



Causes and consequences of coexistence in the *Vachellia drepanolobium* ant-plant mutualism

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Causes and consequences of coexistence
in the *Vachellia drepanolobium* ant-plant mutualism

A dissertation presented

by

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to

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Abstract

This thesis focuses on a mutualism between the East African acacia tree *Vachellia drepanolobium* and the species of canopy-dwelling ants that inhabit it. The tree provides the ants with nesting space in the canopy and extrafloral nectar, and in return the ants defend the tree from herbivores. Several different ant species compete vigorously with each other for this canopy nesting space. Despite this, coexistence is maintained among those species at fine spatial scales, apparently by a colonization-competition tradeoff: ant species that are the best at colonizing unoccupied trees are the worst at defending those trees from neighboring competitors, and vice versa.

In Chapter 1, to determine the causes of these different competitive and colonizing abilities among the ant species, I used RADseq, a next-generation sequencing technique, to determine the colony structure of each ant species. This enabled me to reject a primary hypothesis for why the ant species differ in competitive ability, which is that the more competitive ants have more than one egg-laying queen per colony, and therefore these queens can produce much larger colonies than other ant species that have only one queen. By sampling about 6-8 worker ants per tree, I could estimate the number of queens in the colony as well as the number of times that each queen had mated. I found that more competitive species do not have more egg-laying queens in their colonies.

In Chapter 2, I looked for alternative explanations for differences in competitive and colonizing ability. I measured how individual colonies divide their biomass among different

castes, and how these castes are distributed throughout the tree. I found that the three main ant species inhabiting *V. drepanolobium* differ significantly in colony composition as well as the distribution of castes throughout the tree, and some of these differences are likely to shape competitive and colonizing abilities.

I also performed a broader scale survey of *V. drepanolobium* and its mutualist ant species across their range in Kenya. In Chapter 3, I show that this mutualism varies widely across Kenya, for both the tree and the ant. I also use the measurements from Chapter 2 to estimate how the biomass of each partner varies in the landscape, and compares to other parties in the system, such as vertebrate herbivores.

In Chapter 4, I present a RADseq-based landscape genomics study of *V. drepanolobium* and its ant inhabitants. This analysis uncovered how differences in ant behavior at small spatial scales were recapitulated at landscape scales. I also explored whether abiotic factors influence the different ranges of the tree and ant species. I found that geography contributed to population structure in the tree and in each of the ants, but that environmental distance had a weak or negligible effect on the population structures of the tree and of two of the three associated ant species. Moreover, the tree had a population structure distinct from its ant inhabitants. These results suggest that population structure in this system may be influenced more by dispersal limitation than by local adaptation of specific partners to each other or to the abiotic environment.

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Introduction

The high plains of East Africa are dominated by the Whistling Thorn Acacia, *Vachellia drepanolobium* (formerly *Acacia drepanolobium*). Ants chew holes in the hollow swellings which the tree produces at the base of its thorns. The wind blowing across these holes produces an eerie whistling, giving the tree its name. The ants in question live in the hollow swellings, or “domatia”, and pay for this housing by defending the tree from its enemies, which range from myriad herbivorous insects through to large, grazing vertebrates like giraffes and elephants. This ant-plant mutualism is a fascinating one, not least because of the coexistence of four primary ant species, all of which associate with *V. drepanolobium*, and all of which may be found nesting in trees within meters of one another. Before introducing the findings of my dissertation research, I will briefly describe the current state of our knowledge of the *V. drepanolobium* mutualism.

The plant: *Vachellia drepanolobium*

Vachellia drepanolobium is an East African thorn tree growing primarily in the highlands of Kenya and Tanzania, but its range extends northward into Ethiopia, Somalia, and Sudan, and westward into Uganda and the eastern parts of the Democratic Republic of the Congo (Brenan 1959). Although the tree rarely grows to great heights, typically standing less than 4 m tall (Young *et al.* 1997b), it can live for quite a long time, possibly as long as 150-200 years (Palmer and Brody 2007). On dark, clay, so-called “Black Cotton” soil, it grows in a near-monoculture, accounting for 95% or more of woody stems (Young *et al.* 1997b). *V. drepanolobium* participates in a defensive mutualism with ants: about 95% of trees (Hocking 1970), and more than 99% of trees 1 m or taller are occupied by ants (Young *et*

al. 1997, Palmer *et al.* 2000). Such ant-plant mutualisms are a widespread phenomenon in the tropics, having evolved in some 50 families of plants (including as many as a dozen independent evolutions in Fabaceae, the family containing *V. drepanolobium*) and five subfamilies of ants (Bronstein *et al.* 2006, Chomicki and Renner 2015). In these mutualisms, plants provide ants with nesting space in hollows of stems (Janzen 1969) or thorns (Janzen 1967), and often with nutrients from extrafloral nectaries (Hocking 1970) or protein- or lipid-rich food bodies (O'Dowd 1982). In return, the ants may defend the tree from herbivores (Janzen 1967), insulate it from competition by pruning away nearby vegetation (Janzen 1969), and/or provide the plant with nutrients that the plant absorbs from the ants' debris piles (Sagers *et al.* 2000).

In the *V. drepanolobium* ant-plant mutualism, ants nest in round swellings about 2-3 cm in diameter that form at the base of about 20% of the acacia's stipular thorns (Hocking 1970). The tree also provides the ants with food in the form of extrafloral nectaries: Hocking, one of the earliest researchers on *A. drepanolobium*, estimated that extrafloral nectar may meet about half of the ants' energy requirements (Hocking 1970). The ants, in turn, defend the tree from a variety of herbivores, from insects to elephants (e.g. Martins Visitacao, 2011, Goheen and Palmer 2010).

The ants: *Crematogaster mimosae*, *C. nigriceps*, *C. sjostedti*, and *Tetraoponera penzigi*

What makes the *V. drepanolobium* ant-plant mutualism particularly interesting is its diversity of ant mutualists. Four different ant species are known to participate in the mutualism. Three of these are members of the myrmicine genus *Crematogaster*: *C. mimosae*, *C. nigriceps*, and *C. sjostedti*, and the fourth is the pseudomyrmicine *Tetraoponera*

penzigi (Young *et al.* 1997b). Each tree is typically occupied by a single colony (although individual colonies often spread across multiple trees), yet most areas feature a mosaic, where trees inhabited each species may be found within meters of each other (Hocking 1970; Young *et al.* 1997b). Three of the species, *C. mimosae*, *C. nigriceps*, and *T. penzigi*, are obligately “phytoecious” associates, nesting only in the domatia of *V. drepanolobium* (Stanton *et al.* 2002). While some have been described on other ant-plants in various parts of Africa (Hocking 1970), these trees are absent or nearly so on the Black Cotton soils of Kenya. The fourth species, *C. sjostedti*, seldom nests in domatia, instead nesting in cavities in the trunk, or in dead wood; nonetheless, it still occupies living trees and can exclude the other ant species from *V. drepanolobium* canopies in places where their ranges overlap (Stanton *et al.* 2002). Some salient differences among the four ant associates are summarized in Table 1.

Furthermore, each of the four species has distinctive behavior, including many apparent tendencies to cheat on the tree. At the Mpala Research Centre in Laikipia, Kenya, where most of the research on this system has taken place, the two most common mutualists are *C. mimosae* and *C. nigriceps*. *C. mimosae* is commonly reported to be the numerically dominant mutualist, occupying more trees than any other species, and sometimes an outright majority of trees in many studied locations (Hocking 1970, Young *et al.* 1997b, Martins 2010). *C. mimosae* colonies often occupy multiple trees (Table 1), and they may have a single queen or multiple, usually related queens (Rubin *et al.* 2013). Of all four ant species, *C. mimosae* ranks highest in its defense of the tree, responding the most quickly and in the greatest numbers to real (Martins Visitacao 2011) or simulated herbivores (Palmer and Brody 2007). However, like the other ant associates, it is not a

Table 1: behavior varies widely among the ant associates of *V. drepanolobium*.

Ant species	Competitive rank (1= most)	Plant defense vs browsers (1=best) ²	Plant defense vs insects (1=best) ²	Abandons tree (1=most often) ³	Number of trees per colony \pm SEM ⁴	Tends scales? ⁵	Other possible cheating behaviors?	Other behaviors or effects on tree?
<i>C. mimosae</i>	2	1	2	2	22.0 \pm 4.8	Yes		May increase rate of photosynthesis ⁸
<i>C. nigriceps</i>	3	1	1	4	4.4 \pm 0.3	No	Sterilizes tree ⁵ ; simulates herbivory ⁶	May increase rate of photosynthesis ⁸
<i>C. sjostedti</i>	1	3	3	1	2.5 \pm 0.2	Yes	Introduces cerambycids ⁷	Increases probability of flowering ⁹ and death ⁷
<i>T. penzigi</i>	4	2	2	3	1.3 \pm 0.3	No		Destroys extrafloral nectaries ⁵

¹ Stanton *et al.* 2002; ² Palmer and Brody 2007; ³ Palmer *et al.* 2000; ⁴ Palmer *et al.* 2010; ⁵ Young *et al.* 1997; ⁶ Young *et al.* 2003; ⁷ Palmer *et al.* 2008; ⁸ King and Caylor 2010; ⁹ Brody *et al.* 2010

perfect mutualist: *C. mimosae* cultivates colonies of sap-sucking scale insects (Hocking 1970), a common ant behavior that has been shown to reduce tree fitness and spread pathogens among trees in other systems (reviewed in Delabie 2001). Furthermore, since *C. mimosae* colonies often spread across multiple trees (Palmer *et al.* 2010), it has a tendency to abandon trees, perhaps as colonies contract in size due to harsher conditions during droughts (Palmer *et al.* 2000). Although such abandoned trees may soon be reoccupied by nearby colonies or by foundress queens, the interim period is a hazardous time for the acacia, as unoccupied *V. drepanolobium* trees have higher mortality than trees occupied by any of the four ants (Palmer *et al.* 2010).

C. nigriceps seems to be the most numerous mutualist at some sites (Hocking 1970, Stapley 1998, Palmer 2004), and it is also usually found in those *V. drepanolobium* plots where *C. mimosae* colonies are the most frequent (Young *et al.* 1997b). *C. nigriceps* colonies generally spread across fewer trees than those of *C. mimosae* (Table 1), and they have been reported to be monogynous, based on anecdotal evidence in Stanton *et al.* (2002). Also like *C. mimosae*, it is a vigilant defender of the tree (Palmer *et al.* 2007; Martins Visitacao 2011), but unlike *C. mimosae*, it is much less likely to abandon its hosts (Palmer *et al.* 2000), perhaps simply because its colonies spread across fewer trees (Palmer *et al.* 2010). It is not known to tend scale insects (Young *et al.* 1997b), and it provides the tree with better protection from certain kinds of herbivores, like gall midges (Schumer *et al.* 2013). However, it also chews up its tree's axillary buds (Stanton *et al.* 1999). The trees do not appear to be able to distinguish this damage from herbivory, and thus in vertebrate herbivore exclosures they continue to mount inducible anti-herbivore defenses (including extra-floral nectar for its ant defenders, which may explain this behavior on *C. nigriceps*'

part: Young *et al.* 2003, Palmer *et al.* 2008). Furthermore, this chewing reduces the likelihood that the tree will put on seeds (Young *et al.* 1997, Martins 2013).

A less common ant is *C. sjostedti*, which is not found at all *V. drepanolobium* sites (e.g. Martins 2010). Its behavior is quite different from those of the other ant species. *C. sjostedti* colonies typically extend across 20 or more trees (Table 1), and Stanton and colleagues speculated that they are highly polygynous (Stanton *et al.* 2002). Unlike *C. mimosae*, *C. nigriceps*, and *T. penzigi*, which nest only in the swollen thorn domatia, *C. sjostedti* prefers to nest in cavities in the trunk, including holes chewed in the wood by cerambycid beetle larvae (Palmer *et al.* 2008). *C. sjostedti* is also the worst defender of all four ant species (Palmer *et al.* 2007) and is likely to abandon a tree (Palmer *et al.* 2000). It herds scale insects, like *C. mimosae*, and it tolerates gall-forming insects (Young *et al.* 1997); it may even attract the cerambycid larvae that hollow out the tree trunk (Palmer *et al.* 2008). Trees inhabited by *C. sjostedti* flower more often than trees occupied by other ant species, but on the whole *C. sjostedti* is so harmful to the tree that Brody *et al.* (2010) suggest that such flowering may be a sort of last hurrah by trees that sense they are heading quickly toward death.

Finally, *T. penzigi* (subfamily Pseudomyrmecinae) is an unusual-looking black ant, easily distinguishable from the three species of *Crematogaster* (subfamily Myrmicinae) by its long, thin body and its ability to sting. *T. penzigi* colonies typically inhabit only a single tree (Table 1), and were thought to be monogynous (Stanton *et al.* 2002). Unlike the *Crematogaster* species, which supplement extrafloral nectar with insect prey found on the tree or while foraging on the ground (Hocking 1970), *T. penzigi* has not been observed

attacking insects on the tree, does not appear to forage off the tree (Palmer 2003), and does not consume the tree's extrafloral nectar: rather, it destroys extrafloral nectaries by chewing them up (Palmer *et al.* 2002). Indeed, *T. penzigi* has never been observed to eat, leading researchers to hypothesize that it merely eats very small things that are not observed, such as pollen, fungal spores, or mites (Palmer 2003), or that it cultivates fungus within its domatia (Martins Visitacao 2011). Stable isotope studies of *T. penzigi* have been ambiguous: Palmer reported a high $\delta^{15}\text{N}$ value (and thus, presumably, a higher trophic level) for *T. penzigi*, comparable to *C. sjostedti* and *C. mimosae* and higher than *C. nigriceps* (2003), while Martins reported a value significantly lower than either *C. mimosae* or *C. nigriceps* (he did not study *C. sjostedti*: Martins Visitacao 2011). The two studies took place at different sites in Kenya, so perhaps the diets of these species vary across their ranges. *T. penzigi* patrols its trees very little, but despite this apparent lack of cooperation, trees inhabited by *T. penzigi* appear to survive fairly well (Palmer *et al.* 2008, Stanton *et al.* 2011), perhaps because *T. penzigi* does not exhibit uncooperative behaviors documented for *Crematogaster* colonies: it does not tend scale insects (Young *et al.* 1997); it is unlikely to abandon its host tree (Palmer *et al.* 2000); and by destroying extrafloral nectaries, it may free the tree from making a substantial energy output (Stanton *et al.* 2011).

Third parties in the *V. drepanolobium* mutualism

Arthropods

Several studies have catalogued substantial arthropod diversity on or around *A. drepanolobium* trees (Hocking 1970, Kuria and Villet 2002, Baker *et al.* 2016). Such insects

include herbivores and seed predators (Martins Visitacao 2011) and the cerambycid beetles and scale insects discussed above. Kuria and Villet (2012) studied the arthropod assemblages found in the canopies of *V. drepanolobium* trees occupied by different ant associate species. They collected arthropods by beating and fogging, and thus collected mainly those organisms in the canopy, but outside the domatia. They found differences in community structure primarily between canopies occupied by the scale-tending *C. sjostedti* and *C. mimosae* on the one hand, and the non-scale-tending *C. nigriceps* and *T. penzigi* on the other (Kuria and Villet 2012). Hocking (1970), collecting mainly in Tanzania, noted many inquilines; i.e., arthropods living in domatia with the ants. He reported that *C. mimosae* had the greatest diversity and abundance of inquilines. Baker *et al.*'s study of domatium-dwelling inquilines at field sites in Laikipia also found that *C. mimosae* hosted the greatest diversity of inquilines, and revealed characteristic communities of inquilines for each of *C. mimosae*, *C. nigriceps*, and *T. penzigi* (they did not include *C. sjostedti*), although many inquilines were found in domatia of all three host ants (Baker *et al.* 2016). Other insect species known to have associations with particular acacia ants include a gall-forming fly (Schumer *et al.* 2013), and brood parasites of the ants such as the butterfly *Anthene usamba* (Martins *et al.* 2013) and the wasp *Trigastrotheca laikipiensis* (Quicke and Stanton 2005).

The ant species inhabiting *V. drepanolobium* appear to supplement extrafloral nectar and/or other plant derived nutrients, such as honeydew, with arthropod prey to different extents, although the evidence is ambiguous and suggests substantial variation within and among species. Palmer found *C. sjostedti* (and *T. penzigi*, which has an unknown diet) enriched in $\delta^{15}\text{N}$, and *C. nigriceps* relatively deficient; *C. mimosae* has $\delta^{15}\text{N}$

levels near *C. sjostedti* and *T. penzigi* when its trees are near termite mounds, but it otherwise has low $\delta^{15}\text{N}$ levels, near those of *C. nigriceps*. This suggests that *C. mimosae* (sometimes) and *C. sjostedti* (always) forage for ^{15}N -rich insect prey to a greater extent than *C. nigriceps* (Palmer, 2003). However, at a different site, Martins found *C. nigriceps* higher in $\delta^{15}\text{N}$ than *C. mimosae* (and both higher than *T. penzigi*) (Martins Visitacao 2011). Termites mounds in the soil play a major role in structuring *V. drepanolobium* habitat, as the microenvironment near the mound is enriched in soil nutrients, which find their way into the acacia trees: *V. drepanolobium* leaves from trees near mounds had a higher nitrogen content than leaves from other trees, and those trees were more likely to fruit (Brody *et al.* 2010). The termites also have an effect the outcomes of competition between nearby acacia-ants, although the mechanism for this is not well understood (discussed below in *What maintains coexistence among the ant species*).

Vertebrates

Vertebrate herbivores are important for maintaining the *V. drepanolobium* ant-plant mutualism. At Mpala, where most studies have taken place, the main browsers of *V. drepanolobium* include elephant, giraffe, and several species of antelope (Palmer *et al.* 2007; Goheen and Palmer 2010) black rhino also feed on *V. drepanolobium*, although these are no longer common in Laikipia (Martins 2010). In the absence of herbivores, *V. drepanolobium* relaxes its defenses: trees show reduction in thorn length as well as the number of domatia and nectaries produced (Young *et al.* 2003, Palmer *et al.* 2008).

The only documented vertebrate predators of the ants are Patas monkeys: acacia ants may make up a substantial proportion of their diet (Isbell and Young, 2007). There are

various frog and lizard species inhabiting the Black Cotton habitat that may well also feed on ants.

Fungi

Baker *et al.* (2017) described the fungal communities isolated from within the domatia of the three obligately phytoecious ant species. Each ant species harbors a distinctive fungal community, even when comparing communities from widely separated localities (about 200 km apart). Fungal communities may be responding to an extended phenotype of the ants in the form of the distinctive contents of each species' domatia. In addition to different inquiline communities, *C. mimosae* domatia contain carton walls; *C. nigriceps* pack their domatia with leaves; *T. penzigi* domatia often contain loose, particulate matter variously described as fibers (Hocking 1970), frass (Isbell and Young 2007), or plant material (Martins Visitacao 2011); *C. sjostedti* domatia are commonly empty, although they may also contain carton walls (Hocking 1970). Some evidence, however, suggests that these communities may additionally be vectored by dispersing alates: alates of *C. nigriceps* and *T. penzigi* were found with fungal hyphae in their infrabuccal pockets (Baker *et al.* 2017). It is possible that these fungal communities may affect the tree. King and Caylor (2010) reported differences in rates of photosynthesis and water use efficiency among *A. drepanolobium* trees occupied by different ant species in open and herbivore-excluded plots. The authors provided little account of how the ants might mediate such changes in the trees' metabolism; perhaps the ants achieve this by inoculating the trees with endophytic fungi, which have been implicated in changing plant metabolisms in diverse systems (reviewed in Yuan *et al.* 2010).

Major research topics

Much of the research in the *V. drepanolobium* system has focused on three interrelated problems: what maintains coexistence among the four ant associates? What are the costs and benefits of the ant-plant association to each partner? How does the system as a whole respond to perturbations in its environment?

What maintains coexistence among the ant species?

Species coexistence has long been a conundrum in population biology (Gause 1934). Biologists have sought explanations for numerous examples that appear to violate the Competitive Exclusion Principle (or Gause's Law), namely that species that compete for limiting resources cannot coexist (Hardin 1960). Such work has often led to valuable insights into factors governing species diversity and distribution. For example, "the paradox of the plankton" has been a classic problem in need of explanation (Hutchinson 1961), and recent metatranscriptomic analysis has revealed much functional differentiation in resource use among diatoms previously thought to be more uniform in their biology (Alexander *et al.* 2015).

The ant associates of *V. drepanolobium* are a classic case of species coexistence. Each tree is occupied by a single colony (although individual colonies may spread across multiple trees). Nevertheless, many areas feature a mosaic of trees inhabited by each one of all four ant species, all found within meters of each other (Hocking 1970, Young *et al.* 1997). These four ant species coexist despite pronounced differences in competitive ability (Palmer *et al.* 2000).

The main hypothesis put forward to explain this coexistence is a competition-colonization hierarchy. The competition-colonization hierarchy hypothesis posits that species vary in their ability to “compete” with neighbors for occupied resources, as well as in their ability to “colonize” unoccupied resources. Less competitive species persist by occupying empty patches and rapidly reproducing before they are expelled by more competitive species. Such dynamics can maintain the coexistence of multiple species, assuming some turnover in patches (Tilman 1994, Yu and Wilson 2001). This idea has been invoked in this system, in which individual trees are the patches, which may either be occupied during the brief windows in which they are unoccupied or abandoned, or may be taken over through inter-colony battles (Stanton *et al.* 2002).

Both observational and experimental evidence support a competitive hierarchy. Palmer *et al.* (2000) observed that most natural transitions occurred in accordance with the following hierarchy: *C. sjostedti* > *C. mimosae* > *C. nigriceps* > *T. penzigi*. The same hierarchy was observed in staged conflicts, when two trees occupied by different species of ants were tied together, and the ants then fought for sole possession of the now-combined canopy. In experimental contests, battles involving *T. penzigi* were often draws (Palmer *et al.* 2000). This may be because *T. penzigi* chew smaller entrance holes in the domatia, making it difficult for *Crematogaster* species to enter their domatia, and because *T. penzigi* destroy extrafloral nectaries, making their tree less valuable to the *Crematogaster* invaders (Palmer *et al.* 2002). Although *T. penzigi* is a better defender than *C. nigriceps*, it was placed below *C. nigriceps* in the hierarchy because *C. nigriceps* is more likely to take over trees from other species than is *T. penzigi*. Supporting this competitive hierarchy is the observation that the average size of trees occupied by each species follows the hierarchy,

with *C. sjostedti* occupying the largest trees (on average), and so on, with *T. penzigi* occupying the smallest trees (Palmer *et al.* 2002).

Stanton *et al.* (2002) proposed a colonization hierarchy running in the opposite direction, with *T. penzigi* best at founding new colonies on unoccupied trees, then *C. nigriceps*, *C. mimosae*, and *C. sjostedti*. Foundress queens fight over individual domatia, and by examining the corpses of defeated foundresses left behind by the victor, Stanton *et al.* (2002) described the queen combat hierarchy of *T. penzigi* > *C. nigriceps* > *C. mimosae*. Foundresses of *C. sjostedti* are extremely rare in domatia, and do not appear to found colonies on empty trees; perhaps colonies are founded elsewhere (i.e., in rotten wood or underground), or they may reproduce by fission (Stanton *et al.* 2002, 2005). In addition to being better fighters, *T. penzigi* foundresses are at less risk of parasitism from a braconid wasp (Stanton *et al.* 2005), and are better defended against patrolling workers from mature colonies of *C. sjostedti* (Palmer *et al.* 2013). Another line of evidence comes from experimentally removing colonies from trees; the tree is then occupied by many foundresses in different domatia, which compete among each other to dominate the tree. In mature trees from which colonies had been removed, *T. penzigi* and (to a lesser extent) *C. nigriceps* foundresses were subsequently overrepresented, while *C. mimosae* foundresses were underrepresented and *C. sjostedti* foundresses nearly absent (Stanton *et al.* 2002). Thus, it appears that the better colonizing species are more likely to establish on the most valuable empty trees. On less valuable saplings, *C. nigriceps* and *C. mimosae* foundresses were overrepresented, and *T. penzigi* slightly underrepresented (Stanton *et al.* 2002, see also Palmer *et al.* 2013). It should be noted that empty trees can also be occupied by mature colonies expanding from nearby trees; such expansions did not show clear patterns

in accordance with either the competition or colonization hierarchy, and empty trees were likely to be occupied by different ant species, depending on the condition and size of the empty tree (Stanton *et al.* 2002).

Other, often complementary hypotheses have been proposed to explain species coexistence in this system. Palmer found that microhabitats are important for structuring the ant community on *V. drepanolobium* trees: the dominance hierarchy was most pronounced in the high-quality habitat near termite mounds (which enrich the soil nearby), whereas the hierarchy was less severe further from the mounds, although dominant ants were still more likely to supplant subordinate species than vice versa (Palmer 2003). Furthermore, in one transitional habitat between Black Cotton soil and a sandier soil type on which *V. drepanolobium* grows poorly, the dominance hierarchy was reversed, at least with respect to *C. mimosae* and *C. nigriceps* (Palmer 2004). These studies suggest the possibility that the competitive hierarchy may differ in different microhabitats, contributing to species coexistence.

Palmer *et al.* (2013) also found that the subordinate ant *T. penzigi* was most successful, at both the foundress queen stage and the mature colony stage, when colonies of the dominant ant *C. sjostedti* were nearby. They argued that because these two species tend to specialize on either large (*C. sjostedti*) or small (*T. penzigi*) trees, their niches overlap the least of any pair of species, and that *C. sjostedti* thus creates an enemy-free space for *T. penzigi* by excluding *C. mimosae* and *C. nigriceps* from areas which *C. sjostedti* workers patrol. Supporting this hypothesis of indirect facilitation, they find that at a nearby site where *C. sjostedti* is absent, *T. penzigi* is also absent (Palmer *et al.* 2013).

What are the costs and benefits of the association?

While the acacia ants clearly benefit from nesting space and extrafloral nectar, the benefits of ant occupancy for the tree are less clear. Two of the ant species, *C. mimosae* and *C. nigriceps*, defend the tree from herbivores ranging in size from insects (Martins Visitacao 2011), to goats (Stapley 1998), to megafauna such as rhino (Martins 2010), elephants (Goheen and Palmer 2010), and possibly giraffe (Martins 2010, but see also Madden and Young 1992 and Palmer and Brody 2013). The ants may also deter parasites such as galling cecidomyiid flies (Schumer *et al.* 2013) or stem-boring cerambycid beetles (Palmer *et al.* 2008). However, *T. penzigi* and *C. sjostedti* defend the tree weakly or not at all (Palmer and Brody 2007, Martins 2010).

Even in the case of ants that do defend the trees, a study by Stanton *et al.* (2011) suggests that herbivore and parasite deterrence over short time scales (i.e., a few years) may not provide the tree with enough benefit for it to recoup its energy investment in domatia and nectar; the ants may instead be a sort of insurance policy for the tree, deterring feeding by elephants which, although uncommon, can be catastrophic for the tree. This study found that trees from which colonies were eliminated grew faster than (in the case of *C. sjostedti*, *C. nigriceps*) or at the same rate as (in the case of *C. mimosae*, *T. penzigi*) control trees. However, the ants in their experimental manipulations were killed with pesticides, and their bodies were not then removed, making it difficult to disentangle the effects of removal of the ants *per se* from possible fertilization effects such as those seen in the *Cecropia-Azteca* ant-plant system (Sagers *et al.* 2000).

Regardless of the benefits (or otherwise) of herbivore defense, each ant species appears to provide some value to the tree. Palmer *et al.* (2010) observed that trees with any ant occupant (even non-defending species) have better survival than uninhabited trees, although they were unable to rule out the possibility that trees which are already in poor condition are more likely to be abandoned by their ant occupants. Their demographic model suggests that, across all size classes, trees occupied by *T. penzigi* may have the greatest survival, despite this ant's non-defending behavior, possibly because *T. penzigi* is less likely than the defending species to abandon its host tree, or because it does not use extrafloral nectar. Palmer *et al.*'s model also predicted that, comparing trees occupied by a single species over the entire lifespan of the tree, those trees occupied by *T. penzigi* should have the highest fitness. However, their model suggests that trees should have even greater fitness when occupied sequentially by all four ant species. They suggest that trees on average are occupied by *T. penzigi* when small, then *C. nigriceps*, *C. mimosae*, and finally *C. sjostedti* at the largest size, and that each ant species provides benefits that are disproportionately valuable to the size class of trees which they tend to occupy. However, this model is dependent on a particular assumption regarding empty trees, *viz.*, that the proportion of empty trees is independent of the species composition of the plot. Altering this assumption changes the model outcome such that the trees have the highest fitness when occupied only by the single best mutualist, *T. penzigi* (Palmer *et al.* 2010).

Palmer and Brody (2013) provided evidence that *C. mimosae* colonies may be larger than optimal for their host tree by experimentally reducing ant colony sizes. Larger colonies provided more defense against elephants, gall making insects, and cerambycid beetles; for the first two, however, smaller colonies provided nearly as much benefit as

larger ones, and trees with smaller colonies reproduced more (Palmer and Brody 2013). Their evidence suggests that the defensive benefits provided by the ants saturate as colony size increases, but the reproductive cost imposed on the tree increases linearly; however, they note that large *C. mimosae* colony sizes may also benefit the tree by reducing the likelihood of takeover by *C. sjostedti*, which ant is even more costly to the tree.

How does the V. drepanolobium mutualism respond to environmental change?

The *V. drepanolobium* system is responsive to changes in both biotic and abiotic environment. A number of studies have taken advantage of the Kenya Long-term Exclosure Experiment (KLEE) plots at the Mpala Research Centre to investigate the effects of changing herbivory regimes (Young *et al.* 1997a). Young *et al.* (2003) found that *V. drepanolobium*'s investment in thorns decreases in the absence of herbivory. Huntzinger *et al.* (2004) found that the absence of herbivory also led to reductions in the host trees' investment in their ant occupants, with *V. drepanolobium* trees producing fewer domatia and extrafloral nectaries in the absence of herbivores. Trees occupied by *C. nigriceps*, however, showed lesser reductions, possibly because of the tendency by this ant species to chew their host's foliage, simulating herbivory (Huntzinger *et al.* 2004). Palmer *et al.* (2008) found that when the absence of herbivory caused trees to reduce their investment in their ant occupants, *C. mimosae* colonies responded by defending their host tree less vigorously and increasing their tendency to attend sapsucking scale insects.

Herbivory may have other effects on the trees as well: King and Caylor (2010) found that in unbrowsed plots, *V. drepanolobium* trees had similar rates of photosynthesis and

transpiration; in the presence of herbivores, however, trees occupied by different species showed differences in both photosynthesis and transpiration.

