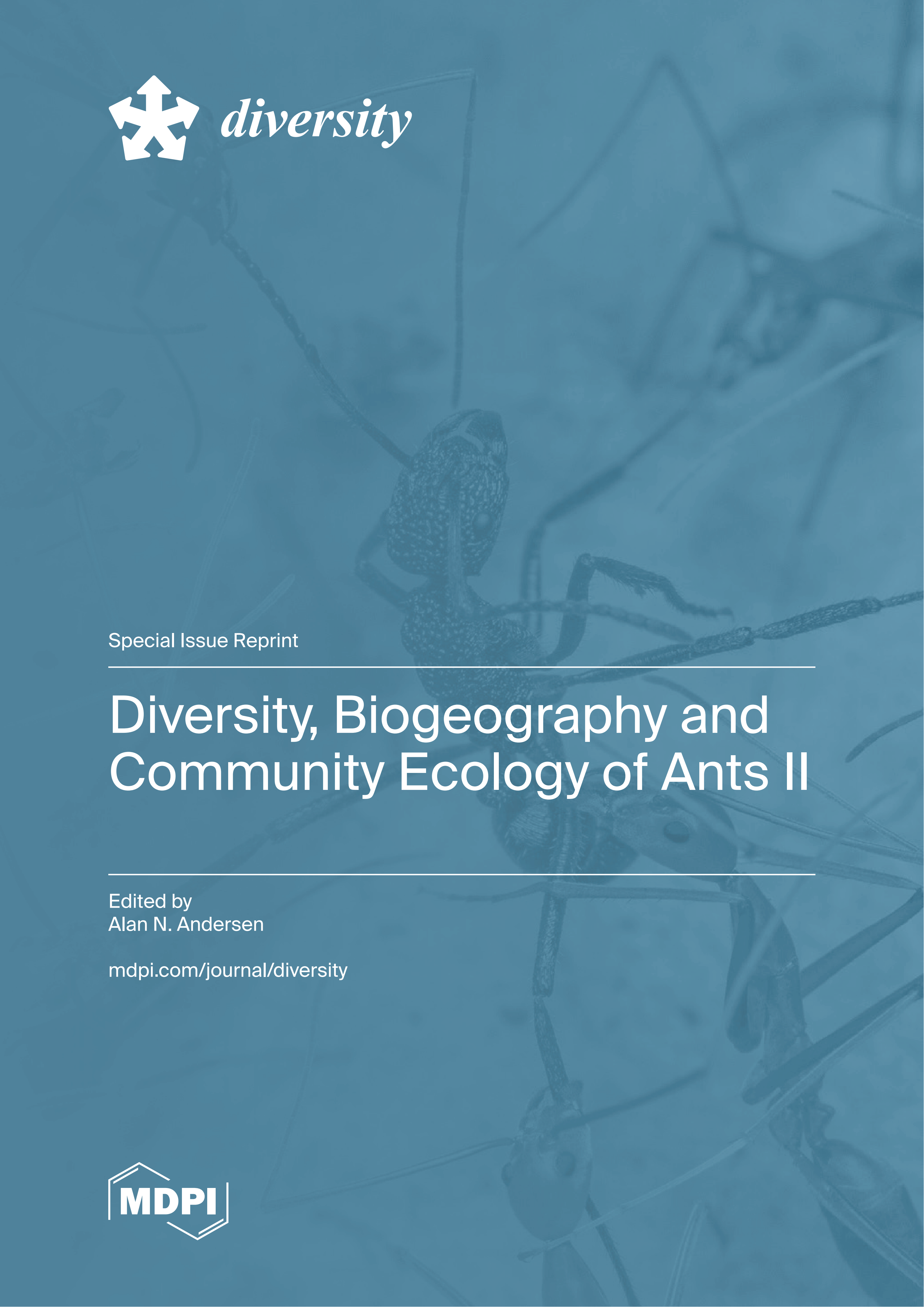

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Diversity, Biogeography and Community Ecology of Ants II

Edited by
Alan N. Andersen

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Guest Editor

Alan N. Andersen

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Contents

About the Editor

Alan N. Andersen

Alan N. Andersen is the Professor of Terrestrial Invertebrates in Charles Darwin University's Research Institute for the Environment and Livelihoods. His primary research interests lie in the global ecology of ant communities, where he integrates community ecology, historical and contemporary biogeography, and systematics to gain a predictive global understanding of ant diversity, behavioural dominance and functional composition in relation to environmental stress and disturbance. He applies this understanding to the use of ants as bioindicators of ecological change. Prof. Andersen is the author of five books and 300 scientific papers. He is a Fellow of the Australian Academy of Science.

Preface

Ants are a highly diverse and ecologically dominant faunal group globally. The ease of sampling combined with their sensitivity to environmental change makes them valuable bioindicators in land management. They are also widely used by researchers as model organisms for studies of diversity, biogeography and community ecology.

This is the second Special Issue of papers published in *Diversity* on the diversity, biogeography and community ecology of ants. It comprises papers published from 2022 to 2024, covering a diverse range of topics including microbial ecology, undescribed diversity, functional ecology, biogeography and climate change.

I am most grateful to all the authors of the papers for their contributions to this Special Issue and to Ms. Emma Li from MDPI for her editorial oversight.

Alan N. Andersen
Guest Editor

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Article

Ant Diversity Declines with Increasing Elevation along the Udzungwa Mountains, Tanzania

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Abstract: Biodiversity patterns along elevational gradients are generally characterised by monotonic decreases or mid-elevational peaks in species richness, while elevational zones may be characterised by distinct assemblages, or higher zones may be subsets of lowland assemblages. Elevational gradients in diversity have been less studied in the Afrotropical region. This study documents ant diversity patterns in three forest types associated with the tropical mountains of Udzungwa; we hypothesise that: (1) ant diversity and activity will show a monotonic decrease from mid-elevation with increasing elevation and (2) that forests associated with different elevations will have a distinct ant assemblage. Pitfall traps were deployed at three targeted elevations (650–800, 800–1400, and 1400–1500 m a.s.l.). Ant species richness declined with increasing elevation from 650 m a.s.l. and formed three elevational assemblages with lower elevation forests having almost twice as many species as sub-montane forests and three times as many as that of the montane forests. In contrast, overall ant activity peaked at 800–1400 m a.s.l. The ant assemblages associated with the lower elevation forest were very distinct, while assemblages associated with the sub-montane and montane forests shared species. Our study reveals valuable and relevant information for biodiversity monitoring and conservation planning as the species associated with each forest type may be used as indicator species for assessing biodiversity responses to climate change and anthropogenic activities on these mountains.

Keywords: ant assemblages; elevational gradients; biodiversity; ants (Hymenoptera, Formicidae); Eastern Arc Mountains

1. Introduction

One of the central goals in ecology is to understand distributional patterns and the abundance of living organisms. This understanding allows ecologists to assess and monitor changes in ecosystems and develop conservation priorities and policies [1,2]. To achieve this goal, ecologists must first quantify and characterise communities across various ecosystems. This involves a description of assemblage structures and their variations across time and space [3].

Environmental gradients have been the target of much research; they facilitate studying the response of biodiversity to climatic conditions and how they vary across space and time [4–10]. The latitudinal gradient of increasing richness from polar regions to the equator [7] has been one of the best documented biodiversity patterns and is relatively consistent across taxa and regions [11]. Elevational gradients have also been widely studied, but changes in biodiversity along this gradient vary depending on the taxa as well as region [12–14].

Altitudinal gradients are powerful model systems [15]. They provide natural experiments for investigating the response of biodiversity to environmental conditions within a small geographic space [16], which makes it particularly easy to test hypotheses about patterns and processes that may occur at a larger scale [17]. The drop in air temperature by 0.6–1.0 °C for every 100 m rise in elevation limits species from moving further up the mountain (especially thermophilic species) and suggests that ecosystems at high altitudes (with the predicted 2 °C increase in air temperature by year 2100) are more vulnerable to climate change, making research on patterns along altitudinal gradients important for conservation [18,19]. Climate change research [20–22] predicts a general increase in annual average temperature globally, which will shift eco-zones upwards and result in the disappearance of habitats. This may result in the extinction of affected species and negatively impact local ecosystem functioning [23]. Therefore, it is important to map and understand biodiversity patterns and identify the factors that generate and sustain high concentrations of biodiversity along elevations to support conservation programmes.

The Udzungwa Mountains are part of the Eastern Arc Mountains, a mountain chain stretching from Southeastern Kenya to Southern Tanzania along the coast [24,25]. This mountain chain, together with other Afromontane areas in Eastern Zaire and Ethiopia, is recognised as a biodiversity hotspot [26] and a conservation site for iconic endemic primate species, such as the Udzungwa red colobus (*Piliocolobus gordonorum* Matschie, 1900) and the Sanje mangabey (*Cercocebus sanjei* Mittermeier, 1986) [27,28]. Arguably, it also hosts the second richest bird diversity in Africa [27]. Much of what is known about the faunal diversity is from avifauna [27,29,30] and mammal studies [25,31,32], and very little is known about invertebrates, with data emerging especially for slugs [33], spiders [3], millipedes [34], dragonflies [35], Lepidoptera [36], and beetles [37].

Ants are a diverse and important group of insects in tropical rainforests [38]. They contribute an estimated 10–20% of animal biomass in terrestrial ecosystems and are of great ecological importance [39] as they are found in all forest strata and serve as herbivores, scavengers, and predators. Furthermore, they are ecosystem engineers [40] and can be used as biological indicators [41]. The species composition and ecological characteristics of ants species vary along environmental gradients [42], therefore making the presence or absence of a particular species a potentially appropriate indicator of environmental stress [42,43]. Climatic variables, especially temperature and precipitation, are some of the main drivers of ant diversity and assemblage composition across gradients [13,16].

For ant species richness, two general patterns have been observed across elevational gradients: either a decline with increasing elevation [38] or mid-elevational peak [12]. While some studies have reported an increase in ant species richness with increasing elevation, other studies have observed no clear pattern [44]. However, most of these studies set their lowest elevation at 500 m asl, so they assessed only partial gradients.

Here, we document the diversity patterns of ant communities in three forest types along an altitudinal gradient in the Udzungwa Mountains and characterise them by testing the following hypotheses: (1) ant diversity will decline with increasing elevation considering that ants are largely thermophilic, and (2) there will be distinct ant assemblage compositions that correspond to the three main forest types at different elevations.

Our findings will contribute to the knowledge on how Afrotropical ants are distributed along the main forest and in elevation. Furthermore, our findings will reveal useful and relevant information for biodiversity monitoring and conservation planning.

2. Materials and Methods

2.1. Study Site Description

The Udzungwa Mountains (−8.503722 35.9076; −7.678377 36.94129) are widely recognised for their outstanding biodiversity and high endemicity [28]. They form the largest mountain block of the Eastern Arc Mountains [45] covering 10,000 km ^{2}. Their long-term climatic stability has allowed the habitats in these mountains to endure for millions of years [24]. The Udzungwa mountain ranges in altitude from 200 to 2500 m a.s.l. along which different habitats are found, including lower elevation rainforest (300–800 m a.s.l.); sub-montane rainforest (700–1400 m a.s.l.) covered by a moist forest consisting of evergreen species; montane rainforest (1400–1800 m a.s.l.); and mountain bamboo forest (2400 m a.s.l.) covered by a mosaic of bamboo (*Sinarundinaria alpina*) and *Hagenia abyssinica*, as described by Shangali et al. [46]. The climate in the Udzungwa Mountains is variable, and the eastern slopes receive 2000–3000 mm of rain per year due to the influence of the Indian Ocean [25,47]. More specifically, the lower elevation forest experiences an average temperature of 22.9 °C and an average humidity of 88%. The sub-montane and montane forests experience an average temperature of 20.02 °C and 17.08 °C, respectively, while the humidity is 94.26% and 96.25%, respectively. The main rainy season is between March and May, and there is a light rainy season between November and February [47]. Our study was conducted in the Udzungwa Mountain National Park and the Uzungwa Scarp Nature Reserve in the southern part of the Udzungwa Mountains [25].

2.2. Ant Sampling

We set up five elevational transects, each with a 50 m × 50 m square plot at targeted elevations (650–800, 800–1400, and 1400–1500 m a.s.l.) for a total of 15 plots (Figure S1). These three elevations correspond to three main forest types: lower elevation forest, sub-montane forest, and montane forest, respectively (Table 1) [48]. The five transects were separated horizontally by 0.1, 1, 20, and 175 km from the first transect (Figure S1). At each 50 m × 50 m square plot, 12 pitfall traps were installed 4 m apart on each side of the plot (Figure S1) for a total of 48 traps. However, it needs to be noted that the lowest sampled elevation was 650 m a.s.l.; therefore, we studied a partial gradient.

Table 1. Description of the three studied forest habitat types in the Udzungwa Mountains, Tanzania.

Habitat Types	Altitudinal Range	Description
Lower elevation forest (lowest elevation)	650–800	Forest with deciduous and semi-deciduous trees, canopy 15–25 m with emergents reaching 50 m
Sub-montane forest (mid elevation)	800–1400	Moist forest with mainly evergreen species, canopy 25–40 m with emergents reaching 50 m
Montane forest (high elevation)	1400–2600	Evergreen moist forest, with canopy height progressively lower with increasing altitude

We combined four contiguous traps to form a sample so that we obtained 12 samples from each plot. This sampling design was part of the application of the Conservation Oriented Biodiversity Rapid Assessment for Tropical Forests (COBRA-TF) sampling protocol [49]. Pitfall traps were partly filled with preservative solution (propylene glycol) and a few drops of liquid soap to break the surface tension and to be protected against rain and falling leaves using lids on stilts about 2–3 cm above the ground. The traps ran for 14 days from October to November in 2014.

Samples were washed and sorted in the laboratory and stored in 96% ethanol at −20 °C. Ants were identified to genus using Fisher and Bolton [50] and then identified to species where possible using online databases, viz., AntWiki (http://www.antwiki.org/, accessed on 22 March 2019) and AntWeb (http://antweb.org/, accessed on 25 March 2019). Number codes were assigned to unidentified ant species. Voucher specimens of all species are deposited in the Natural History Museum of Denmark.

2.3. Data Analysis

Sample coverage for species richness was analysed in iNEXT online software [51]. Sampling completeness based on Chao1 richness estimators was determined using EstimateS (Version 9.1.0) [52], and graphs were drawn using R [53]. Chao1 is a known qualitative measure of alpha diversity that considers the ratio of singletons to doubletons and, therefore, considers rare species. The Simpson diversity index was also used to compare dominance between the three main habitats and plots.

The species composition of the ant communities was visualised by non-metric multidimensional scaling (nMDS) using Sorensen's index dissimilarity matrix based on presence/absence data. The comparison of species composition within the three habitat types was conducted using PERMANOVA in R [53]. The characteristic species of each habitat was determined using the Indicator Value Method (IndVal), which uses the degrees of specificity (uniqueness to a particular site) and fidelity (frequency within the vegetation type/aspect) of each species [54]. An indicator value above 70% shows that a species is both highly specific and has a high fidelity to a given site. The significance of the IndVal values was then tested by random reallocation of replicates among groups [44].

3. Results

3.1. Ant Diversity across Three Main Forest Types along the Elevational Gradient

A total of 31 776 ant specimens belonging to five subfamilies, 34 genera, and 101 morphospecies was collected (Table S1). Myrmicinae was the most diverse subfamily with the highest number (54%) of ant foragers in pitfall traps, 50% of the total number of species (51 species), and 41% of the total number of genera (Table 2). The second most diverse subfamily was Ponerinae with 24% of the species and 29% of the total number of genera, followed by Formicinae with 19% of the species and 18% of the total number of genera. The least diverse subfamilies were Dolichoderinae and Dorylinae with 3% and 4% of the total species richness, respectively. However, Dorylinae had the second highest ant activities. The most speciose genera were *Tetramorium* (19 species), *Pheidole* (8 species), and *Strumigenys* (6 species), while *Camponotus* and *Crematogaster* had five species each (Table S1).

Table 2. Number of genera, species richness, and individuals (activities) of ant subfamilies collected in the study.

Subfamily	Species	Individuals (Activities)
Dolichoderinae		
Technomyrmex	3	5
Dorylinae		
Aenictus	1	2
Dorylus	1	13,260
Parasyscia	2	4
Formicinae		
Camponotus	5	68
Lepisiota	5	209
Nylanderia	1	156
Plagiolepis	3	8
Polyrhachis	2	15
Tapinolepsis	3	10
Myrmicinae		
Calyptomyrmex	1	1
Cardiocondyla	1	6
Carebara	2	126
Catalaucus	1	1
Crematogaster	5	50
Melissotarsus	1	1
Meranoplus	1	7

Table 2. *Cont.*

Subfamily	Species	Individuals (Activities)
Microdaceton	1	1
Monomorium	2	49
Myrmicaria	1	10,512
Pheidole	8	4751
Solenopsis	2	308
Strumigenys	6	83
Tetramorium	19	1261
Ponerinae		
Anochectus	3	5
Bothroponera	4	79
Cryptopone	1	1
Hypoponera	4	11
Leptogenys	4	449
Megaponera	1	210
Mesoponera	3	35
Odontomachus	1	10
Plectroctena	3	82

3.2. Sampling Completeness

Sampling coverage was nearly complete for the three habitat types, lower elevation, sub-montane, and montane forests, as sampling coverage was close to 1 (Table 3). Coverage was the lowest in lower elevation forests (Figure S2).

Table 3. Observed number of species (per habitat and plots), Simpson's diversity index, and sample coverage for each sampling plot in the three habitat types (lower elevation, sub-montane, and montane forests).

Habitat Type	Observed Species per Habitat (Mean SD)	Plot	Altitude m a.s.l	Observed Species Richness per Plot	Simpson's Diversity Index	Sample Coverage
Lower elevation	71 (30.6 ± 8.1)	1	650	31	0.500	0.9963
		2	650	38	0.375	0.996
		7	708	40	0.862	0.993
		10	674	22	0.405	0.989
		13	659	24	0.825	0.974
Sub-montane	44 (19.6 ± 5.3)	3	1005	24	0.297	0.998
		4	993	21	0.447	0.995
		8	978	25	0.623	0.998
		11	1006	15	0.666	0.996
		14	908	13	0.813	0.911
Montane	33 (15.2 ± 6.5)	5	1448	23	0.747	0.994
		6	1482	18	0.271	1
		9	1527	15	0.820	0.995
		12	1552	15	0.466	0.986
		15	1531	5	0.261	0.986

3.3. Species Diversity Patterns

Ant activity, which is represented by the total number of individuals per plot, was the highest at mid-elevation mainly because of *Myrmicaria rustica angustior* (27% of the total activities). At low elevations, *Dorylus helvolus* dominated ant activities and contributed 27% of the total ant activities. Simpson's index suggests that lower elevation forest ant communities were the most diverse (0.664), followed by the mid-elevation ones (0.49), with the high elevation communities being the least diverse (0.44). Species richness was higher at low elevations (31 ± 8), followed by mid-elevations (20 ± 9), and finally high elevations

(15 ± 7) (Table 3). In the low elevation forest, seventy-four percent of the species were found, while 44% and 33% were collected in the sub-montane and montane forests, respectively.

3.4. Ant Assemblage Composition across Gradients

The NMDS showed distinct species assemblages according to their elevations (Figure 1; Figure S3). PERMANOVA confirmed significant differences in species composition between plots at the three elevations (df = 2; Pseudo-F = 2.7863; *p* = 0.002).

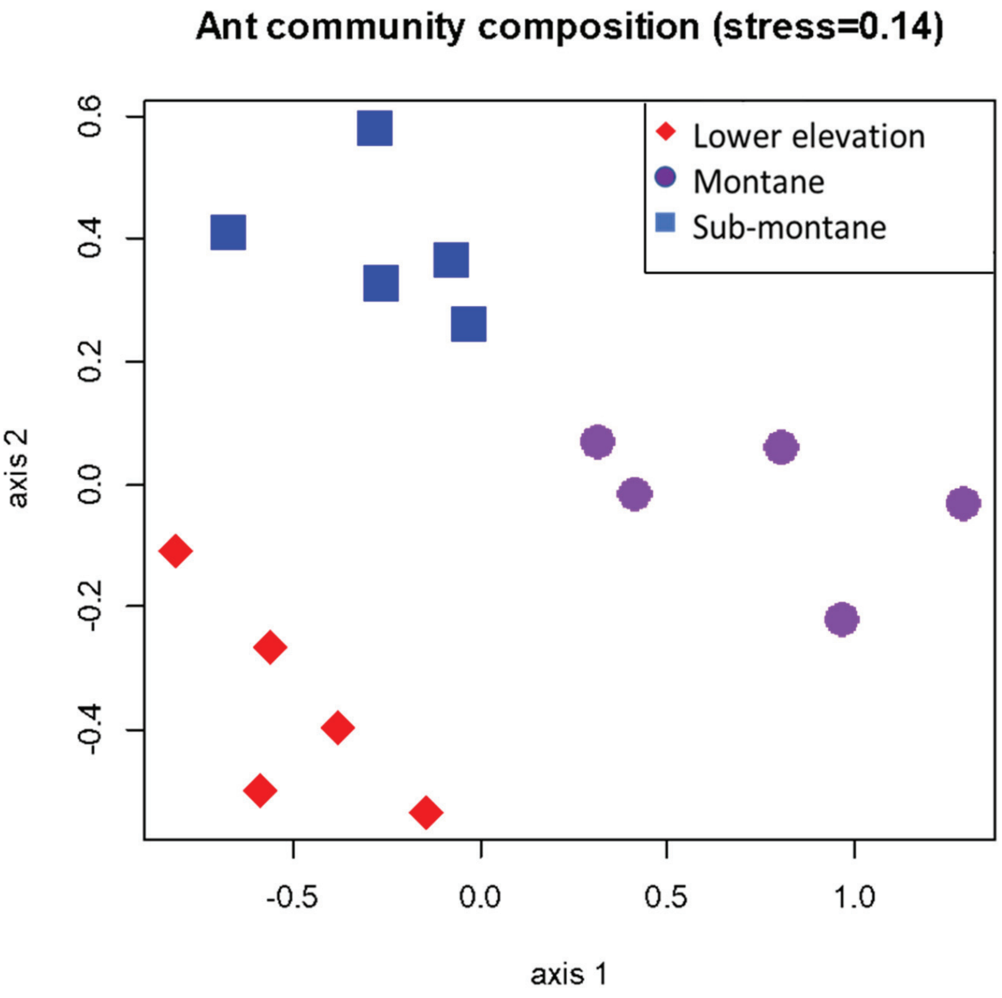

Figure 1. Non-metric multidimensional scaling (NDMS) of community similarity (Bray–Curtis dissimilarity index) based on ant species found in three forests types—lower elevation (red), sub-montane (purple), and montane (blue)—in the Udzungwa Mountains, Tanzania.

3.5. Indicators Species

Fifteen species had a wide distribution and occurred at all elevations; thirty-seven species were associated with the lower elevation forest, eleven were associated with the sub-montane, and nine were associated with the montane forest (Figure S3; Table S1). However, the latter species were not characteristic of the different forest types. Five species were exclusively sampled in the lower elevation forest, while the sub-montane forest and montane forest had two characteristic species each (Table 4).

Table 4. Indicator values (IndVal) of ant species for forest habitat types along the elevational transects. All indicator values are significant ($p < 0.05$).

Habitat Type	Species	Indicator Value (%)
Lower elevation	*Pheidole* sp.05	100
	Megaponera analis rapax	99.52
	Camponotus sp.02. (*etiolipes* gp.)	94.12
	Tetramorium cf. *yarthiellum*	80
	Nylanderia sp.01	70.77
Sub-montane	*Bothroponera* sp.01	85.71
	Myrmicaria rustica angustior	81.23
Montane	*Tetramorium* sp.14	100
	Mesoponera sp.02	82.61

4. Discussion

Ant species numbers declined with increasing elevation from 650 m a.s.l. along the Udzungwa Mountains. This is a widely observed pattern along elevational gradients [55,56] but not for all other taxa studied across the Udzungwa Mountains. Along these mountains, ground dwelling spiders increased with elevation [3], bird richness peaked at mid-elevations [30], and plants and small mammals increased with elevation [24,57]; however, it needs to be noted that all these studies were along a partial gradient. Similarly, in other Afrotropical studies, ant species richness patterns along partial elevation gradients varied between mountains and aspects. A mid-elevation peak was observed along the Maloti-Drakensberg mountains (900 to 3000 m a.s.l.) [16] and northern aspect of Soutpansberg mountains (800 to 1700 m asl) but decreased with elevation on the southern aspect of the latter mountain [13], while a complete gradient by Botes, McGeoch, Robertson, van Niekerk, Davids, and Chown [44] along the Cederberg mountains (sea level—1926 m a.s.l.) reported no clear pattern in their study.

It is widely accepted that ants are generally thermophilic [43]. The distribution of ants is mainly determined by their tolerance to heat [58]; therefore, a decrease in ant species along an altitudinal gradient may be explained by the decrease in temperature with altitude [16,17]. Cooler temperatures may slow down metabolic processes of ants, affect the development of eggs and larvae, affect their foraging activities, and consequently reduce their primary productivity [59]. The limited tolerance of ants to the cold may also cause niche conservatism and limit their species ranges [60]. Mountain areas have bands of climatic conditions that act as barriers, limiting the establishment of species in different areas. Each barrier, therefore, creates a difference in species richness [60,61].

Three highly distinct ant assemblages associated with the three forest types were evident. These findings are similar to those of spiders in the Udzungwa Mountains [3]. However, ant assemblages seem to be more distinct, and lower elevation forests had the most distinct assemblages while montane and sub-montane shared species between them (Figure S3). Tropical species have narrow elevational ranges as the stable local environmental conditions allow them to specialise relative to those specific conditions [62]. The latter may explain the separation of assemblages with respect to elevation zones. The distinct low elevation assemblages may be a result of many tropical lower elevation species possessing narrow fundamental niches limiting their distribution to the lower elevation, while other species may occur in more than one elevation as a result of their wider tolerances [63].

The homogeneous habitat structure, as described by some authors (for example, see Lovett [48]), might be the reason for the similarities between the mid- and high-elevation communities, and therefore resembles the distribution of spider communities on the same mountain [3]. However, other important factors may structure arthropod assemblages that might have contributed to this pattern: for example, how species are specialised to resources and their physiological tolerances to climatic conditions [63].

In the tropics, lowland species are reported to have very narrow fundamental niches, which limits their distribution to the lowland [64], which matches our findings (Figure S2). These species vary from subterranean species, a widely distributed *Technomyrmex pallipes*, to the common rainforest ant *Odontomachus assiniensis*, a predatory species common in evergreen forests as well as the common and generalist ant species *Monomorium mirandum* [65]. Many of the ant species in this study favour the lower elevation forest, as shown by the number of species and species associations.

Fewer ant species were restricted to the montane forest, and only two were characteristic of this forest type. Some species seemed to have larger distributional ranges as they were found in all three vegetation zones. This is typical of tropical species at higher elevations as they tend to be generalists with wider tolerances [66] compared to those in lower elevation forests. However, in the current study, species showing wide tolerances were found across the mountain. Amongst them were both generalists and specialised genera such as *Myrmicaria* (Tropical Climate Specialists), *Pheidole* (Generalised Myrmicinae), and *Solenopsis* (Hot Climate Specialists) [67]. However, all these species may be generalists on these mountains as they have wide geographic distribution and show no habitat preferences.

The two most abundant species were *Myrmicaria rustica angustior* and *Dorylus helvolus*. The former is found in open areas of Afrotropical regions, and together with some other species in the genus *Myrmicaria*, they are well-known honeydew feeders and scavengers [68], while some species are even predatory, feeding on other insects. In the current study, *Myrmicaria rustica angustior* was collected in all habitats and, therefore, did not show any habitat preferences. This can be explained by the fact that the species of genus *Myrmicaria* do not have a specialised diet [69,70]. *Myrmicaria rustica angustior* activity peaked at mid-elevations, perhaps owing to overlapping ranges of the lower elevation and montane forests resulting in the edges providing more open habitats for ants to inhabit [64]. However, another influential species, *Dorylus helvolus,* declined in abundance with increasing elevation. The species of genus *Dorylus* are generalist predators that consume any kind of prey ranging from immatures of other insects to vertebrate carrion, and this may explain their occurrence throughout the mountains [69]. Moreover, the *Dorylus* species are known to move nests in response to prey availability [71] and are most likely to have been influenced by this foraging behaviour as their colonies migrate to new colonies in irregular intervals resulting in new colonies forming through colony fission [69].

5. Conclusions

Conservation plans can benefit from the information that we provide here: The species that we have identified as associated with each forest type may be used as an indicator species for monitoring the response of biodiversity to climate change and anthropogenic activities on these mountains. The importance of the Udzungwa Mountains for conserving Eastern Arc biodiversity has been emphasised [27], but biological data on mainly vertebrates and plants have been used in current conservation strategies. Furthermore, there is an urgent need for further research on the effects of the environmental and climatic factors on the diversity patterns of invertebrate communities in the Eastern Arc Mountains.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/d14040260/s1, Figure S1: The five (50 m × 50 m) plots consisting of 12 pitfall traps on each side of the plot 4 m apart at each elevation (lower elevation rainforest (650–800 m.a.s.l), sub-montane rainforest (800–1400 m.a.s.l), montane rainforest (1400–1500 m.a.s.l)), Figure S2: Interpolation and extrapolation of species diversity at three forest types (lower elevation, sub-montane, and montane) across the studied communities, Figure S3: A Venn diagram showing the number of species restricted and shared within the three forest types of the Udzungwa mountains and Table S1: Checklist of subfamilies and ant species collected in three habitat types of the Udzungwa mountains.

Author Contributions: J.M.-O., N.S. and T.P. designed the study and collected data; T.C.M. identified the ants; C.K. analysed the data and led the writing of the manuscript under the supervision of T.C.M. and S.H.F.; C.K., S.H.F., T.C.M., J.M.-O., N.S. and T.P. read, edited, and agreed to the published version of the manuscript. All authors have read and agreed to the published version of the manuscript.

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diversity

Article

Investigating the Diversity of *Wolbachia* across the Spiny Ants (*Polyrhachis*)

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Abstract: Among insects, *Wolbachia* is an exceedingly common bacterial endosymbiont with a range of consequences of infection. Despite the frequency of *Wolbachia* infection, very little is known about this bacteria's diversity and role within hosts, especially within ant hosts. In this study, we analyze the occurrence and diversity of *Wolbachia* across the spiny ants (*Polyrhachis*), a large and geographically diverse genus. *Polyrhachis* samples from throughout the host genus' phylogenetic and biogeographical range were first screened for single infections of *Wolbachia* using the *wsp* gene and Sanger sequencing. The multilocus sequence typing (MLST) scheme was then used on these singly infected samples to identify the *Wolbachia* strains. A *Wolbachia* phylogeny was inferred from the *Polyrhachis* samples analyzed in this study as well as other Formicidae MLST profiles from the MLST online database. We hypothesized that three key host factors were impacting *Wolbachia* diversity within the *Polyrhachis* genus: biogeography, phylogeny, and species level. The results suggest that the phylogeny and biogeography of *Polyrhachis* hosts have no impact on *Wolbachia* diversity; however, species level may have some limited influence. Additionally, *Wolbachia* strains appear to group according to being either Old World or New World strains. Among the taxa able to form complete MLST allelic profiles, all twenty are seemingly new strains.

Keywords: multilocus sequence typing (MLST); Formicidae; host–microbe associations

1. Introduction

Ants (Formicidae) are a highly diverse family of insects with a global distribution. One of the many factors contributing to the overwhelming ecological success of ants is their many associations with symbiotic microbes. Ants associate with microbial eukaryotes, fungi, viruses, and bacteria; further, many of these associations are understood to have contributed to the diversity of diets, occupied niches, and life history in various ant groups [1]. For example, the ant genus *Cephalotes* is able to survive on a nutrient-poor herbivorous diet due to the microbial symbionts present in its gut [2]. In addition, the functions of these symbionts are of particular interest to researchers, especially in the case of maternally transmitted bacterial symbionts already known to alter host reproduction, development, nutrition, and defense in many arthropods [3]. The *Wolbachia* bacterial genus is a well-known example of such a symbiont. It is estimated that *Wolbachia* infects up to 75% of all insect species [4] and is an incredibly common, heritable maternally transmitted bacterial symbiont of ants [3,5]. Some of the most notable consequences of *Wolbachia* infection in insects are alterations to the host's reproductive abilities—these include parthenogenesis, male killing, male feminization, and cytoplasmic incompatibility [6]. Within ants (Formicidae) specifically, *Wolbachia* has also been found to accelerate the colony life cycle [7] and enhance the host's nutrient uptake [8]. Due to the variety of *Wolbachia's* impacts on its ant hosts, more studies are needed to elucidate the diversity of *Wolbachia* across Formicidae to understand the consequences of its associations with ants [9,10].

While it is now known that *Wolbachia* is a widespread symbiont of insects, it was first discovered as a rickettsial symbiont of the mosquito *Culex pipiens* in the 1920s [11]. All *Wolbachia* strains are divided into supergroups via phylogenetic analysis using one or multiple marker genes (e.g., 16S rDNA, *wsp*, *ftsZ*). Currently, there are twenty-one *Wolbachia* supergroups, ranging from A to U [11–13]. Further, these *Wolbachia* supergroups also appear to have set associations to specific host taxa. For instance, it has been found that the strains in Formicidae hosts are from mostly supergroups A and F with the majority being from supergroup A [9], though there has been a single instance where a supergroup B strain was found associated to an ant host from Mexico, *Pheidole sciophila* [14].

Previously, the standard procedure for sequence typing *Wolbachia* strains was based upon sequencing the *Wolbachia* surface protein gene, *wsp* [15]. After it was determined that *wsp* experiences extensive recombination via swapping of conserved amino acid motifs within hyper-variable regions [16], the Multilocus Sequence Typing (MLST) approach was proposed [6]. MLST was introduced alongside an online database of bacterial and host information (https://pubmlst.org/organisms/wolbachia-spp, accessed on 27 February 2023), and *Wolbachia* sequence types are based upon the allele determination of five different housekeeping genes (*coxA*, *fbpA*, *ftsZ*, *gatB*, and *hcpA*) rather than *wsp* [4]. MLST has become the standard method of sequence typing *Wolbachia* since it provides a more robust approach to assessing *Wolbachia* diversity across a variety of host taxa due to the reliance on five loci rather than the *wsp* locus alone. The MLST scheme has been used by researchers studying this bacterial genus within a wide range of hosts including filarial nematodes and ticks [17], butterflies [18] and ants [1,19–21].

Polyrhachis Smith, 1857 is a large ant genus (over 700 species) that inhabits Africa, Asia, Australia, and Oceania [22,23]. They are commonly called “spiny ants” due to the spinescence of most species, which can vary in shape, length, and numbers; this spinescence is hypothesized to be a defense characteristic against vertebrate and invertebrate predators [24,25]. They exhibit a large variety of nesting techniques including the use of larval silk to weave their nests (a trait limited to few ant genera), nesting inside hollow bamboo, and attaching nests to stones [26,27]. In addition, *Polyrhachis* belongs to the Camponotini tribe, which is well known for their symbiotic relationships with bacteria—in particular *Blochmannia* [28]—and an association with *Wolbachia* has been previously found in Camponotini as well [20,29,30]. *Polyrhachis's* broad biogeographical range spanning across Africa, Asia, Australia, and Oceania [26] makes it a useful host for studying the impacts of host biogeography on *Wolbachia* diversity.

In past studies, specific ant species have been studied for their associations to *Wolbachia* [19,23], and the evolutionary association of *Wolbachia* was evaluated across the entire Formicidae family [9]. Additionally, *Wolbachia* has been studied in other social insects such as bees, termites, and wasps [10]. In one species-specific study, the diversity of *Wolbachia* was analyzed across the geographically diverse giant turtle ant species (*Cephalotes atratus*), and results suggested that *Wolbachia* diversity is affected by geography [19]. In a broader study that analyzed *Wolbachia* across Formicidae, the evolutionary origins of *Wolbachia* infection in ants were illuminated and the biogeographical origin of the symbiosis was inferred to be in Asia [9]. Our intention with this work, investigating *Wolbachia* infection across the *Polyrhachis* genus, is to further explore the notion that *Wolbachia* diversity can be impacted by geography, as well as the evolutionary association between host and microbe via phylogenetic and species level analyses.

In the following study, our primary objective was to analyze and observe patterns of *Wolbachia* infection in *Polyrhachis*. We hypothesized that three factors related to the *Polyrhachis* host will impact the observed diversity of *Wolbachia*: phylogeny, species level, and biogeography. If these factors do impact *Wolbachia* diversity, we would anticipate seeing significant correlations in increases (or decreases) of *Polyrhachis* host diversity with that of its *Wolbachia* symbionts. If, for example, the *Polyrhachis* host phylogeny impacts the observed diversity of *Wolbachia,* we will see phylogenetic signal and potential evolutionary co-diversification between *Polyrhachis* and *Wolbachia.* If species level within *Polyrhachis* impacts the observed diversity of *Wolbachia*, we may observe different kinds of *Wolbachia* infecting different *Polyrhachis* species in statistically significant ways. If biogeography of the *Polyrhachis* host impacts the observed diversity of its *Wolbachia*, we may see a variance in *Wolbachia* infection that is correlated with the different locations where each *Polyrhachis* sample was collected.

2. Materials and Methods

Samples from 102 *Polyrhachis* species (237 *Polyrhachis* samples) were screened for their associated *Wolbachia* strains. These samples were collected from 29 countries (Table 1 shows samples positive for *Wolbachia*; all samples are listed in Supplementary Material File S1). The DNA extractions was performed on whole ant specimens following the DNeasy Blood and Tissue (Qiagen) protocol. The DNA was stored at −20 °C. The sampled *Polyrhachis* species were taken to be representative of the entire host genus and spanned across the entire *Polyrhachis* biogeographical range. To screen for *Wolbachia* and determine which samples contained single infections, sequencing of *Wolbachia*'s *wsp* gene was performed. The *wsp* gene was PCR amplified using Taq DNA Polymerase, primers wsp81f and wsp69r (at 1 μM each), and 1 μL of DNA [17,31] for 36 cycles with an annealing temperature of 59 °C [4]. The thermocycler program was set to the following: the cycle began with denaturation at 94 °C for 30 s, annealing for 45 s, 72 °C for 1.5 min, an elongation step at 70 °C for 10 min, and a hold at 4 °C. Annealing temperatures varied by gene: *coxA* was annealed at 55 °C, *fbpA* at 59 °C, *hcpA* at 53 °C, and both *ftsZ* and *gatB* at 54 °C. The PCR products were first evaluated using gel electrophoresis [32] wherein the presence of a band indicated the infection of at least one *Wolbachia* strain for that sample. *Wolbachia*-positive PCR products were purified using ExoSap (Cleveland, OH, USA) with the manufacturer-recommended thermocycler settings. BigDye Terminator (Applied Biosystems, Waltham, MA, USA) was used to prepare the samples for Sanger sequencing, which was carried out by the Cornell Institute of Biotechnology (Ithaca, NY, USA). The resulting sequence electropherograms were evaluated in Geneious Prime 2022.1 (https://www.geneious.com, accessed on 15 August 2022) to determine whether samples were infected with single or multiple strains of *Wolbachia*.

Table 1. Sample ID, host species, and country of origin for all samples positive for *Wolbachia*. A complete list of samples screened is available in Supplementary Material File S1.

Sample ID	Species	Country	Sample ID	Species	Country
DG06	*(Polyrhachis (Myrmatopa)* sp.	Phillipines	RA0766	*Polyrhachis flavibasis*	Australia
ISR_06	*Polyrhachis (Myrma)* sp.	Thailand	SUL02	*Polyrhachis (Myrma)* sp. *1*	Indonesia
GM 894	*Polyrhachis (Myrmhopla)* sp. *2*	Malaysia	SKY20	*Polyrhachis* sp.	Singapore
GM3990	*Polyrhachis (Myrmhopla)* sp. *4*	Malaysia	SL_28_2	*Polyrhachis illaudata*	Malaysia
GM3589b	*Polyrhachis (Myrmothrinax)* sp.	Malaysia	SKY24	*Polyrhachis* sp.	Singapore
AS4132a	*Polyrhachis (Polyrhachis)* sp.	Cambodia	LEA04	*Polyrachis schistaceae*	Mozambique
CSM0776	*Polyrhachis abbreviata*	Australia	MS1177	*Polyrhachis shixingensis*	China
DG10	*Polyrhachis armata*	Philippines	RA0784	*Polyrhachis* sp.	Solomon Islands
DG14	*Polyrhachis armata*	Phillipines	RA1157	*Polyrhachis illaudata*	Laos
CSM0761	*Polyrhachis australis*	Australia	MJ9286	*Polyrhachis* sp.	Papua New Guinea
DG26	*Polyrhachis bicolor*	Philippines	RA1163	*Polyrhachis illaudata*	Laos
BB012	*Polyrhachis bihamata*	China	MJ 8277	*Polyrhachis* sp.	Papua New Guinea
CSM1806a	*Polyrhachis bihamata*	Malaysia	PH09	*Polyrhachis afrc_cd03*	Democratic Republic of the Congo
CSM1806b	*Polyrhachis bihamata*	Malaysia	PH11	*Polyrhachis laboriosa*	Democratic Republic of the Congo
DG08	*Polyrhachis bihamata*	Phillipines	RA0769	*Polyrhachis "chario5"*	Australia
CSM1846	*Polyrhachis boltoni*	Malaysia	PH14	*Polyrhachis gagates*	South Africa
EMS2584	*Polyrhachis campbelli*	Solomon Islands	RA736a	*Polyrhachis dives-group* sp.	Thailand
DG04	*Polyrhachis carbonaria*	Phillipines	RA0765	*Polyrhachis ammon*	Australia
CSM1854	*Polyrhachis cephalotes*	Malaysia	PH15	*Polyrhachis afr_cd01*	Democratic Republic of the Congo
EMS2617	*Polyrhachis* cf. *bismarckensis*	Solomon Islands	RA736c	*Polyrhachis* cf. *laevissima*	Thailand
CSM1841	*Polyrhachis danum*	Malaysia	PH12	*Polyrhachis revoili*	Democratic Republic of the Congo
BB28	*Polyrhachis hippomanes*	China	MJ 9243	*Polyrhachis* sp. *near bicolor*	Papua New Guinea
JRNG01	*Polyrhachis hookeri*	Australia	TAS 02	*Polyrhachis hexacantha*	Australia

Table 1. *Cont.*

Sample ID	Species	Country	Sample ID	Species	Country
DG03	*Polyrhachis illaudata*	Phillipines	RA0755	*Polyrhachis "BATH3"*	Australia
GM3551	*Polyrhachis illaudata*	Malaysia	SKY21	*Polyrhachis nigropilosa*	Singapore
DG25	*Polyrhachis inermis*	Philippines	RA1162	*Polyrhachis illaudata*	Laos
EMS2637	*Polyrhachis kaipi*	Solomon Islands	RO 122	*Polyrhachis* sp.	Tanzania
ISR_03	*Polyrhachis lacteipennis*	Israel	SOH 02	*Polyrhachis beccari*	Singapore
CSM1868	*Polyrhachis lepida*	Malaysia	PH21	*Polyrhachis schistacea*	Mozambique
DG16	*Polyrhachis near lilianae*	Philippines	RA1158	*Polyrhachis mucronata-group* sp.	Laos
BB48	*Polyrhachis proxima*	China	MJ 9280	*Polyrhachis mucronata-group* sp.	
CSM0655	*Polyrhachis rufifemur*	Australia	MJ8280	*Polyrhachis* sp.	Papua New Guinea
CSM0740	*Polyrhachis rufifemur*	Australia	MJ9242	*Polyrhachis sexspi-sa group*	Papua New Guinea
DG11	*Polyrhachis saevissima*	Phillipines	RA1154	*Polyrhachis mucronata-group* sp.	Laos
DG17	*Polyrhachis saevissima*	Phillipines	RA1160	*Polyrhachis illaudata?*	Laos
KATE02	*Polyrhachis schistacea*	South Africa	TAS04	*Polyrhachis semipolita*	Australia
AS4132b	*Polyrhachis* sp.	Cambodia	LEA05	*Polyrachis schistaceae*	Mozambique
BB026	*Polyrhachis* sp.	China	MJ 8282	*Polyrhachis sexspi-sa group*	Papua New Guinea
CSM1860	*Polyrhachis* sp.	Malaysia	PH16	*Polyrhachis latharis*	Democratic Republic of the Congo
CSM2632	*Polyrhachis* sp.	Uganda	PH22	*Polyrhachis schistacea*	Tanzania
CSM2738	*Polyrhachis* sp.	Uganda	PSW5403	*Polyrhachis andromache*	Australia
CSM2745	*Polyrhachis* sp.	Uganda	PSW6454	*Polyrhachis obesior*	Malaysia
CSM2831	*Polyrhachis* sp.	Australia	RA0735	*Polyrhachis abdominalis*	Singapore
FH1085	*Polyrhachis* sp.	Uganda	RO538	*Polyrhachis* sp.	Tanzania
FH1101	*Polyrhachis* sp.	Uganda	SKY05	*Polyrhachis frustorferi*	Indonesia
FH205	*Polyrhachis* sp.	Kenya	SKY11	*Polyrhachis lamellidens*	Japan
FH987	*Polyrhachis* sp.	Uganda	SKY17	*Polyrhachis hector*	Indonesia
JCM120P	*Polyrhachis* sp.	Palau	TAS 01	*Polyrhachis hexacantha*	Australia
JRNG02	*Polyrhachis* sp.	Australia	TAS03	*Polyrhachis phryne*	Australia
LD01	*Polyrhachis* sp.	Ghana	LEA03	*Polyrachis schistaceae*	Mozambique
AS4121	*Polyrhachis* sp. *near furcata*	Cambodia	MJ 8263	*Polyrhachis* sp.	Papua New Guinea
AS4148a	*Polyrhachis* sp. *near furcata*	Cambodia	MJ 8291	*Polyrhachis* sp.	Papua New Guinea
BB_075	*Polyrhachis* sp. *near sixspi-sa*	China	MJ9275	*Polyrhachis* sp.	Papua New Guinea
CSM0746	*Polyrhachis thais*	Australia	SL32	*Polyrhachis furcata*	Malaysia
IND05	*Polyrhachis thrinax*	India	MJ 9287	*Polyrhachis* sp.	Papua New Guinea
CAB01	*Polyrhachis ypsilon*	Malaysia			

Only singly infected *Polyrhachis* samples (n = 34) were subjected to the Multilocus Sequence Typing (MLST) process wherein the five MLST genes (*coxA*, *fbpA*, *ftsZ*, *gatB*, and *hcpA*) were amplified and sequenced according to the same procedure as was done for the *wsp* gene. Since Sanger sequencing of multiple strains at once creates indecipherable electropherograms (due to the sequences for each strain overlaying each other), multi-infected *Polyrhachis* samples were excluded from the MLST process. Sequence alignments for each locus were created in Geneious Prime 2022.1 (https://www.geneious.com, accessed on 15 August 2022) then checked against reference sequences in the MLST online database (https://pubmlst.org/organisms/wolbachia-spp, accessed on 15 August 2022) to determine closest matching allele types. The closest matching sequence type (ST) for each *Wolbachia* strain able to produce clear electropherograms for all five loci (n = 20) was determined based upon these five alleles.

The five MLST genes were concatenated (2098 bp total length, order: *coxA*, *fbpA*, *ftsZ*, *gatB*, *hcpA*) for each of the 20 remaining samples, then added to a pool of 70 MLST database sequences from other Formicidae-associated *Wolbachia* strains [33]. A *Wolbachia* phylogeny was inferred with these 90 MLST sequences via the IQ-Tree web server 1.6.12 [34] to infer a phylogenetic tree by maximum likelihood and generate bootstrap values. The best fit model of substitution for each locus was determined by the ModelFinder [35] and partition model [36] features available through IQ-Tree web server. Partitions and their best-fit models are shown in Table 2. *Wolbachia* strains for ST124 and ST557 (both supergroup F) from the host species *Ocymyrmex picardi* and *Paratrechina*, respectively, formed the outgroup of the phylogeny. The haplotype network for each MLST gene was constructed with Network 4.5.1.0 [37] using the median joining parameter.

Table 2. Partitioning and best-fit models for each partition as determined by the IQ-Tree web server ModelFinder. The third and fourth columns detail the length of each partition and its position within the concatenated sequence (with a total length of 2098 bp).

Partition	Gene(s)	Position in Concatenation (bp)	Length of Gene(s) in Partition (bp)	Model
1	*coxA*	1–403	403	HKY + F+G4
2	*fbpA*	404–840	437	HKY + F+G4
3	*hcpA*, *ftsZ*	1651–2098, 841–1277	448, 437	TIM3 + F+I+G4
4	*gatB*	1278–1650	373	TIM + F+I+G4

Two mantel tests were performed using the R package vegan [38]. The first of these tested the correlation between phylogenetic distance between *Wolbachia* strains and geographical distances between latitudes and longitudes of collection sites. The second test examined correlations between phylogenetic distances between *Polyrhachis* host species (Blanchard and Moreau, in press) and phylogenetic distances of *Wolbachia* strains. Both the *Wolbachia* and *Polyrhachis* phylogenies were pruned down to seven tips, representing ant host or *Wolbachia* from seven different *Polyrhachis* species: *P. bihamata*, *P. cephalotes*, *P. carbonaria*, *P. thrinax*, *P. shixigensis*, *P. illaudata*, and *P. hexacantha*.

3. Results

Of the initial 237 *Polyrhachis* samples screened for *Wolbachia* using the *wsp* gene, 112 (47%) tested positive (Table 3). Positive samples represent 69 of the 102 tested *Polyrhachis* species. To test the hypothesis of host phylogeny influence on *Wolbachia* diversity, in the subsequent analyses we kept only the *Wolbachia*-positive samples of host species present in the *Polyrhachis* phylogeny generated by Mezger and Moreau [24]; this reduced the sample size to 73 samples. There were 43 different *Polyrhachis* species across the 73 samples. After analyzing the electropherograms to evaluate if the positive samples were single or multiple infections of *Wolbachia*, 34 of the 73 samples (47%) were determined to be single infections. Single and multiple infections of *Wolbachia* in *Polyrhachis* occurred in the same six biogeographical regions (Figure 1A); the singly infected samples were collected from 15 different countries (Figure 1B). Of the 34 singly infected samples, there were 21 different *Polyrhachis* species represented (Figure 1C).

Table 3. The allele, ST, and *Polyrhachis* host information for the 34 *Polyrhachis* samples. Allele numbers are included for each MLST gene that was able to be sequenced; blue-shaded alleles are close matches i.e., new allele variants for those loci. A dash (-) in an MLST gene column indicates that no sequence was able to be produced and thus no allele determination was made, and a dash in the ST column indicates a sample that was unable to be assigned to a sequence type. An asterisk (*) indicates the closest matching ST. The strains from samples CSM2738, MJ9280, MJ9287, MS1177, and SUL02 all had multiple "close matching" STs according to the MLST database, indicated by two asterisks (**). The country of origin and host species are shown in the last two columns.

