Complexity in Mutualisms: Indirect Interactions With Multiple Parties

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A dissertation presented

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Complexity in mutualisms: indirect interactions with multiple parties

Abstract

Ant-plants provide ants with rewards such as housing and food in exchange for protection from herbivores. These protection mutualisms are complex webs of both direct interactions, such as ants feeding on host plant extrafloral nectar, and indirect interactions mediated by 'third party' species, such as ants consuming exudates from hemipterans feeding on the host plant. While some indirect interactions are well understood, in many cases our understanding is hindered by an incomplete picture of the relevant third-party species.

In this dissertation, I explore third-party interactions of three obligately phytoecious ant species on the African ant-plant *Vachellia drepanolobium* (formerly *Acacia drepanolobium*) – *Crematogaster mimosae*, *C. nigriceps* and *Tetraponera penzigi*.

First, I examine relationships between ants and fungi. I show behavioral differences towards fungi among the three ant species, and then use multiplexed amplicon sequencing to characterize their associated fungal communities. Each ant species harbors its own distinctive fungal community, and these communities are similar for each species even at two field sites separated by 200 kilometers. The ants may vector fungi when they colonize new host trees. *T. penzigi* most likely uses fungi as a food source, and fungi may also have nutritional or other growth implications for the host plant.

Second, I investigate relationships between ants and 'myrmecophiles' – i.e. 'ant loving' arthropods that live alongside ants in the domatia. I show that myrmecophile communities differ among the three ant species, but are also highly context dependent, differing strongly between locations and sampling periods. Surprisingly, several species of myrmecophilous Lepidoptera are herbivorous, but are more commonly associated with the 'better' ant mutualists, *C. mimosae*, whose workers defend more effectively against browsing mammalian herbivores.

My results show that plant ants shape both fungal communities and myrmecophile communities in domatia of their *V. drepanolobium* host plants. These third-party species may be viewed as 'extended phenotypes' of the ants, and are essential elements whose effects need to be incorporated into our understanding of the ant-plant protection mutualism.

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To my parents

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It is fitting that this dissertation on complexity in mutualisms should itself be the product of contributions from so many people. Thank you all.

Chapter 1

Introduction

Ant-plant protection mutualisms are widespread, especially in the tropics[27,83,98]. In these mutualisms, plants provide ants with rewards such as housing and food in exchange for protection from herbivores. The exchanges vary widely: some ants provide extrafloral nectar to a range of generalist facultative ant partners; while others provide domatia, nectar and food bodies to specialized obligate ant partners[28,177].

Ant defense adaptations have evolved many times; these protection mutualisms are found in more than 100 angiosperm genera and over 50 ant genera[34,39,219]. Furthermore, the evolution of traits to attract ant mutualists is associated with elevated plant diversification rates. In the legume genus Senna, for example, a single evolution of extrafloral nectaries appears to have promoted diversification by facilitating the colonization of new habitats[120]. An analysis across the vascular plants shows that diversification rates are twice as high in plant families with extrafloral nectaries than in those without[219].

In addition to their ecological importance, ant plants have also featured prominently as model systems for investigating how mutualisms evolve and persist[27,83]; how species coexist[156,239]; how communities may be regulated by multitrophic interactions[51,187]; the chemical ecology involved in recruiting ant mutualists and inducing ant defense against herbivory[30,95,186,221]; the nutritional ecology underpinning the substantial biomass of ants in rainforest canopies[40,81]; and the coevolution of traits in insects and plants[29,84].

Central to many of these questions is an evaluation of costs and benefits relating to the ant-plant

mutualism, but these costs and benefits have long been difficult to quantify. Although the role of ants in protecting plants from herbivores is now well established[33,179,210], even this remained controversial until the observations and experiments of Janzen and others in the 1960s and 1970s[27,28] – despite experiments dating back as early as 1889 suggesting that ants attracted to extrafloral nectaries could reduce insect damage (von Wettstein, 1889[218] as cited in Beattie, 1985[14]).

One of the chief challenges in assessing a variety of costs and benefits is that they need not be mutually exclusive. For example, ants may deter herbivory by multiple species of herbivores, each targeting different parts of the plant. The costs of herbivory may vary among different locations and points in time[91], and over the lifespan of the plant and/or the ant colony[23,166]. Ants may protect host plants by pruning encroaching vegetation[41,178,202], but may also prune the host plant itself with the effect of modifying growth or temporarily sterilizing the plant[238]. Plants may provide for the nutritional needs of their ants through multiple channels – extrafloral nectar, food bodies, fungal patches, and hemipteran intermediaries[165] – but may also obtain nutrition from ants[59,178,211]. Ants may provide other services to theirs hosts, such as removing potentially pathogenic microbes[116]. And different ant symbionts may vary in their efficacy as plant defenders, and draw differently on the resources of the host[27,156]. While in principle these myriad costs and benefits can be subsumed into measurements of long-term reproductive fitness, this is often impractical, owing in part to the dispersal of both partners and the longevity of host plants[155]. Measures such as foliar herbivory, while convenient, prove to be imperfect proxies for reproductive fitness[210].

Although ant-plant protection mutualisms are often described as interactions between ants and host plants, many of the interactions that make up a given mutualism involve 'third party' species (Figure 1.1). Phloem-feeding hemipterans, for example, facilitate the trophic exchange between many plant-ants and their hosts[165]. Aggressive ant defense might be a direct deterrent to herbivores, but might also have the effect of reducing the density of spiders and hymenopterans that would oth-

erwise eat or parasitize the herbivores[139]. And of course protection against herbivores is itself an interaction mediated by the herbivores[157]. The importance of third-parties magnifies the challenge of understanding ant-plant mutualisms: although some interactions such as hemipteran-mediated feeding are relatively well understood, others are often more diffuse and less well known. Treating these indirect interactions as if they were direct may sometimes be a useful simplification, but ignores context dependency arising from variation in the identity or abundance of third parties over time or between locations.

One set of third parties whose role in ant-plant mutualisms has garnered particular attention in recent years is fungi[126]. Chaetothyriales or 'black yeast' fungi have been found in the domatia of about 20 plant genera associating with several unrelated ant species. These fungi appear to play a nutritional role, especially in nutrient recycling for ants, but possibly also in nutrient transfer to the host plant[20,44,45]. A distinct group of these black yeast fungi have also been implicated as a building resource in some ant-plant systems[183]. As in many areas of microbial ecology, developing molecular methods are likely to expose additional roles and complexity surrounding these fungi in coming years.

In this thesis, I explore two sets of third-party species – arthropods and fungi – inside domatia of the African ant plant *Vachellia drepanolobium*.

1.1 The *Vachellia drepanolobium* ant-plant system

Myrmecophily has evolved multiple times in the genus *Vachellia* (formerly *Acacia* subg. *Acacia*)[73,110]. Among the African vachellias, swollen thorn domatia appear to have at least two origins, though the phylogeny of these taxa is not well resolved. Among the neotropical vachellias, which diverged from the Old World species ca. 15 mya, swollen-thorn taxa form a monophyletic group[73].

Vachellia drepanolobium ant-plants are widespread throughout the East African tropics, usually in

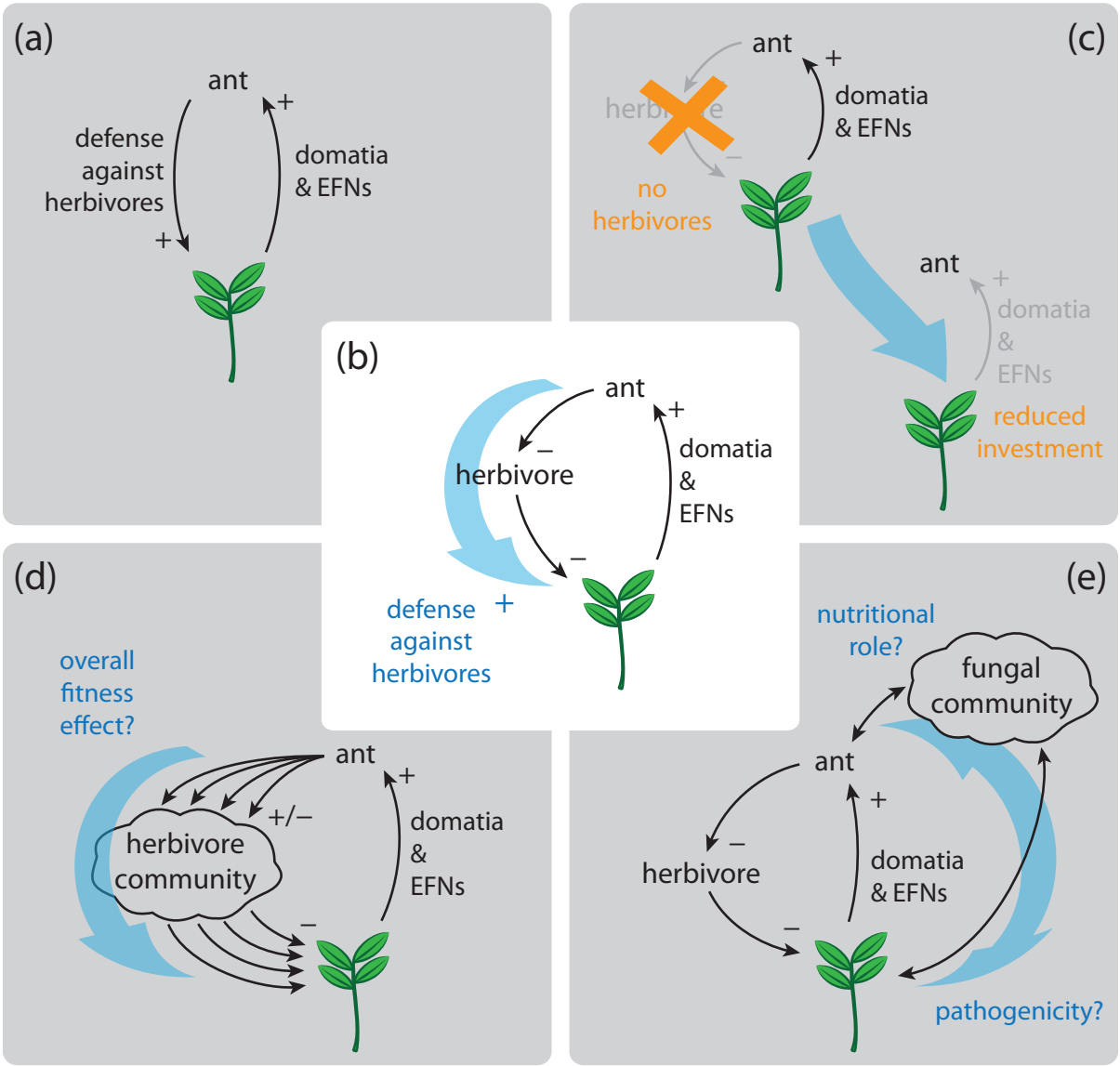

Figure 1.1: (a) Ant-plant protection mutualisms may be usefully regarded as two-party interactions. (b) However, protection against herbivores is really a multi-party interaction. (c) The two-party representation is simple, but may have shortcomings. e.g. the value of protection depends on herbivores being present, and plants may reduce costly investments in ants where herbivores are absent[157]. (d) Ants may have different effects on different herbivores, so the value of protection depends on the herbivores present. (e) Other interactions may also mediated by third-parties. e.g. fungi might play beneficial roles in recycling ant nutrients or transferring nutrients to plants, or may be pathogens of ants or host plants.

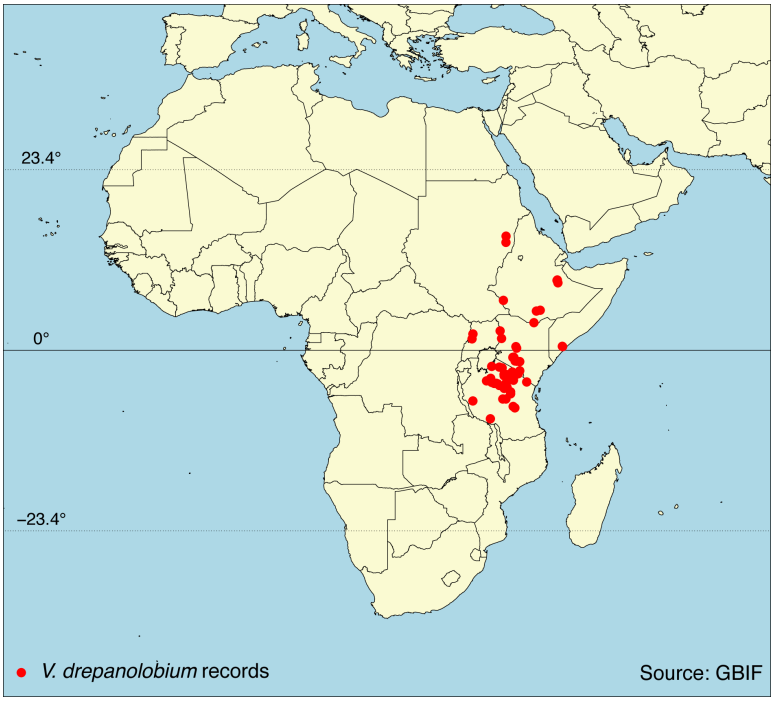

Figure 1.2: *V. drepanolobium* is widespread in the East African tropics. Map shows 159 occurrence records accessed through GBIF on 11 August 2015[69] plus our own sampling sites.

savannas with hardpan grey soil or poorly-drained black cotton soil[46] (Figure 1.2). Where it occurs, it often forms large mono-dominant stands[237] (Figure 1.3).

V. drepanolobium is covered with hollow swollen-thorn domatia that, at least on larger trees, are almost always occupied by ants[237] (Figure 1.4). Three ant species nest obligately in the domatia: *Crematogaster mimosae*, *C. nigriceps*, and *Tetraponera penzigi*[88]. A fourth species, *C. sjostedti*, also associates with *V. drepanolobium* trees but more commonly nests in trunk cavities created by cerambycid beetles or in the ground around the tree bases[158]. Each tree is normally occupied by a single ant species, but different trees, even within meters of one another, may be occupied by different species[158]. While the ants compete for housing on the same species of host plant, several ecological mechanisms (outlined below and reviewed by Palmer *et al.*[156]) have been shown to facilitate their coexistence[151,152,158,196,197,198]. At least 11 other ant species have been recorded visiting *V. drepanolo-*

Figure 1.3: *V. drepanolobium* often forms large mono-dominant stands such as this one.

bium, but not nesting arboreally[88,108,237].

The obligate domatium-dwelling ants engage in a classic protection mutualism[98] with their hosts. In exchange for housing, as well as food in the form of extrafloral nectar, the ants protect their host plant from mammalian herbivores such as giraffe, rhinoceros, antelope and goats[119,199]. The ants vary, however, in the quality of their defense[88,154]. Among the domatium-dwelling ants, the aggressive *C. mimosae* provides better defense than *C. nigriceps*, while *T. penzigi* does little to deter browsers[124]. The ants also impose other costs on their hosts: *C. nigriceps* prunes the plant's axillary buds, shaping growth and temporarily preventing flowering, while *T. penzigi* prunes the extrafloral nectaries, perhaps to reduce the risk of invasion by another ant colony[151,198].

The ants' effects are also evident in the diverse assemblage of organisms on the host plant.

Existing studies have documented dozens of specialized domatium inhabitants that are sometimes preferentially associated with one or more of the ant species[88,140]. These inhabitants range from herbivores to parasitoids and predators. Scale insects, for example, are found with *C. mimosae* and *C. sjostedti*[237], while neither *C. nigriceps* nor *T. penzigi* is typically found with scales. The lycaenid *An-*

Figure 1.4: *V. drepanolobium* often forms large mono-dominant stands such as this one.

thene usamba specializes on trees occupied by *C. mimosae*[125]. The braconid wasp *Trigastrotheca laikipiensis* is a brood parasite of claustral colonies of *C. mimosae* and *C. nigriceps*, but is rarely found with *T. penzigi*[169].

Previous work has also indicated potential associations between the domatium ants and microbes. Martins describes apparent fungivory in *T. penzigi*, based on behavioral observations and stable isotope results, and reports isolating a specific *Chaetomium* strain from several *T. penzigi* domatia in the field[123]. Work in other ant systems has shown roles for fungi as a food source for ants[20], as a building material[183], and as an agent of nutrient recycling and nutrient transfer to the host plant[44].

But despite these promising observations, and evidence of differences in canopy insect communities[109], we have no quantitative data on either the communities of specialized domatium inhabitants or the microbial communities in the domatia. This has left us without a good picture of the strength and importance of associations between the ants and these third parties, which in turn limits our understanding of the potential roles of these third parties in facilitating the ants' existence on the trees,

or in mediating indirect interactions between ants and their plant hosts.

1.2 Dissertation overview

In Chapter 2, I explore relationships between ants and fungi, following previous observations suggesting that *T. penzigi* may engage in trophic or other interactions with fungi in its domatia [123]. First, I find that *T. penzigi* ants forage on and remove more fungi from a cultured isolate in a lab setting than either *C. mimosae* or *C. nigriceps*. Then I use multiplexed amplicon sequencing to characterize domatium fungal communities, and find that community composition differs among the three ant species, but not between two sampling locations in Kenya separated by about 200km. Field-collected domatium samples also differ from field-collected leaf samples and greenhouse-collected domatium samples. Finally, I show that DNA extracted from alates of *C. nigriceps* contains matches for most of the fungal sequences from *C. nigriceps* domatia, suggesting that alates may vector fungi when they colonize new host trees, most likely in the debris that is visible in sections of alates' infrabuccal pockets. These results suggest that *T. penzigi* may use fungi as a food source – perhaps as a way to recycle nutrients, since this species lacks the gut bacterial pouch found in other species of *Tetraponera*. They also indicate that different ant species cultivate different fungal communities in the domatia, with potential nutritional or other growth implications for the host plant.

In Chapter 3, I investigate patterns of association between ants and 'myrmecophiles' – i.e. 'ant loving' arthropods that live alongside ants in the domatia. Using DNA barcoding, I identify over 80 myrmecophile taxa from domatia on almost 500 trees. Myrmecophile incidence and community composition vary significantly among the three obligate domatium dwelling ant species. Surprisingly, I find myrmecophiles are more common with the ant species normally regarded as better mutualists on account of being better defenders against large mammalian herbivores, underscoring a need to better incorporate myrmecophile communities into our understanding of this ant-plant system.

Myrmecophile community composition also varies substantially between collection locations and years, suggesting that the *V. drepanolobium* ecosystem might show more spatial and temporal variation than has previously been appreciated.

In Chapter 4, I examine the trophic relationships among ants, myrmecophiles and their host plants. Using carbon and nitrogen stable isotope analysis, I show that several species of Lepidoptera are herbivorous on the host plant, while several species of spider and Hymenoptera are predators or parasitoids, and derive up to 30% of their carbon from off the host plant. The *Crematogaster mimosae* and *C. nigriceps* ants themselves appear herbivorous and are likely deriving much of their diet from extrafloral nectar or scale insect exudates.

My results point to the role of plant-ants in shaping both fungal communities and myrmecophile communities in *V. drepanolobium* domatia – communities that should therefore be viewed as part of the 'extended phenotype' of the ants. These third-party species may have fitness implications for the host plant, for example through nutrient recycling or herbivory, that should be incorporated into our picture of the ant-plant mutualism, alongside other effects of the ants, such as protection from mammalian herbivores, and pruning of axillary buds and extrafloral nectaries. These results thus highlight the need to incorporate both direct and indirect effects of ant mutualists when assessing their contributions to host plant fitness.

Chapter 2

Different ant species host distinctive fungal communities in domatia of the African ant plant, *Vachellia (Acacia) drepanolobium*

2.1 Summary

Three species of ants nest obligately in the swollen-thorn domatia of the African ant-plant *Vachellia drepanolobium*. In this study, we investigate observations that one of these species, *Tetraponera penzigi*, may engage in trophic or other interactions with fungi in its domatia. First, we found that *T. penzigi* ants would forage on and remove more fungi from a cultured isolate in a lab setting than either of the other two ant species, *Crematogaster mimosae* and *C. nigriceps*. Second, we used multiplexed amplicon sequencing to show that fungal community composition differed among the three ant species, but not between two sampling locations in Kenya separated by about 200km. Taxonomic richness did not vary with ant species or location. These field-collected domatium samples differed from field-collected leaf samples and greenhouse-collected domatium samples in both community composition and taxonomic richness. Third, DNA extracted from alates of *C. nigriceps* contained matches for most of the fungal sequences from that ant species' domatia, suggesting that alates may vector fungi when they colonize new host trees. Fungal hyphae and other debris are visible in sections of these alates' infrabuccal pockets. Our results suggest that *T. penzigi* may use fungi as a food source – perhaps as a way to recycle nutrients, since this species lacks the gut bacterial pouch found in other species of *Tetraponera*. They also indicate that different ant species cultivate different fungal communities in the domatia, with potential nutritional or other growth implications for the host plant.

2.2 Introduction

Vachellia (Acacia) drepanolobium ant plants are widespread in the East African tropics and are typically the dominant tree in black cotton savannahs[237]. While as many as 15 ant species have been documented on *V. drepanolobium* in Kenya[88], three obligately phytoecious species are common throughout its range, nesting in swollen-thorn 'domatia' formed by the swollen bases of stipular thorns (Figures 2.1a and 2.1b): *Tetraponera penzigi*, *Crematogaster mimosae* and *C. nigriceps*. A fourth species, *C. sjostedti*, is also common on *V. drepanolobium* in some locations, but typically nesting in trunk cavities or in the ground at the tree base[158]. Each tree is normally occupied by one colony[158], although sometimes a single colony may extend over several trees, and it is not uncommon for adjacent trees to host different ant species in a complex mosaic throughout the range of *V. drepanolobium*.

The four ant species engage in a protection mutualism with their host plants – defending against large herbivores in exchange for housing (Figure 2.1d) – but the exchange varies among the species[119]. The ants differ in the extent to which they patrol and deter herbivores[88,124]. The *Crematogaster* ants take advantage of extrafloral nectaries on their host plants, while *T. penzigi* destroys them (Figure 2.1f)[155,237]. *C. nigriceps* prunes axillary buds, stimulating terminal growth but eliminating flowering[237]. Demographic modelling indicates positive synergistic effects of these multiple ant species over time[155], suggesting that plants obtain different kinds of benefits each ant species.

While much of the existing work on *V. drepanolobium* has focused on direct interactions among ants, plants and large herbivores, indirect interactions may also have important fitness consequences. *C. mimosae* and *C. sjostedti* ants, for example, tend phloem-feeding scale insects[88,237], presumably offsetting the protection those ants offer against large herbivores. But other ant associations are less well known. For example, we have little quantitative data on the diverse community of ant-associates or 'myrmecophiles' – such as lepidopterans and spiders[88] – that live in the domatia alongside the ants.

Recent observations have raised the possibility that fungi are important players in the *V. drepanolobium* system. Martins[123] describes *T. penzigi* engaging in behavior resembling fungus-tending, and suggests that a *Chaetomium* sp. fungus isolated from colonies in the lab and field might represent a new case of fungal agriculture (Figures 2.1c and 2.1e). Indeed, the diet of *T. penzigi* is otherwise unknown: they do not tend scale insects; few workers are normally found outside of the domatia; and unlike the two *Crematogaster* species they are not known to prey on insects, despite forfeiting extrafloral nectar by destroying nectaries[88,152,155,237]. Some authors have suggested that *T. penzigi* might feed on pollen and fungal spores[152,157], but this remains untested. Existing observations do not, however, establish whether putative fungus-tending behavior is specific to *T. penzigi*, nor whether such behavior has any effects in the field.

In this study we explore ant-fungal associations in the *V. drepanolobium* system with three lines of enquiry. Firstly, we test the hypothesis that the three obligate domatium-dwelling ant species would show different recruitment to fungi isolated from ant colonies. Secondly, we hypothesize that the ants inhabiting the domatia of *V. drepanolobium* might culture fungal communities, and explore this hypothesis by using multiplexed amplicon sequencing to characterize the fungi living in the domatia. Thirdly, after finding differences in fungal communities among the ants, we hypothesize that alates may carry fungi with them when dispersing. We use additional amplicon sequencing to compare fungi in surface-sterilized alates and in domatia, and light microscopy to examine alate infrabuccal pockets.

2.3 Results

Recruitment experiment

To test whether *T. penzigi*, *C. mimosae* and *C. nigriceps* behave different towards fungi, we presented colonies from each species with a *Phoma* fungal culture isolated from a *V. drepanolobium* domatium

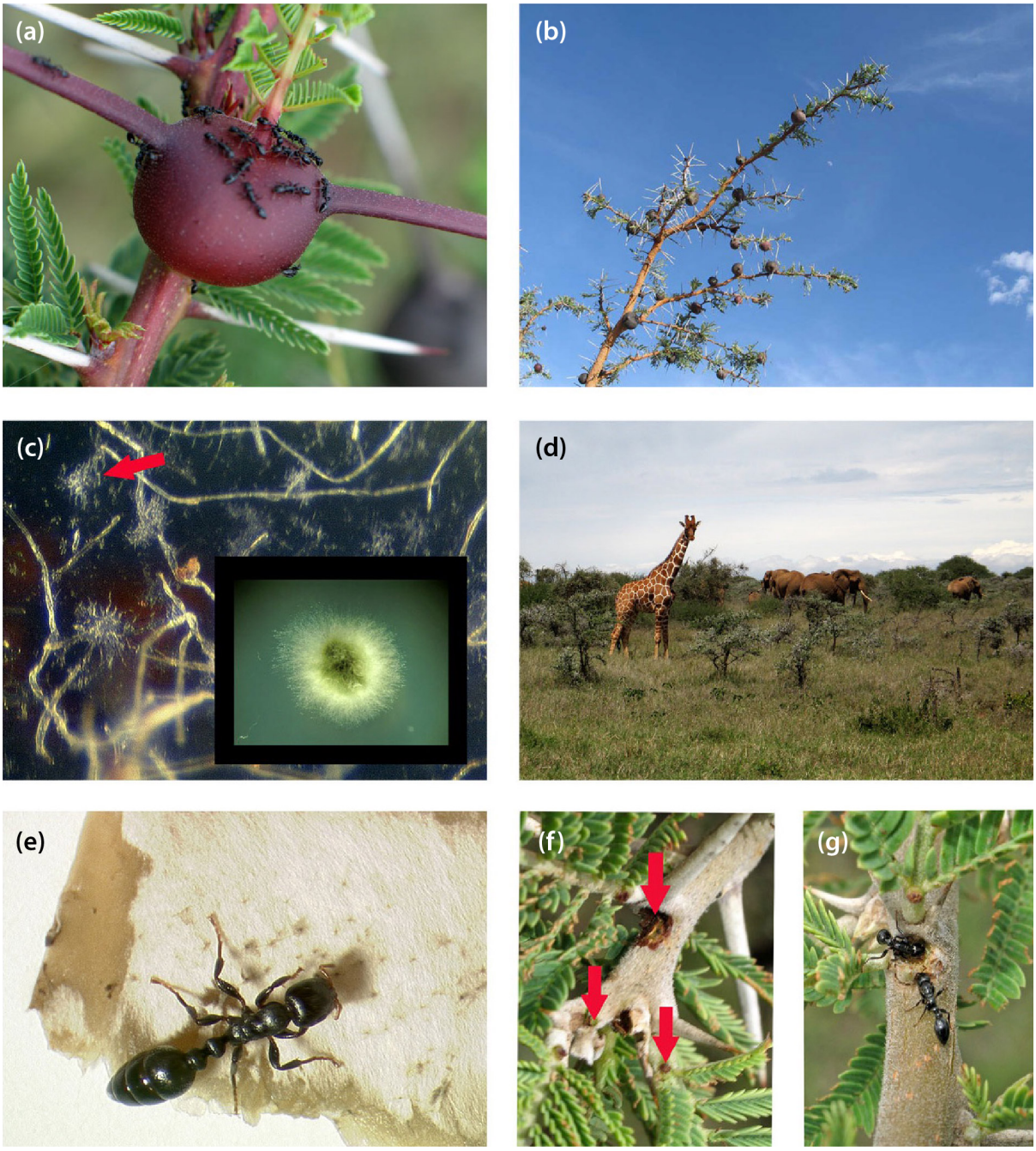

Figure 2.1: (a) *T. penzigi* workers on domatium. (b) *V. drepanolobium* sapling. (c) Details of *Chaetomium* fungus growing on glass tube maintained by *T. penzigi* colony in lab. (d) Resident any colonies defend host plants against damage by large mammalian herbivores. (e) *T. penzigi* grazing on *Chaetomium* isolate. (f) and (g) *T. penzigi* destroys extrafloral nectaries and axillary buds on its host plants. Images: Dino Martins.

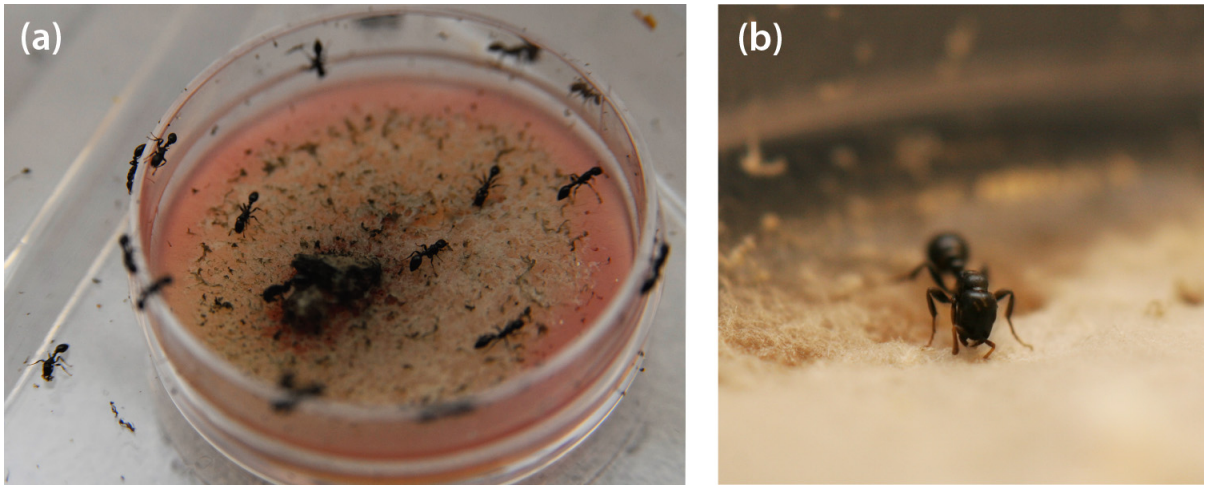

Figure 2.2: (a) *T. penzigi* workers have almost completely removed this *Phoma* isolate. (b) A *T. penzigi* worker chewing on a *Phoma* fungal isolate.

and grown on PDA media, plus a control consisting of only PDA. Although *T. penzigi* colonies did not recruit more workers to the fungal culture, they removed significantly more mycelium than *C. mimosae* or *C. nigriceps* colonies (Figures 2.2 and 2.3). Ant species remained a significant predictor of fungus removal in a generalized least squares model with recruitment to the fungus plate and colony size included as predictors ($LR = 10.4$, $p < 0.01$); the presence of a queen or of brood were not significant. This result did not appear specific to the *Phoma* isolate used in the experiment – similar results were obtained in preliminary trials with a range of fungal isolates.

Domatium fungal communities

Since the three obligate domatium-dwelling ants differed in their behavior toward the fungal isolates, we used multiplexed amplicon sequencing to investigate how communities of fungi inside ant-occupied domatia varied among the ant species. For this sequencing, we sampled the contents of 56 domatia from different *V. drepanolobium* trees in Kenya, split among the three ants and between two distant sampling locations. Each domatium's contents was typical for the ant occupant: old *Vachellia* leaflets for *C. nigriceps*, carton lamellae for *C. mimosae*, and loose fibrous particles for *T. penzigi*[88]

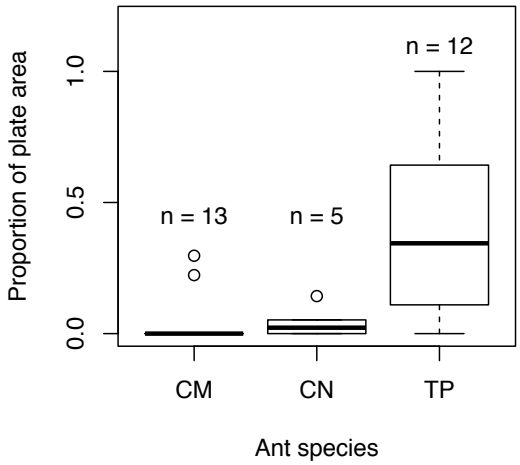

FIGURE 2.3: *T. penzigi* ants (TP) removed significantly more fungus than either *C. mimosae* ants (CM) or *C. nigriceps* ants (CN).

(Figure 2.4). For comparison, we also sampled the contents of 13 ant-occupied domatia from *V. drepanolobium* trees in our greenhouse in Cambridge MA, plus leaves from 6 trees at Mpala.

We obtained 407769 sequences across more than 660 operational taxonomic units (OTUs) from the 75 domatium and leaf samples. Many OTUs showed low abundance, with just 84 OTUs represented by $\geqslant 100$ sequences each. Sequencing depth varied between 1119 and 21345 sequences per sample, with a median of 3980 sequences.

OTU richness varied substantially among the samples, but not with ant species. Rarefaction curves indicated that the leaf samples contained more fungal OTUs than the Kenyan domatium samples, which in turn contained more OTUs than the greenhouse domatium samples (Figure 2.5). At a rarefaction depth of 1000 sequences, these differences were highly significant (leaves vs Kenyan domatia: $t_{5.3} = 6.0, p < 0.01$; Kenyan vs greenhouse domatia: $t_{66.8} = 12.8, p < 0.01$). However, the Kenyan domatia contained similar numbers of OTUs irrespective of ant or location (ant: $F_{2,53} = 2.2$, $p = 0.13$; location: $t_{48.4} = 0.33, p = 0.74$).

Fungal community composition varied significantly among sample types when evaluated us-

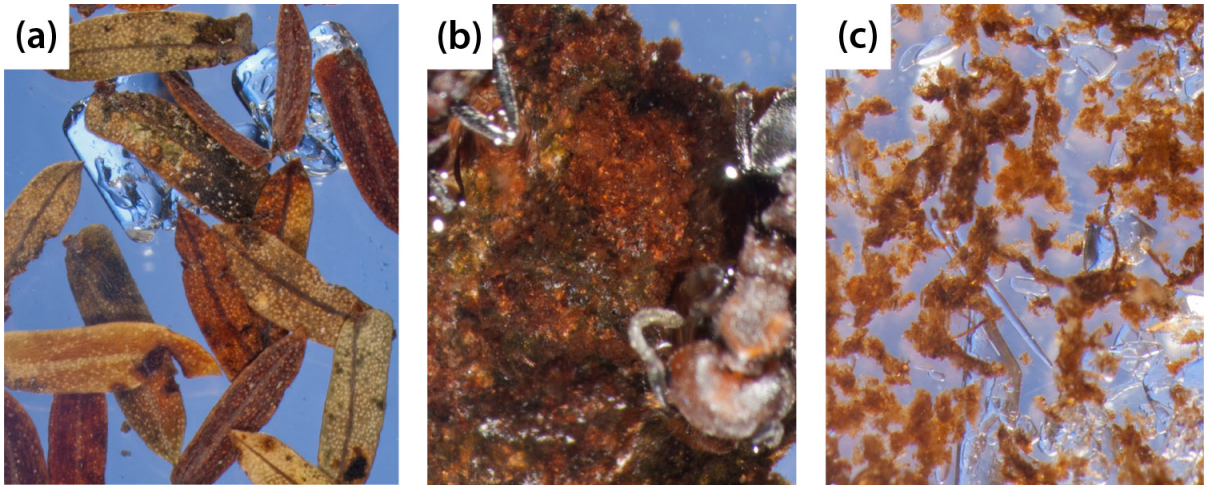

FIGURE 2.4: Typical domatium contents. (a) old *Vachellia* leaflets for *C. nigriceps*, (b) carton lamellae for *C. mimosae*, and (c) loose fibrous particles for *T. penzigi*.

ing Sørensen distances between samples rarefied to 1000 sequences. The greenhouse domatia and the leaf samples were consistently distinct from the Kenyan domatium samples (Figure 2.6; adonis pseudo-$F_{2,72} = 9.4, p < 0.01$), reflecting in part the differences in alpha diversity among these sample types. But the Kenyan domatium communities also differed among the ants (adonis pseudo-$F_{2,53} = 5.2, p < 0.01$), even though there was no significant difference in alpha diversity. Community composition also varied with sampling location among the Kenyan domatia (adonis pseudo-$F_{1,54} = 2.2, p < 0.01$), but location had less explanatory power than ant species (adonis $R^2 = 0.04$ for location vs $R^2 = 0.17$ for ant).

The community differences among the ants were apparent even after aggregating the fungi by class (Figure 2.7). Although most abundant OTUs were present with more than one ant, some OTUs showed stronger associations with some ants than with others (Figure 2.8; $H2' = 0.5, p < 0.01$).

Fungi carried by alates

To see whether the ants carry fungi in their infrabuccal pockets, we extracted and where possible sequenced DNA from the heads of 21 surface-sterilized alates of *C. nigriceps* that we had collected

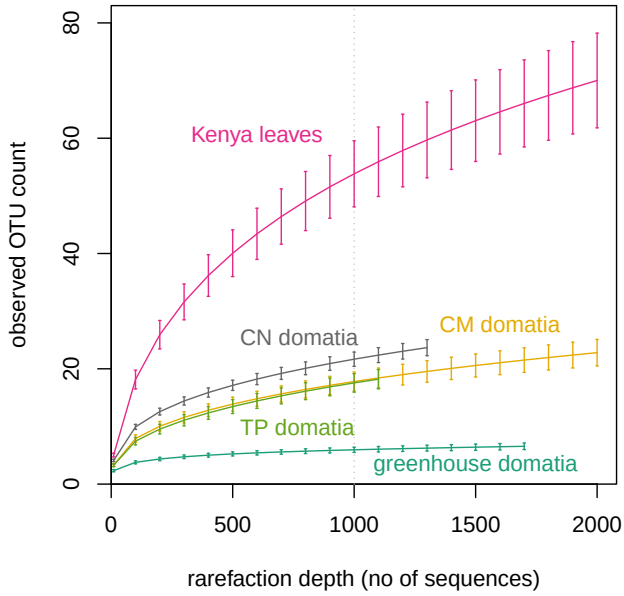

Figure 2.5: Kenyan domatium communities differ in taxonomic diversity compared to leaf sample communities and greenhouse domatium communities. Error bars show standard errors.

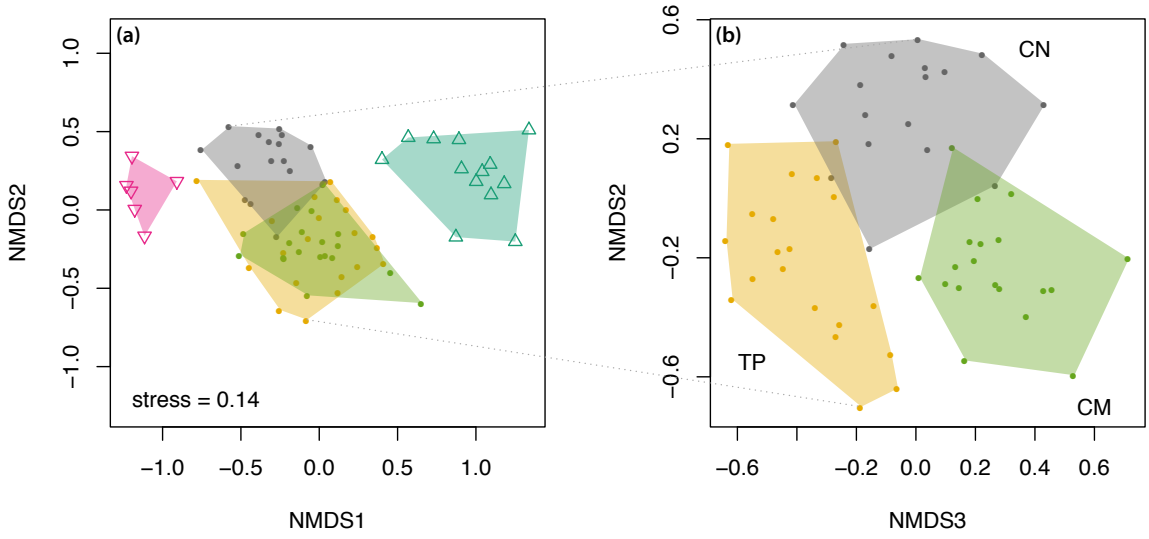

Figure 2.6: Non-metric multidimensional scaling based on Sørensen distances for rarefied dataset. Greenhouse domatia and leaf samples were distinct from Kenyan domatium samples. Kenyan domatium communities also differed among ant species.

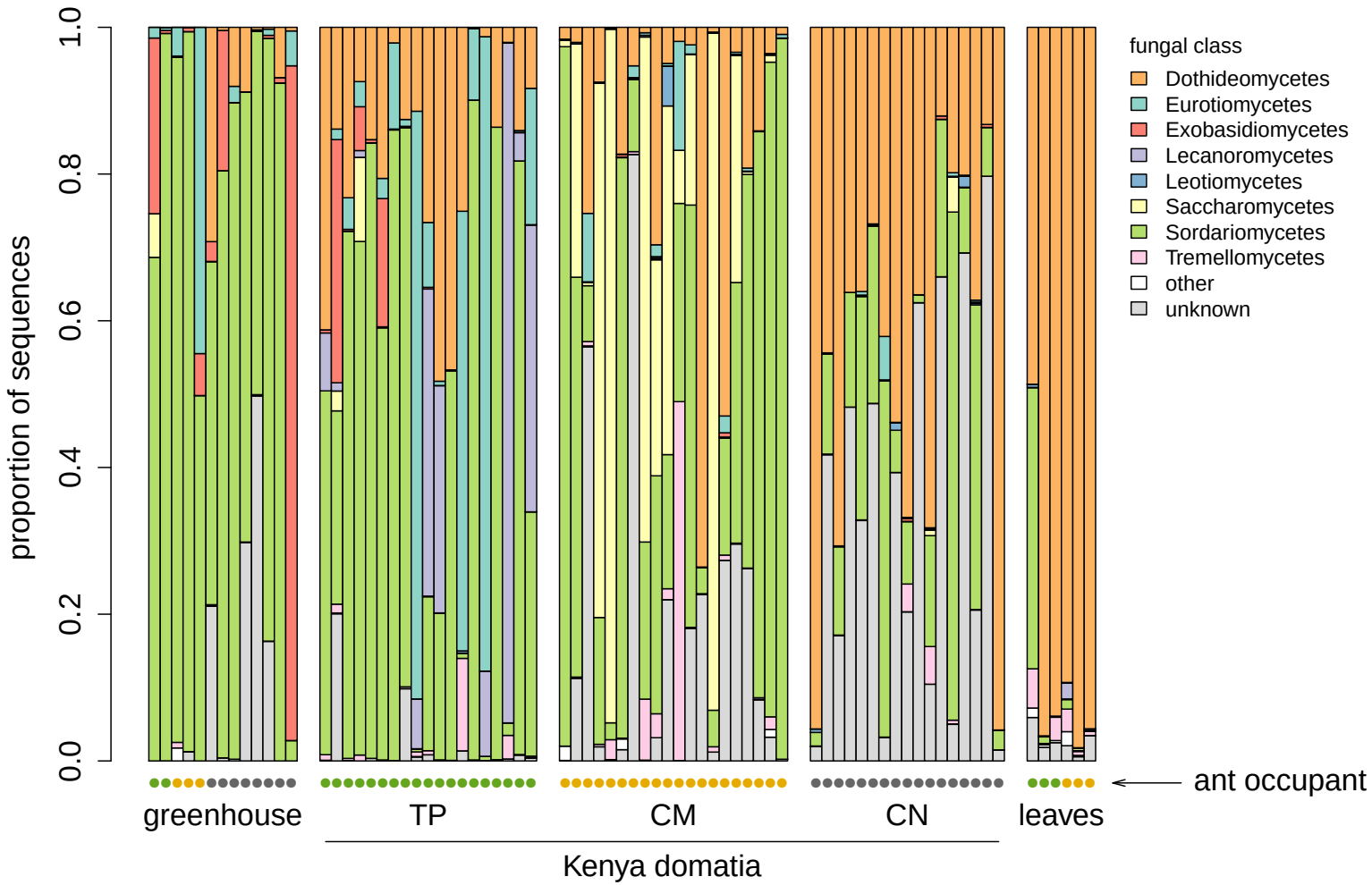

FIGURE 2.7: Relative abundances of fungi aggregated to class differ between sample types and between ant occupants. Each column represents one sample.

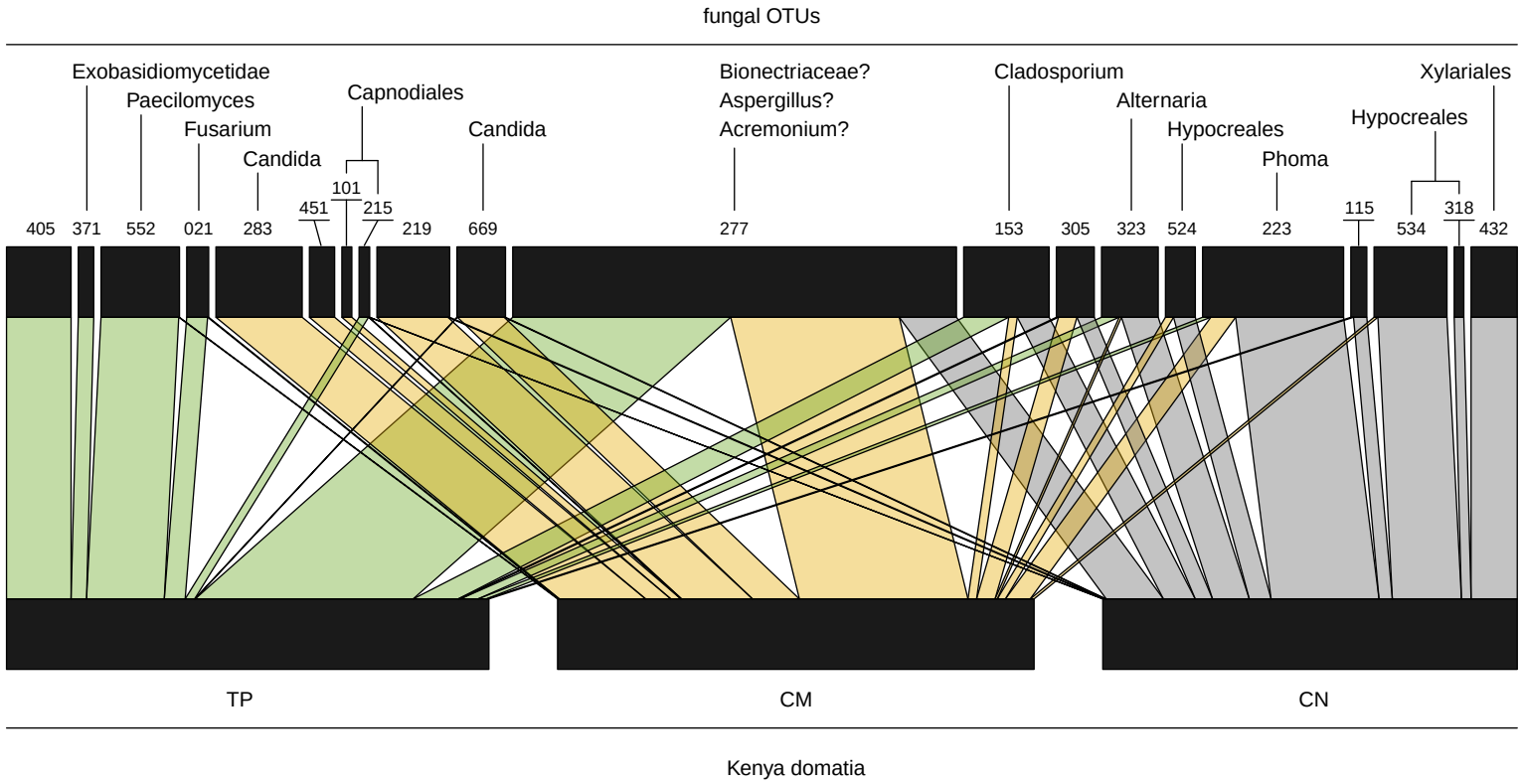

Figure 2.8: Bipartite graph of ant species against most abundant fungal OTUs in rarefied dataset. Connections reflect sequence counts.

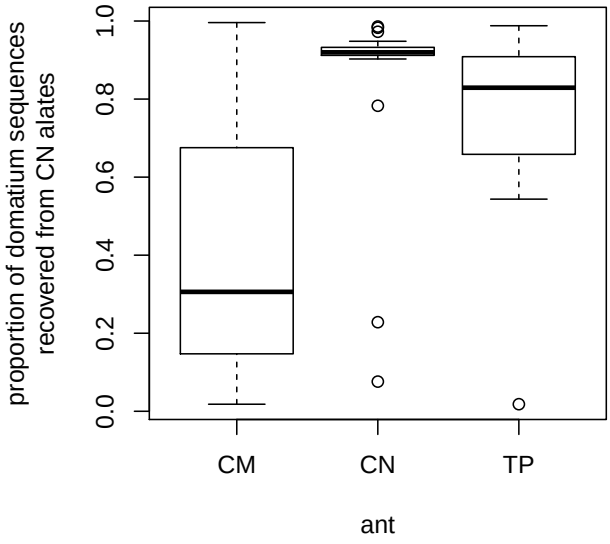

Figure 2.9: Proportion of each Kenyan domatium's sequences that were also recovered from the pooled alate samples.

previously in Kenya, with the assumption that fungal hyphae in the infrabuccal pocket would be captured in an extraction from the whole head. Of these alates, 10 contained sufficient sample to provide $\geqslant$ 1000 fungal sequence reads. Individual alates showed low alpha diversity, and did not recapitulate the typical *C. nigriceps* domatium community. However, in aggregate, the fungi from the 10 alates contained matches for, on average, 83% of the sequences we obtained from *C. nigriceps*-occupied domatia, compared to 76% of sequences from those occupied by *T. penzigi*, and 44% of those occupied by *C. mimosae* (Figure 2.9).

Sagittal sections through the heads of female *T. penzigi* and *C. nigriceps* alates showed a pellet of mixed debris in the infrabuccal pocket, supporting our assumption that fungi present in the alate heads are being carried in the infrabuccal pocket (Figure 2.10).

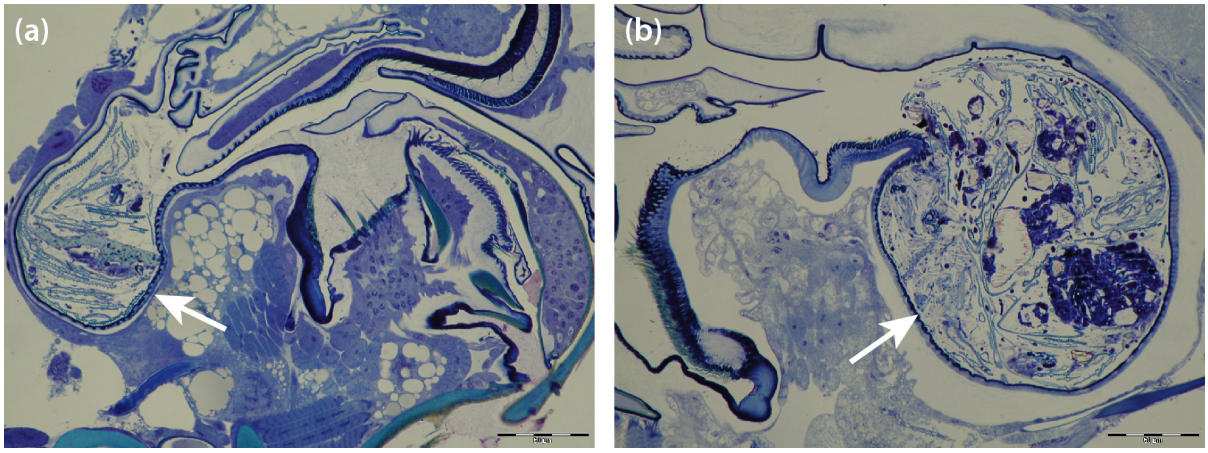

Figure 2.10: Sagittal view of (a) *T. penzigi* and (b) *C. nigriceps* alates showing debris in the infrabuccal pockets (arrowed). Scale bars show 50µm.

2.4 Discussion

Our sequencing results show that domatium fungal community composition differed among *V. drepanolobium* trees occupied by different ant species. To our knowledge, this is the first record of such ant-specific fungal community-level differences on the same myrmecophytic host species. *V. drepanolobium* domatia that have never been occupied by ants have no entry holes (as these are created initially by foundress queens), and we have never successfully amplified fungal ITS from unopened domatia, indicating that the presence of the fungal community accompanies ant occupation. The ants' communities showed similar differentiation at our two Kenyan sampling sites, suggesting that the communities assemble in a consistent way.

The differentiation in fungal communities among the ants appears broad-based, highlighting the utility of culture-independent community sequencing for examining microbial community structure. In contrast to recent work in other ant-plant-fungal systems [126], Chaetothyriales fungi are not prominent in our sequence libraries, nor indeed is the *Chaetomium* that initially caught our attention [123].

The differences in fungal community composition and richness between leaves and domatia indi-

cate that domatium communities are shaped by processes specific to the domatia, and can be viewed as 'extended phenotypes' of the ants that inhabit them. First, fungal dispersal may influence communities – including both passive dispersal (e.g. by air or water movement, or on ants' legs and bodies) and active vectoring by ants (discussed further below). Ant-specific differences in the domatia, such as in the size and number of domatium holes, or in typical foraging locations, may tend to favor different fungi. Second, the different substrates contributed by the ants – for *T. penzigi*, *C. nigriceps* and *C. mimosae* these are loose fibrous particles, old dried leaflets and carton, respectively – may have been exposed to different fungi prior to being handled by ants, and are likely to select differently for fungal growth. Third, the ants might play an active role in manipulating community composition. For example, weeding and grooming, or applying metapleural gland secretions, may help remove entomopathogens[36,57,147] or phytopathogens[116]. The fungal community could also be shaped by the untargeted trimming of fungal growth to keep the ants' living space free from obstructions and entanglements[12], or by ant-mediated changes to domatium characteristics such as chemistry or humidity.

Our sequencing results suggest that fungi may also be dispersed in ants' infrabuccal pockets. These structures are typically filled with debris that workers ingest while foraging, feeding, or cleaning[12,171]. This debris may be periodically expelled onto the ants' waste piles, potentially aiding microbial dispersal[12,55]. Infrabuccal pockets may also allow microbes to be carried by dispersing alates – leafcutter ant foundresses, for example, use the infrabuccal pocket to carry a fungal inoculum when starting a new colony[36,171]. A substantial fraction of the fungal sequences from *C. nigriceps* domatia were recovered from *C. nigriceps* alates, suggesting that the ants could be vectoring fungi in their infrabuccal pockets (Figure 2.9) when dispersing between host trees.

Fungi may comprise part of the diet of the domatium-dwelling ants on *V. drepanolobium*. Our recruitment experiment showed *T. penzigi* readily removing mycelium, perhaps to feed larvae as has been described in other pseudomyrmecines[229]. But while the accumululation of plant material in do-

matia resembles the collection of fungal garden substrate prepared by lower attine ants, no obvious fungal cultivars are growing on the material in *V. drepanolobium* domatia. The response of *T. penzigi* to a range of fungi in our recruitment experiment and other observations, and the broad-based shift in the fungal community revealed by our sequencing, also suggests that fungivory in *T. penzigi* is less specialized than that observed in attine ants or in Chaetothyriales-based ant-plants [141,216]. Nonetheless, even small quantities of fungi may help relieve nitrogen or other nutrient constraints for the phytoecious ants associated with *V. drepanolobium* [40] by digesting ant waste or other domatium contents and rendering nutrients available to workers [44]. This is especially likely to be important for *T. penzigi*, which is rarely observed foraging for insect prey, does not associate with scale insects, and destroys its host plant's extrafloral nectaries [88,152,155,237]. Further, while some ants overcome nitrogen constraints through gut bacterial associations [184], *T. penzigi* does not possess the bacterial gut diverticulum previously described in other *Tetraponera* ([17] and Appendix A), and fluorescence microscopy and sequencing indicate relatively few bacteria (unpublished; also Sanders 2015 and [184]).

Domatium fungi might also play a role in host plant nutrition. Nutrients released by digesting plant material or ant waste may become available to the host via the domatium wall – a flux known in other systems [59] and possibly even facilitated by fungi [44]. Indeed, we might expect partner fidelity feedback to select for such fungi-mediated contributions by ants to their host plants.