Several recent studies have described the impact of fire on the *V. drepanolobium* mutualism. Kimuyu *et al.* (2014) report that fire almost always destroyed ant colonies on trees less than 1 meter tall, and often killed colonies on larger trees. Furthermore, fire affected different species differently, with *C. mimosae* and *C. nigriceps* colonies more likely to survive a fire than *C. sjostedti* or *T. penzigi*. Greater survival for *C. mimosae* and *C. nigriceps* was likely due to these species evacuation behaviors, in which smoke triggers them to relocate the colony temporarily underground (Jaffe and Isbell 2009, Sensenig *et al.* 2017). Furthermore, even when colonies survived, burns reduced the worker populations (Sensenig *et al.* 2017). Over a short time scale (4-16 months), fire led to a decreased presence of *T. penzigi* relative to unburned plots, and an increase in *C. sjostedti* (but the latter only when megaherbivores were also excluded: Pringle *et al.* 2015). Over a longer time period (6-7 years) after fire, *C. nigriceps* was more common, and the other three species less common, than in unburned plots (Sensenig *et al.* 2017).

Other social insects play an important role in the system as well. Termite mounds affect not only the dynamics of the ants (discussed above), but also the reproduction of the tree: Brody *et al.* (2010) found that trees near termite mounds are much more likely to fruit. The invasive ant *Pheidole megacephala* has also invaded the *V. drepanolobium* mutualism in recent years (Martins Visitacao 2011). Riginos *et al.* found that colonies of all three *Crematogaster* species were largely eliminated by brood-raiding *P. megacephala* invaders, while *T. penzigi* colonies were able to coexist with the invaders. Furthermore,

because neither *T. penzigi* nor *P. megacephala* defend the tree against megaherbivores, elephant damage increased in invaded areas (Riginos *et al.* 2015).

Thesis overview

Several lines of evidence, described above, support a competition-colonization hierarchy among the ants; however, little is known about what characteristics or behaviors cause the ants to be better or worse at colonizing or competing. In the first two chapters of my thesis, I test two hypotheses put forward (e.g., in Palmer 2004) regarding what colony-level traits which may underlie this hierarchy.

In Chapter 1, I use genetic markers derived from RADseq, a next-generation sequencing technique, to test the hypothesis that more competitive species are more competitive because they have more egg-laying queens per colony, which in turn permit larger colony sizes. I find no evidence that this is the case: neither queen number nor the mating frequency of the queens is associated with the dominance hierarchy.

In Chapter 2, I test the hypothesis that the different ant species experience a similar tradeoff between competing (by producing workers) and colonizing (by producing reproductives), and how they invest their resources determines where they are on both the competitive and colonizing hierarchy. I do this by dissecting colonies on individual trees to determine how biomass is divided between workers and reproductives, finding evidence that such a tradeoff may indeed underlie the competition-colonization hierarchy. I also describe the reciprocal impacts both partners have on each other; namely, how the ants affect the growth of their host tree, and how the characteristics of the host tree influence

the organization of the ant colony. I argue that these data provide more support for proposals that this mutualism is based on reciprocal exploitation (e.g., Palmer and Brody 2007).

In Chapters 3 and 4, I expand our understanding of the *V. drepanolobium* mutualism to regions outside of Laikipia. Almost all of the studies on this system have been performed at or near the Mpala Research Centre; however, the few studies to take place outside of this region suggest that the system may look very different in other parts of East Africa (e.g., Hocking 1970, Martins Visitacao 2011). Mutualisms can vary widely across their geographic ranges, and having an understanding of this variation is important when considering how the different partners may have evolved (Thompson 2005).

In Chapter 3, I survey the demography of this system throughout Kenya, including both highlands sites similar to those in Laikipia and also relatively low-lying sites in the Rift Valley. I find that the system varies dramatically across Kenya, in terms of *V. drepanolobium* growth patterns, and also presence, absence, and biomass of the difference species of ant mutualists. I compare the biomass of the ants to that of the herbivores against which they defend, as well as to the biomass which *V. drepanolobium* devotes to other herbivore-defense strategies. Although the absolute biomass of acacia-ants in this system is relatively low compared to the few published estimates for ants, comparing their biomass to that of thorns or tannins shows that they have an ecological influence disproportionate to their mass.

In light of the diversity shown in Chapter 3, it is important to know the population structure of the mutualists to know whether there are opportunities for local adaptation to

this variety of conditions. I use RADseq markers to study this in Chapter 4, carrying out a population genomic study of *V. drepanolobium*, and of the three ants that are obligately phytoecious associates, *C. mimosae*, *C. nigriceps*, and *T. penzigi*. I find little evidence for local adaptation, particularly in the tree, in which single genetic clusters are found in a variety of abiotic and biotic environments. Furthermore, the population structures of the plant and the ants are quite different, limiting the opportunity for local coevolution. I argue that this diversity of structures means that individual lineages of each partner have not been evolving under constant biotic or abiotic conditions; rather, any adaptations that have evolved in this system must allow these organisms to persist in a variety of environments, and alongside a variety of other symbiotes.

Chapter 1: Colony relatedness revealed by RADseq markers shows that polygyny cannot explain the superior competitive ability of dominant ant associates in the African ant plant, *Vachellia drepanolobium*.

Abstract

The *Vachellia drepanolobium* ant-plant symbiosis is a classic case of species coexistence, in which four species of tree-defending ants compete for nesting space in host trees. Species coexistence in this system has been explained by trade-offs in the ability of the ant associates to compete with their neighbors for occupied trees and establish new colonies on unoccupied trees. Proximal reasons for how and why the ant species vary in competitive or colonizing abilities are largely unknown. In this paper, we use RADseq derived SNPs to identify relatedness of workers in colonies to test the hypothesis that competitively dominant ants reach large colony sizes due to polygyny, i.e., the presence of multiple egg-laying queens in a single colony. We found that variation in queen number is not associated with competitive ability; in fact, the most dominant species, unexpectedly, was usually monogynous. We also used these markers to investigate variation in mating behavior among the ant species, and found that different species varied in the number of males fathering the offspring of each queen. Finally, we show that among *Crematogaster mimosae* and *Tetraponera penzigi*, the two commonly polygynous species, the manner of polygyny varies: in *C. mimosae*, queens in the same colony are often related, while this is not the case for *T. penzigi*. These results demonstrate the effectiveness of RADseq-derived SNPs for parentage analysis, and shed light on factors influencing the evolution of species coexistence in an ant-plant mutualism.

Introduction

Species coexistence, the question of how different species can coexist while competing for limiting resources, is a central question in ecology (e.g. Hutchinson 1961, Huston 1979). A classic example of species coexistence is found among four ant species that inhabit the ant plant *Vachellia* (formerly *Acacia*) *drepanolobium*. The Whistling-Thorn Acacia, *V. drepanolobium*, is an East African savannah tree that engages in a defensive mutualism with ants, providing the ants with extra-floral nectar and housing in hollow, swollen-thorn 'domatia' in return for defense from herbivory (Hocking 1970; Young *et al.* 1997b; Figure 1.1). At a well-studied field site in Kenya, four species of ants are commonly hosted by the acacia. Three of them, *Crematogaster mimosae*, *C. nigriceps*, and *Tetraponera penzigi*, are obligate phytoecious inhabitants of *V. drepanolobium*; the fourth, *C. sjostedti*, is also free-living, nesting under bark and in rotting wood (Stanton *et al.* 2002). Almost every mature tree of *V. drepanolobium* is occupied by a single ant colony, and competition among ants for these trees is intense (Palmer *et al.* 2000). In light of this, much research has focused on how these four species are able to coexist on a single resource (Palmer 2003, Palmer *et al.* 2000, Stanton *et al.* 2002, Young *et al.* 1997b), in violation of theory suggesting that this situation should be ecologically unstable (Gause 1934, Hardin 1960).

The best-supported hypothesis to explain this coexistence is that a colonization-competition trade-off exists among the ant species, in which ants must specialize either in colonizing new resources (i.e., unoccupied trees) or in competing for occupied resources. Observations of naturally-occurring transitions from occupancy by one ant species to another have shown a competitive hierarchy among the ants, with *C. sjostedti* as the most dominant, followed by *C. mimosae*, *C. nigriceps* and *T. penzigi*; this hierarchy has also been

supported by experiments in which contests were staged by tying the canopies of neighboring trees together (Palmer *et al.* 2000). The degree to which a single colony extends over multiple trees (i.e., a form of polydomy, where a single colony spreads across multiple locations) is also correlated with the competitive hierarchy, with *C. sjostedti* occupying the greatest number of adjacent trees, and *T. penzigi* occupying the least (Palmer *et al.* 2010). Furthermore, more competitive ant species also occupy larger, presumably more valuable trees (Young *et al.* 1997b, Palmer *et al.* 2000).

Under the competition-colonization trade-off hypothesis, a colonization hierarchy runs counter to the competitive hierarchy, allowing species coexistence. Colonization ability has been measured using several metrics: production of foundress queens, recruitment of these foundresses to empty trees, ability of foundresses to win fights with each other over individual domatia on newly colonized trees, speed with which foundresses can produce workers that occupy the rest of the tree, vulnerability to parasitism, and risk of destruction by mature colonies (Palmer *et al.* 2000, Stanton *et al.* 2002, Stanton *et al.* 2005, Palmer *et al.* 2013). This research has supported the existence of a colonization hierarchy in which *T. penzigi* is the best colonizer, followed by *C. nigriceps*, *C. mimosae* and *C. sjostedti*: the reverse order of the competitive hierarchy.

While differences in competitive and colonizing ability among *V. drepanolobium*'s ant associates have been well described, we still have little understanding of the mechanistic basis for these differences in ability among the four ant species. A primary hypothesis proposed for why some species are better competitors than others is that more competitive species are polygynous, i.e., they have multiple queens per colony (Palmer 2004, Rubin *et al.* 2013). Collectively, these queens are able to lay more eggs, thereby



Figure 1.1: Ants inhabit *V. drepanolobium* trees on black cotton soils. On black cotton soils, *V. drepanolobium* may account for 95% or more of trees, as shown on the left-hand side (Young *et al.* 1997b). Ants live in hollow, swollen thorns and patrol the tree against herbivores (right). Photos: Naomi E. Pierce (left) and Dino J. Martins (right).

producing larger worker populations that can outcompete their neighbors. Polygyny is known to promote competitive ability in other ant systems, including another acacia-ant mutualisms (Kautz *et al.* 2009, McGlynn 2010), and it is common among highly competitive invasive ant species (Tsutsui and Suarez, 2003). In the *V. drepanolobium* system, higher competitive ability seems to be driven by larger worker populations (Palmer 2004, Ruiz-Guajardo *et al.* 2017), but it is unknown whether higher worker populations are the result of polygyny or some other difference(s) in the intrinsic rate of growth of the different ant species. The only research to date on queen number in this system is that of Rubin *et al.* (2013), who used microsatellite markers to show that *C. mimosae* colonies are commonly polygynous. However, no further work on the remaining three species has been done to test the hypothesis that queen number underlies competitive ability in these ants.

To learn more about the colony structure of all four common ants in this system, we genotyped multiple same-colony workers for each of the four species of ant associates using double-digest Restriction-site Associated DNA sequencing (RADseq). This reduced-representation genomic sequencing method generates DNA sequences of sites near

restriction enzyme cut sites, providing a repeatable subset of the genome at a relatively low cost (Peterson *et al.* 2013). Using hundreds to thousands of single-nucleotide polymorphisms for each worker, we reconstructed intra-colony relationships, and were able to examine the degree of polygyny and polyandry of the four ant species inhabiting *V. drepanolobium*.

Methods

Collections

From February-April 2012, ants were collected from *V. drepanolobium* trees tagged in the long-term monitoring plot of the Center for Tropical Forest Science-Forest Global Earth Observatories (CTFS-ForestGEO) at the Mpala Research Centre, Kenya. For each of the four species of ant, about 15 trees were selected, and at least eight workers collected into 95-100% ethanol. Because (female) workers were collected, all selected individuals were diploid. In June 2015, the sizes of the selected trees were measured using two metrics: height of the tallest part of the canopy, and diameter of the stem at 0.5 meters height.

DNA extraction and sequencing

We extracted DNA from each worker using an AutoGenprep 965 Tissue/ES Cell DNA Extraction Kit. Genomic DNA was stored at -20° C before use.

The amount of genomic DNA was then increased by whole genome amplification, using the REPLI-g mini kit in 15 or 20 µL reactions.

We used the double-digest restriction-site associated DNA sequencing (RADseq) protocol of Peterson *et al.* (2012). We modified their protocol in a number of respects: we started with an (amplified) genomic DNA mass of 150 ng, which we then digested with the

restriction enzymes EcoRI-HF and BfaI. Bead cleanups throughout the protocol were performed with a MagNA bead solution described by Rohland and Reich (2012). We used the 48 inline indices for EcoRI described in the Sequences-S1 spreadsheet in the supplement of Peterson *et al.* (2012). We chose a range of 264-336 bp for the size selection step, which we performed using 2% ethidium bromide cassettes on a Sage Science Pippin Prep machine. The final PCR was set for 10 cycles.

These libraries were then sequenced in 100 bp, single-end reads on Illumina HiSeq 2000 and 2500 at the Harvard University Bauer Core Facility.

DNA sequence alignment and base-calling

To demultiplex the Illumina libraries, as well as to align reads across worker ants and call single nucleotide polymorphisms (SNPs), we used the program Stacks version 1.21 (Catchen *et al.* 2011, Catchen *et al.* 2013). Reads were demultiplexed using the *process_radtags* function of stacks, rescuing barcodes and RAD-tags, and disabling checking if the RAD site was intact.

We quality filtered reads using the FASTX-Toolkit version 0.0.13 (http://hannonlab.cshl.edu/fastx_toolkit/). For each read, the first seven base pairs, including the EcoRI-HF restriction site and two often-low-quality bases, were removed using the *fastx_trimmer* tool, because the low sequence diversity in this region produced low quality scores. The trimmed reads were then quality-filtered using the *fastq_quality_filter* tool, removing any reads with a quality score of less than 25 at more than 2% of bases.

We then aligned all reads for all workers within each species using the *denovo_map.pl* script of Stacks, allowing 2 mismatches between loci when processing a

single individual, and 1 mismatch when building the catalog. To build the final matrix of SNPs, we culled individuals for which sequencing had failed or had produced too low coverage to be useful (i.e., had less than 20% SNP coverage when run through a preliminary *populations* run with $-r = 0.5$; see below), leaving, on average, about 6 worker libraries per tree. We called SNPs using the *populations* program of Stacks. In order to maximize our ability to make comparisons among workers from the same tree (within-tree comparisons), we assigned all the workers from the same tree to their own processing group (i.e., a “population” in the Stacks terminology). A SNP was only processed for the ants from a single tree if it was present in at least half of the individuals in the processing group ($-r$ cutoffs were set to produce about 1000 SNPs total per species: for *C. sjostedti*: 0.6, *C. mimosae*: 0.6, *C. nigriceps*: 0.9, *T. penzigi*: 0.5), and a SNP was only processed for all ants if it was found in five or more (about one-third) of the total number of processing groups. Heterozygosity was calculated using the adegenet package version 2.0.1 (Jombart 2008, Jombart and Ahmed 2011).

Relatedness of ants within and between trees

We then compared the average relatedness of each worker ant to every other ant of the same species. We compared the relatedness values across species, for both (i) workers from the same tree (within-tree), and (ii) from different trees (between-tree). We determined relatedness values using the Queller and Goodnight method (1989) implemented in the R package *related* (Pew *et al.* 2015). We chose the Queller-Goodnight method because in simulations it performed better than four other methods of calculating relatedness also implemented in *related*: those of Li *et al.* (1993), Lynch and Ritland (1999), Ritland (1996), and Wang (2000). We then used Family-Sim version 1.0

(https://github.com/timothyfrasier/C_software/blob/master/FAM-SIM_v1.0_LINUX_1.tar.gz), along with the number of alleles recovered for each species, and the allele frequencies observed in our data. For each species we simulated 1000 pairs of each of the following relationships between diploid individuals: parent-offspring, full-sib, half-sib, and unrelated. The Queller-Goodnight method had the highest correlation between the true relatedness (0.5, 0.5, 0.25, and 0, respectively) and the relatedness recovered using that method in *related*. Correlations are shown in the File S1. For within-tree comparisons, only relatedness values between workers from the same tree were averaged together (across all workers from the same tree); we then took the mean of these (across all trees of a single ant species) to find the average within-tree relatedness for each species. For between-tree comparisons, only relatedness values between workers from different trees were averaged together (across all workers from the same pair of trees); we then took the mean of these to find the average between-tree relatedness for each species.

Within-tree relationships: polygyny and polyandry

To determine the relationship between individuals collected from the same tree, we used the program COLONY version 2.0.5.9 (Jones and Wang 2010). COLONY estimates a number of paternal and maternal genotypes, assigning each reconstructed father or mother as a parent of one or more of the observed, genotyped individuals. We ran COLONY under the default run parameters, except changing the mating system to female polygamy and the ploidy to haplodiploidy. In addition, because *T. penzigi* showed more genetic diversity within colonies, the run length was set for “very long” for *T. penzigi*. We set each locus as codominant, with the allele frequency as “unknown,” with an allelic dropout rate of 0.0001 and an additional error rate of 0.0025. For *C. nigriceps* and *T. penzigi*, our data set included

the genotype of a queen that we happened to collect, each from one tree for each species respectively. These two reproductive females were identified as queens by their lack of wings and physogastric abdomens. The genotypes for these queens were included in the offspring genotypes with the worker genotypes. They were also given to COLONY as possible maternal genotypes (alongside any inferred maternal genotypes), with a prior probability of their being the mother of one of the offspring set at 0.5. We used the most likely sibship configuration output, using this to ask how many different mothers (i.e., queens) and fathers were estimated to give rise to the workers within each tree. Furthermore, for each estimated queen, we also looked for multiple mating by recording the number of different males estimated to be the father(s) of her worker offspring. For this purpose, only data from those queens that had at least four offspring among the genotyped workers were used. Finally, for each tree, we calculated a metric of queen dominance by calculating the proportion of the genotyped workers that were daughters of the queen with the most offspring in that sample; we did this for the male that sired the most worker offspring as well.

Maternal relatedness

We recovered the estimated maternal genotypes from COLONY runs, using the same parameters as above, but excluding the possibility that workers from different trees shared a parent. Genotypes for any allele to which COLONY assigned a less than 0.90 probability were set as missing data. To determine whether the mothers reconstructed from each tree were related to each other, we ran these putative maternal genotypes in COLONY again, using the same parameters as above, and recorded whether each pair of estimated mothers were full siblings, half siblings, or unrelated.

Statistical analyses

If colonies with more queens are better able to compete for large and valuable trees, this could produce a correlation between colony structure and tree size. We looked for this by testing whether tree size impacted within-tree relatedness, queen number, male number, and males per queen, using the Pearson correlation test when the data were approximately normal (or could be transformed to normality: males per queen data were square-root transformed), and Spearman's rank correlation otherwise. We also took into account tree size and ant species by using the `lm` function of R to perform an analysis of covariance (ANCOVA), with tree height or diameter as the covariate. For each test, there was no significant interaction between ant species and tree size, so we did not include an interaction term in the results presented below. Tree size was measured in two ways, height and diameter at 0.5 meters above the ground; we present only the results for height here, since height and diameter were strongly correlated and the results of the tests qualitatively identical. The results of tests on tree diameter may be found in File S2 of the Supporting Information.

Since we found no significant effect of tree size on any of these factors, we did not include tree size as a covariate in our final analyses. Comparisons among ant species for between-tree relatedness, within-tree relatedness, queen number, male number, and males per queen were done using Analysis of Variance (ANOVA) when the data were relatively normally distributed, or Kruskal-Wallis tests (KWT) otherwise. Post-hoc comparisons were done using Tukey's honest significant difference test (Tukey HSD) for ANOVA and the Nemenyi post-hoc test for KWT.

For between-tree relatedness, we compared each species' distribution to zero using Student's t-test.

For maternal relatedness, we compared the proportion of sibling queens within nests to the proportion of sibling queens between nests using Fisher's exact test.

Statistics were done in R version 3.2.3 (R Core Team 2015). Nemenyi post-hoc tests were performed using the *PMCMR* package (Pohlert 2014).

Results

Sequencing and base calling

After DNA sequence filtering, alignment, and SNP calling, each worker was sequenced at several hundred SNPs, and across all workers from all trees, we produced genotypes for 900-1500 SNPs for each species (Table 1.1).

Relatedness of ants within and between trees

The average relatedness of ants within and between trees for each species is shown in Table 1.2 and Figure 1.2A. The average relatedness between trees was not significantly different from zero for any of the ant species (Student's t-test, $p > 0.5$ for all). There were significant differences in the distributions of relatedness among the four species (KWT, $p < 10e-4$; distributions and the results of Nemenyi post-hoc tests are shown in Figure 1.2A).

Tree height did not correlate with within-tree relatedness either across all ant species (Pearson correlation test, $p = 0.3$) nor within species (ANCOVA, $p = 0.3$). However, the average within-tree relatedness did vary among species (ANOVA, $p < 10e-8$), as shown in Table 1.2 and Figure 1.2B. Post-hoc comparisons show that *C. nigriceps* and *C. sjostedti* had higher within-tree relatedness than *C. mimosae* and *T. penzigi* (Tukey HSD test, $p <$

Table 1.1: Results of RADseq genotyping: total reads, SNPs per worker ant, total SNPs, and average observed heterozygosity across all SNPs.

Species	Trees	Workers/ Tree	Reads/ Worker	Average SNPs per worker	SNPs across all workers	H_{obs}
<i>C. sjostedti</i>	15	5.7	356,000	440	1199	0.10
<i>C. mimosae</i>	14	6.1	370,000	478	1182	0.13
<i>C. nigriceps</i>	18	6.6	422,000	787	1535	0.17
<i>T. penzigi</i>	16	6.2	386,000	294	915	0.11

Table 1.2: Relatedness of workers within- and between- trees in the CTFS-ForestGEO plot at Mpala.

Species	Trees sampled	Between-tree relatedness	Within-tree relatedness	Queens per tree	Queen dominance	Males per tree	Males per queen	Male dominance
<i>C. sjostedti</i>	15	-0.11±0.02	0.50±0.03	1.2±0.1	0.96±0.02	4.5±0.4	4.3±0.5 (n=15)	0.39±0.07
<i>C. mimosae</i>	14	-0.02±0.01	0.30±0.04	2.6±0.4	0.70±0.07	5.0±0.3	4.4±0.5 (n=10)	0.36±0.07
<i>C. nigriceps</i>	18	-0.05±0.01	0.59±0.02	1.1±0.1	0.99±0.01	2.7±0.3	2.6±0.3 (n=18)	0.73±0.05
<i>T. penzigi</i>	16	-0.00±0.02	0.26±0.05	3.6±1.7	0.48±0.06	5.3±0.4	2.7±0.4 (n=6)	0.31±0.06

Values shown are means ± standard error.

Queen dominance is the proportion of genotyped workers who are offspring of the queen with the greatest number of offspring among the genotyped workers. Male dominance is analogous, but for the male with the most offspring.

To calculate the number of males with which each queen mated, we only considered those queens with at least four offspring among the workers; the number of these queens is given after the number of males per queen in parentheses.

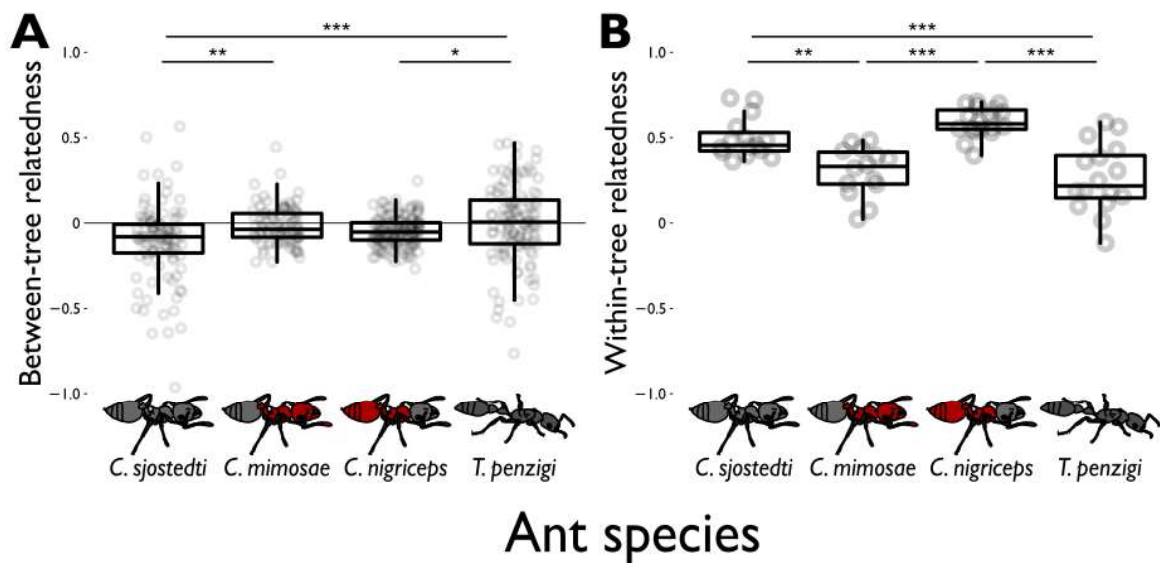


Figure 1.2: Average relatedness of workers between trees is near-zero, but relatedness within trees is high, and differs among ant species. A) shows relatedness between ants on different trees (between-tree comparisons); B) shows relatedness between ants on the same tree (within-tree comparisons). The species are arranged left to right in order from most to least competitively dominant. Box plots show the median and inter-quartile range for each species. Dots underlying each box plot show the average relatedness between each pair of trees (A) or within each colony (B); they are jittered horizontally to better show their distribution. Lines above the boxplots denote significant differences between species as follows: * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$. In Figure 1.2A, although the distributions are significantly different among the species, none are significantly different from zero.

0.01); this pattern corresponds to the pattern of higher numbers of queens in *C. mimosae* and *T. penzigi* colonies, discussed below.

Within-tree relationships: polygyny and polyandry

Across all four ant species, we found no overall correlation between the number of queens in a tree and the height of that tree (Spearman's rank correlation, $p = 0.3$). Nor did we find evidence for a correlation between queen number and height within any single ant species (ANCOVA, $p = 0.5$).

However, we did find a strong effect of ant species on degree of polygyny (KWT, $p < 10e-6$). *T. penzigi* and *C. mimosae* typically had multiple queens per colony, while *C.*

sjostedti and *C. nigriceps* both had only slightly more than one queen per colony (Table 1.2, Figure 1.3A). Post-hoc tests found significant differences between *C. mimosae* and *T. penzigi* on the one hand, and *C. sjostedti* and *C. nigriceps* on the other (Nemenyi tests, $p < 0.05$). In both *C. mimosae* and *T. penzigi*, despite averaging multiple queens per colony, a single queen appeared to be the mother of a disproportionate number of offspring, accounting for 70% and 48%, on average, of the genotyped workers. Finally, for the two queens recovered during sampling, both the *C. nigriceps* and *T. penzigi* queens were recovered as sisters to their colony-mates, rather than as their mothers.

Across all species, we found no correlation between the number of males fathering the workers in each tree and the height of that tree (Spearman's Rank Correlation, $p = 0.7$), or any correlations for one or more individual species (ANCOVA, $p = 0.4$). The number of paternal genotypes present in a tree differed between ant species (KWT, $p < 10e-3$). For *C. sjostedti*, *C. mimosae*, and *T. penzigi*, the workers collected from each tree were fathered by about 5 males, whereas for *C. nigriceps*, workers were fathered by about half that many males (2.7 males per tree; Table 1.2, Figure 1.3B). Post-hoc tests found significant differences only between *C. nigriceps* and the other three species (Kruskal-Nemenyi tests, $p < 0.05$).

We found no correlation between the number of males with which each queen had mated (including only queens that were the mothers of at least four genotyped progeny) and the height of the tree occupied by those ants (Pearson correlation test, $p = 0.051$). We also did not find evidence for a correlation between queen polyandry and tree size for any single ant species (ANCOVA, $p = 0.2$).

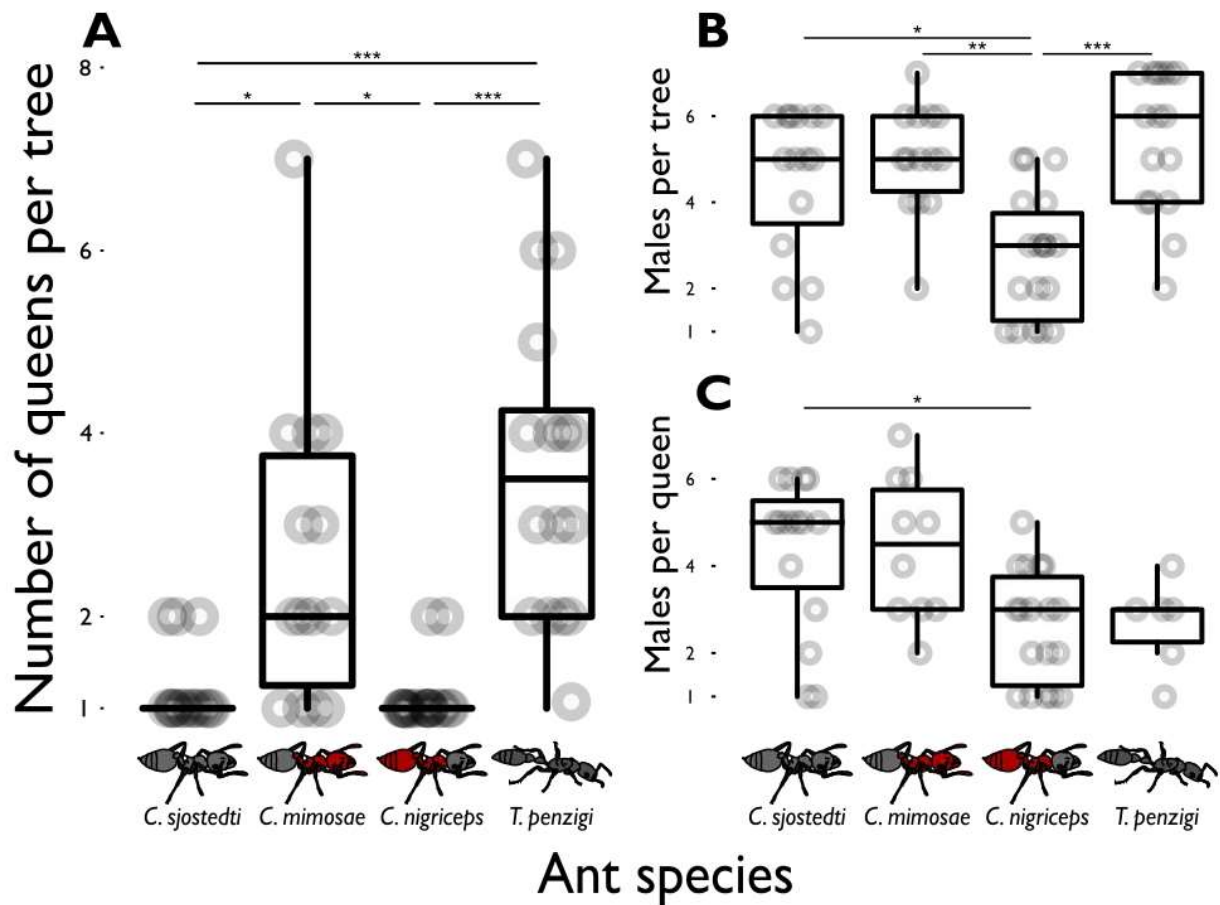


Figure 1.3: Ant species vary in number of queens per tree (polygyny), number of males per tree (polyandry), and number of male mates per queen (mate number). Figure 1.3A shows the number of queen genotypes recovered from each tree; B shows the number of male genotypes recovered from each tree; C shows the number of male genotypes recovered from the offspring of each recovered queen (including only those recovered queens with a minimum of four offspring sampled in our data set). The species are arranged left to right in order from most to least competitively dominant. Boxes show median and inter-quartile ranges. Dots behind each plot show the number of genotypes recovered from each tree (from each queen for C); the values are jittered slightly to help display the data. Lines above the boxplots denote significant differences between species as follows: * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

The queens of different species differed in number of matings (ANOVA test, $p < 0.01$). Queens of *C. sjostedti* and *C. mimosae* averaged 4-5 mates per queen, while *C. nigriceps* and *T. penzigi* were around 2-3 (Table 1.2, Figure 1.3C). Post-hoc tests found significant differences between [*C. sjostedti* and *C. mimosae*] and [*C. nigriceps*], but not

between these and *T. penzigi*, for which the sample size was quite small (Tukey HSD tests, $p < 0.05$).