Sample ID	MLST Allele Number					ST	Country	*Polyrhachis*
	coxA	*fbpA*	*ftsZ*	*gatB*	*hcpA*			Host Species
AS4121	2	51	45	20	47	61 *	Cambodia	*P. (Myrmhopla)* sp.
AS4132b	2	51	45	20	47	61 *	Cambodia	*P. (Polyrhachis)* sp.
AS4148a	2	51	45	20	47	61 *	Cambodia	*P. (Myrmhopla)* sp.
BB012	2	51	45	20	47	61 *	China	*P. bihamata*
CSM1854	2	51	45	20	47	61 *	Malaysia	*P. cephalotes*
CSM2738	33	61	47	34	195	**	Uganda	*P. (Myrma)* sp.
DG04	2	51	45	20	47	61 *	Philippines	*P. carbonaria*

Table 3. *Cont.*

Sample ID	MLST Allele Number					ST	Country	*Polyrhachis*
	coxA	*fbpA*	*ftsZ*	*gatB*	*hcpA*			Host Species
GM3589b	2	356	258	22	343	52 *	Malaysia	*P. (Mymothrinax)* sp.
IND05	2	52	45	20	47	61	India	*P. thrinax*
MJ9243	2	51	45	20	47	61 *	Papua New Guinea	*P. (Myrmhopla)* sp.
MJ9280	33	465	17	3	343	**	Papua New Guinea	*P. (Myrmhopla)* sp.
MJ9287	33	463	17	130	343	**	Papua New Guinea	*Polyrhachis* sp.
MS1177	33	463	17	3	343	**	China	*P. shixingensis*
RA1157	2	51	45	20	47	61 *	Laos	*P. illaudata*
RA1163	2	51	45	20	47	61 *	Laos	*P. illaudata*
RA736c	2	51	45	20	47	61 *	Thailand	*P.* cf. *laevissima*
RO122	2	51	45	20	47	61 *	Tanzania	*P. (Myrma)* sp.
SKY24	32	48	6	57	50	51 *	Singapore	*Polyrhachis* sp.
SUL02	296	97	258	3	343	**	Indonesia	*P. (Myrma)* sp.
TAS02	33	277	17	3	343	481 *	Australia	*P. hexacantha*
CSM0655	32	-	6	57	50	-	Australia	*P. rufifemur*
DG11	2	-	258	22	343	-	Philippines	*P. saevissima*
EMS2584	218	6	-	158	141	-	Solomon Islands	*P. campbelli*
EMS2617	33	-	17	3	343	-	Solomon Islands	*P. bismarckensis*
FH1101	2	-	261	20	47	-	Uganda	*P. (Myrma)* sp.
GM894	32	-	6	57	50	-	Malaysia	*P. (Myrmhopla)* sp.
KATE02	-	-	-	-	-	-	South Africa	*P. schistacea*
MJ8291	-	-	-	-	-	-	Papua New Guinea	*Polyrhachis* sp.
MJ9286	109	-	261	191	83	-	Papua New Guinea	*Polyrhachis* sp.
RA0755	32	-	-	57	50	-	Australia	*Polyrhachis* "BATH3"
RA0784	33	-	17	3	343	-	Solomon Islands	*P. (Myrmatopa)* sp.
RA1158	2	-	258	182	343	-	Laos	*P. (Myrmhopla)* sp.
RA1160	2	-	45	20	47	-	Laos	*P. illaudata*
TAS03	33	-	17	3	343	-	Australia	*P. phryne*

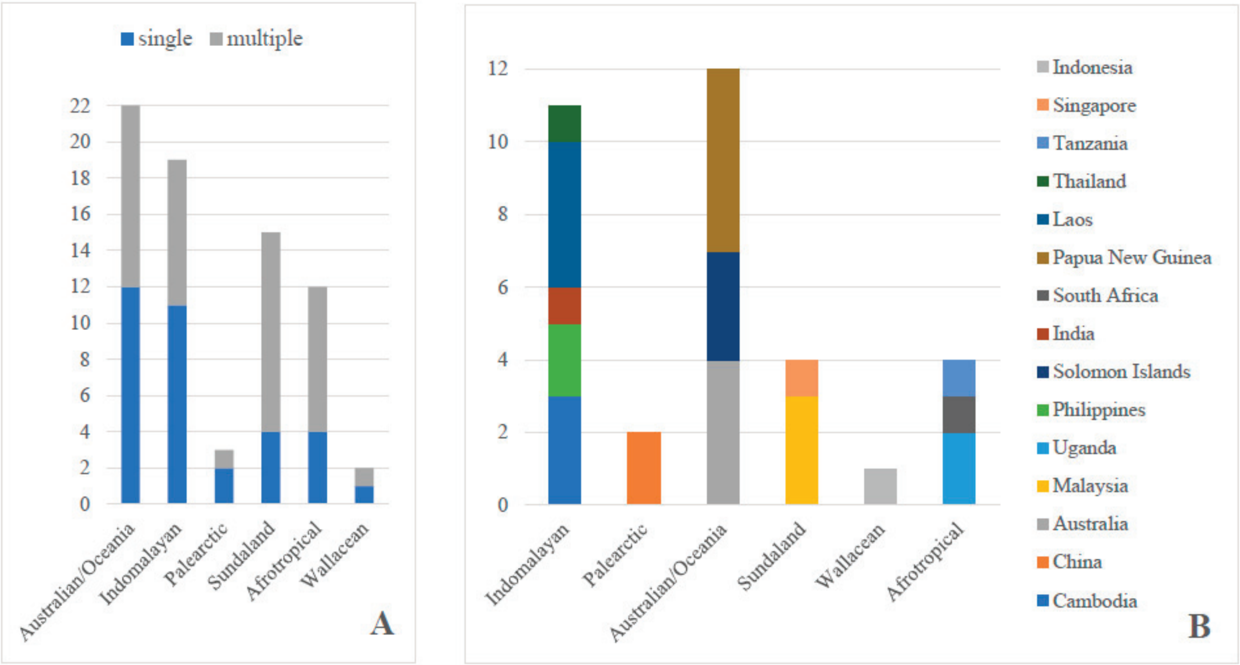


Figure 1. *Cont.*

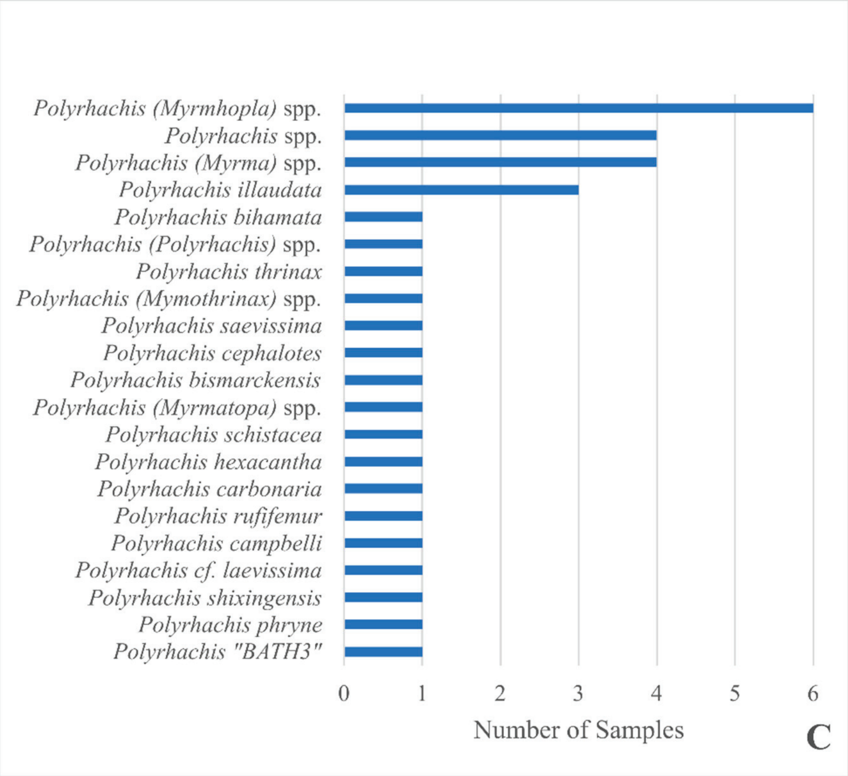
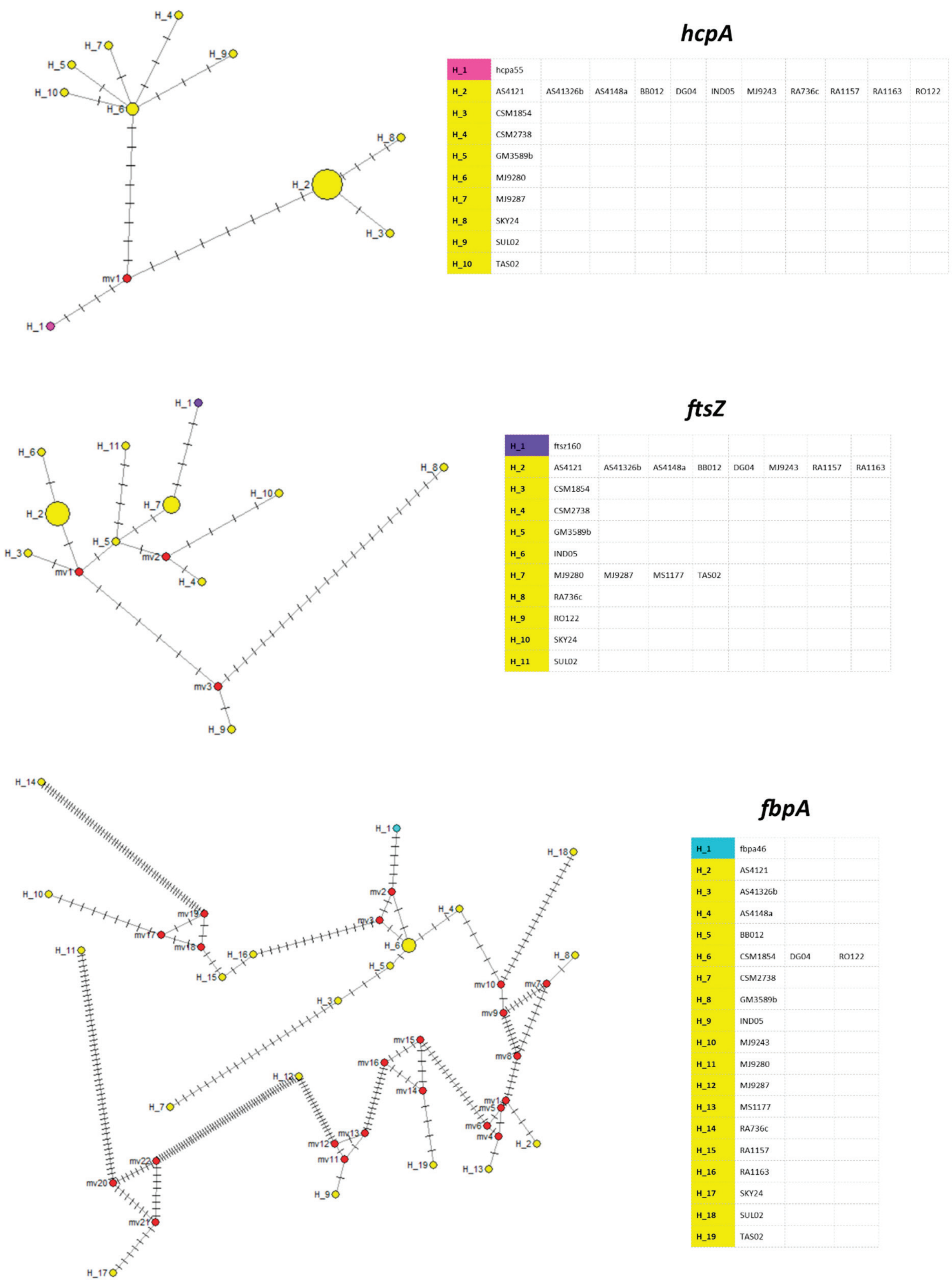


Figure 1. (**A**) Biogeographic distribution of single-strain *Wolbachia* infections and multi-strain *Wolbachia* infections within *Polyrhachis* hosts. The "Sundaland" and "Wallacean" groups are included here as separate categories to better distinguish their geography from the more northern parts of the Indomalayan realm. (**B**) Country distribution of the singly infected *Polyrhachis* samples. (**C**) *Polyrhachis* species distribution of the 34 singly infected samples. There were 21 different host species represented in this sample pool.

Twenty of the singly infected samples were able to produce viable sequences for all five MLST loci. Table 3 shows the allele and ST determinations, as well as host information, for those 20 samples. Further, Figure 2 illustrates nucleotide differences in the form of a haplotype network. Loci with no exact matches to sequences in the MLST database were considered to have new allele variants—every strain identified had at least two loci with new variants. The 14 singly infected samples that were unable to produce complete MLST alignments each had at least one locus with indeterminable Sanger results—two samples, KATE02 and MJ8291 (from *Polyrhachis shistacae* in South Africa and *Polyrhachis* sp. in Papua New Guinea, respectively) were unable to produce sequences for any of the five loci (Table 3).

The *coxA* and *gatB* loci were seemingly the most stable MLST loci for *Polyrhachis*-associated *Wolbachia* strains. Of the five loci, they had the most samples with exact matches to allele variants currently registered in the MLST database, with only five possible new allele variants found at both loci. The *ftsZ* and *hcpA* loci presented a greater number of new allele variants than either the *coxA* or *gatB* loci: seven and eight new allele variants were found at the *ftsZ* and *hcpA* loci, respectively. The *fbpA* locus presented the most genetic change of all five loci when compared to references in the *Wolbachia* MLST database—17 of the 20 samples with complete MLST profiles presented new allele variants, each of which appear to be unique. Additionally, 13 samples with incomplete MLST allelic profiles produced indeterminable sequences for the *fbpA* locus, and for 10 of these samples, *fbpA* was the only locus unable to be properly sequenced (Table 3). Ultimately, due to each strain having at least one new MLST allele variant, it appears that all 20 samples present new *Wolbachia* STs not yet seen in the MLST database.



hcpA

H_1	hcpa55										
H_2	AS4121	AS41326b	AS4148a	BB012	DG04	IND05	MJ9243	RA736c	RA1157	RA1163	RO122
H_3	CSM1854										
H_4	CSM2738										
H_5	GM3589b										
H_6	MJ9280										
H_7	MJ9287										
H_8	SKY24										
H_9	SUL02										
H_10	TAS02										

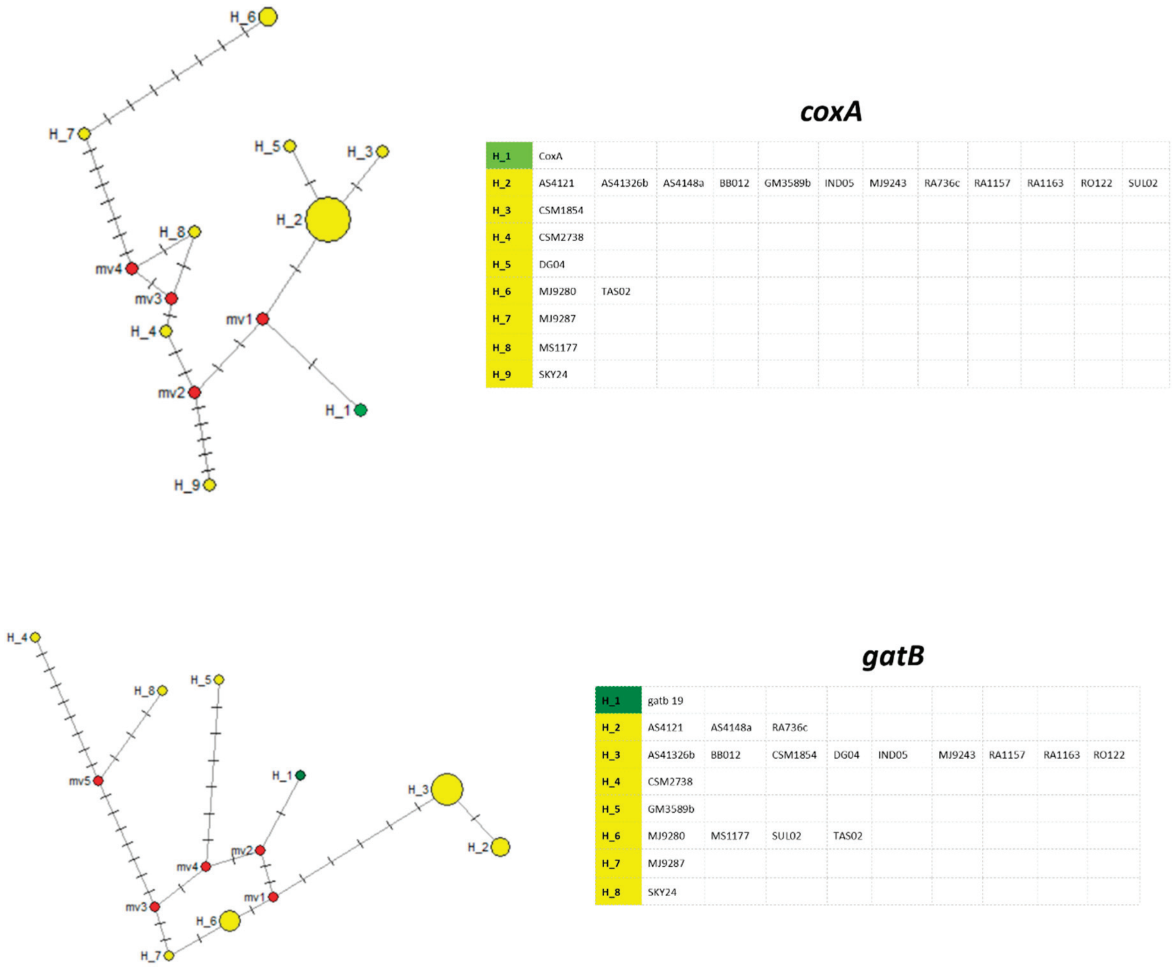
ftsZ

H_1	ftsz160							
H_2	AS4121	AS41326b	AS4148a	BB012	DG04	MJ9243	RA1157	RA1163
H_3	CSM1854							
H_4	CSM2738							
H_5	GM3589b							
H_6	IND05							
H_7	MJ9280	MJ9287	MS1177	TAS02				
H_8	RA736c							
H_9	RO122							
H_10	SKY24							
H_11	SUL02							

fbpA

H_1	fbpa46		
H_2	AS4121		
H_3	AS41326b		
H_4	AS4148a		
H_5	BB012		
H_6	CSM1854	DG04	RO122
H_7	CSM2738		
H_8	GM3589b		
H_9	IND05		
H_10	MJ9243		
H_11	MJ9280		
H_12	MJ9287		
H_13	MS1177		
H_14	RA736c		
H_15	RA1157		
H_16	RA1163		
H_17	SKY24		
H_18	SUL02		
H_19	TAS02		

Figure 2. *Cont.*



coxA

H_1	CoxA											
H_2	AS4121	AS41326b	AS4148a	BB012	GM3589b	IND05	MJ9243	RA736c	RA1157	RA1163	RO122	SUL02
H_3	CSM1854											
H_4	CSM2738											
H_5	DG04											
H_6	MJ9280	TAS02										
H_7	MJ9287											
H_8	MS1177											
H_9	SKY24											

gatB

H_1	gatb 19								
H_2	AS4121	AS4148a	RA736c						
H_3	AS41326b	BB012	CSM1854	DG04	IND05	MJ9243	RA1157	RA1163	RO122
H_4	CSM2738								
H_5	GM3589b								
H_6	MJ9280	MS1177	SUL02	TAS02					
H_7	MJ9287								
H_8	SKY24								

Figure 2. Haplotype network figures for all samples able to be assigned a sequence type. The haplotype size represents the frequency found, and black bars between haplotypes represent the numbers of nucleotide differences between haplotypes. Red dots (labelled "mv" and numbered) were added by the program as a hypothetical haplotype.

The phylogeny inferred with 90 *Wolbachia* MLST sequences (20 are the *Polyrhachis*-associated from this study, 70 are other Formicidae-associated strains from the MLST database) is shown in Figure 3. Bootstrap values ≤ 70% were hidden. No samples exhibited close relationships to any *Wolbachia* strains from the outgroup, supergroup F. Thus, all *Wolbachia* found in *Polyrhachis* belong the Supergroup A. The *Polyrhachis* strains from this study were organized into 13 genotypes, seven of which contain only one *Polyrhachis*-associated strain (either independently or with another Formicidae-associated strain). Only two of these genotypes contain samples from the same country of origin: *P.* (*Myrmhopla)* sp. and *P.* (*Polyrhachis*) sp. from Cambodia, and the two *P. illaudata* samples from Laos. Additionally, the samples from Laos are also the only grouping which contains *Wolbachia* strains from the same host species (*Polyrhachis illaudata*). In addition, all *Polyrhachis*-associated *Wolbachia* strains grouped with other *Polyrhachis*-associated strains, which suggests that there is a specificity of *Wolbachia* for *Polyrhachis* species. Six biogeographical ranges are represented in the phylogeny by the *Polyrhachis*-associated strains and all *Polyrhachis*-associated strains were grouped together with other Old World samples. Additionally, distinct clades formed to separate *Wolbachia* into Old World and New World groupings. The clades "a" and "c" contain several samples from the same biogeographical region–the Old World. Clade "b" are mixed, however contain two subclades: "b1" with samples from the Old World, and "b2" with samples from the New World (Figure 3).

Figure 3. *Wolbachia* phylogeny. Samples are named here with the following convention: "Sequence-Type_Genus_species_Country". White diamonds indicate bootstrap values ≥ 70%. The bolded taxa are the 20 *Wolbachia* strains from this study; each of the 13 genotypes they formed are numbered and highlighted in light teal. All 20 taxa belong to the supergroup A clade. The supergroup F clade is the outgroup. Stars next to the taxa within each sample indicate biogeography: Afrotropical (n = 2, blue), Australian/Oceania (n = 4, red), Palearctic (n = 2, green), Indomalayan (n = 8, purple), Sundaland (n = 3, yellow), Wallacean (n = 1, orange). Only two of the 13 genotypes formed by the *Polyrhachis*-associated strains contain multiple samples from the same biogeographical region (seven and 12). Clades "a," and "c" are the Old World clades that formed within the supergroup A taxa. Within clade "b," "b1" represents Old World taxa while "b2" represents the New World taxa that seemingly evolved from Old World taxa. The red-highlighted taxon (ST111_*Odontomachus_clarus*_USA) is the only strain to not group according to being from an Old World or New World sample.

Results of the Mantel tests indicated no correlation between both *Wolbachia* phylogenetic distance and geographic distance (Mantel statistic r: -0.030; *p*-value: 0.531) and *Polyrhachis* phylogenetic distance and *Wolbachia* phylogenetic distance (Mantel statistic r: 0.117; *p*-value: 0.302).

4. Discussion

By using such a large and biogeographically diverse host genus like *Polyrhachis*, we were able to study whether host geography, phylogeny, and species level have any observed impact on *Wolbachia* diversity. Although 12 of the strains with complete allelic profiles best matched to ST61, they are all seemingly unique since their ST determination is based upon apparently new allele variants at multiple loci. For instance, RO122 (*Polyrhachis* [subgen. *Myrma*] sp. from Tanzania) was best matched to ST61 while having a possible variant at the *ftsZ* locus, but CSM1854 (*Polyrhachis cephalotes* from Malaysia) was also best matched to ST61 while having possible new variants at the *coxA*, *ftsZ*, and *hcpA* loci (Table 3). Indeed, these two samples were divided into their own clades in the *Wolbachia* phylogeny and there appears to be no tendencies for other strains with the same best matching STs to be grouped into clades. Therefore, our results suggests that each strain is a new ST (for a total of 20 new *Wolbachia* strains being found across the *Polyrhachis* genus), implying that across *Polyrhachis* there is an incredible diversity of *Wolbachia*.

The inferred *Wolbachia* phylogeny indicates that all strains identified in *Polyrhachis* aare from supergroup A, since there were no ant samples from this study that nested within the outgroup clade. Since the 20 strains included in the phylogeny span across the entire *Polyrhachis* geographic range, this phylogeny also suggests that the *Wolbachia* found within this host genus will likely belong to supergroup A, independent of the host's geographic range.

Some studies have seen that *Wolbachia* strains may group according to being Old World or New World [7,16], and it appears that the inferred phylogeny follows this trend as well. The blue boxes in Figure 3 represent Old World clades ("a," "b," and "c") that formed among the Supergroup A taxa—taxa not included in these boxes are strains from New World samplings. Clade "b" was further divided into clades "b1" and "b2"—"b1" being Old World taxa that seemingly evolved from Old World taxa, and "b2" being New World taxa that evolved from Old World taxa. Both taxa within the supergroup F outgroup are from Old World hosts. The only taxon that did not group according to the New World and Old-World clades is an ST111 strain from another study from an *Odontomachus clarus* host in the United States (highlighted red in Figure 3). This New World taxon grouped most closely into Old World clade "b1" and closest to *Polyrhachis* clade six. All other strains sourced from New World hosts formed exclusive New World clades. To understand why this *O. clarus* strain best fit into an Old World clade—and close to a *Polyrhachis* clade—rather than with other New World samples, more sampling of *Wolbachia* from that host genus would be necessary. Clades "a" and "b" appear to share a more recent common ancestor than they do with clade "c." Interestingly, all *Polyrhachis*-associated strains fell into the more closely related "a" and "b" clades; however, the majority were grouped into clade "b" with only clades one and two being part of "a." Ultimately, all but one taxon grouped according to being New or Old World, but it was not a perfect split-grouping since there were multiple clades of either type. Regardless, this still supports the trend seen in previous studies of *Wolbachia* [7,16] wherein strains will form clades according to Old or New World geography.

Among the 70 database MLST profiles used to infer the *Wolbachia* phylogeny, there was one strain also sourced from a *Polyrhachis* host (ST51_*Polyrhachis_vindex*_Philippines). This strain showed close relation to the strain from sample SKY24 (*Polyrhachis* sp. from Singapore), and together they form a distinct clade (clade one, Figure 3). However, since these samples are sourced from different hosts and different countries, this clade suggests that *Wolbachia* diversity is not significantly impacted by host species level or biogeography. Rather, this clade (as well as the other 12 clades) suggests that strains are likely to be more closely related if they are from the same host genus since no clades formed with strains sourced from different host genera. Although they did not form a single, unified clade, the fact that all 13 clades contain exclusively *Polyrhachis*-associated strains suggests that the host's genus has some degree of influence on the associated *Wolbachia* diversity.

Of the 13 clades that the *Polyrhachis*-associated *Wolbachia* strains formed within the phylogeny, two had biogeographical consistency across the clade—clade 12 with two samples from Laos and clade seven with two samples from Cambodia—with both clades being from the Indomalayan biogeographical range (Figure 3). Interestingly, clade 12 contains the only two representative samples for the host species *Polyrhachis illaudata* (RA1163, RA1157), but based on their allelic profiles from Table 3 they are perhaps more likely to be closely related STs rather than the exact same STs. Although both strains were flagged as having possible new allele variants at the *fbpA* and *ftsZ* loci (and RA1163 with an additional variant at the *coxA* locus), they are not flagged for the same nucleotide modifications at either loci. Further verification of the genetic alterations that indicate these loci as having new allele variants would need to be conducted in order to distinguish these samples as different STs.

Polyrhachis illaudata was also the only host species able to have multiple samples with complete MLST allelic profiles sequenced. Both *P. illaudata*-associated strains formed a single clade (clade 12, Figure 3), suggesting that *Wolbachia* strains from the same host species will be more related than strains sampled from different host species. If all sampled *Polyrhachis* hosts receive expanded sampling across multiple colonies, it will be possible to determine whether this trend is true to other *Polyrhachis* hosts beyond *P. illaudata*. Thus, current results suggest that the species level of *Polyrhachis* hosts potentially impacts the observed *Wolbachia* diversity within this host genus.

The samples sourced from Cambodian *Polyrhachis* hosts present an interesting case. There was a third sample from Cambodia, AS4121 (*Polyrhachis* [subgen. *Myrmhopla*] sp.), not included in clade seven (Figure 3) with the other two Cambodian samples, AS4148a (*Polyrhachis* [subgen. *Myrmhopla*] sp.) and AS4132b (*Polyrhachis* [subgen. *Polyrhachis*] sp.)—this is seemingly because AS4148a and AS4132b both have the same new allele variant at the *gatB* locus whereas AS4121 has an already documented *gatB* allele variant (Table 3). Yet the host of AS4121 is more closely related to the host of AS4148a since they both belong to the subgenus *Myrmhopla*, while the host of AS4132 is subgenus *Polyrhachis* [24]. This instance suggests, then, that neither geography nor host phylogeny impacts the association of *Wolbachia* strains since more closely related hosts do not share similar *Wolbachia* strains and strains with hosts from the same country and geographical region do not appear in the same clade. Indeed, the Mantel test results support this since there was no correlation found between *Wolbachia* phylogenetic distance and geographical distance or between *Wolbachia* phylogenetic distance and *Polyrhachis* phylogenetic distance. However, this does not necessarily exclude the possibility that host species level could still be an impactor on *Wolbachia* diversity as seen in the *P. illaudata* clade that formed (clade 12, Figure 3).

Overall, the trends seen among the samples from Cambodia appear across the phylogeny—there is no consistent grouping of *Wolbachia* strains according to how related their host species are. For example, SUL02 and RO122 are both from subgenus *Myrma* of *Polyrhachis*, and MJ9280 and MJ9243 are both from subgenus *Myrmhopla*. Yet, in both cases, the two taxa are distantly related into two separate clades (SUL02 clade six, RO122 clade 13; MJ9280 clade 5, MJ9243 clade nine). From the perspective of host geography, there is rarely consistency for samples sourced from the same region to have more closely related strains. The three samples from Sundaland—GM3589b (*Polyrhachis* [subgen. *Myrmothrinax*] sp. from Malaysia), CSM1854 (*Polyrhachis cephalotes* from Malaysia), and SKY24 (*Polyrhachis* sp. from Singapore)—have perhaps the most distinct case of exhibiting that host geography may have no impact on the strain similarity of associated *Wolbachia*. Despite two of the three samples being from the same country, the three samples are split into three distant clades (6, 10, and one, respectively) in the phylogeny which, again, suggests that the geography of *Polyrhachis* hosts is not structuring *Wolbachia* diversity. Previous studies across genera in butterflies [18] and termites [39] similarly concluded that host geography did not impact which *Wolbachia* strains would be associated to the host. The study in termites also found that distantly related host species could have more closely related *Wolbachia* strains [39] as seen in this study, thereby supporting the notion that the phylogeny of *Polyrhachis* hosts also has no strong impact on *Wolbachia* associations. In contrast, these results may contradict the results of Kelley et al. [19], which found that the association of *Wolbachia* to *Cephalotes atratus* was impacted by host biogeography. Yet, this may not be a true contradiction if it can be confirmed that across a single *Polyrhachis* species, host biogeography impacts *Wolbachia* diversity (which is seemingly seen in the *P. illaudata* clade [clade 12, Figure 3]) since the study by Kelley et al. [19] took place in a single host species.

For the third host factor (host species level), some results suggest that it has an impact on *Wolbachia* diversity. However, as discussed with the case of *P. illaudata*, expanded sampling of each *Polyrhachis* species is required to verify the observed trends. In the initial sample pooling, there were multiple instances where the same host species was sampled from several colonies. However, once removing samples containing multiple strains of *Wolbachia* the sample pool was reduced by over 50% and many of these multi-colony samplings were lost. These samples were removed because multiple strains in one sample cannot be parsed into individual strains.

We found that *Polyrhachis*-associated *Wolbachia* strains will form exclusive clades distinct from strains of other host genera. In other words, *Polyrhachis*-sourced strains of *Wolbachia* will only form clades with other *Polyrhachis*-associated strains. It was also found that samples of the same host species were sometimes grouped into the same clade. This suggests that there is some level of restructuring occurring at the hosts' species level. Beyond the *Polyrhachis* genus, there also appears to be separation of *Wolbachia* strains based upon being either Old World or New World, wherein taxa from the Old World will not typically be grouped into a closely related clade with New World taxa and vice versa.

Ultimately, the results of this study suggest that host biogeography and phylogeny do not have any significant impact on which strains of *Wolbachia* will be associated to the *Polyrhachis* host species, though our findings suggest that the *Polyrhachis* species level may have some effects on *Wolbachia* strain. Further work on the impact of geography of *Wolbachia* infection would benefit from incorporation of more data on the host's current range and historical biogeography, which were not included in this study. Additionally, horizontal transfer of *Wolbachia* between hosts is not common, but has been observed, primarily in related hosts [7]. Horizontal transfer events may affect the results of phylogenetic analyses, particularly in comparisons of the host's and *Wolbachia* phylogenies.

Our findings from this study, particularly our observation that some *Wolbachia* strains may be associated with particular *Polyrhachis* species, highlight the impacts that microbial diversity can have on ant diversity, and vice versa. The presence of vertically-transmitted symbionts like *Wolbachia* suggests the possibility of a microbial impact on evolution; coevolution of ants and microbes over long time-scales has already been observed in some ant genera, in some cases allowing the ants to pursue diets or occupy niches not previously available to them. While our findings about *Polyrhachis* help to elucidate more of the ways that symbionts can impact ant diversity, still, little is known about the microbial partners of the majority of ant genera. Studies of this nature are crucial in understanding the many factors that contribute to present-day ant diversity and may provide insights into the ways that the associates of ants may shape the evolutionary future of their hosts.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d15030348/s1, File S1: Sample ID, host species, and country of origin for all for each of the initial 237 samples screened for *Wolbachia*. The presence of *Wolbachia* is indicated by the *wsp* column ("+" indicates positive for *Wolbachia*, "−" indicates negative for *Wolbachia*).

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Impact of Nesting Mode, Diet, and Taxonomy in Structuring the Associated Microbial Communities of Amazonian Ants

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Abstract: Studies of ant biodiversity are important to understand their group better, as well as to extend our knowledge on the evolution of their associated organisms. Host-associated microbial communities, and particularly bacterial communities, are shaped by different host factors such as habitat, diet, and phylogeny. Here, we studied the structures of bacterial and microbial eukaryote communities associated with Amazonian ants collected from two habitats: the rainforest and the city. We collected 38 ant species covering a large taxonomic range, and we used 16S rRNA and 18S rRNA amplicon sequencing to study the impact of the host's ecological and phylogenetic factors on their microbial communities. Our results show that (1) habitat does not structure ant microbial communities, (2) ant diet and nesting mode impact bacterial communities, while only nesting mode structures microbial eukaryote communities, and (3) microbial diversity is not correlated with host phylogeny, although several ant genera have conserved bacterial communities. As we continue to uncover the diversity and function of insect-associated microbes, this work explores how host ecology and evolutionary history shape ant microbial communities.

Keywords: Formicidae; insect; 16S rRNA; 18S rRNA; amplicon sequencing; metabarcoding

1. Introduction

One of the major goals in community ecology is to understand the diversity, maintenance, and consequences of biological interactions between different communities, especially in increasingly fragmented ecosystems [1]. Studies of ant biodiversity are not just relevant to an understanding of this globally dominant faunal group but also to an understanding of the evolution of associated organisms. Every organism has biological needs that can only be met under specific environmental conditions. Habitat filtering is one of the processes invoked to explain why, in a given habitat, only individual species possessing suitable traits for this specific habitat can persist [2,3]. Organisms rely on specific factors to survive and thrive. When organisms living in the same local environment depend on the same resources, they engage in competition for those resources, often leading to competitive exclusion [4]. In contrast, when certain organisms living in the same local environment do not depend on the same resources, they can coexist without competition, and this process is described as niche differentiation [3,5].

Microbial communities are ubiquitous and can be found in all types of environments, even the most extreme [6,7]. Environmental factors have been shown to have strong impacts on microbial richness and diversity in species-rich biomes [8–13]. Microbial communities are also often found in associations with diverse host organisms. In insects, symbiotic bacteria have shaped the evolution of their hosts and are key components in providing basic functions to their host [14,15]. Mutualistic bacteria are known to supplement nutritionally insufficient host diets [16–19]. They also help maintain and improve their host's health and immune system [20,21] and participate in broadening their host's environmental tolerance [22–26]. On the other hand, parasitic bacteria can manipulate host reproduction [27] and limit host dispersion [28,29].

Compared to the study of insect-associated bacteria, scientific research on insect-associated microbial eukaryotes is not as widespread. Indeed, scientists tend to study insect-associated eukaryotes only when an insect shows outward signs of infection. Common signs of insect infection include behavioral and morphological changes such as climbing on the top of the canopy or a change in color and size. Many microbial eukaryotes including pathogenic fungi, nematodes, and protists have been identified as being associated with insects. Ascomycota fungi infect a wide range of insect hosts, and after killing an insect, often feed on its cadaver [30]. Basidiomycota and Chytriodiomycota fungi are known parasites of scale insects [31,32] and insect eggs [33,34], whereas Zygomycota occur on various insects without showing signs of host pathogenicity [35–38]. Regarding nematodes, several *Heterorhabditis* and *Steinerma* species have been reported as pathogens of coleopterans [39], dipterans [40], lepidopterans [41], and orthopterans [42]. Other nematodes also use insects as vectors but do not necessarily kill them. This is the case for several *Brugia* [43,44], *Dirofilaria* [45], *Onchocerca* [46], and *Wuchereria* [47] species, which use mosquitoes or midges as vectors to transmit diseases. Finally, among the stramenophiles, oomycota [48,49] are known to infect insects. Mutualistic associations between eukaryotes and insects are also widespread, especially between insects and fungi [50]. One of the most common examples is the cultivation of fungi for nutrition, which exists in several species of ants [51], beetles [52], and termites [53]. Mutualistic non-nutritional associations have also been demonstrated in numerous tripartite symbioses between the ascomycete (order: Chaetothyriales) fungi–plant–ant [54–56]. Another important aspect of mutualism between insects and fungi is the dispersion of fungi. Many insects have even evolved to have specific organs to carry fungal spores [57,58] or carry them in the gut [59]. Finally, insects also benefit from antimicrobial molecules produced by some fungi [60]. Mutualistic associations between insects and nematodes are rare, but a few examples are known [61], and some nematodes may have mutualistic associations with bacteria within insect hosts [62]. However, relying on observational methods limits the study of microbial eukaryote diversity in insects.

Among insects, ants represent a species-rich clade with different ecologies and provide many ecosystem services [63]. Symbiotic bacteria are thought to be partly responsible for their evolutionary success. For example, it has been hypothesized that symbiotic bacteria allow ants to dominate rainforest canopies [64,65]. Many studies have shown that symbiotic bacteria in ants differ depending on the ant diet, especially between herbivorous and carnivorous ants [66]. Predatory army ants possess specialized Firmicutes and Entomoplasmatales gut bacteria, which are common to all lineages of army ant with a symbiosis dating from the Cretaceous period and which are likely socially transmitted by trophallaxis or coprophagy [67,68]. The functional role of these bacteria in army ants is not yet known due to the lack of available symbiont genomes from army ants, but a nutritional role for these symbionts has been suggested [68]. Herbivorous turtle ants rely on symbiotic gut bacteria to recycle nitrogen into amino acids [69,70], while the Camponotoni tribe benefits from amino acid production by the obligate intracellular symbiont *Blochmannia* [19,71]. In addition, many studies have studied the bacterial communities of specific ant clades, such as *Daceton* [72], *Paraponera* [73], ponerine ants [74], *Pseudomyrmex* [75], *Solenopsis* [76], and spiny ants [77]. Several bacteria symbionts are associated across these multiple ant clades. For example, Acetobacterales, Entomoplasmatales, and Rhizobiales are bacterial orders commonly found in ant guts. Their role in ants is not fully understood, but Acetobacterales might be involved in larval immune function [78] and development [79], while Entomoplasmatales might be involved in chitin processing of insect prey [80], and Rhizobiales might be involved in protein degradation [81] and urea recycling pathways [82]. The bacteria symbiont *Wolbachia* is also found across several ant clades and may induce reproductive changes in hosts [83] and vitamin B supplementation [84], although in most cases, there appear to be no positive or negative effects of *Wolbachia* infection for ants. Several microbial eukaryotes are also known to be associated with ants. The fungal class Sordariomycetes contains several species that infect ants, like the pathogen fungus infecting fungus-growing ants [85] or the fungus causing zombie ants [86]. Among the nematodes, many families have been shown to infect different ant species [87].

In this work, we aimed to study the factors structuring the microbial communities associated with Amazonian ants. We collected 38 ant species in French Guiana from a wide phylogenetic range spanning several ant subfamilies. We focused on the microbial communities associated in terms of both abundance and diversity using qPCR and 16S rRNA and 18S rRNA amplicon sequencing. Our sampling strategy had three objectives. First, a wide phylogenetic range was chosen to test correlations between microbial communities and the evolutionary history of their hosts. Second, we collected species possessing different ecological traits (diet and nesting mode) which were assessed for their correlations with bacterial communities. Third, among the 38 collected ant species, nine species were collected from both rainforest and city habitats to evaluate the impact of the environment on microbial communities. The results of this work contribute to our understanding of the different factors structuring the microbial communities associated with Neotropical ants.

2. Materials and Methods

2.1. Sample Collection

Samples were collected in March 2018 from two sites in French Guiana in the Nouragues Rainforest Reserve and the city of Cayenne (Figure S1). A total of 49 ant colonies were collected, representing 38 ant species from 18 genera spanning eight subfamilies. Several workers were collected from each colony with pincers and stored in small individual tubes containing ethanol. Then, tubes were kept at −20 °C until DNA extraction. Collected ant samples were identified up to the species level, when possible, in the field with a magnifying glass. Identifications were further refined in the lab with a binocular magnifier using the key to subfamilies of the Neotropical region from Baccaro et al. [88] The list of collected samples and different phylogenetic (subfamily, genera) and ecological factors (habitat, nesting mode, and diet) chosen is presented in Supplementary File S1. For 44 of the 49 sampled colonies, we also collected a sample of the nest material or foraging area. Nest samples were collected and stored in small sterile bags. Then, bags were kept at −20 °C until DNA extraction. Vouchers for all samples were deposited in the Cornell University Insect Collection (Ithaca, New York, NY, USA).

2.2. DNA Extractions

DNA extractions from single ants and nest samples were performed using the DNeasy PowerSoil Kit (Qiagen, Germantown, MD, USA) following the manufacturer's protocol with a few modifications before the first step. For the DNA extractions, a single worker from each collected nest was used, and between 0.1 and 0.25 g of nest material was used. First, ant samples and nest samples were introduced into sterile 1.5 mL Eppendorf tubes. Then, 1 mL of liquid nitrogen was added to each Eppendorf tube, and each sample was immediately manually crushed in the tube with a sterile micropestel. To avoid any contamination between samples during this step, a new sterile micropestel was used for each sample tube. The crushed samples were then transferred to tubes with 500 µL PowerSoil bead solution. Following the procedure suggested by Rubin et al. [89], 60 µL of solution C1 and 100 µg of proteinase K were added to each PowerSoil tube, and tubes were incubated at 56 °C overnight. The extraction then proceeded following the DNeasy PowerSoil Kit protocol. The same protocol was followed with 4 blank tubes containing no ant or nest sample, and these served as negative controls. Filtered pipette tips and sterile techniques were used in every step to avoid contamination [90]. All DNA extractions were quantified via Qubit to verify the success of the DNA extraction. The Qubit quantification was performed with the High Sensitivity Assay Kit (Life Technologies Corp., Carlsbad, CA, USA).

2.3. DNA Amplification

Amplification of the V4 region of bacterial 16S rRNA for the ant and nest dataset and V1–V2 region of 18S rRNA of the ant dataset as well as Miseq sequencing of each DNA dataset were performed by the Argonne National Laboratory (Lemont, IL, USA). Negative controls were also processed following the same protocol. Following the protocol suggested in the Earth Microbiome Project (EMP) (http://www.earthmicrobiome.org/protocols-and-standards/16S/, accessed on 9 December 2019), amplifications for the V4 region of 16S rRNA were performed using 515F (5′-GTGCCAGCMGCCGCGGTAA) and 806R (5′-GGACTACHVGGGTWTCTAAT) primers, as described by Caporaso et al. [91], and amplifications for the V1–V2 region of 18S rRNA were performed using F04 (5′-GCTTGTCTCAAAGATTAAGCC) and R22 (5′-GCCTGCTGCCTTCCTTGGA) primers, as described by Creer et al. [92]. Each PCR reaction contained 12 μL of DNA-free PCR water, 10 μL of 5 Prime HotMasterMix 1X, 1 μL of 5 mM forward primer, 1 μL of 5 mM Golay barcode tagged reverse primer, and 1 μL of extracted DNA. The amplification conditions were as follows: 94 °C for 3 min, with 35 cycles at 94 °C for 45 s, 50 °C for 60 s, and 72 °C for 90 s, and a final cycle of 10 min at 72 °C. Each PCR reaction was performed in triplicate. Electrophoresis with 1% agarose gel was performed to confirm the efficiency of the amplification. Amplification samples from ants and their nests were pooled separately with each pool containing a 100 μL samplr, and these were cleaned using the QIAquick PCR Purification Kit (Qiagen, USA) following the manufacturer's instructions. The molarity of the pool was determined and diluted down to 4 nM, denatured, and then diluted to a final concentration of 6.75 pM with 10% PhiX for sequencing. Three separate runs (one for the 16S rRNA ant dataset, one for the 16S rRNA nest dataset and one for the 18S rRNA ant dataset) were performed with the MiSeq Illumina V3 Reagent Kit 600 Cycles (300 × 300) using the custom sequencing primers and procedures described in the Supplementary Methods by Caporaso et al. [91] for 16S rRNA and Creer et al. [92] for 18S rRNA.

2.4. Bacterial Quantification

Bacterial quantification of each sample was performed through qPCR quantification (Thermo Fisher Scientific, Waltham, MA, USA) on real-time CFX Connect equipment (Bio-Rad). A SYBRAdvanced 2X (Bio-Rad) SYBR green supermix and 2 μL of extracted DNA were used to verify the total amount of bacteria present in each sample. Amplification of the V4 region of 16S rRNA in the qPCR was performed using 515F and 806R primers, as described by Caporaso et al. [91], following the protocol suggested in the Earth Microbiome Project (EMP) (http://www.earthmicrobiome.org/protocols-and-standards/16S/, accessed on 9 December 2019). Each qPCR reaction was performed in triplicate. Standard curves were generated from serial dilutions of linearized plasmids containing *E. coli* 16S rRNA inserts, following the same parameters of Rubin et al. [89]. All triplicate qPCRs values were satisfactory and had R2 values from 70% to 100%. The mean triplicate value for each sample was used in the analysis. Negative controls were also analyzed following the same protocol.

2.5. Bacterial and Microbial Eukaryote Diversity

Demultiplexing of sequences and taxonomic assignments was performed separately for the ant and nest sequences but following the same protocol. Demultiplexed sequences were analyzed using Qiime2-2019.1 [93] with the plugin demux (https://github.com/qiime2/q2-demux, accessed on 22 August 2020). Sequence quality control and feature table construction were performed through the dada2 plugin [94]. Taxonomic assignment was conducted with the SILVA_132_QIIME database [95], and the ASVs (amplicon sequence variants) were selected with 99% identity. To generate the taxonomy table, paired-end sequence reads were trimmed in the V4 region of 16S rRNA with the 515F/806R primers and in the V1–V2 region of 18S rRNA with the F04/R22 primers. Thereby, our own classifier was created using the "feature-classifier fit-classifier-naive-bayes" command.

Once the classifier was obtained, the reads (rep-seqs) were classified by taxon using the "feature-classifier classify-sklearn" command [96].

The filtration of contaminants (blank samples) in the datasets was performed with the Decontam package [97] of R software version 4.02 [98]. In this package, the prevalence method was used to remove the contaminant sequences from our samples. The decontaminated datasets were inserted back into Qiime2 [93] to filter all mitochondria and chloroplast sequences from the datasets. Hymenopteran sequences were also excluded from the 18S rRNA table so that sequences coming from the ant host did not appear in our analyses. The alignment was performed using the align-to-tree-mafft-fasttree command [99] to reconstruct the microbial phylogenies.

The alpha and beta diversity analyses were performed by using the "qiime diversity core-metrics-phylogenetic" command. Beta diversities were visualized using the visualization interface https://view.qiime2.org/, accessed on 22 August 2020. Alpha diversity metrics computed were the Shannon index, the Pielou's evenness index, the Faith's phylogenic diversity, and the number of ASVs. Beta diversity metrics computed were the Jaccard similarity index, Bray–Curtis dissimilarity, unweighted unifrac distance, and weighted unnormalized unifrac distance. The Jaccard similarity index gauges the similarity and diversity without accounting for the abundance [100]. The Bray–Curtis dissimilarity is a measure of the overabundant taxa [101]. The unweighted unifrac distance measures the unique branch length [102], and the weighted unnormalized unifrac distance estimates the abundance but does not correct for different evolutionary rates between taxa [103].

2.6. Statistical Analysis

Statistical analyses of bacterial quantification were performed using one-way ANOVAs (Analysis of Variance) with PAST software version 4.02 [104]. Statistical analyses of alpha diversity and beta diversity were performed on Qiime2-2019.1 [93] using the "diversity alpha-group-significance" command with the pairwise Kruskal–Wallis methods, and the "diversity beta-group-significance" command with the pairwise PERMANOVA method and 999 permutations. The bacterial quantification and alpha diversity results were visualized using PCoAs (Principal Coordinates Analysis) generated with the R packages "ggpubr" version 0.3.0 [105] and "PMCMR" version 4.3 [106]. The beta diversity results were visualized using PCoAs with the R packages "ggplot2" version 3.3.0 [107] and "ggfortify" version 0.4.10 [108]. The correlation between ant evolutionary history and microbial composition was determined using Mantel tests using the R package phytools [109]. The contribution of each ASV to each sample at the order level (for the 16S rRNA datasets) and at the phyla level (for the 18S rRNA dataset) was determined using the "qiime taxa barplot" and "qiime taxa collapse" commands on Qiime2-2019.1 [93]. SIMPER (Similarity Percentage) analyses were performed using the PAST software version 4.02 [104] and visualized using boxplots with the R packages "ggpubr" version 0.3.0 [105] and "PMCMR" version 4.3 [106].

3. Results

3.1. 16S rRNA Assessing Sequencing Quality

A total of 49 ant samples and 44 nest samples were sequenced with four control samples, two for each sample type (Supplementary File S1). For the ant samples, a total of 1,879,666 reads were sequenced. After rarefaction at a sampling depth of 4500 reads, three samples were removed due to a low number of reads, resulting in a total of 5908 ASVs recovered from the 46 ant samples, ranging from 7548 to 80,226 reads, with a mean frequency of 40,842 reads per sample. The rarefaction curve shows that sequences from every sample reached a plateau, indicating that most of the bacterial diversity was recovered (Figure S2A). Three samples (CSM3695a, CSM3695b and PJF10) were excluded from the dataset because they did not reach the minimum sampling depth of 4500 reads. Nest samples were rarefied to 9000 reads (Figure S2B), and in total, 19,934 ASVs were obtained from 2,350,607 reads sequenced in the 44 nest samples, ranging from 9130 to 76,765 reads, with a mean frequency of 53,442 reads per sample.

3.2. 16S rRNA Alpha Diversity

First, we tested for dissimilarities in bacterial alpha diversity across ant habitat, nesting mode, diet and subfamily. There was no differences in bacterial alpha diversity between rainforest ants and city ants (Figure S3; *Shannon: H = 0.017, p-value = 0.895; Pielou: H = 0.002, p-value = 0.965; Faith: H = 0.213, p-value = 0.644; ASV: H = 0.148, p-value = 0.700*), However, there were differences in bacterial alpha diversity across nesting modes (Figure S4; *Shannon: H = 15.563, p-value = 4.17E-04; Pielou: H = 13.742, p-value = 0.001; Faith: H = 12.159, p-value = 0.002; ASV: H = 16.350, p-value = 2.817E-04*), diets (Figure S5; *Shannon: H = 4.316, p-value = 0.116; Pielou: H = 6.431, p-value = 0.040; Faith: H = 0.424, p-value = 0.809; ASV: H = 0.456, p-value = 0.796*) and subfamilies (Figure S6; *Shannon: H = 18.096, p-value = 0.012; Pielou: H = 17.280, p-value = 0.016; Faith: H = 12.113, p-value = 0.097; ASV: H = 17.834, p-value = 0.013*).

The SIMPER (Similarity Percentage) analysis was performed to determine the most abundant bacterial orders in the ant samples. The taxa bar plot of the bacterial relative abundance exhibited very diverse patterns across ant genera (Figure 1), and across the habitat, nesting mode, and diet (Supplementary File S2). The 15 most abundant bacterial orders found in the host samples were, in order, Rickettsiales, Rhizobiales, Enterobacteriales, Acetobacterales, Lactobacillales, Burkholderiales, Xanthomonadales, Erysipelotrichales, Flavobacteriales, Pseudomonadales, Corynebacteriales, Entomoplasmatales, Opitutales, Sphingomonadales, and Micrococcales (Supplementary File S3).

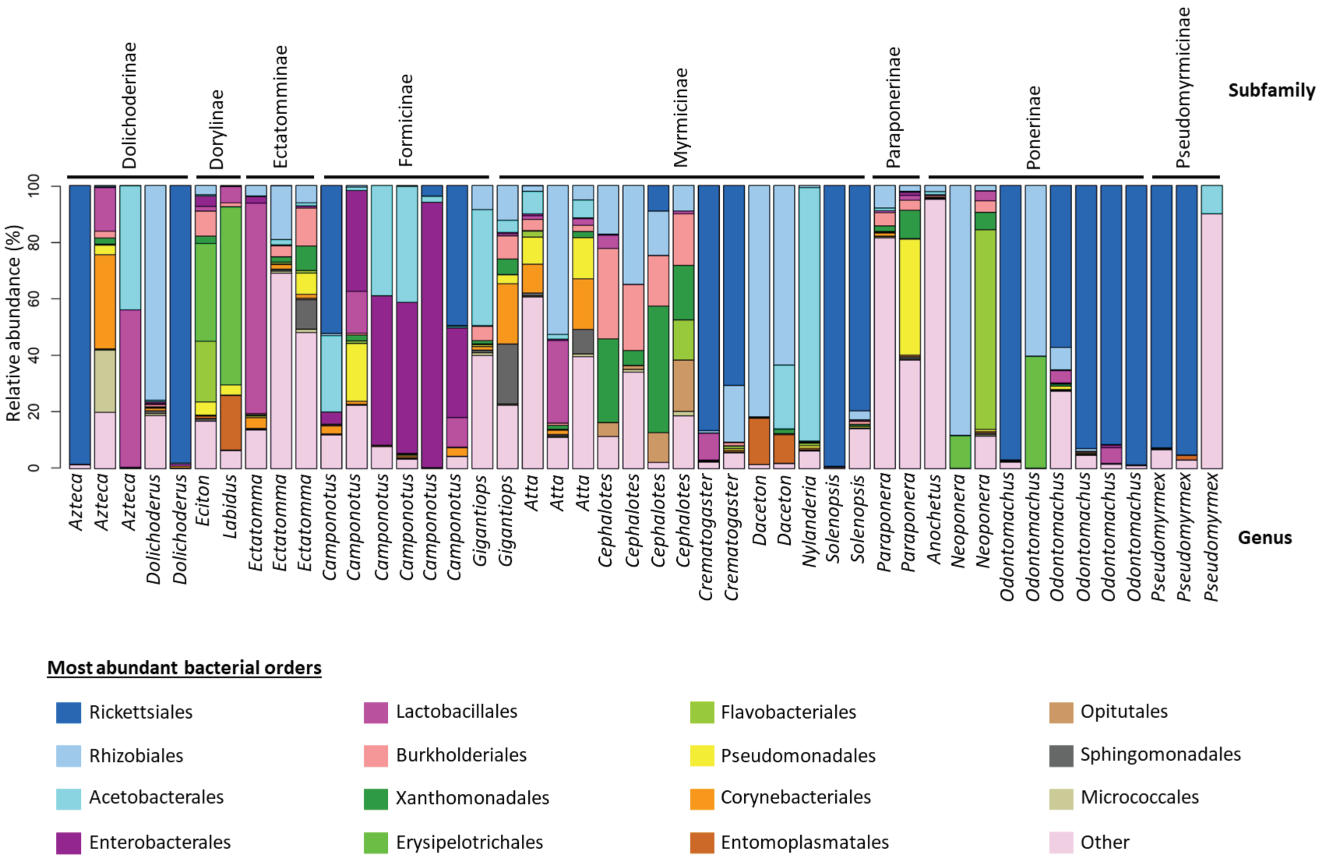


Figure 1. Taxa bar plot based on the relative abundance classified by ant subfamily and genus. Relative abundance bars are colored by bacterial order percentage. Only the 15 most abundant bacterial order associated with Amazonian ants are listed. The less abundant bacterial orders are grouped under the term "Other".

Rickettsiales were present (at least 10% of relative abundance) in every habitat, diet, and nesting mode tested (Supplementary File S2). Rhizobiales were also very common, being found across carnivorous and herbivorous ants (carnivorous: 21.38%; herbivorous: 23.63%) as well as in ants nesting in trees (21.36%) and in the ground (10.25%) and in rainforest ants (16.87%). Acetobacterales and Enterobacteriales were present in omnivorous ants (respectively, 13.76% and 12.71%) and ants nesting in rotten logs (respectively, 24.3% and 28.69%). Acetobacterales were also found in city ants (10.93%). Burkholderiales and Xanthomonadales were common in herbivorous ants (respectively, 11.00% and 10.98%), while Erysipelotrichales were only found in carnivorous ants (9.29%).