In summary, different species of ants inhabiting *V. drepanolobium* cultivate distinctive and specialized fungal communities. These fungi may contribute to the nutrition of the ants and their plant hosts, as well as perform other useful roles such as producing antimicrobial compounds or reinforcing the *C. mimosae* carton wall. Future work investigating mechanisms of fungal transmission, and the nutritional and/or other roles played by these fungi, will help incorporate fungi-mediated ant-plant interactions into our understanding of the *V. drepanolobium* system. Other microbial communities – such as epiphytic, endophytic, mycorrhizal and extrafloral-nectary fungi and bacteria – may likewise

have important roles in the *V. drepanolobium* system and represent promising areas for research.

2.5 Experimental procedures

Recruitment experiment

Thirty ant colonies from our Kitengela field site in Kenya were presented with a plate containing a *Phoma* sp. fungal culture on PDA media, plus a control plate containing only PDA. We selected the fungus arbitrarily from several isolates from ant colonies on *V. drepanolobium*. We recorded the number of ants visiting the plates over 8 hours, and then determined the proportion of the dish contents that had been removed.

Fungal community sequencing

We sampled the contents of 56 ant-occupied domatia from different *V. drepanolobium* trees in Kenya. The samples were split roughly equally among *C. mimosae*, *C. nigriceps* and *T. penzigi* ant occupants, and were also split between our Kitengela field site and our Mpala Research Centre field site (approximately 190km apart). For comparison with the 56 Kenyan domatium samples, we also sampled the contents of 13 ant-occupied domatia from different *V. drepanolobium* trees grown from seed in our greenhouse in Cambridge MA, plus leaves from 6 trees at Mpala.

To see whether the ants carry fungi in their infrabuccal pockets, we extracted and where possible sequenced DNA from the heads of 21 surface-sterilized alates of *C. nigriceps* collected at Kitengela, assuming that fungal hyphae in the infrabuccal pocket would be captured in an extraction from the whole head.

DNA extracts from all samples were sent to Research and Testing Laboratory (Lubbock TX) for PCR and multiplexed 454 pyrosequencing, using ITS1F and ITS4 primers to target fungi.

We demultiplexed and denoised the data using Ampliconnoise in QIIME. We isolated the ITS1

region and picked operational taxonomic units (OTUs) using uclust with a similarity threshold of 95%. We assigned putative taxonomic descriptions to OTUs using blast. Statistical analyses were performed in R.

Alate microscopy

To examine the contents of *T. penzigi* and *C. nigriceps* infrabuccal pockets, we collected female alates from our Kitengela field site in Kenya as they departed from domatia for their mating flights. The alates' heads were fixed prior to sectioning and staining with methylene blue and thionin.

Chapter 3

Myrmecophile communities vary between three ant mutualists on the African ant plant, *Vachellia (Acacia) drepanolobium*

3.1 Abstract

Ant colonies represent unique habitats for diverse communities of specialized inhabitants or 'myrmecophiles'. But since few studies provide quantitative data on these communities, our knowledge of myrmecophile abundances, interactions and ecological importance remains limited. This study uses DNA barcoding to identify over 80 myrmecophile taxa from domatia on almost 500 trees of the widespread African ant-plant *Vachellia drepanolobium*. Three ant species nest obligately in the domatia of this ant-plant. Myrmecophile incidence and community composition varied significantly among the three species. Myrmecophiles were more common with the ant species normally regarded as better mutualists on account of being better defenders against large mammalian herbivores, underscoring a need to better incorporate myrmecophile communities into our understanding of this ant-plant system. Myrmecophile community composition also varied substantially between collection locations and years, suggesting that the *V. drepanolobium* ecosystem might show more spatial and temporal variation than has previously been appreciated.

3.2 Introduction

Ants are some of the most ecologically and evolutionarily successful organisms, accounting for a quarter of insect biomass in some areas [107]. Their success supports greater abundance and diversity

throughout the ecosystem, on account of their effectiveness as predators, scavengers, herbivores, mutualists and ecosystem engineers[24,185].

The success of ants is partly attributable to their ability to establish and defend a stable nest environment. This 'homeostatic fortress'[92] often represents a highly modified environment relative to its surroundings. It is environmentally controlled (e.g. humidity, temperature)[102]. It is heavily guarded against intrusion by either conspecifics or predators, with entry controlled by highly evolved communication via pheromones and tactile signaling. And this stable protected environment is long-lived, maintained by successive overlapping generations of ant workers.

But the fortress is not impregnable: many specialized 'myrmecophiles' spend part or all of their lives in intimate association with ants[90,106]. These myrmecophiles may be mutualists – for example Anthene emolus caterpillars exchanging food secretions for protection by their *Oecophylla smaragdina* hosts[58]. They may be commensals, providing little benefit to ants that might afford them some protection from predators or parasitoids, but imposing little cost beyond the space they occupy – such as is likely for *Deloneura ochrascens* caterpillars and their *Crematogaster castenea* hosts[97,162]. Or they may be detrimental to their hosts – such as *Phengaris* caterpillars eating on the brood of their *Myrmica* hosts, or being fed by host workers[6].

An ant colony is a highly unique habitat for these myrmecophiles. Myrmecophiles need to be able to avoid the keen defenses of the ants: through chemical[135,217], acoustic[13] or tactile[160] mimicry, with physical defenses[228], or by inducing the ants to drop their defenses through mutualism[195]. But beyond the colony's defenses, the unique environment of the nest is likely to further select for life history traits adapted to that environment – for example, ant parasites with low costs and ant mutualists with low benefits. In addition to selecting for traits in individual myrmecophiles, the ant nest might also be expected to shape myrmecophile interactions in a way that produces a distinct signature at a community level[92].

But despite strong theoretical predictions and a wealth of natural history information on individual myrmecophile species[90,106,226,227,228] or interaction networks[159,161,175,235], we have limited quantitative data on myrmecophile communities. Such data are necessary in order to determine absolute and relative abundances, to calculate covariation between species that may reflect interactions or environmental preferences, to identify patterns involving groups of species that may not manifest for any individual species, and to parse out biotic and abiotic correlates of variation in the myrmecophile community.

This study addresses this gap by examining communities of myrmecophiles associated with three species of ant that nest obligately in *Vachellia* (formerly *Acacia*) *drepanolobium* trees.

Study system

V. drepanolobium ant-plants are the dominant trees in black cotton savannahs across East Africa. At least 15 species of ant have been recorded previously on these trees, including 4 close associates: *Crematogaster mimosae*, *C. nigriceps* and *Tetraponera penzigi*, all of which are obligately phytoecious; and *C. sjostedti*, which is associated with *V. drepanolobium* in some locations but also occupies domatia on *V. seyal*[88,108,237] The ant associates nest arboreally and engage in a classic protection mutualism[98] with their plant hosts. *C. mimosae*, *C. nigriceps*, and *T. penzigi* nest within the hollow swollen stipular thorns known as 'domatia' that cover the tree. *C. sjostedti* more commonly nests in trunk cavities or in the ground around the bases of the trees. Each tree is typically occupied by a single ant species[237] but different trees, even in close proximity, may be occupied by different species. In addition to housing, host plants also supply their ants with food in the form of extrafloral nectar from glands near the base of the leaves.

But despite broad similarities in the obligate ants' lifestyles, closer scrutiny reveals numerous differences in their interactions with their host plants. For example, the ants differ significantly in the extent to which they patrol the plant and deter browsing by large herbivores[88,124,154]. *C. nigriceps*

prunes the plant's axillary buds, shaping growth and temporarily preventing flowering, while *T. penzigi* prunes the extrafloral nectaries, perhaps to reduce the risk of invasion by another ant colony[151,198]. Long-term demographic data and modeling indicate synergistic (i.e. non-additive) effects when a plant hosts different ant species over its lifetime[155], presumably arising as a function of these and other differences among the ants.

Existing work has also shown differing effects of the obligate ants on the diversity of insects living in the tree canopy. *Acacidiplosis* gall midge parasites, for example, are found more frequently with *C. mimosae* ants than with *C. nigriceps* ants[190]. A recent study using fogging and beating methods, and a morphospecies approach, found that canopy insect communities on trees occupied by *C. mimosae* or *C. nigriceps* were distinct from those on trees occupied by *C. sjostedti* or *T. penzigi*[109] (see Appendix B for a comparison of these datasets). In contrast, different vertebrate grazing and browsing regimes appear to have little effect on canopy insect communities[109,157], even though terrestrial arthropods in the same ecosystem may be indirectly affected by ungulate exclusion[167].

Several studies have documented specialized domatium inhabitants on *V. drepanolobium*. Hocking, for example, recorded at least 44 species in actively ant-occupied domatia, and 24 in domatia without ants[88]. Numerous lepidopteran larvae, including tortricid moths, occur in domatia, often protected by a silk tube or cap[2,3,4]. Larvae of the dipteran *Melanagromyza acaciae* develop in domatia after oviposition in growing thorns[194].

Many of these records of domatium inhabitants suggest preferential or even exclusive association with particular ant species. The lycaenid *Anthene usamba*, for example, specializes on trees occupied by *C. mimosae*[125]. *Hockiana insolitus* scales associate with *C. mimosae*[176], and *Ceroplastes* scales with *C. mimosae* and *C. sjostedti*[237], while neither *C. nigriceps* nor *T. penzigi* is typically found with scales. The braconid wasp *Trigastrotheca laikipiensis* is a brood parasite of claustral colonies of *C. mimosae* and *C. nigriceps*, but is rarely found with *T. penzigi*[169].

Table 3.1: Samples were collected from two sites in Kenya, around 210km apart

Site name	Location	Latitude	Longitude	Elevation
Kitengela	undeveloped land outside Nairobi National Park, Kajiado North County, Kenya	1°23.8′S	36°49.5′E	1650m asl
Suyian	Suyian Ranch, Laikipia County, Kenya	0°31.3′N	36°43.3′E	1880m asl

This study builds on and draws together our understanding of these various individual species by comparing entire myrmecophile communities across hundreds of *V. drepanolobium* trees collected at two locations in two field seasons. Each community is clearly delineated by tree and separated from less closely associated taxa by its location inside domatia. Our study had four goals:

(i) to catalogue and quantify myrmecophile diversity in *V. drepanolobium* domatia;

(ii) to explore the structure of the myrmecophile communities, focusing especially on variation with ant species, but also with date and location of collection;

(iii) to identify myrmecophile taxa associated with each ant species; and

(iv) to establish a myrmecophile DNA barcode library, to assist with identification, delineation of taxa, and matching insect life stages in this and future studies.

3.3 Materials and methods

Sampling locations

Myrmecophiles were collected from 480 ant colonies at two field sites in Kenya (Table 3.1 and Figure 3.1). The two sites are approximately 210km apart. Collections took place during two field seasons: in March 2012, we sampled 204 trees at Kitengela; in August 2013, we sampled 123 trees at Kitengela and a further 153 at Suyian.

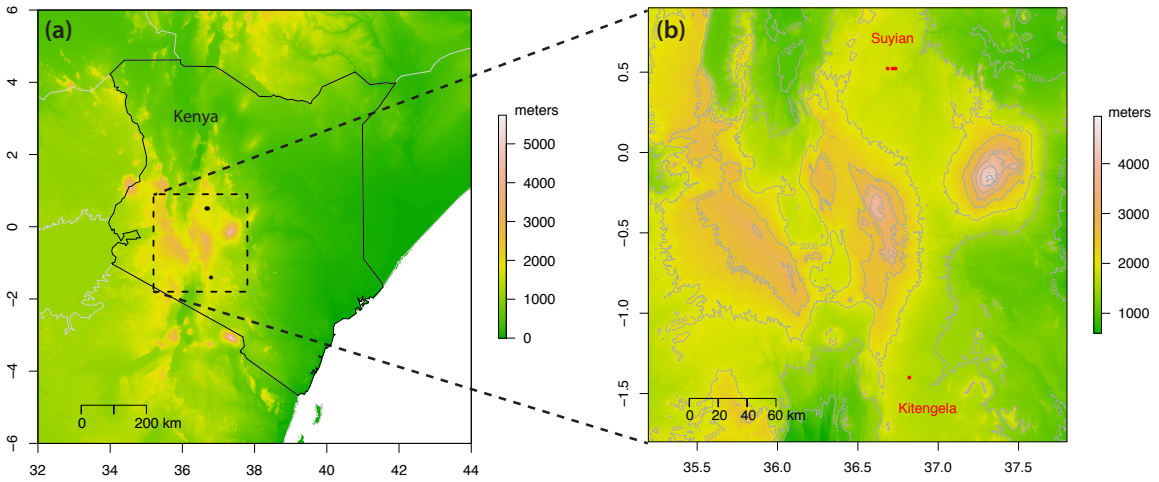

FIGURE 3.1: Samples were collected from two sites in Kenya, around 210km apart

Sampling methods

Most trees that we surveyed were 'fully sampled' – i.e. we collected all myrmecophiles from every domatium on the tree. We selected trees for full sampling with two purposes in mind: to collect myrmecophiles and to obtain queenright colonies for unrelated lab experiments. We therefore favored trees that were of moderate size (around 0.5-1.0m in height) that appeared to be occupied by a single ant species, as these were likely to represent a single colony with workers. We also favored trees that were either not close to any other trees, or close to a tree occupied by a different ant species, to minimize the chances that the queen was located on a tree other than the one we sampled. For each tree, we collected all domatia into one large ziplock bag, using secateurs to cut the branch above and below the domatia, and to trim thorns so that they did not pierce the bag. This procedure allowed us to process each tree quickly, minimizing the opportunities for myrmecophiles to evacuate the domatia. The ziplock bag was placed into an insulated cooler box in order to help keep ants and myrmecophiles alive while we sampled from other trees.

Forty-two of the trees that we surveyed were 'subsampled' – i.e. we collected myrmecophiles from

a subset of the domatia. These trees were all located at Kitengela and were surveyed in 2013. They made up around 34% of the Kitengela trees from 2013, and around 9% of the total trees surveyed across 2012-13. The trees selected for subsampling were chosen haphazardly from among those on a transect for an unrelated study. These trees were generally much larger than the fully sampled trees (157cm vs 57cm) and it was infeasible to collect and survey all domatia. We therefore haphazardly selected 40 domatia per tree and placed them in a large ziplock bag, for later processing as per the fully sampled trees.

For each tree, we counted the number of domatia that we processed (i.e. either the total number on the fully sampled trees, or the number that we collected on the subsampled trees). We also recorded tree height, GPS location and the species of ant occupant.

Sample processing

Upon return to the field station, we carefully cut open all domatia with secateurs or a sharp knife. Ant workers and brood were placed into pre-prepared colony boxes for transport back to the lab. Myrmecophiles – i.e. any other inhabitants of the domatia – were transferred into ethanol and stored at -20°C in preparation for molecular work. Some of these myrmecophiles were clearly resident in the domatia, e.g. pupae or larvae in silk tubes attached to the domatium wall. Others may have been only temporarily present, e.g. other ant species foraging or sheltering in old abandoned domatia. We made no attempt to exclude temporary residents from our sampling.

Upon return to the lab, each myrmecophile was assigned a unique identifier and photographed using a Nikon D200 digital camera mounted on an Olympus SZX12 stereomicroscope.

Additional samples

In addition to our sampling in 2012-13, we collected myrmecophiles at Suyian in June 2014 for a related project using stable isotope analysis to examine myrmecophile trophic interactions (see Chap-

ter 4). We also obtained the following specimens of Lepidoptera from David Agassiz's personal collection: *Phthoropoea chalcomochla* Agassiz (Tineidae); *Dichomeris* sp. (Gelechiidae); and *Endotera cyphospila* (Meyrick) =*nodi* Agassiz, *Hystrichophora bopprei* Agassiz, *Hystrichophora griseana* Agassiz, *Endotera cyaneana* Agassiz, and *Hystrichophora vittana* Agassiz (all Tortricidae). Locations and collecting methods for these species are detailed by Agassiz[3].

These two sets of additional samples are generally excluded from the statistical analysis in this paper. However, we have included their sequence data in defining myrmecophile taxa for this study, and also in our analysis of within-taxon genetic diversity.

Molecular methods

Total genomic DNA was extracted from each sample using one of three extraction protocols.

We extracted DNA from the March 2012 myrmecophile samples with a Chelex bead rapid extraction method. A 5% Chelex slurry was prepared by mixing 2g of 200-400 mesh Chelex resin beads with 20mL TE buffer and 20mL H_2O. To extract DNA from each sample, a small piece of tissue was placed in a 1.5mL microcentrifuge tube with 50μL 5% Chelex and 1μL Proteinase K (20 mg/mL), and ground with a micropestle. The tube was incubated for 1 hour at 57°C, followed by 5 min at 95°C. Samples were then centrifuged for 15 min at 13000 rpm. The supernatant was then used directly as the DNA template in PCRs.

For the August 2013 and June 2014 samples we used an automated DNA extraction with an AutoGenPrep 965 (essentially a phenol-chloroform extraction with ethanol precipitation). Samples were prepared for DNA extraction by macerating a small amount of tissue in extraction buffer, consisting of 200μL reagent M1, 200μL reagent M2 and 0.4mg Proteinase K. Samples were incubated at 55-60°C overnight. Samples were then processed in the AutoGenPrep 965 using the standard Mouse Tail protocol, with 2 DNA washes and resuspending in 50µL of buffer.

For the specimens supplied by David Agassiz, we extracted DNA with the Qiagen Blood and Tis-

sue kit, following the standard manufacturer's protocol.

Following DNA extractions, we amplified cytochrome oxidase I (COI) from each sample using standard barcode primers[61]:

```
LCO1490     5' - GGTCAACAAATCATAAAGATATTGG - 3'
HCO2198     5' - TAAACTTCAGGGTGACCAAAAAATCA - 3'
```

We performed PCRs using 12.5μL Omega BioTek MasterMix, 0.5mM $MgCl_2$ (additional to MasterMix), 0.5μM each primer, 1μL template and water to 25μL. PCR conditions followed a touchdown profile: 3 min at 94°C, followed by 20 cycles of 50 sec at 94°C / 40 sec at 49°C decreasing by 0.5°C per cycle / 80 sec at 72°C, followed by a further 20 cycles of 50 sec at 94°C / 40 sec at 48°C / 80 sec at 72°C, and a final 5 min at 72°C. We used BigDye Terminator v3.1 reactions and sequenced products on an Applied Biosystems Genetic Analyzer 3130xl.

Data analysis

After processing raw DNA sequence data using Sequencher v.5.1, sequences were clustered into putative species or 'operational taxonomic units' (OTUs) at 97% similarity, with a small number of manual adjustments especially for poor quality sequences (see Box 3.1). We cross-checked our OTU assignments against our photographic records, particularly to identify potential sequence contamination. Where possible, we assigned taxonomic identifications to our sequence-based OTUs based on our photographic records and on BLAST searches against the NCBI and BOLD databases.

Statistical analyses were conducted using R[206]. We used χ^2 tests and logistic modeling to explore relationships between overall myrmecophile incidence and collection year, location, sampling regime, ant species, tree height and number of domatia (see Box 3.2). We then briefly examined overall myrmecophile diversity, and per-tree myrmecophile abundance and taxonomic richness. We visualized the community matrix for the most abundant myrmecophile taxa using correspondence analy-

Box 3.1: Delimiting myrmecophile taxa

In this study, individuals were classifed into taxa using DNA barcodes instead of morphological characters[80]. We sequenced a short segment of the mitochondrial cytochrome C oxidase I gene for each individual, and deemed individuals with sufficiently similar sequences to belong to the same taxon. To delimit taxa – i.e. to determine which sequences are 'sufficiently similar' to be called the same taxon – we used the uclust[53] algorithm as implemented in QIIME[32] to find clusters of sequences meeting a 97% identity threshold (Figure 3.2). Our results were not generally sensitive to the choice of identity threshold. Most clusters thus defined likely correspond to true biological species, and where possible we identified the species by searching against publicly available sequence databases. Many species, however, are not represented in these databases and, in some cases, clusters may not correspond exactly with biological species. We therefore refer to each cluster as an 'operational taxonomic unit' (OTU) rather than a species, and assign each OTU a number for identification purposes (e.g. OTU 1, OTU 2 etc).

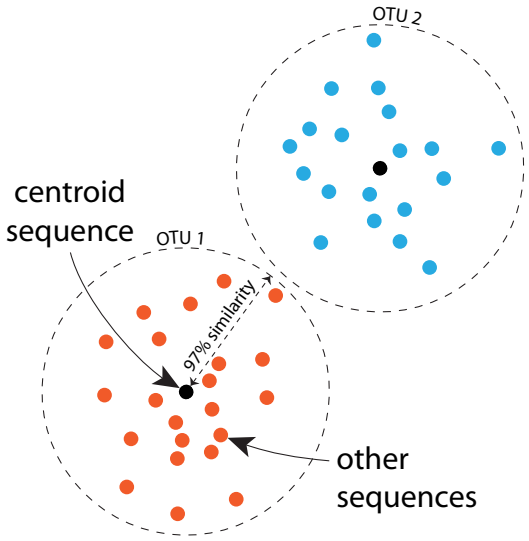

Figure 3.2: The uclust algorithm[53] defines clusters such that each sequence in a cluster exceeds a specified identity threshold, in our case 97%, relative to the centroid of the cluster.

Box 3.2: Quantifying myrmecophiles

After clustering sequences into OTUs (see Box 3.1), myrmecophiles were quantified in a variety of ways. An OTU's incidence refers to the probability that an OTUs is present on a randomly selected tree or, equivalently, the proportion of trees with at least one representative of the OTU. In Figure 3.3, for example, both the spiders and the orange ladybugs have an incidence of 80%, while the striped green beetles have an incidence of 20%. The number of representatives of the OTU on each tree is disregarded. In contrast, an OTU's abundance refers to the number of individuals from that OTU. In Figure 3.3, the abundance of the spiders is 2 individuals per tree, compared to 4 orange ladybugs, and 0.6 striped green beetles per tree

Figure 3.3: Illustrative myrmecophile communities

sis and hierarchical clustering of the taxa based on Spearman correlations, focusing especially on the variation associated with sampling location and year.

To examine the effect of ant species on myrmecophile community composition, we first used canonical correspondence analysis to examined the community variation associated with ant species, conditioning on location, year and sampling regime, both among the most abundant taxa and among the full dataset. We used Dufrêne and Legendre's indicator value (IndVal) analysis to identify myrmecophile taxa showing especially strong associations with the three ants[43,49]. IndVal is the product of

'specificity' and 'fidelity' for each myrmecophile taxon with respect to each ant (or combination of ants), where specificity is the proportion of myrmecophile taxon's individuals associated with the ant species, and fidelity is the proportion of the ant species' trees where the myrmecophile taxon was found. To help avoid problems arising from year or location effects, we conducted separate analyses for each of the three year/location combinations.

We used a probabalistic model to identify OTU pairs that tended to co-occur more or less frequently than expected by chance[214]. Species co-occurrence patterns have long been of interest to ecologists for their potential to reflect biogeographic history, common environmental requirements, and positive or negative interactions between the species themselves. Much of this work has focused on quantifying co-occurrence across entire communities[71,201]. Pairwise co-occurrence analyses have become popular recently[111,150,214,215], though older whole-matrix methods remain appropriate for some questions[191].

Finally we used a Mantel testing approach to look for signs of within-taxon genetic variation associated with physical distance, location or ant species.

3.4 Results

Collection summary

We collected a total of 2361 myrmecophiles from 480 trees at our 2 sites over 2012-13 (Table 3.2).

DNA barcoding and taxon delineation

We obtained COI sequence for 1091 individual myrmecophiles from 2012 and 2013. We classified a further 28 individuals based on visual inspection where we failed to obtain good sequence. We also classified 1270 individuals that we did not attempt to sequence. These individuals belonged to 6 morphotypes, found with high abundance on a relatively small number of trees, for which the cost of

Table 3.2: Summary of myrmecophile collections. The table excludes 15 trees and 124 myrmecophiles for which either ant colony data were not recorded or a single ant species could not be identified for the tree.

		Trees		Myrmecophiles	
Site	Ant	Mar 2012	Aug 2013	Mar 2012	Aug 2013
Kitengela	CM	75	37	135	141
	CN	66	43	105	214
	TP	63	29	70	165
Suyian	CM	–	50	–	1004
	CN	–	54	–	190
	TP	–	48	–	213

sequencing all individuals did not appear to be justified (873 scale insects, 149 snails, 53 thrips, and 132 ants belonging to three taxa). We therefore had a total of 2326 classified myrmecophiles. We were unable to classify the remaining 35 myrmecophiles; however these were retained in the dataset for analyses that did not require classification (e.g. total myrmecophile incidence).

Sequences from the 2012-13 collections clustered into 82 operational taxonomic units (OTUs). Representative photographs of each OTU are included in Appendix B. Rarefaction curves indicate that our current sampling effort falls some way short of exhausting taxonomic diversity even on these small trees (Figure 3.4a). Chao2 richness estimators suggest that our sampling has recovered around half of the true diversity on small trees at these sites.

In addition to the sequence data from the 2012-13 collections, we obtained sequences from 180 samples collected in 2014, and for 6 of the 7 samples from David Agassiz (we were unable to amplify *P. chalcomochla*). These mostly overlapped with OTU clusters that already contained samples from the 2012-13 collections. Just four additional OTUs were present in the 2014 data, and among the samples that we successfully amplified from David Agassiz, only *H. bopprei* was not recovered in the

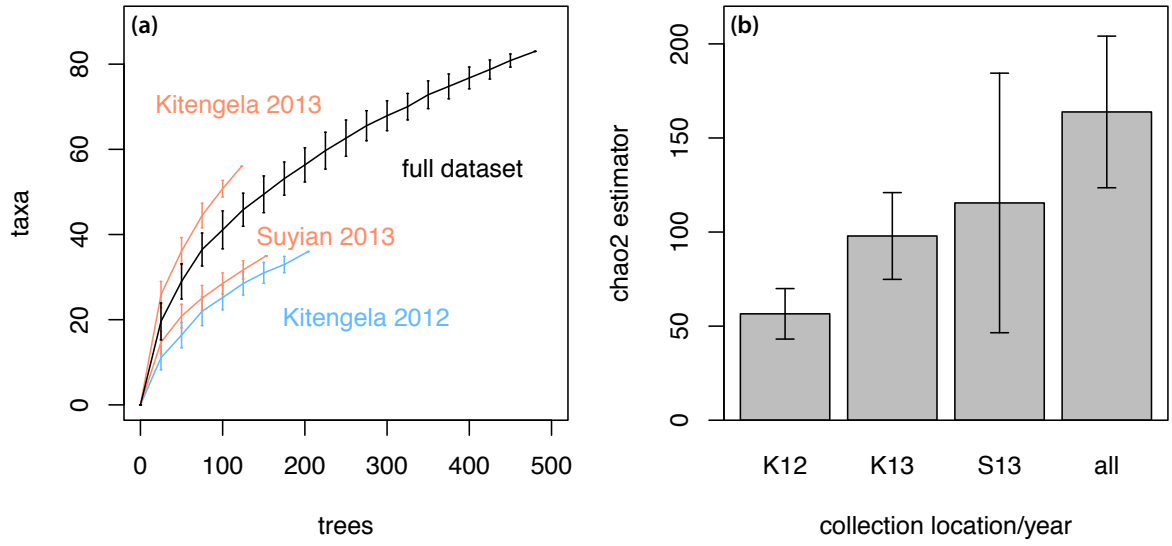

FIGURE 3.4: (a) Rarefaction curves indicate that our present sampling effort falls short of exhausting taxonomic diversity even on these small trees. (b) Chao2 estimators suggest that our present sampling effort has recovered approximately half of the true diversity.

2012-2013 collections.

Sequence data are available through the NCBI and through BOLD. The full set of photographs and other metadata are associated with the sequence data in BOLD. Photographs and data are also available directly from the authors.

Taxonomic assignments

Table 3.3 summarizes the breakdown of our putative taxonomic assignments. Appendix B provides a full listing of OTUs and summarizes the presence/absence data graphically.

Myrmecophile incidence

Myrmecophiles were present on 62% of all trees sampled. However, the proportion of trees with myrmecophiles was substantially higher in 2013 (72% occupied) than in 2012 (49% occupied) (Figure 3.5a). In addition, myrmecophile incidence varied significantly with ant species in both years

Table 3.3: Summary of myrmecophile taxa collected at Kitengela and Suyian in 2012 and 2013, ordered by trees occupied.

Group	OTUs	Trees	Individuals
Lepidoptera	29	219	581
Araneae	16	108	322
Hymenoptera	18	45	234
Hemiptera	2	39	874
Gastropoda	1	23	152
Polyxenida	1	16	35
Coleoptera	8	16	20
Diptera	4	11	30
Blattodea	1	5	5
Thysanoptera	1	3	59
unknown	1	1	14
Total	82	–	2326

(2012: $\chi^2 = 9.99$, $df = 2$, $p = 0.007$; 2013: $\chi^2 = 16.30$, $df = 2$, $p < 0.001$). *C. mimosae*-occupied trees were most likely to contain at least one myrmecophile, and *T. penzigi*-occupied trees least likely. Among the 2013 samples, there was no significant difference in myrmecophile incidence between location conditional on ant species ($\chi^2 = 5.99$, $df = 6$, $p = 0.425$; Figure 3.5b).

Trees with more domatia tended to be more likely to have myrmecophiles (Figure 3.6a). However the number of domatia also varied among trees occupied by different ant species. The median number of domatia on trees occupied by *C. mimosae* was 38 in 2012 and 42 in 2013, compared to 43.5 and 42 respectively on those occupied by *C. nigriceps*, and 60 and 51 respectively on those occupied by *T. penzigi* (Figure 3.6b). But this did not explain the variation in myrmecophile incidence with ant species – rather, the ant species with higher myrmecophile incidence tended to have fewer domatia.

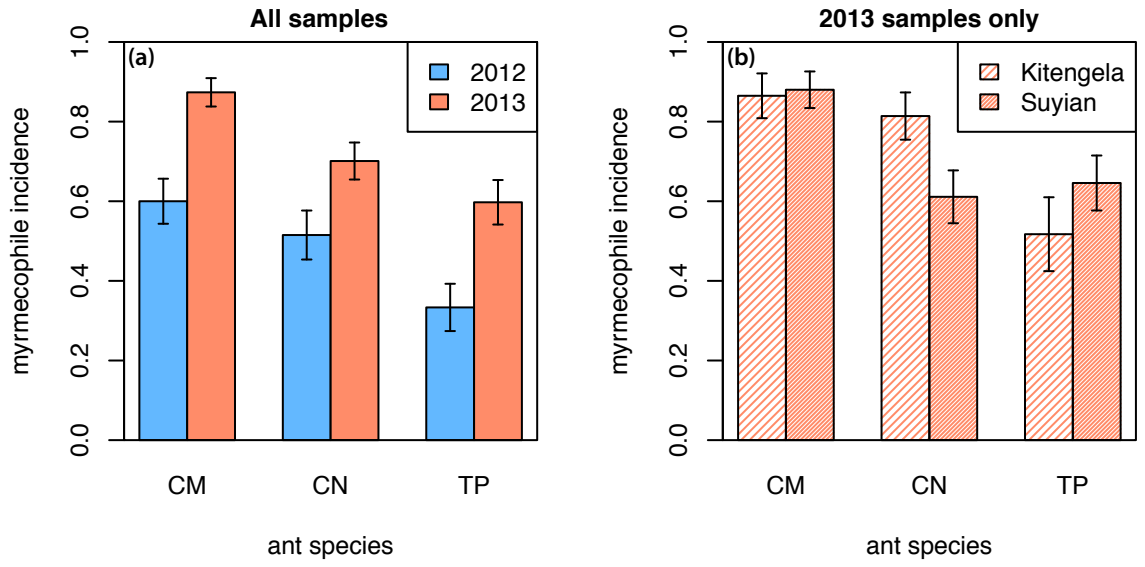

Figure 3.5: Proportions of sampled trees yielding at least one myrmecophile. (a) The proportion occupied differed significantly between sampling years, but also among ant species within each year. (b) Within the 2013 samples, myrmecophile incidence followed broadly the same pattern among ant species at both locations.

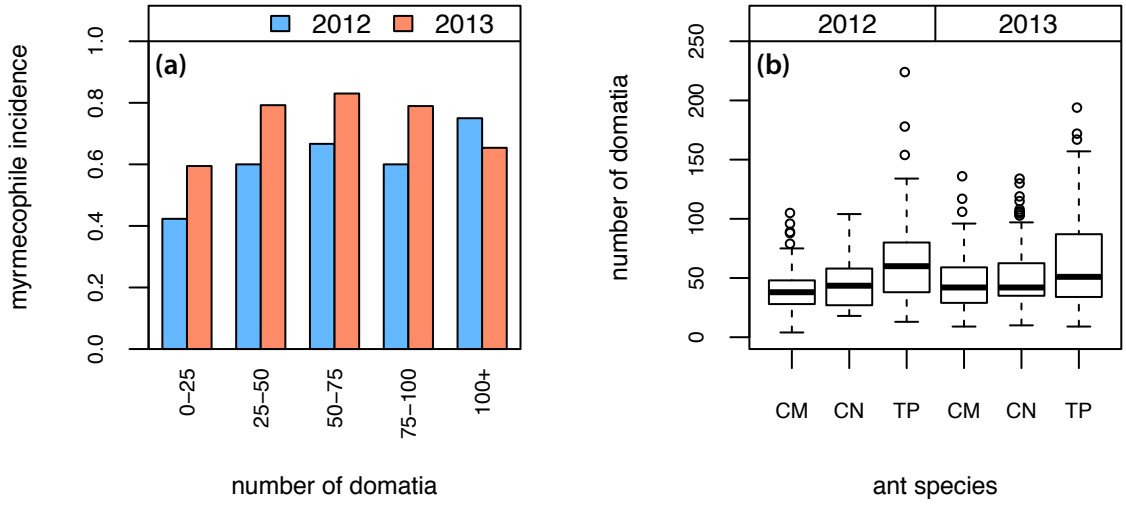

Figure 3.6: (a) Trees with more domatia tended to be more likely to have myrmecophiles. (b) But the number of domatia per tree also varied with ant species, with domatia especially numerous on *T. penzigi* trees.

We used logistic regression to assess the effects of ant species, number of domatia and collection year on the probability that a tree has myrmecophiles. These predictors were all significant, with signs on coefficients corroborating the simpler analyses in Figures 3.5 and 3.6 (see Table 3.4).

Overall myrmecophile diversity

We identified 82 different myrmecophile OTUs in 2012-13. In both years, abundance was highly right skewed, with just 3 taxa recovered from 5% or more of the sampled trees in 2012, and just 11 taxa in 2013 (Figures 3.7a and 3.7b). Individual- and tree-counts were positively correlated across the two years, though with substantial noise (Figures 3.7c and 3.7d; individual counts: $r_s = 0.20$; tree counts: $r_s = 0.30$).

Per tree abundance and richness

The myrmecophile community was relatively sparse on most trees (Figures 3.8a and 3.8b). In the 2012 dataset, the median number of myrmecophile individuals was 2 per tree, for each of the three ant species. In the 2013 dataset, the median number of individuals was 5 for *C. mimosae*, 3 for *C. nigriceps* and 2 for *T. penzigi*. However, the distributions of myrmecophile counts were highly skewed, with some trees yielding dozens of myrmecophiles, especially scale insects on *C. mimosae* trees at

Table 3.4: Results of logit modeling of probability a tree contains myrmecophiles

	estimate	z-statistic	p-value
intercept	-1.524e+03	-3.363	<0.001
collection year	7.578e-01	3.365	<0.001
ant (CN)	-5.801e-01	-2.066	0.039
ant (TP)	-1.516e+00	-5.245	<0.001
no. of domatia	1.036e-02	2.653	0.008

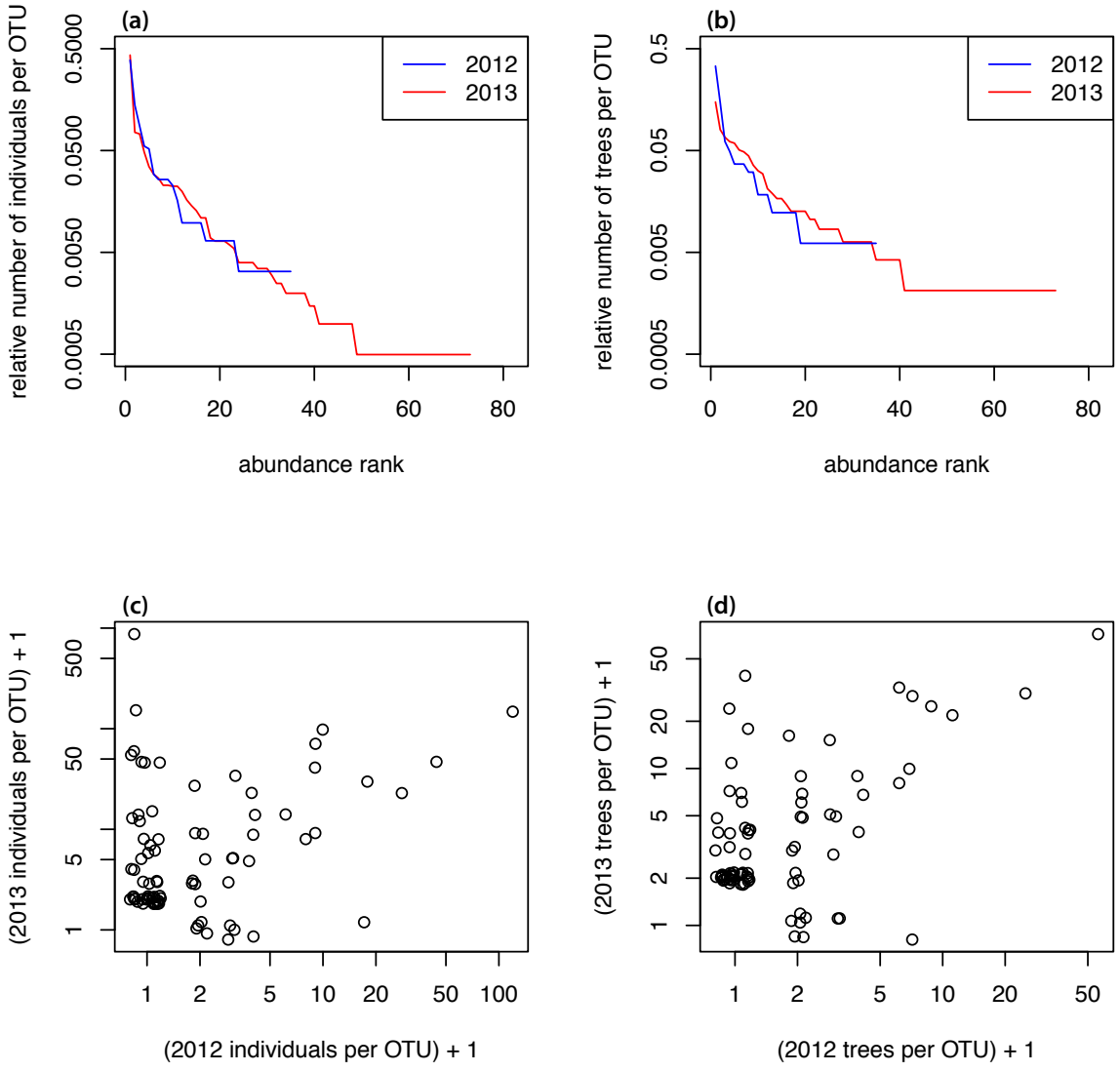

FIGURE 3.7: Rank abundance curves based on (a) number of individuals and (b) number of trees. Scatterplots comparing 2012 and 2013 data with respect to (c) number of individuals and (d) number of trees (+1 to allow for plotting on logarithmic axes; data are jittered to allow visualization of overlying points).

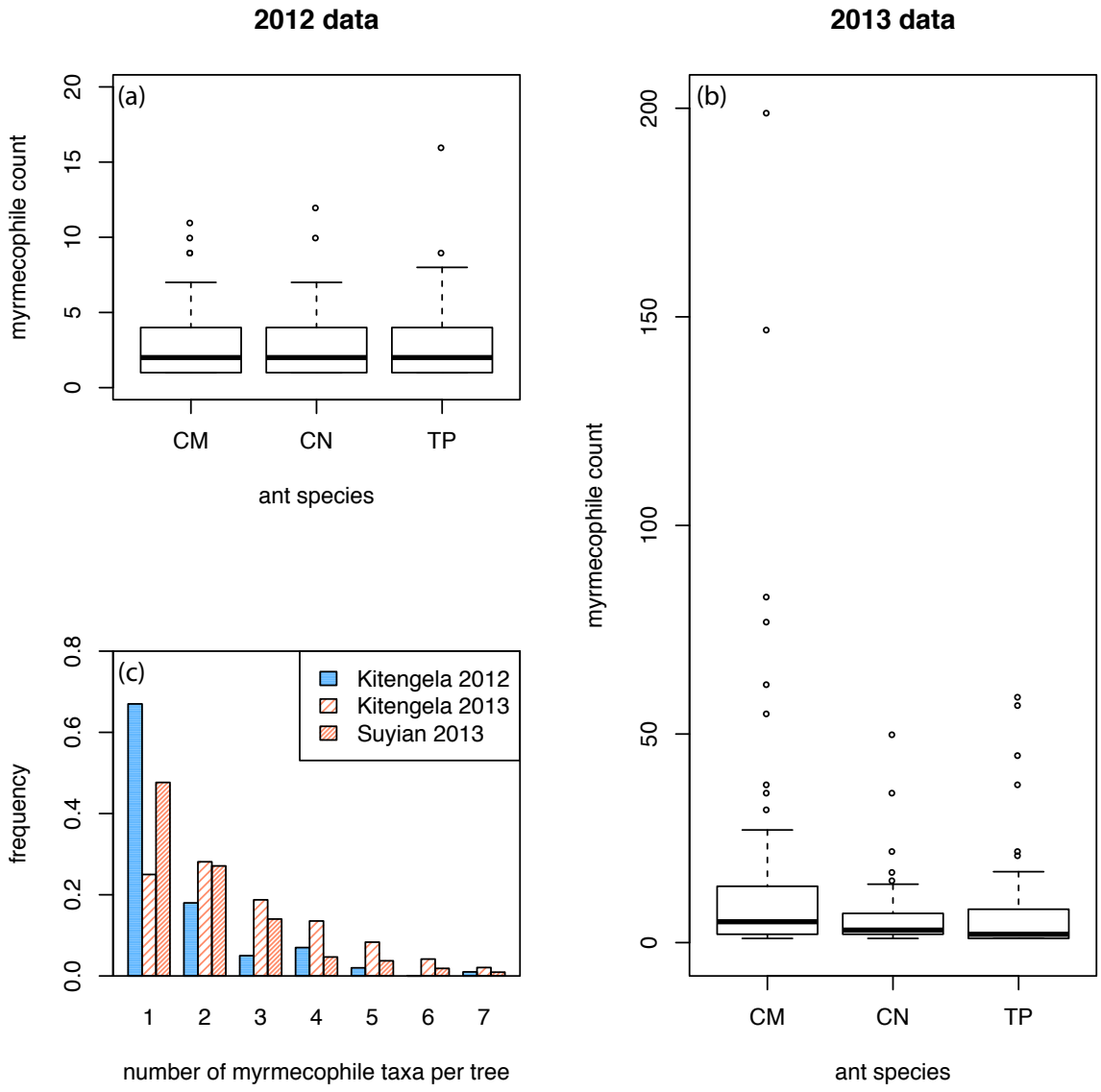

Figure 3.8: Number of individual myrmecophiles per tree in (a) 2012 and (b) 2013. (c) Number of myrmecophile taxa per tree. (a) through (c) exclude trees without myrmecophiles.

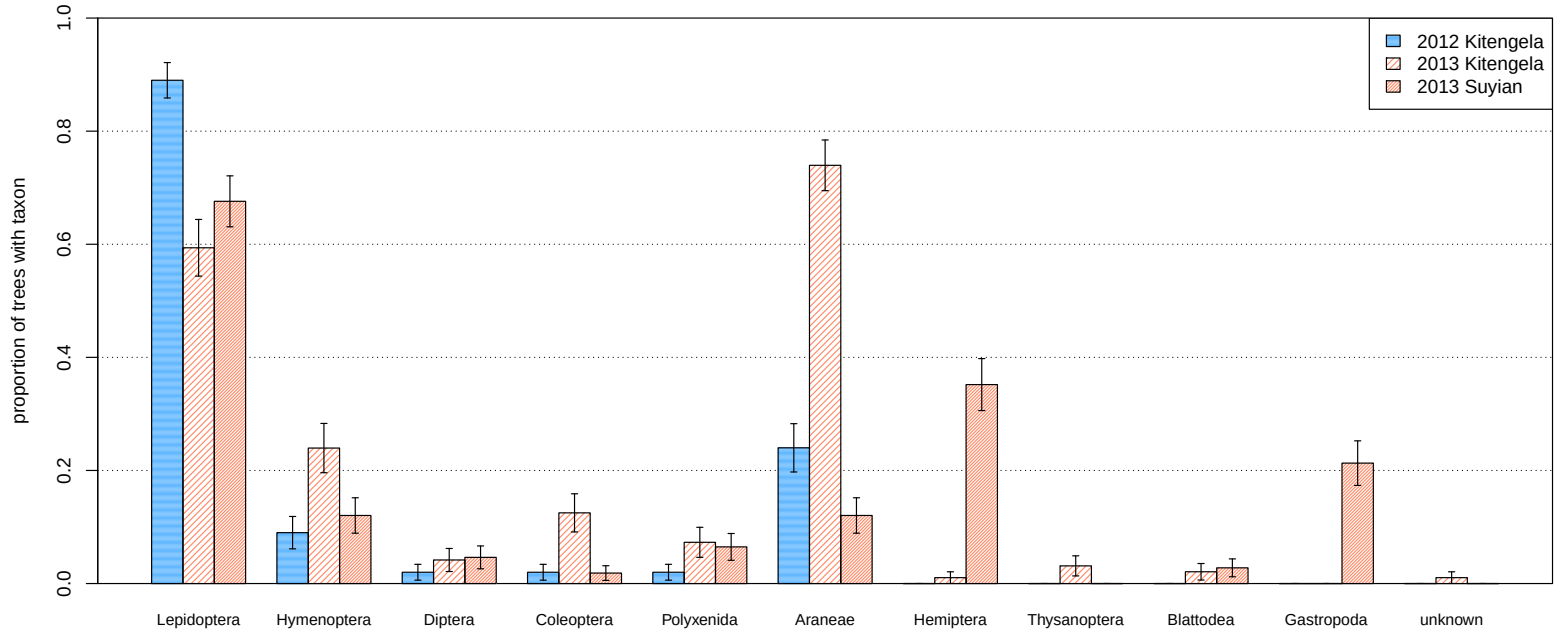

FIGURE 3.9: Proportion of trees with myrmecophiles present from major taxonomic groups. Proportions calculated separately for each combination of location and year.

Suyian in 2013: excluding these the median numbers of individuals per tree in 2013 fell to 3 for *C. mimosae*.

Per-tree taxonomic richness was also low. The median tree contained just 1 myrmecophile taxon in 2012, and 2 taxa in 2013, consistent with the low median numbers of individuals. As for the individual counts, the distributions of taxa per tree were right skewed (Figure 3.8c). However, we never recovered any more than 7 different myrmecophile taxa from a single tree, even on trees with much higher individual counts. In other words, high individual counts were not the result of those trees accumulating a large and rich myrmecophile community, but rather the result of those trees sustaining high numbers of a small set of taxa – especially scale insects.

Variation in myrmecophile communities with year and location

Much of the overall variation in the composition of myrmecophile communities was associated with collection location and year. Several of the major taxonomic groups showed substantial differences between years and locations in the proportion of trees occupied (Figure 3.9).

A clustering of the 15 most abundant taxa (defined as those occurring on $\geqslant 10$ trees) indicated four main groups among these taxa (Figure 3.10). One group comprised taxa found only at Suyian; another comprised taxa found predominantly at Kitengela; and the remaining two groups consisted of taxa found at both collecting locations.

In a correspondence analysis (CA) of these 15 taxa, the strong effect of location was visible (Figure 3.11) and, among the Kitengela samples, the effects of collecting year were also apparent. In addition, larger subsampled trees from Kitengela in 2013 tended to yield non-representative communities relative to fully sampled trees at the same location and time (Figure 3.11 – crosses vs. open circles). Coloring trees by ant species, the same correspondence analysis also indicated non-random associations between myrmecophile taxa and ant species (Figure 3.12).

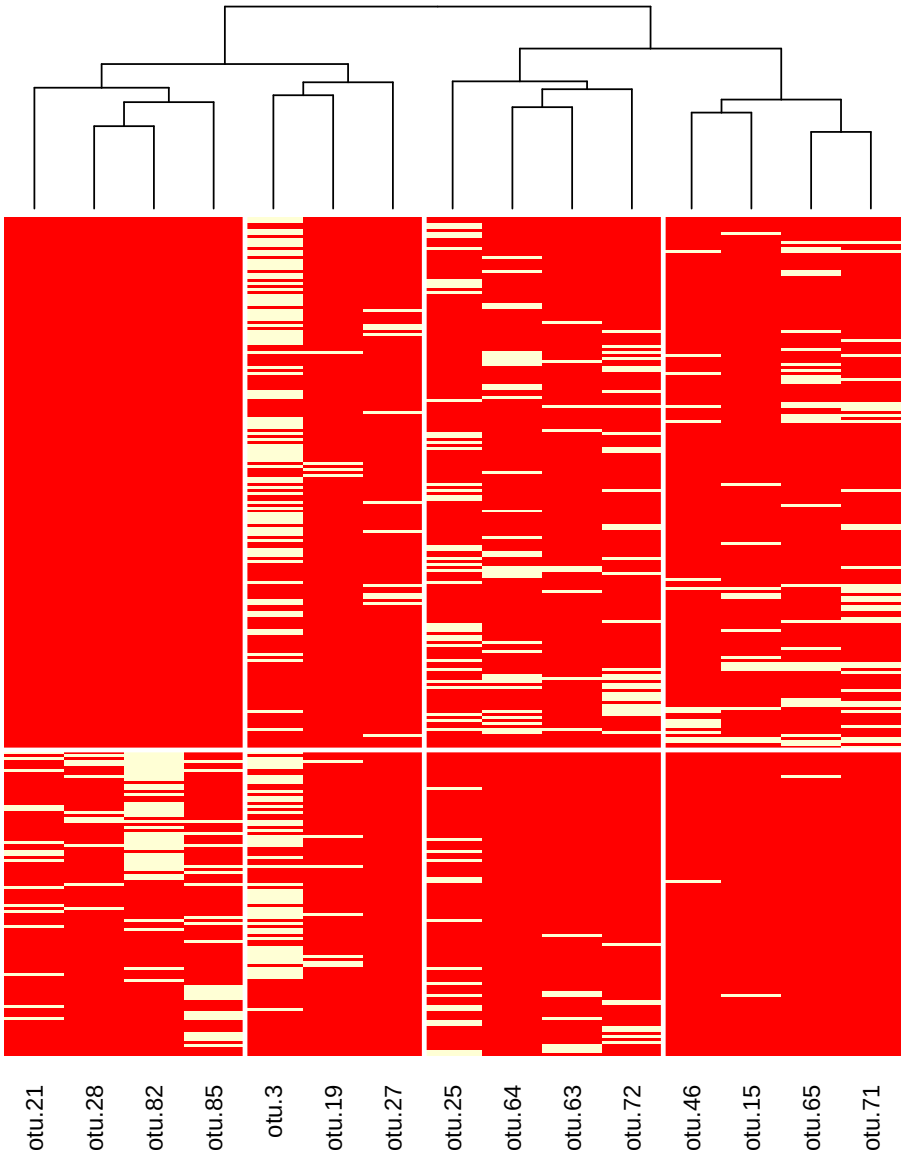

Figure 3.10: Clustering the most abundant myrmecophile taxa (defined as those occurring on $\geqslant 10$ trees) according to their Spearman correlations indicates that these taxa fall into four groups. Dendrogram at top of diagram shows a Ward's minimum variance clustering of the OTUs based on their Spearman correlations. The heatmap below the dendrogram shows presence (white) and absence (red) for each taxon on each tree, with trees (i.e. rows) grouped by collection location.

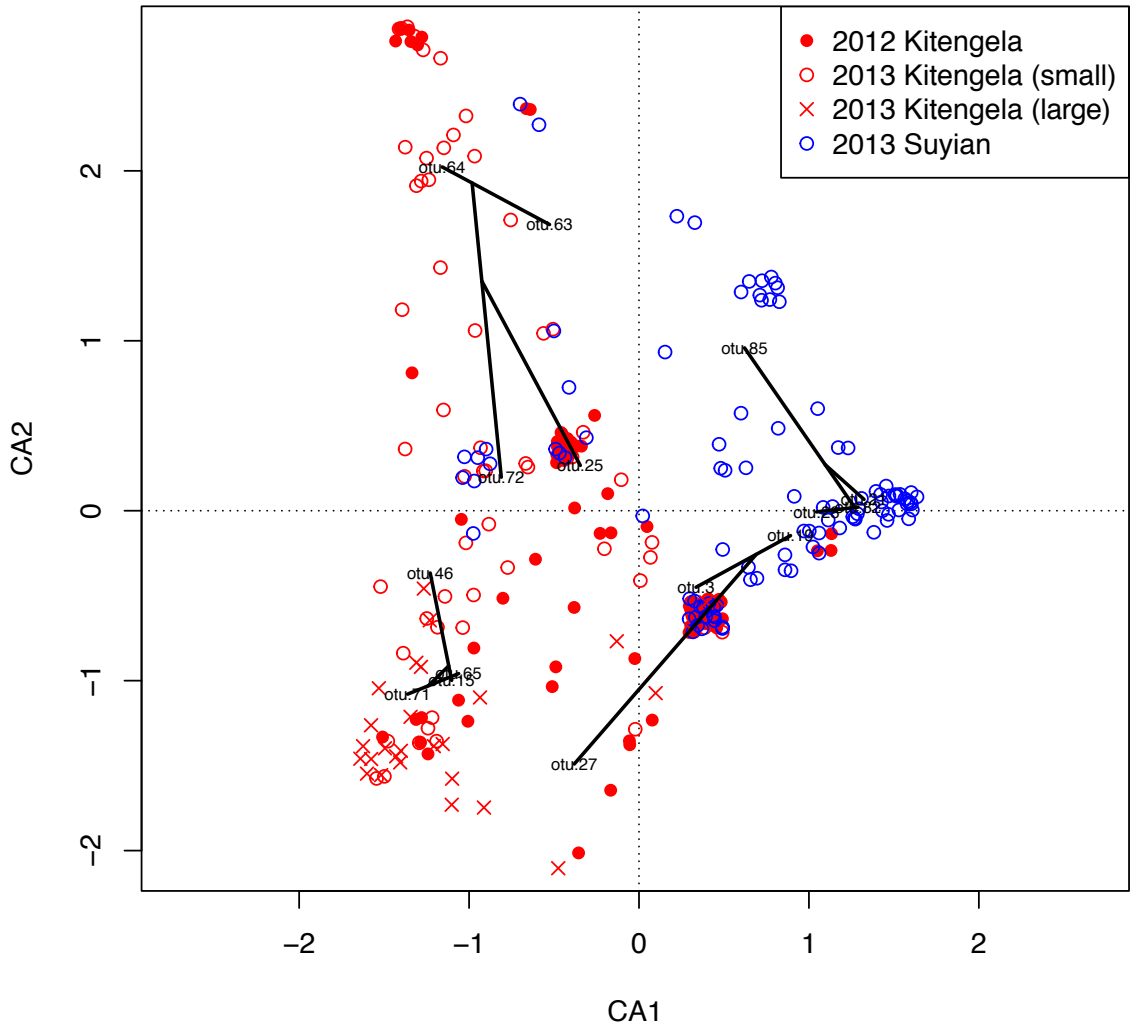

Figure 3.11: Correspondence analysis biplot for myrmecophile taxa found on $\geqslant 10$ trees. Myrmecophile taxa are shown in grey lettering; trees are shown as points. Abundance data were log transformed with adjustment for zeros[9]. Coloring trees by site and using different symbols for different collection years shows separation by location, year and sampling completeness.

