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Evolutionary Ecology and Natural History of Fungus-Growing Ants: Host-Switching, Divergence, and Asexuality.

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Evolutionary Ecology and Natural History of Fungus-Growing Ants: Host-Switching, Divergence, and Asexuality.

by

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This dissertation is dedicated to my family.

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Evolutionary Ecology and Natural History of Fungus-Growing Ants: Host-Switching, Divergence, and Asexuality.

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Host-switch associated divergence is an important generator of diversity among insects. Here, I investigate whether host-switching plays a role in fungus-growing ant divergence. There are over 210 known species of ants that cultivate fungus as their primary food source. This diversity of ants and their fungal cultivars offers a rich comparative system to investigate the complexities of host-switch associated divergence. This work complements previous studies utilizing the fungus-growing ant system to investigate coevolution, speciation, conflict and cooperation.

In chapter one, I introduce the system and discuss features that make it ideal for studying evolutionary and ecological aspects of host-switch associated divergence. In chapter two, I examine whether switching to a new cultivar crop could trigger speciation events in two Central and South American species complexes of fungus-growing ants. Using behavior experiments and molecular phylogenetics, I investigate whether cultivar switches are associated with ant genetic divergence. It appears that, while some fungus-growing ants specialize on a narrow group of fungal cultivars and do not switch, other fungus-growing ant species exchange fungal cultivars more frequently. Varying degrees of host-fidelity will have different consequences for coevolutionary dynamics in this symbiosis. In the course of investigating fungus-growing ant host-associated divergence, I discovered new facets of the system that are the subjects of chapters three and four.

In chapter three, I investigate whether *Mycocepurus smithii* is the first completely asexual ant. Female mating anatomy studies, field and laboratory surveys, and DNA fingerprinting not only support complete asexuality in this widespread ant species, but also rule out bacteria and the fungal cultivars as causative agents inducing asexuality. In chapter four, I reconstruct the phylogenetic placement of a new species of fungus-growing ant in the *Cyphomyrmex longiscapus* species group and discuss their unique nesting biology. In chapter five I detail the first population study of nest architecture and sex ratios in *Mycetosoritis hartmanni*, one of the few North American fungus-growing ants. As these studies attest, after over one hundred years of research on the fungus-growing ant symbiosis, novel aspects of the system continue to emerge, providing a rich resource to test coevolutionary hypotheses within the context of this complex and ancient mutualism.

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Chapter 1

Introduction

1.1 INTRODUCTION

Symbioses are diverse, widespread and extremely influential interactions between organisms whose coevolutionary relationships affect all levels of life (Herre *et al.*1999, Thompson 1994). The fungus-growing ant symbiosis has emerged as a model system for studies investigating coevolution, speciation, host-parasite dynamics, conflict and cooperation (Schultz *et al.* 2002; Mueller *et al.* 2004; Gerardo *et al.* 2006). This ancient symbiosis includes over 210 described species of fungus-growing ants in the strictly Neotropical tribe Attini that depend upon the fungi they cultivate as a primary source of food for their larvae. The tribe is divided into two groups, the phylogenetically basal "lower" attines, and the derived "higher" attines that include the leaf-cutting ant genera. In addition, a suite of microbes is associated with the ant-fungus symbiosis. Because of the diversity of both the ants and their microbial associates, this complex system is a rich resource for ecological and evolutionary studies on a variety of scales, ranging from genotype-genotype interactions (Advani and Mueller 2006) to higher level coevolutionary processes (Currie 2001; Currie *et al.* 2003b). Here, I use the fungus-growing ant system to study both natural history and behavior of single species and questions regarding species-level processes.

During most of the 1900s, work on the attine ant-fungus symbiosis focused primarily on natural history (e.g. Weber 1940, 1972; Wheeler 1907) and taxonomy (Kempf 1968). The publication of a few seminal papers in the mid-1990s permitted more advanced questions about the symbiosis. In 1994, Chapela *et al.* showed that the phylogenies of attine ants and their fungal cultivars were congruent, establishing the association as an ancient,

coevolving mutualism. In 1998, Mueller *et al.* revealed novel aspects of the primitive fungus-growing ant agriculture, including lateral fungal transfers, redomestication from free-living fungi, and more generalist farming by the ants. With the advent of molecular phylogenetics, these studies provided a comparative framework to pursue more advanced questions from fine-scale individual and population-level questions to broad-scale questions on coevolutionary dynamics (Mueller *et al.* 2001; Gerardo *et al.* 2006; Currie *et al.* 2003, speciation (Schultz *et al.* 2002; Himler and Mueller Chapter 2), and asexual versus sexual lifestyles (Mikheyev *et al.* 2006; Himler and Mueller Chapter 3).