The bacterial relative abundance showed different patterns across ant genera but was relatively consistent within the same genus. This was especially true for *Camponotus*, *Cephalotes*, *Crematogaster*, *Daceton*, *Solenopsis,* and *Odontomachus* (Figure 1). *Crematogaster* and *Odontomachus* were found to have very conserved bacterial compositions, consisting primarily of Rickettsiales (respectively 78.58% and 72.87%) and Rhizobiales (respectively 10.85% and 11.60%). Rickettsiales was also found to be the most abundant bacterial order in *Solenopsis* (59.59%). *Camponotus* were dominated by Enterobacteriales (45.19%), but also contained Acetobacterales (18.47%) and Rickettsiales (17.60%). *Daceton* were dominated by Rhizobiales (72.49%) but also had Entomoplasmatales (13.20%) and Acetobacterales (11.31%). *Cephalotes* were associated with Xanthomonadales (24.65%), Burkholderiales (22.71%), and Rhizobiales (19.19%). In addition, some of the most abundant bacterial orders were only found in a few ant genera. Erysipelotrichales (Firmicutes) were only found in *Eciton* (34.47%) and *Labidus* (62.80%). *Eciton* also contained Flavobacteriales (21.37%), while *Labidus* also possessed Entomoplasmatales (19.24%).

3.3. 16S rRNA Beta Diversity Analysis

The bacterial diversity of the ants was compared with the bacterial diversity of their nest samples, and both the Jaccard and unUniFrac distances showed statistical differences (Figure S7A,B; *Jaccard: pseudo-F = 2.967, p-value = 0.001; unUniFrac: pseudo-F = 8.162, p-value = 0.001*). The forest/city dataset showed the same statistical differences (*Jaccard: pseudo-F = 1.919, p-value = 0.001; unUniFrac: pseudo-F = 3.535, p-value = 0.001*). In the rest of the manuscript, we only focused on the analyses of the ant samples.

Then, we tested for dissimilarities in bacterial diversity across ant habitat, diet, nesting mode, and taxonomy. All statistics for each beta diversity metric are reported in Supplementary File S4. The bacterial qPCR quantification analysis revealed no statistical differences in bacterial quantification associated with ants across ant taxonomy, diet, nesting mode, and habitat (Figure S8; *df = 46; Habitat: t-test, t = 1.438, p-value = 0.157; Diet: ANOVA, F = 0.193, p-value = 0.956; Nesting mode: ANOVA, F = 0.128, p-value = 0.957; Subfamily: ANOVA, F = 0.400, p-value = 0.813*). There were no statistical differences either in the forest/city dataset across ant habitat, diet, nesting mode, or taxonomy (*df = 20; Habitat: t-test, t = 1.345, p-value = 0.194; Diet: ANOVA, F = 1.761, p-value = 0.106; Nesting mode: ANOVA, F = 1.823, p-value = 0.141; Subfamily: ANOVA, F = 0.331, p-value = 0.941*).

The bacterial beta diversity did not differ between rainforest ants and city ants (Figure 2A,B, Supplementary File S4; *Bray-Curtis: pseudo-F = 0.920, p-value = 0.619; wUniFrac: pseudo-F = 0.945, p-value = 0.502*); however, the nesting modes ground nesting, rotten log, and tree nesting all showed statistically different bacterial diversity results (Figure 2C,D, Supplementary File S4; *Bray-Curtis: pseudo-F = 1.473, p-value = 0.005; wUniFrac: pseudo-F = 2.217, p-value = 0.002*). Overall, the diet was a strong factor in differential bacterial diversity (Figure 2E,F; *Bray-Curtis: pseudo-F = 1.361, p-value = 0.018; wUniFrac: pseudo-F = 1.675, p-value = 0.029*). Carnivorous ants were shown to have different bacterial communities than herbivorous ants, which had different bacterial communities to omnivorous ants, but no difference was found between carnivorous and omnivorous ants (Supplementary File S4). Concerning ant taxonomy, the results were statistically different across subfamilies (Figure 3A,B; *Bray-Curtis: pseudo-F = 1.368, p-value = 0.018; wUniFrac: pseudo-F = 1.715, p-value = 0.025*). More specifically Dorylinae, Formicinae, Myrmicinae, and Ponerinae had different bacterial compositions to other subfamilies (Supplementary File S4). To test whether these dissimilarities are due to the ants' evolutionary histories, we performed Mantel tests comparing the bacterial diversity with the host phylogeny [110]. However, the Mantel tests showed no correlation between bacterial diversity and ant phylogeny for both the Bray–Curtis and wUniFrac distances (Figure 3C; *Bray–Curtis: F = 1.473, p-value = 0.516; wUniFrac: F = 2.217, p-value = 0.498*). The forest and city datasets gave the same statistical results for every factor tested (Supplementary File S4).

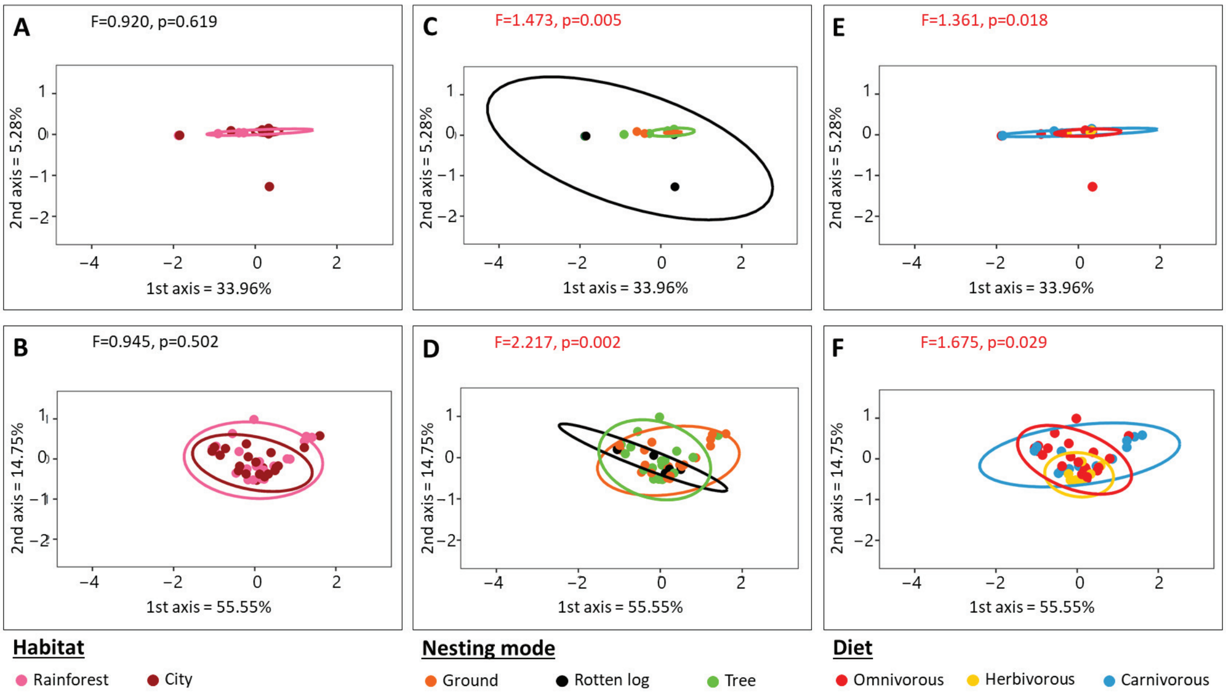


Figure 2. Differences in bacterial communities visualized with PCoAs across ant habitats (**A**,**B**), nesting modes (**C**,**D**), and diets (**E**,**F**). The distances used were the Bray–Curtis (**A**,**C**,**E**) and wUniFrac (**B**,**D**,**F**). The statistical *p*-values were obtained with pairwise PERMANOVAs. The circles represent confidence intervals of 0.95.

3.4. 16S rRNA ASV Similarity Percentage Analysis

Next, we used a SIMPER analysis to identify the most abundant bacterial orders responsible for the dissimilarities observed previously. This analysis identified 15 bacterial orders responsible for the differences. All statistics for the SIMPER analyses are presented in Supplementary File S5. Erysipelotrichales were more abundant in carnivorous ants (Figure S9A), while Rhizobiales were more abundant in herbivorous ants (Figure S9C). However, both were also more abundant in ground nesting ants than in rotten log nesting ants (Figure S9B,D). Lactobacillales were more abundant in ground nesting ants compared to ants nesting in rotten logs, while Rickettsiales had a higher abundance in rotten log nesting ants than in tree nesting ants (Figure S9F,H). Entomoplasmatales were more abundant in carnivorous ants than in herbivorous ants (Figure S9E). Finally, Acetobacterales had a greater abundance in city ants than in rainforest ants (Figure S9G). However, there was no statistical difference in any bacterial order between ant subfamilies. Pairwise Kruskal–Wallis comparisons for all the discussed bacterial orders are presented in Supplementary File S6.

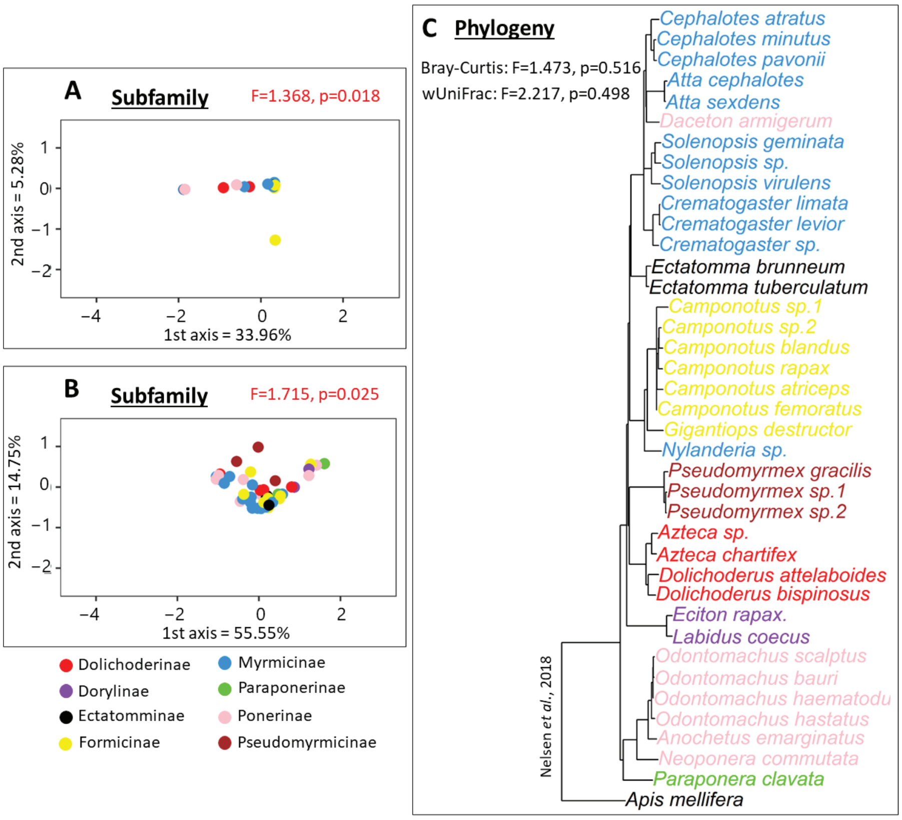


Figure 3. Differences in bacterial communities visualized with PCoAs across ant subfamily (**A**,**B**) and phylogeny (**C**). The distances used were the Bray–Curtis (**A**,**C**) and wUniFrac (**B**,**C**). The statistical *p*-values were obtained with pairwise PERMANOVAs.

3.5. 18S rRNA Assessing Sequencing Quality

We assessed the eukaryote diversity associated with the same 49 Amazonian ants used for the bacterial diversity analysis (Supplementary File S1). In the raw dataset, there was a total of 21,719 reads. After rarefaction of the samples to 199 reads, 33 samples were excluded from the dataset because they did not reach the minimum sampling depth (Figure S10). In total, from the 16 remaining samples, we obtained 118 ASVs from 20,185 reads ranging from 199 to 7815 reads with a mean frequency of 1261 reads per sample.

3.6. 18S rRNA Alpha and Beta Diversity Analysis

Alpha diversity analyses were performed using Qiime2 software to test for dissimilarities in eukaryote diversity in our dataset. There was no differences in microbial eukaryote alpha diversity between rainforest ants and city ants (Figure S11; *Shannon: H = 0.540, p-value = 0.462; Pielou: H = 0.011, p-value = 0.916; Faith: H = 1.103, p-value = 0.294; ASV: H = 1.773, p-value = 0.183*), nesting mode (Figure S12; *Shannon: H = 4.352, p-value = 0.360; Pielou: H = 5.593, p-value = 0.200; Faith: H = 3.643, p-value = 0.456; ASV: H = 2.581, p-value = 0.630*), diet (Figure S13; *Shannon: H = 3.824, p-value = 0.148; Pielou: H = 1.283, p-value = 0.526; Faith: H = 3.107, p-value = 0.212; ASV: H = 4.634, p-value = 0.099*) or subfamily (Figure S14; *Shannon: H = 8.184, p-value = 0.017; Pielou: H = 4.634, p-value = 0.099; DOI, p-value = 0.212; ASV: H = 1.283, p-value = 0.526*).

Beta diversity analyses were performed using Qiime2 software to test for dissimilarities in eukaryote diversity in our dataset. Statistics for the two beta diversity metrics tested (Bray–Curtis distance and wUnifrac distance) are presented in Supplementary File S8.

For the two metrics tested, there were no differences between ant habitats (Figure 4A,B; Bray–Curtis: pseudo-F = 1.042, *p*-value = 0.320; wUnifrac: pseudo-F = 1.295, *p*-value = 0.199), diets (Figure 4E,F; Bray–Curtis: pseudo-F = 1.041, *p*-value = 0.281;

wUnifrac: pseudo-F = 1.113, *p*-value = 0.313), or subfamilies (Figure 5A,B; Bray–Curtis: pseudo-F = 1.043, *p*-value = 0.245; wUnifrac: pseudo-F = 0.718, *p*-value = 0.652). There was also no correlation between the eukaryote diversity and ant evolutionary history, as shown by the Mantel test (Figure 5C; Bray–Curtis: F = 0.074, *p*-value = 0.227; wUnifrac: F = 0.064, *p*-value = 0.603). Ground nesting ants showed statistically different results to tree nesting ants (Figure 4C,D; Bray–Curtis: pseudo-F = 1.121, *p*-value = 0.048; wUnifrac: pseudo-F = 3.143, *p*-value = 0.029). Omnivorous ants also showed statistically different results to carnivorous and herbivorous ants for the Bray–Curtis distance (Figure 4E; F = 1.041, *p*-value = 0.033); however, these differences did not appear for the wUnifrac distance (Figure 4F; F = 1.113, *p*-value = 0.313).

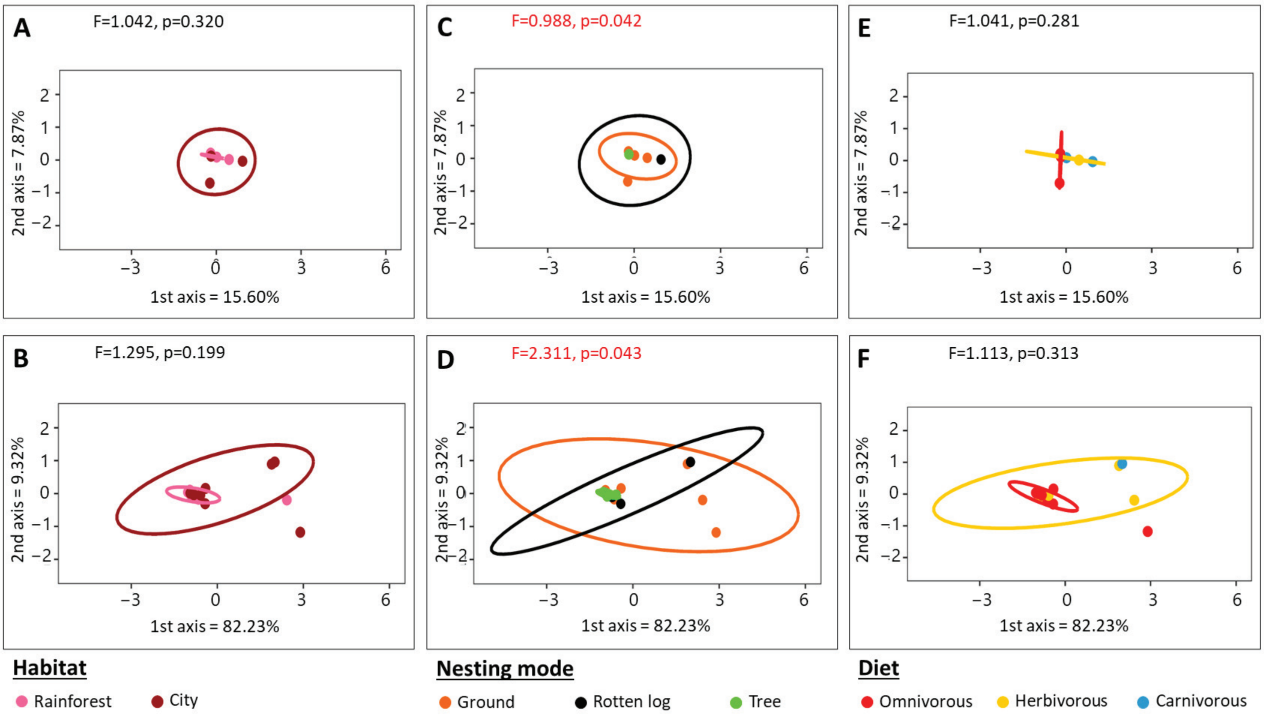
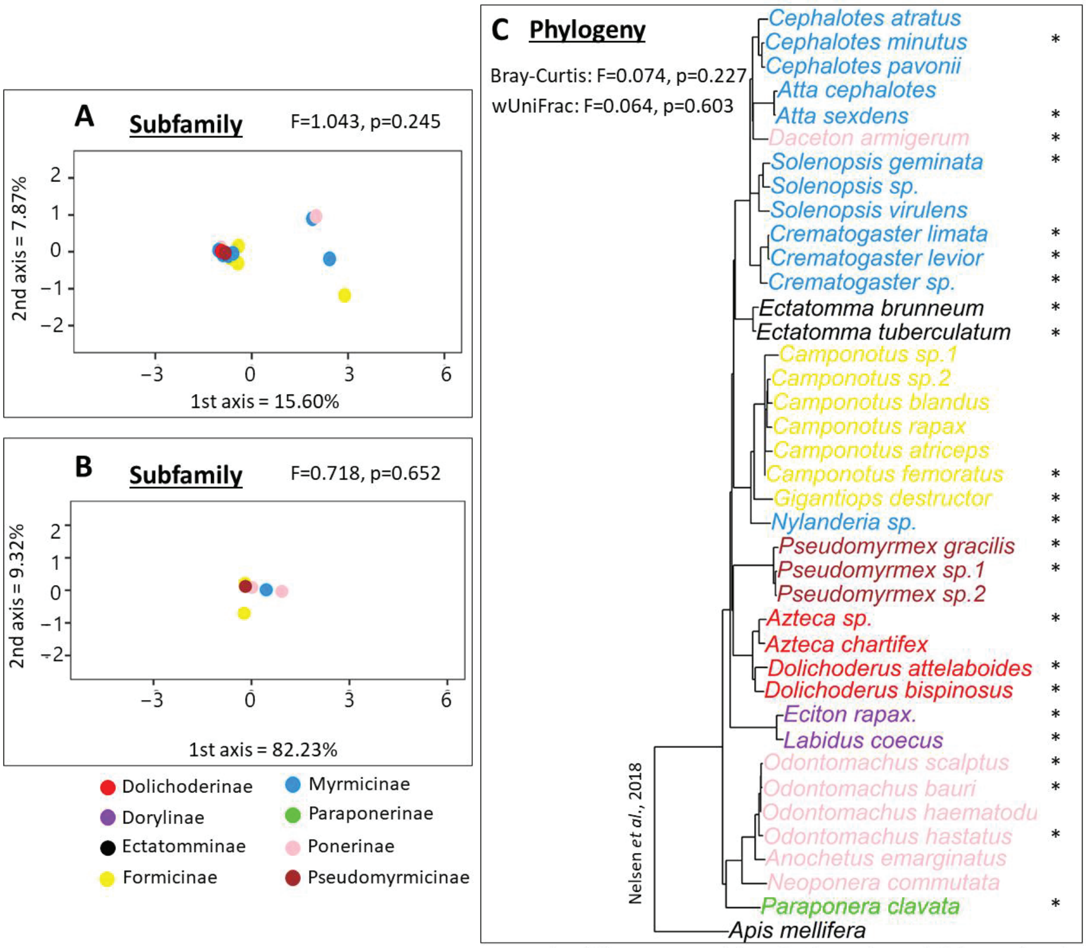


Figure 4. Differences in microbial eukaryote communities visualized with PCoAs across ant habitats (**A**,**B**), nesting modes (**C**,**D**), and diets (**E**,**F**). The distances used were the Bray–Curtis (**A**,**C**,**E**) and wUniFrac (**B**,**D**,**F**). The statistical *p*-values were obtained with pairwise PERMANOVAs. The circles represent confidence intervals of 0.95.

3.7. 18S rRNA ASV Similarity Percentage Analysis

To investigate which eukaryote subphyla are the most commonly associated with Amazonian ants, we used SIMPER analyses. The taxa bar plot of the total eukaryote relative abundance was largely dominated by undetermined eukaryotes for every factor tested (Figure 6A). Overall, besides the undetermined eukaryotes, the 10 most abundant eukaryote subphyla were Nematoda, Eugregarinorida, Chytridiomycota, Mortierellomycotina, Saccharomycotina, Ustilaginomycotina, Pezizimycotina, Basidiobolomycetes, Mucoromycotina, and Arthropoda. All statistics for the SIMPER analyses are presented in Supplementary File S7.



∗ Samples which were removed from the dataset due to their low number of sequences

Figure 5. Differences in eukaryote communities visualized with PCoAs across ant subfamilies (**A**,**B**) and phylogenies (**C**). The distances used were the Bray–Curtis (**A**,**C**) and wUniFrac (**B**,**C**). The stars on the tips of the phylogenies denote samples that were excluded from this analysis due to low sequencing coverage. The statistical *p*-values were obtained with PERMANOVAs.

By focusing on the different ant habitats and nesting modes, undetermined eukaryotes were found to represent half of the total eukaryote relative abundance in city ants and ground nesting ants (49.10% and 61.90% respectively), while they represent almost 75% of the relative abundance in ants nesting in rotten logs and more than 98% in rainforest ants and ants nesting in trees. Nematoda are also very common in city ants and ground nesting or rotten log nesting ants (23.70%, 20.40%, and 23.40%, respectively). Eugragarinorida were present in city ants and in ground nesting ants (14.70% and 12.50%, respectively), while Chytridiomycota were only present in ground nesting ants.

The ant subfamilies Dolichoderinae and Pseudomyrmicinae were entirely associated with undetermined eukaryotes. Formicinae and Myrmicinae also contained mainly undetermined eukaryotes (72.70% and 73.90% respectively), but Formicinae also contained Eugragarinorida (19.60%), while Myrmicinae contained Nematoda and Chytridiomycota (13.50% and 11.30%, respectively). Ponerinae were composed of undetermined eukaryotes and Nematoda (51.40% and 48.60%, respectively).

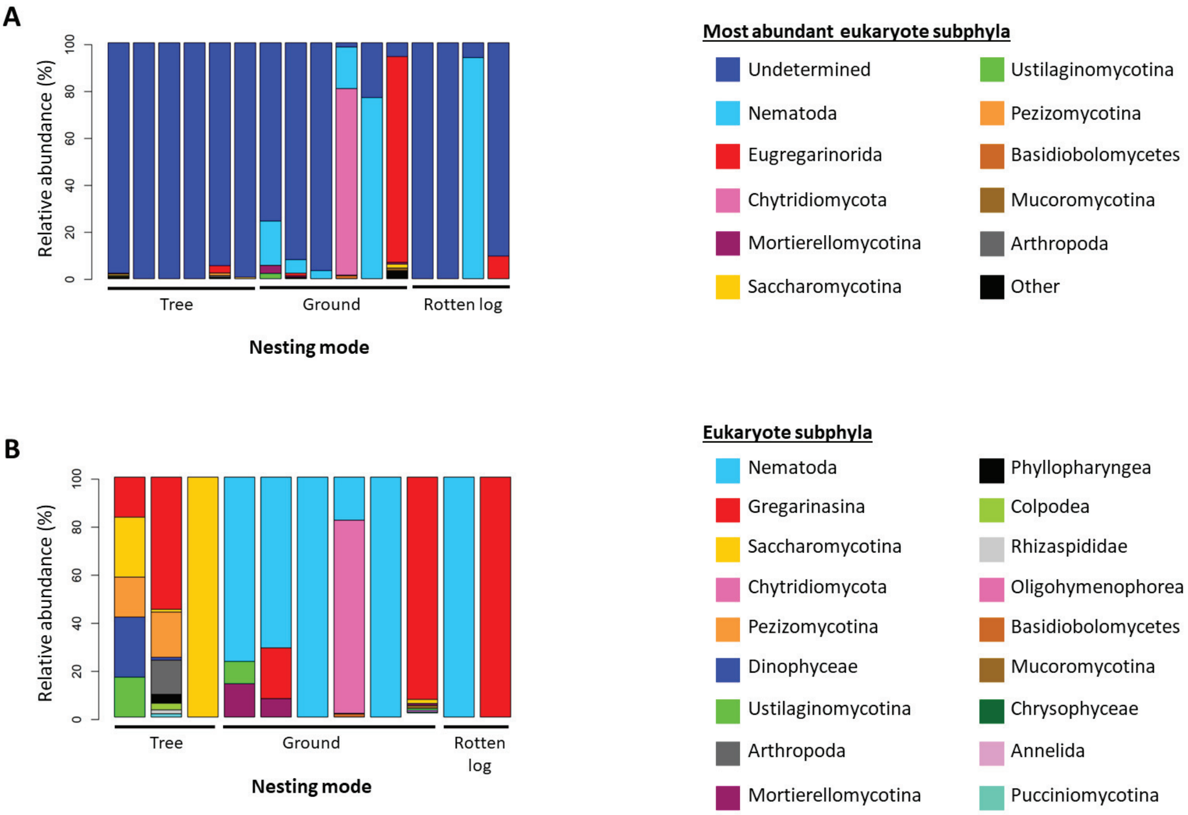


Figure 6. (**A**) Taxa bar plot based on the relative abundance ordered by ant nesting mode with all ASVs. The relative abundance bars are colored by the percentages of eukaryote subphyla. The 11 most abundant eukaryote subphyla associated with Amazonian ants are listed. The less abundant eukaryote subphyla are grouped under the term "Other". The term "Undetermined" represents eukaryote ASVs that could not be identified with the SILVA taxonomy. (**B**) Taxa bar plot based on the relative abundance ordered by ant nesting mode after removing all undetermined eukaryote ASVs. The relative abundance bars are colored by the percentages of eukaryote subphyla. A description of each eukaryote subphyla and examples of genera associated with each subphylum can be found in Supplementary File S11.

We used a SIMPER analysis to determine the contributions of the 11 most abundant eukaryote subphyla to the dissimilarities observed previously. All statistics obtained from the SIMPER analyses are presented in Supplementary File S9. There were no statistical differences in any eukaryote subphyla between ant habitats, diets, or subfamilies. However, there were statistical differences in some eukaryote subphyla between nesting modes. Indeed, undetermined eukaryotes had increased relative abundances in tree nesting ants compared with in ground nesting ants. In contrast, Nematoda had an increased relative abundance in ground nesting ants compared with in tree nesting ants (Figure S15). Pairwise Kruskal–Wallis comparisons for these two eukaryote subphyla are presented in Supplementary File S10.

Due to the high number of undetermined ASVs in our samples, we decided to remove these sequences to obtain a better representation of the identified eukaryote subphyla results (Figure 6B). The most abundant eukaryote subphyla were Nematoda and Gregarinasina. As shown before, Nematoda are more abundant in ground nesting ants than in tree nesting ants (Figure 6B). Most genera are associated with only a few different eukaryote subphyla, with one subphylum being predominant in the sample and the other subphyla having a low abundance. However, *Cephalotes* is the only genus in which the samples were associated with at least five different eukaryote subphyla with no subphylum being dominant, but instead, the different eukaryote subphyla were present with similar relative abundances. A description of each eukaryote subphylum and examples of genera associated with each subphylum can be found in Supplementary File S11.

4. Discussion

4.1. Dietary Niche Structures Bacterial Communities but Not Microbial Eukaryote Communities Associated with Amazonian Ants

Our results show differences in bacterial diversity but not in bacterial relative abundance between carnivorous and herbivorous ants for both the Bray–Curtis and wUniFrac distances. Furthermore, our analyses highlight the finding that some bacterial orders are specific to ants with different diets. Indeed, Erysipelotrichales and Entomoplasmatales are more abundant in carnivorous ants, while Rhizobiales are more abundant in herbivorous ants. These results are in accordance with several previous studies that also highlighted the roles of these bacterial orders in complementing the host diet in ants [19,66,70] as well as in mammals [111]. Additionally, symbiotic bacteria have been shown to drive host evolution [68,112]. One of the possible explanations for this correlation between gut bacteria and the host diet could be the nutrient niche theory.

The structure of the gut bacterial community is hypothesized to be determined by the abundance and diversity of nutrients extracted from the host diet during digestion. The nutrient niche theory posits that gut ecological niches are determined by available nutrients in the gut [113–115]. This means that a specific bacteria species can only assert itself in the host gut if it is able to use a limiting nutrient. The nutrient niche theory has been supported by numerous diet supplementation studies, which have shown that the presence and abundance of specific bacterial species can be altered by experimentally modifying the types and abundance of nutrients present in the gut [116–120].

4.2. Microbial Community Structure Associated with Amazonian Ants Is Influenced by Abiotic Factors and Nesting Modes

Microbial community structure can be influenced by multiple abiotic factors, resulting in different habitat niches. Studies of abiotic factors in rainforests have shown that environmental parameters, like luminosity, humidity, and temperature, vary between the canopy and the forest floor [121,122]. Arthropods and free-living bacteria vertically structure their communities according to their different tolerance levels to these abiotic factors [123]. Some symbiotic bacteria can also confer to their host improved tolerance to these environmental perturbations, and this has previously been shown in several insects [124–128].

Our results indicated differences in microbial alpha and beta diversity, but not in bacterial abundance, between ground nesting ants and rotten log or tree nesting ants. Furthermore, as shown in our diet analysis, some bacterial orders are specific to a particular ant nesting mode. Indeed, Erysipelotrichales, Lactobacillales, and Rhizobiales are more abundant in ground nesting than in rotten log nesting ants, while Rickettsiales showed a higher abundance in rotten log nesting ants than in tree nesting ants. These bacteria could help their hosts to withstand specific environmental conditions, and future experimental work could demonstrate the roles of these bacteria.

The low numbers of currently available 18S rRNA sequences in public databases limit the study of microbial eukaryotes. In our study, the majority of the retrieved 18S rRNA sequences, after excluding any sequence identified as "Hymenoptera", could not be identified further than as being eukaryotes. Among the determined microbial eukaryote ASVs, the identification accuracy was variable with only a few ASVs assigned at the species level, while other AVSs could only be identified at the order or kingdom levels. This major issue has been discussed in previous work [129]. The fact that more undetermined microbial eukaryotes were found in tree nesting ants could come from the fact that, to date, there has been a greater number of studies on eukaryote diversity from soils [129–132], which could cause a lack of sequences from eukaryotes found primarily in the canopy. If this is the case, more studies on global eukaryote diversity from trees and arboreal species would be necessary to increase the eukaryote databases.

4.3. Bacterial Communities in Long-Term Association with Specific Ant Hosts Are Conserved

Several ant genera have well-established long-term relationships with specific bacterial communities. These bacterial communities are usually conserved within the different species of their host genus and often provide benefits to the host, as previously reported for *Camponotus* [19] and *Cephalotes* [70].

By focusing on the differences in bacterial beta diversity, we noticed that, overall, three genera were different from the other ant genera: *Camponotus* (subfamily: Formicinae), *Cephalotes* (subfamily: Myrmicinae), and *Odontomachus* (subfamily: Ponerinae). Looking deeper, we found that *Camponotus* are mainly composed of three bacterial orders: Enterobacterales (especially the bacterial genus *Blochmannia*), Acetobacterales, and Rickettsiales (especially the bacterial genus *Wolbachia*). *Blochmannia* are known to be the main symbiont of *Camponotus*, providing it with nutritional supplementation [133,134]. Some *Camponotus* species have also been shown to be strongly associated with Acetobacterales [135]. *Wolbachia* is a common insect symbiont and can have beneficial effects, like vitamin B supplementation in bedbugs [84,136], as well as negative effects when manipulating host reproduction [137]. Concerning the *Cephalotes* samples, we determined that they were mainly composed of four bacterial orders: Xanthomonadales, Burkholderiales, Rhizobiales, and Opitutales. Our results are in accordance with previous studies on *Cephalotes*, which highlighted that they possess a very stable core microbiome composed of five bacterial orders: Burkholderiales, Opitutales, Pseudomonadales, Rhizobiales and Xanthomonadales [66,69,112,138]. Together these symbionts synthesize amino acids via nitrogen recycling for their host [70]. Finally, focusing on the *Odontomachus* samples, our results show that they are mainly composed of *Rickettsiales* (especially the bacterial genus *Wolbachia*), *Rhizobiales,* and *Erysipelotrichales*. Few studies have focused on the bacterial diversity associated with *Odontomachus,* but a couple of studies have also shown strong associations of *Wolbachia* and Rhizobiales with *Odontomachus* ants [74,139]. To the best of our knowledge, the presence of Erysipelotrichales bacteria has not been previously studied in *Odontomachus* ants, but as it is a common symbiont of carnivorous species [140–142], its presence in predatory *Odontomachus* samples is not surprising.

In contrast to these very conserved microbiomes, we also identified two ant genera which displayed very high levels of species richness: *Ectatomma* and *Paraponera*. These two ant genera are characterized by not being associated with a core microbiome, but instead, have transient bacterial communities. Two studies have reported the bacterial communities associated with *Paraponera clavata* [73,143], but no clear identification of a core microbiome has been revealed, thus suggesting that the bacterial communities found are very variable and originate from the broad diets and habitats of these omnivorous ants.

4.4. Habitat Does Not Participate in Structuring Microbial Communities Associated with Amazonian Ants

As we have shown previously, microbial communities associated with ants are structured by host diet and nesting mode. Yet, these ecological factors may vary in different habitats. For example, ants may need to adapt their feeding habits, as different prey or plants may live in contrasting habitats. This is known as environmental filtering, a process in which the environment selects for and against certain species. Omnivorous ants or ants whose diet does not rely on a specific species could adapt faster and more easily to the resources they find in a new environment. Urbanization, in particular, is known to be associated with a variety of effects on arthropods, like pollution, habitat fragmentation, and a decrease in species richness [144,145]. Environment filtering has also been shown to affect microbial communities present in different habitats [146,147].

We did not find any correlations between the microbial communities from these ants in these contrasting habitats in term of diversity or abundance. Our results corroborate previous work investigating the differences in the microbial diversity of insects collected from different habitats that did not find any differences in the bacterial diversity between insects collected from urban environments and insects collected from rural environments [148,149].

We did find a statistical difference in the abundance of one bacterial order (Acetobacterales) between rainforest ants and city ants. However, this result represents an exception in our study, which suggests that environmental filtering does not affect the structure of ant microbial communities.

5. Conclusions

Our results show that microbial communities associated with Amazonian ants are structured by different factors. The bacterial communities associated with Amazonian ants are structured by the ant diet, nesting mode, and taxonomy, while the microbial eukaryote communities associated with Amazonian ants are only structured by the ant nesting mode. The ant habitat and evolutionary history were not shown to have any impact on structuring their associated microbial communities. Despite the large number of undetermined sequences of microbial eukaryotes, future work focusing on the co-occurrence between bacterial communities and microbial eukaryote communities could reveal microbe–microbe interaction dynamics inside the insect host. To the best of our knowledge, this is one of the first studies to focus on the microbial communities associated with a wide range of Neotropical ants. As such, future work on this topic would be useful to confirm our findings. In particular, since only one nest per species was collected in this study, research focusing on the microbiome of ants from several nests of the same species might increase the robustness of our findings.

Supplementary Materials: The following are available online at https://www.mdpi.com/xxx/s1, Figure S1: Sampling map; Figure S2: 16S rRNA rarefaction curves; Figure S3: 16S rRNA alpha diversity for ant habitat; Figure S4: 16S rRNA alpha diversity for ant nesting mode; Figure S5: 16S rRNA alpha diversity for ant diet; Figure S6: 16S rRNA alpha diversity for ant subfamily; Figure S7: 16S rRNA qPCR; Figure S8: 16S rRNA beta diversity for ants and nests; Figure S9: 16S rRNA SIMPER analysis; Figure S10: 18S rRNA rarefaction curves; Figure S11: 18S rRNA alpha diversity for ant habitat; Figure S12: 18S rRNA alpha diversity for ant subfamily; Figure S13: 18S rRNA alpha diversity for ant nesting mode; Figure S14: 18S rRNA alpha diversity for ant diet; Figure S15: 18S rRNA SIMPER analysis; Supplementary File S1: All samples; Supplementary File S2: 16S rRNA alpha diversity metrics; Supplementary File S3: Bacterial relative abundance; Supplementary File S4: 16S rRNA beta diversity metrics; Supplementary File S5: 16S rRNA SIMPER statistics; Supplementary File S6: 16S rRNA SIMPER pairwise comparisons; Supplementary File S7: 18S rRNA alpha diversity metrics; Supplementary File S8: 18S rRNA beta diversity metrics; Supplementary File S9: 18S rRNA SIMPER statistics; Supplementary File S10: 18S rRNA SIMPER pairwise comparisons; Supplementary File S11: Eukaryote phyla.

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diversity

Article

Unrecognised Ant Megadiversity in Monsoonal Australia: The *Tetramorium spininode* Bolton Group in the Northern Territory

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Abstract: We document unrecognised diversity within the *Tetramorium spininode* Bolton group of the Australian monsoonal tropics, which has a single described species. At the time of its description, *T. spininode* was known from just two collections, but there have since been hundreds of collections from throughout monsoonal Australia. We document morphological and genetic (CO1) variation within the group's fauna of the Northern Territory (NT), in the centre of its range, where collection intensity has been highest. We recognise 20 species among 124 CO1-sequenced specimens, and 32 species in total from the NT. A key to these species is provided. The most intensively sampled regions within the NT are the mesic (>1000 mm mean annual rainfall) Top End in the far north (with 14 species) and the semi-arid (500–900 mm) Sturt Plateau region to its south (13 species). Only one species is known from both regions. Given such high regional turnover and highly patchy sampling, we estimate that at least 40 species of the *T. spininode* group occur in the NT. Similar diversity appears to occur in Western Australia, especially in the Kimberley region, but less in Queensland. Our findings suggest that the total number of species in the *T. spininode* group is likely to be around 100. Our study provides further evidence that monsoonal Australia is an unrecognised global centre of ant diversity.

Keywords: ant diversity; CO1; species delimitation; species group; tropical savanna

1. Introduction

Monsoonal Australia, the northern third of the continent that experiences a seasonal tropical climate, is a global centre of ant diversity but is largely unrecognised as such because the great majority of species are undescribed. An early estimate of the total size of the fauna was 1500 species [1], but it more likely numbers in the several thousand given more comprehensive sampling over recent decades, and, informed by extensive DNA barcoding, the realisation that many taxonomically recognised 'species' are in fact hyperdiverse species groups [2,3]. For example, *Melophorus rufoniger* Heterick, Castalanelli and Shattuck was described as a single species occurring throughout mainland Australia but represents dozens of species just in the monsoonal tropics [4]. The similarly widespread *Monomorium* '*fieldi*' Forel possibly contains 200 species in the region [5].

Tetramorium is another ant genus that has unrecognised Australian hyperdiversity. Inland Australia is believed to harbour up to 500 species or more of the genus [6], but fewer than 10 of these have been described. One such species from the monsoonal tropics is *T. spininode* Bolton, which is characterised by uniquely reflexed petiolar and postpetiolar nodes and a highly distinctive first gastral tergite that has pronounced basal flanges and conspicuous sculpture (Figure 1). As such, it was celebrated as 'the most distinctive and spectacular tetramoriine' ever described [7].

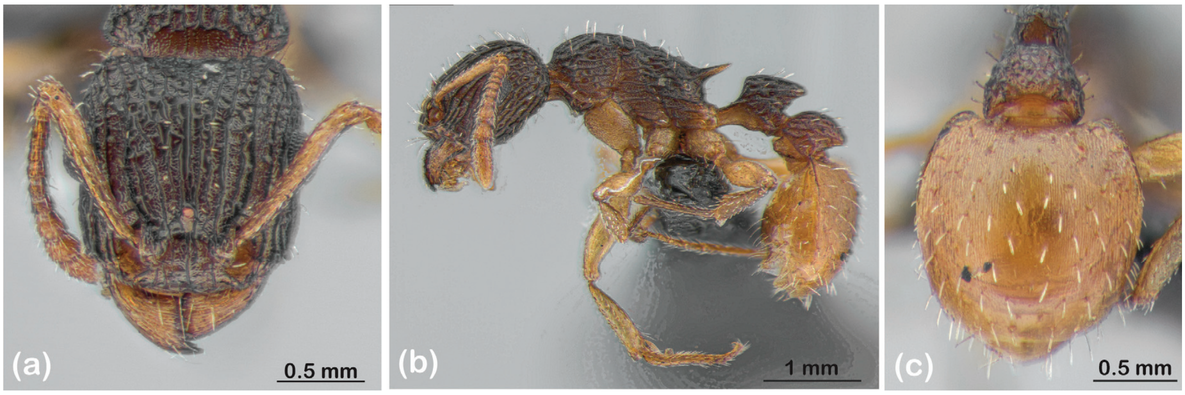

Figure 1. *Tetramorium spininode*. (**a**) Head in frontal view. (**b**) Lateral view. (**c**) Gaster in dorsal view. All images are of sequenced specimen OZBOL 6501-22, collected from the type locality.

At the time of its description in 1977, *T. spininode* was known from just two collections: its type series from the Kimberley region of far northern Western Australia (WA), and a single specimen (differing in colour) from Newcastle Waters, 1000 km southeast in the Northern Territory (NT) [7]. There have since been hundreds of collections of the taxon, almost entirely from the monsoonal zone, and it clearly represents dozens of species [6]. *Tetramorium spininode* was originally placed in the *T. striolatum* Viehmeyer group [7] but given its diversity has since been considered to represent its own species group [1]. The key morphological characters that vary among species are:

- Metapleural lobes (following the nomenclature of their original description [7], although they are actually part of the propodeum), which range from inconspicuous and rounded to prominently spinose (Figure 2);
- Gastral sculpture, which ranges from finely striate (typically longitudinally, but sometimes radiating from the base, and with varying degrees of punctate background sculpture) to spectacularly costate (Figure 3);
- Structure of the first gastral tergite, including the extent of basal flanging (Figure 3) and the presence of an antero-medial ridge (Figure 4);
- Rugosity of the head and mesosoma (Figure 5);
- Body colour, which ranges from uniformly yellowish or orange-brown to bicoloured, with dark brown head, mesosoma and waist, contrasting with yellowish legs and gaster (Figure 5).

Here we assess the extent of unrecognised diversity within the *T. spininode* group by providing an integrated morphological and genetic (CO1) analysis of diversity within the NT, which has by far the highest number of collections of the taxon. We specifically address two questions. First, how many species are likely to be represented by collections of the *T. spininode* group from the NT? Second, what is the extent of geographical turnover of species within the NT? We then draw on this information, along with collections from elsewhere in northern Australia, to discuss the likely total number of species in the *T. spininode* group. A formal taxonomic analysis and species descriptions will be provided in a following paper.

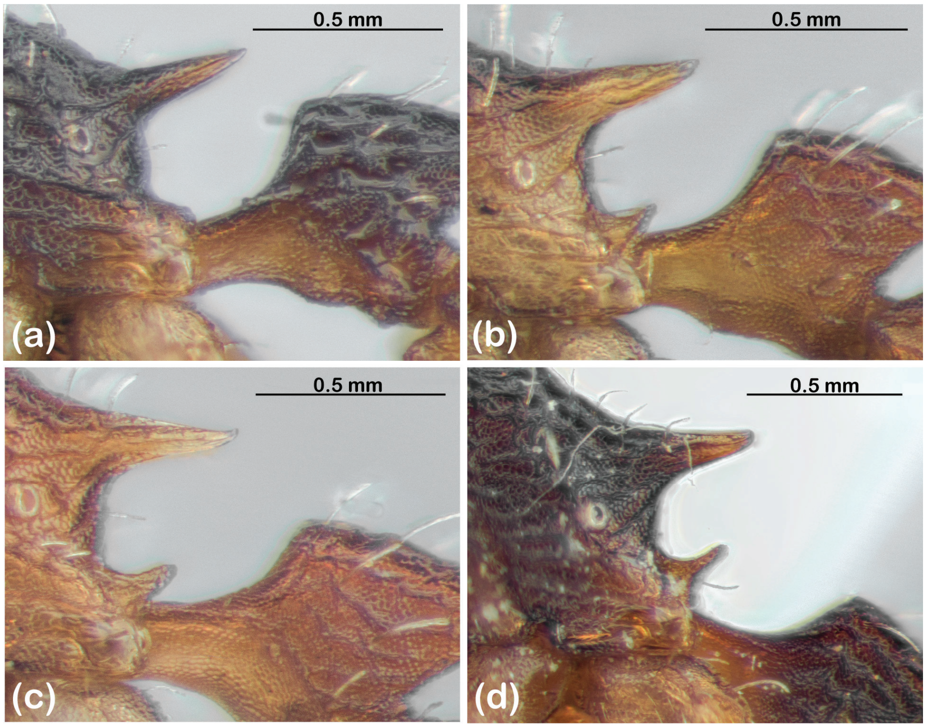

Figure 2. Variation in metapleural lobes among species of the *T. spininode* group. (**a**) *T. spininode*; OZBOL 6501-22. (**b**) sp. 21; OZBOL 8250-22. (**c**) sp. 3; OZBOL 3953-21. (**d**) sp. 14; TET 088-17.

Figure 3. Variation in gastric sculpture among species of the *T. spininode* group from the NT. Species are arranged with increasing sculptural coarseness. (**a**) sp. 15; Arnhem Land, NT (not sequenced). (**b**) sp. 21; OZBOL 8250-22. (**c**) sp. 20; TET 086-17. (**d**) sp. 14; TET 088-17. (**e**) sp. 11; Manbulloo Stn, NT (not sequenced); (**f**) sp. 5; OZBOL 8227-21.

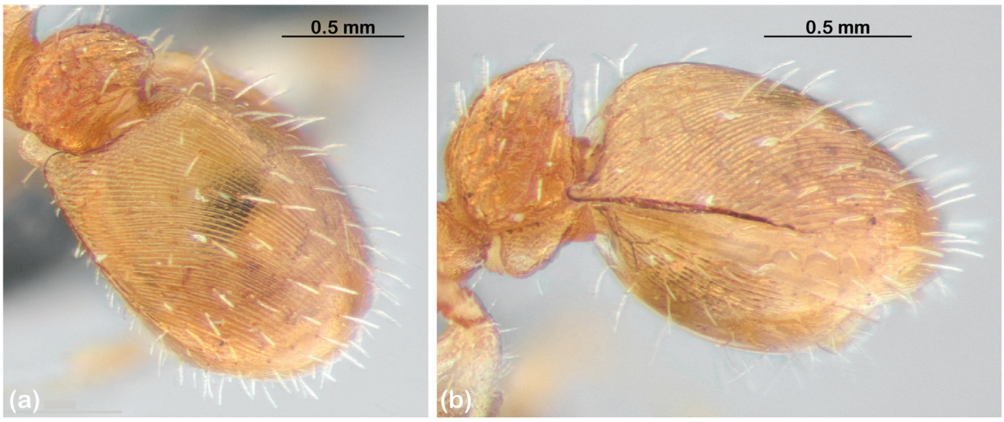

Figure 4. Ridged gaster. (**a**) Oblique view, showing dorsal ridge. (**b**) Lateral view, showing perpendicular anterior face. Both images are of sp. 12; King River, NT (not sequenced).

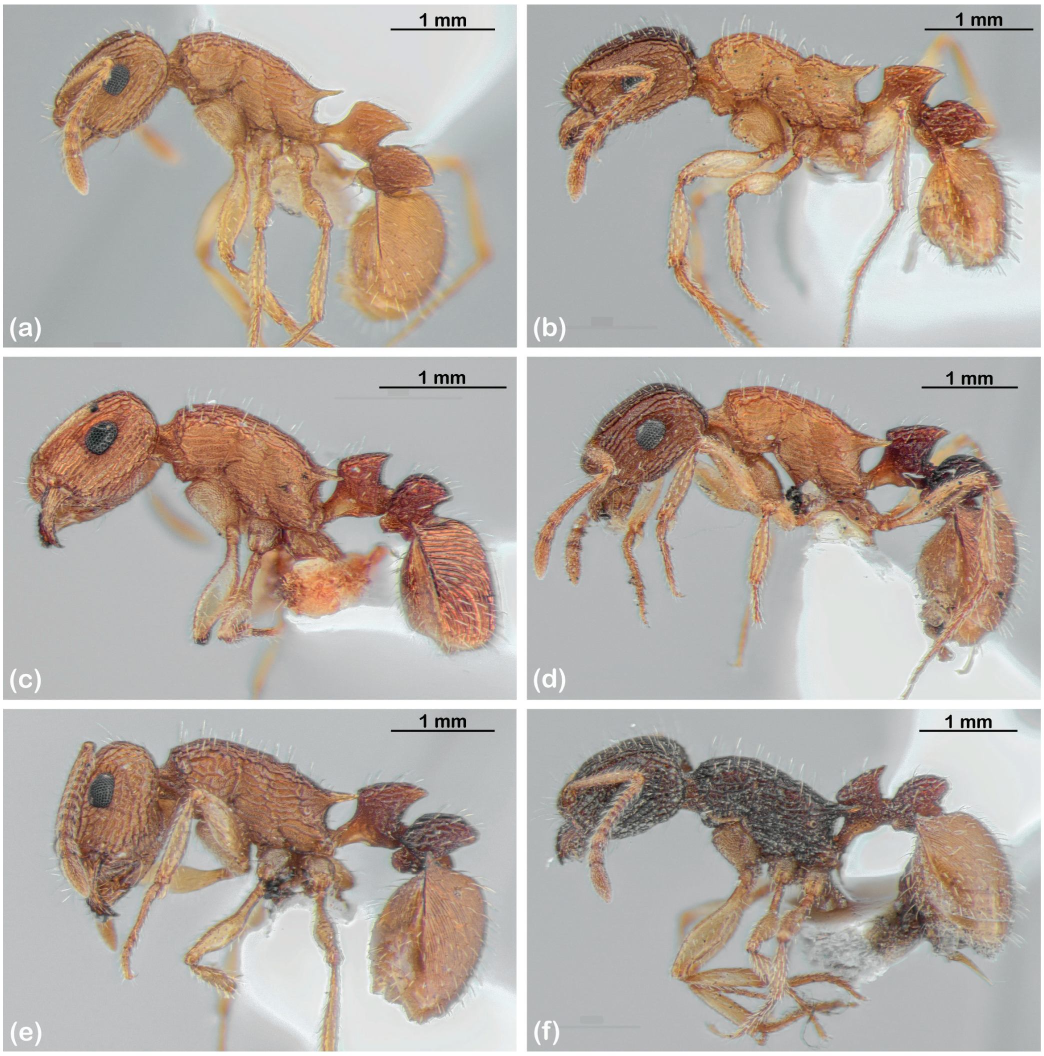

Figure 5. Variation in morphology, sculpture and colour among species of the *T. spininode* group. (**a**) sp. 12; King River, NT (not sequenced); (**b**) sp. 8; nr Manyallaluk, NT (not sequenced); (**c**) sp. 6; Manbulloo Stn, NT (not sequenced); (**d**) sp. 9; nr Beswick, NT (not sequenced); (**e**) sp. 11; OZBOL 3950-21; (**f**) sp. 26; TET 073-17.

2. Materials and Methods

Our study was based on the approximately 800 pinned specimens of the *T. spininode*-group in the ant collection held at the CSIRO laboratory in Darwin (subsequently referred to as the Darwin collection), which holds the vast majority of collected specimens of the taxon. More than 80% of the *T. spininode*-group specimens in the Darwin collection are from the NT. Geographic coverage of samples within the NT is extremely patchy, being heavily concentrated in the central north–south corridor of the monsoonal region (Figure 6). Most samples are from either the very mesic (1000–1500 mm mean annual rainfall) Top End (especially the central Kakadu/Nitmiluk region) or the semi-arid Sturt Plateau region (500–900 mm) to its south (Figure 6). Most other regions were only sparsely sampled or not represented at all.

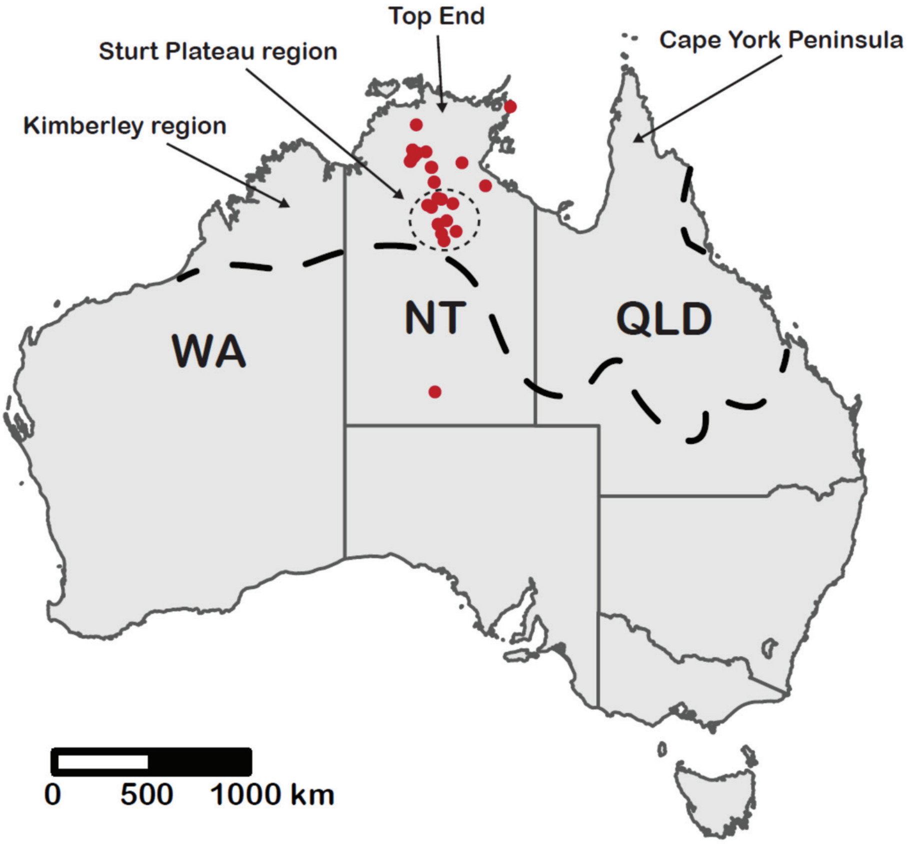

Figure 6. Map of Australia showing collection localities (red dots) for sequenced specimens of the *Tetramorium spininode* group. Many localities represent multiple sites. The four major biogeographic regions in northern Australia are indicated. The dashed line represents the approximate southern boundary of the monsoonal zone, where rainfall is very heavily concentrated in a summer wet season. Total annual rainfall ranges from about 2000 mm on the Tiwi Islands in the Top End to 500 mm on the southern boundary with the central arid zone.