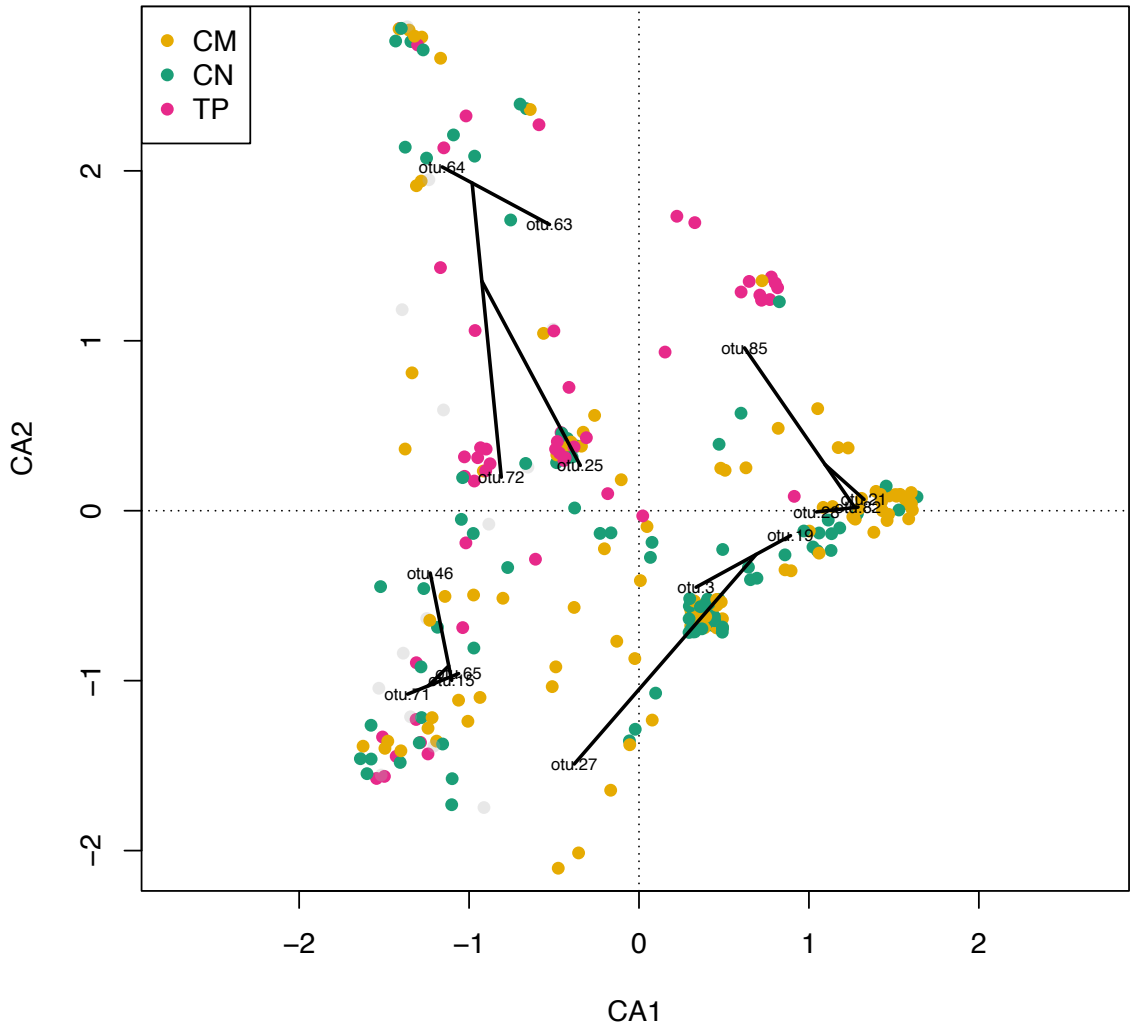

Figure 3.12: Correspondence analysis biplot for myrmecophile taxa found on at least 10 trees. Myrmecophile taxa are shown in grey lettering; trees are shown as points with ellipses denoting standard deviations. Abundance data were log transformed with adjustment for zeros[9]. Coloring trees according to ant occupant shows little separation by ant on the first two CA axes.

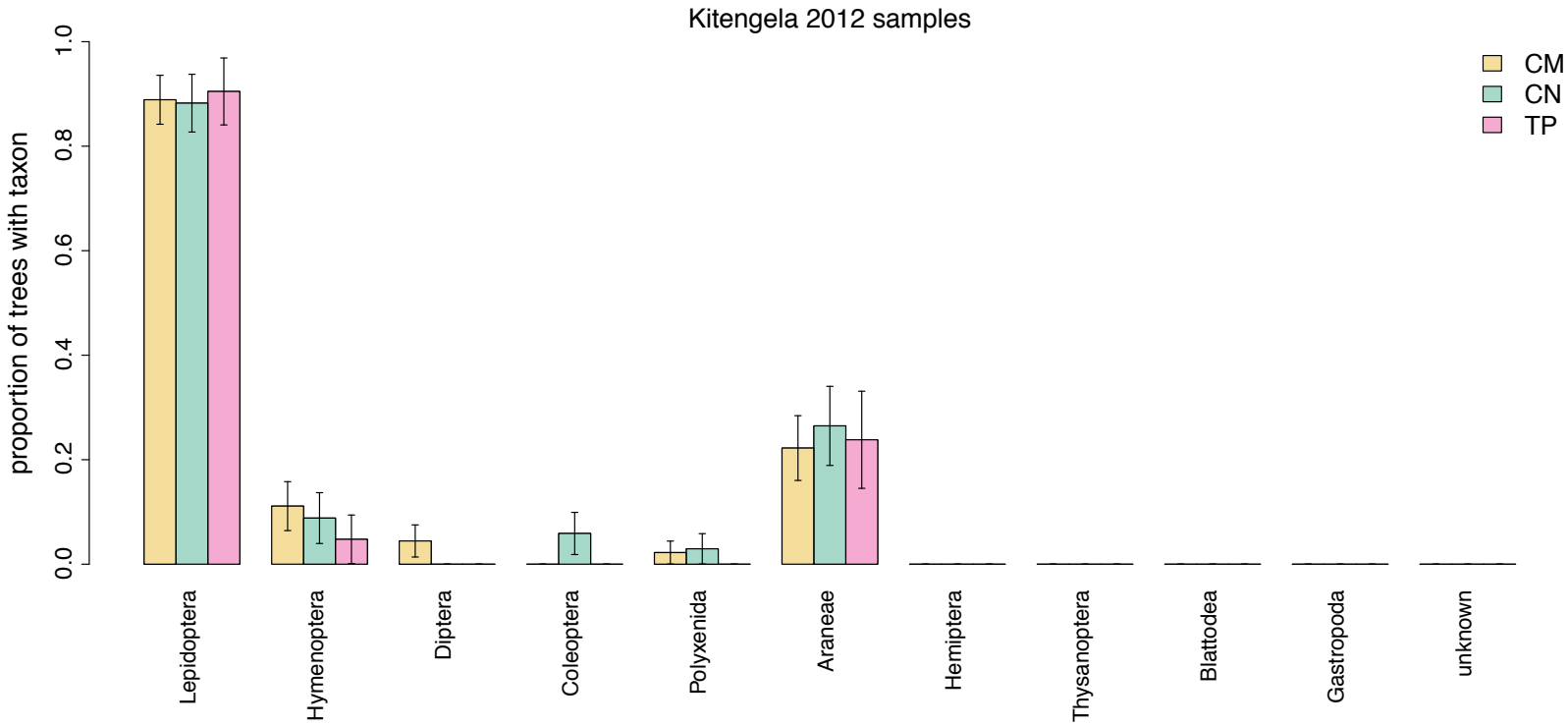

Figure 3.13: Proportion of trees with myrmecophiles present from major taxonomic groups. Proportions calculated separately for each combination of location and year.

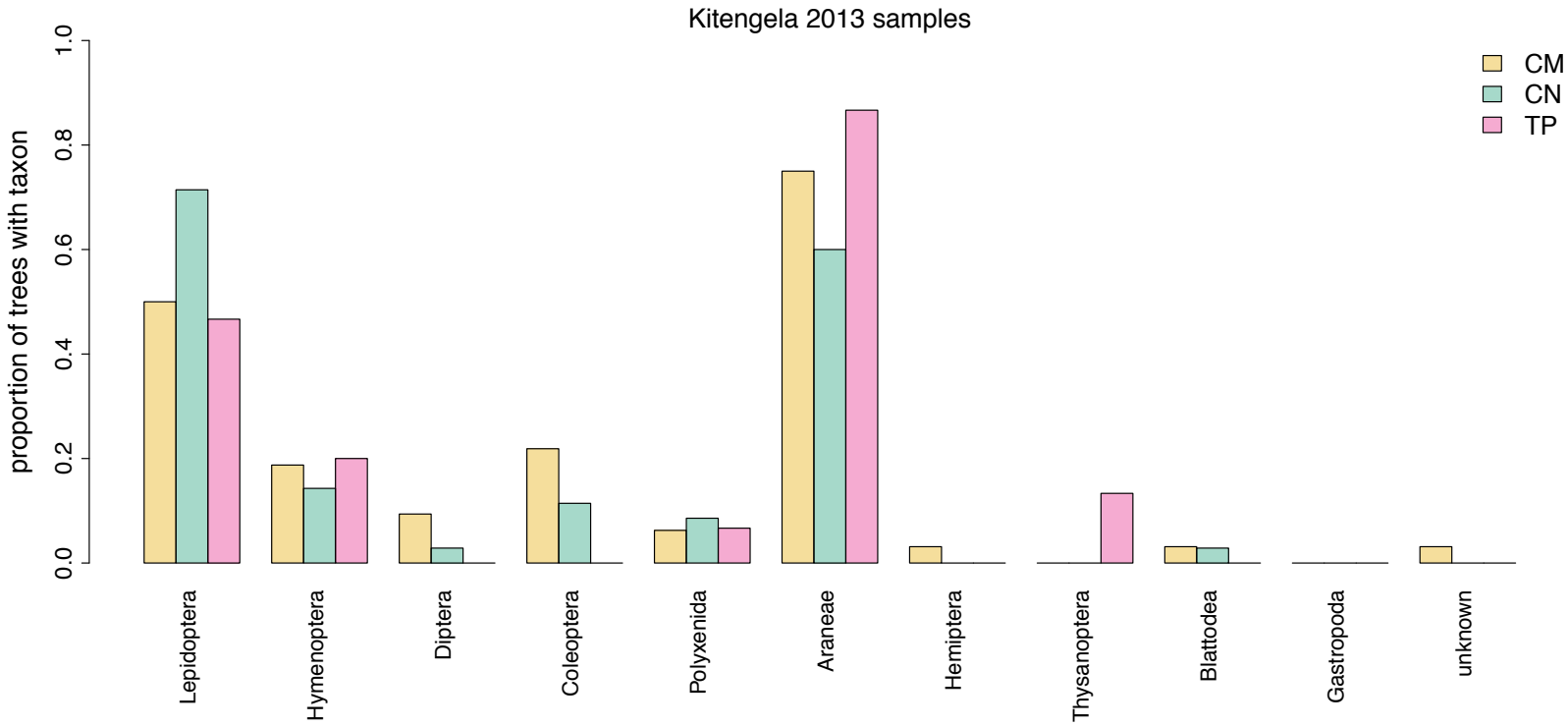

Figure 3.14: Proportion of trees with myrmecophiles present from major taxonomic groups. Proportions calculated separately for each combination of location and year.

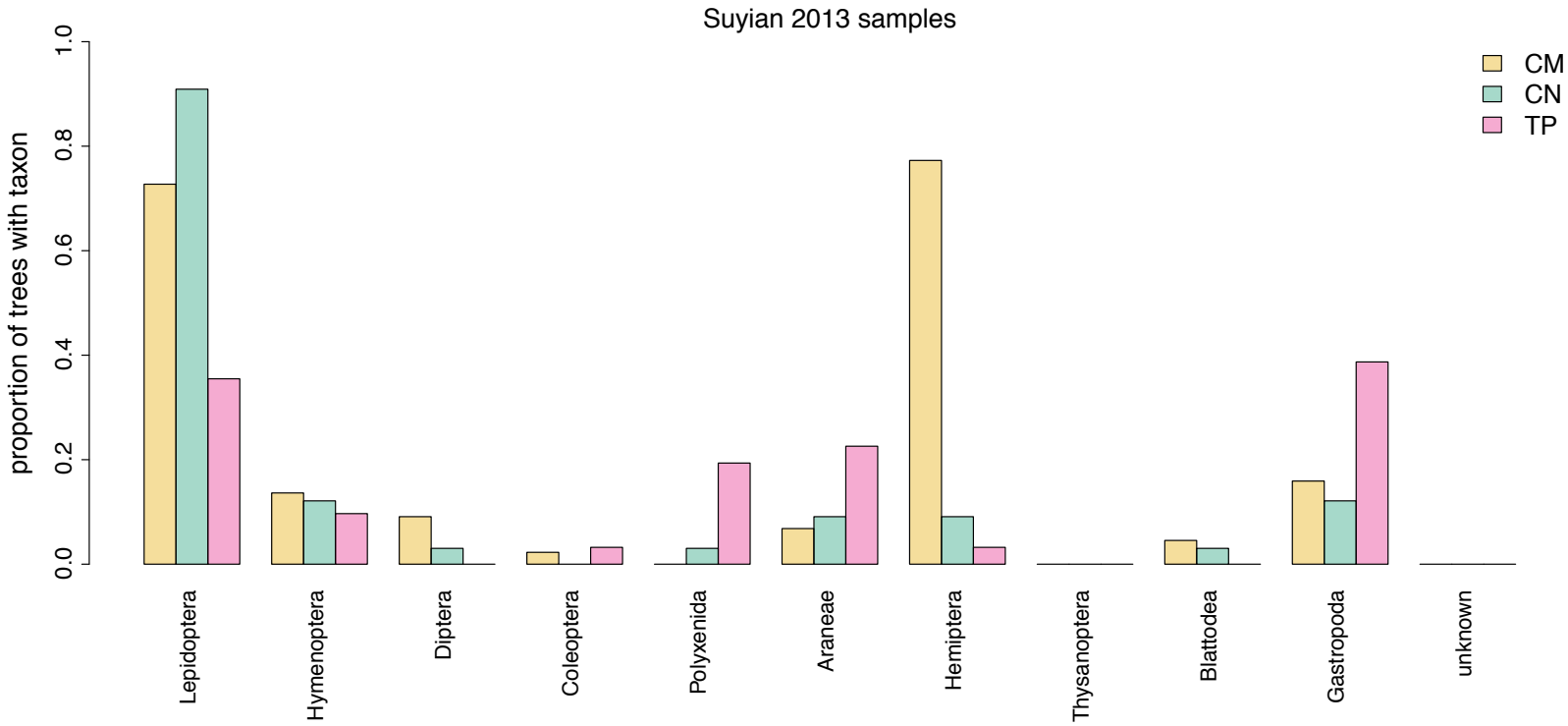

Figure 3.15: Proportion of trees with myrmecophiles present from major taxonomic groups. Proportions calculated separately for each combination of location and year.

Variation in myrmecophile communities with ant species

Although much of the obvious variation in myrmecophile community composition was associated with collection year and location – especially among the most abundant taxa – the ant species occupying the tree also affected the myrmecophile community. Some of this ant-associated variation is visible even in data aggregated at higher taxonomic levels (Figures 3.13 through 3.15).

A canonical correspondence analysis (CCA) based on the full dataset revealed significant ant-associated variation after controlling for collection location and year (Figure 3.16; $F = 3.1367$, $df = 2, 264$, $p = 0.001$). The CCA suggested a number of myrmecophile taxa are specialized to associate with the three ant species, including some of the most abundant taxa (Figure 3.17). For example, the tortricid *Hystrichophora griseana* (OTU 3) was found much less commonly with *T. penzigi* than with *C. mimosae* or *C. nigriceps*; and the scale insect *Hockiana* sp. (OTU 82) was strongly associated with *C. mimosae* but not with the other ant species. This was supported by the significant network-wide specialization measure H2′ (i.e. normalized two-dimensional Shannon entropy $H2^{21}$) ($H2' = 0.24$, $p < 0.001$).

Our IndVal analysis identified 12 myrmecophile taxa significantly associated with either one or two of the ant species in at least one of the analyses; these taxa may be regarded as a candidate set of 'specialists'. In addition, 6 taxa show the highest IndVal results for all three ants combined, and might be regarded as a candidate set of fully generalist taxa. To identify a subset of taxa that are most likely to display true specialization or generalization, we filtered the IndVal results to those taxa found on at least 10 trees (Table 3.5).

Myrmecophile co-occurrence patterns

The co-occurrence of myrmecophile OTUs on trees in our dataset is largely a reflection of the community patterns associated with collecting location, year, and ant species. While we lack the power to

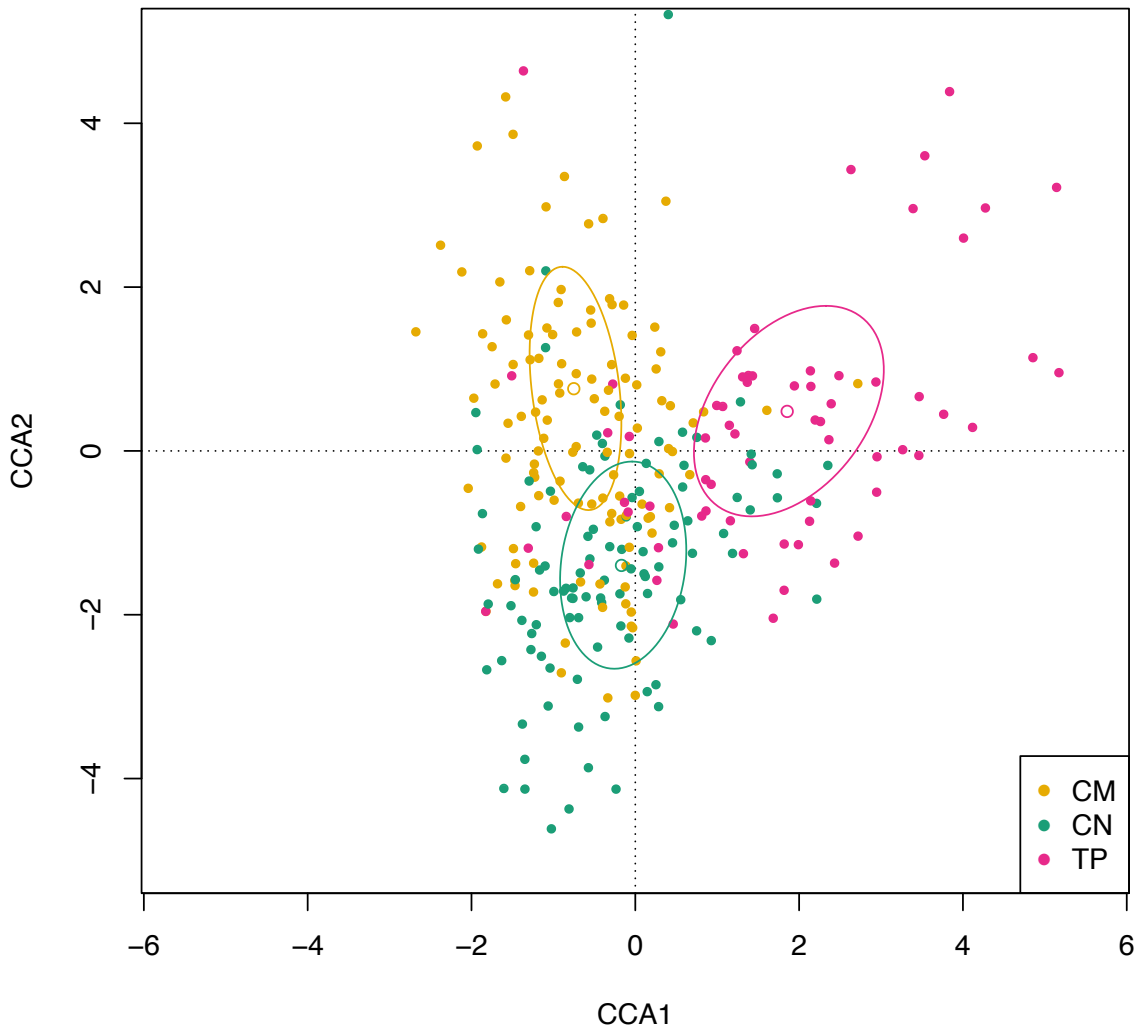

Figure 3.16: Canonical correspondence analysis based on all myrmecophile taxa, using log transformed data with adjustment for zeros[9]. Ant species was the explanatory variable; ordinations conditioned on collection location, year and completeness of sampling.

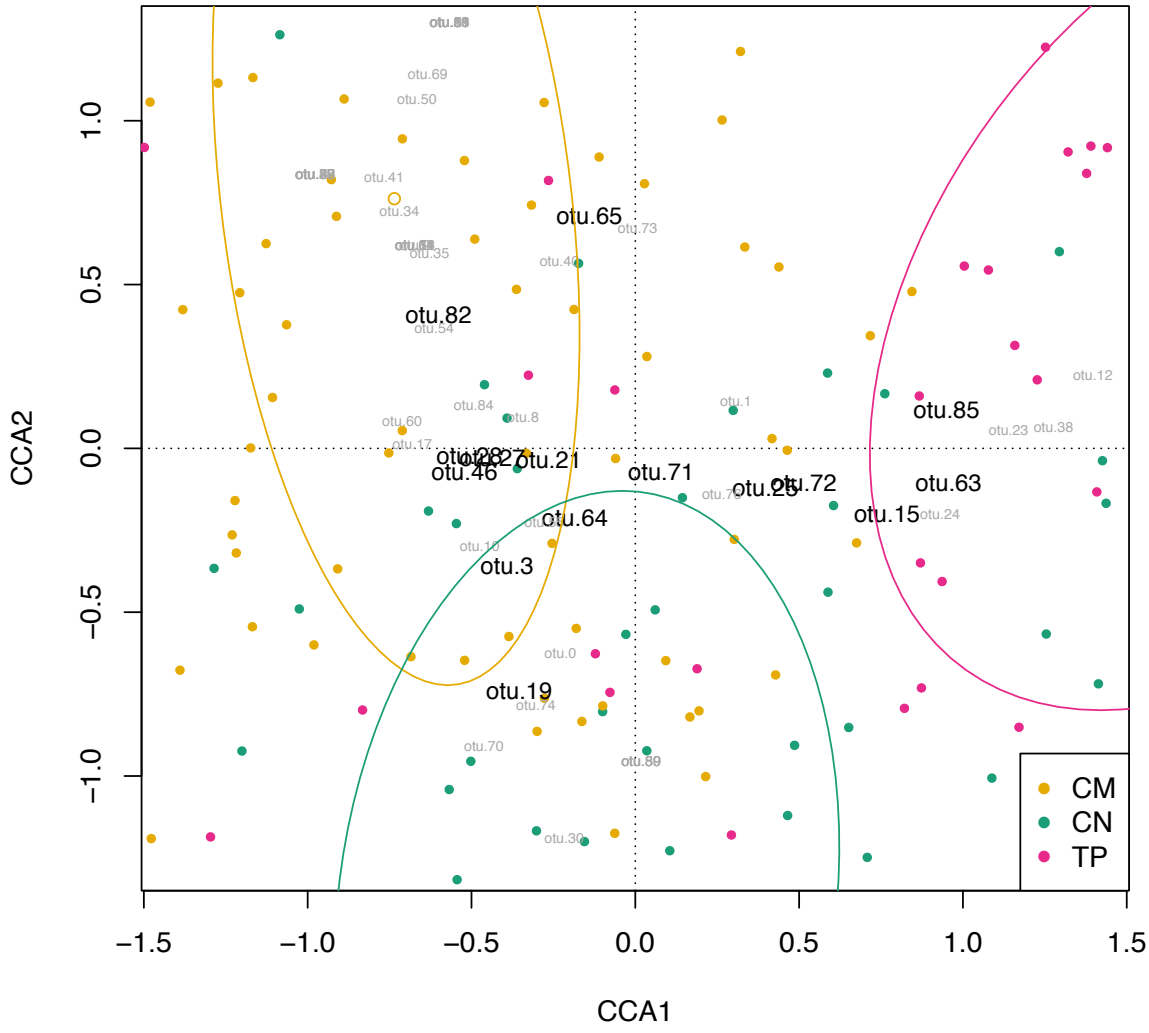

Figure 3.17: Canonical correspondence analysis based on all myrmecophile taxa, using log transformed data with adjustment for zeros[9]. The central portion of the biplot has been enlarged, and the 15 most abundant myrmecophile taxa (occuring on $\geqslant$ 10 trees) are shown in larger text. Ant species was the explanatory variable; ordinations conditioned on collection location, year and completeness of sampling.

Table 3.5: Myrmecophile-ant specialization. Indicator Value calculated for each OTU within each year/location combination. Table only shows significant results for myrmecophiles sampled from $\geqslant$ 10 trees within year/location, plus taxa reporting highest Indicator Value for CM+CN+TP. *p*-values are not corrected for multiple testing. CM = *C. mimosae*, CN = *C. nigriceps*, TP = *T. penzigi*.

OTU	Description	Location	Year	Grouping	A_{ij}	B_{ij}	$\sqrt{IndVal}$	*p*-value	no. trees
82	Hempitera: *Hockiana* sp.	Suyian	2013	CM	0.91	0.77	0.84	0.005	38
72	Eutichuridae: *Cheiracanthium* sp.	Kitengela	2013	TP	0.64	0.60	0.62	0.005	25
85	Gastropoda: unknown sp.	Suyian	2013	TP	0.77	0.40	0.56	0.005	23
3	Tortricidae: *H. griseana*	Kitengela	2012	CM+CN	0.94	0.65	0.78	0.005	55
3	Tortricidae: *H. griseana*	Kitengela	2013	CM+CN	1.00	0.33	0.57	0.020	24
3	Tortricidae: *H. griseana*	Suyian	2013	CM+CN	0.99	0.60	0.77	0.005	47
65	Salticidae: unknown sp.	Kitengela	2013	CM+TP	0.91	0.32	0.54	0.020	20
25	Gelechiidae: *Dichomeris* sp.	Kitengela	2012	CM+CN+TP	1.00	0.24	0.49	NA	24
25	Gelechiidae: *Dichomeris* sp.	Suyian	2013	CM+CN+TP	1.00	0.15	0.39	NA	16
64	Salticidae: *Myrmarachne* sp.	Kitengela	2013	CM+CN+TP	1.00	0.23	0.48	NA	24
71	Eutichuridae: *Cheiracanthium* sp.	Kitengela	2013	CM+CN+TP	1.00	0.28	0.53	NA	28

test many pairs in our dataset, several OTU pairs co-occurred significantly more or less than would be expected by chance (Figure 3.18a).

Sources of genetic variation within myrmecophile taxa

Using a Mantel testing approach, we found a significant difference between Kitengela and Suyian in genetic distance in a number of myrmecophile taxa. Among the 5 taxa present on at least 10 trees per collecting location, location was significantly associated with genetic distance in 4 taxa: OTU 3 (Tortricidae: *H. griseana*, $p = 0.001$), OTU 25 (Gelechiidae: *Dichomeris* sp., $p = 0.001$), OTU 63 (bristly millipedes, $p = 0.001$) and OTU 65 (Salticidae sp., $p = 0.001$); OTU 72 (*Cheiracanthium* sp.) showed no significant association between genetic distance and location ($p = 0.399$). Within the two locations, however, we did not find any significant association between genetic distance and either geographic distance (based on GPS coordinates) or collecting year.

We also found no convincing association between myrmecophile genetic distance and the ant species occupying the tree. Even without correcting for multiple testing, no significant results for ant species were obtained for myrmecophile taxa represented by more than 5 trees per ant species – either within collecting locations and partialling out tree effects, or pooling across collecting locations and partialling out location effects.

3.5 Discussion

Myrmecophile incidence and abundance

Our collection of more than 80 myrmecophile taxa from domatia on almost 500 *V. drepanolobium* trees reveals substantial variation in abundance. Some of these taxa – e g. *Hockiana* sp. scale insects, the tortricid *H. griseana*, snails, the salticid ant-mimic *Myrmarachne* sp. and the gelechiid *Dichomeris* sp. – were found repeatedly, often with extremely high numbers. These taxa are likely to represent

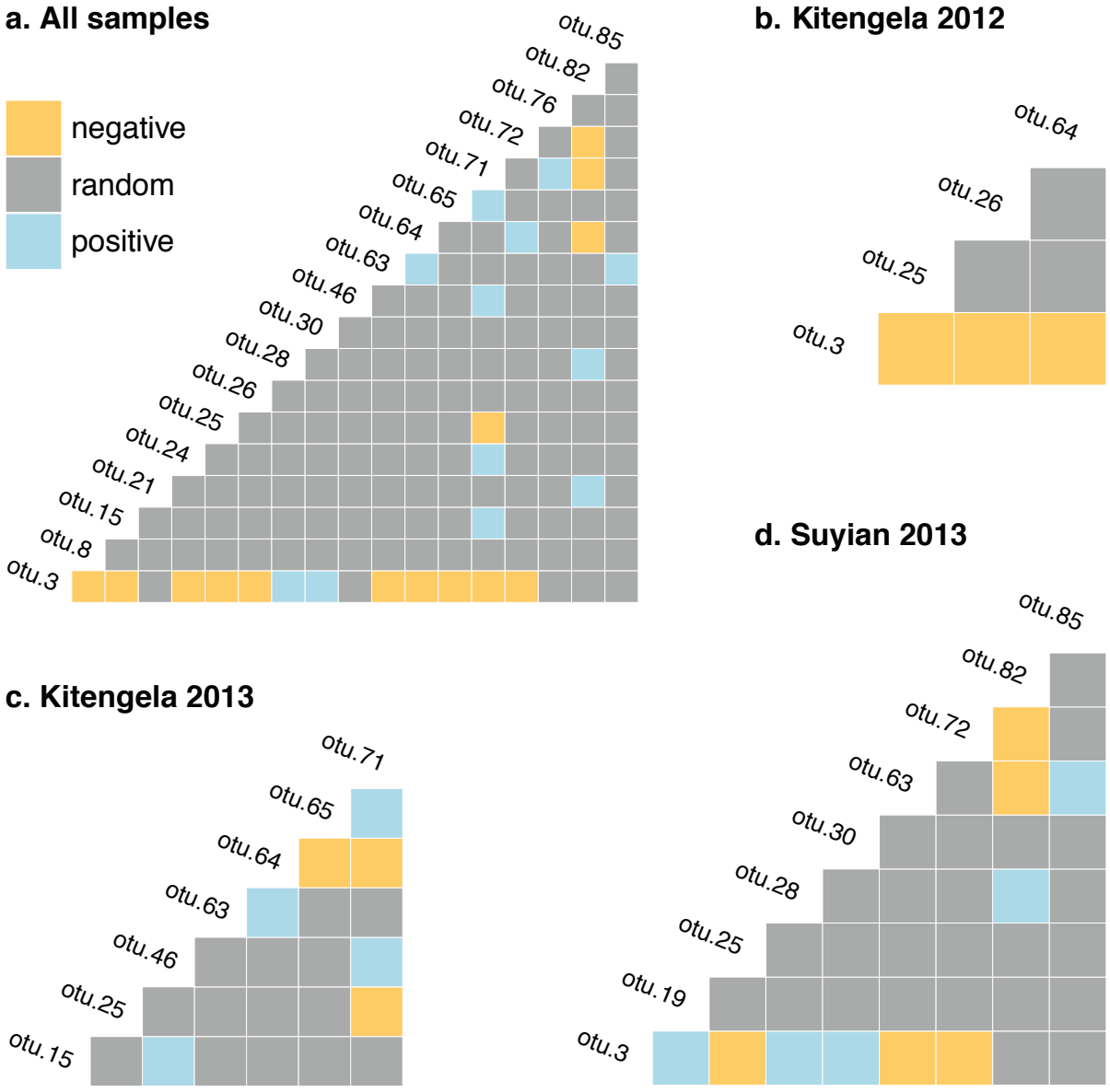

Figure 3.18: Myrmecophile OTU co-occurrence analysis for (a) all samples, (b) Kitengela 2012 samples, (c) Kitengela 2013 samples, and (d) Suyian 2013 samples. Positive/negative sample pairs are those co-occurring significantly more or less frequently than expected at $\alpha = 0.05$.

important ecological components and are particularly good candidates for closer study. However, the many taxa that were found on just one or two trees may still play important ecological roles, especially in aggregate, and need to be considered in any complete picture of the system. Moreover, all of these taxa, irrespective of abundance, are species that have managed to bypass their ant hosts' defenses in order to live alongside them in the domatia, and thus are likely to have specialized and interesting natural history[92].

Our dataset likely underestimates the richness and abundance of myrmecophile communities across *V. drepanolobium* trees in general. Many of the trees in our dataset yielded no myrmecophiles, and even trees with myrmecophiles often had a sparse community. However, our collections were focused on relatively small trees, and we would expect a larger and richer community of myrmecophiles on larger trees. Although we did not find good evidence for a relationship between tree height and myrmecophile incidence, this is likely an artifact of our collection scheme, which was not designed to sample well across a range of tree sizes (see also discussion of community composition and sampling regime below). Our data did show, however, that myrmecophile incidence increased with the number of domatia.

A positive relationship between myrmecophile and number of domatia might arise from several causes. First, more domatia likely implies more ant-occupied domatia. For myrmecophiles that need to interact with the ants (e.g. ant brood predators), this therefore increases the size of the resource. Second, larger and older trees tend to have more domatia, and on those trees a larger fraction of the domatia (e.g. older or damaged domatia) may be abandoned or underutilized by ants. This therefore increases the space available for myrmecophiles that prefer to be associated peripherally with the ant colony (e.g. spiders that prey on insects other than ants but benefit from the 'enemy-free space' available on the ant-plant[99]. Third, domatia represent discrete hiding places for myrmecophiles. Thus, even holding constant the size of the ant colony or the volume of unoccupied domatia, myrme-

cophiles wishing to avoid interactions with other myrmecophiles have more opportunities to occupy a tree with more domatia. Fourth, domatium count may be correlated with other tree characteristics. e.g. healthier trees have both more domatia and more herbivores that may be prey for a myrmecophilic spider. A detailed dataset with better sampling of tree sizes and more detailed information on other characteristics (e.g. foliar nitrogen content) may help disentangle some of these correlated variables. Finally, domatium number may respond to myrmecophiles or herbivory – e.g. trees subject to heavy herbivory may put on more domatia[94,157], and this may be correlated with myrmecophile incidence either because myrmecophiles themselves are responsible for the herbivory or because they prey on the herbivores.

Myrmecophile community variation with location and year

Our dataset shows collecting year and location to be major sources of variation in myrmecophile incidence and community composition (Figures 3.5 and 3.11). This substantial variation is not anecdotally surprising – for example, recent sampling from a small number of trees at Suyian in May 2015 yielded even lower myrmecophile densities than reported here.

Several myrmecophile taxa were present at either Kitengela or Suyian, and virtually absent at the other site. The lepidopteran taxa OTU 15 (Tineidae), OTU 24 (Gelechiidae), and OTU 27 were present in good numbers at Kitengela in 2012 and 2013 but not at Suyian in 2013. This was also the case for the braconid OTU 46 (*T. laikipiensis*), the coleopteran OTU 55, and the spiders OTU 65 (Salticidae), OTU 71 (*Cheiracanthium* sp.) and OTU 76. OTU 72 (also *Cheiracanthium* sp.) showed a similar pattern, except that it was also collected readily from *T. penzigi* trees at Suyian. In contrast, OTU 85 (Gastropoda) and OTU 82 (scale insects), and the lepidopteran taxa OTU 19 (Geometridae), OTU 20 and OTU 28, were virtually absent at Kitengela in both 2012 and 2013, but readily collected from trees at Suyian in 2013.

The tight association between location and OTU 82 (scale insects) accounts for several of the non-

random pairwise OTU co-occurrences (Figure 3.18). OTU 82 appears negatively associated with the higher-abundance myrmecophiles that do not share its affiliation with Suyian, including OTUs 71 and 72 (*Cheiracanthium* spp.), OTU 63 (Polyxenida) and OTU 64 (*Myrmarachne*). At the same time, it is positively associated with OTU 21 and OTU 28, both of which occurred at Suyian but not Kitengela.

Some of the community variation across collection locations and years is likely to reflect seasonal and spatiotemporal climatic variation, especially in rainfall. The 2012 and 2013 samples were collected at different times of year – March and August respectively – with the March sampling at the end of Kenya's relatively long dry season and the August sampling in the shorter dry period between the two main annual rainy seasons (Figure 3.19). Seasonal or year-to-year rainfall variation might therefore account for differences in abundance and composition in our data between the two years. Although the 2013 Kitengela and Suyian samples were collected at the same time of year, the rainfall regime differs between the two sites, with Suyian experiencing a third rainy period in the middle of the short dry season, i.e. just prior to the August sampling. This interaction between site and time of year might therefore explain a portion of the location effect in our dataset.

Some of the variation in location is also likely to reflect long term differences in the myrmecophile fauna at the Kitengela and Suyian locations. This in turn suggests that there might be more heterogeneity among different areas of *V. drepanolobium* savannah than has been recognized up to now, either reflecting site-to-site variation in environmental parameters or patchiness in the distribution of taxa. Indeed, recent work has uncovered significant differences in the relative abundances of the obligate ants on *V. drepanolobium* at different sites (J.Boyle, unpublished), suggesting more site-to-site variation than has previously been appreciated.

The clear variation among sites represents an excellent opportunity to assess mechanisms underlying community assembly and subsequent dynamics of myrmecophiles. For example, why are scale in-

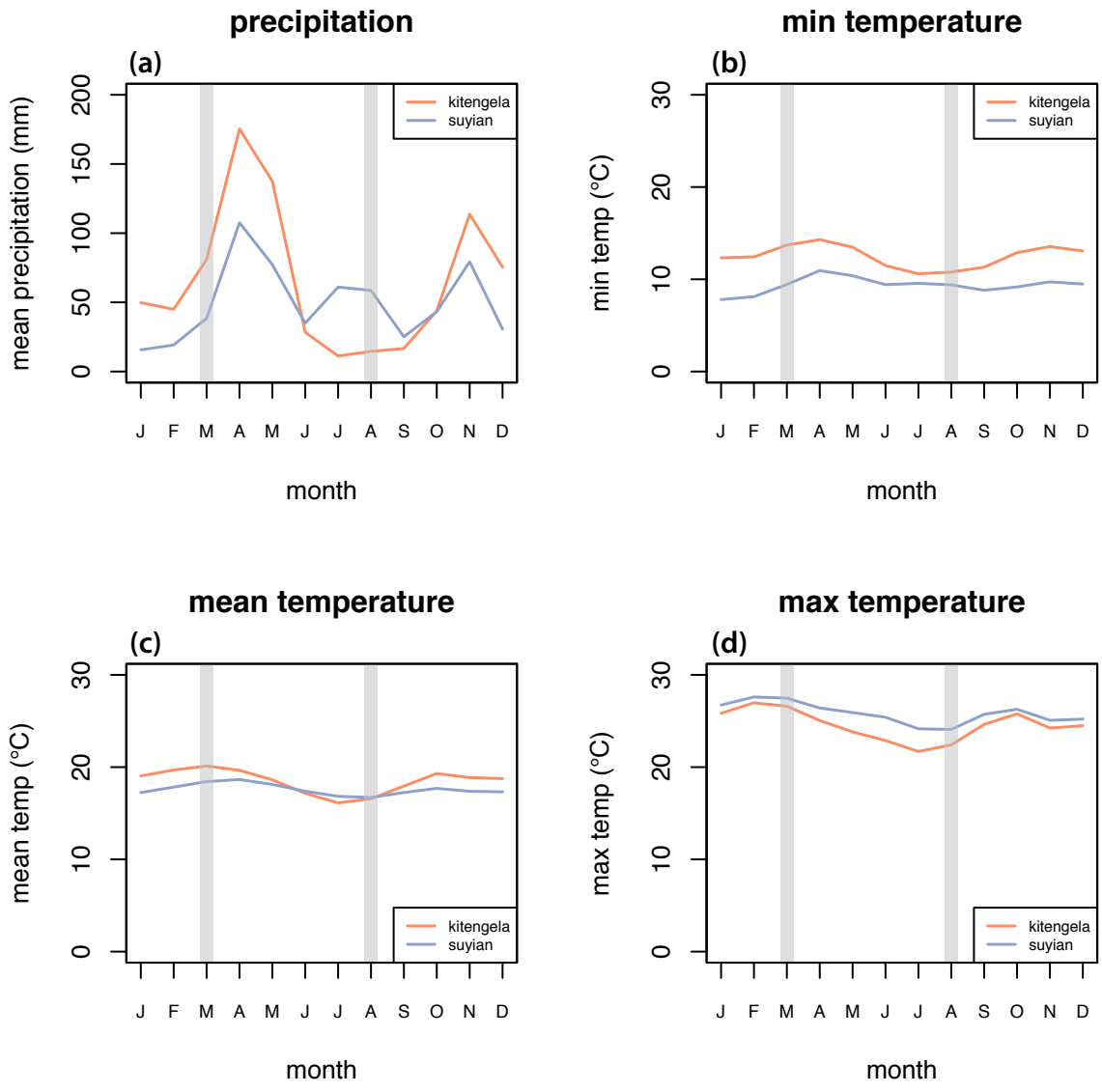

Figure 3.19: Climate differs between the Kitengela and Suyian sites. The rainfall regime at Kitengela is bimodal, but weakly trimodal at Suyian; and monthly temperatures are more variable at Kitengela than at Suyian. March and August sampling times (shaded bars) also represent different points in the annual cycle – March is hotter than August and comes towards the end of a drier period. Climate data taken from the WorldClim database[85].

sects so abundant at Suyian but not at Kitengela? The putative interaction – namely *C. mimosae* ants protecting scales feeding on *V. drepanolobium* as a food source for themselves – seems just as feasible at both sites. Is there something that makes this interaction infeasible or unnecessary at Kitengela, or does the explanation simply come from historical contingency? Are biotic or abiotic conditions unsuitable there for the scales? Interactions among myrmecophiles might also be responsible for some of the differences between sites. For example, the lepidopteran OTU 28 was found at Suyian but not Kitengela, while for OTU 27 the reverse was true. Our DNA sequence data indicate that these are closely related taxa, suggesting similar ecology. Could competitive exclusion between these OTUs explain their distribution at the two sites, or could their distribution point to relevant environmental differences between the sites?

Myrmecophile community variation with sampling regime

Although our collections were not designed to examine the effect of tree size or age, community differences between the sampling regimes raises the possibility of succession in the myrmecophile community as the tree grows and ages, since the subsampled trees were substantially larger than the fully sampled trees (approx 150cm vs 50cm). Our data show that myrmecophile communities on the larger subsampled trees overlapped with, but were not representative of, the smaller fully sampled trees from Kitengela in 2013. But the effect of the sampling regime itself does not appear to fully explain the difference between the two sets of trees: montecarlo resampling of the fully sampled trees did not remove the difference. While future collections from a range of tree sizes will be necessary to thoroughly examine community succession effects, the existing dataset suggests several candidate taxa for investigation.

One particularly promising set of taxa for further work is a small guild of spiders: OTU 64 (*Myrmarachne*), OTU 65 (unidentified Salticidae), and OTUs 71 and 72 (*Cheiracanthium* spp.). Among the Kitengela 2013 samples, OTU 72 appears to be relatively strongly associated with the set of large

subsampled trees, while OTUs 64 and 71 are more strongly associated with the set of small fully-sampled trees, and OTU 65 does not show an obvious preference. These associations are naturally reflected in the OTUs' co-occurrences, especially between OTUs 64 and 71 (negative association) and OTUs 64 and 72 (positive association). The association between OTUs 71 and 72 was negative but only marginally significant ($p \approx 0.06$). But underscoring these associations at Kitengela, OTU 72 (*Cheiracanthium* sp.) was also readily found with *T. penzigi* at Suyian, while the other three spiders were virtually absent at that location. What underlies the distribution of these taxa? Do the *T. penzigi* trees at Suyian share characteristics with the smaller trees occupied by other ants at Kitengela that favor OTU 72 but not the other spiders in this guild (e.g. low prey numbers)? Given their abundance, and the likely ecological similarities among the taxa (and especially between the two *Cheiracanthium* spp.), these spiders represent good candidates for closer examination.

Myrmecophile community variation with ant species

Our data showed clear differences in myrmecophile community abundance and composition among the three ant species. Myrmecophiles were consistently more likely to be present on *C. mimosae* trees and less likely to be present on *T. penzigi* trees, even after controlling for tree size and number of domatia. Isbell and Young found a similar result in their 2007 study, in which they counted larvae from 1-3 randomly chosen domatia per tree; *C. mimosae* domatia were more likely than *C. nigriceps* or *T. penzigi* domatia to contain myrmecophile larvae, although spider webs were found with all three ants[96]. The three ant species also tended to host different species of myrmecophiles, notwithstanding substantial variation among individual trees. The ant-associated variation in myrmecophile community composition appears stronger than variation of taxa living in the canopy of *V. drepanolobium* as assessed with fogging and beating methods and a morphospecies approach[109] – canopy communities associated with *T. penzigi* are distinctive but those associated with the *Crematogaster* ants are less well differentiated.

The increase in myrmecophile incidence from *T. penzigi* to *C. nigriceps*, and from *C. nigriceps* to *C. mimosae*, is particularly interesting in relation to the competitive hierarchy and patrolling tendencies of the ants. Among the three ants in our study, *C. mimosae* is regarded as the most aggressive, and competitively dominant over the other two species[158], while *T. penzigi* appears least reactive to disturbances on their trees. These differences in aggression appear to translate into large herbivore deterrence, with the more aggressive ants providing greater deterrence[124]. However, any benefits for the tree from large herbivore deterrence need to considered in the context of other costs and benefits – especially if more aggressive ants also tend to be better defenders of associates like scale insects[31,237], which represent a cost to the plant. Our data show that differences in myrmecophile communities among the ants are not just restricted to scale insects, but rather are manifest across numerous taxa, including many of the most abundant. These results underscore the importance of expanding our view of each ant species to encompass its set of characteristic associates, as it is the combination of the ants and their myrmecophile that make up the full 'extended phenotype'[42] of the ants.

The mechanisms by which the ants affect their myrmecophile communities remain unknown. Some myrmecophiles may show an affiliation for ant species that they are better adapted to living with, e.g. through mutualism or mimicry, or conversely may avoid ant species that are more likely to be aggressive. The ants may also shape their myrmecophile communities indirectly. For example, *C. mimosae* trees might on average have canopy foliage that is more attractive to herbivores that either are themselves myrmecophiles or attract predatory myrmecophiles.

Associations between myrmecophile taxa and ant species

Associations between ants and specific myrmecophile taxa in our dataset range from highly specific and reliable connections, through loose associations only visible at larger sample sizes, to taxa that appear to be true generalists. Our quantitative data allow the identification of these weaker associations that may have gone unnoticed in more casual observations. But what accounts for this range

of specificity?

Where a myrmecophile taxon tends to be found with some ant species more than others, it may be conceptually useful to consider this association between ant species and myrmecophile abundance to arise from two sources. Firstly, direct associations arise where a myrmecophile is better adapted to some ant species than to others, e.g. chemical mimicry that works for one ant but not for another. In this case, the tightness of the association between ant and myrmecophile will depend on the specificity of the adaptation and the extent to which it is obligate versus facultative. Secondly, indirect associations arise where a myrmecophile does better on some trees than others, and the characteristics that make a tree better or worse are correlated with the ant species, e.g. the myrmecophile eats on insect herbivores which are more abundant with some ants than others, perhaps due to systematic differences in tree health. In this case, the tightness of the association between ant and myrmecophile will depend on the importance of the tree characteristic in question for the myrmecophile, as well as the tightness of the correlation between the characteristic and the ant species. In reality, of course, these two sources may not be completely distinct – for example, a myrmecophile seeking a particular tree characteristic may use ant species as a cue, and may still need specific adaptations to escape detection by the ants.

Two high-abundance taxa show particularly clear ant-associations. OTU 82 (scale insects, likely *Hockiana* sp.[176]) was found commonly and almost exclusively with *C. mimosae*, though only at Suyian. This association, in which *C. mimosae* defends the scale insects for food, is well known[88,158,237], though the strong variation in abundance between locations is an aspect that deserves further attention. A less well recognized but equally tight association is that involving the tortricid OTU 3 (*H. griseana*, likely an obligate associate of *V. drepanolobium*[2,3]), found reliably with *C. mimosae* and *C. nigriceps*, and only rarely with *T. penzigi*. While the diet of *H. griseana* remains unknown, we speculate that the tortricid eats the ant brood; after removing such a tortricid from its domtium and presenting it with

an ant larva, we observed the tortricid biting into the ant (M. Whitaker, pers. obs.). However, the reason for specializing on the two species of *Crematogaster* is less clear. In any case, the high abundances of these scales and tortricids means that they are easily found and likely to have biologically important effects, making them excellent candidates for more targeted investigation into their ecology and distribution.

On the other hand, some taxa show little ant association and are likely generalists with respect to their ant hosts. For example, the moderately abundant gelechiid moth OTU 25 (*Dichomeris* sp.) and the two *Cheiracanthium* sp. spiders OTU 71 and OTU 72 were found with all three ants, though OTU 72 shows some greater association with *T. penzigi* – especially at Suyian – possibly reflecting a tendency to be found on smaller trees than its congener OTU 72. More surprisingly, we found little evidence of ant specialization in the salticid OTU 64 (*Myrmarachne*), which is an extremely convincing visual mimic of *C. mimosae* ants (Figure 3.20). It is unclear why selection should favor close mimicry of a specific ant if the spiders are not found preferentially with *C. mimosae*. Perhaps the spiders benefit from deceiving predators such as birds who recognize it as a legitimate aggressive domatium-dwelling ant without noticing that it differs from other ants on the tree, which would immediately give away its true identity.

In between these two extremes, several myrmecophile taxa showed weaker evidence for ant association – either because of weaker specialization or a limited number of observations. OTU 63 (Polyxenida) and OTU 85 (snails), for example, were found with all three ants, but were found more commonly and in higher numbers with *T. penzigi*. OTU 65 (Salticidae sp.) likewise occurred with all ants, but more often with *C. mimosae* and *T. penzigi*. Diptera and Coleoptera were mostly found with *C. mimosae* and *C. nigriceps* colonies although individual taxa were not abundant enough to assess separately. The small number of Blattodea in our dataset were also found only with *C. mimosae* and *C. nigriceps*, in line with previous findings that Blattodea were relatively common with *Crematogaster*[109].

Figure 3.20: The myrmecophile spider OTU 63 (*Myrmarachne*) (left) is an good visual mimic of *C. mimosae* ants (right), but surprisingly was not found to be any more closely associated with *C. mimosae* than with either *C. nigriceps* or *T. penzigi*.

Lepidoptera, aside from OTU 3 (*H. griseana*), showed a mix of putative ant associations. OTU 12 (Pyralidae), OTU 15 (Tineidae), OTU 24 (Gelechiidae), and OTU 26 appear relatively *T. penzigi*-associated. Meanwhile, OTU 19 (Geometridae), OTU 21, OTU 27 and OTU 28 may tend to be associates of *C. mimosae* and *C. nigriceps*. The lycaenid taxa OTUs 16 and 17, thought to be *C. mimosae* associated[125], were in fact found also with *C. nigriceps*. However, in the 2014 dataset (not included in the analysis here), most examples were found with *C. mimosae*, suggesting that such association with *C. nigriceps* is relatively unusual, or perhaps that those trees had been subject to turnover of the ants.

Many Hymenoptera were found with *C. mimosae* and *C. nigriceps* ants colonies. These included several chalcid wasps, ichneumonids and braconids that are likely to be parasitoids of lepidoptera living in the domatia. (Indeed, a number of our hymenopteran sequences came from specimens of lepidopteran pupae and larvae that we did not realize had been parasitized until we received the barcode data.) The preferential association of these taxa with *C. mimosae* and *C. nigriceps* might thus be driven in part by the relative abundance of taxa such as OTU 3 (*H. griseana*) with those ants. The braconid OTU 46 (*Trigastrotheca laikipiensis*), reported to be a claustral brood parasite of *C. mimosae* and *C. nigriceps*[169], was indeed not observed with *T. penzigi*; however ant associations were not well

documented for this species, as many came from trees with multiple foundresses.

The high abundance and strong ant-association of OTU 3 (*H. griseana*) accounts for many of the non-random pairwise co-occurrence measures among the myrmecophiles (Figure 3.18). The *Crematogaster*-associated OTU 3 appears negatively associated with several other taxa that are found with all three ants, including OTU 25 (*Dichomeris* sp.), OTU 64 (*Myrmarachne*) and OTU 71 (*Cheiracanthium* sp.), or that are found relatively often with *T. penzigi*, including OTU 15 (Tineidae), OTU 24 (Gelechiidae), OTU 26, OTU 63 (Polyxenida) and OTU 71 (*Cheiracanthium* sp.). On the other hand, it shows a positive association with the lepidopteran OTU 28 and the chalcid wasp OTU 30, both of which were only found with *C. mimosae* and *C. nigriceps* ants.

Within-taxon genetic differentiation

Our detection of at least some genetic differentiation between our sampling locations in two lepidopterans, two spiders and a millipede makes intuitive sense. Our sampling sites are some 210km apart and we might thus expect some degree of isolation by distance. Conversely, distances within each site are in the order of meters to hundreds of meters, and dispersal over these distances seems unlikely to be seriously impeded. The apparent lack of genetic differentiation between individuals on trees with different ant species is also perhaps unsurprising. Any such differentiation is unlikely to arise from spatial isolation – the trees occupied by the different ants occur in the same areas, such that dispersal between trees occupied by different ants is unlikely to be seriously hampered. It is also unlikely to be fostered by vertical transmission, since new ant colonies are founded by single foundresses rather than by colony fission. Therefore, any differentiation would likely arise from preferential association between particular myrmecophile genotypes and specific ant species. However, if there were some advantage to preferential association, we would only see this in our dataset if the lineages have not yet sufficiently diverged, i.e. if the preferential association has arisen recently and/or if the preferential association is weak. Once the lineages have diverged sufficiently, then they would ap-

pear in our dataset as two ant-specialized OTUs. Although we have many individuals in our dataset, most taxa are relatively rare, limiting our ability to detect specialization and thus to test for phylogenetic pairs of specialized species. A larger myrmecophile dataset may yet reveal evolutionary patterns of specialization.

Conclusions

The long-lived stable environments of ant colonies not only facilitate the ecological success of ants, but also represent unique habitats for communities of specialized 'myrmecophiles'. Despite a large literature on the natural history of myrmecophiles, few datasets provide quantitative data on myrmecophile communities, leaving a gap in our understanding of myrmecophile abundances and interactions, and their ecological importance. The data that do exist suggest that the taxonomic distribution of myrmecophiles varies substantially between ant species, e.g. among the doryline army ant *Eciton burchellii*[175], the formicine red wood ant *Formica rufa*[159], the ponerine army ant *Leptogenys distinguenda*[235] and the camponotine weaver ant *Camponotus* sp. aff. *textor*[161]. Future meta-analyses and quantitative studies of myrmecophile communities across ant species from a variety of habitats, geographic locations, and with a variety of lifestyles, will be invaluable to identify broader patterns that are difficult to identify within any single ant species.

This study identifies over 80 myrmecophile taxa in domatia on *V. drepanolobium* trees occupied by 3 species of obligate ant mutualist. Some are rare, but others are highly abundant. While many trees had no or few myrmecophiles, some had extremely high numbers; however, our focus on small trees almost certainly underestimates myrmecophile incidence on typical trees. Myrmecophiles were most commonly found with *C. mimosae*, and least commonly with *T. penzigi*; moreover, myrmecophile community composition varied significantly with ant species. As *C. mimosae* is usually regarded as the most mutualistic of these ants, being the best defenders of the trees against large herbivores[158], this finding underscores the importance of incorporating myrmecophiles into our understanding of

this ant-plant system. The composition of the myrmecophile community also varied substantially between collection locations and years, suggesting that the *V. drepanolobium* ecosystem might show more spatial and temporal biotic variation than has previously been appreciated.

Our community-level findings on the *V. drepanolobium* myrmecophiles suggest several promising directions for future inquiry.

First, sampling across additional locations at multiple time points would improve our understanding of spatiotemporal variation in myrmecophile communities. Sampling from a wider range of tree sizes will be useful for exploring community succession. Recording additional environmental parameters (e.g. foliar nitrogen content, canopy density) and examining spatial information (e.g. proximity to termite mounds) will help identify additional myrmecophile community determinants. This in turn will be invaluable for improving our understanding of variation within the *V. drepanolobium* system.

Second, collecting and identifying additional life stages, and connecting those samples to existing datasets such as this one using sequence data, will substantially increase the value of both the existing natural history knowledge and this community ecology work. Our natural history understanding is likely to benefit from a quantitative picture of the ecological context in which the organisms live. At the same time, DNA barcoding can help identify the juvenile stages of organisms that have only to date been identified from adults. However, current sequence databases are sparsely populated, frequently failing to provide a good match even to insect family.

Third, it would be useful to examine the myrmecophile-ant interactions more closely. How do the myrmecophiles employ mimicry, physical protection or mutualism to bypass the ants' defenses? If ants are so good at defending their nests against intrusion against conspecifics, how is it that some myrmecophiles can associate with multiple ant species? How are the myrmecophiles distributed at a fine spatial scale over the tree? For example, alates tend to be located in more distal domatia, while

workers and brood tend to be located on higher branches[96]. Which taxa share domatia with the ants, and which are more peripherally associated with the colony? We might expect this to shed some light on the myrmecophiles' life histories – e.g. with ant parasites and mutualists located in higher and more distal domatia, and commensals distributed more uniformly or in the proximal domatia. This information is likely to help in further parsing the myrmecophile community data – we might, for example, expect myrmecophiles found in domatia with ant workers and brood to show tighter associations with ant species than those found in domatia that are not actively ant occupied.

Finally, what are the ecological roles played by the myrmecophiles? We might, for example, use stable isotope data to help uncover trophic interactions. In combination with quantitative information on how ants structure the myrmecophile community, this is likely to shed useful light on the roles of these myrmecophiles as part of the ants' extended phenotypes.

