or different sex. In addition, the mere frequency of the polymorphism can make it more or less favourable.

Insects have long served as suitable models to measure the adaptive significance of mitochondrial or mito-nuclear variation. Generally, their generation time is short, making it possible to fully replace the existing nuclear background with the desired one by multi-generational backcrossing in a relatively short time [41,52,54,55]. The experimental model used in this study, *Drosophila obscura*, possesses a high level of sympatric mtDNA variation across the species range [56]. Therefore, it gives an excellent possibility to test the adaptive significance of sympatric mtDNA variation, and consequently forces that maintain it. Our preliminary work on desiccation resistance, in this model species [57], identified the importance of temperature-specific effects on mito-nuclear variation, and to a lesser extent, the effects of SSS. In the present study, we use four Experimental Blocks (EBs), each representing variation collected within a specific natural site. Each block has three distinct mtDNA haplotypes and three distinct nuclear genetic backgrounds that are combined in nine possible experimental mito-nuclear introgression lines (MNILs). These MNILs were subjected to measurements of a set of life-history traits (both larval and adult) at two different temperatures. This way, we disentangle the specific effect of mtDNA on fitness and its dependence on nuclear genetic background, experimental temperature and sex of the individual. Ultimately, we discuss the importance of mito-nuclear interactions, genotype-to-environment interactions, and sex-to-genotype interactions, and we identify balancing selection mechanisms that maintain sympatric variation in mtDNA in natural populations of this model species.

2. Materials and Methods

Starting material for this study consisted of isofemale lines (IFL) of *Drosophila obscura* that were constructed from females collected in the wild and that were previously genotyped for the *Cyt b* gene [56]. These IFLs were maintained in the laboratory on a standard corn-meal medium for multiple generations. All IFLs used were previously tested and were negative on *Wolbachia*. In addition, IFLs used in this experiment were jointly analysed for microbiota composition, and no maternally transmitted bacteria were found [56]. Four experimental blocks (EB) were formed, each from a specific locality. Three distinct sympatric haplotypes per block, representing IFLs from the same population were chosen in order to construct 9 MNILs.

The first EB consisted of three haplotypes O1, O2 and O8, from the ST population collection site 1 [56]. Experimental block II was formed by backcrossing haplotypes O2, O9 and O10, which originated from the ST population collection site 2. Combinations of haplotypes O2, O4 and O3 belonging to the population SS, were used to make MNILs for the EB III. The MNILs of the fourth EB were constructed using haplotypes O2, O3 and O18 which come from the SG population. Specific IFLs were renamed to avoid confusion between EBs, as given in Table 1.

Table 1. *D. obscura Cyt b* haplotypes used in the experiment (left) and their corresponding IFL names (right) per experimental block. ST, Tara Mountain; SS, Balkan Mountains; SG, Goč Mountain.

I EB Population ST Site1		**II EB Population STSite2**		**III EB Population SS**		**IV EB Population SG**	
O2	A	O9	D	O2	L	O2	O
O1	B	O2	E	O4	M	O3	P
O8	C	O10	F	O3	N	O18	Q

For each population, three selected IFLs were backcrossed for 14 generations, creating nine MNILs with all combinations of mitochondrial haplotypes (mtDNA) and nuclear genetic backgrounds (nuDNA). For the backcrossing procedure, we kept the vials with fly pupae in the dark such that we can collect virgin flies every morning. There is scarce information on the reproductive behaviour of *D. obscura*; thus, we tested the methodology for collecting virgin individuals used for *D. subobscura* [25,52]. From the pilot experiment, we concluded that *D. obscura* flies will not mate in the first 24 h after eclosion when being in total dark, since no flies oviposited upon being collected from a dark box every 24 h. Each MNIL was created by mating 10 virgin females of a specific haplotype with 20 virgin males with the desired nuclear genetic background. The full backcrossing design is shown in Figure 1. After 14 generations, flies were sequenced for the *Cyt b* gene again to verify that all of the final 36 MNILs possess the appropriate haplotype.

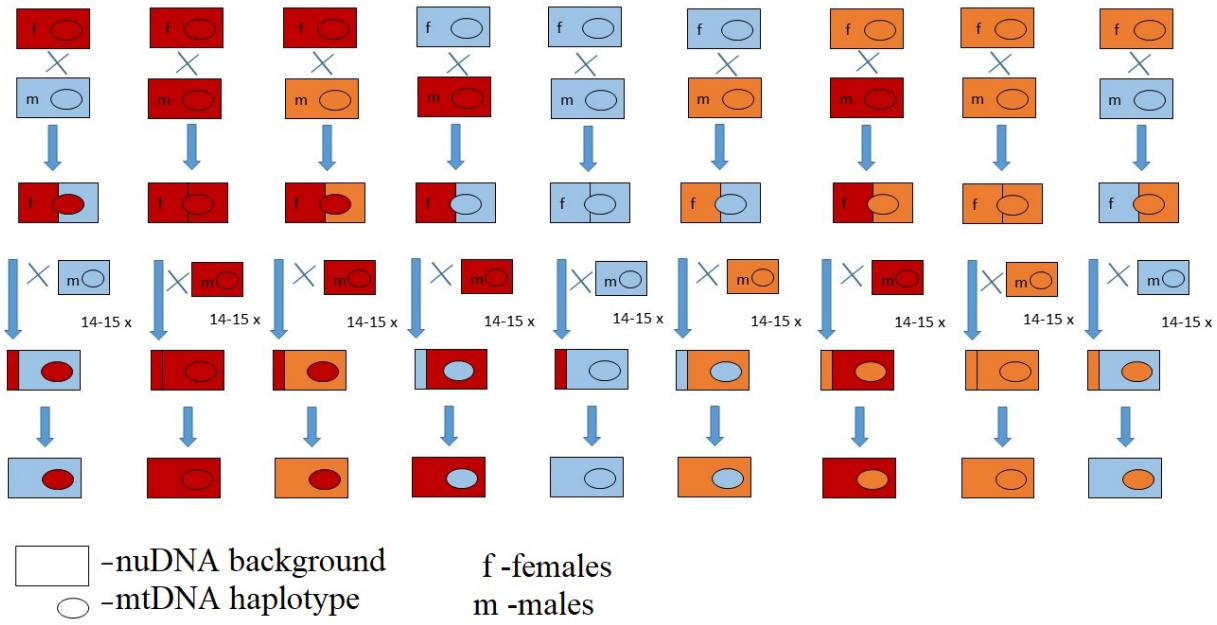

Figure 1. Full backcrossing design for each experimental block.

In each block, we wanted to compare different mitochondrial haplotype triplets; in some, we compared specific single nucleotide polymorphisms that distinguish between

most prevalent haplogroups, while some pairs differ in 6 or more mutations [56]. The scheme of differences between haplotypes in each of the four EBs is given in Figure 2.

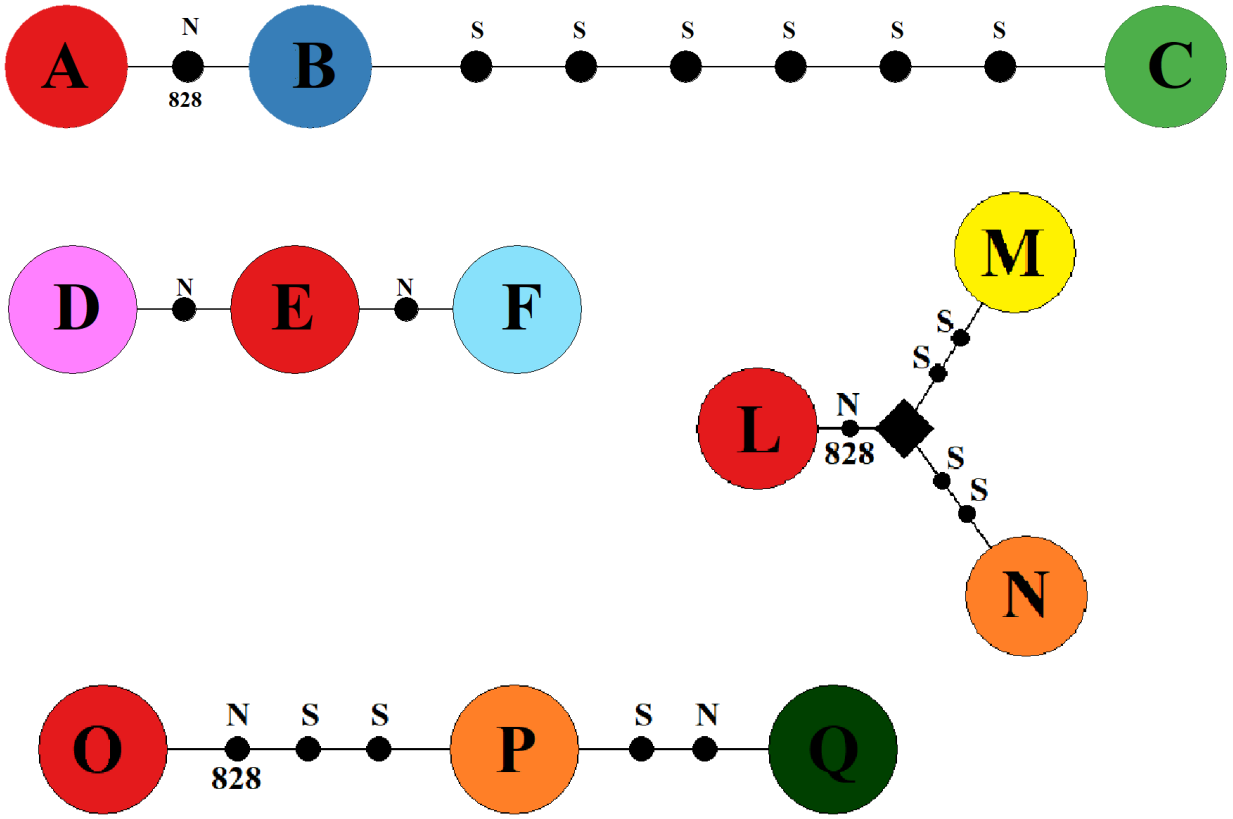

Figure 2. Haplotypes A, B and C from EB I; haplotypes D, E and F from EB II; L, M and N from EB III; haplotypes O, P and Q from EB IV; identical haplotypes are coloured with the same colour. S, synonymous mutation; N, nonsynonymous mutation; 828 A > G, a specific nonsynonymous substitution that separates the two groups of haplotypes.

In order to compare the fitness of the different combinations of mito-nuclear haplotypes, we conducted two grand experiments with several fitness components tested, in different generations due to a large number of individual flies of both sexes needed for each experiment. All experiments were conducted at two different temperatures. In all experiments for every block, we modelled three comparisons of haplotype pairs as well as the whole block with three different haplotype pairs compared together.

2.1. Desiccation Resistance

Desiccation resistance was the first conducted experiment after the 14th generation of backcrossing, two experimental temperatures were 16 and 19 °C, while the air humidity was set at 30%. For every specific MNIL, we had up to 40 flies per sex, for each of the two experimental temperatures. In some MNILs, we could not collect 40 flies per sex of the desired age. We had 38.8 females and 37.6 males on average for each MNIL per temperature. Individual virgin flies 5 to 7 days old were placed in small plastic modular containers with small holes for air circulation, where each module is being capped with the next making a total of two columns with 20 connected plastic containers per sex for each MNIL, for easier inspection. Containers were placed in two different rooms with regulated temperature and air-humidity levels. After the experiment was set, the flies were inspected every hour until effectively all flies had died. Mortality was determined by the inability of the *Drosophila* to keep an upright position or stand up after the plastic container had been shaken. In 4 EBs, each containing 9 MNILs, two temperatures, two sexes and up to 40 individuals had around 5500 flies tested for desiccation resistance.

2.2. Egg-to-Pupa-to-Adult Viability, Developmental Time and Sex Ratio Experiment

This experiment was conducted after the 15th generation of backcrossing, on two developmental temperatures (15 °C and 20 °C). For each MNIL, 40 virgin females and 60 virgin males 6–7 days old were placed in a bottle with a standard corn-meal medium for 5 days to mate. Another bottle with the same number of flies was kept as a backup. After 5 days flies were relocated to the new empty bottle which had a Petri dish instead of the cap. New bottles were placed upside down, standing on the Petri dish with corn-meal medium and yeast paste for flies to oviposit eggs. Each morning during the set-up phase, we replaced the old Petri dishes with the new ones. From the Petri dishes, we collected the oviposited eggs under binoculars and placed 20 eggs per vial for the viability experiment. For every MNIL, we made 30 replicas containing 20 eggs for each of the two experimental temperatures. Vials containing eggs were kept at two thermo-regulated chambers under constant temperature, light and dark cycles (15 °C and 12 h light/12 h dark regime and 20 °C and 16 h light/8 h dark regime) to imitate different seasons. Every two days we shuffled the vials inside the chambers to make sure that the temperature is constant for each vial. To sum up, with 4 EBs, each containing 9 MNILs, two temperatures, and 30 replicated vials with 20 transferred eggs we had over 43,000 eggs ($4 \times 9 \times 2 \times 30 \times 20 = 43{,}200$).

To measure developmental time 10 vials out of the 30 replicas for each temperature for each of the 36 MNIL, were randomly chosen, to be checked every day for hatched flies. Emerging adult flies were counted and sexed every day (in an air-conditioned room of the desired temperature) until the end of the experiment, with the number of pupae inside each vial counted as well. For the rest of the replicas, the number of adult flies, males, females and pupa was counted at the end of the eclosion after day 36.

2.3. Statistical Analysis

All statistical analyses were conducted using R v.4.0.3 [58]. All Figures were made in R using the ggplot2 package [59].

Desiccation data were analysed using a Cox proportional hazards model [60] using survival package v.3.2-13 [61] for each EB individually as well as for each of the three pairwise comparisons per EB, with no censoring since all flies died in the experiment. Mitochondrial haplotype (mtDNA), nuclear background (nuDNA), sex and temperature were used as fixed effects with all interactions of the four factors used as well (mtDNA × nuDNA × sex × temp). The proportional hazards assumption was checked with the cox.zph function, and where violated, the corresponding factors were stratified. Cox proportional models for EBs I and III were already presented at the 1st international electronic conference on Entomology [57]. Here, we present extended models with all factors and interactions included, in order to make the results of all four blocks comparable.

Developmental time data were analysed with a general linear model using the lmer function from the lme4 package in R [62]. Full model with REML estimation and type III sums-of-squares was fitted with all interactions of fixed effects factors (mtDNA, nuDNA, sex and temperature) and replica number as random effect factor for each EB as well as for each pairwise comparison inside the EBs. LmerTest package [63] was used to obtain p values in ANOVA model fits.

Viability per vial was analysed with generalised linear models using the glm function in R. Egg to pupa (EtP), pupa to adult (PtA) and egg to adult (EtA) viability was scored for each pair of haplotypes compared respectively and for all four blocks apiece. Models had mitochondrial haplotype, nuclear background and temperature as fixed effects and all interactions of the three factors. All three component models, (EtA, EtP and PtA) had binomial error distribution and used the number of eggs/pupae per vial as the denominator.

Sex ratio data calculated as a proportion of males was analysed using the same general linear model with binomial error with the total number of eclosed adults per vial as the denominator. All pairwise comparisons were modelled individually, but also pulled together within each EB.

3. Results

3.1. Desiccation Resistance

Mean desiccation resistance times for four EBs are given in Supplementary Figures S1–S4. When modelling desiccation resistance, we found sex to have a significant effect on survival times as we expected. This is because female *D. obscura* flies are generally bigger than their counterpart males. Greater surface area to volume ratio means increased exposure to the environment, which makes survival in dry conditions harder. Flies of both sexes survived longer on the lower temperature in all four EBs. In all EBs, the differences between the groups are much more pronounced at 16 °C.

Here, we jointly analysed Cox proportional model data on desiccation resistance from four EBs, two of which (I and III) were presented at the First International Electronic Conference on Entomology [57] (https://sciforum.net/paper/view/10522, accessed on 22 December 2021; I = A, II = B, III = C, IV = L, V = M, VI = N). The ANOVA of Cox proportional hazards models for each EB, both pairwise comparisons and the whole block are given in Tables 2–5. Across all blocks, there were some comparisons that had their proportional hazards assumption violated. All factors whose hazard functions were not proportional to other factors in the comparison were stratified accordingly. In the first EB, mitochondrial haplotype had a significant effect on desiccation resistance in two out of three comparisons [57], as well as in the second EB. In the third [57] and fourth EB, mtDNA was significant in only one out of three haplotype comparisons. In total, mtDNA significantly influenced desiccation resistance in six out of twelve comparisons. The nuclear background was also highly significant in five out of the seven un-stratified comparisons. In the first EB, sex was highly significant in all comparisons, while in the latter three blocks, it was significant in only one comparison per block (In total: 6/11). Furthermore, the combination of sex and mtDNA also influenced survival in the desiccation experiment, as it was significant in two out of three comparisons in the first three EBs and one out of three pairs in the fourth EB. Temperature, as was expected, had the most substantial effect on desiccation survival time, as it was highly significant in all comparisons in which its proportional hazard assumption was not violated (7/7 comparisons). The interaction term of temperature and mitochondrial haplotype had an impact on desiccation resistance, being significant in all three pairwise comparisons in EBs I and III [57], while in the II and IV EB, it was statistically significant in two-thirds and one-third of comparisons, respectively. Mito-nuclear haplotype combination mtDNA × nuDNA had an even bigger effect on the desiccation resistance than the mitochondrial haplotype. In EBs I and III, it was highly significant in all three comparisons [57], while in EBs II and IV comparisons it was significant in one comparison respectively. Sex × mtDNA × nuDNA interaction did not prove to be influential for survival under desiccation stress, as in the first two EBs no comparisons showed significance, and only one out of three comparisons in EBs III and IV. Mito-nuclear haplotype and temperature interaction, conversely, influenced the flies survival time significantly in eight out of twelve total pairwise comparisons with all of the comparisons in the third EB being highly significantly influenced by it. The combination of mitochondrial haplotype, sex and temperature produced different results in different EBs, as it showed no connection to survival time in the first two EBs, while it showed significant influence on survival time under desiccation stress in two out of three comparisons in each of the last two EBs. The highest interaction term with all four factors (mtDNA × nuDNA × sex × temp) had a mild effect on survival time as it was significant in one out of three comparisons in the EBs I, II and III and two out of three pairs in EB IV.

Table 2. The effect of mitochondrial haplotype (MT), nuclear genetic background (NU), sex, temperature (T) and their interactions on desiccation resistance for the experimental block (EB) I of *D. obscura*. LogLik, log-likelihood; Chisq, chi-squared value; Df, degrees of freedom; strata, variable is stratified; *p* values that are significant at $p < 0.05$ are given in bold. Reduced Cox proportional hazards models for pairwise comparisons with some interactions missing for this EB was presented at the 1st International Electronic Conference on Entomology, giving qualitatively indistinguishable results [57].

EB I	**AB**				**AC**				**BC**				**ABC**			
	LogLik	**Chisq**	**Df**	***p***	**LogLik**	**Chisq**	**Df**	***p***	**LogLik**	**Chisq**	**Df**	***p***	**LogLik**	**Chisq**	**Df**	***p***
MT	−2531.4	9.240	1	**0.0024**	−2966.2	13.56	1	**0.00023**	−2994.8	2.284	1	0.13072	−6307.2	5.74	2	0.05684
NU		strata			−2955.7	21.16	1	$\mathbf{4.2 \times 10^{-6}}$		strata				strata		
sex	−2521.6	19.570	1	$\mathbf{9.7 \times 10^{-6}}$	−2949.9	11.54	1	**0.00068**	−2989.2	11.348	1	**0.00076**		strata		
temp(T)		strata				strata			−2952.2	73.989	1	$\mathbf{2.2 \times 10^{-16}}$	−6294.2	26.03	1	$\mathbf{3.4 \times 10^{-7}}$
MT:NU	−2506.3	30.534	1	$\mathbf{3.3 \times 10^{-8}}$	−2941.9	16.07	1	$\mathbf{6.1 \times 10^{-5}}$	−2949.7	4.846	1	**0.02771**	−6264.9	58.51	4	$\mathbf{6.0 \times 10^{-12}}$
MT:sex	−2503.6	1.352	1	0.2450	−2936.6	10.55	1	**0.00116**	−2943.6	12.341	1	**0.00044**	−6264	1.79	2	0.40841
NU:sex	−2503.5	0.104	1	0.7473	−2936.6	0.00	1	0.95096	−2942.6	1.997	1	0.15766		strata		
MT:T	−2504.2	4.209	1	**0.0402**	−2934	5.24	1	**0.02209**	−2938.1	8.856	1	**0.00292**	−6255.5	17.14	2	**0.00019**
NU:T		strata			−2934	0.01	1	0.93708	−2930.1	16.104	1	$\mathbf{6.0 \times 10^{-5}}$	−6254.9	1.09	2	0.57892
T:sex	−2498.3	10.356	1	**0.0013**	−2927.2	13.43	1	**0.00025**	−2916.3	27.490	1	$\mathbf{1.6 \times 10^{-7}}$	−6233	43.86	1	$\mathbf{3.5 \times 10^{-11}}$
MT:NU:sex	−2477.9	0.056	1	0.8122	−2926.4	1.61	1	0.20438	−2916.3	0.004	1	0.94981	−6202.2	61.54	4	$\mathbf{1.4 \times 10^{-12}}$
MT:NU:T	−2477.9	40.789	1	$\mathbf{1.7 \times 10^{-10}}$	−2925.7	1.43	1	0.23108	−2913.7	5.255	1	**0.02188**	−6200.9	2.60	4	0.62690
MT:T:sex	−2476.7	2.378	1	0.1230	−2925.4	0.59	1	0.44138	−2912.2	3.115	1	0.07760	−6200.6	0.56	2	0.75397
NU:T:sex	−2476.6	0.151	1	0.6972	−2925.1	0.62	1	0.43070	−2911.7	0.856	1	0.35477	−6200.1	0.99	2	0.61096
MT:NU:T:sex	−2476.6	0.163	1	0.6866	−2925	0.16	1	0.69248	−2907.9	7.542	1	**0.00603**	−6194.1	12.01	4	**0.01725**

Table 3. The effect of mitochondrial haplotype (MT), nuclear genetic background (NU), sex, temperature (T) and their interactions on desiccation resistance for the experimental block (EB) II of *D. obscura*. LogLik, log-likelihood; Chisq, chi-squared value; Df, degrees of freedom; strata, variable is stratified; *p* values that are significant at $p < 0.05$ are given in bold.

EB II	**DE**				**DF**				**EF**				**DEF**			
	LogLik	**Chisq**	**Df**	***p***	**LogLik**	**Chisq**	**Df**	***p***	**LogLik**	**Chisq**	**Df**	***p***	**LogLik**	**Chisq**	**Df**	***p***
MT	−2993.6	4.84	1	**0.02777**	−2601.3	0.61	1	0.43587	−2964.5	28.42	1	$\mathbf{9.7 \times 10^{-8}}$	−7897.5	53.47	2	$\mathbf{2.5 \times 10^{-12}}$
NU	−2993.6	0.00	1	0.94573		strata			−2945.6	37.76	1	$\mathbf{8.0 \times 10^{-10}}$	−7869.6	55.87	2	$\mathbf{7.4 \times 10^{-13}}$
sex	−2992	3.24	1	0.07200	−2591.6	19.53	1	$\mathbf{9.9 \times 10^{-6}}$	−2944.5	2.16	1	0.14148		strata		
temp(T)		strata				strata							−7860.8	17.61	1	$\mathbf{2.7 \times 10^{-5}}$
MT:NU	−2991.1	1.74	1	0.18733	−2591	1.27	1	0.26037	−2926.3	36.45	1	$\mathbf{1.6 \times 10^{-9}}$	−7840.7	40.11	4	$\mathbf{4.1 \times 10^{-8}}$
MT:sex	−2988.3	5.54	1	**0.01859**	−2587.5	7.00	1	**0.00816**	−2926.3	0.01	1	0.90468	−7832.5	16.34	2	**0.00028**

Table 3. *Cont.*

EB II	DE				DF				EF				DEF			
	LogLik	**Chisq**	**Df**	***p***	**LogLik**	**Chisq**	**Df**	***p***	**LogLik**	**Chisq**	**Df**	***p***	**LogLik**	**Chisq**	**Df**	***p***
NU:sex	−2973.7	29.23	1	$\mathbf{6.4 \times 10^{-8}}$	−2584.9	5.02	1	**0.02511**	−2919.9	12.83	1	**0.00034**	-7818.8	27.50	2	$\mathbf{1.1 \times 10^{-6}}$
MT:T	−2973.4	0.61	1	0.43353	−2580.8	8.23	1	**0.00412**	−2916.2	7.44	1	**0.00638**	−7812.6	12.39	2	**0.00204**
NU:T	−2963.6	19.60	1	$\mathbf{9.6 \times 10^{-6}}$		strata			−2910	12.40	1	**0.00043**	−7790	45.24	2	$\mathbf{1.5 \times 10^{-10}}$
T:sex	−2961.2	4.74	1	**0.02946**	−2578.8	3.96	1	**0.04666**	−2904.3	11.41	1	**0.00073**	−7781.2	17.49	1	**0.00003**
MT:NU:sex	−2961	0.45	1	0.50107	−2578.8	0.02	1	0.87763	−2904.3	0.01	1	0.92003	−7762.1	38.16	4	$\mathbf{1.0 \times 10^{-7}}$
MT:NU:T	−2961	0.06	1	0.81429	−2577.1	3.57	1	0.05874	−2882.4	43.78	1	$\mathbf{3.7 \times 10^{-11}}$	−7760.9	2.44	4	0.65468
MT:T:sex	−2960.6	0.84	1	0.35998	−2576.6	0.98	1	0.32177	−2882.1	0.53	1	0.46709	−7755.2	11.46	2	**0.00325**
NU:T:sex	−2954.1	12.92	1	**0.00032**	−2575.9	1.35	1	0.24562	−2878.8	6.58	1	**0.01032**	−7749.9	10.63	2	**0.00491**
MT:NU:T:sex	−2952.2	3.76	1	0.05246	−2574.6	2.65	1	0.10334	−2872.9	11.73	1	**0.00061**	−7738.2	23.39	4	**0.00011**

Table 4. The effect of mitochondrial haplotype (MT), nuclear genetic background (NU), sex, temperature (T) and their interactions on desiccation resistance for the experimental block (EB) III of *D. obscura*. LogLik, log-likelihood; Chisq, chi-squared value; Df, degrees of freedom; strata, variable is stratified; *p* values that are significant at $p < 0.05$ are given in bold. Reduced Cox proportional hazards models for pairwise comparisons with some interactions missing for this EB was presented at the 1st International Electronic Conference on Entomology, giving qualitatively indistinguishable results [57].

EB III	LM				LN				MN				LMN			
	LogLik	**Chisq**	**Df**	***p***	**LogLik**	**Chisq**	**Df**	***p***	**LogLik**	**Chisq**	**Df**	***p***	**LogLik**	**Chisq**	**Df**	***p***
MT	−2873.1	2.08	1	0.14912	−3278.7	4.07	1	**0.04362**	−2798.6	**0.09**	1	0.76424	−6391.6	5.60	2	0.06090
NU	−2853.4	39.27	1	$\mathbf{3.7 \times 10^{-10}}$	−3219	119.33	1	$\mathbf{2.2 \times 10^{-16}}$	−2780.6	**36.17**	1	$\mathbf{1.8 \times 10^{-9}}$		strata		
sex	−2851.2	4.48	1	**0.03431**	−3218.3	1.44	1	0.23044	−2779.1	**2.94**	1	0.08623	−6295.7	191.83	1	$\mathbf{2.2 \times 10^{-16}}$
temp(T)	−2769	164.44	1	$\mathbf{2.2 \times 10^{-16}}$	−3149.4	137.81	1	$\mathbf{2.2 \times 10^{-16}}$	−2761.2	**35.71**	1	$\mathbf{2.3 \times 10^{-9}}$	−6295.6	0.15	1	0.70212
MT:NU	−2764.4	9.08	1	**0.00259**	−3144.6	9.59	1	**0.00195**	−2756.8	**8.74**	1	**0.00312**	−6271.9	47.28	4	$\mathbf{1.3 \times 10^{-9}}$
MT:sex	−2764.4	0.03	1	0.86929	−3142.3	4.55	1	**0.03293**	−2756.8	**0.06**	1	0.80429	−6263.5	16.84	2	**0.00022**
NU:sex	−2761.3	6.26	1	**0.01236**	−3137.7	9.26	1	**0.00234**	−2755	**3.65**	1	0.05622	−6246.6	33.81	2	$\mathbf{4.6 \times 10^{-8}}$
MT:T	−2754.5	13.65	1	**0.00022**	−3134.7	6.01	1	**0.01423**	−2752	**5.98**	1	**0.01446**	−6245.2	2.87	2	0.23812
NU:T	−2752.1	4.69	1	**0.03030**	−3131.8	5.67	1	**0.01723**	−2751.9	0.23	1	0.63140	−6237.3	15.86	2	**0.00036**
T:sex	−2752.1	0.03	1	0.85993	−3129.3	5.11	1	**0.02385**	−2750.3	**3.26**	1	0.07112	−6235.9	2.68	1	0.10149
MT:NU:sex	−2751.9	0.45	1	0.50464	−3127.3	3.92	1	**0.04782**	−2750.1	0.42	1	0.51560	−6209.7	52.40	4	$\mathbf{1.1 \times 10^{-10}}$
MT:NU:T	−2749.1	5.48	1	**0.01923**	−3123.6	7.48	1	**0.00625**	−2737.8	24.51	1	$\mathbf{7.4 \times 10^{-7}}$	−6205.7	7.96	4	0.09311
MT:T:sex	−2748.8	0.69	1	0.40653	−3117	13.18	1	**0.00028**	−2731.5	12.62	1	**0.00038**	−6196.4	18.62	2	$\mathbf{9.1 \times 10^{-5}}$
NU:T:sex	−2747.1	3.43	1	0.06399	−3116.9	0.17	1	0.67903	−2730.3	2.45	1	0.11786	−6195.3	2.18	2	0.33614
MT:NU:T:sex	−2746.7	0.72	1	0.39545	−3114.6	4.54	1	**0.03320**	−2729.8	0.98	1	0.32162	−6192.6	5.49	4	0.24058

Table 5. The effect of mitochondrial haplotype (MT), nuclear genetic background (NU), sex, temperature (T) and their interactions on desiccation resistance for the experimental block (EB) II of *D. obscura*. LogLik, log-likelihood; Chisq, chi-squared value; Df, degrees of freedom; strata, variable is stratified; *p* values that are significant at $p < 0.05$ are given in bold.

EB IV	**OP**				**OQ**				**PQ**				**OPQ**			
	LogLik	**Chisq**	**Df**	***p***	**LogLik**	**Chisq**	**Df**	***p***	**LogLik**	**Chisq**	**Df**	***p***	**LogLik**	**Chisq**	**Df**	***p***
MT	−2915.2	0.86	1	0.35422	−2983.5	2.02	1	0.15542	−2976.2	5.10	1	0.02397	−6261.5	10.45	2	**0.00537**
NU	−2915.2	0.02	1	0.88632		strata				strata				strata		
sex		strata			−2972.6	21.85	1	**2.9×10^{-6}**	−2976.1	0.07	1	0.79310	−6050.6	421.85	1	**2.2×10^{-16}**
temp(T)	−2810.4	209.51	1	**2.2×10^{-16}**	−2824.3	296.56	1	**2.2×10^{-16}**	−2888.9	174.40	1	**2.2×10^{-16}**		strata		
MT:NU	−2809.9	0.99	1	0.32078	−2824.3	0.00	1	0.96923	−2882.8	12.31	1	**0.00045**	−6042.6	16.07	4	**0.00293**
MT:sex	−2801.8	16.30	1	**5.4×10^{-5}**	−2822.7	3.21	1	0.07312	−2879.1	7.36	1	**0.00667**	−6034.5	16.05	2	**0.00033**
NU:sex	−2801.8	0.01	1	0.93663	−2818.3	8.78	1	**0.00304**	−2879.1	0.07	1	0.79796	−6032.6	3.96	2	0.13834
MT:T	−2800.4	2.72	1	0.09938	−2816.2	4.26	1	**0.03900**	−2879	0.06	1	0.80007	−6017.3	30.56	2	**2.3×10^{-7}**
NU:T	−2800.4	0.01	1	0.91035	−2815	2.31	1	0.12894	−2878.2	1.75	1	0.18603		strata		
T:sex	−2797.4	5.96	1	**0.01462**	−2814.6	0.80	1	0.36981	−2878	0.35	1	0.55487	−6015.4	3.78	1	0.05190
MT:NU:sex	−2796.4	2.03	1	0.15397	−2814.6	0.07	1	0.79290	−2874.8	6.38	1	**0.01152**	−6005.6	19.63	4	**0.00059**
MT:NU:T	−2795.5	1.87	1	0.17163	−2810.6	8.00	1	**0.00469**	−2864.6	20.40	1	**6.3×10^{-6}**	−5998.3	14.43	4	**0.00603**
MT:T:sex	−2788.6	13.76	1	**0.00021**	−2805.3	10.55	1	**0.00116**	−2864.6	0.06	1	0.81162	−5990.1	16.49	2	**0.00026**
NU:T:sex	−2785.2	6.81	1	**0.00907**	−2798.7	13.27	1	**0.00027**	−2862.5	4.16	1	**0.04134**	−5969.5	41.23	2	**1.1×10^{-9}**
MT:NU:T:sex	−2781.5	7.34	1	**0.00674**	−2794.8	7.64	1	**0.00570**	−2861.4	2.21	1	0.13668	−5962.8	13.45	4	**0.00929**

When analysing EBs as a whole (comparison of all three haplotypes per EB), nuDNA and temperature as individual factors were significant in all blocks in which they were not stratified. Sex was significant in two out of three blocks. Mitochondrial haplotype was significant in two out of four blocks, while the combination of mtDNA and nuDNA was highly significant in all four EBs. Interaction terms of mtDNA × sex, as well as mtDNA × temperature, were highly influential on desiccation resistance in three of four EBs each. Conversely, mito-nuclear interaction with sex was significant only in the fourth EB. The combination of mito-nuclear haplotype and temperature was clearly associated with desiccation stress survival time in all EBs. Genotype × sex × temperature, both only mitochondrial, and the combination of mtDNA and nuDNA were statistically significant in three EBs each.

3.2. Developmental Time

Mean developmental times for all four EBs are presented in Supplementary Figures S5–S8. Overall, males and females had similar developmental times across all EBs, with some genotype combinations being favoured in males and others in females. Temperature, conversely, had the most substantial influence on the developmental times across all EBs, with all groups developing notably faster on the higher temperature, as was expected.

ANOVA results of GLM of developmental time for four EBs are given in Tables 6–9. Temperature was significant in all comparisons across all EBs. Mitochondrial haplotype, as well as nuclear background and sex, showed different impacts on the developmental time in different EBs. In the first EB, mtDNA was significant in one comparison, while nuDNA and sex were significant in two. Both mtDNA and nuDNA were highly significant in all three comparisons in EB II, while sex was in only one. Sex showed no significant influence on developmental time in the third EB, while both nuDNA and mtDNA were significant in one out of three comparisons. EB IV had two-thirds of comparisons statistically significant for mtDNA and one-third for nuDNA, while sex was highly significant in all three pairwise comparisons. Mito-nuclear interaction affected the developmental time in two comparisons in EBs I and IV and one comparison per block in II and III. Sex × mtDNA interaction was not as influential as sex and mtDNA are individually, as it had an effect in only one pairwise comparison in EBs II and IV. Moreover, sex × mito-nuclear interaction was also not important for the developmental time as it showed no effect in three EBs, while in the third EB, it was significant in two comparisons. Genotype × temperature interaction (mtDNA × temp and mtDNA × nuDNA × temp) is not pivotal for the developmental time as they were both statistically significant in only three comparisons out of the total twelve, across all EBs. Sex × mtDNA × temperature was also proven to be noncrucial for development time as it showed statistical significance in only one comparison in the second EB. In addition to that, the highest interaction term with all four factors included showed significant influence on developmental time in only two pairwise comparisons out of the total twelve.

Table 6. The effect of mitochondrial haplotype (MT), nuclear genetic background (NU), sex, temperature (T) and their interactions on developmental time for the three pairwise comparisons from experimental block (EB) I and the whole EB I of *D. obscura*. SSq, sum of squares; ddf, denominator degrees of freedom; *p* values that are significant at $p < 0.05$ are given in bold.

EB I	**AB**				**AC**				**BC**				**ABC**			
	SSq	**ddf**	**F**	***p***	**SSq**	**ddf**	**F**	***p***	**SSq**	**ddf**	**F**	***p***	**SSq**	**ddf**	**F**	***p***
MT	0.450	70.0	0.62	0.4320	5.2	72.3	6.46	**0.0132**	0.1	69.6	0.10	0.7585	0.5	158.7	0.31	0.7374
NU	0.350	70.0	0.49	0.4870	7.8	72.3	9.61	**0.0028**	18.3	69.6	27.55	$\mathbf{1.6 \times 10^{-6}}$	33.6	158.6	21.55	$\mathbf{5.3 \times 10^{-9}}$
sex	5.400	931.4	7.43	**0.0065**	3.8	1042.2	4.67	**0.0309**	1.4	1028.8	2.18	0.1405	6.3	2214.3	8.12	**0.0044**
temp(T)	1726	70.0	2374	$\mathbf{2.2 \times 10^{-16}}$	1675	72.3	2073	$\mathbf{2.2 \times 10^{-16}}$	1353	69.6	2041	$\mathbf{2.2 \times 10^{-16}}$	3835	158.7	4913	$\mathbf{2.2 \times 10^{-16}}$
MT:NU	3.120	70.0	4.30	**0.0419**	5.3	72.3	6.50	**0.0129**	1.5	69.6	2.20	0.1428	14.4	158.5	4.63	**0.0015**
MT:sex	0.210	931.4	0.29	0.5928	1.3	1042.2	1.56	0.2123	1.1	1028.8	1.73	0.1892	1	2214.2	0.65	0.5217
NU:sex	1.670	931.4	2.30	0.1298	2.1	1042.2	2.56	0.1100	0.5	1028.8	0.68	0.4113	2.5	2213.9	1.60	0.2019
MT:T	1.740	70.0	2.39	0.1263	37.4	72.3	46.29	$\mathbf{2.5 \times 10^{-9}}$	0.1	69.6	0.13	0.7176	5.1	158.7	3.25	**0.0416**
NU:T	0.950	70.0	1.31	0.2571	21.0	72.3	26.04	$\mathbf{2.6 \times 10^{-6}}$	7.4	69.6	11.12	**0.0014**	34.9	158.6	22.35	$\mathbf{2.8 \times 10^{-9}}$
T:sex	0.490	931.4	0.67	0.4131	3.7	1042.2	4.53	**0.0336**	5.6	1028.8	8.42	**0.0038**	4.6	2214.3	5.95	**0.0148**
MT:NU:sex	1.550	931.4	2.14	0.1441	0.0	1042.2	0.01	0.9115	0.0	1028.8	0.01	0.9201	1.9	2213.6	0.60	0.6591
MT:NU:T	14.96	70.0	20.58	$\mathbf{2.3 \times 10^{-5}}$	0.2	72.3	0.24	0.6277	3.9	69.6	5.84	**0.0183**	55.4	158.5	17.75	$\mathbf{4.6 \times 10^{-12}}$
MT:T:sex	2.310	931.4	3.18	0.0749	0.0	1042.2	0.00	0.9446	1.2	1028.8	1.75	0.1857	0.3	2214.2	0.20	0.8185
NU:T:sex	1.810	931.4	2.49	0.1146	0.0	1042.2	0.01	0.9263	1.9	1028.8	2.91	0.0885	3	2213.9	1.93	0.1452
MT:NU:T:sex	1.990	931.4	2.74	0.0984	0.2	1042.2	0.25	0.6154	0.0	1028.8	0.05	0.8181	7.6	2213.6	2.44	**0.0446**

Table 7. The effect of mitochondrial haplotype (MT), nuclear genetic background (NU), sex, temperature (T) and their interactions on developmental time for the three pairwise comparisons from experimental block (EB) II and the whole EB II of *D. obscura*. SSq—sum of squares; ddf, denominator degrees of freedom; *p* values that are significant at $p < 0.05$ are given in bold.

EB II	**DE**				**DF**				**EF**				**DEF**			
	SSq	**ddf**	**F**	***p***	**SSq**	**ddf**	**F**	***p***	**SSq**	**ddf**	**F**	***p***	**SSq**	**ddf**	**F**	***p***
MT	5.54	70.66	5.63	**0.020**	9.28	70.7	8.72	**0.0043**	32.1	71.14	33.26	$\mathbf{1.9 \times 10^{-7}}$	45.3	160.0	22.66	$\mathbf{2.2 \times 10^{-9}}$
NU	3.93	70.66	4.00	**0.049**	20.78	70.7	19.52	$\mathbf{3.5 \times 10^{-5}}$	3.9	71.14	4.07	**0.0474**	12.7	160.0	6.33	**0.0023**
sex	6.36	954.08	6.46	**0.011**	0.25	996.9	0.24	0.6271	0.9	996	0.91	0.3396	3.7	2233.5	3.72	0.0538
temp(T)	2474	70.66	2514	$\mathbf{2 \times 10^{-16}}$	3028	72.0	2845	$\mathbf{2.2 \times 10^{-16}}$	3265	71.14	3378	$\mathbf{2.2 \times 10^{-16}}$	6008	160.1	6008	$\mathbf{2.2 \times 10^{-16}}$
MT:NU	0	70.66	0.00	0.982	0.08	70.7	0.08	0.7843	8.3	71.14	8.64	**0.0044**	8.2	160.0	2.06	0.0889
MT:sex	1.72	954.08	1.75	0.186	3.69	996.9	3.46	0.0631	6.5	996	6.71	**0.0097**	5.8	2233.1	2.90	0.0554

Table 7. *Cont.*

EB II	DE				DF				EF				DEF			
	SSq	**ddf**	**F**	***p***	**SSq**	**ddf**	**F**	***p***	**SSq**	**ddf**	**F**	***p***	**SSq**	**ddf**	**F**	***p***
NU:sex	3.57	954.08	3.63	0.057	2.36	996.9	2.21	0.1371	1.6	996	1.67	0.1960	14.6	2233.0	7.28	**0.0007**
MT:T	0.96	70.66	0.98	0.326	6.81	72.0	6.40	**0.0136**	14.6	71.14	15.15	**0.0002**	19.6	160.0	9.81	$\mathbf{9.5 \times 10^{-5}}$
NU:T	1.77	70.66	1.80	0.184	26.3	72.0	24.71	$\mathbf{4.4 \times 10^{-6}}$	15.4	71.14	15.95	**0.0002**	38.4	160.0	19.20	$\mathbf{3.4 \times 10^{-8}}$
T:sex	1.6	954.08	1.63	0.202	0.51	999.3	0.48	0.4875	0	996	0.02	0.8837	2.2	2233.5	2.20	0.1382
MT:NU:sex	1.38	954.08	1.41	0.236	0.05	996.9	0.04	0.8339	0.2	996	0.21	0.6484	5.2	2231.9	1.31	0.2643
MT:NU:T	0.05	70.66	0.05	0.826	0.09	72.0	0.08	0.7771	0.3	71.14	0.32	0.5719	0.5	160.0	0.14	0.9685
MT:T:sex	1.52	954.08	1.54	0.215	3.48	999.3	3.27	0.0710	4.9	996	5.03	**0.0251**	3.4	2233.1	1.69	0.1850
NU:T:sex	3.33	954.08	3.38	0.066	3.42	999.3	3.21	0.0735	7.4	996	7.66	**0.0057**	7.2	2233.0	3.62	**0.0269**
MT:NU:T:sex	0.01	954.08	0.01	0.943	0	999.3	0.00	0.9865	6.5	996	6.77	**0.0094**	12.2	2231.9	3.05	**0.0162**

Table 8. The effect of mitochondrial haplotype (MT), nuclear genetic background (NU), sex, temperature (T) and their interactions on developmental time for the three pairwise comparisons from experimental block (EB) III and the whole EB III of *D. obscura*. SSq, sum of squares; ddf, denominator degrees of freedom; *p* values that are significant at $p < 0.05$ are given in bold.

EB III	LM				LN				MN				LMN			
	SSq	**ddf**	**F**	***p***	**SSq**	**ddf**	**F**	***p***	**SSq**	**ddf**	**F**	***p***	**SSq**	**ddf**	**F**	***p***
MT	0.08	352.01	0.08	0.774	2.56	79.85	1.64	0.2040	10.17	263.43	10.84	**0.0011**	1.13	508.12	0.48	0.6201
NU	0.08	352.01	0.08	0.772	0.77	79.85	0.50	0.4832	19.82	263.43	21.13	$\mathbf{6.7 \times 10^{-6}}$	20.16	350.54	8.55	**0.0002**
sex	0.68	684.68	0.75	0.388	4.85	623.7	3.11	0.0783	0.35	782.68	0.37	0.5435	5.25	1563.83	4.45	**0.0350**
temp(T)	840	352.01	917	$\mathbf{2 \times 10^{-16}}$	2147	79.85	1376	$\mathbf{2 \times 10^{-16}}$	1447	263.43	1543	$\mathbf{2.2 \times 10^{-16}}$	2672	550.98	2267	$\mathbf{2.2 \times 10^{-16}}$
MT:NU	2.89	352.01	3.15	0.077	0.84	79.85	0.54	0.4657	9.71	263.43	10.35	**0.0015**	23.37	334.23	4.96	**0.0007**
MT:sex	0.41	684.68	0.44	0.506	0.03	623.7	0.02	0.8810	1.43	782.68	1.52	0.2174	0.09	1566.61	0.04	0.9619
NU:sex	2.61	684.68	2.84	0.092	0	623.7	0.00	0.9913	2.93	782.68	3.12	0.0776	1.56	1582.77	0.66	0.5158
MT:T	0.11	352.01	0.12	0.731	5.7	79.85	3.65	0.0595	2.61	263.43	2.78	0.0968	1.05	508.12	0.45	0.6408
NU:T	0.19	352.01	0.20	0.653	6.99	79.85	4.48	**0.0375**	11.09	263.43	11.82	**0.0007**	5.67	350.54	2.41	0.0917
T:sex	0.31	684.68	0.34	0.558	0.77	623.7	0.49	0.4830	2.09	782.68	2.23	0.1356	0.14	1563.83	0.11	0.7348
MT:NU:sex	4.55	684.68	4.97	**0.026**	0.06	623.7	0.04	0.8425	5.77	782.68	6.15	**0.0134**	8.51	1580.6	1.81	0.1252
MT:NU:T	8.14	352.01	8.89	**0.003**	0.6	79.85	0.39	0.5365	0	263.43	0.00	0.9458	10.77	334.23	2.29	0.0600
MT:T:sex	2.8	684.68	3.06	0.081	1.96	623.7	1.26	0.2626	0.07	782.68	0.07	0.7913	3.37	1566.61	1.43	0.2395
NU:T:sex	0.08	684.68	0.09	0.770	2.56	623.7	1.64	0.2009	0.31	782.68	0.33	0.5684	1.94	1582.77	0.82	0.4396
MT:NU:T:sex	1.14	684.68	1.25	0.265	0.39	623.7	0.25	0.6165	0.96	782.68	1.02	0.3117	2.41	1580.6	0.51	0.7280

Table 9. The effect of mitochondrial haplotype (MT), nuclear genetic background (NU), sex, temperature (T) and their interactions on developmental time for the three pairwise comparisons from experimental block (EB) IV and the whole EB IV of *D. obscura*. SSq, sum of squares; ddf, denominator degrees of freedom; p values that are significant at $p < 0.05$ are given in bold.

EB IV	**OP**				**OQ**				**PQ**				**OPQ**			
	SSq	**ddf**	**F**	p	**SSq**	**ddf**	**F**	p	**SSq**	**ddf**	**F**	p	**SSq**	**ddf**	**F**	p
MT	4.32	74.1	5.23	**0.02510**	3.07	82.0	2.99	0.0877	28.92	66.4	45.15	$\mathbf{5.0\times 10^{-9}}$	22.88	172.1	14.38	$\mathbf{1.7\times 10^{-6}}$
NU	25.84	74.1	31.28	$\mathbf{3.6\times 10^{-7}}$	2.46	82.0	2.39	0.1263	0.73	66.4	1.15	0.2881	19.83	171.9	12.47	$\mathbf{8.8\times 10^{-6}}$
sex	9.05	1146	10.95	**0.00097**	20.83	884.2	20.23	$\mathbf{7.8\times 10^{-6}}$	9.46	1014	14.76	**0.0001**	29.81	2318	37.47	$\mathbf{1.1\times 10^{-9}}$
temp(T)	1690	74.1	2045	$\mathbf{2.2\times 10^{-16}}$	1235	82.0	1199	$\mathbf{2.2\times 10^{-16}}$	1251	66.4	1953	$\mathbf{2.2\times 10^{-16}}$	3075	172.6	3865	$\mathbf{2.2\times 10^{-16}}$
MT:NU	3.18	74.1	3.85	0.05362	23.28	82.0	22.61	$\mathbf{8.4\times 10^{-6}}$	9.90	66.4	15.46	**0.0002**	28.23	171.0	8.87	$\mathbf{1.6\times 10^{-6}}$
MT:sex	0.01	1146	0.01	0.91750	0.08	884.2	0.07	0.7870	2.70	1014	4.21	**0.0404**	2.04	2315	1.28	0.2779
NU:sex	6.47	1146	7.82	**0.00524**	0.10	884.2	0.10	0.7572	0.89	1014	1.39	0.2392	6.15	2314	3.87	**0.0211**
MT:T	2.19	74.1	2.64	0.10813	0.00	82.0	0.00	0.9813	0.17	66.4	0.27	0.6049	2.55	172.1	1.60	0.2045
NU:T	5.55	74.1	6.72	**0.01149**	0.08	82.0	0.08	0.7833	8.87	66.4	13.84	**0.0004**	18.35	171.9	11.53	$\mathbf{2.0\times 10^{-5}}$
T:sex	0.15	1146	0.18	0.66836	0.20	884.2	0.20	0.6581	0.12	1014	0.18	0.6711	0.38	2318	0.48	0.4905
MT:NU:sex	0.03	1146	0.04	0.83715	0.85	884.2	0.82	0.3643	0.10	1014	0.16	0.6907	2.65	2311	0.83	0.5049
MT:NU:T	2.1	74.1	2.55	0.11480	0.00	82.0	0.00	0.9927	0.23	66.4	0.36	0.5500	2.12	171.0	0.67	0.6167
MT:T:sex	1.21	1146	1.47	0.22586	0.41	884.2	0.40	0.5279	0.71	1014	1.11	0.2914	0.52	2315	0.33	0.7202
NU:T:sex	0.15	1146	0.18	0.66802	2.99	884.2	2.90	0.0888	0.03	1014	0.05	0.8156	1.03	2314	0.65	0.5230
MT:NU:T:sex	6.44	1146	7.79	**0.00534**	0.02	884.2	0.02	0.8987	0.11	1014	0.18	0.6740	7.18	2311	2.26	0.0607

When modelling EBs as a whole (comparing all three haplotypes per EB), temperature and nuclear background had the biggest impact on developmental time, being highly significant in all four blocks. Sex was significant in three EBs, while mtDNA and mtDNA× nuDNA interactions with sex showed no connection to the developmental time in none of the four blocks modelled. The combination of mtDNA, temperature and sex was also nonsignificant in all four blocks. While mtDNA was a significant factor in two EBs, mtDNA × nuDNA interaction was significant in three. Interaction terms containing temperature and genotype (mtDNA and mtDNA × nuDNA) showed a statistically significant influence in two and one EBs respectively. The highest interaction term with all four factors was significantly influencing developmental time in half of the four EBs modelled.

3.3. Viability

Egg-to-Adult

Mean egg-to-adult (EtA) viability scores for the four EBs are presented in Supplementary Figures S9–S12. In the viability experiment, we noted that mito-nuclear combinations that consisted of an mtDNA haplotype on its own nuclear background (e.g., DD, LL, QQ), usually scored the lowest viability, except in the first EB where they were usually most viable.

The results of ANOVA on EtA viability from all pairwise and whole block comparisons are presented in Table 10. The nuclear genetic background had the most substantial effect on the EtA viability as it was highly significant in all pairwise comparisons across all EBs. Temperature as well was crucial for EtA viability, being statistically significant in a total of nine comparisons across all EBs. The effect of mtDNA on viability was more variable, conversely. In the first EB, it showed a significant effect in only one of three comparisons, while in the II and III, it was significant in two of the pairs. In the fourth EB, mtDNA was significant in all pairwise comparisons. We found that mito-nuclear interaction showed substantial influence on EtA viability as it was statistically significant in nine out of the total twelve comparisons across all EBs. Different mtDNA haplotypes on different temperatures had significantly different EtA viability in eight out of twelve comparisons, making this interaction also important. Mito-nuclear genotype × temperature interaction had different results in different EBs. In EB II its effect was significant in all comparisons, while in the IV EB, it was significant in none. EBs I and III had one and two out of three comparisons statistically significant respectively.

When modelling EBs as a whole (comparing all three haplotypes per EB), mtDNA was significant in all four EBs, furthermore, nuDNA, temperature as factors as well as their interaction terms with mtDNA showed statistical significance in all four EBs. The three-factor interaction term was also highly significant in all EBs.

The model for the egg-to-pupa (EtP) viability had similar results as the EtA model with all factors discussed being highly statistically significant. Pupa-to-adult (PtA) viability was high, with most of the individuals that reached the pupal stage reaching adulthood. Results for EtP and PtA viability are given in Supplementary Tables S1 and S2.

3.4. Proportion of Males

The mean percentage of males for all genotype combinations inside all four EBs on two temperatures are given in Supplementary Figures S13–S16. A skewed proportion of males towards females was observed in a few experimental MNILs. This effect was most pronounced in MNILs with M nuclear genetic background with almost all combinations of mito-nuclear haplotypes on both experimental temperatures having as little as 5% of hatched individuals male. The only exception is the MM combination at 15 °C where the percentage of males spikes up to around 12%, which is still considered low (Supplementary Figure S15). In the fourth EB, distortion in the sex ratio was also detected, although not as pronounced and not as obvious as in the EB III, as there were significant differences between the same MNILs on two experimental temperatures (Supplementary Figure S16). In only one case did the proportion of males decrease to as low as 12% in the QO MNIL at

20 °C, while that same MNIL on the lower temperature had a proportion of males around 40%. This effect was most noticeable on the O nuclear background, but also in the OQ MNIL which has Q nuclear background and O mtDNA haplotype.

Table 10. The effect of mitochondrial haplotype (MT), nuclear genetic background (NU), temperature (T) and their interactions on Egg-to-adult viability for three pairwise comparisons and the experimental block (EB) analysed as a whole for each of the four experimental blocks. DF, degrees of freedom; Dev, deviance; p values that are significant at $p < 0.05$ are given in bold.

EB I		**AB**			**AC**			**BC**			**ABC**	
	Df	**Dev**	p	**Df**	**Dev**	p	**Df**	**Dev**	p	**Df**	**Dev**	p
MT	1	1.11	0.2932	1	7.24	**0.0071**	1	1.874	0.1711	2	7.041	**0.0296**
NU	1	40.66	$\mathbf{1.8\times 10^{-10}}$	1	132.6	$\mathbf{2.2\times 10^{-16}}$	1	171.0	$\mathbf{2.2\times 10^{-16}}$	2	288.7	$\mathbf{2.2\times 10^{-16}}$
temp(T)	1	0.18	0.6732	1	20.15	$\mathbf{7.2\times 10^{-6}}$	1	57.03	$\mathbf{4.3\times 10^{-14}}$	1	48.41	$\mathbf{3.5\times 10^{-12}}$
MT:NU	1	17.38	$\mathbf{3.1\times 10^{-5}}$	1	78.50	$\mathbf{2.2\times 10^{-16}}$	1	2.71	0.0997	4	101.2	$\mathbf{2.2\times 10^{-16}}$
MT:T	1	18.39	$\mathbf{1.8\times 10^{-5}}$	1	1.45	0.2285	1	15.77	$\mathbf{7.1\times 10^{-5}}$	2	16.51	$\mathbf{2.6\times 10^{-4}}$
NU:T	1	3.23	0.0723	1	70.44	$\mathbf{2.2\times 10^{-16}}$	1	15.24	$\mathbf{9.5\times 10^{-5}}$	2	62.91	$\mathbf{2.2\times 10^{-14}}$
MT:NU:T	1	11.82	**0.0006**	1	1.839	0.1751	1	2.761	0.0966	4	40.30	$\mathbf{3.7\times 10^{-8}}$
EB II		**DE**			**DF**			**EF**			**DEF**	
	Df	**Dev**	p	**Df**	**Dev**	p	**Df**	**Dev**	p	**Df**	**Dev**	p
MT	1	15.51	$\mathbf{8.2\times 10^{-5}}$	1	10.55	**0.0012**	1	1.458	0.2273	2	11.51	**0.0032**
NU	1	66.97	$\mathbf{2.8\times 10^{-16}}$	1	34.21	$\mathbf{4.9\times 10^{-9}}$	1	35.74	$\mathbf{2.3\times 10^{-9}}$	2	130.5	$\mathbf{2.2\times 10^{-16}}$
temp(T)	1	29.32	$\mathbf{6.1\times 10^{-8}}$	1	87.20	$\mathbf{2.2\times 10^{-16}}$	1	40.59	$\mathbf{1.9\times 10^{-10}}$	1	200.5	$\mathbf{2.2\times 10^{-16}}$
MT:NU	1	2.596	0.1071	1	23.32	$\mathbf{1.4\times 10^{-6}}$	1	13.11	**0.0003**	4	76.41	$\mathbf{1.0\times 10^{-15}}$
MT:T	1	31.34	$\mathbf{2.2\times 10^{-8}}$	1	9.333	**0.0023**	1	19.69	$\mathbf{9.1\times 10^{-6}}$	2	23.56	$\mathbf{7.7\times 10^{-6}}$
NU:T	1	0.811	0.3678	1	19.71	$\mathbf{9.0\times 10^{-6}}$	1	62.83	$\mathbf{2.3\times 10^{-15}}$	2	31.95	$\mathbf{1.2\times 10^{-7}}$
MT:NU:T	1	149.9	$\mathbf{2.2\times 10^{-16}}$	1	15.07	**0.0001**	1	36.52	$\mathbf{1.5\times 10^{-9}}$	4	173.4	$\mathbf{2.2\times 10^{-16}}$
EB III		**LM**			**LN**			**MN**			**LMN**	
	Df	**Dev**	p	**Df**	**Dev**	p	**Df**	**Dev**	p	**Df**	**Dev**	p
MT	1	1.085	0.2976	1	28.27	$\mathbf{1.1\times 10^{-7}}$	1	6.41	**0.0114**	2	18.83	$\mathbf{8.1\times 10^{-5}}$
NU	1	238.3	$\mathbf{2.2\times 10^{-16}}$	1	56.36	$\mathbf{6.0\times 10^{-14}}$	1	94.99	$\mathbf{2.2\times 10^{-16}}$	2	522.3	$\mathbf{2.2\times 10^{-16}}$
temp(T)	1	313.8	$\mathbf{2.2\times 10^{-16}}$	1	18.26	$\mathbf{1.9\times 10^{-5}}$	1	225.9	$\mathbf{2.2\times 10^{-16}}$	1	266.4	$\mathbf{2.2\times 10^{-16}}$
MT:NU	1	8.779	**0.0030**	1	17.70	$\mathbf{2.6\times 10^{-5}}$	1	16.53	$\mathbf{4.8\times 10^{-5}}$	4	55.97	$\mathbf{2.0\times 10^{-11}}$
MT:T	1	32.16	$\mathbf{1.4\times 10^{-8}}$	1	27.78	$\mathbf{1.4\times 10^{-7}}$	1	1.581	0.2087	2	37.28	$\mathbf{8.0\times 10^{-9}}$
NU:T	1	0.115	0.7341	1	2.159	0.1418	1	125.2	$\mathbf{2.2\times 10^{-16}}$	2	106.3	$\mathbf{2.2\times 10^{-16}}$
MT:NU:T	1	28.06	$\mathbf{1.2\times 10^{-7}}$	1	50.24	$\mathbf{1.4\times 10^{-12}}$	1	1.9	0.1681	4	126.0	$\mathbf{2.2\times 10^{-16}}$
EB IV		**OP**			**OQ**			**PQ**			**OPQ**	
	Df	**Dev**	p	**Df**	**Dev**	p	**Df**	**Dev**	p	**Df**	**Dev**	p
MT	1	20.38	$\mathbf{6.3\times 10^{-6}}$	1	78.53	$\mathbf{2.2\times 10^{-16}}$	1	87.32	$\mathbf{2.0\times 10^{-16}}$	2	200.8	$\mathbf{2.2\times 10^{-16}}$
NU	1	9.33	**0.0023**	1	103.0	$\mathbf{2.2\times 10^{-16}}$	1	327.1	$\mathbf{2.0\times 10^{-16}}$	2	447.3	$\mathbf{2.2\times 10^{-16}}$
temp(T)	1	45.95	$\mathbf{1.2\times 10^{-11}}$	1	2.752	0.0971	1	0	0.9581	1	17.14	$\mathbf{3.5\times 10^{-5}}$
MT:NU	1	7.112	**0.0077**	1	0.01	0.9197	1	128.7	$\mathbf{2.0\times 10^{-16}}$	4	148.3	$\mathbf{2.2\times 10^{-16}}$
MT:T	1	0.061	0.8051	1	57.04	$\mathbf{4.3\times 10^{-14}}$	1	1.42	0.2339	2	61.24	$\mathbf{5.0\times 10^{-14}}$
NU:T	1	0.299	0.5844	1	6.415	**0.0113**	1	5.57	**0.0183**	2	2.32	0.3130
MT:NU:T	1	2.017	0.1555	1	0.134	0.7144	1	0.48	0.4900	4	23.16	**0.0001**

The results of ANOVA on the percentage of males from all pairwise and whole block comparisons are presented in Table 11. The results on percentage of males indirectly reflect influence of sex interacting with other factors on egg-to-adult viability. None of the factors modelled for the EB I were significant for this fitness component, both in pairwise comparisons inside the first block and when modelling the block as a whole. Mitochondrial haplotype was significant in three out of the nine remaining pairwise comparisons in the latter three EBs. The nuclear background showed influence on the proportion of males in two out of three comparisons for each of the III, and IV EBs. The temperature conversely,

showed an effect on the proportion of males in two out of three pairwise comparisons inside EBs II and III. Out of the interaction terms, mito-nuclear interaction proved to be most influential as it was significant in five out of twelve comparisons, with mtDNA × temp being significant only in two comparisons within the second and fourth EB. The three-factor interaction term was significant in three out of six pairwise comparisons within EBs III and IV and none in the first two EBs.

Table 11. The effect of mitochondrial haplotype (MT), nuclear genetic background (NU), temperature (T) and their interactions on the percentage of males for three pairwise comparisons and the experimental block (EB) analysed as a whole for each of the four experimental blocks. DF, degrees of freedom; Dev, deviance; p values that are significant at $p < 0.05$ are given in bold.