Maternal Relatedness

For *C. sjostedti* and *C. nigriceps*, only a few of the inferred queens came from the same tree; however, some of these queens were related to each other (Table 1.3). *C. mimosae* had multiple inferred queens per tree. These were also commonly siblings, at a higher rate than that found between queens from different tree (Fisher’s Exact Test, $p < 0.05$). However, *T. penzigi* showed a different pattern: despite having multiple inferred queens per tree, these queens were very rarely siblings, and the rate was not significantly different from the rate of sibship between queens from different trees ($p > 0.2$). The queen we collected from a *T. penzigi* colony was determined by COLONY to be a sister to the workers in that tree.

Table 1.3: Relatedness of inferred queens

Species	Number of trees	Number of inferred queens	SNPs across all inferred queen genotypes	Average SNPs per inferred queen genotype	Proportion of related queens from the same tree	Proportion of related queens from different trees
<i>C. sjostedti</i>	15	17	1199	713	0.50 (1/2)	0.07 (10/134)
<i>C. mimosae</i>	14	35	1182	688	0.27 (12/45)	0.12 (69/550)
<i>C. nigriceps</i>	18	21	1535	932	0.33 (1/3)	0.03 (7/207)
<i>T. penzigi</i>	16	49	915	669	0.03 (2/65)	0.01 (15/1111)

For each proportion, the raw number of related queens and possible comparisons are given in parentheses.

Discussion

We found no evidence that the genetic structure of colonies—in terms of polygyny, polyandry, or within-tree relatedness—underlies competitive ability, either within or between species, in the ant associates of *V. drepanolobium*.

Between species, there was no association between polygyny and competitiveness: Our results do not support the hypothesis that the competitive *C. sjostedti* and *C. mimosae*

are polygynous and the less competitive *C. nigriceps* and *T. penzigi* are monogynous (Stanton *et al* 2002, Palmer 2004). Palmer (2004) noted seven *C. sjostedti* queens in a single colony fragment, but we found little evidence for polygyny in this species. It is possible that the observed queens were not yet mated, but were still in the process of budding off from their natal colony. *C. sjostedti* alates and foundress queens are rarely found, and this species is believed to reproduce primarily via colony budding (Stanton *et al.* 2002).

Our results also show that *T. penzigi* colonies are polygynous. A previous report indicated that this species was monogynous, based on the dissection of colonies (cited in Stanton *et al.* 2002). We have also observed that small trees containing colonies of *T. penzigi* tend to have only one laying queen, based on the collection of the ants inhabiting about 20 small (< 1 meter high) trees of *T. penzigi* in 2016 (Boyle and Pierce, *unpublished data*). It is possible that the multiple maternities of workers observed in a single colony of *T. penzigi* are a relic of the colony founding event, as young trees of *V. drepanolobium* typically have a foundress queen in every domatium, and these foundresses are disproportionately *T. penzigi* (Stanton *et al.* 2002). Possibly the victorious foundress and/or her workers eliminate the competing queens, but tolerate their worker offspring, or continue to raise their brood, similar to slave-making ants. This scenario would result in colonies with a single queen, but a worker population with multiple mothers. This would also be consistent with our finding that a disproportionate number of workers are the offspring of a single queen (about half). Even if *T. penzigi* colonies are socially monogynous, however, it remains the case that polygyny does not underlie the competitive hierarchy, as only the second of the two more competitive species showed evidence of polygyny.

The nature of polygyny also appears to differ among the different species of ants. In the polygynous colonies of *Crematogaster* species multiple queens are often related to each other, possibly due to daughter queens remaining in their natal nests. This was suggested by Rubin *et al.* for *C. mimosae* (2013), and it may also be the case for the uncommonly polygynous *C. sjostedti* and *C. nigriceps*. These *Crematogaster* colonies also appear to have accepted unrelated queens, although this appeared to be less common in these ant species than in *T. penzigi*. Queens in polygynous colonies of *T. penzigi*, in contrast to the other three species, were rarely related to each other. We did directly sequence one *T. penzigi* queen, which we found to be sister to the workers collected with it; this may represent an exception to the general rule, or possibly an artefact of COLONY having difficulty distinguishing parent-offspring from sibling relationships. However, in the vast majority of cases, *T. penzigi* queens were unrelated to each other, which would be expected if the multiple maternity of *T. penzigi* workers is a relic of competing foundresses.

The number of queens and males for each set of genotyped workers is not an exact estimate of the true genetic diversity within that tree. When the number of queens and/or males is high relative to the number of workers sampled per tree, as for *C. mimosae* and *T. penzigi*, then our estimates are likely to be underestimates. Further work with larger worker sample sizes will be necessary to infer the absolute degrees of polygyny and polyandry in these species. However, to evaluate whether different colony structures underlie competitive ability, we only need to know if the competitively dominant species are more polygynous and polyandrous than the competitively subordinate species. Our results provide a strong enough estimate of the relative degree of polygyny (or polyandry) among these four ant species to rule out this possibility.

Furthermore, colonies of *C. sjostedti* and *C. mimosae* are well known to occupy multiple trees (and *C. nigriceps* moderately so; see Palmer *et al.* 2010), and if workers from different trees are not thoroughly mixed, then sampling from a single tree will only capture a subset of the genetic lineages in a colony. However, there is good reason to believe that the workers do mix well, as *C. sjostedti* and *C. mimosae* ants readily move along the ground between trees Young *et al.* (1997b). Moreover, Palmer created artificial barriers to the flow of workers between different trees of the same colony, and found that this reduced competitive ability to the extent that the colonization hierarchy could be reversed (2004). Both observations suggest that workers show sufficient movement among trees within a single colony such that sampling from single trees is adequate to capture the diversity of an entire polydomous colony.

Our results also do not support the hypothesis that polygyny plays a role in intra-specific contests. Across the four species, more competitive ants occupy larger, more valuable trees; however, we did not find an association between queen number and tree size, either within or across ant species. However, since single ant colonies can occupy multiple trees, it is still possible that more polygynous colonies spread across a greater number of trees of the same size class than their less polygynous conspecifics.

Our work demonstrates the utility of RADseq data in determining family structure, especially in non-model systems. RADseq has previously been used to identify parent-offspring relationships where the pool of possible parents is known and genotyped (Kess *et al.* 2016), and to search for related individuals within a population (Hellmann *et al.* 2016, Kjeldsen *et al.* 2016). To our knowledge, our study is the first to use RADseq data to determine the number of parents giving rise to a set of (potentially sibling) offspring, and

the first to use RADseq data to determine any kind of kinship relationship in a social insect. This information is important in social insects, for which polygyny and polyandry are both of particular relevance in the evolution and maintenance of eusociality (Boomsma *et al.* 2009). This analysis can also answer other questions, such as quantifying extra-pair paternity. Although RADseq produces single-nucleotide polymorphisms, which are individually less informative than microsatellite markers, it produces a great many of them, allowing relationships to be resolved with the same or greater specificity as with microsatellites. For instance, Weinman *et al.* found that 102 SNPs had power comparable to 15 microsatellites for identifying parentage in a cooperatively breeding bird, and in fact the SNPs performed better when parents were related (2014), as appears to be the case among some of the ant associates of *V. drepanolobium*. Furthermore, RADseq is especially useful in the many systems for which microsatellite markers have not yet been identified, as no pre-existing genomic information is needed in order to recover RADseq markers (unlike microsatellites, for which loci must be identified and primers designed beforehand, a potentially expensive and time-consuming process). In our case, RADseq markers allowed us to conclude that polygyny does not drive competition in the ant associates of *V. drepanolobium*, and we should investigate other factors that could promote differences in competitive ability between species, such as distribution of colony biomass among different castes and foraging differences (Palmer 2004), or differences in relative rates of development, in diet, and/or abilities to form cohesive colonies across multiple trees.

Interactions among *V. drepanolobium*'s ant associates are not uniform across East Africa. For example, Hocking noted that the proportion of trees occupied by particular ants varied widely from site to site, and he rarely found *C. sjostedti* in his sites in the southern

part of the *V. drepanolobium* range (1970). This ant, however, is the competitively dominant species at the Mpala Research Centre where most of the research on the system has taken place. Similarly, competitively dominant ants in some habitats may be subordinate in others: Palmer found one site with a different soil profile where the competitive hierarchy was partially reversed, with *C. nigriceps* dominant over *C. mimosae* (2004). To understand how and why the competitive hierarchy changes across the range of *V. drepanolobium*, it will be necessary to understand more about which factors promote competitive ability between the different ant species.

Chapter 2: Differences in resource allocation underlie a competition-colonization hierarchy in *V. drepanolobium* acacia ants.

Abstract

In the *Vachellia drepanolobium* ant-plant mutualism, several ant species compete for nesting space in the canopy of *V. drepanolobium* trees, providing defense against herbivory in return. Previous research has described a colonization-competition hierarchy which allows these species to coexist: the species which are the best at founding colonies on empty trees are the worst at defending them against neighbors, and vice versa. However, the mechanistic basis for these differences in colonizing and competing abilities is not known. Here we give evidence that the colonization-competition hierarchy arises from a colony-level tradeoff between survival and competition on the one hand (mediated by worker ants) and reproduction on the other (by the reproductive caste). Finally, we describe the reciprocal impacts of the partners on the structure of the other: how the species of ant occupant affect the growth of the tree, and how the structure of the tree affect the arrangement of the ant colony in its canopy. We find that the ant occupants have a greater effect on the short-term growth of the tree than on its long-term woody architecture, and that the ant species organize their colonies based on particular architectural features of the *V. drepanolobium* canopy.

Introduction

The problem of species coexistence has been an important one in ecology for decades (Hutchinson 1961, Huston 1979). Many species appear to compete with each other for limiting resources, yet still coexist, in contradiction to Gause's Law that such coexistence should be impossible (Gause 1934, Hardin 1960). One solution to this problem is the colonization-competition hierarchy. In such a hierarchy, a number of coexisting species compete for a limiting resource, but the species vary among themselves along two axes: different species are better or worse at locating unused resource patches; and different species are better or worse at displacing their competitors from occupied patches. If the species that are better at displacing other species from occupied patches (good "competitors") are poorer at locating unused patches (poor "colonizers"), and vice versa, then two or more species can coexist, assuming some turnover in the resource for which they compete, and that the competitor species can outcompete adults of the colonizing species (Tilman 1994, Yu and Wilson 2001).

Such dynamics have been proposed as a coexistence mechanism in a number of systems (Tilman 1994, Mordecai *et al.* 2016), including defensive mutualisms between ants and plants, in which multiple ant species compete for nesting space in trees, which they in turn defend from herbivores (Janzen 1975). In the *Vachellia drepanolobium* (formerly *Acacia drepanolobium*) system, four species of ant compete for nesting space in hollow swollen-thorn domatia: *Crematogaster mimosae*, *C. nigriceps*, *C. sjostedti*, and *Tetraponera penzigi*. At one well-studied site in central Kenya, these ants show consistent differences in their ability to displace other colonies from neighboring trees, in nature as well as in experimentally staged contests (Palmer *et al.* 2000). These ants have a hierarchy from *C.*

sjostedti as the most competitive, then *C. mimosae*, *C. nigriceps*, and *T. penzigi*. Evidence from contests between founding queens and experiments on incipient colonies suggests a colonization hierarchy in the opposite direction, with *T. penzigi* being the best competitor, then *C. nigriceps*, *C. mimosae* and *C. sjostedti* (Stanton *et al.* 2002, Palmer *et al.* 2013).

The colonization-competition hierarchy is especially important in the *V. drepanolobium* system, because the different ant species have a variety of different behaviors that range from mutualistic to parasitic, even within the same ant species. *C. nigriceps*, for instance, rigorously defends its host against vertebrate herbivores, but also prunes foliage, potentially castrating the tree by preventing flowering (Young *et al.* 1997b, Martins 2010). The dynamics of a colonization-competition hierarchy ensure that most trees are likely to experience several transitions in their ant occupant over the course of their lifetimes, meaning that they experience a variety of different behaviors from the mutualists (Palmer *et al.* 2010). Indeed, Palmer *et al.* even argued that this variation provided a net benefit to the tree, as the different parasitic behaviors of the different ants are experienced, on average, during the phase in a tree's ontogeny when they exact only small costs relative to the benefits those species provide (Palmer *et al.* 2010).

While much work has been done describing these differences in competition and colonization ability, and on working out their consequences for the ant-plant mutualism, we know very little about what causes the ants to vary in these abilities. A number of possibilities have been proposed. One such proposal was that more competitive species have more queens, allowing them to lay more eggs, which develop into larger, more competitive worker populations (Palmer 2004; Rubin *et al.* 2013). While it is true that competitive species are more competitive because they have more workers (Palmer 2004),

this is not because these species have more queens (Chapter 1). Another proposal is that the ants experience a common tradeoff between investing in competitive ability or in colonizing ability (Palmer 2004). In this chapter, I test this hypothesis by examining how ant colonies invest their resources.

The eusocial nature of ants makes them an excellent system in which to study this question, because the different tasks of the colony are performed by morphologically distinct castes. In this case, competition is mediated through sterile workers (Palmer 2004), while colonization is performed by a winged reproductive caste; therefore, by measuring investment in these castes, we can investigate whether different species invest differently in colonization and competition. While we are not able to measure directly the energy costs of maintaining individuals of different castes, by examining how much biomass is devoted to these castes, we can measure an important part of a colony's past investment in producing individuals of these castes.

Workers, however, do more than compete with other colonies; in addition, mutualistic and parasitic behaviors toward the tree host are also performed by workers, whether these behaviors are foliage defense, castration, or tending scale insects, to give a few examples (Young *et al.* 1997b). Therefore, investment in workers, as well as their distribution on the tree, could have important consequences for the ant-tree mutualism. To investigate this, we recorded not only how much biomass different ant colonies invested into worker and reproductive castes, we also recorded where on the tree these individuals were found, and in relation to what tree structures (e.g., leaves, nectaries, domatia). This allows us to study the reciprocal impacts of the ant colony and the tree: i.e., how does the

ant colony affect the growth of the tree? And how does the structure of the tree affect the distribution of the ant colony?

Methods

Collections were made at from May-July 2014 at Suyian ranch in Laikipia, Kenya (0.507° N, 36.694° E). We measured tree architecture of selected trees and collected colony fragments of their inhabiting ants. A total of 223 focal trees were chosen, representing all four ant inhabitants and a range of sizes from saplings to 2 meters height or more, as shown in Table 2.1 and Figure 2.1. Because *C. sjostedti* colonies do not inhabit domatia, but instead nest in excavations in the tree's trunk or soil, we focused our efforts on trees inhabited by one of the three domatia-dwelling ant species.

We determined the size of the focal tree by measuring both height and trunk diameter. The height of the tree was measured as the distance above ground of the highest point in the canopy. The diameter of the trunk was recorded at 0.5 m above the ground. If a tree had multiple stems, these were recorded separately.

The sizes of the five nearest *V. drepanolobium* trees within 5 m of the focal tree were also measured. We recorded which species of ant occupied each tree, as well as its distance to and bearing from the focal tree. For these purpose, if the canopies of multiple trees were entangled, all such entangled trees were considered a single unit, because a single ant colony can move freely among the different canopies without having to go along the ground. The height of the tallest of the entangled trees was recorded, along with the trunk diameter of each such tree.

A branch tip was selected as follows: a random compass heading was chosen. Among the branch tips within a few degrees of that heading from the trunk, a single tip was

chosen haphazardly from those tips that showed recent growth and/or mature domatia. If no branch tips were within a few degrees of the chosen heading, then we continued clockwise around the tree until an appropriate branch tip was found. This tip was marked with flagging tape. The part of the tree that would be surveyed was all the branch which connected the selected branch tip to the base of the trunk (Figure 2.2).

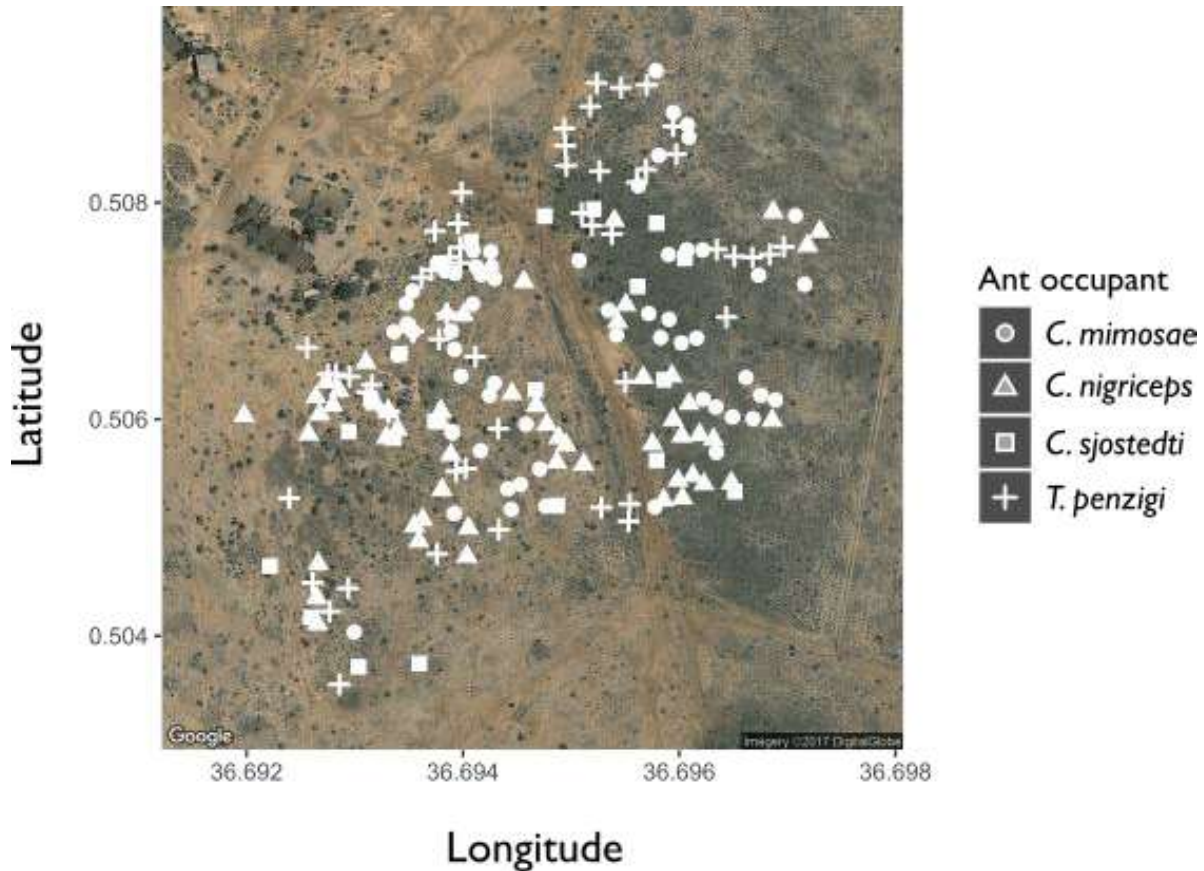


Figure 2.1: Distribution of the *V. drepanolobium* trees sampled for this experiment. Each point represents an individual tree; the shape represents which species of ant occupied that tree. GPS coordinates were not recorded for 25 trees (not shown). The figure was produced using imagery from DigitalGlobe via Google Maps, as implemented in the ggmap R package (Kahle and Wickham 2013).

Table 2.1: Sampling effort was spread across several size classes

Ant species	Trees <1 m	1-1.5 m	Trees > 1.5 m
<i>C. mimosae</i>	33	24	15
<i>C. nigriceps</i>	24	24	13
<i>T. penzigi</i>	35	22	14
<i>C. sjostedti</i>	11	5	5

The following morning at dawn, when cool temperatures made the ants mostly inactive, we euthanized the ants in the chosen part of the tree by injecting each domatium with ethanol. We then recorded the characteristics of the selected branch as shown in Figure

2.3. The branch was divided into segments. Each segment was

separated from the next by the branching-off of a stem at least 5 mm or more in diameter.

For each segment, we recorded length, diameter at the proximal (i.e., nearer the trunk) and distal (i.e., nearer the branch tip) ends, the diameter of the stem branching off from its

proximal end, and the number of branching-off stems < 5 mm in diameter. We also

recorded the number of leaves coming from that segment, and their condition, which we

quantified by estimating what proportion of the original area of those leaves was still green and had not suffered herbivory. We also counted the number of living extrafloral nectaries

on those leaves. We considered a red or green nectary to be living; scars where extrafloral nectaries had been destroyed by *T. penzigi* (or another organism) were not counted. We

recorded the number of flowers and seed pods growing from that segment. Finally, we

recorded the number of domatia on each segment and where they were located along the length of that segment.



Figure 2.2: A schematic of the sampling scheme. A branch tip (circled in red) was selected. The branches connecting that tip to the root were measured (shown in black), as were the domatia attached to those branches (in dark brown).

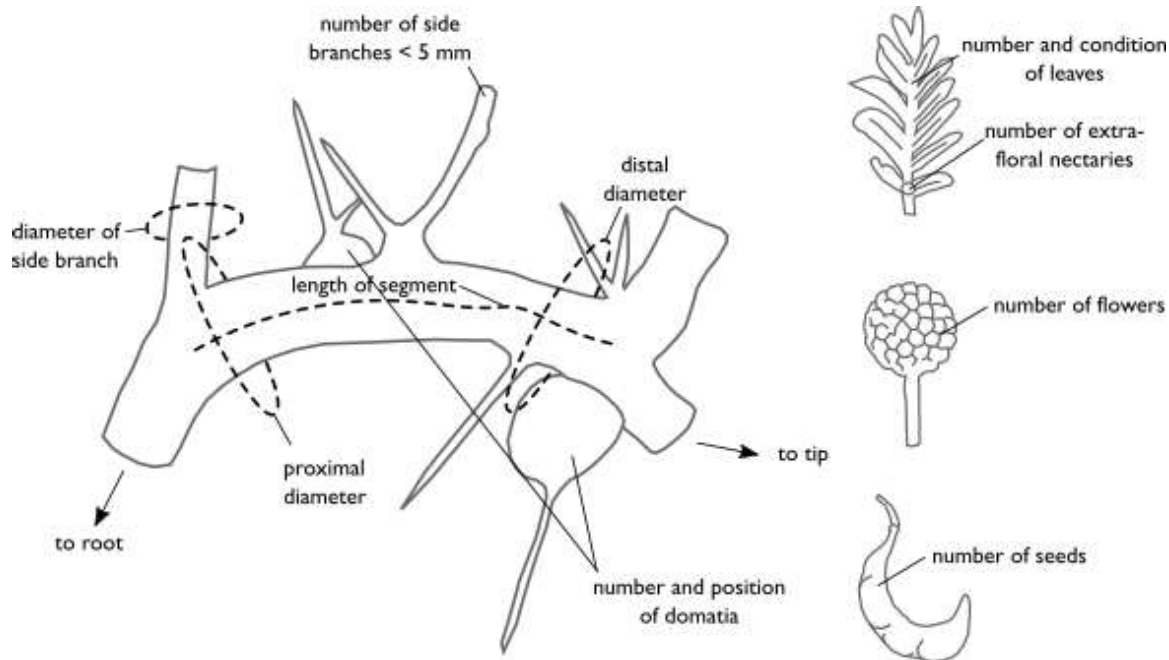


Figure 2.3: Characteristics measured for each segment of the focal branches.

In some cases, a domatium had decayed sufficiently that it crumbled at the touch, or had already been destroyed. In these cases, we recorded the presence and location of the domatium (destroyed domatia leave a scar on the branch), but did not record any additional information about them.

We removed the intact domatia and allowed the ethanol to evaporate before storing them in drierite to desiccate the ants inside. We also recorded the size of each domatium: each domatium has two thorns arising from it, and we recorded the diameter of the domatium at two places relative to the positions of the thorns as shown in Figure 2.4. We also recorded the approximate age of the domatium, assigning it to one of five categories, from youngest to oldest: 1 = green, with flexible walls, 2 = red-brown, with firm walls,

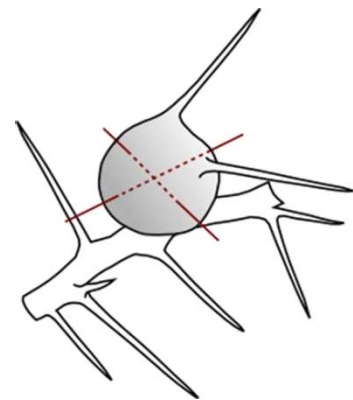


Figure 2.4: domatium diameter was measured along two axes, parallel and perpendicular to the domatium's two thorns, as shown in red.

3 = dark brown or black, fully lignified, 4 = gray, 5 = gray, with thin and deteriorating walls. Finally, we opened each domatium and recorded its contents. Contents were divided into several categories: adult worker ants, adult males, adult female alates, adult female queens, immature workers, immature reproductives (we could not distinguish small, early-instar reproductive larvae from worker larvae; larvae < 1.5-2 mm were considered workers). In the case of immature individual ants, the median stage (egg, larva, pupa) was estimated, along with the average length of larvae. We recorded the dry biomass of each category separately. When the biomass of a single category was less than the minimum mass recordable by our scale (about 4-5 mg), we simply recorded the number of individuals, and used the average biomasses for each caste and developmental stage (see below) to calculate their mass, capping this value at 6 mg. If any non-antinquilines were inhabiting the domatia, their presence and dry biomass were also recorded.

Individual-level biomass

In order to convert between number of individuals of each caste at various developmental stages and their mass, we weighed a number of exemplars of each caste and stage for each species. For each group (workers, queens, alate females, alate males, reproductive brood, worker brood) for each species, we randomly chose 10 domatia that contained that group (or as many domatia as possible, if there were fewer than 10 across all trees). In the case of worker and reproductive brood, we had further categorized each set of brood as having an average stage, from eggs, to 1.0 mm larvae, 1.5 mm larvae, etc, to pupae. We measured the worker or reproductive brood for up to 10 domatia with each average stage of brood.

For each selected domatium, we counted the number of individuals of the chosen caste and stage, and weighed them to the nearest 0.01 mg in order to find the average mass

of an individual in that domatium. We then averaged this number across all 10 domatia selected for that stage and species.

For workers and female and male alates, we used R 3.2.3 (R Core Team 2015) to perform analyses of variance (ANOVA) to compare the mean masses of each caste across species, and the Tukey HSD test to compare between pairs of species.

Colony-level biomass

We used an analysis of covariance (ANCOVA) to test whether tree height (as a covariate) and ant species affect the total ant biomass of the colony fragment collected from each tree. We found no significant main effect of height, but a significant interaction between height and ant species. We then divided the data set into trees less than or equal to the median tree height in the data set, and trees taller than the median, performing an ANOVA test followed by the Tukey HSD test to compare the colony biomasses of different species on shorter and taller trees. For all tests, ant biomass data was cube-root transformed to improve the normality of the distribution.

We also tested whether these factors affected the presence or absence of inquilines on the tree using logistic regression to model how tree height and ant occupancy effected the presence or absence of inquilines. Using AIC, we chose among models including only height as a predictor variable, only ant species, both height and ant, and both height, ant, and an interaction between them.

Caste investment

We then compared how the different ant species distribute their biomass between workers and reproductives, and between adults and brood. Since *C. sjostedti* does not nest in the domatia of *V. drepanolobium*, we excluded this species from these analyses.

We used an ANCOVA, with tree height as a covariate, to test for differences between ant species in the proportion of the colony biomass devoted to workers and worker brood, comparing species means with Tukey HSD tests as implemented in the multcomp package (Hothorn *et al.* 2008).

We found no reproductives in a large number of the colonies we sampled, presumably in part because we were sampling only a fraction of the domatia on a tree, and not all the domatia. Because a large number of data points were zero, parametric tests were inappropriate. Instead, we analyzed whether ant occupant or tree height had an effect on the probability that a given branch had alates present (either male or female). We chose the lowest AIC-score model among models including only height as a predictor variable, only ant species, both height and ant, and both height, ant, and an interaction between them. We repeated this analysis for the presence or absence of alate brood. We also performed an ANCOVA on the proportion of alates and alate brood in the colony, taking into account only those tree on which alates and alate brood, respectively, had been found.

Ants' effect on trees: woody architecture

We used an ANCOVA to determine whether the woody architecture of the focal trees varied with ant species or tree size. The following characteristics were calculated for each branch across all of its segments: total number of intact domatia, average number of intact domatia per cm, average domatium diameter, average domatium stage (both considering only intact domatia, and also including destroyed domatia, which were considered as stage 5 domatia), branching rate (i.e., the number of stems of any diameter branching off from the segments of the focal branch over its length), and average branch diameter. These values were

transformed where necessary to make their distributions roughly normal, then fit to a model including tree height and ant occupant as independent variables.

We did not find evidence of an interaction between ant occupant and tree height for any of the ANCOVAs, so we report here the results of a model including only the main effects of ant and tree height.

Since trees experience turnover in ant occupant over their lifespans (Palmer *et al.* 2010), measurements across a whole tree might include the effects of different species that occupied that tree in the past. To control for this and measure primarily the effect of the current ant occupants, we repeated the above analyses, but used custom perl scripts to prune the data to include measurements only from the terminal 50 cm of each tree. Using Okello *et al.*'s estimate that *V. drepanolobium* increases in height at a rate of 28.6 cm/yr (2001), this 50 cm stretch represents roughly two years of tree growth, which is less than the approximately 6.5 years between changes in ant occupancy suggested by the data in Palmer *et al.* (2000) for colonies monitored in 1998 at a field site neighboring our own.