1.2 RESEARCH OVERVIEW

My work with fungus-growing ants began with a natural history study on one of the five primitive fungus-growing ant species that occurs in the United States. Over a reproductive season, I studied the sex ratio and nest architecture of this previously unstudied local Texas species. However, my primary interests lay in pursuing evolutionary questions of divergence due to host switching in the fungus-growing ant system. Previous work demonstrated that several primitive (lower) attine species cultivate multiple, sometimes distantly related, fungal crops (Mueller *et al.* 1998). This indicated that either these ant species are generalist farmers cultivating multiple crops, or that they are cryptic species complexes with each ant species specialized on its own phylogenetically distinct cultivar. To investigate the link between host switching, specialization, and divergence, I focused on two ant systems that may comprise cryptic species complexes, *Cyphomyrmex* and *Apterostigma*, each with evidence of a recent switch to a novel host. I utilized a combination of genetic and behavioral information to detect patterns of host-switch associated divergence in a comparative framework.

To study host-switch associated divergence in target fungus-growing ant complexes, I conducted extensive surveys across Central America. During the course of these surveys, I made two discoveries that are the subjects of chapters three and four. In chapter three, I

report data establishing that *Mycocepurus smithii* is the first known completely asexual fungus-growing ant. In chapter four, I discuss the discovery of a new species of fungus-growing ant, based on phylogenetic analysis and nesting biology. A member of the *Cyphomyrmex longiscapus* species group, it occupies a unique phylogenetic position between lower attines and derived leaf-cutter attines.

1.3 FUTURE RESEARCH PROSPECTS

Cultivar switch experiments are a useful tool to elucidate the sensory parameters under which fungus-growing ants interact with their mutualist fungi. Several recent studies have revealed new aspects of the system: Adams *et al.* (2000) demonstrated that ants deprived of their garden in the laboratory, simulating garden loss in the field, employed a variety of tactics to survive and acquire new cultivar. Another use of cultivar switch experiments showed that fungus-growing ants prefer cultivars most closely related to their own cultivar type, not that of most closely related ants (Mueller *et al.* 2004). This demonstrated that ants have evolved cultivar-specific preferences. These previous experiments assumed a myrmecocentric perspective where the ants control the mutualism (Mueller 2002), but Mehdiabadi *et al.* (2005) tested a hypothesis of mycological control where the cultivar may manipulate colony sex ratio, thereby directly influencing cultivar transmission between generations. Another set of cultivar-switch experiments demonstrated that ant cultivar preferences are sensitive to the genotypic level, whereby ants prefer and selectively cultivate specific cultivar clones or strains (Advani and Mueller 2006). Such fine-scale preference has implications for the evolution of selfish cultivar strains that promote their selection and cultivation by the ants. Thus a body of experimental work on cultivar switch experiments has developed to inform future studies for testing hypotheses of cultivar-switch-induced diversification in the tribe in a comparative framework.

Practical concerns regarding cultivar switch experiments include selecting species that are easy to collect entire colonies in the field, survive well in the laboratory, and are sufficiently large to permit multiple replicates made from the same colony. Fortunately, the fungus-growing ant symbiosis contains such a diversity of ant species, that there are several viable candidates to use for experiments to compare with the *Cyphomyrmex longiscapus*/*C. muelleri* model system. To further pursue cultivar-switch-induced divergence at an experimental level, future studies should examine attine ant cuticular hydrocarbon profiles (Howard and Bloomquist 2005) and colony fitness (Mehdiabadi *et al.* 2005) after switching fungal cultivars. The ideal experiment to establish whether cultivar switching causes potentially immediate reproductive isolation would involve switching entire colonies onto a novel cultivar type, allowing these colonies to produce sexual reproductives, and conduct their own assortative mating experiment. Both observation and molecular genetics could be used to establish whether queens from switched colonies mated with males from colonies on their traditional cultivar, or with males from colonies on the novel cultivar. Unfortunately, it is difficult to induce natural mating of any ant species in captivity, and this has yet to be done with attine ants.