Chapter 4

Ants that defend against mammalian herbivores harbor insect herbivores in domatia of the African ant-plant *Vachellia (Acacia) drepanolobium*

4.1 Abstract

Three different ant species live obligately in domatia of the African ant-plant, *Vachellia drepanolobium*. Dozens of other arthropods, or 'myrmecophiles', also inhabit the domatia alongside the ants, with different myrmecophiles tending to accompany different ants. However, the roles of these mymecophiles remain largely unknown, although it seems likely that some may be more consequential than others. Using carbon and nitrogen stable isotope analysis, this study shows that 9 lepidopteran and 1 dipteran myrmecophiles are herbivorous on the host plant; in addition, 8 spider and 2 hymenopteran myrmecophiles are predators or parasitoids, and up to 30% of their carbon comes from prey that is not herbivorous on the host plant. The *Crematogaster mimosae* and *C. nigriceps* ants themselves appear to be herbivorous and are likely deriving much of their diet from extrafloral nectar or scale insect exudates. The herbivorous myrmecophiles in this study tend to be more abundant with *C. mimosae* and *C. nigriceps* ants than with the third obligate ant associate, *Tetraponera penzigi* – even though the *Crematogaster* ants are more aggressive and usually thought to be better mutualists for their host plant. These results highlight the need to take into account indirect effects of the ants on their host plants when evaluating the roles of the different ants in the mutualism.

4.2 Introduction

Ant-plant protection mutualisms are widespread, especially in the tropics[27,83,98,177]. In these symbioses, ants protect their host plants from herbivory in exchange for housing (e.g. domatia) and food (e.g. extrafloral nectar and food bodies). Such mutually beneficial arrangements have evolved repeatedly, and consequently are found in more than 100 angiosperm genera and over 50 ant genera[34,39,219]. The exchanges vary widely, from tight obligate relationships to diffuse facultative associations.

The nutritional ecology of arboreal plant-ants contrasts with that of non-arboreal ants. Though ants are often regarded as predatory[60], the biomass of arboreal ants often far exceeds what could be supported by available prey, leading John Tobin to point out that perhaps a great many canopy ants might be herbivorous[208,209]. Plant ants are thus often functionally herbivorous, relying on food supplied by their host plants or on exudates from insects feeding on plant fluids[40]. Where ants consume plant-supplied food, such as extrafloral nectar or food bodies, this may give host plants some scope to preferentially attract better ants mutualists or to incentivize better defense when necessary by manipulating the quantity or nutritional composition of the food[104]. Where ants rely on exudates from insects such as hemipterans, the ants often protect and herd the insects in a close symbiotic relationship. More recently, research has focused on fungi cultivated by plant-ants as a food source[20,44], and preliminary studies of plant-ant associated bacteria have identified putatively nitrogen-fixing taxa[54], in line with results for gut bacteria in other arboreal ants[130,184].

In most ant-plant systems, the fitness interests of the ant and plant are broadly aligned. Many ant-plant symbioses probably evolved as byproduct mutualisms, in which ants happened to visit plants in search of food such as herbivorous insects, and plants benefited from further attracting those ants with shelter and additional food[126]. Once established, many ant-plant symbioses have likely been maintained through partner fidelity feedback, in which each party's fitness is tied to the fitness of

the other. Ants have little incentive to shirk their duties patrolling their hosts, as herbivore damage to the plant might also endanger the ant colony; likewise, plants have little incentive to reduce their investment in the ants, as a weaker ant colony might render the plant vulnerable to herbivory.

But some plant ants manipulate their hosts while at the same time defending them from herbivores. *Allomerus* ants, for example, temporarily sterilize their *Cordia nodosa* host plants in order to direct more plant resources to ant housing[238]. *Crematogaster nigriceps* prunes axial buds of its host plant, again temporarily sterilizing the host and also redirecting plant growth to minimize contact with neighboring plants that may host a different ant species or colony[198]. Such manipulations appear to enhance the fitness of the ants at the short term expense of the plant. It is less clear whether the plant's fitness is reduced or enhanced in the long term – either through partner fidelity feedback, or because the forced short term reallocation of plant resources from reproduction to growth actually enhances long term reproductive success.

Exploitation and parasitism of ant-plant mutualisms

Ant plant symbioses are also vulnerable to exploitation by unrelated ants. While partner fidelity feedback keeps mutualistic ant partners acting cooperatively, there is no such constraint on an unrelated ant, since its fitness is not – at least initially – tied to the fitness of a particular host plant. *Camponotus planatus* and *Pseudomyrmex gracilis* ants, for example, provide relatively little defense to their neotropical *Vachellia* host plants, and are not evolutionarily derived from the plants' usual *P. ferrugineus* ant mutualists[82,172]. Ant plants may thus associate with multiple ants that vary in quality as partners, raising interesting evolutionary questions about how such mutualisms are maintained, and ecological questions about how multiple ant species can coexist in ostensibly overlapping niches[27,156].

Ant plant mutualisms may also be parasitized by species other than ants. The salticid spider *Bagheera kiplingi*, for example, consumes Beltian food bodies from its *Vachellia* ant-plants[134]. *Phyllobaenus* beetle larvae both feed on the brood of *Pheidole* ant mutualists of *Piper* ant plants and also deceive the

host plant into producing food bodies that the beetle larvae consume instead[115].

But other attendees may be commensal or beneficial to the ant-plant mutualisms. Sap-sucking insects such as scales and aphids, for example, appear to be an important feature of some ant-plant mututalisms[165]. By tapping into plant resources and effectively directing those resources to the ants, they may relieve the ants' nutritional constraint, and thus facilitate the mutualism without the plant having to produce specialized food sources. Attendees such as predators or parasitoids of herbivores may raise the fitness of the ant and the plant by augmenting the ant defense and removing herbivores that would otherwise harm the host plant[182].

Study system

Vachellia (formerly *Acacia*) *drepanolobium* is an East African ant-plant that dominates the black cotton savannahs where it occurs[237]. It associates with four ant species: *Crematogaster mimosae*, *C. nigriceps*, and *Tetraponera penzigi* nest obligately in the many hollow, swollen stipular thorns known as 'domatia' that cover the trees; *C. sjostedti* more commonly nests in cavities bored by cerambycid beetle larvae in the trunk or in the ground around the bases of the trees[88,158]. In addition to housing, the host plant provides food in the form of extrafloral nectar secreted from glands near the base of leaves. Each tree is generally occupied by a single ant colony (i.e. a single species), but neighboring trees often host different species[158].

In exchange for housing and food, the ants protect their hosts from mammalian herbivores such as giraffe, rhinoceros, antelope and goats[119,199]. However, the ants vary in the quality of their defense[88,154]. Among the domatium-dwelling ants, the aggressive *C. mimosae* provides better defense than *C. nigriceps*, while *T. penzigi* provides little deterrence to large herbivores[124]. In addition to their patrolling behavior, closer scrutiny also reveals other differences in their interactions with their host plants. *C. nigriceps* prunes the plant's axillary buds, shaping growth and temporarily preventing flowering, while *T. penzigi* prunes the extrafloral nectaries, perhaps to reduce the risk of invasion by

another ant species[151,198]. Plants live longer than ant colonies in this system, and long-term demographic data and modeling indicate synergistic (i.e. non-additive) effects when a plant hosts different ant species over its lifetime[155], presumably arising as a function of these and other differences among the ants.

Numerous species of insects, spiders and even mollusks are found on *V. drepanolobium* trees along with the ants. Hocking, for example, recorded at least 44 species in actively ant-occupied domatia, and 24 in domatia without ants[88]. Monod and Schmitt list some 20 species, excluding ants, recorded by several authors[140]. Our own DNA barcode study identified over 80 species living in domatia (Chapter 3).

The abundance of these arthropods varies among the ants. Domatia occupied by *C. nigriceps* are less likely to contain myrmecophiles than those occupied by *C. mimosae*, and those occupied by *T. penzigi* less likely again[96] (see also Chapter 3). Invertebrates are more common on the branches and trunks of trees occupied by *C. mimosae* than on trees occupied by *C. nigriceps*[190].

The composition of the insect communities also varies among the ants. Canopy insect communities on trees occupied by *C. mimosae* or *C. nigriceps* differ from those on trees occupied by *C. sjostedti* or *T. penzigi*[109]. The composition of the domatium myrmecophile community also varies among the three ants (Chapter 3). Indeed many of the ant associates occur preferentially or even exclusively with one or more of the ant species. The lycaenid *Anthene usamba*, for example, is only found with *C. mimosae*[125]. *Hockiana insolitus* scales are likewise found with *C. mimosae*[176], and *Ceroplastes* scales with *C. mimosae* and *C. sjostedti*[237]. Trees occupied by *C. mimosae* are significantly more likely to be infested with *Acacidiplosis* gall midges than those occupied by *C. nigriceps*[190].

Several studies have described aspects of the diet or other natural history of species inhabiting the domatia. For example, the braconid wasp *Trigastrotheca laikipiensis* and the lycaenid *A. usamba* parasitize ant brood[125,169]. Larvae of the dipteran *Melanagromyza acaciae* develop in domatia after

oviposition in growing thorns[194]. *H. insolitus* and *Ceroplastes* scales presumably tap into plant sap and feed ants[125,237] (but see also Schneider *et al.*[188]).

For the most part, however, the roles played by insects in the canopy or myrmecophiles in the domatia of *V. drepanolobium* remain unknown. For example, the larvae of Lepidoptera such as tortricid moths occur in domatia, often protected by a silk tube or cap[2,3,4]. Are these larvae parasites of the ants, perhaps feeding on ant brood, or are they herbivores? If they are herbivorous, do the ants gain trophically – say by preying on the larvae or feeding on exudates – or are the larvae true parasites of the ant plant mutualism?

Stable isotope analysis

This study uses carbon and nitrogen stable isotopes to examine trophic relationships among *C. mimosae* and *C. nigriceps* ants, their *V. drepanolobium* host plants, and several myrmecophiles taxa that inhabit the domatia. These analyses involve quantifying the abundance of the ^{14}N and ^{15}N isotopes of nitrogen, and the ^{12}C and ^{13}C isotopes of carbon, in small samples of ant, myrmecophile and plant tissue. These data are expressed as ratios relative to a standard:

$$\delta^{15}N \equiv \left(\frac{{}^{15}N_{sample} / {}^{14}N_{sample}}{{}^{15}N_{standard} / {}^{14}N_{standard}} - 1 \right) \times 1000 \tag{4.1}$$

and

$$\delta^{13}C \equiv \left(\frac{{}^{13}C_{sample} / {}^{12}C_{sample}}{{}^{13}C_{standard} / {}^{12}C_{standard}} - 1 \right) \times 1000 \tag{4.2}$$

where multiplying by 1000 serves to express the difference from the standard in permil (‰).

An ant or myrmecophile's $\delta^{15}N$ and $\delta^{13}C$ values reflect the isotopic mix in its diet. For example, if a herbivore consumes two food plants, one with low $\delta^{13}C$ and one with high $\delta^{13}C$, then the herbivore's own $\delta^{13}C$ will tend to lie somewhere between the two, depending on the contributions of the

two plants to the herbivore's carbon budget. In our savannah study system, virtually all trees are *V. drepanolobium*[237] which has a low $\delta^{13}C$ of around $-28‰$, as is characteristic of C_3 photosynthesizers. The $\delta^{13}C$ of C_4 plants including many of the understory grasses[237] is typically around $-14‰$[122]. A herbivorous myrmecophile's $\delta^{13}C$ then largely reflects the relative contributions of C_3 and C_4 plants, which we might regard as a proxy for the host plant and other carbon sources respectively.

But a myrmecophile's $\delta^{15}N$ and $\delta^{13}C$ will also normally deviate slightly from its dietary sources – a phenomenon referred to as fractionation. Fractionation among herbivores and plant sap feeders varies widely due to variation in nutrient limitations and the relative contributions of assimilative and metabolic fractionation[213,234]. Among higher trophic levels, however, metabolic fractionation dominates, and rates of fractionation are more consistent (though with non-trivial variation[63,129,213]). Nitrogen shows a particularly strong trophic signal, with the lighter ^{14}N isotope preferentially excreted, leaving higher-order consumers enriched in ^{15}N by around 3 or 4‰ relative to their diets[22,40,138,207].. $\delta^{13}C$ typically shows a smaller increase of around 1‰ between trophic levels[213].

Stable isotope analysis thus provides information on ant and myrmecophile diet, without the need to observe feeding directly. It also has the advantage of integrating diet over time, thus accommodating dietary variation. Using this approach, our goals for this study were:

(i) to characterize trophic relationships among brood, workers and host plants, for both *C. mimosae* and *C. nigriceps*;

(ii) to characterize trophic differences between *C. mimosae* and *C. nigriceps*;

(iii) to identify likely trophic relationships for a set of domatium dwelling myrmecophiles.

This information in turn sheds light on the likely relationships among the ants, myrmecophiles and host plants, informing our understanding of the *V. drepanolobium* ant-plant mutualism.

4.3 Materials and methods

Sampling locations

We collected samples at our Suyian Ranch field site in Laikipia County, Kenya (0°31.3′N 36°43.3′E, 1880m asl; Figure 4.1). Collections took place in June 2014.

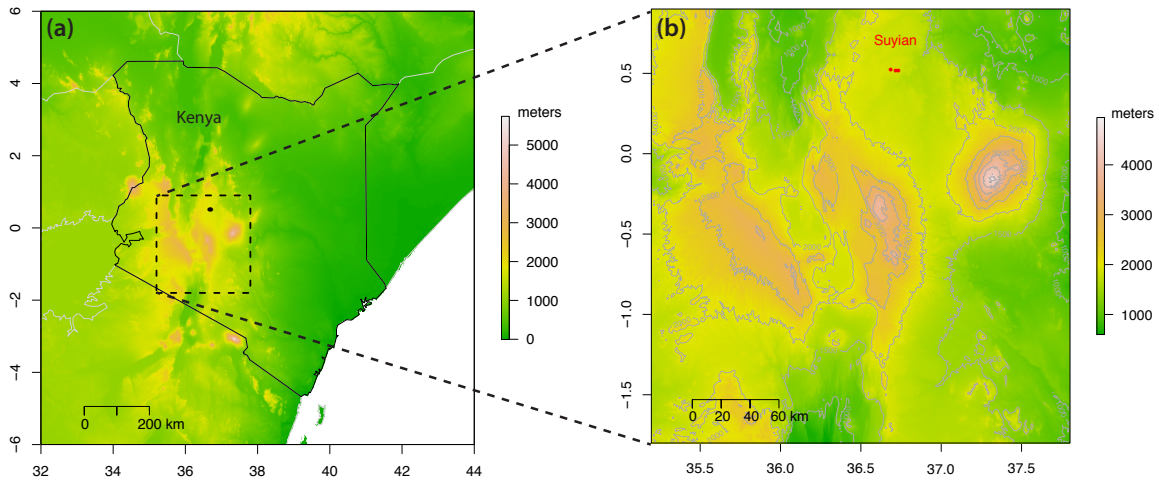

Figure 4.1: Samples were collected from Suyian Ranch in Laikipia County, Kenya

Sampling and sample storage

Trees were selected for sampling by haphazardly choosing trees approximately 1m in height with visibly active ant colonies. Only trees occupied by *C. mimosae* and *C. nigriceps* were selected for sampling, because sampling was conducted in conjunction with an unrelated project focusing on those ant species. Moreover, based on data from previous collections (see Chapter 3), in which the relative abundance of myrmecophiles with the ants was quantified, we expected to encounter many of the most abundant taxa with this sampling approach.

For each tree, we avoided older domatia towards the center of the tree, as these tended to contain

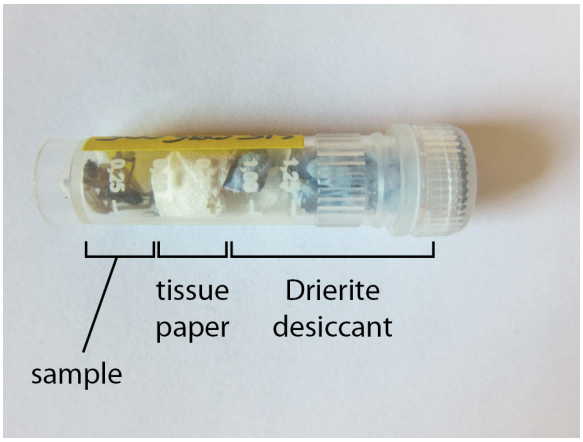

Figure 4.2: Sample storage method. Placing the sample in the tube first, below a loose wad of tissue paper and before the desiccant, simplifies changing the desiccant whenever necessary.

fewer myrmecophiles, and searched exhausitively or haphazardly through the remaining domatia. Trees that yielded no myrmecophiles were not recorded. Each myrmecophile found was placed in a 2mL Sarstedt screw cap micro tube with a small quantity of Drierite indicating desiccant (Figure 4.2; $CaSO_4$ desiccant; W.A. Hammond Drierite Co. Ltd, Xenia OH), since the ethanol preservation that we normally favor for preserving DNA can affect stable isotope signatures[207]. We dissected the gut from all lepidopteran larvae prior to storage, for a separate project; other myrmecophiles were stored whole. The Drierite was replaced as often as necessary as the samples dried out and the desiccant absorbed the moisture. For each tree with myrmecophiles, we collected samples of leaves (3-5 primary leaflets) and around 10 ant workers (and in many cases, ant brood) to help control for variation among host plants.

DNA barcoding and taxon delineation

In order to connect these samples to existing myrmecophiles samples (see Chapter 3), we extracted genomic DNA and sequenced the mitochondrial cytochrome oxidase I (COI) barcode region for each sample. While we have found COI barcoding to be useful for assessing myrmecophile diversity

in general, it was especially helpful for these samples, as the drying process altered the appearance of the samples, making identification difficult, and in some cases gut dissections rendered the sample unrecognizable.

Total genomic DNA was extracted from each sample using an AutoGen Gene Prep DNA preparation robot – essentially a phenol-chloroform extraction with ethanol precipitation. Samples were prepared for DNA extraction by macerating a small amount of tissue in extraction buffer, consisting of 200µL reagent M1, 200µL reagent M2 and 0.4mg Proteinase K. Samples were incubated at $55-60^{\circ}$C overnight. Samples were then processed in the AutoGenPrep 965 using the standard Mouse Tail protocol, with 2 DNA washes and resuspending in 50µL of buffer.

We amplified COI using standard barcode primers[61]:

```
LCO1490     5' - GGTCAACAAATCATAAAGATATTGG - 3'
HCO2198     5' - TAAACTTCAGGGTGACCAAAAAATCA - 3'
```

We performed PCRs using 12.5μL Omega BioTek MasterMix, 0.5mM $MgCl_2$ (additional to MasterMix), 0.5μM each primer, 1μL template and water to 25μL. PCR conditions followed a touchdown profile: 3 min at 94°C, followed by 20 cycles of 50 sec at 94°C / 40 sec at 49°C decreasing by 0.5°C per cycle / 80 sec at 72°C, followed by a further 20 cycles of 50 sec at 94°C / 40 sec at 48°C / 80 sec at 72°C, and a final 5 min at 72°C. We used BigDye Terminator v3.1 reactions and sequenced products on an Applied Biosystems Genetic Analyzer 3130xl.

After processing raw DNA sequence data using Sequencher v.5.1, sequences were added to those from previous myrmecophile collections (see Chapter 3) and clustered into putative species or 'operational taxonomic units' (OTUs) at 97% similarity, with a small number of manual adjustments especially for poor quality sequences. Where possible, we assigned taxonomic identifications to our sequence-based OTUs based on photographic records from our previous collections and on BLAST searches against the NCBI and BOLD databases.

Preparations for stable isotope analysis

Following DNA barcoding, myrmecophile samples were selected for stable isotope analysis from among the successfully identified samples – in particular, we excluded some samples from taxa that were over-represented in our collection. Leaf and ant samples were also selected for analysis so that, as far as possible, each myrmecophile could be compared to its own host plant and to ants from the same host. For a subset of host plants, ant workers and brood (mostly pupae) from the same plant were analyzed, in order to capture any potential variation across ant life stages.

Myrmecophile and plant samples were carefully weighed into tinfoil capsules (5×9mm capsules, Costech Analytical, Valencia CA) in preparation for stable isotope analysis. We used approximately 1mg of tissue for ant and myrmecophile samples, with ants from the same host plant pooled where necessary to make up the required mass; we used approximately 4mg of tissue for plant leaf samples, again pooling leaflets from the same plant as required. For ant worker and spider samples, we avoided using the gaster or abdomen in order to avoid influence of gut contents on the data[207], although this was not possible with the ant larvae; lepidopteran samples had already had their guts removed prior to preparation for stable isotope analysis.

Samples were sent to the UC Davis Stable Isotope Facility for analysis. Each sample was analyzed for carbon and and nitrogen isotopes with a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK). Throughout this paper, delta values for carbon are expressed relative to Vienna PeeDee Belemnite, and those for nitrogen relative to atmospheric air.

Analysis of stable isotope data

We compared the stable isotope data for ant brood, ant workers, and host plants to identify both relationships among brood, workers and plants , and overall differences among the sample types in $\delta^{13}C$

and δ^{15}N. We paid particular attention to differences between *C. mimosae* and *C. nigriceps* ants, since existing data on the ants have suggested different orderings[123,152]. We examined differences among myrmecophile taxa, ants and host plants in order to infer trophic relationships. Since host plant stable isotope values are strongly correlated with those of ants and myrmecophiles living on the plant, we used linear regression to control for variation among host plant. Finally, since spiders showed substantial variation in δ^{13}C, we implemented a simple two-member mixing model for spiders to estimate the proportion of their carbon budget derived from C_3 and C_4 plants. Statistical analyses were conducted using R[206].

4.4 Results

Sequencing and stable isotope sample summary

We obtained DNA barcodes from 180 myrmecophiles in 20 taxa. We had seen 16 of these taxa previously in our 2012-13 barcoding study (see Chapter 3), but 4 were not in that dataset. Sequence data are available through the NCBI and through BOLD, or directly from the authors.

After excluding some samples from over-represented taxa, we obtained stable isotope data for 115 myrmecophiles spread across the 20 taxa (Table 4.1). We also sent 49 ant worker samples and 47 plant leaf samples, such that 95 myrmecophiles were paired with both ant worker and leaf samples. In addition, we sent 24 samples of ant brood, of which 23 were paired with both ant worker and leaf samples, in order to examine variation between ant and host plant, and between ant life stages.

Relative abundance of taxa

Although our stable isotope dataset does not provide abundance information for individual species, we were able to obtain this information from our larger collection of myrmecophiles from 2012 and 2013 (see Chapter 3 for methods and discussion). The Lepidoptera in our stable isotope dataset were

on average more abundant with *C. mimosae* and *C. nigriceps* ants than with *T. penzigi* ants (Figure 4.3). The abundance of Araneae did not differ significantly with ant species.

Ant worker and brood stable isotopes

C. mimosae workers differed from *C. nigriceps* workers in δ^{13}C, though the difference was small (Figure 4.4). δ^{13}C was approximately 1.1‰ higher in *C. mimosae* workers than in *C. nigriceps* workers ($t = 3.9$, $df = 41.3$, $p < 0.001$). δ^{15}N did not differ significantly between the species ($t = 0.85$, $df = 43.1$, $p = 0.402$). Workers did not differ significantly from brood in either δ^{15}N or δ^{13}C in either ant species.

Both species showed higher δ^{15}N and δ^{13}C than their host plants. δ^{15}N was about 1.8‰ higher than host plants in both species (paired *t*-tests *C. mimosae*: $t = 6.17$, $df = 10$, $p < 0.001$; *C. nigriceps*: $t = 6.81$, $df = 11$, $p < 0.001$). δ^{13}C was about 2.1‰ higher in *C. mimosae* workers than in host plants (paired *t*-test $t = 12.7$, $df = 10$, $p < 0.001$), and about 1.8‰ higher in *C. nigriceps* than in host plants (paired *t*-test $t = 11.8$, $df = 11$, $p < 0.001$).

Interestingly, the host plants of *C. mimosae* showed δ^{13}C values around 0.8‰ higher than those of *C. nigriceps* ($t = 2.6$, $df = 43.5$, $p = 0.013$), though no difference in δ^{15}N ($t = 0.21$, $df = 41.1$, $p = 0.83$). *C. mimosae*-occupied plants also had slightly higher C:N ratios than those occupied by *C. nigriceps* ($t = 2.16$, $df = 39.6$, $p = 0.04$).

Matched samples of ant workers, ant brood and plant leaves showed strong linear relationships between the three sample types for both δ^{15}N and δ^{13}C (Figure 4.5). Slopes for all six regressions were significantly different from zero (δ^{15}N workers vs brood $t = 18.9$, $df = 22$; δ^{15}N workers vs plant $t = 10.4$, $df = 21$; δ^{15}N brood vs plant $t = 14.7$, $df = 21$; δ^{13}C workers vs brood $t = 8.1$, $df = 22$; δ^{13}C workers vs plant $t = 7.7$, $df = 21$; δ^{13}C brood vs plant $t = 9.9$, $df = 21$; all tests $p < 0.001$) and not significantly different from one. However, different intercepts among the regressions reflect the pairwise differences seen in Figure 4.4.

Table 4.1: Summary of samples by ant species (CM = *C. mimosae*, CN = *C. nigriceps*)

	Trees				
OTU	CM	CN	Order	Family	Species
0	2	5	Lepidoptera	Tortricidae	*Endotera cyaneana*
1	3	0	Lepidoptera	Tortricidae	*Endotera cyphospila*
3	12	14	Lepidoptera	Tortricidae	*Hystrichophora griseana*
11	1	0	Lepidoptera	UnknownLep1f	UnknownLep1s
17	16	1	Lepidoptera	Lycaenidae	Lycaenid1s
19	1	0	Lepidoptera	Geometridae	Geometrid1s
23	1	0	Lepidoptera	UnknownLep2f	UnknownLep2s
25	1	2	Lepidoptera	Gelechiidae	*Dichomeris* sp.
28	20	6	Lepidoptera	UnknownLep3f	UnknownLep3s
44	1	0	Hymenoptera	Braconidae	Braconid1s
47	1	0	Hymenoptera	Pompilidae	Pompilid1s
52	0	2	Diptera	UnknownDip1f	UnknownDip1s
65	7	4	Araneae	Salticidae	Salticid1s
67	2	0	Araneae	Salticidae	Salticid2s
69	3	0	Araneae	Clubionidae	*Clubiona*
71	1	0	Araneae	Eutichuridae	*Cheiracanthium*
72	0	4	Araneae	Eutichuridae	*Cheiracanthium*
74	1	1	Araneae	UnknownAra1f	UnknownAra1s
75	0	2	Araneae	UnknownAra2f	UnknownAra2s
76	1	0	Araneae	Gnaphosidae	Gnaphosid1s
ant workers	28	21	–	–	–
ant brood	12	12	–	–	–
plant leaves	27	20	–	–	–

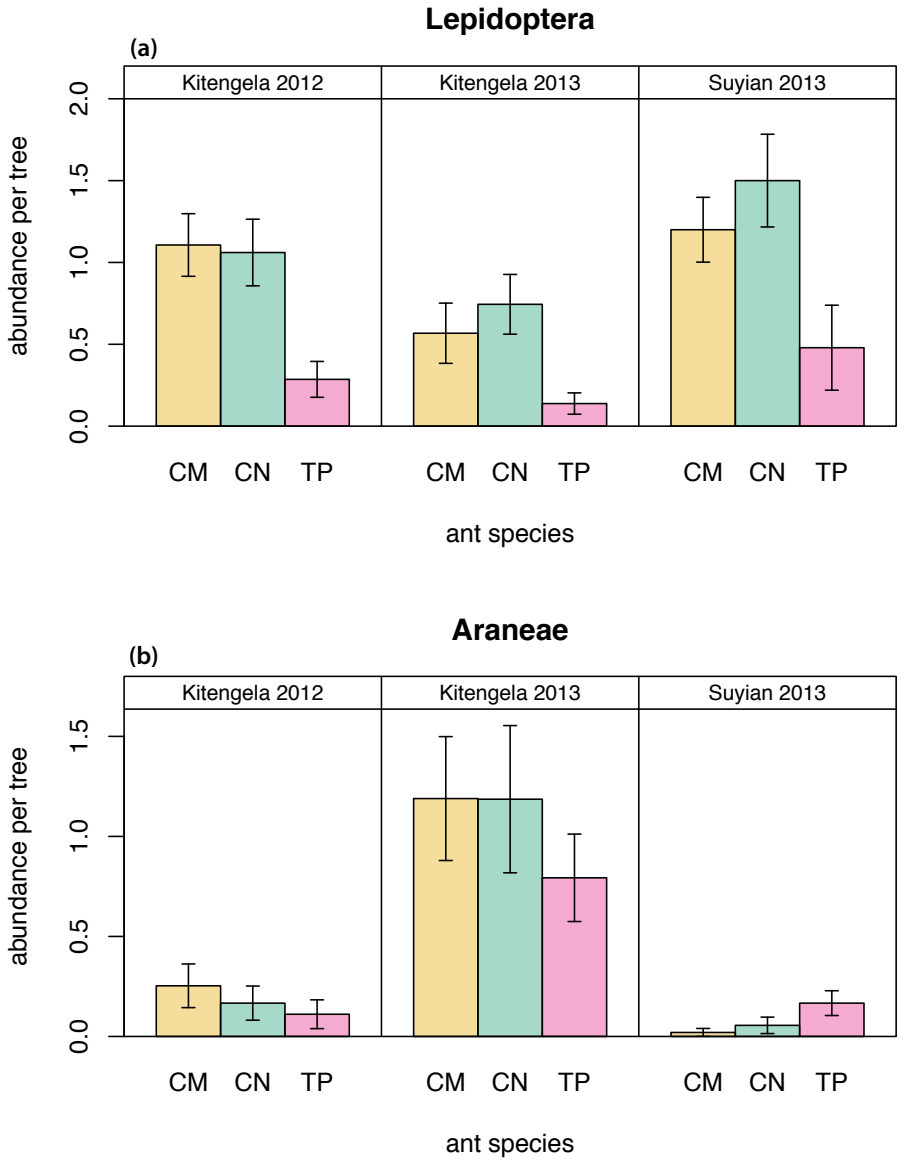

Figure 4.3: Lepidoptera were more abundant with CM and CN ants than with TP, while Araneae abundance was similar for the three ants. Figure shows per-tree abundance of Lepidoptera and Araneae taxa in the stable isotopes dataset, based on data from our previous myrmecophile surveys (see Chapter 3 for methods and analysis of those data). Error bars show standard errors.

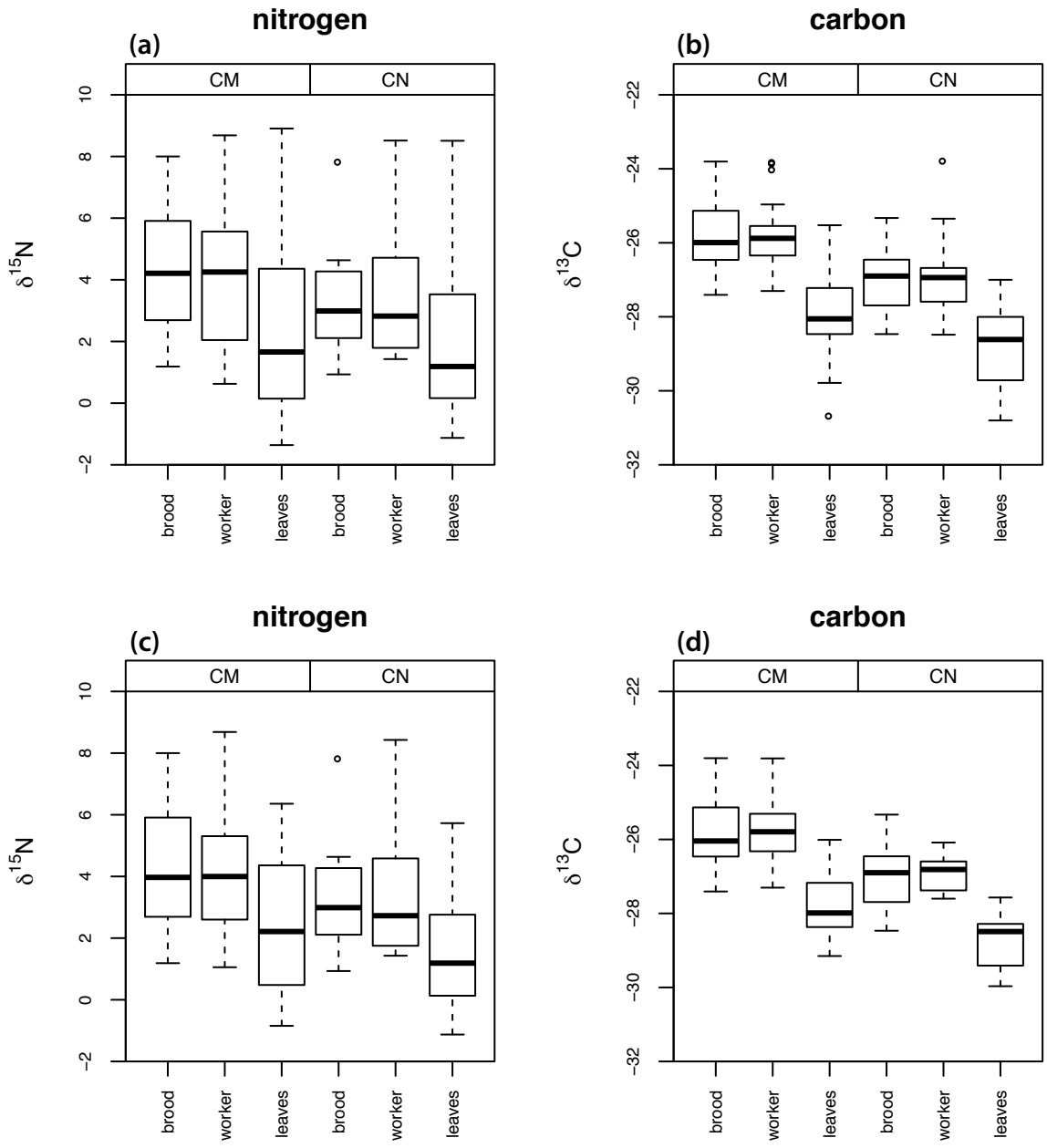

Figure 4.4: (a) and (b) Stable isotope values for ant brood (CM: $n = 12$; CN: $n = 12$), workers (CM: $n = 28$; CN: $n = 21$), and plant leaf samples (CM: $n = 27$; CN: $n = 20$). These panels use the full data set; workers, brood and leaves were not necessarily sampled from the same trees. (c) and (d) Stable isotope values for matched samples of ant brood, ant workers and plant leaves. Within each of the two and species, workers, brood and leaves all come from the same trees (CM: $n = 11$ trees; CN: $n = 12$ trees). CM = *C. mimosae*; CN = *C. nigriceps*.

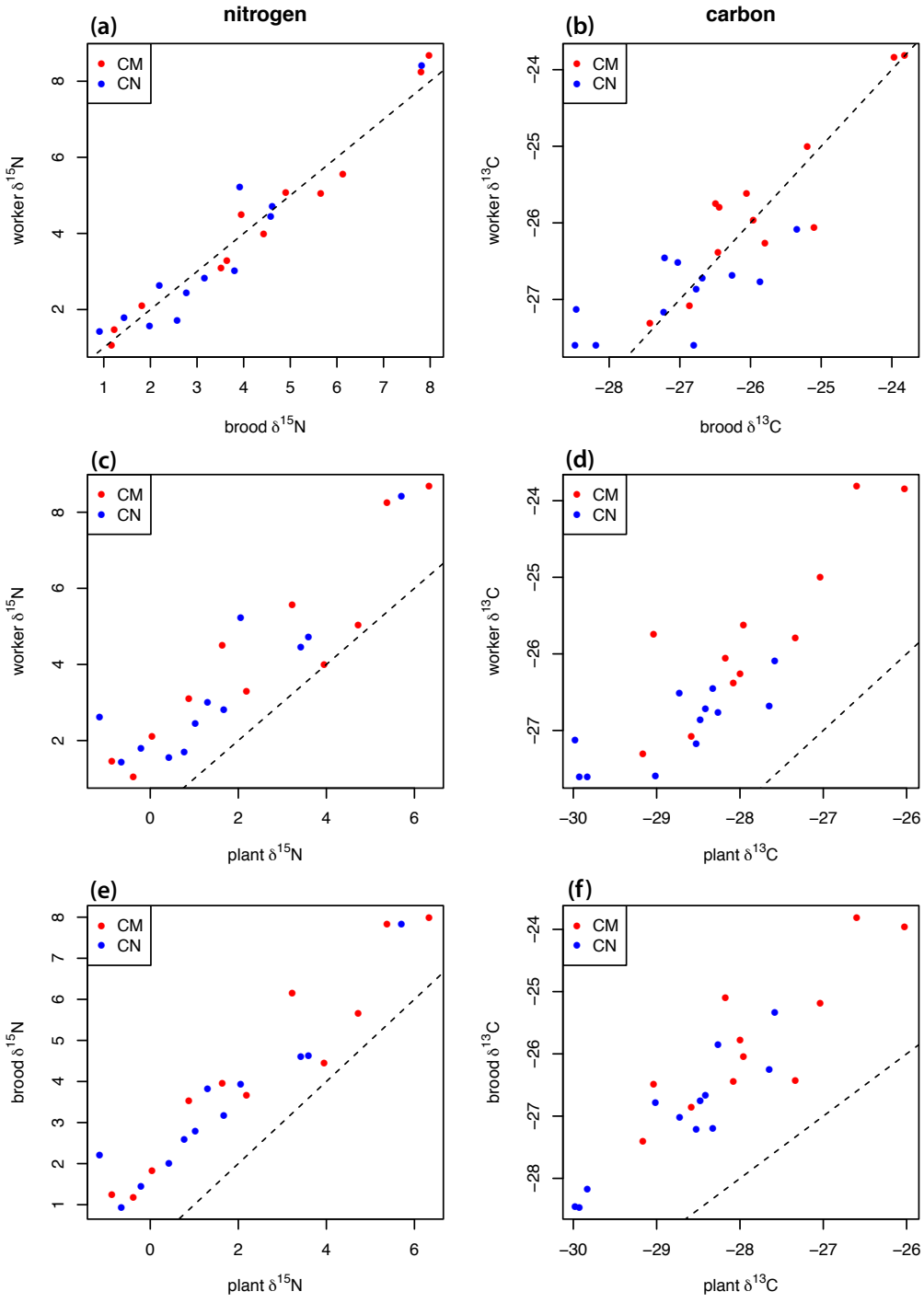

FIGURE 4.5: Stable isotope values for matched samples of ant brood, ant workers and plant leaves (CM: $n = 11$ trees; CN: $n = 12$ trees). For reference, dashed lines indicate equal x and y values. CM = *C. mimosae*; CN = *C. nigriceps*.

Myrmecophile stable isotopes

On average, our Lepidoptera samples showed δ^{15}N values around 0.7‰ higher than their host plants and δ^{13}C values around 1.4‰ higher than their host plants, placing them mostly between plants and ant workers (Figure 4.6). Spiders showed δ^{15}N values around 4.3‰ higher than their host plants, and δ^{13}C values around 4.0‰ higher, putting them substantially higher than the ant workers. Our Diptera samples showed similar δ^{13}C values to the Lepidoptera, but relatively low δ^{15}N; our Hymenoptera samples were placed close to the spiders. Among the spiders, OTUs 65, 67, 69 and 76 appear to cluster, with OTUs 72, 74 and 75 tending to show higher δ^{13}C values.

Covariation of stable isotopes with host plant

As with the ant brood and workers (Figure 4.5), δ^{15}N and δ^{13}C values covaried strongly in myrmecophiles and their host plants (Figure 4.7). However, the slope of the relationship between myrmecophile and plant stable isotope values was significantly greater for Lepidoptera than for Araneae. Regressing myrmecophile δ^{15}N on plant δ^{15}N and myrmecophile type (Araneae/Lepidoptera) showed a significant interaction between myrmecophile type and slope ($F = 6.7$, $df = 1, 91$, $p = 0.011$); the same was true for δ^{13}C ($F = 17.4$, $df = 1, 91$, $p < 0.001$).

We used linear regression to reduce the effect of host plant variability on myrmecophile and ant stable isotope values. For the myrmecophiles, we used OLS to estimate the model

$$Y_{ij} = \beta_{0j} D_j + \beta_{1j} D_j X_j + \varepsilon_{ij} \quad \text{for} \quad j \in \{\text{Araneae}, \text{Lepidoptera}\} \tag{4.3}$$

where Y_{ij} is the myrmecophile's δ^{15}N or δ^{13}C value, X_j is the host plant's δ^{15}N or δ^{13}C value, D_j is a dummy variable denoting the two sets of myrmecophiles, and $\varepsilon_{ij} \approx \mathrm{N}(0, \sigma^2)$. We then calculated corrected δ^{15}N and δ^{13}C values as

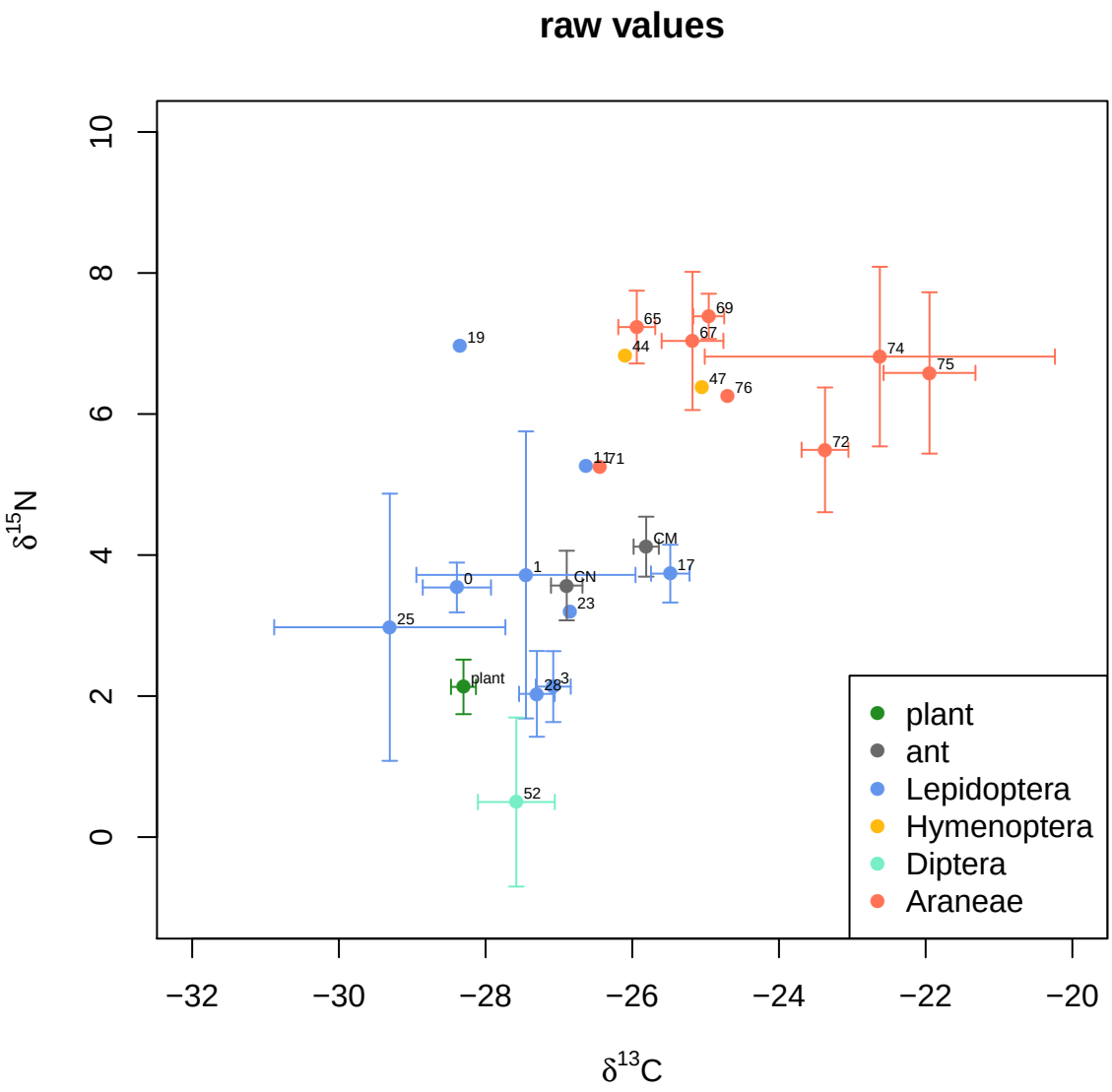

Figure 4.6: Stable isotope values for myrmecophiles, ant workers and and plant samples. Numbers adjacent to centroids denote OTU numbers. CM denotes *C. mimosae*; CN denotes *C. nigriceps*; plant denotes *V. drepanolobium* samples. See Table 4.1 for OTU numbers and sample sizes. Errors bars show standard errors.

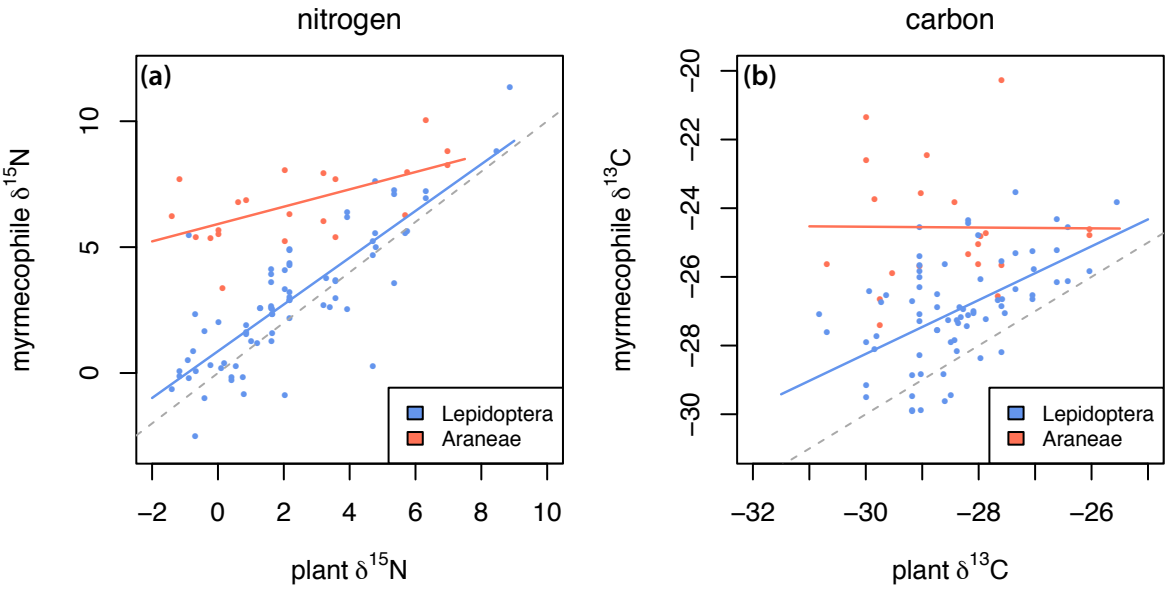

Figure 4.7: Stable isotope values for Araneae ($n = 21$) and Lepidoptera ($n = 74$) myrmecophile samples, plotted against values for leaves from each myrmecophile's host plant. Lines show predicted values from OLS regression. For both nitrogen and carbon, variation in the plant is associated with less variation in Araneae than in Lepidoptera.

$$\tilde{Y}_{ij} = \hat{\beta}_{0j} D_j + \hat{\beta}_{1j} D_j \bar{X} + \hat{\varepsilon}_{ij} \quad \text{for} \quad j \in \{\text{Araneae}, \text{Lepidoptera}\} \tag{4.4}$$

where the $\hat{\beta}_{0j}$, $\hat{\beta}_{1j}$ and $\hat{\varepsilon}_{ij}$ are our estimated parameters and residuals, and $\bar{X}$ is the mean plant δ^{15}N or δ^{13}C in our dataset (i.e. $\delta^{15}\text{N} = 2.13‰$ and $\delta^{13}\text{C} = -28.30‰$). For the purposes of model fitting, we treated the Hymenoptera samples as if they were Araneae, and the Diptera samples as if they were Lepidoptera, as their δ^{15}N and δ^{13}C values suggest trophic similarity, but we did not have sufficient data to estimates separate slopes for those groups. While we might ideally have estimated separate effects for each species, this was not feasible with our dataset owing to the small number of data points for several taxa. We fit a second, analogous model to the ant workers – i.e. with $j \in \{C.\ mimosae, C.\ nigriceps\}$ – and determined corrected δ^{15}N and δ^{13}C values in the same way.

The corrected values show stronger clustering of spiders and of Lepidoptera (Figure 4.8), providing stronger support for trophic similarity within each of those groups and trophic separation between them. The spiders, however, still show substantial variation especially in δ^{13}C. *C. mimosae* and *C. nigriceps* ants still show a significant difference in δ^{13}C after controlling for the effect of host plant ($F = 11.9$, $df = 1, 38$, $p = 0.0013$) but no significant difference in δ^{15}N ($F = 0.50$, $df = 1, 38$, $p = 0.48$).

A simple two-member mixing model for spiders

In our dataset, the mean δ^{13}C for Lepidoptera was -27.4‰, representing a roughly 1‰ enrichment over the C_3-photosynthesizing *V. drepanolobium* host plants at -28.3‰. *V. drepanolobium* comprises most of the trees in this system, but the understory comprises several species of grasses and herbs[237]. We would expect C_4 photosynthesizers in the understory to have δ^{13}C values of approximately -14‰[122]. Assuming similar carbon fractionation for herbivores of these plants as for Lepidoptera on *V. drepanolobium*, then these C_4 herbivores should have δ^{13}C values of approximately -13‰.

A spider that obtains a fraction θ of its carbon from C_3 herbivores, with δ^{13}C of around -27.4‰, and the remaining fraction $(1-\theta)$ from C_4 herbivores, with δ^{13}C of around ?13‰, will have a mean dietary δ^{13}C of

$$\delta^{13}C_{\text{spider diet}} = (-27.4)\theta + (-13.0)(1-\theta). \tag{4.5}$$

If spiders have similar carbon fractionation values to the herbivores, i.e. about 1‰, then the spider's own δ^{13}C will then be approximately

$$\delta^{13}C_{\text{spider}} = [(-27.4)\theta + (-13.0)(1-\theta)] + 1.0. \tag{4.6}$$

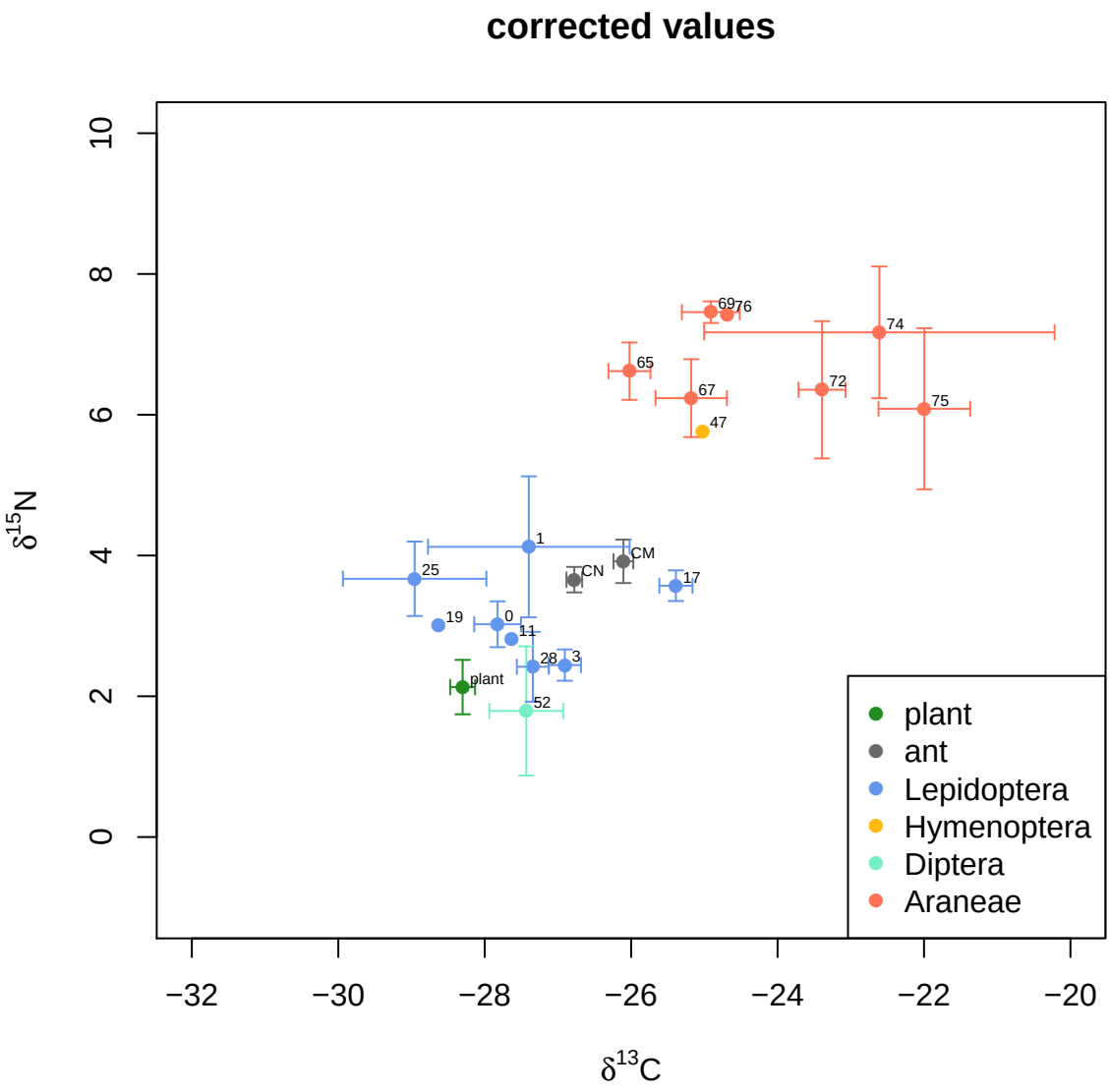

Figure 4.8: Stable isotope values for myrmecophiles, ant workers and and plant samples, standardized to the mean plant stable isotope values from our dataset (i.e. $\delta^{15}\text{N} = 2.13‰$ and $\delta^{13}\text{C} = -28.30‰$). Numbers adjacent to centroids denote OTU numbers. CM denotes *C. mimosae*; CN denotes *C. nigriceps*; plant denotes *V. drepanolobium* samples. See Table 4.1 for OTU numbers and sample sizes. Errors bars show standard errors. Note that some taxa present in the raw data (Figure 4.6) are absent due to missing plant stable isotope data.

Since we have empirical data on $\delta^{13}C_{spider}$, this allows us to estimate the fraction of carbon obtained from C_3 herbivores as

$$\theta = -\frac{12.0 + \delta^{13}C_{spider}}{14.4} \tag{4.7}$$

The estimated carbon contribution of C_3 herbivores ranges from around 1 for those taxa with the lower $\delta^{13}C$ values in Figures 4.6 and 4.8 to around 0.7 to those with the higher $\delta^{13}C$ values. These should be a good approximation of the carbon contribution of herbivores on the *V. drepanolobium*; it does not allow us to distinguish the host plant from other *V. drepanolobium* plants, but the host plant is likely to dominate, especially light of the correlation of spider and host plant stable isotope values.

4.5 Discussion

Ants are functionally herbivorous

Carbon and nitrogen stable isotope data indicate that the *C. mimosae* and *C. nigriceps* ants in our dataset, like many arboreal ants[40], are largely relying on a herbivorous diet sourced from their host plants. Extrafloral nectar is probably a major diet component, supplemented by scale insect exudates in *C. mimosae*. Data for ant workers and brood show no evidence of dietary shifts between life stages.

However, the two ant species are towards the top end of the range of $\delta^{15}N$ and $\delta^{13}C$ seen among the herbivorous myrmecophiles. Since extrafloral nectar is usually a poor nitrogen source, the ants likely supplement it with insect prey[181], or perhaps other detritus that is likely to have a similar isotopic profile to the host plant[87,163]. However, the ant stable isotope data suggest that the contribution of these other sources to the ants' diet is small. Another possibility is that plant tissues vary in stable isotope content and dietary differences – say between feeding on leaves, extrafloral nectar or insect exudates derived from plant phloem – may tend to separate the ants from the other herbivores. Consistent with this, Rudolph and Palmer report slightly higher mean $\delta^{13}C$ for extrafloral nectar com-

pared to our measurements for leaves, and slightly lower values for *C. nigriceps* fractionation, though the differences are small (EFN δ^{13}Cmean $\pm$ sd $= -26.96 \pm 1.62$ compared to our leaf samples from *C. nigriceps* trees $\delta^{13}C = -27.78 \pm 1.02$; *C. nigriceps* carbon fractionation was $\approx 1.4‰$ in Rudolph and Palmer[181] compared to 1.8‰ in our study).