Ants' effects on trees: new growth

We investigated whether tree size and ant identity affected a number of traits having to do with new growth. We calculated the following characteristics across all the segments of each branch: total number of leaves, extra-floral nectaries, flowers, and seeds. We also estimated the average leaf condition over all leaves on the branch. To get a rough estimate of photosynthetic area, we multiplied the number of leaves by the average leaf condition (we assume that the leaves of trees occupied by each species of ant are of roughly the same size). Finally, leaves may have more than one (or no) extrafloral nectaries, so we also calculated the average number of extra-floral nectaries. For leaves and extrafloral

nectaries, we used the statistical methods described above to compare these values among trees of different sizes and ant occupants. For seeds and flowers, most branches had no seeds or flowers. We therefore combined seeds and flowers into a single category, presence or absence of any reproductive organs, and used logistic regression to model how tree height and ant occupancy effected the presence or absence of flowers and seeds. Using AIC, we chose among models including only height as a predictor variable, only ant species, both height and ant, and both height, ant, and an interaction between them.

Finally, as with woody architecture, we repeated the above tests, including only the terminal 50 cm of each branch.

Trees' effects on ants: colony distribution

We then wanted to determine which plant characteristics affect the distribution of ants across tree branches. The plant characteristics of interest were correlated with each other, so in order to visualize the structure of this correlation, we performed a hierarchical clustering of the variables using the *ClustOfVar* package in R (Chavent *et al.* 2012) using the following variables across all branch segments with at least 1 domatium: length of branch segment, proximal diameter of branch segment, distal diameter of branch segment, distance of the center of the segment from the branch tip, distance of the center of the segment from the root, leaf condition on that segment, number of domatia on that segment, and the total volume of the domatia on that segment. The volume of each domatium was calculated as $4/3\pi(d/2)^2$, where d is the average of the two diameters recorded for that domatium. Variables were transformed if necessary to make the distributions roughly normal, and then rescaled so that the distribution had a mean of 0 and standard deviation of 1. In addition, we included as categorical variables the presence or absence of the

following: leaves, extrafloral nectaries, flowers, seeds, and off-shooting branches less than 5 mm in diameter.

Due to the intercorrelated nature of the plant variables, we dissected their effect on ant colony structure using Redundancy Analysis (RDA), implemented in the *vegan* package in R (Oksanen *et al.* 2016). We performed a separate analysis for each ant species. The constraining matrix included the plant characteristics described above, except that the transformations were done separately for each of the four data sets for *C. sjostedti*, *C. mimosae*, *C. nigriceps*, and *T. penzigi*.

The community matrix included the following measurements of the ant colony on each branch segment, Hellinger transformed: total mass of workers found in the domatia on that branch segment, and the same for male alates, female alates, worker brood, alate brood, andinquilines. In both the constraining (plant characteristics) and community (ant characteristics) matrices, only those branch segments containing at least one intact domatium were considered.

In order to determine which of the plant characteristics in the constraining matrix were relevant to the ant composition described by the community matrix, we performed forwards model selection using *vegan*'s `ordistep()` function. In all cases, possible RDA models were conditioned on the tree identity, to remove the risk of pseudoreplication due to having multiple branch segments from the same tree in the data set.

Queen distribution

The most important individual in an ant colony is the queen; however, our sampling method recovered only a handful of queens, so it is difficult to draw any conclusions about where the queen is on the tree. In order to determine the location of queens, we

exhaustively sampled 63 *V. drepanolobium* trees in July of 2016, representing 22 colonies of *C. mimosae* (average tree height: 102 cm), 19 colonies of *C. nigriceps* (average tree height: 97 cm), and 22 colonies of *T. penzigi* (average tree height: 92 cm). We opened every domatium on the focal trees in order to be sure that we had located all the queens on that tree. When we found a queen, we recorded the diameter of the domatium, and the distance between that domatium and the ground, measured along the branches. We compared the three domatium-dwelling ants to each other using ANCOVA and Tukey HSD tests, using total tree height as a covariate. If we found multiple queens on a single tree, we averaged the domatium diameter and distance from the root across all queens for that tree.

Results

Individual-level biomass

The average masses for castes of the different species is shown in Table 2.2. The tree-defending species, *C. mimosae* and *C. nigriceps*, had larger workers than *C. sjostedti* and *T. penzigi*. For both male and female reproductives, average mass following the competition-colonization hierarchy, with better-colonizing species producing smaller individuals (Figure 2.5).

Colony-level biomass

An ANCOVA showed no significant main effect of tree height on ant colony biomass ($p = 0.7$), but a significant effect of ant species ($p < 0.0001$) and a significant interaction between ant species and tree height ($p = 0.04$). For the competitively dominant ant species *C. sjostedti* and *C. mimosae*, the mass of ants in a single branch increased with tree size, while for the subordinate *C. nigriceps* and *T. penzigi*, the mass of ants in a single branch decreased with tree size (Figure 2.6). This does not mean that the total colony biomass

across a whole tree decreases with tree height for *C. nigriceps* and *T. penzigi*. Since larger trees have disproportionately more branches, there is a positive relationship between tree height and total colony biomass across all branches for all four ants (Chapter 3).

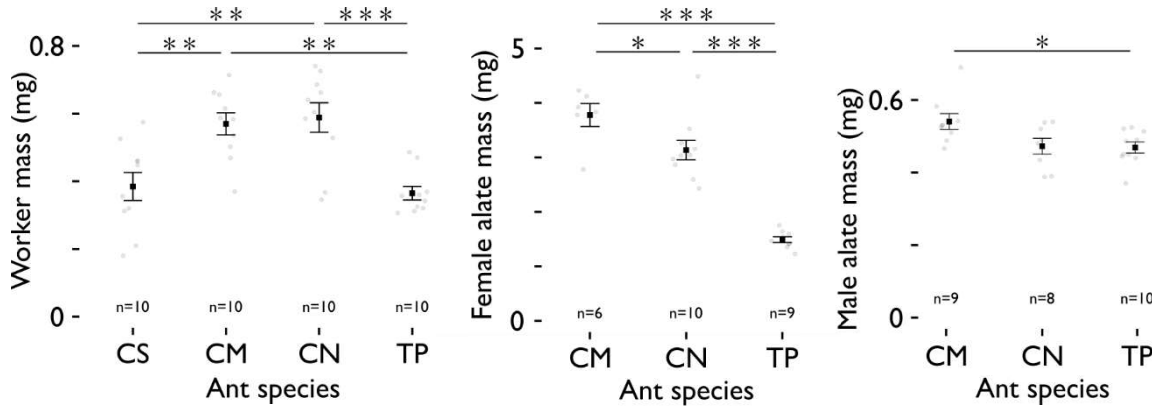


Figure 2.5: Ants of different species have different sizes. *C. mimosae* and *C. nigriceps*, which defend their host trees from herbivores, have larger workers (left) than the non-defending *C. sjostedti* and *T. penzigi*. In the case of female and male alates (center and right-hand plots, respectively), masses of individual reproductives follow the competitive hierarchy. Dot and whisker plots show the mean plus or minus standard error. Points show the average mass of an individual in a single domatium. Stars indicate significant differences between species, * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

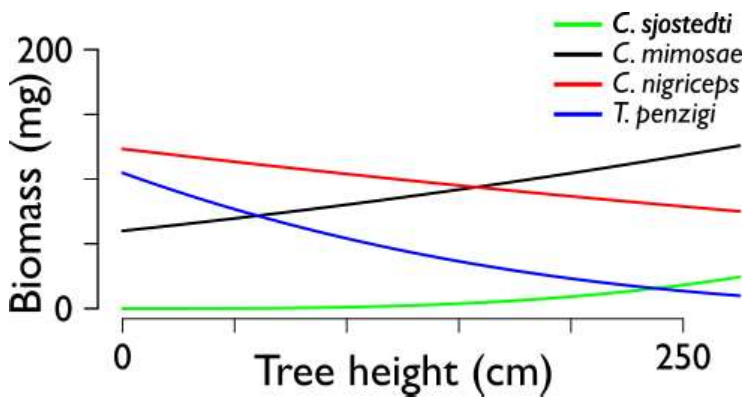


Figure 2.6: total ant biomass on a branch is affected by an interaction between ant species and tree height. For the competitively dominant *C. sjostedti* and *C. mimosae*, biomass increases with tree size, whereas it decreases with tree size for the subordinate *C. nigriceps* and *T. penzigi*. Biomass was cube-root transformed and then fit to a straight line, which was then back-transformed to produce the cubic functions shown here.

Following on this, we looked at shorter and taller trees separately, using a Bonferroni-corrected critical $p = 0.025$. For shorter trees, we found that *C. sjostedti*, which does not commonly occupy domatia, had less ant biomass in the domatia than the other species. For taller trees, we found that *T. penzigi* had relatively smaller colonies,

significantly smaller than *C. nigriceps*, and not significantly different from the *C. sjostedti* biomasses in the domatia (Figure 2.7).

Table 2.2: Average individual mass for castes and developmental stages across ant species

Caste	Average stage	<i>C. sjostedti</i>	<i>C. mimosae</i>	<i>C. nigriceps</i>	<i>T. penzigi</i>
Worker		0.38 ±0.04	0.57 ±0.03	0.59 ±0.04	0.36 ±0.02
Queen		n/a	4.77 ±0.28	2.95 ±0.77	3.08 ±0.76
Alate female		n/a	3.78 ±0.21	3.13 ±0.18	1.49 ±0.05
Alate male		n/a	0.54 ±0.02	0.47 ±0.02	0.47 ±0.02
Worker brood	Egg	n/a	0.03 ±0.01	0.03 ±0.01	0.05 ±0.03
	0.5 mm larva	n/a	0.04 ±0.01	0.03 ±0.01	n/a
	1.0 mm	n/a	0.13 ±0.04	0.07 ±0.01	0.13 ±0.02
	1.5 mm	n/a	0.28 ±0.04	0.26 ±0.04	0.14 ±0.02
	2.0 mm	n/a	0.38 ±0.03	0.28 ±0.05	0.28 ±0.03
	2.5 mm	n/a	0.67 ±0.06	0.41 ±0.06	0.27 ±0.02
	3.0 mm	n/a	n/a	²	0.34 ±0.05
	Pupa	0.14 ¹	0.59 ±0.04	0.32 ±0.04	0.49 ±0.13
Reproductive brood	1.5 mm larva	n/a	n/a	2.54 ±0.19	n/a
	2.5 mm	n/a	n/a	n/a	1.04 ±0.58
	3.0 mm	n/a	n/a	2.97 ±0.12	1.37 ±0.54
	3.5 mm	n/a	n/a	2.88 ±0.11	1.95 ¹
	4.0 mm	n/a	n/a	1.91 ±0.21	1.60 ±0.17
	4.5 mm	n/a	n/a	n/a	0.63 ¹
	Pupa	n/a	0.55 ±0.04	1.42 ±0.31	0.88 ±0.13

All masses are given as mean ± standard error.

¹ Only one domatium was found with this stage, so no standard error is given.

² We estimated *C. nigriceps* 3.0 mm worker brood mass as 0.36 mg by averaging 2.5 mm and pupa masses for that species. We estimated *C. mimosae* 2.0 mm alate brood mass as 0.38 mg, using the value for workers of the same size.

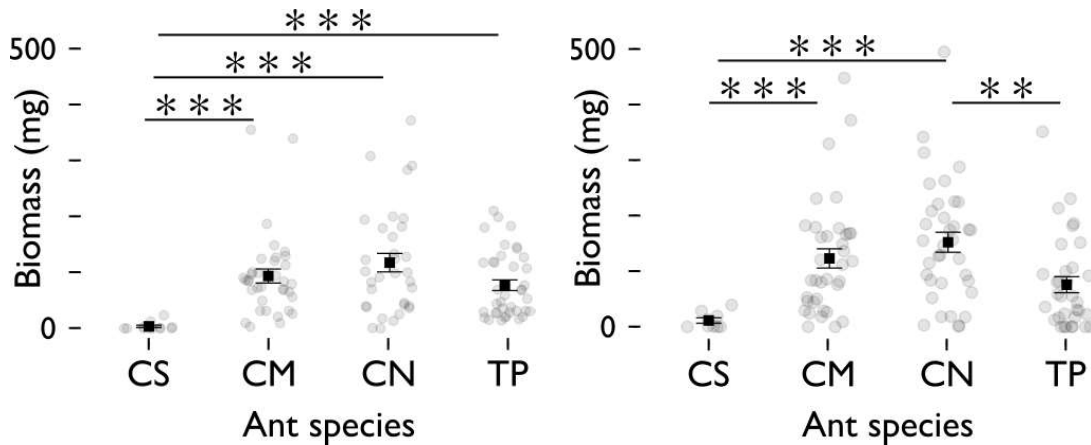


Figure 2.7: relative biomass among the different ant species changes for short (left) and tall (right) trees. For trees less than or equal to the median height in our data set, *C. sjostedti* colony fragments had much lower biomass than those of the other species, with no significant differences among the three. In larger trees, *T. penzigi* colonies had lower biomasses, not different from *C. sjostedti*, and lower than *C. nigriceps*.

For inquilines, the model with the lowest AIC showed a positive effect of height on the likelihood of a tree harboring inquilines, but no effect of ant species.

Caste investment

Our analyses showed that both tree size and ant species (but not an interaction between the two) affect how biomass is distributed among castes and developmental stages (summarized in Table 2.3, Figure 2.8). Across all species, taller trees have a greater proportion of adult workers, a lower proportion of worker larvae, and are more likely to harbor alates and alate brood. Within species, investment in workers followed the competitive hierarchy of *C. mimosae* > *C. nigriceps* > *T. penzigi*, and investment in alates and alate brood followed the colonization hierarchy, with *T. penzigi* > *C. nigriceps* > *C. mimosae*, although not all comparisons are statistically significantly different (see Figure 2.8). *T. penzigi* also has more of its colony biomass devoted to worker brood than does *C. mimosae* or *C. nigriceps*. This may be because *T. penzigi* are more rapidly growing, because adult *T. penzigi* workers die more quickly than *Crematogaster* workers, or because small reproductive brood was indistinguishable from small worker brood, and included in the

Table 2.3: Colony composition varies among ant species

Dependent variable	Transformed	Effect of tree height	p-value	Effect of ant	p-value
Workers as proportion of colony biomass	arcsine	positive	0.04	yes	<1e-12
Worker brood as proportion of colony biomass	square-root	negative	<1e-4	yes	<1e-10
Probability of presence of alates		positive		yes	
Alates as proportion of colony biomass*	cube-root	no	0.9	yes	0.02
Probability of presence of alate brood		positive		yes	
Alate brood as proportion of colony biomass*	fourth root	positive	<0.03	no	<1e-4

For presence/absence tests, we analyzed the results using logistic regression; see methods for details.

* These tests considered only those trees with any alates or alate brood, respectively.

latter category. In the case of alates and alate brood, *T. penzigi* colonies were more likely to produce alates than *C. mimosae*. We found no difference between the likelihood of producing alates and alate brood between colonies of *C. mimosae* and *C. nigriceps*, but among those colonies that produced alates, *C. nigriceps* colonies had a disproportionate mass of alates compared to those of *C. mimosae* ($p = 0.02$). Comparing only colonies which

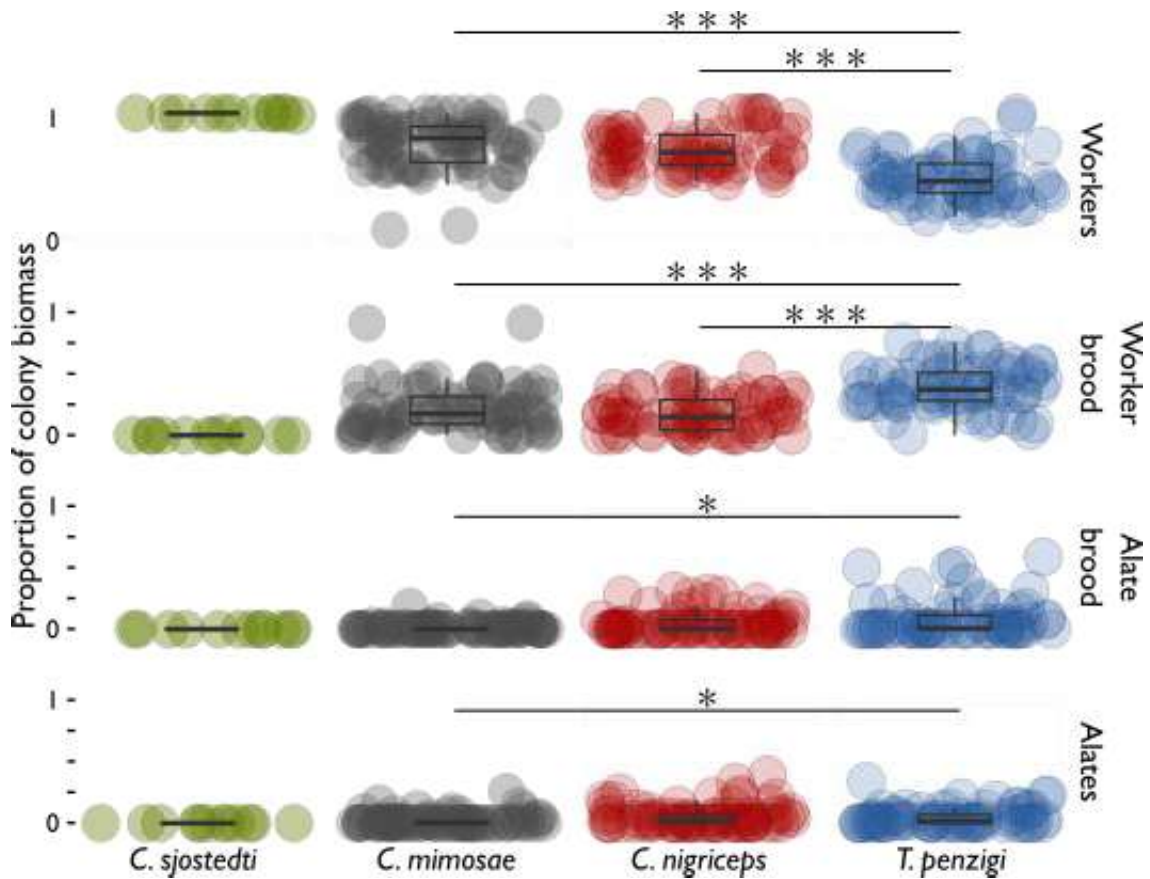


Figure 2.8: Differences in colonies' investment in different castes and developmental stages reflect the competition-colonization hierarchy. Each column shows the distribution of that species' biomass among (top to bottom) adult workers, worker brood, alate brood, and alates. Box plots show the median and interquartile ranges for each distribution; the underlying points show raw values from individual trees. Since *C. sjostedti* brood and reproductives are not found in domatia, this species was not considered in statistical tests. Investment in workers follows the competitive hierarchy. Lines with stars separate statistically significantly different proportions of biomass for workers and worker brood. For alates and alate brood, these lines indicate that *T. penzigi* colonies are more likely than *C. mimosae* colonies to produce these categories.

produced alate brood, *C. mimosae* colonies produced less alate brood than either *C. nigriceps* ($p < 0.01$) or *T. penzigi* ($p < 0.0001$).

Ants' effects on trees: woody architecture

The woody architecture of a *V. drepanolobium* tree changes considerably with size and age. Larger trees have larger and later-stage domatia than smaller trees, and they also have more domatia overall, but fewer per unit length of branch. The latter difference is likely driven by domatia in older parts of the tree, as there was no effect of height on number of domatia per unit length in the terminal 50 cm of the branches. Naturally, larger trees had thicker branches, but they did not vary in branching rate across the whole tree. However, in the terminal 50 cm, larger trees did have a lower branching rate, but there was no effect of height on branch diameter.

Ant occupant is correlated with the number and stage of domatia, both across the whole tree and in the terminal 50 cm, with *C. nigriceps*-occupied trees having the most, earliest-stage domatia. We also found some evidence that, in the terminal 50 cm of the tree, *T. penzigi*-occupied trees had thinner branches than *C. mimosae*-occupied trees. We found no evidence that trees with different ant occupants had different branching rates, or different size domatia. The results of this section are summarized in Table 2.4 and Figure 2.9.

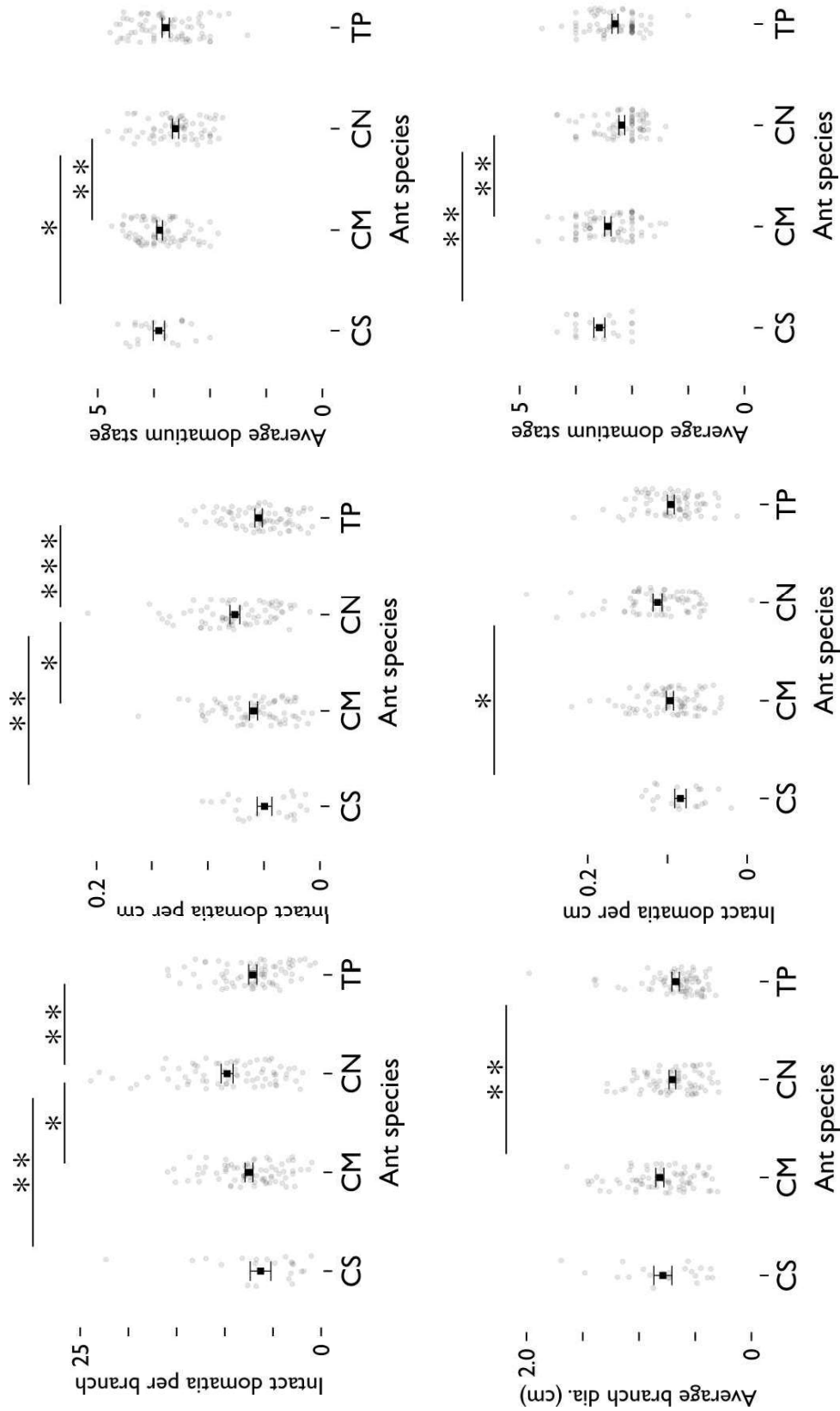


Figure 2.9: Trees occupied by different ant species have different woody architectures, both throughout the entire tree (top row), and in the terminal 50 cm of the tree (bottom row). Squares and bars show mean plus and minus standard error for each species. Bars signify significant differences between trees occupied by different species at * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

Table 2.4: Effects of tree height and ant occupant on *V. drepanolobium* woody architecture.

Dependent variable	Part of tree considered	Transformed	Effect of tree height	p-value	Effect of ant	p-value
Number of intact domatia	whole branch	square-root	positive	<1e-6	yes	<1e-3
Intact domatia per cm	whole branch	square-root	negative	<1e-15	yes	<0.01
	terminal	square-root	none	0.07	yes	0.02
Average domatium diameter	whole branch	no	positive	<1e-15	no	0.1
	terminal	no	positive	<1e-15	no	0.2
Average domatium stage (all domatia)	whole branch	no	positive	<1e-7	yes	<0.01
	terminal	no	none	0.5	yes	<1e-3
Average domatia stage (intact domatia only)	whole branch	no	positive	<1e-15	no	0.08
	terminal	no	positive	<1e-3	yes	<0.01
Branching rate	whole branch	square-root	none	0.2	no	0.1
	terminal	square-root	negative	<1e-3	no	0.08
Average branch diameter	whole branch	square-root	positive	<1e-15	no	0.3
	terminal	fourth root	none	0.4	yes	0.02

Ants' effects on trees: new growth

Ant occupant had a strong effect on leaves: trees occupied by *C. mimosae* put on more leaves than other trees, while trees occupied by *C. nigriceps* had leaves in better condition. When these measures were combined into the single metric of leaf area, *C. mimosae* and *C. nigriceps* (the ant species whose workers defend foliage) had a higher leaf area than *C. sjostedti* and *T. penzigi* (whose workers do not defend foliage). Leaves were also affected by tree height: taller (presumably older) trees had leaves in poorer condition. Taller trees also had fewer leaves per unit length, but overall had more total leaves, which in turn gave taller trees a larger total leaf area.

In the case of extrafloral nectaries, trees with *T. penzigi* had fewer active nectaries overall and fewer per leaf, which was expected due to the nectary-pruning behavior of workers of *T. penzigi* (Palmer *et al.* 2002). Leaves on *T. penzigi* trees had scars where extrafloral nectaries were usually found, meaning that trees occupied by *T. penzigi* still put on nectaries, but they are subsequently destroyed by *T. penzigi* workers. We did not find any differences in extrafloral nectary number among trees occupied by the three

Crematogaster species. Larger trees had more extrafloral nectaries, not only because larger trees have more leaves, but also because larger trees produce more nectaries per leaf.

When looking only at the terminal 50 cm of each branch, ant species still had an effect on these characteristics, but tree height did not, suggesting that the effects of tree height on foliage is driven by the areas of the tree further from the branch tips, i.e., large trees have more poor-condition foliage because the trunk and the middle of the canopy, where there are relatively few defending ants, make up a greater proportion of the whole tree. In the case of extrafloral nectaries per leaf, we found no main effect of height on nectaries, but did find an interaction between height and ant ($p = 0.02$), such that larger trees occupied by *C. sjostedti* and *T. penzigi* each had more extrafloral nectaries than smaller trees.

The effects of height and ant occupant on leaves and extrafloral nectaries are summarized in Table 2.5 and Figure 2.10.

We found that a tree's likelihood of putting on flowers and/or seeds is affected by ant occupant, tree height, and the interaction between the two. Larger trees were more likely to reproduce, regardless of their ant occupant. However, size had a stronger impact on reproduction for trees occupied by *C. sjostedti* and *T. penzigi* than for those occupied by *C. mimosae* and *C. nigriceps* (Figure 2.11). This pattern was the same when considering data from entire branches and from just the terminal 50 cm.

Table 2.5: Effects of tree height and ant occupant on *V. drepanolobium* new growth

Dependent variable	Part of tree considered	Transformed	Effect of tree height	p-value	Effect of ant	p-value
Number of leaves	whole branch	cube-root	positive	<0.001	yes	<0.001
Leaves per cm	whole branch	cube-root	negative	<0.01	yes	<0.001
	terminal	square-root	none	0.4	yes	<0.001
Leaf condition	whole branch	arcsine	negative	0.02	yes	<0.001
	terminal	arcsine	none	0.06	yes	<0.001
Leaf area (number x condition)	whole branch	cube-root	positive	<0.01	yes	<0.001
	terminal	square-root	none	0.1	yes	<0.001
Number of extrafloral nectaries	whole branch	fourth root	positive	<0.01	yes	<0.001
	terminal	fourth root	none	1	yes	<0.001
Extrafloral nectaries per leaf	whole branch	square-root	positive	0.04	yes	<0.001
	terminal	square-root	none*	0.2	yes	<0.001
Presence of flowers and/or seeds	whole branch		positive		yes	
	terminal		positive		yes	

For presence of flowers and/or seeds, we analyzed the effects of height and ant slightly differently, and so no p-value is reported; see methods for details.

*An interaction term was found between height and ant; see results section.

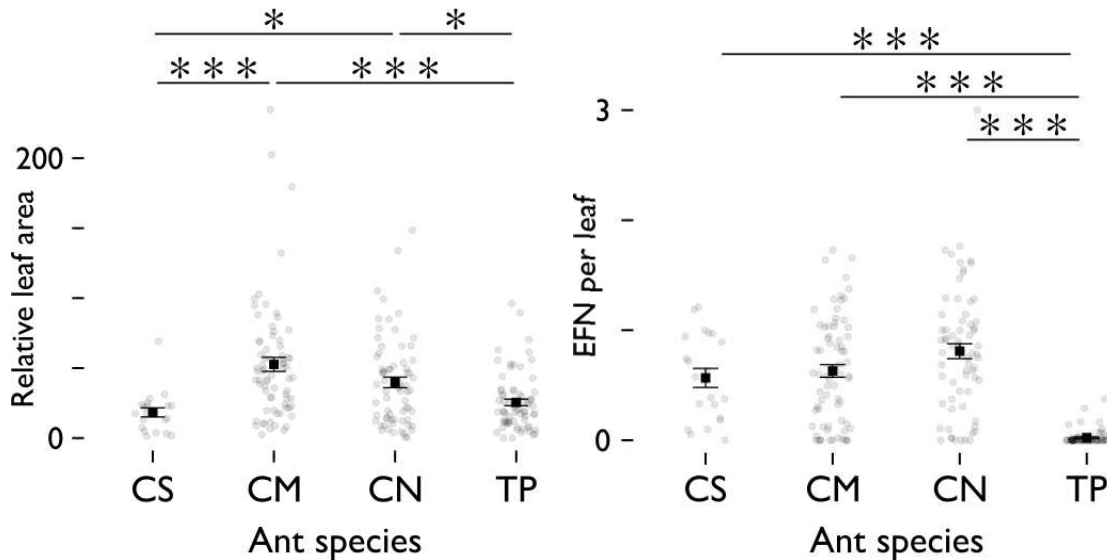


Figure 2.10: Trees occupied by different ant species show differences in new growth. Trees occupied by *C. mimosae* and *C. nigriceps*, which defend the tree, have more relative leaf area per branch than trees occupied by the non-defending *C. sjostedti* and *T. penzigi* (left). In addition, trees occupied by *T. penzigi* have fewer extrafloral nectaries due to *T. penzigi* nectary-pruning

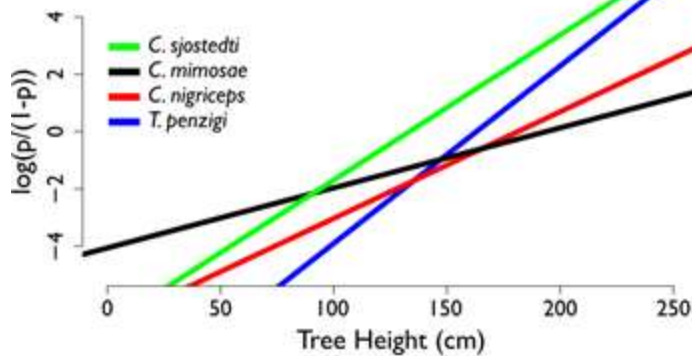


Figure 2.11: Tree reproduction is influenced by an interaction between ant occupant and height. The y-axis gives the logarithm of the odds of the tree having either flowers and/or seeds. Large trees occupied by *C. sjostedti* or *T. penzigi* are more likely to put on reproductive structures.