EB I		**AB**			**AC**			**BC**			**ABC**	
	Df	**Dev**	p	**Df**	**Dev**	p	**Df**	**Dev**	p	**Df**	**Dev**	p
MT	1	0.02	0.8808	1	0.04739	0.8277	1	1.33724	0.24750	2	0.36	0.834
NU	1	0.00	0.9872	1	2.74073	0.0978	1	0.00149	0.96920	2	1.12	0.571
temp(T)	1	0.30	0.5828	1	0.09581	0.7569	1	0.00376	0.95110	1	0.24	0.626
MT:NU	1	0.01	0.9396	1	0.48097	0.4880	1	0.30348	0.58170	4	2.47	0.651
MT:T	1	0.09	0.7636	1	1.0408	0.3076	1	0.0083	0.92740	2	0.16	0.922
NU:T	1	1.78	0.1825	1	0.48283	0.4871	1	0.67503	0.41130	2	0.35	0.841
MT:NU:T	1	1.92	0.1659	1	0.06717	0.7955	1	1.32005	0.25060	4	5.53	0.237
EB II		**DE**			**DF**			**EF**			**DEF**	
	Df	**Dev**	p	**Df**	**Dev**	p	**Df**	**Dev**	p	**Df**	**Dev**	p
MT	1	4.51	**0.03379**	1	1.6082	0.20470	1	0.1452	0.70314	2	12.25	**0.002**
NU	1	0.62	0.43183	1	0.1475	0.70100	1	2.5504	0.11027	2	28.76	$\mathbf{6\times 10^{-7}}$
temp(T)	1	7.31	**0.00686**	1	1.6117	0.20430	1	15.36	$\mathbf{8.9\times 10^{-5}}$	1	0.31	0.578
MT:NU	1	1.09	0.29647	1	0.0122	0.91210	1	4.9746	**0.02572**	4	19.09	**0.001**
MT:T	1	4.97	**0.02586**	1	15.2255	$\mathbf{9.5\times 10^{-5}}$	1	0.5121	0.47425	2	0.74	0.691
NU:T	1	8.00	**0.00468**	1	0.2847	0.59360	1	10.6933	**0.00108**	2	5.59	0.061
MT:NU:T	1	1.10	0.29332	1	0.5458	0.46000	1	0.3765	0.53949	4	6.17	0.187
EB III		**LM**			**LN**			**MN**			**LMN**	
	Df	**Dev**	p	**Df**	**Dev**	p	**Df**	**Dev**	p	**Df**	**Dev**	p
MT	1	0.00	0.97074	1	0.3288	0.56638	1	12.92	**0.00032**	2	11.7	**0.003**
NU	1	472.89	$\mathbf{2.2\times 10^{-16}}$	1	0.0007	0.97957	1	633.78	$\mathbf{2.2\times 10^{-16}}$	2	1125.3	$\mathbf{2\times 10^{-16}}$
temp(T)	1	8.40	**0.00375**	1	2.7945	0.09459	1	4.59	**0.03210**	1	5.5	**0.019**
MT:NU	1	15.68	$\mathbf{7.5\times 10^{-5}}$	1	3.479	0.06215	1	23.35	$\mathbf{1.4\times 10^{-6}}$	4	33.1	$\mathbf{1\times 10^{-6}}$
MT:T	1	0.35	0.55523	1	0.8008	0.37085	1	3.57	0.05889	2	1.0	0.610
NU:T	1	8.54	**0.00348**	1	1.3167	0.25119	1	13.88	**0.00019**	2	9.3	**0.010**
MT:NU:T	1	14.50	**0.00014**	1	0.0018	0.96654	1	5.64	**0.01751**	4	16.9	**0.002**
EB IV		**OP**			**OQ**			**PQ**			**OPQ**	
	Df	**Dev**	p	**Df**	**Dev**	p	**Df**	**Dev**	p	**Df**	**Dev**	p
MT	1	35.30	$\mathbf{2.8\times 10^{-9}}$	1	3.409	0.06486	1	0.0482	0.82617	2	2.20	0.334
NU	1	90.58	$\mathbf{2.2\times 10^{-16}}$	1	7.96	**0.00478**	1	0.4858	0.48582	2	188.33	$\mathbf{2\times 10^{-16}}$
temp(T)	1	1.13	0.28824	1	2.405	0.12093	1	0.5486	0.45890	1	0.68	0.409
MT:NU	1	66.80	$\mathbf{3.0\times 10^{-16}}$	1	102.83	$\mathbf{2.2\times 10^{-16}}$	1	0.9611	0.32692	4	168.34	$\mathbf{2\times 10^{-16}}$
MT:T	1	39.46	$\mathbf{3.3\times 10^{-10}}$	1	44.23	$\mathbf{2.9\times 10^{-11}}$	1	0.1021	0.74928	2	27.32	$\mathbf{1\times 10^{-6}}$
NU:T	1	0.48	0.49005	1	140.156	$\mathbf{2.2\times 10^{-16}}$	1	4.3152	**0.03777**	2	31.88	$\mathbf{1\times 10^{-7}}$
MT:NU:T	1	11.23	**0.00081**	1	0.674	0.41178	1	3.7143	0.05395	4	169.68	$\mathbf{2\times 10^{-16}}$

When analysing EBs as a whole (comparing all three haplotypes per EB), more statistical power does not result in more statistical significance as we obtain the same results.

Mitochondrial haplotype was significant only in II and III EB, while the temperature was only in III. The nuclear background was still significant in EBs II, III and IV, as well as mtDNA × nuDNA. Temperature × mtDNA interaction was still only significant in IV EB, and the three-factor interaction influenced the proportion of males in III and IV EB only.

4. Discussion

We chose haplotypes for this experiment based on the differences in the *Cyt b* gene. One of our goals was to see if there are principal differences between the number and type (synonymous vs. nonsynonymous) of polymorphisms and the degree of fitness differences achieved by bearers of different mito-nuclear combinations.

First, we wanted to examine whether crossing lines with different *Cyt b* 828A > G variants, a nonsynonymous substitution that divides two large groups of haplotypes in *D. obscura* [56], will result in a lower relative fitness. This was completely confirmed in EB I where we compared MNIL A which has G on bp 828 to MNILs B and C which have A, additionally, B and C have six synonymous mutations between them. Practically all fitness components in this EB (except pct. of males that had no factor significant in EB I) showed that the greater the difference in mtDNA sequence the greater the significance and impact on MNILs relative fitness. Comparison with the most different haplotypes (A and C) had both the mtDNA and mtDNA × nuDNA interactions as significant in the largest number of components assayed and the most similar haplotype comparison (B and C) had those factors as significant in the fewest components tested.

Contrary to these results, in other EBs fitness differences between haplotypes with aforementioned 828A > G, and other substitutions were not as correlated with the magnitude and type of sequence variation. Although we obtained predictable results in some components tested both for mtDNA and cytonuclear interaction effects, it was nowhere near the uniformity seen in the first EB, where it was almost as a rule. For some components, synonymous mutations proved more influential on fitness than nonsynonymous ones. Moreover, in some pairwise comparisons, combinations of more distant haplotypes showed greater relative fitness than combinations of close haplotypes.

It has been known for some time that even synonymous mutations have fitness consequences, which may sometimes be greater than nonsynonymous ones [64]. It is also generally assumed that the greater the number of mutations the bigger the phenotypic differences between the MNILs, but that may not always be the case. The discrepancy in our work between different MNILs could be because the lines we used were sequenced only for the *Cyt b* gene, and all other differences between mitochondrial DNA were not known. Thus, what looks more similar or more divergent when we look only in *Cyt b* haplotypes may not be the case for the whole mitochondrial genome. In our experiment, we had the same haplotype pairwise comparison within the third and fourth EB, as LN and OP are the same combinations of *Cyt b* gene haplotypes but coming from different populations. In almost all the components tested, we had contrasting results when comparing these two sets of identical haplotype comparisons. Apart from all the differences outside the *Cyt b* gene that were not screened in our work, these MNILs, with the same *Cyt b* haplotype originate from two different populations, and in turn should have completely different nuclear genetic backgrounds. This makes their comparisons difficult and further explains the discrepancy in our results.

During the backcrossing procedure that preceded the experiments, in IFL M we noted an unusually high percentage of females, while in O IFL, the observed portion of females was slightly elevated. The effect was apparent enough that we had difficulties collecting half as many M male flies, for the crossing procedure, as only about 5% hatched individuals were male. This effect is associated with the nuclear genome since the sex ratio experiment showed that this distortion is present only in MNILs that had M nuclear background. Maternally transmitted microorganisms can be excluded as a factor that causes this distortion by male killing or feminization, since it is not associated with a particular mtDNA haplotype which is expected to be transmitted jointly with maternally transmitted

microorganisms. In addition, known maternally transmitted organisms were excluded with molecular genetic techniques including microbiome sequencing [56]. Compared to other MNILs from the third EB, MNILs with M nuclear background did not show lower viability that could be caused by embryonic lethality in males. Rather, at 15 °C, they showed much higher EtA viability indicating the exclusion of Y chromosomes before fertilization.

This sex ratio distortion (SRD), in particular MNILs, which is associated with nuclear genetic background is probably caused by a meiotic drive mechanism. This sex ratio distortion is frequently found in *Drosophila* [65]. The first-ever case of the meiotic drive has been documented in *D. obscura* [66]. Gershenson found sex distortion in two out of nineteen IFLs formed by females collected in the wild. Studious experiments on deviations in the percentage of males that hatched from crossing different *D. obscura* lines led him to a conclusion that a gene localized on the X chromosome prevents the genesis of functional spermatozoa without X chromosome. The meiotic drive has been confirmed in a broad range of phyla, and papers on different *Drosophila* species showed that there are more than a few molecular mechanisms for it to cause SRD [67,68].

Although the majority of MNIL with M nuclear background had around 5% of males eclosed in viability experiment, MM MNIL, scored a twofold increase (12%) at a lower temperature. This observation is in line with the findings that sex ratio distortion is extremely temperature-sensitive, as spermatogenesis in higher temperatures results in a higher percentage of X chromosome bearing sperm [69,70]. Although the sex ratio was initially skewed in some MNILs, probably due to meiotic drive, our experimental design enabled us to capture and quantify the effects that mtDNA and nuDNA had on the survival of hatched individuals of a specific sex even in these lines with intrinsic skewed sex ratio.

This study supports a growing body of evidence of non-neutrality of mitochondrial DNA variation [26,71,72], and more importantly, our results give weight to the adaptive significance of intra-population variation in mtDNA [51,73,74]. As we hypothesized, almost all of our models for different fitness components showed that mito-nuclear interactions are more important as units for selection to act on than mitochondrial haplotypes on their own, as our results suggest. This should come as no surprise, considering that the *Cyt b* gene is part of the respiratory complex III, which includes subunits coded by both genomes. In addition, as noted previously, haplotypes probably have differences in genes that comprise the other three of four complexes that have subunits coded by both genomes. If an mtDNA haplotype is coupled with a non-matching nuDNA background, a decline in adaptive value is expected, as the subunits from two genomes have to be co-adapted for the optimal energy production in the mitochondria.

As expected, our experimental model identified sex-specific differences in the fitness of bearers of different mtDNA haplotypes. While this effect was significant in seven out of twelve pairwise comparisons in the desiccation experiment, this male-specific mutational load was noticeable in only two comparisons for the developmental time component. This effect was indirectly measured as an effect of mtDNA in the sex ratio component where it was significant in three out of twelve pairwise comparisons. This observation is in line with the mother's curse hypothesis, a phenomenon frequently found while measuring the effects of mtDNA on life history [47,48,50,55]. Due to maternal inheritance of mtDNA, mutations that are disadvantageous only in males, and have no effect or are advantageous to females, cannot be purged by natural selection.

One of our goals was to test whether different combinations of mitochondrial and nuclear genomes show different fitness ranks depending on the sex of the individual. This finding would support the theoretical presumptions that SSS is responsible for maintaining stable sympatric mitochondrial and mito-nuclear variation [45,46]. Experimental support for this type of balancing selection comes from several previous experiments. When analysing cytonuclear interactions between the X chromosome and mitochondrial DNA, Rand [35] observed the action of SSS in *D. melanogaster*. In a viability experiment with 25 mtDNA haplotypes scored on three nuclear genetic backgrounds, Dowling et al. [50] found the interaction of mtDNA × nuDNA × sex significant, but only in the first out of three

repeated EBs. In the second EB, they could not test this interaction due to missing data, but in the third EB and in overall analyses (across EBs), they did not find evidence of sex specificity of mito-nuclear effects on viability. Jelic et al. [52] analysed a series of key life-history traits of *Drosophila subobscura* MNILs made from three sympatric mtDNA haplotypes. They unequivocally found sex-specific effects (mtDNA × nuDNA × sex) in two experimental modules for adult longevity and indirectly in one module (mtDNA × nuDNA) for egg-to-adult viability when analysing the proportion of males hatching [52]. Conversely, they found no evidence of SSS in any of the experimental modules for desiccation resistance. Similarly, using seed beetles *Acanthoscelides obtectus,* Đorđević et al. performed mortality assays and tested mito-nuclear effects on survival [55]. Although they did not obtain significant mtDNA × nuDNA interaction in their models, mtDNA × nuDNA × sex interaction was significant in model for variance in lifespan, but not in two survival models. In their work, they also analysed the effects of mtDNA and nuDNA as well as sex on the activity of METC complexes. Variation in ETC activity was significantly influenced by sex-specific mito-nuclear interactions in METC complexes I and II, contrastingly they did not find this interaction significant in complexes III and IV. In our experimental design, the role of SSS on mito-nuclear variation was scored directly in desiccation resistance and developmental time as a significant effect of interaction between mtDNA × nuDNA × sex. Conversely, a significant interaction between mtDNA × nuDNA for a sex ratio of hatched adults is also an indirect measure of SSS. In our model species, we found the interaction between mito-nuclear genotype and sex to be statistically significant only in two out of twelve pairwise comparisons for developmental time and desiccation resistance experiments each (if desiccation resistance is scored jointly with previously analysed blocks [57]). Signature of SSS was observed in five out of twelve comparisons in the sex ratio experiment. Our findings support theoretical presumptions that SSS is involved in the maintenance of sympatric mtDNA variation.

The aim of this study was also to test whether different mitochondrial haplotypes or combinations of mitochondrial and nuclear genomes show different fitness ranks depending on the experimental temperature. This finding would support the idea that temperature variation may promote stable sympatric genetic variation. Temperature is of key importance in metabolic processes. Numerous papers on the subject point to the particular sensitivity of OXYPHOS enzyme complexes to temperature [54,75–77]. The connection between mtDNA variation and the temperature has been observed as a clinal shift of haplotype frequencies along latitude [78–80] and altitude [81,82]. Additionally, the optimal function of subunits coded by two different genomes may depend on the thermal environment that the reactions are taking place. While some combinations of mtDNA and nuDNA may be supreme in one thermal environment that may not be the case in others.

Using seed beetles Callosobruchus maculatus, Immonen et al. [54] had different mtDNA haplotypes compete on two different experimental temperatures. Their results claim that temperature is influencing mtDNA evolution to some extent, most likely through mito-nuclear interactions. Similarly, another study [22] measuring EtA development time on the same model organism and two temperatures showed the significance of G × G × E interactions. This effect of temperature-specific fitness of MNILs was found once more, [41] using the same experimental lines in another experiment, measuring metabolic rate. Similarly, Rand [83] using Drosophila as a model found that altered dietary or oxygen environments modify the fitness of mito-nuclear haplotypes.

Research that analyses the fitness of sympatric mitochondrial variation in regard to the extrinsic environment including temperature is scarce [14]. For example, Dowling et al. [50] analysed a single panmictic laboratory population and showed that multiple mitochondrial haplotypes can be preserved within it. Their experiment consisted of three repeated measures (three blocks) of the same experiment, and as they suggest relative fitness of the cytonuclear combination is dependent on the environment that they exist in, as they find the effect of the block to be substantial. They attributed this to the unforeseeable heterogeneity of environmental factors across blocks [50]. The effects of temperature or other

environmental factors on fitness ranks of mito-nuclear genotypes between populations suggest [14,22,50] that this mechanism could potentially support sympatric variation as well. Although the haplotypes used by Immonen et al. [54] originate from geographically distant populations, their haplogroups have all been found to segregate sympatrically in the same West African population, which gives more support to the above claims of temperature-specific epistasis.

Data for our model species included temperature as an environmental factor in the experiments, and we showed that the genotype × temperature interaction has a significant effect on *Drosophila* fitness in all components assayed in this work. This effect was especially important in desiccation resistance (if analysed jointly with [57]), and viability experiments, with both mtDNA × nuDNA × temp and mtDNA × temp interactions being highly significant in both pairwise and whole block models. The abundance of this type of interaction in our data supports the presumption based on interpopulation research [22,41,54] that genotype-by-environment interactions are also important for maintaining stable intra-population mtDNA variation in nature [22,41] and compels for further research to be performed on this phenomenon.

Taken together, the fitness assays performed on *D. obscura* show the complexity of maintenance of sympatric mtDNA variation. Different balancing selection mechanisms may operate simultaneously in upholding joint genomic polymorphism in the same model. Our results give more weight to environment-mediated selection compared to SSS. However, the question stands as to what extent these results could be extrapolated to variation in natural habitats. Our experiment was performed on arbitrarily chosen temperatures, compared to the continual and unpredictable variation in nature. While sex is a discrete variable, the temperature is continuous, and different results could have been observed if fitness was compared at other experimental temperatures or other environmental conditions.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/insects13020139/s1, Figure S1: Mean survival times in hours for the desiccation experiment for all combinations of genotypes and sex from the EB I on two experimental temperatures. Figure S2: Mean survival times in hours for the desiccation experiment for all combinations of genotypes and sex from the EB II on two experimental temperatures. Figure S3: Mean survival times in hours for the desiccation experiment for all combinations of genotypes and sex from the EB III on two experimental temperatures. Figure S4: Mean survival times in hours for the desiccation experiment for all combinations of genotypes and sex from the EB IV on two experimental temperatures. Figure S5: Mean developmental times in days for all combinations of genotypes and sex from the EB I on two experimental temperatures. Figure S6: Mean developmental times in days for all combinations of genotypes and sex from the EB II on two experimental temperatures. Figure S7: Mean developmental times in days for all combinations of genotypes and sex from the EB III on two experimental temperatures. Figure S8: Mean developmental times in days for all combinations of genotypes and sex from the EB IV on two experimental temperatures. Figure S9: Mean egg-to-adult viability scores for all combinations of genotypes from the EB I on two experimental temperatures. Figure S10: Mean egg-to-adult viability scores for all combinations of genotypes from the EB II on two experimental temperatures. Figure S11: Mean egg-to-adult viability scores for all combinations of genotypes from the EB III on two experimental temperatures. Figure S12: Mean egg-to-adult viability scores for all combinations of genotypes from the EB IV on two experimental temperatures. Figure S13: Mean proportion of males for all combinations of genotypes from EB I on two experimental temperatures. Figure S14: Mean proportion of males for all combinations of genotypes from EB II on two experimental temperatures. Figure S15: Mean proportion of males for all combinations of genotypes from EB III on two experimental temperatures. Figure S16: Mean proportion of males for all combinations of genotypes from EB IV on two experimental temperatures. Table S1: The effect of mitochondrial haplotype (MT), nuclear genetic background (NU), sex, temperature (T) and their interactions on egg-to-pupa viability (EtP) for three pairwise comparisons and the experimental block (EB) analysed as a whole for each of the four experimental blocks. Table S2: The effect of mitochondrial haplotype (MT), nuclear genetic background (NU), sex, temperature (T) and their interactions on pupa-to-adult viability (PtA) for three pairwise comparisons and the experimental block (EB) analysed as a whole for each of the four experimental blocks.

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Article

Phenology and Potential Fecundity of *Neoleucopis kartliana* in Greece

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Simple Summary: The silver fly *Neoleucopis kartliana* Tanasijtshuk (Diptera, Chamaemyiidae) is the most abundant predator of the giant pine scale (GPS), *Marchalina hellenica* (Hemiptera, Margarodidae), and is considered a major factor in controlling GPS populations in Greece and Turkey. GPS has recently been detected in Australia. While generally not harmful to trees in its area of origin, GPS has a detrimental impact on pine trees in Australia and, therefore, needs to be controlled. As part of an evaluation of the silver fly for importation to Australia where it may be used as a biological control agent against GPS, we studied several aspects of the fly's life history, namely its seasonal occurrence and number of generations per year (phenology), its acceptance of artificial food sources as adult flies, and the number of eggs females produce over their lifetime. We found that the fly has three generations per year and feeds on all life stages of GPS (eggs, nymphs, and adults). Adults readily feed on a mixture of sugar and dry yeast, and females emerge with no or few eggs and develop more as they age.

Abstract: *Neoleucopis kartliana* Tanasijtshuk (Diptera, Chamaemyiidae) is the most abundant predator of the giant pine scale (GPS), *Marchalina hellenica* (Hemiptera, Margarodidae) in Greece. GPS is native to Greece and Turkey, where it is not considered a pest of *Pinus* spp., but a valuable resource for pine honey production. However, its introduction to new areas leads to high population densities of the scale, linked to declines in tree health and insect biodiversity. To assess the potential use of *N. kartliana* for a classical biological control program in Australia, we studied selected life-history traits of the silver fly, namely its phenology in northern Greece, feeding preferences of adult flies on artificial food sources, and potential fecundity of female flies. The silver fly was present in every site in northern Greece studied and was found to have at least three generations per year in this area. The fly's overall sex ratio was 1:1, and adult females emerged with no or few mature eggs in their ovaries, but egg production was exponential until at least the eighth day after emergence. These findings increase our knowledge about the biology of *N. kartliana* and aided in the evaluation of the silver fly as a classical biological control agent against invasive GPS in Australia.

Keywords: Chamaemyiidae; Margarodidae; voltinism; egg development

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1. Introduction

The family Chamaemyiidae (Diptera) is a group of small flies, commonly known as silver flies, whose larvae prey on sternorrhynchous Hemiptera, particularly adelgids,

aphids, mealybugs, scales [1,2], and psyllids [3]. The majority of chamaemyiids have one to three generations [4] and inhabit grassland, lowland, and montane habitats [5]. Larvae feed on adult soft-bodied hemipteran species, as well as on their nymphs and eggs [6], and they either pupariate on twigs and branches where their prey is found [7] or drop from the tree and pupariate in the soil [8]. Regarding the oviposition in Diptera, factors such as environmental temperature [9], quality and quantity of larval [10] and adult diet [11–13], mating [14], adult population density [9,12], age [13,15], photoperiod [16], and relative humidity [17] affect female egg production. Several Chamaemyiidae species have been successfully utilized in classical biological control programs throughout the world, e.g., Hawaii [18], New Zealand [19], and Chile [20,21]. Despite the potential of Chamaemyiidae as biological control agents against soft-bodied hemipteran species, the family has been understudied, and the biology and morphology of many species are not adequately described [1].

Neoleucopis kartliana Tanasijtshuk (Diptera, Chamaemyiidae) has been successfully used as a biological control agent against giant pine scale (GPS), *Marchalina hellenica* (Gennadius) (Hemiptera, Margarodidae) on the island of Ischia (Italy) [22]. GPS is a univoltine sap-sucking insect native to the eastern Mediterranean region, particularly Greece and Turkey. The scale feeds on *Pinus* spp., especially *P. brutia* and *P. halepensis*, but it can also infest *Abies cephalonica* Loudon (Pinales: Pinaceae) [23]. In its native range, it is considered an economically important insect for the apiculture industry rather than a major pest of *Pinus* spp., since it rarely causes tree mortality [24,25]. GPS excretes a sweet, glutinous substance called honeydew, which is collected and converted by bees into pine honey and represents 60–65% of the annual honey production in Greece [23,26]. Due to its importance to apiculture, GPS has been deliberately introduced to new areas of Greece and to the Italian island of Ischia [27], where, on several occasions, it became a pest, reaching high population densities associated with the decline in tree health and reduction in insect biodiversity on pines [25]. In late 2014, GPS was detected in Australia (Melbourne and Adelaide) on a novel host: the North American species *Pinus radiata* D. Don (Pinaceae), which represents 74.5% of the nation's softwood plantation estate [28]. Since its discovery, GPS population densities have increased dramatically, causing significant damage to untreated *P. radiata* in urban and peri-urban settings and threatening the pine forest industry of Australia [29]. The combination of GPS invading a novel environment without its natural enemies and the availability of suitable host trees increases the likelihood of GPS damaging susceptible trees and plantations if not controlled.

Recent research on the scale's natural enemy complex has shown that the silver fly *N. kartliana* is the most abundant predator among the natural enemies of GPS in its native range [29,30], suggesting the potential of *N. kartliana* as a classical biological control agent in Australia [29,31]. The species was previously studied by Gaimari et al. [32], who presented an extensive description of the morphology and biology of the silver fly in southern Greece. Here, we add to the knowledge about the species by (1) investigating the phenology of *N. kartliana* in northern Greece and (2) presenting novel data on the egg development in female flies (egg load or potential fecundity).

2. Materials and Methods

To study the phenology and occurrence of *N. kartliana* in northern Greece, we collected GPS-infested pine tree twigs and branches every 7–10 days between 6 November 2019 and 21 October 2021 from Kedrinos Lofos in Thessaloniki (57 sampling repetitions). No sampling took place between 4 March and 20 May 2020 because of the closure of the laboratory due to the COVID-19 pandemic. Additionally, to investigate the presence of the fly in different regions, we collected infested twigs and branches from eight sites in northern Greece: Stratoni-Stratoniki, Parthenonas, Katerini-Makriyalos, Pyrghetos-Tempi, Edessa (2–3 sampling repetitions each), Arnea, Alexandroupoli, and Thassos (1 sampling repetition each) (Figure 1). The samples were then transferred to the Laboratory of Forest Entomology (Forest Research Institute, Hellenic Agricultural Organization Demeter) at Vassilika

(Thessaloniki, Greece), where random samples of GPS-infested twigs were examined under a stereoscope Zeiss Stemi 508 (Zeiss, Oberkochen, Germany, magnification range 6.3–50×) to determine and count all silver fly and GPS stages present on the twig. The silver fly's developmental stages were evaluated according to the descriptions of Gaimari et al. [32] and the life stage of GPS according to those of Hodgson and Gounari [33]. Additionally, any species found on the infested branches were collected and kept in ethanol for future identification to further contribute to the description of the natural enemy complex of GPS. For a graphical analysis of GPS and *N. kartliana* phenology, the number of individuals per developmental stage and species was calculated as a percentage relative to other stages (n = minimum 100 for GPS).

Figure 1. Sampling locations for *Neoleucopis kartliana* in northern Greece.

For the investigation of selected life-history traits of adult *N. kartliana*, all remaining GPS-infested branches potentially containing *N. kartliana* were transferred to well-ventilated cages (60 × 60 × 60 cm) that were placed inside a climate chamber Termaks KB8400F (Termaks, Bergen, Norway) set to 23 °C and 60% relative humidity. In order to resemble the conditions from dusk to dawn, the climate chamber had a 16:8 h light:dark photoperiod with a gradual transition (lasting one hour) from 0% light to 100% light, and vice versa. The cages were inspected every 1–2 days in search of any *N. kartliana* adults.

To determine the overall sex ratio, emerging *N. kartliana* adults were individually collected in small falcon tubes (5.5 cm length and 1.5 cm diameter), and their sex was identified by visual inspection of their genitalia according to the descriptions provided by Gaimari et. al. [32] using a stereoscope (Zeiss Stemi 508, magnification range 6.3–50×).

To study the acceptance of artificial food sources as substitutes for GPS honeydew, which was presumed to be their natural food source [30] in the manner of other Chamaemyiid species [34], adults (n = 270) were gradually transferred to smaller cages (30 × 30 × 30 cm, mean number of adults per cage 15 ± 5) between 12 August and 5 October 2020. These individuals were provided with water (through a constantly soaked cloth strip laid loosely on a vial) and five different media simultaneously: (1) pine honey; (2) pine honey mixed with dry yeast diluted in water (2 mL:1 gr:100 mL); (3) water-diluted

pasteurized milk (50:50) provided through soaked cotton on a petri dish (8.5 cm diameter); (4) dry yeast diluted with sugar; and (5) raw, moist yeast mixed with sugar. Artificial food sources (4) and (5) were both provided in different rates (5–50%) and different liquidity states on cotton laid over petri dishes. All food sources were renewed every 2–3 days, and the behavior of the flies was observed twice per day (morning and noon) every 1–2 days. Cotton was used as a substrate for all artificial food sources to resemble the cotton-like wax excreted by GPS under which the honeydew is naturally produced.

To investigate the development of eggs in the ovaries of adult *N. kartliana* females over time (often called egg load or potential fecundity), branch samples from Kedrinos Lofos (Thessaloniki) were placed in cages and positioned near a natural light source for at least two hours. Thus, emerging flies were attracted to the light source, promptly collected in small falcon tubes (5.5 cm length and 1.5 cm diameter), and isolated in small containers (7 cm height and 5 cm diameter) in which they were provided with water and artificial food source (4) (see paragraph above for more details on the artificial food sources). The containers were placed in a climate chamber with the conditions as described above for infested branches. After 3, 6, or 8 days of rearing, females were killed by placing them into 99% ethanol for several minutes. Flies were then dissected under a microscope, and the eggs were counted either immediately after emergence (n = 25), or 3 (n = 35), 6 (n = 34), and 8 (n = 38) days after emergence. To be considered mature, eggs had to carry the stripe pattern typically visible on oviposited eggs [32], which was visible at 40× magnification, confirming that the eggshell was fully developed.

The influence of female age on the number of mature eggs in the ovary was analyzed using a negative binomial generalized linear model fitted with the *nb.glm* function of the MASS package [35] in R [36]. Female age was taken as a continuous independent variable and the number of eggs as the dependent variable. The Poisson distribution was not used because the residuals were overdispersed, as indicated by Pearson's chi-squared test, which was resolved by using a negative binomial distribution.

3. Results

Neoleucopis kartliana was present at all sites (Figure 1) in this study; however, its abundances varied widely between sites and over the season.

The fly was observed in every subadult developmental stage on the branches (eggs, larvae, puparia). Eggs were usually located inside or close to the cotton-like wax produced by GPS. Larvae were spotted either inside the ovisacs of GPS or close to other developmental stages (first, second, and third instar nymphs and adults). Puparia were found either inside the wax of GPS or in bark crevices, without the presence of GPS being necessary.

The data from Kedrinos Lofos (Thessaloniki) (n = 1124 individuals) suggested that, unlike its univoltine prey, the silver fly has three generations per year in northern Greece (Figure 2). The fly's eggs were found during all developmental stages of GPS. However, the graphical analysis of *N. kartliana*'s generations was based on the relative abundance of larvae and puparia only, as they are greater in size and could therefore be more easily detected compared to the eggs. *Neoleucopis kartliana* larvae were observed feeding on all developmental stages of GPS. In the first *N. kartliana* generation, larvae (young and mature) preyed mostly on GPS eggs and adults; in the second fly generation, larvae preyed on the first-instar nymphs of GPS; while in the third fly generation, larvae preyed on the second- and third-instar nymphs of GPS (Figure 2). Although *N. kartliana* larvae did not extensively prey on the third instar nymphs of GPS due to overwintering as puparia, early emerging larvae of the subsequent fly generation (first) were found preying on third instar nymphs of GPS and remained attached to their prey during the scale's ecdysis.

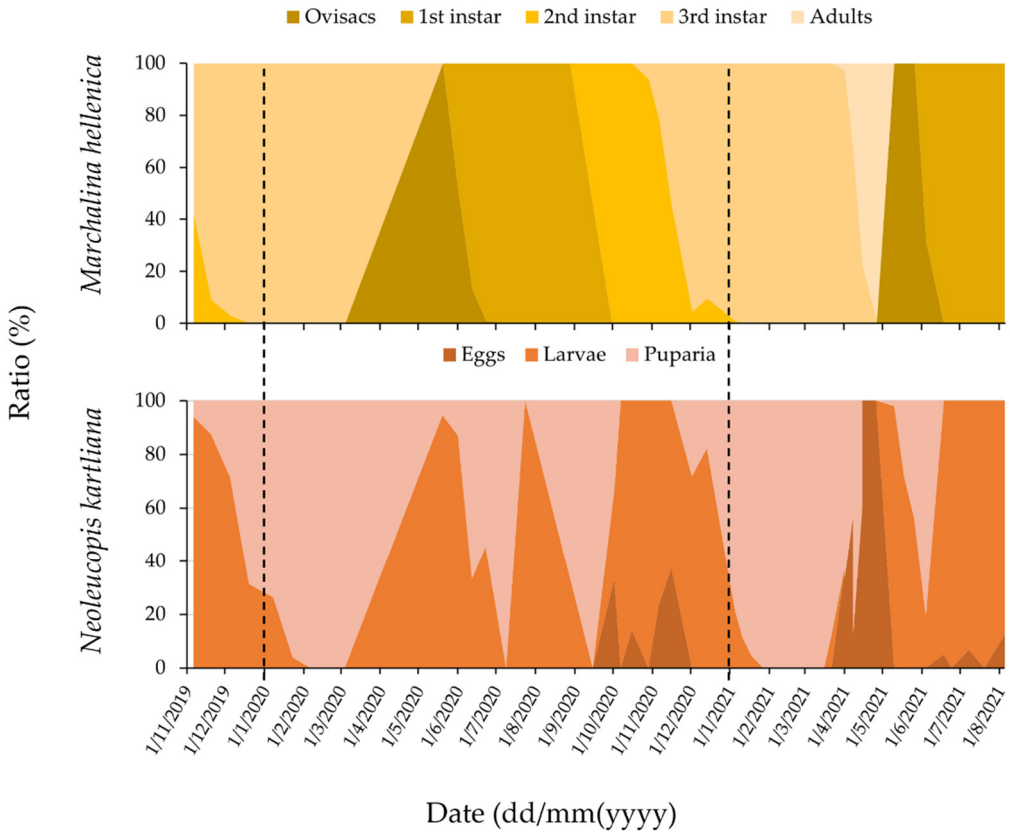

Figure 2. Ratio (%) of different developmental life stages of (upper panel) *Marchalina hellenica* and (lower panel) *Neoleucopis kartliana* in Kedrinos Lofos (Thessaloniki) between November 2019 and August 2021. The area between the two dashed lines is one full year (2020), in which *M. hellenica* underwent one full generation and *N. kartliana* underwent three.

A total of 6031 *N. kartliana* adults were sexed to estimate the overall sex ratio. With 50.8%:49.2% males:females, the sex ratio was almost 1:1, and no apparent difference in the sequence of emergence was observed between the sexes.

Data on the artificial food preference of adults could not be retrieved from this test, because the flies tended to frequently roam inside the cage. However, whenever flies were observed feeding, they were found on only two of the media provided. Adults introduced into the cage containing the different artificial food sources were observed to mainly feed on dry yeast diluted with sugar and, to a lesser extent, on the mixture of honey and yeast. When introduced into the cage, the flies roamed, inspecting the various artificial food sources. However, most flies soon gathered, attached their mouthparts, and fed only on the food sources mentioned above. Adults survived approximately two weeks in captivity with a sole providence of artificial food sources; however, this should not be considered as the fly's lifespan, as it was not estimated individually, but rather in groups of 15 $\pm$ 5 adults.

There was a significant effect of female age on the number of mature eggs found in the ovaries (χ^2 = 112.77; df = 1; $p < 0.0001$). Dissections showed that within 24 h of emergence, females had either zero (n = 21) or one to two (n = 4) eggs in their ovaries. However, until the eighth day after emergence, eggs matured in an exponential manner (Figure 3), and a mean of 25.7 eggs was found, with a maximum of 79 mature eggs found in one female.

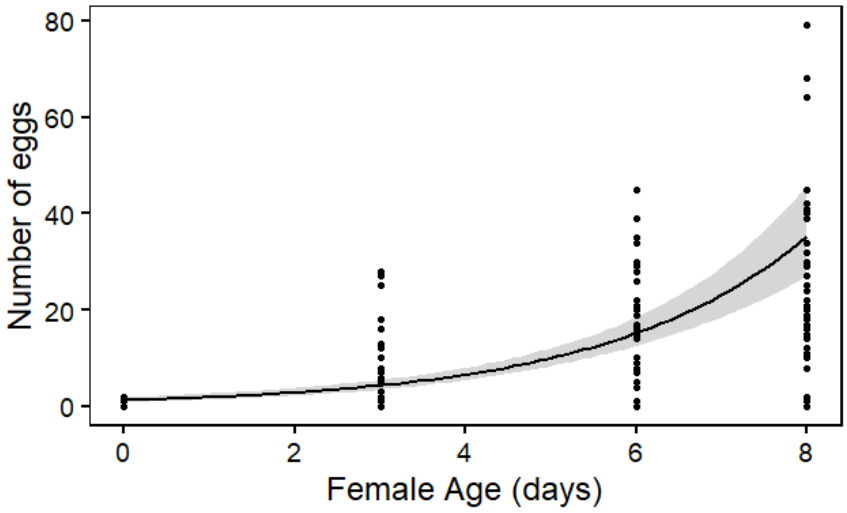

Figure 3. Number of mature eggs in ovaries of *Neoleucopis kartliana* at different ages. The regression line indicates the predictions of the negative binomial generalized linear model that are back-transformed from the log scale. The gray area around the line shows 95% confidence intervals.

4. Discussion

Results from this study support the proposed use of the predatory fly *N. kartliana* as a classical biological control agent to minimize the impact and spread of *M. hellenica* in Australia [17]. The silver fly has a high intrinsic growth rate, allowing it to undergo three generations per year in northern Greece, while its host is univoltine. It seems to prey indiscriminately on every developmental stage of the scale and was found to be present in all sites studied in northern Greece. Furthermore, potential fecundity of the fly was found to increase exponentially in the first eight days after emergence with an average of 25.7 eggs and females holding up to 79 eggs in their ovaries.

N. kartliana appears to have at least three generations per year, confirming the observations of Gaimari et al. [32], who suggested that *N. kartliana* has two to three generations annually. Additionally, other species of the genus *Neoleucopis* have been described to be at least bivoltine, e.g., *N. pinicola* [37] and *N. atratula* [38,39]. Multivoltinism is an attribute that may considerably increase the chances of adaptation to novel environments because it imparts the capability of surviving and reproducing under various environmental conditions [40]. In a study on introduced biological control agents, Crawley et al. [41] found that insects with the highest intrinsic growth rates that typically also had other characteristics of r-selected species (smaller body size and faster maturity resulting in several generations per year) were more likely to establish successfully than those with a slower growth rate. Accordingly, Hokkanen and Sailer [42] suggested that there is a positive correlation between success in biological control and the agent's power of increase over that of the prey, supporting that, in general, successful natural enemies have two generations for every host generation. An example supporting this theory is the parasitoid *Aphytis melinus* (DeBach) (Hymenoptera, Aphelinidae), which was successfully used as a biological control agent against the California red scale, *Aonidiella aurantii* (Maskell) (Hemiptera, Diaspididae), a worldwide pest of citrus [43]. According to Murdoch et al. [43], apart from prey specificity, another key feature leading to the success with this species appears to be the rapid development of *A. melinus* compared to the development of the pest, since the parasitoid has three generations for each scale generation. However, recent research suggests that, when considering the multivoltinism of a biological control agent in a more holistic context of biological control programs, agent-related life-history traits play a rather minor role, compared to those related to how and when agents are released [44]. Nevertheless, we show here that the three generations of *N. kartliana* allow this predator to feed on all life stages of the scale, which would maximize its impact on *M. hellenica* populations. This

finding confirms those of previous studies on the feeding habits of Chamaemyiidae. For example, Satar et al. listed six *Leucopis* species that were observed preying on several developmental stages of aphids in Turkey [45].

We found that adult *N. kartliana* males and females emerged simultaneously, similar to *Leucopis argenticollis* and *L. piniperda* [46] (later both assigned to a new genus, *Leucotaraxis* [47]). Additionally, our data suggest that *N. kartliana* adults follow the Fisherian sex ratio (1:1) [48], which was also found for *N. pinicola* following laboratory rearing of field-collected puparia [37].

Neoleucopis kartliana adults survived for two weeks in captivity, successfully feeding solely on artificial food sources that consisted of water and a mixture dry yeast and sugar, as was also offered successfully to other silver flies [45]. The adults rejected or showed little interest in the alternative artificial food sources (pine honey, pine honey with dry yeast, milk, and a mixture of raw yeast and sugar). Flies generally require both sugar and protein to fully develop their reproductive systems and produce eggs, and different sources of protein can have various effects on longevity and fertility [12,17]. Although the traditional protein source used for dipteran species is milk powder, yeast could replace milk powder without a considerable loss of viability or egg production [12]. Chamaemyiidae flies are known to feed on honeydew produced by their host [49], which is a sugar-rich secretion of aphids and scale insects [50]. Because it is difficult and laborious to keep honeydew-producing scale insects alive on their host plants (especially when they are trees), replacing the natural food source with artificial ones can increase the efficiency of adult rearing. Gaimari and Turner [1] suggested the use of a mixture of honey and yeast as an artificial diet for adult *Leucopis* spp. While a mixture of sugar and yeast was used as an artificial diet for the *N. kartliana* adults in this study, our results do not contradict those of Gaimari and Turner, since *N. kartliana* adults did feed on the diet suggested by the authors but ultimately preferred the mixture of yeast and sugar. The comparative impact of the different food sources on the longevity and fecundity of *N. kartliana* remains to be studied.

Neoleucopis kartliana is oviparous, corresponding with most dipteran species [51]. Our results showed that females emerge with no or very few (one to two) mature eggs in their ovaries. The few eggs that were found in some freshly emerged females may have been developed in the few hours between emergence and dissection. Clearly, most of *N. kartliana*'s eggs mature after its emergence, making it a strongly synovigenic species (i.e., producing eggs throughout its adult life), which are typically relatively long-lived and dependent on external food sources, as shown for parasitoids [52]. The presence of mature eggs in an ovary is considered as the definitive characteristic for female sexual maturity [13], suggesting that there should only be a very short or no pre-mating period after emergence of females. This study showed that, over the span of 8 days, egg load increased exponentially to a mean of 25.7 eggs and a maximum of 79 mature eggs per female. Possibly, the eighth day after the emergence of *N. kartliana* is the transition point between sexually immature and mature female flies, similar to another Chamaemyiid species, *Leucopis palumbii* Rodani, that reaches sexual maturity at 8–10 days after emergence [53]. While this illustrates a promising intergenerational population growth rate, the silver fly's fecundity in nature has not yet been estimated, and it remains unclear if more eggs can be produced over a female's lifetime and if factors such as mating, food source, or environmental temperature affect egg production and, ultimately, realize the fertility of *N. kartliana*. Nevertheless, the maximum of 79 eggs produced by female flies in captivity signifies prospects for an increase in egg production above the current mean of 25.7 eggs if adjusted methods for rearing are practiced.

5. Conclusions

Phenological observations revealed that *N. kartliana* has at least three generations per year in northern Greece and is preying indiscriminately on every developmental stage of the univoltine GPS. According to laboratory observations, the silver fly is oviparous and produces eggs without mating occurrence, and can survive for about two weeks in

captivity when provided with artificial food sources consisting of water, sugar, and dry yeast. Adult females emerge with no or very few mature eggs in their ovaries, after which egg production increases exponentially until at least the eighth day after emergence. This investigation of the silver fly's life-history traits helps to better understand its biology and contribute to its evaluation as a classical biological control agent of the invasive GPS in Australia. However, research on the fly's prey specificity, mating behavior, rearing, as well as definite lifespan and egg load throughout its lifespan remains to be conducted in order to further understand its behavioral ecology and safe use as a biological control agent and to optimize its chances of establishment, as other Chamaemyiid biological control agents have failed to establish in Australia [54].

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Article

Longer mtDNA Fragments Provide a Better Insight into the Genetic Diversity of the Sycamore Lace Bug, *Corythucha ciliata* (Say, 1832) (Tingidae, Hemiptera), Both in Its Native and Invaded Areas

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Simple Summary: The sycamore lace bug (*Corythucha ciliata* Say, 1832) is one of the most abundant and widespread pests on plane trees (*Platanus* spp.) across the globe. The native range of the species is in North America, but it has been introduced to Europe (1964), South America (1985), Asia (1995), Australia (2006), and Africa (2014). To understand the genetic background behind this successful colonisation, we analysed a fragment (1356 bp) of the mitochondrial DNA. The 327 individuals revealed 17 haplotypes forming two separated groups. One group includes North American and Japanese individuals, while the other group comprises North American, European, and Asian individuals. We found a much higher genetic diversity in the native area (North America, 12 haplotypes) than in the invaded areas (Europe, five and Asia, four). The longer DNA fragment provided detailed information about the genetic structure of the species both in its native range and in the invaded areas, but the shorter DNA fragment could not provide a clear link between the genetic variation and the geographic origin.

Abstract: The sycamore lace bug (*Corythucha ciliata* Say, 1832) is of North American origin, but after its introduction to Europe (1964), South America (1985), Asia (1995), Australia (2006), and Africa (2014), it became an abundant and widespread pest on plane (*Platanus* spp.) trees. We analysed a 1356 bp long fragment of the mtDNA (COI gene) of 327 sycamore lace bug individuals from 38 geographic locations from Europe, Asia, and North America. Seventeen haplotypes (17 HTs) were detected. *C. ciliata* populations from North America exhibited higher haplotype diversity (12 HTs) than populations from Europe (6 HTs), Asia (4 HTs), or Japan (2 HTs). The haplotypes formed two haplogroups separated by at least seven mutation steps. One of these mutation steps includes HTs from North America and Japan. Another includes HTs from North America, Europe, and Asia. Haplotypes from Asia Minor, the Caucasus, and Central Asia are linked to haplotypes from Europe, while haplotypes found in Japan are linked to haplotypes found in North America only. The incorporation of published data from the GenBank into our dataset (altogether 517 individuals from 57 locations, but only 546 bp long fragment of the mtDNA) did not show any structure according to the geographic origin of the individuals.

Keywords: *Corythucha ciliata*; mtDNA; COI fragment length; population genetics; phylogeny; invasive insect

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1. Introduction

Understanding the success of an invasive insect species requires knowing the source and pathway(s) of invasion and the spatial distribution of intraspecific diversity [1,2].

Without that knowledge, establishing efficient control measures proves difficult. Several studies conducted in recent decades have investigated various genetic markers to address this concern [3–7].

The native range of the sycamore lace bug (SLB) (*Corythucha ciliata* Say, 1832) is in North America, where its main hosts are *Platanus* spp. [8]. The invasion history of sycamore lace bug across Europe is well documented. The first record was in Italy (1964), followed by a high-speed spread across the continent: 1970, Croatia (ex-Yugoslavia, Zagreb); 1972, Slovenia (Ljubljana); 1973, Serbia; 1975, South France and Switzerland; 1976, Hungary; 1978, Spain; 1982, Austria; 1983, Germany and Czechoslovakia; 1987, Bulgaria; 1990, Romania; 1994, Portugal; 1988, Greece; 1996, Russia; 2005, Moldova; 2006, United Kingdom and Belgium; 2007, Turkey and Ukraine; 2008, Netherlands and Georgia; 2009, Poland; 2011, Macedonia; and 2012, Luxemburg. No continent has remained unaffected: South America, Chile (1985); Asia, South Korea (1995), China (2002), Japan (2003), and Uzbekistan (2017); Australia (2006); and Africa, South Africa (2014) [9–44]. *C. ciliata* has spread via anemochore and antropochore transportation [10–12,17,22,45,46].

Plane trees (taxon names noted according to the Catalogue of Life [47]; *Platanus occidentalis* L., *P. orientalis* L., and *P. hybrida* Brot.), are major host plants [8,48] of *C. ciliata,* but feeding has also been recorded on *Fraxinus* sp., *Morus alba* L., *Broussonetia papyrifera (L.) Vent*., *Carya ovata* (P. Mill.) K. Koch, and *Chamaedaphne* sp. [8]. However, another publication [48] reports other possible host plant species as well (*Quercus laurifolia* L., *Liquidambar sytraciflua* Michx., and *Euphorbia pulcherrima* Willd. Ex Klotzsch), but their list does not include *Broussonetia*, *Carya*, *Chamaedaphne*, or *Fraxinus* as hosts. Torres-Miller [49] only detected it on *P. occidentalis* from West Virginia.

In North America, heavy infestations and damages were reported on ornamental and shade plane trees, especially in the eastern part of the United States [50–53]. Coyle et al. [54] investigated the sycamore lace bug because *P. occidentalis* is an important tree species in the managed hardwood forests of North America. They concluded that the species does not cause considerable damage under traditional forest conditions. The sycamore lace bug was found mainly on *Platanus* species in the invaded areas [11,21,22,41,45,46,55,56]. However, nymphs and adults were also detected on maples (*Acer*) and ash trees (*Fraxinus*) from Georgia [18]. Sycamore lace bug individuals cause aesthetic damage by sucking sap from the leaves, but they may play a role as a vector of various diseases as well (e.g., *Erysiphe platani* (Howe) U. Braun & S. Takam., 2000). Plane trees are widely used as ornamental tree species in the northern hemisphere [57–60].

Imagos overwinter under tree bark. The sycamore lace bug is well adapted to the extreme cold temperatures (−30 °C) [21], but adults can tolerate extreme high temperatures (35–41 °C) as well, [61,62] which allows the species a wide potential distribution area. Ju et al. [63] revealed phenotypic synchronicity between SLB individuals and their host.

Various methods to control SLB have been investigated. Yoon et al. [20]) examined the effect of bistrifluron (chitin synthesis inhibitor), and Pavela et al. [64] applied azadirachtin (active ingredient of the neem tree *Azadirachta indica* A. Juss.) with good results. Several studies assessed the natural enemy complex of SLB [65]. Entomopathogenic fungi (e.g., *Beauveria bassiana* [Bals.-Criv.] Vuill., 1912), nematodes (e.g., *Steinernema* sp., *S. carpocapsae* [Weiser, 1955]) and generalist predatory insects (e.g., *Chrysoperla lucasina* [Lacroix, 1912] [Neuroptera: Chrysopidae]) proved to be possible biological control agents [66–68].

A few genetic studies have been conducted on *C. ciliata* in the last decade. One single individual was analysed with microsatellites markers from China [69]. The gene expression profiles were studied [14,62,70] as a part of a DNA barcode library construction project [71,72]. Kocher et al. [73] published a whole mitogenome compared with the avocado lace bug (*Pseudacysta perseae*, Heidemann 1908) using a genome skimming approach. Yang et al. [69,74] analysed ten populations from China including one outgroup population for Slovenia. There are currently 33 COI fragment data entries for *C. ciliata* in the GenBank [75]. Some preliminary results on *C. ciliata* were published in 2020 [76], but this subset of data incorporates only 22 locations, 117 individuals, and a short fragment

(546 bp) of the COI gene. Further, Besedina et al. [56] analysed 20 individuals of three populations from Krasnodar (Russia) using RAPD-PCR markers and revealed high DNA polymorphism. However, their main conclusion was that there is no genetic difference between the studied populations.

Several studies used a fragment that was longer (>1100 bp) than the barcode fragment of COI. Some examples of this method include the plant bug *Adelphocoris fasciaticollis* Reuter, 1903 (Hemiptera: Miridae) [77], hoverflies (*Merodon* sp., Diptera: Syrphidae) [78], *Anopheles darlingi* Root, 1926 (Diptera: Culicidae) [79], *Scarabaeus* (Coleoptera: Scarabaeidae) [80], and *Pyllonorycter platani* (Staudinger, 1870 (Lepidoptera: Gracillariidae) [7] to reveal the population genetic structure of the investigated insect taxa. Forensic studies use a longer fragment of COI to identify the Diptera species as well [81].

Our aims were (i) to reveal the genetic structure of *Corythucha ciliata* both in its native and invaded area, (ii) to explore the species' phylogeographic pattern across three continents (Europe, Asia, and North America), (iii) to revisit the possible introduction events of the species, and (iv) to reanalyse our data with the already published datasets.

2. Materials and Methods

2.1. Sampling and Molecular Methods

We collected nymphs and imagos from 38 populations of *C. ciliata* from Europe, Central Asia, Japan and North America and one Hungarian population of *Corythucha arcuata* (Say, 1832) (Figure 1, Table S1). All samples were stored in 96% ethanol at 4 °C. DNA was extracted from entire bodies using GenElute Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich), following the manufacturer protocol. Eluted DNA was stored at −20 °C.

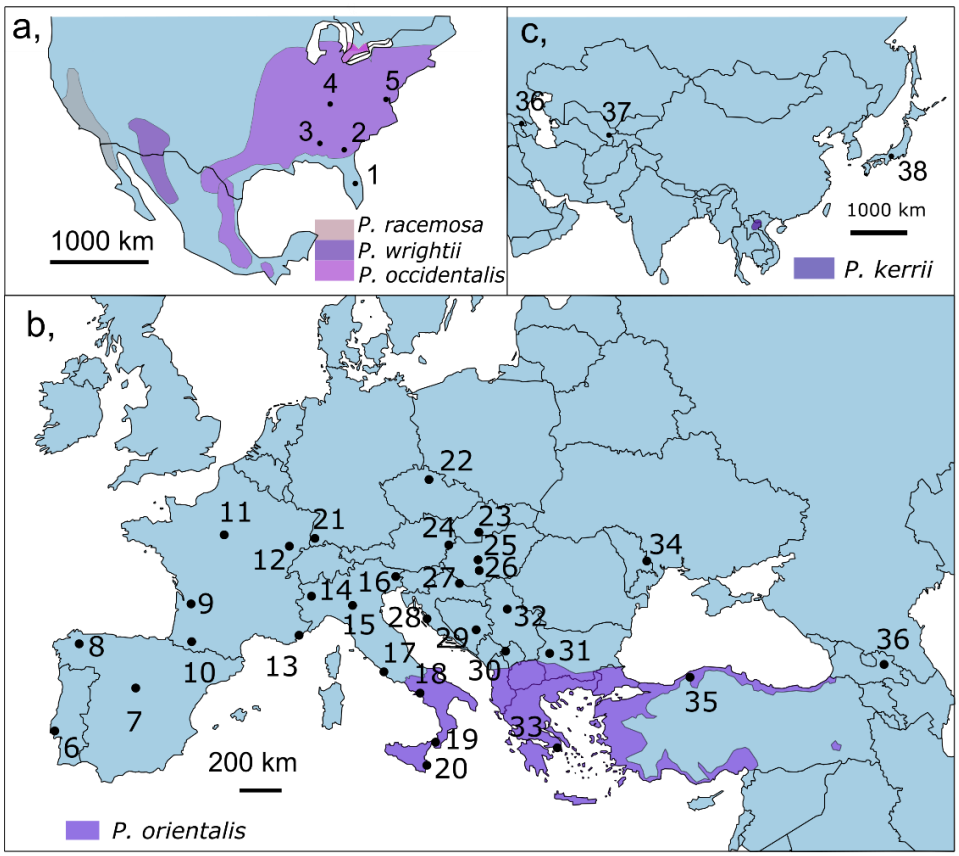

Figure 1. The sampling locations (1–38) of *Corythucha cilata* and the native range of *Platanus* spp. in (**a**) North America, (**b**) Europe, and (**c**) Asia. The native range of *Platanus racemosa* Nutt. ex Audubon, *P. wrightii* S. Wats., *P. occidentalis*, *P. orientalis* and *P. kerrii* Gagnep. are presented based on the map published by Feng et al. [82].

A 1356 bp long region of the COI gene was amplified for 327 individuals by using Pat (5′-TCC AAT GCA CTA ATC TGC CAT ATT A-3′), and LCO1490-J-1514 (5′-GGT CAA ATC ATA AAG ATA TTG G-3′) primers [83,84]. PCR conditions included an initial denaturation step at 94 °C for 2 min, followed by 34 cycles at 94 °C for 30 s, 46 °C for 1 min, and 72 °C for 1 min 30 s with a final extension step that lasted 10 min at 72 °C.

Sequences were generated at the Eurofin's Laboratory (Ebersberg, Germany). All sequences are available at NCBI GenBank (accession numbers OM033605-621).

2.2. Data Analysis

Three hundred and twenty-seven individuals were used for mitochondrial DNA (COI) analyses (Table S1). Sequences were visualized using FinchTV 1.4.0 [85] and then aligned using ClustalX [86]. After haplotypes were identified, those represented by only a single individual were verified by additional sequencing of an independent amplicon. *Corythucha arcuata* (Say, 1832) sequence (OM033622) was used as an outgroup. Genetic distances were estimated using the Kimura 2-parameter and computations were completed in MEGA 5.02 [87].

2.3. Phylogenetic Analyses

We applied jModeltest 2.1.2 [88,89] to select the best model of nucleotide substitution with Akaike Information Criterion (AIC) [90]. Maximum likelihood (ML) analysis was performed under GTR+I model with MEGA 5.02. The level of support for individual nodes was evaluated by bootstrapping with 5000 replicates.

Population structure: Patterns of molecular diversity based on the mtDNA sequences between and within populations were assessed by estimating nucleotide diversity (π) [91], transition/transversion ratio, and haplotype diversity (h) [92,93] using the software Arlequin version 3.5.1.2 [94].

Genetic distances between groups (continents; natural-invaded area) and within groups were estimated using the Kimura 2-parameter and computations were completed in MEGA 5.02 [87].

Demographical expansion: Population dynamics analyses were performed on different geographical scales: overall dataset, between natural and invaded area, within natural and within invaded area, between continents, and within continents, with special emphasis on European populations. Arlequin 3.5.1.2 with 10,000 permutations [94] was used for the estimation of Tajima's D statistics [95] and Fu's Fs [96]. With small sample sizes (as in the case of 546 bp dataset: <60 individuals); we also used DnaSp 5.10 [97] to estimate R2 [98].

Phylogeographical analysis: Spatial analysis of molecular variance (SAMOVA) was performed using SAMOVA v1.0 [99]. The program was run 1023 iterations. K values were tested, starting from two until the value for which FCT reached a plateau [100]. In addition, alternative groups (e.g., natural and invaded area) were tested with Analysis of Molecular Variance (AMOVA) [101–103] with Arlequin 3.5.1.2 [94]. The statistical significance of variance components in AMOVA was tested with 1000 permutations. Statistical parsimony network (SP) [104] was constructed with TCS 1.2.1 [105] and edited using tcsBU [106].

QGIS 2.18.11 [107] was used to project haplotype distributions and frequencies onto maps. Annotations on the maps, phylogenetic trees, and haplotype networks were edited using Inkscape 1.0.2-2 [108].

3. Results

3.1. Long Fragments of the COI Gene

Seventeen haplotypes were detected on the 1356 bp long fragment of the COI gene from 327 individuals from 38 localities (Figure 2, Table S1). The variable sites numbered 26 (1.92%). Approximately half of them were located on the barcoding part of the gene. Haplotypes were differentiated from each other by 1–10 polymorphic sites.

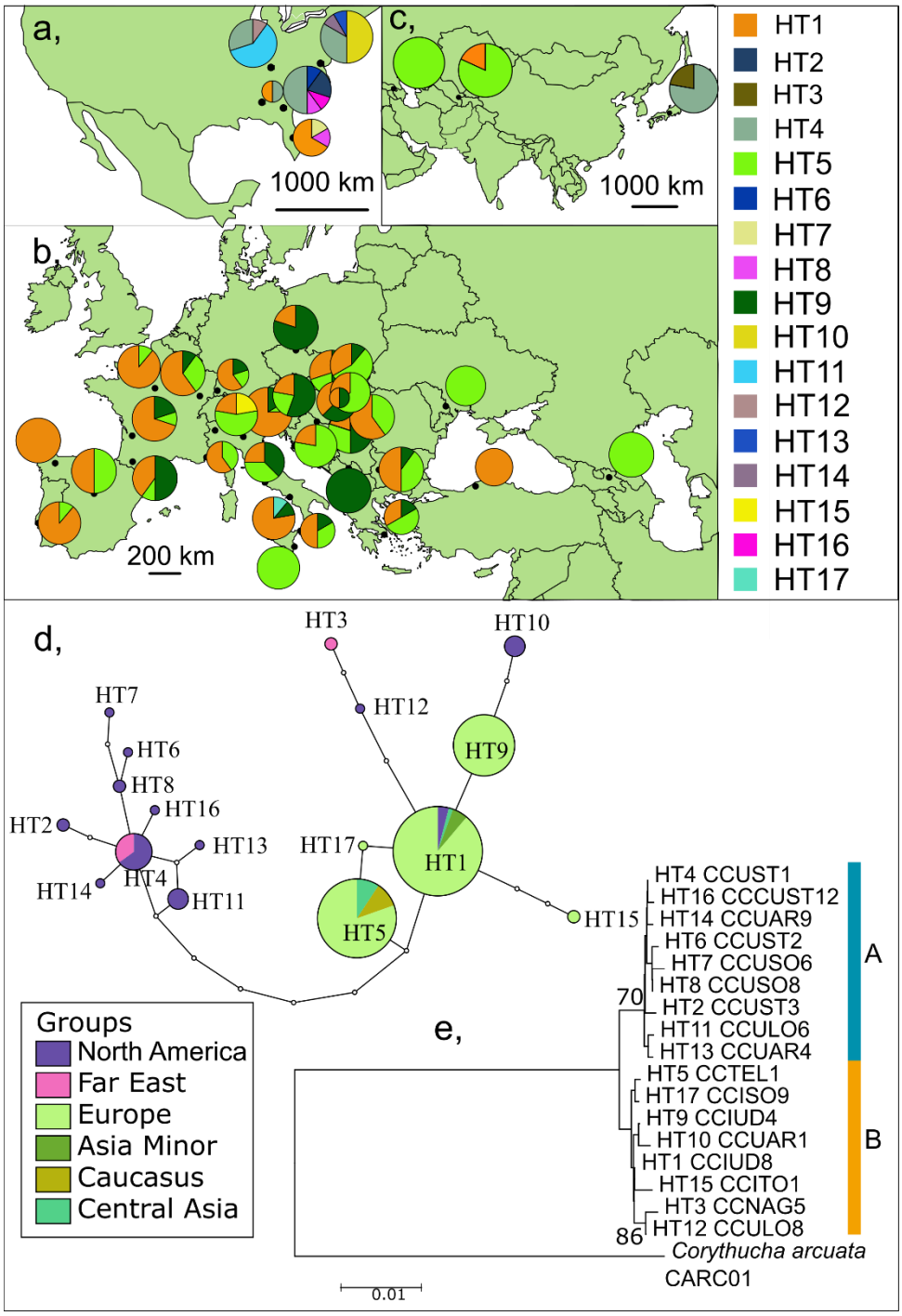

Figure 2. Distribution and phylogenetic relationship of *Corythucha ciliata* haplotypes on the long fragment of COI: (**a**) in North America, (**b**) in Europe, and (**c**) in Asia; (**d**) statistical parsimony networks for all haplotypes (empty circles indicate missing or theoretical haplotypes); and (**e**) ML consensus tree of all COI haplotypes. Bootstrap support values expressed in percentages are indicated near the nodes (>60%).

The haplotypes formed two haplogroups (A and B), which were separated by at least seven mutation steps (Figure 2). The 14 intermediate haplotypes were not present in our data set. The topology of the phylogenetic tree was similar to the haplotype network. Haplogroup A includes nine haplotypes from North America (HT2, 4, 6–8, 11, 13–14, and 16) and one haplotype from Japan (HT4), while haplogroup B includes only three haplotypes from North America (HT1, 10, and 12), five from Europe (HT1, 5, 9, 15, and

17), two from Western and Central Asia (HT1 and 5), and one from Japan (HT3). The most abundant haplotypes are HT1 (38.23% of the total dataset), HT5 (29.66%), and HT9 (17.74%). HT5 and HT9 were only detected from Europe and Central Asia. HT3 is unique from Japan. Most haplotypes were detected from North America only (HT2, HT6–8, HT10–14, and HT16; six of these are singletons). HT4 (6.12%) was found both in Japan and North America. HT15 and HT17 are unique haplotypes from Europe. The average sequence divergence between the haplotypes of the SLB was 0.07–1.04%, much lower than the interspecific divergence between *C. arcuata* and *C. ciliata* 8.49–8.93%.