We first conducted a preliminary sorting of NT specimens based on morphological variation. This sorting was then refined through analysis of CO1 sequences obtained from 124 of the specimens that covered a wide range of morphological variation among relatively recent (<15 years) collections (Supplementary Table S1). We also sequenced a specimen of *T. spininode* collected from its type locality, along with a specimen of the *T. striolatum* group for use as an outgroup in CO1-tree construction. DNA extraction (from foreleg tissue) and CO1 sequencing were conducted through the Barcode of Life Data (BOLD) System (for extraction details, see http://ccdb.ca/resources; accessed on 20 March 2023). Each sequenced specimen was assigned a unique identification code that combines the batch within which it was processed, its number within the batch and the year of sequencing (e.g., OZBOL6501-22 for the *T. spininode* specimen). All specimens were labelled with their respective BOLD identification numbers and sorted to the species documented here, in the Darwin collection.

DNA sequences were checked and edited in MEGA [8]. Sequences were aligned using the UPGMB clustering method in MUSCLE [9] and then translated into (invertebrate) proteins to check for stop codons and nuclear paralogues. The aligned sequences were trimmed accordingly, resulting in 1056 base pairs. MUSCLE was also used to construct a maximum-likelihood tree.

There is no specific level of CO1 divergence that can be used to define a species, but it is typically 1–3% within a species [10]. We delimited species based on the integration of morphological variation, CO1 clustering and distance, and geographic distribution [11]. We followed a species concept based on reproductive isolation and evolutionary independence as evidenced by morphological differentiation between sister (i.e., most closely related) clades (considering all available samples from the same collections as those of sequenced specimens) and sympatric distribution.

We imaged representative morphological characters and specimens using a Leica DMC5400 camera mounted on a Leica M205C dissecting microscope. We took image montages using the Leica Application suite v. 4.13 and stacked them in Zerene stacker.

3. Results

3.1. Species Richness

We recognise 32 species (spp. 1–32) of the *T. spininode* group among specimens from the NT, 20 of which were represented among the 124 sequenced specimens (Figure 7). Species that were not sequenced are represented only by older (>15 years) collections. A key to species (including *T. spininode*) is as follows:

1. First gastral tergite costate 2
   First gastral tergite striate 8
2. Metapleural lobes strongly developed and dentiform or spinose 3
   Metapleural lobes weakly developed and rounded 6
3. Metapleural spines short and broadly triangular 4
   Metapleural spines long and narrow 5
4. Body reddish brown contrasting with yellowish legs; sides of head behind eyes regularly rugose (Forest Hill and Manbulloo stns) . . . . sp. 1
   Body uniformly yellowish; sides of head behind eyes irregularly sculptured (Manbulloo Stn) . . . . sp. 2
5. Sides of head behind eyes regularly rugose; gastral costae more strongly radial anteriorly (Hayfield Shenandoah Stn) . . . . sp. 3
   Sides of head behind eyes irregularly sculptured; gastral costae less strongly radial anteriorly (Lorella Springs) . . . . sp. 4
6. Gastral costae transverse anteriorly (throughout Sturt Plateau) . . . . sp. 5
   Gastral costae never transverse 7
7. Small species with short petiolar and postpetiolar nodes that are not so strongly reflexed (Katherine region; Figure 5c) . . . . sp. 6
   Larger species with more strongly reflexed petiolar and postpetiolar nodes (Groote Eylandt, Gulf region) . . . . sp. 7
8. Metapleural lobes strongly developed and dentiform or spinose 9
   Metapleural lobes not strongly developed, rounded or very weakly dentate 26
9. Mesosoma with a deep metanotal groove (Manyallaluk; Figure 5b) . . . . sp. 8
   Mesosoma with at most a weak metanotal notch 10
10. First gastral tergite with a rounded medial ridge such that in profile it is angled anteriorly, with a perpendicular anterior face 11
    First gastral tergite without a medial ridge such that its profile lacks a distinct anterior face 15
11. Mesosoma conspicuously rugose throughout most of its lateral faces 2
    Mesosoma without conspicuous rugae on most of its lateral faces 13

12. Petiole and postpetiole conspicuously darker than mesosoma and gaster (Manyallaluk, Beswick; Figure 5d) . . . . . . sp. 9
    Petiole and postpetiole concolorous with mesosoma and gaster (Nitmiluk NP) . . . . . . sp. 10
13. Ventral processes of postpetiole yellowish, distinctly lighter than node 14
    Ventral processes of postpetiole reddish to dark brown, concolorous with node (Forest Hill and Manbulloo stns; Figure 5e) . . . . . . sp. 11
14. First gastral tergite more strongly flanged, and lateral striations strongly radiating towards the medial line (King River; Figure 4) . . . . . . sp. 12
    First gastral tergite less strongly flanged, and lateral striations less strongly radiating towards the medial line (Lakefield, Manbulloo and Mataranka stns . . . . . . sp. 13
15. Mesosoma yellowish brown, concolorous with gaster 16
    Mesosoma reddish or blackish brown, distinctly darker than gaster 20
16. Gastral striations coarse 17
    Gastral striations fine, often faint 18
17. Metapleural spines long and narrow (Kakadu NP) . . . . . . sp. 14
    Metapleural spines short and triangular (Vermalha Stn) . . . . . . sp. 15
18. First gastral tergite primarily punctate anteriorly, with only feeble striations (Kakadu NP) . . . . . . sp. 16
    First gastral tergite finely but conspicuously striate throughout 19
19. First gastral tergite with punctate background sculpture, giving it a somewhat matt appearance; rugae on frontal area finer and more closely approximated (Kakadu NP) . . . . . . sp. 17
    First gastral tergite with feeble background sculpture, giving it a shiny appearance; rugae on frontal area very coarse and widely separated (Kakadu NP, Nitmiluk NP, Arnhem Land) . . . . . . sp. 18
20. Head, mesosoma and waist orange-brown; metapleural lobes spinose; first gastral tergite without striations on its posterior half medially (Gove) . . . . . . sp. 19
    Head, mesosoma and waist darker reddish or blackish brown 21
21. First gastral tergite conspicuously striate throughout 22
    First gastral tergite mostly smooth and shiny medially on posterior half 24
22. Metapleural spines especially long and narrow; first gastral tergite with feeble background sculpture, giving it a shiny appearance (Kakadu NP) . . . . . . sp. 20
    Metapleural spines not so long and narrow; first gastral tergite with punctate background sculpture, giving it a more matt appearance 23
23. Basal flanges of first gastral tergite weakly developed; in dorsal view, lateral margins of first gastral tergite only weakly curved (Sturt Plateau region, Kakadu NP) . . . . . . sp. 21
    Basal flanges of first gastral tergite strongly developed; in dorsal view, lateral margins of first gastral tergite strongly curved (eastern Top End) . . . . . . sp. 22
24. Metapleural spines short and broadly triangular (Kakadu NP) . . . . . . sp. 23
    Metapleural spines long and narrow 25
25. First gastral tergite with very feeble background punctation, giving it a shiny appearance (Cobourg Peninsula, Blue Mud Bay) . . . . . . sp. 24
    First gastral tergite with stronger background punctation, giving it a more matt appearance (Limmen NP) . . . . . . sp. 25
26. Mesosoma dark brown, contrasting with light honey-brown legs and gaster 27
    Mesosoma yellowish or reddish brown, more or less concolorous with legs and gaster 28
27. Anterior half of pronotal dorsum with regular, coarse rugae (Kimberley, WA) . . . . . . *spininode*
    Anterior half of pronotal dorsum irregularly sculptured (Kakadu NP) . . . . . . sp. 26
28. Colour darker reddish brown, rugae on frontal area coarser 29
    Colour paler yellowish or orange-brown; rugae on frontal area not so coarse 31
29. Lateral striations on first gastral tergite diverging from the base (Tandidgee Stn) . . . . . . sp. 27
    Striations on first gastral tergite parallel throughout 30
30. Gastral striations finer (Sturt Plateau, Victoria River District, Gulf region) . . . . . . sp. 28
    Gastral striations coarser (Newcastle Waters and Hidden Valley stns) .. . . sp. 29
31. Striations on first gastral tergite parallel throughout (Vermhala Stn) . . . . . . sp. 30
    Lateral striations on first gastral tergite either diverging or converging from the base 32

32. Lateral striations on first gastral tergite diverging from the base (Tandidgee, Newcastle Waters and Henbury Stations, Alice Springs, Uluru NP) . . . . sp. 31
    Lateral striations on first gastral tergite converging from the basal lobes (Limmen NP) . . . . sp. 32

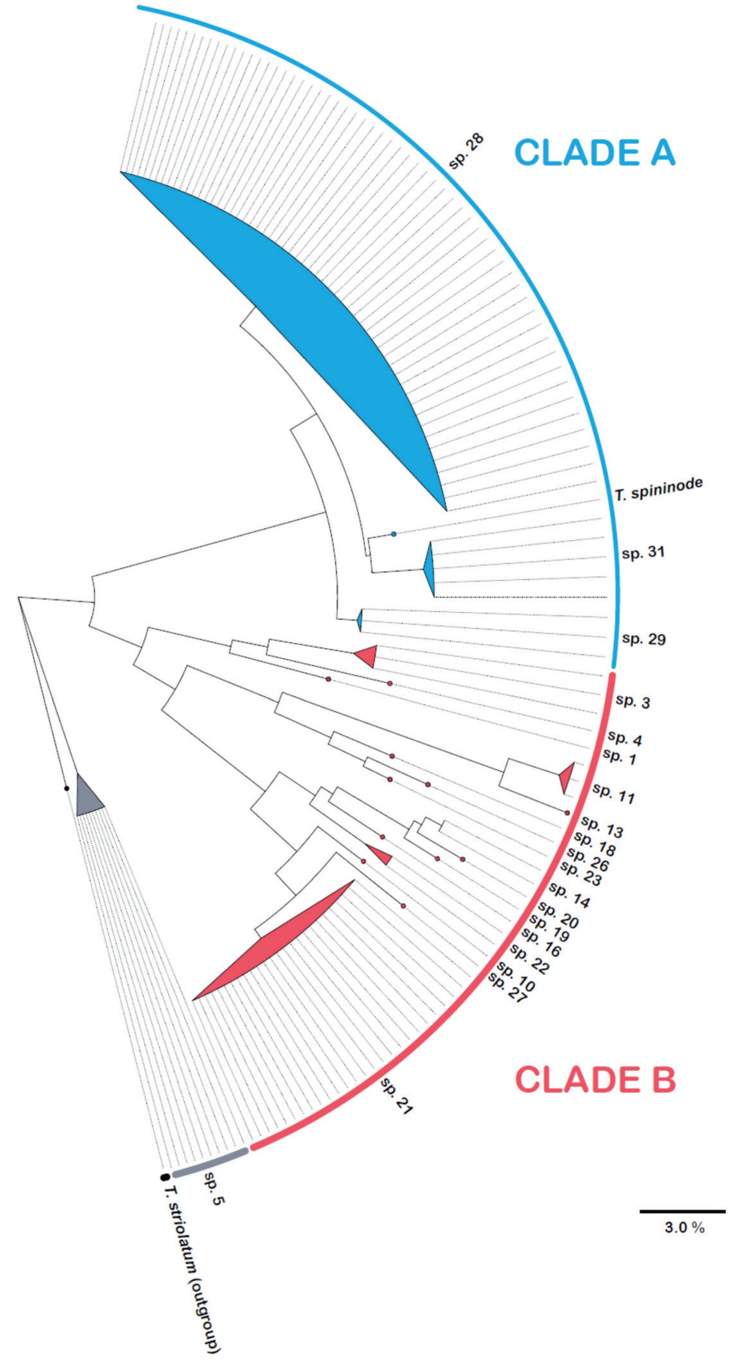

Figure 7. Summary CO1 tree constructed by maximum likelihood showing the 20 sequenced species of the *T. spininode* group from the NT, along with *T. spininode* from WA. All species other than sp. 5 belong to one of two major clades (A and B). The full tree is shown in Figure S1.

The CO1 tree is structured into three primary clades: clade A (three NT species and *T. spininode*), clade B (sixteen species) and sp. 5 (Figure 7). Key morphological characters show strong structure within the CO1 tree. Species 5 has a unique combination of a lack of metapleural spines and costate gastral sculpture. All species in clade A lack a metapleural spine, whereas, with one exception (sp. 27, which is morphologically very similar to sp. 28

in clade A), the metapleural lobe is spinose in all species in clade B. The three sequenced species with costate gastral sculpture other than sp. 5 (sp. 1, sp. 3 and sp. 4; all with spinose metapleural lobes) form a distinct subclade within clade B. The non-sequenced sp. 2 shares these characters and therefore likely belongs in this clade. The two other non-sequenced species with costate gasters (spp. 6 and 7) lack spinose metapleural lobes and so are likely allied to sp. 5. The two sequenced species with a ridged gaster (sp. 11 and sp. 13; both also with spinose metapleural lobes) form a subclade within clade B. The three non-sequenced species with a ridged gaster (sp. 12, sp. 14 and sp. 15) also have spinose metapleural lobes and therefore likely belong in this subclade. There is also geographic structure among the characters. For example, none of the 7 species with costate gastral sculpture are among the 14 species known from the mesic (>1000 mm mean annual rainfall) Top End. On the other hand, a subclade of five species within clade B (sp. 14, sp. 16, sp. 19, sp. 20 and sp. 22), all with spinose metapleural lobes and a finely striate gaster, is known only from the Top End.

3.2. Species Turnover

There is almost complete species turnover between the mesic Top End (14 species) and the semi-arid Sturt Plateau region to the south (13 species), with only 1 (sp. 21) in common. Of the four species from the Gulf region (sp. 4, sp. 7, sp. 28 and sp. 32), only one (sp. 28) has been recorded elsewhere. The two remaining species (sp. 8 and sp. 9) are from the Beswick/Manyallaluk area southeast of Katherine, and neither is known from elsewhere. Only one species (sp. 31) is known to extend beyond the NT monsoonal zone into the central arid zone (Figure 6).

4. Discussion

We have recognised 32 species of the *T. spininode* group from NT specimens. None of the species are *T. spininode*, and so all are undescribed. None are cryptic in the sense of being unable to be differentiated morphologically [12], and there was a very strong match between morphological and CO1 variation.

Sampling was heavily concentrated in two regions, the mesic (>1000 mean annual rainfall) Top End and semi-arid (500–900 mm) Sturt Plateau region to the south, and this is where most (26) of the species were collected. A similar number of species occur in each region (14 and 13, respectively), and only one is known from both. The remaining six species are all known from very restricted locations. Such high rates of geographic turnover suggest that many additional species occur in regions that have not been sampled. It is likely that the total number of species occurring in the NT is at least 40 and possibly > 50.

Diversity in the *T. spininode* group appears to be at least as high in WA as it is in the NT. The Darwin collection has 19 sorted species from WA, from just 21 collection localities. Notably, none of these have spinose metapleural lobes, and six have a spectacularly costate gaster (Figure 8). The one NT species extending into the arid southern NT (sp. 31) also occurs in WA's Great Sandy Desert and the southern Kimberley, but otherwise, there appears to be no overlap between the WA and NT species in the Darwin collection. Diversity appears to be lower in Queensland (Qld). Species 38 (occurring throughout the semi-arid NT) appears to occur throughout northern Qld (extending into Cape York Peninsula), but none of the other Qld species are known from the NT. As in WA, none of the Qld species in the Darwin collection have spinose metapleural lobes.

Assuming that the total NT fauna consists of more than 40 species and a similar number occurs in WA, given that the group occurs throughout northern Qld and that geographic turnover is extremely high, the total number of species in the *T. spininode* group would appear to be around 100. We acknowledge that this figure might change somewhat (either up or down) following more detailed taxonomic analysis, but we believe that our finding of extremely high unrecognised diversity is robust. Such a finding further builds the case that monsoonal Australia should be recognised as a global centre of ant species diversity. Unrecognised diversity occurs throughout the monsoonal fauna of *Tetramorium*, especially in the *T. striolatum* group, which is far more diverse than the *T. spininode* group [6].

The initial figure of about 1500 ant species occurring in monsoonal Australia [1] was based on estimates that have now proven to be far too low. For example, the *Melophorus rufoniger* group was initially estimated to include ten species and the *Monomorium nigrius* group twenty [1], whereas their true diversity is an order of magnitude higher [4,5]. The original estimate for the number of species in the *T. spininode* group was 8 and for the *T. striolatum* group 15 [1], both just small fractions of the actual faunas. All these findings point to Australia's monsoonal ant fauna numbering in the several thousand species, which would make it by far the most species-rich known anywhere in the world.

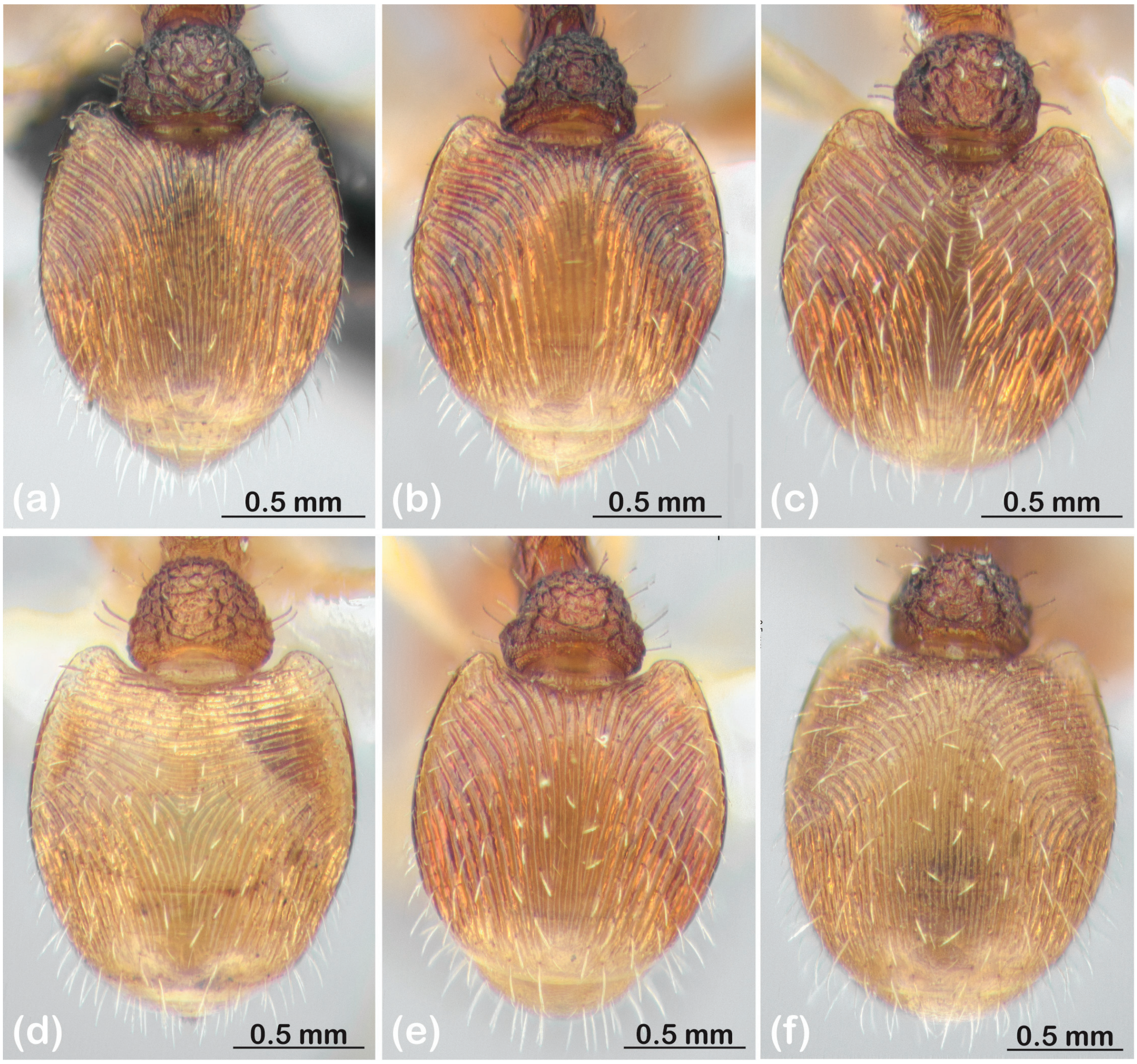

Figure 8. Variation in coarse gastral sculpture of six species (**a**–**f**) of the *T. spininode* group from the Kimberley region of WA.

5. Conclusions

The extraordinary hyperdiversity of ants in monsoonal Australia is not reflected in a recent analysis of ant global diversity patterns, either within Australia or internationally [13]. This is because the analysis is based on described species only. The analysis shows ant diversity within Australia to be highest along the eastern coast and in the temperate southeast and southwest. These are the areas where the great majority of the Australian population lives and where taxonomic effort has been heavily concentrated. The Australian map is one of sampling intensity and taxonomic effort rather than of species richness. The global analysis shows species richness in monsoonal Australia as being lower than for most of the United States, and that the Neotropics is the clear global hotspot for ant diversity [13]. A very different picture would emerge if undescribed species were included. A high number of undescribed species is characteristic of tropical ant faunas throughout the world, but there is no evidence that the extreme level of unrecognised diversity in monsoonal Australia occurs anywhere else. For example, an extensive CO1 analysis of the fauna of Brazil's southern Atlantic Forest revealed that its size was likely only 6–10% larger than currently recognised [14]. The Australian monsoonal tropics thus appears to represent a major biogeographic anomaly in terms of global patterns of ant diversity. This is even more remarkable given that the vast majority of species are epigaeic, with relatively few specialist arboreal or cryptobiotic species that feature so strongly in tropical rainforest [3,15].

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/d15040476/s1, Figure S1: full CO1 tree, showing all sequenced specimens, Table S1: list of specimens sequenced in this study and their collection locations. Specimens are identified by their BOLD ID codes and arranged according to species.

Author Contributions: A.N.A. conceived the study, led the development of the Darwin ant collection and wrote the first draft of the manuscript. F.B. prepared the figures and contributed to the writing of the paper. B.D.H. helped develop the Darwin ant collection and contributed to the writing of the paper. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The CO1 data presented in this study are available on request from the corresponding author.

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

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Nutrient Supplementation to Arboreal Ants: Effects on Trophic Position, Thermal Tolerance, Community Structure and the Interaction with the Host-Tree

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Abstract: Carbohydrates and proteins are essential to maintain the basic functions of animals. Over the course of one year we conducted a factorial experiment to determine the influence of carbohydrate (sucrose) and protein supplementation on the thermal tolerance, trophic position, overall abundance, species richness and composition, and on the strength of the protective effects of arboreal ants on their host tree (*Caryocar brasiliense*). Using *Azteca* ants as a model we found evidence of dietary and thermal plasticity among arboreal ants as colonies supplied with protein increased their trophic level relative to colonies that received no protein. Colonies that received sucrose increased their thermal tolerance on average by 1.5 °C over a six-month period, whereas those that did not receive sucrose did not change their thermal tolerance. Overall ant abundance was lower in control trees than in those that received any nutrient addition treatment. Species richness was also lower in control trees, but those receiving sucrose presented more species than those receiving only protein. There was greater similarity in species composition between the trees that received sucrose than between these and those receiving only protein or just water as control. Trees whose ant colonies received sucrose presented lower levels of leaf damage than those that did not. Overall, these results indicate that food resources can modulate the population and community ecology of arboreal ants as well as their interaction with the host trees. Interestingly, although arboreal ants are thought to be N-limited, it was the supplementation of sucrose—not protein—that elicited most of the responses.

Keywords: nutritional ecology; ecological stoichiometry; cerrado; formicidae; diet; nitrogen; sucrose

1. Introduction

Nutrition influences every aspect of an animal's life, either directly by building the components of the organisms and affecting their performance, or indirectly by influencing the interaction between species and the environment at multiple scales [1]. Rather than simply maximizing food intake, consumers must regulate foraging in a way that matches the optimal mixture of macronutrients and micronutrients required for survival [2,3], and this becomes even more challenging when considering that nutrient availability varies between and within habitats [4]. Nutritional interactions between organisms and the environment might influence species physiology and behavior, working as a bottom-up force that alters species relations and the structure of biological communities [5–7].

Carbohydrates and proteins are essential compounds that act in a complementary way to maintain the basic functions of animals, such as growth, survival, and reproduction [8,9]. The availability of carbohydrates and protein depends on the type of food resource, which, in turn, varies temporally and spatially. For example, for organisms in the canopy of tropical forests there is often a greater availability of carbohydrates than of proteins [10]. According to the Compensation Hypothesis [11,12], the attractiveness and utility of a food resource to a given organism is conditional to its availability in the environment in question.

Thus, for consumers in the forest canopy, protein may be more attractive than the abundant carbohydrate [13,14].

Ants are one of the most ubiquitous and diverse groups of arthropods in the canopy of tropical forests and savannas [15]. In general, arboreal ants have a carbohydrate-based diet that follows the high availability of sugary plant and insect exudates in the canopy [16–18]. However, most arboreal ants are omnivorous [17,19,20], whose foraging behavior and trophic level can vary according to the availability of different food sources in the environment [21]. Although some studies suggests that ants are more constrained by protein sources in the canopy, and, therefore, more attracted to it [10,20], there is also evidence that, in fact, the most limiting resource for arboreal ants are carbohydrates [22]. It has been suggested that if selective pressures across ants' evolutionary history favored species that feed on the most abundant resource, then carbohydrates could be a limiting nutrient for arboreal ants, even considering its high abundance in the canopy [22].

Carbohydrates fuel the more energetically costly activities of the colonies and is related to the maintenance of the workers' body functions, whereas protein is fundamental for the growth and development of larvae and pupae [23]. In this sense, both nutrients act in complementary ways to ants' colony growth and survival, which ultimately could affect species interactions and the structure of communities [24,25]. Moreover, both carbohydrates and protein can interact and influence numerous other aspects of an ant's life. For example, carbohydrates can provide energy for thermoregulation [26] and increase evaporative cooling effects [27], while protein can influence the production of heat shock proteins, which are essential to cope with thermal stress [28,29]. Therefore, nutrition can alter the critical thermal maximum (CT_{max}) of ant workers [6] which, in turn, can affect their foraging schedules, disrupt transitive hierarchies [30,31] and/or affect their mutualistic interactions with plants [32].

Many species of arboreal ants nest or forage on plants that have extrafloral nectaries (non-reproductive organs and plant tissues that produce carbohydrate-rich nectar, EFNs hereafter). This interaction can be considered mutualistic when ants prey on herbivores and, thus, increase plant fitness, while benefiting from nesting and food resources [18]. Several non-exclusive hypotheses have been proposed to explain why ants defend EFN-producing plants. The Fuel for Foraging Hypothesis [33–35] proposes that the ant visitors are fueled by carbohydrates provided in the EFNs, increasing ant's foraging and aggressiveness. The Ownership Hypothesis [36,37] proposes that a valuable resource elicits ownership behavior, so that ants would defend the plant against its natural enemies while protecting and dominating the food resource. Lastly, the Deficit Hypothesis [38] proposes that the nutritional imbalances of EFNs (high C:N) increases the ants' need for protein, increasing the chance that they attack an herbivore on the plant.

Although there are studies showing how the availability of carbohydrates alters the foraging behavior and aggressiveness of ants [39,40], ultimately benefiting the plant [41,42], none of these studies have explored how protein availability (and its interaction with carbohydrates) affects the outcome of the interaction between ants and plants. Furthermore, relatively few studies have evaluated how nutrient supplementation affects the thermal tolerances and trophic position of individual colonies, as well as the structure of the ant communities foraging on trees.

Here, we evaluated the influence of nutrient supplementation on the ecology of arboreal ants. For this, we performed a one-year long field experiment in which carbohydrate (sucrose) and protein were supplied to ants associated with an EFN-producing savanna tree species. We addressed four questions. At the level of individual ant colonies we asked: (1) What are the individual and combined effects of carbohydrate and protein supplementation on the thermal tolerance of arboreal ants? We expected that thermal tolerance would vary among colonies subject to different nutrient supplementation treatments, since carbohydrates and protein are known to influence the thermal tolerances of insects in different ways [28,29]. In addition, (2) we evaluated what are the individual and combined effects of carbohydrate and protein supplementation on the trophic position of arboreal ants? According to the Compensation Hypothesis, which states that animals tend to forage in a way to correct nutritional imbalances [11,12], we expected that colonies supplemented with the most limiting resource would have the greatest change in their trophic positions.

At the community level we asked: (3) What are the individual and combined effects of carbohydrate and protein supplementation on the overall abundance, species richness, and composition of arboreal ants on trees? Given that competition over food resources is a strong structuring force among arboreal ant communities [43–46], we would expect that both protein and sucrose would generate an increase in the overall abundance and species richness of ants on trees. Furthermore, considering that the attractiveness of food resources varies between species [20], we also expected that species composition would be different across the nutrient supplementation treatments. Finally, we asked: (4) Does nutrient supplementation affect the protective effects of ants against the herbivores of their host tree (level of foliar damage)? We expected that, if the Ownership Hypothesis [34,36,37] is correct, then colonies receiving complementary food resources (i.e., both carbohydrate and protein) would better protect their host trees. On the other hand, if the Deficit Hypothesis [38] is correct, then the supplementation of protein should diminish the ants' need for protein and, therefore, their predatory activities. In this sense, the most protected trees would be those in which colonies receive carbohydrates only. Similarly, if the Fuel for Foraging Hypothesis [33–35] is correct, access to carbohydrates would fuel foraging and aggressive behavior of ants, increasing protection on trees supplemented with carbohydrates.

2. Materials and Methods

2.1. Study Area

This study was conducted at the Reserva Ecológica do Panga, a 404 ha reserve located 30 km south of Uberlândia, Minas Gerais, Brazil (19°10′ S, 48°23′ W). The mean annual temperature of the region is 22 °C and the mean annual rainfall is 1650 mm. All observations and experiments were performed on *Caryocar brasiliense* trees located within an area of approximately five hectares covered by the dominant vegetation type of the reserve (woodland savanna, locally known as *cerrado sensu stricto*), and which is characterized by a superior layer of trees and shrubs and a ground layer formed by grasses, herbs, and small shrubs [47]. *Caryocar brasiliense* is a common and widespread species in the savannas of central Brazil which produces EFNs on its leaves and inflorescences.

2.2. Supplementation Experiment

We selected and marked 67 medium sized (3–4 m in height) *C. brasiliense* trees that were separated from each other by at least 12 m. This distance was large enough to ensure that ants from a given experimental tree were not foraging in another tree. In fact, foraging by predominantly arboreal species was largely restricted to the host tree. This was because the savanna where our experiment took place was relatively open and, therefore, there was very little connectivity between the crowns of different trees [48]. Each of the selected trees received one of the following nutrient supplementation treatments, (1) carbohydrate only (20% water solution of sucrose) (n = 17 trees); (2) protein only (20% water solution containing equal parts of isolate whey protein, calcium caseinate and egg white powder) (n = 16); (3) carbohydrate and protein (mixture containing equal parts of the carbohydrate and protein solutions) (n = 19); and (4) control trees (water only) (n = 15). The amounts of carbohydrate and protein added to the water solutions are similar to those used in previous studies [20,25] and were chosen because they mimic the concentration of these two elements in natural food sources [49,50]. A total of six sealed plastic cups (6 cm high, 5 cm in diameter), with access holes (five 6 mm diameter holes, drilled on the sides of each cup), were wired onto the branches of each experimental tree. Each cup was filled with 60 mL of water, sugar, protein, or sugar and protein solution. A piece of folded tissue paper in the cup served both as a feeding platform and a structure that ants could use to escape the liquid if they fell in. Solutions and tissue platforms were changed weekly. The experiment had a duration of one year (August 2020–July 2021).

2.3. Critical Thermal Maximum (CT_{max})

To evaluate the influence of the nutrient supplementation treatments on ant's resistance to heat, we measured the CT_{max} of 21 colonies of an unidentified *Azteca* species (hereafter *Azteca*) just prior to and again six months after the beginning of the experiment. The CT_{max} of the ants was determined using dry bath equipment (Kasvi Dry Bath model K80-S01/02). In each test, 20 workers of the same colony were placed individually in Eppendorf tubes sealed by cotton and placed randomly in the dry bath equipment. The initial temperature of the test was 36 °C, which was increased by two degrees every 10 min of exposure until death or permanent loss of muscle coordination in the workers. Tests were carried out within a maximum period of up to four hours after the collection of the ants in the field. We considered the CT_{max} of the species as the average temperature of death or permanent loss of muscle coordination of the 20 workers.

2.4. Stable Isotope Analyses

We determined the isotopic signature ($\delta^{15}N$) and the carbon and nitrogen ratios (C:N ratio) of ants from 18 *Azteca* colonies. For this, we collected approximately 20 workers from each colony 10 months after the beginning of the experiment. We removed the gaster of each worker during sample preparation to avoid the effect from recently ingested food items on the analysis [16,51]. Ant samples were dried in an oven at 60 °C for 48 h and then crushed with an agate mortar and pestle. The dried samples were put into small tin capsules in precisely weighed amounts (1.25–1.5 mg) then molded into a spherical shape, put on ELISE dishes, and sent to the University of California Stable Isotope Facility, in Davis, CA, USA, for analysis. The results were expressed in delta notation per thousand, with an internationally acknowledged standard as reference.

2.5. Sampling of the Ant Fauna

To estimate the abundance and diversity of arboreal ants in the experimental trees ($n = 67$), two observers counted the number and determined the identity of the ants foraging on the main trunk and branches of each tree for a period of two minutes during the morning (7:00 a.m.–9:30 a.m.), and again in the afternoon (12:00 a.m.–2:30 p.m.), and evening (7:00 p.m.–9:30 p.m.) of the same day. This was done once in May 2021, and again one month later by the same two observers. When necessary, ant specimens were collected and stored in alcohol for later identification in the laboratory. Voucher of all species collected are deposited at the Zoological Collection from the Federal University of Uberlândia (UFU) in Uberlândia, Brazil.

2.6. Artificial Nests

We also assessed the effect of nutrient supplementation on the occupation of artificial wooden nests by arboreal ants. For this we wired six nests to the branches of about half of the experimental trees (32 of the 67 trees). The nests were made of bamboo (~100 mm in length and 10 mm in diameter). Three of the bamboo nests had an opening of 6 mm^2 and the other three an opening of 8 mm^2, which were the most used nest entrance sizes in an experiment conducted earlier [48]. The bamboo nests were installed in October 2020 and removed in August 2021. Each nest was sealed with adhesive tape and transported to the laboratory, where they were opened and the identity and number of ants was recorded, as well as the presence of eggs, larvae, pupae, and alates.

2.7. Herbivory Measurements

We estimated the level of leaf damage on 59 of the 67 experimental trees. For this, one branch from each tree was randomly selected and 10–14 newly initiated leaves (with no signs of damage) from this branch were marked using plastic-coated wires placed around the leaf petiole. This was done in September 2020 when *C. brasiliense* was flushing new leaves. After 90 days, we collected the marked leaves and produced a scanned image of each one. The area damaged by chewing insects and the total area of each leaf was determined using the ImageJ software [52].

2.8. Statistical Analyses

We analyzed the individual and interactive effects of the nutrient supplementation treatments on the isotopic signature (δ^{15}N) and on the carbon to nitrogen ratio (C:N) of *Azteca* ant workers using two-way ANOVAs, in which the predictor variables were carbohydrate (with or without) and protein (with or without). The same model was used to evaluate the magnitude of change (the effect size) of the CT_{max} of the *Azteca* ants. The magnitude of change was calculated as the logarithm of the response ratio using the formula log (CT_{max} after supplementation/CT_{max} before supplementation). Similarly, two-way ANOVAs were employed to evaluate the effects of nutrient supplementation on the species richness and overall abundance (i.e., the abundance of all species combined) of ants foraging on trees, as well on the proportion of artificial nests colonized by ants and the abundance of ants in nests. Finally, a two-way Anova was run to evaluate effects on leaf herbivore damage in *C. brasiliense* trees. When a significant interaction between the effects of carbohydrate supplementation and protein supplementation was detected, we performed a posteriori, pairwise multiple comparisons among individual treatments using the Tukey method.

Data on ant abundance were log (x + 1) transformed prior to the analyses to meet the assumption of data normality and homoscedasticity. Similarly, data on leaf herbivory was arcsine square root transformed prior to the analysis. A Gaussian error distribution was assumed in most models, except the one on species richness in which a quasi-Poisson model was run, and the one on the proportion of artificial nests colonized by ants in which a binomial distribution was assumed. Model assumptions were checked by evaluating the plot of the residuals against the fitted values and the normal probability plot. Analyses were performed in R v.4.1.1 [53], using the packages “car” [54] and “emmeans” [55].

To evaluate the differences in ant species composition among trees subject to different nutrient addition treatments we performed a two-way cluster analysis. For this, we first built a matrix containing information about the total number of trees (from each nutrient addition treatment) in which each ant species was recorded. The analysis was run in PCORD 7.0 (MJM Software Design, Gleneden Beach, OR, USA) using the Bray–Curtis index of similarity (with individual species data relativized by the species total) and the group average linkage method [56]. In this analysis, we used only data for those species that occurred in at least three of the 67 trees sampled, since the occurrence of a rare species in a giving treatment could be more related to its low abundance in the community than to its nutritional requirements.

3. Results

3.1. Effects on the Trophic Position and Thermal Tolerance of Azteca

Protein supplementation presented a significant effect on the δ^{15}N and C:N ratio of the *Azteca* colonies, whereas the supplementation of carbohydrate (sucrose) did not have an effect (Figure 1). Colonies that received protein (i.e., only protein or protein together with carbohydrate) presented, on average, a δ^{15}N 4% greater and a C:N ratio 7.2% lower than those that did not. Conversely, while sucrose supplementation affected the critical thermal maxima (CT_{max}) of *Azteca*, protein did not have an effect (Figure 2). On average, colonies receiving sucrose (sucrose only or sucrose together with protein) increased their CT_{max} in 1.59 °C, whereas the mean absolute change in the CT_{max} of the colonies that did not receive sucrose was close to zero (−0.022 °C).

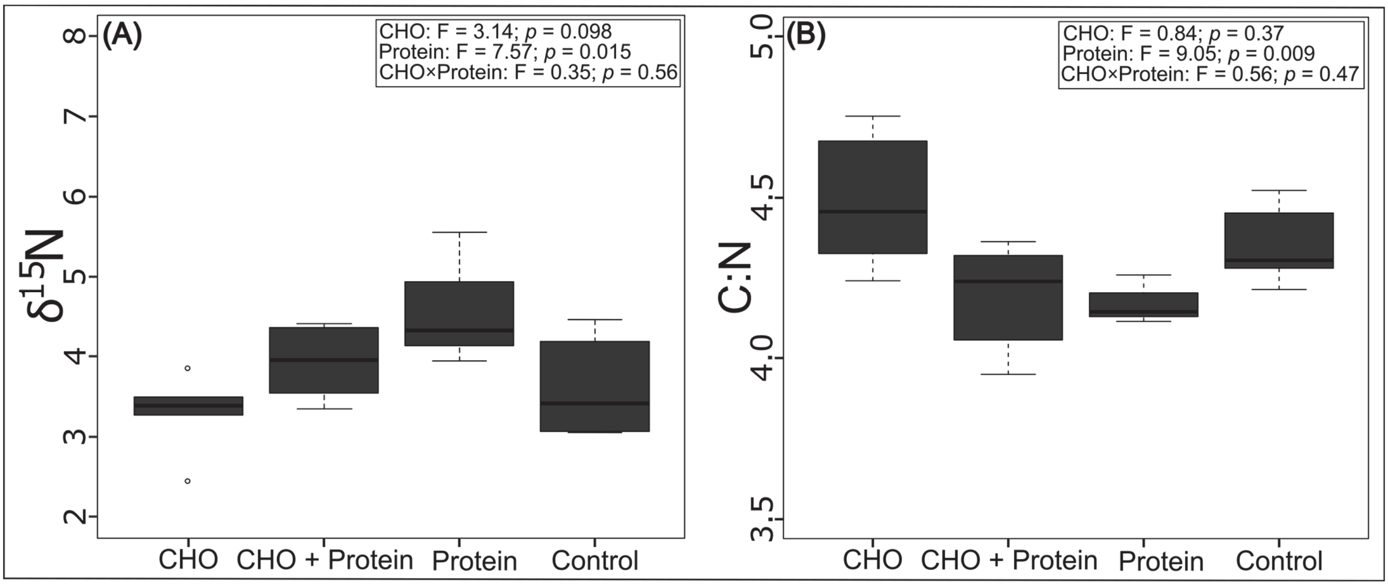

Figure 1. Effects of carbohydrate and protein supplementation on the δ^{15}N (**A**) and the carbon to nitrogen ratio (**B**) of *Azteca* sp. colonies. Circles represent outlier values.

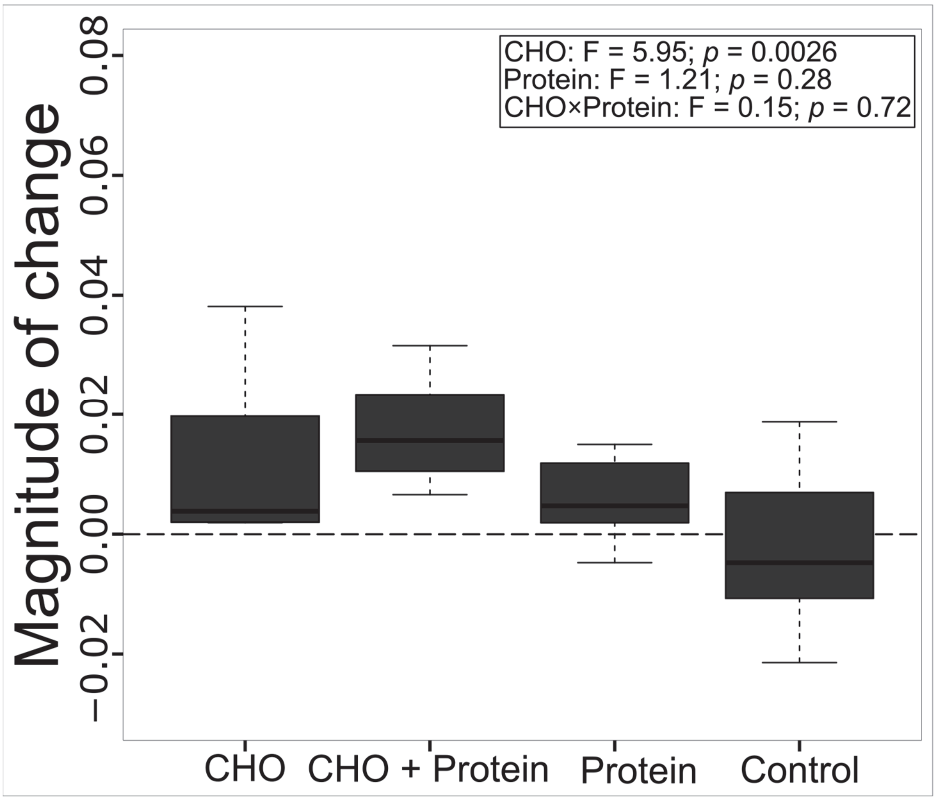

Figure 2. Effect of carbohydrate and protein supplementation on the thermal tolerance of *Azteca* sp. Values represent the magnitude of the difference (effect size) in the CT_{max} of ant workers prior and six months after the beginning of the experiment. The dashed line represents the null expectation.

3.2. Effects on Overall Ant Abundance, Species Richness and Composition

There was a significant interaction between the effects of carbohydrate and protein supplementation on ant species richness per tree. Control trees had fewer ant species than those that received carbohydrate, protein, or both (Tukey test, $p \leq 0.008$). In addition, there was a difference between those that received only carbohydrate or only protein, with the former presenting more species than the latter (Tukey test, $p = 0.042$) (Figure 3). Similarly, there was a significant interaction between the effects of carbohydrate and protein on the overall abundance of foraging ants per tree. Overall ant abundance in control trees was significantly lower than in trees from all the remaining treatments (Tukey test, $p \leq 0.006$) (Figure 3).

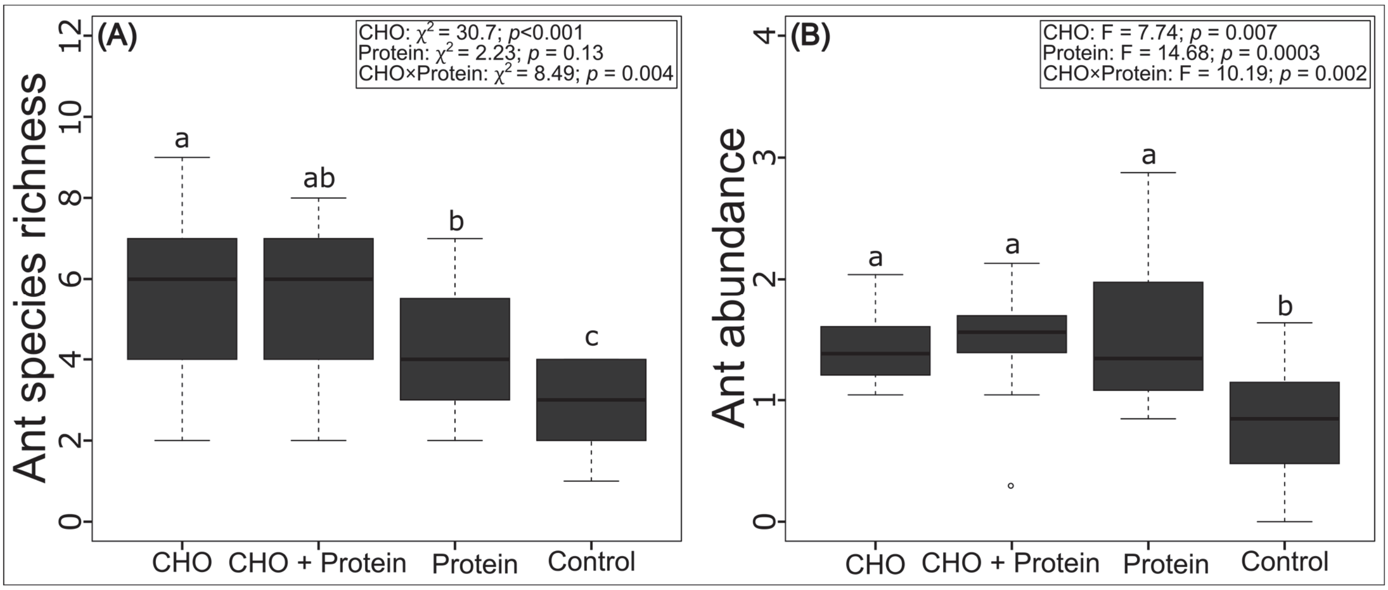

Figure 3. Effects of carbohydrate and protein supplementation on the species richness (**A**) and overall abundance (**B**) of ants (log transformed) foraging in *Caryocar brasiliense* trees. Since there was a significant interaction between the main effects, a multiple comparison test was performed. Different letters above box plots indicate significant differences among mean values.

We recorded a total of 43 ant species from 19 genera in the 67 experimental trees (Table S1). In total, 23 species were rare being recorded in just one or two trees. Excluding the rare species, we found that the similarity in species composition was comparatively greater between trees that received sucrose only and those that received sucrose and protein than between these two and those that received protein only or the control trees (Figure 4). Five species (*Azteca* sp. 1, *Camponotus senex*, *C. bonariensis*, *Pseudomyrmex gracilis*, *P. curacaensis* and *Tapinoma* sp. 7) were relatively widespread being found in trees from any treatment. *Brachymyrmex* nr. *aphidicola*, *Camponotus melanoticus*, *C. fastigatus*, *Dorymymrex* sp. 10, *Ectatomma tuberculatum* and *Pheidole radoszkozwskii*, were found mainly in trees receiving sucrose (alone or in combination with protein) whereas *Atta laevigata*, *Camponotus blandus*, *Camponotus substitutus*, *Neoponera villosa* and *Solenopsis basalis* in trees that received protein (alone or in combination with sucrose) (Figure 4).

3.3. Colonization of the Artificial Nests

From 192 artificial nests, 44 (22.9%) were colonized by a total of four species of ants, *Camponotus senex*, *Camponotus melanoticus*, *Camponotus bonariensis* and *Pseudomymex gracilis*. From these 44 occupied nests, 6 had only ant workers, and 38 contained workers and ant brood (eggs, larvae and/or pupae). Neither carbohydrate nor protein supplementation affected the proportion of artificial nests colonized by ants (Figure 5). However, nests in tree supplemented with carbohydrates had significantly more ant workers per nest than those in trees without carbohydrates, whereas protein supplementation did not affect the abundance of ants in the artificial nests (Figure 5).

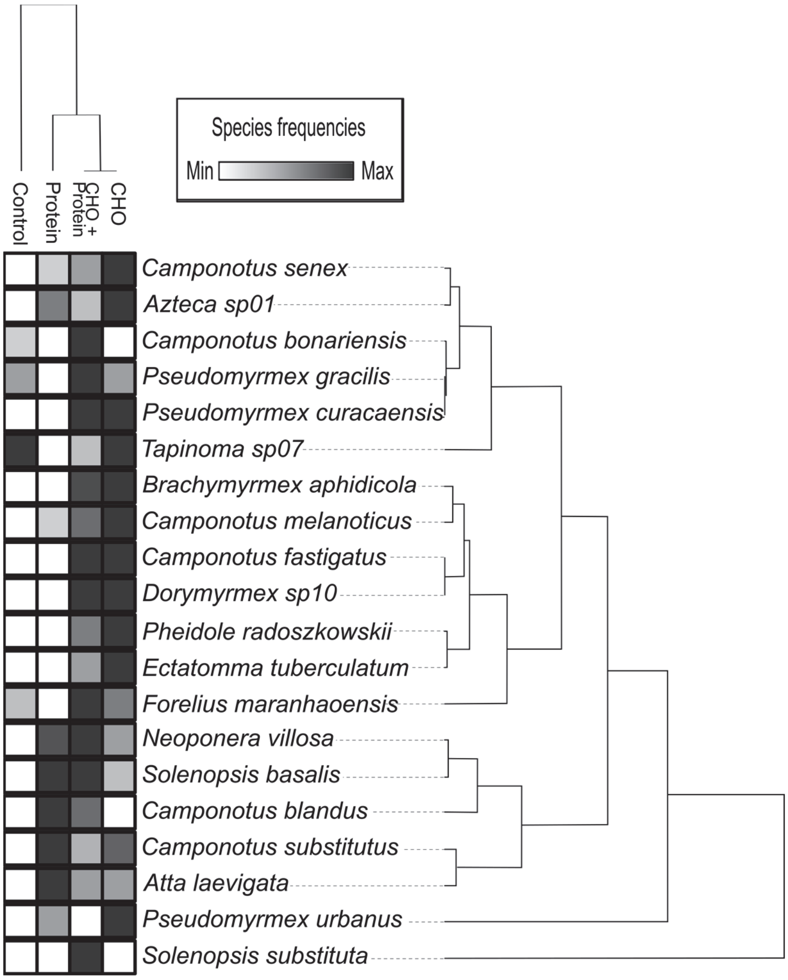

Figure 4. Two-way cluster dendrogram showing the relative frequency of different ant species in trees subject to different nutrient supplementation treatments.

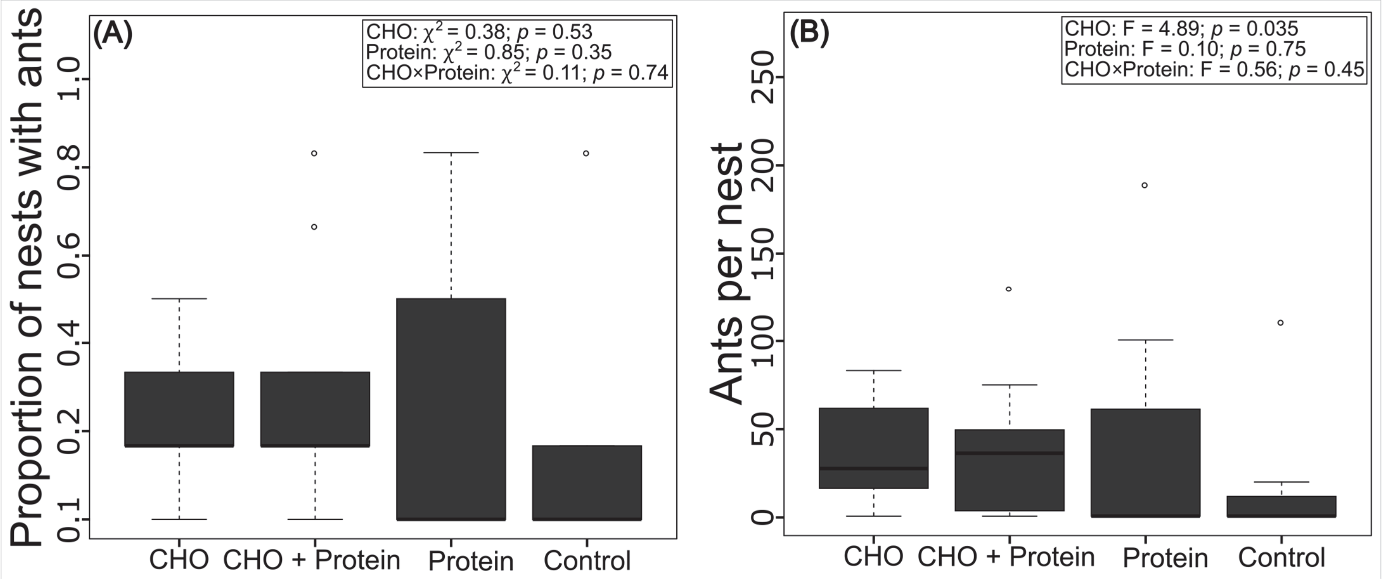

Figure 5. Effects of carbohydrate and protein supplementation on the proportion of artificial nests colonized by ants (**A**) and the overall abundance of ants in the colonized nests (**B**). Circles represent outlier values.