Differences in stable isotope values between ant species

Our data showed no significant difference in δ^{15}N values between *C. mimosae* and *C. nigriceps*, and only a small difference in δ^{13}C, suggesting similar diets for the two ants. Palmer et al. also found limited differentiation between *C. mimosae* and *C. nigriceps* colonies located >10m from termite mounds, with values similar to those in our study[152] (see also Appendix C). Martins reports higher δ^{15}N in *C. nigriceps* than in *C. mimosae*, and higher δ^{13}C in *C. mimosae* than in *C. nigriceps*[123]. While these differences were statistically significant, values of δ^{15}N in that study showed an order of magnitude less intercolony variation than those in our study, despite comparable sample sizes, suggesting that multiple samples might have been drawn from colonies spanning multiple trees. (see Appendix C). On the other hand, Palmer *et al.* found that *C. mimosae* showed higher δ^{15}N than *C. nigriceps* among colonies <10m from mounds, and values for both species were elevated relative to colonies further from mounds ([152]; see also Appendix C). Given the tight coupling between ant and host plant stable isotopes, this may in part reflect higher plant δ^{15}N values closer to mounds[62]. But in addition, these results suggest that both *C. mimosae* and *C. nigriceps* may subsist on a functionally herbivorous diet when necessary, and in situations with better prey availability – such as near termite mounds – *C. mimosae* tends to adopt a more carnivorous diet than *C. nigriceps*.

Variation in plant stable isotope values

Part of the difference in δ^{13}C between *C. mimosae* and *C. nigriceps* was explained by differences in host plant δ^{13}C, but the two ant species were still significantly different after controlling for host plant

differences. But what explains the differences in plant $\delta^{13}C$ values between the ant species? One possibility is that the ants preferentially occupy trees with different characteristics such as size or age[237] that are reflected in $\delta^{13}C$.

On the other hand, it is also possible that the different ant occupants are responsible for differences in $\delta^{13}C$ in their host plants. Possible mechanisms might include, firstly, different ants modifying photosynthesis – a major source of carbon isotope variation in plants[122] – in a way that alters the plant's $\delta^{13}C$. Different ant occupants are known to have different effects on photosynthetic rate in *V. drepanolobium*, with *C. mimosae* and *C. nigriceps* occupied trees showing greater photosynthesis in the presence of browsing than *C. sjostedti* or *T. penzigi* occupied trees[105]. While this is insufficient to account for the difference in $\delta^{13}C$ between the ant species, since *C. mimosae* and *C. nigriceps* appear to have similar effects, it is possible that other aspects of photosynthesis are altered. Such regulation of plant metabolism is not unknown: aphid-colonized plants have higher $\delta^{15}N$ relative to uncolonized controls because of higher nitrate reductase activity in colonized plants[234].

A second possibility is that different ants draw on plant extrafloral nectar to different extents. Since different plant tissues and fluids may have different isotopic contents[35,79], differences in EFN flux will tend to be reflected in differences in plant $\delta^{13}C$. A similar sink function has been described for nitrogen e.g. for coccids feeding on *Euphorbia*[143].

A third possibility is that some nutrient flow occurs from ant to plant. While insects such as aphids may inject proteins in the course of feeding[233], in our case it seems most likely if nutrients including carbon can be absorbed through the domatium wall. Some other species of ant plant source nitrogen from inside domatia e.g. *Piper* gets small amounts of nitrogen from ants[59,178]. While carbon is usually readily available to plants as atmospheric CO_2, supplementing this supply with carbon from inside domatia might have advantages such as limiting water loss through exposed leaf stomata – potentially a substantial advantage in the xeric conditions at our field sites. The ant plant *Dischidia major*, for ex-

ample, obtains almost 40% of the carbon in its sac domatium leaves from ants[211]. While *V. drepanolobium* domatia are not modified leaves, and are therefore unlikely to have specialized structures such as stomata for taking in carbon, some plants are known to take in CO_2 from tissue surfaces without specialized structures, such as inside hollow stems[18], and this kind of uptake has been suggested in other ant plants[178]. Coupled with dietary differences among the ants that might generate differences in ant $\delta^{13}C$, such as feeding on non-tree-derived carbon sources, such nutrient update could allow the different ants to generate consistent differences in $\delta^{13}C$ in their host plants.

Myrmecophile trophic relationships

Stable isotope data indicate that all nine species of Lepidoptera in our dataset are herbivorous on their *V. drepanolobium* host plants. There is no evidence that they are preying on ant brood or on other myrmecophiles. On average, $\delta^{15}N$ values were around 0 to 2‰ higher than host plant leaves, while $\delta^{13}C$ values ranged from around 1‰ below host plants to around 2‰ above. These values are consistent with herbivory, and are further supported by the strong correlations between myrmecophile and host plant isotope values (Figure 4.7). OTU 17 (Lycaenidae) showed consistently higher $\delta^{13}C$ than other Lepidoptera, similar to ant workers, possibly reflecting dietary differences with other Lepidoptera, but no difference in $\delta^{15}N$ suggesting similar trophic position. One possible explanation is that most of the lepidopteran larvae consume leaf material, while the ant larvae are more likely to consume extrafloral nectar which may have a different stable isotope signature than leaves[11,35]; if ant workers feed lycaenid larvae (OTU 17) via trophallaxis, this would explain the elevated $\delta^{13}C$ in this species relative to other Lepidoptera.

Our results suggest that shelter is the primary benefit to Lepidoptera residing in domatia, rather than predation on ants. Consistent with this, many larvae and pupae are found within domatia but within a protective tube or below a tough membrane separating them from the ants[3]. When ant workers and lepidopteran larvae are placed together, ants typically attack and kill the larvae[3]. On

the other hand, we have also observed lepidopteran larvae attacking and consuming ant larvae when placed together (Whitaker pers. obs.). Taken together with the stable isotope data, these observations suggest that the lepidopteran larvae are herbivorous, but capable of attacking in response to a threat, e.g. from ants that have breached the larva's protective membrane. However, it is unclear how the membranes are constructed in the first place, nor how they might be enlarged by the larvae if necessary for growth. Agassiz reports sharp ovipositors on several species, including our OTU 0 (*Endotera cyaneana*), OTU 3 (*E. cyphospila*) and OTU 25 (*Dichomeris* sp.), and speculates that eggs may be injected into soft, growing plant tissue[3]. It is possible that larvae have some capacity for camouflaging themselves from ant workers while constructing or enlarging their protective membrane; but the stable isotope data suggest that they do not take advantage of any such camougflage to prey on ants.

All eight spider taxa in our dataset appear predatory and one trophic level higher than the Lepidoptera and ants. δ^{15}N was on average about 3.6‰ higher for the spiders than for the Lepidoptera – a difference roughly equal to the 3.4‰ step seen on average between consecutive trophic levels[22,138]. But δ^{13}C was more variable among taxa. Some taxa – e.g. OTUs 65, 69, 67 and 76 – showed δ^{13}C values 1 to 2‰ higher than the Lepidoptera, consistent with typical fractionation between trophic levels[213]. Other taxa – e.g. OTUs 72, 74 and 75 – showed δ^{13}C values up to 5.5‰ higher than Lepidoptera. A simple mixing model suggests that taxa towards the upper end of this range of δ^{13}C values may be deriving up to 30% of their carbon from C_4 plants (Figure 4.9), by consuming herbivores that are consuming understory grasses. The lower correlations between spider and host plant isotope values (Figure 4.7) compared to Lepidoptera are consistent with the host plant contributing less to spider diet. While our data do not allow us to identify prey species, the variation in the contribution of C_4 plants to the carbon budget suggest variation in prey species or foraging modes among the taxa, perhaps facilitating coexistence of these species.

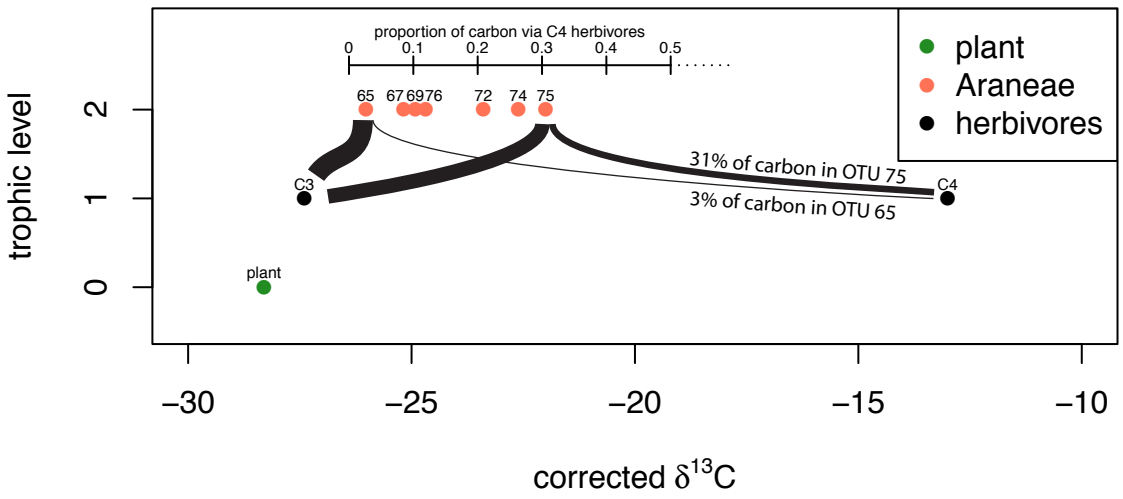

Figure 4.9: A simple two-member mixing model suggests that some spider taxa (e.g. OTU 65, Salticidae) derive virtually all of their carbon from prey that are herbivorous on C_3 plants, most likely the host plant. Others (e.g. OTU 75) derive up to about a third of their carbon from prey that are herbivorous on C_4 plants such as understory grasses.

Our dataset also included a small number of samples from an unidentified species of Diptera and two unidentified species of Hymenoptera – one pompilid wasp and one braconid wasp. Many pompilids and braconids are parasitoids and, consistent with this, $\delta^{15}N$ values for both taxa in our dataset placed them at the same trophic level as the spiders. The dipteran, on the other hand, showed similar stable isotope values to the Lepidoptera in our dataset, suggesting a herbivorous or detritivorous lifestyle.

Cheating, exploitation, parasitism and mutualism

Data from our previous myrmecophile collections indicate that the Lepidoptera shown here to be herbivores are more abundant on *C. mimosae*- and *C. nigriceps*-occupied trees than on *T. penzigi*-occupied trees (Figure 4.3). In contrast, *C. mimosae* and *C. nigriceps* are usually regarded as more aggressive ants, reflected in the ants' ability to deter large herbivores, and *T. penzigi* shows little reac-

tion to disturbances on its trees[124,158]. These higher abundances of herbivores, moreover, echo the distribution of plant-feeding scale insects with the more aggressive *C. mimosae* and *C. sjostedti* ants[237] (see also Chapter 3).

The abundance of the carnivorous spiders, however, did not differ significantly among the ants. Previous studies of *Pseudocedrela kotschyi* plants and their *Camponotus* spp. ants found that ants were negatively associated with spiders and hymenoptera that might have otherwise have contributed to reducing herbivory. We might likewise have expected more effective ant defense here to be partly offset by a reduction in spider abundance, but found no evidence[139].

Why are there more herbivores in the domatia of supposedly more aggressive ants? One possibility is that ant optimal behaviors trade off defense against insect herbivores with defense against mammalian herbivores. For example, if the marginal effect of insect herbivores on plant fitness is low for a plant that is already well defended against mammalian herbivores, and high for a plant that is poorly defended, then ants that are poor defenders against mammals should allocate more resources to defending against insects. In other words, timid ants may be good defenders against arthropod herbivores[67,114], even if they are poor defenders against mammals, and vice versa for aggressive ants. But the presence of insect herbivores could, alternatively, be a sign of diligent ant defenses if the herbivores are under bottom-up control: i.e. trees occupied by the *Crematogaster* ants are healthier, either due to ant patrolling or otherwise, and the healthier trees attract more herbivores (Figure 4.10a). A third possibility is that herbivores are under top-down control and ants that effectively deter mammalian herbivores also deter predators that would prey on insect herbivores (Figure 4.10b)[31].

Overlaid on any ecological differences among the ants is specialization in some myrmecophile taxa. For example, the highly abundant tortricid Hystrichophora griseana (OTU 3) was found on 37% of the *C. mimosae* and *C. nigriceps* trees in our 2012-2013 dataset, but only 4% of the *T. penzigi* trees. It seems unlikely that there is enough variation in tree quality or defense among the ant species,

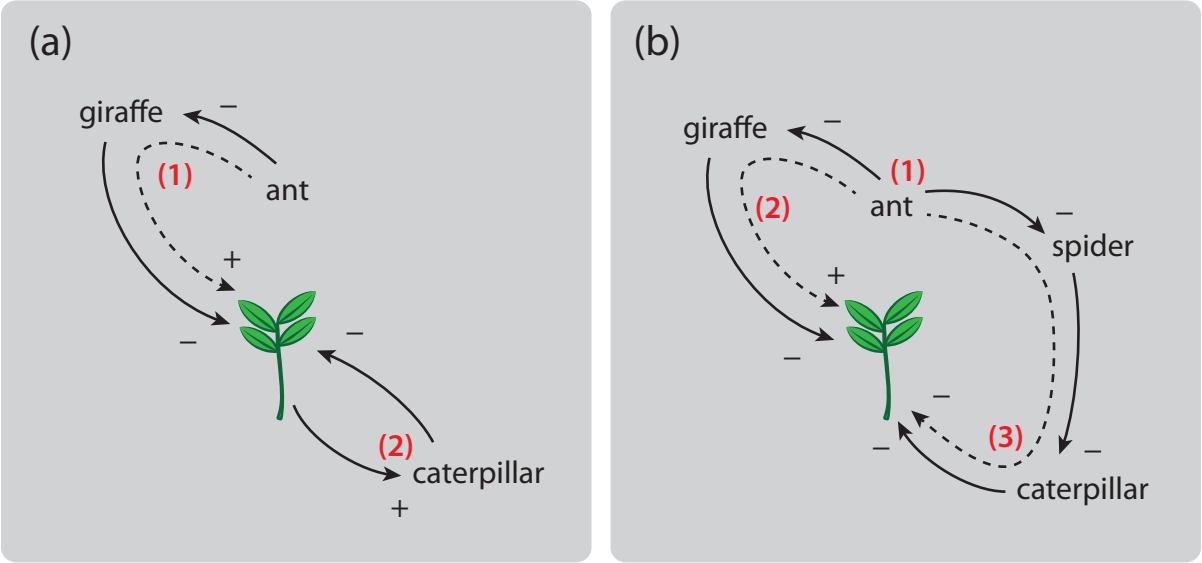

Figure 4.10: Two potential explanations for the greater abundance of herbivorous myrmecophiles with ants that are better defenders against herbivory by large mammals such as giraffe. (a) Better defenders deter mammals, and thus benefit their plant (1). Healthier plants support more myrmecophiles, partly offsetting the benefits of ant protection (2). (b) Better defenders deter mammals, but also predators and parasitoids, such as spiders and wasps (possibly not domatium residents) (1). Ants benefit their host plants by deterring mammals (2). But since predators and parasitoids would otherwise have reduced insect herbivory, this represents a cost to the host plant (3).

relative to within each species, to account for such a dichotomous pattern. Myrmecophile ant specificity might reflect myrmecophiles being able to evade colony defense for some but not all ants, or myrmecophiles using ant species as a cue to identify better quality or better defended trees.

The fitness effects of the Lepidoptera on the ants remain unknown. Unlike known ant parasites such as the braconid *Trigastrotheca laikipiensis*[169], our stable isotope data show that the Lepidoptera are not preying on the ants, despite sharing domatia, but are instead drawing nutrition from the plant. Is their presence negative to the ants by virtue of the cost imposed on the host plant, or is it possible that the ants even benefit from the presence of herbivorous myrmecophiles? Trees respond to mammalian browsing with an increased number of extrafloral nectaries and domatia[94,157], and natural or artificial herbivory increases thorn length[236]. In addition, pruning of axillary buds by *C. nigriceps* is

associated with more shoots, healthier leaves, and more numerous and larger nectaries[198], suggesting stimulation by pruning, perhaps through release of apical dominance. Ants might then benefit from the presence of herbivorous myrmecophiles if their presence results in a transfer of resources from plant to ant that more than offsets any ant fitness cost arising from decreased host plant fitness. Such ants would experience little pressure to evict herbivorous myrmecophiles and might even favor them. Indeed, such indirect ant-herbivore mutualisms even suggests an additional explanation for the higher abundance of myrmecophiles with *C. mimosae* and *C. nigriceps* ants – the *Crematogaster* ants might favor a low density of myrmecophiles for their stimulating effects on extrafloral nectar, but *T. penzigi* would be less likely to favor myrmecophiles as it would indirectly bear costs of herbivory on the plant yet derive little benefit from extrafloral nectar, since it destroys nectaries[237].

The net fitness effects of herbivorous myrmecophiles on the plant are also not obvious. While herbivory necessarily imposes a direct cost from tissue loss, the fitness cost to the plant may be small, e.g. if the tissue is non-photosynthetic tissue from inside the domatia rather than the leaves. The absolute volume of herbivory may also be small. Palmer and Brody found no significant difference in invertebrate leaf herbivory between *C. mimosae* and *T. penzigi*, and lower leaf herbivory with *C. nigriceps*[154]. Moreover, any direct costs may be more than offset by positive fitness effects, e.g. from release from apical dominance or from recycling nutrients into new growth[5]. If plants respond to insect herbivory by boosting inducements to their mutualistic ants, then plants might also experience higher long-term fitness because its mutualistic ants benefit and provide better defense against future insect or mammalian herbivores – just as plant fluid feeders such as aphids and scale insects may facilitate ant protection[165]. Alternatively, a fitter ant colony might be more resistant to eviction by a less mutualistic ant partner[153]. Indeed, a potential parallel already exists with large mammalian herbivores of *V. drepanolobium*, in which reductions in extrafloral nectar and in the number of domatia in herbivore exclusion plots leads to mutualism breakdown[94,157].

Conclusions

This study uses stable isotopes to investigate trophic relationships for several associates of the ant plant *V. drepanolobium*. The ants themselves, like many plant ants, appear functionally herbivorous, and they share their domatia with several species of herbivorous Lepidoptera and carnivorous spiders. Surprisingly, the herbivorous Lepidoptera are more abundant (see Chapter 3) with *C. mimosae* and *C. nigriceps* ants, which are normally regarded as better host plant mutualists. Host plants also showed small differences in isotopic ratios between the two ants, perhaps reflecting either host plant selection or ant mediated effects on the plant.

Including myrmecophiles in our picture of the *V. drepanolobium* system opens up new possibilities for understanding variation in the system over space and time, since myrmecophiles may have different responses than either the ants or their host plants to outside forces such as climate, nutrient availability, and other indigenous or introduced species. Our results suggest several promising lines for further study. Additional collections and stable isotope analyses will be useful for teasing out variation at multiple scales – for example, between geographic locations, among different trees at a site, within individual trees, or over the lifetime of a tree or ant colony. Experimental work is needed to explore interactions among plants, ants and myrmecophiles. For example, ant and herbivore exclusion experiments in screened enclosures or greenhouses would allow growth effects on plants to be quantified, as well as plant responses such as extrafloral nectary production. Finally, detailed work on the natural history and chemical ecology of some of the more abundant myrmecophile taxa will be invaluable for understanding the nature and mechanics of interactions between those myrmecophiles and their ants.

Appendix A

Supplementary material for Chapter 2

A.1 Recruitment experiment: detailed methods and results

We ran a recruitment experiment to quantify ant preferences towards fungi. Ant colonies were collected in March 2012 from our Kitengela field site in Kenya (1°23.8′S, 36°49.5′E, 1650m asl) and transported to Cambridge, MA, USA where they were housed in glass containers with sides coated with Insect-A-Slip Insect Barrier until the choice trial experiment was conducted in May 2012. Colonies were supplied with water continuously, and with Bhatkar-Whitcomb ant diet[16] approximately every two days.

For the experiment, we tested a total of 30 different colonies split into 2 groups of 15 assayed on consecutive days. Each ant colony's glass container was placed next to a foraging arena comprising a $\sim$ 25cm diameter plastic dish whose sides were also coated with Insect-A-Slip. A balsa wood bridge led from the floor of the glass container to the center of the foraging arena. This entire setup for each colony was placed inside a large lidded plastic box.

Each colony was presented with two 60mm plastic petri dishes on opposite sides of the foraging arena. Each petri dish contained Difco potato dextrose agar (PDA) media. One of the two plates had previously been inoculated with a single fungal strain isolated from a *C. mimosae*-occupied domatium and incubated at room temperature for $\sim$ 3 days to allow the fungus to grow. We selected this fungus arbitrarily from among several isolates, and tentatively identified it as *Phoma* sp. based on ITS and LSU sequences. We were also able to isolate the same fungus from *T. penzigi* domatia. The location

of the fungus and control plates was alternated between colonies to avoid potential artefacts from e.g. phototaxis.

Every hour, we recorded the number of ants present at each petri dish (inside the rim) and, of those, the number that were stationary and apparently manipulating the dish contents (agar and/or fungus). Observations continued for 8 hours, after which the dishes were removed and photographed. The photographs were used to determine the proportion of the dish contents' area that had been chewed or removed by the ants.

T. penzigi colonies tended to remove a larger fraction of the fungus than did *C. nigriceps* or *C. mimosae* colonies (Figure A.1a). This did not appear to be simply an artefact of the *T. penzigi* colonies being more vigorous than the *Crematogaster* colonies since *C. nigriceps*, rather than *T. penzigi*, showed the highest recruitment to both fungus and control plates (Figures A.1b and A.1c). To formalize this result, we estimated a generalized least squares model, with different variance parameters for each ant species to deal with heteroskedasticity. Ant species was a significant predictor of fungus removal even with recruitment to the fungus plate and colony size included in the model ($LR = 10.4, p < 0.01$). The presence of a queen or of brood were not significant predictors.

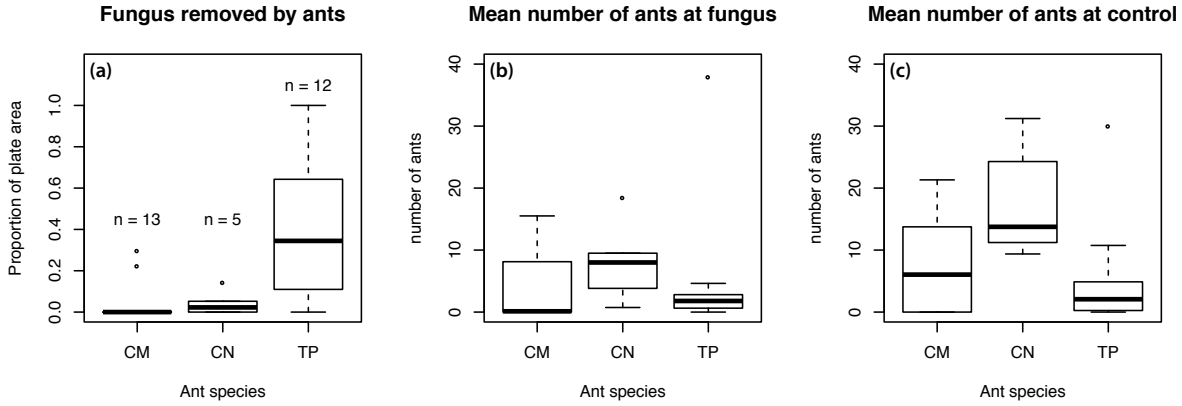

Figure A.1: (a) *T. penzigi* colonies removed a larger proportion of the *Phoma* fungus culture presented to them than either *C. mimosae* or *C. nigriceps*. (b) and (c) *T. penzigi* colonies were not simply larger and more active than the *Crematogaster* colonies – *C. nigriceps* generally had more workers at both fungus and control plates.

Table A.1: Sampling locations

Site name	Location	Latitude	Longitude	Elevation
Mpala	Mpala Research Center, Laikipia County, Kenya	0°18.8′N	36°52.5′E	1800m asl
Kitengela	undeveloped land outside Nairobi National Park, Kajiado North County, Kenya	1°23.8′S	36°49.5′E	1650m asl
Greenhouse	Museum of Comparative Zoology Labs, Harvard University, Cambridge MA, USA	42°22.8′N	71°06.9′E	50m asl

A.2 Multiplexed amplicon sequencing: detailed methods

Sampling locations

We sampled from two field sites in Kenya and from our greenhouse in Cambridge MA, USA (Table A.1). The two Kenyan study sites are approximately 190km apart.

Sample summary

Table A.2 shows the breakdown of the samples used for the fungal community analysis in this paper, split by ant species, location and sample type.

Sampling procedure – domatium and leaf samples

At Mpala and Kitengela, we selected trees by walking transects and haphazardly choosing a set of trees approximately every 100m, with every set including one tree occupied by each of *Crematogaster mimosae* (CM), *C. nigriceps* (CN) and *Tetraponera penzigi* (TP). Some flexibility was required in the tree selection since trees were not uniformly distributed across the landscape, nor were ants uniformly distributed on trees. We removed around five domatia from each tree and placed them in a new plastic zip-closure sandwich bag for later processing. We selected domatia that appeared healthy and were actively used by the host ants. We also sampled several fresh leaves from the same trees,

Table A.2: Number of samples by ant species, location and sample type

	Domatia			Leaves	Alates
Ant	Mpala	Kitengela	Greenhouse	Mpala	Kitengela
C. mimosae	13	7	3	3	-
C. nigriceps	9	8	8	-	10*
T. penzigi	13	6	2	3	-

* We extracted from 21 surface sterilised *C. nigriceps* alates, but we were unable to PCR amplify from 6 of these. DNA from the 15 remaining alates was sent for 454 sequencing. Two of those were unable to be sequenced, and we excluded a further 3 alates that yielded low 454 sequence counts. Data from the remaining 10 alates were used in our analysis.

keeping them separate from the domatium samples.

We opened domatia with sterile instruments and transferred their contents to preservative on the same day that the domatia were collected. Almost all domatia contained ants. In addition, domatia of the three ant species typically contained different 'substrates': CM domatia typically contained carton lamellae; CN domatia contained old *Vachellia* leaflets; and TP domatia usually contained the loose fibrous particles that Hocking suggests may be derived from the domatium's medullary parenchyma[88]. Only one of the five domatia collected from each tree was used for molecular work. Where a domatium did not contain both ants and substrate, its contents were discarded and the next domatium from the same tree was examined, until we had found a suitable domatium. The fresh leaf samples were also placed in preservative, separate from the domatium contents. In both cases, the preservative was a pH 5.2 buffered ammonium sulfate solution. Samples were kept at room temperature for around three weeks while in the field, and then at -20°C upon return to Cambridge MA, USA.

In addition to the field samples, we also collected a small number of samples from *Vachellia drepanolo-*

bium plants grown from seed in our greenhouse in Cambridge MA, USA. Seeds were originally collected from the Kitengela field site in September 2010, and planted in March 2011. In April 2012, we introduced to each plant an ant colony that had been collected from Kitengela in March 2012 and transported to Cambridge. The ants occupied the plants for approximately 4 months before domatia and leaves were sampled as per the Kenyan field samples, except that samples were immediately stored at -20°C instead of being kept in preservative at room temperature.

Sampling procedure – alate samples

After determining that domatium fungal communities differed among the ant species, we wanted to investigate the possibility that alates were transporting fungi in their infrabuccal pockets and thus vectoring the fungal community in the same manner as those of attine ants[171]. We therefore decided to examine the diversity of fungi in alate heads using pyrosequencing methods similar to those used for the main analysis in this paper.

For this study, we used 21 CN alates collected opportunistically in the course of carrying out other fieldwork in Kenya at the sampling locations described above. Alates were stored at 20°C in 100% ethanol.

DNA extractions – domatium and leaf samples

We prepared domatium and leaf samples for DNA extraction by placing each sample in a sterile 1.5mL microcentrifuge tube, removing excess preservative with a pipette, rinsing the sample with molecular grade water, and then removing excess water. For fresh leaf samples and the old leaflet samples from CN domatia, we counted out 40 leaves for the extraction. For the fibrous particle samples from TP domatia and the carton lamellae from CM domatia, we measured out 80 to 100mg wet weight. We used the whole sample wherever less material was available.

We homogenized each sample in an MPBio Lysing Matrix A tube (including ceramic sphere; MP

Biomedicals LLC, Santa Ana CA) using an MPBio FastPrep-24 benchtop homogeniser. Each tube contained the sample plus 1000μL of Qiagen Buffer AP1 with 4μL Qiagen Proteinase K (20mg/mL; Qiagen Inc, Valencia CA) added. Other researchers have found Proteinase K to be important for successful extraction from samples preserved in ammonium sulfate[15]. We homogenized samples for 40 sec at speed setting 6.0, removed the samples to ice for 5 min, then homogenized for a further 40 sec at the same speed.

We incubated the homogenized samples for 10 min at $60-65^{\circ}$C, inverting the tubes 2-3 times during incubation. We further incubated the tubes overnight at 55°C in a shaking incubator.

We continued the extraction protocol the next day, following the Qiagen DNeasy Plant Mini Kit protocol with the following modifications. We added 325μL Buffer AP2 to the lysate, since the lysate volume was larger than that in the original protocol. After precipitation, we split the lysate for each sample into two portions and passed each portion through a separate QIAshredder column. After adding Buffer AP3/E, we passed the combined flow-through from both QIAshredder columns through a single DNEasy Mini spin column. We washed the adsorbed DNA with a single 500μL volume of Buffer AW, and eluted with two 100μL volumes of Buffer AE.

DNA extracts were quantified with a NanoDrop and a Qubit fluorometer, precipited in ethanol and resuspended in Buffer AE at 20ng/μL, or at a lower concentration if required in order to have a minimum 20μL once resuspended.

DNA extractions – alate samples

We extracted DNA from all 21 CN alates following a standard phenol-chloroform extraction protocol. Using forceps and a scalpel blade, alate heads were removed and placed in individual 2 mL Sarstedt tubes with five 0.5mm glass beads. We homogenized these samples in a Mini-Beadbeater-8 (BioSpec Products, Bartlesville OK) at full speed for 1 min. We then added 400μL CTab buffer, and incubated samples overnight at $60-65^{\circ}$C. Flame sterilized tools were used to handle the alates at

all times. Some samples were surface sterilized prior to DNA extraction with 1 min in 100% ethanol followed by 1 min in 10% bleach.

Following incubation, we added 400μL of 25:24:1 phenol:chloroform:isoamyl alcohol and repeatedly inverted the tubes for 1 min to mix. We then centrifuged samples for 15 min at 13000 rpm. We removed 300μL supernatant to a new tube and added 300μL of 24:1 chloroform:isoamyl alcohol before again mixing for 1 min and centrifuging for 15 min at 13000 rpm. We transferred 200μL supernatant to a new tube, and added 500μL of 100% ethanol, 75μL sodium acetate, and 3μL glycogen to precipitate DNA.

After precipitating overnight at 20°C, we centrifuged samples for 15 min at 13000 rpm and removed supernatant. We then washed the pellet in 500μL of 70% ethanol and centrifuged the samples again for 10 min at 13000 rpm. We removed the ethanol with a pipette, air dried the pellets and resuspended DNA in 100μL of molecular grade water. Extracts were quantified with a NanoDrop and a Qubit fluorometer. Extracts were stored at 20°C or 80°C until PCR amplification and sequencing.

PCR amplifications – alate samples

Since the alate samples were small and likely varied in the amount of fungal material that they contained – particularly if some were not carrying an infrabuccal pellet – we used PCR amplifications to assess the presence of fungal material in each alate sample. PCR products were visualised using agarose gel electrophoresis and scored for either successful or unsuccessful amplification. Each reaction consisted of 2.5μL Omega BioTek 10X buffer, 1.0μL $MgCl_2$ at 25mM, 0.25μL dNTPs at 25mM, 1.2μL each primer at 10μM, 1U Omega BioTek Taq polymerase, 1μL DNA template and molecular grade H_2O to 25μL. Reaction conditions were 2 min at 94°C, followed by 34 cycles of 35 sec at 95°C / 55 sec at 55°C / 45 sec at 72°C, and a final 7 min at 72°C. We used the primers ITS1F[64] (`5'-CTTGGTCATTTAGAGGAAGTAA-3'`) and ITS4[230] (`5'-TCCTCCGCTTATTGATATGC-3'`) to capture the broad fungal community while excluding non-fungal taxa.

Multiplexed amplicon pyrosequencing – all samples

Amplification and pyrosequencing were performed by Research and Testing Laboratory (RTL), Lubbock, TX. We sent 75 domatium and leaf samples for sequencing, plus the 15 CN alates that we successfully PCR amplified and that passed NanoDrop/Qubit quality checks.

Samples were first amplified using forward and reverse fusion primers. The forward primer was made up of the Roche A linker (454 Life Sciences, Branford CT), an 8bp multiplex identifier and the ITS1F fungal primer[64] (`5'-CTTGGTCATTTAGAGGAAGTAA-3'`). The reverse primer consisted of a biotin molecule, the Roche B linker, and the ITS4 primer[230] (`5'-TCCTCCGCTTATTGATATGC-3'`). PCRs comprised 1μL of each primer at 5μM, 1μL of extract, Qiagen HotStarTaq Master Mix plus water to a total volume of 25μL. RTL performed reactions on ABI Veriti Thermo Cyclers (Applied Biosytems, Carlsbad CA) with the following conditions: 5 min at 95°C, followed by 35 cycles of 30 sec at 94°C / 40 sec at 54°C / 60 sec at 72°C, and a final 10 min at 72°C. PCR products were then pooled equimolar.

Pooled PCR products were cleaned with Diffinity RapidTips (Diffinity Genomics, West Henrietta NY), and size selected using Agencourt AMPure XP beads (BeckmanCoulter, Indianapolis IN) following Roche 454 protocols. RTL then hybridized 150ng of DNA to Dynabeads M-270 (Life Technologies, Grand Island NY) to create single stranded DNA, again following Roche 454 protocols. Single stranded DNA was diluted and used in emulsion PCR reactions, which were subsequently enriched. Sequencing was performed on a Roche GS FLX 454 pyrosequencer with Titanium chemistry following standard manufacturer protocols.

Bioinformatics – all samples

RTL provided demultiplexed sff files generated using the Roche sffinfo tool. The demultiplexing operation filters out sequences whose terminal regions (i.e. primer, linker or barcode sequences) fail to

match the known set of valid sequences, and thus functioned as a basic quality filter.

We processed the prefiltered sequence data ourselves using a combination of software tools. We denoised sequences with Ampliconnoise[170] as implemented in the QIIME 1.6 bioinformatics pipeline[32]. We then isolated the ITS1 region from our reads using an open-source software utility provided by Nilsson *et al.*, as OTU clustering may be distorted by the inclusion of conserved flanking regions[144]. Because our unidirectional sequencing started from the end of 18S, virtually all sequences contained ITS1; any remaining sequence (i.e. 18S, 5.8S or ITS2) was discarded from our analysis. Previous studies suggest that ITS1 and ITS2 yield similar results for this kind of analysis[19], so we do not consider our decision to sequence from the ITS1 primer likely to be highly consequential.

We picked OTUs based on the ITS1 fragments using uclust[53] in QIIME with a similarity threshold of 95%. We used the full dataset for the clustering – i.e. the 75 domatium and leaf samples, plus the 10 alates samples remaining after excluding 2 that could not be sequenced and a further 3 with low sequence counts (each yielded <500 sequences).

For comparison, we also tried several variants on this workflow, including open reference OTU picking in QIIME and ESPRIT complete-linkage based hierarchical clustering[203] instead of uclust, different similarity thresholds, and clustering with full-length sequences rather than just ITS1. Results were broadly similar to those presented here.

We assigned putative taxonomic descriptions to a representative sequence from each OTU using blast via QIIME's assign_taxonomy.py script. In short, we downloaded nucleotide (db=nuccore) sequences from NCBI with the query

```
"fungi[Organism] NOT (environmental sample[filter] OR
metagenomes[orgn])"
```

to help exclude unidentified environmental sequences, and built a blast database from those sequences. We then used our own Python script to parse the NCBI taxonomy database and return a

QIIME-compatible taxonomy mapping file for our blast database. Our Python code is freely available and may be applied to any fasta file using NCBI GI numbers as identifiers (i.e. including any sequence set downloaded from the NCBI using gquery or e-direct).

Data analysis – domatium and leaf samples

We performed statistical analyses of the OTU data using a combination of QIIME, the statistics package R[206] and the R packages vegan[148], biom[131], RcolorBrewer[142], spatstat[10] , randomForest[117], plyr[232], bipartite[48] and shape[192], plus our own custom scripts.

We used a rarefaction curve approach to assess differences in taxon richness and diversity between groups of samples. We resampled our OTU table 1000 times at predefined per-sample depths. For each sample and for each sampling depth, we calculated the mean number of observed species across the 1000 resamplings. We then used these averages to find group means and standard errors at each sampling depth, so that our standard errors primarily reflect between-sample variation.

To formally test for differences in diversity evident in the rarefaction curves, we used simple parametric statistical tests after rarefying each sample to 1000 sequences, since sampling depth may affect observed taxonomic richness and beta diversity comparisons[93]. Although some authors argue that rarefying is statistically inefficient[132], we were relatively unconcerned about type II errors in this dataset, as we effect sizes appeared reasonably large. Rarefying to 1000 sequences allowed us to retain all domatium and leaf samples in the analysis, since the minimum of sequences per sample was 1119 sequences. As the rarefaction curves do not cross, our choice of rarefaction depth is unlikely to have much influence over our qualitative assessment of alpha diversity, which is our main interest here.

We used distance-based ordination to assess variation in fungal community composition within and among our sample types and sampling locations. Although phylogenetic distances such as UniFrac are commonly used in work on bacterial communities and may be more informative[118], the ITS region is not easily aligned among highly diverged taxa such as those in our samples[164]. Moreover, no

multiple alignment is available as a scaffold akin to the Silva or Greengenes datasets for bacterial 16S. We therefore chose to use Sørensen[193] and Bray-Curtis[25] distances for our ordinations. Using a single rarefaction to 1000 sequences, we visualised distances using non-metric multidimensional scaling (NMDS) plots in three dimensions. We then used adonis[7] to test the significance of the separation between sample types and locations in the full multidimensional space implied by the distance matrices.

We used bipartite graphs to help visualise associations between particular fungal OTUs and the three ant species. Since part of our interest in these fungal communities is to identify fungal taxa that may have ecological relevance, we focused on relatively abundant taxa by applying several filters to our OTU table. Although we cannot rule out apparently rare OTUs playing an important role, this would require, for example, sequencing biases to have reduced the apparent abundance of a common fungus, or for a rare fungus to have unusually large effects. We therefore chose to focus on the more abundant taxa. We included only OTUs with > 100 total sequences, and that were present in $\geqslant 2$ samples from the same ant species. These OTUs together comprised more than 90% of the rarefied dataset. Instances where an OTU contributed $\leqslant 10$ sequences to a sample were omitted from the graph for clarity (but an OTU may still be shown on the graph where it contributed > 10 sequences to other samples).

Data analysis – alate samples

We assessed alpha and beta diversity of the alate samples using rarefaction curves, distance-based ordinations, and permutational statistical tests as described above for the domatium and leaf samples.

To gauge the possibility that alates might contribute to the domatium communities by vectoring fungi, we also pooled the CN alate samples and determined the proportion of sequences in each ant-occupied Kenyan domatium that was found among the pooled alates. This pooling approach allows for the possibility that individual alates only take or contribute a fraction of the fungal community,

and also helps overcome the limitations of applying our extraction and amplification methods to the tiny alate samples. Our opportunistic sampling did not allow such pooling at a finer scale (e.g. within a tree or within an ant colony).

A.3 Multiplexed amplicon sequencing: detailed results

PCR amplifications and pyrosequencing data – all samples

Our PCR amplifications were successful for 15 of the 21 surface sterilised CN alate samples that we tested (71%). We sent these 15 samples for 454 sequencing, along with the 75 domatium and leaf samples. Two of the 15 CN alate samples could not be amplified by RTL; we therefore received sequence data for a total of 88 samples.

Although PCR products were pooled in equimolar amounts per sample prior to multiplexed sequencing, sequence yields for the domatium and leaf samples after denoising and filtering ranged from 1119 to 21345 sequences per sample. The CN alates ranged from 263 to 11159 sequences per sample. Three CN alate samples yielded fewer than 500 sequences and were excluded from the analysis prior to OTU clustering; after removing these samples, the lowest sequence count for the alate samples was 1068 sequences. The distribution of per-sample sequence counts in the final dataset used for the analysis was broadly similar across the sample types (Figure A.2).

The final dataset comprised 451755 sequences, including 407769 from the domatium and leaf samples and 43986 from the CN alates. After picking OTUs using uclust with a 95% similarity threshold, our library separated into 673 OTUs; of these, 666 were present among the domatium and leaf samples, and 27 were present among the CN alate samples. Many OTUs were present at low abundance, with just 84 represented by $\geqslant 100$ sequences each.

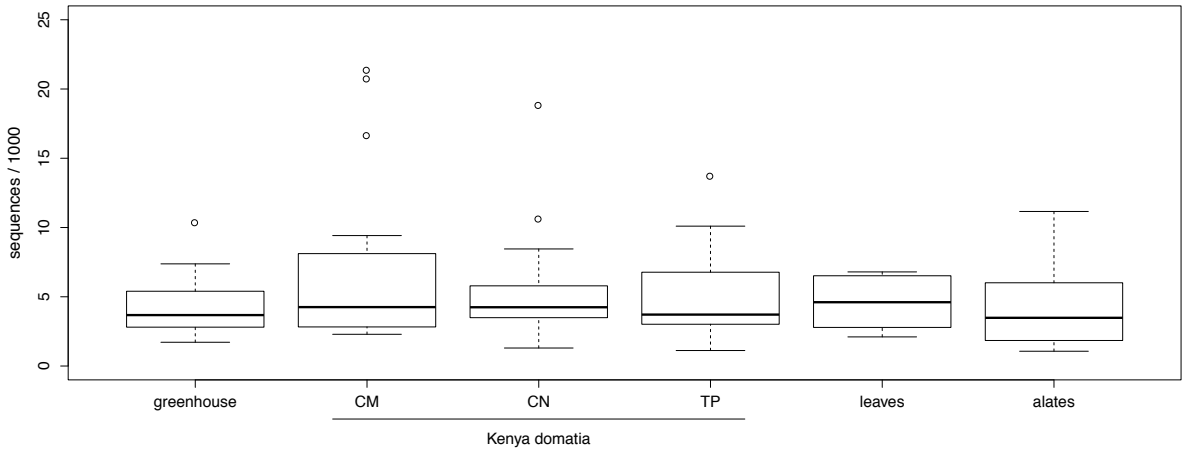

FIGURE A.2: Per-sample sequencing depth

Alpha diversity – domatium and leaf samples

OTU richness varied substantially among the samples. Rarefaction curves showed that Kenyan leaf samples contained more fungal OTUs than the Kenyan domatium samples, which in turn contained more OTUs than the greenhouse domatium samples (Figure A.3). At a rarefaction depth of 1000 sequences, these differences were highly significant (leaves vs Kenyan domatia: $t_{5.3} = 6.0, p < 0.01$; Kenyan domatia vs greenhouse domatia: $t_{66.8} = 12.8, p < 0.01$). Among the Kenyan domatia, however, there was little variation between the two sampling locations ($t_{48.4} = 0.33, p = 0.74$ at 1000 sequences rarefaction depth) or among the three ant species ($F_{2,53} = 2.2, p = 0.13$ at 1000 sequences rarefaction depth).

Beta diversity – domatium and leaf samples

Our NMDS plots showed clear separation between Kenyan leaves, greenhouse domatia and Kenyan domatia with either binary (Sørensen) or quantitative (Bray-Curtis) distance measures (Figures A.4 and A.5). Our adonis tests provided statistical confirmation of the separation (Sørensen: pseudo-$F_{2,72} = 9.4, p < 0.001$ with 1000 permutations; Bray-Curtis: pseudo-$F_{2,72} = 5.4, p < 0.001$ with

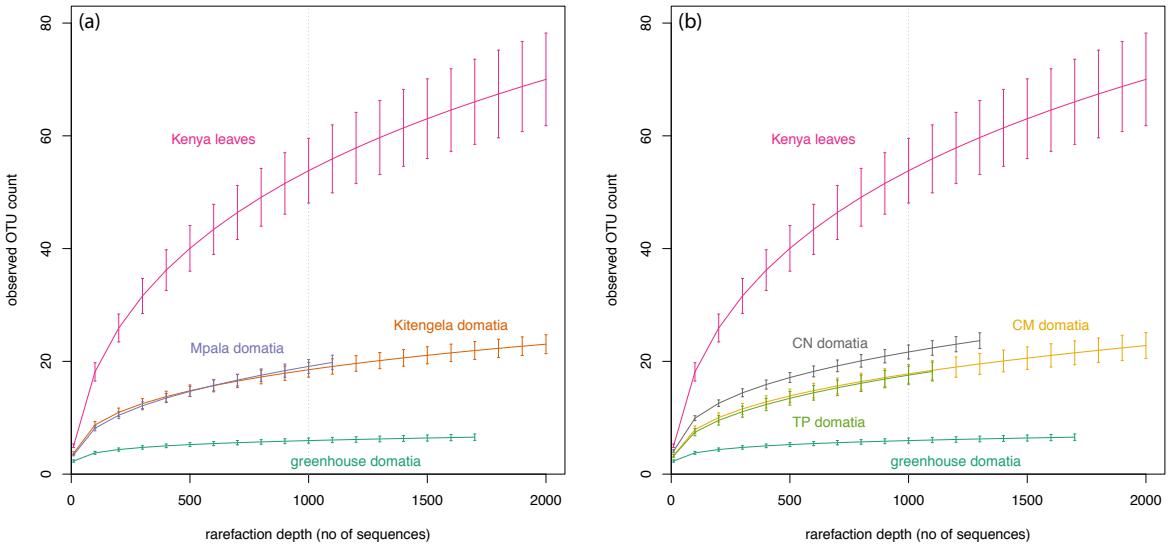

FIGURE A.3: Rarefaction curves for observed number of OTUs. Panel (a) breaks down Kenyan domatia by sampling location. Panel (b) instead breaks down Kenyan domatia by ant occupant.

1000 permutations).

The Kenyan domatia also separated clearly by ant occupant (Figures A.4 and A.5), and to a lesser extent by location (not shown). Both ant occupant and location were significant among the Kenyan domatia (Sørensen: adonis pseudo-$F_{2,53} = 5.2$, $p < 0.001$ for ant and $F_{1,54} = 2.2$, $p = 0.007$ for location; Bray-Curtis: pseudo-$F_{2,72} = 6.4$, $p < 0.001$ for ant and $F_{1,54} = 1.7$, $p = 0.05$ for location; 1000 permutations for each test).

These differences in community composition do not appear to be driven primarily by differences in OTU richness or multivariate dispersion. OTU richness was similar among ant occupants and between locations for the Kenyan domatia (see above). Multivariate dispersion[8] for the Kenyan domatia was not significantly different among ants or between locations, though it varied among the greenhouse domatia, Kenyan domatia and leaf samples.

These differences also do not appear to be artifacts of the single rarefaction to 1000 sequences. Repeated rarefactions produce similar separation between the sample groups (e.g. Figure A.6) and

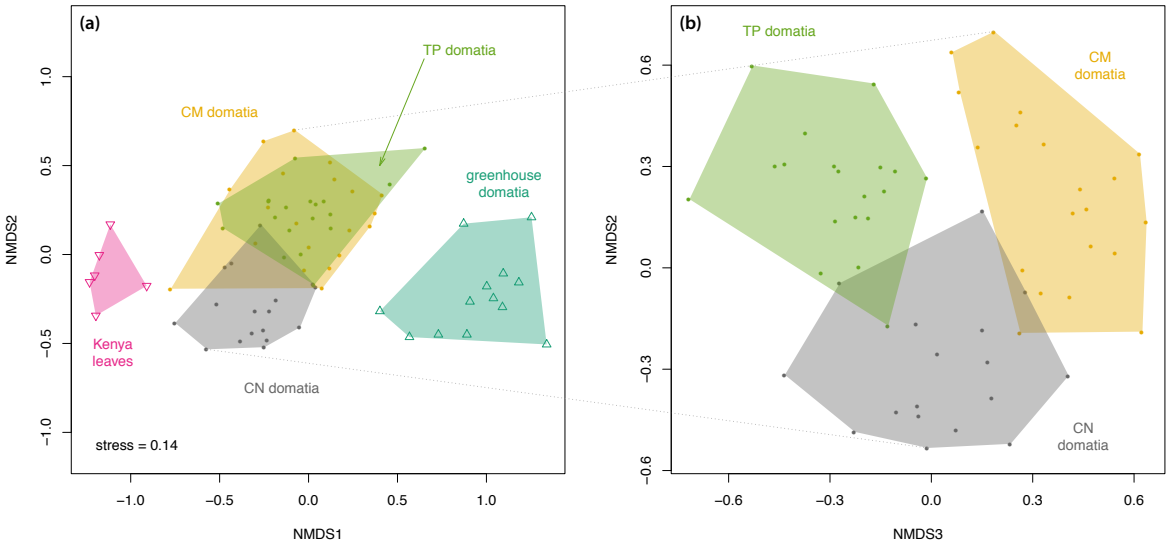

FIGURE A.4: Three-dimensional non-metric multidimensional scaling ordinations for the domatium and leaf samples, based on Sørensen (i.e. binary) distances between communities rarefied to 1000 sequences per sample.

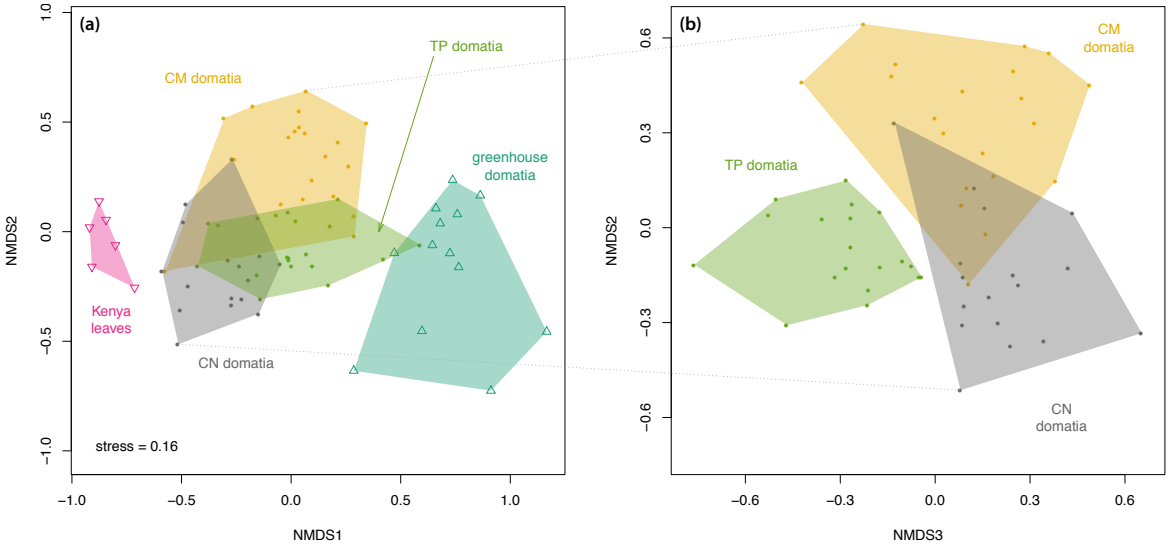

FIGURE A.5: Three-dimensional non-metric multidimensional scaling ordinations for the domatium and leaf samples, based on Bray-Curtis (i.e. quantitative) distances between communities rarefied to 1000 sequences per sample.

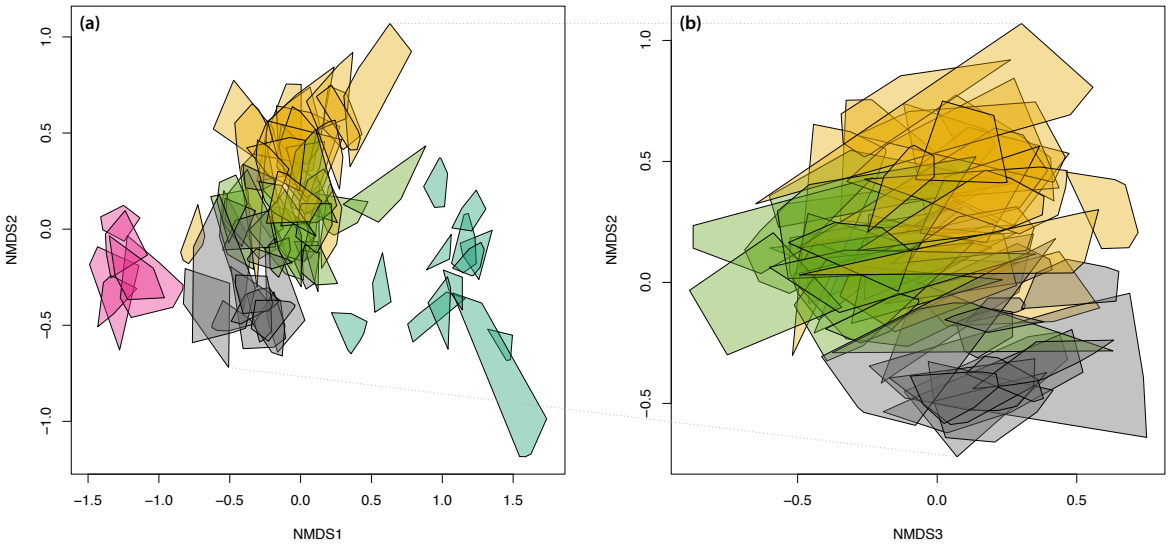

Figure A.6: Superimposed non-metric multidimensional scaling ordinations for the domatium and leaf samples. The OTU table was rarefied 10 times each to 1000 sequences per sample, and pairwise Sørensen distances calculated for each rarefaction. NMDS ordinations were calculated for each of the 10 resulting distance matrices, and superimposed after Procrustes alignment. Each polygon is a projection of the convex hull representing a single sample. Although the rarefactions introduce substantial noise, the samples still clearly separate by sample type.

qualititatively similar statistical results (not shown).

Taxonomy assignment – domatium and leaf samples

Our taxonomic assignments showed differences between the sample types but also highlighted the degree of within-group variability (Figure A.7). In the absence of phylogenetic distances for our samples (see above), examining the taxonomic breakdowns provides a useful complement to our beta diversity analyses described.

Although we have found our taxonomy assignments to be useful in describing broad patterns in our data, it was generally difficult to be confident in our assignments to species level, and therefore difficult to draw conclusions about the natural history of the fungi in our samples based on taxonomy. Although the ITS region may be a good general purpose barcode for fungi[189], we typically found a

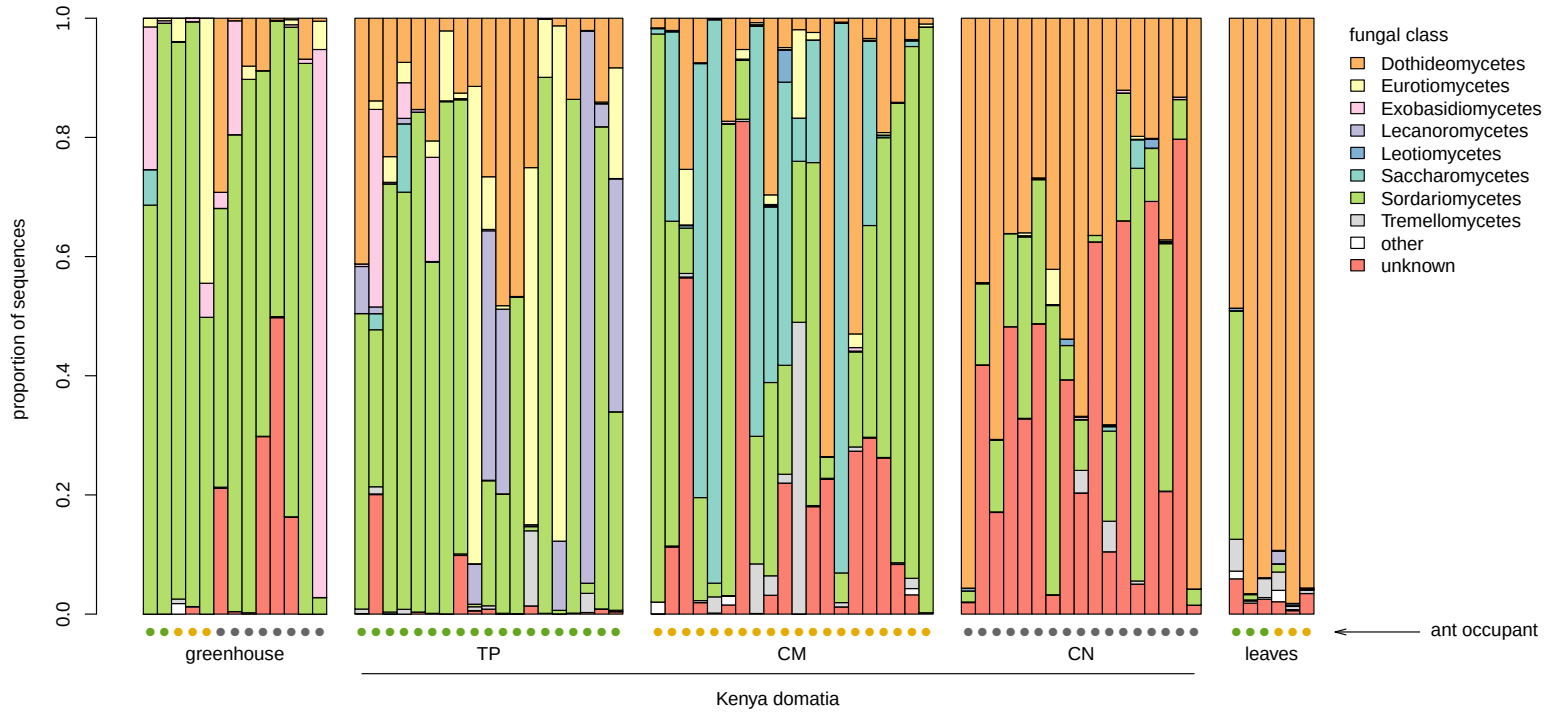

FIGURE A.7: Taxonomic breakdown of samples at class level. Each column represents one sample.

diversity of fungal taxa among the top blast hits for any given sequence in our dataset. Our results were typically stable at higher taxonomic levels (e.g. family or class) but not at lower levels (e.g. genus or species).