The genetic distance between populations was 0.00–0.65%; within populations, 0.00–0.52%; and the overall mean distance (TOTAL DATASET) was 0.20%. Overall, haplotype diversity (h) was 0.73, and nucleotide diversity (π) was 0.20% (Table 1).

Table 1. Summary of genetic diversity indices for the long fragment of the COI gene: (n) number of individuals sampled; (No) number of haplotypes; (Nex) number of exclusive haplotypes; (S) number of polymorphic sites; (h) haplotype diversity; (π) nucleotide diversity; (n.r.) not relevant; (E) Europe; (A) Asia; and (NA) North America.

Group	n	No.	Nex	S	ts/tv	h ± SD	π (%) ± SD
Invaded	287	7	4	16	14/2	0.6717 ± 0.0124	0.1196 ± 0.0785
E	250	5	3	6	6/0	0.6542 ± 0.0123	0.0937 ± 0.0655
A	37	4	1	12	10/2	0.6562 ± 0.0555	0.2401 ± 0.1406
Native/NA	40	12	10	21	20/1	0.8462 ± 0.0378	0.3630 ± 0.2005
Total	327	17	n.r.	26	25/2	0.7320 ± 0.0136	0.1945 ± 0.1152

3.1.1. Genetic Diversity and Structure in the Native Range—North America

Altogether 12 haplotypes were detected among the sequences of the 40 specimens collected in North America (five sampling locations). Ten of these haplotypes (HT2, HT6–8, HT10–14, and HT16) were unique. HT4 was the most common (32.50%) and was found in all populations except Orlando (Figure 2, Table S1). This haplotype was also found in Japan. HT1 and HT8 revealed two populations. All the other haplotypes were detected at single locations. Both haplogroups A and B were represented in this continent (Figure 2). Haplotype diversity (h) was 0.85, and nucleotide diversity (π) was 0.36% (Table 1).

Neutrality test results showed that neither Tajima's D nor Fu's Fs values were significant. Mismatch distributions showed multimodal (SSD = 0.041) waves, associated with a constant population size [109,110].

3.1.2. Genetic Diversity and Structure in the Invaded Range

Average sequence divergence between invaded and natural groups (0.46%) was higher than at the intrapopulation level (invaded: 0.12%; natural: 0.35%). The genetic distance within the natural group was nearly three times higher (0.35%) than within the invaded group (0.12%).

Fu's Fs and Tajima's D values were not significant. Mismatch distribution (SSD = 0.020) shows a multimodal shape, which suggests a constant population size [109,110].

Europe

Five haplotypes were detected among the sequences of the 250 specimens collected in Europe (29 locations). Two of these were common (HT1 44.40% and HT5 31.20%) while HT9 (23.20%), HT15 (0.80%), and HT17 (0.40%) were unique for Europe. HT9 was common in the populations from Central Europe and the Balkan Peninsula. Europe is represented in haplogroup B only (Figure 2). Haplotype diversity (h) was 0.65, and nucleotide diversity (π) was much lower than in North America 0.09% (Table 1). Intrapopulation divergence was 0.10% within the European group.

Mismatch distributions show a slightly unimodal (SSD = 0.015) shape for the European dataset. Unimodal distributions are generally associated with a sudden/recent population expansion or bottleneck [109,110].

Asia

Four haplotypes were observed among the 37 specimens collected in Asia (four locations) with one being (HT3) unique to the continent. The population from Japan differs from the other Asian populations unambiguously because HT3 and HT4 were only observed there, while no other Asian or European haplotype was detected there. HT1 (24.32%) and HT5 (51.35%), common in Europe, were found from Asia Minor, the Caucasus and Central Asia, and are included in Haplogroup B. Haplotypes of the Japanese population are present in both haplogroups. Diversity indices are slightly lower than the North American values ($h = 0.66$, $\pi = 0.24\%$).

The results of the neutrality tests (Tajima's D, Fu's Fs, and mismatch distribution) did not provide significant values.

Intrapopulation divergence was two times higher in the Asian (0.22%) group than in the European (0.10%). The Asian group was better differentiated (due to the population from Japan) than the European group.

The FCT values reached a plateau at K = 4 (FCT = 0.702) by SAMOVA, but the four groups did not consist with the geographic distribution.

Results of AMOVA demonstrated that the largest variation (44.64%) occurs among natural and invaded groups (Table 2), while 31.08% of variation occur among populations within groups and 24.28% within populations.

Table 2. Analysis of molecular variance (AMOVA) for the natural and invaded groups of *C. ciliata*, long COI fragment (*** $p < 0.001$).

Groups	Source of Variation	var%	Fixation Indices
Natural	Among groups	Va = 44.64	FCT = 0.446 ***
Invaded	Among populations within groups	Vb = 31.08	FSC = 0.561 ***
	Within populations	Vc = 24.28	FST = 0.757 ***

3.2. Short (Barcoding) Fragments of the COI Gene including Already Published Data

Our data (327 individuals, 38 localities) were supplemented by the results of Yang et al. [74] (190 individuals, 19 localities). The consolidated dataset (517 individuals, 57 localities) contains, altogether, twelve haplotypes on the 546bp long barcoding fragment of the COI gene (Figure 3, Table 3, and Table S1). Yang et al. [74] has described five of these haplotypes; the remaining seven are new. The number of variable sites was 11 in this case (2.01%). The pairwise genetic distances between the haplotypes were 0.18–1.48%.

The haplotype SLB2 was detected in 43.52% of the individuals across the entire invaded area, but it could not be confirmed in the native area. The other common haplotype was SLB5 (37.33%), which was present across all continents. In our samples from Europe (SLB2; SLB5), Asia Minor (SLB5), Caucasus (SLB2), and Central Asia, (SLB2, SLB5) we detected only two haplotypes with various patterns. There were only two, albeit different, haplotypes from Japan (SLB1; SLB3). Yang et al. [74] detected five haplotypes from China (SLB1–5), where only SLB4 was unique. We revealed altogether eight haplotypes from North America (SLB1, SLB5, SLB6–12)—all of the later ones were unique. Neither the ML tree nor the haplotype network supports the existence of well-defined haplogroups on the barcode fragment of COI (Figure 3). We observed a moderate value of the haplotype diversity indices and a low value of the nucleotide diversity in the short fragment of COI ($h = 0.66$, $\pi = 0.26\%$) (Table 3).

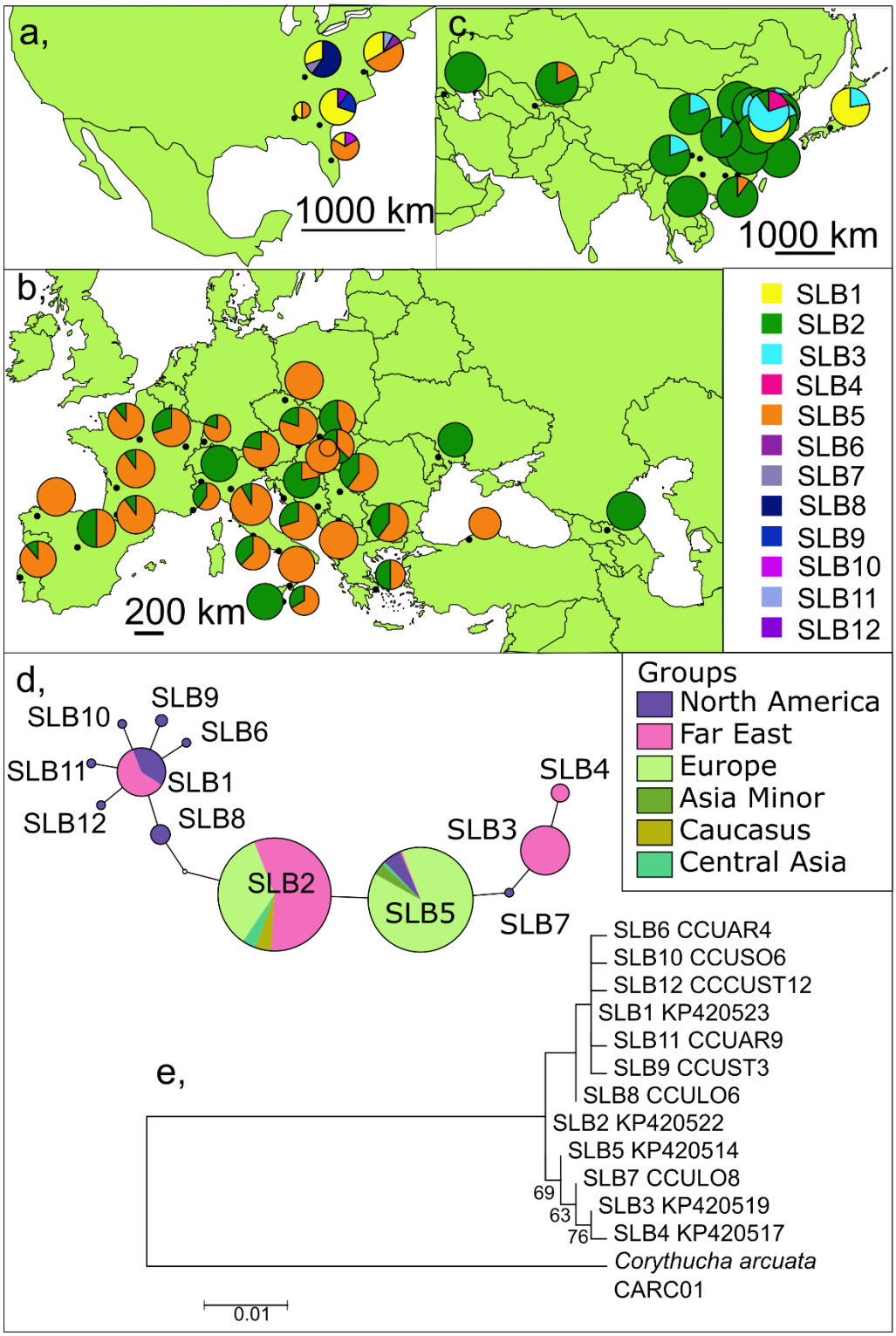

Figure 3. Distribution phylogenetic relationship of *Corythucha ciliata* mitochondrial haplotypes on the barcoding fragment of COI: (**a**) in North America, (**b**) in Europe and (**c**) in Asia; (**d**) Statistical parsimony networks for all haplotypes (empty circles indicate missing or theoretical haplotypes); and (**e**) ML consensus tree of all COI haplotypes. Bootstrap support values expressed in percentages are indicated near the nodes (>60%).

Table 3. Summary of genetic diversity indices for the barcoding fragment of the COI gene: (n) number of individuals sampled; (No) number of haplotypes; (Nex) number of exclusive haplotypes; (S) number of polymorphic sites; (h) haplotype diversity; (π) nucleotide diversity; (n.r.) not relevant; (E) Europe; (A) Asia; and (NA) North America.

Group	n	No.	Nex	S	ts/tv	h ± SD	π (%) ± SD
Invaded	477	5	3	7	6/1	0.6232 ± 0.0125	0.2120 ± 0.1517
E	250	2	0	1	1/0	0.4310 ± 0.0222	0.0789 ± 0.0791
A	227	7	2	7	6/1	0.5368 ± 0.0331	0.2996 ± 0.1964
Native/NA	40	9	7	10	10/1	0.7551 ± 0.0456	0.3933 ± 0.2479
Total	517	12	n.r.	11	11/1	0.6600 ± 0.0122	0.2552 ± 0.1737

None of the neutrality tests (including the Tajima's D, Fu's Fs indices, and the mismatch distribution) provided significant results.

The FCT values reached a plateau at K = 8 (FCT = 0.652) by SAMOVA, but the set of the eight groups did not consist with the geographic distribution.

AMOVA analysis detected the largest variation (57.47%) among natural and invaded groups (Table 4), while only 15.97% of variation occurs among populations and 26.56% within populations. The fixation index among groups was more than 1.5 times higher than among populations within groups (FCT = 0.575, $p < 0.01$; FSC = 0.376, $p < 0.01$), indicating that there may be factors limiting the gene flow among regions.

Table 4. Analysis of molecular variance (AMOVA) for two groups (natural and invaded area) of *C. ciliata*, barcoding fragment of the COI (*** $p < 0.001$).

Groups	Source of Variation	var%	Fixation Indices
Natural	Among groups	Va = 57.47	FCT = 0.575 ***
Invaded	Among populations within groups	Vb = 15.97	FSC = 0.376 ***
	Within populations	Vc = 26.56	FST = 0.734 ***

3.2.1. Invaded Versus Natural Range

Invaded Area

Altogether five haplotypes were observed in the invaded area, where SLB2 and SLB5 were the two most common haplotypes (47.17% and 38.16%). SLB2-SLB4 were detected only from the invaded area.

In the invaded area we detected moderate haplotype diversity with low nucleotide diversity indices (h = 0.62, π = 0.21%). Haplotype diversities were moderate and nucleotide diversities were low in both the Far East (h = 0.53, π = 0.32%) samples and in the European samples (h = 0.42, π = 0.08%).

We observed 0.51% average sequence divergence between invaded and natural populations. Within-group divergence of the invaded area was approximately half (0.21%) of the natural range of within-group divergence (0.40%). Sequence divergence was 0.30% within the Asian group and 0.08% within the European group, while the sequence divergence between Asia and Europe was 0.25%.

The neutrality tests (Tajima's D, Fu's Fs, mismatch distribution, and R2) usually were not significant in the most of investigated groups (invaded, Europe, Asia, Far East, etc.) except the invaded group, where the mismatch distribution (SSD = 0.019) had an unimodal shape. This is a common finding in populations that experienced recent population expansion or bottleneck [109,110].

The question of genetic diversity and the term of the invasion is interesting because approximately 60 years have passed since the invasion began in Europe. Regardless, we revealed only two haplotypes (257 individuals; h = 0.42; π = 0.08%) in Europe. Only 20 years have passed since the invasion began in the Far East, yet we revealed five haplotypes (199 individual; h = 0.53, π = 0.32) there.

Native Range

Nine haplotypes were detected in the native range where SLB1 (40%), SLB5 (27.5%), and SLB8 (15%) were the most common and SLB6–12 were unique. From North America, we observed high haplotype diversity with low nucleotide diversity (h = 0.76, π = 0.39%) and revealed average sequence divergence (0.40%) that was nearly two times higher than in the invaded area (0.21%). Fu's Fs and Tajima's D values were not significant. Mismatch distribution (SSD = 0.035) shows multimodal shape, which is usually associated with constant population size [109,110].

4. Discussion

4.1. Genetic Diversity of SYCAMORE Lace Bug

We detected moderate haplotype diversity (17 HTs) on the long (1356 bp) fragment of the COI gene in the *Corythucha ciliata* populations. Interspecific divergence of the COI gene in the plant bugs (Miridae) was reported as 6.30% [111]. Park et al. [112] detected more than 3% interspecific divergence for lace bugs (Tingidae), to which *C. ciliata* belongs. The interspecific divergence values between *C. arcuata* and *C. ciliata* varies 8.49–8.93% in our study.

Intraspecific distances for other Heteropteran species were reported 0–7.72% (mean distance 0.74%) [112], and for *Apolygus* species (Miridae) 0.40% [111]. In our study, the overall mean distance was 0.20% and the distance between populations was 0.00–0.65%. Jung et al. [111] revealed that in some cases the average interspecific genetic distance between closely related species was 32 times higher than the average intraspecific distance (e.g., genus *Scolopocelis*). In our study, we also detected 44 times higher interspecific divergence. COI sequences of *C. ciliata* showed higher genetic differentiation than avocado lace bug (*P. perseae*), where altogether nine haplotypes from 469 individuals with 16 polymorphic sites were found [113]. The haplotype diversity is relatively high (h = 0.73) on the 1356 bp long fragment of COI, but with low nucleotide diversity (π = 0.20%), which predicts a population bottleneck followed by rapid population growth and accumulation of mutations [114]. While we found slightly higher values of all diversity indices in the native group than in the introduced one, we found also high haplotype diversity with low nucleotide diversity values (h = 0.85; π = 0.36%), which also supports the above conclusion [114]. Furthermore, these results show that we have incomplete information about the past and current distribution and about the genetic structure of SLB in North America. This is reflected in discontinuous distribution records (e.g., the occurrence in the eastern part of the Rocky Mountains) [8] as well. In the invaded regions, we found relative high haplotype diversity with low nucleotide diversity (Europe h = 0.65, π = 0.09%; and Asia h = 0.66, π = 0.24%), which also suggests a population bottleneck followed by rapid population growth and accumulation mutations [114]. Several authors [1,7,74,115] report the loss of genetic diversity for invasive species under the process of biological invasion.

The genetic structure (Figure 2) together with the known invasion history of the SLB [9–44] suggest that there was only a single introduction event in Europe.

4.2. Long Versus Short (Barcoding) Fragments of the COI

Several papers have analysed the applicability of COI fragments of different lengths and locations on the mtDNA. Roe & Sperling [114] suggest the use of a longer COI fragment in pilot studies on any taxon. Maggioni et al. [115] experienced that COI-3′ regions were slightly more variable than 5′. Therefore, they recommend using this part of the mtDNA to assess the intraspecific geographic structure of Odonata species.

We detected significantly higher values in most of the diversity indices (No, S, ts, tv, and h) values on the longer fragment than on the barcode fragment. Our study could not reveal all possible links and connections in the invaded areas of *Corythucha ciliata*. New populations need to be included in future analyses, particularly in Asia.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/insects13020123/s1: Table S1, COI haplotypes distributions.

Author Contributions: Conceptualization, F.L., K.T., H.K. and V.T.; methodology, F.L. and V.T.; software, B.B. and V.T.; validation, F.L. and V.T.; formal analysis, V.T.; investigation, F.L., K.T., H.K. and V.T.; resources, F.L.; data curation, V.T.; writing—original draft preparation, F.L. and V.T.; writing—review and editing, F.L. and V.T.; visualization, V.T.; supervision, F.L.; project administration, V.T.; funding acquisition, F.L. and V.T. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The following data are available in Supplementary Materials, Table S1: GenBank accession numbers for nucleotide sequences generated for this study (accession numbers OM033605-621). [dataset] Yang, W.Y.; Tang, X.T.; Ju, R.T.; Zhang, Y.; Du, Y.Z. 2017. The Population Genetic Structure of *Corythucha ciliata* (Say) (Hemiptera: Tingidae) Provides Insights into Its Distribution and Invasiveness. *Sci. Rep.* **2017**, *7*, 635; (GenBank accession numbers KP420514-523).

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

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Article

The Handsome Cross Grasshopper *Oedaleus decorus* (Germar, 1825) (Orthoptera: Acrididae) as a Neglected Pest in the South-Eastern Part of West Siberian Plain

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Simple Summary: The handsome cross grasshopper is one of the most abundant and widely distributed grasshopper species over the steppe and semi-desert territories of Eurasia. In many areas, especially in east Mongolia and north-east China, it is a very common and dangerous pest on agriculture fields and pasturelands. However, the species was relatively rare in the steppes of west Siberia until the 1960s, but by the end of the last century, the geographic and ecological distribution of the handsome cross grasshopper was changed significantly. Nowadays, it often occurs across all steppes of the south-eastern part of West Siberian Plain, its abundance is high, and its populations are found in the forest-steppes and also on the eastern side of the Ob River. The authors tried to use ecologo-geographic modelling to estimate how species distribution may change in the near future.

Abstract: *Oedaleus decorus* is a widely distributed acridid over the Eurasian semi-arid territories, from the Atlantic coast to the Pacific coast. In many semi-arid territories, *O. decorus* was and is the most important pest, but in the south-eastern part of West Siberian Plain, it was not considered a pest until the 1960s. We compared two sets of data on the acridid distribution in the region: before 1960 and from 1961 until 2021. Until the 1960s, the species occurred mainly in the southern steppes. Since the 1960s, its distribution changed significantly. Nowadays, it occupies almost all local steppes and the southern part of the forest-steppes and can be also found on the eastern side of the Ob River. These shifts may be explained by both climatic changes and changes in human activities. During upsurges the densities of *O. decorus* were often more than one to two adults per square meter. It is often abundant in the same habitats and in the same periods as the Italian locust (*Calliptamus italicus*)—one of the most important acridid pests. This means during joint outbreaks these two species can simultaneously damage almost all spectrum of plants.

Keywords: distribution; range; dispersal; South Siberia; dynamics; population; plant protection; outbreak; Italian locust

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1. Introduction

The south-eastern part of West Siberian Plan lies in the central section of the range of *Oedaleus decorus* (Germar, 1825) or the handsome cross grasshopper [1,2]. This acridid species is widely distributed over the Eurasian semi-arid areas (mainly across forest-steppes, steppes, semi-deserts, Mediterranean and mountain dry grasslands), from the Atlantic coast to the Pacific one [3–8]. It also occurs in North Africa, on the Canary Islands, and on Madeira [3]. *O. decorus* commonly prefers different dry grasslands, from dry meadows to some types of the deserts with grasses [9–12]. The species is usually

univoltine with overwintering eggs. It prefers to feed on different grasses and commonly avoids forbs [12–17]. During outbreaks, it can seriously damage cereals, pastures, and hayfields [13,18,19], and it may also harm cotton, alfalfa, different legumes, sugar beet, vegetables, vine, and some fruit trees [13]. In many semi-arid territories, *O. decorus* was and is the important pest [18–21], especially in the eastern part of its range [22–24]. However, in the south-eastern part of West Siberian Plain, one of the main agricultural regions of Russia and Kazakhstan, according Berezhkov [25], until the 1960s this species occurred mainly in the dry (southern) steppes, its abundance was low, and it was not considered as a pest (cf. [19]). Furthermore, in many cases, plant protection services did not and do not still separate grasshoppers' species when they collect data concerning acridid abundance. During the twentieth century, ecosystems of this region were significantly changed by human activity. For instance, in 1954–1956 about 4.4 million ha were ploughed in the Altaj and Novosibirsk Regions during the so-called Virgin Land campaign [26]. Later, in the end of the 20th century, the general area of arable fields significantly decreased from about 11 million to 10.2 million ha (for two regions) [27]. In the second half of the last century, such ecosystems' transformations and, perhaps, global climatic variations as well resulted in species distribution changes [1,2,28]. The aims of this publication are to show how the distribution of *O. decorus* over West Siberian Plain has shifted, to estimate what climatic factors are essential for the species, how its distribution can change in the coming years, and to discuss some forecasts of its dispersal until 2040.

2. Materials and Methods

2.1. Study Territory

Original data were collected from 1979 until 2021 in the south-eastern part of West Siberian Plain. We also used some additional data collected in the adjacent north-eastern and eastern parts of the Kazakh Uplands (Saryarqa) and in the steppes on the western side of the Irtysh River in 1980. The south-eastern part of West Siberian Plain borders the Irtysh River to the west and south-west and the mountains of south Siberia to the east and south-east. Its northern boundary is approximately defined by the southern border of the taiga life zone (about 56° N). Originally this area was characterized by grasslands and forests. Later it was mainly converted to agricultural lands (fields and pastures). The forest-steppe life zone occupies mainly the area from 54° N to 55.5° N between the Irtysh and Ob Rivers and also on the eastern side of the Ob River. The steppes are between 50.5° N to 54° N, but in the western and central parts of the region. There are some flood-plains with meadows and forest patches, sandy plots, solonchaks (with saline soils), and swamps as well. Average temperatures are relatively low (mean temperatures of the warmest month are between 17 to 22 °C, the same for the coldest month—from −17 to −20 °C), and annual precipitation amounts range between 280 to 520 mm [29].

2.2. Field Studies

Grasshoppers' distribution patterns were characterized by quantitative and qualitative samples collected in natural and transformed ecosystems, usually in July and August when adults were dominated [4,28]. Samples captured during a fixed period of time were done in every habitat studied [4,30,31]. Using this method, insects were collected with a standard net (40 cm diameter) over a period of 10–30 min. Results for each habitat were transformed to an hour. As a rule, hand collecting and sweep netting were used to get additional information as well. In many cases, especially during locust outbreaks, we also counted acridid densities on arbitrarily placed plots 0.25×0.25 m^2 (in some cases—0.5×0.5 m^2), mainly in the zonal habitats [31,32]. We used the GLONASS/GPS navigators to determine geographical coordinates of localities.

Several fixed sites were selected to study long-term dynamics of the species populations [32,33]. All plots were covered by more or less typical steppe zonal vegetation; however, in some cases, vegetation cover was damaged by moderate grazing or haymaking. As a rule, the acridid abundance was counted in the first half and middle of July, while

adults prevailed. In this study, we analyzed data for 2000–2008, 2015, and 2018 when all three sites were explored (except of Yarovoe in 2018).

1. SE Aleksandrovskij settlement (Novosibirskaya Oblast (Novosibirsk Region), 53.67° N 78.25° E, northern steppe, in 2003 the local control model plot was moved about 100 m southwards, because the main part of the area was ploughed).
2. SW Yarovoe town (Altai Krai (Altaj Region), 52.85° N 78.57° E, dry steppe (actually very old crested wheatgrass field)).
3. E Ust-Volchikha settlement (Altai Krai (Altaj Region), 51.93° N 80.28° E, dry steppe).

In 2004, several series of adults of *O. decorus* were specially collected in some localities of the Kulunda steppe as well to compare some phenotypic variations of its local populations.

2.3. Data Analysis

Besides our original data, we also analyzed some old data, especially collected by the expeditions of Novosibirsk State University (1961–1980), and, in this case, checked some previous species identifications. These samples were collected by the methods described in Section 2.2. For localities explored before 2000, we used Google Earth Pro (©Google, 2020) to get geographical coordinates of localities. The main part of studied specimens is in the collections of Novosibirsk State University and the Institute of Systematics and Ecology of Animals (Novosibirsk). We also used data from different publications for the end of the 19th century [34] and the first half of the 20th century [25,35–41] and data for some specimens from the collections of Zoological Institute, Russian Academy of Sciences (Saint Petersburg, Russia), Novosibirsk State University, and Berezhkov's collections at Tomsk State University (Tomsk, Russia). The geographic coordinates for almost all old localities of grasshoppers (151) were determined (Table S1). These data were compared with dataset for 1961–2021 (194 localities for all acridid insects) (Table S2).

Maps of species distribution were produced on the basis of geographic coordinates with MapInfo 15.2.4 (© Pitney Bowes Software Inc., Lanham, MD, USA; now—© Precisely, Burlington, MA, USA). A Lambert conformal conic projection (ETRS 89) was used as the basic map.

We used Maxent 3.4.4 software [42–45] to model the species distribution over the south-eastern part of West Siberian Plain for two sets of data, until 1960 and from 1961 until 2021. We selected this software, because it is highly standardized [43] and has a relatively friendly interface.

For ecomodelling, we exploited resources of WorldClim 2 [46,47] such as "Historical climate data" (19 standard annually averaged bioclimatic variables and 12 monthly averaged variables for solar radiation for 1970–2000 at the 30 arcsecond spatial resolution) and "Future climate data" (19 standard averaged bioclimatic variables for 2021–2040 downscaled from two global climate models [47], namely CNRM-ESM2-1 (Centre National de Recherches Meteorologiques and Centre Europeen de Recherche et de Formation Avancee en Calcul Scientifique, France) [48,49] and MIROC6 (Japan Agency for Marine-Earth Science and Technology, Atmosphere and Ocean Research Institute, The University of Tokyo, National Institute for Environmental Studies, and RIKEN Center for Computational Science, Japan) [50,51], at the 2.5 arcminute spatial resolution and for the 3–7.0 Shared Socioeconomic Pathway based on high greenhouse gas emissions [52]).

Both the approach to ecomodelling and the data on climatic variables have some limitations. The MaxEnt models are based only on presence data and depend on the number of occurrences, selected parameters of modelling, and selected sets of variables [42–44]. These limitations can be especially important for an analysis of small samples [53]. Besides, the WorldClim dataset includes spatially interpolated climatic data, and their reliability depends partly on densities of weather stations (relatively low for south Siberia) [46]. This is why results of our ecomodelling should be qualified as the preliminary ones. In any case, we tried to use the full sets of applicable bioclimatic variables to compare results for the same territory, but for different periods, to estimate accuracy of our models by

using the AUC (the area under the receiver operating characteristic curve) values for training and test (25%) data and producing sets of 20 replicates with cross-validation, and to estimate significance of climatic variables by their predictive contributions and Jackknife tests. We generated the models with the following parameters: features—auto, output format—cloglog [44], regularization multiplier = 1.

The Spearman rank-order correlation coefficient was used to compare long-term dynamics patterns of local populations of *O. decorus* and the Italian locust (*Calliptamus italicus* (Linnaeus)). We also analyzed several series of the handsome cross grasshopper adults, including at least five specimens and estimated frequencies of brown and green morphs relative to the species abundance, because in the populations of the so-called Mongolian locust (*Oedaleus decorus asiaticus* Bey-Bienko), the brown morphs are mainly associated with high levels of species abundance and the green ones are common at low density [54]. We used a multivariate regression analysis to compare several rows of data as well. These parts of the analysis were mainly conducted using PAST 4.02 [55].

3. Results

3.1. Taxonomical Notes

In the first half of the 20th century, some specimens of the genus *Oedaleus* Fieber from the West Siberian Plain were misidentified as *O. infernalis* Saussure [25]. We checked several such specimens from Berezhkov's collections (Tomsk State University, Tomsk, Russia). All of them proved to be *O. decorus*. To distinguish similar specimens and the true individuals of *O. infernalis* from East Asia, Bey-Bienko [56] described the new species, namely, *O. asiaticus* Bey-Bienko, from South Siberia. Later Ritchie [3] revised the genus and showed that *O. asiaticus* is conspecific with *O. decorus*. He noted very significant variability of individuals of *O. decorus* from different parts of its range. Some orthopterists [4,21,57,58] follow this proposal, and our data support this point of view [59]. However, some others [60] do not. Molecular data do not allow us to solve this problem up to date. Fries et al. [61] showed that *O. decorus* and *O. asiaticus* are very close forms, but regarded them as separate species. Kindler et al. [62] discussed that this species complex includes three distinct, but closely related species or subspecies. Later, Schmid et al. [63] also described evident similarity between *O. decorus decorus* from Europe and *O. decorus asiaticus*. That is why we prefer to follow the most comprehensive revision of the genus [3,59].

3.2. Shifts in Species Distribution

Until the middle of the 20th century, *O. decorus* was mainly distributed in the southern parts of the region (Figures 1 and 2) and was commonly associated with the dry steppes (Figure 3B). Its northernmost localities were found near the 53th parallel north in the so-called Kulunda steppe between the Irtysh and Ob Rivers [25]. The only known exception was the locality in the vicinities of Omsk (about 55° N—the forest-steppes of the Irtysh River basin) [37,40,41]. Bey-Bienko [41] found this species near Omsk on the dry southern slope on the eastern side of the Irtysh River and noted that it was very rare.

Our data show that in the 1970s, the species occurred already over the whole Kulunda steppe (up to 54° N) (Figures 1 and 4). In the 1990s, *O. decorus* crossed the 54th parallel, and its populations were observed in the southern parts of the forest-steppe. In 1999, its specimen was found in the southern part of Novosibirsk, in Novosibirsk Scientific Center of Russian Academy of Sciences (so-called Akademgordok) [64], on the eastern side of the Ob River. This locality became the north-easternmost one on the West Siberian Plain. Later, several colonies of this species were observed in the forest-steppes on the eastern side of the Ob River. They were commonly associated with dry transformed habitats, e.g., with overgrazed meadow steppes, lawns, and roadsides.

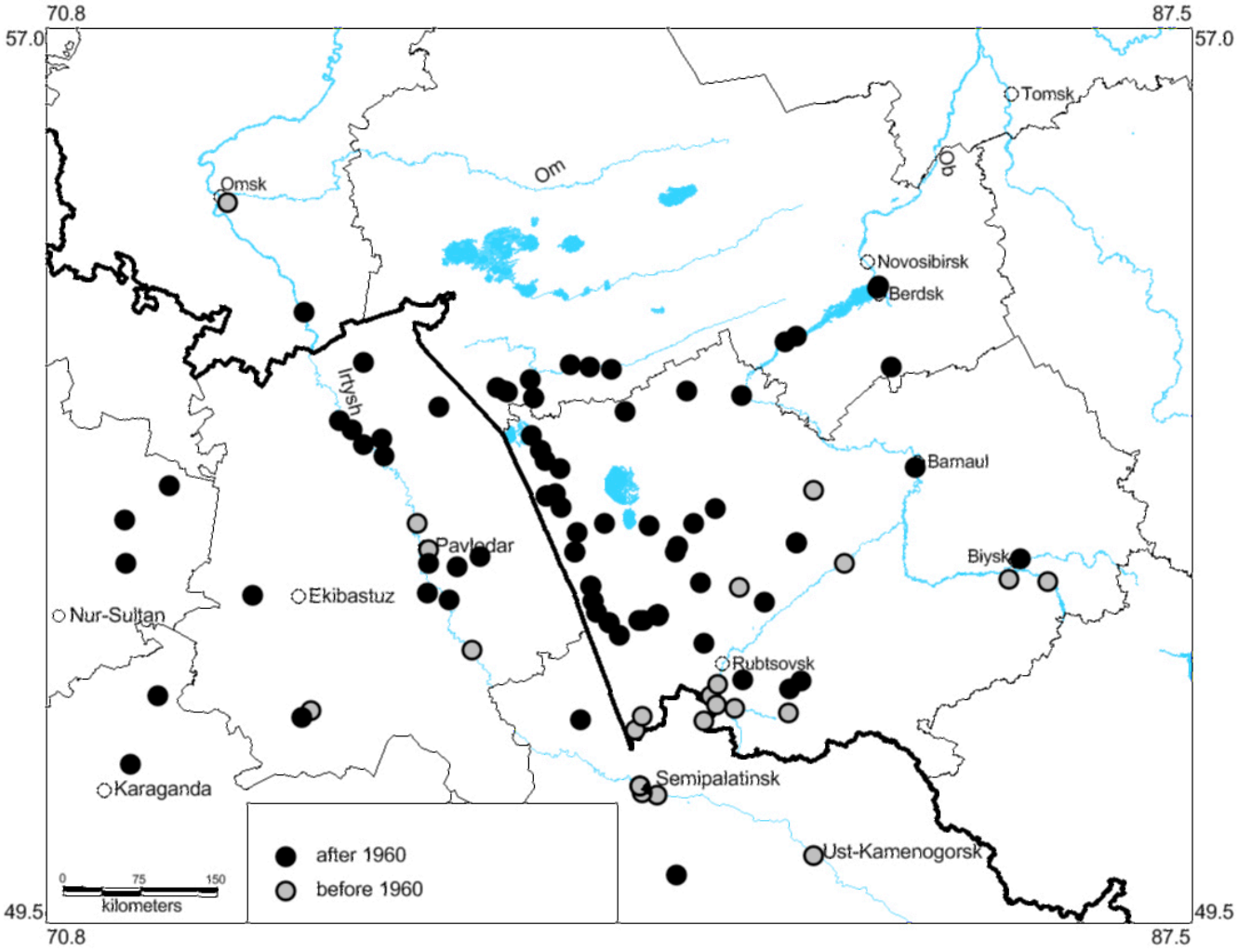
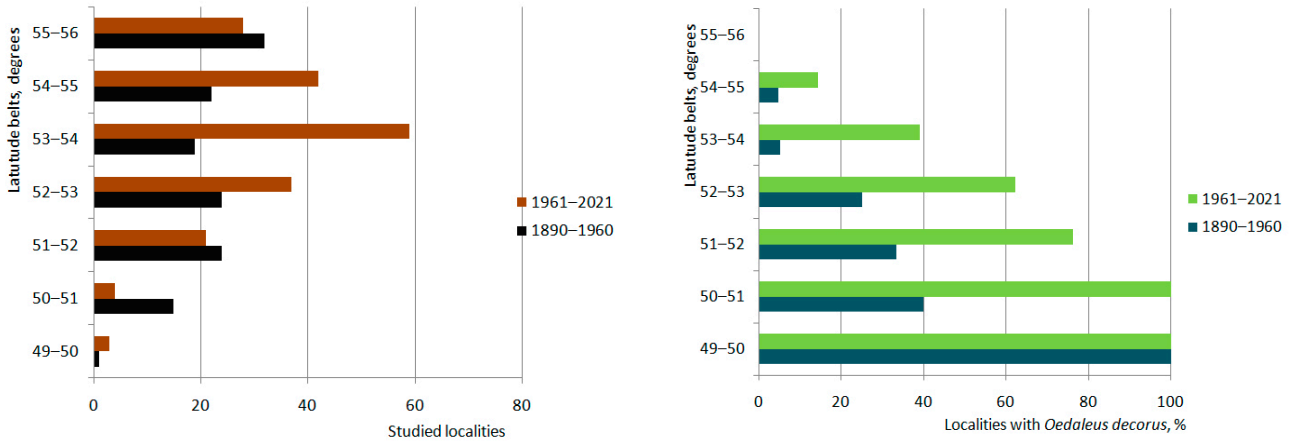


Figure 1. Distribution of *Oedaleus decorus* over the south-eastern part of the West Siberian Plain until 1960 and after 1961.



(**A**) (**B**)

Figure 2. Latitudinal distribution of all studied localities (**A**) and the localities with *Oedaleus decorus* (**B**) over the south-eastern part of West Siberian Plain until 1960 and after 1961 (Tables S1 and S2).

(**A**) (**B**)

Figure 3. Typical habitats of *Oedaleus decorus* in the Kulunda steppe: (**A**)—typical steppe (near the Kuchuck River, Altai Krai (Altaj Region), 52.41° N 80.03° E); (**B**)—dry steppe (near Ust-Volchikha settlement, Altai Krai (Altaj Region), 51.93° N 80.28° E).

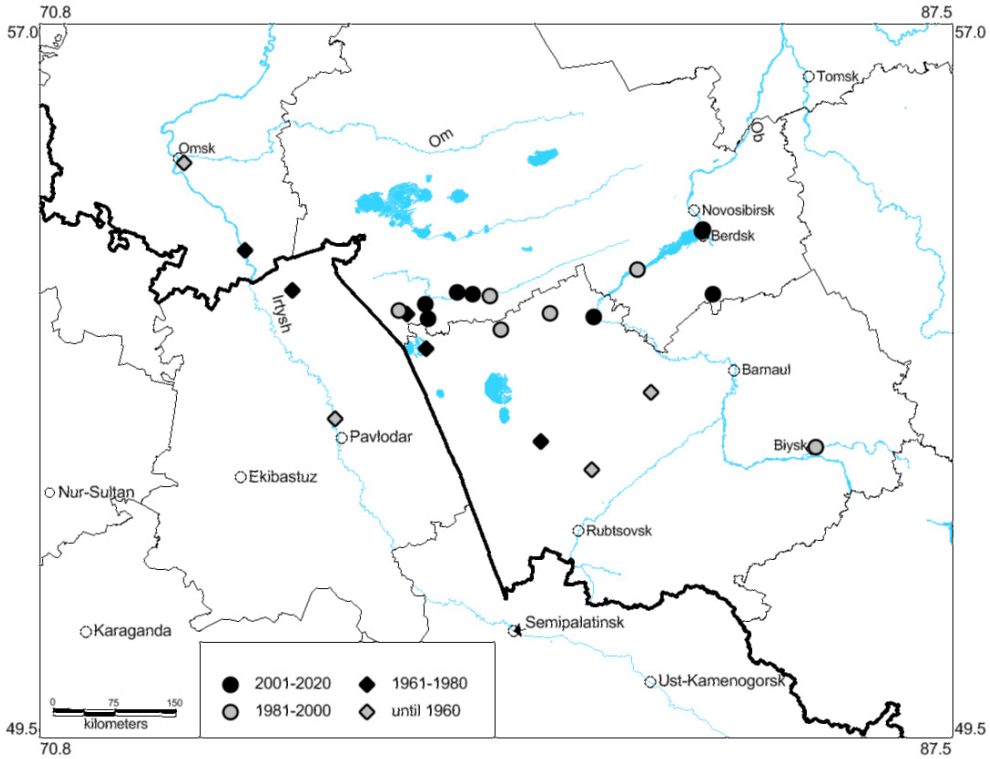


Figure 4. Changes of the known northernmost and north-easternmost localities of *Oedaleus decorus* in the south-eastern part of the West Siberian Plain from the beginning of the 20th century.

The multivariate regression analysis of species occurrences relative to latitudinal bands (Figure 2) shows very significant correlations between these parameters for each period: until 1960 (r = –0.888, p = 0.008) and from 1961 until 2021 (r = –0.987, p = 0.00004) and explicit difference between two periods (overall statistics: R^2 = 0.895, F = 522.6, MSE = 175.8, Wilks' lambda = 0.0038).

Hence, *O. decorus* has become one of the most widely distributed grasshopper over the south-eastern parts of West Siberian Plain and occupies all steppe areas, the southern

parts of the forest-steppes between the Irtysh and Ob Rivers, and the forest-steppes on the eastern side of the Ob River where it colonized some transformed habitats [1,2].

3.3. Ecological Models of the Species Distribution

The analysis of the predicted distribution of *O. decorus* based on the occurrence data until 1960 relative to climatic data for 1970–2000 (Figure 5) allows us to compare such backward forecasts with the actual distribution of species in the end of the 20th century. This analysis shows that (1) its northernmost population (for this period) near Omsk (Figures 1, 2 and 4) looks like the very local and limited one [41] (perhaps temporal), (2) conditions for this species may be suitable not only in the southern part of the Kulunda steppe, but in its central and eastern territories as well and also in some areas on the eastern side of the Ob River. Perhaps, the old mention of *O. infernalis* for Meret settlement (now in Novosibirsk Region near the boundary of Altaj Region, on the eastern side of the Ob River, 53.57° N 82.40° E) [65] really belongs to *O. decorus*. If this is right, the first colony (or colonies) of the species occurred on the eastern side of the Ob River more than a century ago.

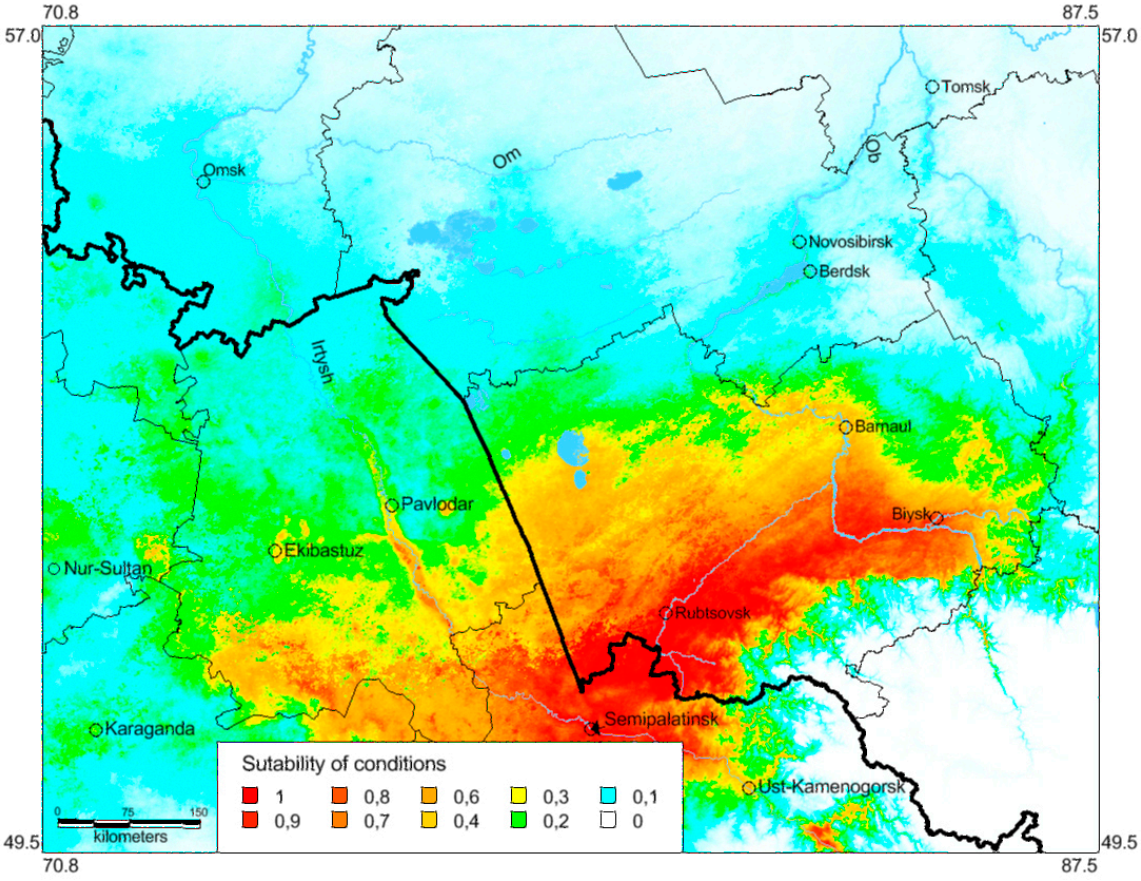


Figure 5. Predicted probabilities of suitable conditions for *Oedaleus decorus* in the south-eastern part of the West Siberian Plain (distribution data before 1961 and bioclimatic variables for 1970–2000; point-wise mean for 20 replicates).

This model shows that in the end of the 20th century, global warming could result in the species distribution shifts northward and north-eastward. However, the actual distribution pattern (Figure 1) indicates that *O. decorus* occupied all local steppes of the Altaj, Novosibirsk, Omsk, and Pavlodar Regions and penetrated into the southern parts of the forest-steppes in the Novosibirsk Region. It also became widely distributed across the forest-steppes on the eastern side of the Ob River. Such deviations can be partially

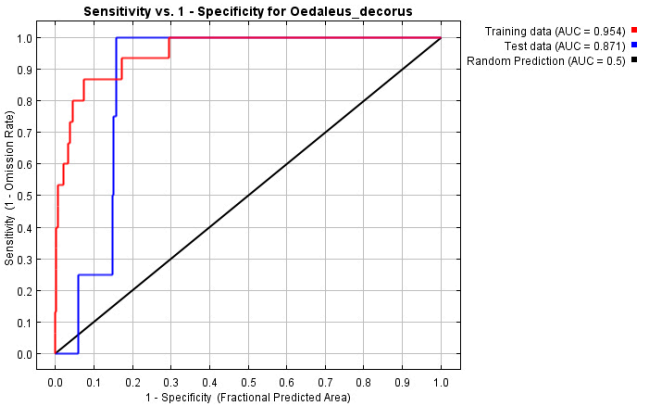
explained by species dispersal through transformed habitats, e.g., roadsides, abandoned fields, overgrazed meadow steppes, dry lawns.

The model performance is high (Figure 6A,B), because the AUC value for training data is 0.954, for the test set—0.871, and for 20 replicates—0.901. In this model, the solar radiation in July is the most important factor (Table 1), besides, the solar radiation in October and April, the annual mean temperature and the precipitation of wettest month are significant as well. The Jackknife test allows to add also the mean diurnal range of temperatures (Figure 7A). Almost all factors are essential for development of nymphae and adults (and certainly for grass vegetation as well), while the solar radiation in October may be associated with autumnal high survival rates of eggs in eggpods.

Table 1. Predictive contributions for two periods (until 1960 and 1961–2021) and for two climatic models for 2021–2040.

Variable	Variable Explanation	Distribution Until 1960		Distribution 1961–2021		CNRM-ESM2-1		MIROC6	
		Percent Contribution	Permutation Importance	Percent Contribution	Permutation Importance	Percent Contribution	Permutation Importance	Percent Contribution	Permutation Importance
bio_1	annual mean temperature	**10.9**	12.4	0.4	1.3	**5.1**	17.1	3.2	18.4
bio_2	mean diurnal range (mean of monthly (max temp—min temp))	2.7	25.7	0.1	0.9	0.3	0.5	0.2	1.2
bio_3	isothermality (bio2/bio7) (×100)	3.5	1.5	1.8	1.1	0.2	0.1	0.6	0.2
bio_4	temperature seasonality (standard deviation ×100)	0.1	0	0.2	0	0.1	0	0.1	0.1
bio_5	max temperature of warmest month	1.4	2.1	5.5	4.5	**7.6**	11	**9.9**	13.9
bio_6	min temperature of coldest month	0	1.1	0	0	0	0	0	0
bio_7	temperature annual range (bio5-bio6)	0.2	1.4	0.1	1	0.1	0.1	0.1	0.1
bio_8	mean temperature of wettest quarter	1	0.3	**31.3**	17.9	**57.9**	26.9	**60.4**	21.3
bio_9	mean temperature of driest quarter	0.7	2.4	0.4	0.3	3.8	0.1	**3.5**	0.4
bio_10	mean temperature of warmest quarter	0.3	0	0.2	0.4	**6.4**	1.2	**6.2**	0.3
bio_11	mean temperature of coldest quarter	0	0	0	0	0	0.2	0	0.1
bio_12	annual precipitation	0.4	0.1	0	0.1	0.2	4.8	0.2	1.9
bio_13	precipitation of wettest month	**8.7**	8.4	2.4	0	2.5	0.1	2.1	0.3
bio_14	precipitation of driest month	2.4	1.8	0.1	0.5	0.5	2.4	0.5	3.1
bio_15	precipitation seasonality (coefficient of variation)	0.8	0.6	0.6	0.8	0.2	1.8	0.4	3.1
bio_16	precipitation of wettest quarter	0.1	0	2.2	5.9	3.6	19.2	3.3	18.9
bio_17	precipitation of driest quarter	4.1	0.1	0	0.1	1.4	0.9	1.7	0.9
bio_18	precipitation of warmest quarter	0	0	2.4	1.4	**5.9**	0.9	**4.3**	1.1
bio_19	precipitation of coldest quarter	0.5	0.1	0.8	0.3	4.1	12.6	3.3	14.8
srad_1	solar radiation, January	0	0	0.1	0.4	n.a.	n.a.	n.a.	n.a.
srad_2	solar radiation, February	0	0	0	0	n.a.	n.a.	n.a.	n.a.
srad_3	solar radiation, March	0	0	0	0	n.a.	n.a.	n.a.	n.a.
srad_4	solar radiation, April	**11.9**	0.6	**4.7**	25.3	n.a.	n.a.	n.a.	n.a.
srad_5	solar radiation, May	0	0	0.1	1.7	n.a.	n.a.	n.a.	n.a.
srad_6	solar radiation, June	0.3	0	0.3	0	n.a.	n.a.	n.a.	n.a.
srad_7	solar radiation, July	**36.9**	30.4	**25**	0.4	n.a.	n.a.	n.a.	n.a.
srad_8	solar radiation, August	0	0	0.8	0	n.a.	n.a.	n.a.	n.a.
srad_9	solar radiation, September	0		0	0	n.a.	n.a.	n.a.	n.a.
srad_10	solar radiation, October	**12.8**	11.1	**13.4**	22.9	n.a.	n.a.	n.a.	n.a.
srad_11	solar radiation, November	0.4	0	**7.1**	12.7	n.a.	n.a.	n.a.	n.a.
srad_12	solar radiation, December	0	0	0	0	n.a.	n.a.	n.a.	n.a.

In bold and highlighted green—five most significant variables for each group; n.a.—not available.



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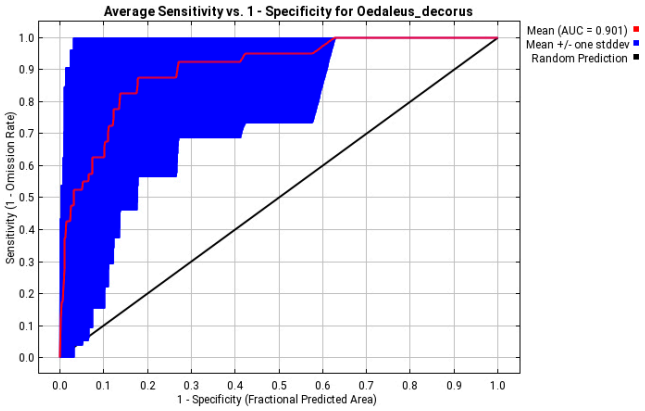

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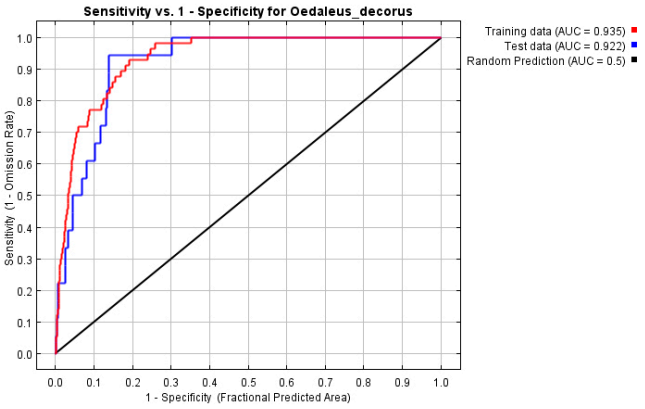

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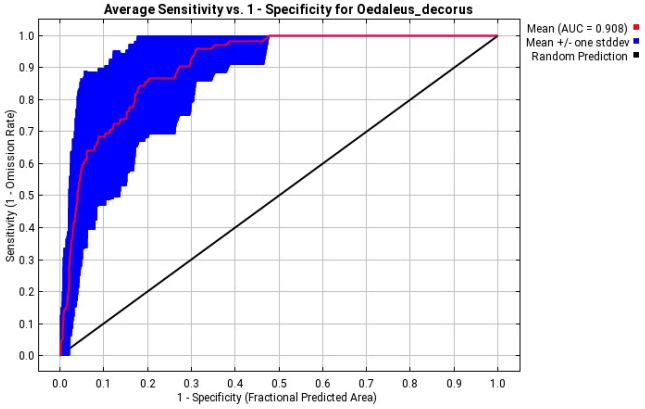

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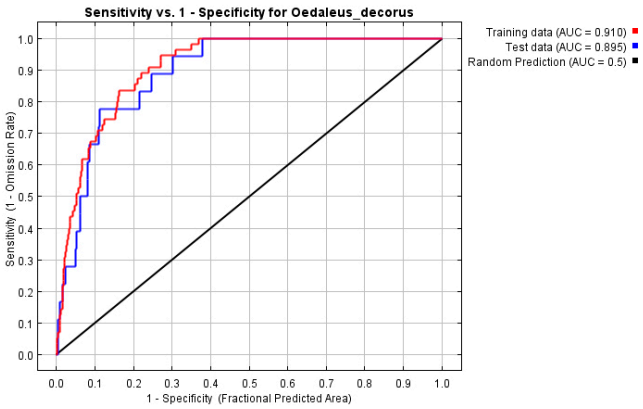

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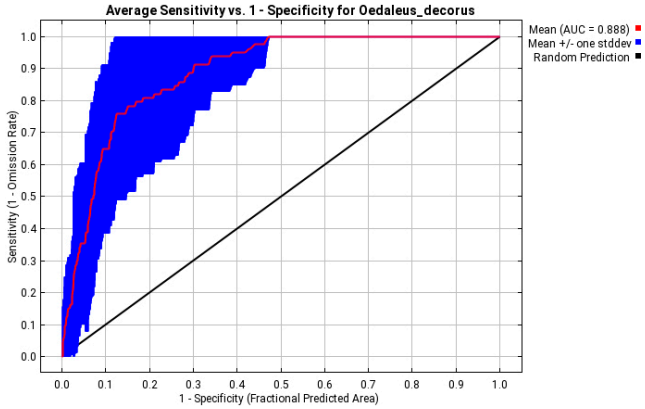


(**F**)

Figure 6. *Cont.*

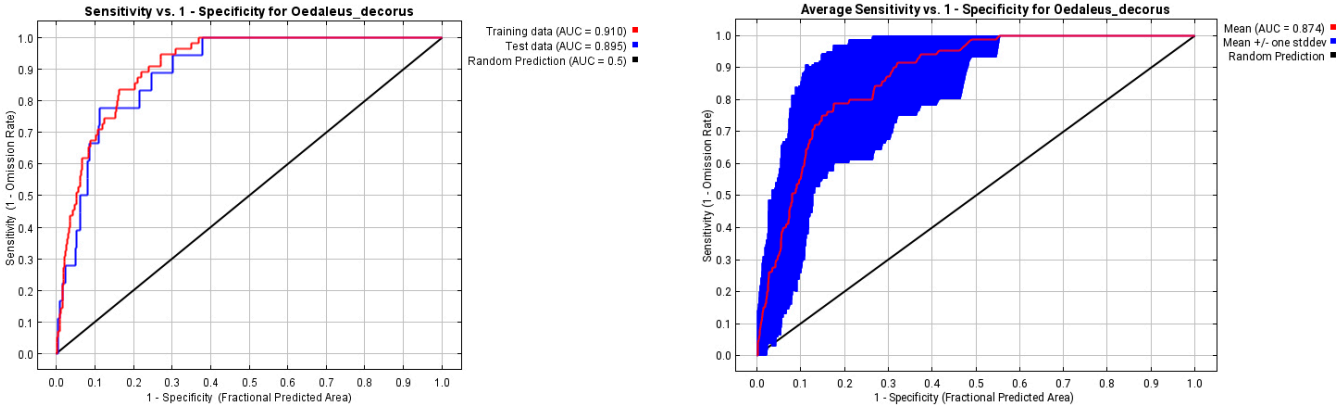


Figure 6. Reliability tests of the *Oedaleus decorus* distribution models for the south-eastern part of the West Siberian Plain: (**A**,**B**)—distribution data before 1961 and bioclimatic variables for 1970–2000; (**C**,**D**)—distribution data from 1961 until 2021 and bioclimatic variables for 1970–2000; (**E**,**F**)—distribution data from 1961 until 2021 and forecasts of bioclimatic variables from the climatic model CNRM-ESM2-1 2021 (**G**,**H**)—distribution data from 1961 until 2021 and forecasts of bioclimatic variables from the climatic model MIROC6; (**A**,**C**,**E**,**G**)—for training and test (25%) data; (**B**,**D**,**F**,**H**)—for 20 replicates with cross-validation.

The modern known distribution of *O. decorus* (for 1961–2021) matches to the predicted probabilities of suitable conditions (cf. Figures 1 and 8). All steppes and the southern parts of the forest-steppes in Novosibirsk and Altaj Regions of Russia and Pavlodar Region of Kazakhstan are applicable for the handsome cross grasshopper. In the local steppes, high levels of habitat suitability show opportunities of the species upsurges. Besides, there are large areas on the eastern side of the Ob River where populations of this species may occur. Importantly, it can disperse across these territories by dry verges and lawns [64]. Perhaps, *O. decorus* is able to use some vehicles for migrations as well (cf. [59]).

The performance of this model is also high (Figure 6C,D) (the AUC values for training and test data are 0.935 and 0.922, respectively; for 20 replicates—0.908). For this set of data, two factors, namely, the mean temperature of the wettest quarter and the solar radiation in July, are the most important factors. Three other factors are relatively significant: the solar radiation in October, November, and April. The Jackknife test allows to add the mean temperature of the warmest quarter as well (Figure 7B). One can hypothesize that the first factor and the mean temperature of warmest quarter may determine grass development and may be indirectly associated with grasshoppers' sustainability. Autumnal and vernal solar radiation can be important for high survival rates of dormant eggs. Importantly, this group of factors looks more significant than prior to 1960. Such a shift may be explained by global and local warming, especially in the end of the 20th century, and by a corresponding increase of warm season duration.

Ecological modelling of the species distribution in 2021–2040 based on two climatic models (CNRM-ESM2-1 and MIROC6) for the 3–7.0 Shared Socioeconomic Pathway based on high greenhouse gas emissions (Figure 9) shows that the local part of the range may significantly shift northwards and north-eastwards and *O. decorus* will able to distribute over the territories of the modern forest-steppes and south taiga. Besides, it will able to penetrate into two new regions, Tomsk and Kemerovo, as well. Actually, these possible changes may be mainly associated with some significant shifts of life zones northward and north-eastward.