3.4. Leaf Herbivory

Damage by leaf chewing insects was 1.5 times lower in trees that received carbohydrates than in those that did not, whereas leaf damage in trees receiving protein was not significantly different from those that did not (Figure 6).

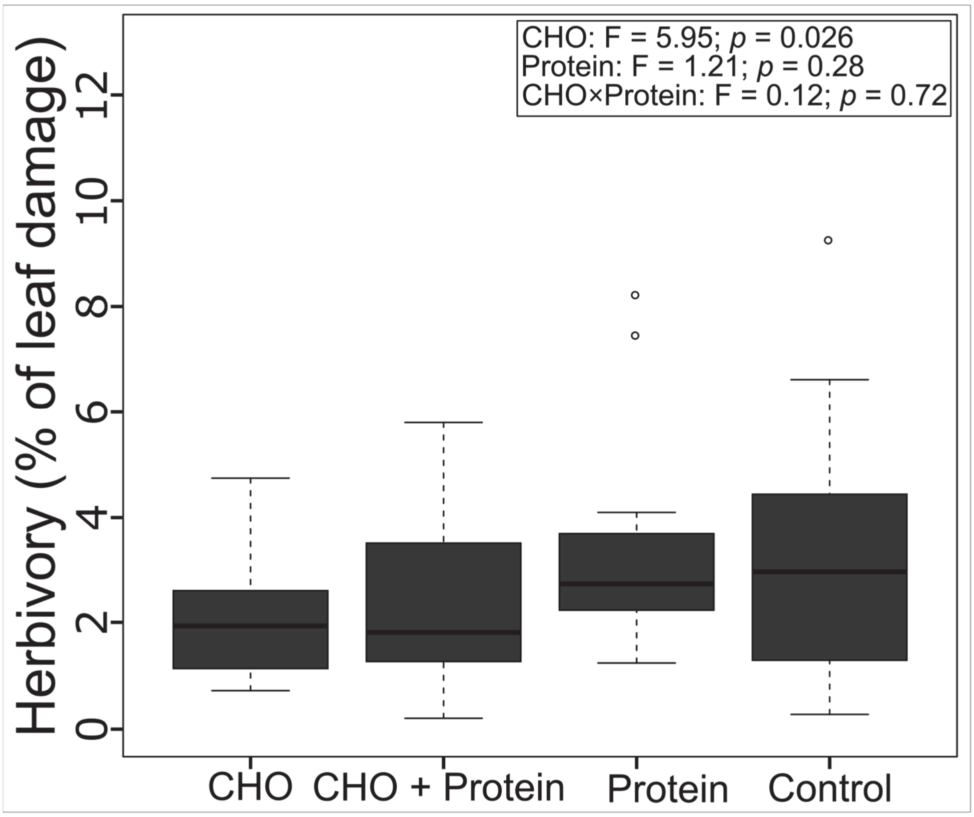

Figure 6. Indirect effect of carbohydrate and protein supplementation on the amount of leaf damage by chewing insects in *Caryocar brasiliense*. Circles represent outlier values.

4. Discussion

4.1. Colony Level Effects

The results of this study showed that colonies of *Azteca* that received protein had a lower C:N ratio and a higher $\delta^{15}N$ than those that were not supplemented with protein. These findings reinforce the notion that omnivorous ants, such as *Azteca*, have some degree of dietary plasticity, depending on the relative availability of different nutrients in the environment [21,57]. Ants of the genus *Azteca* are numerically and behaviorally dominant, with polydomous nests that commonly are distributed through numerous branches of the same plant [58–60]. They are usually associated with coccoid Hemipterans in the vegetation from which they obtain carbohydrate-rich honeydew [17,61], which may help to explain why only the experimental supplementation of protein—but not that of sucrose—affected the trophic level of *Azteca*.

In addition, we found evidence of thermal plasticity in *Azteca* since colonies increased their CT_{max} after receiving sucrose for six months. These results support earlier findings with laboratory colonies of *Azteca chartifex* [6], which showed that *Azteca* support higher temperatures when fed with carbohydrates. The observed increase in the CT_{max} of *Azteca* colonies can be explained because, in insects, carbohydrates is stored in the hemolymph as disaccharides [62], and thus can be used to generate ATP [63], which is essential to cope with thermal stress [64]. In addition, carbohydrates can also be stored as glycogen in muscle and fat bodies [65], and this can generate ATP and metabolic water, and increase evaporative cooling effects through the releasing of bound water, thus improving desiccation resistance [27]. Irrespective of the exact mechanism, it is clear that a carbohydrate-rich diet can help arboreal ants to expand their thermal niche and tolerate higher temperatures, potentially increasing foraging performance and competitive ability [cf. 6]. On the other hand, although protein is necessary to the production of heat shock proteins [28,29], a higher content of protein over carbohydrates on an insect diet is also related to reduced performance and increased mortality [66–69], since the elimination of nitrogenous waste products is highly costly [66,69] and can increase physiological stress [70].

4.2. Community Level Effects

Relative to control trees, trees receiving carbohydrate and/or protein had more ant species and more ant workers foraging on their leaves and branches. Surprisingly however, and in contrast to a similar study that involved the supplementation of these same nutrients to arboreal ants over the short term (7 days) [20], we found that trees receiving only sucrose had more species than those receiving only protein. Nevertheless, in agreement with this same study [20], we found significant variation in species composition between trees subject to different nutrient addition treatments, reinforcing the view that different ant species present different levels of attractiveness to carbohydrates or protein sources. In fact, predatory arboreal ants like *Neoponera villosa* and *Solenopsis basalis* [71] were more much frequent in trees supplemented with protein than in those receiving sucrose only.

Contrary to expected, the nutrient addition treatments did not affect the rate of occupancy of the artificial nests placed in the experimental trees. However, nests in trees that were supplemented with carbohydrates had more ants than those that did not receive carbohydrates. This result is in line with a laboratory study in which *Cephalotes pusillus* colonies provided with a diet rich in extrafloral nectar had five times more individuals per colony as compared to control colonies [72]. One possibility is that the increased availability of carbohydrates on trees has reduced the mortality of ant workers [35,69], ultimately increasing colony size. The fact that protein supplementation did not elicit a similar response can perhaps be explained because all the species (three *Camponotus* and one *Pseudomyrmex*) that colonized the artificial nests can overcome their N-deficiencies by means of endosymbiotic bacteria [73,74].

4.3. Effect on the Host Tree

We detected significant lower levels of leaf damage in trees that received sucrose, whereas protein supplementation did not have an effect. These results lend some support to the Fuel for Foraging Hypothesis, since, according to this hypothesis, carbohydrates can fuel more metabolically expensive behavior, increasing aggression and improving foraging performance by ants, which ultimately can lead to an increase in the protection of the host plants [33,35]. Carbohydrate supplementation also increased the number of ant workers occupying the artificial nests, and thus this may well have enhanced the level of protection provided by ants, given that larger colonies are more effective in defending their partner plants against herbivores than smaller ones [75]. Similarly, it is relatively well known that some ant species are more aggressive towards insect herbivores than others (e.g., [76,77]), and in this sense the larger number of ant species foraging in trees supplemented with carbohydrates may have increased the chances that the more aggressive ants were present in these trees [78]. It is also possible that carbohydrate supplementation, by increasing the CT_{max} of arboreal ants, may have allowed these ants to forage in hotter periods (when, otherwise, they would probably not be active), thus increasing the chances of encountering potential herbivores and ultimately benefiting the host plant [32]. Finally, it is important to mention that herbivory levels were very low among all surveyed trees, and, in this sense, it is likely that the differences in leaf damage we detected may not have a significant influence on plant fitness. However, considering that herbivory pressure can vary temporally and spatially [79,80] and that ant–plant mutualisms are highly facultative [81], the relevance of carbohydrate availability to the strength of ant–plant interactions may be more important in other contexts, like just after an intense fire [80] or across the different ontogenetic stages of the host-plants [57].

5. Conclusions

Overall, the results of this study indicate that the availability of food resources can modulate the population and community ecology of arboreal ants as well as the mutualistic interaction between these ants and their host trees. The supplementation of carbohydrates as well as of protein increased the number of ant workers and the number of ant species foraging on trees. However, and although arboreal ants are thought to be N-limited [18,82,83], it was the supplementation of sucrose—not protein—that elicited most of the responses. Only sucrose-supplemented colonies had an increase in their thermal tolerance, and trees with more carbohydrate available were visited by more ant species, had larger colonies, and were better protected than those that did not receive carbohydrates. In this sense, our results give further support for the idea that carbohydrates can be more important than protein for arboreal ants despite the higher availability of sources of carbohydrate than of protein in the tree canopy [22].

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d15060786/s1, Table S1: List of the ant species recorded in this study in trees supplemented or not with carbohydrate (CHO) or protein. Numbers represent the number of trees in which the species was recorded.

Author Contributions: L.A.Z. and H.L.V. conceived the study and wrote the first draft. L.A.Z., R.E.O.S.L. and A.Q. performed the field work. K.C.N. and R.E.O.S.L. helped with the data analysis and ant species identifications. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The data that support the findings of this research are available from the authors upon request.

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Morphological Strategies in Ant Communities along Elevational Gradients in Three Mountain Ranges

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Abstract: Species traits often vary in a coordinated manner, making up an ecological strategy comprised of suites of interrelated traits. Environmental gradients, such as those along elevational gradients, provide an ideal venue in which to examine variation in ecological strategies with the environment. We examined variation in the morphological strategies of ants along elevational gradients on thirteen mountains across three mountain ranges in central and south-eastern Australia. We pitfall-trapped ants, counted and identified workers and measured morphological traits. Most species showed a hump-shaped relationship between occurrence and elevation, and several responded to microhabitat variables. Morphological traits varied along two key axes: “gracility”, where high values indicated longer-legged species with dorsally positioned eyes and smooth, bare cuticles; and “size and darkness”, where species with high values were larger and darker. Analysis of assemblage-weighted means revealed that gracility decreased with temperature and increased with precipitation, suggesting links with desiccation tolerance. Size and darkness increased with UV-B, declined with increasing canopy cover and peaked at mid-temperatures. We thus detected strong shifts in dominant morphological strategies along our elevational gradients. However, the multifunctionality and interrelatedness of traits and the covariance of climatic factors may make isolation of the function of individual traits difficult. Further, the predictive power of our models may be limited in the context of novel environments predicted under global change.

Keywords: cuticle structures; ecological strategy; Formicidae; gracility; lightness; microhabitat; precipitation; size; temperature; UV-B

1. Introduction

In recent decades, species traits have come to the fore as a potential solution to improving generality in ecology [1]. Recognition of the interrelatedness of traits means that research is increasingly focused on ecological strategies, which comprise suites of traits that vary in a coordinated fashion [2,3]. Local assemblages consist of a subset of species from the regional pool, filtered by their traits [4]. Local assemblages often support a large proportion of global variation in traits, e.g., 74% of variation in CTmax (critical thermal maximum) [5], because local habitats support a large variety of niches. At larger scales, macroecological studies have revealed that further variation in traits can be driven largely by climate [6,7].

In animals, latitudinal gradients in body size, form and colour are long-established examples of how traits vary with the environment at large scales [8,9]. However, the mechanisms driving relationships of these traits with environmental gradients are often unclear, with patterns in different studies often contradictory [10,11]. For example, body size tends to increase in colder environments, but it is unclear whether this is due to thermal inertia, resistance to starvation, desiccation tolerance, or combinations of these mechanisms [12,13]. Ectotherms are often darker in cold environments, but competing hypotheses suggest that this could be due to more rapid absorption of solar radiation (thermal melanism), protection from harmful solar radiation (photo-protection) or increasing desiccation tolerance [14]; in contrast, Gloger's rule suggests darker colouration is associated with warm and wet climates due to a range of other mechanisms, including camouflage, parasitism resistance and photo-protection [15]. Recent studies suggest that body size and lightness (i.e., the opposite of darkness) are associated and may form a thermoregulatory trade-off in ectotherms, with size-related thermal inertia counter-balanced by lightness-related thermal inertia [16–19]. This suggests that, in some cases, suites of traits vary in a coordinated fashion and may be better considered as an ecological strategy [2,3].

As anthropogenic climate change accelerates [20], it is increasingly important that we understand the relationship between species' ecological strategies and climate if we are to predict the consequences for ecosystems. Conditions change rapidly along elevational gradients [21], with climates becoming colder and wetter and solar radiation exposure increasing as elevation increases. Elevational gradients are thus expected to act as a microcosm for the influence of climate change. Climate drives animal species responses directly, through impacts on their morphology, life history, physiology and behaviour [22–25]. However, it also has indirect effects on species, particularly by altering the structure of their habitats [26,27], which, in turn, drive microclimates, and regulate interactions with other species [28]. Changes in species traits with elevation have been documented across a wide variety of taxa and ecosystems [17,22,25]. Responses are often expressed through changes in the relative abundance of species [17], with many ecological strategies able to coexist in any local assemblage, but the environment determining those that become most abundant. Although plant-based studies commonly consider these changes in terms of ecological strategies, this approach has only rarely been used for animals outside of metabolic scaling and pace of life theories [29–31]. Gibb et al. [3] recently suggested that social insects such as ants may be particularly amenable to the ecological strategy approach due, in part, to similarities with plants. For example, both taxa can be considered central place foragers with expendable resource-harvesting units, i.e., leaves and worker ants [3,32].

Ants are diverse and ecologically important in most habitats globally [33,34], comprising a large percentage of animal biomass [35,36]. Their morphological traits have been relatively well-studied and previous studies have detected key morphological changes in response to climate, habitat structure and elevation [13,37]. For example, species living in colder environments are generally larger than those in warmer environments [8,13], those at higher elevations are both larger and darker than those at lower elevations [17], thermophilic and open-habitat ants have longer legs than non-thermophilic and closed-habitat ants [38,39], while correlated cuticle traits such as pilosity and sculpturing are indicative of desiccation tolerance [40,41]. Studies have attempted to deal with this covariation in traits using multivariate approaches to identifying categorical trait-based functional groups, e.g., [42,43]. More recently, we highlighted that ant traits often co-vary, so may be better considered as part of a continuous ecological strategy [3]. This thinking can also be applied to suites of morphological traits: for example, in ants, species with longer limbs generally also have longer antennae and dorsally positioned eyes, making up a 'gracile' morphological strategy [3,44].

Here, we examine morphological change along a suite of elevational gradients in three mountain ranges in central to south-eastern Australia. We ask the following questions: (1) What are the key axes of variation in morphological strategies of ants? (2) How does ant assemblage composition vary with elevation and habitat? and (3) How do morphological strategies of local ant assemblages respond to climatic and habitat factors that vary with elevation?

2. Materials and Methods

2.1. Study Sites

Field work was conducted along multiple elevation transects (total of 13 mountains) across three of the highest elevation regions in Australia: the MacDonnell Ranges (Mt Giles, 1389 m a.s.l., Mt Sonder 1380 m, and Mt Zeil, 1531 m) in central Australia, the Snowy Mountains in the Great Dividing Range (transects along Mt Back Perisher 2014 m, Mt Franklin 1646 m, Mt Grey Mare 1870 m, Mt Kosciuszko 2228 m and Mt Nattung 1625 m) in south-eastern Australia, and in Tasmania (Legges Tor 1572 m, Stacks Bluff 1527 m, Mt Field 1434 m, Mt Mawson 1318 m and Ironstone Mountain 1444 m) (Figure 1, Table S1). Mean annual temperatures at the study sites range from 17.9 to 21.7 °C for the MacDonnell Ranges, 3.8 to 13.7 °C for the Snowy Mountains, and 4.2 to 10.8 °C for Tasmania. Mean annual precipitation ranges from 231 to 432 mm for the MacDonnell Ranges, 800 to 1878 mm for the Snowy Mountains, and 660 to 1856 mm for Tasmania. UV-B of the highest month ranged from 7281 to 7473 $\mathrm{mW.cm^{-2}}$ for the MacDonnell Ranges, 6406 to 6669 $\mathrm{mW.cm^{-2}}$ for the Snowy Mountains, and 5330 to 5844 $\mathrm{mW.cm^{-2}}$ for Tasmania. Tasmanian sites were sampled in December 2012, the Snowy Mountains in January 2013, and the MacDonnell Ranges in May 2013.

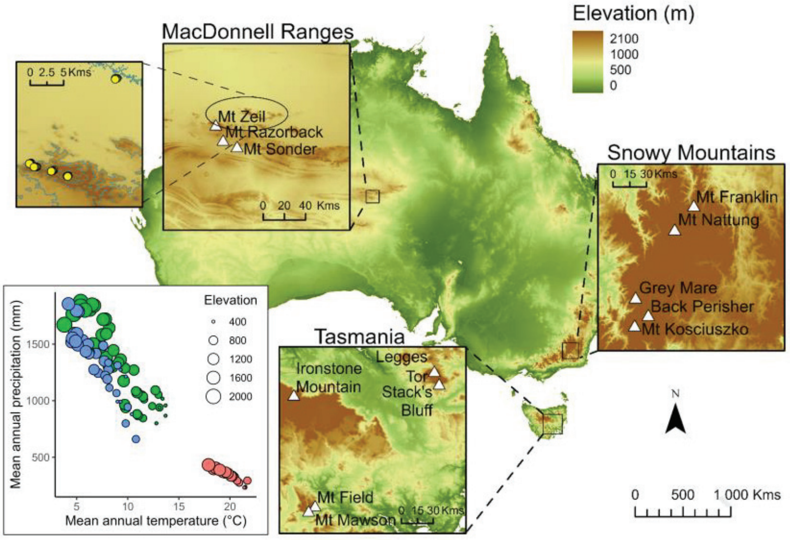


Figure 1. Map of the three study mountain ranges, showing location in Australia and close-up of Mt Zeil transects (contour interval 200 m). Inset shows the mean annual temperature and precipitation by mountain range and elevation, with MacDonnell Ranges in pink, the Snowy Mountains in green and Tasmania in blue. Contours for the mountain-scale map are in 200 m intervals.

2.2. Sampling Methods

We conducted pitfall trapping along gradients at elevations between 400 m and 2000 m above sea level in 200 m elevational intervals on each of the mountains, where the maximum elevation was determined by the height of the mountain. Ten pitfall traps (8 cm in diameter and 7 cm in depth) were placed in a two by five formation along the mountain side, with traps separated by ten metres. Traps were buried flush with the ground, filled with 100% propylene glycol to 1 cm depth and left out for seven days each.

At each site, we recorded the site aspect and noted coarse woody debris cover (low, medium or high) and dominant vegetation. At each pitfall location, we used a cover estimator chart to make visual estimates of the percentage canopy, summed percentage of the canopy and understorey cover (cover as observed by epigaeic organisms) and the percentage of bare ground (including rock and bare soil), leaf litter, grass, other ground cover plants and moss within a 1 m radius of the pitfall trap location.

In the laboratory, we separated ants from other material and transferred them to vials with 80% ethanol (20% water). We counted ants and identified ants to genus and morphospecies in the laboratory and the identity of reference specimens was determined by Prof. Alan Andersen (Charles Darwin University, Darwin, Australia). We considered both occurrence (number of pitfalls per site in which a species was present) and abundance (total number of workers of each species collected per site).

2.3. Morphological Measurements

We measured a subset of the suite of morphological traits recommended and tabulated in Parr et al. [45], consistent with the Global Ant Database (globalants.org, 01/01/2014) [13] on six specimens for monomorphic species and ten specimens for polymorphic species. The traits measured were head width across eyes (mm), mandible length (mm), hind femur length (mm), inter-ocular width (mm), sculpturing (ordinal measure, 0–3), pilosity (count of hairs crossing the mesosoma profile) and the dominant colour of the mesosoma (see [45] Tables 2 and 3 for details). Eye position was calculated as the residual of (head width—inter-ocular width) with head width.

2.4. Statistical Analysis

Statistical analyses were conducted using R [46]. To describe habitats and reduce the dimensionality of habitat characteristics measured at each pitfall trap, we used principal component analysis (PCA) (prcomp on R) to determine the key axes of variation, standardising all variables. We calculated the site means of habitat variables from the ten pitfall measures for each transect at each elevation for every mountain to use in the analysis. The two principal component axes contributing most to the variation in habitat characteristics among the sites were used in hierarchical modelling of species communities (HMSC).

We used PCA to determine the key axes of morphological variation among species (“ant morphological strategy axes”). Head width was used as a measure of body size and we calculated the residuals of other continuous morphometric measures (femur length, mandible length, eye position) and pilosity (count) with head width to use in the analysis, as these traits are generally highly correlated [45]. The final analysis included head width across the eyes, residual mandible length, residual hind femur length, residual pilosity, residual eye position and lightness of the mesosoma. These traits were reduced to two key morphological strategy axes of “gracility” and “size and darkness” (described in results).

To examine the effects of elevation (made up of the terms “elevation” and “elevation^2”, to account for humped relationships with elevation) and habitat variation (principal components PC1 and PC2 from the habitat PCA) on the distributions of ant species and their traits on each mountain range (accounting for the random effect of transect), we used hierarchical modelling of species communities HMSC, Rpackage“hmsc” [47]. HMSC is a joint species distribution model with a hierarchical structure that allows us to model species responses to environmental covariates, and to ask how these depend on species traits and phylogenetic relationships [48,49]. To model the relationship between species’ traits and their environmental responses to each covariate, we included a matrix of the two key morphological strategy axes “gracility” and “size and darkness”.

We accounted for possible phylogenetic correlations in the species’ responses to the environmental variables, in addition to that explained by traits included in the analyses [48], by including an ant phylogeny in the model. Phylogenetic relationships were accounted for by including a species-level phylogeny constructed by Economo et al. [50], which was simplified to genus level by retaining the middle species in each genus (i.e., that occupying the most central of the branches occupied by that genus). We then created polytomies to represent the study species. We excluded rare species (those present at <5 sites) from the HMSC analysis as rare species cannot be modelled reliably [51]. The ant phylogeny was converted into a phylogenetic correlation matrix and used to test for evidence of phylogenetic structure in the residual variance in species environmental responses (after accounting for their traits). The estimated phylogenetic correlation parameter, rho (ρ), takes values from 0 to 1, depending on whether residual variance in environmental responses is independent (0) to fully structured (1) by the phylogenetic relatedness of taxa. Because species assemblages and habitat components differed substantially among mountain ranges, we conducted separate HMSC analyses for each of the three mountain ranges. We assessed MCMC convergence by examining the potential scale reduction factors [52] of the model parameters (i.e., the beta (species–environment relationship) and gamma (trait–environment relationship) parameters of HMSC) [49]. We initially modelled both species occurrence and abundance data, but were unable to obtain satisfactory model convergence for species abundance, perhaps due to its high among-site variability, so we present only the species occurrence results.

We fitted the HMSC models with the "sampleMcmc" function in the "Hmsc" package. We sampled the posterior distribution with four Markov Chain Monte Carlo (MCMC) chains, each of which was run for 37,500 iterations, of which the first 12,500 were removed as burn-in. The chains were thinned by 100 to yield 250 posterior samples per chain. For species–environment models for each mountain range, we extracted the signs of the beta parameters (positive, negative or no response) with at least 95% posterior support. To explore the link between species traits and their environmental niches for each mountain range, we extracted the signs of the gamma parameters (positive, negative or no response) with at least 95% posterior support. We also show relationships with a lower level of statistical support (75% posterior probability) for species–environment and trait–environment models. Finally, we extracted the median value of the rho parameter with its 95% credible interval (i.e., the Bayesian 95% credible interval).

We calculated the assemblage-weighted means (AWM) for the first two ant morphological strategy axes (PC1 and PC2 from the principal component analysis of ant morphology). For assemblage-weighted means, the trait value for each individual in the community is weighted equally, such that numerically dominant species have a greater influence on the AWM than rarer species. AWMs were calculated for each plot without missing values (n = 215):

$$AWM = \sum_{i=1}^{S} p_i x_i$$

Here, S is the number of species in the assemblage, p_i is the proportional abundance of each species, and x_i is the trait value (ant morphological strategy axis PC1 or PC2).

We also used linear mixed effects models lmer from the lme4 package on R [53] to understand the broader assemblage-wide responses of morphological strategy to the climate. Here, we tested the effect of climate (mean annual temperature, mean annual precipitation and mean UVB of the highest month) and habitat (PC1 and PC2 from the principal component analysis of habitat) on the assemblage-weighted means (AWM) of ant morphological strategy axes (PC1 and PC2 from the principal component analysis of ant morphology). Mean annual temperature and mean annual precipitation were taken at 30 arcsec resolution from WorldClim [54], which covers the period 1970–2000. Mean UVB of the highest month was obtained at 15 arcmin resolution from the glUV dataset [55], which covers the years 2004–2013. We also tested key traits for each component (pilosity and eye position for PC1 and lightness and head width for PC2) individually. Mountain peak, nested within mountain range, was included as a random factor. We used the "dredge" function on MuMIn [56] to determine the best model and models within 2 AIC of the best model.

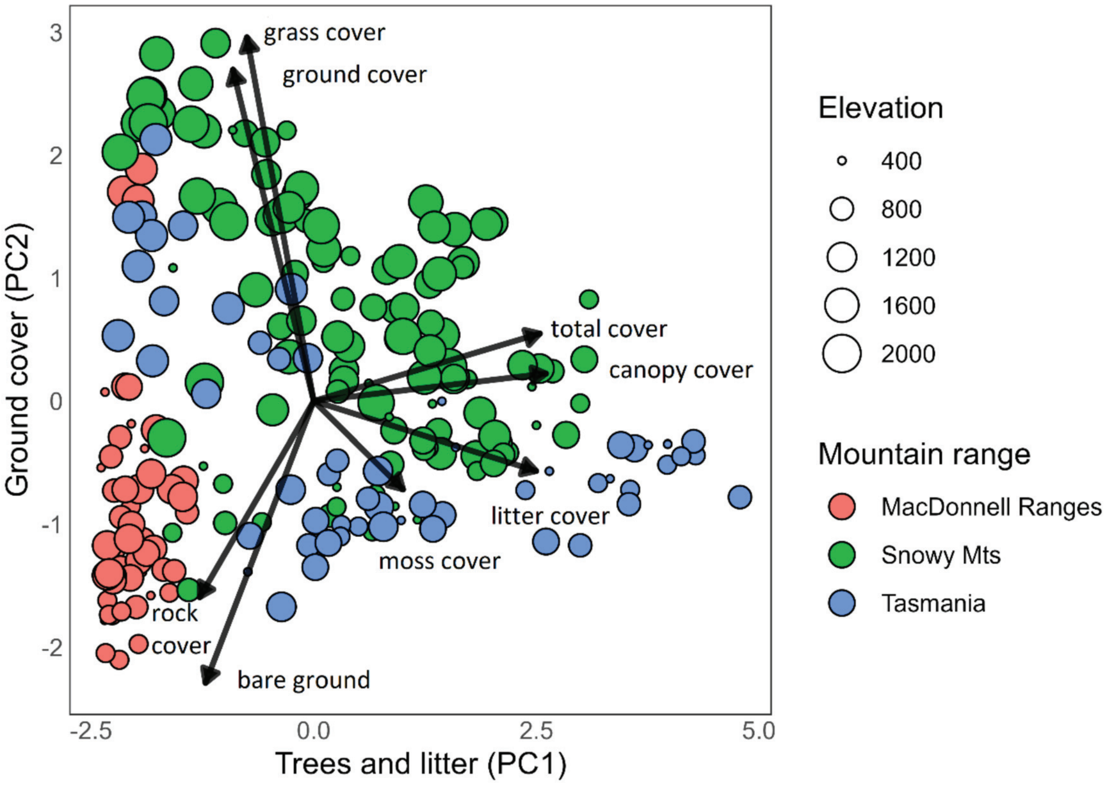
3. Results

3.1. Microhabitat Composition among Mountain Ranges and Elevations

Principal component analysis of the habitat characteristics data revealed that the first two major axes of variation contributed to 40% and 16% of the variation in habitats, respectively (Table S2). PC1 was positively associated with canopy cover and leaf litter cover (Figure 2a). PC2 was positively associated with rock and bare ground and negatively associated with ground cover and grass (Table S2). The MacDonnell Ranges sites varied minimally along PC1, mostly lacking tree cover and leaf litter. At the MacDonnell Ranges, there were no clear patterns of covariance between elevation and PC2, with sites at most elevations tending toward lower ground cover. An exception to this was a few high elevation sites that had high grass and ground cover (Figure 2a). Both the Tasmanian mountain ranges and the Snowy Mountains exhibited a broader spread along PC1, indicating that canopy and litter cover were more extensive at these sites, particularly in Tasmania. These mountain ranges also included sites with a broad range of ground cover types. High litter cover at sites with abundant canopy cover meant that those sites had little bare ground or ground vegetation (ground cover or grass cover). The highest elevation sites tended to have little canopy cover or leaf litter, but abundant ground vegetation.

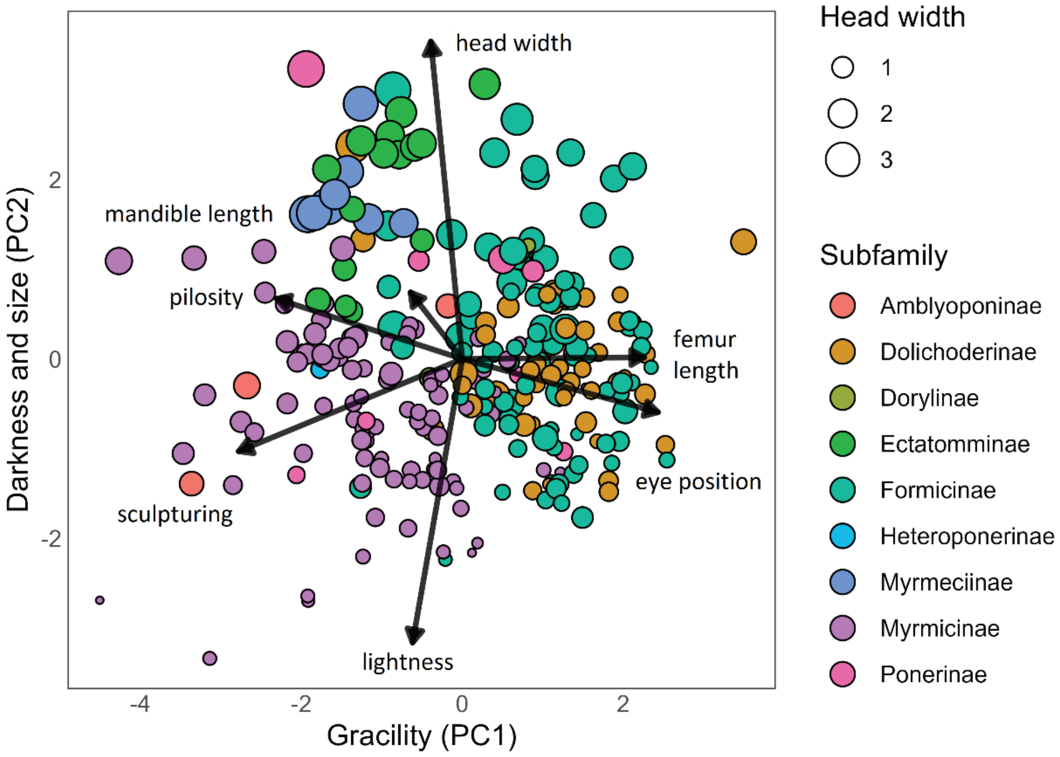
3.2. Effects of Elevation and Microhabitat on Ant Assemblage Composition

Species responses to elevation varied among mountain ranges. In the two cooler mountain ranges (Tasmania and the Snowy Mountains), most species showed humped relationships between abundance and elevation (negative relationship with the polynomial term for elevation), with many species increasing in abundance between low and mid-elevations, but declining at high elevation (Figure 3). In the hot arid mountain range (MacDonnell Ranges), several species declined with increasing elevation (negative relationship with the linear term for elevation) and humped patterns were less common. Declines with elevation were most common in the subfamily Myrmicinae, which includes genera that tend to be small and light-coloured (Figure 2b), such as *Meranoplus*, *Monomorium*, *Pheidole*, *Strumigenys* and *Tetramorium*. In Tasmania, many species declined with increasing canopy and litter cover and with increasing ground cover. In the other mountain ranges, patterns were mixed, with similar proportions of positive and negative responses to habitat measures. Across mountain ranges, several taxa, including *Rhytidoponera* and *Iridomyrmex*, responded negatively to increasing canopy and litter cover, but different species within the same genus often showed different patterns. In response to increasing ground cover, we observed declines in taxa such as the thermophilic *Melophorus* and some dolichoderines.



(**a**)

Figure 2. *Cont.*



(**b**)

Figure 2. Principal component plots for: (**a**) habitat characteristics of mountain ranges by elevation; (**b**) morphological traits of ants by body size (head width) and subfamily.

3.3. Morphological Trait Variation among Subfamilies

Principal component analysis of ant morphological traits revealed that PC1 and PC2 contributed to 28.7% and 19.0% of variation in the data, respectively (Figure 2b). PC1 was positively associated with femur length and eye position and negatively associated with cuticle sculpturing and pilosity. PC2 was positively associated with mesosoma darkness and head width. Subfamilies clustered within this space, with formicines and dolichoderines tending to have eyes more dorsally positioned and to have a more gracile body form, with longer legs (femur length). *Myrmecia* (the sole genus in Myrmeciinae) were characterised by large body size and long mandibles. Ectatommines also tended to be large and dark. Myrmicines were characterised by small size, robust body form, more laterally positioned eyes, lighter colour and greater pilosity.

HMSC analyses of the trait–environment relationships on each mountain (again using elevation, the polynomial degree 2 term for elevation, and the two habitat principal components) revealed several relationships between species' traits and their predicted environmental niches (Figure 4). Size and darkness had a positive relationship with species' elevational distributions in the Snowy Mountains at >0.95% posterior probability, with less evidence of a peak at higher elevations. Size and darkness also decreased with PC1 (trees and litter) at all mountain ranges at >0.75 posterior probability. Gracility increased with elevation in the MacDonnell Ranges and Snowy Mountains with >0.75% posterior probability. The median estimated phylogenetic independence parameter indicated that the phylogenetic signal was very weak in the MacDonnell Ranges (ρ = 0.06, posterior probability = 0.27%) and Tasmania (ρ = 0.08, posterior probability = 0.29%) and somewhat stronger, but still with low support in the Snowy Mountains (ρ = 0.36, posterior probability = 0.81%). This suggests a relatively small contribution of phylogenetic relatedness to the outcome of the analysis.

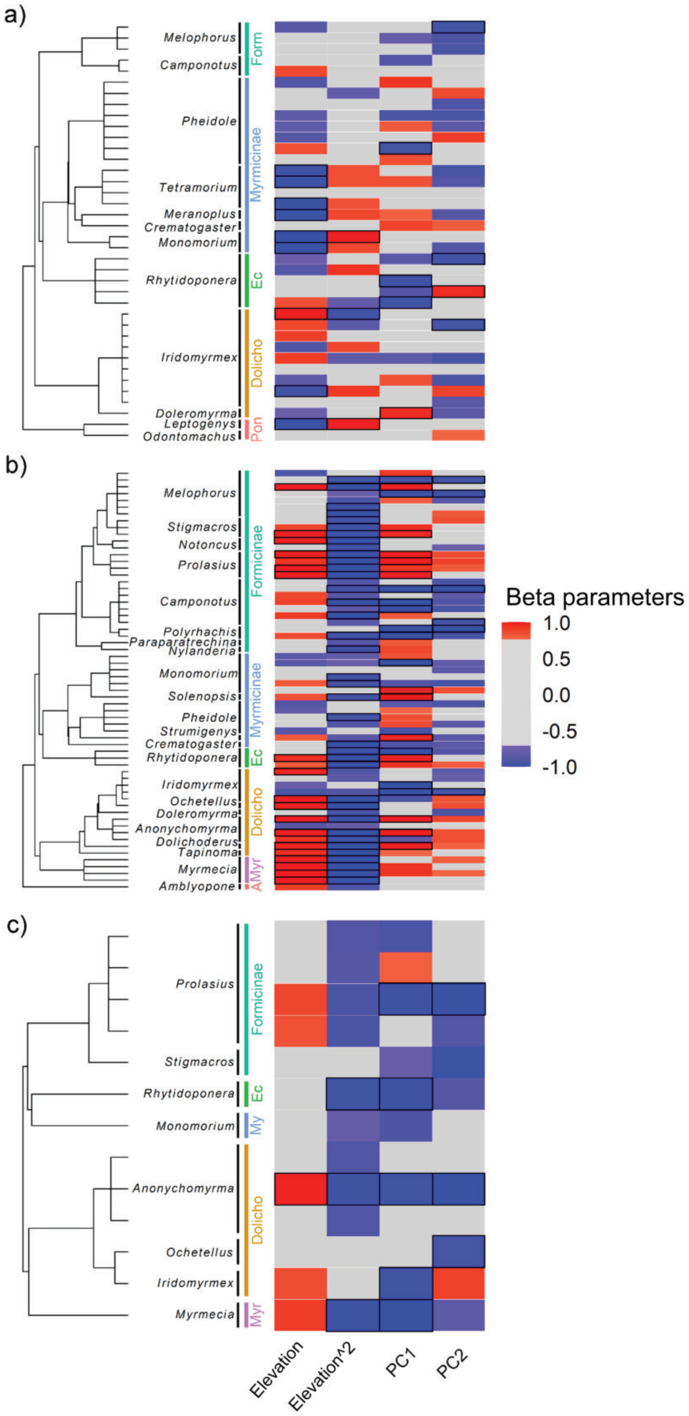


Figure 3. β parameters (species responses to environmental covariates) for the MacDonnell Ranges (**a**), Snowy Mountains (**b**), and Tasmania (**c**). β parameters with at least 75% posterior probability of being positive (red) or negative (blue) in the HMSC model are shown. Grey colours show species that were present, but did not gain strong statistical support and highlighted boxes are species that gained particularly strong support of ≥0.95% posterior probability. Environmental covariates included were elevation, elevation^2 (to account for humped relationships with elevation), and the two habitat principal component axes, representing trees and litter (PC1) and ground cover (PC2). Species are ordered according to their phylogeny, shown on the left; only species with greater than four occurrences were included in this analysis. Abbreviated labels: 'Form' = Formicinae, 'Ec' = Ectatomminae, 'Dolicho' = Dolichoderinae, 'Myr' = Myrmeciinae, 'A' = Amblyoponinae, 'Pon' = Ponerinae, 'My' = Myrmicinae.

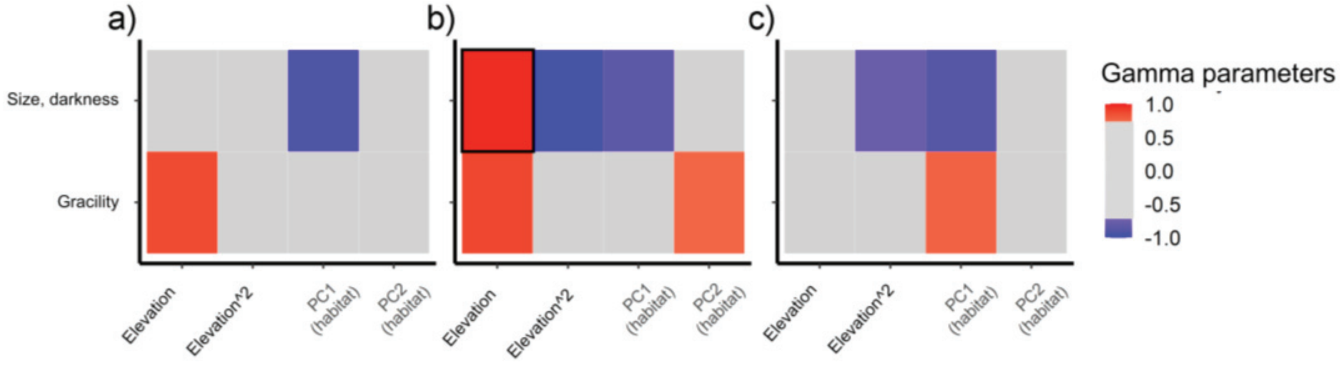


Figure 4. Relationships between species' traits (PC1 "gracility" and PC2 "size and darkness") and their environmental niches (beta parameters per environmental covariate) from hierarchical modelling of species communities models for: (**a**) the MacDonnell Ranges; (**b**) the Snowy Mountains; and (**c**) Tasmania. γ parameters with at least 75% posterior probability of being positive (red) or negative (blue) in the HMSC model are shown. Grey colours show trait-covariate combinations that were included, but did not gain strong statistical support. Highlighted boxes are trait-covariate relationships that gained particularly strong support of ≥0.95% posterior probability. Environmental covariates included were elevation, elevation^2 (to account for a humped relationships with elevation), and the two habitat principal component axes, representing trees and litter (PC1) and ground cover (PC2).

3.4. Effect of Climate and Microhabitat on Morphological Strategies across Mountain Ranges

Model selection on linear mixed models revealed that, across the suite of sites, climatic variables were generally more important than habitat variables in determining the assemblage-weighted mean of morphological strategy axes. Compared with the HMSC traits models, these models did not correct for phylogenetic similarity, but weighted species based on their numerical dominance, therefore indicating the ecological success of morphological strategies. Models within 2 AIC of the best models for both PC1 "gracility" and PC2 "size and darkness" all included mean annual temperature and UV-B (Tables 1 and 2, Figure 5a,c,e). The fit of the best model for gracility ($R^2_m = 0.47$ and $R^2_c = 0.50$, including UVB, MAT and MAP) was better than the fits of the best models of key component pilosity ($R^2_m = 0.27$ and $R^2_c = 0.32$, including UVB, MAP and both habitat components) and eye position ($R^2_m = 0.36$ and $R^2_c = 0.39$, including MAT and canopy and litter cover). The fit of the best model for size and darkness ($R^2_m = 0.24$ and $R^2_c = 0.33$, including MAT, UV-B and canopy and litter cover) was less than the fit to lightness alone ($R^2_m = 0.37$ and $R^2_c = 0.49$, including only MAT and UV-B), but not size alone ($R^2_m = 0.24$ and $R^2_c = 0.33$, including MAT and canopy and litter cover). Gracility (numerical dominance by gracile species) declined with increasing mean annual temperature and displayed a weak U-shaped relationship with UV-B. Gracility also increased with mean annual precipitation. Models within 2 AIC of the best models for size and darkness included UV-B, habitat PC1, mean annual temperature and sometimes mean annual precipitation. Size and darkness (numerical dominance by large, dark species) peaked at intermediate mean annual temperatures, declined with increasing canopy and litter cover (habitat PC1) and increased with UV-B (Tables 1 and 2, Figure 5b,d,f).

Table 1. Estimates, degrees of freedom, log likelihoods, AICc, delta and weight from best models testing the effect of climate and habitat on assemblage-weighted mean ant morphological strategies. "Poly" refers to the polynomial term, which is used to account for humped relationships between assemblage-weighted means and climatic factors.

Best Models	Intercept	poly(MAP,2)	poly(MAT,2)	poly(UVB,2)	MAP	MAT	UV-B	Habitat PC1	Habitat PC2	df	logLik	AICc	Delta	Weight
PC1 "gracility"														
Model 1	0.43			+	0.27	−0.60				8.00	−128.42	273.5	0.00	0.064
Model 2	0.43			+		−0.93				7.00	−130.14	274.8	1.28	0.034
Model 3	0.43			+	0.26	−0.63		0.02		9.00	−128.26	275.4	1.85	0.025
PC2 "darkness and size"														
Model 1	−0.09		+				0.21	−0.14		8.00	−99.72	216.1	0.00	0.079
Model 2	−0.09		+		−0.19		0.27	−0.12		9.00	−98.69	216.2	0.11	0.074

Table 2. Test statistics and significance of best models for the relationship between assemblage weighted mean "gracility" and "darkness and size" and the environment.

Source	χ^2	df	*p*-Value
PC1 "gracility"			
Mean Annual Temperature (MAT)	10.3	1	**0.0013**
Mean Annual Precipitation (MAP)	3.8	1	0.0516
UVB (Polynomial, 2)	67.5	2	**<0.0001**
PC2 "darkness and size"			
Mean Annual Temperature (MAT)	24.1	2	**<0.0001**
PC1 (trees and litter cover)	11.1	1	**0.0009**
UVB	11.8	1	**0.0006**

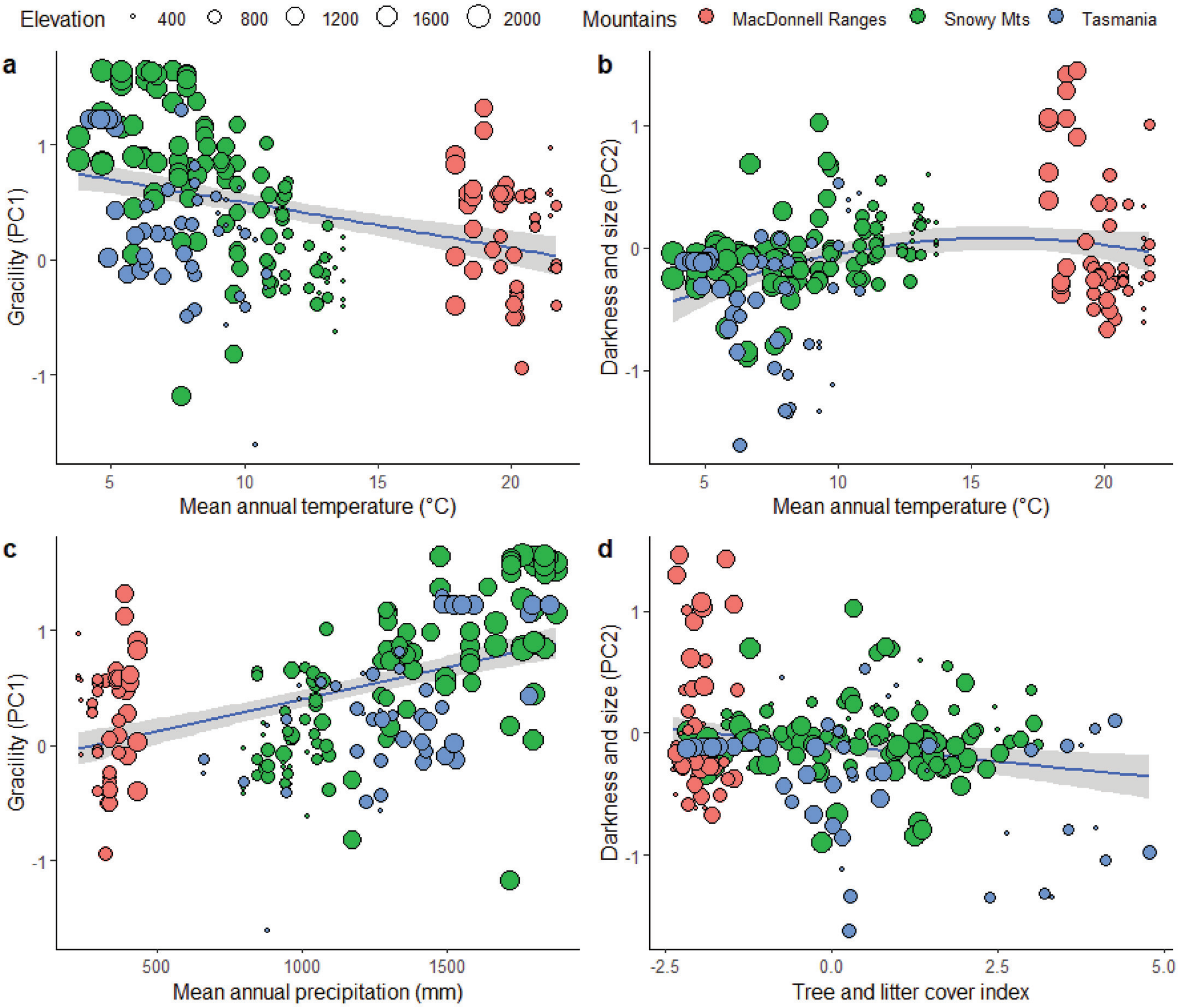


Figure 5. *Cont.*

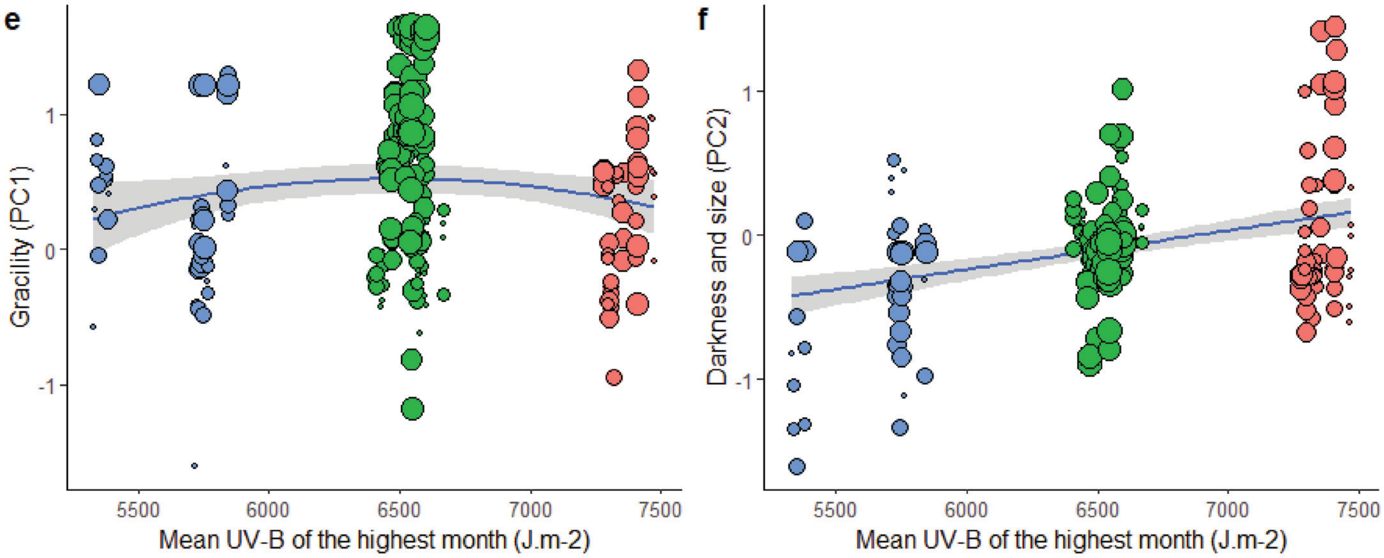


Figure 5. Predicted relationships between ant trait principal components "Gracility" (**a**,**c**,**e**) and "Darkness and size" (**b**,**d**,**f**) and predictor variables: mean annual temperature (**a**,**b**), mean annual precipitation (**c**), (tree and litter cover index (**d**) and mean UV-B of the highest month (**e**,**f**). Size and colour of points indicate elevation and location (as per the legend). J.m^{-2} is joules per square metre; grey areas are the standard error of the mean.

4. Discussion

We examined associations between ant assemblage composition, ant morphological strategies and the environment along elevational gradients on thirteen mountains across three mountain ranges, two in temperate south-eastern Australia and the third in arid central Australia. These gradients captured environmental variation in climate, openness and litter cover as well as extreme trait variation (Figure 2a). Individual species responded idiosyncratically to elevation and habitat structure, but we observed a general trend toward humped occurrence patterns at the temperate sites, where climatic conditions varied more across the gradient. Ant morphologies occupied two key axes of "gracility" and "size and darkness", suggesting that suites of traits are interrelated, presenting important morphological strategies. When we accounted for species abundances by considering assemblage-weighted mean responses of ant morphological strategies, we detected clear responses to the environment. Although assemblages in all mountain ranges supported a broad range of morphological strategies, our study thus suggests that dominant morphological strategies are regulated by climate.

4.1. Assemblage Composition along Elevational Gradients

Species assemblages varied in their response to elevation among mountain ranges. In the temperate mountain ranges, many species increased in abundance with elevation to a point, then decreased (showing a humped relationship). A decline with increasing elevation was particularly common among the myrmicines, which are smaller and lighter in colour than most other subfamilies present and were most diverse in leaf litter environments. Although several species declined with elevation in the MacDonnell Ranges, overall patterns were more idiosyncratic, reflecting lower variation in climatic variables among sites: mean annual temperatures varied less than 4 °C among sites in the MacDonnell Ranges, but more than 10 °C for the Snowy Mountains and 6 °C in Tasmania. Similarly, mean annual precipitation in the MacDonnell Ranges varied less than 200 mm among sites, while that in the Snowy Mountains varied by 1000 mm and Tasmania by 1200 mm. This is also reflected in the low variation in microhabitats in the MacDonnell Ranges, compared with the south-eastern mountain ranges.

At all sites, greater ground cover was associated with a lower occurrence of several species (at >95% probability), particularly species within the thermophilic genus *Melophorus*. Patterns were more idiosyncratic when a broader band of certainty was considered (>75%). Greater tree and litter cover was associated with a reduced occurrence of several ant species in Tasmania, consistent with the finding that cool forest environments are less favourable than grasslands and shrublands for ants [36]. More broadly, *Iridomyrmex* and *Rhytidoponera* declined in occurrence with increasing canopy cover, consistent with known preferences of *Iridomyrmex* and large species of *Rhytidoponera* for open habitats [28,57]. Globally, ant species richness increases with temperature and precipitation [58]. Although mean annual temperatures may be higher at low elevations, high canopy cover may reduce access to solar radiation relative to high elevation sites, which lack canopy cover. Ants do not need to be active year-round, so the occurrence of warmer conditions during summer days may favour ant species able to optimise their foraging activity during those times.

4.2. Changes in Morphological Strategy in Relation to the Environment

4.2.1. Gracility

Changes in species dominance across mountain ranges and elevations were linked with the morphological strategy axes of "gracility" and "size and darkness". Our "gracility" axis aligns well with a global-scale description of the major axes of morphological variation in ants [3]. Increasing "gracility" indicated species with relatively longer legs, more dorsally positioned eyes and barer (less pilose), less sculptured cuticles (Figure 2b). Formicines and dolichoderines tended to be more gracile, while myrmicines, amblyoponines and ponerines were less gracile. Dominance by gracile species declined with increasing temperature and decreasing rainfall, suggesting that robust, hairy morphologies are favoured in hot and arid conditions, probably because they protect against desiccation [41]. Fits for the composite "gracility" variable were better than those for its key components. Extreme long-leggedness is usually associated with thermophily as it allows ants to raise the body into cooler air layers and increase running speeds, resulting in reduced foraging times and therefore less heat exposure [38]. In contrast, gracility tended to increase with increasing elevation (Figure 4), and was low in the (hotter) MacDonnell Ranges, and at lower elevation (hotter, drier) sites in the Snowy Mountains and Tasmania (Figure 5a,c). However, long-limbed species are also likely to have a larger surface area to volume ratio, increasing rates of water loss [59]. In ants, high pilosity and sculpturing are associated with reduced water loss rates and increased temperature tolerance [41], suggesting another mechanism through which low "gracility" increases desiccation resistance. Even sparsely distributed hairs increase boundary layer resistance to water loss in insects, and hydrophobic hairs situated over spiracles may further reduce water loss [60–63]. The suite of covarying traits that comprises "gracility" may therefore indicate a spectrum of desiccation tolerance. Species that are desiccation-tolerant may trade-off benefits accrued from high gracility, such as forager speed [38], to increase foraging times in more arid environments.