Plants' effects on ants: colony distribution

The distribution of ant biomass was strongly biased toward the distal ends of the branches. However, a number of plant characteristics, such as number of leaves and domatia volume, also show similar distributions (Figure 2.12). The structure of correlation among the different plant traits is visualized in a cluster dendrogram in Figure 2.13, which shows several modules: the first consists of the new-growth-related characteristics of presence of leaves, presence of extrafloral nectaries, and condition of leaves, all of which are tightly correlated. Also correlated are distance from the tip of the branch and root-end and tip-end diameter of the branch segment, which correspond to how close that segment is to the thin branch tips or the thicker branches close to the trunk. Less tightly correlated are presence of off-shoot branches less than 5 cm, and number of domatia, which are both closely correlated with total segment length. Finally, domatia volume on a segment is loosely correlated with the presence of flowers and seeds, as well as the total distance of the segment from the root, all of which are likely rough metrics of how large a tree is, as larger trees put on more flowers and seeds, as well as having larger average distances to the base of the tree.

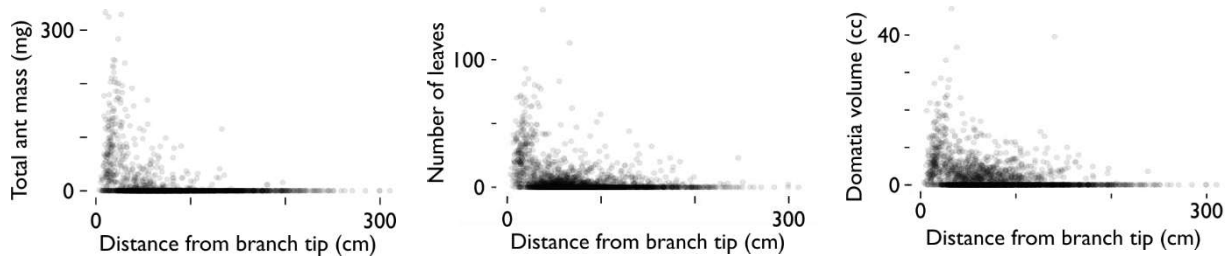


Figure 2.12: Both ant and plant characteristics are correlated with position on the tree. Total ant biomass is biased toward the distal regions of branches (left), as both new growth and woody features (as exemplified here by leaves and domatia volume, respectively). Each point represents an individual branch segment.

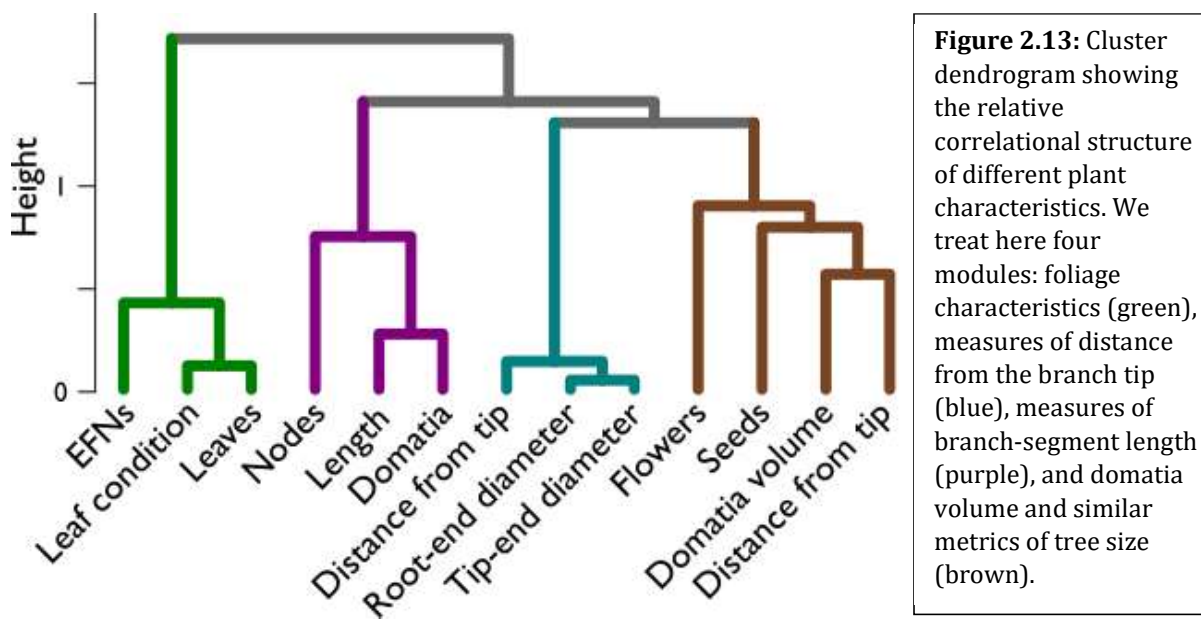


Figure 2.13: Cluster dendrogram showing the relative correlational structure of different plant characteristics. We treat here four modules: foliage characteristics (green), measures of distance from the branch tip (blue), measures of branch-segment length (purple), and domatia volume and similar metrics of tree size (brown).

Model fitting with redundancy analysis allowed us to disentangle which of these correlated variables best explained the distribution of the ant colonies of each species (Table 2.6; Figure 2.14). For all species of ants, the best model included domatia volume as a predictor, and for all species but *C. sjostedti*, one or more of the tightly-correlated variables indicating distance from the branch tips were selected. The only other variables to be selected in any model were leaf condition, which was part of the best model for *T. penzigi*, and presence of extrafloral nectaries for *C. mimosae*.

Overall, ant colony distributions are correlated primarily with tree architectural traits, with most of the colony biomass occurring on the terminal ends of the branches, where there is a larger amount of nesting space in terms of domatia volume. Domatia on the terminal ends of branches are also younger and contain more living tissue than those that are closer to the trunk, which are typically more lignified. Plant foliage traits are correlated with colony distribution for only two of the ant species, and even in those species, leaf condition still explains less variation in colony distribution than the selected architectural traits.

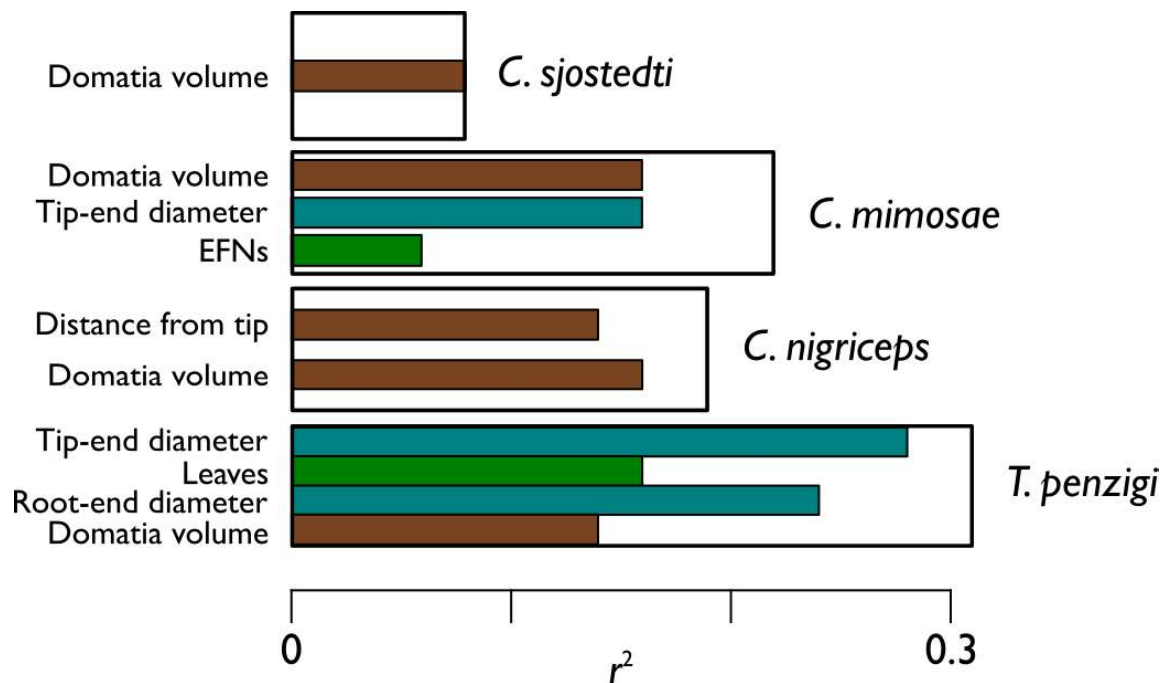


Figure 2.14: Plant characteristics explain the distribution of ants on *V. drepanolobium* trees. Large bars show the r^2 for the complete model; i.e., the proportion of variability in the distribution of ants explained by the chosen plant characteristics. Small, colored bars show the r^2 for each individual characteristic, after controlling for the effect of the other factors. Because some variation is equally-well-explained by more than one plant characteristic, the sum of these colored bars is less than the total r^2 .

Table 2.6: Ant distribution is explained by different plant characteristics

Ant Species	Factor	r^2	r^2 (conditioned)
<i>C. sjostedti</i>	Full model	0.08	-
	Domatia volume	0.08	-
<i>C. mimosae</i>	Full model	0.22	-
	Domatia volume	0.16	0.05
	Tip-end branch diameter	0.16	0.04
	Presence of extrafloral nectaries	0.06	0.01
<i>C. nigriceps</i>	Full model	0.19	-
	Distance from branch tip	0.14	0.05
	Domatia volume	0.16	0.03
<i>T. penzigi</i>	Full model	0.31	-
	Tip-end branch diameter	0.28	0.02
	Presence of leaves	0.16	0.01
	Root-end branch diameter	0.24	0.01
	Domatia volume	0.14	0.01

The r^2 column gives the proportion of variation in ant distribution explained only by that factor. Since some variation is explained equally well by more than one factor, the sum of these r^2 is greater than the r^2 of the total model. The r^2 (conditioned) column shows the r^2 for that factor conditioning on the other factors in the model; this leaves only that variation attributable solely to the factor in question.

We used biplots (Figure 2.15) to explore how individual plant characteristics chosen in the model-fitting step influence particular ant castes and developmental stages. Each axis of the plot represents a compound variable made up of linear combination of the plant characteristics chosen during model selection. The length of the arrows along that axis shows how strongly that characteristic loads into that axis. The points, meanwhile, show how strongly a given axis impacts the distribution of a particular caste and stage of ants: those placed far from the origin are more strongly influenced by plant traits, whereas the distribution of those castes and stages near the origin are weakly or not at all influenced by the plant characteristics (Figure 2.15, Table 2.7).

In all species, the first RDA axes placed workers and worker brood far from the origin, suggesting that the plant characteristic discussed disproportionately impact the distribution of workers.

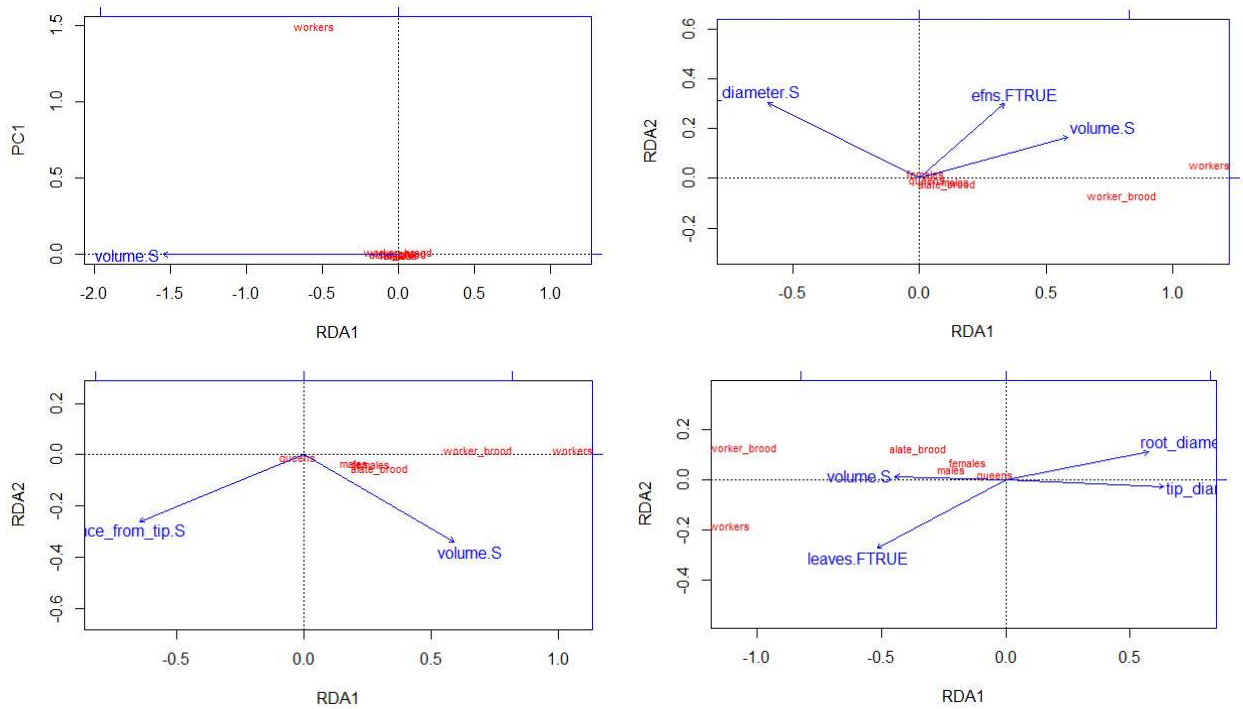


Figure 2.15: Plant characteristics impact ant distributions for each species of ant, *C. sjostedti* (top left), *C. mimosae* (top right), *C. nigriceps* (bottom left), and *T. penzigi* (bottom right). Axes represent composite variables composed of linear combinations of the chosen plant characteristics which explain the most (RDA1) and second-most (RDA2) of the variability in the ants' distribution. Arrows show the degree to which each plant trait loads onto RDA1 and RDA2 (horizontal and vertical length, respectively). Distance of points from the origin horizontally and vertically show how strongly each caste and stage's distribution is effected by RDA1 and RDA2, respectively.

Queen distribution

By exhaustively sampling whole colonies, we were able to find differences among species in where the queen is housed on the tree. *T. penzigi* queens are found in smaller domatia, approximately 5 mm smaller in diameter than the domatia inhabited by *C. mimosae* and *C. nigriceps* (although only the comparison between *C. mimosae* and *T. penzigi* is statistically significant). Furthermore, *T. penzigi* queens are found further out along the branches, often near the tips, than are *C. nigriceps* queens, which are often found near the trunk. *C. mimosae* queens are found spread throughout the tree (Table 2.8; Figure 2.16).

Table 2.7: Effect of each RDA axis on the distribution of different castes and developmental stages.

Ant species	Caste and stage	Distance from origin along:			
		RDA1	RDA2	RDA3	RDA4
<i>C. sjostedti</i>	Workers	-0.55			
	Worker brood	-0.00			
	Queens	0			
	Female alates	0			
	Male alates	0			
	Alate brood	0			
<i>C. mimosae</i>	Workers	1.15	0.05	-0.01	
	Worker brood	0.80	-0.07	0.01	
	Queens	0.03	-0.01	0.01	
	Female alates	0.02	0.02	0.04	
	Male alates	0.14	-0.01	0.01	
	Alate brood	0.11	-0.02	-0.02	
<i>C. nigriceps</i>	Workers	1.06	0.02		
	Worker brood	0.68	0.01		
	Queens	-0.02	-0.02		
	Female alates	0.26	-0.03		
	Male alates	0.20	-0.03		
	Alate brood	0.30	-0.05		
<i>T. penzigi</i>	Workers	-1.11	-0.18	0.02	0.00
	Worker brood	-1.06	0.13	-0.04	-0.01
	Queens	-0.04	0.02	0.01	0.01
	Female alates	-0.15	0.07	0.07	-0.02
	Male alates	-0.22	0.04	0.01	-0.01
	Alate brood	-0.35	0.13	0.03	0.03

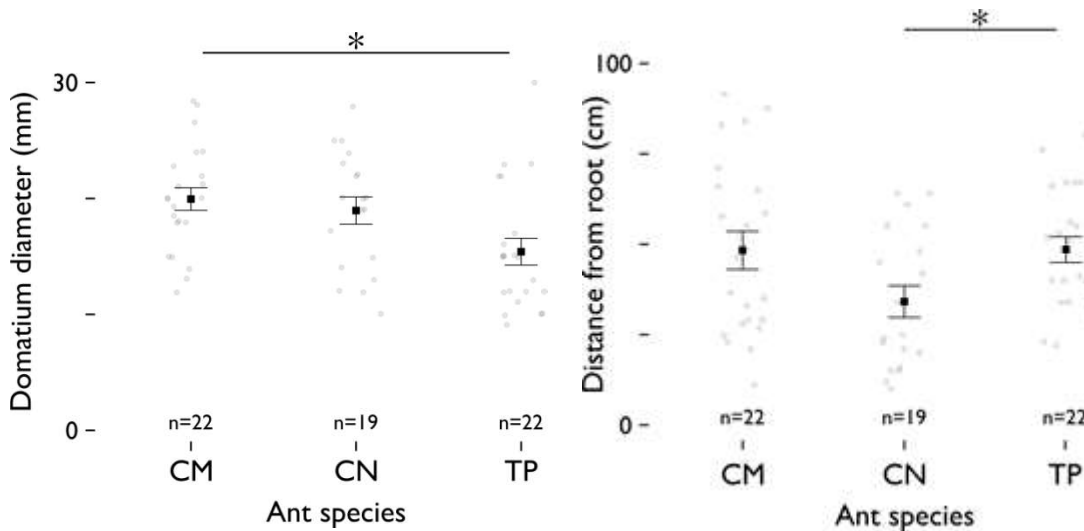


Figure 2.16: Queens of different ant species are found in different parts of the tree: *T. penzigi* queens are found in smaller domatia (left-hand plot), and *C. nigriceps* queens are found nearer to the root (right-hand plot). Squares and bars represent mean and standard error; mean values for each tree are shown underlying each dot and whisker plot. Horizontal lines show significant differences between species; * $p < 0.05$.

Table 2.8: Location of queens varies among ant species

Dependent variable	Transformed	Effect of tree height	<i>p</i> -value	Effect of ant	<i>p</i> -value
Domatium diameter	none	positive	<0.01	yes	0.02
Distance from root	square-root	positive	<0.01	yes	0.03

Discussion

We found support for the hypothesis that differences in caste investment underlie the competition-colonization hierarchy. Across the three domatia-occupying ants considered in this study, investment in workers corresponded to the competitive hierarchy of *C. mimosae* > *C. nigriceps* > *T. penzigi*, while investment in reproductives corresponded to the colonization hierarchy of *T. penzigi* > *C. nigriceps* > *C. mimosae*. On small trees, colonies of all three species had similar biomasses, suggesting that the colonization hierarchy being a reversal of the competition hierarchy is not a coincidence; rather, it derives from the ants' three different solutions to a tradeoff between investment in competition through workers and investment in colonization through reproductives.

On larger trees, we see more pronounced differences in colony size, with *T. penzigi* colonies being smaller than *C. nigriceps* colonies. This may be because large investments in reproduction come at the expense not only of competitive ability, but also of colony maintenance, since all future generations of workers must be raised by current workers. We do, in fact, see that the correlation between tree size and colony biomass follows the competitive hierarchy: the most competitive species show the largest increases in biomass with tree size, and the least competitive species show the smallest. This could be because larger trees are occupied by older colonies, representing how investment in workers early in the colony's life allows it to reach larger colony sizes later.

We also found that the ratio of brood to workers was higher in *T. penzigi* than in the other species. High juvenile:adult ratios are often a sign of a growing population, or one with high adult mortality (Ricklefs 2008). This may result from *T. penzigi*'s position at the bottom of the competitive hierarchy; under the competitive-colonization tradeoff framework, it persists by producing many ephemeral colonies which quickly grow to the size needed to reproduce.

Our results are similar to those of Isbell and Young, who found that the number of workers per domatium increased with the competitive hierarchy; they, however, did not find evidence that reproductives or immatures (worker and alate brood combined) varied among the ant species (2007). They did not include tree size as a covariate, so it may be that *T. penzigi* and *C. nigriceps* had relatively fewer alates in their analysis due to these species inhabiting, on average, smaller trees. It could also be that these discrepancies come from the time of year at which sampling took place. An important caveat to our analyses is that they represent a single time point within a seasonal cycle. However, if reproductives are produced disproportionately at different times of year, then this could bias our results. Isbell and Young found wide variation in numbers of ants per domatium across a 22-month period, although this variation did not appear to be linked to climate, at least for rainfall (2007). Therefore, if for instance *C. mimosae* alates represented a large proportion of biomass at a different time of year, this could invalidate our observation of a tradeoff between competition and colonization. Further research needs to be done to determine whether and how colony makeup changes over the annual wet-dry cycles.

Despite this caveat, interspecific differences in caste investment remains an attractive explanation for the competition-colonization hierarchy. Unlike other

explanations, like the hypothesis that more competitive species were more polygynous (see Chapter 1), in this case, a single hypothesis simultaneously explains both hierarchies and why they run counter to each other. Moreover, the hypothesis that organisms experience tradeoffs between reproduction and physical condition is a common one, particularly among non-eusocial species (Stearns 1992, Flatt and Heyland 2011). That the colonization-competition hierarchy has a generalizable explanation suggests that similar mechanisms may underlie coexistence in other systems as well.

Interspecific differences in resource use among the ant species are broad. However, the evidence for interspecific differences in the ants' interaction with their host tree varied greatly depending on what aspects of the association were examined. Identity of the ant associate had a strong effect on the host tree's new growth, with trees occupied by *C. mimosae* and *C. nigriceps* having more leaves or better-condition leaves, respectively. This agrees with previous observations that these species aggressively defend the tree, while the other ant species do not (Palmer and Brody 2007, Martins 2010). Lifewise, the nectary-pruning behavior of *T. penzigi* reported by Palmer *et al.* (2002) was also observed in this data set: while we found no differences in extrafloral nectary number among trees occupied by the *Crematogaster* species, trees occupied by *T. penzigi* had fewer active nectaries.

However, we found fewer effects of ant species on the woody architecture of their host trees. Along with Stanton *et al.* (1999), we found that *C. nigriceps* trees have a higher density of domatia. We were unable, however, to replicate several of Stanton *et al.*'s other findings, i.e. variation in domatia diameter and variation in branching rate (our sampling methodology also made it impossible to compare our results with their fourth metric of

canopy architecture, “apical dominance index”). We did not find any evidence that domatia diameter or branching rate varied across species.

Our results may vary from Stanton *et al.*'s for at least three reasons. First, their statistical analyses on architectural traits do not include tree size as a covariate, and they do not report whether they attempted to size match their focal trees for that particular study. We show here that smaller trees have higher branching rates, if only toward the terminal parts of the branches. At the Mpala Research Centre, where the study of Stanton *et al.* took place, *C. nigriceps* disproportionately occupy small trees (Palmer *et al.* 2010). This would explain why *C. nigriceps* trees observed in their study have a higher branching rate than *C. mimosae* and *C. sjostedti*, and why this affect disappears in our analysis when tree size is accounted for. However, this explanation would suggest that *T. penzigi*-occupied trees should also have high branching rates in their analysis, which does not appear to be the case, and it would also predict that *C. nigriceps* (and *T. penzigi*) trees should have smaller domatia, since we found that smaller trees have smaller domatia. This was not the case. Thus, our inclusion of tree height as a covariate in our analyses does not appear to explain most of our departures from their data.

Another possibility is that their data set had greater power to detect differences than ours; however, they surveyed only about 10% more trees than we did (242 vs 223), so we do not expect that our data sets differ greatly in power.

Finally, our results may vary from Stanton *et al.*'s due to location. Our site was about 30 km distant from theirs, and had likely experienced different pressures from herbivores than theirs: average tree size was smaller at Suyian than at Mpala (Chapter 3). Both partners in this mutualism change their behavior under different herbivory regimes

(Palmer *et al.* 2008). Possibly *C. nigriceps* ants do less pruning at Suyian than at Mpala, resulting in fewer architectural differences among the trees at Suyian.

At this site at least, ant occupancy does not seem to have a lasting effect on most aspects of the architecture of the tree, despite having clear impacts on the new growth. This may mean that the short-term costs or benefits provided by particular ant species do not strongly translate into long-term costs or benefits. It may also be the case that while long-term costs or benefits are felt by the tree (Palmer *et al.* 2010; Stanton *et al.* 2011), they are expressed not as differences in mode of growth, but simply in amount; that is, trees in worse condition grow in the same patterns, but simply grow less than trees in better condition.

We did, however, find that many tree traits, both in new growth and woody architecture, change with tree height even when ant occupant is accounted for. Larger trees tend to have fewer structures, i.e., leaves and domatia, per unit length of branch, but because their branches are longer, they have more total leaves, more total domatia, and more extrafloral nectaries, both overall and per leaf. Furthermore, we only surveyed a single branch from tip to root, but the branching structure of the trees means that larger trees have disproportionately more branches than smaller trees, further compounding the value of large trees. Thus, while large trees often appear to be in somewhat poorer condition (as shown here in leaf condition and domatia stage), they still represent resources for the ants that are disproportionately valuable relative to smaller trees.

While the ants show a remarkable variation in behaviors such as defending foliage, pruning foliage or extrafloral nectaries, or tending scale insects, we found that the three domatia-nesting species arranged their colonies on the tree in broadly similar ways. We

found, in agreement with Isbell and Young (2007), that the colonies were biased toward the outer areas of the canopy. In addition, we identified the tree traits that best predict the arrangement of the colony. In all three species, colony distribution was affected by domatia volume, and by the location of the branch relative to the tip, with colony distributions biased toward areas of the branch with plentiful nesting space, and those that are near the branch tips. Contrary to our expectations, foliage does not have a strong impact on colony distribution, despite the fact that two of the three species defend foliage and consume extrafloral nectaries. In *C. nigriceps*, no foliage trait was a good predictor of colony distribution. Presence of extrafloral nectaries and presence of leaves affected colony distribution in *C. mimosae* and *T. penzigi*, respectively, but even in these species, the various architectural traits together (and usually individually) explained more variation in ant distribution than did the foliage traits.

This suggests that the *V. drepanolobium* mutualism may be one of reciprocal exploitation; that is, ant mutualists arrange their colony not so as to defend the tree, keeping workers near foliage to protect it, but rather they simply occupy the areas of the tree with the best nesting space. The plant then benefits because the foliage disproportionately grows from branches with many domatia, near the tips, and so the ants defend the tree's foliage as a byproduct of defending their nest site. Alternatively, the ants may indeed benefit by defending foliage, through partner fidelity feedback or similar mechanisms. Under this scenario, architectural traits may be simple cues that, because of their correlation with the presence of foliage, allow the ants to arrange their colony in the areas of the tree where they can best benefit the tree. Whichever is the case, it does seem

that architectural traits like domatia volume and distance from the branch tip are more important in explaining ant colony distribution than are foliage traits.

Finally, we find that queens do show distinct interspecific differences in their location within the canopies of their host acacias. In *C. mimosae* and *C. nigriceps*, queens were found in larger domatia, often close to the trunk, while in *T. penzigi*, queens were found in smaller domatia nearer the branch tips. This may make *T. penzigi* queens more vulnerable to disturbance, since they are in a part of the tree more exposed to herbivores. Since *T. penzigi* is at the bottom of the competitive hierarchy, however, this may be less of a risk, since the colony may be evicted by a more competitive species relatively quickly. Meanwhile, the more competitive *C. mimosae* and *C. nigriceps* may invest more in longevity, keeping the queen in a less disturbed area of the tree, even though this means that brood must be shuttled from the queen's domatium to the outer parts of the tree, where we generally found it.

The ant associates of *V. drepanolobium* thus display both variation and remarkable similarity in their colony-level behaviors. We see variation in colony-level investment in workers versus reproductives; variation which we argue here underlies the competitive-colonization tradeoff in this system. We also see variation in the effects of the various ants on their trees' foliage, but less variation in their effects on woody architecture, and we find that the ants use similar tree characteristics to organize their colonies on the canopy. These results tell us much about the *V. drepanolobium*-ant mutualism at Suyian; however, there is still much more to learn. We do not yet know how generalizable these results are to different sites in this system, and comparison of some of our data to Stanton *et al.* suggests that some characteristics of the system may vary from site to site.

Chapter 3: Demography and biomass of the *V. drepanolobium* ant-plant mutualism varies widely across its geographic range.

Abstract

Vachellia drepanolobium is a well-studied East African acacia tree which provides nesting space to defensive ant mutualists from several different ant species. Research in this system has led to insights on mechanisms of species coexistence, the boundaries of mutualism and parasitism, the effects of disturbance on symbiosis, and more. However, most of the research in this system to date has taken place within a relatively small portion of the range of *V. drepanolobium*, particularly Laikipia plateau. Here we present a systematic survey of *V. drepanolobium* plant-ant system at a number of sites throughout the Kenyan range of this mutualism. We find the ecology of both partners varies widely across their ranges, in terms of the size and density of the tree host, and the presence, numerical dominance, and biomass of the ant associates. We also find that the total ant biomass in this system is quite low, especially compared to biomass estimates for the vertebrates against which they provide protection, and to the biomass of other anti-herbivore defenses like thorns and secondary defensive compounds.