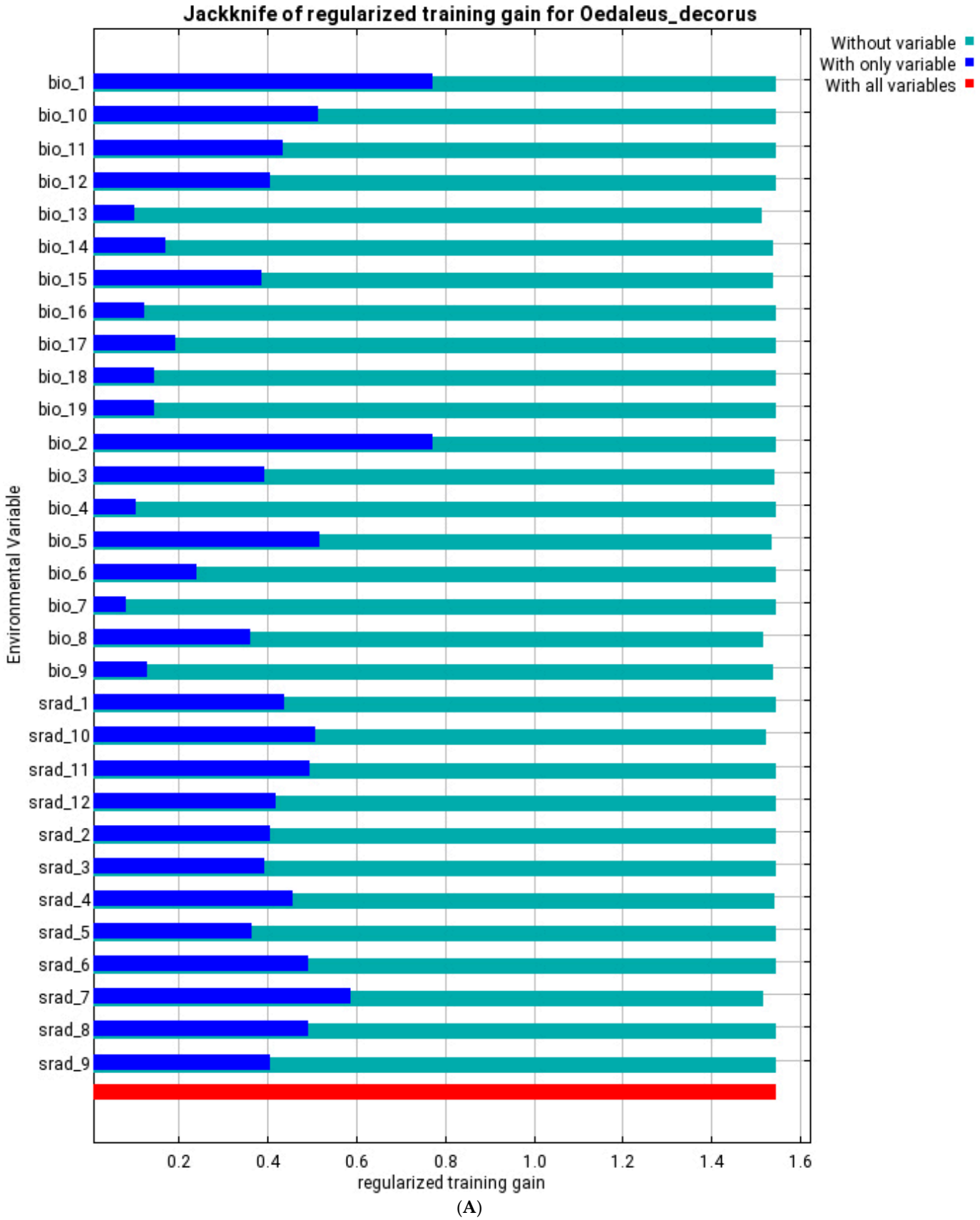
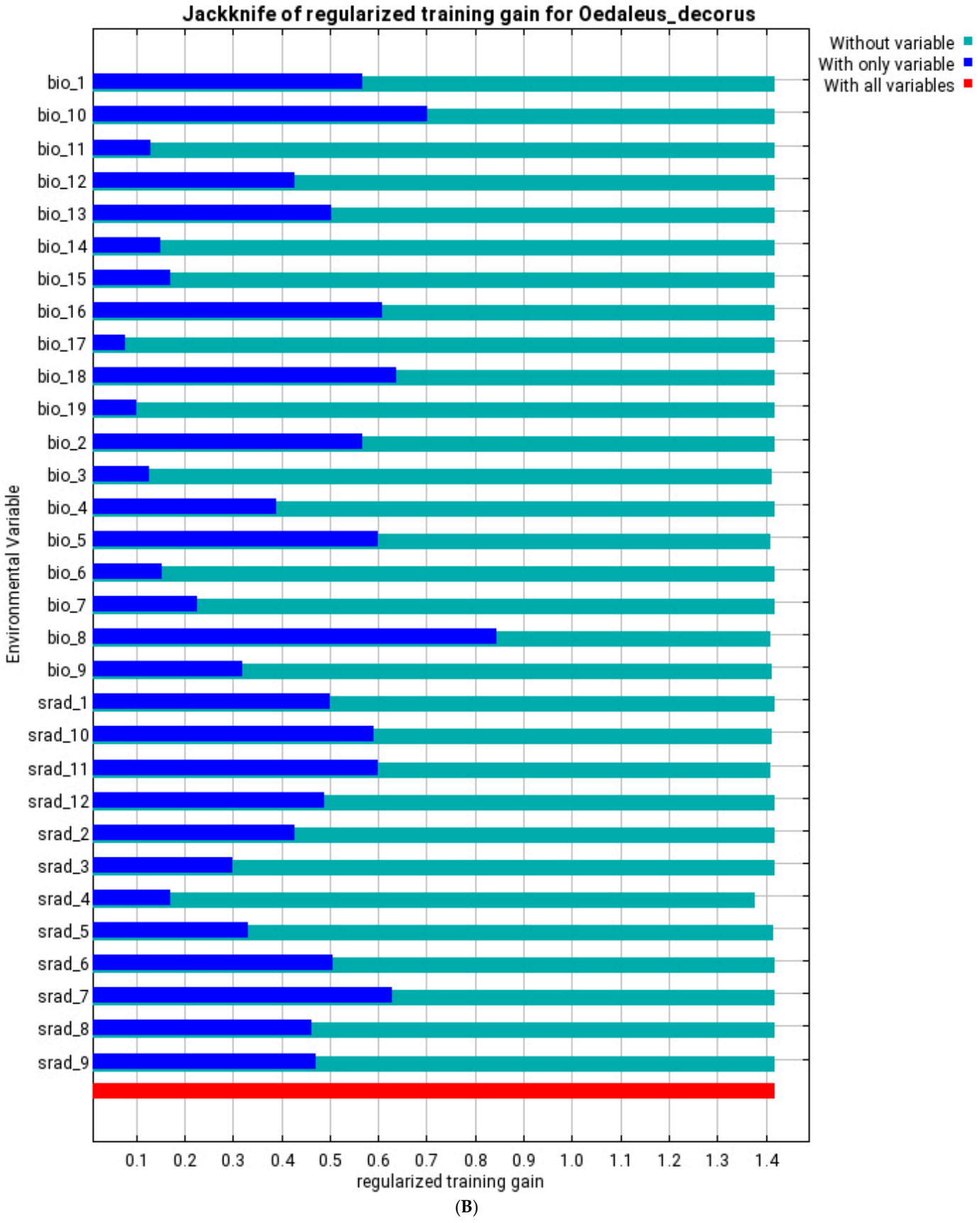


Figure 7. *Cont.*



(**B**)

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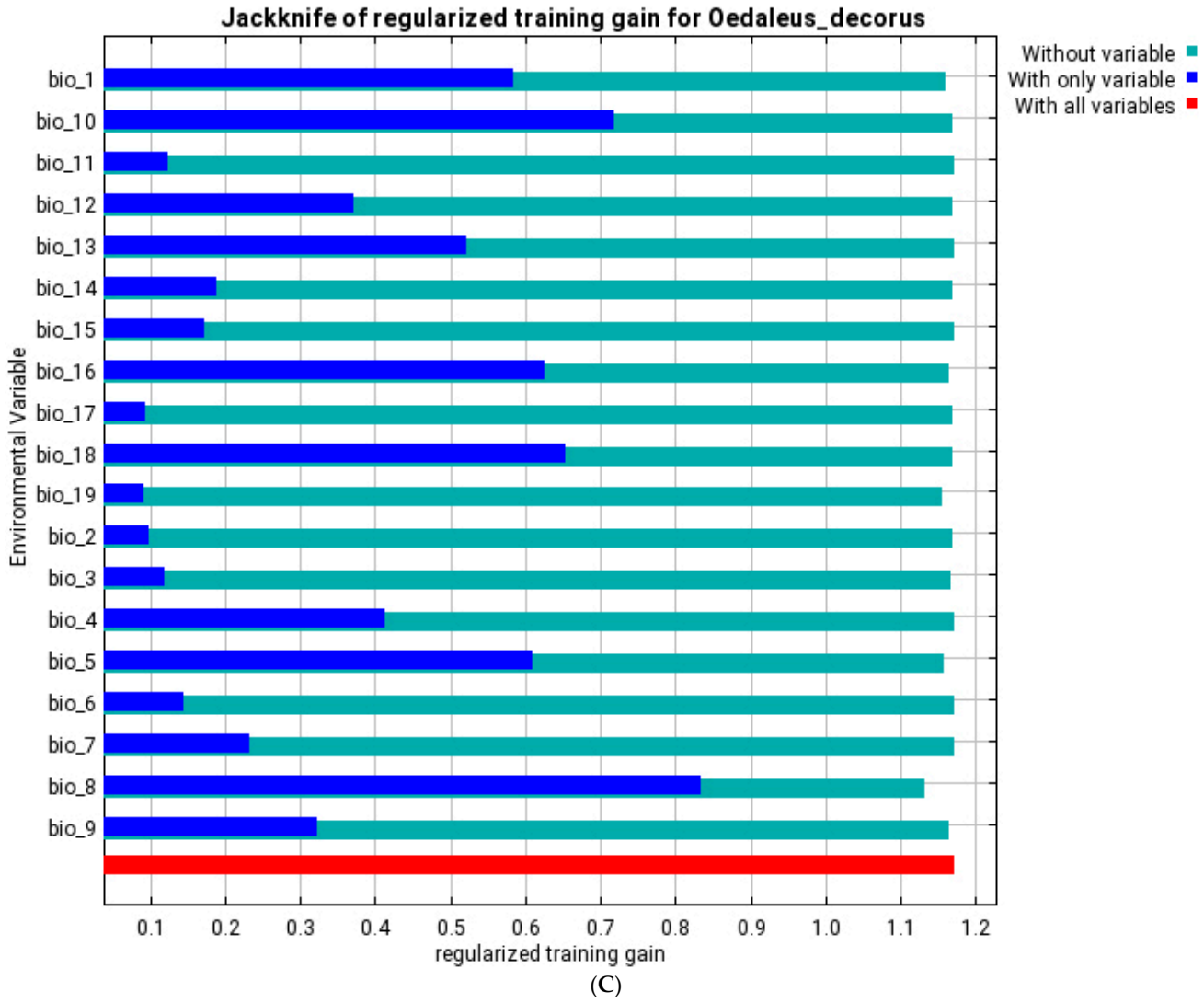

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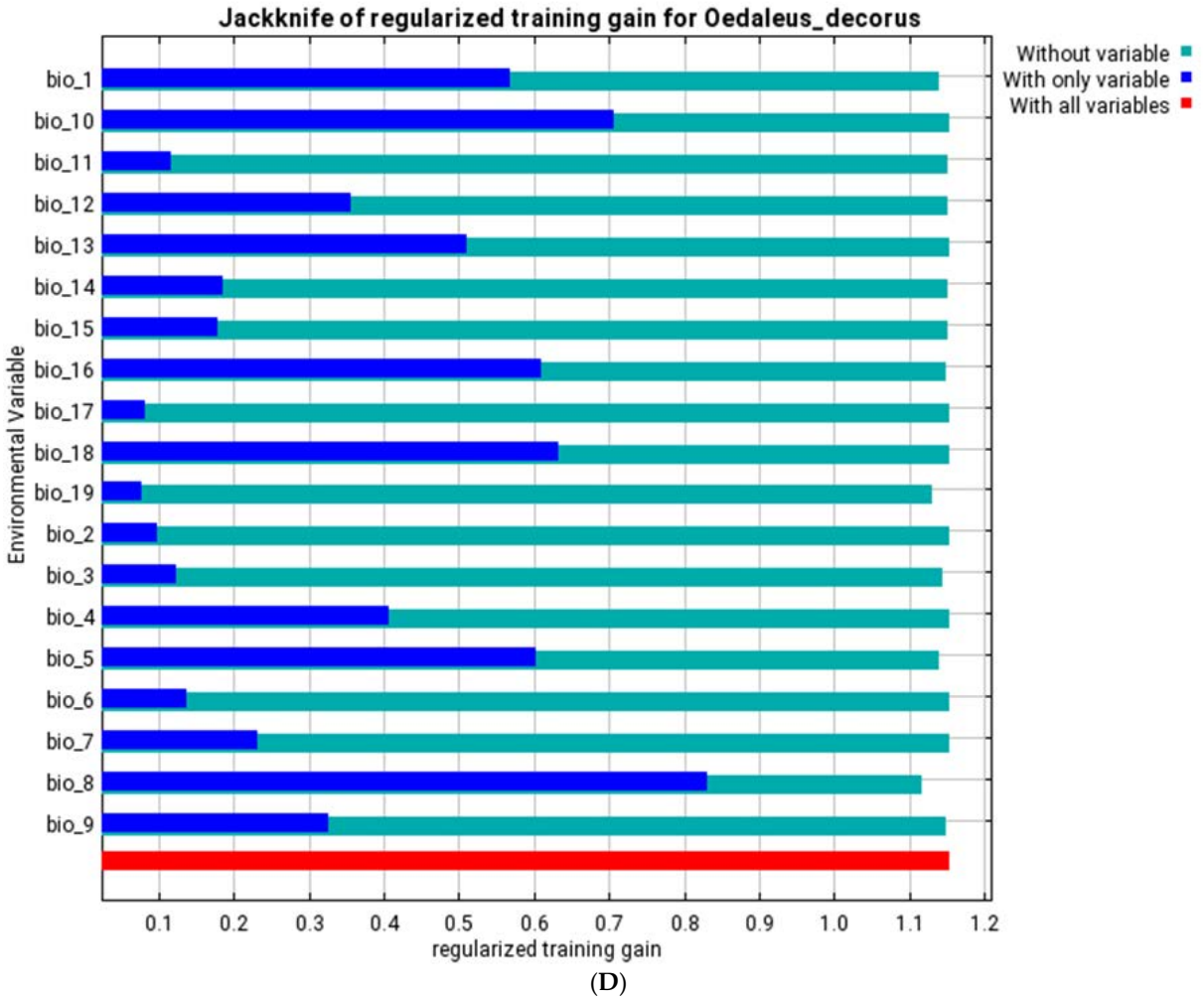


Figure 7. Jackknife of regularized training gain for the *Oedaleus decorus* distribution models for the south-eastern part of the West Siberian Plain: (**A**)—distribution data before 1961 and bioclimatic variables for 1970–2000; (**B**)—distribution data from 1961 until 2021 and bioclimatic variables for 1970–2000; (**C**)—distribution data from 1961 until 2021 and forecasts of bioclimatic variables from the climatic model CNRM-ESM2-1; (**D**)—distribution data from 1961 until 2021 and forecasts of bioclimatic variables from the climatic model MIROC6.

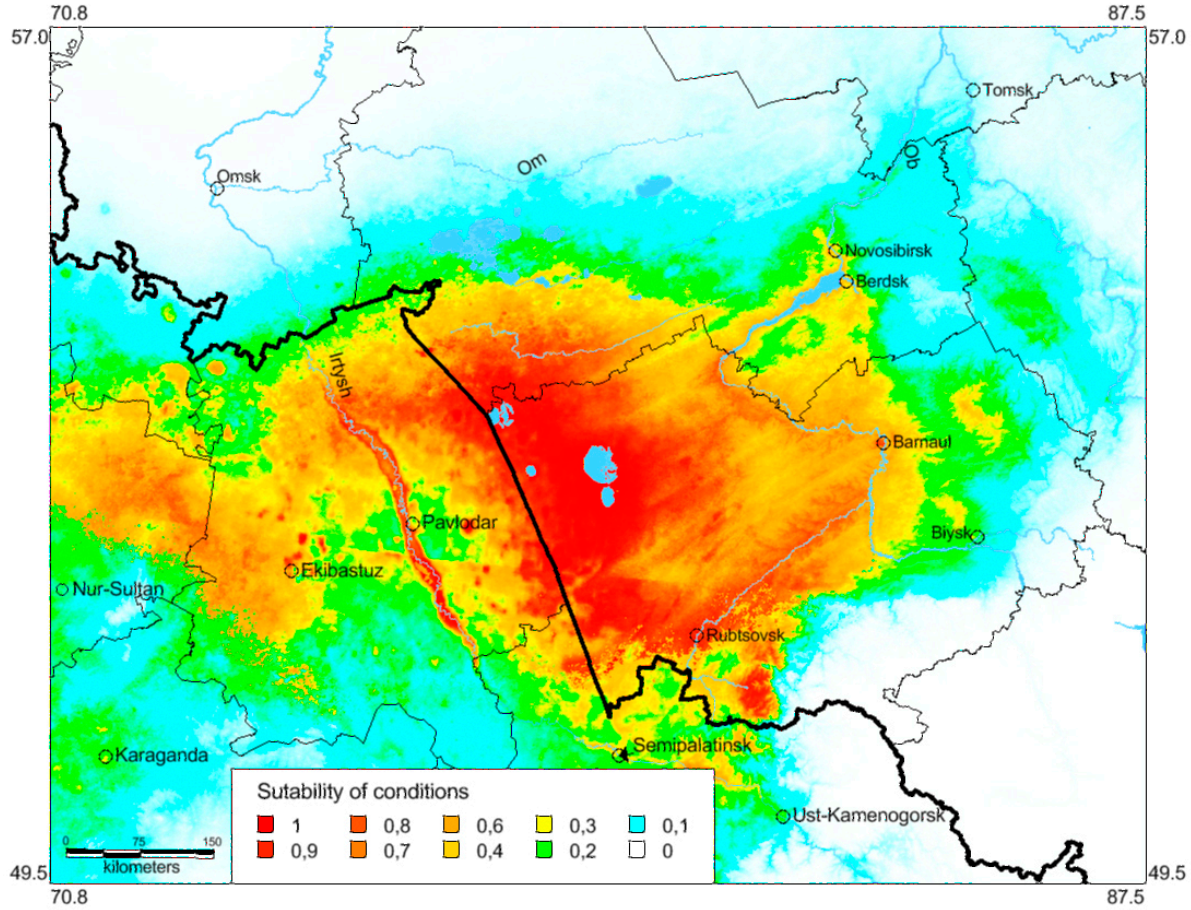
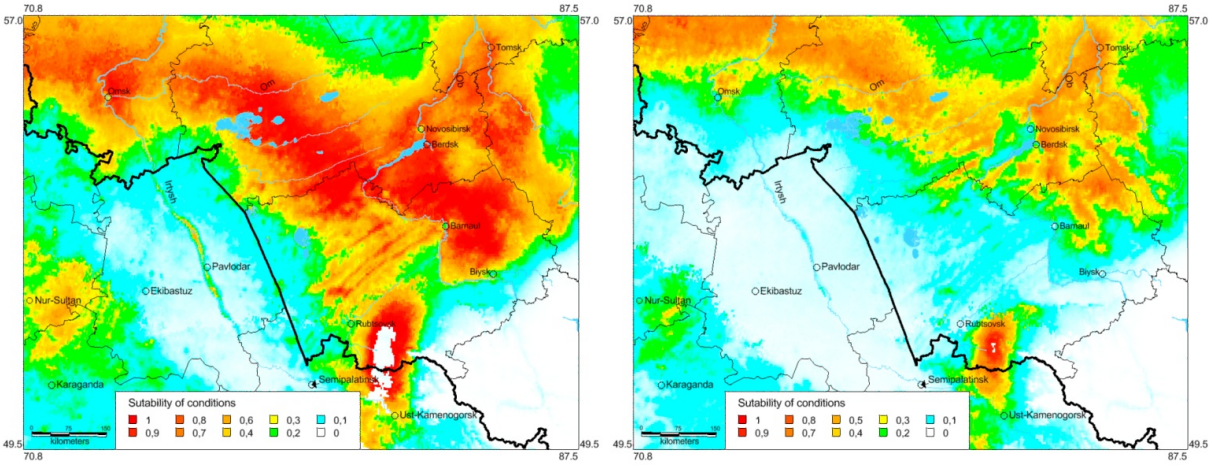


Figure 8. Predicted probabilities of suitable conditions for *Oedaleus decorus* in the south-eastern part of the West Siberian Plain (distribution data from 1961 until 2021 and bioclimatic variables for 1970–2000; point-wise mean for 20 replicates).



(**A**) (**B**)

Figure 9. Predicted probabilities of suitable conditions for *Oedaleus decorus* in the south-eastern part of the West Siberian Plain for 2021–2040 (distribution data from 1961 until 2021 and forecasts of bioclimatic variables from the two global climate models CNRM-ESM2-1 [48,49] (**A**), and MIROC6 [50,51] (**B**), and for the 3–7.0 Shared Socioeconomic Pathway based on high greenhouse gas emissions [52]; point-wise mean for 20 replicates).

According to the MIROC6 model, the species distribution will shift further north, but the level of condition suitability will decrease. The difference between results of ecomodelling for 2021–2040 may be explained by some peculiarities of the last model, because it can slightly overestimate thermal trends in some regions of Asia [66].

Performance of these predictions may be relatively high (the AUC value for training data is 0.910 and for the test sets—0.895; for 20 replicates—0.874 (MIROC6) and 0.888 (CNRM-ESM2-1)) (Figure 6E–G). The main factor for both models is the mean temperature of the wettest quarter, the same as for the contemporary period. Several other factors are also relatively significant: the maximal temperature of warmest month, the mean temperature of warmest quarter, the precipitation of wettest quarter, the annual mean temperature, and the mean temperature of the driest quarter (Table 1). This means, in the near future, the main factors determining the species distribution will remain almost the same. All these factors determine chiefly development of the species during summer.

In July of 2021, we tried to collect some field data supporting these models. We crossed the central part of the so-called Baraba steppe (actually the forest-steppe region between Omsk and Novosibirsk) from the Kochki settlement in the southern forest-steppe (54.3° N 80.4° E) to the Chulym settlement in the northern one (55.13° N 81.03° E) (Table S2). We found no populations of *O. decorus* along this transect.

3.4. Peculiarities of the Long-Term Dynamics of Species Populations

In the 1920s, the abundance of *O. decorus* was relatively low. Bey-Bienko [41] noted that this species was rather numerous only on the sandy dunes in the southern part of the Kulunda steppe in Kazakhstan, and was rare in some adjacent habitats with dominance of short grasses and sagebrushes. However, *O. decorus* was very common in the semi-deserts of East Kazakhstan.

In the end of the 20th century and in the beginning of the 21th century, the handsome cross grasshopper became very abundant in the steppe habitats, especially during warm and dry summers. During the Italian locust outbreaks, its abundance could be very high and comparable with the abundance of *Calliptamus italicus* (Table 2). The Spearman rank-order correlation coefficient for the long-term dynamics of populations of *O. decorus* and *C. italicus* is relatively high for each plot studied (Table 2) and very high if we summarize data for all plots ($r_s = 0.917$, $p < 0.001$).

Table 2. Dynamics of the abundance (ind. per hour) of *Oedaleus decorus* relative to the abundance of the Italian locust in the Kulunda steppe.

Year	Aleksandrovskij	Yarovoe	Ust-Volchikha
2000	1146/2682	252/582	474/144
2001	210/246	54/450	24/234
2002	6/0	0/24	72/42
2003	6/+	0/4	6/6
2004	0/+	0/4	0/+
2005	0/24	54/6	0/0
2006	0/18	60/30	6/0
2007	18/84	150/60	20/+
2008	60/78	144/6	40/0
2015	+/+	4/0	204/480
2018	+/6	?	17.2/17.1
Spearman's correlation	0.586 ($p = 0.058$)	0.609 ($p = 0.063$)	0.518 ($p = 0.102$)

Abundance of *O. decorus*/abundance of the Italian locust; +—one or several specimens were found beyond counts (in such cases, the value 0.01 was used to compute correlations); ?—no data; years of acridid outbreak (commonly associated with the Italian locust) are in bold (cf. [32,33]).

We also estimated correlations between average densities of both species, but for relatively short rows of observations after acridid outbreak (2004–2008, 2015, and 2018)

(Table 3). In this case, the Spearman rank-order correlation coefficient is non-significant for each plot and is moderate, but significant for summarized data (r_s = 0.446, p = 0.049).

Table 3. Dynamics of the average density (ind. per m^2 ± s.e.) of *Oedaleus decorus* relative to the average density of the Italian locust in the Kulunda steppe.

Year	Aleksandrovskij	Yarovoe	Ust-Volchikha
2004	0/0.32 ± 0.32	0.32 ± 0.16/+	0/0.32 ± 0.32
2005	0.43 ± 0.30/0.32 ± 0.23	0.13 ± 0.13/0.38 ± 0.22	0/+
2006	0/+	0.53 ± 0.24/0.21 ± 0.15	0.16 ± 0.16/0.16 ± 0.16
2007	0.48 ± 0.32/0.32 ± 0.23	0.96 ± 0.48/0.64 ± 0.32	0.16 ± 0.16/+
2008	0.42 ± 1.06/0.48 ± 0.27	0.27 ± 0.18/0.51 ± 0.25	0.32 ± 0.32/ 0
2015	+/0	0/0	0.64 ± 0.64/ 1.28 ± 0.89
2018	0/+	?	0.32 ± 0.32/0.32 ± 0.32

Density of *O. decorus*/density of the Italian locust; +—one or several specimens were found beyond counts (in such cases, the value 0.0001 was used to compute correlations); ?—no data.

This means that the contemporary long-term dynamics of the *O. decorus* local populations resembles the dynamics of the Italian locust. As a result, these two species may be members of one temporal distribution guild [67].

3.5. Frequency of the Color Morphs during the Species Outbreak

The handsome cross grasshopper is characterized by high variability in size and color pattern; however, two main color forms, namely brown and green, are very common (Figure 10). Cease et al. [54] tried to check possible associations between color form frequencies, population density levels, and migratory polyphenism for the Mongolian locust (*O. decorus asiaticus*). We analyzed our data (unfortunately, only several limited series) to estimate characters of correlation between frequencies of the brown and green forms and the species abundance in field conditions.

(**A**) (**B**)

Figure 10. Main color forms of *Oedaleus decorus* in the Kulunda steppe: (**A**)—brown; (**B**)—green.

The multivariate regression model shows very week non-significant correlations between these parameters: however, the correlation is positive for the brown form (r = 0.214, p = 0.553) and negative for the green one (r = –0.325, p = 0.359) (overall statistics: R^2 = 0.070, MSE = 0.050, Wilks' lambda = 0.853) (Figure 11).

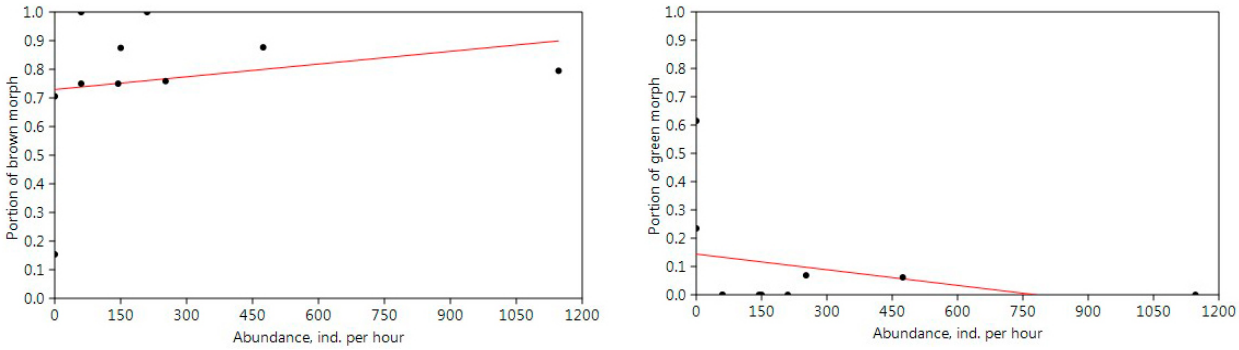


Figure 11. Multivariate regression model for two main color form variations of *Oedaleus decorus* in the Kulunda steppe: (**A**)—brown; (**B**)—green.

Our estimation conforms with the observations for *O. d. asiaticus* [54]. The brown morph is more or less common despite level of abundance. During species outbreaks, a population may consist only of brown adults; however, when the species abundance is very low, the portion of this morph can be very high as well (Figure 11A). The green individuals present in populations with low or moderate abundances (Figure 11B) and in low proportions. However, in other regions, there are several common color forms, e.g., the grey one [68]. Besides, the species demonstrates typical cryptic behavior [68]. This means that diversity of its color patterns may be determined by specificity of a habitat, and individuals of *O. decorus* can select microlandscapes depends on their color forms [68], though Vorontsovsky [9] emphasized that, in the Orenburg Region, the color forms of *O. decorus* were not associated with specific types of habitats.

4. Discussion

Species distribution model approaches have been used to discuss and solve some issues related to species conservation problems and invasions. Several models were generated for grasshoppers and other orthopteran groups (e.g., [69–71]); however, there are a few publications concerning possible pest acridids. Olfert et al. [72] tried to model the distribution shifts of the economically important species, namely the migratory grasshopper or *Melanoplus sanguinipes* (F.), in North America. Recently, two articles concerning ecomodelling of the famous desert locust (*Schistocerca gregaria* (Forsk.) were published [73,74].

O. decorus is a good species for ecomodelling. It is very widely distributed in the southern part of the Palaearctic Region, from south and central Europe, through north Kazakhstan, south Siberia and Mongolia to north and north-east China, and from North Africa, through Asia Minor, Levant, Caucasus, Iran, Kopet Dagh (Turkmen-Khorasan Mountain Range), north Afghanistan, Tien Shan, Pamiro-Alay, south Kazakhstan, Xinjiang, to E China [1,3–8]. The species is also found on the Canary Islands and on Madeira [3]. In Europe, the northern boundary of its range crossed France, Switzerland, Slovakia [3], north Ukraine, and European Russia (Orel, Tambov, and Samara Province) [75]. Uvarov [75] also noted that the species occurred in the Moscow Province; however, this mention is uncertain [76]. In the steppes of south Siberia, the species was distributed over their western (up to Kurgan and Omsk) (*O. d. decorus*) and eastern parts (Minussinsk and Transbaikal areas—*O. d. asiaticus*) [76].

In the end of the last century and in the beginning of the 21th century, the species distribution over Central Europe did not change significantly [77]. *O. decorus* was also found in the Lipetsk and Tambov Regions of European Russia [78]. The northern boundary of its range remained almost the same in the mountains of South Siberia as well [1,4,5,59]. However, in the Volga River Basin (the eastern part of European Russia), in the second half of the 20th century, several sporadic colonies of this species were found in the taiga life zone of the Kirov Region [79]. In 2014, the species was also found in the Kologrivski

Forest Nature Reserve (the taiga life zone, Kostroma Region, about 58°N) [80]. *O. decorus* also became widely distributed in the Republic of Tatarstan [81]. Similar changes are described for the south-eastern part of West Siberian Plain as well (see Results). Such changes, especially the relatively wide dispersal of the species through territories on the eastern side of the Ob River, were predicted more than 30 years ago [4,64].

O. decorus is the usual and often abundant form in dry grasslands of extra-tropical Eurasia, including human-dominated landscapes. It is well represented in many collections, and their known localities are relatively numerous. In this context, importantly, our studies are restricted territorially, and we used several relatively limited sets of data (see Section 2). This is why our results should be interpreted as the preliminary ones.

O. decorus has become one of the most common and widely distributed species not only in the dry steppes of the south-eastern parts of West Siberian Plain, but in the typical and meadow steppes of the region as well. During upsurges the density of its adults is commonly about one to two per square meter. However, our data show that sometimes its density may be more than ten adults per square meter. In many cases, some overlapping of outbreaks of the Italian locust and *O. decorus* was observed, especially in the dry steppes and the semi-deserts of Russia and Kazakhstan. As a result, these two species may damage almost all spectrum of cultivated plants, pastures, and hayfields. Further, in the steppes, during outbreaks hoppers and especially adults of *O. decorus* can actively migrate from one habitat to others as well [21,22].

The main trends in the species distribution over the region are supported by ecological modelling. Our results show that the main ecological factors determining the species distribution are the factors defining its development in the middle of summer. Importantly, our analysis demonstrates that the monthly averaged levels of solar radiation can be very useful variables for forecasts. It is understandable, because really the level of solar radiation can be characterized as a complex factor, which influences on both species development and net primary production. Continuation of global warming may result in further northward and north-eastward shifts of range boundaries of *O. decorus* and in changes of distribution and abundance of its populations, especially in the optimal parts of its population system where the species occupies normally all suitable habitats, its average abundance is relatively high and its colonies are common even during depressions [31,82]. In this context, it is important that *O. decorus asiaticus* has become one of most important pests in the eastern parts of the steppe life zone [22–24,54,83,84], and a similar possible scenario may be realized for *O. decorus decorus* in the south-eastern part of the West Siberian Plain in the nearest future.

5. Conclusions

Since the 1960s, in the south-eastern parts of the West Siberian Plain, the distribution pattern of the handsome cross grasshopper changed significantly. Nowadays, *O. decorus* occupied almost all local steppes and the southern part of the forest-steppes, up to 55°N, and spread over the forest-steppes on the eastern side of the Ob River too. These changes may be explained by both climatic changes (especially northward) and some transformations of human activities (mainly for eastward spreading). One can suppose that in the nearest future, this species will continue to spread eastward and penetrate into the so-called Kuznetsk steppe in the Kemerovo Region. Furthermore, the ecologo-geographic modelling demonstrates evident opportunities for *O. decorus* to penetrate northwards (at least up to 58° N) and north-eastwards as well.

The species has become very common and occurs in almost all steppe habitats in the steppe life zone and on plots with xerophytic and mesoxerophytic vegetation (especially overgrazed meadows, roadsides, and dry lawns) on the eastern side of the Ob River. In 1999–2002, the abundance of *O. decorus* was very high in the Kulunda steppe between the Irtysh and Ob Rivers. This period of its long-term dynamics may qualified as the local outbreak. It is vitally important that local populations of *O. decorus* often became very abundant in the same habitats and in the same periods as the populations of the Italian

locust—one of the most important pests in the region. In this case, the situation for plant protection services may be very complicated, because the handsome cross grasshopper (*O. decorus*) is more or less a typical grass feeder, while the Italian locust prefers dicots. This means during joint outbreaks, these two species can simultaneously damage almost all plants. Besides, one can also suppose that local steppe colonies of *O. decorus* may evolve and some gregarization processes may develop, resulting in their transformation to more or less typical locust populations as described for populations of the Mongolian locust (*O. decorus asiaticus*) in Inner Mongolia [23,54,83,84].

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/insects13010049/s1, Table S1. Studied localities for all acridid species and known records of *Oedaleus decorus* before 1960. Table S2: Studied localities for all acridid species and known records of *Oedaleus decorus* after 1960.

Author Contributions: Field collecting of grasshoppers, geographic coordinate determination, specimen identification, all authors; Conceptualization, methodology, supervision, project administration, funding acquisition, writing, M.G.S.; Old data analysis, K.V.P.; Coordinate checking, map producing, modelling, V.V.M.; General discussion, K.V.P., O.V.Y., N.S.B. and V.D.Z. All authors have read and agreed to the published version of the manuscript.

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insects

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Review

How Did Seal Lice Turn into the Only Truly Marine Insects?

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Simple Summary: Sucking lice are permanent and obligate ectoparasites throughout their whole life cycle. Echinophthiriids escorted their mammal hosts during their passage from fully terrestrial to amphibian life. Seal lice synchronize their reproduction cycle with that of their mammalian hosts. Echinophthiriids tolerate long immersion periods and extreme hydrostatic pressures. Diving lice can reach kilometers under the surface and survive, during the months their hosts remain in the open ocean. In the present work, we describe and discuss how some of these adaptations allow seal lice to cope with the amphibious habits of their hosts and how they can help us to understand why insects are so rare in the ocean.

Abstract: Insects are the most evolutionarily and ecologically successful group of living animals, being present in almost all possible mainland habitats; however, they are virtually absent in the ocean, which constitutes more than 99% of the Earth's biosphere. Only a few insect species can be found in the sea but they remain at the surface, in salt marshes, estuaries, or shallow waters. Remarkably, a group of 13 species manages to endure long immersion periods in the open sea, as well as deep dives, i.e., seal lice. Sucking lice (Phthiraptera: Anoplura) are ectoparasites of mammals, living while attached to the hosts' skin, into their fur, or among their hairs. Among them, the family Echinophthiriidae is peculiar because it infests amphibious hosts, such as pinnipeds and otters, who make deep dives and spend from weeks to months in the open sea. During the evolutionary transition of pinnipeds from land to the ocean, echinophthiriid lice had to manage the gradual change to an amphibian lifestyle along with their hosts, some of which may spend more than 80% of the time submerged and performing extreme dives, some beyond 2000 m under the surface. These obligate and permanent ectoparasites have adapted to cope with hypoxia, high salinity, low temperature, and, in particular, conditions of huge hydrostatic pressures. We will discuss some of these adaptations allowing seal lice to cope with their hosts' amphibious habits and how they can help us understand why insects are so rare in the ocean.

Keywords: adaptation; Anoplura; Echinophthiriidae; extreme environments

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1. Introduction

When one searches for information about marine insects, the literature usually refers to sea-skaters, a small group of Heteroptera of the family Gerridae ("water-striders"), belonging to the genus *Halobates* [1]. Scientists have identified 46 different species, which live in association with the bi-dimensional world of the sea surface [2]. Only five species of *Halobates* live in the open ocean. It is worth mentioning, however, that sea-skaters remain on the surface and never dive below it. As a consequence, they are not truly exposed to marine conditions, even though the sea surface can also be hostile because of the exposure to UV rays, and passive displacement. Technically speaking, *Halobates* should be considered

terrestrial insects that live in the open ocean, not so different from their relatives skating on the surface of ponds on the mainland.

Insects first appeared more than 420 Mya during the Silurian–Ordovician epoch and, during the next 300 million years, they dispersed and diversified, colonizing nearly every available mainland habitat. Intriguingly, the most ecologically and evolutionarily successful group of organisms on Earth is virtually absent from the greatest available habitat, i.e., the ocean, which constitutes more than 99% of our biosphere. This lack of insects in the ocean, as well as their occasional occurrence in marine ecosystems, contrasts with their richness on land, leading to a variety of scientific hypotheses and assumptions, which we shall explore further in this paper [3]. Yet, there exists a particular group of insects that managed to survive underwater at great depths during long immersion periods, i.e., seal lice.

Lice (of the order Phthiraptera) are the only group of insects that have become obligate and permanent parasites throughout their entire life cycle, living as ectoparasites among the feathers, fur, or hairs of vertebrate hosts [4,5]. Throughout their evolutionary history, sucking lice (suborder Anoplura) have established associations and co-evolved with mammals, being present in most Mammalian genera, with the exception of those belonging to the orders Monotremata, Cetacea, Sirenia, Pholidota, Edentata, and Proboscidea. Across the great diversity of anopluran lice, the family Echinophthiriidae shows the unique characteristic of infesting amphibious hosts, such as pinnipeds (walruses, seals, and sea lions) and the North American river otter [6,7].

Pinnipeds are diving mammals and many of them forage at significant depths [8]. The most extraordinary diver is the southern elephant seal, which can dive more than 2000 m deep [9]. On the other hand, during the feeding periods (i.e., most of the year), pinnipeds can spend several months in the open sea [10] without returning ashore. Despite the extreme constraints imposed by these habits on echinophthiriid lice, they have managed to adapt to the amphibian biology of their hosts [11]. The survival of an originally terrestrial louse in the deeps of the ocean implies that this insect gradually evolved to tolerate the particular physical conditions of extreme environments, such as high hydrostatic pressure, hypoxia, low temperature, and high salinity.

The underlying mechanisms that allow echinophthiriids to live in association with deep-diving hosts have only recently started to be investigated. This review provides a critical discussion of the state of knowledge about the adaptations of echinophthiriids to survive where no other known insect is capable of surviving. This synthesis is relevant and timely because, until recently, there was a widespread belief that the ectoparasitic lice that live on semi-aquatic mammals would perish if their hosts went to sea. The discovery of adult elephant seals ashore in Antarctica with living adult lice clinging to their bodies has finally disproved this theory [12], meaning that these insects have traveled with their host, probably for months, in the open sea and survived. This finding made us think about echinophthiriids' unique adaptations to withstand the harsh environments of the ocean. It also pushes us to forsake the notion that insects are not naturally suited to surviving in the ocean.

2. Materials and Methods

2.1. Evolution

According to molecular and paleontological data, pinnipeds diverged from their carnivorous ancestors about 45 Mya, with the separation of the Feliformia and the Caniformia [13]. Molecular analysis also supports the monophyly of the Pinnipedia, with a basal split between Otariidea (sea lions, fur seals, and walruses) and Phocidae (seals) [14]. Evidence suggests a North American origin for pinnipeds, which was followed by a Pacific dispersal of otariids into the Southern hemisphere and an Atlantic dispersal for phocids. During the colonization of the marine environment, pinnipeds lost most of their parasites [15]. Yet, the fact that pinnipeds kept their contact with the terrestrial environment, allowed some parasites like echinophthiriid lice to accompany this evolutionary process [15,16]. The family Echinophthiriidae comprises five genera and 13 species (Table 1), including *Antarctophthirus,* the ectoparasites of sea lions, Antarctic seals, the north-

ern fur seal, and the walrus; *Echinophthirius* from true seals in the Northern hemisphere; *Latagophthirus* from the North American river otter; *Lepidophthirus* from elephant and monk seals; and *Proechinophthirus* from northern and southern fur seals [6,7,17].

Table 1. Seal-louse associations of the family Echinophthiriidae (Anoplura).

Louse Genus	Species	Host
Antarctophthirus	*A. callorhini*	Northern fur seal
	A. carlinii	Weddell seal
	A. lobodontis	Crabeater seal
	A. mawsoni	Ross seal
	A. microchir	Steller, Californian, South American, Australian, and New Zealand sea lion
	A. ogmorhini	Leopard seal
	A. trichechi	Walrus
Latagophthirus	*La. rauschi*	North American river otter
Lepidophthirus	*Le. macrorhini*	Elephant seals
	Le. piriformis	Monk seals
Echinophthirius	*E. horridus*	Northern true seals
Proechinophthirus	*P. fluctus*	Northern fur seal
	P. zumpti	Southern fur seals

A phylogenomic analysis, including *A. microchir* from Southern and Australian sea lions, *A. carlinii* from Weddell seals, *A. lobodontis* from crabeater seals, *A. ogmorhini* from leopard seals, *L. macrorhini* from southern elephant seals, and *P. fluctus* from the northern fur seal, supports the monophyletic origin of the echinophthiriids and the terrestrial origin of this host–parasite association (Figure 1) [11]. These results agree with the pioneering ideas of Kim [4,18]. Based on morphological phylogenetic analysis, Kim was the first to suggest that the terrestrial ancestors of pinnipeds were already infested by ancestral sucking lice. Therefore, lice adapted to the new environmental conditions imposed by their hosts. This is likely one of the primary reasons why lice became the only insects to colonize the deep sea, probably acquiring unique morphological, physiological, behavioral, and ecological adaptations in the process to cope with the amphibious lifestyle of their hosts. Next, we discuss the main adaptations that allowed lice to coevolve alongside their hosts.

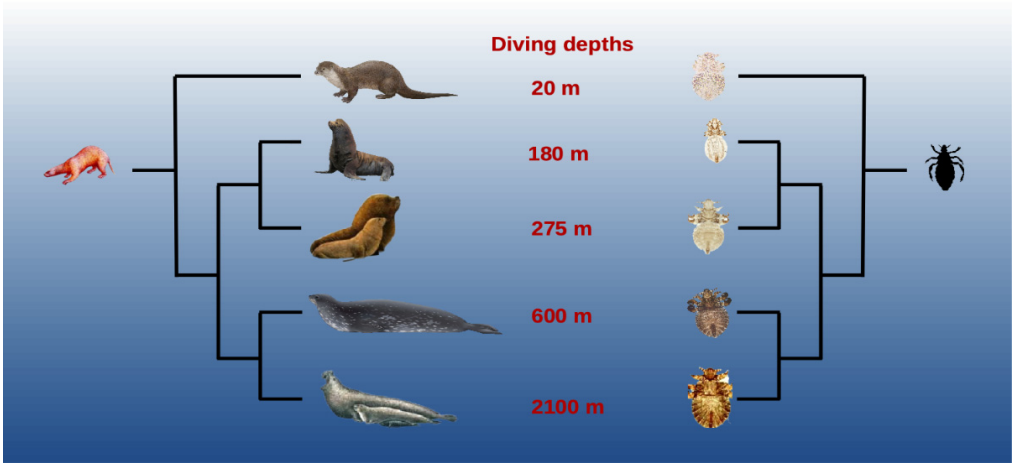

Figure 1. Schematic phylogenetic tree comparing the evolutionary histories of pinnipeds (**left**) and their lice (**right**), modified from Leonardi et al. (2019). Host-louse associations: 1—North American river otter—*Latagophthirus rauschi*; 2—Northern fur seal—*Proechinophthirus fluctus*; 3—Southern sea lion—*Antarctophthirus microchir*; 4—Weddell seal—*A. carlinii*; 5—Southern elephant seal—*Lepidophthirus macrorhini*. Seal images are from Pieter Folkens and the NOAA; *Le. macrorhini* and *La. rauschi* photos are from phthiraptera.org.

2.2. Morphological Adaptations

Echinophthiriids present some unique morphological adaptations for underwater life. Firstly, all species have the tibia-tarsi of second and third pairs of legs that are strongly adapted to clinging. The first pair of legs in most species is smaller and more slender than the others. Probably, these legs play a sensory role in insects where, according to the literature, eyes are absent [4,19,20]. However, the first pair of legs of *L. macrorhini* is robust, and the tarsal claws are modified into well-developed hooks [21]. It has been suggested that this species utilizes its claws to perforate the skin and dig into the host epidermis, in order to stay attached during elephant seal molting [21]. Regarding the absence of eyes, a series of studies in different species is required to determine the presence of specific structures or pigments capable of detecting light.

Secondly, according to Kim (see Figure 343 in [22]), the louse spiracles present an elaborated closing device that could have a double function, i.e., to preserve the atmospheric air into the tracheal system and to prevent the entry of seawater during immersions. However, due to the extremely high hydrostatic pressure seen during deep dives, the tracheal system may entirely collapse [23]; some oxygen could be conserved at a cellular level, either dissolved or associated with (as yet unknown) respiratory pigments. Thus, the elaborated system for closing spiracles would be more related to avoiding the entry of water, rather than retaining air in the tracheal system.

Finally, the abdomens of seal lice are membranous and considerably thicker than the typical Anopluran abdomen [19]. It has been identified for *A. carlinii* that the ventral surface cuticle is at least half as thin as the dorsal side and it is especially thin in the head. [19]. A thin cuticle could enable gas exchange and cutaneous respiration, a possibility that remains to be investigated. We will discuss this point in more detail later on.

Scales, or specialized and modified spines [24], are a distinctive feature of echinophthiriids (Figure 2), and their density and size increase as they develop [19,20,25,26]. The initial nymphal stage, which remains in the case of non-swimming juvenile hosts, is devoid of scales [20,22]. The specific role of scales has been discussed many years ago, and two different possible functions have been proposed, both related to adaptations for surviving underwater [22,27,28]. Murray [26] postulated that scales would protect the cuticle from mechanical damage (e.g., by high hydrostatic pressure) and against desiccation, whereas Hinton [29] proposed that scales could form a "plastron" (i.e., a physical gill formed by a thin layer of air, retained by hydrophobic structures) making underwater respiration possible. These two hypotheses are not mutually exclusive, and recent studies did provide support to both of them. On the one hand, Leonardi and Lazzari [30] reported that sea lion lice showed higher survival rates and shorter recovery times when they were submerged for variable time periods, between 1 to 15 days, in normoxic rather than in hypoxic water, supporting aquatic respiration and, by extension, Hinton's hypothesis. On the other hand, experiments aimed at determining tolerance to high hydrostatic pressure also revealed that adults, which have their bodies fully covered with scales, performed better than nymphs with fewer scales over their bodies [23]. It should be noted, however, that the effects of high hydrostatic pressure would include collapsing air-filled cavities, as tracheal tubes, and, when the pressure is very high, affecting cellular and molecular integrity. Since the lice body is mostly incompressible, it is hard to make a link between scales and tolerance to high pressure, as suggested by Murray [26].

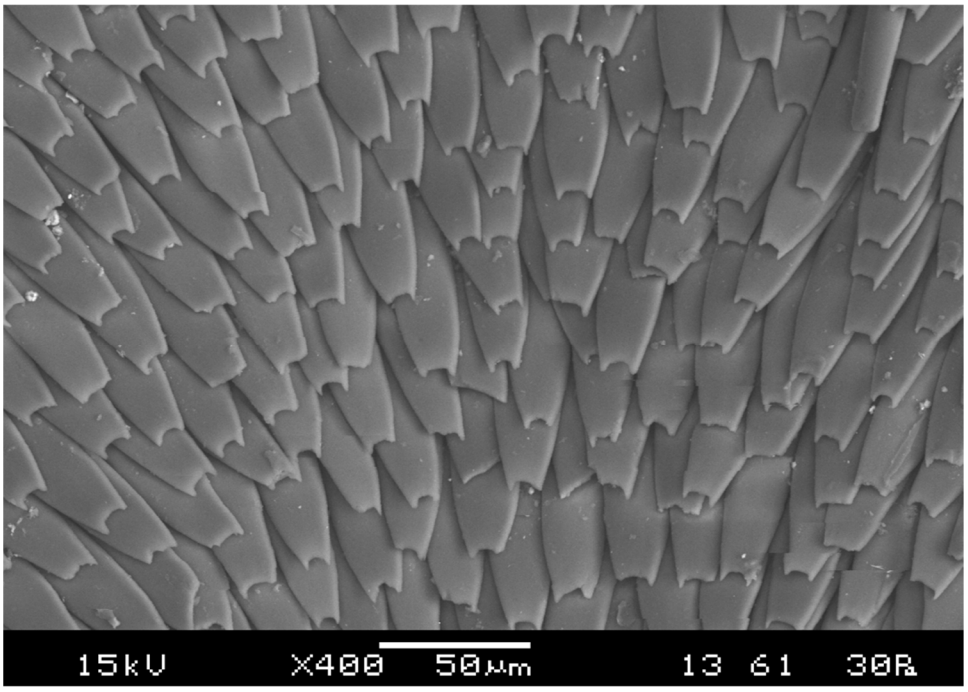

Figure 2. Scanning electron micrography of a female *Lepidophthirus macrorhini*, depicting the disposition of scales.

2.3. Reproductive Synchronization with Hosts

One of the greatest constraints for echinophthiriids is that their eggs do not survive underwater [30,31]. Consequently, lice reproduction can only occur during those periods when hosts remain on land for enough time, i.e., during their reproduction and molting season. So, the reproductive events and the number of lice generations per year are constrained by the haul-out behavior of their hosts. Indeed, there is an adaptive reproductive schedule of seal lice according to the biology and ecology of their hosts [28]. For instance, in the case of *A. microchir* from South American sea lions, the reproductive season is the only moment of the life cycle when the host spends enough time ashore, and only newborn pups remain outside the water long enough to allow lice to reach the imaginal state [27,30]. Instead, in the case of *A. lobodontis* from the crabeater seal, reproduction and transmission would only be possible with juvenile hosts [32].

2.4. Tolerance to Immersion

A series of experiments have been conducted on nymphs and adults, to evaluate lice survival under different conditions of immersion and temperature, using the protocol depicted in Figure 3 (for details, see [30]). It was observed that the first nymphs (N1) were unable to survive underwater but the rest of the instars and adults tolerated submersions lasting for several days [30]. Previous contributions by Murray and Nicholls [33] had already reported the death of eggs and the survival in seawater of advanced nymphs and adults; however, N1 were not included in their experiment. According to the findings of a recent study, N1 can only withstand immersion for a few days. The reduced tolerance to immersions of N1 compared to more advanced instars explains the reduction of N1 in the South American sea-lion pup population when they start to swim, as alleged by Leonardi and Lazzari [30]. Murray and co-workers had previously arrived at a similar conclusion [31,33], as well as Kim [22], from the absence of N1 in old pups and adult pinnipeds on northern fur seals. The incapacity of N1 to survive underwater was suggested to be associated with the absence of abdominal scales [20,22,27], which are abundantly present in the tolerant instars.

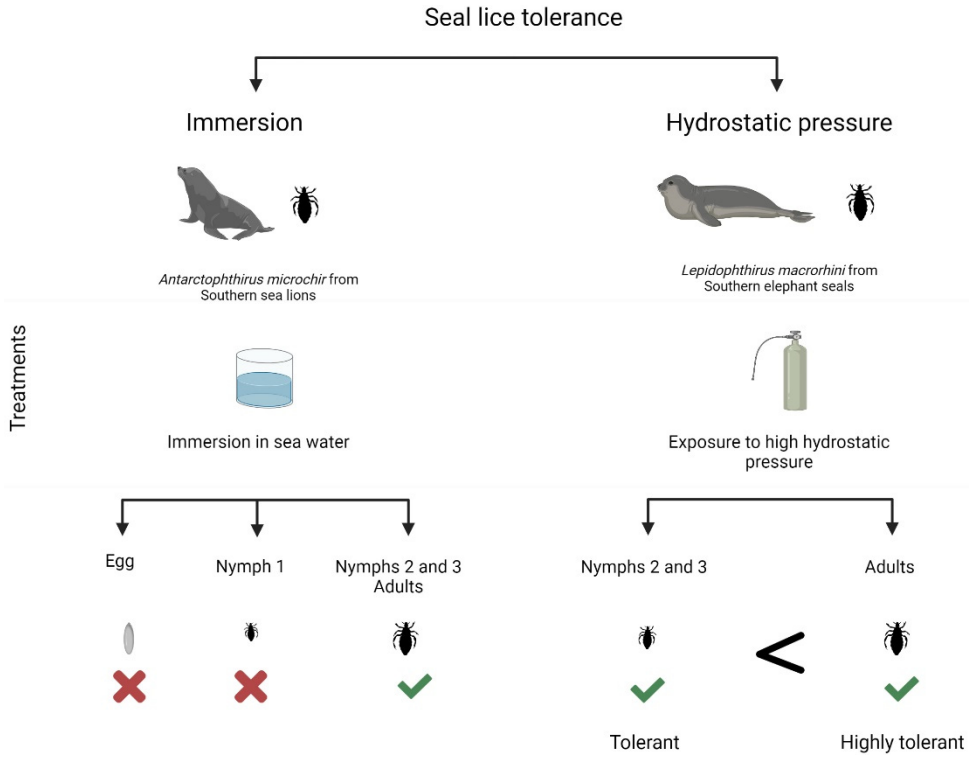

Figure 3. Experimental design and main results testing the tolerance to immersion (**left**) and high hydrostatic pressure (**right**). ✓ indicates survival; ✕ death.

When seal lice emerge in seawater, they experience a reduction in oxygen availability, and a rapid and large drop in temperature (20–25 °C difference between air and water in summer), as well as an increase in hydrostatic pressure. It has been reported that contact with seawater triggers reflex immobility (akinesis) in *A. microchir* and in *L. macrorhini*, which is immediate (in seconds) in the former and requires several minutes in the latter [23,30]. Thus, it seems that the tolerance to immersion depends on a reflex reduction in metabolism and activity (i.e., quiescence) triggered by the physical contact of lice with seawater. This rapid response would help to spare energy, nutrients, and oxygen, consequently allowing the survival of lice for a long time underwater. Furthermore, their differential survival when submerged in normoxic or in hypoxic water [30] strongly suggests that echinophthiriids would be able to exchange gases with the surrounding water, a capacity never before reported in the group. The adaptations and mechanisms that underpin this ability are currently unknown, and more anatomical and physiological research is required.

2.5. Tolerance to Hydrostatic Pressure

In another series of experiments, depicted in Figure 3, it was found that lice from elephant seals can tolerate hydrostatic pressures equivalent to 2000 m in depth [23], which represents a depth equivalent to seven times the Eiffel Tower or the Empire State Building beneath the surface of the sea. Serendipitously, a louse was observed to survive to a pressure of 450 kg cm^{-2} (eq. 4.5 km in depth) when accidentally exposed to this hydrostatic pressure for some minutes. This represents a 50% higher hydrostatic pressure than that supported by the deepest marine mammal, i.e., the Cuvier's beaked whale [34], for which a maximum diving depth of 3000 m was reported. The same study also revealed that in addition to tolerating high compression, lice supported rapid changes in hydrostatic pressure, which can be thought of as the natural equivalents of the rapid dives and climbs to the surface performed by their hosts [23]. Another significant finding was that seal lice can tolerate hydrostatic pressure by themselves, i.e., they do not need to be associated with the host mammal to do so. It can, therefore, be assumed that this ability is an intrinsic feature of echinophthiriids [23] and that hiding in the hosts' fur is not crucial to their survival.

When penguins are in the water, they trap a blanket of air within their feathers and a warm skin temperature; thus, it appears that their lice do not encounter true marine conditions and can continue to spawn whether the bird is on land or at sea [35].

2.6. Ecology

During the 1960s and 1970s, Murray and Kim conducted the first studies on the ecology and life cycles of echinophthiriids. Murray focused on lice from two Antarctic seals, i.e., *A. carlinii* from Weddell seals [31,35] (Murray, 1964; Murray et al., 1965) and *L. macrorhini* from the southern elephant seal [33,35–37]; while Kim studied *A. callorhini* and *P. fluctus* from the northern fur seal [22,25,38]. In these pioneering studies, the authors first showed that the reproduction and transmission of echinophthiriids can only occur when their hosts are on land; consequently, their life cycle adjusts precisely to the reproduction cycle of their hosts. The main consequence of this adjustment is a temporal restriction of reproduction, which limits the number of lice generations [27].

As is the case in all lice species, spreading requires close contact between potential hosts. In the particular case of echinophthiriids, transmission occurs during the time that seals spend ashore mating, nursing, molting, or resting [22,28,39]. For most species, it has been reported that the main method of transmission for seal lice occurs from the mother to the newborn pup during nursing [27,38]. However, for other seals, the pattern seems to be different. *Antarctophthirus lobodontis,* from the crabeater seal, is more abundant in juveniles, and lice move between individuals rather than just between mothers and their offspring [32]. As this occurs with reproduction, the strategies of each echinophthiriid species adjust precisely to the particularities of its host species, which reflects a long coevolutionary process.

3. Conclusions

The particular biology of seal lice makes them a fascinating example of adaptation. Their long evolutionary history in association with their amphibious hosts has exposed them to selective pressures that no other insect undergoes. The research into the specific morphological, physiological, and behavioral adaptations that enable them to tolerate the harsh environments they encounter during their ectoparasitic life is only just getting underway. A major piece of information that was recently acquired is particularly revealing: the fact that they do not die during the long excursions into the open sea of their deep-diving hosts.

This premise is not as simplistic as it appears. It puts aside the conservative idea that only those remaining on the mainland would somehow survive and wait during most of the year for the return of their hosts ashore for the next reproductive season. With their capacity to survive in extreme environments being confirmed beyond any doubt, we can now focus on the next scientific challenge, i.e., explaining how this is possible. The previous sections presented some hypotheses to be tested and research leads to follow, which should help to decipher the puzzle.

This review helps us identify some key questions to be investigated next, in order to understand better the morphological and physiological adaptations of seal lice to the amphibious life of their host; for example: (1) can seal lice breathe underwater through cuticular diffusion or a plastron? (2) Does the tracheal system completely collapse during dives? (3) Do they reduce their metabolism when submerged, sparing oxygen and energy? (4) Are they capable of keeping an oxygen reserve associated with respiratory pigments? (5) Does high hydrostatic pressure trigger molecular mechanisms that aid in the tolerance of high pressures, as in the synthesis of piezolytes?

Beyond their fascinating biology, seal lice encourage us to forsake the notion that "insects are not made to survive in the ocean", based on arguments concerning their respiratory system, osmoregulation, or their lack of transparency [40,41]. So far, seal lice have not revealed any unusual structural or physiological adaptations associated with their extraordinary endurance. Their secret appears to be a well-balanced set of traits that they share with a variety of other insects.

So, if insects are able to live in the oceans, a legitimate question is: why are they virtually absent? The study of seal lice suggests that the answer to this question could well not be related to morphological or physiological constraints but probably for evolutionary and/or ecological reasons [41]. Despite the constraints imposed by their biology, we expect that in the near future, these insects will continue to offer more information about their adaptations to marine life.

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Life History Traits in Two *Drosophila* Species Differently Affected by Microbiota Diversity under Lead Exposure

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Simple Summary: Microbiota have a significant functional role in the life of the host, including immunity, lifespan and reproduction. *Drosophila* species are attractive model organisms for investigating microbiota diversity from different aspects due to their simple gut microbiota, short generation time and high fertility. Considering such an important role of the microbiota in the life of *Drosophila*, we investigated the extent to which lead (Pb), as one of the most abundant heavy metals in the environment, affects the microbiota and the fitness of this insect host. The results indicate that different factors, such as population origin and sex, may affect individual traits differently and this could be species-specific. In addition, there are members of microbiota that help the host to overcome environmental stress and they could play a key role in reducing the fitness cost in such situations. Studying the influence of microbiota on the adaptive response to heavy metals and the potential implications on overall host fitness is of great pertinence.

Abstract: Life history traits determine the persistence and reproduction of each species. Factors that can affect life history traits are numerous and can be of different origin. We investigated the influence of population origin and heavy metal exposure on microbiota diversity and two life history traits, egg-to-adult viability and developmental time, in *Drosophila melanogaster* and *Drosophila subobscura*, grown in the laboratory on a lead (II) acetate-saturated substrate. We used 24 samples, 8 larval and 16 adult samples (two species × two substrates × two populations × two sexes). The composition of microbiota was determined by sequencing (NGS) of the V3–V4 variable regions of the 16S rRNA gene. The population origin showed a significant influence on life history traits, though each trait in the two species was affected differentially. Reduced viability in *D. melanogaster* could be a cost of fast development, decrease in *Lactobacillus* abundance and the presence of *Wolbachia*. The heavy metal exposure in *D. subobscura* caused shifts in developmental time but maintained the egg-to-adult viability at a similar level. Microbiota diversity indicated that the *Komagataeibacter* could be a valuable member of *D. subobscura* microbiota in overcoming the environmental stress. Research on the impact of microbiota on the adaptive response to heavy metals and consequently the potential tradeoffs among different life history traits is of great importance in evolutionary research.

Keywords: *Drosophila melanogaster*; *Drosophila subobscura*; egg-to-adult viability; developmental time; microbiota diversity; lead exposure

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1. Introduction

The intestines of animals are occupied by diverse communities of microorganisms that can affect different aspects of host health. The microbiota plays a key role in many

aspects of host life, including development, digestion, behavior and the immune system [1–3]. Simple gut microbiota of *Drosophila* species, short generation time and high fecundity are some of the reasons that make them an attractive model for studying the significance of gut microbiome from different aspects. *Drosophila* hosts only a small number of bacterial populations in its gut, but includes species present in the human microbiota as well. *Drosophila* gut microbiota in laboratory is represented by a low-diversity bacterial community [4,5], but it has great implications on its overall health. The gut microbiome of *Drosophila* contributes to a variety of host traits, such as innate immunity [6], lifespan [7–9], nutrition and reproduction [10] and behavior [11,12]. Shifts in microbiota could lead to serious consequences on host physiology, causing even mortality [13]. Thus, it is important to investigate the factors that shape the composition and diversity of microbiota and their possible implications on the host.

One of the greatest problems that animals currently face within the natural environment is pollution. Due to the anthropogenic factors, the presence of pollutants is widespread in the air, soils and water. Heavy metals are pollutants commonly found in nature, with great impact on plants and animals. Recently, it has been reported that heavy metals, such as arsenic, cadmium, lead and mercury, have detrimental effects on the diversity of terrestrial invertebrates at levels below those considered safe for humans [14]. The population-level phenotypic variability of different species depends on the interaction between genetic and environmental variability. The variability in the components of adaptive value (fitness) is an aspect of phenotypic variability. Lead is one of the widespread heavy metals that has previously been reported to have a major negative impact on *Drosophila* fitness [15]. However, it has been shown that its negative impact can be modified depending on the population genetic background [16], genome heterozygosity [17] and genetic variation [18]. Since *Drosophila* species are mainly exposed to lead through food intake, it was suggested that gut microbiota could also have an impact on the species resistance to lead toxicity. Our previous research suggested that bacterial diversity increased in two *Drosophila* species after extended exposure to a lead-saturated substrate. This increase in bacterial diversity underlined certain bacterial genera, such as *Komagataeibacter* and *Acetobacter*, that could be good lead-tolerant members of microbiota [5].

The results obtained in our previous study suggested the difference in shifts of microbiota composition between natural populations of two *Drosophila* species under laboratory conditions on standard and lead-saturated substrate after 13 generations [5]. In the present paper we investigate the microbiota of the same lab-reared *Drosophila* species (*D. melanogaster* and *D. subobscura*), but from each of the two distinct localities on standard and lead-saturated substrate after an additional 22 generations (35 in total). Further, we explore if the changes in life history traits (egg-to-adult viability and developmental time) are associated with microbiota content enabling different responses to lead (Pb) in those experimental groups. The influence of lead exposure was discussed for the population, species, substrate and sex levels for both microbiota and fitness components. The microbiota was analyzed using NGS sequencing of the V3-V4 16S rRNA gene and assessed through diversity indices and taxonomical analysis.

The aim of this study was to determine the impact of prolonged lead exposure in two lab-reared *Drosophila* species, each from two populations on microbiota composition and life history traits, to find potential cause-and-effect relationships between them and to differentiate the response of different origin, species and sex to stress factors.