A major problem with taxonomic assignment is that fungal ITS databases generally remain either poorly populated or curated. Global fungal diversity is estimated at around 1.5 million species[78] but the number of described species accounts for less than 10 per cent of that estimated diversity. Our NCBI-derived fungal database contained almost 1.2 million sequences. However, many of these sequences do not cover the ITS region, and coverage of fungal taxa is uneven, so that many fungi still lack representation even in the largest publicly available dataset. Moreover, many sequences in the database lack good taxonomic information, often because they are uncultured environmental sequences, or are even misidentified. While the problems of misidentification can be solved by relying on well-curated fungal datasets such as the UNITE database[1,103], such datasets remain small.

Bipartite analysis – domatium and leaf samples

Bipartite graphs showed that few of the most abundant fungal taxa associated with just one ant species (Figure A.10). However, many fungi appeared to have stronger associations with some ant species than with others. This was supported by the significant network-wide specialisation measure H2′ (i.e. normalised two-dimensional Shannon entropy H2[21]) ($H2' = 0.5, p < 0.01$).

Alpha and beta diversity – alate samples

Taxonomic diversity of the alate samples was slightly lower than that in the greenhouse domatia and significantly less than that of the Kenyan domatia (Figure A.8).

The composition of the alate fungal communities was substantially different than that of the domatium or leaf communities (Figure A.9), consistent with the low alpha diversity for the alate samples relative to the leaves and domatia. There was little evidence that alate fungal communities were

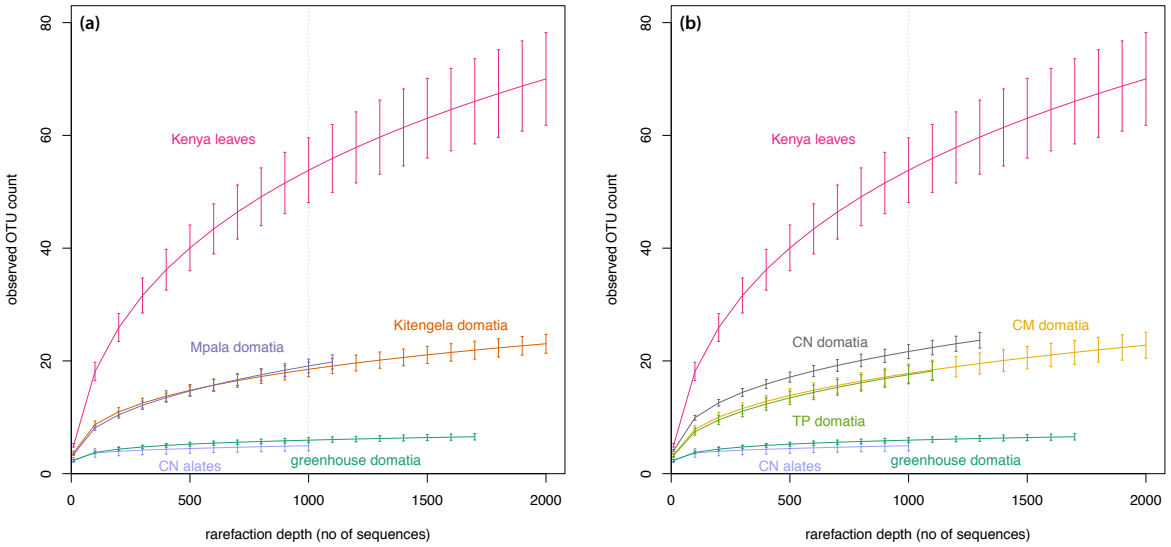

FIGURE A.8: Rarefaction curves for observed number of OTUs. Panel (a) breaks down Kenyan domatia by sampling location. Panel (b) instead breaks down Kenyan domatia by ant occupant. Plots are the same as in Figure A.3 but with the addition of alate samples.

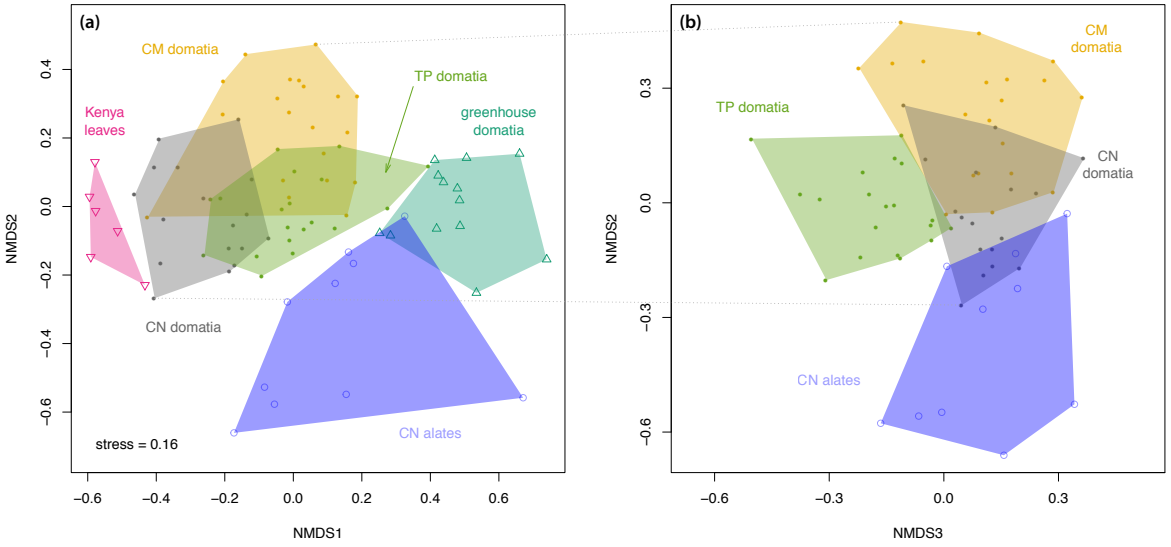

FIGURE A.9: Three-dimensional non-metric multidimensional scaling ordinations for the domatium, leaf and alate samples, based on Sørensen (i.e. binary) distances between communities rarefied to 1000 sequences per sample.

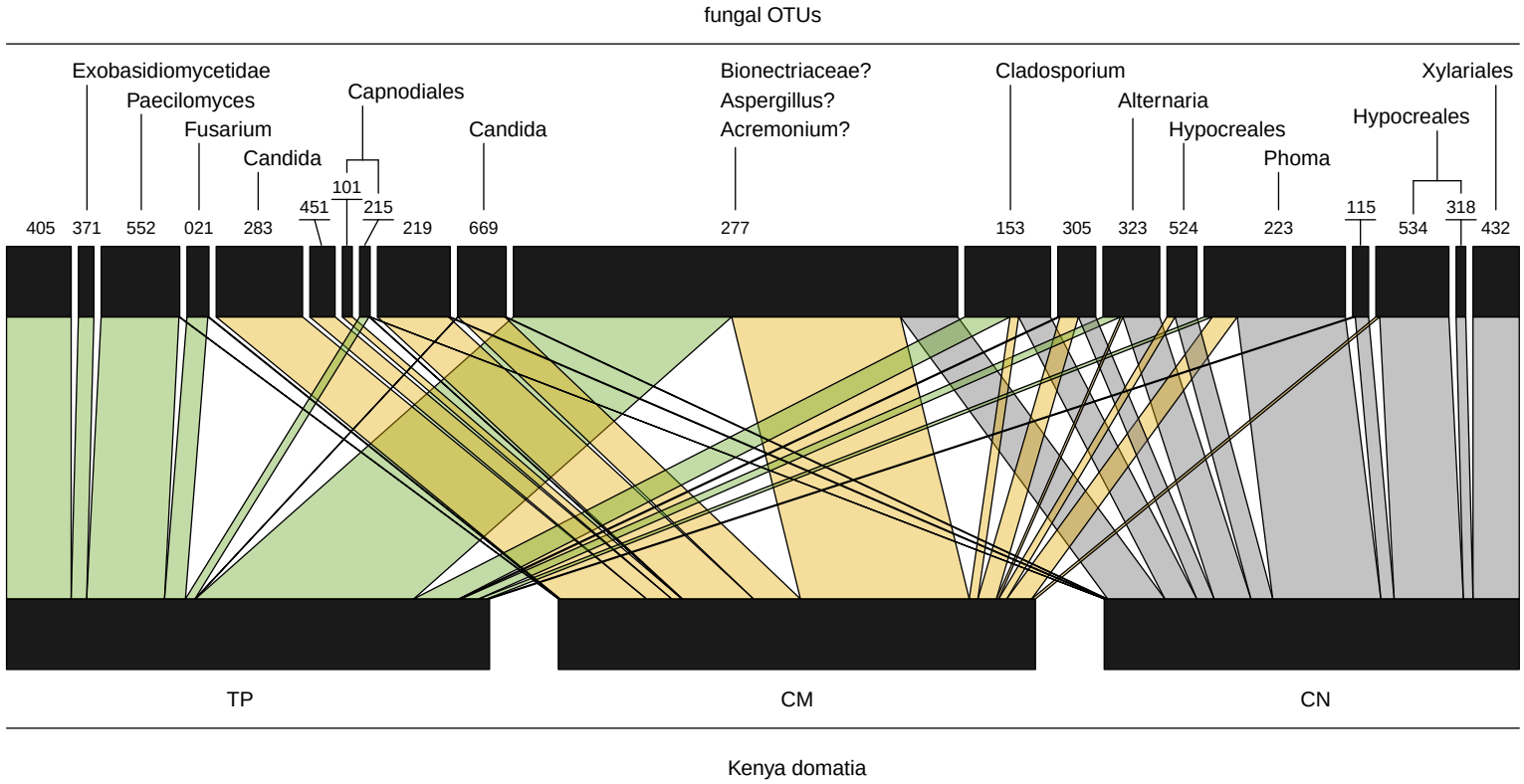

Figure A.10: Bipartite graph showing major fungal species recovered from Kenyan domatia.

any closer to CN domatium communities than to the domatium communities of the other two ant species.

These results show that the alate fungal communities do not simply recapitulate those of the CN domatia, but rather are distinct, low-diversity sets of fungi. Further, there is little evidence that the alate communites are on average any closer to the CN domatia than to the domatia of CM or TP ants. Although the alate samples were preserved and extracted using different methods than the domatium and leaf samples, other studies suggest that this is unlikely to account for the entirety of the differences in composition [112,180].

Overlap between alate and domatium samples

Although individual alate fungal communities are distinct from individual domatium communities, it is also useful to consider the alate as a pooled sample. Although our alates came from different colonies, we might imagine the pooled alates to be representive of a set of alates that had emerged from a single CN colony, each bearing some fungi in its infrabuccal pouch. Each individual alate might contain a different subset of the colony's fungi, but together we might expect them to largely cover the community of fungi present in the colony.

We therefore pooled the alates and, for each domatium, asked what proportion of the fungal sequences could conceivably have been transferred to at least one of the alates – in other words, for what proportion of each domatium's fungal sequences was there at least one match in the pooled alate sample? On average, over 83% of the sequences in each CN domatium were also recovered from the pooled alates, compared to 76% for the TP domatia and 41% for the CM domatia ($F_{2,34.6} = 10.2$, $p < 0.001$) (Figure A.11).

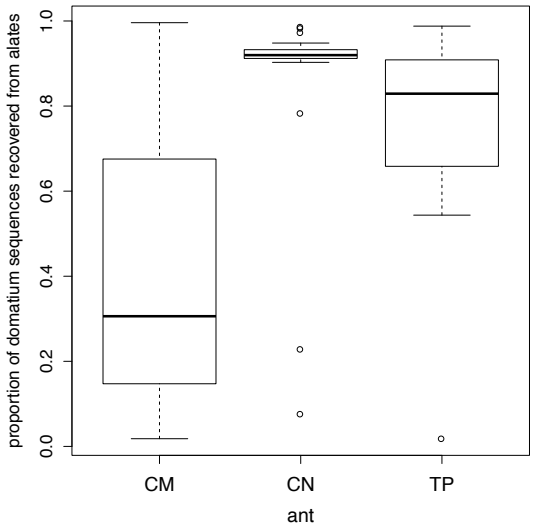

Figure A.11: Proportion of each Kenyan domatium's sequences that were also recovered from the pooled alate samples.

A.4 Microscopy: detailed methods and results

To examine the contents of *T. penzigi* and *C. nigriceps* infrabuccal pockets, we collected female alates as they departed from domatia for their natal flight and subsequent journey to establish a new colony at our Kitengela field site in Kenya (1°23.8′S, 36°49.5′E, 1650m asl). For comparison, we also collected *T. penzigi* workers and plant material from the inside of the domatium wall. The ant heads and domatium wall material were fixed in 2% glutaraldehyde overnight at 4°C. The glutaraldehyde was then exchanged for a pH 7.3 buffer containing 50mM sodium cacodylate and 150 mM saccharose. We exchanged the buffer for fresh buffer after 10 minutes and kept the sample at 4°C until we could perform sectioning. The heads were embedded in Araldite and sectioned with a Reichert Ultracut E microtome. Semithin 1 mm sections were stained with methylene blue and thionin and viewed in a Zeiss Axioskop microscope.

Debris pellets were visible in the infrabuccal pockets of both *T. penzigi* (Figure A.12) and *C. nigriceps* alates (Figure A.13). *T. penzigi* workers also had debris in their infrabuccal pockets resembling

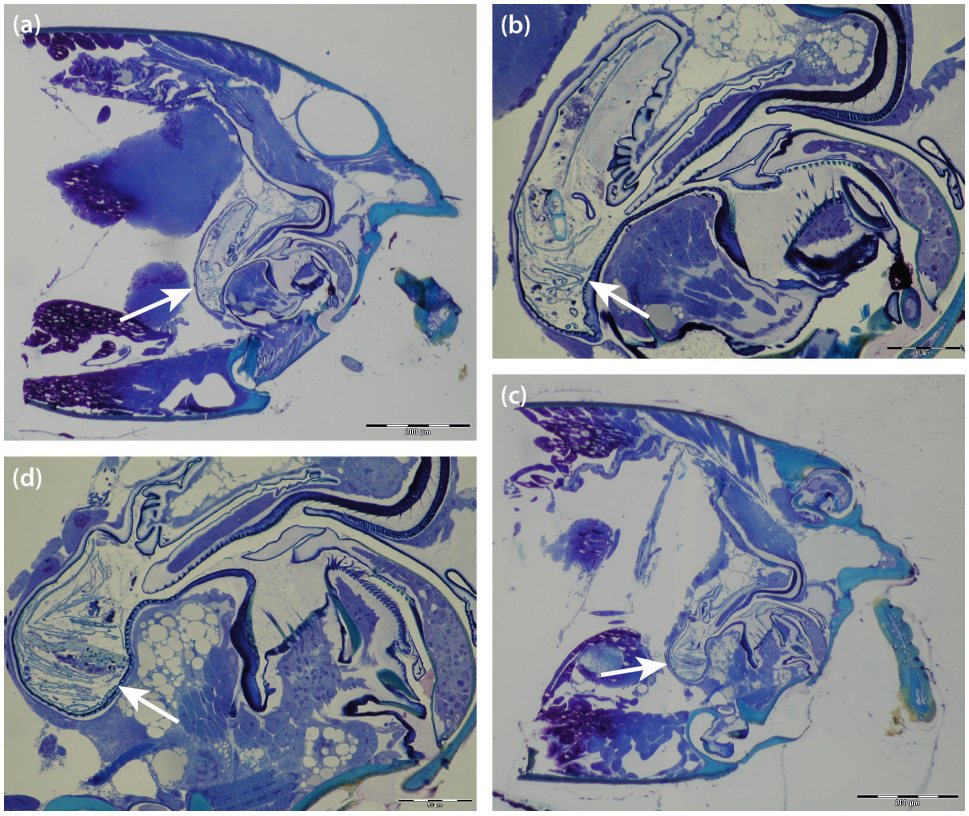

Figure A.12: Sagittal sections through *T. penzigi* alate heads. (a) and (b) show the same worker at different magnifications; likewise for (c) and (d). Arrows indicate debris pellet in the infrabuccal pocket.

plant cells from the domatium wall (Figure A.14).

To examine the morphology of the *T. penzigi* digestive tract, we dissected several workers and examined them under an Olympus SZX12 stereomicroscope. Workers were briefly immersed in ethanol before being dissected immediately in pH 7.4 phosphate buffered saline. We dissected the workers' gasters by gently pulling on the last abdominal segment with forceps to extend the digestive tract and then removing the anterior sclerites. No evidence of a pouch or other specialized structure that might contain bacterial symbionts was visible at the midgut-hindgut junction (Figure A.15).

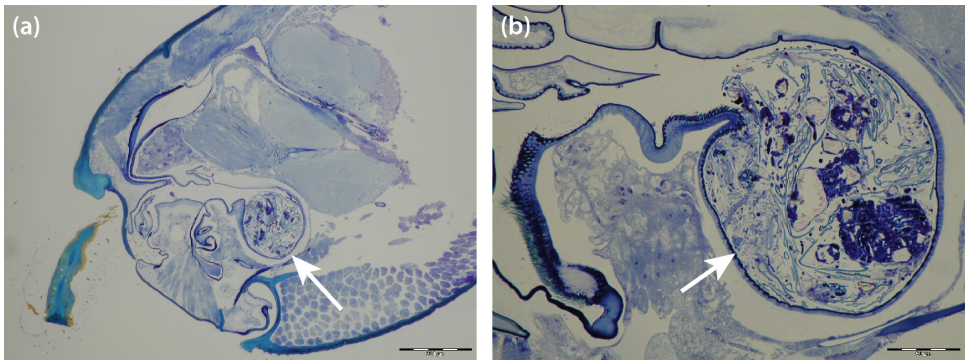

Figure A.13: Sagittal sections through *C. nigriceps* alate heads. (a) and (b) show the same worker at different magnifications. Arrows indicate debris pellet in the infrabuccal pocket.

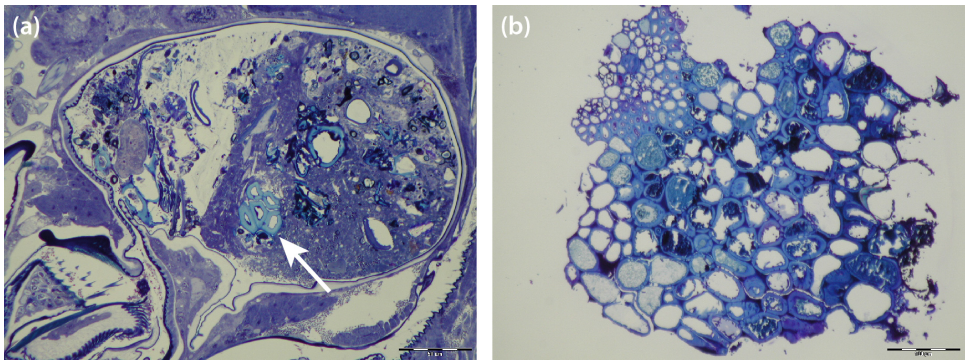

Figure A.14: (a) Sagittal section through *T. penzigi* worker infrabuccal pocket showing debris pellet. Arrow indicates debris resembling plant cells. (b) Section through cells from the inside of the domatium wall.

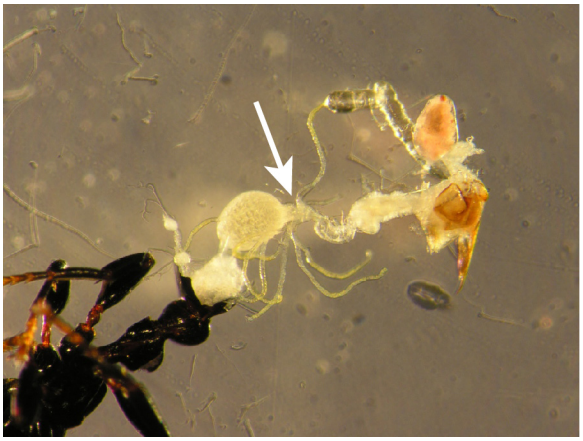

Figure A.15: Dissected *T. penzigi* worker gut. No evidence of a bacterial pouch is present at the junction of midgut and hindgut, indicated with an arrow.

Appendix B

Supplementary material for Chapter 3

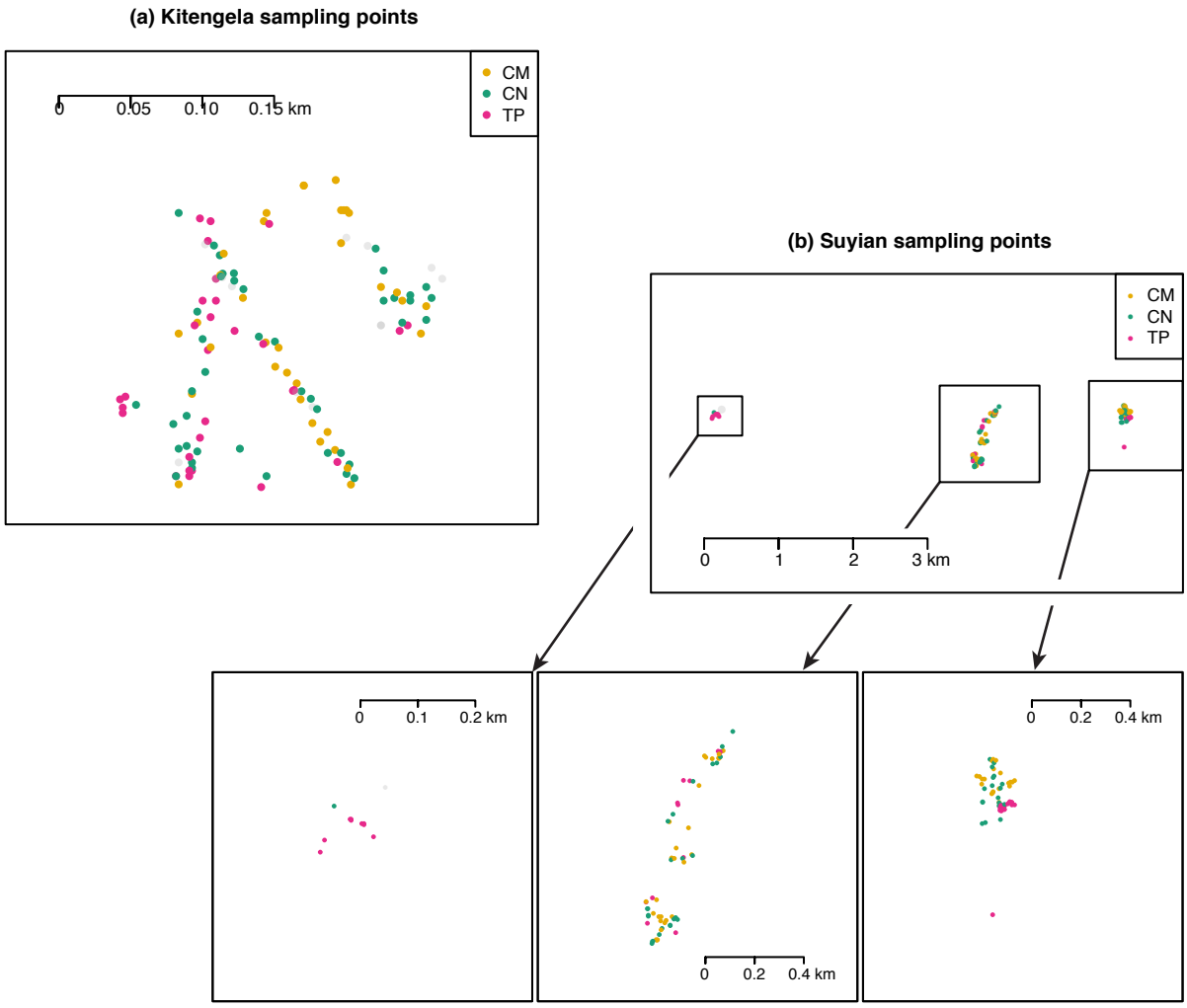

Figure B.1: Maps of sampling locations at (a) Kitengela and (b) Suyian

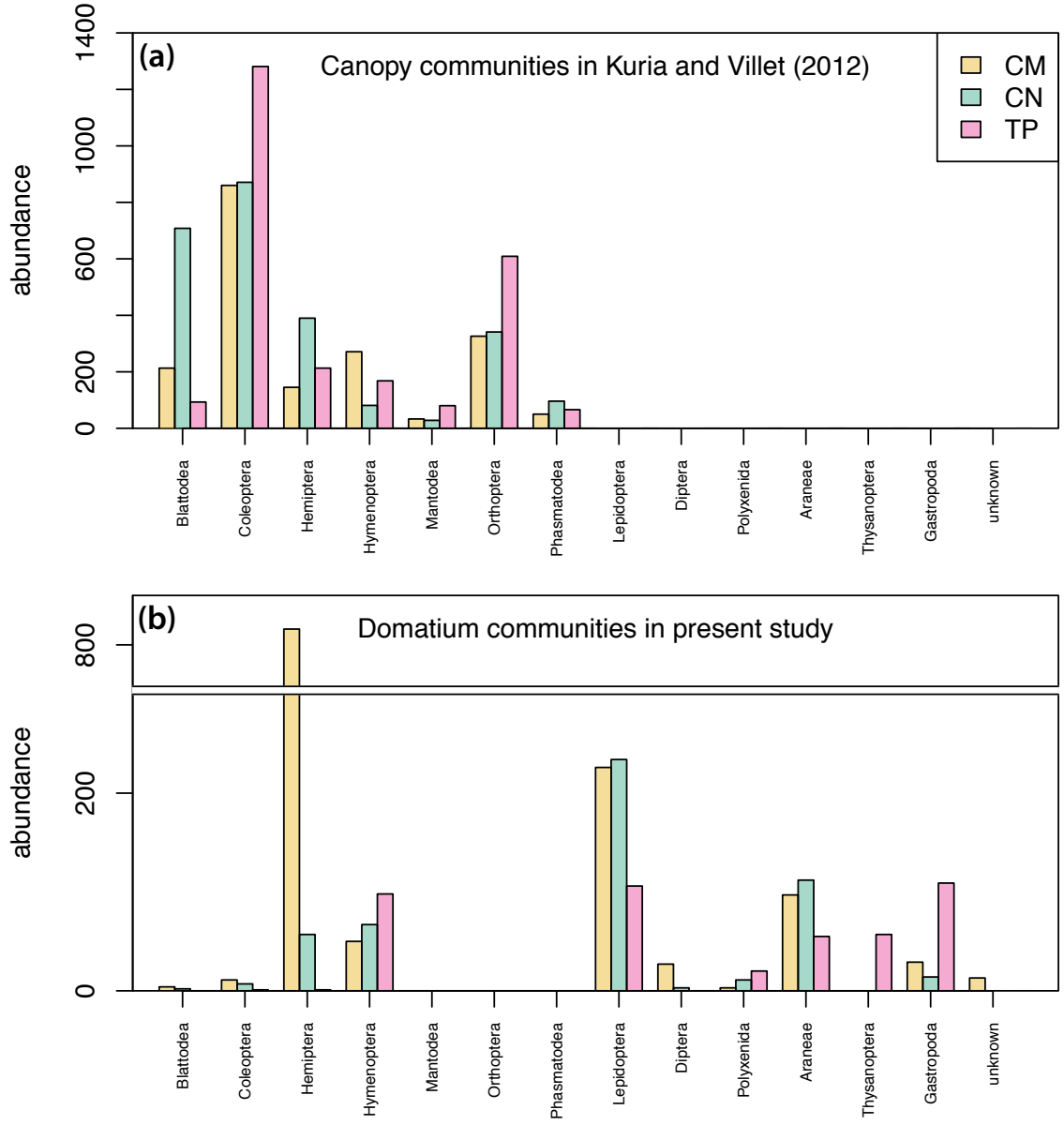

Figure B.2: Domatium communities show little taxonomic overlap with canopy communities as assessed by Kuria and Villet in 2012 using fogging and beating methods and a morphospecies approach[109]. Each order is broken down here by the ant species occupying the tree.

Table B.1: Summary of myrmecophile taxa collected over 2012-2014 at Kitengela and Suyian

			Kitengela 2012		Kitengela 2013		Suyian 2013		Total	
OTU	**Family**	**Species**	**trees**	**indivs**	**trees**	**indivs**	**trees**	**indivs**	**trees**	**indivs**
Arthropoda : Insecta : Lepidoptera										
0	Tortricidae	*Endotera cyaneana* Agassiz	2	2	2	2	0	0	4	4
1	Tortricidae	*Endotera cyphospila* (Meyrick) =nodi Agassiz	1	1	2	2	0	0	3	3
2	Tortricidae	*Hystrichophora vittana* Agassiz	0	0	1	1	0	0	1	1
3	Tortricidae	*Hystrichophora griseana* Agassiz	55	119	24	39	47	108	126	266
4	Tortricidae	*Hystrichophora bopprei* Agassiz	0	0	0	0	0	0	0	0
5	Noctuidae	NA	0	0	0	0	1	1	1	1
6	NA	NA	1	2	0	0	0	0	1	2
7	NA	NA	0	0	0	0	1	1	1	1
8	NA	NA	0	0	6	7	0	0	6	7
9	NA	NA	0	0	0	0	1	1	1	1
10	NA	NA	2	2	0	0	0	0	2	2
11	NA	NA	0	0	0	0	1	1	1	1
12	NA	NA	2	2	4	4	0	0	6	6
13	NA	NA	0	0	1	1	0	0	1	1
14	NA	*Tytroca leucoptera?*	0	0	0	0	1	2	1	2
15	Tineidae	*Phthoropoea chalcomochla?*	6	27	8	20	1	2	15	49

Table B.1: Summary of myrmecophile taxa collected over 2012-2014 at Kitengela and Suyian (continued)

			Kitengela 2012		Kitengela 2013		Suyian 2013		Total	
OTU	**Family**	**Species**	**trees**	**indivs**	**trees**	**indivs**	**trees**	**indivs**	**trees**	**indivs**
16	Lycaenidae	NA	1	1	0	0	0	0	1	1
17	Lycaenidae	NA	0	0	1	2	1	1	2	3
18	Geometridae	NA	0	0	1	1	2	3	3	4
19	Geometridae	NA	3	3	1	1	7	7	11	11
20	NA	NA	0	0	1	1	0	0	1	1
21	NA	NA	0	0	0	0	17	46	17	46
22	NA	NA	0	0	1	1	0	0	1	1
23	NA	NA	3	3	2	3	1	10	6	16
24	Gelechiidae	NA	3	5	6	13	0	0	9	18
25	Gelechiidae	*Dichomeris* sp.	24	43	13	22	16	24	53	89
26	NA	NA	6	16	0	0	0	0	6	16
27	NA	NA	5	7	7	7	0	0	12	14
28	NA	NA	0	0	0	0	10	13	10	13
29	NA	NA	0	0	1	1	0	0	1	1
Arthropoda : Insecta : Hymenoptera										
30	NA	NA	2	2	0	0	4	4	6	6
31	Chalcidoidea	NA	0	0	0	0	1	12	1	12
32	Chalcidoidea	NA	0	0	1	1	0	0	1	1

Table B.1: Summary of myrmecophile taxa collected over 2012-2014 at Kitengela and Suyian (continued)

OTU	Family	Species	Kitengela 2012		Kitengela 2013		Suyian 2013		Total	
			trees	indivs	trees	indivs	trees	indivs	trees	indivs
33	Chalcidoidea	NA	1	3	0	0	0	0	1	3
34	Chalcidoidea	NA	1	8	0	0	1	8	2	16
35	Chalcidoidea	NA	1	3	4	4	0	0	5	7
36	Megachilidae	NA	0	0	1	1	0	0	1	1
37	Formicidae	*Crematogaster* sp.	0	0	0	0	3	45	3	45
38	Formicidae	*Technomyrmex* sp.	0	0	2	50	1	4	3	54
39	Formicidae	*Tapinoma* sp.	0	0	1	45	0	0	1	45
40	Ichneumonidae	NA	0	0	0	0	4	6	4	6
41	Ichneumonidae	NA	2	2	0	0	0	0	2	2
42	Ichneumonidae	NA	1	1	0	0	0	0	1	1
43	Braconidae	NA	0	0	1	1	0	0	1	1
44	Braconidae	NA	0	0	0	0	0	0	0	0
45	Braconidae	NA	0	0	3	5	0	0	3	5
46	Braconidae	*Trigastrotheca laikipiensis*[169]	1	1	14	22	1	4	16	27
47	Pompilidae	NA	0	0	0	0	0	0	0	0
48	Diapriidae	NA	0	0	1	1	0	0	1	1
49	Crabronidae	NA	0	0	1	1	0	0	1	1

Table B.1: Summary of myrmecophile taxa collected over 2012-2014 at Kitengela and Suyian (continued)

OTU	Family	Species	Kitengela 2012		Kitengela 2013		Suyian 2013		Total	
			trees	indivs	trees	indivs	trees	indivs	trees	indivs
Arthropoda : Insecta : Diptera										
50	Syrphidae	*Microdon* sp.	1	1	1	1	0	0	2	2
51	Phoridae	NA	0	0	1	1	0	0	1	1
52	NA	NA	0	0	0	0	0	0	0	0
53	NA	NA	0	0	2	2	0	0	2	2
54	Agromyzidae	*Melanagromyza acaciae*[88,194]	1	3	0	0	5	22	6	25
Arthropoda : Insecta : Coleoptera										
55	Chrysomelidae	NA	1	1	6	8	0	0	7	9
56	Chrysomelidae	NA	0	0	1	1	0	0	1	1
57	Tenebrionidae	NA	0	0	0	0	1	1	1	1
58	Cerambycidae	NA	0	0	2	2	0	0	2	2
59	Curculionidae	NA	0	0	1	1	0	0	1	1
60	Anthicidae	NA	1	1	2	2	0	0	3	3
61	NA	NA	0	0	1	2	0	0	1	2
62	NA	NA	0	0	0	0	1	1	1	1
Arthropoda : Diplopoda : Polyxenida										
63	NA	NA	2	2	7	12	7	21	16	35

Table B.1: Summary of myrmecophile taxa collected over 2012-2014 at Kitengela and Suyian (continued)

			Kitengela 2012		Kitengela 2013		Suyian 2013		Total	
OTU	**Family**	**Species**	**trees**	**indivs**	**trees**	**indivs**	**trees**	**indivs**	**trees**	**indivs**
Arthropoda : Arachnida : Araneae										
64	Salticidae	*Myrmarachne* sp.	8	9	24	97	0	0	32	106
65	Salticidae	NA	10	17	20	28	1	1	31	46
66	Salticidae	*Hyllus dotatus?*	0	0	0	0	1	1	1	1
67	Salticidae	NA	1	1	0	0	0	0	1	1
68	Salticidae	NA	1	1	0	0	0	0	1	1
69	Clubionidae	*Clubiona* sp.	1	1	1	2	0	0	2	3
70	Clubionidae	*Clubiona* sp.	0	0	5	6	1	1	6	7
71	Eutichuridae	*Cheiracanthium* sp.	6	8	28	70	0	0	34	78
72	Eutichuridae	*Cheiracanthium* sp.	5	8	25	31	7	9	37	48
73	Eutichuridae	*Cheiracanthium* sp.	0	0	2	2	1	1	3	3
74	NA	NA	1	1	4	4	0	0	5	5
75	NA	NA	0	0	0	0	0	0	0	0
76	Gnaphosidae	NA	1	1	6	6	2	2	9	9
77	NA	NA	0	0	1	1	0	0	1	1
78	NA	NA	0	0	1	1	0	0	1	1
79	Thomisidae	NA	0	0	1	11	0	0	1	11
80	Theridiidae	NA	0	0	1	1	0	0	1	1

Table B.1: Summary of myrmecophile taxa collected over 2012-2014 at Kitengela and Suyian (continued)

OTU	Family	Species	Kitengela 2012 trees	Kitengela 2012 indivs	Kitengela 2013 trees	Kitengela 2013 indivs	Suyian 2013 trees	Suyian 2013 indivs	Total trees	Total indivs
Arthropoda : Insecta : Hemiptera										
81	Cixiidae	NA	0	0	1	1	0	0	1	1
82	Stictococcidae	*Hockiana* sp.	0	0	0	0	38	873	38	873
Arthropoda : Insecta : Thysanoptera										
83	NA	NA	0	0	3	59	0	0	3	59
Arthropoda : Insecta : Blattodea										
84	NA	*Blattella lobiventris?*[88]	0	0	2	2	3	3	5	5
Mollusca : Gastropoda										
85	NA	*Succinea concisa?*[88]	0	0	0	0	23	152	23	152
Arthropoda : Insecta										
86	NA	NA	0	0	1	14	0	0	1	14

Figures B.3 through B.83 show representative images of myrmecophile taxa where available.

Figure B.3: OTU 0 – *Endotera cyaneana* (Lepidoptera:Tortricidae)

Figure B.4: OTU 1 – *Endotera cyphospila* (Lepidoptera:Tortricidae)

Figure B.5: OTU 3 – *Hystrichophora griseana* (Lepidoptera:Tortricidae)

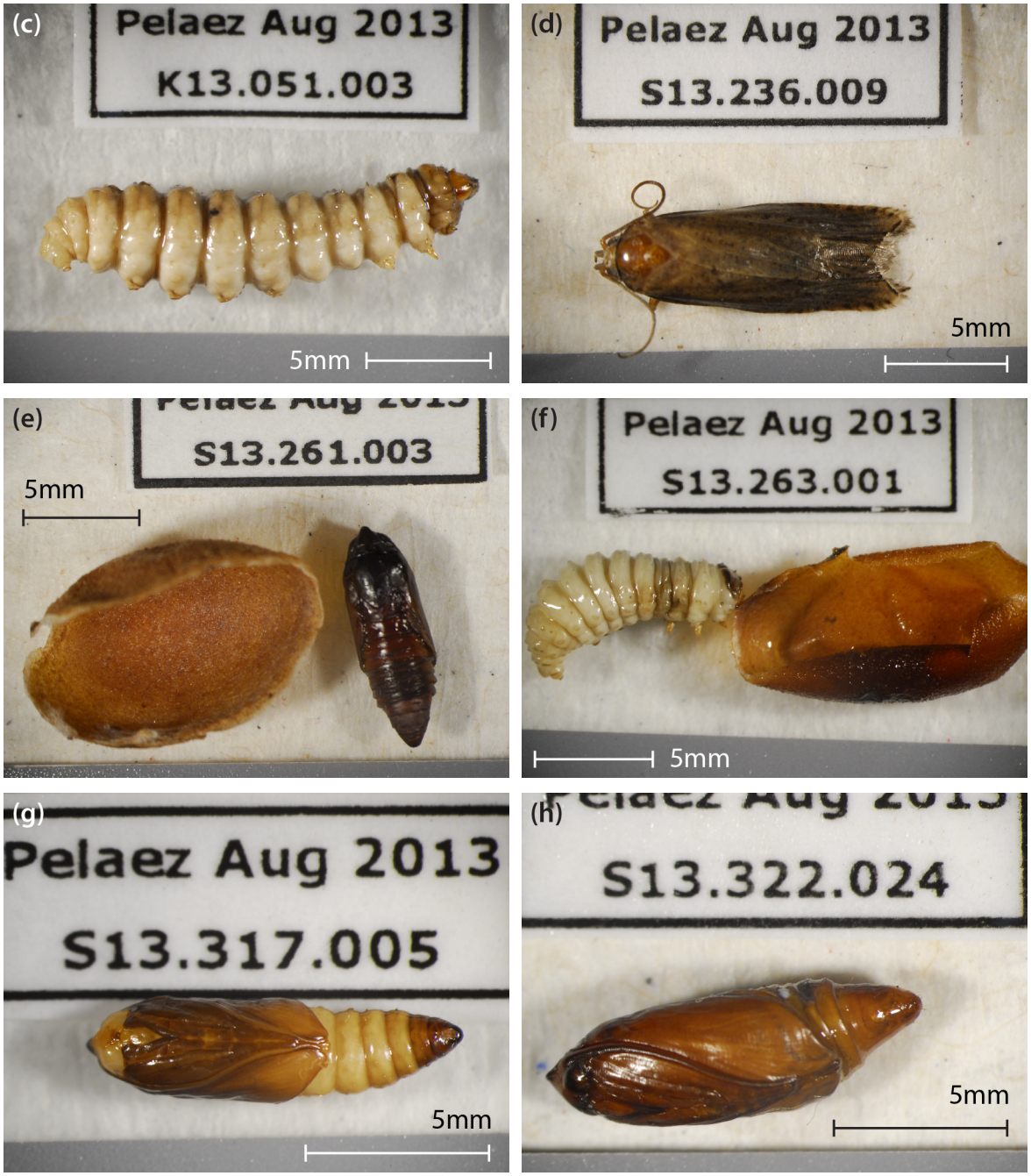

Figure B.5: OTU 3 – *Hystrichophora griseana* (Lepidoptera:Tortricidae) (continued)

Figure B.6: OTU 5 – unidentified species (Lepidoptera:Noctuidae)

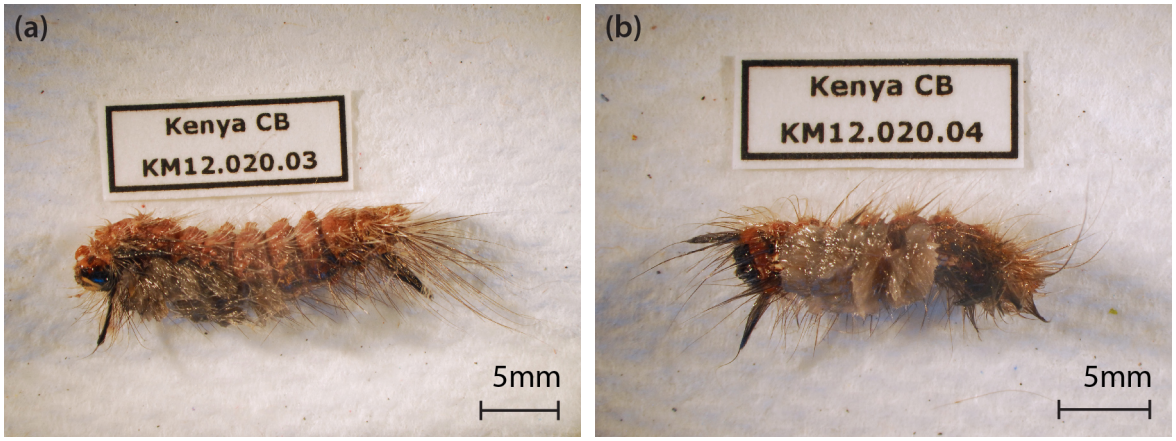

Figure B.7: OTU 6 – unidentified Lepidoptera

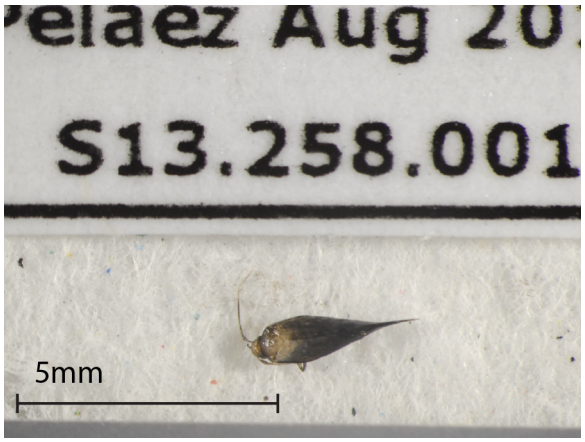

Figure B.8: OTU 7 – unidentified Lepidoptera

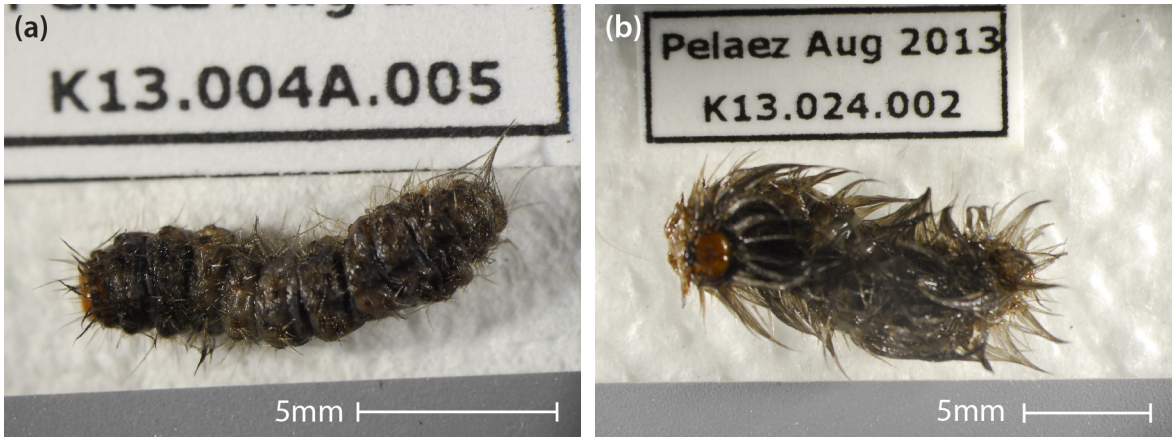

Figure B.9: OTU 8 – unidentified Lepidoptera

Figure B.10: OTU 9 – unidentified Lepidoptera

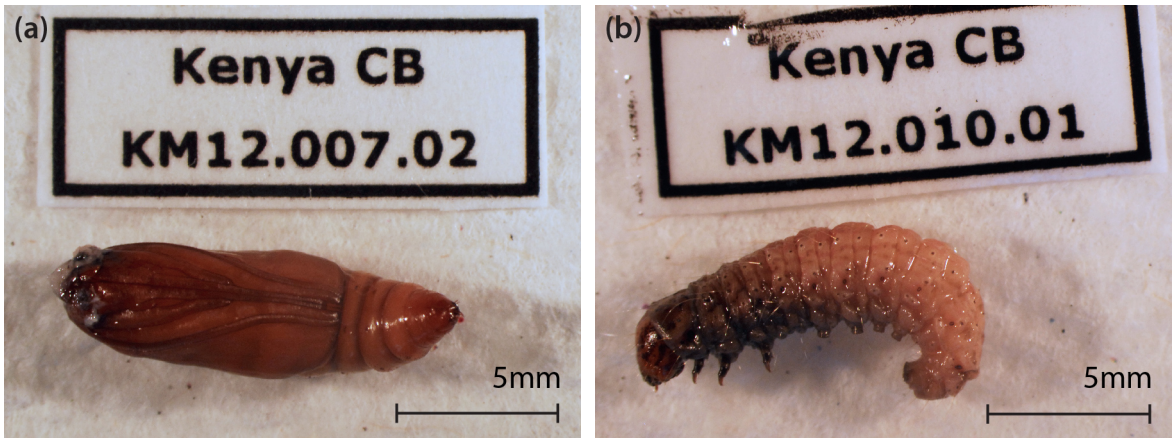

Figure B.11: OTU 10 – unidentified Lepidoptera

Figure B.12: OTU 11 – unidentified Lepidoptera

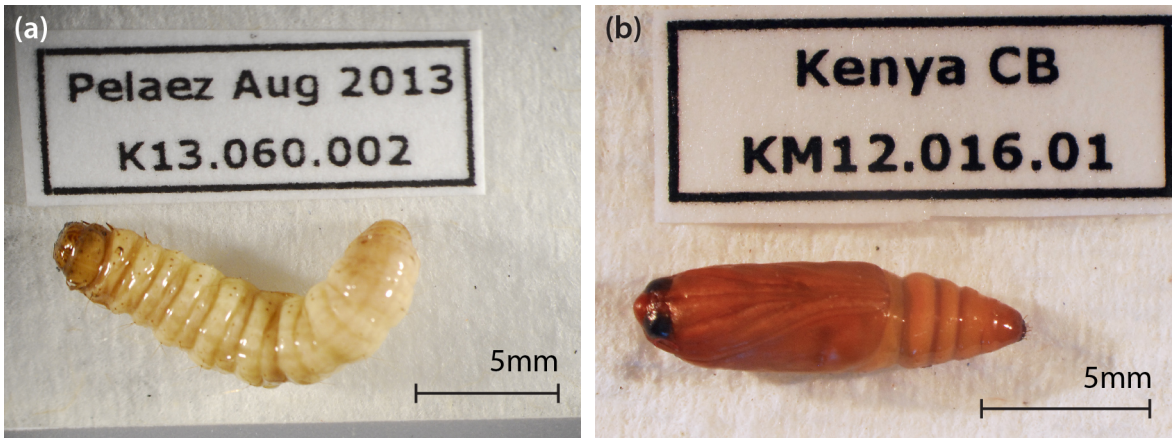

Figure B.13: OTU 12 – unidentified Lepidoptera

Figure B.14: OTU 13 – unidentified Lepidoptera

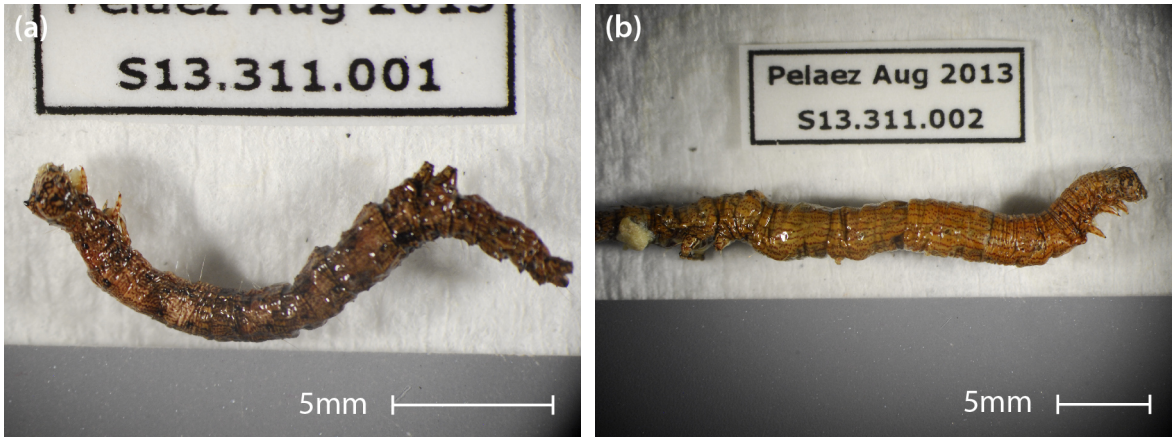

Figure B.15: OTU 14 – unidentified Lepidoptera

Figure B.16: OTU 15 – unidentified species (Lepidoptera:Tineidae)

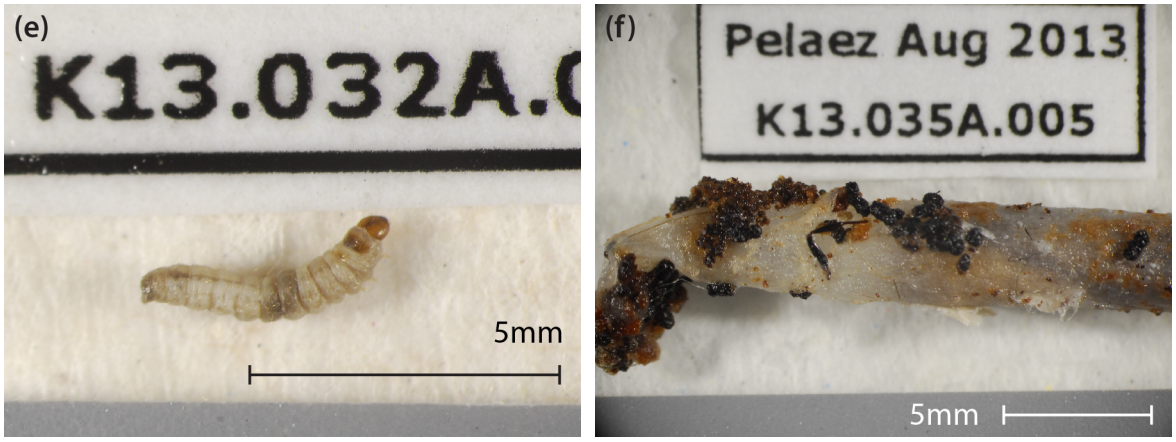

Figure B.16: OTU 15 – unidentified species (Lepidoptera:Tineidae) (continued)

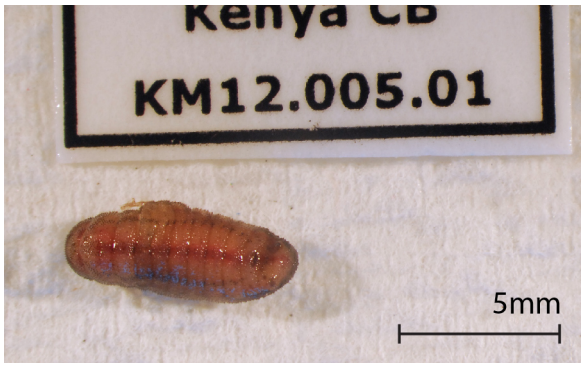

Figure B.17: OTU 16 – unidentified species (Lepidoptera:Lycaenidae)

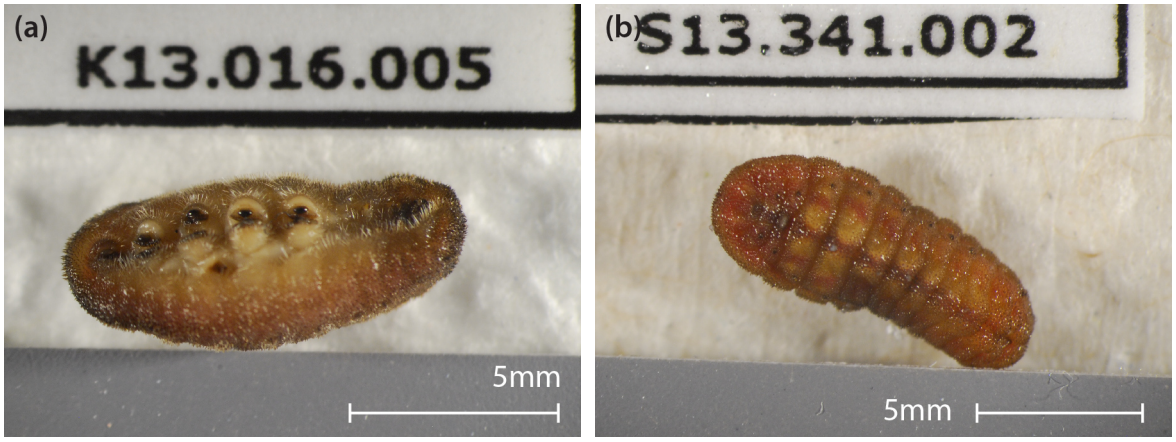

Figure B.18: OTU 17 – unidentified species (Lepidoptera:Lycaenidae)

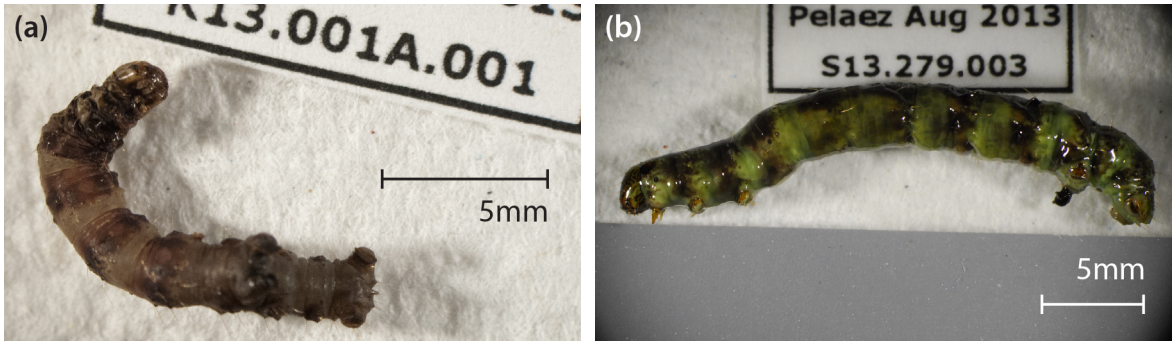

Figure B.19: OTU 18 – unidentified species (Lepidoptera:Geometridae)