Introduction

In eastern Africa, dark, clay, “Black Cotton” soils are typically dominated by the savannah thorn tree, *Vachellia drepanolobium* and several species of associated ant mutualists. The ants nest in hollow, swollen-thorn domatia, and receive extrafloral nectar from the host tree, in return for which they defend the tree from herbivores (Hocking 1970, Madden and Young 1992). Because ants of different species compete to occupy *V. drepanolobium* canopies, the system has been used to investigate possible mechanisms of species coexistence (e.g., Stanton *et al.* 2002, Palmer 2004, Chapters 1 and 2). Because the ant species vary in the benefits they provide to the tree and the costs they impose, the system has yielded insights into the boundaries between parasitism and mutualism (Palmer *et al.* 2010, Martins 2010, Stanton and Palmer 2011, Chapter 2). And because of the human impact on the savannah, much work has been done elucidating how the system as a whole responds to environmental perturbations (Palmer *et al.* 2008, Riginos *et al.* 2015).

The *V. drepanolobium* system has been well studied in the Laikipia plateau of central Kenya, particularly at the Mpala Research Centre (e.g. Stanton *et al.* 1999, Palmer *et al.* 2010). However, little has been done to characterize this system outside of Laikipia, with a handful of exceptions: two of the earliest studies in this system took place in northern Tanzania and/or southern Kenya (Hocking 1970, Madden and Young 1992). Subsequently, Stapley had field sites in the same region (1998; 1999), as did Martins (2010, 2013, Martins *et al.* 2013) and Baker *et al.* (2016). However, most of these studies do not provide systematic surveys comparable to what has been done in Laikipia (e.g., Young *et al.* 1997a, Palmer *et al.* 2010). We do not, therefore, know whether the *V. drepanolobium* system in Laikipia is representative of the system as a whole.

This study describes *V. drepanolobium* and its ant associates at a variety of sites across Kenya. At each site, we describe both sides of the mutualism, recording tree sizes and densities, as well as which ants are present at each site and in what numbers. The sites spread across geographically diverse area: two sites are in Laikipia (including one at the Mpala Research Centre), three sites are in the highlands area south of Nairobi, three sites are in the Rift Valley, and one site lies on each of the western and eastern edges of the Rift. The Rift Valley is a central feature of Kenyan geography. The Eastern Rift Valley runs through the middle of Kenya, from Lake Turkana in the north to the Tanzanian border in the south. Its formation began approximately 20 million years ago as the African tectonic plate began to split apart, a process that continues to this day (Chorowicz 2005). The difference between the relatively low-lying sites in the Rift and the highlands sites outside the Rift can be as much as a kilometer (Table 3.2), and the valley is separated from the highlands by a steep escarpment. The Rift Valley thus may be a barrier to gene flow in organisms including plants (Omondi *et al.* 2010, Ruiz Guajardo *et al.* 2010) and insects (Lehmann *et al.* 1999), as well as vertebrates (Arctander *et al.* 1999, Ahlering *et al.* 2012).

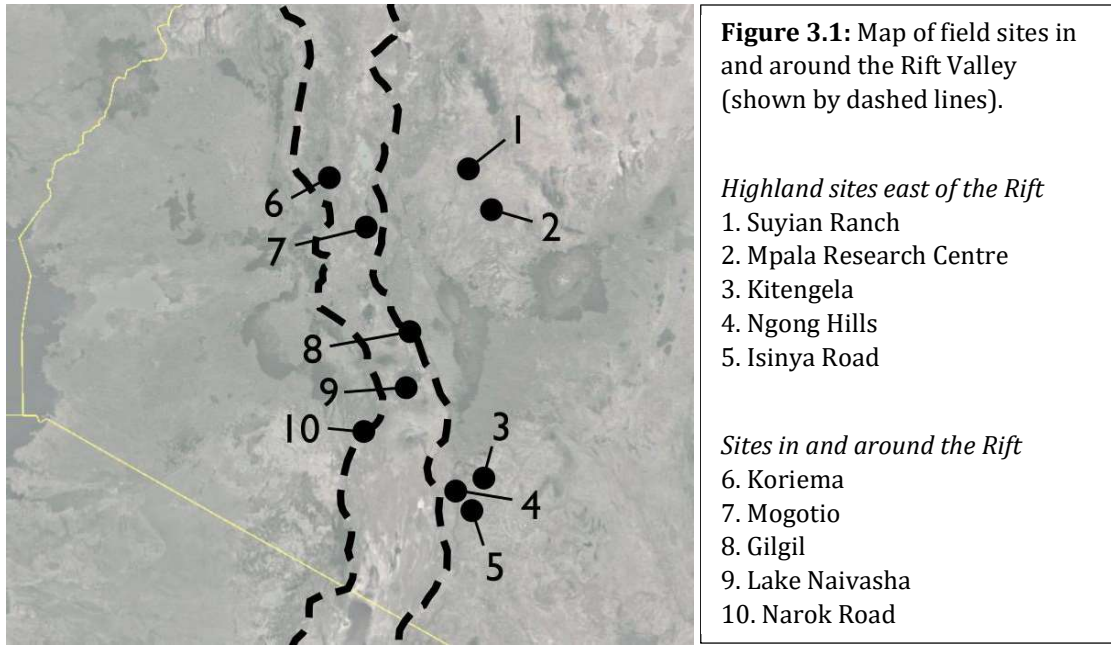
Finally, we combine the demographic data described here with the biomass data collected in Chapter 2 to estimate the total biomass per land area of the three obligately phytoecious ant associates. Ants are well-known to play an important role in most ecosystems, and Hölldobler and Wilson famously estimate that the biomass of ants on earth approximates that of humans (2008). More fine-grained estimates of ant biomass, however, are rare, likely due to the difficulties of finding and accessing nest sites. However, the arboreal nesting habits of the ant associates of *V. drepanolobium* allow for precisely these estimates. We the combine them with with previous estimates of *V. drepanolobium* biomass

to estimate the biomass of each partner at the different sites. Estimating ant biomass facilitates direct comparisons to other species, including to the herbivores against which they defend the tree, and permits comparisons between the ants and other forms of defense against herbivory, such as thorns or phytotoxins.

Methods

Site survey

Between 2013-2016, we surveyed *V. drepanolobium* and its ant associates at 10 sites in Kenya (Figure 3.1). These sites included five in the highlands east of the Great Rift Valley and five in or around the Rift itself. At most of the sites, we surveyed a transect at least 0.15 ha in area, measuring every tree greater than 1.0 cm diameter at 0.5 meters above the ground. At Koriema and Gilgil, the patch of *V. drepanolobium* was smaller than this, so we surveyed every *V. drepanolobium* tree, and then calculated the area they occupied. Within these transects, we recorded the height, stem diameter at 0.5 meters above the ground, and ant occupant of each tree. At the Mpala site, we surveyed 6.7 hectares of the Mpala CTFS-ForestGeo plot for tree number and ant occupant, and a 0.24 ha subset of that area for tree number, ant occupant, and tree height and diameter. When the canopies of two trees were entangled, we counted this as a single tree for our purposes, since ants could move freely between them without having to leave the canopy; in these cases, and when a single tree had multiple stems 0.5 meters above the ground, we recorded the diameter of the single largest stem, and the height of the tallest point in the combined canopy.



Ant biomass estimates

To estimate the ant biomass at each site, we used the data set collected in Chapter 2, which included the ant biomass along a single branch for each of 223 trees. These branches were divided into segments, each of which began or ended at a node at which two branches of at least 5 mm diameter separated (see Figure 3.2). We estimated the total size of the tree by assuming that the tree was symmetric at each node; i.e., that the part of the tree we did not measure was equivalent to the part we did measure (Figure 3.2). The biomass observed at each branch segment was multiplied by x , where:

$$x = \begin{cases} 1 + \sum_{i=1}^{s-1} 2^{i-1} p^i, & s > 1 \\ 1, & s = 1 \end{cases}$$

Here s is the number of the branch segments between that segment and the base of the tree, inclusive; i.e., the basal branch segment (the trunk) of the tree has $s = 1$, the next segment connected to it has $s = 2$, etc. The branch for which we measured ant biomass was

alive from tip to trunk; however, most *V. drepanolobium* trees have many dead or broken branches, which are typically not occupied by ants. The constant p represents the probability that an unobserved branch was not dead or broken, adjusting our estimate to account for dead branches in the unobserved part of the tree. To estimate p , we surveyed 61 additional trees, divided evenly across a variety of size classes and all four ant occupants. For each tree, we selected a focal branch using the method described in Chapter 2. For each node at which two branches of at least 5 mm diameter separated, we recorded whether or not the “non-focal” branch was either broken off, or entirely dead (which we defined as having no green leaves growing from anywhere on the branch). Across all trees, $p = 0.57$.

For each species, we then calculated a line of best fit between the biomass estimates for each tree and the tree heights, after first \log_{10} transforming both height and (biomass+1). We used these functions to estimate the total biomass on the trees at each of the 10 field sites. If a tree at those sites was occupied by more than one ant, or by a different species of ant, we used the estimates for *C. sjostedti*, which were low because this species does not typically nest in *V. drepanolobium* domatia.

Tree biomass estimates

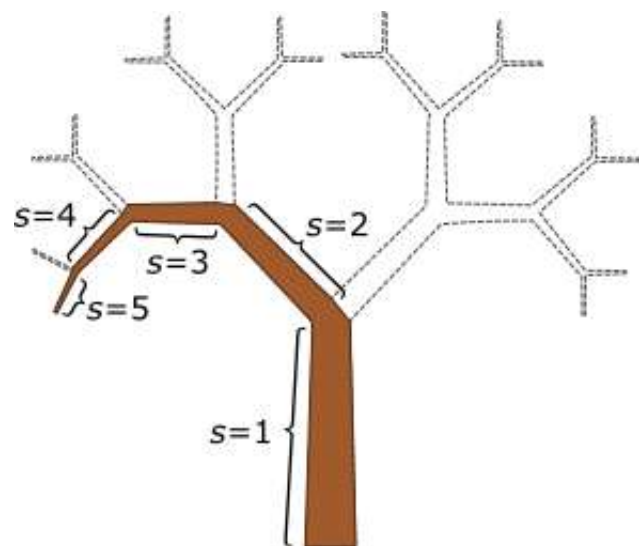


Figure 3.2: Sampling scheme of individual trees. The brown area represents the branch segments (numbered 1-5) for which biomass was recorded. We assumed that the tree was symmetric at each node, as shown, except that we took into account the possibility that unsampled branches were broken or dead.

To estimate the biomass of the trees at each site, we used the correlations between ln(dry biomass) and ln(stem diameter) calculated by Okello *et al.* for *V. drepanolobium* (2001), as shown in Table 3.1.

Table 3.1: Lines of best fit from Okello *et al.* 2001 used to estimate *V. drepanolobium* biomass

Biomass category	Equation
Total biomass	$\ln(\text{total biomass} + 1) = 2.2949 * \ln(\text{stem diameter}) + 4.7997$
Non-swollen thorn biomass	$\ln(\text{thorn biomass} + 1) = 1.7896 * \ln(\text{stem diameter}) + 2.5554$
Leaf biomass	$\ln(\text{leaf biomass} + 1) = 0.9164 * \ln(\text{stem diameter}) + 3.9627$

Biomass is in units of grams; stem diameter in centimeters.

Okello *et al.* measured stem diameter at the base of the tree, while we measured stem diameter 0.5 m above the base, except for the 223 trees at Suyian used to estimate ant biomass, where we recorded diameter at both locations. We used these data to calculated a line of best fit between the stem diameters at each point on the trunk, which was (diameter at base = diameter at 50 cm * 0.856 + 1.269, $r^2 = 0.81$). For some of the larger trees, this line of best fit results in a diameter at the base smaller than the diameter at 50 cm; in this case, we set the diameter at the base to be equal to the diameter at 50 cm. We used these diameters when using Okello *et al.*'s equations to estimate biomass.

Results

Site survey

Plots varied substantially along several axes (Table 3.2, Figure 3.3). Tree density varied about five-fold, from 220 *V. drepanolobium* trees per hectare at Gilgil to a maximum of 1090 at the Isinya Road site; trees generally grew more densely in the eastern highlands than in the sites within the Rift. Average tree height also varied; at most sites the average height was 1-3 m, but at Koriema and Mogotio in the northwestern part of its range, *V. drepanolobium* trees averaged 4-5 meters, and grew up to 10 meters in height.

Likewise, there was substantial variation in ant communities among sites. *C. sjostedti* was only found on *V. drepanolobium* trees in the northeastern sites of Suyian and Mpala. *C. mimosae* was more widespread, but commonly found only in the Eastern Highlands sites. In and around the Rift, *C. mimosae* was found rarely at Narok, and outside of that site, only a single colony was found (near the Lake Naivasha site). *C. nigriceps* and *T. penzigi*, however, were found at every location throughout the studied range. Furthermore, which ant was numerically dominant varied as well: for all four ant species, there was at least one site at which that species occupied the plurality of trees.

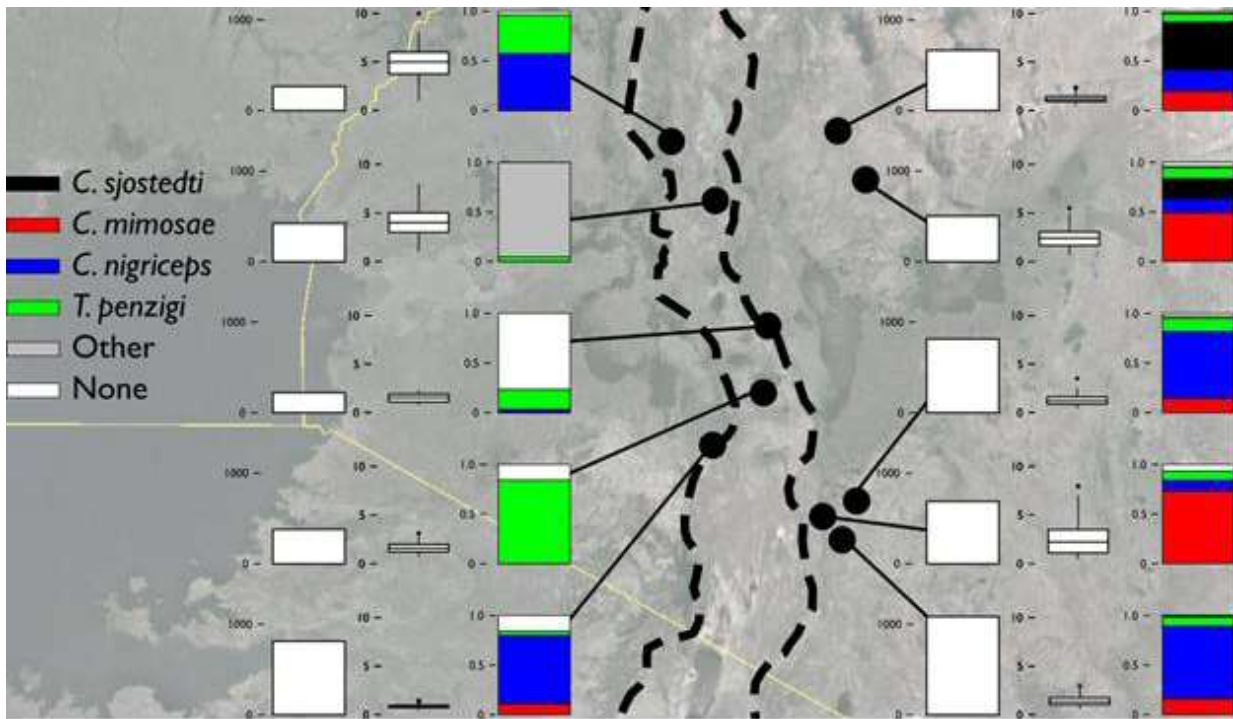


Figure 3.3: *V. drepanolobium* ant-plant mutualism varies across Kenya. Three plots summarize the characteristics of each site. The leftmost bar shows tree density, in trees/hectare. The middle box and whisker plot shows the distribution of tree sizes (in meters) at each site. The rightmost bar shows the proportions of those trees occupied by each species of ant.

Table 3.2: Demography of *V. drepanolobium*-ant association varies throughout Kenya

Site	Lat. (°)	Long. (°)	Alt. (m)	Plot size (m ²)	Trees surveyed	Tree density (trees/ha)	Tree height (m)
Suyian Ranch	0.50675	36.69235	1900	8000	533	666	1.20
Mpala Re-search Center	0.28961	36.87106	1807	67200 ²	3391 ²	505	2.41
Kitengela	-1.39645	36.82562	1673	2000	163	815	1.30
	-1.47344	36.64562	1947				
Ngong Hills ³	-1.47590	36.64340	1921	2000	138	690	2.67
	-1.60041	36.75144	1811				
Isinya Road ³	-1.60114	36.75115	1801	1500	163	1090	1.41
Koriema	0.47942	35.85226	1375	1050	28	270	5
Mogotio	0.17323	36.08356	1238	1040	44	420	4
Gilgil	-0.47670	36.35554	1938	1350	30	220	1.54
Lake Naivasha	-0.82773	36.33415	1195	2000	76	380	1.65
Narok Road	-1.10153	36.06934	2073	2250	183	813	0.87
	Proportion of trees occupied by:						
Site	<i>C. sjostedti</i>	<i>C. mimosae</i>	<i>C. nigriceps</i>	<i>T. penzigi</i>	None ¹	Other ¹	
Suyian Ranch	0.48	0.19	0.21	0.08	0.02	0.01	
Mpala R.C.	0.21	0.49	0.14	0.11	0.04	0.02	
Kitengela	0	0.14	0.67	0.13	0.03	0.02	
Ngong Hills ³	0	0.73	0.11	0.09	0.07	0	
Isinya Road ³	0	0.16	0.74	0.09	0	0.01	
Koriema	0	0	0.57	0.39	0.04	0	
Mogotio	0	0	0 ⁴	0.05	0	0.95 ⁴	
Gilgil	0	0	0.03	0.20	0.77	0	
Lake Naivasha	0	0 ⁵	0 ⁵	0.84	0.16	0	
Narok Road	0	0.11	0.69	0.04	0.16	0	

¹ Trees occupied by “none” indicate trees with no mature colonies. “Other” includes trees occupied by a different species, as well as those trees with more than one species of ant in the canopy, or those too tall to determine the canopy occupant.

² Height and diameter measurements come from 69 trees in a 2400 m² subset of this area.

³ At Ngong Hills and Isinya Road, we sampled from two separate transects within a short distance of each other. Both locations are given, but we pooled the height and ant occupancy data.

⁴ At Mogotio, trees were occupied by a *Lepisiota* sp., or by *Lepisiota* and *T. penzigi* together, or in one case, by *Lepisiota*, *T. penzigi*, and *C. nigriceps*.

⁵ At Lake Naivasha, only *T. penzigi* was found at the site we surveyed. However, we found *C. nigriceps* and a single colony of *C. mimosae* nearby along the South Lake Road.

Biomass estimates

As shown in Figure 3.4, the estimated biomass on the single trees measured at Suyian ranged from 0-50 grams of ants. Since the average worker mass of these ants is

approximately 0.5 mg (see Chapter 2), each gram of worker ants represents roughly 2000 ants. The total colony mass estimates were relatively small for most colonies (less than 10 g), but did increase with tree size across all four species. However, this effect was much more pronounced for *C. mimosae* and *C. nigriceps* than for the other species.

Several sites included trees larger than any of the trees we used to estimate the function of how mass increases with tree height. However, even for the tallest of these trees (8-10 meters in height), our average biomass estimates were within the range of 0-50 g: i.e., a range of masses similar to those we observed in our training data set.

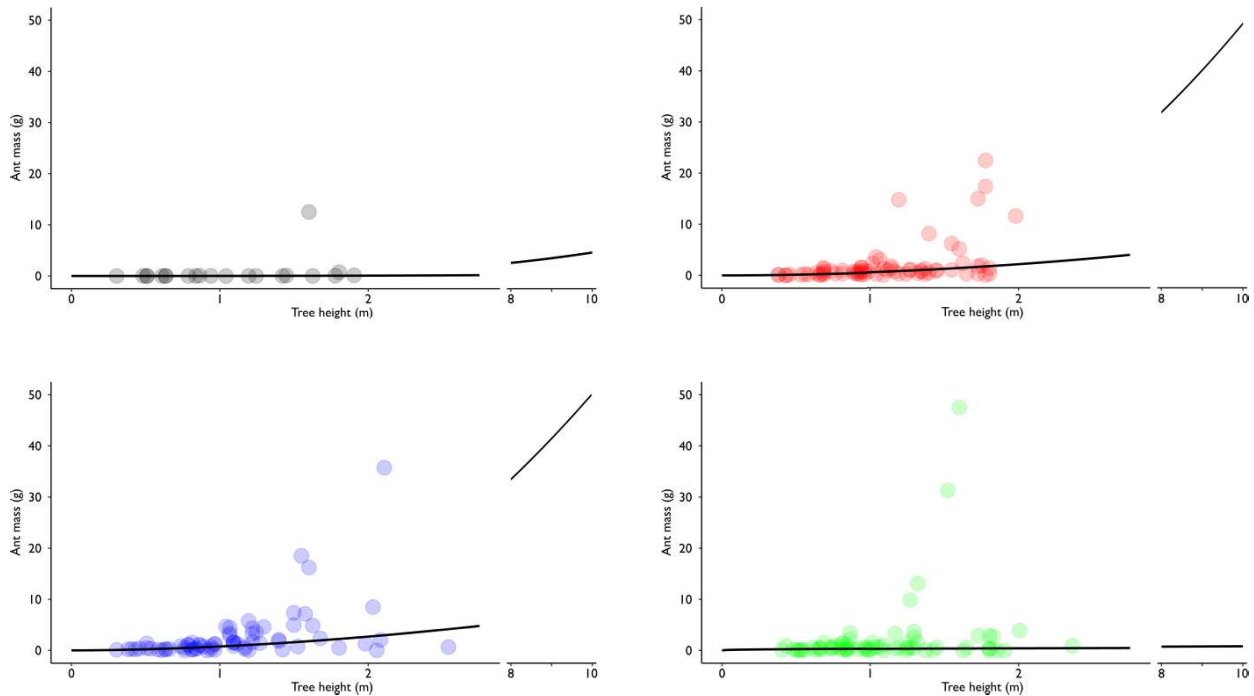


Figure 3.4: The power functions shown here were used to estimate how much ant biomass was contained on trees of a given height. *C. sjostedti* is in the upper left, *C. mimosae* upper right, *C. nigriceps* lower left, *T. penzigi* lower right. For each species, circles show the estimated biomass for individual trees of a variety of heights. The power curve estimated is also shown, both across the size range of trees used to calibrate the curve (0.3-2.5 m), and across the maximum tree height observed across all sites (8-10 m, although tree this large were rare and confined to the sites in the northern part of the Rift Valley).

Our estimates of total ant biomass varied from site to site (Table 3.3, Figure 3.5). In the sites in the Eastern highlands, ant biomass ranged from a low of 33 kg/km² (at Suyian)

to a high of 273 kg/km² (at the Ngong Hills). The ant biomass averaged across these five sites was 128 kg/km². Estimates from sites in the Rift were much lower except for Koriema; however, our method is likely to be less accurate at calculating the biomass at some of these sites: for one, the tree sizes at Koriema and Mogotio were very large (Figure 3.2), much larger than the trees at Suyian which were used to calibrate the function connecting ant mass and tree height; this could explain the large biomass of ants found on the (large) trees at the Koriema site.

In the interest of completeness, we have included the low numbers for the biomass of *C. sjostedti* at the two sites at which it was found. However, this does not mean that the biomass of this species is low at these two sites. Our method was only able to capture ant biomass inhabiting the swollen thorns, and while virtually the entire colony of the obligately phytoecious species of *C. mimosae*, *C. nigriceps*, and *T. penzigi* nest in the thorns, *C. sjostedti* colonies nest mainly in the ground or in hollows in the trunk of the tree (Palmer *et al.* 2000). Our method only estimates the *C. sjostedti* biomass found in the domatia, which is a small fraction of the entire colony biomass.

Table 3.3: Ant biomass by species at 10 sites throughout Kenya

Site	Ant biomass (kg/km ²) comprised of:				Total ant bio-mass (kg/km ²)	Total tree bio-mass (kg/km ²)	Leaf biomass (kg/km ²)	Thorn biomass (kg/km ²)
	<i>C. sjostedti</i>	<i>C. mimosae</i>	<i>C. nigriceps</i>	<i>T. penzigi</i>				
Suyian Ranch	0.6	11.1	19.6	1.9	33.0	226,000	12,600	11,100
Mpala Research Center	0.1	79.8	12.6	0.9	93.4	247,000	7700	9600
Kitengela	0	10.2	73.1	4.0	87.4	258,000	14,700	12,700
Ngong Hills	0	233.8	35.9	3.4	273.1	848,000	18,200	27,800
Isinya Road	0	25.9	121.1	3.6	150.6	355,000	20,200	17,600
Koriema	0	0	255.7	6.1	261.7	531,000	9900	17,400
Mogotio	0	0	0	0.9	24.2	532,000	12,900	19,100
Gilgil	0	0	1.2	1.7	2.9	72,000	4200	3600
Lake Naivasha	0	0	0	12.3	12.3	162,000	7700	7400
Narok Road	0	3.4	35.6	1.1	40.1	113,000	11,100	6800
Average of all sites	0.1	36.4	55.5	3.6	97.9	334,000	11,900	13,300

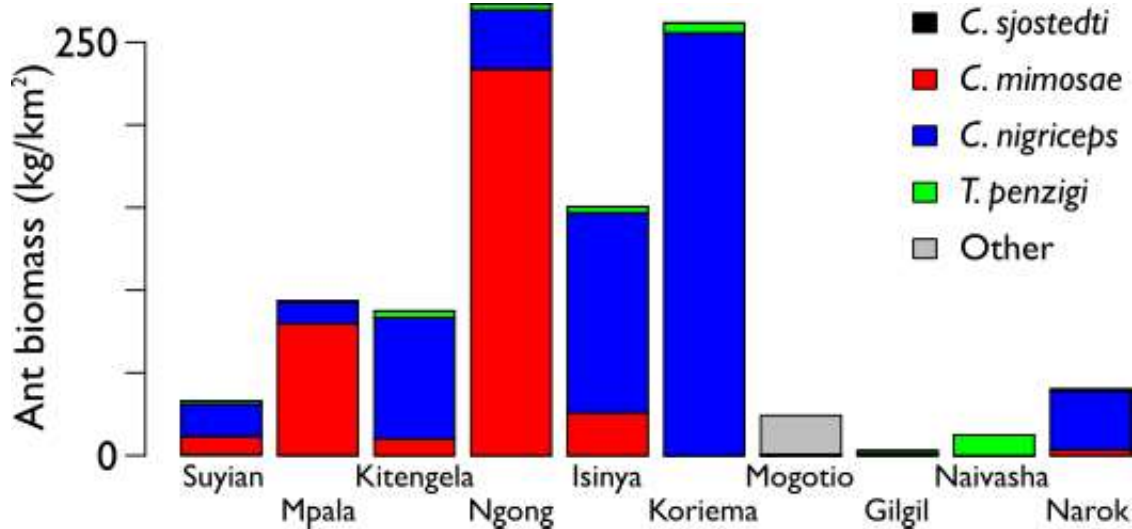


Figure 3.5: Ant biomass varies among sites, but is typically higher in the Eastern Highlands sites (Suyian-Isinya), than in or around the Rift (Koriema-Narok). Except for the Naivasha site, most biomass was from *C. mimosae* and *C. nigriceps*. “Other” includes both other ant species, as well as trees occupied by more than one species of ant.

Tree biomass estimates

Tree biomasses likewise varied across sites (Table 3.3, Figure 3.6). The site with the greatest total *V. drepanolobium* biomass was the Ngong Hills site, which had relatively large trees growing at fairly high densities. The sites at Koriema and Mogotio, which featured even larger trees, but growing at lower densities, had somewhat lower total biomasses, followed by the other Highlands sites, which features smaller trees growing more densely. The least total biomass was found in the southern Rift sites, which had small trees, usually growing sparsely. These patterns were less pronounced for leaf biomass, probably because leaf mass increases less steeply with tree size than does total biomass.

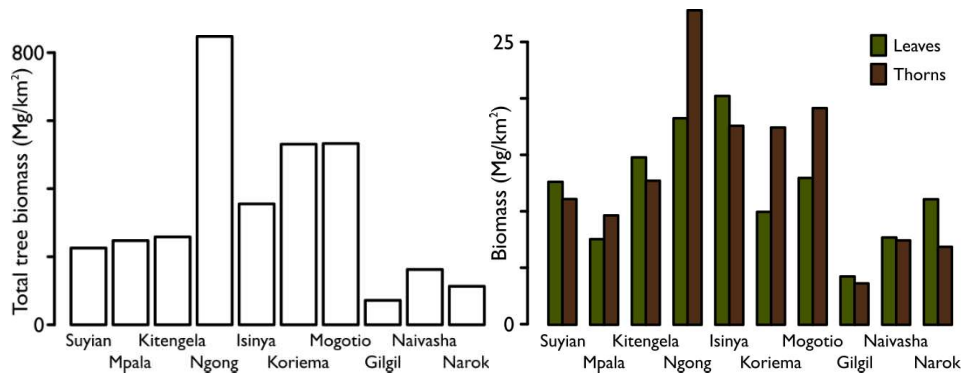


Figure 3.6: Total tree biomass (left) and leaf and thorn biomass (right) varies at different sites across Kenya.

Discussion

The *V. drepanolobium* system shows considerable variability across its range in central and southern Kenya. Much of this variation comes from comparing the sites in the Eastern Highlands to the sites in the Rift Valley, where the tree is less common, patches are smaller and sparser, and tree sizes vary greatly. However, there is substantial variation even among the eastern highlands sites, among which densities, average sizes, and tree biomass vary by a factor of 2 or more. Nor is the variability confined to the plant; ants show equally broad differences. In particular, the four ant species found at Mpala are commonly found together only in Laikipia: we did not find *C. sjostedti* outside of Laikipia, and we found *C. mimosae* only very rarely outside of the Eastern Highlands (although it is also found at lower elevations in Tanzania: Hocking 1970). Even when comparing sites with the same species of ants, how the trees were divided among the ants varied: among our five highlands sites, numerical dominance varied, with the plurality, or even majority of trees being occupied by any one of *C. sjostedti*, *C. mimosae*, or *C. nigriceps*, depending on the site, and with *C. mimosae* and *C. nigriceps* having greater biomass than the other species depending on the site as well.

Many interspecific associations show considerable variation across their geographic range (Thompson 2005), and the *V. drepanolobium* mutualism appears to be another such system. In light of the data presented here, it is clear that the dynamics of this mutualism vary broadly across the Kenyan landscape. For instance, *T. penzigi* does not defend trees against large herbivores at Mpala (Palmer and Brody 2007) or Kitengela (Martins 2010); if this is true in the Rift as well, then *V. drepanolobium* at sites like Gilgil or Lake Naivasha, where *T. penzigi* dominates, are undefended against herbivores. Likewise, if the competitive hierarchy is the same throughout the range, then *C. nigriceps* is a subordinate ant in the eastern part of the study area, but the dominant ant at sites in the Rift where *C. sjostedti* and *C. mimosae* are rare or absent.