Analysis of the gracility–UVB relationship suggests that gracility may peak at mid UVB. However, this weak response may be a result of the dominance of relatively gracile *Iridomyrmex* in the Snowy Mountains. *Iridomyrmex* are named for their iridescent cuticle, with the iridescence arising from cuticle nanostructures [64]. Iridescent hairs increase the reflectivity of *Cataglyphis bombycina* ants in the visible and near-infra-red and emissivity in the mid-infra-red ranges, enhancing their survival in the hot arid climate of the Saharan desert [65,66]. Structural iridescence in *Iridomyrmex* cuticles might have similar effects in the high UV environments of central Australia, although the function of cuticular iridescence is unclear: experiments with tiger beetles (*Cicindela*) were unable to show that cuticular iridescence influences heat gain from solar radiation [67].

4.2.2. Size and Darkness

Along the second morphological strategy axis, size and darkness increased together, with myrmeciines and ectatommines tending to be larger and darker, while myrmicines, dolichoderines and many formicines were smaller and lighter in colour. Darkness and body size are thought to be linked due to interrelated thermal benefits, whereby size-related thermal inertia is counter-balanced by lightness-related thermal inertia [16,18]. However, UV-B is hypothesised to increase cuticle darkness without affecting body size, suggesting the potential for the decoupling of these traits where UV-B and temperature are positively correlated, e.g., [14], this study. Dominance by larger and darker ant species was associated with several environmental factors in the assemblage weighted models, including mean annual temperature, tree and litter cover and UV-B (Figure 5). Consistent with this, large, dark species occurred more at high elevations (in the Snowy Mountains) and less in sites with high tree and litter cover in the HMSC (Figure 4). Mean annual precipitation appeared in the next-best model for size and darkness. The best models for assemblage darkness achieved a better fit than the composite “size and darkness” variable, although those for assemblage size (head width) did not. The best models for assemblage darkness included MAT and UV-B, but not precipitation, suggesting that Gloger’s rule (ants are darker in warmer, wetter environments) [9] was not supported. Melanin, the pigment that makes ant cuticles appear dark, absorbs UV wavelengths such that cuticles containing large melanin concentrations may protect against UV-B wavelength light penetration [17,68,69]. Consistent with the photo-protection hypothesis [14], dominance by larger and darker ant species was strongly associated with increasing UV-B, despite large variation in this axis within mountain ranges. This continent-scale result is consistent with Law et al.’s [14] local-scale study, which showed that ants occupying higher strata in a tropical rainforest were darker. Other studies of ants at global scales have shown weaker relationships between lightness and UV-B [17,70], suggesting there is greater evidence for thermal melanism.

Ectotherms are commonly reported to be larger and darker in cooler environments [13,18,71]. While temperature and “size and darkness” were negatively correlated in the MacDonnell Ranges, increasing temperature was associated with increasing size and darkness for the Snowy Mountains and Tasmanian mountain ranges, resulting in a humped relationship between temperature and the size/darkness morphological axis. The decline in ant size/darkness with increasing temperature in the MacDonnell Ranges is consistent with the thermal melanism hypothesis [71,72] because darker species heat up faster, while larger body size increases thermal resistance [18,59]. However, the increase in size and darkness with increasing temperature in the south-eastern sites contradicts the thermal melanism hypothesis. It also contrasts with Bishop et al.’s [17] finding that assemblage darkness in ants declines with increasing temperature at low UV-B, but not at the most extreme UV-B. The anomaly in the two south-eastern mountain ranges might be associated with their high tree lines: 1800–2000 m in the Snowy Mountains and about 1200 m on mountain peaks in Tasmania [73,74]; and greater ground cover at high elevation sites. Both factors would increase shading and insulation at ground level and protect ants from temperature extremes. While ground cover (habitat PC2) did not appear in the best models, greater tree and litter cover (habitat PC1) was associated with decreased darkness and size. Microhabitats filter the larger-scale climates experienced by species and are therefore a critical consideration in understanding trait–environment links at a scale relevant to species [75,76]. Increasing tree and litter cover likely protects species from harmful UVB and reduces their opportunity to use dark colours to rapidly absorb heat. Litter also creates complex habitats that can be difficult for larger species to traverse [37,77].

5. Conclusions

This study builds the case for the contribution of coordinated variation in traits among ants to ecological strategies. We have identified broad morphological strategies of ants in response to climatic and habitat variables that change in a coordinated fashion along elevational gradients. A variety of functions have been attributed to the traits we have examined here, many of which have been experimentally tested and verified, e.g., pilosity and sculpturing are associated with desiccation tolerance [41]. The multifunctionality of traits suggests that trait covariance may differ among systems if there is no physiological trade-off despite correlations among traits. For example, although size-related thermal inertia is counter-balanced by lightness-related thermal inertia, lightness is also predicted to respond to UV-B, independent of body size. Elevational gradients are unique in the covariance of several climatic variables, e.g., temperature and UV-B are negatively correlated along elevational gradients, but positively correlated along latitudinal gradients, so it is unclear whether the covariance of the suites of traits examined in this study is universal (e.g., through trade-offs). However, this is important information if we are to predict species responses to future novel climates, where associations among climate variables may differ from those found in contemporary environments. We argue that our focus on the coordinated variation of traits as ecological strategies, measured as continuous variables, allows for a predictive science that can better equip us for global change than the placement of taxa into categories, e.g., functional groups [43,78].

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d16010048/s1, Table S1: Locations and conditions of sampling sites. Abbreviations are as follows: MAT = mean annual temperature, MAP = mean annual precipitation, and UVB = mean UVB of the highest month. Table S2: Eigenvalues and % variance explained by principal components and contribution of habitat characteristics and morphological traits to the primary and secondary principal components from PCA.

Author Contributions: Conceptualization: H.G., N.J.S. and R.R.D.; Methodology: H.G., I.O. and M.P.; Data Analysis: M.M.J., P.C. and H.G.; Writing—Original Draft Preparation: H.G.; Writing—Review and Editing: all authors. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was approved by the Ethics Committee of La Trobe University (AEC11-46 01.09.2011).

Data Availability Statement: Data will be deposited in the Global Ant Database (globalants.org).

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Diversity and Resilience of Seed-Removing Ant Species in Longleaf Sandhill to Frequent Fire

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Abstract: Prescribed fire is used globally as a habitat restoration tool and is widely accepted as supporting biotic diversity. However, in fire-prone ecosystems, research has sometimes documented post-fire reduction in ant diversity and accompanying changes in seed removal behavior. This is concerning because ants provide important ecosystem services that can aid in restoration efforts, including seed dispersal. In this study, we examined the immediate impacts of fire in the well-studied ant community of longleaf pine forests (LLP) in the SE USA. We surveyed seed-removing ant species in a LLP sandhill ecosystem to investigate the effects of prescribed fire and coarse woody debris (CWD), a nesting and foraging resource, on ant community composition and ant–seed interactions. Seed-removing ants comprised a significant portion of detected ant species (20 of 45); eight of these species are documented removing seeds for the first time. Following an experimentally applied low-intensity summer burn, decreases in seed remover detection were observed, along with reductions in the number of seeds removed, across both burned and unburned areas; neither prescribed fire nor proximity to CWD significantly influenced these factors. Together, these results show that seed-removing ant species constitute a substantial proportion of the LLP sandhill ant community and are relatively robust to habitat changes mediated by low-intensity prescribed burning during the growing season. Considering ant community resiliency to fire, we can infer that using prescribed fire aligns with the goals of restoring and maintaining biotic diversity in this fire-prone ecosystem.

Keywords: Formicidae; *Crotalaria rotundifolia*; *Pinus palustris*; leaf litter; National Ecological Observatory Network (NEON); *Pheidole*; restoration; seed dispersal; tuna–honey bait; *Aphaenogaster*

1. Introduction

Prescribed fire is used globally as a habitat restoration and conservation tool [1,2]. Successful long-term restoration and conservation of fire-prone habitats are contingent upon understanding the relationships between fire and the organisms each habitat supports. Research in various ecosystems has investigated the effects of prescribed fire on the diversity and composition of many floral and faunal groups, including ants.

Ants provide many important ecosystem services that can aid ecosystem restoration efforts [3,4], including soil turnover, aeration, decomposition, and seed dispersal [5]. Ant–seed interactions include both predation and mutualism—many ant species consume seeds, disperse seed, or both [6]. Both dispersing and predatory relationships can be characterized along a spectrum ranging from specific—occurring between select plants and ant species with specialized diets, e.g., phylogenetically constrained seed granivores are highly specialized seed predators; other species are keystone dispersers of plants with nutritious "elasiosome" seed appendages [7–9]—to diffuse, occurring between a diversity of plants and opportunistic, omnivorous generalist ants [10,11]. Proposed benefits of seed dispersers—ants that remove but do not consume seeds—include increasing plant fitness by carrying seeds farther than passive dispersal limits [12] and by depositing seeds in locations with nutrient-rich soil [13]. By moving seeds below ground or into wood, ants may potentially benefit plants by reducing seed loss to rodent predation and fire [14]. In contrast, seed-predating ants can decrease plant fitness by directly consuming seeds and preventing germination by caching seeds far belowground [15]. Of course, seed predators occasionally act as seed dispersers—seeds cached near the soil surface may germinate before consumption, and discarded seed may germinate in refuse piles [16–18]. By these processes, seed predation and dispersal shape plant communities [8,19–21].

A large body of literature documents the general effects of fire on ant communities (reviewed by Andersen [22]). This research shows that the largest community composition differences are seen in habitats managed with highly contrasting fire regimes. Ants that are most susceptible to direct mortality from fire are those that nest in flammable substrates such as twigs and leaf litter [22]. Studies in fire-prone ecosystems have shown that reductions in ant diversity and abundance are common in temperate forests for as long as six months post-fire, with litter-dwelling ants more adversely affected than soil dwellers [23]. Other ant species are impacted indirectly by changes in habitat mediated by fire [22,24]. One way that fire effects on ant communities impact the broader ecosystem is through seed dispersal. Observed changes in seed dispersal behaviors post-fire include increased seed removal rates [25–27], changes in the composition of seed-removing ant species [25], and shifts in the relative activity of seed-removing species [26,27].

Fire management may affect ant–seed interactions by altering the availability of coarse woody debris (CWD), a resource that contributes to overall biodiversity in forest systems [28–30]. Many invertebrates, such as termites and saproxylic beetles depend on decaying wood, and other taxa use it for nesting or foraging [31,32]. For ants, CWD is known to provide nesting habitat to species in the genera *Camponotus*, *Formica*, *Pheidole* and *Aphaenogaster*, among others [33–35], but the importance of wood presence to other ant taxa is unclear. Ant species richness has been shown to increase in the immediate vicinity of logs as compared to 3 m away [36], and the presence of both fire and CWD has been shown to benefit specific ant species [36]. CWD may offer wood-nesting ants protection from heat- and fire-inflicted mortality, as it does for termites [37]. Additionally, decaying CWD retains moisture during and after burning, which maintains a more hospitable microclimate for ants [31]. For this reason, CWD may be favored by wood-nesting species such as *Aphaenogaster*, a genus generally associated with high-humidity logs [34] and other taxa attracted to cool, humid microclimates. Consequently, CWD may increase the survival of wood-associated ants both during and after fire and mitigate the disruption of seed dispersal after prescribed burns.

In the Southeastern U.S., prescribed fire is commonly used to manage and restore biodiverse longleaf pine forest (LLP) ecosystems [1,38], which are characterized by the presence of *Pinus palustris* Mill. (Pinales: Pinaceae). At present, about only 3% of LLP habitat that existed before European settlement remains intact, and many sites have experienced fire suppression for decades [39]. In areas undergoing restoration and conservation, frequent, periodic prescribed fire is consistently applied. In LLP ecosystems, frequent fire maintains a diverse herbaceous groundcover [1,39–42] and provides a habitat for open-canopy pine specialist animal species, including the gopher tortoise, red-cockaded woodpecker, upland snakes, and brown-headed nuthatch [43,44]. While multiple studies have addressed ant diversity in LLP ecosystems [45–50], few have identified which ant species interact with plant seeds [51–54], and little is known about the impact of restoration activities (including prescribed burning) on seed-removing ants.

Studies specific to LLP ecosystems suggest that prescribed burning has short-term negative impacts on the native ant community. Izhaki et al. [46] detected a decrease in ant abundance and richness following a winter burn, with a return to pre-burn richness and abundance 6 months post-fire. However, ant genera respond variably to fire [45,46], which may influence competition for resources post-burn. Higher disturbance regimes are correlated with the presence of non-native invasive species such as the red-imported fire ant [45,50], which competes with native ants for seeds [52,53]. While fire is generally thought to reduce CWD volumes, studies have shown variable effects of fire frequency on the volume of CWD in LLP ecosystems.

Prescribed fire and CWD resource use interact with seasonal changes to influence ant community dynamics in LLP ecosystems. It is well established that ant activity varies seasonally [55]. For example, in the well-studied LLP-dwelling *Pogonomyrmex badius* (Latreille), foraging intensity is correlated with energy demands related to colony phenology, with foraging peaking when reproductive castes are being produced [56]. For *P. badius* and others (e.g., *Camponotus* and *Temnothorax* spp.,) peak foraging occurs during the wet, growing season [56,57], while others (e.g., *Prenolepsis imparis* Say [58]) are more active in the dry, dormant season. Previous research on ant community response to prescribed fire in LLP has been limited to dormant season burns [45] or growing season burns without informative controls [46,50]. Based on these studies, it is not possible to disentangle the effects of fire management from seasonal peaks of activity related to abiotic/biotic factors, including the presence of CWD. Growing season burns are preferred in restoration efforts as they induce flowering in the dominant groundcover wiregrass species (*Aristida stricta* Michx./*Aristida beyrichiana* Trinius and Ruprecht) (Poales: Poaceae) [59]. However, growing season burns likely coincide with periods of high energy demands for colonies of many under-studied ant species.

To explore these dynamics, we conducted a two-year study assessing ant community dynamics in relation to fire in a LLP sandhill ecosystem in north-central Florida. We investigated whether prescribed fire and CWD were associated with discernable changes in ant community composition and seed removal behaviors over time. This field study was designed to test the effects of conservation management on ground-dwelling ant species by (1) identifying the seed-removing ant species in LLP sandhill habitat and (2) determining the influence of prescribed fire and the presence of CWD on seed-removing ant species community composition and activity within the context of seasonal change. We expected seed-removing species to be resilient to prescribed fire, resulting in relatively short-term, minor declines in seed-removing activity post-fire, and even smaller declines in seed-removing activity in areas where CWD was present.

2. Materials and Methods

2.1. Study Site

This study was conducted at Ordway-Swisher Biological Station (OSBS), a >9500 acre biological research station owned and operated by the University of Florida in Putnam County, Florida (29.67° N, 82.03° W). The site was xeric sandhill LLP savanna with intact, biodiverse groundcover managed with growing season burns every 2–4 years, on average, for at least the past 20 years [60]. Sandhill, one of six recognized LLP ecosystem types, is found throughout the Southeastern United States Coastal Plain [39] and is characterized by widely dispersed *P. palustris*, a midstory of oaks and shrubs and a dense, grassy, and herbaceous groundcover, dominated by wiregrass [59]. The seeds of at least seven plant species in sandhills at OSBS are known to be dispersed by ants (Table S1).

2.2. Experimental Design

We implemented a Before-After-Control-Impact (BACI) study by conducting ant sampling before and after application of prescribed fire to half our study plots. We established 10 plots, each of which consisted of two 5 × 5 m paired subplots spaced 10 m apart, at least 15 m from other plots, and at least 25 m from management unit edges (Figure 1). Each plot was considered independent from others; plots were distanced farther apart than the average foraging distance of an individual ant colony in eastern North American forests, and thus far enough to attract ants from separate nests [61]. For each plot, subplots were selected with CWD naturally present in one and naturally absent in the other. CWD was defined as, minimally, one segment of a dead, decaying oak or pine tree trunk or branch at least 6 cm in diameter and 1 m in length. All downed wood meeting these criteria were summed for a measure of total CWD volume (Table S2). The average volume of CWD per plot was 960.1 $cm^3 \pm 173.7$ cm^3 SE.

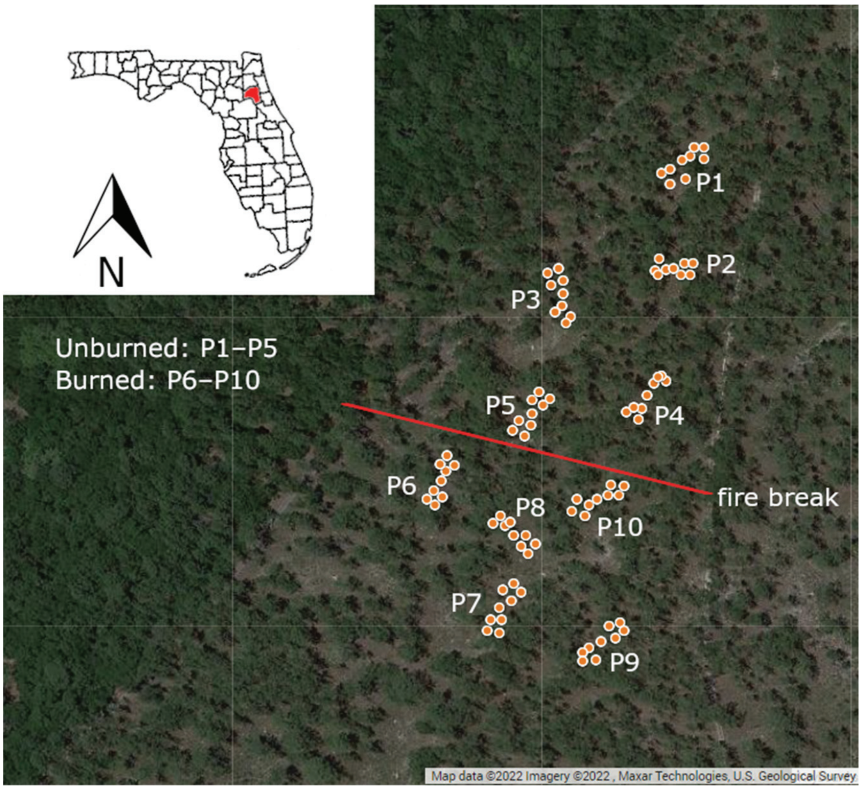


Figure 1. Diagram of the 10 treatment plots, each containing a pair of treatment subplots, at Ordway-Swisher Biological Station in Putnam Co. FL. Red line represents the division between burned and unburned sites. One experimental burn was conducted south of the red line; north of the line remained unburned. Each 25 m^2 subplot is represented by four orange circles, each represents the Global Positioning System referenced locations of a subplot corner.

Prescribed Fire

A low-intensity prescribed fire was lit during the growing season to align with the timing of historically prevalent lightning-ignited fires [62]. A fire break bisected the unburned (UB) and prescribed burn (PB) study plots (Figure 1). A backing fire was lit midmorning on 24 July 2017 along the fire break and progressed 15–30 m south, after which a series of strip fires were used to carry the fire [63] across half of the experimental plots. High relative humidity (>70%) in combination with a relatively low Keetch–Byram Drought Index (287) resulted in a low-severity fire. Ignition was completed after approximately 2 h [64]. A combination of ignition methods promoted fire behavior to vary across the management unit, creating independently altered microsites. The fire was heterogeneous in intensity, severity, and patchiness across the landscape as is typical in LLP ecosystems where subtle variation in elevation, soil moisture, and fuel loading interact to affect the fire regime [65–68].

2.3. Specimen Sampling

Over 15 months, we sampled a LLP sandhill ant community by conducting 12 rounds each of seed trials and tuna–honey bait trials as well as 400 leaf litter sample collections. Activity and composition of seed foragers and non-seed foragers were monitored before and after prescribed burning (sampling periods: 1–3 months pre-, 1–4 months post-, and 10–12 months post-burn (Table 1), hereafter referred to as pre-, post-, and 1-year post-burn), in areas with and without CWD. Ants were sampled using three complementary methods: (1) baiting with *Crotalaria rotundifolia* seed, (2) baiting with a tuna–honey mixture, and

(3) systematic leaf litter sampling. Seed baiting identified seed-removing species specifically, whereas other sampling methods captured the breadth of the larger ant community.

Table 1. Sampling period dates by sampling method. Prescribed burn was conducted on 24 July 2017.

	Sampling Period		
Sampling Method	**Pre-Burn 2017**	**Post-Burn 2017**	**1-Year Post-Burn 2018**
Seed trials	13 June to 9 July	2 Aug to 6 Nov	22 May to 28 June
Tuna–honey trials	4 May to 5 July	31 July to 15 Oct	5 June to 25 July
Leaf litter sampling	12 June to 9 July	1 Aug to 26 Sept	31 May to 30 July

2.3.1. Ant Identification and Characterization

Collected ants were identified to the species level using MacGown [69] as a reference and stored in 95% EtOH at −20 °C at the University of Florida (Gainesville, FL, USA). A representative collection of all identified species was deposited at the Florida State Collection of Arthropods (Gainesville, FL, USA). Ant species in LLP known to forage for seeds were identified by a literature review (Table 2). Species lacking literature records of ant–seed interactions were considered newly identified seed removers. We considered an ant to be removing a seed when the ant had lifted the seed off the substrate and moved a minimum distance of approximately 2.5 cm with the seed retained in its mandibles.

Table 2. Seed-removing ant species present in LLP ecosystems identified in this and other studies.

Species Name	**Our Study**	**Stamp and Lucus [51]**	**Stuble et al. [52]**	**Cumberland and Kirkman [54]**	**Studies in Non-Longleaf Habitat**
Aphaenogaster floridana M.R. Smith	✓				
Aphaenogaster treatae Forel	✓	✓			Disperser: Hilley and Thiet [70]
Brachymyrmex depilis Emery			✓	✓	
Crematogaster lineolate (Say)			✓		
Cyphomyrmex rimosus (Spinola)			✓		
Dorymyrmex bossutus (Trager)	✓				
Dorymyrmex bureni (Trager)	✓		✓	✓	
Forelius pruinosus (Roger)	✓	✓	✓		
Forelius sp A	✓				
Formica pallidefulva Latreille	✓				Disperser: Beattie and Culver, Bale et al., Giladi, Ness [71–74]
Nylanderia arenivaga (Wheeler)	✓		✓		
Nylanderia parvula (Mayr)	✓				Disperser: Beattie and Culver [71]
Nylanderia wojciki (Trager)	✓				
Odontomachus brunneus (Patton)	✓				
Pheidole bilimeki Mayr	top	✓			
Pheidole dentata Mayr	✓	✓	✓	✓	Disperser: Giladi [74]
Pheidole metallescens Emery	✓	top			
Pheidole morrisi Forel	✓	✓			
Pheidole navigans Wheeler	✓				

Table 2. *Cont.*

Species Name	Our Study	Stamp and Lucus [51]	Stuble et al. [52]	Cumberland and Kirkman [54]	Studies in Non-Longleaf Habitat
Pogonomyrmex badius (Latreille)	✓	✓			Predator: Harmon and Stamp, Smith, Tschinkel and Domínguez [75–77]
Solenopsis carolinensis Forel			✓		
Solenopsis geminata (Fabricius)	✓				Predator: Horvitz, Carroll, and Risch, Horvitz and Schemske, Kaspari, Cuautle et al., Chauhan et al., Motzke et al., Hernandez-Cumplido et al. [78–86]
Solenopsis invicta Buren			top	top	
Solenopsis nickersoni Thompson	✓				
Solenopsis truncorum Forel			✓		
Tapinoma sessile (Say)			✓		
Trachymyrmex septentrionalis (McCook)	✓				

Studies previously identifying species as dispersers or predators are listed as "Studies in non-longleaf pine habitat". The top seed remover for each study is noted. Stuble et al. and Cumberland and Kirkman [52,53] did not identify all ants to the species level. Some species were identified to the genus level (*Pheidole, Nylanderia, Monomorium*); therefore, those ants cannot be added to this table.

2.3.2. Seed Trials

Seed removal trials served to identify seed-removing ants and monitor their activity in subplots with and without CWD. Each plot was sampled four times during each of the experiment's three sampling periods. Seed trials took place between 8:00 and 13:00 UTC when ant activity was high. During each seed trial, we placed ten *Crotalaria rotundifolia* (Walter) Poiret (Fabales: Fabaceae) seeds on each of two 3 × 5 in index cards, spaced 1 m apart in the center of each subplot. Observers watched ant activity within each plot for 30 min, alternating between subplots at 5 min intervals. Ants observed removing seeds were collected using an aspirator.

Seeds of the common, native herbaceous perennial *C. rotundifolia* (commonly called rabbitbells) were used because fruits are continuous and abundant from March to December [87]. *Crotalaria* seeds do not possess a nutritious elaiosome appendage; their attractiveness to ants may be a result of chemical mimicry, but its mechanism remains unknown. Despite their lack of elaiosomes, *C. rotundifolia* seeds are known to attract a wide variety of genera [51,53]. Ants characteristically carry these seeds by using mandibles to grasp an indentation in the seed coat; this is where the seed attaches to the fruit wall before detaching upon ripening [51]. Seeds were collected from ripe fruit and stored in a refrigerator set to 4 °C for up to 4 months until use. While other ant-dispersed plants were present, seeds could not be collected in the quantity necessary for seed trials.

In addition to spacing plots at least 15 m apart to avoid overlapping recruitment to baits, we tested for spatial autocorrelation by performing Mantel tests on the plot level pre-, post, and one-year post-burn composition of seed removers. Plot distances were calculated from the distances between the midpoints of each plot. In each test, we failed to reject the null hypothesis that plots were not spatially autocorrelated (pre: $p = 0.071$, post: $p = 0.59$, one-year post: $p = 0.054$), indicating that they can be considered independent replicates [88].

2.3.3. Tuna–Honey Bait Trials

Tuna–honey baits were used to monitor generalist ants attracted to fat, protein, water, and sugar. Singly or combined, tuna and honey are commonly used to estimate and monitor ground-foraging ant richness and composition [89,90]. Each subplot was sampled four times during each sampling period. Tuna–honey bait stations consisted of a 2 cm mound of a 5:2 tuna to honey mixture atop an index card. Because tuna–honey baits can become dominated by individual fast-recruiting species and we wanted to capture the breadth of generalist ants, we set up four bait stations in each subplot, each located 1 m toward the plot center from each outer corner. We did not quantify the amount of tuna–honey bait removal (as with seed trials) after the initial establishment of all bait stations; rather, each subplot was observed once approximately every 40 min over a 4-hour period for a total of 5 separate collections. Ants at each station were collected by aspirator and pooled by subplot.

2.3.4. Leaf Litter Sampling

Leaf litter sampling was conducted to assess overall community composition in the burned and unburned areas and provide a more complete characterization of ant community composition than baits alone [89]. Each 50 m transect sample consisted of 10 individual 1 m^2 leaf litter collections, sifted and bagged every 5 m along the transect. During each sampling period, litter samples were collected from 20 transects: 10 from the burned area and 10 from the unburned area. Each transect location was assigned using a stratified random protocol, which maintained a 25 m buffer at the perimeter of the management unit and did not allow intersection with subplots. Arthropods were extracted from each sample over 48 hours using the mini-Winkler method [91] in a greenhouse at the University of Florida (Gainesville, FL, USA) set to 26 °C.

2.3.5. Corroborative Samples

To validate the completeness of community capture using the three sampling techniques described above, we compared our resulting community composition with the ant species recovered from pitfall traps administered at OSBS by the National Ecological Observatory Network (NEON). Four NEON sampling sites were chosen for comparison; each had a fire management history similar to our study unit and sites were spaced across OSBS to isolate the effects of ecosystem type and limit similarity in species composition attributable to proximity effects (Figure S1). Pitfall traps (3/site; 11 surveys) were administered following NEON protocol [92] between May and September 2017, coincident with our pre- and post-burn sampling periods. All ants were identified to species-level as described above and are retained by NEON.

2.4. Statistical Analyses

Differences in ant species diversity and composition across treatments and plots over time were evaluated using R [93]. Species accumulation curves and sample coverage estimates for each sampling method were created to assess the completeness of sampling efforts using the iNEXT R package [94] (Figure 2). To visualize how species' abundances varied across sampling periods, heat maps depicting average frequency of species detection were produced for each sampling technique (Figures S2 and S3).

To contextualize the diversity of seed-removing species within the broader ant community, seed-removing ant composition was compared to leaf litter sampling composition. For every ant species detected by litter sampling we calculated individual ant species' contribution to beta diversity (SCBD) with the adespatial R package [95] using the method developed by Legendre and De Cáceres [96]. With these calculations, we identified the seed-removing species whose contribution to beta diversity was higher than the community mean as well as how much seed-removing species as a subset contributed to the beta diversity of the overall community.

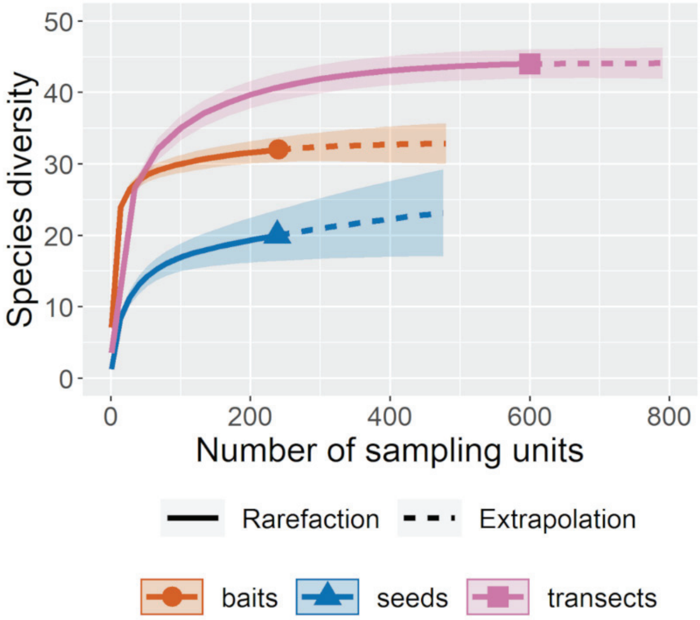


Figure 2. Ant species richness accumulation and extrapolation by sampling type: leaf litter quadrats, tuna–honey bait recruitment, and seed bait removals.

To investigate seed-removing species' resilience to prescribed fire we assessed the interactive effect of burn treatment and sampling period on the relative abundance and composition of these species compared to the ant community response as a whole. Using leaf litter sampling data, we defined "composition" as the relative detection rate of each species by transect, i.e., the proportion of samples the species was present in out of the total samples (10) per transect. Nonparametric multivariate analyses of variance (npMANOVA) were conducted on resultant matrices with 999 permutations using the adonis function in the vegan R package [97]. Results were visualized with non-metric multidimensional scaling (NMDS) using Bray–Curtis dissimilarity distance measures. Homogeneity of multivariate dispersions was tested using the betadisp function in the vegan R package [97]. Pairwise comparisons of sampling period were conducted with the RVAideMemoire R package [98] using the Bonferroni correction for multiple comparisons.

We evaluated the effects of prescribed fire, CWD, and sampling period on seed trial species richness using a generalized linear model (GLM) with Poisson distribution. Separately, we tested the effect of these variables on the proportion of seeds removed using a GLM with a quasibinomial distribution to account for overdispersion. For each GLM, model selection was determined by comparing AICc or quasi AICc (QAICc) values of all possible variable combinations with the MuMIn R package [99] and choosing the model where the change in AICc or QAICc was less than 2. Significant effects were determined with Wald chi-square tests (type II ANOVA) and then evaluated by pairwise Tukey comparisons with the emmeans R package [100]. To further address whether CWD affects seed removal, we determined if potential wood nesters removed more seed than non-wood nesters with a Wilcoxon rank sum test, and whether potential wood-nesting species were detected more frequently than non-wood nesters in CWD subplots (using tuna–honey bait trial data) with a Welch two sample *t*-test.

3. Results

Over the course of this study, we detected 45 total ant species, 20 of which removed plant seeds (Table S3, Figure 3). Species accumulation curves indicate that richness was well sampled across sampling methods (Figure 2). Sample coverage estimates were 99.95% for both leaf litter and tuna–honey bait sampling and 98.66% for seed removal sampling. Several species found in this study were not found in the pitfall samples collected by NEON.

Overall, the three sampling methods in this study captured nearly all species recovered from NEON pitfall samples with the exception of six (largely nocturnal) species (Table S3).

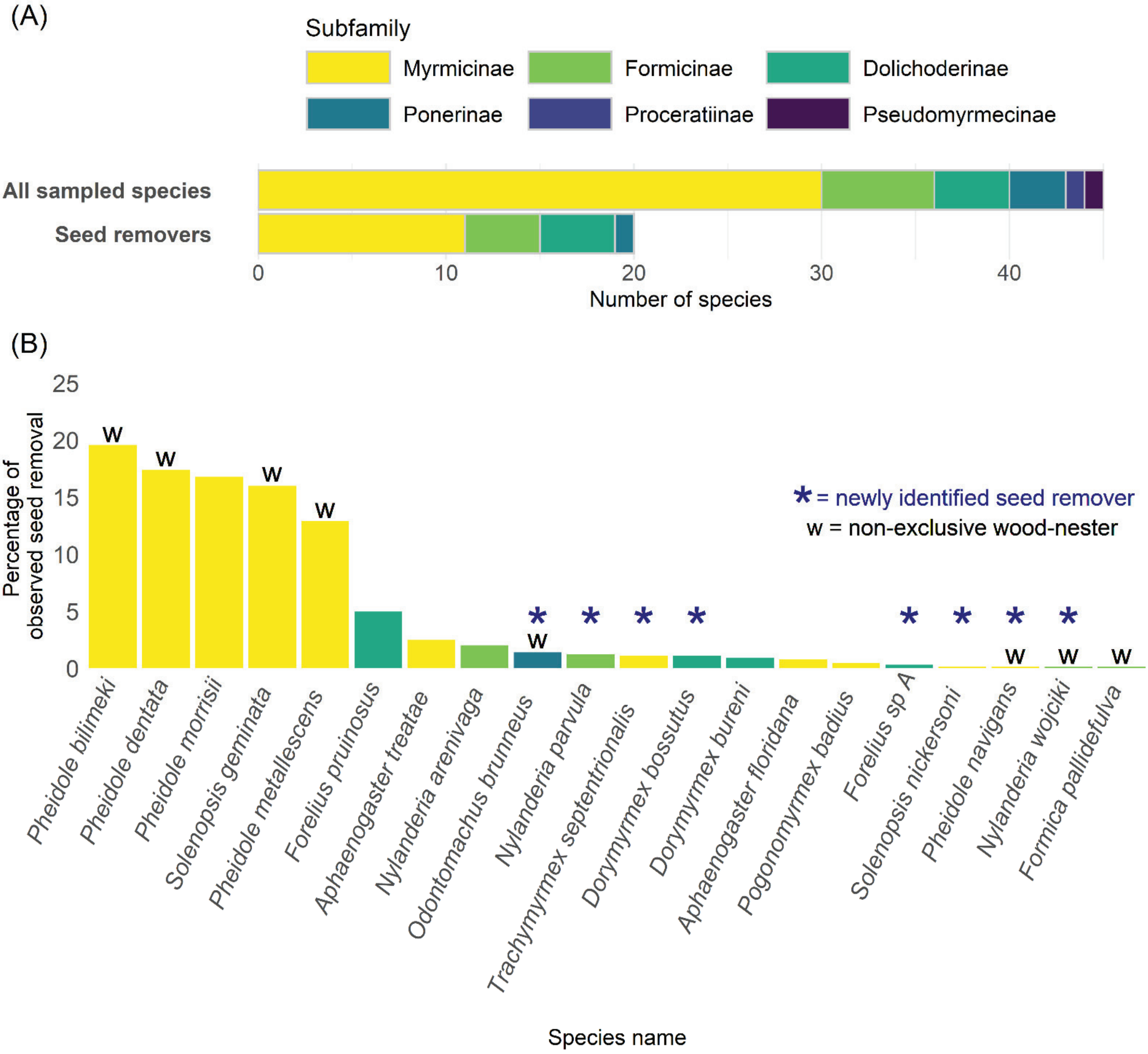


Figure 3. (**A**) Subfamily breakdown of all species sampled compared to the subset of species identified as seed removers. (**B**) Seed-removing ant species and the percent of seeds each species removed out of 644 total seeds removed across the entire study. Asterisks denote new observations of seed removal for a species. "w" identifies the species as a non-exclusive wood nester.

3.1. Seed-Removing Species

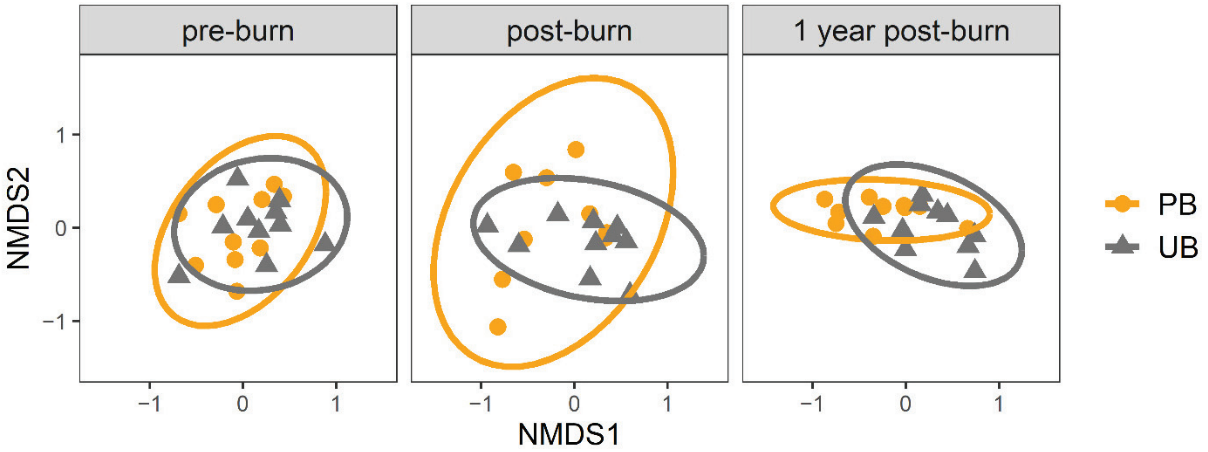
Seed removers were a subset of species collected at tuna–honey baits, which were themselves a subset of the more extensive community of ant species recovered from leaf litter samples (Figure 3, Table S3). Collectively, ants in the subfamily Myrmicinae removed the majority of seeds (Figure 3A): *Pheidole bilimeki* Mayr was responsible for 19.4% of the seeds removed over the course of the study, *Pheidole dentata* Mayr: 17.4%, *Pheidole morrisii* Forel: 16.7%, *Solenopsis geminata* (Fabricius): 16.1%, and *Pheidole metallescens* Emery: 12.9% (Figure 3B). Other species observed removing less than 5% of seeds included two Forelius spp. (dolichoderines), three *Nylanderia* spp. (dolichoderines), and two *Aphaenogaster* spp. (myrmicines). Seed-removing behavior is reported for the first time for eight ant species (Table S3; Figure 3B). The top five seed removers—*Pheidole bilimeki*, *Pheidole dentata*, *Pheidole morrisii*, *Solenopsis geminata*, and *Pheidole metallescens*—were also the most frequently encountered seed-removing species, each present at 13–27% of seed trials. These five species were also among the most common at tuna–honey baits, with each species present at over 25% of tuna–honey baits; *P. bilimeki* and *P. dentata* were present at $\geq$70%. High prevalence at both seed and tuna–honey baits suggests these five species are among this habitat's most abundant generalists. Each top *Pheidole* species was also detected in 11–28% of the total transects, making the genus among the most commonly sampled species across methods. *Solenopsis geminata* was less common in leaf litter samples (present in 4% of transects) than in tuna–honey baits and seed trials. The remaining seed-removing species were detected at $\leq$6% of the seed baits, indicating they were less abundant competitors for seed resources in our sampling.

Many seed-removing species were large drivers of beta diversity. Beta diversity measures the variation in community composition among samples; species with high species' contribution to beta diversity (SCBD) vary the most in abundance among samples and are neither rare nor relatively widespread [101,102]. In leaf litter samples, 10 of the 16 species that contributed more than the mean SCBD were seed removers (Figure S4). This indicates that many seed-removing species were detected regularly by litter sampling, but detections fluctuated over time and/or space.

Eight seed-removing ant species nest non-exclusively in CWD (Figure 3B). Collectively, wood-nesting species were not detected in CWD subplots more often than species that do not nest in wood ($t(10.5) = -1.4$, $p = 0.9$). On average, potential wood nesters were approximately 3.5% more likely to be detected in CWD than no CWD plots, while non-wood nesters were approximately 2.6% more likely to be detected in plots without CWD than with CWD. Two seed-removing species were detected in subplots with CWD at least 15% more often, on average, than in subplots without CWD: *P. bilimeki* (21%) and *Odontomachus brunneus* (Patton) (17%), which are both potential wood nesters.

3.2. Community Resiliency to Fire

Prescribed fire was associated with differences in community composition for both the overall litter ant community and among seed-removing species (Figure 4). Multivariate permutation analysis indicated that burn treatment explained significant differences in the composition of the seed removers within the litter ant community ($F = 4.6$; df = 1, 53; $p = 0.001$) (Table 3, Figure 4A). Differences in composition between burned and unburned areas can be partly attributed to greater variability in species detection rates in burned habitat, i.e., variances between burn treatments were heterogeneous ($F = 6.8$; df = 1, 57; $p = 0.012$). Similarly, multivariate permutation analysis indicated that burn treatment explained significant differences in the composition of the overall litter ant community ($F = 6.3$; df = 1, 53; $p = 0.001$), with heterogeneous variances ($F = 5.1$; df = 1, 57; $p = 0.028$) also attributable to greater variation in composition among burned than unburned litter transects (Table 3, Figure 4B). The sampling period was an additional significant predictor of overall litter ant community composition ($F = 2.3$; df = 1, 53; $p = 0.001$). Variances between sampling periods were also heterogeneous ($F = 3.7$; df = 2, 56; $p = 0.031$), with the most composition variability among transects in the post-burn sampling period. The composition of the overall litter community was different between pre-burn and 1-year post-burn sampling ($p = 0.033$) and post-burn and 1-year post-burn sampling ($p = 0.048$) across burn treatments. Although burn treatment and sampling period (for the overall ant community) were found to be significant predictors of community composition, these predictor variables explained a small proportion of the variability in the observed data (burn treatment $R^2 = 0.9$ for the overall ant community and $R^2 = 0.07$ for seed removers; sampling period $R^2 = 0.07$ for the overall ant community). Importantly, we did not detect interactive burn treatment and sampling period effects for either the seed-removing subset or the overall community, suggesting that prescribed growing season fire does not significantly alter the LLP ecosystem ant species assemblage.


(A) Seed-removing ants

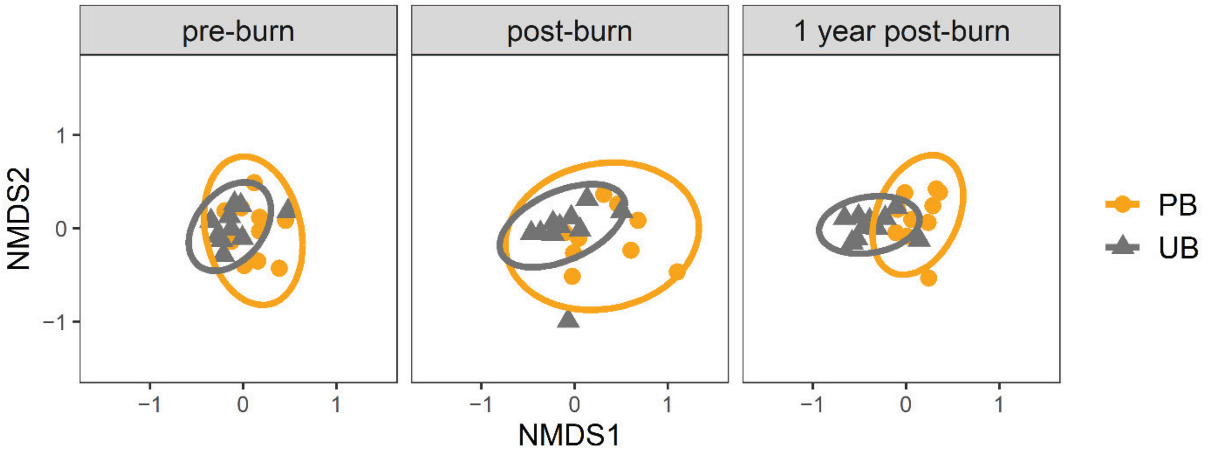

(B) All litter ants

Figure 4. NMDS for ant community composition. Burn treatment is labeled "PB" for prescribed burn and "UB" for unburned. (**A**) Seed-removing species: species frequency of occurrence in transects using Bray–Curtis distance metric. (**B**) Overall community: species frequency of occurrence in transects using Bray–Curtis distance metric.

Table 3. Non-parametric multivariate analyses of variance of leaf litter community composition.

		Seed Removing Species					**Whole Ant Community**				
	DF	**Sum Sqs**	**Mean Sqs**	**F-Model**	**R^2**	***p*-Value**	**Sum Sqs**	**Mean Sqs**	**F-Model**	**R^2**	***p*-Value**
Burn treatment	1	0.8	0.8	4.6	0.07	0.001 *	0.71	0.71	6.3	0.09	0.001 *
Sampling period	2	0.45	0.22	1.3	0.04	0.225	0.51	0.26	2.3	0.07	0.002 *
Burn x Sampling period	2	0.31	0.15	0.89	0.03	0.585	0.29	0.14	1.3	0.04	0.179
Residuals	53	9.3	0.17		0.86		6.0	0.11		0.8	
Total	58	10.8			1		7.5			1	

Asterisks (*) denote significant predictor variables.

3.3. Seed Removal Activity

3.3.1. Ant Richness

Seed-removing ant richness remained stable regardless of burn treatment or CWD proximity. The best-fitting GLM model had sampling period as the sole predictor variable, predicting ~32% of deviance ($\chi^2 = 12.4$, $p = 0.002$). Comparisons across burn and CWD treatments determined that seed-removing species richness was 1.9 times greater pre-burn than post-burn ($z = 3.49$; $p = 0.0014$), with an average of approximately six species removing seeds per sampling round compared to approximately three species, on average, post-burn. Moreover, species richness of seed-removing ants was 1.6 times less post-burn than 1-year post-burn ($z = -2.55$; $p = 0.03$). Species richness of seed-removing ants pre-burn and 1-year post-burn did not differ ($z = 0.99$, $p = 0.58$) (Figure 5A).

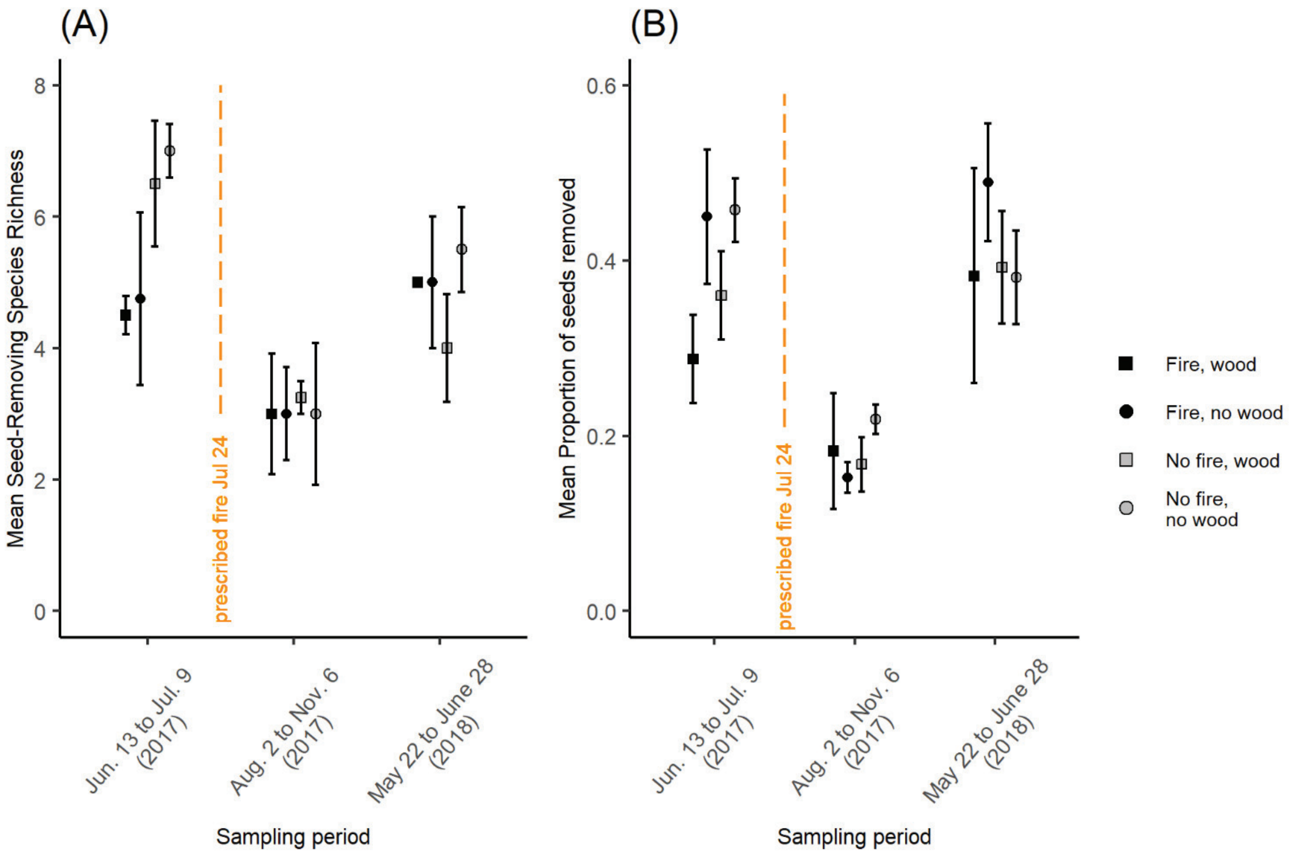


Figure 5. Sampling period effects in seed trials. Error bars are ±1 SE. (**A**) Effect on species richness. (**B**) Effect on number of seeds removed.

3.3.2. Proportion of Seeds Removed

The number of seeds removed was unaffected by prescribed fire or CWD alone or prescribed fire in combination with CWD. The best-fitting GLM model included sampling period and CWD as predictors, with sampling period as the sole significant explanatory variable explaining ~46% of the total deviance ($\chi^2 = 36.2$, $p < 0.0001$) (Table 4). Pairwise Tukey comparisons across burn and CWD treatments indicated that the proportion of seed removed pre-burn and 1-year post-burn was greater than twice that of post-burn ($z = 5.08$; $p < 0.0001$, and $z = -5.64$; $p < 0.0001$, respectively). By contrast, the proportions of seeds removed pre-burn and 1-year post-burn were not significantly different ($z = -0.64$; $p = 0.82$) (Figure 5B). Although CWD was included in this model, it was a nonsignificant predictor, explaining approximately 4% of total deviance ($\chi^2 = 3.6$, $p = 0.06$). Additionally, across subplots, wood-nesting species did not remove more seeds, on average, than non-wood nesters ($W = 40.5$, $p = 0.73$).

Table 4. GLM model analysis of deviance: Proportion of seeds removed by ants across time in subplots with and without coarse woody debris (CWD).

	DF	Deviance	Residual DF	Residual Deviance
Null model			47	547.83
Sampling period *	2	252.88	45	294.94
Coarse woody debris (CWD)	1	22.93	44	272.01
Sampling period x CWD	2	10.0	42	262.01

Asterisks (*) denote significant predictor variables.

4. Discussion

Pine forests of the Southeastern United States Coastal Plain have a rich assemblage of ant species. This study detected a total of 45 ant species within one LLP sandhill site (<4 hectares) in north-central Florida, with an additional six species in similarly managed sandhill habitats detected by NEON pitfall traps. These 51 species represent approximately 20% of the total ant diversity currently documented in Florida. Seed-removing ants were prevalent throughout the site, regardless of treatment, and accounted for nearly half (44.4%) of the total species detected at the LLP site sampled (Figure 3A).

4.1. Seed-Removing Species

This study indicates that seed removal is a relatively common behavior among ant species in longleaf pine sandhill habitat. We documented a high number of ant species (20) removing seeds; these species represent the four most common ant subfamilies we detected (Myrmicinae, Formicinae, Dolichoderinae, Ponerinae) and were found to be abundant using three detection methods: seed baits, tuna–honey baits, and leaf litter sampling. The eight newly documented seed removers include the poorly studied LLP sandhill endemic ant species, *Dorymyrmex bossutus* (Trager) and an unnamed *Forelius* species (designated sp A by Deyrup [35]), as well as the closely related and less habitat-restricted species, *Dorymyrmex bureni* (Trager) and *Forelius pruinosus* (Roger), respectively. The most unexpected seed remover was *O. brunneus*, a trap-jaw ant and arthropod predator, which uses its snapping jaws to stun prey [35]. We observed these ants exhibiting snapping behavior upon discovering seeds, which were thrown into the air, and then retrieved. Three tropical species in this genus are known to collect seeds [103], but this marks the first record of this behavior by *O. brunneus*. In contrast to the richness of seed removers detected here, the majority of seed dispersal in other ecosystems (e.g., North American deciduous forests and Australian chaparral) is performed by a primary keystone species, a single species, or species complex that is mutualistic with myrmecochorous plants [7,104].

The twelve ant species previously documented removing seeds in longleaf pine habitat include both seed specialists and ants with a broad generalist diet. *Solenopsis geminata* and *P. badius* are well-known granivores [78–86], but most are generalists not previously considered to be seed dispersers or predators (Table 2). In *Pheidole* (the genus containing four of the five top seed removers), major workers possess large mandibles that can grind seeds [35]. Despite their morphology, *P. dentata* has been previously identified as a seed disperser [74]. Other seed removers in our study that were previously identified as dispersers include *Formica pallidefulva* Latreille, *Nylanderia parvula* (Mayr), and *Aphaenogaster treatae* Forel (Table 2 and references therein). However, additional knowledge of species-specific seed behavior beyond the classification as predators or dispersers is required to assess seed fate.