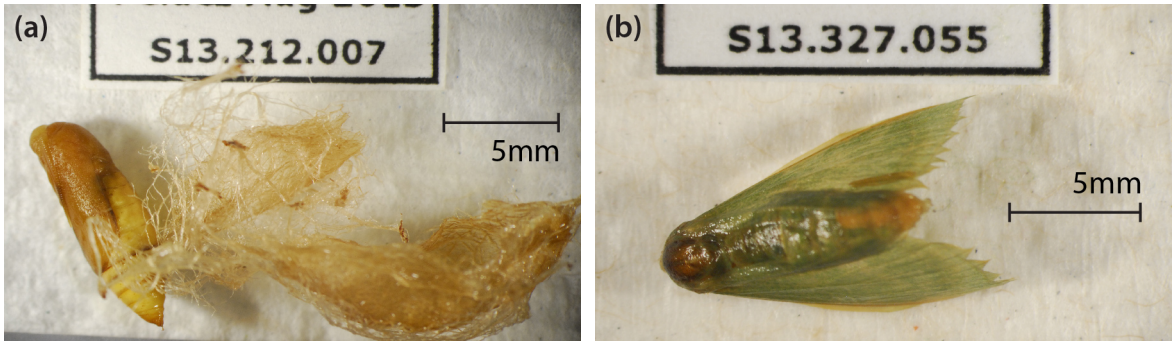

Figure B.20: OTU 19 – unidentified species (Lepidoptera:Geometridae)

Figure B.21: OTU 20 – unidentified Lepidoptera

Figure B.22: OTU 21 – unidentified Lepidoptera

Figure B.23: OTU 22 – unidentified Lepidoptera

Figure B.24: OTU 23 – unidentified Lepidoptera

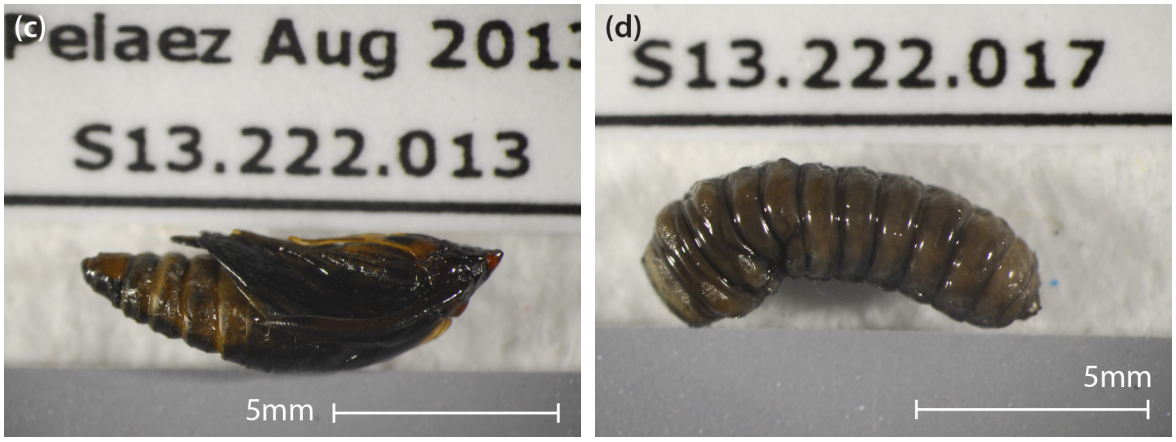

Figure B.24: OTU 23 – unidentified Lepidoptera (continued)

Figure B.25: OTU 24 – unidentified species (Lepidoptera:Gelechiidae)

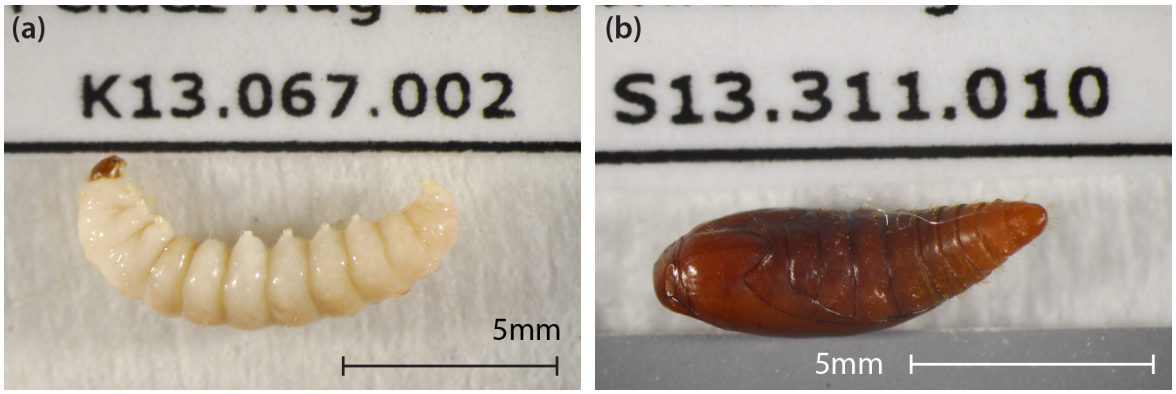

Figure B.26: OTU 25 – *Dichomeris* sp. (Lepidoptera:Gelechiidae)

Figure B.27: OTU 26 – unidentified Lepidoptera

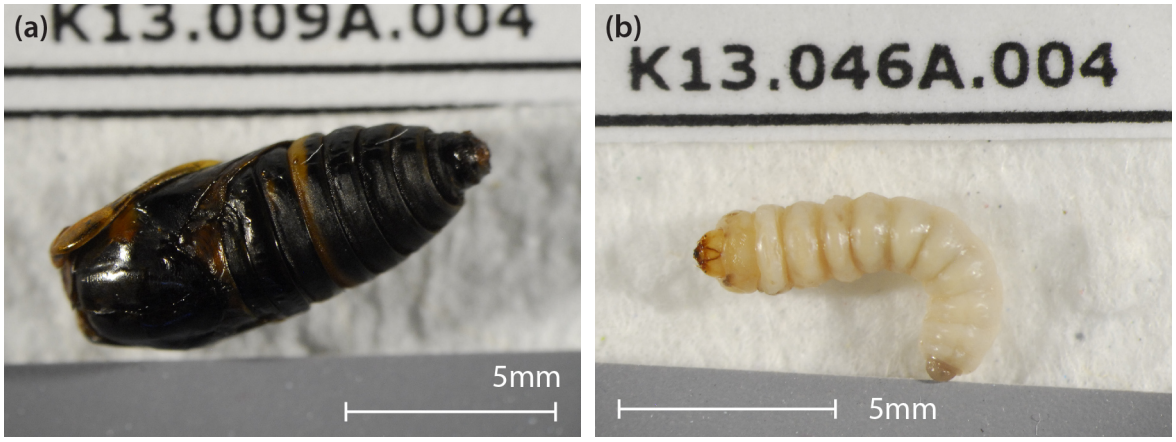

Figure B.28: OTU 27 – unidentified Lepidoptera

Figure B.29: OTU 28 – unidentified Lepidoptera

Figure B.30: OTU 29 – unidentified Lepidoptera

Figure B.31: OTU 30 – unidentified Hymenoptera

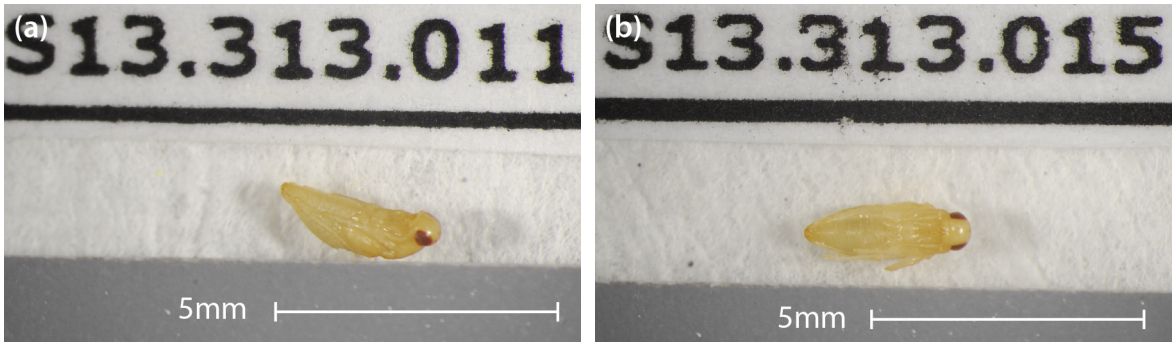

Figure B.32: OTU 31 – unidentified species (Hymenoptera:Chalcidoidea)

Figure B.33: OTU 32 – unidentified species (Hymenoptera:Chalcidoidea)

Figure B.34: OTU 33 – unidentified species (Hymenoptera:Chalcidoidea)

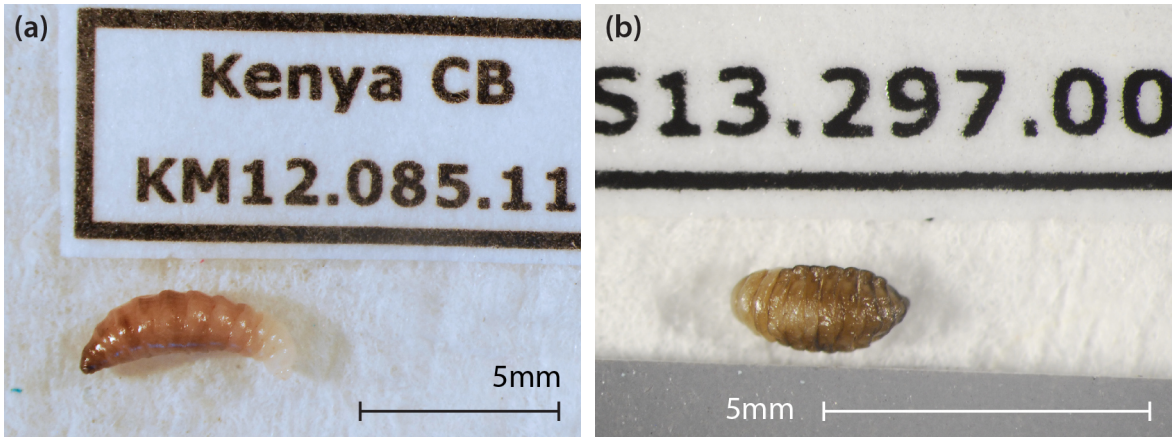

Figure B.35: OTU 34 – unidentified species (Hymenoptera:Chalcidoidea)

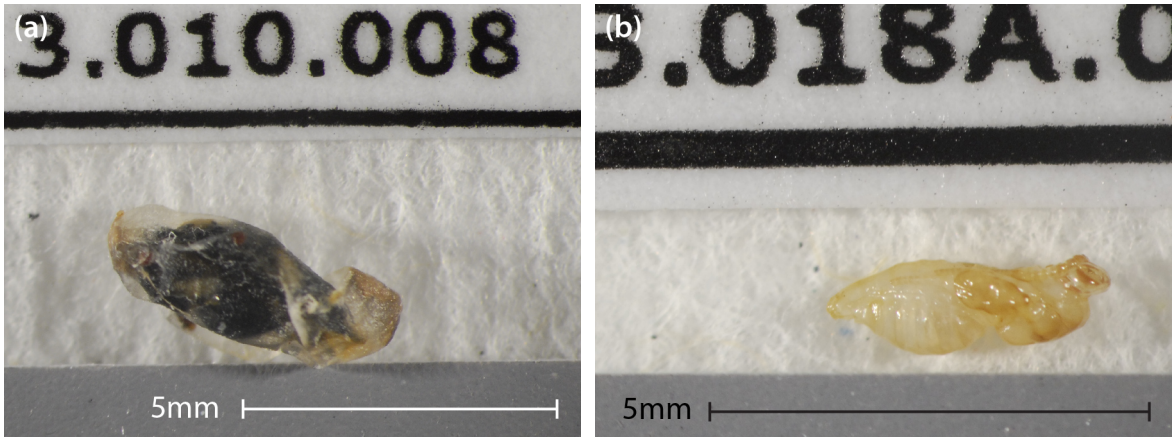

Figure B.36: OTU 35 – unidentified species (Hymenoptera:Chalcidoidea)

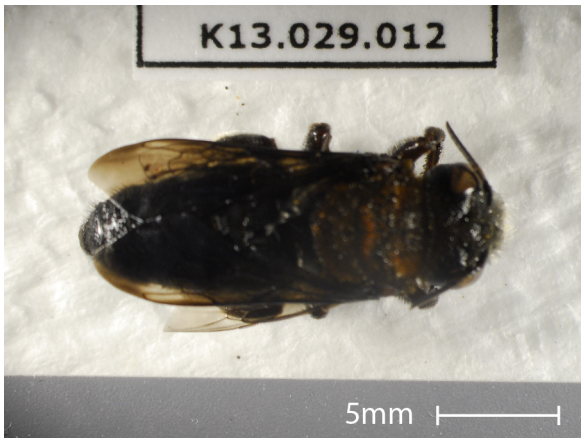

Figure B.37: OTU 36 – unidentified species (Hymenoptera:Megachilidae)

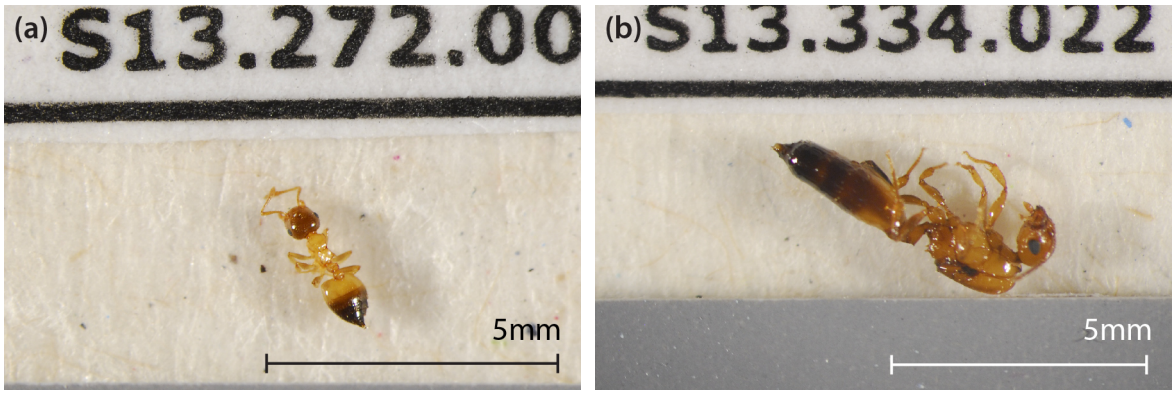

Figure B.38: OTU 37 – *Crematogaster* sp. (Hymenoptera:Formicidae)

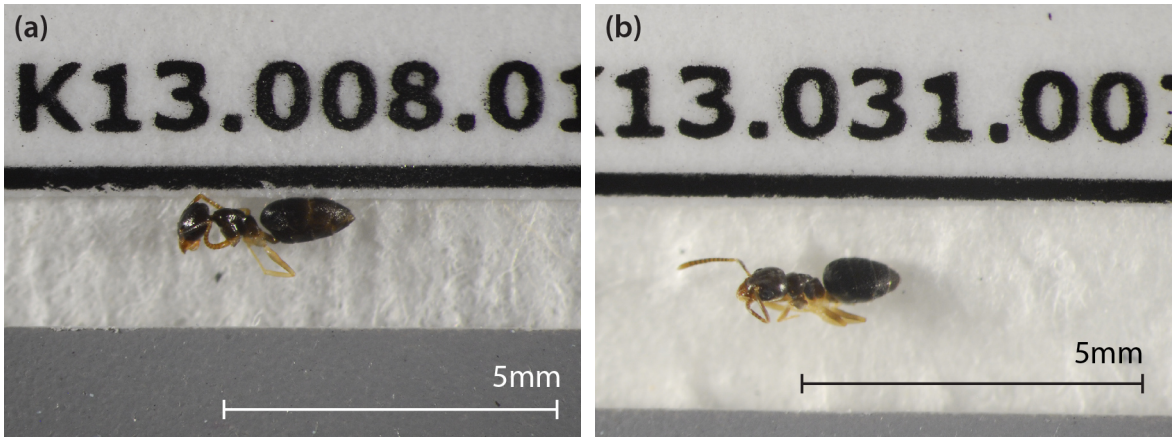

Figure B.39: OTU 38 – *Technomyrmex* sp. (Hymenoptera:Formicidae)

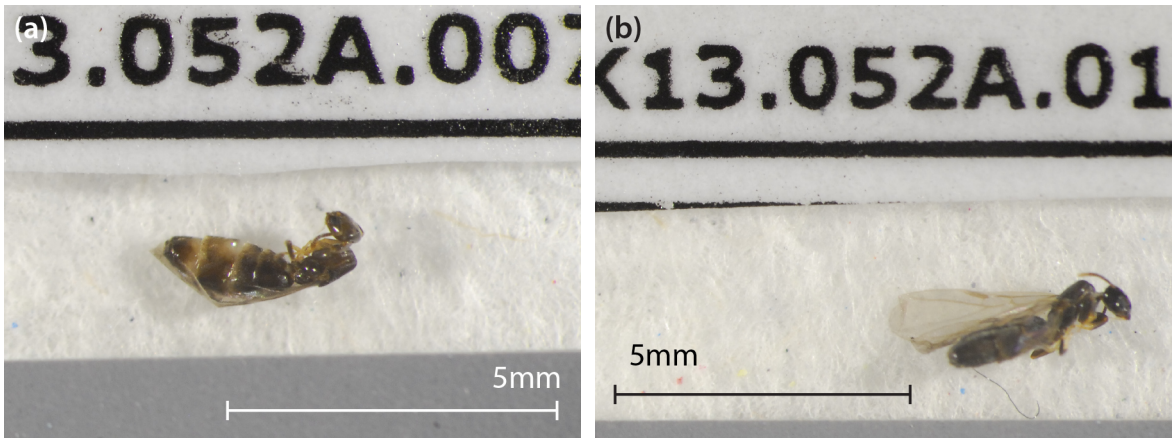

Figure B.40: OTU 39 – *Tapinoma* sp. (Hymenoptera:Formicidae)

Figure B.41: OTU 40 – unidentified species (Hymenoptera:Ichneumonidae)

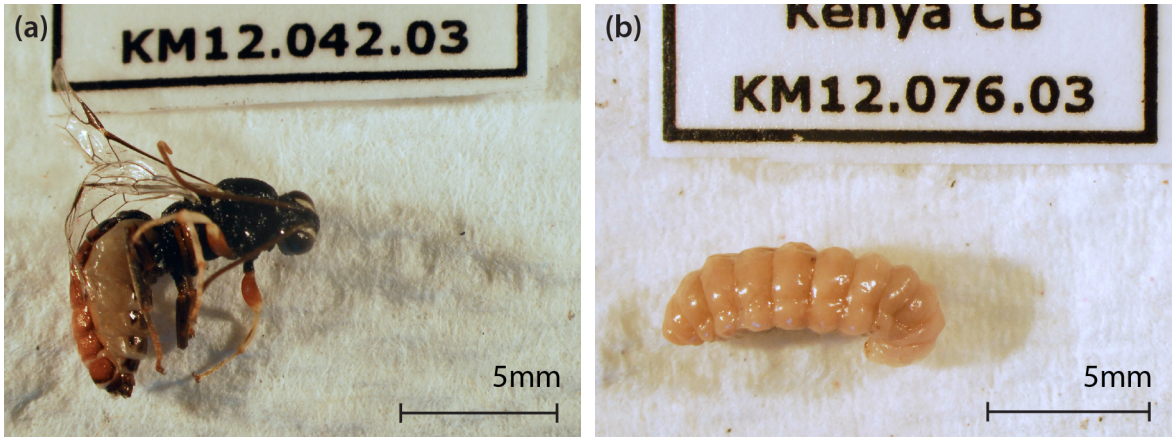

Figure B.42: OTU 41 – unidentified species (Hymenoptera:Ichneumonidae)

Figure B.43: OTU 42 – unidentified species (Hymenoptera:Ichneumonidae)

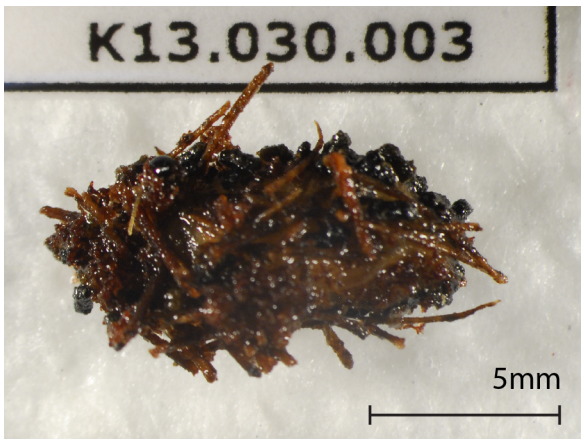

Figure B.44: OTU 43 – unidentified species (Hymenoptera:Braconidae)

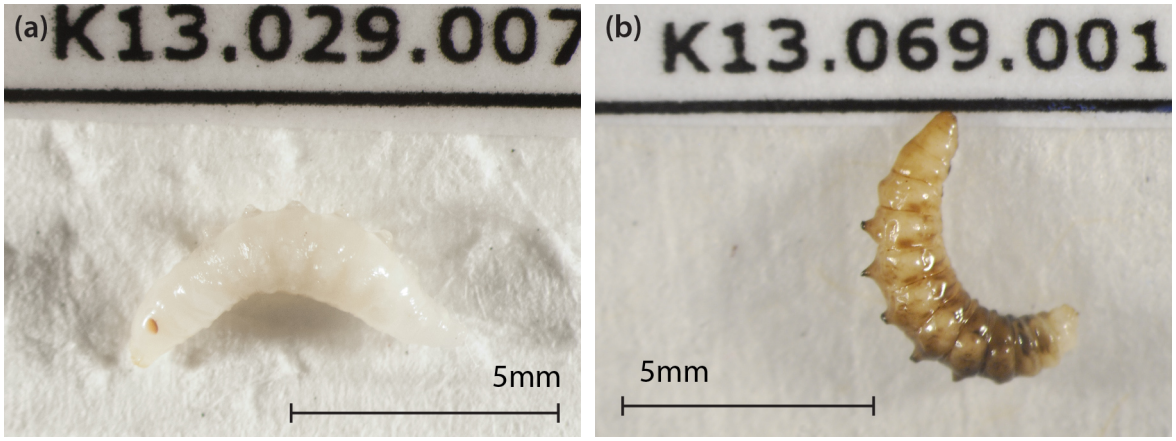

Figure B.45: OTU 45 – unidentified species (Hymenoptera:Braconidae)

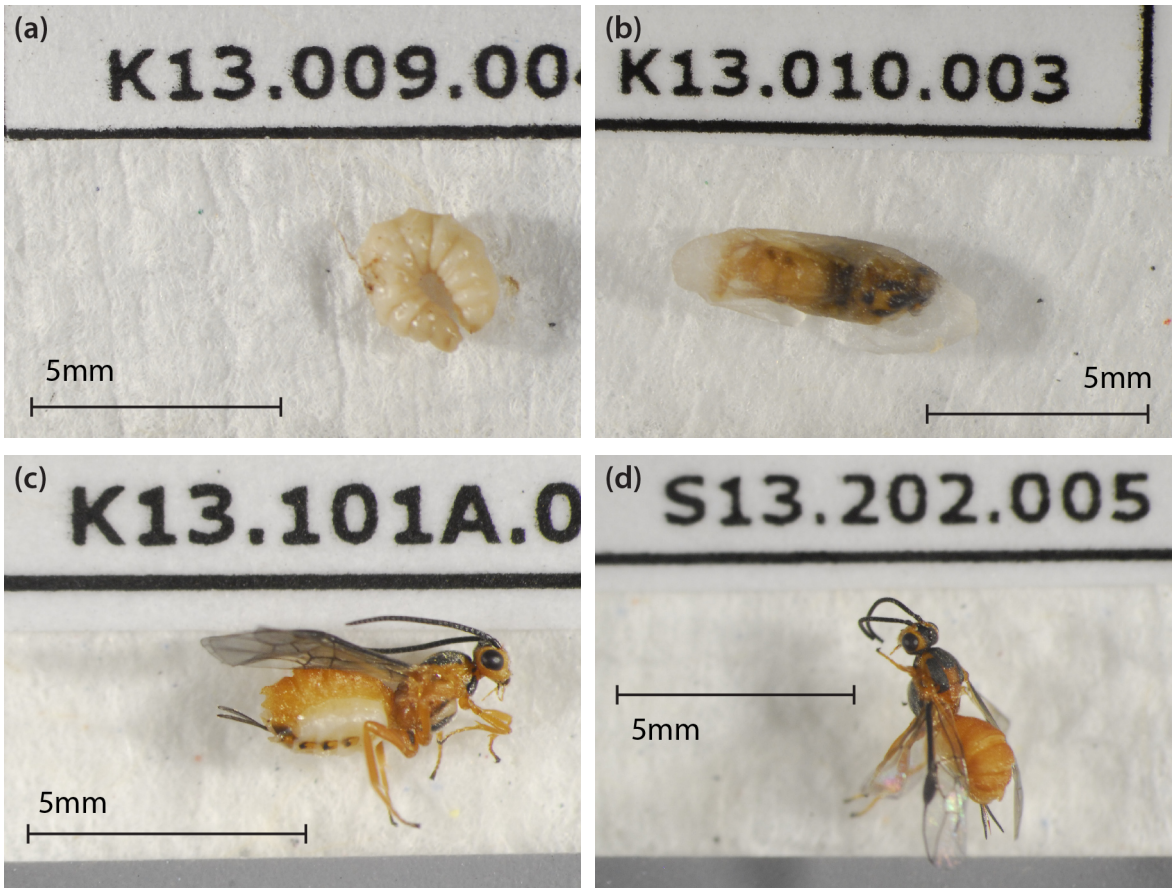

Figure B.46: OTU 46 – *Trigastrotheca laikipiensis* (Hymenoptera:Braconidae)

Figure B.47: OTU 48 – unidentified species (Hymenoptera:Diapriidae)

Figure B.48: OTU 49 – unidentified species (Hymenoptera:Crabronidae)

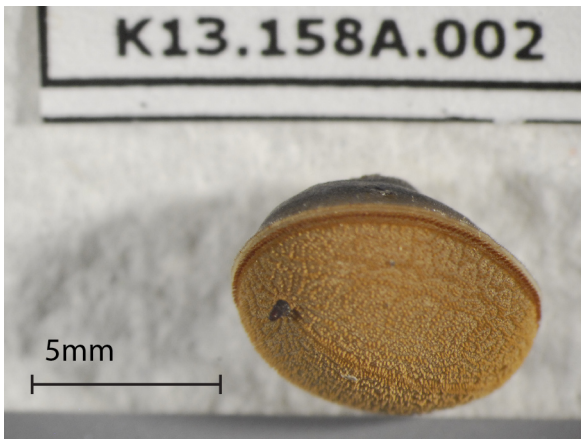

Figure B.49: OTU 50 – *Microdon* sp. (Diptera:Syrphidae)

Figure B.50: OTU 51 – unidentified species (Diptera:Phoridae)

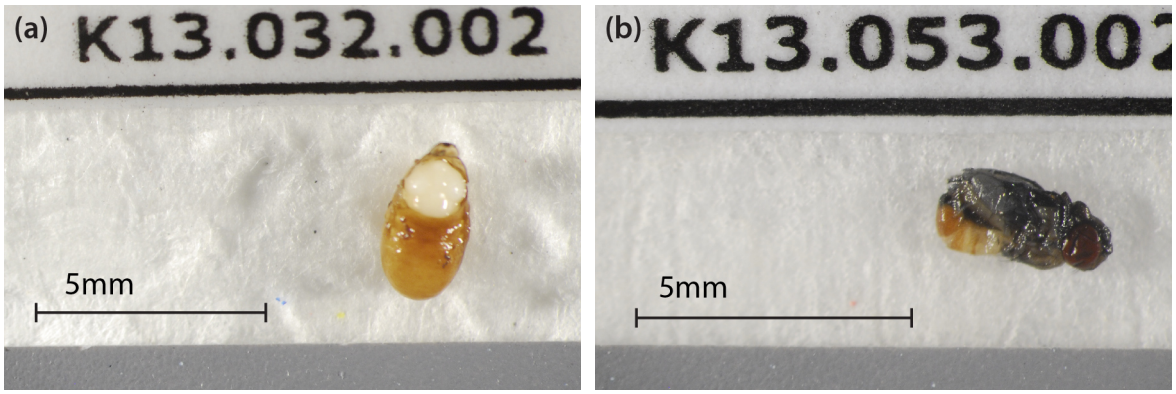

Figure B.51: OTU 53 – unidentified Diptera

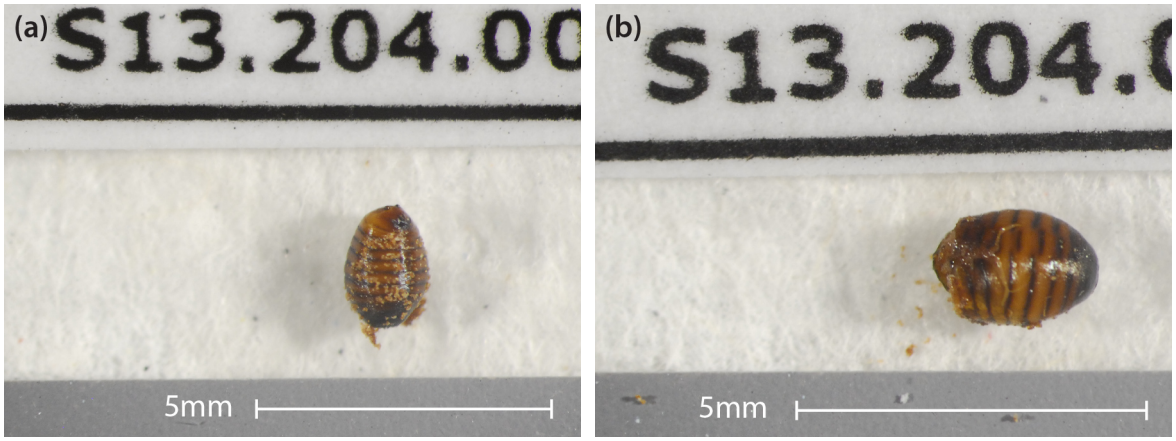

Figure B.52: OTU 54 – *Melanagromyza acaciae* (Diptera:Agromyzidae)

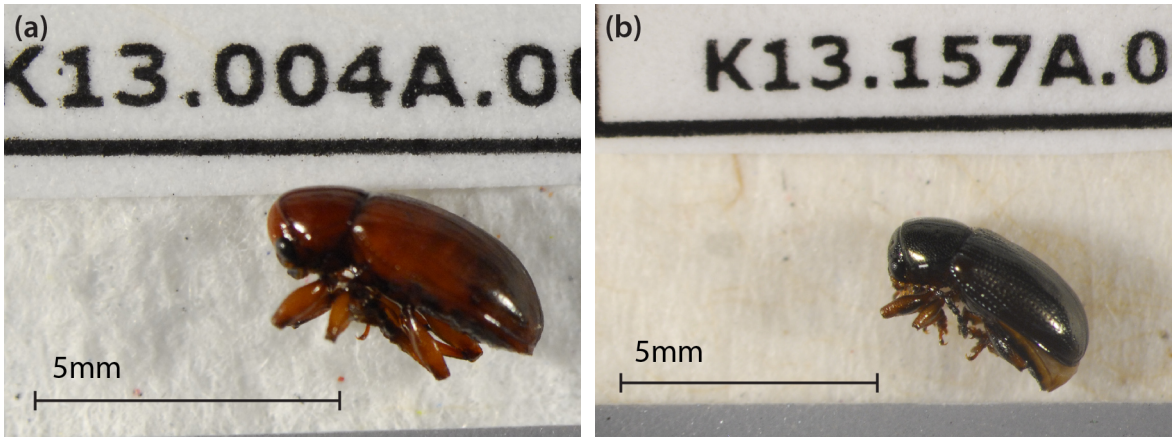

Figure B.53: OTU 55 – unidentified species (Coleoptera:Chrysomelidae)

Figure B.54: OTU 56 – unidentified species (Coleoptera:Chrysomelidae)

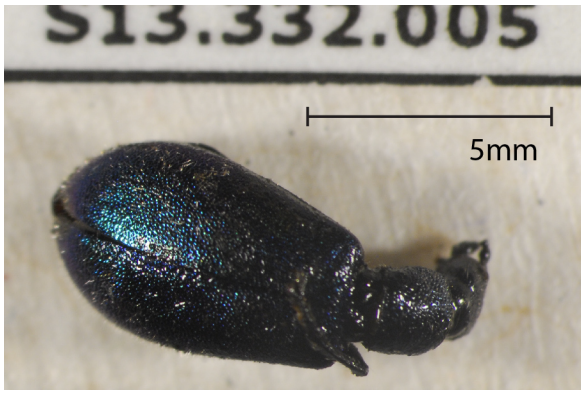

Figure B.55: OTU 57 – unidentified species (Coleoptera:Tenebrionidae)

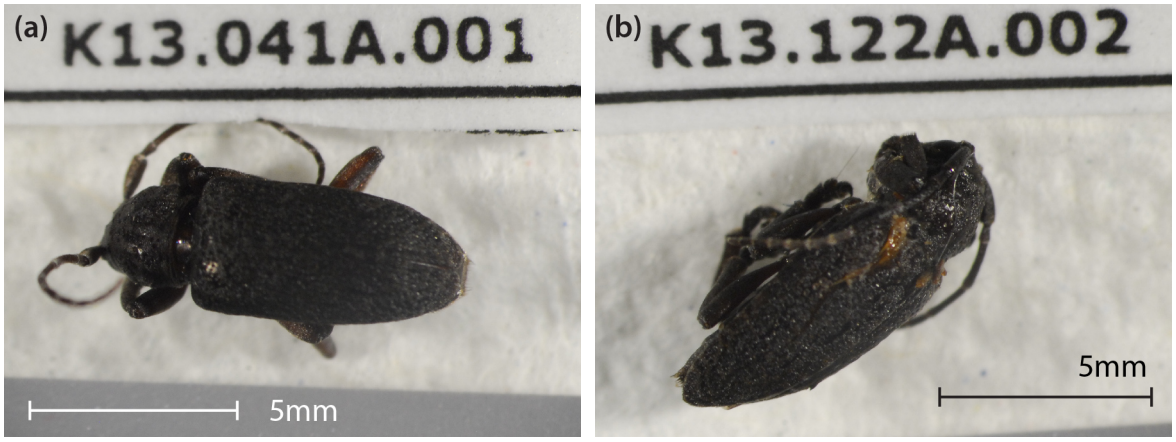

Figure B.56: OTU 58 – unidentified species (Coleoptera:Cerambycidae)

Figure B.57: OTU 59 – unidentified species (Coleoptera:Curculionidae)

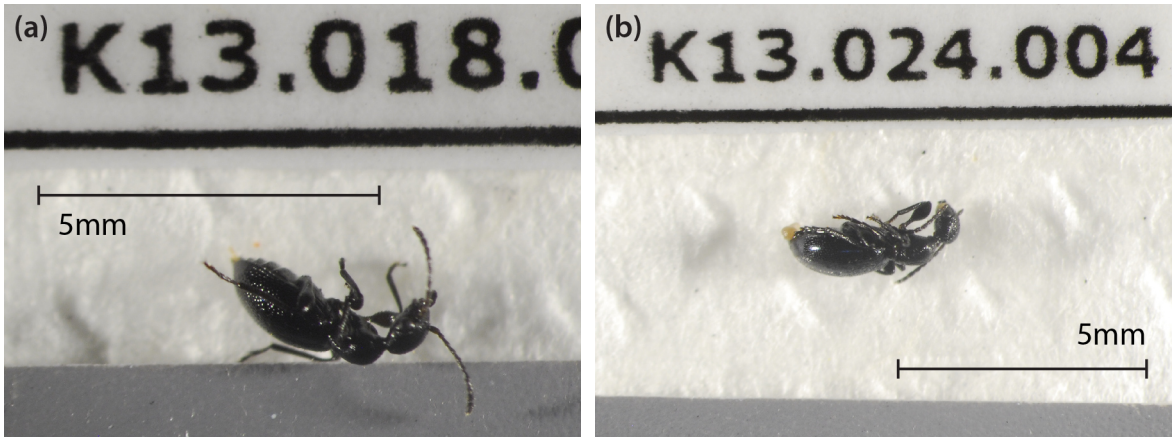

Figure B.58: OTU 60 – unidentified species (Coleoptera:Anthicidae)

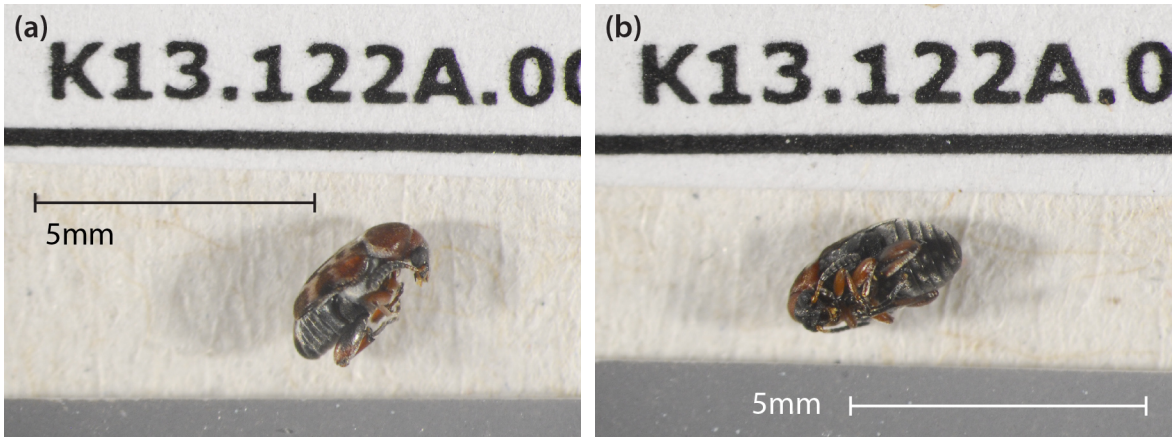

Figure B.59: OTU 61 – unidentified Coleoptera

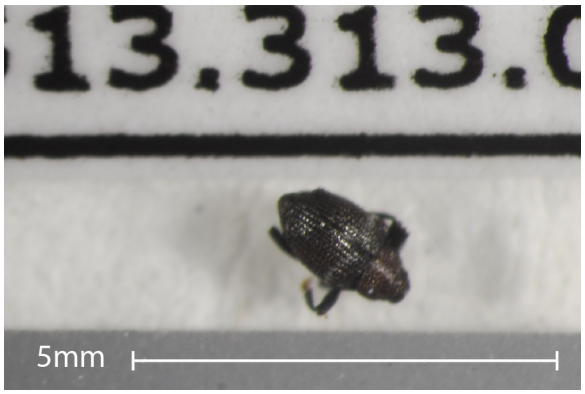

Figure B.60: OTU 62 – unidentified Coleoptera

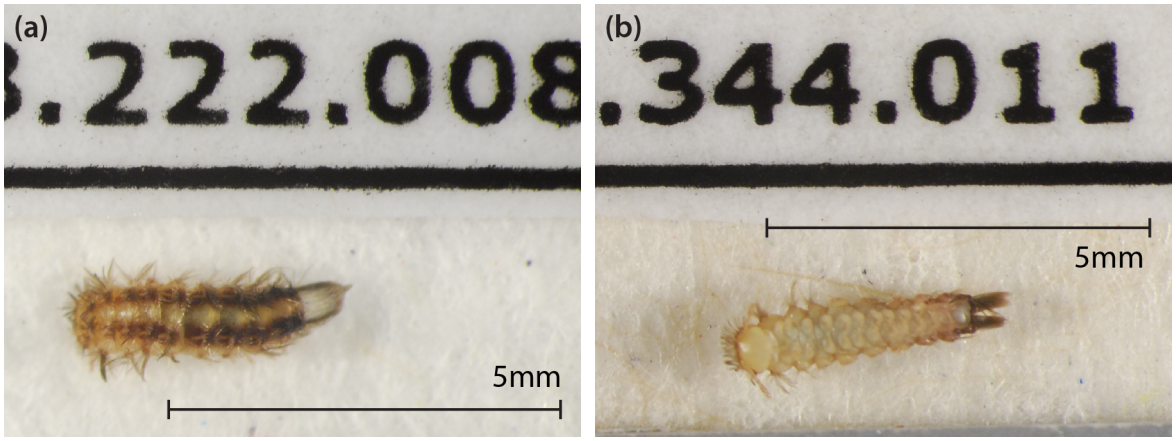

Figure B.61: OTU 63 – unidentified Polyxenida

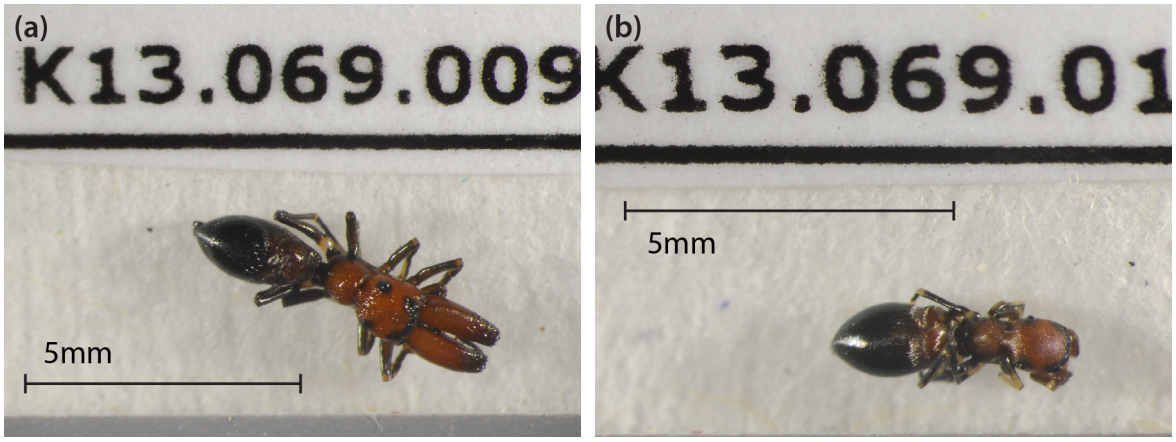

Figure B.62: OTU 64 – *Myrmarachne* sp. (Araneae:Salticidae)

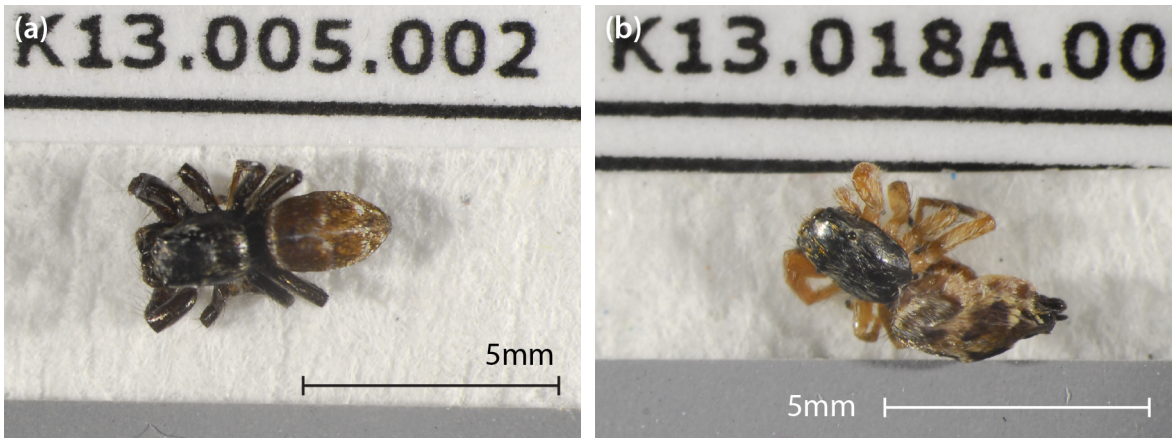

Figure B.63: OTU 65 – unidentified species (Araneae:Salticidae)

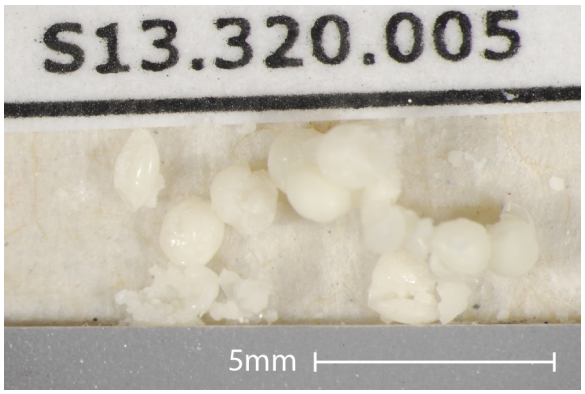

Figure B.64: OTU 66 – unidentified species (Araneae:Salticidae)

Figure B.65: OTU 67 – unidentified species (Araneae:Salticidae)

Figure B.66: OTU 68 – unidentified species (Araneae:Salticidae)

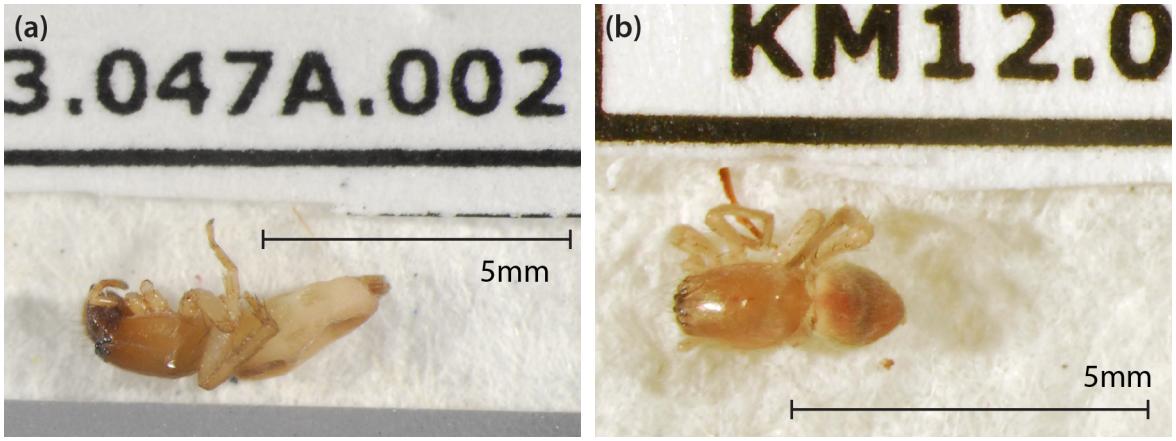

Figure B.67: OTU 69 – *Clubiona* sp. (Araneae:Clubionidae)

Figure B.68: OTU 70 – *Clubiona* sp. (Araneae:Clubionidae)

Figure B.69: OTU 71 – *Cheiracanthium* sp. (Araneae:Eutichuridae)

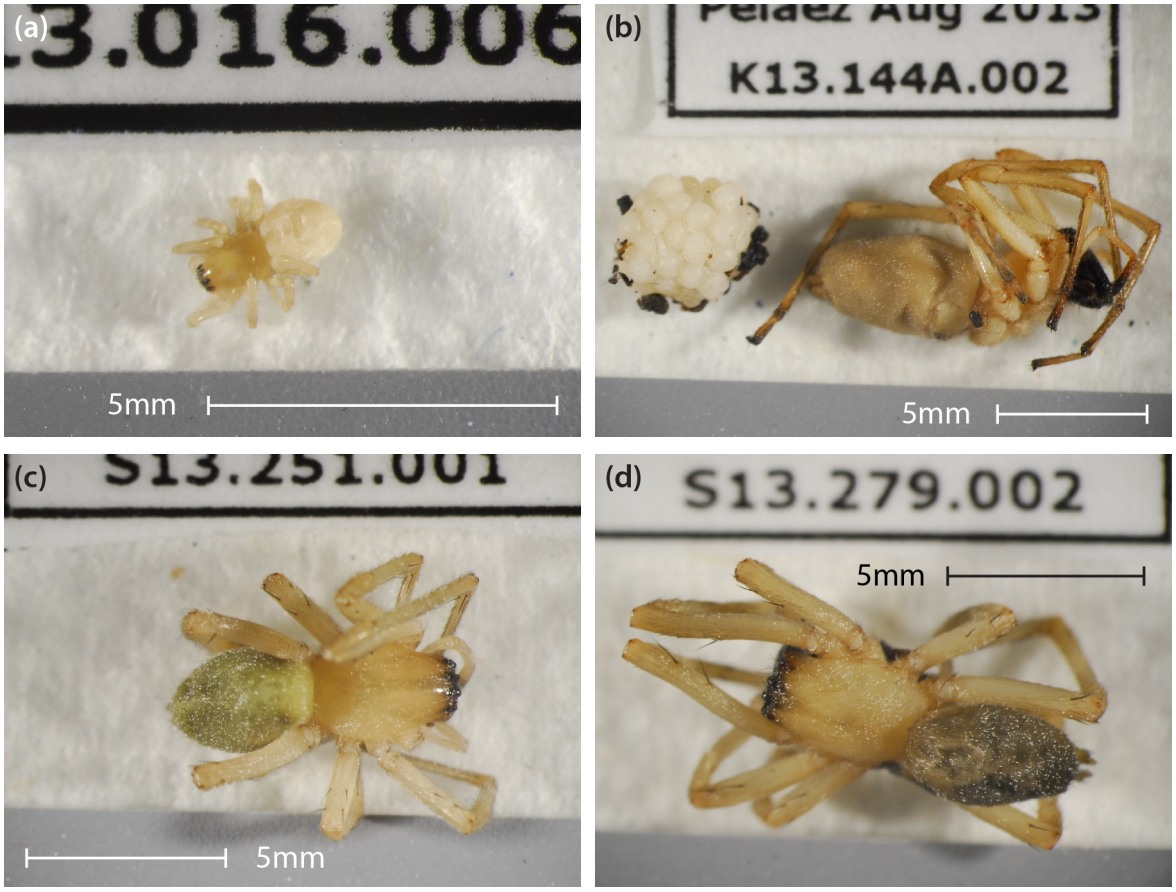

Figure B.70: OTU 72 – *Cheiracanthium* sp. (Araneae:Eutichuridae)

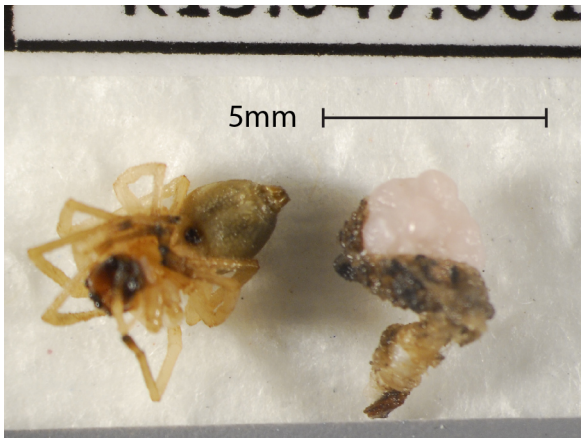

Figure B.71: OTU 73 – *Cheiracanthium* sp. (Araneae:Eutichuridae)

Figure B.72: OTU 74 – unidentified Araneae

Figure B.73: OTU 76 – unidentified species (Araneae:Gnaphosidae)

Figure B.74: OTU 77 – unidentified Araneae

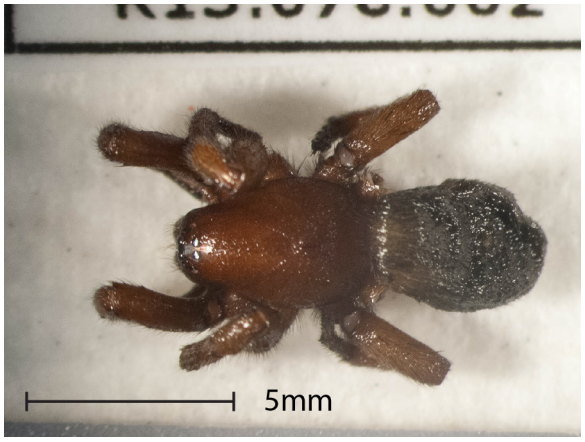

Figure B.75: OTU 78 – unidentified Araneae

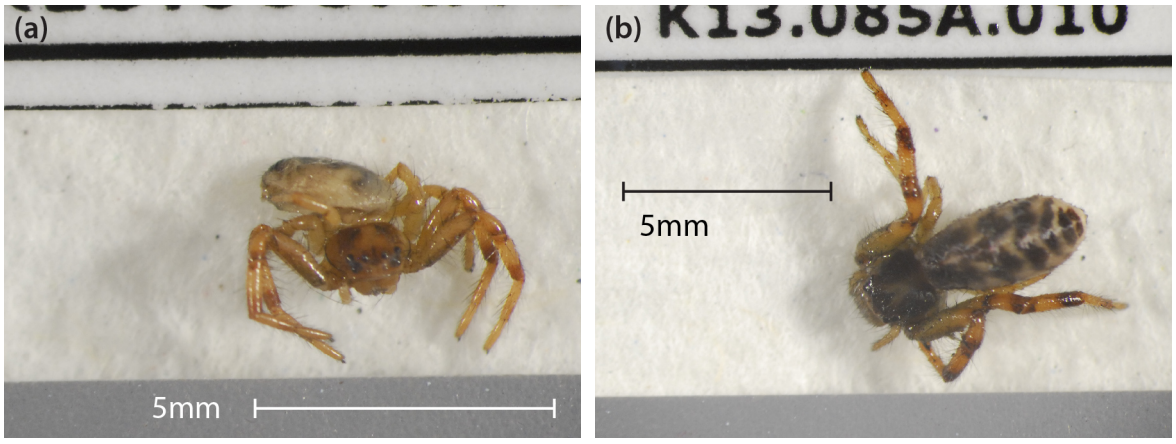

Figure B.76: OTU 79 – unidentified species (Araneae:Thomisidae)

Figure B.77: OTU 80 – unidentified species (Araneae:Theridiidae)

Figure B.78: OTU 81 – unidentified species (Hemiptera:Cixiidae)

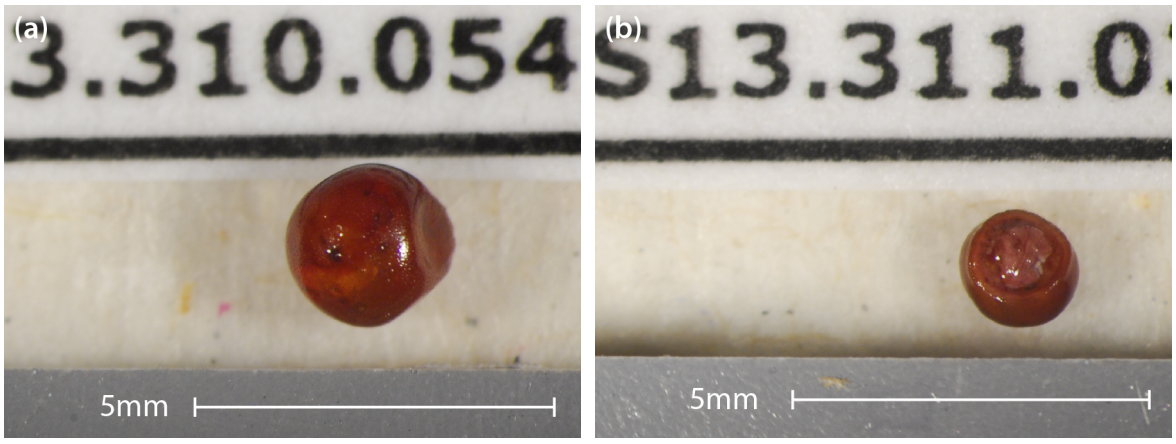

Figure B.79: OTU 82 – *Hockiana* sp. (Hemiptera:Stictococcidae)