2. Materials and Methods

2.1. Sample Collection and Laboratory Maintenance

In this study, both species (*D. melanogaster* and *D. subobscura*) were sampled from two localities in Serbia: Kalna (43.4217 N, 22.4159 E) and Slankamen (45.1415 N, 20.2586 E). Flies of both species were collected with a sweeping net using fermented apple traps. They were maintained in the laboratory at 19 ± 0.5 °C, 12 h:12 h light-dark cycle in 240 mL bottles containing 40 mL of the control substrate (standard-St) or the Pb-acetate-saturated

substrate (labeled as C3). The control substrate consisted of standard molasses corn meal diet (14 g agar, 208 g corn meal, 188 g sugar, 40 g dry active yeast, 5 g Nipagin diluted in 60 mL of 96% ethanol in 2.2 L distilled water) and the Pb-acetate-saturated substrate contained 1000 μg/mL of lead acetate. The standard substrate flies were grown originally for 45 and 30 generations (*D. subobscura* and *D. melanogaster*, respectively). After that, they were maintained for another 35 generations on the standard substrate and the Pb-acetate-saturated substrate. Both species were maintained at 19 °C; *D. melanogaster* is successfully reared at 19–25 °C, while for *D. subobscura* 19 °C is the optimal temperature. Since *D. subobscura* generally does not lay eggs in laboratory conditions without dry yeast powder, the powder was added to the substrate surface in both species in order to maintain equal conditions (detailed description is given in Beribaka et al. [5]). For the same purposes, the Nipagin, which was reported to modify the microbiota associated with the flies to some extent, was added in all substrates in the same amount at the same time [19].

2.2. Experimental Setup

On the first day of the experiment, 30 pairs of flies per bottle were transferred to a fresh substrate for 5 days; they were then removed from the bottles. After eclosion started, virgin males and females of *D. melanogaster* were collected every 12 h and once every 24 h for *D. subobscura* and separated by sex. Bottles with eclosing *D. subobscura* were additionally held in the dark to avoid possible mating. When 150 virgin males and females from each group were collected, 30 females and 30 males per bottle (5 bottles per group) within each group were placed to mate for 3 days. After that, the bottles were covered with Petri dishes that contained substrate and yeast dissolved in distilled water on the substrate surface and turned upside-down to enable flies to lay eggs. The eggs were collected every 8 h from the Petri dishes and transferred into 50 mL vials with 15 mL of the substrate and 1-2 drops of dissolved yeast added to the surface. For each group, 30 vials with 30 eggs were established (additional 5 vials per group were added for larvae collection). The F1 generation eclosion was recorded every 24 h until there were no new individuals in the vial for a period of 72 h; the flies were then stored in EtOH for NGS (separated by sex) at −20 °C. The egg-to-adult viability was calculated per vial as the percentage of individuals that emerged from the 30 eggs. The egg-to-adult developmental time was calculated using the formula:

$$DT = \frac{\Sigma n_d * d}{\Sigma n_d} \quad (1)$$

where n_d is the number of flies emerging in d days after the eggs were laid.

The third instar larvae were collected from the additional 5 vials per group and after 64 h of incubation they were rinsed with distilled water and stored in EtOH at −20 °C for NGS.

The samples were labeled as follows: *D. melanogaster*—Dmel, *D. subobscura*—Dsub, Kalna population—K, Slankamen population—Sl, standard (control) substrate—St, Pb-saturated substrate—C3, males—M, females—F and larvae—L.

2.3. Statistical Analysis of Life History Traits

Prior to the analyses of life history traits, all data were tested for normality by the Shapiro-Wilk test. Since the data for both egg-to-adult viability and egg-to-adult developmental time were normally distributed, we used the three- and four-factor ANOVA test to ascertain statistical significances. For egg-to-adult developmental time, the analyses were performed for males and females separately. The Bonferroni post-hoc test was used to identify the exact statistically significant differences. All the tests were performed in Statistica, ver. 10.0.228.2 [20].

2.4. Total DNA Extraction and 16S rRNA Sequencing

Total DNA was extracted from pools of 25 males, 25 females and 40 larvae for each experimental group, with 24 samples in total. DNA isolation was performed according to

the modified protocol by Kapun et al. [21]. The samples were homogenized using handheld motor homogenizer in a 1.5 mL Eppendorf tube in 300 µL of Solution A (1 M Tris HCl, pH 9, 0.5 M EDTA, pH 8 and 1% SDS). Then 4.5 µL of Proteinase K (20 mg/mL) were added, and the samples were vortexed and incubated for 30 min at 56 °C, after which they were incubated for another 30 min at 70 °C and, finally, for a few minutes at 37 °C. Afterwards, 3 µL of RNAse A (10 mg/mL) were added and incubated for 30 min at 37 °C. Next, 42 µL 8 M potassium acetate was added and the mixture was kept in the freezer for 30 min. The supernatant was collected after centrifugation at 13,000 rpm for 15 min. One volume of phenol-chloroform-isoamyl alcohol (25:24:1) was added and the mixture was centrifuged for 5 min at 13,000 rpm. After the supernatant was collected, the previous step was repeated with 0.75 volume of pure chloroform. Afterwards, 2.5 volume of 95% ice-cold ethanol was added and centrifuged at 10,000 rpm for 5 min to pellet the DNA. The pellet was washed with 1 mL of 70% ethanol, centrifuged at 13,000 rpm for 5 min and then the supernatant was discarded. The pellet was dried for 30 min and left to resuspend in 50 µL of TE buffer. DNA quality was assessed using the Qubit fluorometer (Thermo Fisher Scientific, Waltham, MA, USA).

The sequencing was performed by Eurofins Genomics Europe Sequencing GmbH (Konstanz, Germany) using the standard procedure InView—Microbiome Profiling 3.0 with MiSeq. The V3-V4 hypervariable region of the 16S rRNA gene was amplified using the forward primer 5′-TACGGGAGGCAGCAG-3′ and reverse primer 5′-CCAGGGTATCTAATCC-3′ [22,23]. The samples passed a quality check and fastq data were delivered for further processing. All sequence data were submitted to the GenBank (SRA) database under accession numbers SRX12591286-SRX12591309 (PRJNA616141 BioProject).

2.5. Amplicon Sequence Variant Inference and Taxonomy Assignment

The delivered sequences already had the adapters and linkers removed. The primers were removed using cutadapt 3.4 (https://cutadapt.readthedocs.io/en/stable/guide.html, accessed on 2 December 2021) in paired-end mode and reads without detected primers were discarded. Afterwards, dada2 [24] was used to filter and trim the reads. Apart from the default filter and trim options, the forward reads were trimmed to 270 bases while the reverse reads were trimmed to 200 bases and all the reads shorter than 150 bases were removed, as well as all those which had more than two estimated errors for the forward or reverse reads. After error estimation and dereplication, denoising was performed in selfConsist mode (the algorithm alternated between sample inference and error rate estimation until convergence). Sequence pair merger was performed with a minimal overlap of 20 bases without mismatches. Chimeric sequences were removed using default options in removeBimeraDenovo (https://rdrr.io/bioc/dada2/man/removeBimeraDenovo.html, accessed on 2 December 2021). After the inspection of amplicon sequence variants (ASV) length distribution, all ASV with a length between 400 and 428 were kept. Taxonomy assignment up to the genus level was performed with IDTAXA algorithm [25] using default parameters and the SILVA v138 database (https://www.arb-silva.de/documentation/release-138/, accessed on 2 December 2021). Species level classification was performed with a SILVA species assignment train set (https://zenodo.org/record/3731176, accessed on 2 December 2021) using exact sequence matching without mismatches; multiple matches were allowed (multiple species output). One ASV which had low read counts was not classified at the Kingdom level and was removed from further processing.

2.6. Microbiome and Statistical Analysis

Sequence diversity within samples (alpha diversity) was estimated using the phyloseq 1.36.0 R package [26] at the ASV level after rarefaction to even depth (sample with the lowest number of reads) and shown through estimators Shannon, Gini-Simpson and invSimpson indices. Observed and estimated richness was determined according to the number of observations (Observed) and the Chao1 index. Comparison of alpha diversity among *D. melanogaster* and *D. subobscura* samples, as well as the impact of the growth

substrate on Chao1 and Shannon indices within each species, was determined using the Wilcoxon Rank-Sum test. For further analysis, prevalence filtering was performed.

For beta diversity and differential abundance estimation between samples, genus level aggregation was used (reads from ASV classified as the same genus were aggregated). Since many low occurrence genera were present in only one or a few samples, prevalence filtering was performed where all genera present in less than four samples were removed. Beta diversity was estimated using Double Principal Coordinate Analysis (DPCoA) [27] of the prevalence filtered data after rarefaction to even depth. Since DPCoA relies also on phylogenetic distances of sequences apart from abundances, a maxim likelihood (GTR+G(4)+I) phylogenetic model was estimated using a multiple sequence alignment of the microbial 16S sequences constructed to take into account RNA secondary structures. To assess if the microbial composition differs among species and among fixed effects (sex and substrate) within species, permutational multivariate analysis of variance (PERMANOVA) using distance matrices via R package vegan 2.5-7 [28] was performed on the DPCoA distance matrices.

To perform differential abundance estimation, a Wilcoxon Rank-Sum test was used to test for differences between the median relative abundances of each genus in each *Drosophila* species. *p*-value adjustment for multiple comparisons was performed according to the Benjamini-Hochberg method [29]. In addition to this, similar tests were performed to check if sex, substrate or population affect abundance of taxa within each species; however, in all cases no taxa were found significant with the nonparametric test. The R package metacoder 0.3.5 [30] was used to create a heat tree which visualizes taxonomic categories significantly differing among groups. The analyses were performed using the R (version 4.1) [31].

3. Results

3.1. Life History Traits Analysis

We investigated the influence of the composition of microbiota, different feeding substrates and population origin on two *Drosophila* life history traits, egg-to-adult viability and developmental time. Mean values revealed that egg-to-adult viability was the highest in Dmel_K_St (0.88) and the lowest was in Dmel_Sl_C3 (0.51), while developmental time was the highest in Dsub_Sl_C3_M (23.06) and the lowest was in Dmel_K_St_F (19.24) (Table 1).

The analysis of different effects showed that population origin, substrate, species and sex were significant for differences in both life history traits, but when it comes to the interactions between these factors, not all interactions were significant (Table 2).

The post-hoc analysis revealed that egg-to-adult viability did not differ significantly between populations in *D. subobscura* on both the standard and the lead-saturated substrate. There were also no significant differences between the substrates within either population for *D. subobscura*. *D. melanogaster* showed no significant differences between the populations on the lead-saturated substrate (Figure 1A). All other interactions showed statistically significant differences ($p < 0.05$) in comparisons.

The results obtained for developmental time included sex as another potential factor that could be differentially affected regarding the population, species and substrate. The post-hoc analysis revealed that, on the standard substrate, there were no significant differences between males and females for both species and both populations. *D. subobscura* showed this pattern on the lead-saturated substrate for both populations as well, while in *D. melanogaster* sex differences were significant on the lead-saturated substrate for both populations. In *D. melanogaster*, both sexes from the same substrate but different populations did not differ significantly (Figure 1B). In *D. subobscura*, this pattern was observed only for lead-saturated substrate (Figure 1C). All other interactions showed statistically significant differences ($p < 0.05$) in comparisons.

Table 1. Mean values of egg-to-adult viability and developmental time in all samples.

Sample	Egg-to-Adult Viability	Developmental Time
	Mean ± SE	**Mean ± SE**
Dmel_K_St_M	0.8763 ± 0.0068	19.567 ± 0.0482
Dmel_K_St_F		19.238 ± 0.0578
Dmel_Sl_St_M	0.7333 ± 0.0180	19.782 ± 0.0727
Dmel_Sl_St_F		19.381 ± 0.0588
Dmel_K_C3_M	0.5890 ± 0.0270	21.513 ± 0.1788
Dmel_K_C3_F		20.744 ± 0.1617
Dmel_Sl_C3_M	0.5077 ± 0.0298	21.075 ± 0.1359
Dmel_Sl_C3_F		20.537 ± 0.1727
Dsub_K_St_M	0.7297 ± 0.0166	20.580 ± 0.0593
Dsub_K_St_F		20.870 ± 0.0503
Dsub_Sl_St_M	0.8053 ± 0.0165	21.420 ± 0.0715
Dsub_Sl_St_F		21.514 ± 0.0795
Dsub_K_C3_M	0.7790 ± 0.0180	22.687 ± 0.0913
Dsub_K_C3_F		22.547 ± 0.0859
Dsub_Sl_C3_M	0.7243 ± 0.0164	23.058 ± 0.1088
Dsub_Sl_C3_F		22.955 ± 0.0957

Dmel—*Drosophila melanogaster*; Dsub—*Drosophila subobscura*; K—Kalna population; Sl—Slankamen population; St—standard (control) substrate; C3—lead-saturated substrate; M—male; F—female.

Table 2. Results of the factorial ANOVA test on (a) egg-to-adult viability and (b) developmental time for different effects.

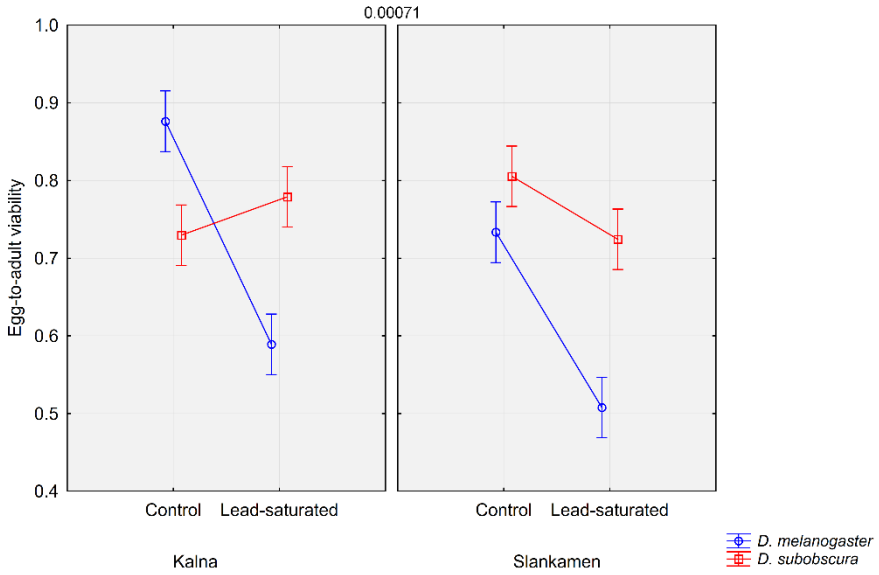
Trait	Effect	df	SS	MS	F	*p*
(a) egg-to-adult viability	Population	1	0.1550	0.1550	13.21	0.000343
	Substrate	1	1.1125	1.1125	94.79	0.000000
	Species	1	0.4133	0.4133	35.22	0.000000
	Population × Substrate	1	0.0177	0.0177	1.51	0.220906
	Population × Species	1	0.2257	0.2257	19.23	0.000018
	Substrate × Species	1	0.8688	0.8688	74.03	0.000000
	Population × Substrate × Species	1	0.1382	0.1382	11.78	0.000709
(b) developmental time	Population	1	7.3	7.3	22.3	0.000003
	Substrate	1	305.5	305.5	931.2	0.000000
	Species	1	356.7	356.7	1087.1	0.000000
	Sex	1	6.7	6.7	20.6	0.000007
	Population × Substrate	1	5.5	5.5	16.7	0.000052
	Population × Species	1	12.2	12.2	37.2	0.000000
	Substrate × Species	1	1.7	1.7	5.3	0.022203
	Population × Sex	1	0.0	0.0	0.0	1.000000
	Substrate × Sex	1	2.7	2.7	8.3	0.004196
	Species × Sex	1	8.9	8.9	27.1	0.000000
	Population × Substrate × Species	1	0.2	0.2	0.5	0.476053
	Population × Substrate × Sex	1	0.5	0.5	1.6	0.199702
	Population × Species × Sex	1	0.2	0.2	0.6	0.445763
	Substrate × Species × Sex	1	0.0	0.0	0.0	0.906872
	Population × Substrate × Species × Sex	1	0.0	0.0	0.0	0.869297

The modified table was presented as a poster presentation at IECE2021 conference.

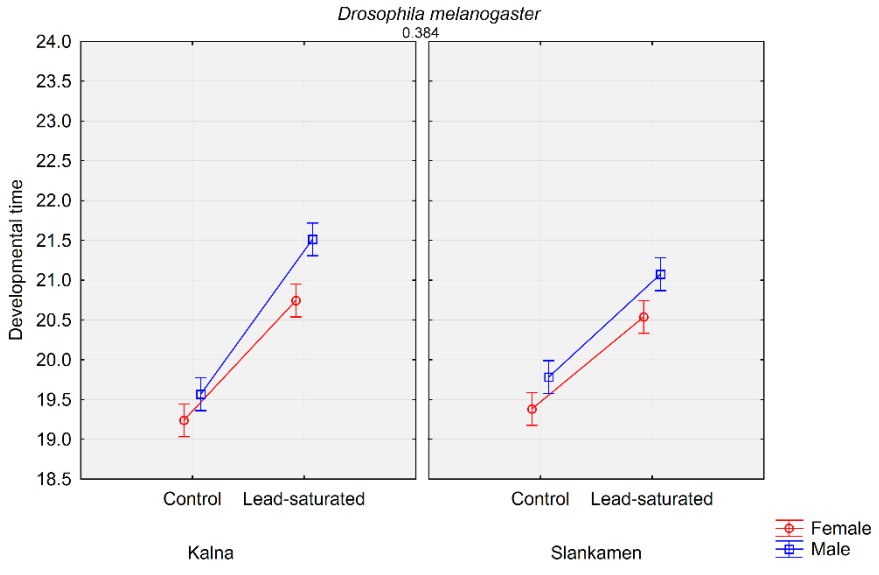
3.2. Taxonomic Abundance of Microbial Communities

A total of 2,706,363 reads in 24 samples were obtained after quality filtering and these were used for ASV inference (Supplementary Table S1). All but one ASV were classified up to the order level (100 ASVs), 81 ASVs were classified up to the genus level and 49 to the species level (Supplementary Tables S2–S4). One ASV could not be assigned to Bacteria, and since it was present in traces only in one sample it was removed from further analysis.

The microbiota of two *Drosophila* species, *D. melanogaster* and *D. subobscura*, was classified into six phyla. At the phylum level, the most abundant phyla in total were Proteobacteria and Firmicutes with 86.6% and 13.3%, respectively; other four phyla accounted for less than 1% in total (Figure 2A).

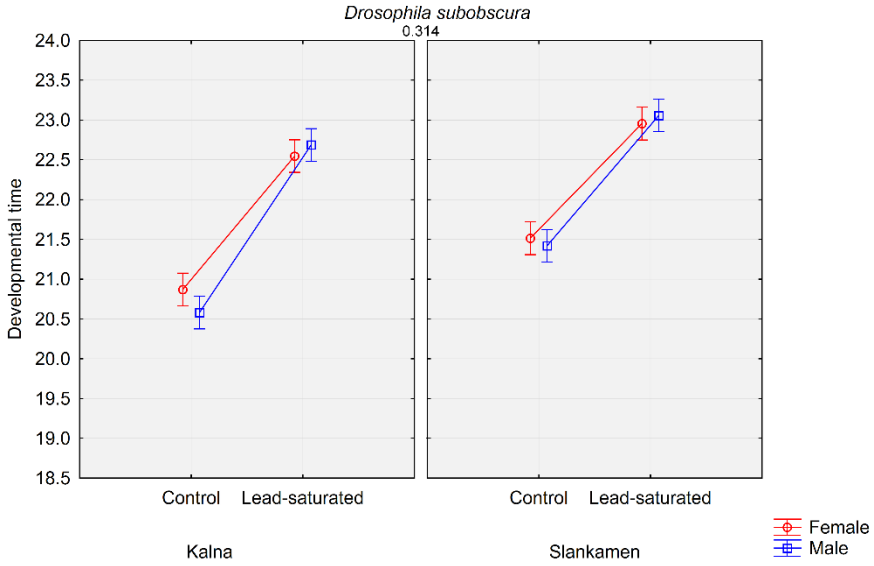


(A)



(B)

Figure 1. *Cont.*



(**C**)

Figure 1. The LS mean plots of the analyzed traits depict changes that have occurred in different groups and subgroups; (**A**) egg-to-adult viability; (**B**) developmental time in *D. melanogaster;* (**C**) developmental time in *D. subobscura*. The numbers above the graphs indicate the *p*-values of the comparisons. The modified figure (**A**) was presented as a poster presentation at IECE2021 conference.

Phylum Proteobacteria was dominant in almost all samples (23 of 24). On the other hand, Firmicutes was highly represented only in *D. melanogaster*, while its prevalence in *D. subobscura* was less than 2% out of total Firmicutes. Phylum Firmicutes was 99.9% represented by the genus *Lactobacillus*. Other most prevalent genera in all the samples were *Wolbachia, Komagataeibacter* and *Acetobacter* (Figure 2B). *Wolbachia* (36.9%) was present in all the samples of *D. melanogaster* flies, ranging from 2% to 21.6%, while it was present only in traces in two *D. subobscura* samples (<0.01%). The second most represented genus was *Komagataeibacter* (25%), with 95.9% found in *D. subobscura* samples. The increase in abundance of *Komagataeibacter* on the lead-saturated substrate was observed in five of six comparisons. Another highly represented genus from the Acetobacteraceae family was *Acetobacter*, with 65.5% in *D. subobscura* and 33.5% in *D. melanogaster* samples. Within each group, *Acetobacter* was the most prevalent in larvae samples. Another genus represented by >10% in total was *Lactobacillus* (13.3%), with 98.2% found in *D. melanogaster* samples, with higher prevalence in adults. All other genera (such as *Vibrionimonas, Staphylococcus, Sphingomonas* and *Acinetobacter*) were abundant with less than 1% in total. In *D. subobscura* samples, rare genera such as *Staphylococcus, Sphingomonas, Vibrionimonas, Acinetobacter* and a genus from family Xanthobacteraceae were present only in larvae samples. Interestingly, in *D. melanogaster* samples, *Staphylococcus, Sphingomonas* and *Vibrionimonas* were completely absent from larvae samples, but also from all lead-saturated substrate samples.

3.3. Alpha Diversity Analysis

Alpha diversity was measured using several metrics: Observed, Chao1, Shannon, Gini-Simpson and invSimpson indices (Table 3).

In terms of alpha diversity, the Shannon index did not show statistically significant differences among groups of samples, but the Chao1 estimator was significantly lower in *D. subobscura* samples (Figure 3). When control versus lead substrate samples were compared within each of the species, no significant differences in Chao1 were observed in any of the samples included. However, if larvae samples were excluded, the Wilcoxon Rank-Sum test indicated a significantly lower Chao1 index in lead substrate samples for both species (Figure 3).

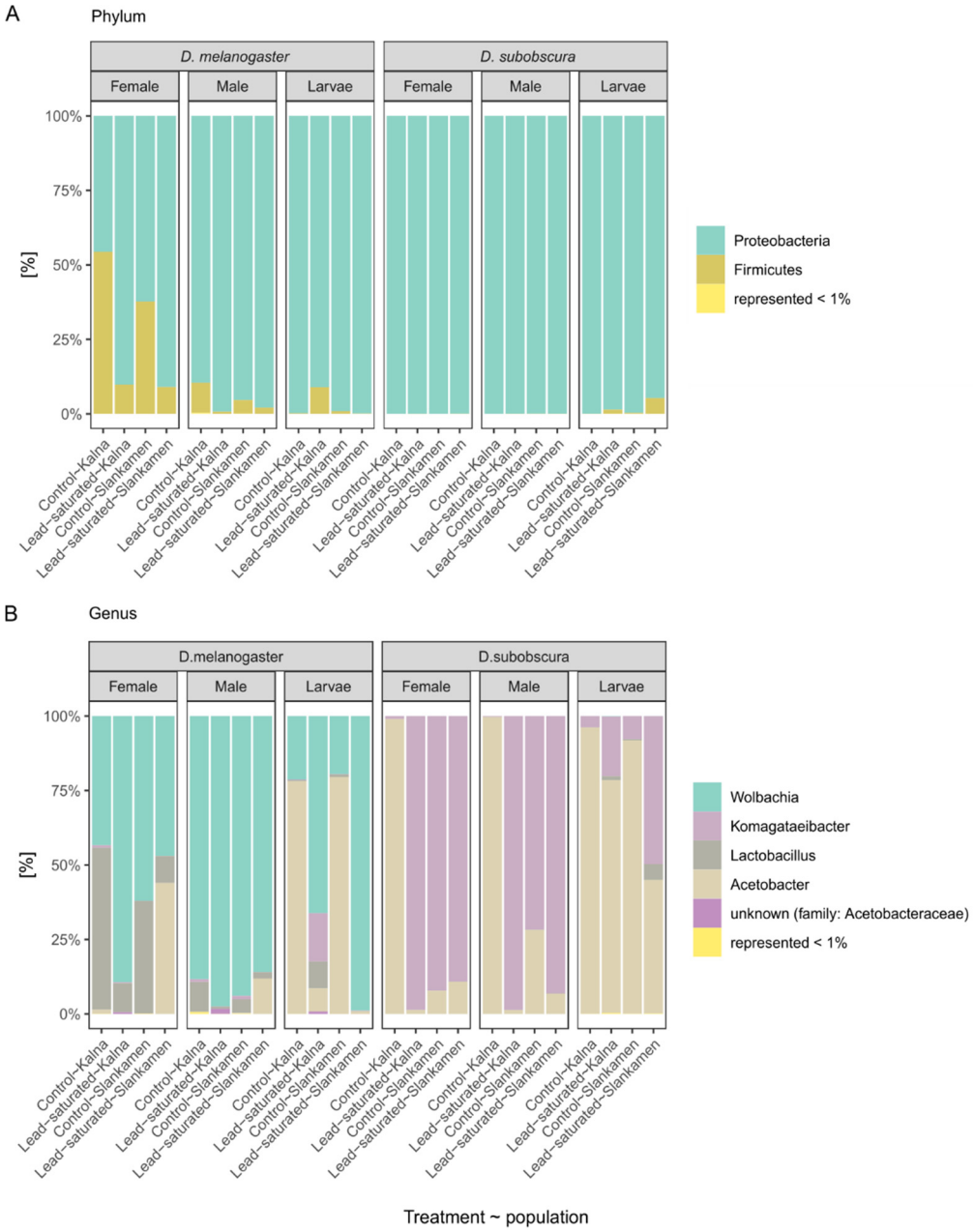


Figure 2. Relative abundance of the most prevalent (**A**) phyla and (**B**) genera in *Drosophila* species (*D. melanogaster* and *D. subobscura*) from two populations (Kalna and Slankamen) on the control substrate and the lead-saturated substrate in larvae and adult males and females. The modified figure (**B**) was presented as a poster presentation at IECE2021 conference.

According to the Shannon and Simpson diversity indices, the highest diversity in adults was observed within the *D. melanogaster* female samples from the Kalna (standard) and from Slankamen (standard and lead-saturated substrate). Regarding the larvae samples, the highest diversity was observed in *D. melanogaster*, the Kalna population on lead and both *D. subobscura* samples on lead (Kalna and Slankamen). The lowest diversity in adults was found in both sexes and from both substrates in *D. subobscura* from Kalna population. Larvae samples with the lowest diversity were *D. melanogaster* from Slankamen on lead and *D. subobscura* from the Kalna population on the standard.

Table 3. Alpha diversity of ASVs represented in the microbial community of *Drosophila*.

Sample	Observed	Chao1	se.chao1	Shannon	Gini-Simpson	InvSimpson
Dmel_K_St_M	36	36.6	1.18	0.57	0.21	1.27
Dmel_K_St_F	28	33.6	5.34	1.29	0.64	2.76
Dmel_Sl_St_M	20	20	0	0.34	0.12	1.13
Dmel_Sl_St_F	16	17	2.29	1.17	0.56	2.29
Dmel_K_C3_M	10	10	0	0.14	0.05	1.05
Dmel_K_C3_F	11	11	0	0.46	0.20	1.24
Dmel_Sl_C3_M	10	10	0	0.49	0.24	1.31
Dmel_Sl_C3_F	9	9	0	1.07	0.58	2.41
Dsub_K_St_M	5	5	0	0.05	0.02	1.02
Dsub_K_St_F	5	5	0	0.11	0.03	1.04
Dsub_Sl_St_M	7	7	0.46	0.60	0.41	1.69
Dsub_Sl_St_F	5	5	0.22	0.29	0.15	1.17
Dsub_K_C3_M	3	3	0	0.07	0.03	1.03
Dsub_K_C3_F	3	3	0	0.08	0.03	1.03
Dsub_Sl_C3_M	4	4	0	0.25	0.13	1.15
Dsub_Sl_C3_F	3	3	0	0.35	0.20	1.25
Dmel_St_K_L	6	6	0	0.56	0.35	1.53
Dmel_St_Sl_L	6	6	0.46	0.55	0.33	1.48
Dsub_St_K_L	8	8	0	0.18	0.08	1.09
Dsub_St_Sl_L	10	10	0.47	0.42	0.19	1.24
Dmel_C3_K_L	6	6	0.46	1.02	0.52	2.08
Dmel_C3_Sl_L	7	7	0	0.07	0.02	1.02
Dsub_C3_K_L	20	20	0	0.61	0.35	1.54
Dsub_C3_Sl_L	18	21	4.15	0.89	0.55	2.22

Dmel—*Drosophila melanogaster*; Dsub—*Drosophila subobscura*; K—Kalna population; Sl—Slankamen population; St—standard (control) substrate; C3—lead-saturated substrate; M—male; F—female; L—larvae.

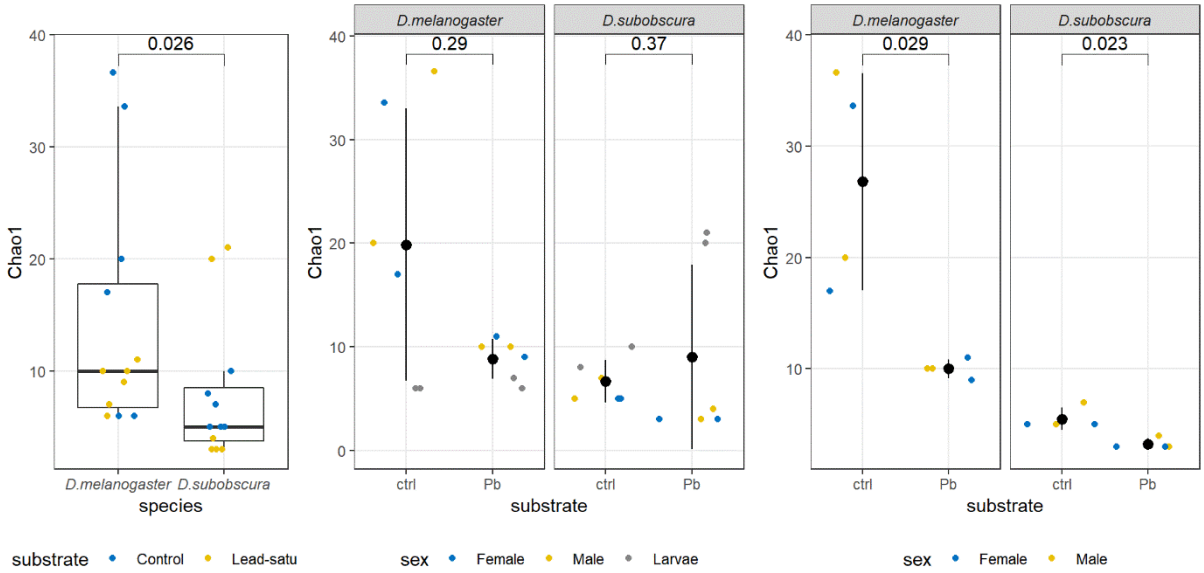


Figure 3. Differences in the Chao1 index between the species and the treatments estimated with the Wilcoxon Rank-Sum test. The number above the data indicates the *p*-value of the comparison.

Similar to the Shannon and Simpson diversity indices, observed richness and Chao1 estimators were highest in adult *D. melanogaster* samples on the standard in the Kalna population, both sexes and the Slankamen population on the standard in males. The lowest richness in adults was observed in seven out of eight *D. subobscura* samples. Larvae richness was higher in *D. subobscura* on lead (both population) than in all *D. melanogaster* larvae samples.

3.4. Beta Diversity Analysis

Beta diversity was estimated using Double Principal Coordinate Analysis (DPCoA) on prevalence filtered taxa at the genus level aggregation after rarefication to even depth (Figure 4).

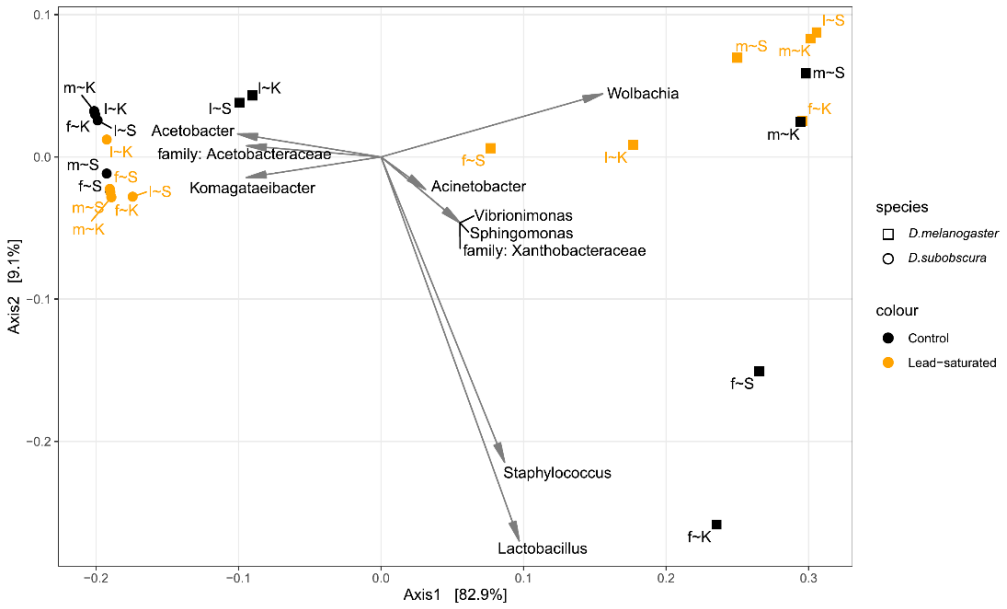


Figure 4. Biplot of Double Principal Coordinate Analysis (DPCoA) of bacterial composition in different *Drosophila* samples (*D. melanogaster* and *D. subobscura*; control and lead-saturated substrate indicated in the legend) using prevalence filtered taxa at the genus level aggregation. f—female; m—male; l—larvae; S—Slankamen population; K—Kalna population.

Samples belonging to the two *Drosophila* species were separated by the first DPCoA axis. All *D. subobscura* samples clustered together, while *D. melanogaster* samples were dispersed on the biplot. Partial separation between the control substrate and the lead-saturated substrate of *D. subobscura* samples was observed by the second DPCoA axis. In addition, the larvae from both control *D. melanogaster* populations were positioned close to *D. subobscura* samples.

To investigate the source of variation in microbial composition, or more precisely to partition it among fixed effects such as species, sex, substrate and their interaction, PERMANOVA using DPCoA distances among the samples was performed on all samples and separately for *D. melanogaster* and *D. subobscura* sample subsets. When DPCoA distances among all samples are taken into account, the greatest source of variation was due to species and all interactions were significant (Table 4). When *D. melanogaster* samples were analyzed separately, substrate, sex and sex × substrate interaction were statistically significant ($p < 0.05$), indicating that the substrate had a different effect depending on the sex. For the *D. subobscura* samples DPCoA distance matrix, the substrate, sex and sex × substrate interaction did not have a significant effect.

Differential abundances of the most prevalent genera indicate the dominance of the Acetobacteraceae family in *D. subobscura* samples, whereas the Lactobacillaceae family was predominant in *D. melanogaster* (Figure 5). Substrate and sex had no effect if all samples were taken into account, nor when *D. subobscura* and *D. melanogaster* were analyzed separately.

Table 4. Results of PERMANOVA using DPCoA distances among all *Drosophila* samples.

Effect	df	SS	MS	F	R^2	*p*
Species	1	0.893082	0.893082	132.2773	0.651498	0.001
Substrate	1	0.046581	0.046581	6.899272	0.033981	0.02
Sex	2	0.100009	0.050004	7.406304	0.072956	0.008
Species × Substrate	1	0.034041	0.034041	5.041911	0.024833	0.029
Species × Sex	2	0.08629	0.043145	6.390341	0.062948	0.013
Substrate × Sex	2	0.065261	0.032631	4.833024	0.047608	0.014
Species × Substrate × Sex	2	0.06453	0.032265	4.778869	0.047074	0.02
Residuals	12	0.081019	0.006752		0.059103	
Total	23	1.370812			1	

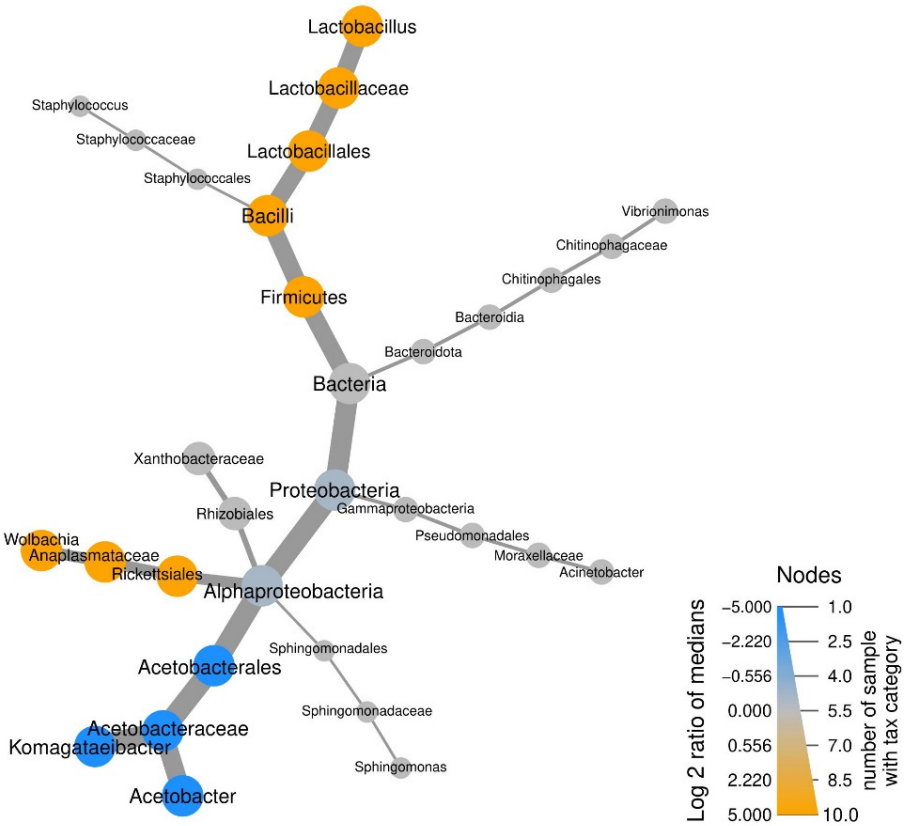


Figure 5. Differential heat tree of microbial communities' abundances in *Drosophila* species with adjusted *p*-values < 0.05. Orange-colored taxa are more abundant in *D. melanogaster* while blue-colored taxa are more abundant in *D. subobscura*. The size of the circle indicates the number of samples with taxonomic category.

4. Discussion

In this study we observed that the overall bacterial diversity and richness were higher in adult *D. melanogaster* compared to *D. subobscura* samples. Life history analysis showed significant differences in population origin, substrate, species and sex effects on egg-to-adult viability and developmental time. The most prevalent genera in all the samples were *Wolbachia*, *Komagataeibacter*, *Acetobacter* and *Lactobacillus*. The genus *Lactobacillus* was dominantly abundant in *D. melanogaster* species on the standard substrate, while *Komagataeibacter* genus was dominant in *D. subobscura* on lead-saturated substrate. *Komagataeibacter* genus proved to be a species-specific member of *D. subobscura* microbiota that could be beneficial in overcoming environmental stress.

Comparing the results obtained from the composition of microbiota and life history traits of two *Drosophila* species reared on different substrates, several potential cause-and-

effect relationships were discovered. The overall microbial diversity and evenness in *D. subobscura* reared in the laboratory was lower than in *D. melanogaster*, as in our previous study which was done only on the Kalna population [5]. With the addition of the Slankamen population in this research it can be seen that the Kalna population in *D. melanogaster* was mainly advantageous regarding the microbial diversity and richness compared to the Slankamen population. Contrary to that, *D. subobscura* originating from the Slankamen population showed higher diversity than the Kalna population. A similar pattern was observed for both adults and larvae, which indicates that the change of microbiota diversity due to the lead exposure could be population-specific. Microbial richness estimated by the Chao1 estimator was lower in *D. subobscura* in overall analysis, but also in both species on the substrate saturated with lead, when only males and females were included. Beta diversity showed that the differences in bacterial diversity were most expressed on the species level, but also revealed that microbial composition of *D. melanogaster* larvae was more similar to *D. subobscura* adult samples than to *D. melanogaster*. *D. subobscura* samples showed strong clustering and indicated a minor impact of lead exposure to variation in microbial composition. This could be due to the increase in the *Komagataeibacter* genus within the lead-saturated samples, which has been reported as a good probiotic candidate due to its high level of glucose conversion rate and survival rate in the presence of acidic pH and bile salt [32].

Life history traits also varied significantly between the populations and kept a similar pattern as microbiota in *D. melanogaster* on the standard substrate, whereby egg-to-adult viability was higher in the Kalna population compared to Slankamen. *D. subobscura* did not show significant differences in egg-to-adult viability regarding the origin, nor regarding the substrate composition, as was shown by Tanasković et al. [17]. On the other hand, developmental time results revealed that *D. subobscura* underwent more changes in this trait regarding the microbial composition, population origin and substrate than *D. melanogaster*. Namely, developmental time varied significantly between *D. subobscura* males and females on the standard substrate in both populations, but also within each sex on the standard substrate with different population origin. Males took longer to develop on the standard substrate compared to females, but both males and females originating from the Slankamen population took longer to develop than the Kalna population. This could be due to pre-adaptation to the polluted environment, as Kalna is probably more polluted area compared to Slankamen. *D. melanogaster* did not show significant differences in developmental time regarding the origin, nor regarding the substrate composition. This indicates that in some species, specific traits could be more susceptible to lead toxicity and changes in microbiota than others.

Microbial composition analysis indicated the dominance of the Acetobacteraceae family in *D. subobscura* samples and the Lactobacillaceae family in *D. melanogaster* species. Thus, *D. melanogaster*, the sample with the highest diversity and richness in microbiota species and the highest representation of genus *Lactobacillus*, showed the highest egg-to-adult viability and the shortest developmental time (Dmel_K_St, females), while the sample which showed microbial diversity albeit poor richness and domination by the genus *Acetobacter* exhibited lower egg-to-adult viability (Dmel_Sl_C3, females). Additionally, it was observed that in larvae of this species, the genus *Acetobacter* was dominant on the standard substrate, while its presence was significantly lower on the substrate with lead. *Lactobacillus* and *Acetobacter* are commonly found in lab-reared *D. melanogaster* [4,33,34]. Both genera can promote growth via different pathways, and in certain conditions, the presence of *Lactobacillus plantarum* helps larval growth and reduces their developmental time, so this could be a reason for short developmental time in Dmel_K_St [35–37]. The presence of 98.2% of total *Lactobacillus* ASVs in *D. melanogaster* suggested that *Lactobacillus* could be a species-specific member of *D. melanogaster* gut microbiota. The *Acetobacter* genus was present in larvae samples of both species, with the highest abundance on the standard substrate, but there was a decrease in egg-to-adult viability when *Acetobacter* was accumulated by adults (Dmel_Sl_C3 and Dsub_K_St). Previous studies have shown

that lead toxicity can drive oxidative stress in many organisms [38–42]. Oxidative stress in *D. melanogaster* can cause various health defects, including reduced lifespan, retarded development, decreased pupation, emergence and survival rates, impaired mobility and reduced egg production [38,43–45]. In addition to the fact that lead has been proven to affect various life history traits, highly reduced viability in *D. melanogaster* on the lead-saturated substrate compared to *D. subobscura* could be a cost of faster development [46,47] and presence of endosymbiotic bacteria (*Wolbachia*), which also have been confirmed to affect the gut microbiota [48,49]. Short developmental time tends to have various fitness costs besides the reduced egg-to-adult viability, such as lower pathogen resistance [50], borderline larval storage of metabolites and reduced adult size [47]. Prolonged developmental time could be a potential mechanism of resistance to heavy metal exposure, providing a higher egg-to-adult viability.

Another possible factor that is greatly involved in shaping the microbiota is temperature [51]. *D. melanogaster* is successfully reared at 19–25 °C, unlike *D. subobscura,* which has a much tighter temperature range in the wild; 19 °C is the optimal rearing temperature in the lab. Heat-stressed *D. subobscura* flies showed changes in bacterial diversity and structure compared to non-stressed flies, and this response demonstrates that the gut microbiota contributes to heat tolerance, which could have important consequences on host fitness [52]. The sub-optimal rearing temperature for *D. melanogaster* could affect the metabolic strategy during the development, but also the growth of the species-specific microbiota. Additional experimental temperature manipulation would probably give a more complete answer in that sense.

Although the *D. subobscura* showed lower richness in microbiota species and lower diversity (Kalna population, both sexes and both substrates), the samples that were predominantly represented by the genus *Komagataeibacter* mainly maintained similar levels of egg-to-adult viability. *D. subobscura* samples from standard substrate, where *Komagataeibacter* genus was highly abundant (Slankamen population), also showed an increase in egg-to-adult viability compared to the population with low prevalence of *Komagataeibacter*. This indicates that the high prevalence of the genus *Komagataeibacter* was beneficial for flies' viability in lab-rearing conditions, but also that it could be the key to the higher tolerance to lead exposure in *D. subobscura*. We previously reported the increase of *Komagataeibacter* in lab-reared flies after 13 generations, where its abundance drastically increased on the lead-saturated substrate, pointing to its higher tolerance to this heavy metal if compared to the other members of the microbial community [5]. After 35 generations, its prevalence has been maintained on the lead-saturated substrate, indicating a good heavy metal adaptation of *D. subobscura* species [15]. Measuring of the concentration of lead by inductively coupled plasma optical emission spectrometry (ICP-OES) in *D. melanogaster* and *D. subobscura* flies maintained for more than 30 generations in the control and lead-saturated substrate conditions showed that *D. subobscura* flies on lead-saturated substrate accumulated more lead than *D. melanogaster* (unpublished data). Moreover, the amount of lead accumulated was higher in males than in females in *D. subobscura*, whereas in *D. melanogaster* it was the opposite. The resistance of *D. subobscura* to increased accumulation of lead could be due to the prevalence of the *Komagataeibacter* genus, which could be an example of stable gut-colonizing bacteria in *D. subobscura*, since it has been proven to have a strong anti-oxidant ability in vitro [53] and it is considered to be a good probiotic candidate [32].

Taxonomic analysis revealed that the rare genera (<1%) in *D. subobscura* were present only in larvae, whereby *Staphylococcus* and *Acinetobacter* were present on the lead-saturated substrate, and *Sphingomonas, Vibrionimonas* and a genus from the family Xanthobacteraceae were present in larvae from both substrates. Interestingly, in *D. melanogaster* most of them were almost completely absent in larvae from both substrates, but also in the majority of adults from the lead-saturated samples. These findings suggest a different dynamic of developmental stages, as well as variability in substrate utilization and degradation by larvae in two species. These implicate the modulation in adaptive strategies under different environmental conditions in the two species.

5. Conclusions

In this study, we observed different patterns of life history traits in accordance with population origin and sex, but also the dominance of different gut microbiota members. The population origin showed a significant influence on life history traits, though each of the traits in the two species was affected differentially. Sex differences were also expressed, but only in *D. subobscura* on the standard substrate, indicating that influence of population origin and sex on life history traits could be species-specific. The presence of the heavy metal caused shifts in developmental time in *D. subobscura*, but maintained the egg-to-adult viability at a similar level. This could be explained by the domination of the *Komagataeibacter* in *D. subobscura* gut microbiota, usually a rare member of the microbiota community. The egg-to-adult viability increased in *D. subobscura* on standard substrate when *Komagataeibacter* was highly abundant, indicating that it could be a valuable member of *D. subobscura* microbiota in overcoming environmental stress. Research of the impact of microbiota on the adaptive response to heavy metals and the potential implications on host fitness is of great importance. Further research could reveal the extent to which species, sex, origin, lead exposure and specific members of microbiota, individually or through interactions, affect the life history traits. It could also help to identify the exact members of the gut microbiota that enable the best possible response to a particular environmental change.

Supplementary Materials: The following are available online at http://www.mdpi.com/article/10.3390/insects12121122/s1, Table S1: Number of sequences before and after filtering, Table S2: Bacterial phylum taxonomy contingency table of microbiota in all samples, Table S3: Bacterial genus taxonomy contingency table of microbiota in all samples, Table S4: Bacterial species taxonomy contingency table of microbiota in all samples.

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insects

MDPI

Article

Alientoma, a Dynamic Database for Alien Insects in Greece and Its Use by Citizen Scientists in Mapping Alien Species

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Simple Summary: Biological invasions have become one of the most intimidating environmental and economic threats of our time as a result of the globalisation and the rise in international commerce, with alien insects representing one of the most abundant groups of organisms introduced into Europe. Over the last decade, citizen science has emerged as a valuable tool for the early detection and monitoring of alien species worldwide. The aim of this study is to introduce a dynamic checklist and database of these organisms in Greece, where a large number of alien insect species have been detected. Alientoma—derived from "alien" and the Greek word "entoma", meaning insects—was created to provide information on alien species (i.e., status, distribution, taxonomy, common names, and impacts) to the public as well as to the scientific community in order to inform and assist in the mitigation of their adverse impacts. This project was promoted through news agencies, both in the press and television, while it also maintained a strong social media presence. Since its launch, 1512 sessions were performed by individuals mainly from Greece and Cyprus. An initial network of citizen scientists has been established and is expected to grow in the near future.

Abstract: Invasive alien species have been increasingly acknowledged as a major threat to native biodiversity and ecosystem services, while their adverse impacts expand to human health, society and the economy on a global scale. Insects represent one of the most numerous alien organismic groups, accounting for about one fifth of their total number. In Greece, a large number of alien insects have been identified, currently reaching 469 species. In recent decades, the contribution of citizen science towards detecting and mapping the distribution of alien insects has been steeply increasing. Addressing the need for up-to-date information on alien species as well as encouraging public participation in scientific research, the Alientoma website—derived from "alien" and the Greek word "entoma", meaning insects, is presented. The website aims towards providing updated information on alien species of insects to the public as well as the scientific community, raising awareness about biological invasions and addressing their distribution and impacts inter alia. By maintaining a dynamic online database alongside a strong social media presence since its launch, Alientoma has attracted individuals mainly from Greece and Cyprus, interacting with the website through a total of 1512 sessions. Alientoma intends to establish a constantly increasing network of citizen scientists and to supplement early detection, monitoring and management efforts to mitigate the adverse impacts of alien insects in Greece.

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1. Introduction

Biological invasions have led to a rapidly growing environmental crisis as a result of climate change and human-mediated activities such as globalisation and the constant rise in international commerce [1–3]. This excessive movement of people and goods has resulted in the introduction of alien species beyond their native range, affecting native biodiversity, ecosystem services, society, human health and economy on a global scale. Invasive alien species (IAS) have been deemed to be a major driver of biodiversity loss inter alia through interspecific resource competition with native species, predation, transmission of pathogens and ultimately inhibition of ecosystem services [4–6]. Regarding the socioeconomic implications, biological invasions pose a serious threat in agriculture and the urban landscape [7,8], fisheries and tourism [9,10], and public health [11–13].

The spread of invasive alien species has long been perceived as an impediment in biodiversity conservation, evidenced by a global need to tackle their spread and to mitigate their adverse impacts, which was expressed at the Convention on Biological Diversity (CBD) in 1992. In Europe, guidelines for "the prevention and management of the introduction and spread of IAS" were issued for EU State Members in EU Regulation 1143/2014. This influential document has led efforts in the prevention of spread, detection, prioritisation, management and eradication of IAS in the old continent [14,15].

The number of alien organisms in Europe currently reaches approximately 14,000 species, with insects accounting for about one fifth of their total number [16]. These species have managed to reach regions outside their natural range of distribution, either actively or passively, through their introduction as biological control agents, their escape from confinement or transport, and even as contaminants and stowaways [17–20]. Insects are well known for a series of direct and indirect impacts on the invaded habitats and indigenous organisms [21], human welfare [22], as well as society and the economy [23,24]. Even though the impacts of alien insects have been receiving more and more research interest (i.e., by identification of disastrous consequences for ecosystems and socioeconomic parameters [25–27]), the list of IAS of Union Concern includes just one insect: the Asian hornet *Vespa velutina nigrithorax* Buysson, 1905 [28–30].

The alien insect fauna of Greece was first assessed by Avtzis et al. (2017) [31], compiling a checklist for 266 species alongside analyses of feeding behaviour, invaded habitats and origin. This endeavour was supplemented by Demetriou et al. (2021), raising the number of alien insects in the country to 469. Further increases in the number of alien insects in Greece were predicted, with new records of alien species being constantly discovered. Conventional research methods have to be integrated with novel approaches such as interactively involving the public in biodiversity monitoring of alien species.

Public participation in scientific research has gradually emerged as an impactful source of information, providing data that ultimately supplement our knowledge on the presence, distribution, abundance, behaviour and impacts of alien species around the world [32–35]. Citizen science records have aided efforts in unravelling the distribution of injurious invaders such as *Chlorophorus annularis* (Fabricius, 1787), damaging bamboo furniture [36], the jasmine pest *Corythauma ayyari* (Drake, 1933) [37], the invasive oak processionary moth *Thaumetopoea processionea* (Linnaeus, 1758) [38] and the marmorated stinkbug *Halyomorpha halys* (Stål, 1855) [39]. Although the validity and taxonomical accuracy of observational data alongside spatiotemporal biases in biodiversity monitoring by citizen scientists have been regarded as drawbacks of such approaches [40–44], citizen science has been proved to assist biosecurity surveillances by detecting alien insects inter alia [45]. Recently, the role of citizen science in environmental policy-making decisions has been gaining increasing interest, highlighting the valuable public input in biodiversity monitoring of invasive species [46].

In Greece, citizen scientists and researchers have joined forces multiple times, reporting on the presence and distribution of alien insects. Nature enthusiasts have provided evidence for the first sightings of alien species in the country, such as the giant mantises *Hierodula tenuidentata* Saussure, 1869 [47]; *Sphodromantis viridis* Forsskål, 1775 [48]; the lantana plume moth *Lantanophaga pusillidactylus* (Walker, 1864) [49]; the feather-legged fly *Trichopoda pennipes* (Fabricius, 1781) [50]; and the broad-headed bug *Nemausus sordidatus* (Stål, 1858) [51]. Additionally, citizen science records have depicted the expansions in the range of and updated distributions of alien insects such as *Leptoglossus occidentalis* Heidemann, 1910, reaching Crete [52] seven years after its initial discovery on the Greek mainland [53] as well as the box-tree moth *Cydalima perspectalis* (Walker, 1859) [54], where public participation unveiled the infestation of native box-tree stands [8].

The growing necessity to accumulate information about alien insects around the globe and to mitigate their impacts has led to the emergence of a number of online databases over the years, such as DAISIE (Delivering Alien Invasive Species Inventories for Europe) [18] and EASIN (European Alien Species Information Network) [16]. These online tools have acted as an effective method to raise awareness and have greatly facilitated the monitoring of biological invasions [55–57]. The compilation of up-to-date information has acted as an important driver for the implementation of science-based policies in the past [58–60], yet these pre-existing projects are often not region-specific oriented. In acknowledgment of this need, a comprehensive information system for these organisms needs to be established on a national level.

In this publication, we introduce Alientoma—derived from "alien" and the greek word "entoma", meaning insects—a project aiming to create a dynamic checklist and database of alien insects in Greece. Our goal is to promote public participation in scientific research of alien species by building an active community of citizen scientists able to identify and record alien insect species throughout Greece. Species occurrence records and relevant data submitted through the Alientoma data collection form will be used to monitor the spread of these species in Greece and to assist in the mitigation of their adverse impacts.

2. Materials and Methods

2.1. Construction of the Website

The catalogue of 469 alien insect species in Greece, which constitutes the backbone of this endeavour, was compiled after a thorough literature survey, review of museum collections and online databases [61]. In addition to the checklist, profiles of 50 species (an initial selection of easily recognisable species; all species were scheduled to be implemented) and details on their status, distribution, taxonomy, common names (also in Greek) and impacts were integrated into the website of Alientoma, which was created using the template for taxonomic websites of Scratchpads 2.0 [62]. Basic insect terminology, photographic material and specific diagnostic characteristics to distinguish them from their native or alien lookalikes were also provided for each species.

One of our approaches to promote the engagement of citizens in scientific research was adopting social media strategies. Social media have been recognised as an effective way for organisations to create interest and to build relationships with the public [63]. At the same time, maintaining a strong presence on the internet through our own social media pages allowed us to not only interact with citizen scientists on a higher level but also share educational material to a wider community and increase awareness. Shared material included taxon descriptions, identification keys, photographic material and notes on the ecology and the current distribution of the alien species. Moreover, public outreach efforts were made through news agencies, both in the press and television.

2.2. Web Analytics

Alientoma was registered with Google Analytics (GA) to monitor website traffic as well as insights on user preferences and serviceability. Although this service is primarily targeted for proprietary websites that provide a deeper understanding of customers visiting

business websites, it is a free tool that can be also used for analysing visitors' information for any website [64]. The major metrics utilised for Alientoma were users' visits, origin and behaviour. Public interaction with the pages of the website was analysed by the Bounce rate, where "a bounce is calculated specifically as a session that triggers only a single request to the Analytics server, such as when a user opens a single page on your site and then exits without triggering any other requests to the Analytics server during that session" [65]. GA provides the opportunity to verify individual users based on the unique IP addresses and cookies acceptance registered in the system. Indeed, there is always the possibility that some of these users might be the same people logging in from different machines, but overall, this accounts for a small percentage of the true unique visitors of such websites [66]. Especially for behaviour, GA gives information regarding the sequence of pages visited per user during their visit. This metric offers an understanding on which pages are most accessed and thus indicates the path from which most users retrieve information.

2.3. Data Collection, Validation and Dissemination

Users can submit their observation data in a collection form provided in our website. After submission, photographic material, geographic coordinates, collection dates, contact details as well as the number of observed individuals are gathered and critically examined to ensure all necessary information are complete and the species of interest has been correctly identified. This form is easily accessible through our website and frequently provided by us under appropriate social media posts, so that citizens are aware of our database and can voluntarily share their records with us. Apart from the data collection form, citizen science records are also gathered from a separate project (https://www.inaturalist.org/projects/alien-insects-of-greece) hosted within the iNaturalist [67] umbrella project.

While citizen scientists' participation is greatly encouraged, referred standards are to be strictly followed regarding data collection. Submitted data are validated by the authors using keys and species descriptions or comparing with museum specimens. For this, it is essential that the data (i.e., photographic material) are of high quality and depicting key characteristics of the species in question. In the case that species identification cannot be fully resolved using only photographic material, citizens are encouraged to provide a specimen for inspection, and only then is the record added in our database.

Collected data are archived, and their submission to GBIF is planned. Special attention is given to records of alien species new to Greece, and it is our effort that, upon meticulous examination, these are published in peer-reviewed scientific journals. Regarding records of species already included in the checklist, georeferenced maps depicting their up-to-date distribution will be shared through our website when enough data are collected.

3. Results

3.1. Interface

The Alientoma website provides visitors with a wide variety of features. The home page provides a welcoming message highlighting the scope of the project (Figure 1). Citizen scientists and academics can easily navigate through eight additional website tabs. The public can familiarise themselves with the terminology used in invasion biology and learn about citizen science and its contribution to the scientific research of alien insects, through the corresponding tabs "Terminology" and "Citizen Science". Visitors can learn more about the scientific community handling the database in the "Alientoma Team" tab.

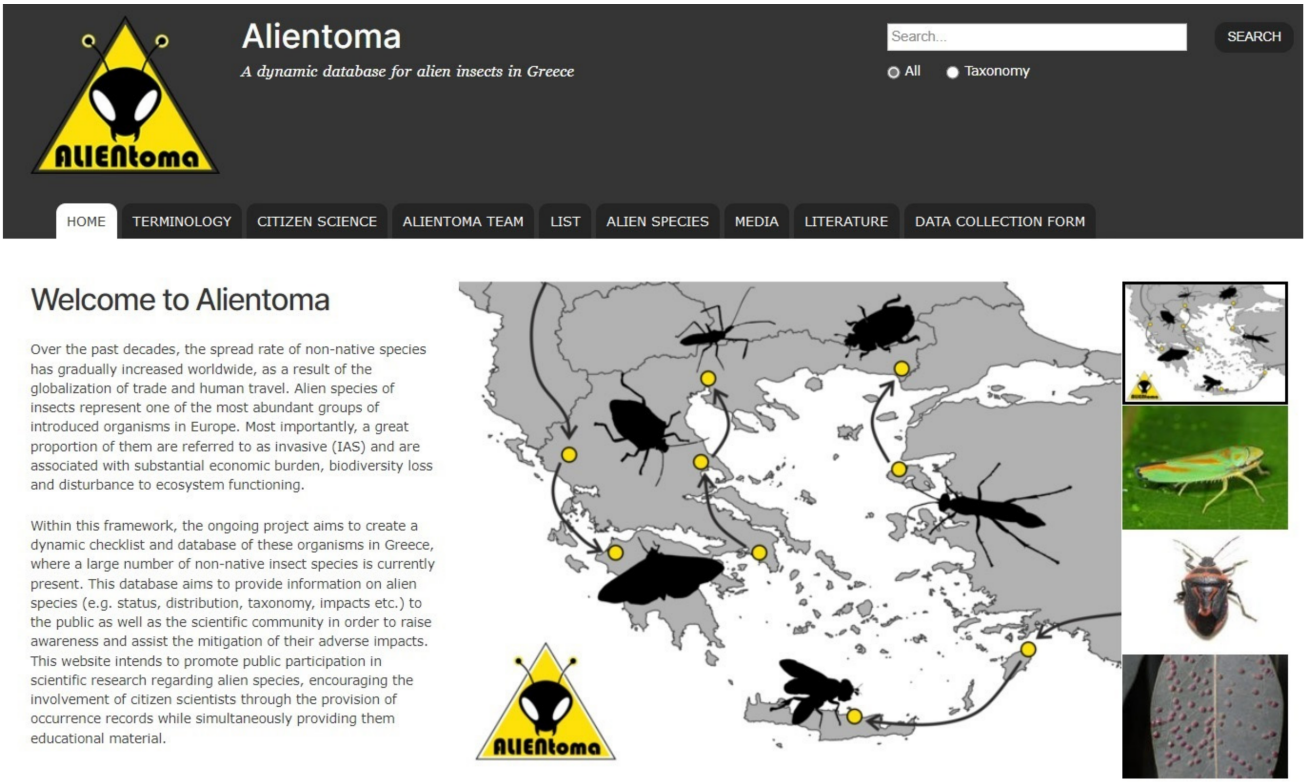

Figure 1. Main page of the Alientoma website.