Finally, exciting work remains to be done on the asexual ant, *Mycocepurus smithii*, including 1) chromosome counts, 2) quantification of allele sequence divergence, and 3) assessment of other degenerate features associated with the loss of sex, such as degenerate spermatogenesis genes.

1.4 SYNOPSIS

Viewed as a whole, these four chapters provide new insight into different levels of the attine symbiosis. At the individual-species level, I demonstrate phylogenetic evidence of a new Neotropical ant species and elucidate the nesting biology of a Texas attine ant species. With broader evolutionary implications, I present the surprising discovery of an asexual fungus-growing ant species, and I demonstrate variability in host-symbiont

relationships. These variable relationships, where some attine ant species are highly cultivar-faithful while other attine ant species frequently switch to novel symbionts, have profound implications for ecological and coevolutionary dynamics. Overall, these studies provide deeper insights into various aspects of the fungus-growing ant system, providing data for comparative studies and generating predictions for ant-cultivar associations in a phylogenetic context. This and other recent work shows that, despite over 100 years of study on fungus-growing ants, new discoveries continue to emerge that can inform current evolutionary theory on topics such as the maintenance of sexual reproduction, species interactions, and fine-scale individual and genotype-level interactions.

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Chapter 2

Speciation by cultivar switching in fungus-growing ants

Abstract. Fungus-growing ants and the fungi they cultivate as their primary food source constitute an example of an integrated, dynamic, and ancient mutualism. It has been suggested that ant diversification in this mutualism could occur through cultivar switching. This research examines whether switching to a new fungal crop could trigger speciation events in Central and South American species complexes of fungus-growing ants. I investigate whether fungal switches in these ants are consistently associated with ant speciation events and shifts in host preference. Specifically, in two different fungus-growing ant complexes, each with evidence of a recent switch to a novel host, I utilize a combination of genetic and behavioral data to detect patterns of host-switch associated divergence. I test the hypothesis that cryptic ant species within a species complex form monophyletic groups, each specialized on a distinct fungal cultivar, as expected under cultivar-switch-associated divergence. Results reveal more complex and diffuse ant-cultivar relationships than previously suspected in this symbiosis. In one species complex (*Cyphomyrmex longiscapus* and *C.muelleri*), the ants remain discretely associated with their particular cultivar as demonstrated in their fungal preferences, while in the other species complex (*Apterostigma auriculatum*), the ants do not form monophyletic groups based on cultivar type and do not demonstrate strong cultivar preferences, invalidating cultivar-switch-induced divergence for this second complex of *A.auriculatum*. Instead, data indicate that *A.auriculatum* ants switch frequently between cultivars. Such diffuse associations have implications for coevolutionary dynamics of the fungus-growing ant system.

2.1 INTRODUCTION

Host switching has been implicated in diversification of a broad range of taxa including vertebrate lice (Hafner *et al.* 1994; Clayton and Johnson 2003), primate parasites (Mu *et al.* 2005), avian malarial parasites (Ricklefs and Fallon 2002), and bird brood parasites (Sorensen *et al.* 2004). This phenomenon has been well studied in phytophagous insects, which feed on plant sap. For example, pea aphids are hemipteran pests on legumes, and Via *et al.* (1999; 2000) and Ferrari *et al.* (2006) have shown that sympatric subspecies of pea aphids that specialize on different hosts have diverged genetically. Another example is the apple maggot fly, *Rhagoletis pomonella* (Feder 1998). These tephritid fruit flies originally used hawthorn fruits as their host, but in the late 19th century, some switched to using apples in North America (Feder 1998). Since this switch, *Rhagoletis* flies using apples have diverged from *Rhagoletis* flies using hawthorn fruits in many characteristics related to their new host plant, including mating and egg laying sites, plant-scent preference, and time of emergence (Feder *et al.* 1994; Berlocher and Feder 2002). The two races have also diverged at neutral genetic loci, suggesting limited gene flow between the two races (Bush 1994; Feder *et al.* 1994; Feder 1998).