This study is also one of the few to quantify the absolute biomass of individual ant species. Hölldobler and Wilson famously estimate that, across the entire earth, the biomass of ants as a group is roughly equivalent to that of humans (2009); however, more fine-grained estimates are rare. Most studies quantify relative biomasses, by collecting ants in traps or by recording the number that pass over an area in a given time (e.g. Hoffmann and Parr 2008, Gibb and Hochuli 2003). These estimates are useful for comparing relative biomasses of ants to each other, but they cannot give absolute biomasses in a given area without also knowing what proportion of the ants in an area are captured using these methods, which is a difficult parameter to estimate. Even when this is accomplished (e.g., Abbott 2005), the resulting estimates include only those ants which leave the nest, which is usually only workers. Getting total colony biomass requires access to nests, which are usually cryptic and/or subterranean, and thus difficult to find. Even when found, considerable trouble is involved in excavating the nest in a way that allows the recovery of

the ants for biomass estimates (e.g. Tschinkel 2005, Cerquera and Tschinkel 2010). Studies which not only quantify biomass within a nest, but also count the number of nests in a given area, are thus extremely rare. Here, we take advantage of the fact that three of the species of acacia-ant nest entirely in the tree's swollen thorns, allowing us easy access to the entire colony.

The three domatium-dwelling acacia-ants combined to account, on average, for a biomass of 96 kg/km². This is about an order of magnitude less biomass per area than those ants for which estimates exist, *Anoplolepis gracilipes*: 1850 kg/km² (workers only, Abbott 2005), and *Solenopsis invicta*: 1500-2800 kg/km² (Macom and Porter, 1996). However, both species are notorious invasives, which may replace entire ant communities (Hoffmann and Parr 2008). We do not know enough about the biomasses of non-invasive ants to compare our estimates to other native species.

Having an absolute estimate of biomass per area permits comparisons to many other (non-ant) species. The best-studied browsers of *V. drepanolobium* are elephant (*Loxodonta africana*) and giraffe (*Giraffa camelopardalis reticulata*), but other browsers include Grant's gazelle (*Gazella granti*), oryx (*Oryx beisa*), steenbok (*Ramphicerus campestris*), and eland (*Taurotragus oryx*) (Young and Okello 1998). Estimates of the biomasses of these species in Laikipia vary from study to study (Table 3.4). Our estimates of acacia-ant biomass at the two Laikipia sites were 33 kg/km² and 93 kg/km², which is roughly comparable to the total biomasses of either giraffe, eland, or grant's gazelle; it is substantially less than the elephant biomass reported by Augustine at Mpala Research Centre (2010), although the number of elephants/km² reported there (1.7) is substantially higher than the average in this region (0.3) reported by Ihwagi *et al.* (2015), so the average

elephant biomass across Laikipia may be closer to hundreds of kg/km² than thousands. The studies of Mizutani (1999) and Georgiadis *et al.* (2007) include both black cotton soil areas where *V. drepanolobium* dominates, as well as other areas where *V. drepanolobium* is rare, and Augustine’s study (2010) focuses on a red soil area with little *V. drepanolobium*, so these values are not exact estimates of herbivore pressure on *V. drepanolobium*, especially since these herbivores tend to spend more time on in red soil habitats, moving onto black cotton soils during periods of drought (Dino Martins, *pers. comm.*). The unpalatability of the *V. drepanolobium* tree is likely an important reason why browsers prefer habitats with other trees, and black cotton soil sites are often nearby red soil areas, so these studies provide an estimate of the potential browsing pressure on *V. drepanolobium* trees were they undefended.

Table 3.4: Estimates of herbivore biomasses in Laikipia, Kenya are comparable to the 33-93 kg/km² of acacia ants found in this region.

Species	Mizutani (1999) ¹	Georgiadis <i>et al.</i> (2007) ²	Augustine (2010) ³
Elephant	30.5 (0-108.7)	n/a	2882 (1420-5858)
Giraffe	43.4 (0-105.5)	32-302	244 (101-593)
Eland	155.5 (0-344.2)	27-210	125 (52-300)
Grant’s gazelle	19.3 (12.8-29.2)	10-42	n/a
Oryx	0.8 (0-2.5)	n/a	n/a
Steenbok	6.2 (3.1-12.2)	n/a	5 (3-9)

All values are in kg/km².

¹ Values shown are estimates and 95% confidence interval for the “Hills & Valley” region, which includes *V. drepanolobium* savanna.

² Values shown are the minimum and maximum estimates from the three different ranch types studied.

³ Values shown are estimates and 95% confidence interval for presence of herbivores on red soils (where *V. drepanolobium* is rare).

Regardless, order-of-magnitude comparisons of the biomasses of the guild of acacia-ants to the guild of possible browsers of *V. drepanolobium* highlights the impressive defensive abilities of these ants: acacia-ants in the tens of kg/km² defend against herbivores whose total biomass is in the hundreds or thousands of kg/km². The ant biomass can also be usefully compared to two other anti-herbivore defenses of *V.*

drepanolobium: thorns and secondary compounds in the leaves. The biomass of *V. drepanolobium* thorns is about 100 times larger than the biomass of the acacia-ants (Table 3.3). Tannin content of *V. drepanolobium* leaves varies from 8-20%, depending on the conditions under which it is measured (Ward and Young 2002, Rubanza *et al.* 2005), whereas the biomass of the ants relative to the leaves is about 1% (Table 3.3). The amount of biomass devoted to ant defenses is thus about 1% of the biomass devoted to thorns, and about 5-10% of the biomass devoted to tannins.

We do not know the relative contributions of defensive ants, thorns, and secondary defensive compounds in deterring grazing of *V. drepanolobium*. However, the differences in biomass between ants and thorns or tannins are one or two orders of magnitude, so it seems likely that the ants are a relatively biomass-efficient investment in herbivore defense. Furthermore, all of the biomass invested in tannins and thorns comes from the tree, meaning that the tree pays an opportunity cost of not investing in growth or reproduction. However, the ant biomass does not come entirely from the tree, as the ants forage off the tree and prey on insect herbivores (Hocking 1970, Martins Visitacao 2011). Hocking estimated that about half of the energy budget of the ant colonies in this system comes from extrafloral nectar (1970). Some of the remainder may come from the tree indirectly, as the species of *Crematogaster* tend sapsucking scale insects to various extents (Young *et al.* 1997b); nevertheless, the biomass of the ants represents only a partial opportunity cost to their host trees.

The ants do have costs that *V. drepanolobium*'s intrinsic defenses do not: for one, the biomass put into thorns and tannins represent a one-time cost paid when the structures are first grown (although these defenses are themselves inducible: Ward and Young 2002,

Goheen *et al.* 2007), while the ant colony requires an ongoing investment in extrafloral nectar to maintain. Furthermore, defensive ability varies among the ants: investment in an ant colony may result in strong anti-herbivore defense if the colony is *C. mimosae* or *C. nigriceps*, but less if the tree is occupied by *C. sjostedti* or *T. penzigi*. Even *C. mimosae* and *C. nigriceps* exact other costs on the tree, as *C. nigriceps* prunes reproductive structures, and *C. mimosae* has the greatest tendency to tend scale insects (Young *et al.* 1997b). The variability of benefits and the ongoing cost of maintaining a colony means that the ants may not be as efficient of an investment in defense as biomass makes them appear. For instance, Stanton *et al.* suggested that the ants may, in fact, be costly on average, but represent a sort of insurance policy against the catastrophic damage than can be inflicted by elephants (2011). The *V. drepanolobium* system is fertile ground for more research on the relative costs and benefits of three different defensive mechanisms: thorns, tannins, and ants. However costly the defensive benefits of the ants are for their acacia hosts, it is nevertheless impressive that the investment is in such a small biomass relative to that invested in thorns and tannins.

Chapter 4: Population genomics show varying effects of landscape on the partners in the *V. drepanolobium* ant-plant mutualism.

Abstract

A well-studied ecological system is the mutualism between the *Vachellia drepanolobium* acacia tree and the ants which inhabit it and protect their host trees from herbivores. This system has provided insight on the boundaries between mutualism and parasitism, the response of symbioses to environmental perturbations, species coexistence, and more. In Chapter 3, we showed that the *V. drepanolobium* system in Laikipia (where most of the work in this system has taken place), is not representative of the system as a whole. In light of this, we used a landscape genomics approach to test whether the ecological diversity of this system is mirrored in the genetic structure of the different partners, finding that population structure does not always separate sites with very different ecology. We also compare the population structure of the host plant and its ant symbionts, finding broadly similar patterns of structure among the ants, but different patterns when comparing them to the tree. This suggests that lineages of these mutualists must adapt to a variety of ecological conditions.

Introduction

Dark, clay, “black cotton” soils in East Africa are dominated by a symbiosis between the thorn tree *Vachellia drepanolobium* and several species of associated ants, which defend the tree from herbivores in return for nest space (Hocking 1970, Young *et al.* 1997b). Well-studied on the Laikipia Plateau in Kenya, this system has been used to study ecological questions such as defining the boundaries between parasitism and mutualism, explaining species coexistence, and investigating how symbioses respond to environmental change (e.g., Stanton *et al.* 2002, Palmer *et al.* 2010, Riginos *et al.* 2015).

In Chapter 3, we extended our understanding of this system by surveying the sites in and outside of the Rift Valley, and we found wide differences from site to site in tree size and density, as well as which ant species were present and which were dominant. In light of the dramatic variation of this system across Kenya, here we report a population genomic study of the *V. drepanolobium* tree and its three mutualist ants, *Crematogaster mimosae*, *C. nigriceps*, and *Tetraponera penzigi*. The fourth major mutualist at Mpala, *C. sjostedti*, is found only in Laikipia (see Chapter 3), and was not included in the study.

We use population genetic data to ask a number of questions about this system. Firstly, do the different partners in this mutualism share similar population structures, or does gene flow among populations of the host tree show different patterns than those found in its symbiote ant species? How mutualism affects the landscape genetics of the partners is still an unresolved issue: relatively few studies, mainly on microbe-macrobe mutualisms, investigate this question. Despite the paucity of studies, previous work has found a wide range of possible outcomes: population structures may be broadly congruent, as in a fungus-beetle symbiosis (Roe *et al.* 2011) or some lichen (Werth *et al.* 2012, Widmer

et al. 2012), or not, as in a cnidarian-dinoflagellate mutualism (Thornhill *et al.* 2013), a lichen which showed less population structure than its algal symbiont (Werth and Sork 2010), or the leaf-cutter ant-fungus mutualism, in which many host ant species associate with a single fungal symbiont (Mikheyev *et al.* 2006). To our knowledge, this is the first study to compare the population genetic structure of the partners in an ant-plant mutualism (but see Bänfer *et al.* 2006 for a comparison of the phylogenetic structure of mutualists in the diffuse, multispecies *Macaranga* ant-plant).

A population genetic comparison of the partners in the *V. drepanolobium* mutualism is particularly interesting because of the documented behavioral variation in the ant partners. The various behaviors of the ants have been well-described in Laikipia; at a landscape scale, do we see signs of these behaviors, particularly hierarchy in short-range colonization ability? Do the different partners adapt to local environments, both physical environments (i.e., temperature, rainfall), and mutualistic environments (i.e., the presence or absence of certain species or genotypes)?

Answering these questions will enable us to assess how applicable the substantial body of research from Mpala is to the *V. drepanolobium* system at other sites in Africa. These questions are also important for understanding mutualisms more generally, especially non-pairwise, multi-partner mutualisms. Mutualisms are often not geographically homogenous, pairwise interactions (Stanton 2003, Thompson 2005). However, our understanding of the consequences of this complexity on population structure and gene flow are still lagging. Here we address this question by comparing the population structures of *V. drepanolobium* and its three common ant associates.

Methods

Collections

Between 2012-2016, at sites around Kenya, we collected tissue from *V. drepanolobium* and its three primary ant associates, *C. mimosae*, *C. nigriceps*, and *T. penzigi*. These sites included five sites in the highlands east of the Rift Valley, and five in or adjacent to the Rift (Figure 4.1). We did not include *C. sjostedti* in our population genetic study, because we found it on *V. drepanolobium* trees only in Laikipia, at two adjacent sites, Suyian Ranch and Mpala Research Centre (as described in Chapter 3). *V. drepanolobium* leaf samples were dried and stored in desiccant, while workers of each species of ants were collected into 95-100% ethanol or into a buffered salt solution.

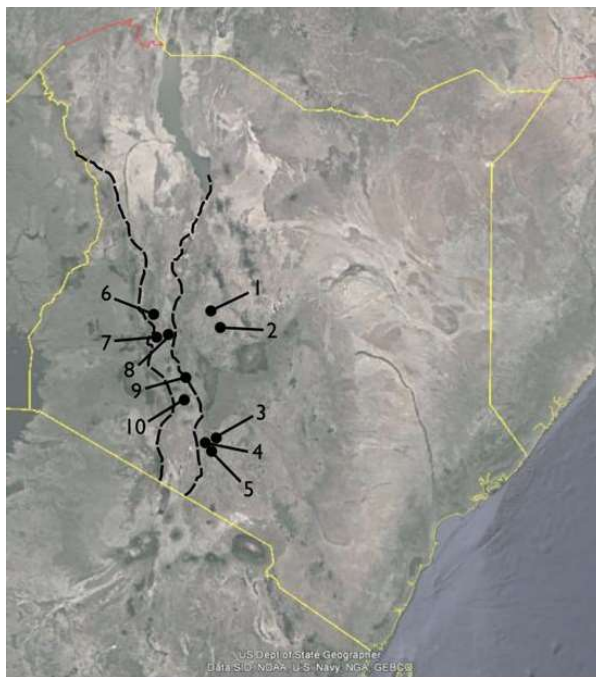


Figure 4.1: Collection sites in Kenya

Northern highlands east of the Rift

1. Suyian Ranch

2. Mpala Research Centre

Southern highlands east of the Rift

3. Kitengela

4. Ngong Hills

5. Isinya Road

In and around the Rift: north

6. Koriema

7. Mogotio-Koriema Road

8. Mogotio

In and around the Rift: south

9. Gilgil

10. Lake Naivasha

Dashed lines represent the approximate edges of the Eastern Rift Valley.

Map image: Google Earth.

DNA extraction and sequencing

We extracted DNA from each ant using an AutoGenprep 965 Tissue/ES Cell DNA Extraction Kit. For extractions of *V. drepanolobium* gDNA, we performed the lysis step using

the CTAB buffer of Cullings (1992), to which was added 5 $\mu\text{L}/\text{mL}$ β -mercaptoethanol. Genomic DNA was stored at -20°C before use.

The amount of genomic DNA was then increased by whole genome amplification, using the REPLI-g mini kit in 15 or 20 μL reactions.

We used the double-digest restriction-site associated DNA sequencing (RADseq) protocol of Peterson *et al.* (2012). We modified their protocol in a number of respects: we started with an (amplified) genomic DNA mass of 150 ng, which we then digested with the restriction enzymes EcoRI-HF and BfaI (for the ants) or EcoRI-HF and MspI (for *V. drepanolobium*). Bead cleanups throughout the protocol were performed with a MagNA bead solution described by Rohland and Reich (2012). We used the 48 inline indices for EcoRI described in the Sequences-S1 spreadsheet in the supplement of Peterson *et al.* (2012). We chose a range of 264-336 bp for the size selection step, which we performed using 2% ethidium bromide cassettes on a Sage Science Pippin Prep machine. The final PCR was set for 10 cycles.

These libraries were then sequenced in 100 bp, single-end reads on an Illumina HiSeq 2000 and 2500 at the Harvard University Bauer Core Facility.

DNA sequence alignment and base-calling

To demultiplex the Illumina libraries, as well as to align reads across worker ants and call single nucleotide polymorphisms (SNPs), we used the program Stacks version 1.21 (Catchen *et al.* 2011, Catchen *et al.* 2013). Reads were demultiplexed using the *process_radtags* function of stacks, rescuing barcodes and RAD-tags, and disabling checking if the RAD site was intact.

We quality filtered reads using the FASTX-Toolkit version 0.0.13 (http://hannonlab.cshl.edu/fastx_toolkit/). For each read, the first seven basepairs, including the EcoRI-HF restriction site and two often-low-quality bases, were removed using the *fastx_trimmer* tool. The trimmed reads were then quality-filtered using the *fastq_quality_filter* tool, removing any reads with a quality score of less than 25 at more than 2% of bases.

We then aligned all reads for all individuals within each species using the *denovo_map.pl* script of Stacks, allowing 2 mismatches between loci when processing a single individual, and 1 mismatch when building the catalog. To build the final matrix of SNPs, we culled individuals for which sequencing had failed or had produced too low coverage to be useful. We called SNPs using the *populations* program of Stacks. In order to explore the effects of missing data on our results, we produced three different SNP data sets: in the first data set, a SNP was only processed if it was found in 55% of the individuals within each species (the “-r” parameter in Stacks), producing a variable number of SNPs for each species, but similar amounts of missing data. In the second data set, we adjusted the “-r” parameter to produce a similar number of markers within each species, but with variable amounts of missing data. We used the same individuals in the first and second data set. In the third data set, we used a subset of the individuals from the first two data sets, and included SNPs only if they were found in 100% of individuals within each species. This produced a matrix with no missing data. In all data sets, we filtered out sites with an unusually high heterozygosity (i.e., over 0.6), and outputted only a single, randomly-selected SNP per RADseq fragment.

Population statistics

We divided the 10 sites into four populations based on their geography; these populations also broadly corresponded to the genetic clustering found by DAPC (described below). As described in Figure 4.1, these corresponded to northern and southern populations in and around the Rift, and northern and southern populations in the highlands east of the Rift. For *C. mimosae*, only a single colony was found in any of the Rift sites; we included the representative worker from this colony in the southern highlands population. For each population, we used Arlequin 3.5.2.2 (Excoffier and Lischer 2010) to calculate summary statistics and pairwise F_{ST} between each population, after first translating Stacks' genepop-format output files into Arlequin using PGDSpider 2.0.9.0 (Lischer and Excoffier 2012) in combination with custom perl scripts. Since we analyzed several data sets with varying degrees of missing data, we set Arlequin's internal missing-data threshold to 1 (i.e., to include all loci with any data).

Analysis of molecular variance (AMOVA)

We used an AMOVA to compare how much genetic variation was partitioned by the four geography-based populations (F_{CT}) versus how much population-level variation was partitioned by the collection sites within each population (F_{SC}). We used Arlequin to perform locus-by-locus AMOVAs, presenting the average F_{SC} and F_{CT} across all loci. We also report whether each measure is significantly greater than zero, as determined with 10,000 permutations.

Genetic clustering analysis

To divide our individuals into genetic clusters, we used the adegenet 2.0.1 package (Jombart 2008, Jombart and Ahmed 2011), in R version 3.2.3 (R Core Team 2015). For each

data set, we identified a number of clusters and assigned individuals to clusters using `find.clusters()`, with a maximum possible cluster number of 20, retaining all principle components, and selecting the cluster number with the lowest BIC score.

For *V. drepanolobium*, we tested whether trees assigned to a particular genetic cluster were more commonly occupied by colonies of a particular ant species. For each genetic cluster, we performed a multinomial exact test of goodness of fit test to determine whether the distribution of ant occupants within each genetic cluster was different from expected. To determine the expected ant-occupancy frequencies for each of the tree's genetic clusters, we averaged the frequency of each ant species at the sites at which that cluster was found, weighting each site by the number of trees from that cluster which were present there. We considered only those sites where trees from multiple genetic clusters coexisted, and we tested association with four categories of ant occupant: *C. mimosae*, *C. nigriceps*, *T. penzigi*, and trees empty or occupied by a different species. When the expected value was zero for one of these species of ant occupant, we dropped that species from the test. We performed all tests using the `xmulti()` function from the `XNomial` package in R (Engels 2015).

Isolation by distance and environment

We then asked whether geographic structure was associated with environmental differences among sites, which would suggest adaptation to the local environment. To distinguish between isolation by environment and isolation by distance, we used the Multiple Matrix Regression with Randomization approach of Yang (2013), as implemented in the `MRM()` function of the R package `ecodist` (Goslee and Urban 2007), testing significance with 1000 permutations. We characterized the environment using the

bioclimatic variables for each site from the WorldClim database at 10 degree-minutes precision (Hijmans *et al.* 2005). Following He *et al.* (2016), we created clusters of climatic variables, such that each variable had a correlation of > 0.9 (or < -0.9) with at least one other variable in that cluster, and with no variables outside the cluster. We then chose one exemplar variable from each cluster, selecting Annual Mean Temperature, Temperature Annual Range, Annual Precipitation, Precipitation of the Warmest Quarter, Precipitation of the Wettest Quarter, and Precipitation of the Driest Quarter. In the case of the Lake Naivasha site, specimens were collected from five nearby locations along the South Lake Road. Since each location had different climate variables, we treated them as separate sites for this analysis. For each species, the climate data from all sites were then scaled to have a mean of zero and standard deviation of one; environmental distance was then estimated as the Euclidian distance between each site for these six variables. Geographic distances between each site were calculated using the `distGeo` function from the `geosphere` package (Hijmans 2016). Genetic distances between each site were calculated using Nei's distance, as implemented by the `dist.genpop()` function of `adegenet`.

Coalescent analysis

To quantify the degree to which the four mutualists moved through the landscape in different ways, we used `fastsimcoal2.5.2.21` (Excoffier and Foll 2011, Excoffier *et al.* 2013), which uses a coalescent approach to estimate population parameters from site frequency spectra (SFS), which summarize the distribution of minor allele frequencies within and between populations. We used Arlequin to produce site frequency spectra for each species, using data set 3, since this requires a data matrix without missing data. The stacks data set used to produce site frequency spectra includes only single-nucleotide polymorphisms, and

not sites that are invariant across all populations. We calculated the number of these sites for each species as $L_v * 89 / (S / (L_v + L_i))$, where L_v and L_i are the number of loci with and without any variable sites, respectively; S is the number of variable sites across all loci; and 89 is the length of the sequenced fragment. This value, rounded to the nearest integer, was added to the number of completely invariant sites for each site frequency spectrum.

These SFS were then used to estimate a number of parameters for each species, including effective population size, growth rates for each population, and migration rates between each population (see Figure 4.2). We used pairwise F_{ST} values for each species to determine which populations coalesced (for *V. drepanolobium*, the other populations coalesced with the Northern Highlands population; for *C. nigriceps*, with the Southern Highlands site; for *T. penzigi*, with the Southern Rift site). The following settings were used to estimate parameters for each run: 100,000 simulations were performed to estimate the expected derived SFS; a minimum and maximum of 10 and 20 conditional maximization cycles, respectively, were used to estimate parameters, with a stop criterion of 0.001. Initial parameters were drawn from a log-uniform prior from 10 to 10^9 for population sizes, a uniform prior from 10 to 10,000 for divergence times, and a log-uniform prior from 10^{-8} to 10^{-3} for migration rates. We performed 100 runs for each species. We present here the results from the run with the highest likelihood, as well as the parameters from all 100 runs, weighted by their likelihood using the Hmisc package in R (Harrell 2016).

Results

DNA sequence alignment and base-calling

As seen in Table 4.1, we successfully genotyped individuals of each species at between 142 and 5258 loci, depending on the species and the data set.

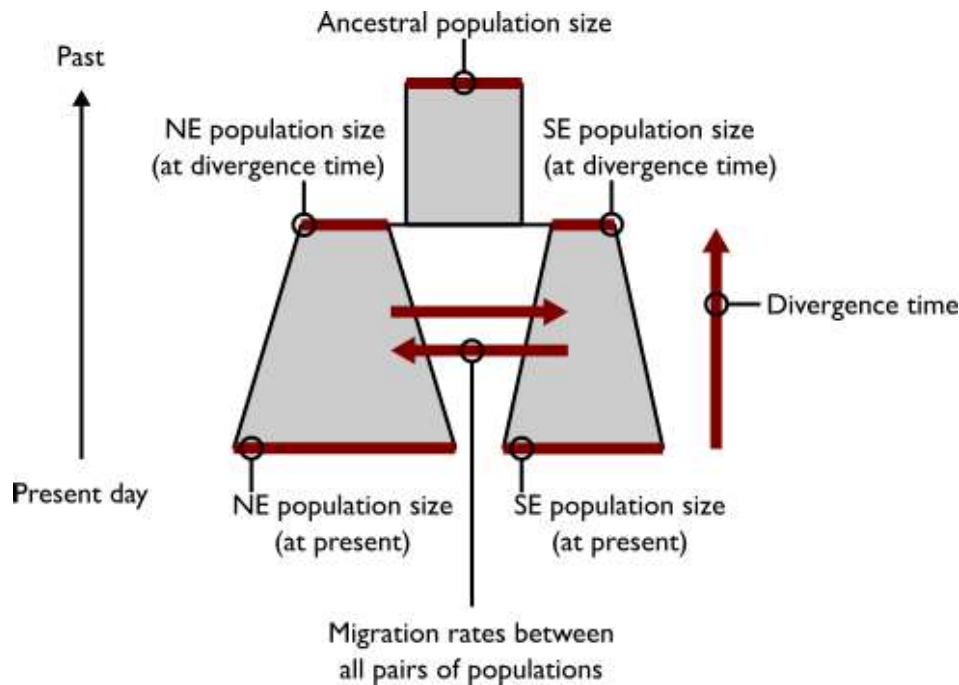


Figure 4.2: Populations parameters estimated by fastsimcoal2, for *C. mimosae*. Estimated parameters are shown in red. Population growth rates were calculated as $\log(\text{Population size at divergence time}/\text{Population size at present})/\text{Divergence time}$. Growth rates could be positive or negative depending on the estimates of population sizes. For simplicity, we assumed that the ancestral population(s) were static, without migration between them.

Table 4.1: results of RADseq sequencing and base-calling

Species	Data set	Stacks -r ¹	Individuals	Loci	Missing data
<i>V. drepanolobium</i>	1	0.55	162	169	0.38
	2	0.5	162	464	0.44
	3	1	37	142	0
<i>C. mimosae</i>	1	0.55	62	1485	0.35
	2	0.6	62	1012	0.32
	3	1	26	559	0
<i>C. nigriceps</i>	1	0.55	108	5258	0.24
	2	0.9	108	665	0.06
	3	1	76	761	0
<i>T. penzigi</i>	1	0.55	110	2816	0.26
	2	0.8	110	981	0.14
	3	1	63	372	0

¹ The -r parameter in stacks denotes the minimum proportion of individuals in which a SNP must be found in order to be included in the data set.

Population statistics

The three data sets showed broadly similar patterns of population structure, despite differences in the individuals and SNPs making up each set. These statistics are given in Tables 4.2 and 4.3 and visualized in Figure 4.3.

V. drepanolobium populations generally had lower values of θ than any of the ants, suggesting relatively small effective population sizes. θ values should be compared across species with caution, since θ is a product of effective population size multiplied by four times the mutation rate, which is unknown for the four species in question (or eight times the mutation rate for *V. drepanolobium*, which is tetraploid: Bukhari 1997). Assuming that the mutation rate is similar in all populations within a species, population sizes were larger in the Eastern Highlands populations than in the Rift populations. F_{ST} for *V. drepanolobium* were relatively low, suggesting that there are fewer barriers to gene flow for the tree than for its ant associates. Most of the structure in *V. drepanolobium* populations was between the Northern Rift (NW) population and the other three populations; F_{ST} values between the other three populations were much lower. Low levels of gene flow between the Northern Rift population and the two highlands populations was expected, considering the important role the Rift Valley plays in the genetics of other African acacias (Omondi *et al.* 2010, Ruiz Guajardo *et al.* 2010). However, this does not explain the high F_{ST} between the two Rift sites, or the low F_{ST} between the Southern Rift and the highlands regions. This suggests that the barrier effect of the Rift may vary across its length, or possibly that the Rift does not constitute a strong barrier to gene flow for *V. drepanolobium*, and some other feature of the landscape separates the Northern Rift population from the other three.

Among the ants, these summary statistics suggested that *C. nigriceps* and *T. penzigi* have roughly similar population structures, and are both distinctly different from *C. mimosae*. *C. mimosae*, which is the worst colonizer of the three at Mpala, also spreads the least widely across the area studied, as it is only commonly found in the Eastern Highlands sites. However, it shows much less population structure between the two Eastern Highlands sites ($F_{ST} = 0.122-0.165$) than do the other two ants ($F_{ST} = 0.326-0.472$), suggesting that the Eastern Highlands region poses lower barriers to gene flow for *C. mimosae* than it does for *C. nigriceps* and *T. penzigi*.

All three ants also contrast with *V. drepanolobium*, not only in their larger values of θ , but also in which populations contain the most diversity. *V. drepanolobium* populations are larger in the east than in the west; in the ants, the larger populations are the two southern ones (or, for *C. mimosae*, the one southern one).

Table 4-2: Population genetic summary statistics each species.

Stat. ¹	Data set	<i>V. drepanolobium</i>			<i>C. mimosae</i>			<i>C. nigriceps</i>			<i>T. penzigi</i>				
		NE	SE	NW	SW	NE	SE	NW	SW	NE	SE	NW	SW		
H ₀	1	0.0791	0.0391	0.0574	0.0344	0.120	0.128	0.204	0.135	0.198	0.156	0.164	0.104	0.203	0.118
	2	0.0794	0.0476	0.0750	0.0356	0.114	0.118	0.166	0.132	0.214	0.155	0.162	0.102	0.253	0.113
	3	0.0745	0.106	0.222	*	0.170	0.131	0.237	0.135	0.211	0.153	0.169	0.121	0.256	0.202
H _E	1	0.0968	0.0957	0.114	0.122	0.178	0.184	0.266	0.170	0.269	0.198	0.192	0.198	0.281	0.214
	2	0.0976	0.102	0.176	0.171	0.166	0.171	0.204	0.151	0.254	0.174	0.178	0.176	0.284	0.189
	3	0.0879	0.148	0.237	*	0.211	0.158	0.316	0.146	0.224	0.169	0.195	0.173	0.326	0.246
θ _π	1	4.27	2.40	1.22	1.60	55.9	91.8	93.2	245	161	432	87.0	157	41.8	112
	2	12.8	5.53	5.62	3.33	38.8	61.6	16.1	33.1	26.4	53.5	29.0	65.1	21.9	49.8
	3	8.08	8.12	2.85	*	59.6	60.3	26.2	43.8	25.3	67.8	10.9	37.9	11.1	40.4
θ _S	1	14.7	13.9	5.10	10.7	196	210	236	499	192	627	155	352	61.6	259
	2	45.8	40.8	19.0	26.0	128	142	23.8	55.3	24.1	73.1	46.0	119	22.6	89.2
	3	21.6	15.1	3.97	*	79.5	94.6	24.1	69.2	25.7	92.2	12.6	53.6	9.58	43.9
F _{SC}	1		0.0611	(<i>p</i> < 1e-5)		0.120	(<i>p</i> < 1e-5)		0.0420	(<i>p</i> < 1e-5)			0.212	(<i>p</i> < 1e-5)	
	2		0.0689	(<i>p</i> < 1e-5)		0.118	(<i>p</i> < 1e-5)		0.0222	(<i>p</i> < 1e-5)			0.194	(<i>p</i> < 1e-5)	
	3		0.0531	(<i>p</i> = 0.05)		0.0928	(<i>p</i> < 1e-5)		0.0112	(<i>p</i> < 1e-5)			0.215	(<i>p</i> < 1e-5)	
F _{CT}	1		0.0858	(<i>p</i> = 1e-5)		0.105	(<i>p</i> < 1e-5)		0.474	(<i>p</i> < 1e-5)			0.457	(<i>p</i> < 1e-5)	
	2		0.104	(<i>p</i> < 1e-5)		0.102	(<i>p</i> < 1e-5)		0.506	(<i>p</i> < 1e-5)			0.450	(<i>p</i> < 1e-5)	
	3		0.187	(<i>p</i> < 1e-5)		0.132	(<i>p</i> < 1e-5)		0.473	(<i>p</i> < 1e-5)			0.475	(<i>p</i> < 1e-5)	

¹ Observed and expected heterozygosities (H₀ and H_E) are calculated across variant sites within each population. F_{SC} corresponds to the proportion of genetic variation within each of the four geographically-determined populations which is partitioned among sites; F_{CT} corresponds to the proportion of total genetic variation partitioned among the four populations. After each is a *p*-value from an AMOVA indicating whether the partitioning of genetic variation is significantly greater than zero.