The "List" section includes the current checklist of alien and cryptogenic insect species recorded in Greece, while "Alien species" provides a taxonomic overview of the alien insects of Greece; by clicking on each insect order, the families and subsequently species are shown. In each species profile, information is fed to four labels. In "Overview" (1), the nomenclature of the species, its common names, its first year recorded and published, the species status, photographic material and an occurrence map retrieved from GBIF are depicted. Under "Description" (2), the species morphology, identification from similar looking species, distribution, as well as ecology and impacts are discussed. The last two labels provide photographic material (3) and the literature (4) utilised during text preparation for each species. The latter two features can also be accessed through the home page under the "Media" and "Literature" tabs, respectively. Finally, citizen science records are submitted in the final tab under the respective name.

3.2. Web Analytics

Shortly after its launch (22 May 2021; International Day for Biological Diversity), the website was registered to the web analytics service offered by Google that tracks and reports website traffic Google Analytics (GA). Since then, GA has managed to pinpoint some interesting facts regarding the use and visibility of Alientoma to the general public. Until today (19 October 2021), 1036 individual users have visited the website, registering 1512 sessions and visiting 7246 pages (4.79 pages/session). Of those visitors, 14.1% have at some point returned to the website. Most of the new users visited the website almost immediately after its launch, with a peak of 101 individuals visiting the website on 22 May, after the advertisement of the website on several social media pages.

The vast majority of the users of Alientoma are from Greece (849 users), with users from Cyprus (65), the United States (23) and India (14) following (Figure 2). Analysing their behaviour, almost half of the users had no interaction with the website but 51.92% indicated some interaction with at least one of the website's pages. The Bounce rate showed 0%, with sessions from Denmark, Belgium, Ukraine (1 session) and New Zealand (3 sessions). Regarding the users showing the highest number of visits, the bounce rate was 47.24% for

Greece, 39.68% for Cyprus, 69.57% for the US and 64.29% for India, meaning that especially the latter ones were mostly random, accidental visits to the website. When looking into the users' interaction flow, we see that the majority of the users holds interaction to at least 4 pages per session, with the maximum pages per session reaching 12.

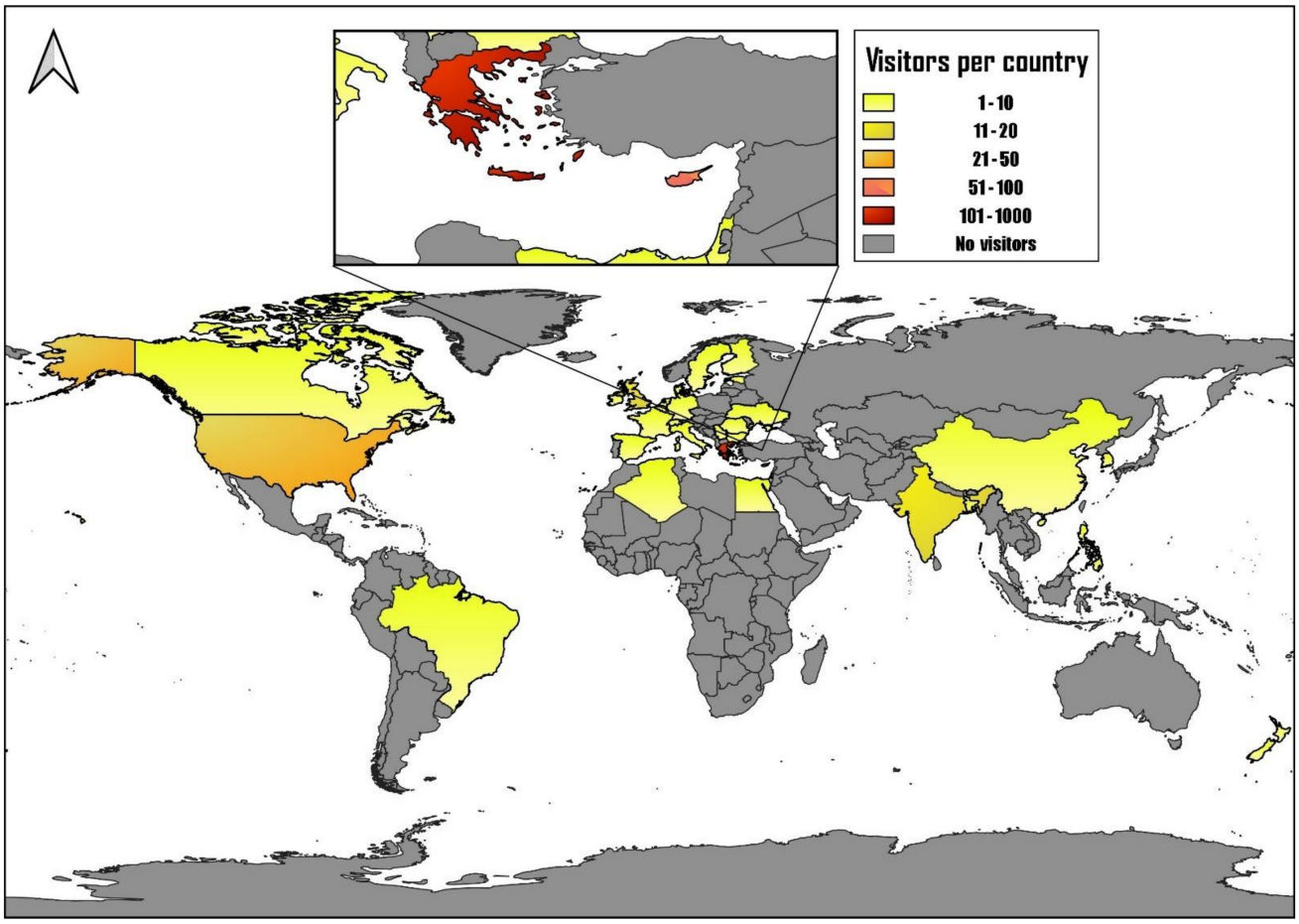

Figure 2. Alientoma users' country map.

3.3. Preliminary Results of the Data Collection Form

Until today, 54 records have been submitted through the Alientoma data collection form by 38 citizen scientists from all geographic regions of Greece with the exception of the Ionian Islands. Upon validation, 18 species of alien species were identified, while one record concerned a misidentified native species. Among the 18 observed species, the leafhopper assassin bug *Zelus renardii* Kolenati, 1856, was the most frequently observed, with 11 records, followed by the box-tree moth *Cydalima perspectalis* (Walker, 1859), with 6 records, and by the Asian ladybird *Harmonia axyridis* (Pallas, 1773) and the giant Asian mantis *Hierodula tenuidentata* Saussure, 1869, with 5 records each.

4. Discussion

In recent decades, citizen science has been rapidly growing largely due to technological advances (i.e., smartphones and GPS), providing vast sums of data regarding alien species [34]. These data constitute an important contribution towards understanding biological invasions and addressing the drawbacks of citizen science compared with traditional and novel surveillance methods [68]. The identification of species throughout photographic material has been regarded as a weakness of citizen science methods [40,41], although participants can be educated and trained in promptly and accurately identifying alien species [44,69]. In the case of Alientoma, species recognition relies on their robust

identification through photographic material attached to the "Data collection form". Thus, morphologically distinctive taxa such as *Xylotrechus chinensis* (Chevrolat, 1852) recently spreading from Crete to Continental Greece [70] or taxonomically unique species, i.e., *Hermetia illucens* (Linnaeus, 1758) [71], can be easily recorded and monitored. However, in the case of taxa with ambiguous taxonomy, experts are consulted to ensure high taxonomic accuracy. Furthermore, the uploaded species profiles provide a description of the species and how they can be distinguished from native counterparts or morphologically similar-looking species. This information can promote biodiversity monitoring training for citizen scientists, leading to the provision of less doubtful data and photographs where diagnostic morphological characters of alien species are evident [72].

Data mining and monitoring of alien species throughout social media has been also increasing in popularity [73,74]. The social media page constructed for Alientoma on Facebook promotes public participation in the study of alien insects of Greece by encouraging citizen scientists' involvement, promoting educational material and assisting in the identification of uploaded material. This direct form of communication with citizen scientists contributes towards raising public awareness about alien species, forming an initial community of citizen scientists and shedding light on the impacts and public perception of observed alien insects [71,75].

The establishment of an efficient early warning system has been stated as a necessary step towards mitigating the future impacts of alien insects in Greece [61]. Such a scheme for insects has yet to be found in the country and will be integrated to the Alientoma website in the near future. Initiatives regarding marine invasions have been already implemented in Greece, providing multiple novel records for alien species detected by volunteers, shell collectors, fishermen and other citizens [76,77]. Alien insects with a high probability of arrival will be pooled in a separate section, indicating possible future invaders from nearby European countries and trading partners [78]. Alien insects of great economic significance and indisputable morphology such as *Anoplophora chinensis* (Forster, 1771); *A. glabripennis* (Motschulsky, 1854); or the small hive beetle *Aethina tumida* Murray, 1867, will be targeted, aiming to alert the public about their introduction risks. Educational material (species profiles, posters and identification guides) highlighting the morphology, ecology, distribution and impacts of these species will be constructed, raising public awareness. Data collection for possible propagules will be mediated through the data collection form and social media profile. This section aims to elicit feedback through the participation of citizen scientists, enhancing the possibility of early detection of an introduced species [69].

Regarding the web analytics, the large Bounce rate for Greece, of almost 50%, is rather troubling. Although it is understandable that not everyone is interested in biological invasions or insects, the website was mostly advertised in biodiversity-recording social media pages and media reporting on environmental issues. Thus, future Alientoma endeavours should focus on catching the eye of website visitors, addressing their interests and finding out what they are seeking when visiting the website (e.g., identification guides, information about species, health risks or socioeconomic impacts) and will provide interactive teaching activities through game-based learning, short videos, and individual acknowledgement of their contributions to enhance community building [46,79]. In addition, at a later stage, we will provide translation in English for the data provided per species to maximise visibility, accessibility and use for non-Greek speaking visitors.

Regardless of data collection, this database has set the creation of updated distributional maps for each species as a fundamental goal, where all collected data will be depicted. These maps will be open to the public for every means of scientific use, with the hopes of being an additional helping hand to citizen scientists, researchers and the public as whole, raising awareness and assisting future projects regarding the treatment of alien insects and their impacts.

5. Conclusions

The Alientoma database has managed to attract more than one thousand visitors in a 5-month period while hosting only a portion of the current checklist of 469 alien insect species. Public engagement is constantly increasing as a result of our promotion in both conventional and social media platforms. An initial network of citizen scientists, trained to identify and record alien insects throughout Greece, has been established and is expected to expand in the future when more species profiles and an early warning system are implemented in our database.

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Immediate and Delayed Mortality of Four Stored-Product Pests on Concrete Surfaces Treated with Chlorantraniliprole

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Simple Summary: We examined the mortality caused by the anthranilic diamide, chlorantraniliprole, at four different doses applied on concrete (0.01, 0.05, 0.1, and 0.5 mg a.i./cm^2) in *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae) adults and larvae, *Rhyzopertha dominica* (F.) (Coleoptera: Bostrychidae) adults, *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae) adults, and *Acarus siro* L. (Sarcoptiformes: Acaridae) adults and nymphs. Mortality data were recorded after 1, 2, 3, 4, and 5 days to determine the immediate mortality. Furthermore, after the 5-day mortality counts, still living individuals were conveyed for 7 days to untreated concrete surfaces to estimate the delayed mortality. The highest immediate mortality was recorded for the larvae of *T. castaneum*, reaching 96.7%, followed by the adults of *A. siro* (92.2%) after 5 days of exposure to 0.5 mg a.i./cm^2. Complete (100.0%) delayed mortality was noticed for *T. castaneum* (adults and larvae), *S. oryzae*, and *A. siro* (both as adults) at 0.5 mg a.i./cm^2. *Rhyzopertha dominica* adults and *A. siro* nymphs exhibited 98.6% and 96.3% delayed mortality at the same dose, respectively. Overall, our results demonstrate that chlorantraniliprole is effective against all the species tested, causing varying immediate and delayed mortality rates at the developmental stages tested.

Abstract: Chlorantraniliprole is an effective pesticide against a plethora of pests, but its efficacy against stored-product pests is very poorly explored. In this study we treated concrete surfaces with four different doses of chlorantraniliprole (0.01, 0.05, 0.1, and 0.5 mg a.i./cm^2) against the red flour beetle, *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae) adults and larvae, the lesser grain borer, *Rhyzopertha dominica* (F.) (Coleoptera: Bostrychidae) adults, the rice weevil, *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae) adults, and the flour mite, *Acarus siro* L. (Sarcoptiformes: Acaridae) adults and nymphs, to examine the immediate mortalities after 1, 2, 3, 4, and 5 days of exposure. Additionally, the delayed mortality of the individuals that survived the 5-day exposure was also evaluated after a further 7 days on untreated concrete surfaces. We documented high mortality rates for all tested species and their developmental stages. After 5 days of exposure to 0.5 mg a.i./cm^2, *T. castaneum* larvae and *A. siro* adults exhibited the highest immediate mortality levels, reaching 96.7% and 92.2%, respectively. Delayed mortality was also very high for all tested species and their developmental stages. Nymphs of *A. siro* displayed a 96.3% delayed mortality followed by the adults of *R. dominica* (98.6%) after exposure to 0.5 mg a.i./cm^2. All other tested species and their developmental stages reached complete (100.0%) delayed mortality, where even 0.01 mg a.i./cm^2 caused ≥86.6% delayed mortality in all species and their developmental stages. Taking into consideration the effectiveness of chlorantraniliprole on this wide range of noxious arthropods, coupled with its low toxicity towards beneficial arthropods and mammals, this pesticide could provide an effective management tool for stored-product pests in storage facilities.

Keywords: anthranilic diamide; concrete surface; insect; mite; pests

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1. Introduction

The red flour beetle, *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae) is a serious insect pest of high economic importance, infesting several stored cereals and foodstuffs worldwide [1]. It is a secondary pest commonly found in mills, warehouses, grocery stores, bakeries, and pet stores [2–5]. The lesser grain borer, *Rhyzopertha dominica* (F.) (Coleoptera: Bostrychidae) seems to have been originally feeding on dried fruits and forest trees [6], and it is currently identified as one of the most destructive insect pests of stored products globally [2,6,7]. As a primary pest, its larvae and adults can attack the sound kernels in storage facilities, while it can occasionally infect ripe grains in the field [8]. The rice weevil, *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae) is a major pest of stored products worldwide, capable of infesting a wide range of commodities such as wheat, maize, barley, sorghum, rye, oats, rice, millet, cottonseed, dallisgrass seed, vetch seed, beans, nuts, flour, pasta, and cassava [2,6,8]. As this species is an internal feeder and completes its entire development inside kernels [6], its larvae remain protected from contact insecticides applied on the external part of the grain [9]. The flour mite, *Acarus siro* L. (Sarcoptiformes: Acaridae) is the most significant mite, infesting mainly cereal products like flour and other commodities like cheese, hay, medicinal herbs, spices, baby food, and fishmeal [8,10–12]. It can be found worldwide in farms, warehouses, mills, and empty grain bins, tainting the products with a musty smell [8,12]. Based on the biological traits presented before, the successful management of these species is restrained by specific difficulties. For instance, several insecticides are not effective against *R. dominica* that has developed resistance to specific formulations (including phosphine) [7,13–19]. Similarly, *S. oryzae* has become resistant to many insecticides in different parts of the world [19,20], indicating that the management of this species in storage facilities requires special care. It is well documented that *T. castaneum* presents resistance to a wide spectrum of insecticides around the globe [20–24]. Finally, *A. siro* is resistant to numerous pesticides. For instance, etrimfos, pirimiphos-methyl, fenitrothion, and chlorpyrifos-methyl were not totally (100%) efficient against *A. siro* individuals throughout a 36-week-long trial [25], while, similarly, beta-cyfluthrin, deltamethrin, chlorpyrifos, and a mix of *s*-bioallethrin and deltamethrin did not result in killing *A. siro* individuals after 21 days of exposure [26]. *Acarus siro* can survive the exposure to diatomaceous earth (DE), such as 3 g/kg Dryacide, from a sample taken from the surface of the bins, for up to 40 weeks [27]. As it becomes evident, the need to examine and find new alternative insecticidal active ingredients against these stored-product pests is imperative.

Chlorantraniliprole is a novel insecticide that belongs to the chemical group of anthranilic diamides [28,29]. It has a unique mode of action, activating the ryanodine receptor in insects' muscles and releasing the cellular calcium that causes the termination of feeding, increased lethargy, the paralysis of muscles, eventually leading to death [29–32]. In addition, chlorantraniliprole presents low mammalian toxicity and can kill a wide range of insect pests without harming beneficial arthropods [30,31,33,34]. For instance, it is very effective against species of agricultural importance belonging to different orders, e.g., Coleoptera, Lepidoptera, Hemiptera, Diptera, Isoptera, and Thysanoptera [35–41]. Furthermore, recent studies have verified the insecticidal activity of chlorantraniliprole applied on different commodities (i.e., barley, maize, oats, peeled rice, whole rice, and wheat) against the Mediterranean flour moth, *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae), the psocid, *Liposcelis bostrychophila* Badonnel (Psocoptera: Liposcelididae), *R. dominica*, *S. oryzae*, and the confused flour beetle, *Tribolium confusum* Jacquelin du Val (Coleoptera: Tenebrionidae) [42], or on maize against the larger grain borer, *Prostephanus truncatus* (Horn) (Coleoptera: Bostrychidae) [43]. However, there is limited knowledge about the efficacy of chlorantraniliprole applied on surfaces against stored-product pests. For example, this compound was examined as a surface treatment on concrete against the different life stages (i.e., eggs, young and old larvae, pupae and adults) of *T. confusum* [44]. Therefore, the objective of the present study is to simultaneously evaluate the immediate and delayed mortality of chlorantraniliprole applied on concrete surfaces against four important stored-

product pests, i.e., *T. castaneum* adults and larvae, *S. oryzae* adults, *R. dominica* adults, and *A. siro* adults and nymphs.

2. Materials and Methods

2.1. Insect and Mite Species

The insect species used in the bioassays were obtained from colonies maintained under laboratory conditions since 2003. *Tribolium castaneum* was cultured on wheat flour containing 5% brewer's yeast at 25 °C and at 65% relative humidity in continuous darkness [45]. Adults of this species were <2 weeks old and larvae were 3rd–4th instar [46]. *Rhyzopertha dominica* and *S. oryzae* were reared on whole wheat at 25 °C at 65% relative humidity, and <2-week-old adults of these species were examined [47]. The initial population of *A. siro* was collected from wheat in Greek storage facilities in 2004, and since then it has been reared under laboratory conditions. The *A. siro* rearing medium consisted of a mixture of oat flakes, wheat germ, and the extract of dried yeast at a 10:10:1 *w*/*w* ratio. The colonies were kept at 25 °C and at 80% relative humidity. For the tests, the nymphs and adults of *A. siro* were selected according to their external morphology, i.e., the bodies of adults are larger and bear longer hairs than the bodies of nymphs [48]. All the above species were reared in continuous darkness.

2.2. Insecticidal Formulation

The formulation of chlorantraniliprole, Coragen® SC (suspension concentrate) with a 200 g/L active ingredient a.i., which was provided by Dupont (Halandri, Greece), was used in the experiments.

2.3. Bioassays

Chlorantraniliprole was examined at four doses: 0.01, 0.05, 0.1, and 0.5 mg a.i./cm^2. The experiments were carried out in a completely randomized block design, with three replicates and three subreplicates. Petri dishes that were 8 cm in diameter and 1.5 cm high, with a surface area of 50.27 cm^2 each, were used. The concrete surface was made one day before the beginning of the tests by filling the bottoms of the Petri dishes with the CEM I 52.5 N material (Durostick, Aspropyrgos, Greece). To prevent the escape of the exposed individuals, the upper internal walls of all dishes were covered with polytetrafluoroethylene (60 wt% dispersion in water) (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany).

Spraying was conducted with the use of an AG-4 airbrush (Mecafer S.A., Valence, France), where 1 mL of an aqueous solution that contained the appropriate volume of the formulation corresponding to each dose was applied on concrete surface as a fine mist. The airbrush was cleaned with acetone after the spraying of each dose. A quantity of 0.5 g clean and pesticide-free whole wheat kernels without infestation was placed on each concrete surface after spraying, as food, for *R. dominica*, *S. oryzae*, and *A. siro*. For *T. castaneum*, the same quantity of soft white wheat flour (a variety mixture made from the endosperm only) was used in the experiments. An additional series of dishes were prepared and sprayed with distilled water (1 mL per dish) with a different AG-4 airbrush, as described above, to serve as controls. Subsequently, 10 individuals were transferred into each dish, and all dishes were put inside incubators set at 25 °C and at 65% relative humidity in continuous darkness. Mortality was determined under an Olympus stereomicroscope (Olympus SZX9, Bacacos S.A., Athens, Greece) after 1, 2, 3, 4, and 5 days of exposure in the treated dishes. After the 5th day of exposure, surviving individuals of each treated or untreated dish were transferred to new unsprayed concrete surfaces that also contained the same quantity of food for each species. The dishes were transferred again into the incubators with the same conditions for an additional period of 7 days. After this interval, the new dishes were opened and the number of the dead individuals per dish was counted, as described above.

2.4. Data Analysis

For all tested species, the immediate or delayed mortality of the controls was low (<5%), so no correction was considered necessary. The repeated measures model was used to separately analyze data for the immediate mortality of each tested species and developmental stage [49]. The repeated factor was the exposure interval, while mortality was the response variable. The dose was the main effect. Data for delayed mortality were submitted to a two-way ANOVA, with the species/developmental stage and dose as the main effects. The associated interaction of the main effects was also considered in the analysis. Prior to the analysis, the transformation of data to a log (x + 1) scale was carried out in order to normalize the variance [50,51]. Means were separated by the Tukey–Kramer honest significant difference (HSD) test at the 0.05 significance level [52]. All analyses were conducted using the JMP 14 software [53].

3. Results

3.1. Immediate Mortality

Between and within the exposure intervals, all main effects were significant for all tested species and life stages, except for *R. dominica* adults (Table 1).

Table 1. MANOVA parameters for main effects and associated interaction for mortality of *Tribolim castaneum* adults and larvae, *Sitophilus oryzae* adults, *Rhyzopertha dominica* adults, and *Acarus siro* adults and nymphs between and within exposure intervals (error DF = 32).

Effect	DF	*T. castaneum* Adults		*T. castaneum* Larvae		*S. oryzae* Adults		*R. dominica* Adults		*A. siro* Adults		*A. siro* Nymphs	
Between exposure intervals													
Source		*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
Intercept	1	4928	<0.01	1293.2	<0.01	2189.7	<0.01	447.9	<0.01	1587.6	<0.01	5554.6	<0.01
Dose	2	36.7	<0.01	9.0	0.01	8.8	0.01	3.6	0.02	10.7	<0.01	19.4	<0.01
Within exposure intervals													
Source		*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
Exposure	4	6285.2	<0.01	112.6	<0.01	6638.0	<0.01	101.9	<0.01	83.2	<0.01	68.2	<0.01
Exposure × dose	8	9.1	<0.01	2.4	0.01	5.8	<0.01	0.7	0.75	3.1	0.01	2.7	0.01

The mortality of *T. castaneum* adults was very low after 3 days of exposure in all tested doses and did not exceed 35.6% at 0.5 mg a.i./cm^2 (Table 2). Mortality increased further, reaching 56.7% at 0.5 mg a.i./cm^2, while at the other doses it ranged between 30.0% and 44.4% after 4 days of exposure. One day later, chlorantraniliprole killed 81.1% of the exposed adults at the highest dose (0.5 mg a.i./cm^2) while it caused moderate mortality at the lower doses (i.e., 0.01, 0.05, and 0.1 mg a.i./cm^2), ranging from 42.2% to 61.1%.

After 2 days of exposure, the mortality of T. castaneum larvae remained at low levels, reaching 33.3% mortality (Table 3). One day later, mortality increased further, ranging between 21.1% and 37.8% at 0.01, 0.05, and 0.1 mg a.i./cm^2, and 60.0% at 0.5 mg a.i./cm^2. After 4 days of exposure, mortality reached 77.8% at 0.5 mg a.i./cm^2, while at 0.05 and 0.1 mg a.i./cm^2, it was moderate (48.9% and 58.9%, respectively). After 5 days of exposure, chlorantraniliprole killed almost all larvae (96.7%) at 0.5 mg a.i./cm^2.

Regarding the immediate mortality of *S. oryzae*, after 3 days of exposure, it was very low at all tested doses and did not exceed 23.3% at 0.5 mg a.i./cm^2 (Table 4). The overall mortality did not exceed 45.6% after 4 days of exposure. At 5 days post-exposure, 80.0% of the exposed adults died on concrete treated with 0.5 mg a.i./cm^2, while mortality rates at all the other doses ranged between 45.6% and 71.1%.

Table 2. Mean immediate mortality (% ± SE) of *Tribolium castaneum* adults exposed on concrete treated with chlorantraniliprole at four doses (0.01, 0.05, 0.1, and 0.5 mg a.i./cm^2), for 1, 2, 3, 4, and 5 days. Within each row, means followed by the same uppercase letter are not significantly different (in all cases DF = 4, 44, Tukey–Kramer HSD test at $p = 0.05$). Within each column, means that are followed by the same lower-case letter are not significantly different (in all cases DF = 3, 35, Tukey–Kramer HSD test at $p = 0.05$). Where no letters exist, no significant differences were recorded. Where dashes exist, no analysis was performed.

Exposure	1 Day	2 Days	3 Days	4 Days	5 Days	*F*	*p*
Dose (mg a.i./cm^2)							
0.01	0.0 ± 0.0 D	0.0 ± 0.0 Db	12.2 ± 1.5 Cc	30.0 ± 1.7 Bc	42.2 ± 2.8 Ac	1045.9	<0.01
0.05	0.0 ± 0.0 D	0.0 ± 0.0 Db	18.9 ± 2.0 Cb	36.7 ± 3.3 Bbc	54.4 ± 2.9 Ab	790.0	<0.01
0.1	0.0 ± 0.0 C	2.2 ± 1.5 Cb	28.9 ± 3.1 Ba	44.4 ± 2.9 ABab	61.1 ± 3.1 Ab	132.0	<0.01
0.5	0.0 ± 0.0 D	11.1 ± 2.0 Ca	35.6 ± 2.9 Ba	56.7 ± 2.9 ABa	81.1 ± 1.1 Aa	160.0	<0.01
F	-	21.8	20.8	14.8	29.8		
p	-	<0.01	<0.01	<0.01	<0.01		

Table 3. Mean immediate mortality (% ± SE) of *Tribolium castaneum* larvae exposed on concrete treated with chlorantraniliprole at four doses (0.01, 0.05, 0.1, and 0.5 mg a.i./cm^2) for 1, 2, 3, 4, and 5 days. Within each row, means followed by the same uppercase letter are not significantly different (in all cases DF = 4, 44, Tukey–Kramer HSD test at $p = 0.05$). Within each column, means that are followed by the same lower-case letter are not significantly different (in all cases DF = 3, 35, Tukey–Kramer HSD test at $p = 0.05$). Where no letters exist, no significant differences were recorded.

Exposure	1 Day	2 Days	3 Days	4 Days	5 Days	*F*	*p*
Dose (mg a.i./cm^2)							
0.01	2.2 ± 1.5 Cb	7.8 ± 3.6 Cb	21.1 ± 3.5 Bc	38.9 ± 2.0 ABc	66.7 ± 2.4 Ac	30.3	<0.01
0.05	5.6 ± 1.8 Cab	13.3 ± 3.3 BCab	26.7 ± 2.9 ABbc	48.9 ± 3.1 Abc	77.8 ± 4.3 Abc	20.2	<0.01
0.1	8.9 ± 2.6 Cab	22.2 ± 4.0 BCa	37.8 ± 5.2 ABb	58.9 ± 5.1 Ab	84.4 ± 4.8 Aab	15.1	<0.01
0.5	14.4 ± 2.9 Ca	33.3 ± 4.1 Ba	60.0 ± 4.4 ABa	77.8 ± 4.0 Aa	96.7 ± 1.7 Aa	25.2	<0.01
F	4.3	5.8	12.2	20.0	12.3		
p	0.01	0.01	<0.01	<0.01	<0.01		

Table 4. Mean immediate mortality (% ± SE) of *Sitophilus oryzae* adults exposed on concrete treated with chlorantraniliprole at four doses (0.01, 0.05, 0.1, and 0.5 mg a.i./cm^2), for 1, 2, 3, 4, and 5 days. Within each row, means followed by the same uppercase letter are not significantly different (in all cases DF = 4, 44, Tukey–Kramer HSD test at $p = 0.05$). Within each column, means that are followed by the same lower-case letter are not significantly different (in all cases DF = 3, 35, Tukey–Kramer HSD test at $p = 0.05$). Where no letters exist, no significant differences were recorded. Where dashes exist, no analysis was performed.

Exposure	1 Day	2 Days	3 Days	4 Days	5 Days	*F*	*p*
Dose (mg a.i./cm^2)							
0.01	0.0 ± 0.0 C	0.0 ± 0.0 Cb	8.9 ± 2.0 B	25.6 ± 2.9 Ab	45.6 ± 3.8 Ac	96.4	<0.01
0.05	0.0 ± 0.0 D	0.0 ± 0.0 Db	15.6 ± 1.8 C	30.0 ± 5.3 Bb	62.2 ± 2.8 Ab	333.6	<0.01
0.1	0.0 ± 0.0 C	0.0 ± 0.0 Cb	18.9 ± 4.8 B	36.7 ± 2.9 Aab	71.1 ± 3.1 Aab	82.8	<0.01
0.5	0.0 ± 0.0 D	3.3 ± 1.7 Ca	23.3 ± 2.4 B	45.6 ± 2.4 ABa	80.0 ± 1.7 Aa	106.0	<0.01
F	-	4.0	2.6	4.8	21.3		
p	-	0.02	0.07	0.01	<0.01		

After 4 days of exposure, the percentages of dead *R. dominica* adults was low for all tested doses, reaching 32.2% at 0.5 mg a.i./cm^2 (Table 5). One day later, mortality increased without significant differences among doses, ranging from 34.4% to 50.0%.

Table 5. Mean immediate mortality (% ± SE) of *Rhyzopertha dominica* adults exposed on concrete treated with chlorantraniliprole at four doses (0.01, 0.05, 0.1, and 0.5 mg a.i./cm^2), for 1, 2, 3, 4, and 5 days. Within each row, means followed by the same uppercase letter are not significantly different (in all cases DF = 4, 44, Tukey–Kramer HSD test at p = 0.05). Within each column, means that are followed by the same lower-case letter are not significantly different (in all cases DF = 3, 35, Tukey–Kramer HSD test at p = 0.05). Where no letters exist, no significant differences were recorded.

Exposure	1 Day	2 Days	3 Days	4 Days	5 Days	*F*	*p*
Dose (mg a.i./cm^2)							
0.01	0.0 ± 0.0 C	2.2 ± 1.5 BC	7.8 ± 2.8 B	18.9 ± 2.6 A	34.4 ± 3.8 Ab	29.8	<0.01
0.05	1.1 ± 1.1 C	4.4 ± 2.4 BC	8.9 ± 2.6 B	25.6 ± 2.9 A	40.0 ± 4.1 Aab	22.0	<0.01
0.1	2.2 ± 1.5 C	8.9 ± 2.6 B	15.6 ±2.4 AB	26.7 ± 3.3 A	43.3 ± 2.4 Aab	22.9	<0.01
0.5	4.4 ± 2.4 D	10.0 ± 3.3 CD	16.7 ± 4.1 BC	32.2 ± 4.3 AB	50.0 ± 4.1 Aa	13.2	<0.01
F	1.4	2.2	2.6	2.2	3.1		
p	0.25	0.11	0.07	0.11	0.04		

The mortality levels of *A. siro* adults was very low at doses ≤0.1 mg a.i./cm^2, reaching 38.9%, while at 0.5 mg a.i./cm^2, moderate mortality was noted (54.4%) 3 days post-exposure (Table 6). One day later, mortality at 0.01 mg a.i./cm^2 remained at low levels (38.9%), but at doses ≥0.1 mg a.i./cm^2, it ranged between 50.0% and 72.2%. After 5 days of exposure, chlorantraniliprole killed 92.2% of the exposed adults at the highest dose, while at 0.01 mg a.i./cm^2, moderate mortality was recorded (65.6%).

Table 6. Mean immediate mortality (% ± SE) of *Acarus siro* adults exposed on concrete treated with chlorantraniliprole at four doses (0.01, 0.05, 0.1, and 0.5 mg a.i./cm^2), for 1, 2, 3, 4, and 5 days. Within each row, means followed by the same uppercase letter are not significantly different (in all cases DF = 4, 44, Tukey–Kramer HSD test at p = 0.05). Within each column, means that are followed by the same lower-case letter are not significantly different (in all cases DF = 3, 35, Tukey–Kramer HSD test at p = 0.05). Where no letters exist, no significant differences were recorded.

Exposure	1 Day	2 Days	3 Days	4 Days	5 Days	*F*	*p*
Dose (mg a.i./cm^2)							
0.01	0.0 ± 0.0 Dc	7.8 ± 2.2 Cb	24.4 ± 3.4 Bb	38.9 ± 3.5 ABc	65.6 ± 2.9 Ac	67.7	<0.01
0.05	5.5 ± 1.8 Cb	17.8 ± 3.6 Bab	33.3 ± 5.0 ABab	50.0 ± 6.2 Abc	74.4 ± 3.4 Abc	20.2	<0.01
0.1	10.0 ± 1.7 Cab	27.8 ± 4.3 BCa	38.9 ± 4.6 ABab	62.2 ± 4.7 Aab	82.2 ± 2.8 Aab	14.5	<0.01
0.5	17.8 ± 3.2 Ca	35.6 ± 6.7 BCa	54.4 ± 7.5 ABa	72.2 ± 5.5 Aa	92.2 ± 2.2 Aa	13.4	<0.01
F	14.2	4.5	4.3	9.0	14.2		
p	<0.01	0.01	0.01	0.01	<0.01		

Regarding *A. siro* nymphs, after 3 days of exposure, mortality was low at all tested doses, reaching 46.7% at 0.5 mg a.i./cm^2 (Table 7). Nymphal mortality ranged between 37.8% and 61.1% at 4 days post-exposure. Moderate mortality was recorded at doses ≥0.05 mg a.i./cm^2, reaching 73.3% at 0.5 mg a.i./cm^2 after 5 days of exposure.

Table 7. Mean immediate mortality (% ± SE) of *Acarus siro* nymphs exposed on concrete treated with chlorantraniliprole at four doses (0.01, 0.05, 0.1, and 0.5 mg a.i./cm^2), for 1, 2, 3, 4, and 5 days. Within each row, means followed by the same uppercase letter are not significantly different (in all cases DF = 4, 44, Tukey–Kramer HSD test at p = 0.05). Within each column, means that are followed by the same lower-case letter are not significantly different (in all cases DF = 3, 35, Tukey–Kramer HSD test at p = 0.05). Where no letters exist, no significant differences were recorded.

Exposure	1 Day	2 Days	3 Days	4 Days	5 Days	*F*	*p*
Dose (mg a.i./cm^2)							
0.01	2.2 ± 1.5 Cb	17.8 ± 2.2 Bc	27.8 ± 3.2 ABb	37.8 ± 2.2 Ac	45.6 ± 3.4 Ac	53.2	<0.01
0.05	7.8 ± 1.5 Ca	24.4 ± 3.4 Bbc	38.9 ± 3.9 ABab	47.8 ± 3.6 ABbc	56.7 ± 3.3 Abc	22.2	<0.01
0.1	13.3 ± 1.7 Da	31.1 ± 3.1 Cab	40.0 ± 3.3 BCa	54.4 ± 3.4 ABab	64.4 ± 4.4 Aab	51.5	<0.01
0.5	16.7 ± 1.7 Da	36.7 ± 2.9 Ca	46.7 ± 1.7 BCa	61.1 ± 2.6 ABa	73.3 ± 3.7 Aa	73.2	<0.01
F	15.8	7.6	5.6	10.9	9.8		
p	<0.01	0.01	0.01	<0.01	0.01		

3.2. Delayed Mortality of the Tested Species

The main effect dose was significant for all species (Table 8). Delayed mortality was high for *T. castaneum* adults, given that >91.0% of the adults died on concrete treated with 0.01, 0.05, or 0.1 mg a.i./cm^2, while 100.0% was recorded at 0.5 mg a.i./cm^2 (Table 9). The same trend was noted for larval delayed mortality of this species. Complete mortality was noticed at 0.5 mg a.i./cm^2. Moreover, 100.0% delayed mortality was noted for *S. oryzae* adults at 0.1 and 0.5 mg a.i./cm^2. *Rhyzopertha dominica* adults reached 96.7% and 98.6% delayed mortality at 0.1 and 0.5 mg a.i./cm^2, respectively. Concerning *A. siro*, adults were more susceptible than nymphs. All adults died at 0.1 and 0.5 mg a.i./cm^2. However, 94.4% and 96.3% of the exposed nymphs died on concrete treated with 0.1 and 0.5 mg a.i./cm^2, respectively.

Table 8. ANOVA parameters for main effects and the associated interaction for delayed mortality of *Tribolium castaneum* adults and larvae, *Sitophilus oryzae* adults, *Rhyzopertha dominica* adults, and *Acarus siro* adults and nymphs (total DF = 202).

Effect	DF	Species	
Source		***F***	***p***
Species	5	1.5	0.21
Dose	3	8.1	<0.01
Species × dose	15	0.4	1.00

Table 9. Mean delayed mortality (% ± SE) of *Tribolium castaneum* adults and larvae, *Sitophilus oryzae* adults, *Rhyzopertha dominica* adults, and *Acarus siro* adults and nymphs exposed on concrete treated with chlorantraniliprole at four doses (0.01, 0.05, 0.1, and 0.5 mg a.i./cm^2). Within each row, means followed by the same lowercase letter are not significantly different (in all cases DF = 3, 35, Tukey–Kramer HSD test at $p = 0.05$). Where no letters exist, no significant differences were recorded.

Dose (mg a.i./cm^2)	0.01	0.05	0.1	0.5	DF	*F*	*p*
Species/Life Stage							
T. castaneum adults	91.0 ± 2.9	95.4 ± 3.1	97.8 ± 2.2	100.0 ± 0.0	3, 35	2.5	0.08
T. castaneum larvae	88.0 ± 4.2	93.5 ± 4.3	97.2 ± 2.8	100.0 ± 0.0	3, 25	1.2	0.32
S. oryzae adults	97.8 ± 2.2	98.2 ± 1.9	100.0 ± 0.0	100.0 ± 0.0	3, 35	0.7	0.58
R. dominica adults	86.6 ± 3.4 b	92.6 ± 2.6 ab	96.7 ± 2.4 a	98.6 ± 1.4 a	3, 35	4.3	0.01
A. siro adults	88.2 ± 6.0	92.6 ± 4.9	100.0 ± 0.0	100.0 ± 0.0	3, 32	1.9	0.15
A. siro nymphs	90.3 ± 4.2	92.6 ± 3.8	94.4 ± 3.7	96.3 ± 3.7	3, 35	0.4	0.75

4. Discussion

Our results indicate that chlorantraniliprole is a promising pesticide for the effective control of the adults and larvae of *T. castaneum*, the adults of *S. oryzae* and *R. dominica*, and the adults and nymphs of *A. siro*, when applied on concrete surfaces. In the present study, chlorantraniliprole killed 81.1% of *T. castaneum* adults, 96.7% of *T. castaneum* larvae, 80.0% of *S. oryzae* adults, 50.0% of *R. dominica*, 92.2% of *A. siro* adults, and 73.3% of *A. siro* nymphs after 5 days of exposure to 0.5 a.i./cm^2. In a former study, Kavallieratos et al. [42] investigated the efficacy of two chlorantraniliprole formulations (WG and SC) as grain protectants against *L. bostrychophila*, *R. dominica*, *S. oryzae* adults, *E. kuehniella* larvae, and *T. confusum* adults and larvae. The 10 mg a.i./kg of grain of the WG formulation killed 95.6% of *S. oryzae* adults after 7 days of exposure on treated whole rice, while at the same dose, the SC formulation caused the mortality of 99.4% exposed individuals. Both chlorantraniliprole formulations killed 100.0% of *S. oryzae* adults after 14 days of exposure to the 10 mg a.i./kg of grain. Concerning *R. dominica*, the WG formulation at 10 mg a.i./kg of grain killed 88.3% of the adults after 7 days of exposure and 100.0% after 14 days of exposure on whole rice and wheat, respectively. The SC chlorantraniliprole

was also effective at causing 83.3% and 96.7% mortality rates 7 and 14 days post-exposure, respectively, to treated whole rice. Furthermore, both chlorantraniliprole formulations effectively controlled *E. kuehniella* larvae, *L. bostrychophila* adults, and *T. confusum* adults and larvae on several commodities (e.g., hard wheat, barley, maize, peeled rice, whole rice, and oats). Similarly, Saglam et al. [44] documented that 0.1 mg a.i./cm^2 sprayed on concrete killed 66%, 100.0%, and 64% of *T. confusum* adults, young larvae, and old larvae, respectively. It also delayed the adult emergence on the first 5 days of the experiment. Chlorantraniliprole (WG and SC) is also effective as maize protectant against *P. truncatus*. In a recent study, Boukouvala and Kavallieratos [43] found that 10 mg a.i. WG/kg of grain and 10 mg a.i. SC/kg of grain killed 98.9% and 96.1% of the exposed individuals after 14 days of exposure, respectively, at 30 °C. Although chlorantraniliprole is not yet authorized for the management of stored-product pests, the aforementioned findings are valuable inputs for its potential use. These research efforts are of particular importance as the number of registered plant protection products in stored-product protection is considerably reduced [54]. Therefore, chlorantraniliprole can be clasified as an a.i. that could potentially recieve registration to be used in storage facilities as a grain protectant and/or as a structural treatment.

Surface treatments constitute an effective approach for the management of stored-product pests. To date, a plethora of pesticides have been applied onto several types of surfaces under different scenarios. For instance, when Kavallieratos et al. [55] treated polypropylene storage bags with alpha-cypermethrin, the immediate mortality of *R. dominica* and *S. oryzae* adults was 37.8% and 40.0% at 5 days post-exposure, respectively. Adults of *T. castaneum* that were exposed to plywood, tiles, and concrete treated with chlorfenapyr exhibited different levels of survival (40.0%, 25.5%, and 2.5%, respectively), concluding that the type of the treated surface affects the efficacy of the tested formulation [56]. Similarly, Vassilakos et al. [57] evaluated ceramic tiles, plywood, concrete, and galvanized steel treated with spinetoram against *T. confusum*, *S. oryzae*, *S. granarius*, *R. dominica*, the rusty grain beetle, *Cryptolestes ferrugineus* (Stephens) (Coleoptera: Laemophloeidae), and *Oryzaephilus surinamensis* (L.) (Coleoptera: Silvanidae) adults. The different treated surfaces led to different mortality rates among all the exposed pests. For example, 0.05 mg spinetoram/cm^2 killed 77.4%, 68.1%, 89.0%, and 59.2% of *T. confusum* adults on ceramic tiles, plywood, concrete, and galvanized steel, respectively, at 7 days post-exposure. Furthermore, the mortality of *E. kuehniella* larvae caused by thiamethoxam, alpha-cypermethrin, and deltamethrin treated on woven polypropylene (0.10 mg a.i./cm^2) ranged between 40.0% and 82.2% [58]. Our findings document that chlorantraniliprole caused even higher mortality rates than the aforementioned formulations in some of the stored-product pests, as in the cases of *T. castaneum* larvae and *A. siro* adults. The fact that chlorantraniliprole killed almost all young larvae of *T. castaneum* under the short exposure scenario followed in our study is of particular importance. This is because chlorantraniliprole can cause the rapid collapse of the larval population upon contact to treated concrete and can consequently prohibit the emergence of adults which, as flyers and walkers, easily colonize stored food commodities [8,59,60]. Whether chlorantraniliprole can perform similarly to the older larvae of *T. castaneum* merits further investigation. In an earlier study, Saglam et al. [44] showed that when the old larvae of *T. confusum* came into contact with concrete surfaces treated with chlorantraniliprole, they exhibited tolerance to this toxicant, taking into account that around 50% of larvae survived after 14 days of exposure. Our study provides new information about the management of *A. siro* on surface treatment, given that there is limited knowledge, mostly coming from previous decades. For example, the DEs SilicoSec and Diasecticide killed 28–98% and 64–88% of *A. siro* adults after 24 h of exposure to three slurry doses (2.5, 5, and 10 g/m^2) [61]. Although we obtained low mortality rates after 24 h of exposure with *A. siro* adults, 92.2% finally died 5 days post-exposure, which is also considered a short exposure interval.

In our study, the delayed mortality was extremely high, ranging between 96.3% and 100.0% in all tested species and their developmental stages. This is an important finding

as it provides a plausible scenario of the arthropods moving from treated to untreated areas that may lead to their colonization by the pests [55,62–64]. This highly toxic effect of delayed mortality is also evident in the cases of several other pesticides. For instance, alpha-cypermethrin caused moderate-to-high delayed mortality in *R. dominica* (from 37.8% to 46.7%) and *S. oryzae* adults (from 32.2% to 60.0%) [55]. Chlorfenapyr killed 56.7% of *S. oryzae* adults after being transferred onto untreated surfaces (polypropylene bags) [55]. As with immediate mortality, delayed mortality may differ depending on the surface that the pesticide is applied. For example, 0.1 mg spinetoram/cm^2 killed 100.0% of *R. dominica* on concrete, but 97.0% on galvanised steel [57]. The presence of untreated food lowered the delayed mortality rates of *S. oryzae* adults from 100.0% to 98.8% and from 91.4% to 78.7% with the *T. confusum* adults [57]. In addition to the aforementioned parameters that affect the rates of delayed mortality, the species and the developmental stage of the exposed pest are equally important, something that was clearly demonstrated both in the current study and in previous investigations. For example, thiamethoxan killed 3.3% of *T. molitor* adults and 35.6% of *T. molitor* large larvae, while the same pesticide killed 63.3% of *T. granarium* adults and 1.1% of *T. granarium* old larvae [62].

5. Conclusions

In conclusion, our study provides new data towards the efficacy of chlorantraniliprole on concrete at four doses against important arthropod pests of stored products. On the basis of our findings, chlorantraniliprole can be a useful management tool since it caused high levels of both immediate and delayed mortality to the majority of the tested species and their developmental stages. However, further experimentation is required to assess the efficacy of chlorantraniliprole applied on other types of surfaces (e.g., storage bags) under different environmental conditions (temperature and relative humidity), doses, exposure intervals, and other stored-product pests in conjunction with their egg laying capacities.

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insects

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Article

Mealybug (Hemiptera: Pseudococcidae) Species Associated with Cacao Mild Mosaic Virus and Evidence of Virus Acquisition

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Simple Summary: Cacao mild mosaic virus was discovered in Trinidad in 1943, where it was shown to be transmitted by five mealybug species. It was recently detected in Puerto Rico, Brazil, and the USA; however, no information is available on insect vectors in these locations. Mealybugs belong to a diverse group known as Pseudococcidae, and species' composition differs among geographic regions. A study conducted on infected trees in Florida found four species of mealybug present: *Pseudococcus jackbeardsleyi*, *Maconellicoccus hirsutus*, *Pseudococcus comstocki*, and *Ferrisia virgata*. Of these, *P. jackbeardsleyi* and *M. hirsutus* have not been tested for their ability to transmit viruses to cacao. Cacao mild mosaic virus was detected in 34.6 to 43.1% of the insects tested; however, acquisition did not differ among species. Owing to their prevalence (>72%), transmission studies should be conducted to determine the ability of *P. jackbeardsleyi* and *M. hirsutus* to transmit the virus. This research improves our understanding of the mealybugs associated with virus-infected plants in Florida and identifies potential new insect vectors. Knowledge of vector species is essential for selecting the most effective control strategies and minimizing disease spread.

Abstract: *Theobroma cacao* is affected by viruses on every continent where the crop is cultivated, with the most well-known ones belonging to the *Badnavirus* genus. One of these, cacao mild mosaic virus (CaMMV), is present in the Americas, and is transmitted by several species of Pseudococcidae (mealybugs). To determine which species are associated with virus-affected cacao plants in North America, and to assess their potential as vectors, mealybugs (n = 166) were collected from infected trees in Florida, and identified using COI, ITS2, and 28S markers. The species present were *Pseudococcus jackbeardsleyi* (38%; n = 63), *Maconellicoccus hirsutus* (34.3%; n = 57), *Pseudococcus comstocki* (15.7%; n = 26), and *Ferrisia virgata* (12%; n = 20). Virus acquisition was assessed by testing mealybug DNA (0.8 ng) using a nested PCR that amplified a 500 bp fragment of the movement protein–coat protein region of CaMMV. Virus sequences were obtained from 34.6 to 43.1% of the insects tested; however, acquisition did not differ among species, X^2 (3, N = 166) = 0.56, $p < 0.91$. This study identified two new mealybug species, *P. jackbeardsleyi* and *M. hirsutus*, as potential vectors of CaMMV. This information is essential for understanding the infection cycle of CaMMV and developing effective management strategies.

Keywords: DNA barcoding; molecular markers; *Pseudococcus*; *Maconellicoccus hirsutus*; mealybug; cacao; *Badnavirus*; virus vector; Florida

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1. Introduction

Mealybugs (Hemiptera: Pseudococcidae) are phloem feeders that use long, slender mouthparts to uptake plant fluids [1], which reduces the vigor of host plants. They can feed on all plant tissues, and severe infestations cause defoliation and, eventually, plant

death. Some species inject plant toxins during feeding, causing twisted/stunted growth [2]. The damage generated varies among taxa and is determined by their reproductive potential, temperature tolerance, preferred feeding locations, the existence of effective control strategies, and their ability to transmit viruses [3].

On cacao, mealybugs are economically important owing to their role as virus vectors [4]. Viruses are present on every continent where cacao is grown commercially, most of which belong to the Badnavirus genus. At least 11 Badnavirus species are known to infect cacao, some of which cause reduced yield or tree death [5]. Most research focuses on highly virulent viruses in West Africa associated with cacao swollen shoot disease (CSSVD). Infected trees experience severe yield decline and death within a few years [6,7]. Annual production loss due to CSSVD was estimated to be 76,000 metric tons in 2012 [8].

In the Americas, virus symptoms were reported on cacao in 1944 in Trinidad and Venezuela [9,10]. Research in Trinidad described two distinct strains, which were recently characterized and named cacao mild mosaic virus (CaMMV) and cacao yellow vein banding virus (CYVBV) [11]. These viruses were thought to be relatively rare, until CaMMV was detected in Puerto Rico [12] and Brazil [13], and then intercepted in material under quarantine in the USA [14] in the past couple of years. Trees infected with CaMMV experience branch dieback and annual yield reduction of 6.6–19%, comparable to mild to moderate strains of CSSV in West Africa [15,16]. Tree death due to infection with CaMMV has not been confirmed.

Isolates of CaMMV that have been molecularly characterized to date have genome sizes just over 7500 bp and are made up of four open reading frames (ORFs); ORFs1–3 and ORFY [11,13]. Although virus particles have not yet been detected in infected tissue, they are assumed to be bacilliform, made up of a double-stranded DNA genome, as is characteristic of members of the Badnavirus genus [17]. Badnaviruses are transmitted to cacao by multiple mealybug species [6,11]; however, owing to the high diversity of Pseudococcidae, each geographic region has a different species composition. In West Africa, *Pseudococcus njalensis* (Laing) is the main vector, owing to its abundance in the area [18], while *Planococcus citri* (*Pl. citri*) (Risso) is the most significant vector of cacao virus in Trinidad, where it was shown to be capable of transmitting both virus species present, CaMMV and CYVBV [4].

Numerous factors influence the importance of each species as virus vectors; that is, prevalence, mobility, uptake efficiency, and feeding preferences. For example, lower transmission rates of cacao swollen shoot virus (CSSV) with *Ferrisia virgata* (Cockerell) than with *Pseudococcus njalensis* were attributed to differences in feeding behavior, where *F. virgata* fed directly from phloem less frequently [19]. Detailed feeding studies showed that *F. virgata* frequently ingested sap from xylem tissue, where virus titer is presumed to be lower.

Successful transmission requires mealybugs to uptake the virus from the phloem during feeding and, subsequently, deposit it in the phloem of a different plant [19]. The ability to transmit viruses varies among related species. For example, *Pseudococcus longispinus* can transmit two strains of CSSV to cacao, but cannot transmit either CaMMV or CYVBV, the two cacao-infecting viruses present in Trinidad [20]. Mealybugs can acquire CaMMV, and are infectious, after as little as 33 min of feeding on infected material. Infectivity can persist in up to 23 h in *P. citri* and eight hours in *D. brevipes* following separation from the infected plant [20].

Previous studies showed that cacao infecting Badnaviruses generally have semi-persistent transmission characteristics [20]. However, circulative transmission has been reported in *P. njalensis*, with CSSV being transmitted after a molt [21]. Overall, these viruses have both non-persistent (stylet-borne) and persistent (circulative) characteristics [22].

In Trinidad, where CaMMV was first discovered, the virus was shown to be transmitted by five mealybug species: *Pl. citri*, *Dysmicoccus brevipes*, *D. sp. near brevipes*, *Ferrisia virgata*, and *Pseudococcus comstocki*. However, no research has been done on this in nearly 70 years. Vector information is not available from other countries, as CaMMV was only

recently detected outside of Trinidad [12]. The purpose of this study was to identify mealybug species associated with cacao plants infected with CaMMV in Florida, USA (Figure 1), and to determine their ability to acquire the virus. This was done by identifying specimens using genetic markers and assaying individuals for the presence of CaMMV using a nested PCR. This information is essential for understanding the infection cycle of CaMMV and identifying potential new vectors.

(**a**) (**b**)

Figure 1. Signs of virus infection and mealybug infestation on *Theobroma cacao*: (**a**) leaves with mosaic symptoms characteristic of cacao mild mosaic virus and mild distortion caused by *Maconellicoccus hirsutus*, and (**b**) severe bunchy top caused by *M. hirsutus*.

2. Materials and Methods

2.1. Insect Sampling and DNA Extraction

In January 2021, female mealybugs were collected from 23 randomly selected *Theobroma cacao* trees in Florida confirmed to be infected with CaMMV following nested PCR testing, as described in Puig [14], followed by Sanger sequencing of the amplicons. Specimens were collected from the pods, stems, leaves, and flowers of each tree (up to 5 individuals from each tissue × tree combination), and placed in microcentrifuge tubes containing 70% ethanol. DNA was extracted from individual specimens using the Qiagen DNeasy Blood and Tissue Kit using a shortened, 10 min lysis step, as described in Albo et al. [23]. The final resuspension step was done with 50 µL of elution buffer for adults and 30 µL for the smaller nymphs. DNA was quantified using a Qubit 4 Fluorometer and the 1× dsDNA High Sensitivity Assay Kit (Life Technologies Corp., Carlsbad, CA, USA).

2.2. Mealybug Identification

To identify mealybugs, the cytochrome c oxidase subunit I (COI) region, the internal transcribed spacer region (ITS2), and the ribosomal DNA subunit 28S (28S) were amplified and sequenced using the primer pairs, MFCO1/MRCO1, ITS2-M-F/ITS2-M-R, and D10F/D10R, respectively (Figure 2; Table 1). PCRs were performed in 25 µL volumes, consisting of 12.5 µL 2× Immomix Red (Bioline Reagents Ltd., London, UK), 1 µL each of 10 µM forward and reverse primers, 1 µL of DNA template, and 9.5 µL sterile nuclease-free water, as presented in Puig et al. [24].

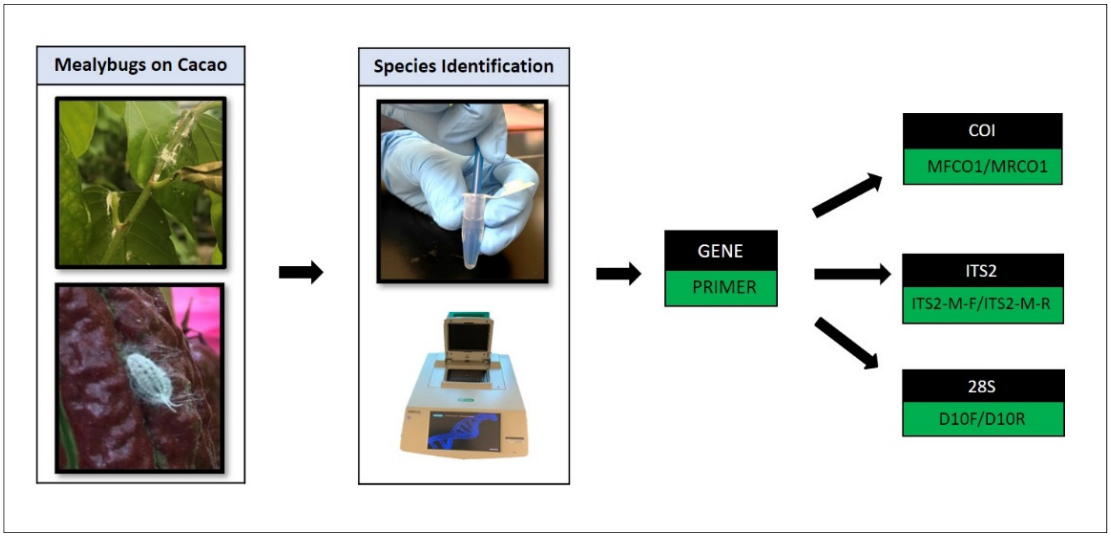

Figure 2. Mealybugs feeding on virus-infected trees of *Theobroma cacao* were identified using COI, ITS2, and 28S markers amplified and sequenced using MFCO1/MRCO1, ITS2-M-F/ITS2-M-R, and D10F/D10R primers.

Table 1. Genes, primer sequences, and amplicon sizes for the markers targeted in this study.

Gene	Primer	Sequence (5′–3′)	Amplicon Size (bp)	Reference
COI	MFCO1 MRCO1	ATATCTCAAATTATAAATCAAGAA ATTACACCTATAGATAAAACATAATG	379	[25]
ITS2	ITS2-M-F ITS2-M-R	CTCGTGACCAAAGAGTCCTG TGCTTAAGTTCAGCGGGTAG	~800	[26]
28S	D10F D10R	GTAGCCAAATGCCTCGTCA CACAATGATAGGAAGAGCC	738–767	[27]

Amplification was visualized on a 1% (*w*/*v*) agarose gel at 150 V for 35 min. PCR products were purified with Qiagen PCR Purification Kit (Hilden, Germany) and sent to Eurofins for Sanger sequencing. Sequences were edited and aligned using Geneious®11.1.2 (Biomatters Ltd., Auckland, New Zealand), and analyzed in BLASTn for identification. Specimen identification was determined based on BLASTn results of the COI sequences because this genetic region is considered the most biologically informative. The samples from which COI sequences were not obtained were identified by aligning their ITS2 or 28S sequences with corresponding sequences from samples that had been identified using COI. A representative subset of sequences generated in this study was deposited in GenBank and is publicly available (Supplementary File S1). A chi-square test of independence was used to determine whether there was a significant association between mealybug species and the tissue on which they were found (flower, leaf, pod, and stem), using the PROC FREQ in SAS Ver. 9.3 (SAS Institute, Cary, NC, USA).

2.3. Detection of CaMMV in Mealybugs

Presence of CaMMV was determined by running a nested PCR for each sample with virus-specific primers (Table 2) [14]. The initial reaction was 20 µL with 10 µL Sigma-Aldrich© JumpStart™ REDTaq® ReadyMix™ (Sigma-Aldrich, St Louis, MO, USA), 7.6 µL molecular grade water, 0.8 µL of each 10 µM primer (Mia1145F and Mia1926R), and 0.8 ng of DNA template. Amplification conditions were an initial 2 min step at 94 °C followed by 22 cycles of 94 °C (25 s), 34 °C (25 s), and 72 °C (1 min), and then a final extension at 72 °C for 12 min.

The final reaction was also a 20 µL volume, as described above, with primers Mia1396F and Mia1876R and 0.8 µL of amplified PCR product as the template. Amplification conditions were an initial 2 min step at 94 °C followed by 37 cycles of 94 °C (20 s), 53 °C (20 s),

and 72 °C (1 min), and then a final extension at 72 °C for 10 min. Amplified PCR products were visualized, purified, and sequenced as described above. Sequences were aligned and edited using Geneious®11.1.2 and analyzed in BLASTn for identification. The number of specimens of each species from which CaMMV was amplified and sequences was analyzed using a chi-square test of independence, as described previously, to determine whether there was a significant association between mealybug species and virus acquisition.

Table 2. Primer pairs designed to amplify the movement protein–coat protein (MP–CP) genome region of CaMMV were used to detect the virus in individual mealybug specimens.

Gene	Primer	Sequence (5′–3′)	Amplicon Size (bp)	Reference
Cacao mild mosaic virus MP–CP	Mia1145F	YAACTTTGAGGACCAGATC	806	[14]
	Mia1926R	YCTAAGTATCCARCTYCTTCCAAGR		
Cacao mild mosaic virus MP–CP	Mia1396F	ACCGTGTCTAYCAGCACTGGA	503	[14]
	Mia1876R	CTGGRATWGCTCTTACKCCATGW		

2.4. Phylogenetic Comparison

To determine the diversity of virus strains detected in mealybugs, a phylogenetic analysis was performed with MP–CP sequences in MEGA X [28], following alignment using the MUSCLE algorithm [29]. The Kimura two-parameter model was chosen using the MEGA X models function to assess various nucleotide substitution models [30]. Initial trees for the heuristic search were generated by applying neighbor-join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach, and then choosing the topology with a superior log likelihood value. The rate variation model allowed for some sites to be evolutionarily invariable ([+*I*], 42.48% sites). Tree branch lengths were measured in the number of substitutions per site.

Codon positions included were first + second + third + noncoding, and positions with less than 95% site coverage were eliminated. Fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position (partial deletion option). There were 60 nucleotide sequences and 401 positions in the final dataset.

3. Results

3.1. Insect Sampling and DNA Extraction

A total of 170 mealybugs were collected from pods, leaves, stems, and flowers of cacao trees in a Florida greenhouse. These trees were determined to be infected with CaMMV following nested PCR testing of three leaves per plant, followed by Sanger sequencing of amplified fragments. The detection of multiple virus strains in several trees suggests that mixed infections are common [31]. Four of these were discarded owing to low DNA concentrations and omitted from subsequent analyses. Three of the 20 selected trees that had no mealybugs present were omitted and were replaced with three additional, randomly selected trees. No mealybugs were found on the tree roots inspected.

3.2. Mealybug Identification

Sequences of all three markers yielded consistent species matches for *Pseudococcus comstocki* (Kuwana) (*n* = 26) and *Maconellicoccus hirsutus* (*n* = 55), with coverage and identities ranging from 96 to 100% (Table 3). For *Pseudococcus jackbeardsleyi* (Beardsley) (*n* = 65) and *Ferrisia virgata* (*n* = 20), only COI sequences provided unambiguous identification. 28S sequences of both species were close matches to multiple genera available in GenBank. In contrast, no close matches were found for the ITS2 sequences obtained from *P. jackbeardsleyi*.

Table 3. BLASTn results for COI, ITS2, and 28S sequences obtained in this study. Species determinations were made based on the best matches obtained with COI sequences. The results shown are from one representative of each species. Bold font denotes incorrect organisms among top matches (adapted from Puig et al. [24]).