Fungus-growing ants (Tribe Attini) may constitute another example of host-switch-associated diversification. Fungus-growing ants are obligate mutualists with the fungus that they cultivate as their primary source of food (Mueller *et al.* 2001). Each ant species is believed to specialize on its own phylogenetically distinct clade of fungal cultivar (Chapela *et al.* 1994; Mueller *et al.* 1998; but see Green *et al.* 2004). While the mechanism of host-switch divergence in fungus-growers is unknown, one hypothesis is that divergence could occur via ant species-specific associations with particular fungal cultivars, similar to the plant-insect cases listed above. When an attine colony switches to a different fungal cultivar, these ants may acquire a different species-specific smell, evident in their cuticular hydrocarbon profile, from their new fungus that results in assortative mating. Attine ants have coevolved with a phylogenetically restricted group of fungi since the origin of this symbiosis approximately 50-60 million years ago (Mueller

et al. 2001). Vertical transmission of the cultivar between parent and offspring nests has led to specialization of ant species on specific clades of fungi (Chapela *et al.* 1994, Mueller *et al.* 1998). This specialization produces preferences by the ants for their own cultivar, or for those most closely related to their native fungal garden (Mueller *et al.* 2004). This ant-cultivar mutualism and subsequent coevolution has resulted in phylogenetic congruence at ancient (higher) levels between attine ants and their cultivar mutualists (Chapela *et al.* 1994; Villesen *et al.* 2004), however, recent work at the population level shows that the symbionts do not always align at fine-scales (Bot *et al.* 2001; Mikheyev *et al.* 2006) suggesting that these ants do occasionally switch to novel fungal cultivar hosts. Attine ants may lose their gardens for a number of reasons (Currie *et al.* 1999; Reynolds and Currie 2004), and unless they quickly replace the fungal cultivar, that colony will die. During the process of requisition, attine ants may acquire new cultivar from local colonies (Green *et al.* 2002) or from free-living fungal populations (Mueller *et al.* 1998); either way, they may obtain a cultivar different from their original one. Several fungus-growing ant species can switch fungal cultivars between species (horizontal transmission), challenging the strict vertical transmission once believed to be true for the fungus-growing ants and their affiliated fungi (Mueller *et al.* 1998; Bot *et al.* 2001; Green *et al.* 2002; Mikheyev *et al.* 2006). When switches do occur, reproductive isolation may be maintained if the ants acquire a strict preference for their new fungus. Here I combine detailed population-level phylogenetic analyses of both the ants and their cultivars with behavioral fungal-switch experiments to determine whether ants that may have recently switched to a novel cultivar exhibit strict specialization, as a first step towards understanding whether host-switches could elicit reproductive isolation and speciation in the fungus-growing ant system.

To investigate the link between host switching, specialization and divergence, I focus on two ant systems that may comprise cryptic species complexes: the *Cyphomyrmex longiscapus* and *C. muelleri* system, and the *Apterostigma auriculatum* system. These species are ideal study organisms because both can be easily collected in the field,

maintained in the lab, and experimentally manipulated. Both contain ant lineages that cultivate two phylogenetically divergent fungal cultivars (Mueller *et al.* 1998). This could mean these ant species are generalist farmers, raising several phylogenetically distantly related fungal crops, or they could be cryptic species complexes, with each ant species specialized on its own particular fungal crop. In the *Cyphomyrmex* case, the cultivars are very distantly related, occurring in two different clades, Clade 1 and 2 (see Figure 1 in Mueller *et al.* 1998), while the *Apterostigma* cultivars are different but more closely related in Clade 1. Thus these two different cases represent different phylogenetic degrees of potential cultivar switching; *Cyphomyrmex* ants may have switched between very distantly related fungal cultivars, while *Apterostigma* ants may have switched between more closely related fungal cultivars. In addition, these ant genera represent historically different places in the attine phylogeny: *Apterostigma* is a "paleo-attine" genus, one of the most phylogenetically basal among attines, while *Cyphomyrmex* occurs at a phylogenetically intermediate location between the primitive "lower" attine group and the "higher" attine group, which includes leaf-cutting ants (Schultz and Meier 1995; Wetterer *et al*. 1998; Schultz *et al.* 2002; Brady *et al.* in preparation). Comparison of fungal preferences between these ants and their associated fungi may reveal important insights into evolutionary differences in ant-fungal associations and diversification.