* No *V. drepanolobium* individuals from the SW population had high enough coverage to be included in data set 3.

Table 4.3: Pairwise FST between populations for each species

Comparison	Data Set	<i>V. drepanolobium</i>	<i>C. mimosae</i>	<i>C. nigriceps</i>	<i>T. penzigi</i>
NE-SE	1	-0.0313	0.122	0.335	0.364
	2	-0.0553	0.133	0.427	0.340
	3	0.0552	0.165	0.364	0.326
NE-SW	1	-0.207		0.127	0.596
	2	-0.286		0.393	0.597
	3	*		0.285	0.617
NE-NW	1	0.0460		0.638	0.690
	2	0.147		0.674	0.712
	3	0.230		0.705	0.761
SE-SW	1	-0.0388		0.253	0.318
	2	-0.0532		0.280	0.304
	3	*		0.229	0.315
SE-NW	1	0.120		0.630	0.554
	2	0.189		0.620	0.560
	3	0.203		0.638	0.561
SW-NW	1	0.129		0.466	0.673
	2	0.138		0.458	0.669
	3	*		0.484	0.656

* No *V. drepanolobium* individuals from the SW population had high enough coverage to be included in data set 3, and thus there are no pairwise comparison between the SW population and the others.

Analysis of molecular variance

As shown in Table 4.2, AMOVA tests revealed significant genetic variation partitioned both among our geographic populations (F_{CT}), and also among sites within those populations (F_{SC}). This was true for all species and all data sets, except for data set 3, in which F_{SC} for *V. drepanolobium* was not significant. The species also varied in the magnitude of genetic variation partitioned among sites and populations. For *V. drepanolobium* and *C. mimosae*, both species has similar F_{SC} and F_{CT} values. However, for *C. nigriceps* and *T. penzigi*, F_{CT} values were much higher than F_{SC} values. This suggests that a greater proportion of total genetic variation is partitioned by large-scale geography in the latter two species than in the former two species. In addition, the absolute values of F_{SC} were quite high for *T. penzigi*, suggesting that both inter-population variation and intra-population variation are important in this species.

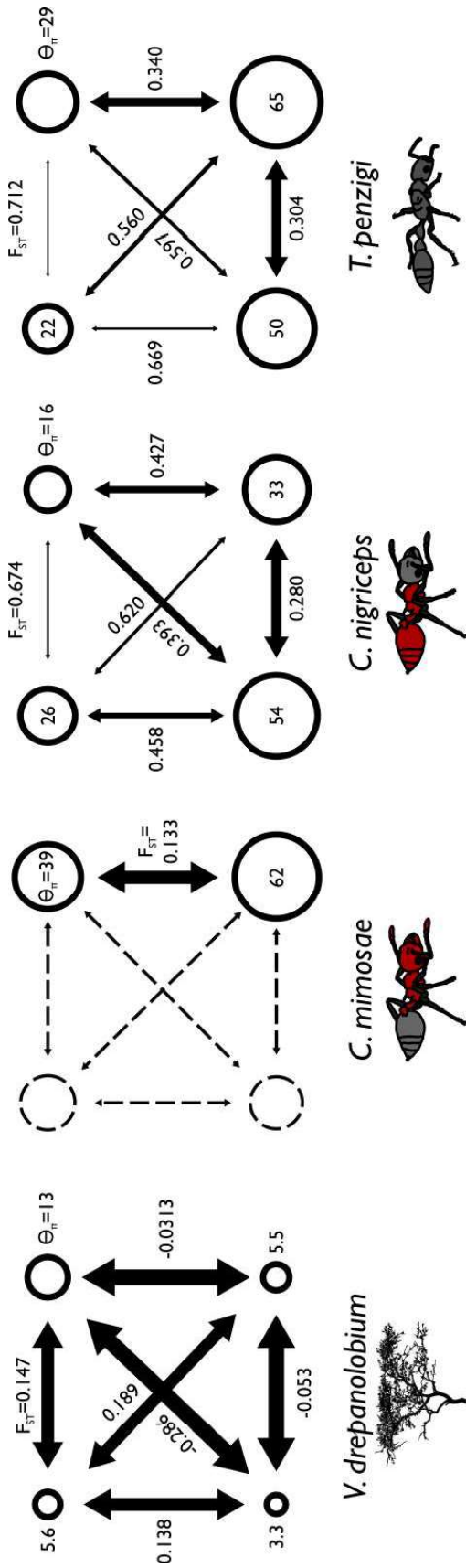


Figure 4.3: Summary statistics show differences in the population structure of the different species. The area of each circle is proportional to the θ_{π} value for each population; the arrows are scaled inversely to F_{ST} . Compared to the ants, *V. drepanolobium* has relatively low θ values, and also low F_{ST} values, showing high gene flow. The values shown are for data set 2.

Genetic clustering analysis

For *V. drepanolobium*, there was only weak correlation between the genetic clusters identified and geography. All three data sets show some degree of separation between the sites in the Northern Rift and the other sites: a majority of individuals fall into a single cluster at both Northern Rift sites, and individuals from this cluster are rare outside the Northern Rift. To the east, Northern Highlands sites are dominated by members of a single geographic cluster; however, members of this cluster also commonly found in other sites. Southern sites both within and outside of the Rift are mixtures of several different genetic clusters.

The ant species show much stronger associations between genetic clustering and geography. Across all data sets, each of the four geographic areas was dominated by a single cluster that was rarely, if ever found outside that region. The only exception to this was *T. penzigi*. In this species, the two genetic clusters were common in the Southern Rift sites, and the Ngong Hills site was dominated by one of these clusters, rather than clustering with the other Southern Highlands sites. In data set 3 for *T. penzigi*, the Ngong Hills site formed its own genetic cluster, distinct from both the Southern Rift and other Southern Highlands sites. Finally, the representative individual from the sole *C. mimosae* colony found in the Rift (at Lake Naivasha) clustered with the individuals from the Southern Highlands sites. Because the results were qualitatively similar across all three data sets, we present the results from data set 4 in Figure 4.4.

We found no evidence that trees assigned to different genetic clusters were associated with particular ant occupants (multinomial exact tests, $p > 0.1$ for all); this equally the case for all three data sets.

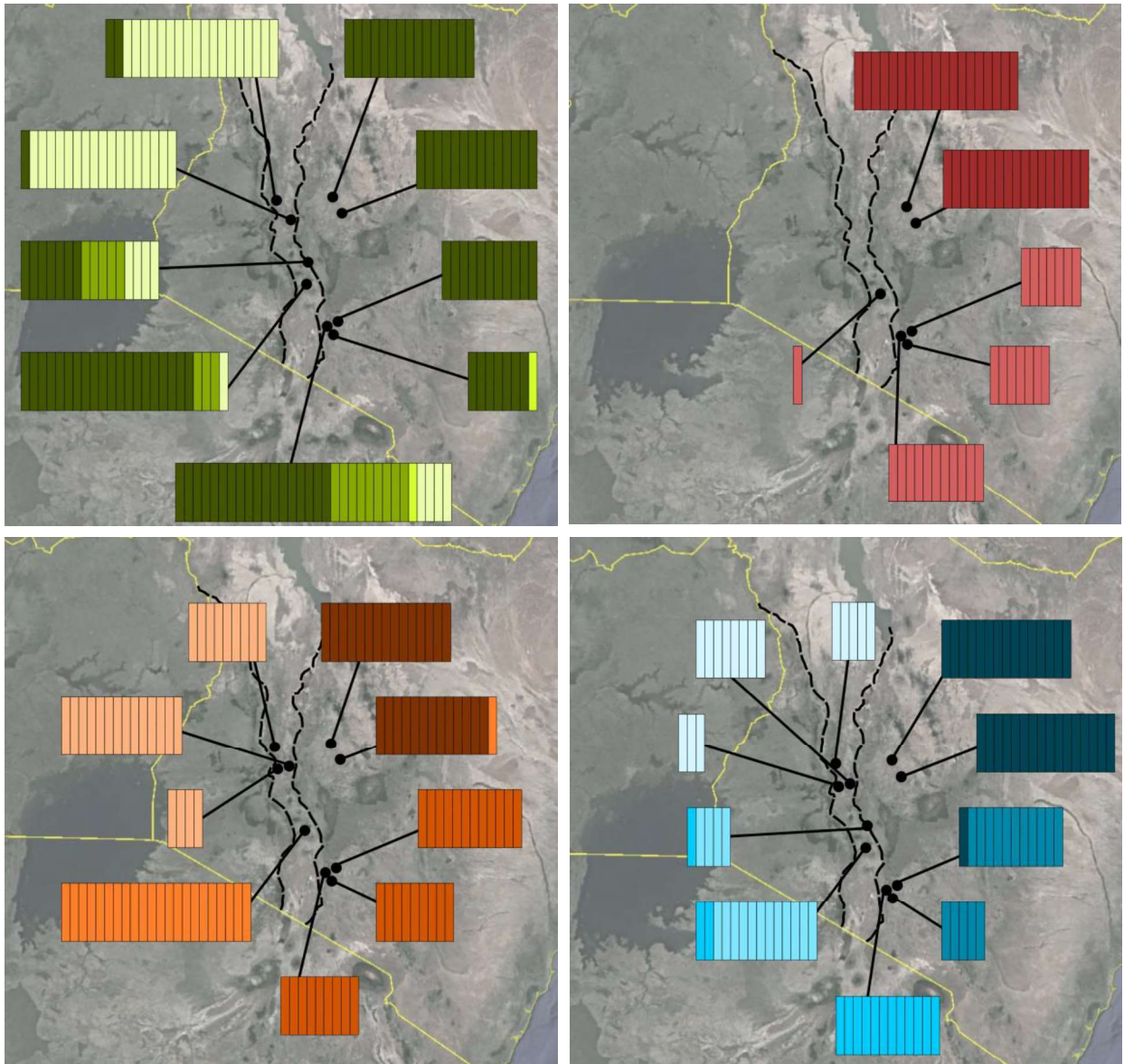


Figure 4.4: ants showed more populations structuring than did *V. drepanolobium*. Facets show the population clustering of (clockwise from upper left) *V. drepanolobium*, *C. mimosae*, *C. nigriceps*, and *T. penzigi*. Colors show what genetic cluster was assigned to each individual (narrow vertical bars) at each site. Images from Google Earth.

Isolation by distance and environment

Within each of the four species, the three data sets produced similar results. For both *V. drepanolobium* and *C. mimosae*, we found no evidence of isolation by distance or isolation by environment in any of the data sets (all p -values > 0.1). In the case of *C. nigriceps*, we

found evidence of both isolation by distance and isolation by environment (all p -values < 0.05). For *T. penzigi*, we found evidence of isolation by distance ($p < 0.01$ in all three data sets), but no evidence of isolation by environment ($p > 0.1$ in all three data sets).

Coalescent analysis

Population parameters estimated by fastsimcoal2 coalescent simulations revealed different dynamics in the various species (presented in Tables 4.4 and 4.5, and in Figure 4.5). In this analysis, which takes into account population growth and migration, population sizes in the *V. drepanolobium* tree were larger relative to the ants than the previously-determined θ values would suggest. However, we still observe the general pattern that *V. drepanolobium* population size was largest in the northeastern population, while ant population sizes were larger in the southern populations.

In addition, we find that *V. drepanolobium* populations are declining in the eastern highlands sites, but expanding in the Northern Rift location. On the other hand, *C. mimosae* and *T. penzigi* populations are generally increasing, although *T. penzigi* are decreasing in the Northern Rift; three of the four *C. nigriceps* sites are also in decline, while the Southern Highlands population is increasing. It is unsurprising that *C. nigriceps* thrives in the Southern Highlands sites, because further to the south and east, some sites have only *C. nigriceps* (Stapley 1999), suggesting that southern Kenya and Tanzania may form a source population for *C. nigriceps*.

Finally, divergence times vary among the different species, with *C. mimosae* having a noticeably shorter divergence time than the other species. This may be because *C. mimosae* has expanded more recently, or it may be that the ant species expanded together, but that fewer generations of *C. mimosae* have passed in the intervening time, which may well be

the case if *C. mimosae* is slower to reproduce, which may likely be the case at Mpala at least, given its position on the colonization-competition hierarchy (Stanton *et al.* 2002, Chapter 2). Further work on the generation time of the plant and ants will be necessary for these numbers to be interpreted accurately.

For individuals of the host tree, *V. drepanolobium*, migration was asymmetric, with more gene flow going from the northern populations to the Southern Highlands population than in the other direction. Migration rates between the northern populations was roughly symmetric, but relatively low.

For colonies of *C. nigriceps* and *T. penzigi*, migration was relatively low between the Northern Rift population and the other sites, when compared to migration rates between the other three sites. In the case of *C. nigriceps*, although gene flow to and from the Northern Rift was low, there was more gene flow going toward the southern populations than from the southern populations into the Northern Rift. For *T. penzigi*, migration rates were higher in the opposite direction, from the Southern Rift to the Northern Rift. We also saw asymmetrical migration in *T. penzigi* between the Northern and Southern Highlands (toward the Northern Highlands), and between the Northern Highlands and Southern Rift (toward the Southern Rift).

Table 4.4: Population parameter estimates from fastsimcoal2

Species	Population	Population size (in thousands)		Growth rate (forward in time)		Divergence time (10 ³ generations)	
		Mean	Best	Mean	Best	Mean	Best
<i>V. drepanolobium</i>	NE	247±325	200	$-(2.5±1.2)*10^{-4}$	$-6.1*10^{-5}$	44±0.3	45
	SE	172±28	97	$-(1.6±1.1)*10^{-4}$	$-1.2*10^{-4}$		
	NW	118±11	94	$(1.7±0.05)*10^{-4}$	$1.6*10^{-4}$		
<i>C. mimosae</i>	NE	75±1	84	$(9.1±1.0)*10^{-5}$	$1.4*10^{-4}$	26±1	15
	SE	110±90	130	$(1.7±0.05)*10^{-4}$	$1.9*10^{-4}$		
<i>C. nigriceps</i>	NE	5±0.1	6	$-(1.2±0.1)*10^{-4}$	$-1.2*10^{-4}$	40±1	40
	SE	69±0.3	69	$(2.0±0.1)*10^{-4}$	$2.0*10^{-4}$		
	NW	11±0.1	11	$-(3.1±0.2)*10^{-5}$	$-3.2*10^{-5}$		
	SW	30±1	28	$-(1.9±0.1)*10^{-4}$	$-2.0*10^{-4}$		
<i>T. penzigi</i>	NE	5±0.1	4	$(5.0±0.5)*10^{-5}$	$2.1*10^{-5}$	49±1	53
	SE	55±1	53	$(1.9±0.1)*10^{-4}$	$1.6*10^{-4}$		
	NW	1±0.02	0.9	$-(4.2±0.2)*10^{-4}$	$4.6*10^{-4}$		
	SW	26±0.3	24	$(4.3±0.1)*10^{-4}$	$4.9*10^{-4}$		

Mean values for each parameter are the mean ± standard error, weighted by the likelihood for that run. Divergence time is the number of generations backwards in time until all populations have coalesced.

Table 4.5: Migration rate estimates from fastsimcoal2

Migration	Data set	<i>V. drepanolobium</i>	<i>C. mimosae</i>	<i>C. nigriceps</i>	<i>T. penzigi</i>
NE→SE	Mean	$(4.1±0.5)*10^{-5}$	$(3.0±0.1)*10^{-5}$	$(2.9±0.2)*10^{-6}$	$(4.6±0.5)*10^{-6}$
	Best	$2.0*10^{-5}$	$2.0*10^{-5}$	$2.8*10^{-6}$	$2.0*10^{-6}$
SE→NE	Mean	$(7.4±0.5)*10^{-6}$	$(1.6±0.05)*10^{-5}$	$(4.7±2.7)*10^{-6}$	$(2.2±0.2)*10^{-5}$
	Best	$1.5*10^{-5}$	$1.8*10^{-5}$	$2.9*10^{-6}$	$2.1*10^{-5}$
NE→SW	Mean			$(9.0±0.5)*10^{-6}$	$(1.8±0.1)*10^{-5}$
	Best			$9.3*10^{-6}$	$1.5*10^{-5}$
SW→NE	Mean			$(1.6±0.1)*10^{-5}$	$(2.3±0.3)*10^{-7}$
	Best			$1.6*10^{-5}$	$1.4*10^{-7}$
NE→NW	Mean	$(7.2±4.1)*10^{-7}$		$(2.9±0.05)*10^{-7}$	$(2.7±0.7)*10^{-6}$
	Best	$1.8*10^{-9}$		$2.9*10^{-7}$	$1.0*10^{-6}$
NW→NE	Mean	$(6.2±0.8)*10^{-7}$		$(3.3±1.1)*10^{-7}$	$(1.9±0.2)*10^{-6}$
	Best	$3.1*10^{-10}$		$2.8*10^{-7}$	$7.8*10^{-7}$
SE→SW	Mean			$(1.6±0.03)*10^{-5}$	$(7.6±0.2)*10^{-5}$
	Best			$1.6*10^{-5}$	$9.3*10^{-5}$
SW→SE	Mean			$(2.0±0.1)*10^{-5}$	$(2.6±0.1)*10^{-5}$
	Best			$2.1*10^{-5}$	$2.3*10^{-5}$
SE→NW	Mean	$(2.5±1.4)*10^{-6}$		$(8.9±5.3)*10^{-8}$	$(7.3±1.3)*10^{-7}$
	Best	$3.8*10^{-6}$		$5.5*10^{-8}$	$3.6*10^{-7}$
NW→SE	Mean	$(2.4±0.3)*10^{-5}$		$(5.1±0.6)*10^{-7}$	$(7.6±0.4)*10^{-7}$
	Best	$1.6*10^{-5}$		$4.8*10^{-7}$	$7.0*10^{-7}$
SW→NW	Mean			$(6.5±0.4)*10^{-7}$	$(2.0±0.05)*10^{-5}$
	Best			$6.5*10^{-7}$	$2.1*10^{-5}$
NW→SW	Mean			$(3.9±0.1)*10^{-6}$	$(4.7±1.1)*10^{-7}$
	Best			$3.9*10^{-6}$	$8.3*10^{-8}$

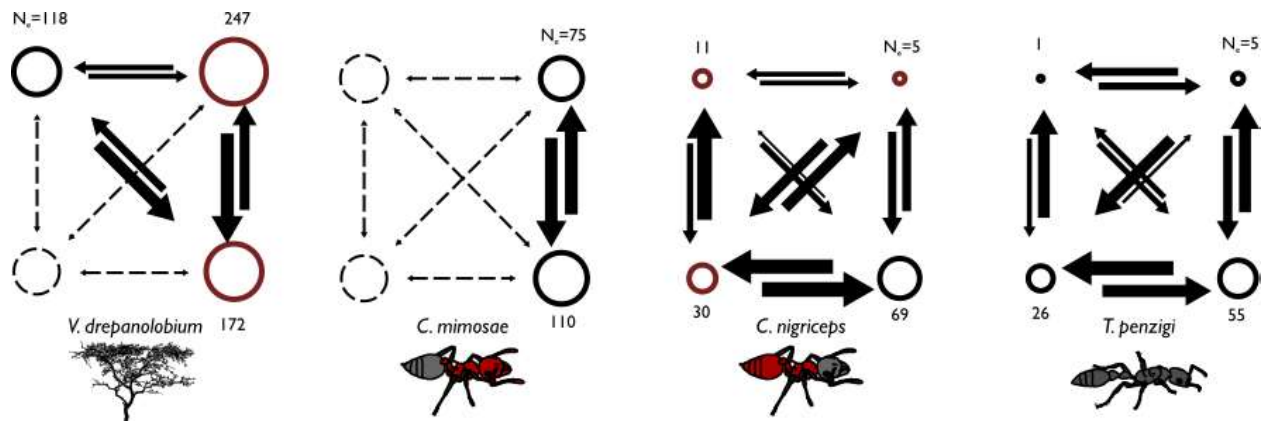


Figure 4.5: fastsimcoal2, like summary statistics, shows differences in the population structure of the different species. The area of each circle is proportional to the effective population size for each population; red circles show populations with a negative growth rate, black circles positive. The arrows are scaled with number of migrants. The values shown are for data set 2.

Discussion

We found that population structure varied widely among *V. drepanolobium* and its ant associates. Although all of the species showed substantial population structuring, *V. drepanolobium* had less structure than did its ant inhabitants: clusters of genetically similar individuals were spread across several different regions in Kenya, and most sites had individuals from more than one cluster, producing relatively low values of F_{ST} . These clusters were not associated with particular ant inhabitants, and the overall pattern is different from the patterns observed for the different ant species, which exhibited much stronger geographic structuring. Most genetic clusters for the ant species were found only in a single region of Kenya, and comprised almost all of the individuals within that region, leading to relatively high F_{ST} values.

Not only did the degree of population structuring among partners vary, but so did the characteristics of the populations themselves. In *V. drepanolobium*, the largest population was that found in sites in the Northern Highlands, which served as a source of

migrants to the Southern Highlands sites, although both Highlands populations appeared to be in decline.

Interestingly, although the tree populations are declining in these sites, the ant populations are growing (for all three species in the Southern Highlands and for *C. mimosae* and *T. penzigi* in the Northern Highlands). This apparent contradiction may be due to changing patterns of herbivory and disturbance. Single ant colonies of all species can spread across multiple trees (Palmer *et al.* 2010), so the population sizes of the ants and the trees are not necessarily linked. For instance, Palmer *et al.* found that in the absence of herbivores, the average number of trees occupied by a single *C. mimosae* colony declined, possible due to the trees producing fewer rewards for the ants (2008). Furthermore, if trees grow less densely in a patch, this might hinder the ability of single colonies to spread across multiple trees that are more distantly spaced from each other. In this scenario, a smaller number of trees could be occupied by more ant colonies, each on fewer trees, resulting in a net increase in the number of colonies, and thus the population size of the ant.

The ant species also differed from their host trees in other respects. While the largest population of *V. drepanolobium* was in the Northern Highlands area, the largest populations for all three ant species were the southern populations. This may be due to *C. sjostedti*, which may occupy as many as half of the trees in the Northern Highlands sites, but is absent in the southern sites. Thus, a larger population of trees in the Northern Highlands might be able to support fewer *C. mimosae*, *C. nigriceps*, and *T. penzigi* colonies due to competition from *C. sjostedti*. Other factors might also decrease the number of trees which single colonies in the southern sites occupy. More work on the degree of polydomy in different areas of Kenya is needed fully to interpret these results.

The ants differed among themselves as well. *C. nigriceps* and *T. penzigi*, the better colonizers at small scales at Mpala, were also the most widespread throughout Kenya: both species were found at almost every site in both the Highlands and in the Rift. *C. mimosae*, on the other hand, was commonly found at the Highlands sites, but only very rarely in the Rift. *C. nigriceps* and *T. penzigi* are thus also better colonizers at a Kenya-wide scale. However, when looking only at the Highlands sites where all three ants are commonly found, *C. mimosae* has lower F_{ST} values and (mostly) higher migration rates between the Northern and Southern populations; this suggests that *C. mimosae* is better at moving through this landscape than the other ants.

Finally, we found little evidence that genetic structure in this system is associated with environmental differences. Only for *C. nigriceps* did we find significant isolation-by-environment, suggesting that it may be more locally adapted to environmental conditions than are the other species. We also found little evidence that either side of the mutualism shows local coadaptation to the particular partners found at each site. We discount this possibility for several reasons: first, the genetic clusters of the tree are not associated with particular ant species, which is unsurprising considering turnover between ant associates can happen multiple times over a single tree's lifetime (Palmer *et al.* 2010). Second, the genetic clusters of the tree are not distributed in the same pattern as the genetic clusters of the ants, meaning that particular genetic lineages of *V. drepanolobium* interact with multiple different lineages of each ant species, reducing the opportunity for coevolution between particular lineages of *V. drepanolobium* and its mutualist ants. Furthermore, we find that, when considering both *V. drepanolobium* and its associated ants, single populations include sites with extremely different ant composition. For instance, the sites

at Suyian, Mpala, and Kitengela are dominated by a single genetic cluster of *V. drepanolobium*. However, each of these sites is dominated by different ant species, and not all of the sites have the same ant species present (Chapter 3).

These data suggest that the *V. drepanolobium*-ant mutualism is a much more complex system than has previously been appreciated. Both the plants and the ants experience a wide variety of conditions, both biotic and abiotic, over the breadth of the Kenyan landscape. Nor do we find any evidence of local adaptation to these conditions: instead, it seems likely that all the partners in the mutualism have experienced a wide array of different conditions at different places and times. In light of this variability, it would be unwise to assume that species dynamics at a single site are representative of those at all sites. Instead, expanding the horizons of this system to different areas with different ant communities will be important for any further work on selective forces maintaining mutualism in the *V. drepanolobium* savannah.

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Supplement

Table S1: The Queller-Goodnight method of calculating relatedness performed best in simulated data sets.

Species	Queller and Goodnight 1989	Li <i>et al.</i> 1993	Ritland 1996	Lynch and Ritland 1999	Wang 2000
<i>C. sjostedti</i>	0.983	0.982	0.963	0.979	0.981
<i>C. mimosae</i>	0.983	0.981	0.947	0.975	0.981
<i>C. nigriceps</i>	0.986	0.985	0.951	0.975	0.985
<i>T. penzigi</i>	0.968	0.964	0.916	0.959	0.964

The r value given for in each cell is the correlation between the true relatedness value and the relatedness value calculated using each method from simulated data.

Stem diameter produces the same results as tree height when used as a measure of tree size.

When trees had multiple stems at 0.5 meters each greater than 1 cm in diameter, we calculated the final diameter, d , as $d^2 = \sum d_i^2$, where d_i is the diameter of each individual stem at 0.5 meters.

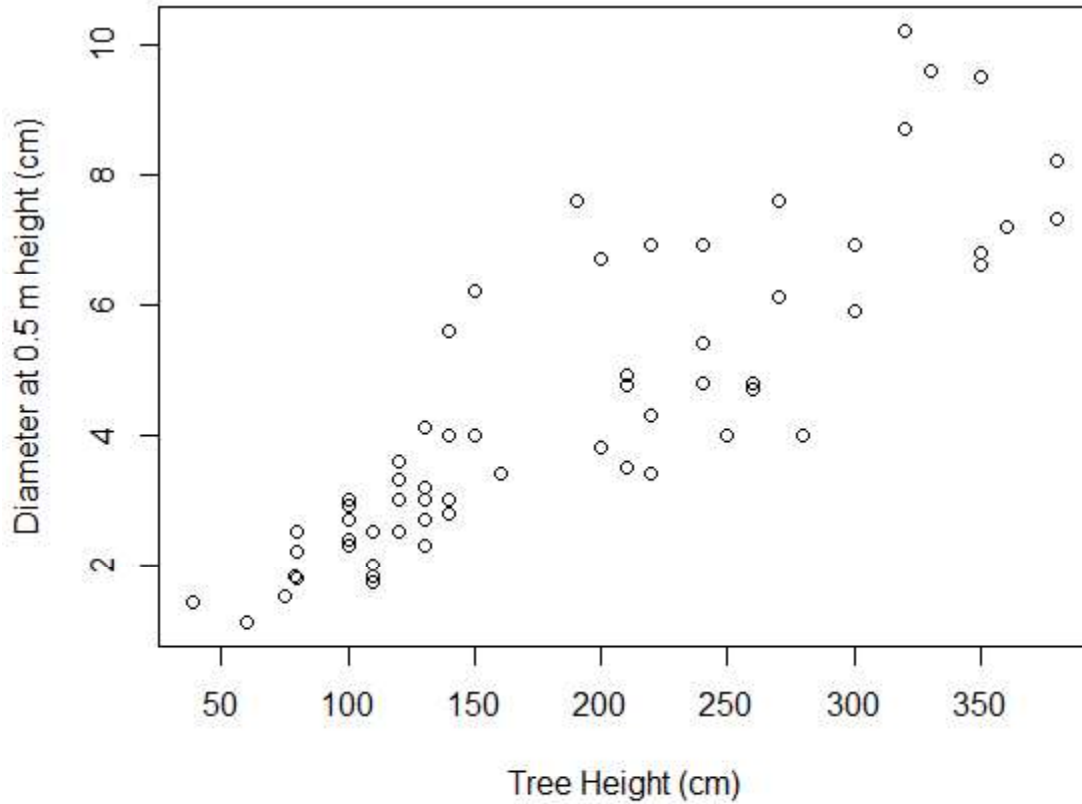


Figure S1: Tree height and stem diameter were closely correlated ($r^2 = 0.74$).

Using stem diameter instead of height did not make a difference for the result of any of the tests performed in Chapter 1 (see Table S2 below).

Table S2: Stem diameter and height were equivalent measures of tree size.

Analysis	<i>p</i> -value	Same as height?
Pearson correlation test between diameter and average intra-colony relatedness	0.06	yes
ANCOVA of diameter and ant species on intra-colony relatedness	0.1	yes
Spearman's rank correlation between diameter and queen number	0.09	yes
ANCOVA of diameter and ant species on queen number	0.07	yes
Spearman's rank correlation between diameter and male number	0.7	yes
ANCOVA of diameter and ant species on male number	0.8	yes
Spearman's rank correlation between diameter and queen mate number	0.2	yes
ANCOVA of diameter and ant species on queen mate number	0.4	yes

A "yes" in the rightmost column indicates that the tests using height and the tests using diameter were both significant or both insignificant at a significance threshold of $p < 0.05$.