	Marker	Genbank Match	GenBank No.	% Ident.	% Coverage
Pseudococcus comstocki	COI	*P. comstocki*	LC121496	98.9	100
	ITS2	*P. comstocki*	KU499509	96.3	100
	28S	*P. comstocki*	JF965413	99.8	98
Pseudococcus jackbeardsleyi	COI	*P. jackbeardsleyi*	KJ187489	99.5	100
	ITS2	***Pseudococcus viburni***	**KF819654**	**79.2**	**90**
	28S	***Pseudococcus viburni***	**AY427376**	**99.1**	**99**
		Oracella acuta	**JF965418**	**98.9**	**99**
		P. jackbeardsleyi	EU188510	99.9	95
Maconellicoccus hirsutus	COI	*M. hirsutus*	MK090645	100	100
	ITS2	*M. hirsutus*	KU883603	99.5	98
	28S	*M. hirsutus*	AY427403	99.5	96
Ferrisia virgata	COI	*Ferrisia virgata*	EU267205	99.2	100
	ITS2	*Ferrisia virgata*	KY423513	77.7	45
	28S	***P. comstocki***	**JF965413**	**97.6**	**99**
		Ferrisia gilli	**AY427398**	**99.1**	**98**
		Ferrisia virgata	AY427373	98.6	98

Pseudococcus jackbeardsleyi was the predominant species found in this study, comprising 38% (n = 63) of all insects collected. It was followed by *M. hirsutus*, which made up over 34% of the specimens examined. Most mealybugs were collected from stems (n = 57), followed by pods (n = 46) (Table 4). A chi-square test of independence showed that there was a significant association between mealybug species and the tissue on which they were found. The proportion of specimens found on flowers, leaves, pods, and stems differed by species, X^2 (9, N = 166) = 20.2, $p < 0.02$.

Table 4. Summary of mealybugs identified in this study and tissues from which they were collected.

Species	# on Pods	# on Leaves	# on Flowers	# on Stems	Total	%
Maconellicoccus hirsutus	25	14	5	13	57	34.3
Pseudococcus jackbeardsleyi	13	12	11	27	63	38.0
Pseudococcus comstocki	6	9	5	6	26	15.7
Ferrisia virgata	2	5	2	11	20	12.0
total	46	40	23	57	166	

3.3. Detection of CaMMV in Mealybugs

To determine the relative rates of virus acquisition, nested PCR was carried with DNA of 166 mealybugs collected from 20 cacao trees infected with CaMMV. Acquisition was determined based on the amplification of a ~500 bp fragment and homology of the resulting sequence to previously obtained CaMMV sequences. Sixty-seven of all mealybugs (40.4%) tested positive for CaMMV, with acquisition rates ranging from 34.6% in *F. virgata* to 43.2% in *P. jackbeardsleyi* (Figure 3). A chi-square test of independence showed that there was no significant association between mealybug species and virus acquisition. The proportion of specimens with detectable levels of CaMMV did not differ by species, X^2 (3, N = 166) = 0.56, $p < 0.91$.

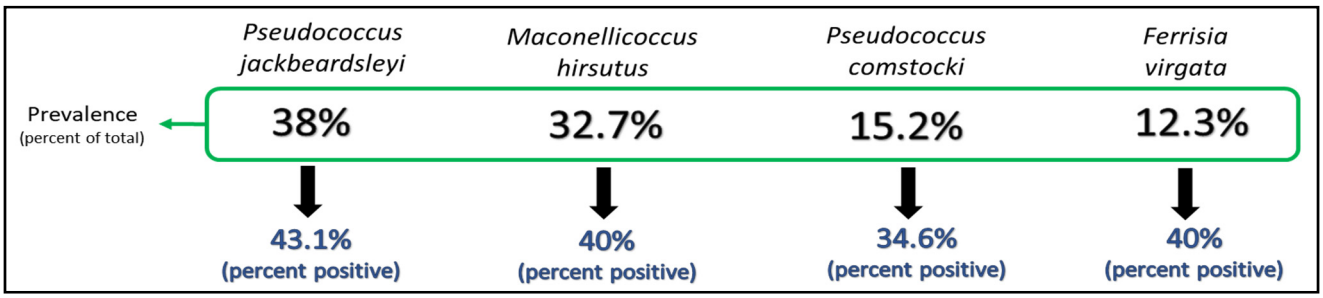

Figure 3. Mealybug species collected from virus-infected cacao trees, listed (left to right) in decreasing order of relative abundance (prevalence). *Cacao mild mosaic virus* sequences were obtained from 34.6 to 43.1% of insects collected.

3.4. Phylogenetic Comparison

The CaMMV sequences (n = 60) generated in this study clustered into four primary groups. Groups I, II, and III shared approximately 99% sequence identity to isolates from Trinidad, Puerto Rico, and Brazil, respectively, as determined following BLASTn analysis (Figure 4). Group IV is the largest (n = 28), and shared approximately 84 to 85% nucleotide sequence identity with strains from Brazil, Puerto Rico, and Trinidad. Group I was the most genetically uniform, with no nucleotide differences among the 21 sequences. A representative subset of sequences generated in this study was deposited in GenBank and is publicly available (Supplementary File S1).

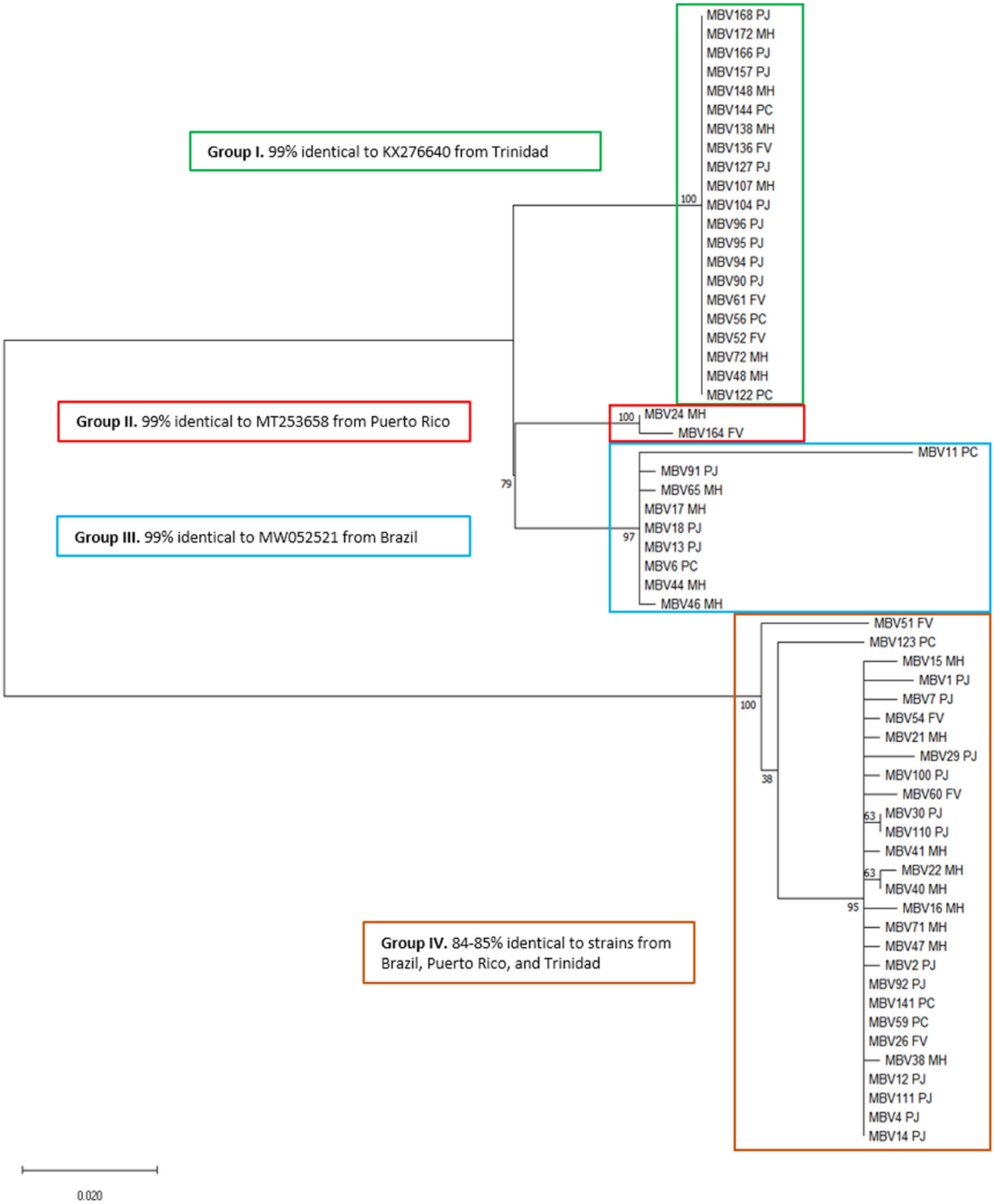

Figure 4. Phylogenetic tree constructed using the nucleotide sequences of the coat protein region of *cacao mild mosaic virus*. Sequence names are followed by the initials of the mealybug species in which they were found; *P. jackbeardsleyi* (PJ), *P. comstocki* (PC), *M. hirsutus* (MH), and *F. virgata* (FV).

4. Discussion

This study identified two new mealybug species, *P. jackbeardsleyi* and *M. hirsutus*, as potential vectors of CaMMV. *P. jackbeardsleyi* has been frequently collected throughout Florida (USA), the Caribbean, and Central and South America on various crops, and is thought to be native to the area. It has only been reported on cacao in Colombia [32]. However, it was described as a species relatively recently, in 1996, and is commonly misidentified as *P. elisae* or *P. landoi* [33]. In contrast, *M. hirsutus* is native to southeast Asia, but has been highly invasive following its introduction into the Caribbean in 1994. Successful control has been obtained in some locations using parasitoid and predator species only when they were released quickly after initial detection [34]. It was introduced to the state of Florida nearly 20 years ago and has become widespread, affecting fruit trees such as papaya, citrus, and soursop [35,36]. *M. hirsutus* has been reported to affect cacao in Trinidad [37] and Brazil [38].

These species have been reported to affect cacao in Africa, the Caribbean, and South America [37–39], but this is the first report of them affecting cacao in Florida. Neither species has been tested for their ability to transmit viruses to cacao, but they are closely related to confirmed vectors, and have been detected on CSSV-infected cacao in Cote d'Ivoire [39]. Most research on mealybugs affecting *T. cacao* is done in West Africa, where the more damaging, and economically significant, viruses are found. However, owing to the status of CaMMV as an emerging disease in the Western hemisphere, it is essential to determine the transmission routes and other significant epidemiological variables.

Cytochrome c oxidase subunit I (COI) is considered the most informative marker for insects; however, it has proven difficult to amplify in mealybugs [26,40]. The COI primers used in this study were designed by Wetten et al. [25] and validated on taxa collected from cacao in Asia, Africa, and the Americas (*Planococcus, Ferrisia, Dysmicoccus,* and *Pseudococcus*). They amplify a small section of the universal barcode region [41] that provided unambiguous identification in the species examined here.

The low homology between ITS2 sequences from *F. virgata* obtained in this study, and those available in GenBank, may indicate high genetic diversity, or the presence of cryptic species. In this study, ITS2 sequences were not considered informative for *P. jackbeardsleyi*, owing to the absence of these sequences in GenBank. However, the ITS2 sequences generated in this study were deposited in GenBank, making this marker valuable for future research.

The presence of multiple virus strains in mealybugs feeding on infected trees suggests that they are being actively transmitted in the greenhouse where this study took place. It is not clear how, or if, these strains interact with each other inside a given tree. Cross-protection has been reported with closely related strains, but does not always occur [42]. Transmission of CaMMV by mealybugs is followed by long latent periods (40–178 days), which reduces the effectiveness of rogueing to control disease [4].

Kirkpatrick [20] observed that *Pl. citri* formed large colonies on pods, but was less likely to feed on young stems or leaves. In contrast, *F. virgata* is the species most frequently observed to feed on leaves. The absence of *Pl. citri* in this study was surprising as this species is a common pest in Florida [43]. Accurate identification of species present in a population is essential for the selection of effective controls. Additional work on the preference of species for certain tissues and environmental conditions could provide useful information for management programs and insecticide applications.

5. Conclusions

The two most prevalent species, *P. jackbeardsleyi* and *M. hirsutus*, make up more than 72% of the mealybug population in this study. Prevalence in a population is a major characteristic affecting the significance of each species in the disease cycle. Neither species were mentioned in previous studies on cacao viruses in the Americas, suggesting a relatively recent association with *T. cacao*. Owing to their prevalence, transmission

studies should be conducted to determine the ability of *P. jackbeardsleyi* and *M. hirsutus* to transmit CaMMV.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/insects12110994/s1, Supplementary File S1: Nucleotide sequences submitted to GenBank.

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Species Complex and Temporal Associations between Coccinellids and Aphids in Alfalfa Stands in Spain

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Simple Summary: Alfalfa is the main fodder crop of the irrigated crop systems of northern Spain, as well as many Mediterranean countries. Alfalfa crops are damaged by some pest species but are also considered to be vast reservoirs of natural enemies of these pests. However, in Europe, the relationships between these pests and their natural enemies have been poorly studied. The aim of this study was to fill this gap in the knowledge. Therefore, we characterized the coccinellid species complex, identifying sixteen species, and the numerical relationships of the two that were most prevalent—*Coccinella septempunctata* and *Hippodamia variegata*—with the different aphid species, which are considered one of the alfalfa pests. The numerical relationships were separately studied in each of the alfalfa growing periods between cuttings (intercuts). Whereas the abundance of *C. septempunctata* was correlated with that of *A. pisum* in the second alfalfa intercut, *H. variegata* was mainly correlated with the abundance of *T. trifolii* in the fourth intercut as well as with the overall aphid abundance in the fifth intercut. This study helps to increase the knowledge on the predator–prey relationships, which is crucial for the proper pest management of alfalfa and the agricultural ecosystems in which it is included.

Abstract: Alfalfa is known to be an important reservoir harboring natural enemies. The reduction in insecticide sprayings in recent years has allowed us to study the coccinellid species complex in this crop and the relationship between these predators and aphids. Alfalfa was sampled by sweep-netting throughout its productive period in several commercial stands each year between 2010 and 2021. The numbers and species of aphids and coccinellids were recorded. Sixteen coccinellid species were found. *Coccinella septempunctata* and *Hippodamia variegata* were, by far, the most prevalent species, with the former dominating during the first and second intercuts, whereas the latter dominated from the third to the fifth intercut. *Acyrthosiphon pisum* and *Therioaphis trifolii* were the most abundant aphid species, peaking in the second and fourth intercuts, respectively. Positive correlations were found between the abundance of *C. septempunctata* and *A. pisum* at the second intercut, between *H. variegata* and *T. trifolii* at the fourth intercut, and between *H. variegata* and the total number of aphids in the fifth intercut. This study helps to increase the knowledge on the predator–prey relationships of this crop and allows for designing strategies of conservation biological control against aphids.

Keywords: *Acyrthosiphon pisum*; *Therioaphis trifolii*; *Aphis craccivora*; *Coccinella septempunctata*; *Hippodamia variegata*; population dynamics; numerical responses

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1. Introduction

Alfalfa, *Medicago sativa* L., is the world's most valuable cultivated forage crop [1]. In Spain, it is a traditional component of crop rotations, covering more than 253,000 ha in 2020 [2], which represents around 20% of the total surface used for this crop in Europe [3]. Spain is the main European exporter of alfalfa (dehydrated or in pellets), especially to Middle Eastern countries and China [4]. Stands usually remain in the field for 4 years and their management consists of regular cuttings during the growing season—usually,

there are five that occur between the end of April and the end of September at intervals of 30–40 days.

Several pests can economically damage this crop—*Hypera postica* (Gyllenhal) (Coleoptera: Curculionidae), *Colaspidema barbarum* (Fabricius) (Coleoptera: Chrysomelidae), *Holotrichapion pisi* (Fabricius) (Coleoptera: Curculionidae), lepidopteran leaf feeders (several species), and aphids (Hemiptera: Aphididae).

The aphid species that occur in Spanish alfalfa stands are *Acyrthosiphon pisum* (Harris), *Aphis craccivora* Koch, and *Therioaphis trifolii* (Monell). Their seasonal occurrence, phenology, and population dynamics have been previously reported [5–7]. Besides the damage inflicted by their alimentary activity, they can transmit a wide range of viruses [8–10]. Although the number of insecticide treatments against aphids has been reduced over the last few years, some sprayings are still applied. In order to develop more sustainable control strategies, it is necessary to know which natural enemies are associated with alfalfa aphids in Spain, and their relationships. Among the most common ones are lady beetles (Coleoptera: Coccinellidae), pirate bugs (Hemiptera: Anthocoridae), damsel bugs (Hemiptera: Nabidae), hoverflies (Diptera: Syrphidae), and parasitoids (Hymenoptera: Braconidae, Aphidiinae) [6,11].

Studies on the occurrence and abundance of natural enemies of the alfalfa aphids in northern Catalonia have been conducted, where the relationships between aphids and parasitoids [7,11], and some heteropteran predators [6] were reported. Relationships between aphids and coccinellids were also investigated in the same area [6], with the results showing that significant numerical relationships only occurred during the growing alfalfa period between the first and the second cuttings. The fact that only two growing seasons were considered in the study and that the data came from a reduced area in northern Catalonia, close to the Pyrenees, could have mediated the results. Therefore, more information from a wider time period and crop cultivation area is needed in order to determine the coccinellid species complex, their numerical relationships with the main alfalfa aphid species, and their potential role as control agents.

The aims of the present work were to (1) characterize the coccinellid species complex in Spanish alfalfa stands, (2) determine their relative occurrence throughout the crop productive period, (3) define the aphid–coccinellid species associations, and (4) determine numerical and temporal relationships between the most abundant coccinellid and aphid species throughout the crop growing season.

2. Materials and Methods

The study was conducted along the Ebro Valley region, in the northeast of Spain, corresponding to 60% of the land surface used for alfalfa cultivation in Spain (and 20% considering Europe) (Figure 1).

A total of 112 alfalfa fields were sampled in the period from 2010 to 2021. All the sampled fields were sown with the Aragon variety, by far the most common one cultivated in the region [12]. Samplings were performed in each of the five growing alfalfa periods between cuttings (intercuts hereafter; see [6,11], from March to September). However, only 99 fields that did not receive insecticide sprayings were included in this study. As the study was part of a more extensive project dealing with the integrated pest management of alfalfa in the region, unequal numbers of fields were sampled in each of those intercuts, and more fields were sampled in the first one, when the most damaging pest *H. postica* occurred [13]. Each field was divided into four sectors, and three samples per sector were collected following the central part of one of the main diagonals and at least 25 m apart (following [6]). Samples were taken with a 38 cm diameter sweep net, sweeping it from side to side five times in a 180° arc. The samples were placed in an icebox and transported to the laboratory, where they were frozen and stored until aphid and coccinellid individuals were identified to the species level, following [14,15], and counted. For each aphid species, all morphs and instars were considered together, whereas the larvae and adults of the coccinellids were distinguished. One sample per intercut was obtained.

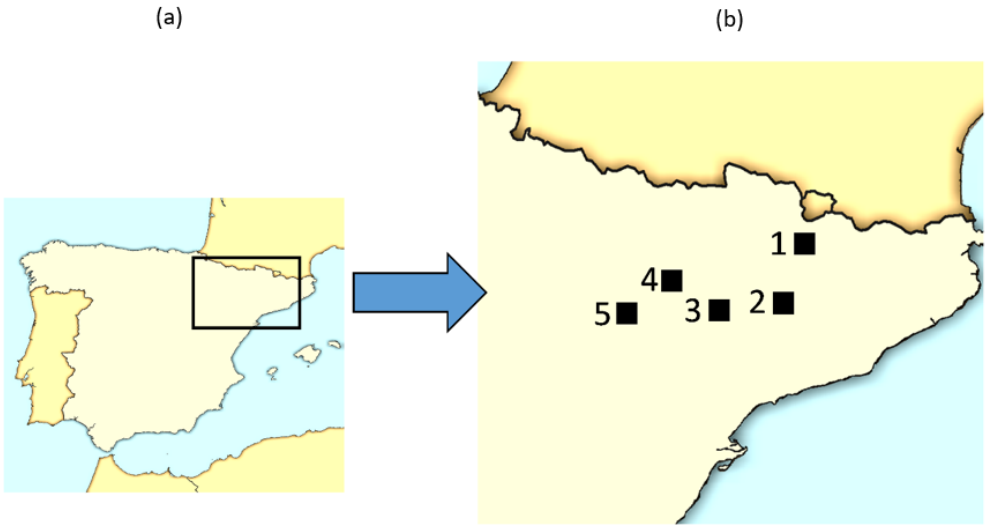

Figure 1. Zones showing the locations of the sampled fields. (**a**) Iberian Peninsula; (**b**) sampling areas in the northeast of Spain: (1) Alt Urgell (42°14′19″ N 1°24′22″ E); (2) Urgell (41°38′40″ N 0°54′46″ E); (3) Segrià (41°37′00″ N 0°37′00″ E); (4) Baja Cinca (41°54′00″ N 0°11′00″ E); and (5) Monegros (41°29′50″ N 0°09′13″ W).

Data Analysis

The total number of coccinellids of each species (adults and larvae) and aphids (all stages together) recorded in each sampling point of one field were averaged, and one field was considered as one replicate. Alfalfa cuttings involve a temporary but drastic change to the system, therefore the five intercuts were considered as separate units. As unequal numbers of fields were sampled in each intercut, the overall relative abundances of the different coccinellid species for the total study period were calculated based on standardized values (the number of each coccinellid species in each intercut was divided by the total number of sampled fields in that intercut).

Statistical analyses were performed with R version 4.0.3 (R Foundation for Statistical Computing, Vienna, Austria). The numerical relationships between coccinellid and aphid species in each intercut were determined by correlation analysis (cor function). Additionally, because we had data on the different *H. postica* larval instar abundances in the first intercut for some years and it has been reported as alternative prey for *Coccinella septempunctata* L. [16,17], the numerical relationship between these two species was also analyzed. The shapiro.test function was used to check for normality before and after transforming the data as (log (x + 1)). The data did not follow a normal distribution; therefore the Spearman correlation test was used. No correlation analyses were performed for the third intercut, as the aphid abundances were very low.

3. Results

3.1. Coccinellid Species Complex and Relative Abundance

A total of 9124 coccinellids were collected throughout the study. Sixteen species/genus were recorded: *C. septempunctata*, *Hippodamia variegata* (Goeze), *Propylea quatuordecempunctata* L., *Scymnus* spp., *Coccinella quinquepunctata* L., *Coccinula quatuordecimpustulata* L., *Exochomus nigromaculatus* (Goeze), *Tytthaspis sedecimpunctata* L., *Hyperaspis* sp., *Adalia bipunctata* L., *Oenopia lyncea lyncea* Olivier, *Coccinella undecimpunctata* L., *Subcoccinella vigintiquattuorpunctata* L., *Chilochorus bipustulatus* L., *Psyllobora vigintiduopunctata* L., and *Stethorus punctillum* (Weise). Whereas *C. septempunctata*, *H. variegata*, *P. quatuordecempunctata*, *C. quatuordecimpustulata*, *Scymnus* sp., *E. nigromaculatus*, *C. quinquepunctata*, *A. bipunctata*, *O. lyncea lyncea*, and *C. undecimpunctata* are aphidophagous predators, *Hyperaspis* sp. and *C. bipustulatus* are mainly coccidophagous, but consume aphids as secondary prey. *Tytthaspis sedecimpunctata*

and *P. vigintiduopunctata* feed on fungus, especially powdery mildew. *Subcoccinella vigintiquattuorpunctata* is herbivorous and *Stethorus punctillum* is a mite predator.

The most abundant species were *C. septempunctata* and *H. variegata* (Table 1), both accounting for nearly 95% of species in the whole study. *Coccinella septempunctata* was the predominant species in the first and the second intercuts, making up around 80% of the collected coccinellids (Table 1). Although it was present throughout the entire alfalfa growing season, its relative abundance decreased drastically from the third to the fifth intercut. The opposite trend was recorded for *H. variegata*, which was also present during the entire growing season, but it became the predominant species from the third to the fifth intercut, when it accounted for 76, 89, and 89% of the collected coccinellids, respectively (Table 1).

Table 1. The numbers of individuals collected and relative abundance (% in parenthesis) of the different coccinellid species per intercut, and the standardized relative abundance (%) for the total sampled period. *C7*: *Coccinella septempunctata*; *Hv*: *Hippodamia variegata*; *P14*: *Propylea quatuordecimpunctata*; *Scy*: *Scymnus* sp.; and *Stet*: *Stethorus punctillum*.

No. Fields	Intercut	*C7*	*Hv*	*P14*	*Scy*	Other Aphidophagous	*Stet*	Others	TOTAL
99	1	1696 (82.57)	230 (11.20)	102 (4.97)	18 (0.88)	3 (0.15)	1 (0.05)	4 (0.19)	2054 (100.00)
77	2	1700 (79.81)	351 (16.48)	47 (2.21)	19 (0.89)	0 (0.00)	12 (0.56)	1 (0.05)	2130 (100.00)
70	3	329 (15.30)	1629 (75.77)	128 (5.95)	44 (2.05)	8 (0.37)	1 (0.05)	11 (0.51)	2150 (100.00)
75	4	108 (5.14)	1882 (89.49)	73 (3.47)	20 (0.95)	3 (0.14)	13 (0.62)	4 (0.19)	2103 (100.00)
37	5	44 (6.40)	611 (88.94)	26 (3.78)	4 (0.58)	1 (0.15)	1 (0.15)	0 (0.00)	687 (100.00)
		Standardized relative abundance (%)							
		37.01	57.07	4.09	1.14	0.17	0.30	0.21	100

Regarding the population structure of *C. septempunctata* (Figure 2), 53% of the collected individuals in the first intercut were adults and the other 47% were larvae. The proportion of larvae increased to 60% in the second intercut. Later in the season, adults occurred more commonly than larvae.

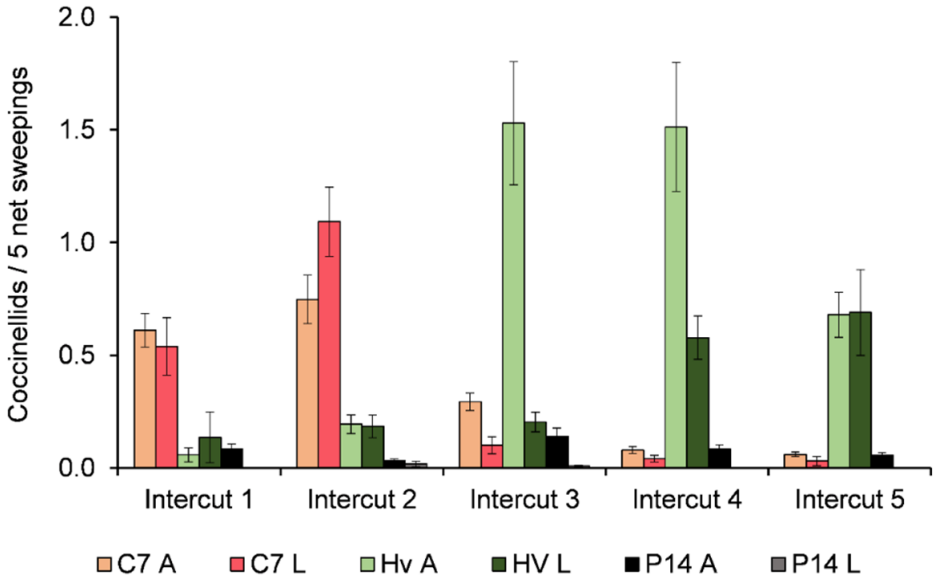

Figure 2. The mean number ± s.e. of adults (A) and larvae (L) per five net sweepings of the three main coccinellid species, *Coccinella septempunctata* (C7), *Hippodamia variegata* (Hv), and *Propylea quatuordecimpunctata* (P14) in each of the five intercuts of the growing season.

Despite the low numbers, the population structure of *H. variegata* in the first intercut was mainly composed of larvae (70% larvae vs. 30% adults), but proportions of both development stages were equal in the second intercut (Figure 2). In the third intercut, many more adults were recorded, accounting for nearly 90% of the collected *H. variegata* individuals. This proportion steadily decreased to 72% and 50% in the fourth and fifth intercuts, respectively.

Propylea quatuordecimpunctata and *Scymnus* sp. were also common species, but only accounted for 4% and 1% of the total number of collected coccinellids, respectively (Table 1). Most of the individuals collected were adults. The occurrences of the other species were sporadic.

3.2. Aphid Abundance

The occurrences of the three previously reported alfalfa aphid species in Spain—*A. pisum*, *T. trifolii*, and *A. craccivora*—were recorded. The former occurred during the whole growing season, but with remarkable differences between the intercuts (Figure 3). This species was prevalent in the first and second intercuts, peaking in the latter. A huge decrease in its population was recorded in the third and fourth intercuts, but it recovered later, at the end of the growing season. An opposite trend in population dynamics occurred with *T. trifolii* (Figure 3), where this species was either not collected or only sporadically collected in the first and second intercuts. A slight increase in its population was recorded in the third intercut, reaching its peak in the fourth. Then, a sharp decrease occurred in the fifth intercut. Despite the values shown in Figure 3, the occurrence of *A. craccivora* was very erratic, with some fields showing high populations whereas, in some others, populations were completely irrelevant. There was an increase in its abundance in the fifth intercut.

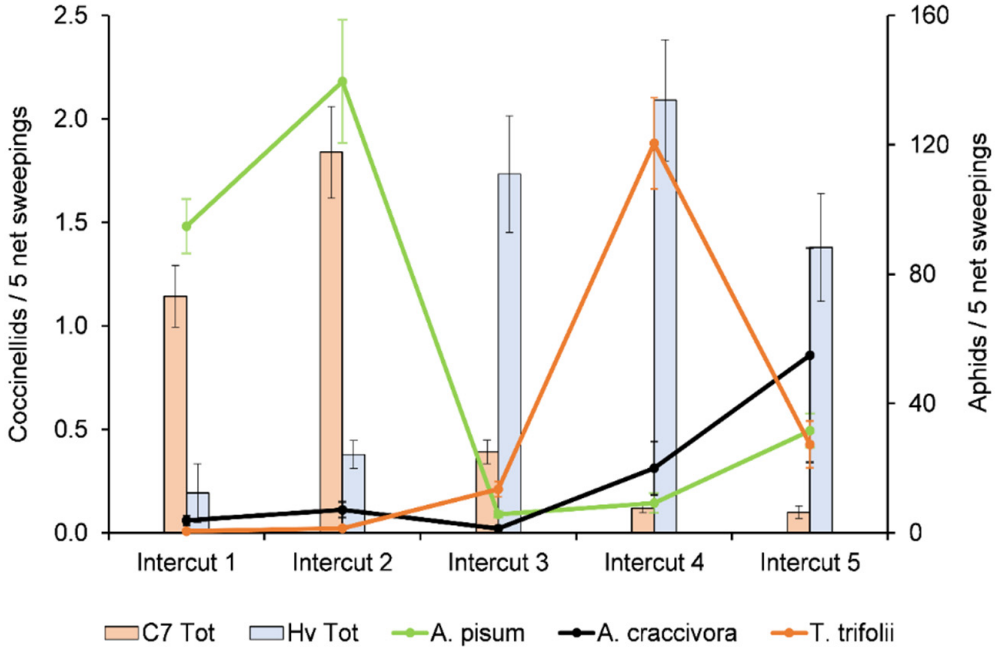

Figure 3. The mean abundance ± s.e. of the alfalfa aphids *Acyrthosiphon pisum*, *Therioaphis trifolii*, and *Aphis craccivora* in each of the five intercuts of the growing season, and the mean number ± s.e. of individuals (adults and larvae) per five net sweepings of the two main coccinellid species (bars), *Coccinella septempunctata* (C7) and *Hippodamia variegata* (Hv).

3.3. Numerical Relationships between Aphid and Coccinellid Species

Based on the relative abundance of the coccinellid species, we restricted the results to *C. septempunctata* and *H. variegata*. Looking at Figure 3, there seems to be a close relationship between the occurrence of *C. septempunctata* and *A. pisum* in the first two intercuts. However, no significant correlations between *C. septempunctata* adults, larvae, or both

together with *A. pisum* were found in the first intercut (Table 2). Conversely, these variables were significantly and positively correlated in the second intercut (Figure 4a, Table 2). A close relationship between *H. variegata* and *T. trifolii* was also shown in the fourth intercut (Figure 3), which was confirmed by the correlation analyses, where *H. variegata* adults, larvae, and both together showed positive correlations with this aphid, the prevalent species at this time (Figure 4b, Table 2). *Hippodamia variegata* larvae were also positively correlated with *A. pisum* and *A. craccivora*. Positive correlations with the total number of aphids were also recorded for this coccinellid species (Table 2). However, these results are likely mediated by the abovementioned positive correlation with *T. trifolii*, the most abundant aphid in this intercut. Regarding the fifth intercut, *H. variegata* was well correlated with *A. pisum*, *A. craccivora*, and the total number of aphids, but not with *T. trifolii* (Figure 4c, Table 2).

3.4. Numerical Relationships between H. postica larvae and C. septempunctata

In the first intercut, no significant correlations were observed ($p > 0.10$) when we evaluated the numerical relationships between *C. septempunctata* and its potential alternative prey *H. postica*. However, after adding the values of the *A. pisum* and *H. postica* larvae abundances, the correlations improved, but statistically significant results were only obtained for *C. septempunctata* adults and *H. postica* L4 plus *A. pisum* (Table 3).

Table 2. Spearman's correlation coefficients (rho) and *p*-values between the most abundant aphid species and coccinellids in four of the five intercuts of the alfalfa growing season (the third intercut was not included due to the low abundance of aphids). C7, *C. septempunctata*; Hv, *H. variegata*; A = adults; L = larvae; and – = no correlation analyses were performed.

	A. pisum		***T. trifolii***		***A. craccivora***		**Total Aphids**	
	rho	***p*-Value**	**rho**	***p*-Value**	**rho**	***p*-Value**	**rho**	***p*-Value**
1st intercut								
C7 (L + A)	0.0041	0.9686	–	–	–	–	–	–
C7 A	0.0484	0.6433	–	–	–	–	–	–
C7 L	−0.0059	0.9548	–	–	–	–	–	–
Hv (L + A)	0.1309	0.2085	–	–	–	–	–	–
Hv A	0.0624	0.5502	–	–	–	–	–	–
Hv L	0.1834	0.0761	–	–	–	–	–	–
2nd intercut								
C7 (L + A)	0.5651	<0.0001	–	–	–	–	–	–
C7 A	0.4202	0.0002	–	–	–	–	–	–
C7 L	0.5211	<0.0001	–	–	–	–	–	–
Hv (L + A)	0.2522	0.0325	–	–	–	–	–	–
Hv A	0.1968	0.0976	–	–	–	–	–	–
Hv L	0.0772	0.5189	–	–	–	–	–	–
4th intercut								
Hv (L + A)	0.0320	0.7936	0.5880	<0.0001	0.3417	0.0078	0.6012	<0.0001
Hv A	−0.2490	0.0391	0.4507	0.0001	−0.0576	0.6382	0.4088	0.0005
Hv L	0.5295	<0.0001	0.4103	0.0005	0.5731	<0.0001	0.4352	0.0002
5th intercut								
Hv (L + A)	0.4433	0.0068	0.1900	0.2671	0.7298	<0.0001	0.7248	<0.0001
Hv A	0.3395	0.0427	0.3818	0.0216	0.4780	0.0040	0.5541	0.0004
Hv L	0.4398	0.0073	0.0111	0.9460	0.6518	<0.0001	0.6310	<0.0001

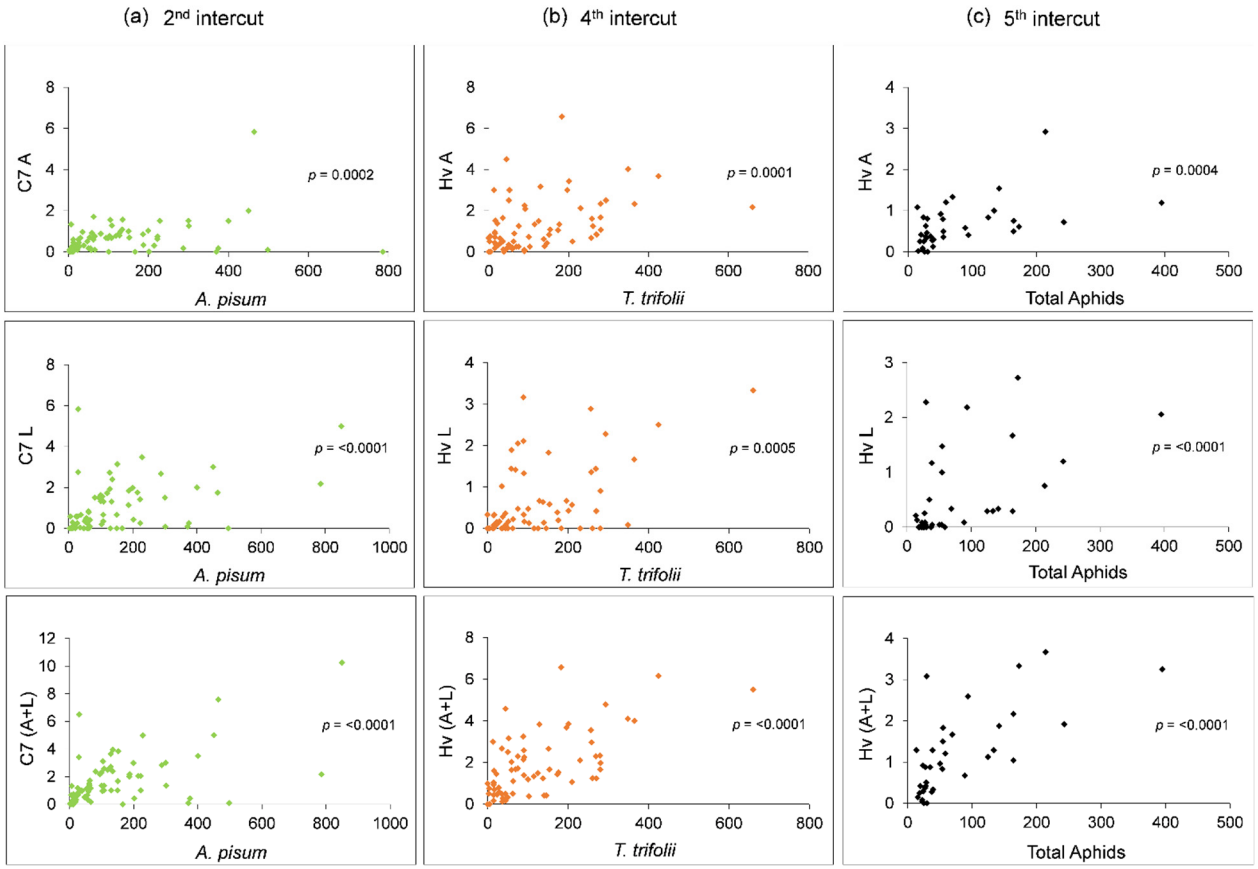

Figure 4. The most representative correlations: (**a**) *A. pisum*–*C. septempunctata* (second intercut); (**b**) *T. trifolii*–*H. variegata* (fourth intercut); and (**c**) total aphids–*H. variegata* (fifth intercut). C7, *C. septempunctata*; Hv, *H. variegata*; A, adults; and L, larvae.

Table 3. Spearman's correlation coefficients (rho) and *p*-values between the different larval instars of *H. postica* and the lady beetle *C. septempunctata* in the first intercut. C7, *C. septempunctata*; A, adults; L, larvae; L1, first instar; L2, second instar; L3, third instar; and L4, fourth instar.

	H. postica* L1-L2 + *A. pisum		***H. postica* L3 + *A. pisum***		***H. postica* L4 + *A. pisum***		***H. postica* Total + *A. pisum***	
	rho	***p*-Value**	**rho**	***p*-Value**	**rho**	***p*-Value**	**rho**	***p*-Value**
C7 (L + A)	0.2408	0.0553	0.2113	0.0936	0.2108	0.0945	0.1982	0.1164
C7 A	0.2005	0.1121	0.2152	0.0876	0.2526	0.0440	0.2046	0.1049
C7 L	0.1351	0.2648	0.0933	0.4422	0.0958	0.4296	0.0788	0.5165

4. Discussion

Alfalfa crops are known to be significant reservoirs of natural enemies [5,11]. However, studies dealing with the predator/parasitoid–prey relationships in this crop are scarce in Europe. Increasing our knowledge in this area is crucial for the proper pest management of alfalfa and the agricultural ecosystems in which it is included. In this eleven-year-long study, new information on the coccinellid species complex and their relationships with the alfalfa aphids was revealed.

Results on the aphid complex species and their temporal occurrence did not differ from those previously reported in the study area [6]. However, our results show a change in the seasonal prevalence, as *A. pisum* was the species that reached the highest abundance values instead of *T. trifolii*, as reported in Pons et al. [6]. The current study considered a wider area that includes regions with milder temperatures during the first and second intercuts, which may have been favorable for *A. pisum* populations.

Sixteen different coccinellid species were recorded. Within them, *C. septempunctata* and *H. variegata* were the most prevalent. These results concur with those of other studies, which also reported these two species as being the dominant coccinellids in alfalfa stands in Europe [5,18]. Although the presence of the harlequin lady beetle, *Harmonia axyridis* Pallas, has been recorded in northeastern Spain [19], and this invasive coccinellid is usually found in urban green areas of the region (authors, unpublished), it was not recorded in this study. Following its invasion in Chile, this coccinellid species rapidly increased in abundance and became dominant in alfalfa stands [20]. If *H. axyridis* is able to colonize alfalfa stands in the study area, the relationships of native coccinellids with aphids will probably change [21].

Regarding the seasonal occurrence of *C. septempunctata* and *H. variegata*, our results show that both species share the same habitat. Contrary to what was reported in other studies [22], there was a clear succession in their prevalence; *C. septempunctata* dominated during the first and second intercut, while *H. variegata* dominated in subsequent intercuts. Three factors may have led to this succession, including the species phenology (crop colonization and the timing of departure), predator–prey species associations, and intraguild predation events. Assuming that both species overwinter as adults outside of alfalfa stands, it seems clear that *C. septempunctata* colonizes alfalfa earlier than *H. variegata*. However, reproduction may occur during the first intercut, and larvae were recorded for both coccinellid species. The non-significant correlation between *C. septempunctata* and *A. pisum*, practically the only aphid species present in the first intercut, suggests that aphids are probably not the only reason for the predominance of *C. septempunctata*. Aggregative and numerical coccinellid responses to aphids can be influenced by several factors [23,24], such as the presence of alternative prey [25]. We found a significant positive correlation between the number of *C. septempunctata* adults and that obtained after the addition of *A. pisum* and *H. postica* fourth instar larvae abundances, suggesting that the weevil can contribute to the reproduction of this species and, thus, to the development of *C. septempunctata* populations, as reported by Richards and Evans [17].

In the second intercut, the abundance of *C. septempunctata* was correlated with that of *A. pisum*, whereas *H. variegata* was not. Although this aphid species was relatively abundant in the first and second intercuts, the competence for prey resources and the intraguild predation events between these two coccinellid species could have contributed to this. The dominance of *C. septempunctata* over other coccinellid species has been reported in several studies [26,27]. In addition, intraguild predation between *C. septempunctata* and *H. variegata* has been already reported [28,29], and was asymmetric for the former. Such interactions could have postponed the proper establishment of *H. variegata* in alfalfa until the departure of *C. septempunctata*.

After the peak of *A. pisum* in the second intercut, its occurrence decreased drastically in the third intercut, as did the occurrence of *C. septempunctata*. This was likely due to the lack of prey leading to the adults leaving the alfalfa stands. Ricci et al. [30] reported that when aphids are scarce, coccinellid adults leave crops in search of new aphid food, which can be a plausible justification for this drop. This is reinforced by Madeira et al. [31] and di Lascio et al. [32], who observed that other common crops in northeastern Spain, such as maize, can have a "sink" effect during its vegetative growing period (coinciding with the third alfalfa intercut) over the alfalfa *C. septempunctata* populations, which do not return after leaving. Summer diapause has also been described as a common trait of Mediterranean *C. septempunctata* populations [33], so it should also be considered as a possible cause for this decrease. It could also be presumed that most of the larvae can be destroyed with the alfalfa cutting, but the records of Ghahramani et al. [22] seem to not corroborate this. However, if the cutting is advanced enough to control the large amount of damage caused by *C. barbarum*, a significant pest that affects the second intercut, larvae of *C. septempunctata* may not be able to reach adulthood and migrate from alfalfa, becoming seriously affected. More studies are needed to elucidate the phenology of this species in the Ebro Valley area.

The highest abundances of *H. variegata* adults were shown during the third and fourth intercuts. These results suggest that significant immigration to the alfalfa stands from the surrounding crops may occur during this period of the year. This massive entry could be related to the growing populations of *T. trifolii*, which were reported to act as an attracting and arrestant stimulus for *H. variegata* [34]. Di Lascio et al. [32] already reported the movement of *H. variegata* individuals between alfalfa and maize crops in this period. The better adaptation for the reproduction of this coccinellid species during the summer [35,36] is probably the cause of its dominance from the third to the fifth intercuts.

The positive correlations recorded for *H. variegata* in the fourth and fifth intercuts partially differ from the results of Pons et al. [6], who only reported a positive correlation between this coccinellid species and *A. craccivora* in the fifth intercut. As mentioned above, the abundant presence of *T. trifolii* during the fourth intercut may have acted as an attracting and arrestant stimulus for *H. variegata* individuals and, thus, may have led to their positive correlation. During the fifth intercut, the dominance of *A. craccivora*, which was already described as very suitable prey for *H. variegata* [37], may explain its positive correlation with this aphid species and, thus, with the total number of aphids. The sharp decrease in *T. trifolli* abundance recorded in this intercut could be due to its own phenology, but the predation of *H. variegata* and, possibly, other specific natural enemies as parasitoids may also have contributed. *Trioxys complanatus* Quilis and *Praon exsoletum* (Nees) have been reported as the parasitoid species associated with *T. trifolii* in the northern area of the study region [11]. The rate of parasitism by these two species was estimated between 5% and 15%, a low to moderate rate compared with the rates of other alfalfa aphids [7]. An extensive study on the aphid parasitism is being carried out in the whole Ebro Valley in order to better know the effective role of parasitoids in alfalfa aphid control.

The abundance of *P. quatuordecimpunctata*, even as the third most frequently occurring species, was very low when compared to *C. septempunctata* and *H. variegata*. The numbers of larvae collected were especially low, and this suggests that adults of this species did not significantly reproduce in alfalfa stands. On the other hand, no correlation with aphids was found (data not shown).

Our study presents previously unreported, positive coccinellid–aphid correlations, such as between *H. variegata* and *T. trifolii*, as well as new information about the coccinellid complex of alfalfa in the Ebro Valley region. More studies are needed in order to increase the knowledge on the predator–prey interactions of this crop in Europe.

5. Conclusions

Based on this eleven-year-long study, we present the first report on the coccinellid species complex in Spanish alfalfa stands and the relationships with aphids. Sixteen coccinellid species were recorded, among which eight were aphidophagous. *Coccinella septempunctata* and *H. variegata* were the prevalent species, but a clear succession between them was observed; the former dominated during the first and second intercuts, whereas the latter dominated from the third to the fifth intercut. Several positive correlations were found, but the most significant were those between the abundance of *C. septempunctata* and *A. pisum* in the second intercut, between *H. variegata* and *T. trifolii* in the fourth intercut, and between *H. variegata* and the total number of aphids in the fifth intercut. This study contributes to increase the knowledge on the predator–prey relationships of this crop, knowledge that remains scarce in Europe.

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Article

Species Diversity and Phylogenetic Relationships of Olive Lace Bugs (Hemiptera: Tingidae) Found in South Africa

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Simple Summary: Olive lace bugs feed on wild and cultivated *Olea europaea*, causing a negative impact on plant vitality and development. These insects are known to affect olive orchards in South Africa, the country where most of the olive and olive products on the continent are produced. However, the diversity of species of these pests is not clear. Morphological analysis and DNA barcoding showed the presence of *Cysteochila lineata*, *Plerochila australis*, *Neoplerochila paliatseasi* and *Neoplerochila* sp. Further analyses of genetic divergence and phylogenetic clustering in 30 species in 18 genera of Tingidae using new and publicly available DNA barcodes showed that the majority of sequences deposited on BOLD Systems were correctly assigned to species. The complete mitochondrial genomes of the four species found in South Africa were sequenced to assess their phylogenetic position within Tingidae. The four olive lace bugs formed one cluster of species, and the genus *Cysteochila* was not monophyletic as *C. lineata* grouped with the other three olive lace bugs but *C. chiniana* was placed in a different cluster. This result suggests that lace bug species that feed on olive trees may have a common ancestor and calls for further research on potential adaptations to *O. europaea*.

Abstract: Olive lace bugs (Hemiptera: Tingidae) are small sap-sucking insects that feed on wild and cultivated *Olea europaea*. The diversity of olive lace bug species in South Africa, the most important olive producer on the continent, has been incompletely surveyed. Adult specimens were collected in the Western Cape province for morphological and DNA-based species identification, and sequencing of complete mitogenomes. *Cysteochila lineata*, *Plerochila australis*, *Neoplerochila paliatseasi* and *Neoplerochila* sp. were found at 12 sites. Intra- and interspecific genetic divergences and phylogenetic clustering in 30 species in 18 genera of Tingidae using new and publicly available DNA barcodes showed high levels of congruity between taxonomic and genetic data. The phylogenetic position of the four species found in South Africa was inferred using new and available mitogenomes of Tingidae. Notably, olive lace bugs formed a cluster of closely related species. However, *Cysteochila* was non-monophyletic as *C. lineata* was recovered as a sister species to *P. australis* whereas *Cysteochila chiniana*, the other representative of the genus, was grouped with *Trachypeplus jacobsoni* and *Tingis cardui* in a different cluster. This result suggests that feeding on *O. europaea* may have a common origin in Tingidae and warrants future research on potential evolutionary adaptations of olive lace bugs to this plant host.

Keywords: *Cysteochila lineata*; DNA barcoding; *Neoplerochila paliatseasi*; *Olea europaea*; *Plerochila australis*

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1. Introduction

Lace bugs (Hemiptera: Tingidae) comprise approximately 2500 species of small phytophagous insects in 300 genera distributed in all tropical and temperate continental and most oceanic regions except for the frigid zones [1]. Lace bug adults and nymphs feed by piercing the abaxial surface of the leaves of living plants to extract sap from cellular tissues [2]. Continuous feeding can result in chlorotic spots that may necrotize with detriment to plant vitality, and heavy infestations may cause premature death of young shoots and defoliation of the host. Lace bugs are generally monophagous, and a species feeds on the same kind of plant or group of closely related plants, including several agricultural crops and ornamentals.

Sub-Saharan Africa has a rich assemblage of native insects associated with Oleaceae, including several species of olive fruit flies and olive flea beetles, and a diversity of parasitoid, hyperparasitoid and olive seed wasps [3–7]. Lace bugs feeding on Oleaceae are only found in sub-Saharan Africa: *Catoplatus dilatatus* Jakovlev (on *Olea* sp.), *Cysteochila pallens* Horvath (on *O. chrysophylla*), *Cysteochila sordida* Stål (on *O. verrucosa*), *Olastrida oleae* Schouteden (on *O. europaea*) [8], *Cysteochila lineata* Duarte Rodrigues (on *O. capensis*), *Cysteochila nervosana* Drake and *Caffrocysta aliwalana* Duarte Rodrigues (on *Olea europaea* subsp. *cuspidata*) [9], and *Plerochila australis* (Distant) and *Neoplerochila paliatseasi* Duarte Rodrigues (on *O. europaea*) [10,11]. The exception to this pattern is *Froggattia olivinia* Froggatt, which is native to Australia and feeds not only on *Notelaea longifolia* (Oleaceae) but also on imported *O. europaea* [8].

In South Africa, cultivated olives are often grown in proximity to African wild olives (*Olea europaea* subsp. *cuspidata*), which may act as a source of both olive pests and their natural enemies. African wild olives and cultivated olives are closely related species; hence, most insects associated with African wild olives have been found to also occur on cultivated olives [5]. Despite the diversity of the native olive-associated entomofauna, South African olive growers face less aggressive threats from phytophagous insects, namely *Bactrocera oleae* Rossi (Diptera: Tephritidae), than their Mediterranean and Californian counterparts. Globally, South Africa is a small producer of boutique olive products mostly sold locally, but the industry also exports table olives and olive oil to neighbouring African countries, the European Union and the United States of America. Lace bugs affecting wild and cultivated olive trees in the Western Cape province, the most important region for olive production on the continent, are commonly referred to as "olive tingids" by local farmers and have been reported to include *P. australis* [12,13] and *N. paliatseasi* [11]. Perceptions on the extent of olive lace bug injury to cultivated olive trees vary from "olive tingids" being a minor pest that does not require management to a threat that impacts olive production and requires insecticide treatment.

The genus *Neoplerochila* was erected by Duarte Rodrigues [14] to hold the species *inflata* Duarte Rodrigues. *Neoplerochila* is only known from Namibia and South Africa and now includes eight species of which *N. millari* Göllner-Scheiding, *N. dispar* Duarte Rodrigues, *N. weenenana* (Drake) and *N. paliatseasi* are found on Oleaceae. The hosts of *N. inflata* Duarte Rodrigues, *N. katbergana* Drake, *N. uniformis* Duarte Rodrigues and *N. youngai* Duarte Rodrigues are presently unknown [10]. *Neoplerochila paliatseasi* is probably distributed countrywide in South Africa, as it was found in the Limpopo, North West and Western Cape provinces [10,11], and in Gauteng province in the present study.

The genus *Plerochila* was erected by Drake (1954) to hold *Plerochila australis* Distant and *P. horvathi* Schouteden as close to the genus *Cysteochila* but differing on the shape of paranota and carinae. *Plerochila* currently includes 17 species restricted to Africa, of which *P. australis*, *P. horvathi* and *P. rutshurica* Schouteden are known to feed on *Olea*. The host plants of most *Plerochila* species are not known [15]. *Plerochila australis* has been reported in Ethiopia, the Democratic Republic of Congo, Kenya, Madagascar, Mauritius, Mozambique, Namibia, Sudan, Tanzania, Uganda and South Africa [15]. In South Africa, *Plerochila australis* is probably distributed countrywide as it was reported in the Western Cape, Northern Cape, Gauteng, Limpopo, Mpumalanga and North West provinces [15].

The Mediterranean Basin and California are currently free from olive lace bugs, but these may become a threat if translocated from their original geographic range. Since our previous report of *N. paliatseasi* in South Africa [11], we learned that the species is indeed present on Madeira Island (Portugal), where it feeds on cultivated olives [16], but it seems to be restricted to that insular region and has not been found in mainland Europe, to the best of our knowledge. Under the right circumstances, olive lace bugs can become a problem, as is the case of the native Australian *F. olivinia*, which moved to cultivated olive trees and African wild olives when imported plants became established in the country starting in the 19th century [17]. *Froggattia olivinia* is now a serious pest of cultivated olives in New South Wales and Queensland, but it has not yet been reported outside Australia.

This work is part of a larger effort to catalogue the diversity of insect species associated with wild and cultivated olives in South Africa. The main objectives were (1) to gain further insights into the diversity of olive lace bugs found in South Africa and (2) to investigate the phylogenetic position of those species within the family Tingidae using new and publicly available mitogenome sequences.

2. Materials and Methods

2.1. Specimen Collection, Morphological Identification and DNA Extraction

Olive lace bugs were collected in nine areas in the Western Cape (cultivated and wild olive trees) and one site in Pretoria (wild olive trees) between November 2015 and March 2020 (Table S1). Additionally, eight olive farms in the Western Cape were visited between October 2020 and March 2021 during the South African olive growing season when olive lace bugs are likely to be present. Specimens were collected from cultivated olive blocks identified as infested by farm workers. The number of trees surveyed at each farm varied from 10 to 50, and every second tree was sampled in any given block. Specimens were collected directly into individual plastic tubes, euthanized by freezing and stored in 100% ethanol at −20 °C until downstream analyses. DNA was extracted from individual specimens using a standard phenol-chloroform method [18] and stored at −20 °C until further use.

Morphological identification of ethanol-preserved specimens was performed by É. Guilbert following original descriptions, photos of type material and collections available [10,15]. Representative specimens of *C. lineata*, *P. australis*, *N. paliatseasi* and a nonspecific *Neoplerochila* were imaged and deposited in the entomological collection of the Iziko Museum (Cape Town) for future reference: *C. lineata* SAM-HEM-A012751, *Neoplerochila* sp. SAM-HEM-A012753, *P. australis* SAM-HEM-A010383 and *N. paliatseasi* SAM-HEM-A011647 (SAMC; Curator Simon van Noort). Codens of institutional depositories of voucher specimens follow Evenhuis (2019) [19]. Images were acquired with a Leica LAS 4.9 imaging system, comprised of a Leica® Z16 microscope (using either a 2× or 5× objective) with a Leica DFC450 Camera and 0.63× video objective attached. The imaging process, using an automated Z-stepper, was managed using the Leica Application Suite V 4.9 software. Diffused lighting was achieved using a Leica LED5000 HDI dome.

2.2. DNA Barcoding

Specimens of *C. lineata* (n = 25), *P. australis* (n = 32), *N. paliatseasi* (n = 11) and *Neoplerochila* sp. (n = 14) were sequenced for the standard COI barcoding region (~650 bp) for assessing the congruency between morphological and DNA-based identifications using genetic clustering analysis and estimates of inter- and intraspecific genetic diversity. New species-specific PCR primers were designed for DNA barcoding of *P. australis* and *C. lineata*, based on their mitochondrial genomes (Table S2). *Neoplerochila paliatseasi* and *Neoplerochila* sp. were barcoded using PCR primers specific to *N. paliatseasi* designed in a previous study [11]. Initial attempts to barcode *C. lineata* were made using the PCR primers specific to *P. australis* and the PCR primers specific to *N. paliatseasi*, but limited success (see Section 3.5) led to the design of species-specific primers for *C. lineata* once the mitogenome of latter species was assembled. All new species-specific PCR primer pairs anneal to the

same COI region as the universal DNA barcoding primers, and were designed to be a perfect match to the COI sequence of each species. PCR amplifications were performed in a total volume of 5 µL containing 1× of KAPA2G Robust HotStart Ready Mix PCR kit (KAPPA Biosystems), 0.5 µM of each primer, 0.5 µL of MilliQ H2O, and 1.0 µL of template DNA (~100 ng), as follows: 95 °C for 3 min; 35 cycles of 95 °C for 15 s, 15 s at 58 °C for *C. lineata* and 54 °C for *N. paliatseasi* and *P. australis*, 72 °C for 1 min; and a final extension at 72 °C for 1 min. PCR products were sequenced using the reverse PCR primers specific to each species with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Waltham, MA, USA) at the Central Analytical Facilities of Stellenbosch University, South Africa.

2.3. Intraspecific and Interspecific Genetic Diversity

All DNA barcodes assigned to Tingidae species were downloaded from the Barcode of Life Database (BOLD) Systems v4 (http://v3.boldsystems.org/, accessed on 20 October 2020) to provide a broader context for the intra- and interspecific divergence and genetic clustering patterns of the four olive lace bug species found in South Africa. The initial dataset included 1141 sequences that were subsequently filtered for (a) sequences identified to species level, (b) sequences with a minimum length of 500 bp overlapping the standard COI barcoding region, and (c) species represented by a minimum of three sequences. The final dataset included 367 sequences representing 30 species in 18 genera, including the new sequences (n = 82) generated in this study. Multiple sequence alignments were performed with the MAFFT algorithm [20] in Geneious Prime v2021.1 (https://www.geneious.com, accessed on 20 October 2020).

Genetic clustering of the COI sequence dataset of 30 species was assessed using a maximum likelihood (ML) tree constructed in IQ-Tree [21], with *Adelphocoris fasciaticollis* (NC_023796.1) (Hemiptera: Miridae) as outgroup. The best partitioning scheme was determined using the edge-linked greedy strategy [22] with automatic model selection [23,24] commands (−m MFP + MERGE). Branch supports were determined using 1000 replicates for both ultrafast bootstrapping and SH-aLRT branch tests [25,26]. The final ML tree was drawn using FigTree v1.4.4 (http://tree.bio.ed.ac.uk/, accessed on 4 September 2021).

Intra- and interspecific genetic divergences were estimated as p-distances (%) under the Kimura 2-parameter model [27] in MEGA X [28], with statistical support calculated from 1000 bootstrap replicates. Intraspecific diversity measures (number of haplotypes, number of polymorphic sites, haplotype diversity and nucleotide diversity) were calculated with Arlequin 3.5 [29]. Median-joining haplotype networks were constructed with Network 10.2, under the default settings [30]. The new sequences COI generated in this study were deposited on GenBank: *C. lineata* (MZ673445 to MZ673468), *N. paliatseasi* (MZ666853 to MZ666863), *Neoplerochila* sp. (MZ673417 to MZ673429) and *P. australis* (MZ676957 to MZ676987) (Table S1).

2.4. Sequencing, Assembly and Annotation of Mitogenomes

The complete mitochondrial genomes for one specimen each of *C. lineata*, *P. australis* and *Neoplerochila* sp. were sequenced using the Ion Torrent™ S5™ platform (ThermoFisher Scientific, Waltham, MA, USA) available at the Central Analytical Facilities of Stellenbosch University, South Africa. Sequence libraries were prepared using the Ion Xpress™ Plus gDNA Fragment Library Kit (ThermoFisher Scientific, Waltham, MA, USA), according to the protocol MAN0009847 REV J.0. Libraries were pooled and sequenced using the Ion 540™ Chef Kit (ThermoFisher Scientific). The NGS reads of each species were mapped against the complete mitogenome of *N. paliatseasi* (MN794065) and assembled using Geneious Prime. Open reading frames were identified with Geneious Prime using the invertebrate mitochondrial genetic code. Transfer RNA genes (tRNAs) and their secondary structures were predicted using ARWEN software (http://130.235.244.92/ARWEN/, accessed on 15 March 2021) [31]. The two ribosomal RNA genes (12S rRNA and 16S rRNA) and the large non-coding region presumed to contain the control for transcription and trans-

lation (AT-rich region) were manually annotated by comparison with the mitogenomes of other Tingidae available on GenBank. The new complete mitogenomes of *C. lineata*, *Neoplerochila* sp. and *P. australis* were deposited on GenBank under the accession numbers MZ935684, MZ935685 and MZ935686.

2.5. Mitogenome Analyses

Nucleotide composition and compositional biases were calculated using Geneious Prime as AT skew = (A − T)/(A + T) and GC skew = (G − C)/(G + C)]. Relative synonymous codon usage was calculated in MEGA X. Repeated regions in the AT-rich region were identified using Tandem Repeats Finder v4.09 [32]. Start codons and overlapping and intergenic spaces were counted manually. Nonsynonymous (Ka) and synonymous (Ks) substitution rates were calculated using DnaSP6 [33].

2.6. Phylogenetic Reconstruction of Tingidae

The phylogenetic position of the olive lace bugs within Tingidae was assessed in the context of the 18 mitogenomes available for the family in GenBank as of October 2020, with *Apolygus lucorum* and *Adelphocoris fasciaticollis* (Hemiptera: Miridae) as outgroups (Table S3). Individual PCGs were extracted from the complete mitogenome sequences and aligned using the translation algorithm in Geneious Prime. Stop codons were removed manually, and individual gene alignments were concatenated to form a single alignment. Poorly aligned regions and gaps in the concatenated alignment were eliminated using GBlocks v0.91b [34]. The final alignment was used to generate three sub-datasets: PCG123 (all codon positions), PCG12 (excluding the 3rd codon position), and an amino acid (AA) alignment.