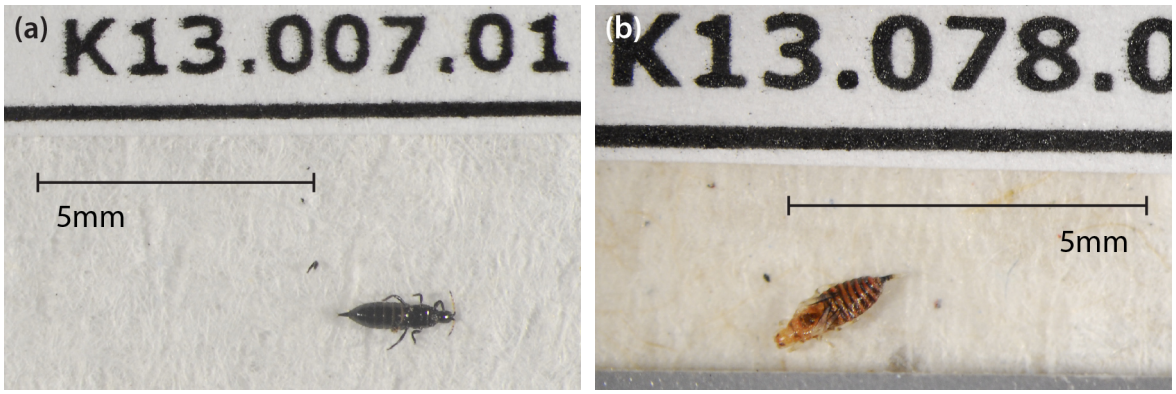

Figure B.80: OTU 83 – unidentified Thysanoptera

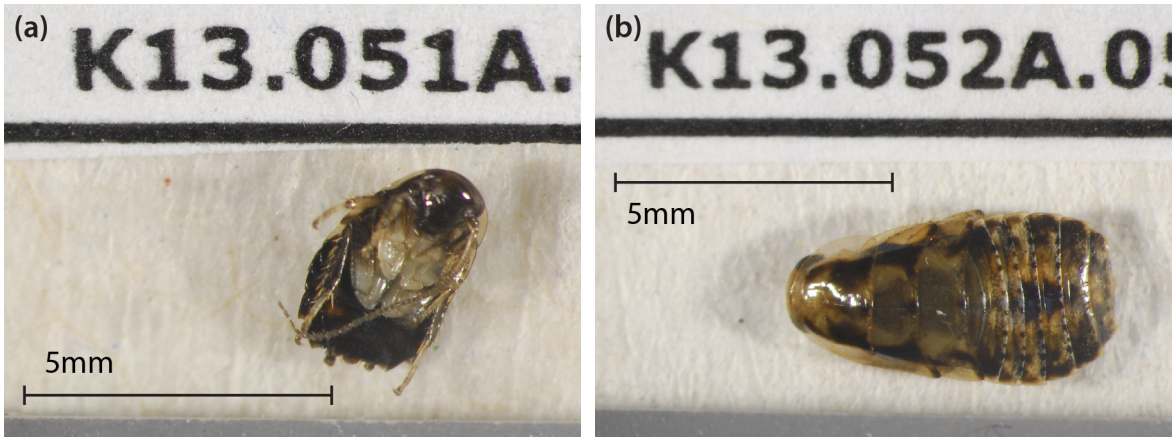

Figure B.81: OTU 84 – unidentified Blattodea

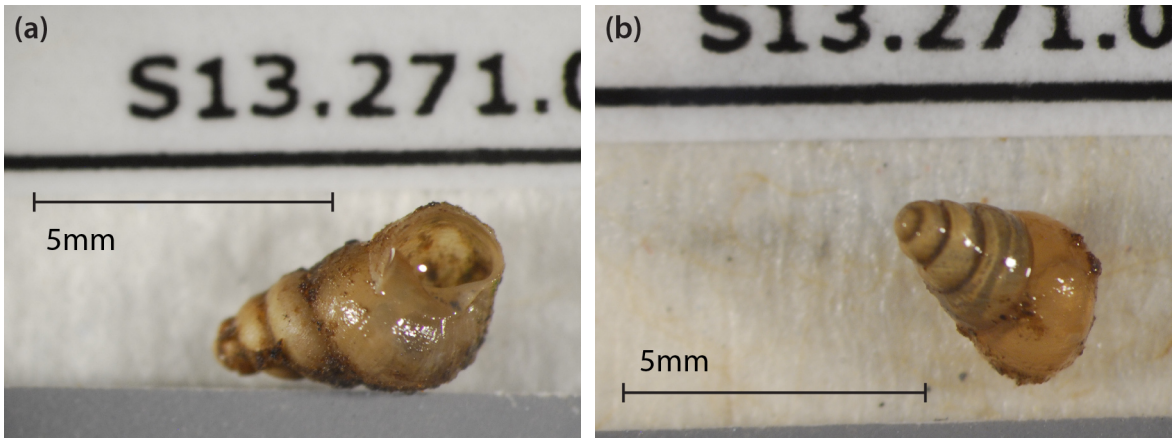

Figure B.82: OTU 85 – unidentified Gastropoda

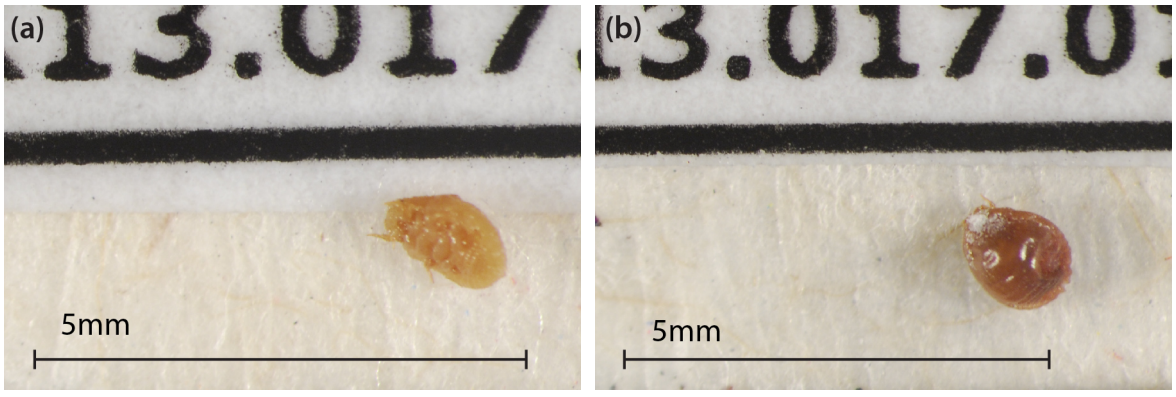

Figure B.83: OTU 86 – unidentified Insecta

Appendix C

Supplementary material for Chapter 4

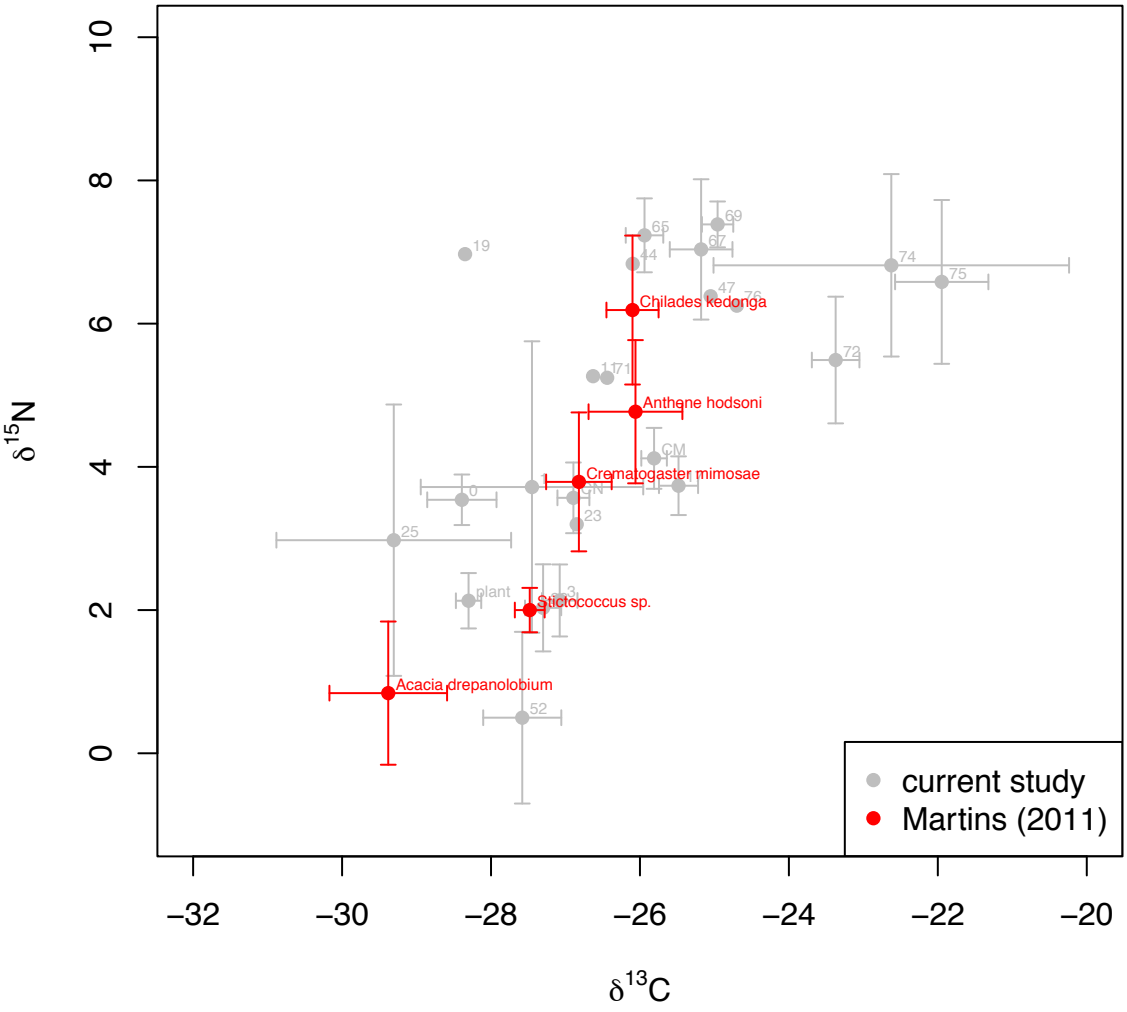

Figure C.1: Comparison of stable isotope data from current study (grey) with data from Suyian in Figure 4.2 from Martins (2011)[123] (red). These data were also published in Martins *et al.* (2013)[125], with the exception of the data for *Chilades kedonga*.

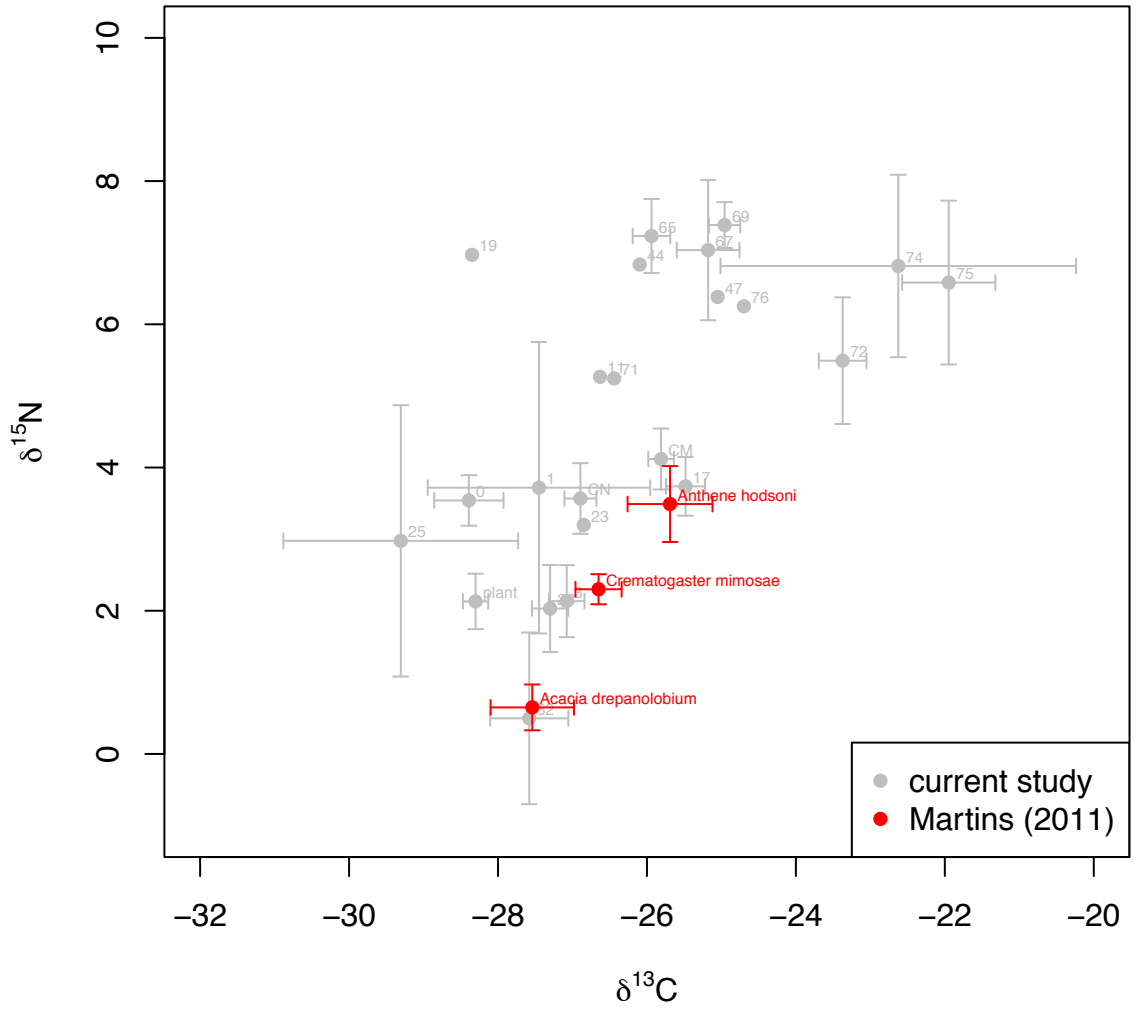

Figure C.2: Comparison of stable isotope data from current study (grey) with data from Kitengela in Figure 4.3 from Martins (2011) [123] (red). These data were also published in Martins *et al.* (2013) [125].

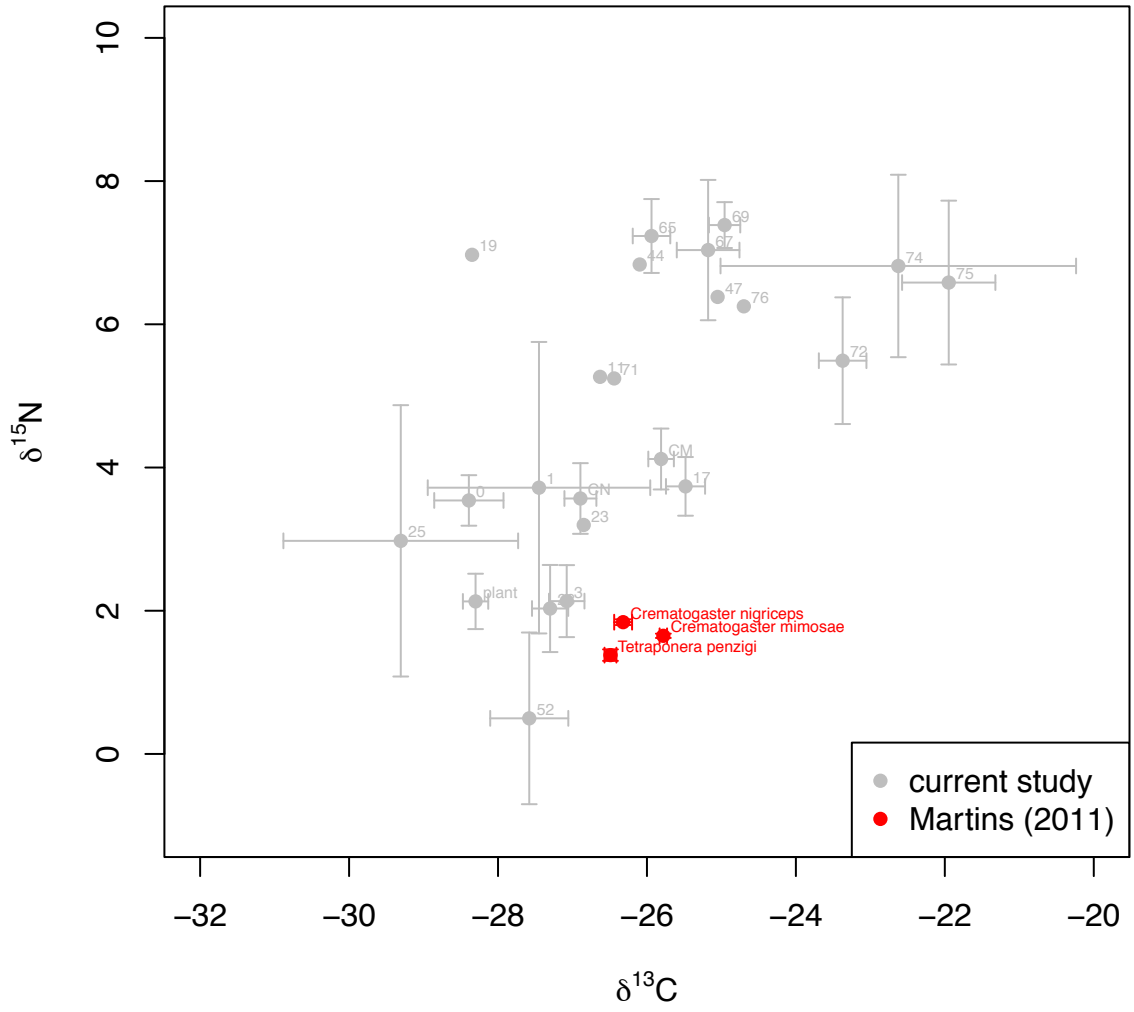

Figure C.3: Comparison of stable isotope data from current study (grey) with data from Figure 5.17 from Martins (2011)[123] (red).

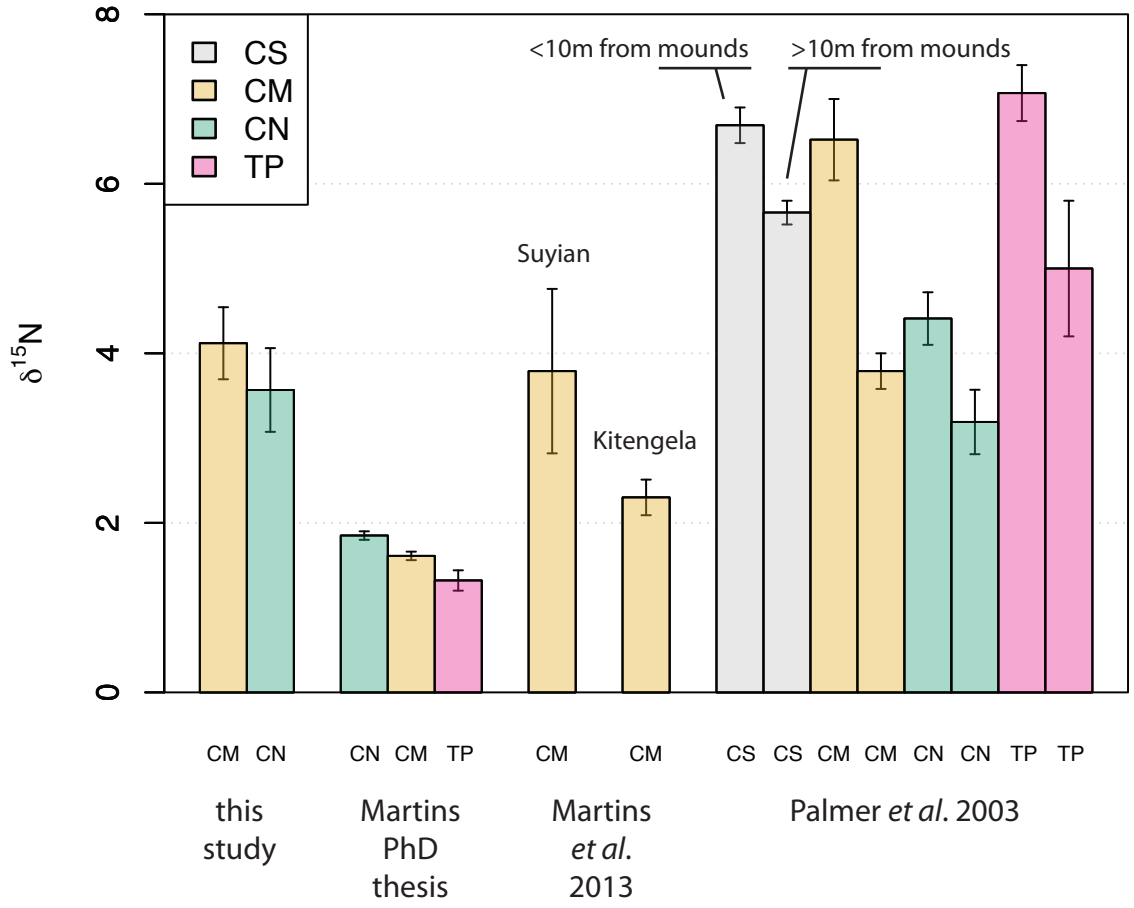

Figure C.4: Comparison of nitrogen stable isotope data for ants from this study with previously published data from Martins (2011)[123], Martins *et al.* (2013)[125] and Palmer (2003)[152]. Each ant species is represented twice in Palmer (2003)[152], with the taller bar in each case representing colonies $<$ 10m from termite mounds, and the shorter bar representing colonies $>$ 10m from termite mounds.

Appendix D

Kin Selection and the Evolution of Social Information Use

Baker, CCM, SRX Dall and DJ Rankin (2012). Kin selection and the evolution of social information use in animal conflict. PLoS One 7(2): e31664. doi: 10.1371/journal.pone.0031664

D.1 Abstract

Animals often use social information about conspecifics in making decisions about cooperation and conflict. While the importance of kin selection in the evolution of intraspecific cooperation and conflict is widely acknowledged, few studies have examined how relatedness influences the evolution of social information use. Here we specifically examine how relatedness affects the evolution of a stylised form of social information use known as eavesdropping. Eavesdropping involves individuals escalating conflicts with rivals observed to have lost their last encounter and avoiding fights with those seen to have won. We use a game theoretical model to examine how relatedness affects the evolution of eavesdropping, both when strategies are discrete and when they are continuous or mixed. We show that relatedness influences the evolution of eavesdropping, such that information use peaks at intermediate relatedness. Our study highlights the importance of considering kin selection when exploring the evolution of complex forms of information use.

D.2 Introduction

Animals frequently rely on information about conspecifics in making decisions regarding mate choice, cooperation or conflicts over resources[37,38,212]. Such information can be provided 'intentionally', as

in the case of signalling, or inadvertently, such as when an individual's actions or their consequences may be observed by others[37,38,101]. Gathering information about conspecifics can both help to promote cooperation, as in the case of image scoring[145,220], or help to resolve conflicts, as in the case of eavesdropping[100]. In image scoring, individuals react to observed cooperation between others by offering help to partners that were previously seen helping others, and refusing help to partners that were unhelpful[146]. A similar situation occurs in eavesdropping, where individuals observe conflicts and use this information by fighting individuals that lost their last encounter and avoiding fights with individuals that won[100]. This type of social information use has been demonstrated in animals[52] and represents a heuristic that may improve an individual's expected outcome from an interaction, but with smaller investments in cognitive capacity and information-gathering than more accurate decision rules, such as full Bayesian updating over a series of interactions.

Almost all social interactions inherently involve interactions with related individuals[74,75]. Such interactions can help to promote cooperation and resolve conflict between individuals[76,77,113,223]. For example, in the case of animal conflict, it has been shown that higher relatedness between partners favours less escalation (i.e. playing 'dove') in the classic hawk-dove game[72]. However, models of social information use in animal conflict and cooperation generally ignore the potential impact that interactions between relatives can have on the evolution of a given behaviour. In a previous model of eavesdropping[100], it was assumed that interactions take place randomly between individuals in an infinitely large population. However, real populations often exhibit population structure: interactions do not take place randomly but rather take place between relatives more commonly than would be predicted by chance in a well-mixed population. Such structure can arise through kin recognition, territorial behaviour, or as a result of limited dispersal. In structured populations, selection should favour individuals that help or avoid conflict with relatives, as well as those that are able to make the most of their interactions with non-relatives. Monitoring simple social cues through eavesdropping

potentially addresses both of these criteria, by allowing players to condition their behaviour on information about individual opponents.

In this paper we examine how relatedness affects the evolution of information use in an eavesdropping game. We model the classic hawk-dove game [127] with eavesdropping [100] and with interactions between relatives. We use two variants of the model – one with discrete strategies and one with continuous strategies – as these variants are known to yield different results in the game without eavesdropping [72]. The discrete strategies version is a direct extension of a previous model of eavesdropping by Johnstone [100], in which hawk, dove and eavesdropping phenotypes each arise from separate genotypes. In the continuous strategies version, each genotype gives rise to a proportion of individuals with the eavesdropping phenotype, a proportion with the hawk phenotype, and a remaining proportion with the dove phenotype. Our results suggest that eavesdropping will be most favoured at intermediate relatedness and highlight the importance of considering population structure in studying animal conflict and the evolution of social information use.

D.3 Model and results

Our model for the evolution of eavesdropping among related individuals is based on the two-player hawk-dove game [128,174]. Animals frequently compete for resources with each other [26], and the hawk-dove game is a well-studied approach to examining these interactions. It has also been used previously to explore the evolution of cooperation [47,127]. We model two variants of the eavesdropping game played among relatives: one with discrete strategies, and one with continuously variable strategies. In any given interaction, each of the two players chooses between the actions *hawk* and *dove*. If both select *hawk* then each wins the resource value v with probability 0.5 but otherwise bears a cost of fighting c, so that the expected payoff is $(v-c)/2$ with $c > v > 0$. (The analysis of the case $v > c > 0$, i.e. the prisoner's dilemma with eavesdropping, is not dissimilar but omitted here for brevity.) If

both select *dove* then each wins the resource with probability 0.5 without bearing the costs of fighting, giving an expected payoff of $v/2$. If one player chooses *hawk* and the other *dove*, then the hawk wins the resource value v with certainty, while the dove receives 0; both hawk and dove in this scenario avoid the cost of fighting c. Note that these expected payoffs would be the same under the common alternative formulation of the hawk-dove game in which the resource is split evenly between a pair of hawks or a pair of doves rather than being randomly assigned; but our eavesdropping strategy assumes the presence of a clear winner to provide a potential source of information to eavesdropping observers, as described below.

We assume an infinite population, where each individual plays a large number of interactions over its lifetime before reproducing clonally. The reproductive success or fitness of an individual is proportional to the average payoff across all interactions during its lifetime. There are no repeated interactions, but we allow for the possibility of eavesdropping: an eavesdropper plays *dove* in any interaction where the opponent's prior encounter was perceived as a win, and otherwise plays *hawk*.

Discrete strategies model

The discrete strategies model envisages three distinct genotypes, each corresponding to a different strategy that may be thought of as a phenotype. An individual with the hawk genotype always plays the action *hawk*; a dove always plays *dove*; and an eavesdropper plays the conditional eavesdropping strategy, which may dictate either *hawk* or *dove* in any given encounter. Johnstone's original model[100] assumes that opponents are drawn randomly from the whole population, so that genotypes encounter one another in proportions determined by their frequencies in the population. We allow for non-random assortment by introducing relatedness as an exogenous parameter reflecting, for example, limited dispersal. The relatedness r measures the probability that a player's opponent has the same genotype as the player, relative to the probability of obtaining the same genotype in a randomly drawn member of the population. This is a standard method of introducing relatedness in simple

game-theoretical models[66,72,173]. Thus, an individual with genotype i plays another type i individual with probability

$$r + (1-r)f_i, \tag{D.1}$$

and plays an opponent of type $j \neq i$ with probability

$$(1-r)f_j, \tag{D.2}$$

where f_i and f_j are the frequencies of genotypes i and j in the population. When $r = 1$, pairs of players always have the same genotype; when $r = 0$, players interact with each genotype in proportion to the population frequencies. Although relatedness may, in principle, be negative[65,222], we restrict our analysis to $r \in [0, 1]$. Note that r measures assortment at the level of the genotype (i.e. hawk, dove or eavesdropper) rather than action (i.e. *hawk* or *dove*) – for example, an eavesdropper meets another eavesdropper with probability $r + (1-r)f_E$, but in a given interaction the two may or may not play the same action, since each player's action depends on the outcome of its opponent's previous encounter.

The probability p_i that a type i individual won its last encounter settles down after relatively few iterations of the game, and is given by

$$p_E = \frac{1 + r + 2f_D(1-r)}{4 - 2f_E(1-r)} \tag{D.3}$$

$$p_H = \frac{1 + f_D(1-r)}{2 - f_E(1-r)} \tag{D.4}$$

$$p_D = \frac{r + f_D(1-r)}{2 - f_E(1-r)} \tag{D.5}$$

with genotype frequencies $f_E + f_H + f_D = 1$.

We use these probabilities to determine each genotype's average payoff as a function of the f_i. We

assume no mutation or drift, and allow the frequencies of the eavesdropper, hawk and dove genotypes to evolve according to standard continuous replicator dynamics[89,205]. Solving for the frequencies that give equal fitness to the three genotypes gives the following long-run equilibrium frequencies:

$$f_E = \frac{4cv(r^2+r+2)+4v^2(r^2+r-2)-c^2r(1-r)}{c(1-r)(8v+c(1-r))} \tag{D.6}$$

$$f_H = \frac{v}{c}-\frac{r}{1-r} \tag{D.7}$$

$$f_D = \frac{(c(1+r)-v(1-r))(c(1-r)-4rv)}{c(1-r)(8v_c(1-r))} \tag{D.8}$$

When $r=0$, the model is identical to Johnstone's[100]. For positive r, all three genotypes still coexist stably, at frequencies given by equations (D.6)–(D.8), as long as

$$r < min\left\{\frac{c}{4v+c}, \frac{v}{v+c}\right\} \tag{D.9}$$

But if (D.9) does not hold, then (D.6)–(D.8) give frequencies outside the range $[0,\,1]$, implying that one or more of the genotypes will be driven to extinction or fixation. For $v/c > 0.5$, eavesdroppers and hawks coexist stably, with doves driven towards extinction over time, if

$$\frac{c}{4v+c} \leqslant r < \frac{4v+c}{7c+4v}, \tag{D.10}$$

and eavesdroppers go to fixation if

$$\frac{4v+c}{7c+4v} \leqslant r < \frac{4v-c}{4v+c}. \tag{D.11}$$

If $r > max\left\{\frac{4v-c}{4v+c}, \frac{v}{v+c}\right\}$, eavesdroppers and doves coexist stably, with hawks driven towards extinc-

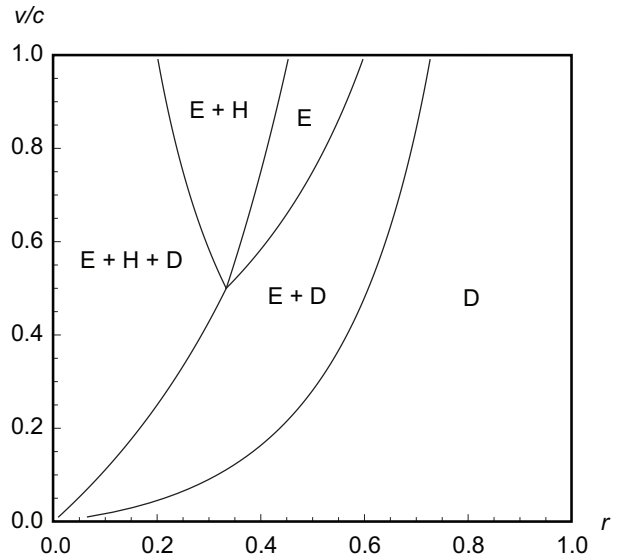

Figure D.1: Phenotypes in equilibrium in the discrete strategies model. Labels indicate genotypes with positive equilibrium frequencies under error-free eavesdropping ($a = 1$) with E = eavesdroppers, H = hawks and D = doves.

tion over time, if

$$8v(1-r) - cr(1+r)^2 > 0 \tag{D.12}$$

otherwise doves go to fixation. Figures D.1, D.2 and D.3 summarize these equilibria as a function of r and v/c.

If eavesdroppers make errors in determining the outcomes of their adversaries' prior encounters, we find that eavesdropping peaks at a lower level of relatedness than in the absence of errors. We model errors by introducing an 'accuracy' parameter $a \in [0.5, 1]$ describing the probability that an eavesdropper correctly perceives an adversary's prior outcome (formal results not shown but available from the authors on request). With probability a, an eavesdropper perceives a win and plays *dove* when the adversary's prior outcome was truly a win, and perceives a loss and plays *hawk* when the prior outcome was truly a loss; with probability $(1-a)$, the eavesdropper perceives a win and plays *dove* when the prior outcome was actually a loss, and perceives a loss and plays *hawk* when the

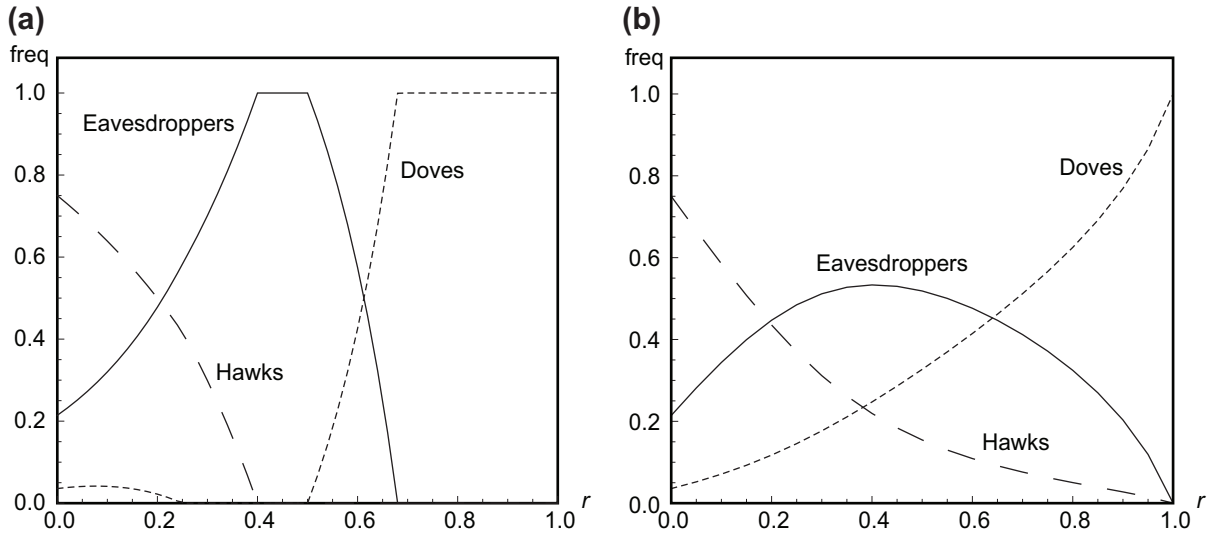

Figure D.2: Equilibrium phenotype frequencies for $v/c = 0.75$ under error-free eavesdropping ($a = 1$). (a) results for the discrete strategies model. (b) results for the continuous strategies model.

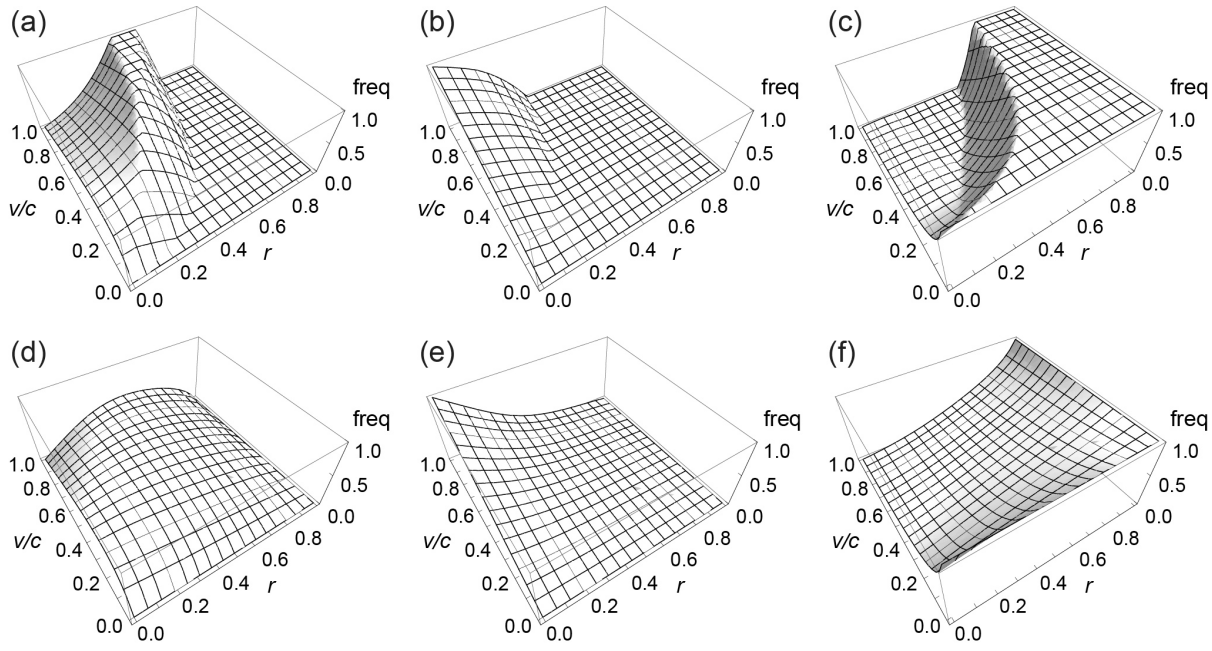

Figure D.3: Equilibrium phenotype frequencies under error-free eavesdropping ($a = 1$). (a) through (c) frequencies of eavesdroppers, hawks and doves in the discrete strategies model. (d) through (f) frequencies of eavesdroppers, hawks and doves in the continuous strategies model.

prior outcome was actually a win. At low relatedness, errors make eavesdropping more attractive if fight costs are low since the population is dominated by eavesdroppers and hawks, and errors allow an eavesdropper to avoid some escalated fights when playing another eavesdropper, although this is partly offset by greater average fight costs when encountering a hawk. If fight costs and/or relatedness are high, eavesdroppers and doves dominate the population, and errors increase the rate of escalated fights among pairs of eavesdroppers, thus selecting against eavesdropping.

In the model with eavesdropping, individual aggression (i.e. *hawk* actions) and escalated conflicts (i.e. *hawk-hawk* encounters) *generally* occur at higher frequency than in the model without eavesdropping (Figure D.4). As in previous work[100], there is an incentive for more aggression than would otherwise occur, since this improves a player's chance of winning future encounters with eavesdroppers. However, at low relatedness, the frequency of escalated conflict is lower than would be expected given the frequency of individual aggression, essentially since eavesdroppers are able to avoid conflict against aggressive opponents. At intermediate relatedness, this is more than offset by the fact that aggressive individuals interact among themselves more often than would be expected by chance, so that the frequency of escalated conflict is higher than might be expected. At high relatedness, doves go to fixation and there is no aggression or escalated conflict at all.

In contrast to the model of Johnstone[100], the discrete strategies model with relatedness *can* produce lower frequencies of individual aggression (i.e. *hawk* actions) and escalated conflict (i.e. *hawk-hawk* encounters) than the model without eavesdropping. This happens within a narrow range of parameters, when fighting is not very costly (v/c close to 1), eavesdropping is very error prone (a close to 0.5) and there is moderate population structure as captured by r. This combination of parameters produces an equilibrium with fewer hawks than in the model without eavesdropping[72]. In the model with eavesdropping, however, individual aggression also includes any eavesdroppers that play the *hawk* action. With a close to 0.5, eavesdroppers are essentially choosing randomly between *hawk*

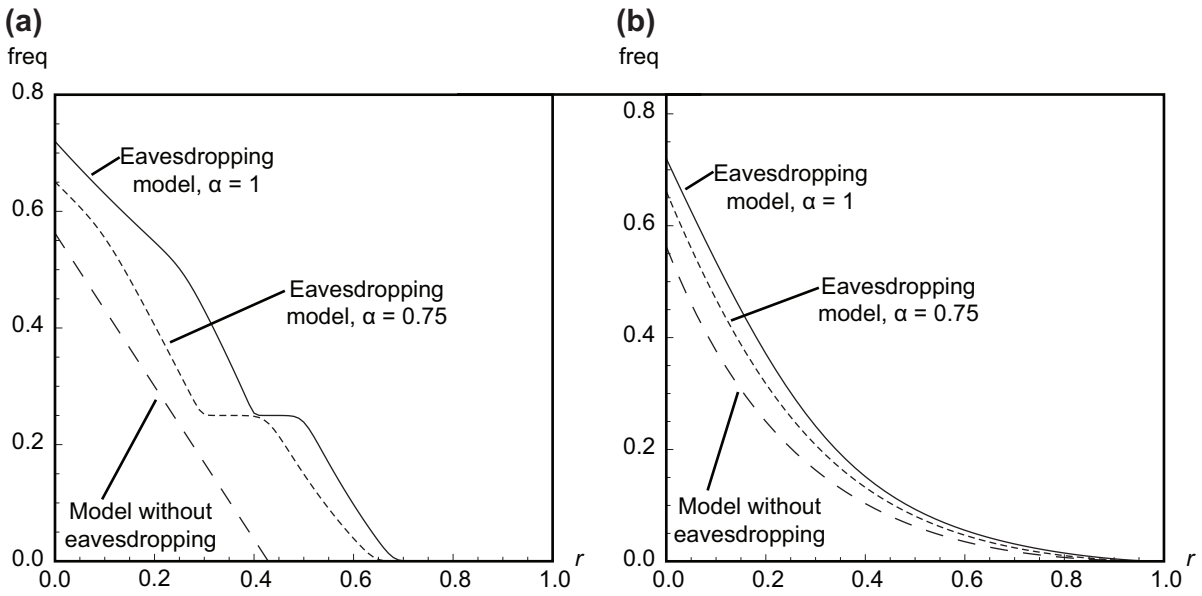

Figure D.4: Frequency of escalated conflicts for $v/c = 0.75$. Escalated conflicts are interactions in which both players play the action *hawk*. (a) frequencies for the discrete strategies model. (b) frequencies for the continuous strategies model.

and *dove* in each interaction, so individual aggression is the frequency of hawks plus approximately half of the frequency of eavesdroppers. Escalated aggression in the model without eavesdroppers is just the frequency of hawk-hawk interactions but, in the model with eavesdroppers, interactions in which eavesdroppers play the *hawk* action must also be taken into account. The frequencies of individual aggression and escalated conflict are lower than in the model without eavesdropping provided that eavesdroppers are sufficiently abundant in equilibrium, and that low costs of fighting ensure that hawks are abundant in the non-eavesdropping model.

Continuous strategies model

In the continuous strategies model, a genotype i displays phenotypic plasticity as described by two parameters, $x_i \in [0, 1]$ and $y_i \in [0, 1]$. We assume phenotypic noise at birth such that, when each genotype i individual is born, it becomes a hawk, dove or eavesdropper at random but with proba-

bilities determined by its genotype (x_i, y_i). Of the genotype i individuals, a proportion x_i takes on an eavesdropper phenotype for life and thus plays the conditional eavesdropping strategy in every interaction. A proportion $(1-x_i)y_i$ takes on the hawk phenotype and thus always plays the action *hawk*; likewise a proportion $(1-x_i)(1-y_i)$ takes on the dove phenotype and so always plays the action *dove*. Since the relevant evolutionary dynamics in our model take place at the genotype level, the fitness of genotype i is the weighted average fitness of its three phenotypes, where phenotype fitness is again measured as the average across all interactions during an individual's lifetime.

As in the discrete strategies model, we allow for non-random assortment among genotypes arising from, say, limited dispersal, by permitting non-zero relatedness. The exogenous relatedness parameter r measures the probability that a player's opponent has the same genotype as the player, relative to the probability of drawing the same genotype randomly from the population. An individual with genotype i plays another type i individual with probability $r+(1-r)f_i$, and plays an opponent of type $j \neq i$ with probability $(1-r)f_j$, where f_i and f_j are the frequencies of genotypes i and j in the population. In the continuous strategies model, genotypes i and j will be a resident genotype close to fixation and a mutant genotype at low frequency, since our equilibrium analysis will concentrate on finding genotypes that cannot be invaded by 'nearby' mutants. We again restrict attention to $r \in [0, 1]$. Note that assortment takes place at the genotype level, and when two players have the same genotype this means that they share the same values for x and y. However, at the time of the interaction, their phenotypes are already determined as eavesdropper, hawk or dove, and the two players may or may not share the same phenotype. Additionally – just as in the discrete model – two eavesdroppers may or may not play the same action against one another.

To analyse our eavesdropping model when we have continuous strategies, we use evolutionary invasion analysis[149], also known as adaptive dynamics[70,136,137]. This assumes homogeneous populations, rare mutants and small phenotypic effects from mutations. It allows us to investigate whether

a mutant is able to invade a monomorphic population with a slightly different genotype and go to fixation. If mutations are small, and rare relative to the time to fixation, the genotype making up the population can move around the genotype space over time via a large number of small evolutionary steps. We thus seek the evolutionary attractors for our genotype space[70,136,137].

As for the discrete strategies model, we start with expressions for the probability that a player has won its last encounter as a function of the resident's genotype, and use these to construct expressions for the expected fitness of a resident and a mutant as

$$w_{res} = w_{E,res}x_{res} + w_{H,res}(1-x_{res})y_{res} + w_{D,res}(1-x_{res})(1-y_{res}) \tag{D.13}$$

and

$$w_{mut} = w_{E,mut}x_{mut} + w_{H,mut}(1-x_{mut})y_{mut} + w_{D,mut}(1-x_{mut})(1-y_{mut}) \tag{D.14}$$

respectively. The fitnesses $w_{E,mut}$, $w_{H,mut}$, and $w_{D,mut}$ are all functions of relatedness r, as a mutant will either interact with an individual of its own genotype (with probability r) or with an individual with the resident genotype (with probability $(1-r)$, since the resident genotype is assumed to be at fixation when the mutant appears). The relative fitness W of a mutant is the difference between the mutant's fitness and the weighted average fitness of the population, i.e. $W = w_{mut} - (sw_{mut} + (1-s)w_{res})$, where s is the frequency of the mutant in the population. To assess the susceptibility of genotypes to invasion by mutants, we assume that the mutant is rare (i.e. $s \to 0$), and so W simplifies to $W \approx w_{mut} - w_{res}$. We can then use this to derive the selection gradients, W_x and W_y, and find equilibrium values of x_i and y_i by solving the first order conditions:

$$W_x = \left.\frac{\partial W}{\partial x_{mut}}\right|_{x_{mut}=x_{res}, y_{mut}=y_{res}} = 0 \tag{D.15}$$

and

$$W_y = \left.\frac{\partial W}{\partial y_{mut}}\right|_{x_{mut}=x_{res}, y_{mut}=y_{res}} = 0. \tag{D.16}$$

for $x \in [0, 1]$ and $y \in [0, 1]$, which provides the parameters for the equilibrium genotype. This approach assumes a homogeneous population (where mutants are at a negligible density relative to resident individuals), that mutants with a positive invasion fitness (that is, those that do better than the resident strategy) will successfully invade and be driven to fixation, and that mutation occurs in small steps, with x_{mut} and y_{mut} deviating only slightly from x_{res} and y_{res}[70,136,137]. This model thus differs conceptually from the discrete strategies model, in which the population was genotypically heterogeneous at equilibrium.

Without population structure ($r = 0$), there is a single equilibrium genotype that gives rise to eavesdropper, hawk and dove phenotypes in accordance with parameters $x^* = 8v(c-v)/(c^2+8cv)$ and $y^* = v(c+8v)/(c^2+8v^2)$. These phenotype frequencies match the genotype frequencies of both our discrete strategies model and Johnstone's eavesdropping model[100].

Our results differ, however, when we incorporate relatedness into the population (i.e. $r > 0$). As relatedness increases, the equilibrium genotype parameter y^* falls, since avoiding escalated conflicts by playing *dove* is always more favourable when interactions with relatives become more common. If the value of the resource v is sufficiently high compared to the cost of fighting c, then the equilibrium eavesdropping frequency x^* peaks at intermediate levels of relatedness before dropping to 0 when relatedness reaches 1 (Figure D.2). Unlike the discrete strategies model, however, eavesdropping never goes to fixation. The peak frequency of eavesdropping occurs at lower relatedness the smaller is v/c; if v/c is sufficiently small then the frequency of eavesdropping decreases monotonically to 0 as r increases (Figure D.3).

When eavesdroppers make errors in determining the outcomes of their adversaries' prior encounters, we find that the equilibrium frequency of eavesdropping peaks at a lower level of relatedness

compared to error-free eavesdropping or, if v/c is small, is lower at all values of r. As with the discrete strategies model, we model these errors by introducing an 'accuracy' parameter $a \in [0.5, 1]$, describing the probability that an eavesdropper correctly perceives an adversary's prior outcome (formal results not shown but available from the authors on request). The equilibrium frequency of eavesdroppers may actually increase with eavesdropping errors when relatedness is low and v/c high, since errors cause eavesdroppers to avoid some escalated fights against other eavesdroppers, and this is favourable. The frequencies of both individual aggression and escalated conflict are always higher in the eavesdropping model than in the model without eavesdropping (Figure D.4), but the frequency of escalated conflict is less than would be expected from simply squaring the frequency of individual aggression. As eavesdropping errors increase (i.e. a approaches 0.5), aggression and escalated conflict converge to the same level as observed in the model without eavesdropping, although eavesdropping still takes place in equilibrium.

D.4 Discussion

Although players cannot distinguish kin from non-kin directly in our model, nor accurately predict what action an opponent will choose in a future encounter, eavesdropping provides scope for a conditional response such that *hawk* is played against opponents that are on average comparatively likely to play *dove*, and *vice versa*.

Our models show that eavesdropping is most successful at intermediate levels of relatedness. By contrast, at high relatedness, individuals maximise their inclusive fitness by always playing *dove*, which consequently goes to fixation. This is similar to the basic hawk-dove game, in the absence of eavesdropping, in which doves also go to fixation if relatedness is high – i.e. if $r \geqslant v/(v+c)$ under discrete strategies, and if $r = 1$ under continuous strategies[72]. It also echoes the classic result in the general 2×2 game where the broad pattern of a stable polymorphism at intermediate relatedness and a

monomorphic equilibrium at high relatedness is associated with negatively additive payoffs[72,121,168]. The formal extension of that result to our models may be useful in placing eavesdropping in the context of more general 3×3 games, but is complicated by the presence of three actions rather than two, and the fact that payoffs are themselves a function of population frequencies (via the probability of winning a previous encounter).

In our models, eavesdropping yields no useful information at high relatedness, since eavesdroppers essentially face a uniform population of adversaries whose members are otherwise interacting only with each other: eavesdropping relatives in the discrete strategies case, or doves in the continuous strategies case. Consequently, rare eavesdroppers play *hawk* and *dove* with equal probability but their choice in any encounter is uncorrelated with their opponent's choice in that encounter. They fare strictly worse than doves under discrete strategies because they sometimes bear the cost of fighting in escalated conflicts, and are unable to increase in frequency under continuous strategies for the same reason. At low relatedness, negative frequency dependent selection means that neither eavesdroppers, hawks nor doves can go to fixation, consistent with Johnstone[100] in which relatedness is zero. A resident population of hawks can be invaded by either doves or eavesdroppers; doves can be invaded by either eavesdroppers or hawks; and eavesdroppers can also be invaded by hawks (and by doves, but only when the relative cost of fighting is high, specifically $v/c \leqslant 4$).

Interacting with relatives relaxes the frequency dependence that maintains all three strategies in equilibrium with zero relatedness. Although this result is borne out qualitatively in both the discrete- and continuous strategies models, the equilibria of the two models differ when relatedness is positive. For example, the discrete strategies model allows eavesdroppers or doves to go to fixation given suitable model parameters; under the continuous strategies model eavesdroppers never go to fixation, and doves only become fixed at $r = 1$. This contrasts with the case where there is no relatedness (i.e. $r = 0$), in which the genotype frequencies under discrete strategies are the same as the respective

probabilities under continuous strategies, and eavesdropping never goes to fixation [100]. This divergence between discrete and continuous models in structured populations is also a known feature of the hawk-dove game without eavesdropping [72].

The equilibrium frequency of eavesdropping may be regarded as a measure of the value of eavesdropping for a given set of model parameters. This differs from the usual measure of the value of eavesdropping, which is the selection gradient given model parameters and genotype frequencies – that is, the fitness of an eavesdropping player relative to hawks or doves under discrete strategies or, in the case of continuous strategies, the change in genotype fitness from a small increase in the proportion of eavesdropping progeny. The selection gradient varies with the frequencies of eavesdroppers, hawks and doves, and also with v/c and r. When the gradient is positive, selection favours an increase in the frequency of eavesdropping which tends to erode the value of eavesdropping, since eavesdropping opponents are comparatively unpredictable [100]. However, the selection gradient will be zero at any equilibrium where eavesdroppers attain frequency strictly between zero and one. It is therefore useful for examining the evolutionary dynamics of eavesdropping, but less helpful for comparing the adaptive value of eavesdropping between different biological settings as captured by parameters v/c and r in our models. The equilibrium frequency of eavesdroppers, on the other hand, is useful for this purpose.

The value of eavesdropping as measured by the selection gradient is closely related to the value of information in our model [86,200]. The value of eavesdropping is the benefit of observing a simple social cue and responding in a specified way – i.e. play *hawk (dove)* against perceived losers (winners) – which may be positive or negative. In contrast, the value of information is the net fitness benefit from responding *optimally* once observations have reduced prior uncertainty, and is always non-negative [133]. The value of eavesdropping is non-negative and equivalent to the value of information if the outcome of fights is a sufficiently reliable predictor of opponents' future actions that it is optimal

to play *hawk (dove)* against perceived losers (winners). But if the outcomes of fights are sufficiently misinformative about the likelihood of an opponent playing *hawk* or *dove* in subsequent fights, the optimal response to the social cue may be either to play *hawk* or to play *dove* unconditionally – in other words, to ignore the social cue. In this case, the value of information is zero, since receiving the cue changes neither choice of action nor outcome. But the value of eavesdropping is negative, since the response conditioned on the social cue yields lower fitness than the best unconditional response that could be employed without such cue. The value of eavesdropping may be negative (and the value of information zero) when cues fail to reduce prior uncertainty sufficiently (are too uninformative) about whether a current opponent won or lost in a previous round, and/or when knowing this fails to improve payoffs from current and future bouts.

The value of eavesdropping (and, correspondingly, the value of information), involves a number of components. Firstly, an immediate direct fitness effect from altering the player's payoff in the current round, by enabling the player to distinguish (albeit imperfectly) opponents who are more likely to play *hawk* from those who are more likely to play *dove*. The size of this effect is determined by the frequencies of eavesdroppers, hawks and doves, the relative payoffs (influencing the value of any available information) which are functions of v/c, as well as the probabilities that each type won its last encounter, since these affect the average ability of an eavesdropping player to predict whether an opponent will play *hawk* or *dove* (the availability of information). Secondly, a fitness effect arising from a mutant effectively facing a different population of opponents than a resident (impacting both value and availability of information). Thirdly, an accumulation of effects in future rounds because an increased probability of winning this round also implies an increased probability of winning against an eavesdropper in the next round, since an eavesdropper plays *dove* if it perceives that its opponent won its last encounter. The net result of all these effects is captured in the relative fitness functions for the different genotypes.

Our models highlight the potential for relatedness to enhance selection for eavesdropping. The relatedness parameter *r* describes the probability of interacting with a similar partner in a given interaction, relative to chance. While we have not specified how such relatedness arises, the mechanisms invoked in the kin selection literature usually involve either kin recognition or limited dispersal[56,76,77,173]. Our *r* is best interpreted as arising from limited dispersal, since we only model interactions with a single level of relatedness. Many species face dispersal limitations, which may help suppress conflict by increasing the relatedness of opponents; however, this effect may be negated to the extent that relatives also compete to reproduce or for other resources[204,224,225]. We have chosen to keep our model simple, and thus assume that any resource competition is relatively global (i.e. non-dispersal limited) compared to the conflict stage captured by our game. Such global competition, with local social interactions, would likely be found in, for example, interactions between nestmates or between young raised on a territory, prior to dispersal and competition for mates or territories[225].

Our models, in which direct assessment of relatedness or strategy is unavailable, predict that eavesdropping will be most favoured at intermediate levels of population structure. More generally, our results highlight the importance of explicitly considering genetic relatedness in addition to the nature and extent of social interactions when exploring the evolution of cognitive abilities. (While more demanding behaviours can easily be found – for example among corvids and primates – eavesdropping likely represents a significant cognitive challenge, at a minimum requiring recognition of individuals and the capacity to process and remember past observations of those individuals.) We suggest that our predictions can be tested directly. Earley and Dugatkin[52] have already demonstrated eavesdropping in the green swordtail *Xiphophorus helleri*. One test of our model would be to repeat the same experimental protocol with *X. helleri* but to vary the degree of relatedness among each trio of lab-raised fish. An alternative test would be to select several *Xiphophorus* species that exhibit different degrees of population structure and to repeat the same experimental protocol across those species.

More indirect evidence might come from examining brain size or cognitive capacity as a function of population structure for each species, since some authors have argued that larger brains evolved in part to process the demands of living in a highly social environment[50,68,231].

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