Evidence for genetic divergence between closely related ant species raising different cultivars could mean that host switching led to reproductive isolation. Recent work resolved *Cyphomyrmex longiscapus sensu lato*, once thought a single ant species that raised two distantly related cultivars, into two cryptic species, *C. longiscapus* Weber and *C. muelleri*, based on ant morphology (Schultz *et al.* 2002), nest architecture (Mueller and Wcislo 1998), AFLP (R.M.M. Adams, unpublished data), microsatellite and allozyme data (Schultz *et al.* 2002). This *Cyphomyrmex* species complex has emerged as a model organism for studies in behavior, ecology and evolution of mutualisms (Mueller *et al.* 1998; Villesen *et al.* 1999; Currie *et al.* 1999; Adams *et al.* 2000; Green *et al.* 2002; Schultz *et al.* 2002; Villesen *et al.* 2002; Mueller *et al.* 2004; Mehdiabadi *et al.* 2005). A

preliminary AFLP survey conducted to differentiate between these two now well-defined *Cyphomyrmex* species revealed lower genetic diversity within *C. muelleri* than in *C. longiscapus* (R.M.M. Adams, unpublished data). The depressed genetic diversity and limited geographic range of *C. muelleri* contained within the larger range of *C. longiscapus* suggest that *muelleri* could be the evolutionarily "younger" species derived from *C. longiscapus*-like ancestors. Since this species complex has recently been analyzed (Schultz *et al.* 2002) further molecular genetic analyses of these ants and their cultivars was unnecessary for the purposes in this study. *A. auriculatum*, however, has not been studied in such detail, therefore I conducted a molecular genetic study of these ants and their cultivars to test for the possibility of cryptic species.

Attine ants' specialization on particular clades of cultivars has been shaped by their coevolutionary history, producing ant preferences for particular fungi. Previous fungal choice tests revealed differences in cultivar preferences based on phylogenetic proximity of the test cultivar to the ants' natal cultivar: one choice test showed that *C. muelleri* ants can distinguish and prefer their own cultivar, or cultivar most phylogenetically similar to their own instead of the distantly related cultivar from the most closely related ant species, *C. longiscapus* (Mueller *et al.* 2004). Thus the ants prefer crops most similar to their own crop, not that of the ant species most closely related to them. In contrast, pilot data showed that *C. longiscapus* exhibits the presumed ancestral unmodified preference for its own cultivar, rejecting *muelleri* cultivar (U. G. Mueller, personal communication.). These pilot data influenced subsequent fungal choice tests such that *C. longiscapus* ants were never tested for fungal preference of *C. muelleri* cultivar, without quantitatively demonstrating this preference asymmetry. Such preference asymmetry is hypothesized if *C. longiscapus* is the ancestral species whose fungal cultivar acceptance is more specific or constrained due to a longer coevolutionary history with its cultivar, while the presumed derived species that may have recently switched to a new cultivar, *C. muelleri*, may prefer its own cultivar but will accept the ancestral cultivar. This preference asymmetry parallels host preferences of the hawthorne/apple *Rhagoletis* races of the

apple maggot fly (Feder *et al.* 1994; Feder 1998). Specifically, in both cases, the evolutionary "younger" species (the one that originated with the host switch) retained some of the ancestral preferences and thus can be switched to the ancestral host, but not vice versa.