Bayesian analyses were performed on the three datasets under the site-heterogeneous mixture model CAT-GTR in Phylobayes MPI in XSEDE v1.8c [35,36] to minimize the effect of mitochondrial compositional heterogeneity on phylogenetic reconstructions [37,38]. Constant sites were removed from the alignments and the minimum number of cycles was set to 30,000 with the burn-in set to 1000. The "maxdiff" was set to 0.3, and the minimum effective size was set to 50. Nodal support was estimated as Bayesian posterior probabilities (BPP). PhyloBayes analyses were run on the CIPRES Science Gateway Portal [39]. The final trees were drawn using FigTree v1.4.4.

3. Results and Discussion

Olive lace bug infestations are known to affect the development, health and fruit yield of cultivated olive trees in South Africa, but the diversity of the species present in the region has been incompletely described. A previous study reported the presence of *P. australis* and *N. paliatseasi* in the Western Cape on *Olea europaea*, but definite morphological identification and genetic data were only generated for *N. paliatseasi* at that point [11]. The present work study follows up by confirming the identity of *P. australis* and by reporting the presence of *C. lineata* and one *Neoplerochila* sp. genetically distinct from *N. paliatseasi*. Furthermore, the phylogenetic position of the four species was assessed in the context of mitogenomes publicly available for other Tingidae.

3.1. Morphological Identification of Olive Lace Bug Species

Images of representative specimens of *C. lineata*, *P. australis*, *N. paliatseasi* and *Neoplerochila* sp. analysed in this study are shown in Figure 1. *Cysteochila*, *Neoplerochila*, and *Plerochila* have a similar habitus with slight differences. All of the species analysed here have wide paranota reflexed onto the pronotum. The paranota are adjoined to the protunum in *C. lineata* and *P. australis* but not in the two *Neoplerochila* species. *Cysteochila lineata* has paranota reaching and partly covering the lateral carinae. *Plerochila australis* differs from the other species by the paranota being less developed and not reaching the lateral carinae. The paranota of both *Neoplerochila* species reach the lateral carinae but do not cover them. The costal area of these species is uniseriate, but *C. lineata* and *P. australis* have small

and round areolae, while *N. paliatseasi* and *Neoplerochila* sp. have larger and subquadrate areolae. *Neoplerochila* sp. is very similar to *N. paliatseasi*: the only morphological difference would be the width of the sutural area, making the width of all the hemelytra quite uniform similar to *N. youngai,* and not narrowed opposite to the sutural area as in *N. paliatseasi*. Therefore, *N. paliatseasi* and *Neoplerochila* sp. could not be distinguished unambiguously, and comparative morphometric analyses for a full description of *Neoplerochila* sp. will have to be performed in the future.

Figure 1. Representative adult specimens of olive lace bug (Hemiptera: Tingidae) species found in South Africa. (**A**) *Cysteochila lineata* Duarte Rodrigues (SAM-HEM-A01275), (**B**) *Neoplerochila paliatseasi* Duarte Rodrigues (SAM-HEM-A011647), (**C**) *Neoplerochila* sp. (SAM-HEM-A012753), and (**D**) *Plerochila australis* (Distant) (SAM-HEM-A010383).

3.2. Distribution of C. lineata, P. australis, N. paliatseasi and Neoplerochila sp.

Olive lace bug specimens were collected spanning a period of five years (2015–2020) over the course of other studies, but a survey of olive farms in the Western Cape was only performed during the South African olive-growing season of 2020. In total, 16 sites were visited, and specimens were collected from wild and cultivated ornamental trees in public and private spaces (Figure 2A). Most wild and cultivated trees from which specimens were collected showed typical symptoms of olive lace bug infestation, such as chlorotic spots and dried-out leaf tips (Figure 2C). Olive lace bugs were found in 12 sites out of the 16 sites visited (75%), including five out of the nine olive farms (56%) (Figure 3A). *Plerochila australis* was the most frequently found species (10 sites; 62.5%), *N. paliatseasi* and *C. lineata* were found at four sites (25%), and *Neoplerochila* sp. was only found at one site (Figure 3B). The four species were not found simultaneously at any site, but two sites had three species, two sites had two species, and 50% of the sites had only one species. Formal questionnaires were not performed, but some olive farmers mentioned using insecticides against olive lace bugs, in which case cultivated trees were sprayed twice a year. In cases of heavy infestations, insecticides have been used up to four times a year to significantly reduce populations. As insecticides represent additional economic and environmental costs, it

will be interesting to investigate if olive lace bugs have efficient natural enemies that may contribute to manage infestations. In Australia, *F. olivinia* is reportedly difficult to manage and control, and low toxicity pyrethrum products are commonly used [17].

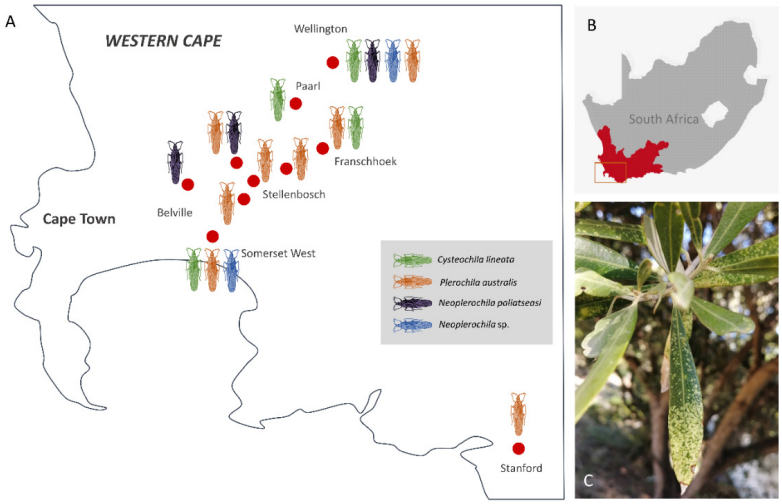

Figure 2. (**A**) Approximate geographic location of broad sampling areas (red dots) of olive lace bugs (*Cysteochila lineata*, *Neoplerochila paliatseasi*, *Neoplerochila* sp. and *Plerochila australis*) in the Western Cape province of South Africa. (**B**) Study area in the Western Cape. (**C**) Characteristic chlorotic spots on the leaves of a cultivated olive tree caused by feeding activity of olive lace bugs.

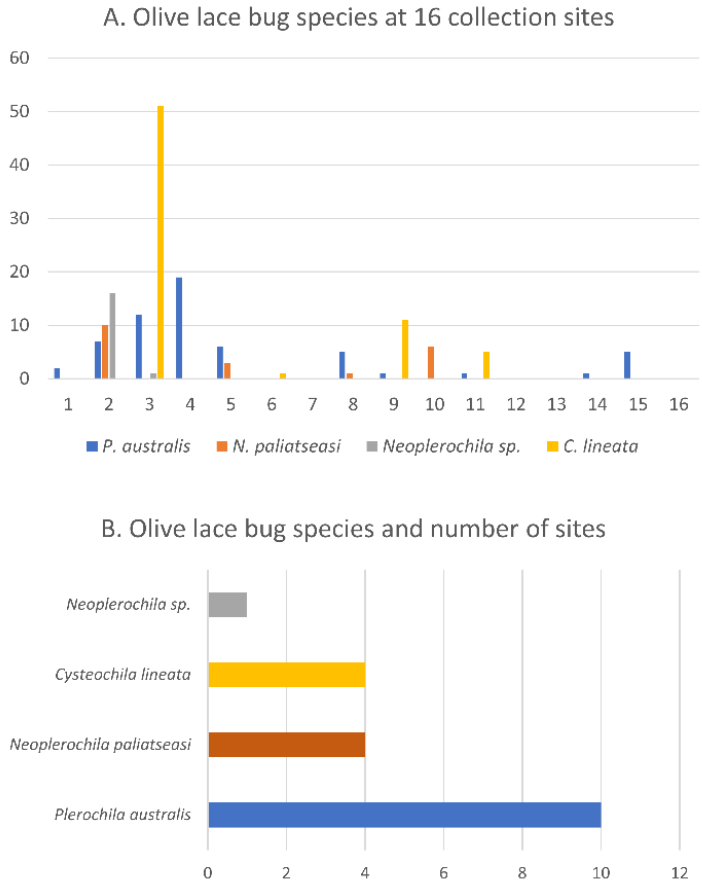

Figure 3. (**A**) Olive lace bug species found at 16 sample collection sites in the Western Cape province of South Africa. (**B**) Olive lace bug species and number of sites where each species was found.

3.3. Haplotype Diversity of C. lineata, P. australis, N. paliatseasi and Neoplerochila sp.

Neoplerochila sp. was the least diverse of the four species as all specimens had the same haplotype. Haplotype diversity is the probability that two randomly selected haplotypes in the sample are different. *Cysteochila lineata*, which was found at four sites, had the highest haplotype diversity (H = 0.963), followed by *Plerochila australis*, the most frequently found species (H = 0.901) (Table 1). The haplotype diversity of *N. paliatseasi* (H = 0.787) was lower than that of *C. lineata* and *P. australis* but can still be considered high. Nucleotide diversity is the probability that two randomly chosen homologous nucleotide sites in the sample are different. The nucleotide diversity of the three species was relatively low, especially the of *N. paliatseasi* ($\pi = 0.005$); therefore, the two *Neoplerochila* were the least diverse species among the four olive lace bugs.

Table 1. Genetic diversity estimates for four species of olive lace bugs (Hemiptera: Tingidae) based on the standard COI barcoding region. k—number of haplotypes; S—number of polymorphic sites.

Species	n	k	S	Haplotype Diversity ± SD	Nucleotide Diversity ± SD
Cysteochila lineata	25	18	26	0.9633 ± 0.0235	0.007644 ± 0.004294
Neoplerochila paliatseasi	17	5	7	0.7868 ± 0.0590	0.005311 ± 0.003243
Neoplerochila sp.	14	1	0	n.a.	n.a.
Plerochila australis	32	14	25	0.9012 ± 0.0324	0.014416 ± 0.007612

None of the species had high-frequency haplotypes or the classic star-like cluster around a central haplotype that is frequently interpreted as a sign of historical population expansion, and the intraspecific haplotype structure broadly reflected the diversity measures (Figure 4). However, the network of *C. lineata* showed several hypothetical haplotypes, and reticulations that were not present in the networks of the other two species. These features often result from the presence of multiple very low-frequency single nucleotide polymorphisms (SNPs) caused by erroneous base-calling due to the presence of double peaks. However, this was not the case as all sequences were of high quality and all SNP positions had single peaks in the electropherograms. It is possible that some of the sequences of *C. lineata* represent NUMTs despite the use of species-specific primers for PCR amplifications (see Section 3.5). If this is the case, the estimates of intraspecific genetic diversity presented here are inflated, but the broad genetic homogeneity and conspecificity of the specimens are not challenged.

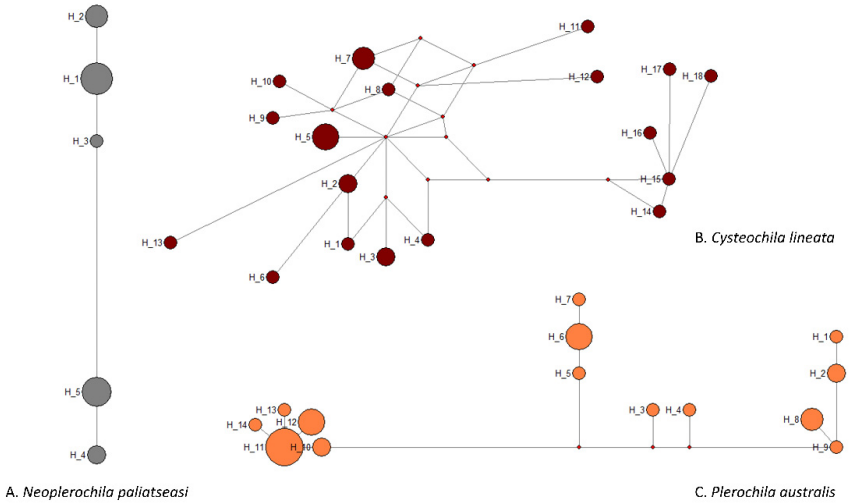

Figure 4. Median-joining network of COI haplotypes of three olive lace bug species (Hemiptera: Tingidae) found in South Africa.

3.4. Genetic Diversity of 30 Species of Tingidae Based on DNA Barcodes

The intra- and interspecific divergence of *C. lineata*, *Neoplerochila* sp. and *P. australis* were assessed in the context of DNA barcodes for other Tingidae retrieved from BOLD Systems. The genetic clustering analyses based on a ML tree showed that all species in the dataset formed monophyletic clusters with high statistical support (Figure 5). Intraspecific genetic divergence was estimated using maximum p-distances (Table S4; Figure 6). Most species (83%) had intraspecific maximum p-distances below 2%, and only four species fell in the range between 2% and 3%, indicating a general trend of consistency in specimen identification. The only evident case of potential misidentification or cryptic diversity was *D. foliacea*, which had an intraspecific maximum p-distance of 9.28% due to the presence of a single highly diverged sequence (BOLD Record GMGMM1352-14) (Figure 5). Based on the results of a search on BOLD Systems using the "Identification" tool implemented on the platform, GMGMM1352-14 was most similar to a sequence deposited as *D. foliacea foliacea* collected in the Netherlands, but this sequence was not publicly available, as is the case for other *Derephysia*. Therefore, it was not possible to infer the monophyly of *Derephysia*. *Plerochila australis* had an intraspecific maximum p-distance of 3.29%, which may indicate ongoing differentiation in this group. The high divergence of *Neoplerochila paliatseasi* and *Neoplerochila* sp. as a single group supported the hypothesis of two distinct species (max p-distance = 7.12%). The choice of thresholds for intra- and interspecific distances is arbitrary, and no fixed value can be universally applied because variation in intraspecific divergence can be due to introgression, incomplete lineage sorting and recent speciation [40]. Nonetheless, estimates of sequence divergence can be useful for inferring patterns of genetic variation that allow for cataloguing of specimens and species into categories and complement morphological and ecological information when observable characters are absent, insufficient or non-informative. In the case of Tingidae, the data analysed here indicate that the range of maximum p-distances between 2% and 3% is a reasonable proxy for inferring conspecificity among sequences. Could this notion be extrapolated to congeneric pairs of species, i.e., are congeneric species of Tingidae consistently less diverged than non-congeneric species? Interspecific p-distances between all species pairs ranged from 5.22% to 28.72% (Table S5). Among congeneric species pairs (*Acalypta*, *Corythucha*, *Gargaphia*, *Neoplerochila*, *Stephanitis* and *Tingis*), these values ranged from 5.21% to 22.83%, and among non-congeneric species pairs, the range was from 13.80% to 28.72%. The least diverged species pairs were indeed congeneric and involved most of the *Corythucha* species pairs, and the pair *N. paliatseasi*/*Neoplerochila* sp. (5.21–12.93%); however, the ranges of genetic divergences between congeneric and non-congeneric species pairs largely overlap, indicating that p-distances are not an adequate proxy for inferring congeners in Tingidae.

3.5. Potential Amplification of NUMTs in Cross-Species PCR

The assessments of genetic diversity and haplotype structure in *P. australis*, *C. lineata*, *N. paliatseasi* and *Neoplerochila* sp. were based on COI sequences obtained using newly designed species-specific PCR primers. However, we first attempted to barcode *C. lineata* using the primers specific to *P. australis* (Ple-F/Ple-R) and *N. paliatseasi* (Neo-F/Neo-R) because the mitogenome of *C. lineata* was the last to be sequenced in the course of this study. These cross-species amplifications of *C. lineata* generated sequences differing by several nucleotides for the same individual and nonspecific PCR products that resulted in low-quality sequences (data not shown). This may have been due to amplifications of nuclear mitochondrial DNA regions (NUMTs). Mitochondrial DNA is undoubtedly useful for assessments of genetic diversity due to its high copy number in the cell, lack of genetic recombination and high evolutionary rate [41], but the presence of NUMTs may confound the analyses. The loss of function of NUMTs results in the accumulation of sequence variation over time, which can be detected in protein-coding genes if amino acid codons are converted into internal stop codons and/or indels that generate frameshift mutations. These degraded versions are considered "older" (paleonumts) than "younger" NUMTs

that are very similar to true mitochondrial DNA [42]. Spurious amplification of NUMTs may lead to overestimation of species diversity and erroneous conclusions in phylogenetic and phylogeographic studies. These problems have been pointed out as a major hindrance to DNA barcoding since the early stages of the development and dissemination of the methodology, which often relies on the use of "universal primers" for generating PCR amplicons across wide ranges of taxa [43]. In our study, the use of species-specific primers eliminated sequence inconsistencies in *C. lineata*, but this solution may not work across all insect taxa. In organisms with large genomes and that are, therefore, more tolerant to the presence of NUMTs, these are likely to be amplified even when species-specific primers are used. For example, a recent study showed that DNA barcoding in Orthoptera is particularly challenging due to the widespread presence of NUMTs, and other mitochondrial markers may have to be employed to infer species diversity correctly [44].

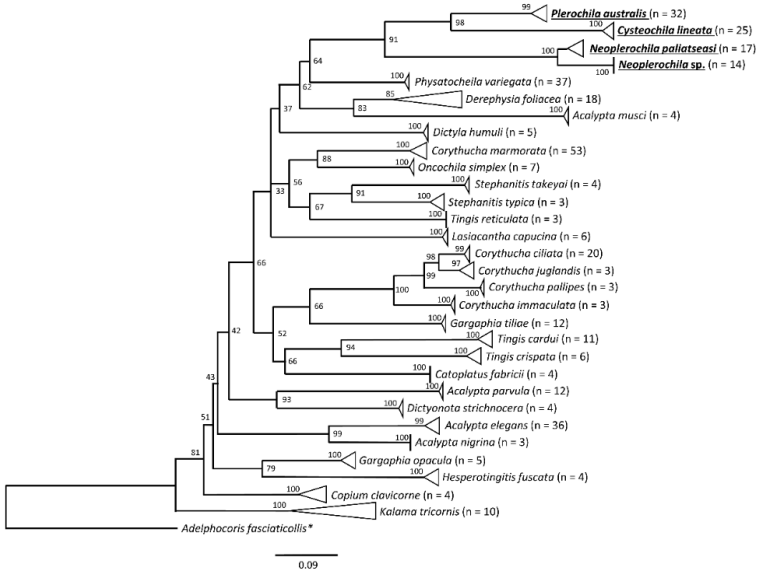

Figure 5. Maximum likelihood tree of lace bug species (Hemiptera: Tingidae) based on a 501-bp alignment of standard COI barcoding sequences. The analyses included 349 sequences representing 30 species in 18 genera retrieved from BOLD Systems and the new sequences of the olive lace bugs *Cysteochila lineata*, *Neoplerochila paliatseasi*, *Neoplerochila* sp., and *Plerochila australis* generated in this study (bold, underlined). Triangles represent collapsed groups of sequences belonging to the same species. * Outgroup (Hemiptera: Miridae).

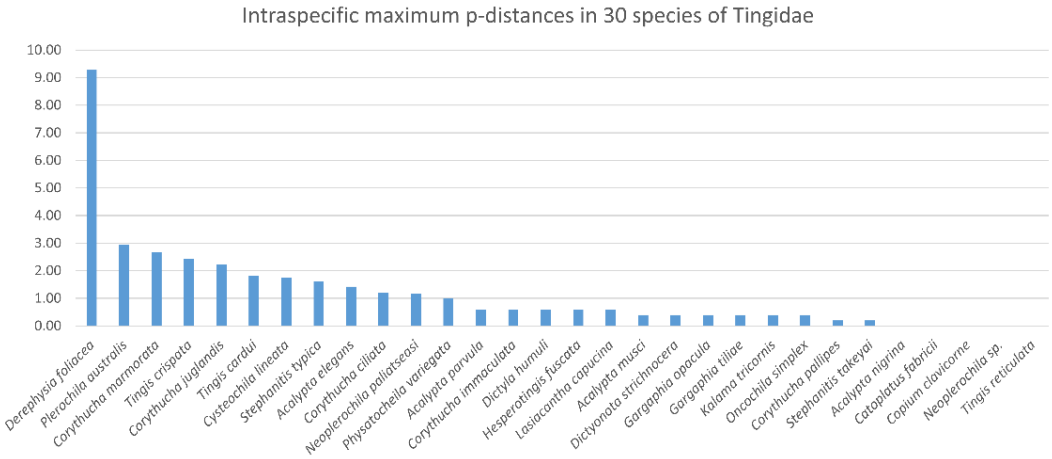

Figure 6. Intraspecific maximum p-distances (%) in 30 species of lace bugs (Hemiptera: Tingidae) based on a 501-bp alignment of the standard COI barcoding region (n = 349).

3.6. Mitogenomics of Olive Lace Bugs

The NGS runs generated 8,298,222 reads with an average length of 176 bp for *C. lineata*, 13,335,929 reads with an average length of 176 bp for *Neoplerochila* sp. and 15,637,818 reads with an average read length of 168 bp for *P. australis*. A total of 80,411 reads from *C. lineata*, 121,545 reads from *Neoplerochila* sp., and 43,401 reads from *P. australis* were mapped to the reference sequence (*N. paliatseasi*, NC_046031). The coverage of the final mitochondrial sequences of *C. lineata* (15,209 bp), *Neoplerochila* sp. (15,339 bp) and *P. australis* (15,208 bp) was 964×, 1384× and 473×, respectively. The quality of mitogenomes is directly associated with read coverage, and low coverage is known to result in sequence gaps and undetermined regions [45]. The read coverage of each final consensus sequence was high and largely exceeded the minimum required for mitogenomic studies (15×) [46]. The average length of the new mitogenomes (15,252 bp) was in line with other Tingidae (15,355 bp), and the three species had the typical set of mitochondrial genes found in Metazoa: 13 protein-coding genes (PCGs), 22 transfer RNA genes (tRNAs), two ribosomal (rRNAs) genes, and a non-coding region presumed to contain the control for replication and transcription (AT-rich region) (Figure 7). Twenty-three genes were located on the majority (J) strand, and 14 genes were located on the minority (N) strand (Table S6). The gene arrangement of the PCGs, tRNAs, rRNA and putative control region (AT-rich region) was conserved in all Tingidae analysed here, and identical to the hypothetical Arthropoda ancestral [47].

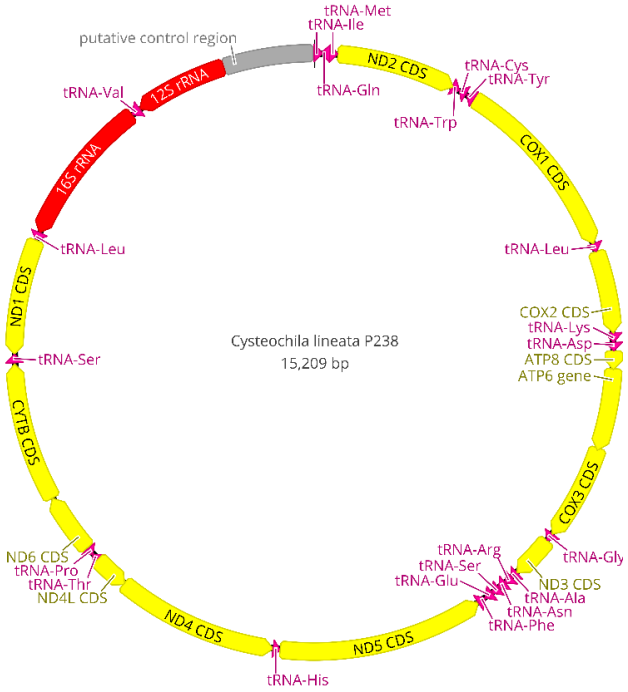

Figure 7. Circular map of the complete mitogenome of *Cysteochila lineata* (Hemiptera: Tingidae). Mitochondrial gene content and arrangement are conserved in Tingidae and identical to the hypothetical Arthropoda ancestor. Arrows indicate the direction of gene transcription.

The set of tRNA genes identified with ARWEN was manually compared with those identified in other Tingidae, and the most probable 22 tRNAs were annotated. All tRNAs had the typical cloverleaf-like structure, except $tRNA^{Ser1}$ (TCT) of *C. lineata* and *P. australis* in that the dihydrouridine (DHU) arm was reduced and replaced with a simple loop, as commonly is the case in Metazoa [48] (Figure 8). In contrast, the DHU arm of $tRNA^{Ser1}$ of *Neoplerochila* sp. and *N. paliatseasi* was complete. A complete DHU arm of $tRNA^{Ser1}$ was also present in the mitogenomes of *Pseudacysta perseae* [49] and *Corythucha ciliata*, the latter of which had a different anticodon (TTC) [50]. The length of the tRNAs ranged

from 63 bp (tRNA^{Ala}) to 76 bp (tRNA^{Thr}) in *C. lineata*, 61 bp (tRNA^{Ala}) to 74 bp (tRNA^{Lys}) in *Neoplerochila* sp., and 63 bp (tRNA^{Cys}) to 75 bp (tRNA^{Lys}) in *P. australis*. The location and average size of 16S rRNA (1228 bp; between tRNA^{Leu1} and tRNA^{Val}) and 12S rRNA (771 bp; between tRNA^{Val} and the AT-rich region) in the four species were in line with the average size of the two genes in other Tingidae (1230 bp and 784 bp, respectively).

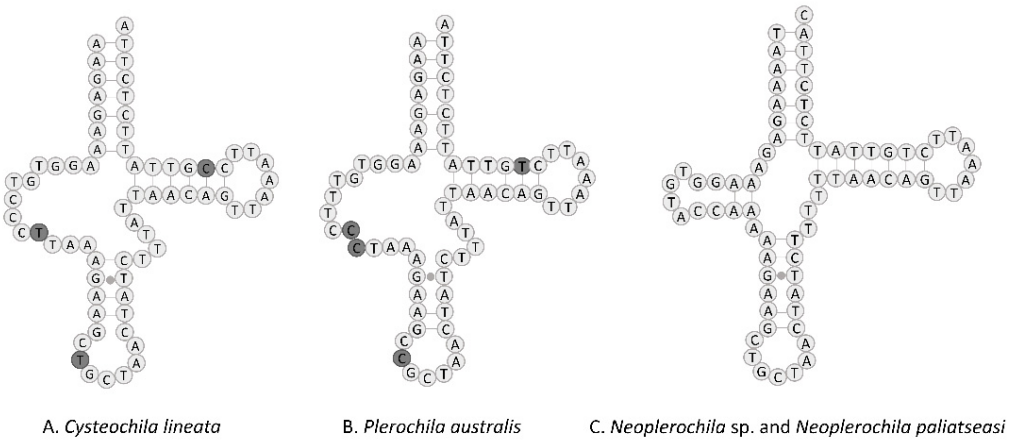

Figure 8. Predicted structure of tRNA^{Ser1} in the complete mitochondrial genomes of four olive lace bugs (Hemiptera: Tingidae) found in South Africa, with nucleotide differences highlighted. Inferred canonical Watson–Crick bonds are represented by lines, and non-canonical bonds are represented by dots.

The large non-coding (AT-rich) region believed to contain the control for replication and transcription was located between 12S rRNA and the I-Q-M tRNA cluster, as in other Tingidae. The sizes of the AT-rich region were similar in the four olive lace bugs and ranged from 733 bp in *C. lineata* to 854 bp in *N. paliatseasi.* In other Tingidae, the AT-rich region ranged from 287 bp in *Tingis cardui* to 2215 bp in *Stephanitis chinensis*. Tandem repeats in the control region are common in animals, most likely as a result of slipped-strand mispairing during DNA replication [51]. *Neoplerochila* sp. had two repeats of a 170-bp motif at the 3′-end of the AT-rich region, which were separated by 4 bp and represented 40% of the region, *P. australis* had two repeats of a 166-bp motif separated by 6 bp representing 45% of the region, but no tandem repeats were identified in *C. lineata.* Tandem repeats in the 3′-end of the control region have also been identified in *P. perseae* (six repeats of 36 bp), *C. ciliata* (two repeats of 189 bp), and *N. paliatseasi* (two repeats of 156 bp) [11,49].

All PCGs in Tingidae started with a canonical ATN except for ND5 in *Corythucha marmorata* (GTG), and the most frequently used start codon was ATG (Figure 9). PCGs in *C. lineata*, *Neoplerochila* sp., *N. paliatseasi* and *P. australis* initiated with ATG in ATP6, COIII, COI, CYTB, ND1, ND4, and ND5; ATT in COII, ND2 and ND6; and ATA in ND4L and ND3, except for ND3 in *C. lineata* (GTG). The alternative start codon GTG has been previously found across a range of insect taxa including some species of Diptera [52], Mecoptera [53], Plecoptera [54], and Hemiptera such as *Sogatella furcifera* (Delphacidae) [55] and *Triatoma dimidiata* (Reduviidae) [56]. Most PCGs in the four olive lace bugs terminated with TAA, except ND4 in *Neoplerochila* sp., ATP6 and ND4 in *P. australis*, and CYTB in *N. paliatseasi*, which terminated with TAG. Incomplete stop codons (TA and T) were present in *C. lineata* (ND5, COII and ND2), *Neoplerochila* sp. (CYTB and ND5), *N. paliatseasi* (COII, ND3 and ND5) and *P. australis* (COII and ND5). Incomplete stop codons are common in animal mitochondrial genes and are presumed to be completed by posttranscriptional polyadenylation [57].

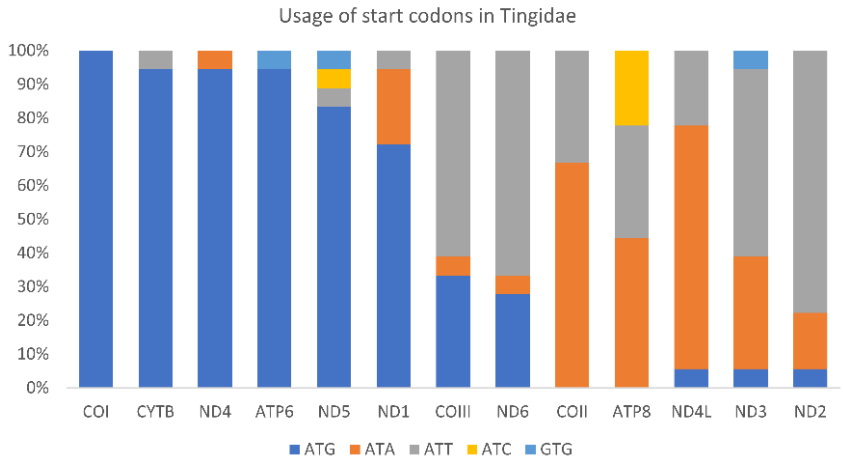

Figure 9. Usage of start codons in the complete set of 13 mitochondrial protein-coding genes in 18 species in the family Tingidae (Hemiptera).

The mitogenomes of *C. lineata*, *Neoplerochila sp.* and *P. australis* were highly compact, with an average of 32 bp of intergenic nucleotides at 11 locations. The longest intergenic spacers were located between $tRNA^{Thr}$ and COI in *C. lineata* (9 bp) and *Neoplerochila* sp. (12 bp) and between $tRNA^{Gln}$ and tRNAMet in *P. australis* (10 bp), in line with *N. paliatseasi* and other Tingidae where the total number of intergenic nucleotides ranged from 1 to 48 bp. Olive lace bugs had an average of 19 gene overlaps, mostly involving tRNAs. The longest overlap (19 bp) was in *C. lineata* between ND4L and $tRNA^{Thr}$, followed by 14 bp between COIII and ATP6 in *Neoplerochila* sp., 14 bp between ATP6 and COIII in *N. paliatseasi*, and 14 bp between $tRNA^{Gln}$ and $tRNA^{Met}$ in *P. australis*. The total number of gene overlaps varied in other Tingidae and was lower than the average for the four olive lace bugs, ranging from 8 bp in *P. perseae* to 17 bp in *C. ciliata*.

Olive lace bugs had the high A+T content typically found in insect mitogenomes, with an average of 75.1% for the total sequences. The A+T content of the AT-rich regions of the three new mitogenomes (*C. lineata*, 78.2%; *Neoplerochila* sp., 76.9%; *P. australis*, 75.2%) was higher than that of their complete sequences, which was also the case of *N. paliatseasi* and all other Tingidae except *C. ciliata* [49]. Olive lace bugs also had a similar A+T content for the combined tRNAs (average = 77.8%), and combined rRNAs (average = 79.2%). The A+T content of the total PCGs in olive lace bugs varied from 65.8% in *C. lineata* to 81.3% in *Neoplerochila* sp. and *N. paliatseasi*. The A+T content of individual PCGs was lowest in COI in all three species (66.4%), and highest in ATP8 (*C. lineata*, 79.5%) and ND4L (*P. australis*, 80.3%; *Neoplerochila* sp., 81.3%) (Table S7).

The mitochondrial GC content varies among species and is influenced by mutation bias, selection and DNA repair bias on the complementary DNA strand [58]. According to the second parity rule, bases in the complementary DNA strand exist at equal frequencies when there are no mutations or selection bias [59]. The presence of AT and GC skews on the same DNA strand may indicate that the species underwent mutations or environmental selection [58]. *Cysteochila lineata*, *Neoplerochila* sp. and *P. australis* had positive AT skews and negative GC skews in most genes and AT-rich regions, except COI and CYTB. ND6 also did not have a positive AT skew and a negative GC skew in *Neoplerochila* sp. and *P. australis*. In the four olive lace bugs, four of 13 PCGs on the N strand had higher AT skews than PCGs on the J strand. The nucleotide bias towards A and T was reflected in codon usage, with AT-rich codons (UUU, UUA, AUU, AUA, UAU, AAU and AAA) representing an average of 42.3% of all codons. Relative synonymous codon usage (RSCU) for each codon is calculated as the relative frequency of a codon within a mitogenome. An RSCU value higher than 1.0 indicates an over-represented codon, whereas an RSCU value lower than 1.0 indicates an under-represented codon [60]. RSCU was higher than 1.0 in all synonymous codons, indicating that AT-rich codons are favoured (Table S8).

The ratio of non-synonymous to synonymous nucleotide substitutions (Ka/Ks) is generally used as an indicator of selective pressure on protein-coding sequences among different species. A Ka/Ks ratio greater than 1 indicates positive selection, which is assumed to have occurred during the evolution of the sequence. Average Ka/Ks were calculated for individual PCGs across the 18 Tingidae species included in this study (Figure 10). All genes had Ka/Ks < 1, which indicates purifying or stabilizing selection, of which ATP8 had the highest Ka/Ks (0.65), and ND1, COI, COII, COIII and CYTB had the lowest Ka/Ks (0.20).

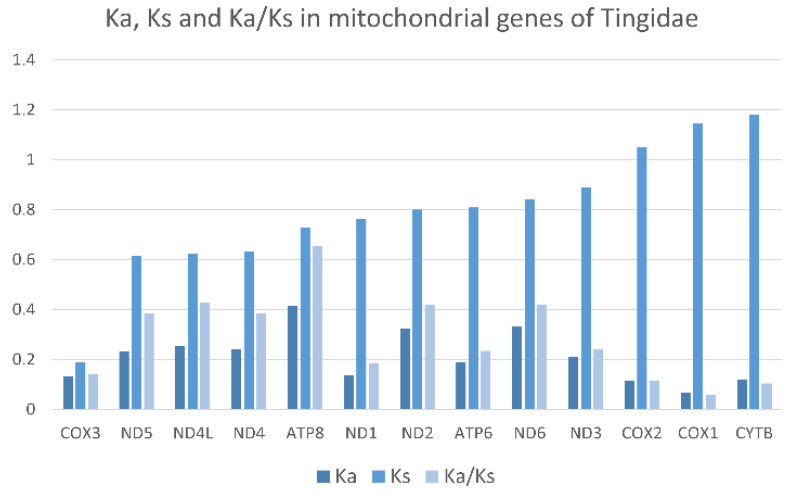

Figure 10. Evolutionary rates in 13 mitochondrial protein-coding genes of 18 species of Tingidae (Hemiptera). Ka—number of nonsynonymous substitutions. Ks—number of synonymous substitutions. Ka/Ks—ratio of the number of nonsynonymous to the number of synonymous substitutions.

3.7. Phylogenetic Position of Olive Lace Bugs within Tingidae

The mitochondrial phylogeny of Tingidae has been recovered inconsistently across different studies, and this instability may be due to atypical sequence heterogeneity and high levels of mutation rates in the family and differences in phylogenetic methodological approaches [49,50,61–64]. Furthermore, patterns of non-monophyly in mitochondrial phylogenies can result from hybridization and introgression events, and incomplete lineage sorting during speciation, which are more likely to occur among recently diverged species than in older lineages species [65]. The phylogenetic positions of *C. lineata*, *N. paliatseasi*, *Neoplerochila* sp. and *P. australis* were recovered using the novel sequences and all Tingidae mitogenomes available on GenBank at the time of this study, except *Eteoneus sigillatus* (KU896784; unverified sequence). Phylogenetic analyses were restricted to PCGs because these have the advantage of being translatable and do not generally contain many length-variable regions at the genera and species level, and the third codon position is mostly neutral and not constrained by selection [66].

The PCG123 and PCG12 trees recovered the same topology with three unresolved nodes, but PCG12 had slighter higher support for some nodes (Figure 11A). The AA tree had only one unresolved node and high statistical support for most nodes (Figure 11B). All trees recovered *Phatnoma laciniatum* (Phatnomini) as basal to Tingini, and the same main clades but a different order of deeper nodes. The genera *Stephanitis* and *Corythucha*, which are represented by more than one species, formed monophyletic clades in agreement with previous phylogenies [11,62]. *Ammianus toi* and *Perissonemia borneenis* were also recovered as sister taxa in agreement with previous reconstructions [11]. The genus *Cysteochila* is represented by two species; however, these did not form a monophyletic clade as *C. lineata* was recovered as a sister species to *P. australis* but *C. chiniana* was placed in a different cluster with *T. jacobsoni* and *T. cardui*. Nevertheless, the four olive lace bugs were placed in the same phylogenetic cluster with high support in both trees. *Cysteochila* is a large genus holding around 136 species closely related to *Plerochila* in which species of *Cysteochila* have been transferred (*C. horvathi* Schouteden, and

C. tzitikamana Drake). In fact, these genera need to be revised, as the morphological characters used to distinguish species show very small differences and many are homoplastic (see [1]). This is also the case for *Neoplerochila*, in which species of *Physatocheila* were transferred (*N. katbergana* Drake and *N. weenenana* Drake).

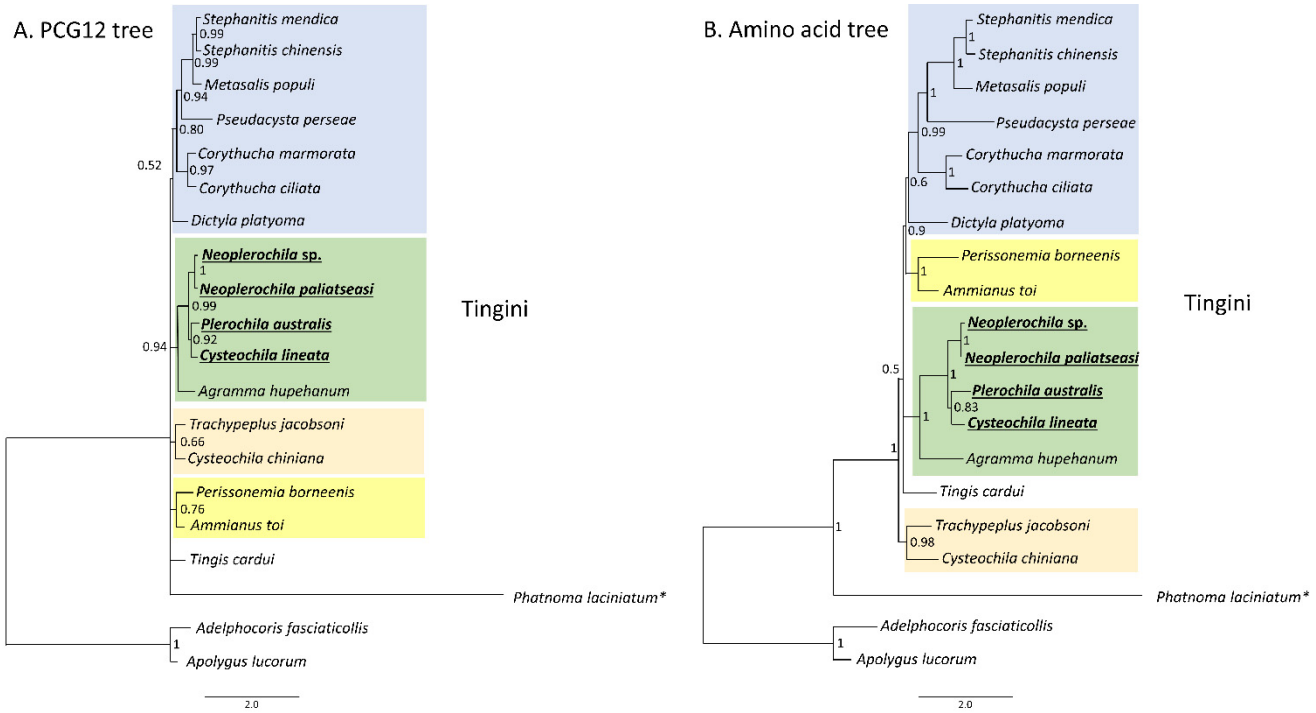

Figure 11. Phylogenetic relationships among 18 lace bug species (Hemiptera: Tingidae) based on 13 mitochondrial protein-coding genes. (**A**) PCG12-only first and second codon positions. (**B**) Amino acid tree. *Adelphocoris fasciaticollis* and *Apolygus lucorum* (Hemiptera: Miridae) were used as outgroups. Nodal statistical support is given as Bayesian posterior probability. * Phatnomatini.

Our results show that *C. lineata*, *P. australis*, *N. paliatseasi* and *Neoplerochila* sp. share a mitochondrial ancestor and suggest that feeding on *O. europaea* may have a common evolutionary origin in lace bugs. To test this hypothesis, it will be necessary to confirm which other lace bug species feed on *O. europaea* and to determine their phylogenetic position within a wider range of Tingidae. Candidate species could include species that have been found on *Olea* such as *Neoplerochila millari*; *N. dispar*; *N. weenenana*; *P. horvathi*; *Cysteochila impressa* Horvath; *Physatocheila namibiana* Duarte Rodrigues; and the Australian *F. olivinia*, a known pest of cultivated olives.

4. Conclusions

Cultivated olive trees were introduced to the Western Cape province of South Africa less than 100 years ago, but African wild olives are widely distributed in this and other regions of South Africa. Previous works have shown that the entomofauna affecting *O. europaea* in sub-Saharan Africa most likely co-evolved with African wild olives. We confirm the presence of four species of olive lace bugs in South Africa (*C. lineata*, *P. australis*, *Neoplerochila* sp. and *N. paliatseasi*), of which *P. australis* was the most frequent. The four olive lace bugs have a close phylogenetic relationship among Tingidae, in agreement with their utilization of *O. europaea*. As relatively few olive lace bug species are adapted to *O. europaea*, it will be interesting to gather further evidence for a common origin of this feeding habit.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/insects12090830/s1, Table S1: Sample list, Table S2: PCR primers, Table S3: Mitogenomes in phylogeny, Table S4: Intraspecific p-distances, Table S5: Interspecific p-distances, Table S6: Mitogenomic features, Table S7: Nucleotide composition, Table S8: Codon usage.

Author Contributions: Conceptualization, E.A. and B.v.A.; formal analysis, V.H., É.G., S.J.S. and J.L.; investigation, V.H., É.G., S.v.N. and J.L.; resources, E.A. and B.v.A.; writing—original draft preparation, V.H.; writing—review and editing, É.G., S.v.N., E.A., J.L. and B.v.A.; supervision, B.v.A.; funding acquisition, E.A. and B.v.A. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The DNA sequences generated in this study were deposited in GenBank under the following accession numbers: *C. lineata* (MZ673445 to MZ673468), *N. paliatseasi* (MZ666853 to MZ666863), *Neoplerochila* sp. (MZ673417 to MZ673429) and *P. australis* (MZ676957 to MZ676987).

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

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The Interaction between *Tribolium castaneum* and Mycotoxigenic *Aspergillus flavus* in Maize Flour

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Simple Summary: It is important to hold cereals in storage conditions that exclude insect pests such as the red flour beetle and fungi, especially mycotoxin-producing ones (as a few strains of *Aspergillus flavus*). This work aims to investigate the interaction between these two organisms when thriving in maize flour. It was observed that when both organisms were together, the mycotoxins detected in maize flour were far higher than when the fungi were on their own, suggesting that the presence of insects may contribute positively to fungi development and mycotoxin production. The insects in contact with the fungi were almost all dead at the end of the trials, suggesting a negative effect of the fungi growth on the insects. Both organisms interacted when in contact. This is the first study on this issue, although further investigation would benefit from clarification on the mechanisms leading to the nature of the detected interactions.

Abstract: *Tribolium castaneum* is one of the most common insect pests of stored products. Its presence makes cereals more susceptible to the spread of the fungi *Aspergillus flavus*, which may produce mycotoxins. The aim of this work was to evaluate the influence of *T. castaneum* adults on the development of a mycotoxigenic *A. flavus* strain in maize flour as well as the influence of this fungus on the insects. Maize flour was exposed to *T. castaneum*, spores of *A. flavus* or to both. The results revealed an interaction between *T. castaneum* and *A. flavus* as the flour exposed to both organisms was totally colonized by the fungus whereas almost all the insects were killed. Aflatoxin B1 (AFB1) revealed a significantly higher concentration in the flour inoculated with both organisms (18.8 µg/kg), being lower when exposed only to *A. flavus*, suggesting that the presence of insects may trigger fungal development and enhance mycotoxin production. The ability of these organisms to thrive under the same conditions and the chemical compounds they release makes the interaction between them a subject of great importance to maintain the safety of stored maize. This is the first work evaluating the interaction between *T. castaneum* and *A. flavus* mycotoxin production.

Keywords: *Aspergillus flavus*; aflatoxin B1; maize flour; *Tribolium castaneum*; food safety

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1. Introduction

Stored products are greatly affected by both abiotic factors such as temperature and humidity and biotic factors such as insects and fungi, which may contribute to their degradation and a loss of quality and quantity. Among the biotic factors, fungi and insects are major threats and both types of organisms may either cooperate or compete to colonize the stored products, as a few authors have postulated (e.g., [1]). The presence of insects on stored food products may lead to food contamination through its body parts, excretions

and secondary metabolites among others, which may be allergenic or even carcinogenic and may also lead to changes in the storage microenvironmental conditions, contributing to the spread of fungi and other microorganisms [2,3].

Several strains of the fungus *Aspergillus flavus* Link are mycotoxigenic and produce aflatoxins (AFs) that can contaminate foodstuff and become harmful to animals and humans mainly through ingestion [4–8]. The main aflatoxins produced by this fungus are aflatoxins belonging to the B type (B1 and B2) although a few authors have also reported the production of aflatoxins belonging to the G type (G1 and G2 [5 and references therein]). As AFB1 is carcinogenic to humans (Group 1, IARC), special attention should be dedicated to its presence in stored products that are used for human consumption in order to protect human health. The extent of fungal growth and aflatoxin production in cereal commodities depend on several biotic and abiotic factors, not only in the field but also during storage, with temperature and water availability playing key roles in these processes [6,9].

Tribolium castaneum (Herbst) is considered to be one of the most important key pests of stored milled grain [10]. It is also a model organism for insect development that has its genome completely sequenced [11]. The adults of this species secrete a mixture of compounds composed mainly of 1,4-benzoquinone, methyl-1,4-benzoquinone and ethyl-1,4-benzoquinone [12–14]. These cuticular secretions play a defensive role towards predators and microbes and a putative regulatory effect on their own population growth [15–17]. This insect has shown resistance to most classes of insecticides, an observation that may be partially attributed to its capacity to produce detoxification enzymes that are encoded by insecticide resistance genes such as cytochrome P450 proteins [13,18–24].

The interaction between insects and mycotoxigenic fungi may trigger fungi growth and mycotoxin production. Insects have been reported to be putative vectors of mycotoxigenic fungi in stored product conditions [25–27]. A few authors have focused mainly on the mechanical damage caused by this insect to grains and its role as a carrier of fungal spores, both contributing actively to fungal dispersion. The insects may also produce metabolic heat that is then transformed into metabolic water, thus augmenting water availability, an essential feature for mycotoxigenic fungal development [6,28–30]. However, it has also been shown that mycotoxins may confer an increased fungal fitness by deterring competitors or mycetophagousness [1].

Future climate change scenarios, predicting increases in temperature in several areas, indicate that food safety issues may be raised regarding not only mycotoxigenic fungi such as *A. flavus*, which are well adapted to warm weather conditions and when exposed to higher temperatures (especially combined with other abiotic factors such as water availability and carbon dioxide levels) may stimulate mycotoxin production [31–33], but also stored grain insect pests such as *T. castaneum*, who may increase their feeding rate or reproduction rate, which is concerning as they are vectors of mycotoxigenic fungi [34]. In addition, *T. castaneum* has also demonstrated adaptive thermal plasticity [35].

This work aimed to evaluate the possible influence of the insect presence in maize flour on the production of aflatoxins by a mycotoxigenic *A. flavus* strain as a contribution to a better understanding of this complex interaction and to the food safety of stored products.

2. Materials and Methods

2.1. Maize Zea mays L. (Poales, Poaceae) Flour Preparation

Maize collected directly from fields was stored at −4 °C and then ground and sieved to obtain maize flour. The initial maize flour moisture content and water activity were determined using adequate equipment (moisture measurement scale PMB202 ADAM (Adam Equipment, Milton Keynes, UK) and Hygrolab C1 (Rotronic, Bassersdorf, Switzerland)). The average values of the moisture content (%) and water activity (A_w) were estimated for different interaction assays and included three replicates for each determination (n = 3).

2.2. Tribolium castaneum Mass Rearing

The red flour beetle, *Tribolium castaneum* (Herbst) (Coleoptera, Tenebrionidae), was obtained from natural populations with less than five years of rearing at the Entomology Laboratory of the Departamento de Ciências e Engenharia de Biossistemas (DCEB) of the Instituto Superior de Agronomia (ISA), University of Lisbon. The cultures were maintained at 26 °C and 65 to 70% relative humidity (RH) in a mixture of wheat flour and baker's yeast (*Saccharomyces cerevisiae* Hansen) in a 95:5 w/w proportion, according to [36]. Mass rearing was performed as previously described [37]. The insects were maintained in a climatic chamber (Fitoclima S600, ClimaPlus 400 (ARALAB, Sintra, Portugal)), at 30 °C ± 2 °C and 70% ± 5% RH. The adults used were eight days old.

2.3. Aspergillus flavus Suspension

A mycotoxigenic strain of *Aspergillus flavus* Link (Eurotiales, Trichocomaceae) obtained from the Minho University Mycotheca (MUM-UMinho) was selected and maintained at 4 °C in the collection of the Laboratory of Mycology of DCEB, ISA, University of Lisbon.

Suspensions of conidia were prepared from *A. flavus*-containing potato dextrose agar (PDA) plates grown for eight days by rubbing the sporulating surface with a bent needle. After filtering through a 60 µm mesh sieve to remove debris, sterile distilled water was added to the suspension to reach a concentration of 10^7 conidia/mL [38,39], based upon cell counts using a hemacytometer.

2.4. Insect/Fungi Interaction Assays

For the interaction assays, 40 g of maize flour was placed in each one of 40 glass flasks (250 mL, Depósito da Marinha Grande, Lisbon, Portugal) and autoclaved at 121 °C for 15 min to eliminate potential fungal and insect contaminations. The experimental procedure for the interaction study included different types of assays: (1) control, with maize flour only; (2) insect, with maize flour inoculated with 80 *T. castaneum* adults; (3) fungus, with maize flour inoculated with 0.5 mL of the fungal conidia suspension (see Sections 2 and 3) and (4) mixed (insects and fungi) assay, with both 80 insect adults and 0.5 mL of the fungal conidia suspension. The flasks were sealed with a plastic lid. Ten replicates were set for each type of assay (n = 10).

The interaction assays were maintained in a climatic chamber at 30 °C ± 2 °C and 70% ± 5% RH for eight weeks. After this period, the living insects were counted and the level of fungal development was evaluated by a direct observation. The maize flour samples were analyzed at the laboratories of the Food and Nutrition Department, National Health Institute Dr. Ricardo Jorge (INSA), Lisbon, Portugal, for the presence of aflatoxins according to the method described in EN15851 [40] with a few modifications [41]. Two replicates of 10 g each collected from different flasks within the same type of assay were analyzed for the aflatoxin content (n = 2). For the mixed assay (insects and fungi), a third sample was added due to the heterogeneous aspect of the maize flour.

2.5. Statistical Analyses

The aflatoxin content of the different types of assays (control, insect, fungus and mixed assay) was compared for detecting significant differences ($p < 0.05$) among them. Assumptions were tested with the Bartlett test for homoscedasticity and the Shapiro–Wilk test for the normal distribution of residues. After this, the data obtained were submitted to an analysis of variance (ANOVA). If the result was considered to be significant ($p < 0.05$), Tukey's honest significant difference test (HSD) was performed. These analyses were completed with RStudio [42] and R-3.1.2.

3. Results

The maize flour was incubated under appropriate conditions of 30 °C ± 2 °C and 70% ± 5% RH for eight weeks on its own (control), inoculated with *A. flavus* conidia (fungus assay), inoculated with *T. castaneum* adults (insect assay) or inoculated with both

organisms (mixed assay). The mixed assay increased the mortality of the adult insects. The insect assay reared only on maize flour achieved a mortality rate of 50.0% ± 52.7% regarding the initial number of adults; in these assays, other developmental life stages were also detected and counted when present with an average number (± standard deviation) of larvae (41.4 ± 45.7), pupae (2.8 ± 4.9) and adults (123.8 ± 140.2). The mixed assay (with insects and fungi) attained a mortality rate of 99.9% ± 0.4%; it was not possible to quantify further developmental stages although it was observed that the insect adults were dead before the next generation could achieve the adult stage.

The fungal assay showed a clearly visible fungal growth. However, a more intense fungal growth was observed in the mixed assay with the development of caking not observed in the other assays (Figure 1).

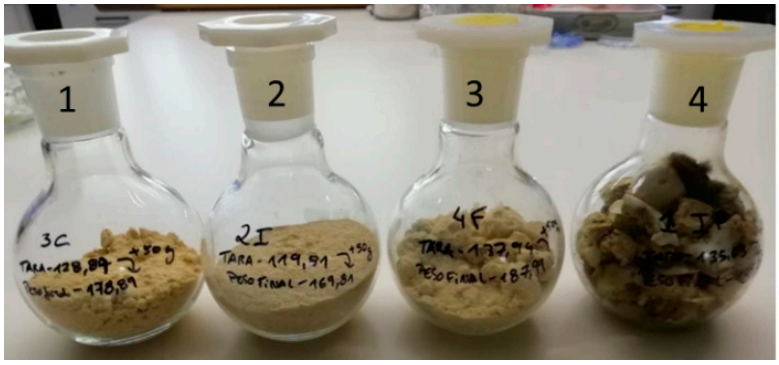

Figure 1. The final aspect of the interaction assays using maize flour: 1—control (containing only maize flour), 2—insect (maize flour and *Tribolium castaneum*), 3—fungus (maize flour and *Aspergillus flavus*) and 4—mixed (maize flour, *T. castaneum* and *A. flavus*).

The assays conducted with insects or with fungi scored higher moisture contents and water availabilities compared with the controls although, visually, it was possible to verify that the mixed assay seemed to have an even higher moisture content and water availability (Table 1). Unfortunately, no data were available on these parameters under this study due to the development of caking during the mixed assay.

Table 1. Average initial and final moisture content (%) and water activity (A_w) values of different interaction assays (n = 3) at room temperature at the moment of A_w measurement (°C): control (containing only maize flour), insects (maize flour and *Tribolium castaneum*), fungi (maize flour and *Aspergillus flavus*).

Assays	Moisture Content (%)		Water Activity (A_w) and Temperature (°C)			
	Initial	Final	Initial A_w	Temp.	Final A_w	Temp.
Control		9.13 ± 0.30			0.48 ± 0.00	22.9 ± 0.06
Insects	8.27 ± 1.00	15.30 ± 0.42	0.60 ± 0.01	23.03 ± 0.15	0.69 ± 0.08	22.7 ± 0.56
Fungi		21.80 ± 0.56			0.83 ± 0.03	22.8 ± 0.38

Regarding the mycotoxin analyses, the results obtained revealed the presence of aflatoxins, mainly AFB1, in the fungal (4.3 µg/kg) and mixed assay (18.8 µg/kg) followed by AFB2 although in lower concentrations in both the fungal (0.3 µg/kg) and mixed assay (0.8 µg/kg). The content of AFB1 was significantly different among the different types of assays ($F = 183.1$; $p < 0.001$) with the mixed assay showing a significantly higher content of aflatoxins (AFB1) than the fungal assay ($p < 0.001$) as well as the control and insect assays ($p < 0.001$ for both); the fungal assay was also considered significantly different from the control and insect assays ($p = 0.037$ for both) (Table 2). The AFB2 content was lower than AFB1 and no significant differences were found among the different types of assays ($F = 2.8$; $p = 0.144$).

Table 2. The average contents (μg/kg) of aflatoxins (aflatoxin B1—AFB1; aflatoxin B2—AFB2; aflatoxin G1—AFG1; aflatoxin G2—AFG2) in the interaction assays: control (containing only maize flour, $n = 2$), insects (maize flour and *Tribolium castaneum*, $n = 2$), fungi (maize flour and *Aspergillus flavus*, $n = 2$) and insects and fungi (maize flour, *T. castaneum* and *A. flavus*, $n = 3$). Limits of detection (LOD) and quantification (LOQ) (μg/kg) are indicated for each aflatoxin analyzed. Different letters following the values of AFB1 and AFB2 in the same column indicate significantly different ($p < 0.05$) values.

Assays	AFB_1	AFB_2	AFG_1	AFG_2
Control	0.018 ± 0.006 a	<0.004 a	<0.007	<0.004
Insects	<0.011 a	<0.004 a	<0.007	<0.004
Fungi	4.306 ± 1.698 b	0.344 ± 0.139 a	<0.007	<0.004
Insects + Fungi	18.883 ± 14.160 c	0.838 ± 0.584 a	<0.007	<0.004
Limit of Detection, LOD (μg/kg)	0.011	0.004	0.007	0.004
Limit of Quantification, LOQ (μg/kg)	0.038	0.013	0.023	0.014
Maximum AFB_1 Content for Maize in EU *	5.000			
Maximum Sum of Aflatoxins (B_1, B_2, G_1, $G_{2)}$ Content for Maize in EU *	10.000			

* Regulation (EC) 1881/2006 [43].

Aflatoxin B1 (AFB1) revealed the highest concentration under the conditions of the mixed assay, exceeding the maximum legal level established for maize to be used as an ingredient in foodstuff (5 μg/kg [43]). AFB2 was also present in the mixed assay but in lower concentrations than AFB1 (0.8 μg/kg). The sum of the aflatoxin (AFB1, AFB2, AFG1, AFG2) levels (19.6 μg/kg) obtained for the mixed assay also exceeded the maximum legal values allowed for total aflatoxins in foodstuff (10 μg/kg [43]).

4. Discussion

The results revealed a possible interspecific competition between the red flour beetle and the mycotoxigenic fungus *A. flavus* in maize flour. The insect mortality in the interaction assays (the presence of both insect adults and fungus in the cereal flour) denoted a possible negative effect of *A. flavus* on the adults of *T. castaneum* as very high insect mortality was scored only when both organisms were present.

The fact that climate change is occurring and that aflatoxins are considered a potential health threat for cereals in Europe due to temperature and humidity increases [33,44–46], thus allowing optimal conditions for *A. flavus* growth, highlights the importance of assessing the potential health risks ahead for consumers and economic losses related to stored products. The increase in insect activity and fungi vectoring, together with an augmented mycotoxigenic production when both organisms are exposed to the higher temperatures recognized in future climate change scenarios, will also cause the spread of this problem to new geographical areas [33,34,47].

Aflatoxin B1, the most abundant aflatoxin detected in this study, has been shown to have a negative influence on the development and fecundity of *Ahasverus advena* Waltl, a mycetophagous insect that may also attack the food products where fungi are growing, although this insect has more tolerance to aflatoxins [48]. The significantly higher levels of aflatoxin B1 and B2 quantified in the mixed assay compared with the fungal assay may also indicate that the insects were possibly contributing to fungal favorable environmental conditions, something that has already been stated by other authors, either by contributing to the fungal dispersion or by altering the environmental conditions in terms of temperature and water availability [6,29,30,49]. For example, maize weevils seem to have an important role in the production of aflatoxins by *A. flavus* [25,27].

It is important to stress that *T. castaneum* excretes defensive secretions, benzoquinones, which compete with other organisms; namely, fungi. *Tribolium castaneum* reaches its maximum benzoquinone excretion value about 40 days after the emergence of adults [13]. Under our assay conditions, the insect adults reached 40 days of age. It therefore seems reasonable to believe that the presence of insects and their benzoquinones might have stimulated the response by *A. flavus* to produce mycotoxins (and/or other secondary de-

fensive metabolites). However, this would need further investigation efforts as mycotoxin production is affected by several abiotic and biotic factors [1,9]. The marked negative effect of fungi on the insects corroborated the competitive nature of their relationship [1] within the conditions of the present study assays. An arms race between these two groups of organisms (insects and fungi) was hypothesized as the fungi and insects are competitors and probably their secondary metabolites are linked to the control of their competing "partners". Based on this result, we considered that there was a competitive exclusion principle [50] when the insect was in the presence of this fungus. Further studies need to be performed to improve our understanding of the potential interaction between insects and fungi and to evaluate the presence of aflatoxins and other mycotoxins in stored products containing insects and fungi as well as their impact on the food safety of stored foodstuff.

It is important to mention that *T. castaneum* is resistant to the entomopathogenic fungi *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin, reducing fungal germination and growth due to benzoquinone-defensive secretions [16,51–53]. Several fungi may also express benzoquinone reductases when in contact with the benzoquinone secretions of tenebrionid beetles [16]. Mycotoxins may also have harmful effects on insects [48] although all these effects should be carefully evaluated in what concerns the concentration of the metabolites (mycotoxins and benzoquinones). In addition, the conditions in terms of abiotic factors such as temperature and water availability as well as food availability should play key roles in defining the type of interactions established in each case between these organisms. Nevertheless, *T. castaneum* has an efficient detoxification system and produces benzoquinones, which are indicated to be one of the reasons for this insect's resistance to entomopathogenic fungi [16–24]. The interaction between this insect and mycotoxigenic fungi is still an intriguing field of research, which may have important outcomes regarding innovative control methods of stored products associating insects and fungi. Elucidating the possible tolerance or resistance mechanism of *T. castaneum* to aflatoxins could be an important contribution to the field of novel mycotoxin control methods in the food industry, which may include the use of enzymes that promote the enzymatic degradation of mycotoxins, and new enzymes are needed [54]. This was the first work evaluating the interaction between *T. castaneum* and *A. flavus* mycotoxin production.

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