Ant species differ in behaviors that influence plant fitness [105], including seed dispersal distance [51,72] and the spatial arrangement of discarded seeds (clumped versus diffuse). For example, researchers have observed *P. metallescens* frequently dropping seeds in depressions upon return to the nest and *P. badius* carrying seeds into nests and later discarding them, uneaten, on the soil outside [51]. In our study, *Pheidole* spp. and *S. geminata* removed the most seeds and were the most numerous at seed baits, but we did not determine seed fate. Less frequently detected ants may be more effective seed dispersers, exerting a greater influence on plant community assembly than ants with higher seed recovery rates if ants which retrieve relatively greater amounts of seeds also exhibit behaviors that negatively or neutrally affect plant fitness. For example, ant body size has been positively correlated with seed dispersal and foraging distances [72], and while singly, each of the two *Aphaenogaster* spp., *O. brunneus*, *P. badius*, and *F. pallidefulva* accounted for less than 5% of the observed seed removal, they were the largest seed-removing ants. Lower incidences of seed removal by other species—e.g., *Forelius* sp A was only observed removing seed twice and four other species (Figure 3B, right of *Forelius* sp A) were observed once—do not suggest they have a large collective impact on plant fitness, but contribute to our observation that generalist ants frequently interact with seeds in this ecosystem.

Daily fluctuations in species' foraging behavior can also determine seed fate. We conducted our seed trials between 8:00 and 13:00 UTC to avoid the warmest part of the day when many ant species decrease foraging activity, but *F. pruinosus* and *P. badius* were observed at later, hotter times when other species became less active. The diversity of ant species attracted to seeds in this habitat suggests that phenology of seed release could determine whether a seed is picked up by *S. geminata* or *Pheidole* spp., or whether more heat-tolerant species have a higher likelihood of seed discovery and retrieval.

Ant recruitment to seed may also depend on plant-specific dispersal mechanisms. Some plants, such as *C. rotundifolia*, use ballistic dispersal for initial seed release with ants as secondary agents of dispersal. In this case, individual seeds will likely be spread out. Other species simply drop seeds so recruitment to seeds concentrated around these plants seems more likely. While our sampling methods were successful at detecting seed removers, natural recruitment was limited. All individuals observed removing seeds were collected before they could recruit nestmates, but some ants were able to retrieve seeds and return to the nest during the 5 min breaks between observation periods. This may have influenced our data on relative seed removal in one of two ways. First, our data on percent seed removal may be skewed towards ants with faster recruitment times, although we cannot determine whether non-collected ants recruited nestmates. For example, in our study, *Pheidole* spp. and *S. geminata* removed the most seed and were the most numerous at seed baits. They also have relatively large colonies and fast recruitment. Second, inhibiting recruitment by removing some workers may have given other species the opportunity to remove seed without being excluded from it by dominantly aggressive species. For instance, we frequently observed *Pheidole* spp. biting each leg of large-bodied *Aphaenogaster* spp. at tuna–honey baits, thereby immobilizing them and preventing food retrieval. While these concerns do not invalidate the data presented here, greater attention to these methodological limitations should be taken into account by future researchers.

4.2. Community Resilience to Fire

Although frequent fire temporarily removes much living plant material and raises the heat index of aboveground microhabitats, ants are resilient in two ways: (1) many species nest far enough belowground to avoid direct mortality from fire, and (2) more susceptible ant species such as litter dwellers can survive in and recolonize from unburned areas.

The soil-nesting habits of the seed removers in LLP sandhill likely contribute to their resilience in this community. We found that ants identified as seed removers were common surface foragers and active across sampling periods, both before and after fire management (Figures 4, S2 and S3), and no significant change in ant community composition caused by fire was detected. The LLP ant community appears to be robust to direct mortality and fire-inflicted habitat change. None of the seed-removing species detected nest exclusively in leaf litter or wood, where they would be most vulnerable to direct fire mortality. All seed-removing species nest in soil, three also nest in wood, and five also nest in wood and leaf litter. Belowground nesting is advantageous in a fire-prone habitat, as exposure to lethal soil temperatures during a fire is typical only at the surface to a couple centimeters deep [106,107]. Soil nest depth is not known for all seed removers but typically ranges between 15 and 25 cm for *D. bossutus* [108] at the most shallow to up to 3 m for *P. badius* [109].

Many herbaceous plants quickly re-sprout after low-intensity fires in LLP sandhill and other fire-prone habitats. As such, ants are not facing a barren habitat post-fire. Re-sprouting and unburnt or partially burnt surviving plants provide food in the form of honeydew resources from sap-sucking insects and by providing habitat for invertebrate ant prey.

Unburned plots or microsites provide reservoirs for aboveground nesters. For the overall community for which sampling period was a significant predictor variable, community composition 1-year post-burn differed significantly from both the pre-burn and post-burn sampling periods. Mean species richness was the highest during the 1-year post-burn sampling period (mean = 16.2 ± 1.16 SE for burned transects and mean = 17.5 ± 0.65 SE for unburned transects), and there was less species turnover among transects than in the post-burn sampling period, suggesting that heterogeneous fire regimes support higher ant diversity.

While no significant effect of fire or CWD was found, beta diversity patterns suggest that burning affects community composition through species turnover. The heterogeneous variances observed between burned and unburned areas for both seed-removing and overall community composition indicate greater species turnover and richness differences among burned than among unburned transects through time. Species turnover, i.e., variability in species detections among transects, is largely driven by species with a relatively high SCBD (Figure S4). Seed removers accounted for 42.7% of beta diversity in leaf litter samples and there was more variance in seed-removing ant community composition, evidenced by the larger spatial extent of dots in the NMDS plots (Figure 4A), than that of the more-inclusive overall ant community (Figure 4B). This likely corresponds to greater habitat heterogeneity in the burned compared to the unburned treatment, where uneven fire contributed to variable microhabitats, with areas left unburned with intact groundcover and burned areas with little to no leaf material in which ants could actively forage or nest. While seed-removing and overall ant composition between burn treatments largely overlapped (Figure 4), they were still determined to be significantly different (Table 3). Four unique species were found in each burn treatment, but all were relatively rare (found in 2% of transects or fewer), indicating that species turnover, rather than unique species distinctions, drove the significant differences between burn treatments across time.

4.3. Seed Removal Activity

Seed removal activity was resilient to fire and CWD influences. Sampling period was the most important predictor of seed-removing species richness and the number of seeds removed (Figure 5, Table 4). While our study defined sampling periods with respect to burn timing, differences observed between sampling periods are the same for both burned and unburned plots and are therefore attributable to seasonality of ant behavior. We observed the same phenological trend across CWD subplots in both burned and unburned plots of high seed-removing ant species richness in early summer, followed by a drop in richness in late summer into fall, and a higher richness during the following spring and summer (Figure 5A). This same pattern was observed for seed removal: the lowest seed removal activity was recorded across burn treatments in the post-burn sampling period (Figure 5B). These same patterns across treatments may indicate a seasonal dip in seed foraging—following a decreased abundance of seed into the cooler months. This trend is unlikely to be explained by a general decrease in ant activity in the late summer and fall, because species richness recovered at tuna–honey baits increased (from 11.5 to 13.25, on average) during the same period.

Coarse woody debris has been shown to be a resource to ants in past studies [31–35]. In particular, Ulyshen et al. [32] found an association between *P. dentata* and burned logs in pine forests. In our study, some seed removers such as *P. bilimeki* and *O. brunneus* were detected more often in plots with CWD, but species richness and the number of seeds removed did not vary with CWD presence. Unlike other studies where species nesting in wood was measured [32], or where specific measured distances from wood was a predictor variable for species richness [36], we did not check CWD for nests, and, while our seed trials were conducted at the center of each subplot, CWD occupied varying space and position within subplot parameters. While this study was unable to establish relationships between seed removers and CWD, future species-specific studies may reveal significant associations that necessitate more direct study. Moreover, this site has been managed for biodiversity for more than 2 decades, making it a relatively undisturbed example of the sandhill ecosystem. Southeastern U.S. pine habitats historically managed for timber resources have densely planted pines that shade out groundcover and inhibit biodiversity until they are restored by thinning and other management practices, so CWD may be more beneficial at LLP sites during the restoration process.

4.4. Conclusions

Overall, these results demonstrate high resilience to fire among ants in LLP sandhill, an important ecosystem that is undergoing extensive biodiversity restoration in the Southeastern United States. Considering ant community resilience to fire, we can infer that using prescribed fire aligns with the goals of restoring and maintaining biotic diversity in this fire-prone ecosystem.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d14121012/s1, Table S1: Ant-dispersed plants detected in longleaf pine sandhill field site, Table S2: Coarse woody debris (CWD) volume measurement, Table S3: Species detection (percent found in out of total sample units) by sampling method, Figure S1: Corroborative National Ecological Observatory Network (NEON) sampling locations, Figure S2: Average proportion of plots per sampling day in which a species was detected, Figure S3: Litter ant detection frequency, Figure S4: Species contribution to beta diversity in leaf litter samples.

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Data Availability Statement: The data presented in this study are openly available at the Knowledge Network for Biocomplexity repository at doi:10.5063/F1891493 [110].

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Patterns of European Ant Communities Reveal a Functionally Coherent Holarctic Fauna

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Abstract: Here we examine the extent to which European patterns of ant diversity and functional composition conform with those documented in North America. Following protocols previously used in North America, ant species distribution and behavioural dominance were quantified at fifteen sites on two environmental gradients, one following elevation (140–1830 m) in France and the other tree cover (0–95%) in Denmark. Pitfall traps were used to assess species distributions, and behaviour at tuna baits was used to inform behavioural dominance. We specifically test three predictions based on North American patterns: (1) Species richness and overall levels of behavioural dominance will decline with increasing thermal stress. (2) Geographic patterns of key taxa in Europe will be consistent with those in North America. (3) Behavioural dominance of European taxa will be consistent with related taxa in North America. We then use our results to classify the European ant fauna into functional groups, as had been done for North American ants. Based on these functional groups, we analyse distributional patterns along our gradients and re-analyse ant community data from published studies to provide a more comprehensive understanding of the structure of European ant communities. Distributional and behavioural predictions of the European ant taxa were consistent with those in North America. Geographical patterns of functional-group composition were very similar to those previously recorded for North America, varying systematically and predictably along the environmental gradients. Our findings indicate that there is a functionally coherent ant fauna throughout the Holarctic.

Keywords: ants; biogeography; community structure; community composition; dominance; environmental gradient

1. Introduction

Global comparisons of biotic composition and organisation represent a major challenge for community ecology because of taxonomic dissimilarity between distant regions. One solution is to classify species into functional groups that have universal currency [1–3], allowing for comparative analyses of communities, regardless of where they occur.

Comparative analyses are particularly challenging for faunal communities, where there are no simple analogues to the life forms that form the basis of vegetation mapping and analysis. A notable exception is for ants, where global-scale functional groups operating at the genus or subgenus level have been identified that parallel plant life forms in relation to environmental stress (factors reducing productivity) and disturbance (factors removing biomass) [4] (Supplementary Material S1). This has allowed for comparisons of community structure and function at multiple spatial scales spanning diverse bioregions [5–7], including comparative analyses of ant community responses to disturbance in the context of using ants as bio-indicators of ecological change [8–10].

The European ant fauna is among the most extensively studied taxonomically, but there have been surprisingly few European studies describing the structure of entire ant communities. Most community-level studies focus on particular aspects, such as competitive interactions among the most common species e.g., [11–15], or have limited samples only (e.g., using only baits, which are biased towards recruit-foraging and aggressive species [16–18]). Similarly, although there have been macroecological studies of ant activity, richness and functional traits [19–22], there has been little attempt to document biogeographical patterns of community composition [23–25], which can be considered either as studies of sites separated widely on a biogeographic scale or studies that incorporate environmental gradients that otherwise emulate largely differing environmental conditions that can be encountered at a biogeographic scale (i.e., desert to a rainforest).

The European ant fauna is very similar taxonomically to that of temperate North America, where almost all European genera and common subgenera also occur. North America supports a richer ant fauna overall, with approximately 1000 species [26] compared with 640 in Europe [27], but North America includes extensive arid and subtropical regions that are very limited or not represented at all in Europe. Patterns of ant community composition along environmental gradients in Europe might, therefore, be expected to be very similar to those in temperate North America. In a very broad sense, this is already known to be true based on the taxonomic composition of regional faunas. For example, in North America, species of behaviourally dominant dolichoderinae (species of *Liometopum* and *Dorymyrmex*, along with the introduced *Linepithema humile*) are restricted to warmer regions, and in Europe this group (represented primarily by species of the *nigerrimum* group of *Tapinoma* and *L. humile*) is similarly distributed, occurring predominantly in the Mediterranean region. In cool-temperate regions of North America, mound-building species of *Formica* (*rufa* and *exsecta* groups) are the leading behaviourally dominant ants, with opportunist species of the *fusca* group of *Formica* being behaviourally submissive [5]; this is also the case in Europe [12]. However, the extent to which the functional composition of the European ant fauna is more broadly consistent with that in North America remains unknown.

Ant compositional patterns across gradients of rainfall, temperature and latitude have been documented for North America [5] based on functional groups in relation to environmental stress and disturbance that were originally developed in Australia and operating at the genus and subgenus level [4]. This scheme has since been widely applied in local studies throughout the world, including in Europe [7,28–31]. However, it has not been used to investigate biogeographic patterns of community composition across Europe.

Here we describe biogeographical patterns of ant species richness, behavioural dominance and functional-group composition in Europe using new data from environmental gradients of thermal stress, following the protocols used in North America [5], complemented by re-analyses of a range of data from published European studies. Temperature is the major factor driving biogeographic variation in ant diversity and composition [32,33], and this incorporates the level of direct insolation, mediated through vegetation cover, as well as ambient temperature [4]. Thermal stress, therefore, refers to temperature at the foraging surface and is determined by the interaction between ambient temperature and vegetation cover. We specifically test three predictions based on North American patterns (Supplementary Material S1): (1) Species richness and overall levels of behavioural dominance will decline with increasing thermal stress. (2) Geographic patterns of key taxa in Europe will be consistent with those in North America. Specifically, species of *Lasius* and the *Formica sanguinea*, *rufa* and *exsecta* species groups will be the most abundant Cold-climate Specialists, occurring primarily at high latitudes or high elevations at lower latitudes; Generalised Myrmicinae (species of *Crematogaster* and *Pheidole*) will show the reverse pattern, occurring primarily at low elevations and latitudes, and Opportunists (species of *Myrmica*, *Tetramorium* and the *Formica fusca* species group) will be widely distributed across environmental gradients. (3) Behavioural dominance of European taxa will be consistent with related taxa in North America. Cold-climate species of the *Formica rufa* and *exsecta* species groups will display the highest behavioural dominance; the Generalised Myrmicinea *Pheidole* and *Crematogaster* will display moderate levels of dominance and Opportunists the lowest.

2. Materials and Methods

2.1. Study Sites

Ants were sampled at 15 sites arranged along two environmental gradients of thermal stress that were selected to represent the major biomes and, therefore, ant taxa of continental Europe (Supplementary Materials S2–S5). Our aim was to document major patterns of functional composition, rather than a comprehensive sampling of European ant species. The first gradient contained eight sites spanning an elevational range in southern France (the low latitude sites), extending from Masif du Cap Canaille (43°10′ N 5°35′ E) at sea level near Marseille to Mont Saint-Guillaume near Embrun in the French Alps (1830 m asl; 44°35′ N 6°27′ E), spanning a distance of approximately 150 km. The gradient was representative of the vegetation structural range of southern Europe, from low shrublands to tall coniferous forest and supports all the major ant genera of central and southern Europe [34] (J Orgeas, pers. comm., 2010). All sites were separated by more than 500 m.

The second gradient contained seven sites within Mols Bjerge National Park (56°13′ N 10°34′ E) in Denmark (the high latitude sites). The gradient spanned the vegetation structural range of northern Europe, from grassland to tall, closed Beech (*Fagus sylvatica*) forest. This study region supports nearly 60% (about 40 out of 70) of all ant species occurring in northern Europe and includes all the major genera [35] (M.G. Nielsen, pers. comm., 2010). Sites were separated between approximately 200–500 m.

2.2. Sampling

To best allow a cross-continental comparison, field sampling followed the exact methodology used previously in North America [5]. It was conducted during sunny and warm weather in August (European summer) 2010, corresponding with the highest levels of ant activity. Pitfall trap catches were used to compare the relative forager abundances of species at each site. At each site, 15 pitfall traps were operated in a 5 × 3 grid with 10 m spacing for 48 h. Traps were 6.5 cm-diameter plastic cups, partly filled with ethylene glycol as a preservative. Specimens were sorted to species level and identified using regional keys and taxonomic revisions [34–36]. Vouchers for all species are lodged at the CSIRO Tropical Ecosystems Research Centre in Darwin, Australia.

Counts of ants at tuna baits were used to quantify the relative behavioural dominance of species at each site. Following the completion of pitfall trapping, a teaspoon of canned tuna in oil was placed at each trap point, and the abundance of each species at the baits was recorded after 5, 15, 30 and 60 min. An ant was deemed to be at a bait if it was observed feeding from it within a 10-s observation period. All baiting was conducted during mild to warm daytime conditions. Voucher specimens were collected from baits for later identification in a laboratory. Opportunistic hand collections were also made at each site to provide additional records of species occurrences.

Environmental data were collected from 1 m quadrats placed centrally over each of the 15 sample locations at each site. The percentage of each ground cover classification was visually estimated; litter depth was measured with a ruler; and tree canopy cover was measured using a hand-held spherical densiometer. These data were averaged for each site. A single maximum tree/shrub height was estimated for each site. Note that the environmental data was only for site description purposes, not for further analysis with ant data.

2.3. Analysis

Species collected from pitfall traps, baits and hand collections were combined to document species occurrences at each site. To investigate sampling completeness, species site occurrences from the 15 pitfall traps and the four baiting times from the 15 baits (n = 75 samples) were used to generate rarefaction curves using EstimateS [37]. Species abundances in pitfall traps and at baits were scored according to a 7-point scale following Andersen (1997a): 1 = 1 ant; 2 = 2–5 ants; 3 = 6–10 ants; 4 = 11–20 ants; 5 = 21–50 ants; 6 = 51–100 ants; and 7 > 100 ants. Species abundances per pitfall trap sample/baiting time were calculated as the sums of abundance scores at individual traps or baits (i.e., maximum of 105 per sample in each case).

We used abundances at baits to characterise relative behavioural dominance based on multiple forms of evidence. Behaviourally dominant species were defined as those whose: (1) abundances at baits increased rapidly to the highest abundance scores; (2) were able to maintain those high abundance scores after 30 and 60 min, resulting in highly right-skewed frequency distributions; (3) relative abundances at baits were notably higher than in pitfall traps [5]. Note that statistics are not needed to make these three determinations, nor are statistics possible for these assessments without site-level replication.

2.4. Functional Group Classification and Re-Analysis of Previous Studies

We classified all European ant taxa into the functional groups established by [4] using data reported here, other distributional records [38], and assignments for North American taxa [5]. Functional group classifications were used to characterise compositional change along our two European bioclimatic gradients, based on pitfall-trap data. The classifications were then used to re-analyse previously published ant community data from other European sites sampled using pitfall traps to test the generality of our findings. The data used for these analyses were: a grassland site in Spain [39]; a heathland site in south west France (summer 1992 sample [40]); two forest sites combined in Spain [39]; shrubland sites F1 and F2 of the present study combined; three woodland sites combined in Italy [7]; woodland sites F3–F6 of the present study combined; forest sites F7–F10 of the present study combined; woodland sites D2 and D3 of the present study combined; forest sites D5 and D7 of the present study combined; and four forest sites combined in Finland (control sites [41]) (Supplementary Material S6). Functional group profiles were graphed, and sites were ordinated by nonmetric multidimensional scaling (nMDS) using Primer 6 [42]. The similarity matrix was constructed using a Bray–Curtis association index based on the percentage contribution data of each functional group at each site or site combination.

3. Results

3.1. The Fauna

A total of 34 species from 13 genera were recorded at the French sites, and 15 species from four genera in Denmark (Supplementary Material S7). Seven species were common to both countries. Pitfall trapping sampled the great majority (40 out of 42) of species collected. The most speciose genera were *Formica* (9 species), *Myrmica* (6), *Camponotus* (6) and *Lasius* (5). In France, the most abundant species in shrubland/woodland sites were *Pheidole pallidula*, *Lasius emarginatus*, *Plagiolepis pygmaea* and *Camponotus cruentatus*, and at forest sites *Formica aquilonia*, *Myrmica ruginodis*, *M. lobicornis*, *C. herculeanus* and *F. lemani*. In Denmark, the most abundant species in grassland/woodland sites were *M. sabuleti, M. ruginodis, F. fusca* and *F. rufa*, and in forest sites *F. rufa* and *M. ruginodis* (Supplementary Material S7).

Species rank-abundance curves from both pitfall catches and bait counts showed pronounced numerical dominance by a small number of species at each site (Supplementary Material S8). Indeed, the most abundant species at each site contributed on average 83% to total abundance in pitfall traps and 98% at baits. Species accumulation curves indicate that almost all species were sampled at Danish sites and the great majority of species at French sites (Supplementary Material S9). Site species richness in France ranged from 3 (site F6) to 13 (F4), and in Denmark from 0 (D7) to 11 (D3) (Supplementary Material S10). Total ant abundance in pitfalls was unimodal along both gradients, being greatest in the low open habitats with a woody overstorey of up to 50% cover (Supplementary Material S10). Site species richness was also strongly unimodal in Denmark and weakly unimodal in France, with highest values at intermediate latitudes (920 m) and vegetation cover (55%).

3.2. Geographic Patterns

Patterns of distribution along the two gradients varied greatly among genera (Figure 1). As predicted, the Cold-climate Specialists *Lasius* and *Formica* (*sanguinea*, *rufa* and *exsecta* species groups) occurred primarily at high latitudes and at high elevations at lower latitudes; Generalised Myrmicinae (*Crematogaster* and *Pheidole*) were found only at low latitudes and elevations; and Opportunists (*Myrmica*, *Tetramorium* and the *Formica fusca* species group) were widely distributed across the bioclimatic gradients.

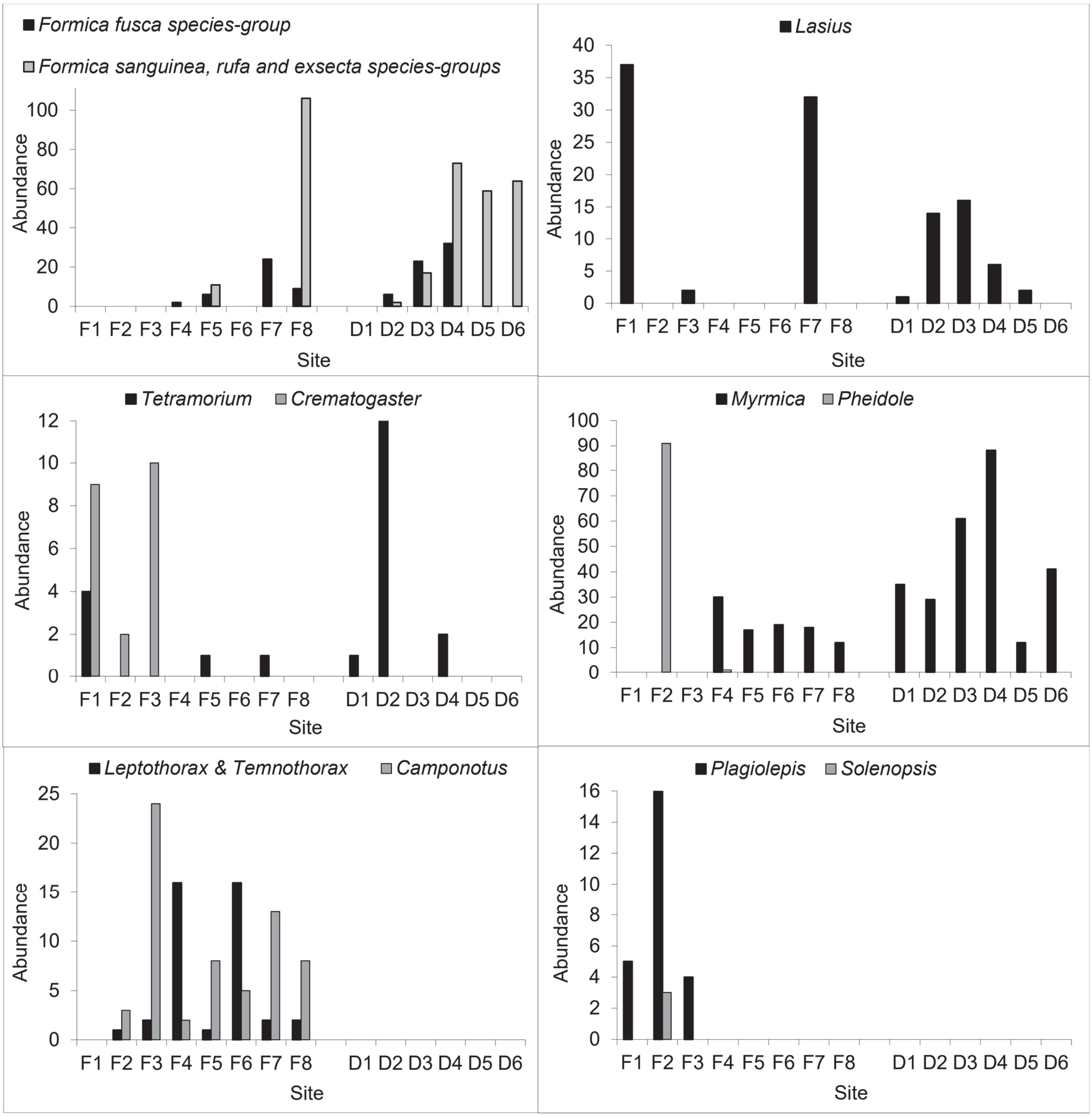

Figure 1. Distribution of major genera along the two bioclimatic gradients in Denmark (D1–D6) and France (F1–F8) of increasing thermal stress. Data are total abundance in pitfall traps. Site details are provided in Supplementary Materials S4 and S5.

Individual species had far more restricted distributions (Supplementary Material S7). For example, within *Lasius*, *L. emarginatus* occurred almost exclusively in the low-elevation open and dry French habitats, *L. fuliginosus* predominantly in the high-elevation French forests and open Denmark sites, and species of the *L. niger* species group exclusively in the Denmark sites. Similarly, *C. herculeanus* was restricted to the high-elevation French forests, whereas most other *Camponotus* species were collected only at the low-elevation French sites.

3.3. Behavioural Dominance

The most abundant species collected in traps at each site were also the most abundant species at baits (Supplementary Material S7). At French sites, with only one exception (F8), numerical dominance at baits was always greater than in pitfall traps, often markedly so (Supplementary Materials S7 and S8). In contrast, numerical dominance at baits was always less than in pitfall traps in Denmark. Total ant abundance at baits tended to increase with time, with maximum values at 60 min (Supplementary Material S11). A notable exception was site F2, where *Pheidole pallidula* was exceptionally abundant, and maximum abundance was attained after 30 min. At French sites, total ant abundance scores at baits after 60 min ranged from about 10 (mean of <1 ant per bait) to 60 (mean of about 15 ants per bait), with lowest scores occurring at mid elevation pine woodland sites. Total ant abundance at baits was consistently very low at Danish sites, with a maximum total score of only about 20 (mean of 2 ants per bait) after 60 min (Supplementary Material S11). There were no consistent patterns of ant abundance at baits along the two gradients.

Patterns of recruitment at baits (Supplementary Material S12) indicate that behavioural dominance was generally greatest at the low-latitude/low-elevation sites (F1–F3), as measured by a greater proportion of baits attended by ants and a greater number of high scores (>20 ants; scores 5–7) compared to other sites. Lowest behavioural dominance occurred at the Danish sites and the French high-altitude sites.

A range of species increased in abundance at baits over time, with the most common overall after 60 min being *Crematogaster scutellaris*, followed by species of *Myrmica*, *Camponotus*, *Lasius* and *Pheidole* (Figure 2). However, the frequency distributions of abundances scores (Figure 3) indicate generally low behavioural dominance. *Lasius emarginatus* had a right-skewed distribution, but it was recorded at only five baits (Figure 3A). *Crematogaster scutellaris*, *Pheidole pallidula* and *Lasius fuliginosus* were common species with relatively even abundance distributions, which is indicative of moderate behavioural dominance. The distributions of all other common species (*Lasius niger* and species of *Camponotus*, *Myrmica*, *Formica* and *Leptothorax*) were skewed to the left, indicating low behavioural dominance. Species of the *fusca* species group (Figure 3H) tended to have lower abundance scores than those from other species groups of *Formica* (Figure 3G), but no species of *Formica* was recorded with an abundance score greater than four (corresponding to a maximum of 20 ants).

3.4. Functional Group Classification

Distributional and behavioural results at baits and pitfall traps were as expected, so, with few exceptions, our functional group classifications of the European ant fauna (Supplementary Material S13) conform to those for North America (Supplementary Material S1). The major exception is that *Lasius flavus* and closely related species are removed from Cold-climate Specialists and now classified as Cryptic Species because they live an almost exclusively subterranean lifestyle. *Plagiolepis* was classified as a Cryptic species in [43], but we consider the European *P. pygmaea* to be an Opportunist.

Some European genera had not previously been classified into functional groups. We classified the social parasites and slave-makers *Rossomyrmex*, *Strongylognathus* and some *Tetramorium* as Cold-climate Specialists because their hosts (primarily species of *Formica*) have a predominantly cold-climate distribution. *Lepisiota* (not occurring in North America) is placed in Opportunists because of its widespread distribution and generalist habits. We classified the *Formica* subgenera *Alloformica* and *Proformica* as Opportunists because of their distributional and behavioural similarity to species of the *F. fusca* species group.

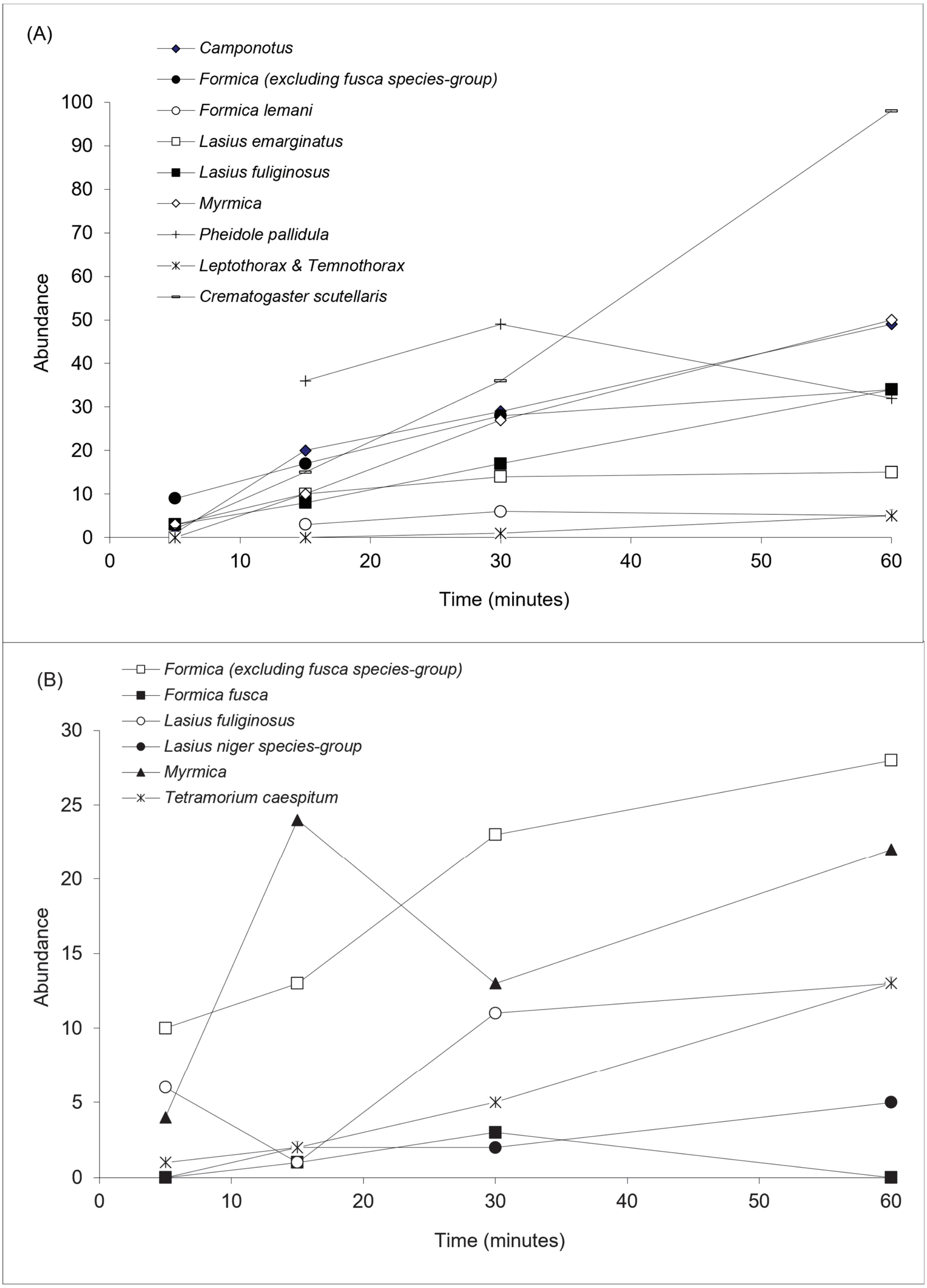

Figure 2. Temporal patterns of abundance at baits for major taxa for all sites combined in France (**A**) and Denmark (**B**).

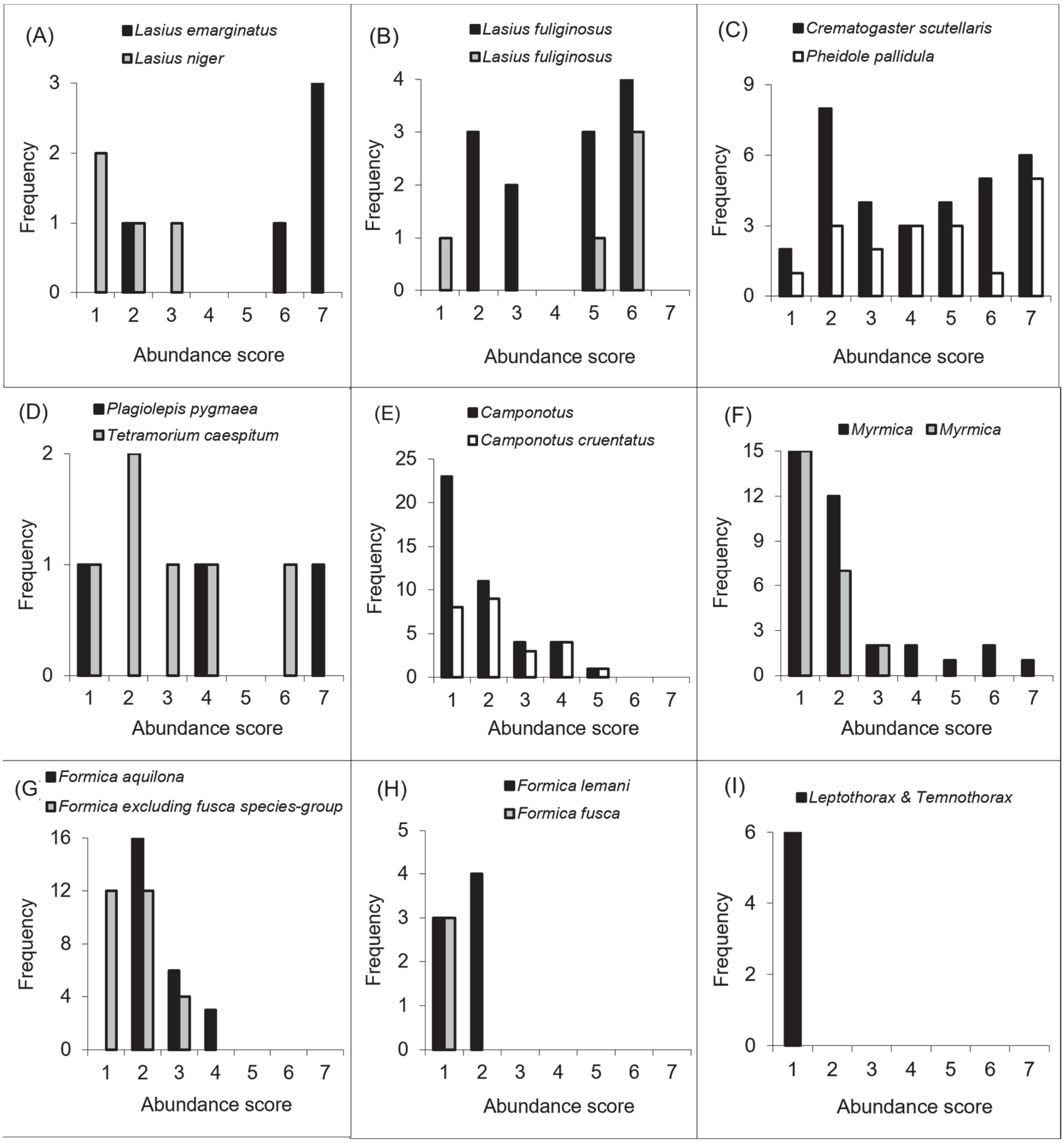

Figure 3. Frequency distributions of abundance scores at baits for all selected taxa (**A**–**I**); data pooled across sites along the vegetation gradients) in France (black or white) and Denmark (grey). Only 30 min and 60 min observation periods are included.

3.5. Biogeographic Patterns of Functional Group Composition

Functional group composition, as found by both pitfall traps and baits, varied systematically and predictably along both gradients (Figures 4–6). Generalised Myrmicinae (*P. pallidula* and *C. scutellaris*) occurred exclusively at warmer (low-elevation/low-latitude) sites, in contrast to the preference of Cold-climate Specialists for cooler sites (Figure 4). Opportunists tended to be most abundant at sites of moderate thermal stress derived from both expected climatic temperatures and levels of vegetation complexity. Subordinate Camponotini occurred throughout the French gradient (except for highest elevation site F8) but was not recorded at all in Denmark. Notably, the fauna of seasonally waterlogged meadow site D1 in Denmark was comprised almost exclusively of Opportunists (species of *Myrmica*, *Tetramorium* and *Formica fusca* species group) (Figure 4).

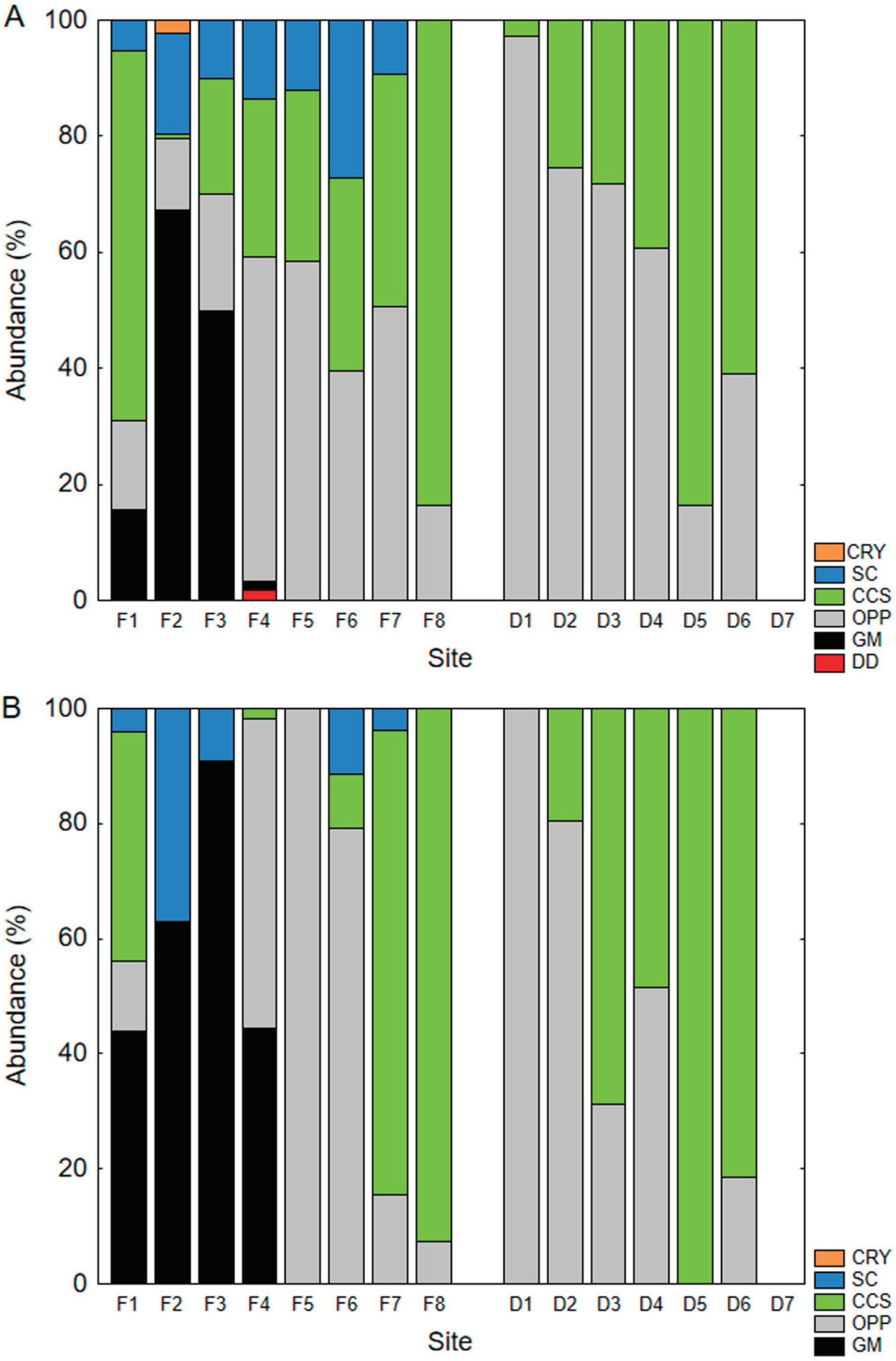

Figure 4. Functional group composition in pitfall traps (**A**) and at baits (**B**) at sites along the two bioclimatic gradients of increasing thermal stress in France (F1–F8) and Denmark (D1–D6). Bait data are for all times combined. The functional groups are: DD, Dominant Dolichoderinae; GM, Generalised Myrmicinae; OPP, Opportunists; CCS, Cold-climate Specialists; SC, Subordinate Camponotini; and CRY, Cryptic Species. Site details are provided in Supplementary Materials S4 and S5.

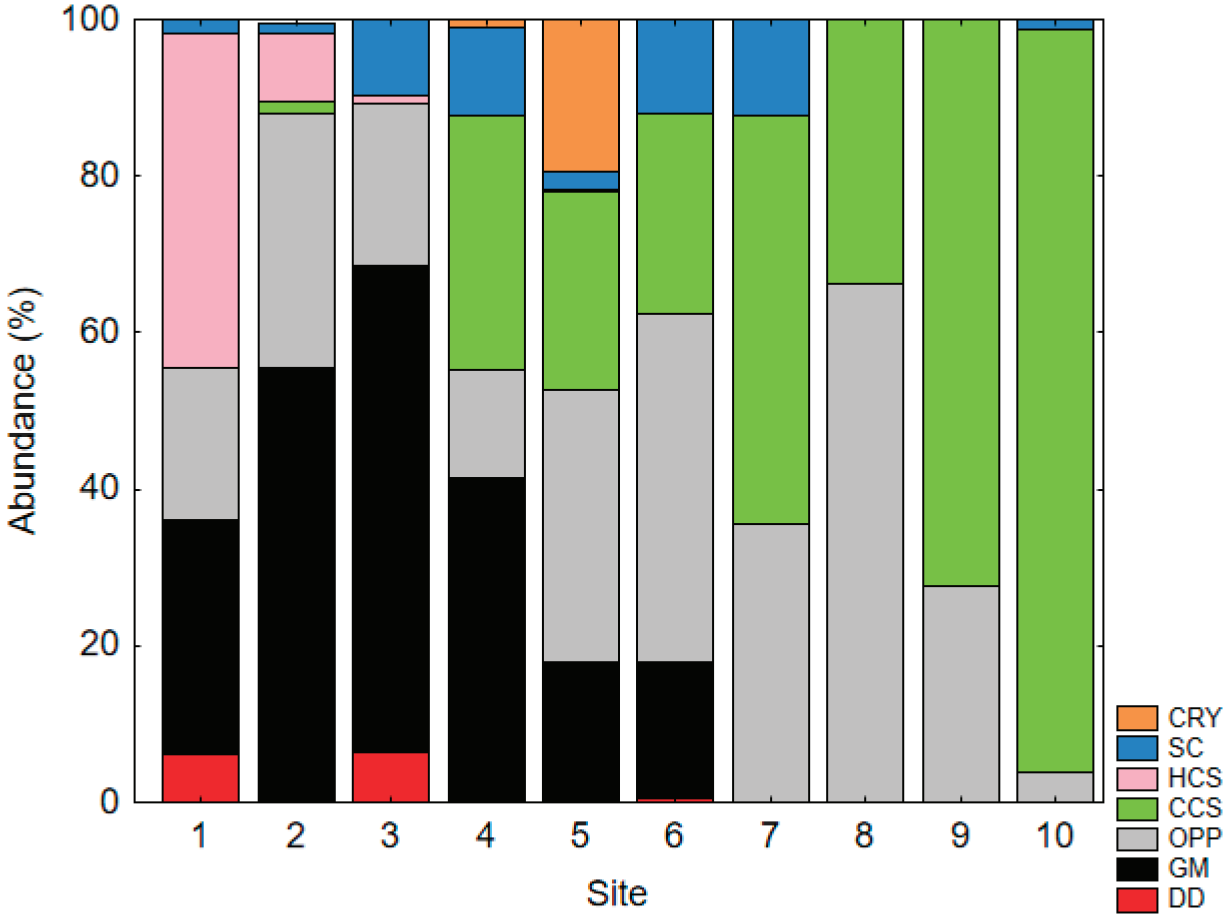

Figure 5. Functional group composition along a gradient of increasing thermal stress, combining results from the present study with published pitfall trap data. 1. grassland site in Spain [39]; 2. unburnt heathland site in south west France, summer unburnt 1992 sample [40]; 3. two Spanish woodland sites [39]; 4. shrubland sites F1 and F2 of the present study combined; 5. three Italian woodland sites combined [7]; 6. woodland sites F3–F6 of the present study combined; 7. forest sites F7–F10 of the present study combined; 8. woodland sites D2 and D3 of the present study combined; 9. forest sites D5 and D7 of the present study combined; 10. four Finnish forest sites combined (the Control sites of [41]). The functional groups are: DD, Dominant Dolichoderinae; SC, Subordinate Camponotini; HCS, Hot-climate Specialists; CCS, Cold-climate Specialists; CRY, Cryptic Species; OPP, Opportunists; GM, Generalised Myrmicinae.

The above biogeographic patterns were maintained when we combined our pitfall data with that from previous studies from throughout Europe, ranging from open grasslands in Spain to boreal forests in Finland (Figure 5). Dominant Dolichoderinae and Hot-climate Specialists occur exclusively in open habitats (grasslands and heathlands) of the Mediterranean region. Species of Generalised Myrmicinae occur primarily in such habitats, and they are increasingly replaced by Cold-climate Specialists with increasing thermal stress. Opportunists are well-distributed across the gradient, tending to be most abundant at moderate levels of thermal stress. NMDS revealed systematic compositional variation along the environmental gradient, with the sites ordering almost uniformly from right to left (Figure 6).

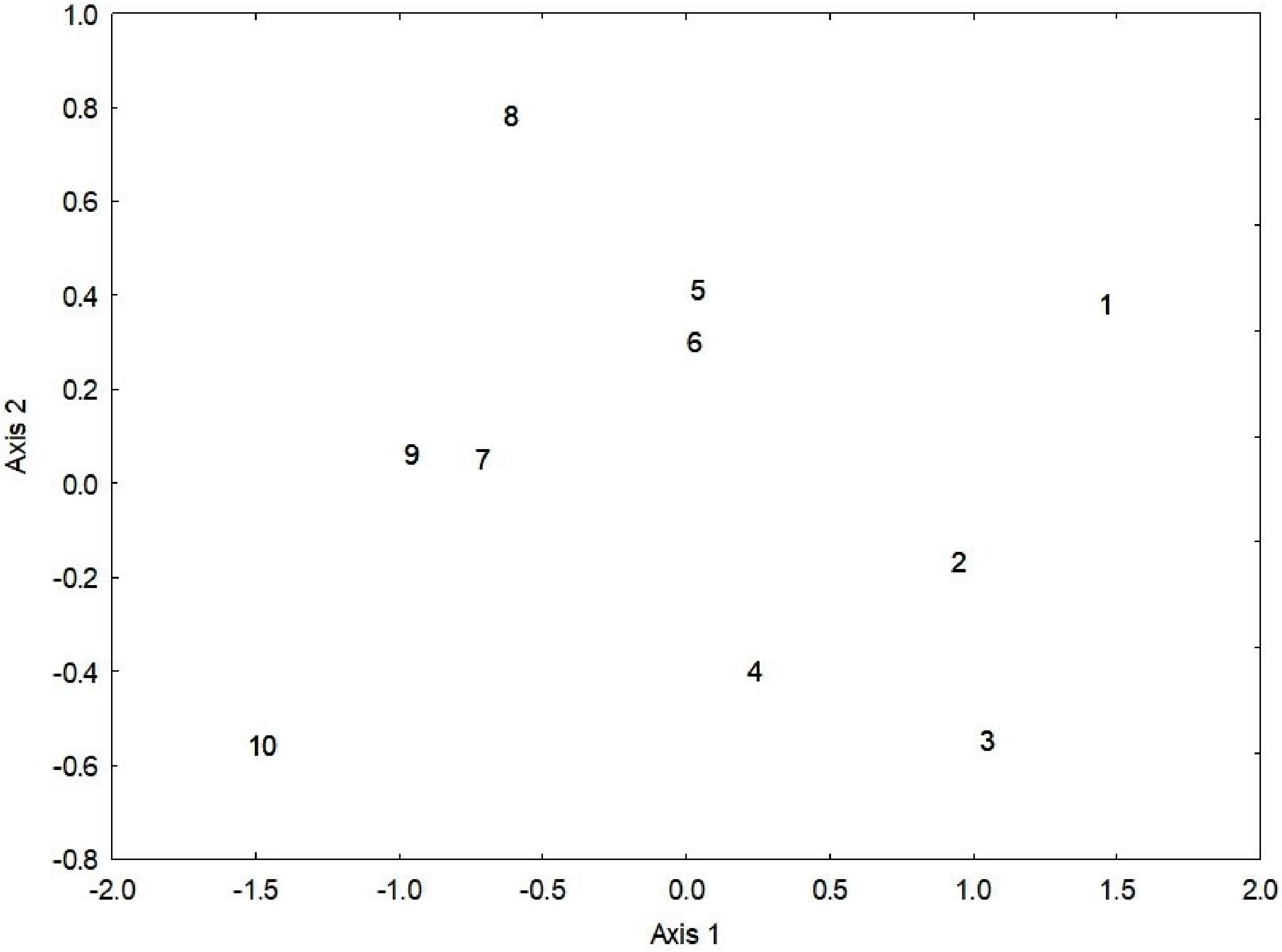

Figure 6. NMDS ordination of functional group composition of sites analysed in Figure 5 (points 1–10) representing an environmental gradient from a Mediterranean grassland (1) to a forest at high latitude (10). 2D Stress = 0.02.

4. Discussion

Our study applied a functional-group approach to investigate patterns of ant community composition in Europe in order to assess if patterns previously documented for North America apply elsewhere in the Holarctic. We first predicted that species richness and overall levels of behavioural dominance would decline with increasing thermal stress. We actually found a unimodal pattern of species richness at both the French and Danish gradients, with richness initially increasing but then decreasing markedly with increasing thermal stress and vegetation complexity. We recorded no ants at the most thermally stressed site (Beech forest) in Denmark. The decline in ant richness with increasing thermal stress is consistent with latitudinal and elevational patterns of ant diversity in Europe [7,19,22–24,44,45] and with broader ant diversity patterns globally [32]. The initial increase in richness along the elevation gradient in France can be attributed to an increase in habitat structural complexity, which has been well-documented as a key factor influencing ant species richness as well as species composition [20,25,46,47]. Overall levels of behavioural dominance as measured by abundance patterns at baits also declined systematically with increasing thermal stress, which is also consistent with patterns elsewhere in the world [4].

Our second prediction was that geographic patterns of key taxa conform to those in North America. As predicted, species classified as Cold-climate Specialists (most commonly from *Formica* (*rufa* species group) and *Leptothorax*) occurred primarily at high latitudes or high elevations at lower latitudes. Generalised Myrmicinae (*Crematogaster* and *Pheidole*) showed the reverse pattern, occurring primarily at low elevations and latitudes Opportunists (species of *Myrmica*, *Tetramorium* and the *Formica fusca* species group) were widely distributed across bioclimatic gradients.

Our third prediction was that variation in behavioural dominance among taxa conforms to that in North America. This proved true in that the Generalised Myrmicines *Pheidole* and *Crematogaster* displayed moderate levels of dominance, as is the case globally [4,5], and other common genera such as *Tetramorium*, *Leptothorax* and *Temnothorax* displayed low behavioural dominance. In North America, the aggressive mound-building species of *Formica* (*rufa*, *sanguinea* and *exsecta* species groups) are behaviourally differentiated from other *Formica* species (particularly from the *fusca* species group), which are behaviourally submissive [5]. This was also the case in Europe, with the *fusca* species group showing poor discovery of, recruitment to, and defence of baits compared with other *Formica* species. However, we were surprised by the lack of high behavioural dominance exhibited by species of the *Formica rufa* group, given that they are widely regarded as the leading dominant species in Europe [12,48,49]. Such species (especially *F. aquilonia* in France and *F. rufa* in Denmark) were common at many of our sites but did not exhibit highly dominant behaviour at baits, having generally low abundance scores with left-skewed frequencies. This might reflect a relative lack of attractiveness to this taxon of the protein-based baits that we used, and the use of liquid-carbohydrate baits may have revealed a different pattern.

We did not record the behaviourally dominant dolichoderine *Liometopum*. We also collected only a single specimen of the *Tapinoma nigerrimum* species group (*T. erraticum*), which has been reported as behaviourally dominant in previous studies [7,16,28,31,39,50]. We classify both as Dominant Dolichoderinae and note that they have very restricted distributions in Europe.

From a global perspective, levels of behavioural dominance in the European ant fauna appear to be very low, even compared with North America [5] and certainly compared with Australia, where highly aggressive species of *Iridomyrmex* and other dolichoderines are ubiquitously dominant [4]. This is despite many European species from a range of functional groups being described in the literature as dominant, including species of *Formica*, *Lasius*, *Crematogaster*, *Pheidole*, *Camponotus* and *Tetramorium* [12–14,18,21,39,51]. However, behavioural dominance is a relative term, and species that are dominant over others locally are not necessarily dominant from a broader perspective [52].

5. Conclusions

For all common taxa, we found that patterns of distribution and behavioural dominance were consistent with their functional group classifications in North America. Such consistency is not surprising given the biogeographic similarity and shared evolutionary history of Europe and North America [51] and points to a functionally coherent ant fauna across the Holarctic. Our study illustrates how a functional-group approach can provide valuable insights into patterns of community organisation at biogeographic scales.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d15030341/s1 Material S1: Summary descriptions of ant functional groups and associated North American taxa, updated from Andersen; Material S2: Map showing the locations of the study sites in Denmark (sites D1-D7) and France (sites F1–F8). The yellow scale bars are 500m; Material S3: Photographs of the study sites. Note that a photograph of site F4 is lost; Material S4: Summary habitat descriptions of the 15 sites distributed along environmental gradients in France (ordered according to elevation) and Denmark (ordered according to cover of woody vegetation), both gradients representing increasing thermal stress for ants; Material S5: Vegetation and ground-layer characteristics of sites along gradients of vegetation complexity in Denmark (D site codes) and France (F site codes). The height and cover of shrubs and trees are visual estimates, whereas all other data are means of 15 × 11 m quadrat samples; Material S6: Map of ten locations where data were sourced for Figure 5; Material S7: Ant species found at each study site in pitfall traps (p) at baits (b); Material S8: Species rank-abundance curves of ants quantified in pitfall traps

(A–C) and at baits (D–F) at sites in France (A, B, D, E) and Denmark (C, F); Material S9: Rarefaction curves of species accumulation at each site in France (A) and Denmark (B) as found in 15 pitfall traps and at 15 baits assessed after 5, 15, 30 and 60 minutes; Material S10: Ant abundance (pitfall trap catches only; black bars) and species richness (all records combined; grey bars) at sites along two bio-climatic gradients of increasing thermal stress in France (F1–F8) and Denmark (D1–D7); Material S11: Ant abundance at baits at each site along the two bio-climatic gradients in France (F1–F8) and Denmark (D1–D6) of increasing thermal stress for four observation times; Material S12: Frequency distributions of ant abundance scores at baits for each site along the vegetation gradients in France (A–H) and Denmark (I–N); Material S13: Assignment of European ant genera to functional groups.

Author Contributions: B.D.H. and A.N.A. designed the experiment. B.D.H. conducted the field work and analyses. B.D.H. and A.N.A. co-wrote the paper. All authors have read and agreed to the published version of the manuscript.

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Predicting the Potential Suitable Area of the Invasive Ant *Linepithema humile* in China under Future Climatic Scenarios Based on Optimized MaxEnt

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Abstract: *Linepithema humile* (Mayr, 1868) (Hymenoptera: Formicidae) is one of “100 of the world’s worst invasive alien species” listed by the International Union for Conservation of Nature and Natural Resources (IUCN). Although native to South America, this ant has spread worldwide via international trade. Currently, *L. humile* has not been found in China, and if it invades China, it might pose a potential risk to the native invertebrates, vertebrates, plants, and human livelihoods. Based on 2432 global occurrence records and ten bioclimatic variables, the optimized MaxEnt model was used to predict the potential suitable areas of *L. humile* in China. We analyzed the important bioclimatic variables affecting the potential suitable areas, and determined the changes in potential suitable areas under future climatic scenarios. Our results indicated that the mean temperature of the coldest quarter (Bio11), precipitation of the coldest quarter (Bio19), mean temperature of the wettest quarter (Bio8), and precipitation of the warmest quarter (Bio18) were the most important bioclimatic variables. Under the current climatic scenarios, the potential suitable area of *L. humile* in China is 80.31×10^4 km^2, which is mainly located in Fujian, Zhejiang, Hunan, Jiangxi, Guangxi, Yunnan, and Hubei. Under future climate scenarios over coming decades, the potential suitable areas of *L. humile* showed an overall increase and a shift to higher latitudes, which indicated the invasion risk of *L. humile* in China will increase under climate change. Our findings provide the theoretical guidance for the early warning and monitoring of *L. humile* in China.

Keywords: invasive alien ant; species distribution model; bioclimatic variable; overfitting; climate scenario

1. Introduction

Biological invasion is the process whereby non-native species are introduced into new areas in which they survive, reproduce, establish, and potentially expand their range [1]. Global trade has been the main driver of biological invasion [2], and with the rapid development of international trade over recent decades, the numbers of invasive alien species (IAS) and the detrimental impacts of invasion have been on the rise [3]. Biological invasions have a considerable effect on the biodiversity, ecological environment, and agricultural production of countries worldwide, and have accordingly become established as one of the five major global environmental issues of the 21st century [4]. Estimates based on recent global studies have put economic losses caused by IAS at up to $1.4 trillion per year, accounting for more than 5% of the global gross domestic product [5]. Therefore, biological invasion control should become one of the main concerns and work centers of all countries.

Ecologically, ants are among the most important insect species. Their small size and multifaceted nesting habits are conducive to human-mediated translocation [6], and as a consequence of the increasing growth in global trade, transportation, and tourism in recent decades, ants have accordingly been anthropogenically distributed beyond their native ranges. Five invasive alien ants (*Linepithema humile*, *Solenopsis invicta*, *Anoplolepis gracilipes*, *Wasmannia auropunctata*, and *Pheidole megacephala*) were listed among "100 of the world's worst invasive alien species" [7].

Linepithema humile is native to South America (Argentina, Uruguay, Brazil, and Paraguay), and has been introduced to six continents and several oceanic islands [8]. Currently, there are no occurrence records of *L. humile* in most of East Asia, including China. The social structure of *L. humile* is polygamous, and many workers and queens form large, high-density nests, thus promoting the spread to new areas of the species [9]. These factors have accordingly contributed to the success of *L. humile* as a strong competitor and invader [9]. *Linepithema humile* is a well-known agricultural and urban pest that can spread rapidly throughout suitable areas, reducing species richness, affecting the growth and development of plants, and causing economic losses in invaded areas. For example, *L. humile* has been found to compete with native ants in San Luis Obispo, California, causing significant declines in the populations of 10 native ant species [10]. Similarly, *L, humile* has been observed to reduce the numbers of arthropods (the main pollinators of inflorescences) in the Western Cape of South Africa by 32% to 62%, thereby severely affecting plant reproduction and growth [11,12]. Moreover, in New Zealand, the cost of controlling *L. humile* has reached millions of dollars [13]. Although to date there have been no reports of *L. humile* populations in China, this invasive ant has biological characteristics similar to those of *S. invicta*. Accordingly, to prevent any future introduction and establishment of *L. humile*, which poses a threat to biodiversity, agricultural production, and human health.

According to the Sixth Assessment Report of the Intergovernmental Panel on Climate Change (IPCC), global warming will rise by 1.5 °C or more in the coming two decades [14]. Insects are poikilothermic temperature animals, which was particularly sensitive to temperature variables [15]. Rapid climate warming affected the growth and development, egg laying, overwintering, distribution patterns, and reproductive rates of insects [16]. Previous studies have shown that global climate warming will affect the distribution of many species worldwide, including invasive alien ants, causing changes in distribution patterns and promoting migration to higher latitudes [17]. For instance, the potential suitable areas of *S. invicta* will increase and move to higher latitudes in China under climate change [18]. Consequently, paying more attention to the response of *L. humile* to climate change is helpful not only in understanding the changes in the distribution pattern but also in formulating early warning and management strategies to prevent the invasion of *L. humile* in China.

Species distribution models (SDMs) are numerical tools that integrate environmental data with data of a species' occurrence or abundance, and can predict the current and future potential suitable areas of the species [19–21]. The MaxEnt model has become the most commonly applied species distribution model, because of its small sample size requirement, simple operation, stable computing results, and short computing time [22–24]. Moreover, it performs well in predicting the regionally suitable distribution of IAS [25,26]. For instance, MaxEnt models were used to study the potential suitable area of the invasive ants *S. invicta* in China [27], and *A. gracilipes* in China and the world [28]. Using the default parameters to build a MaxEnt model is prone to overfitting, and results in less accurate model predictions [29,30]. Therefore, the ENMeval data package in the R software is used to optimize the parameters of the MaxEnt model to avoid model overfitting and improve the rationality and accuracy of species predictions [31].

In this study, we used the optimized MaxEnt model to predict the invasion risk of *L. humile* in China under current and future climatic scenarios. The main objectives of this study were: (1) to determine the important bioclimatic variables affecting the potential suitable area of *L. humile* in China, (2) to assess potential suitable areas of *L. humile* in China under current climatic scenarios, and (3) to determine changes in the potential suitable areas of *L. humile* in China under future climatic scenarios, and centroid shifts of potential suitable areas between current and future climatic scenarios. Our results would provide a scientific basis for continued monitoring and early warning of *L. humile* in China.

2. Materials and Methods

2.1. Global Occurrence Records of *Linepithema humile*

First, 10,371 global occurrence records of *L. humile* worldwide were obtained from the Global Biodiversity Information Facility (GBIF, https://www.gbif.org/, accessed on 27 June 2022) and the Invasive Species Compendium of the Center for Agriculture and Bioscience International (CABI, https://www.cabi.org/, accessed on 28 June 2022). Only one occurrence record was kept for each 5 × 5 km raster interval to prevent overfitting of the model using the ENMtools software for the species occurrence records. Once filtering was complete, 2432 occurrence records were retained for model construction (Figure 1).

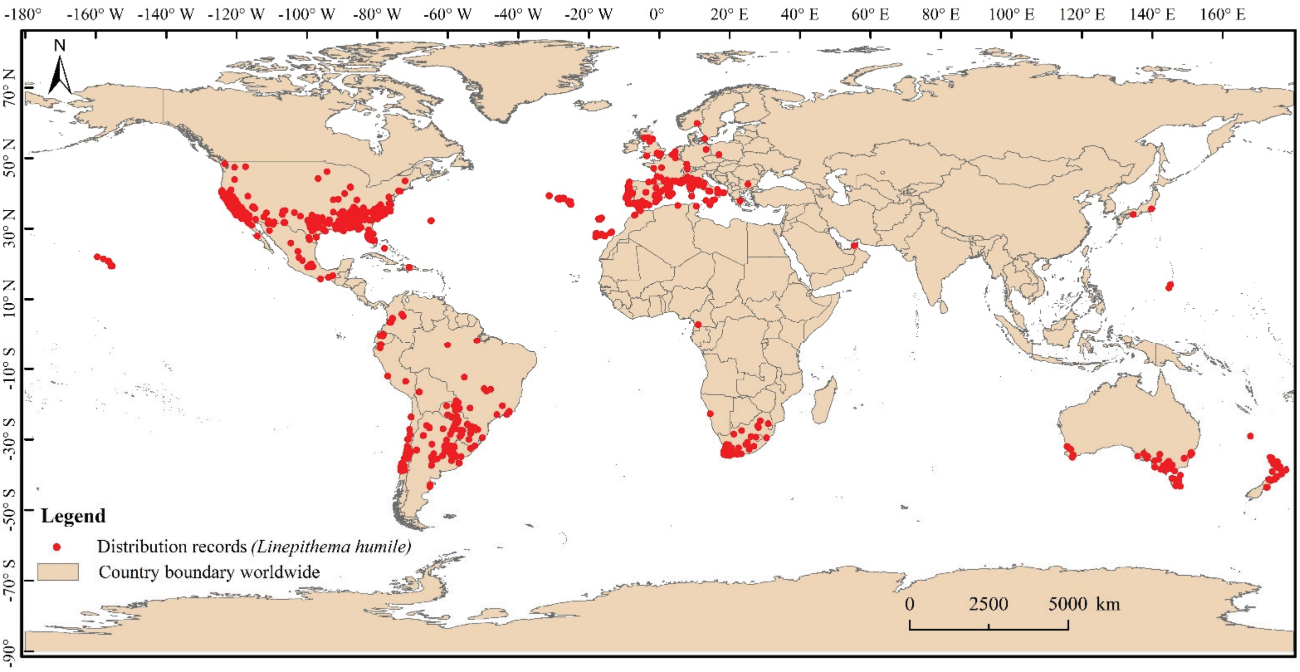

Figure 1. Global occurrence records of the *Linepithema humile*.

2.2. Bioclimatic Variables

A total of 19 bioclimatic variables were downloaded from the World Climate Database (http://www.worldclim.org/ accessed on 27 June 2022) at a resolution of $2.5'$ under current and future climatic scenarios (Table 1). The bioclimatic climate variables under future climate scenarios included three shared socioeconomic pathways (SSP1-2.6, SSP2-4.5 and SSP5-8.5) for two periods (the 2030s and 2050s) under the BCC-CSM2-MR developed by the global climate model of the National Climate Center (Table S1).

In order to avoid the result of the MaxEnt model overfitting due to the multicollinearity between the 19 bioclimatic variables. First, 19 bioclimatic variables were correlated in the spatial analyst tool in ArcGIS software; then the 19 bioclimatic variables and species occurrence records were imported into the MaxEnt model for 10 repetitions. If the two bioclimatic variables had a correlation coefficient with absolute values greater than 0.8 ($|r| > 0.8$), the bioclimatic variable with the higher contribution was retained (Figure S1) [32]. Finally, ten bioclimatic variables were used to construct the MaxEnt model (Table 1).

Table 1. Bioclimatic variables are related to the distribution of *Linepithema humile*.

Variable	Description	Unit	Whether to Use for Modeling
Bio1	Annual mean temperature	°C	No
Bio2	Mean diurnal air temperature area	°C	Yes
Bio3	Isothermality (bio2/bio7) (*100)	—	No
Bio4	Temperature seasonality (standard deviation *100)	—	No
Bio5	Max temperature of warmest month	°C	Yes
Bio6	Min temperature of coldest month	°C	No
Bio7	Temperature annual area (bio5-bio6)	°C	Yes
Bio8	Mean temperature of wettest quarter	°C	Yes
Bio9	Mean temperature of driest quarter	°C	No
Bio10	Mean temperature of warmest quarter	°C	No
Bio11	Mean temperature of coldest quarter	°C	Yes
Bio12	Annual precipitation	mm	No
Bio13	Precipitation of wettest month	mm	No
Bio14	Precipitation of driest month	mm	No
Bio15	Precipitation seasonality (coefficient of variation)	—	Yes
Bio16	Precipitation of wettest quarter	mm	Yes
Bio17	Precipitation of driest quarter	mm	Yes
Bio18	Precipitation of warmest quarter	mm	Yes
Bio19	Precipitation of coldest quarter	mm	Yes

2.3. Optimized MaxEnt Modeling

The results of the MaxEnt model are prone to overfitting when modeling using the program's default settings. Feature combinations (FCs) and regularization multiplier (RM) are the two most important parameters in MaxEnt models [33]. FCs included linear-L, quadratic-Q, hinge-H, product-P and threshold-T. The RM was set 0.5 to 4, at intervals of 0.5. In this study, we used the ENMeval package in R software to adjust the parameters of FCs and RM choose to use the delta AICc minimum parameter combination as the optimal parameter setting for the model [34]. FCs and RM were set to LQHPT and 0.5, respectively.

2.4. Model Evaluation and Potential Suitable Area Delineation

The FC and RM were set up according to the optimal model, and 75% of the occurrence records were selected for simulation training and 25% for model testing. In the MaxEnt model, the maximum number of iterations was set to 500, the background points to 10,000, and the output format to Cloglog, and cross-validated by running 10 replicates. The jackknife method was chosen to test and create response curves to assess the effects of bioclimatic variables on the potential suitable area of *L. humile* in China. The accuracy of the model was examined using the area enclosed by the area of under receiver operating characteristic (ROC) curve (AUC) [35]. The model prediction accuracy evaluation is classified as excellent for 0.8–1, usable for 0.5–0.8, and poor for 0–0.5 [36].

The maximum value of 10 repetitions in the MaxEnt model was selected as the result of this study, and the potential suitable area of *L. humile* in China was cropped according to map of the country. The results were generated based on the presence probability of *L. humile*, with values in the range of 0–1, with larger values indicating higher species presence probability. We used the reclassify tool in ArcGIS software to classify the suitable habitat into four levels, 'highly suitable area' (0.5~1), 'moderately suitable area' (0.3–0.5), 'poorly suitable area' (0.1–0.3) and 'unsuitable area (0–0.1)'.

The spatial distribution of geographical objects can be described by the centroid, and the displacement of geographical objects over a period of time can be expressed using the centroid [37]. In this study, the regularity of potential habitat area displacement of *L. humile* was reflected by the change in the period of the potential habitat area. First, the habitat raster map was transformed into a vector map using the ArcGIS software. Then, the centroid of the potential habitat area was then calculated using the Statistical Analysis Zonal [37].

3. Results

3.1. Model Optimization and Accuracy Evaluation

Our results showed that delat.AICc is lowest when FCs are LQHPT and RM is 0.5, when Mean AUC is greater than the value at default parameters (Figure 2). Under the optimal parameter settings, the mean AUC value of the ten simulations based on the MaxEnt model for the current potential habitat area of *L. humile* was 0.883, indicating excellent model prediction accuracy and high stability (Figure 3).

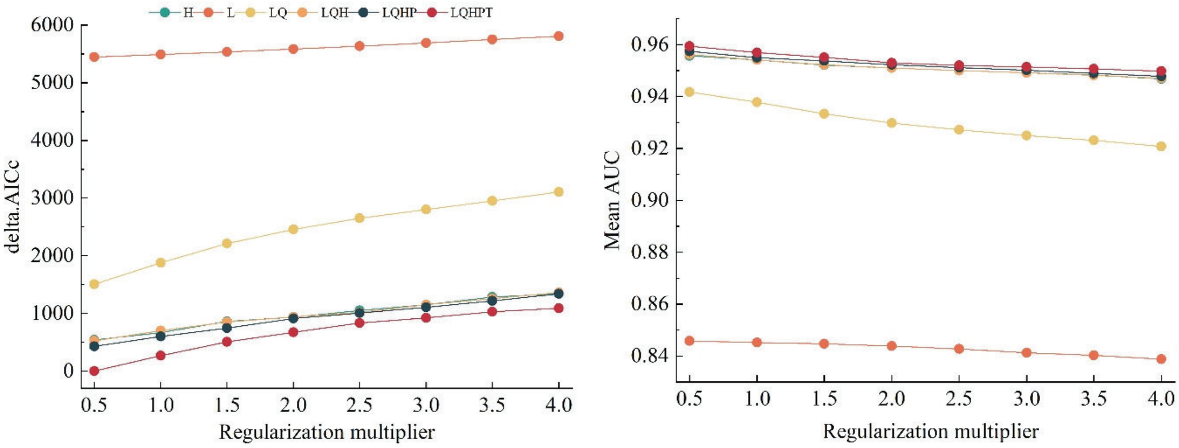

Figure 2. Optimal parameter results for the MaxEnt model.

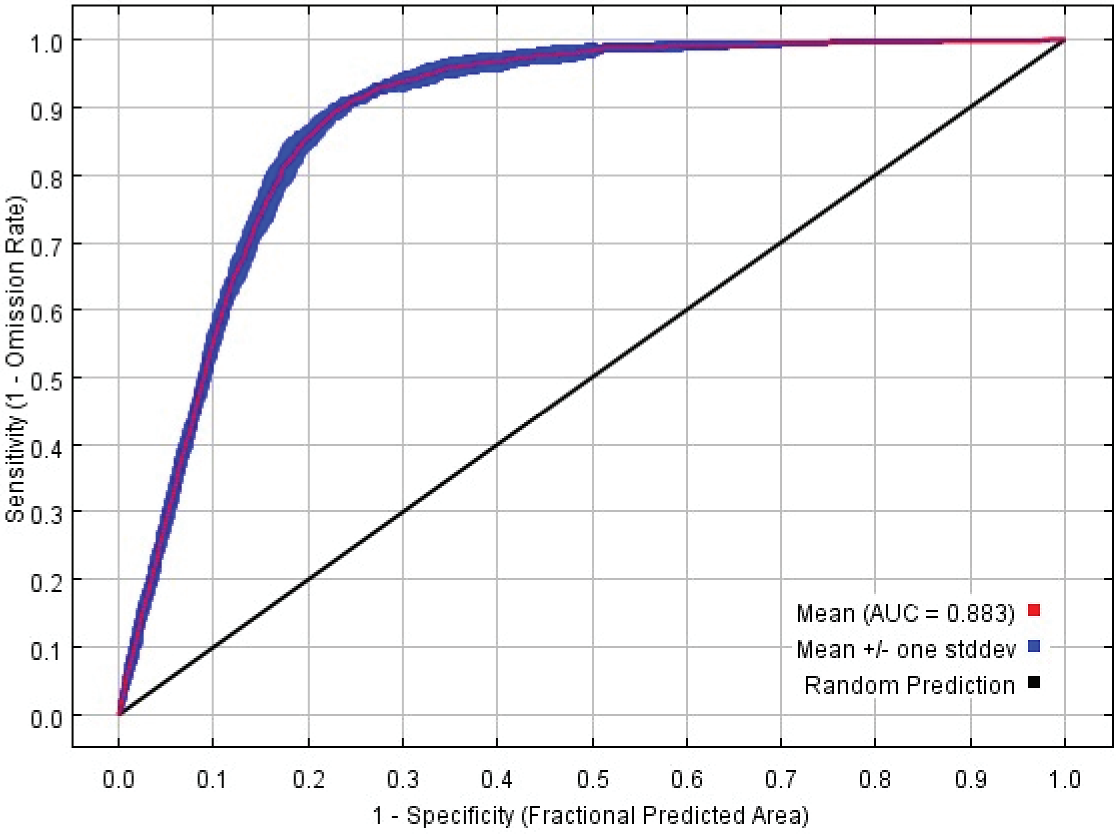

Figure 3. Mean AUC values of the *Linepithema humile* MaxEnt model.

3.2. Bioclimatic Variables Affecting the Potential Suitable Area of Linepithema humile

The mean temperature of the coldest quarter (Bio11), precipitation of the coldest quarter (Bio19), mean temperature of the wettest quarter (Bio8), and precipitation of the warmest quarter (Bio18) were the most important bioclimatic variables based on jackknife and percent contribution (Figures 4 and S2). The important bioclimatic variables affecting the potential suitable area of *L. humile* are two temperature (mean temperature of the coldest quarter, mean temperature of the wettest quarter) and two precipitation variables (precipitation of the coldest quarter, precipitation of the warmest quarter).

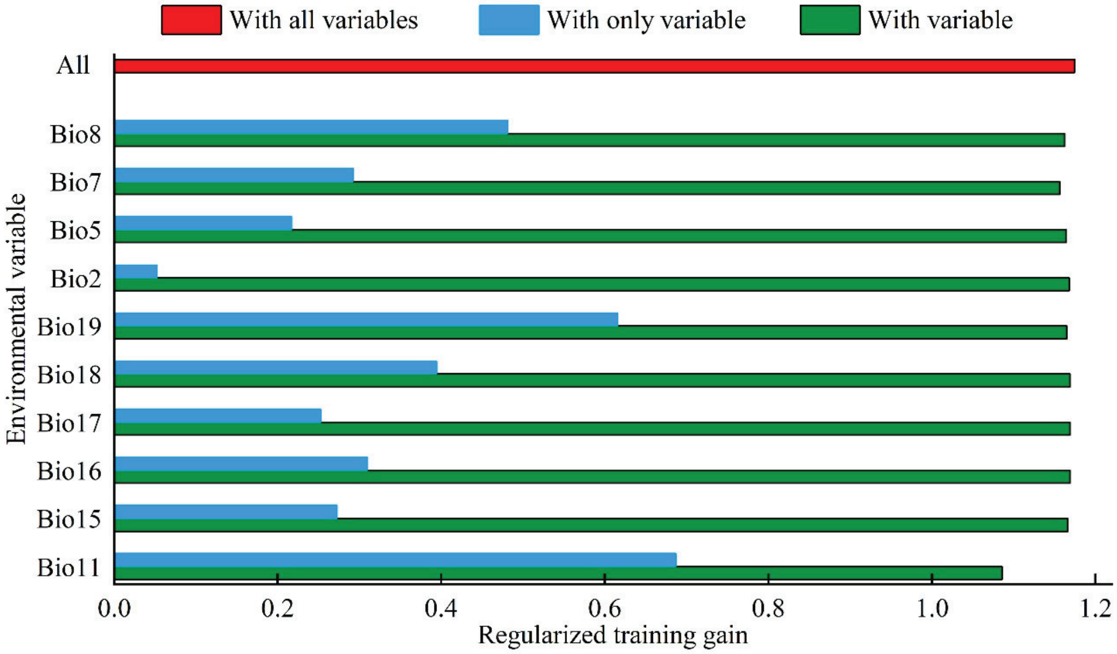

Figure 4. Jackknife test results for *Linepithema humile* bioclimatic variables.

The relationship between the probability of presence of *L. humile* and bioclimatic variables showed in Figure 5. When the probability of presence values of *L. humile* was greater than 0.5, it indicated that the corresponding interval of bioclimatic variables was suitable for the species. For temperature variables, the mean temperature of the coldest quarter (Bio11) and mean temperature of the wettest quarter (Bio8) ranged from 6.83 °C to 19.28 °C, and from 6.97 °C to 28.36 °C, respectively. For precipitation variables, the precipitation of the coldest quarter (Bio19) and precipitation of the warmest quarter (Bio18) ranged from 98.89 mm to 882.65 mm, and from 1.51 mm to 373.27 mm, respectively.

3.3. Current Potential Suitable Areas of *Linepithema humile* in China

The prediction of the potential suitable areas of *L. humile* in China under the current climate is shown in Figure 6. The total suitable area was 80.31 × 10^4 km^2 (Table S2), accounting for 8.36% of the total area of Chinese mainland, which was mainly located in southeast and southwest of China. The highly suitable area was 0.18 × 10^4 km^2, accounting for 0.02% of the total area of the Chinese mainland, which was mainly located in Fujian. The moderately suitable area was 6.37 × 10^4 km^2, accounting for 0.66% of the total area of the Chinese mainland, which was mainly located in Zhejiang, Fujian, Hunan, and Guangxi. The poorly suitable area was 73.76 × 10^4 km^2, accounting for 7.68% of the total area of the Chinese mainland, which was mainly located in Zhejiang, Fujian, Jiangxi, Hunan, Yunnan, and Hubei.

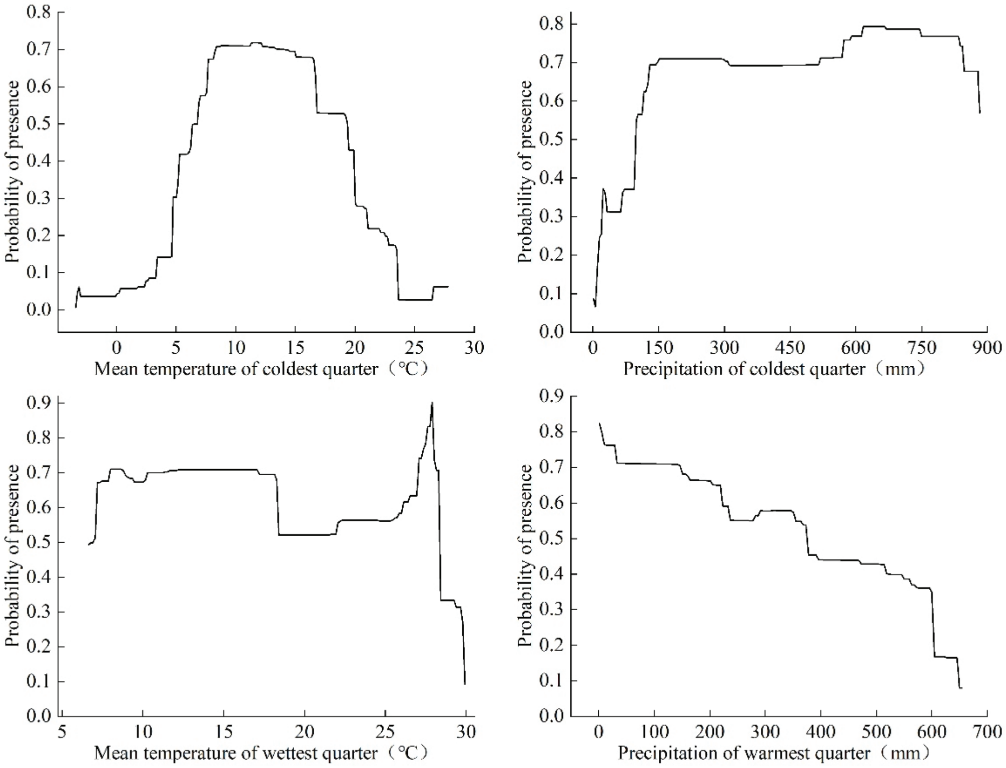

Figure 5. Probability of presence for important bioclimatic variables for *Linepithema humile*.

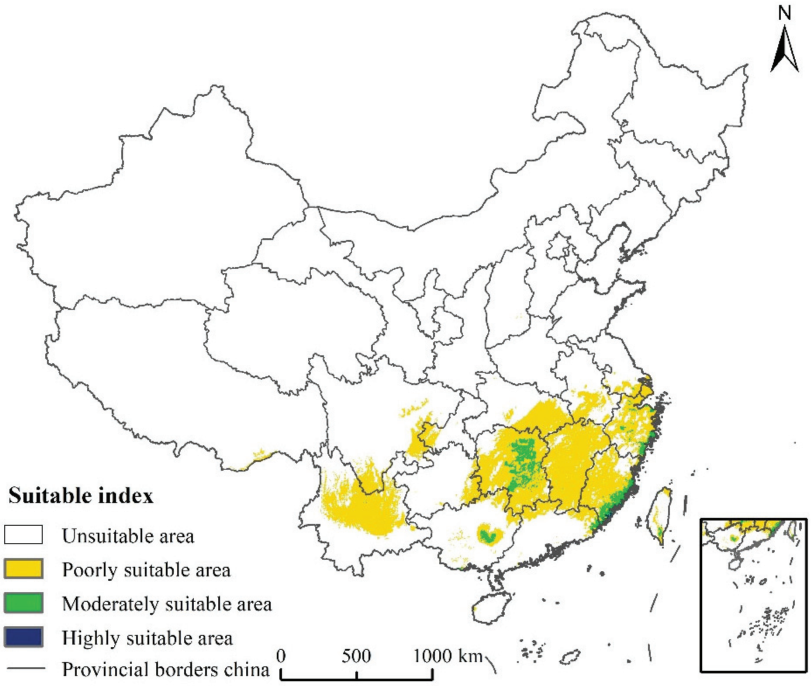

Figure 6. Potential suitable area for *Linepithema humile* under current climatic scenarios in China.

3.4. The Changes Potential Suitable Areas of *Linepithema humile* under Future Climatic Scenarios

The potential suitable areas of *L. humile* under the SSP1-2.6, SSP2-4.5, and SSP5-8.5 scenarios, in the 2030s and 2050s are shown in Figures 7 and 8. The potential suitable areas were mainly located in Jiangsu, Zhejiang, Fujian, Jiangxi, Anhui, Hunan, Hubei, and Yunnan. The results showed that the potential suitable areas increased under the future climatic scenarios. The loss areas were mainly located in Fujian, Jiangxi, Guangxi, Yunnan, and Anhui. The gain areas were mainly located in Jiangsu, Anhui, Hubei, Sichuan, and Henan.

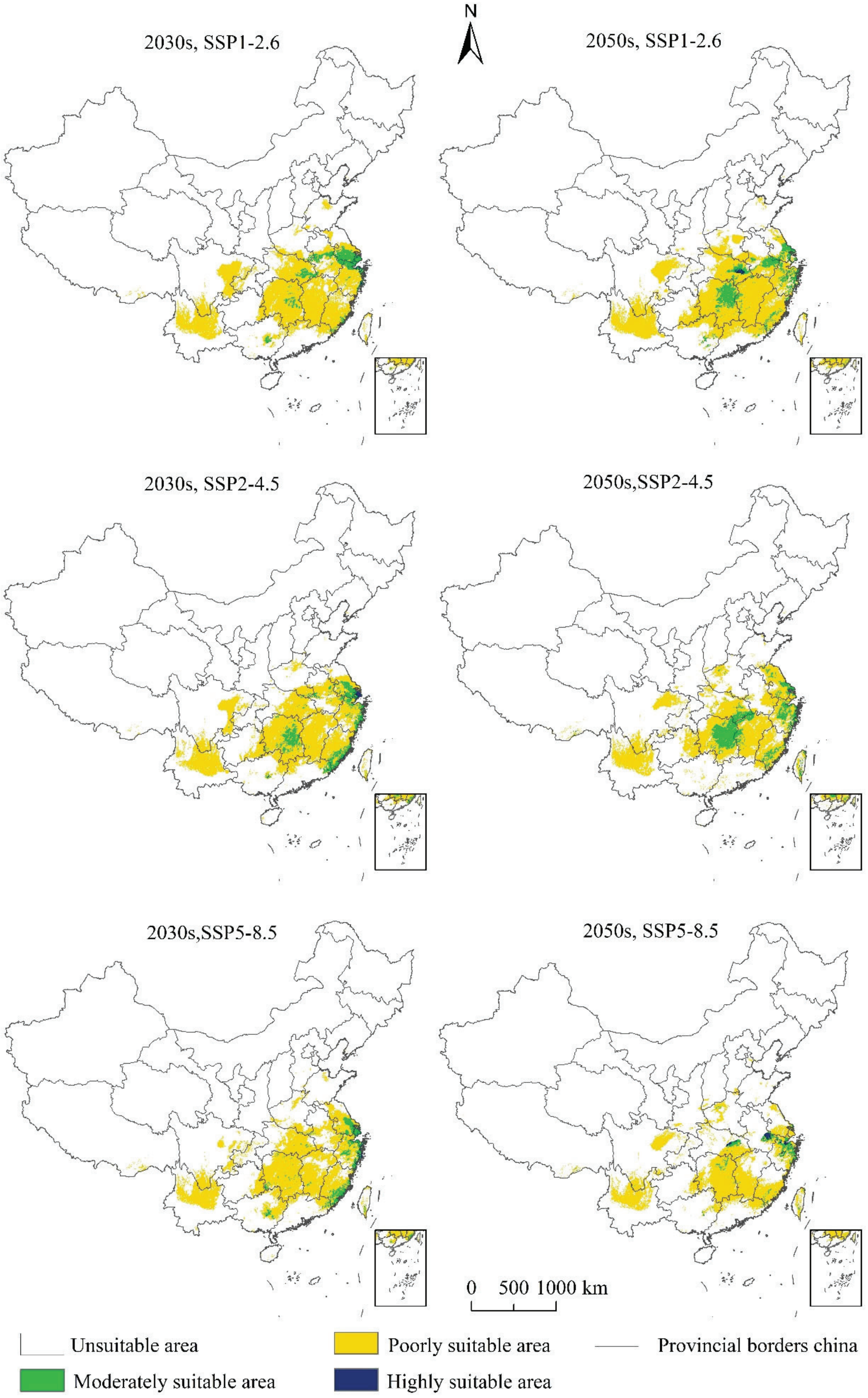

Figure 7. Potential suitable area for *Linepithema humile* under future climate scenarios in China.

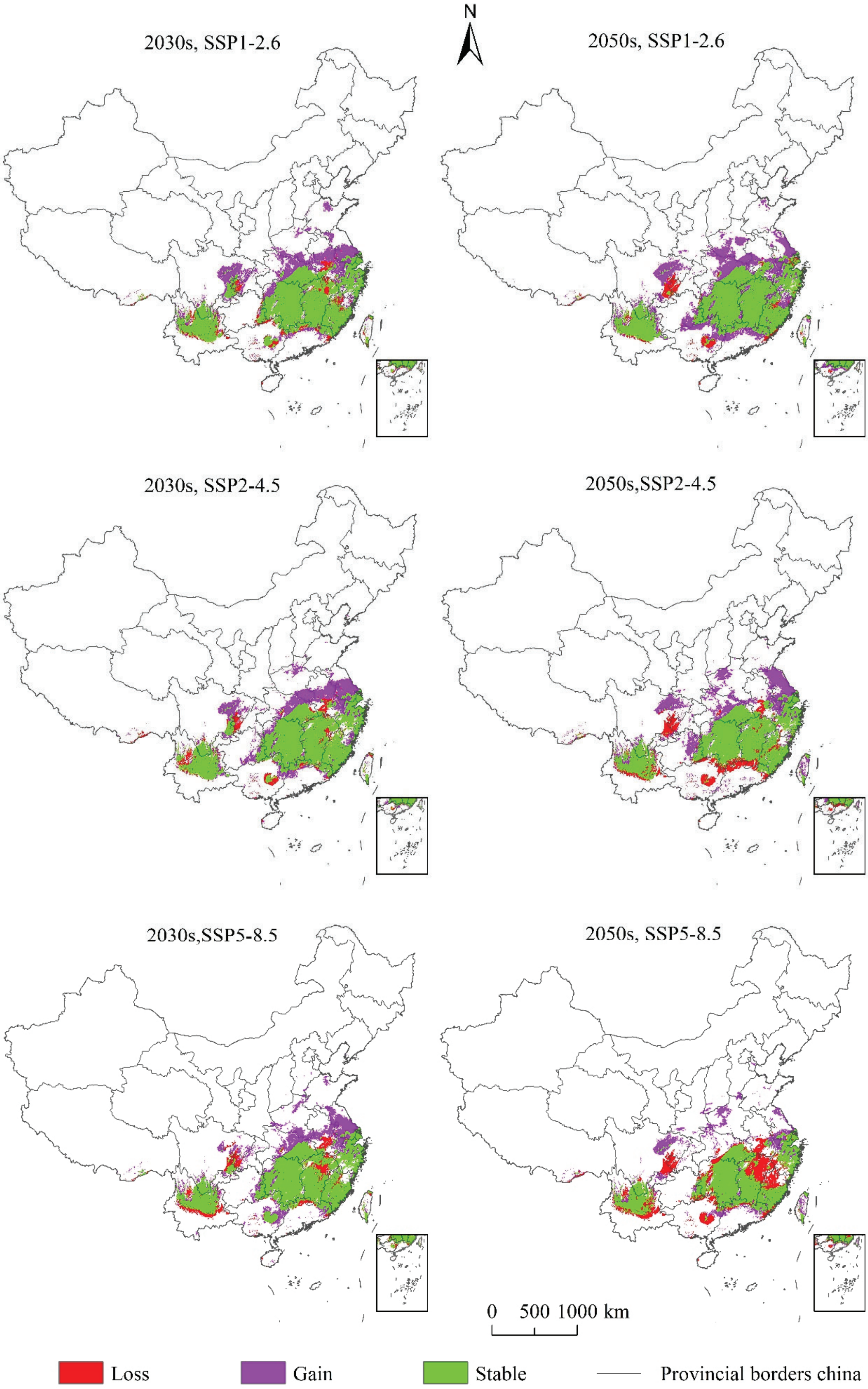

Figure 8. Changes in potential suitable areas for *Linepithema humile* between the current and future climatic scenarios in China.

Under the SSP1-2.6 scenario in 2030s, the total, highly, moderately, and poorly suitable areas of *L. humile* were 104.60 × 10^4 km^2, 0.54 × 10^4 km^2, 9.22 × 10^4 km^2, and 94.84 × 10^4 km^2 (Table S2), respectively, accounting for 10.90%, 0.06%, 0.96%, and 9.88% of the area of Chinese mainland, respectively. Under the SSP1-2.6 scenario in 2050s, the total, highly, moderately, and poorly suitable areas of *L. humile* were 116.10 × 10^4 km^2, 0.76 × 10^4 km^2, 13.43 × 10^4 km^2, and 101.91 × 10^4 km^2, respectively, accounting for 12.09%, 0.08%, 1.40%, and 10.61% of the area of Chinese mainland, respectively. In the 2030s and 2050s, under SSP1-2.6, the total, highly, moderately, and poorly suitable areas gradually increased (Figure 7).

Under the SSP1-2.6 scenario in 2030s and 2050s, the loss areas were 9.24 × 10^4 km^2, and 7.59 × 10^4 km^2 (Table S3), respectively, which was mainly located in Anhui, Jiangxi, Fujian, Guangxi, Chongqing, and Sichuan. The gain areas were 33.21 × 10^4 km^2, and 43.10 × 10^4 km^2, respectively, which were mainly located in Anhui, Jiangsu, Hubei, Sichuan, Henan, and Guangdong (Figure 8).

Under the SSP2-4.5 scenario in 2030s, the total, highly, moderately, and poorly suitable areas of *L. humile* were 102.50 × 10^4 km^2, 0.59 × 10^4 km^2, 11.07 × 10^4 km^2, and 90.84 × 10^4 km^2, respectively, accounting for 10.67%, 0.06%, 1.15%, and 9.46% of the area of the Chinese mainland, respectively. Under the SSP2-4.5 scenario in 2050s, the total, highly, moderately, and poorly suitable areas of *L. humile* were 95.03 × 10^4 km^2, 0.49 × 10^4 km^2, 15.73 × 10^4 km^2, and 78.81 × 10^4 km^2, respectively, accounting for 9.90%, 0.05%, 1.64%, and 8.21% of the area of Chinese mainland, respectively. In the 2030s and 2050s, under SSP2-4.5, the total, highly, moderately, and poorly suitable areas gradually increased.

Under the SSP2-4.5 scenario in 2030s and 2050s, the loss areas were 9.45 × 10^4 km^2, and 16.56 × 10^4 km^2, respectively, which were mainly located in Anhui, Chongqing, Yunnan, Guangxi, Guangdong, and Sichuan. The gain areas were 31.56 × 10^4 km^2, and 31.23 × 10^4 km^2, respectively, which were mainly located in Anhui, Jiangsu, Hubei, Sichuan, Henan, and Guizhou.

Under the SSP5-8.5 scenario in 2030s, the total, highly, moderately, and poorly suitable areas of *L. humile* were 97.69 × 10^4 km^2, 0.87 × 10^4 km^2, 7.22 × 10^4 km^2, and 89.60 × 10^4 km^2, respectively, accounting for 10.17%, 0.09%, 0.75%, and 9.33% of the area of Chinese mainland, respectively. Under the SSP5-8.5 scenario in 2050s, the total, highly, moderately, and poorly suitable areas of *L. humile* were 73.04 × 10^4 km^2, 1.12 × 10^4 km^2, 4.26 × 10^4 km^2, and 67.66 × 10^4 km^2, respectively, accounting for 7.61%, 0.12%, 0.44%, and 7.05% of the area of Chinese mainland, respectively. In the 2030s and 2050s, under SSP5-8.5, the total, moderately, and poorly suitable areas gradually decreased, while the highly suitable area increased.

Under the SSP5-8.5 scenario in 2030s and 2050s, the loss areas were 11.78 × 10^4 km^2, and 26.11 × 10^4 km^2, respectively, which were mainly located in Anhui, Jiangxi, Yunnan, Chongqing, Fujian, and Guangxi. The gain areas were 28.91 × 10^4 km^2, and 18.92 × 10^4 km^2, respectively, which were mainly located in Anhui, Jiangsu, Hubei, Sichuan, and Henan.

3.5. The Centroid Migration of Potential Suitable Areas for Linepithema humile

The centroids of potential suitable areas of *L. humile* in China under current and future climate scenarios are shown in Figure 9. The centroid of potential suitable areas of *L. humile* shifted to northward and high-latitude areas under future climate scenarios.

Under the current climate, the centroid of the potential suitable area was located at the point (112.38° E, 27.28° N). Under SSP1-2.6 scenario, the suitable areas of 2030s and 2050s centroids were to the point (112.70° E, 28.33° N) and the point (112.59° E, 28.11° N), it shifted 0.32° E and 1.05° N from the current to the 2030s, 0.11° E and 0.22° N from the 2030s to the 2050s. Under SSP2-4.5 scenario, the suitable areas of 2030s and 2050s centroids were to the point (112.96° E, 28.13° N) and the point (112.94° E, 28.53° N), it shifted 0.58° E and 0.85° N from the current to the 2030s, 0.02° E and 0.40° N from the 2030s to the 2050s. Under SSP5-8.5 scenario, the suitable areas of 2030s and 2050s centroids were to the point

(112.92° E, 28.19° N) and the point (112.54° E, 27.96° N), it shifted 0.54° E and 0.91° N from the current to the 2030s, 0.38° E and 0.23° N from the 2030s to the 2050s.

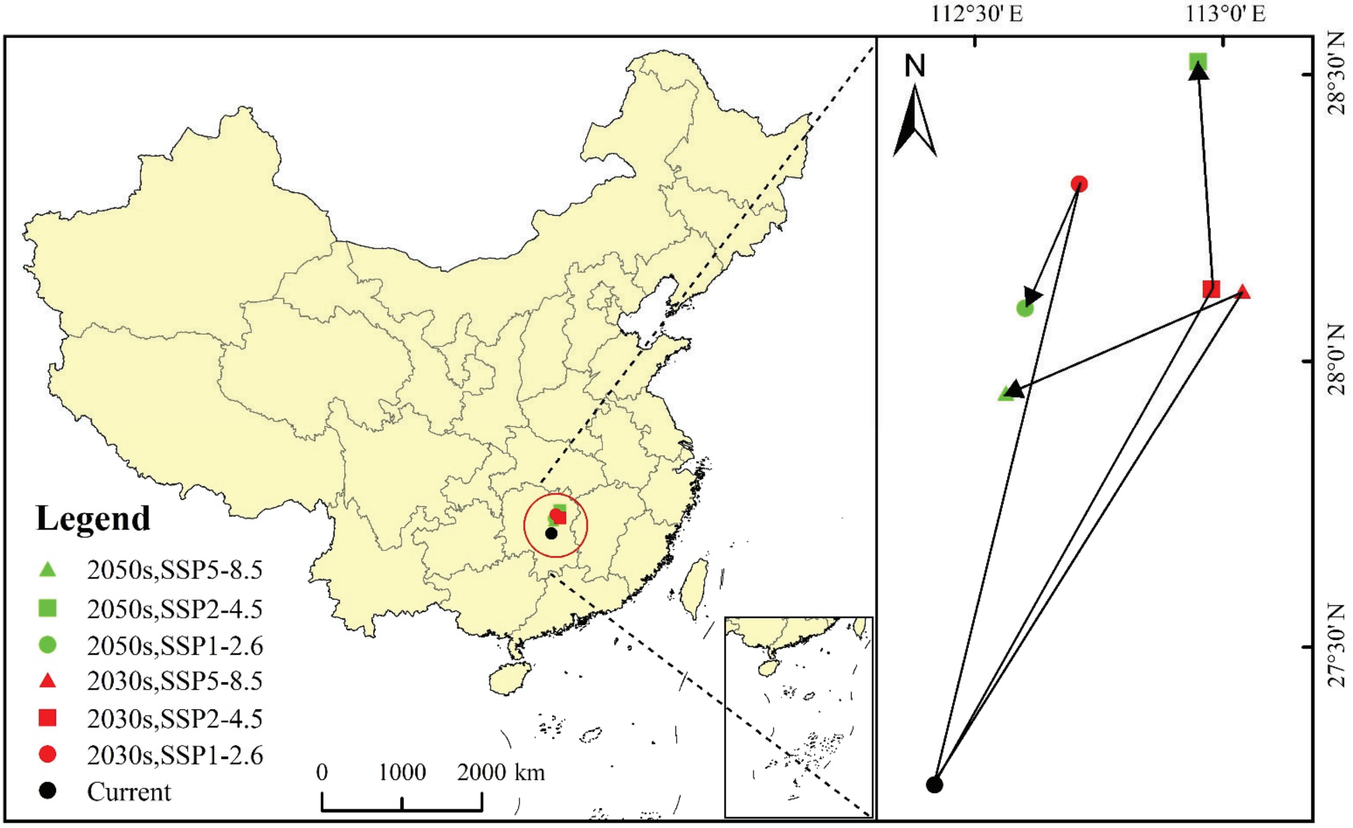

Figure 9. Distribution centroids of *Linepithema humile* under current and future climatic scenarios in China.

4. Discussion

The important bioclimatic variables affecting the potential suitable areas of *L. humile* in China were mean temperature of the coldest quarter (Bio11) and mean temperature of the wettest quarter (Bio8), precipitation of the coldest quarter (Bio19), and precipitation of the warmest quarter (Bio18). Our results showed that the presence probability of *L. humile* gradually increased when the mean temperature of the coldest quarter (Bio11) was −3.42–11.45 °C, and gradually decreased when it was 11.45–27.79 °C. The probability of *L. humile* was less change when the mean temperature of the wettest quarter (Bio8) was 6.63–18.34 °C, gradually increased when it was 18.41–27.88 °C, and gradually decreased when it was 27.88–29.91 °C. This would suggest that they prefer warm, wet climates over cold, wet conditions. This is consistent with previous studies. Temperature within a range from 18 to 32 °C has been established to influence all stages of *L. humile* development from eggs to adults, with development time being shortened at higher temperatures [38]. *Linepithema humile* stops feeding when the temperature is consistently below 5 °C, and the colony eventually starves to death [39]. The oviposition rate of *L. humile* was affected at laboratory temperatures ranging between 10 and 34 °C, and under different polygamous conditions, which oviposition highest at 28 °C [40].

Precipitation variables also had direct and indirect effects on the distribution patterns of *L. humile*. Changes in precipitation affect the humidity and temperature of *L. humile* habitats, and thereby affect growth and development, survival, reproduction, and overwintering [41–43]. Our results indicate that the probability of the presence of *L. humile* was greatly affected by the precipitation of the coldest quarter. Previous study showed that elevated soil moisture increased the populations of *L. humile*, and their ability to compete with native ant species [44]. Cessation of irrigation resulted in a population decline of *L. humile* in San Diego, USA [44].

The prediction of potential suitable areas of IAS is an important element for invasion risk assessment, identifying the potential suitable areas of *L. humile* in China can provide scientific guidance for the early monitoring, prevention, and control of *L. humile*. Our results showed that the potential suitable areas for *L. humile* under the current climate were mainly located in eastern, central and southwestern China. Under future climate scenario, the potential suitable areas generally showed an increasing trend. The mild climate and abundant moisture in the coastal areas of southeastern China provide suitable conditions for the invasion of *L. humile* [45]. Previous studies have indicated that climate warming increases the distribution areas of IAS. For instance, the global distribution areas of Zeuzera pyrina and Ceroplastes cirripediformis will increase under future climate scenarios and will expand to temperate regions worldwide [46,47]. The prediction of distribution areas of the 13 most invasive termites worldwide, indicated that the distribution areas of 12 species would likely increase under future climate scenarios [48]. There is some consistency between the results of the above studies and the those of this study.

As global climate continues to warm, many insects have shifted to high-latitude areas [49]. Our results showed that the centroid of potential suitable area of *L. humile* in China shifted to high-latitude areas under future climate scenarios. Previous studies showed that the potential suitable areas of *S. invicta* and *Hyphantria cunea* in China also shifted to high-latitude areas under all future climate scenarios [50,51]. These studies also verified our results and the hypothesis that insects will move to high-latitude areas with increasing global warming [52,53].

The global dispersal pathways of *L. humile* are mainly natural dispersal, global trade, and accidental dispersal [54,55]. The maximum possible invasion in China would be due to accidental dispersal. There are many vectors for accidental spread, including containers and packaging-wood, human-related waste, sod and mulch, plants or parts of plants, soil, and gravel [56]. When *L. humile* was introduced into a new habitat, they spread rapidly by natural dispersal and vectorial transport. Eastern, central and southwestern coastal areas of China were the potential suitable areas of *L. humile*, which is also the area at the highest risk of invasion, due to the large number of ports receiving international cargo. Zhejiang, Fujian, Jiangxi, Anhui, Hunan, Hubei, Yunnan, and Sichaun areas of China were the potential suitable areas of *L. humile*, which is also the area at the highest risk of invasion, due to the large number of ports receiving international cargo. Therefore, Hangzhou, Fuzhou, Nanchang, Hefei, Changsha, Wuhan, Kunming, and Chengdu customs should strengthen the quarantine of the logs, wooden packaging, and containers that was from Europe and America. If a wild population of *L. humile* is found in China, it should immediately be eradicated using cultural, biological and chemical control measures [56]. Cultural control measures mainly involve applying powder barriers and limiting access to water sources or nesting sites [44]. Biological control measures are more difficult, because of the lack of natural enemies of the species [57]. Chemical control measures are more widely used to prevent *L. humile* from entering an area, such as barrier treatment with contact-residual insecticides and baits, but these only kill actively feeding ants and barely affect the larvae or queen [58]. Finally, a comprehensive risk assessment system for the early warning, quarantine, and control of *L. humile* invasion was established.

5. Conclusions

We are the first to use an optimized MaxEnt models to explore the distribution pattern and limiting bioclimatic variables of *L. humile* in China. The high invasion risk areas were mainly located in Zhejiang, Fujian, Jiangxi, Anhui, Hunan, Hubei, Yunnan, and Sichaun. Moreover, the risk of invasion of *L. humile* in China will further increase under future climate scenarios. The mean temperature of the coldest quarter was the most significant bioclimatic variable affecting the distribution pattern of *L. humile* in China. Our study highlights the importance of climate change on the invasion of *L. humile* and could help formulate targeted early warning, prevention and control policies for *L. humile* in China.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d14110921/s1, Figure S1: Correlation coefficient between Bioclimatic variables; Figure S2: Contribution of different bioclimatic variables to MaxEnt model for *Linepithema humile*; Table S1: Three emission scenarios; Table S2: Potential suitable areas for *Linepithema humile* under different climate change scenarios (10^4 km^2); Table S3: Future changes in suitable area (10^4 km^2).

Author Contributions: M.L., X.X. and W.L.: conception and design of the research. M.L. and X.X.: acquisition of data. M.L., X.X. and H.Z.: analysis and interpretation of data. M.L. and X.X.: statistical analysis. M.L., X.X. and H.Z.: drafting the manuscript. L.X., H.H., B.C., W.L., and F.W.: manuscript revision. All authors have read and agreed to the published version of the manuscript.

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