While the few previous fungal choice tests established that attine ants can distinguish and prefer their own cultivar over others (Adams *et al*. 2000; Mueller *et al*. 2004; Mehdiabadi *et al*. 2005), and even prefer particular fungal clones (genotypes) within a species (Advani and Mueller 2006), such work usually tested only one species of ant (most often *C. muelleri*) without reciprocal switches between ant species to quantify both ant species' response. Reciprocal switches are crucial to determine if asymmetry in fungal preference of closely related ants species' exists. Any asymmetry in ant fungal preferences may reveal the historic direction of a cultivar switch. For example, an exclusive preference by an ant species for one cultivar type could reflect a longer coevolutionary history with that cultivar. To establish ants' fungal preferences as a possible mechanism in attine ant divergence following a cultivar switch, I performed reciprocal fungal switches between two sets of closely related ant species, 1) between *C. longiscapus* and *C. muelleri*, and 2) between two potential *A. auriculatum* ant species. I tested the following hypotheses: for *Cyphomyrmex*, 1) whether the acceptance rate of the interspecific fungus switch of *C. longiscapus* ants on *C. muelleri* fungus was equal to the acceptance rate of the intraspecific fungus switch of *C. longiscapus* ants on *C. longiscapus* fungus from different nests; 2) whether the acceptance rate of the interspecific fungus switch of *C. muelleri* ants on *C. longiscapus* fungus was equal to the acceptance rate of the intraspecific fungus switch of *C. muelleri* ants on *C. muelleri* fungus from different nests; and finally 3) whether the acceptance rate of *C. muelleri* ants on *C. longiscapus* fungus was greater than the acceptance rate of *C. longiscapus* ants on *C. muelleri* fungus. Such asymmetric fungal acceptance may be predicted because the fungal preference of *C. longiscapus*, the putative ancestral species, may be more evolutionarily constrained

compared to that of *muelleri*, the putative derived species. For *Apterostigma*, I tested whether the acceptance rate of A. *auriculatum* ants that normally grow "Fast" type fungus was equal to acceptance rate of *A. auriculatum* ants growing "Slow" type fungus when their cultivars were switched (Mueller *et al.* 2001)

Study Species. *Cyphomyrmex longiscapus* and *C. muelleri* have single-chamber nests of approximately 10-50 workers, occurring in stream embankments with a characteristic soil auricle nest entrance (Mueller and Wcislo 1998; Schultz *et al.* 2002). Each species cultivates a different distantly related type of lepiotaceous G3 fungus (Lepiotaceae, Agaricaceae) (Mueller *et al.* 1998). *C. longiscapus* appears to have a larger range, occurring in wet rainforests of Costa Rica, Panama, and Colombia (Schultz *et al.* 2002). *C. muelleri* has a more restricted range found almost exclusively in central Panama, with one specimen of *C. muelleri* previously reported from Ecuador (Schultz *et al.* 2002), however, recently it was also found on the Osa Peninsula of Costa Rica and at a new site in central Ecuador (RMM Adams, personal communication.).

Apterostigma auriculatum ants are slow moving solitary foragers that live in small nests of 10-50 workers distributed in South and Central America, reaching the northern limit of their range in Costa Rica (Lattke 1997). Their fungal gardens are sessile, occurring on the ground under large rocks, logs, or within large hollow fallen bamboo stems. *A. auriculatum* ants are the only known members of the genus to cultivate the ancestral lepiotaceous (Lepiotaceae, Agaricaceae) G3 fungi (Chapela *et al.* 1994; Mueller *et al.* 1998; Vellinga *et al.* 2003) along with all other fungus-growing ant genera outside the *Apterostigma* genus. *A. auriculatum* ant morphology has not been studied in detail. Lattke (1997) recognizes an *auriculatum* group, which contains *A. auriculatum*, as one of several unresolved species complexes within the genus. Attempts to classify *A. auriculatum* ants into morphotypes according to fungal type grown (see Mycology Methods) did not reveal any obvious distinguishing morphological characteristics (U. G. Mueller personal communication.). Vouchers of representative specimens from all

localities have been deposited at the National Museum of Natural History at the Smithsonian Institution, Washington, DC.

2.2 MATERIALS AND METHODS

Sampling. *A. auriculatum* ants and their fungal cultivars used in this study were collected from five sites in Panama (Gamboa, Pipeline Road, Barro Colorado Island, Fort Sherman, see Green *et al.* 2002 for map of locations, and Coclecito, in western Panama), one in Costa Rica (La Selva), and two in Ecuador (Tiputini and La Selva). For the *Cyphomyrmex* behavior study, 16 colonies were collected from central Panama (Gamboa, Pipeline Road, Barro Colorado Island, and El Llano; see Green *et al.* 2004 for map of locations). When possible, entire queenright colonies were exported live from Panama and Costa Rica for behavior experiments in the laboratory in the United States of America. Ecuador did not permit export of live material so DNA vouchers were exported. Live colonies were maintained at room temperature in multi-chamber artificial colonies at the University of Texas at Austin following the methods of Schultz (1993).

Mycology Methods. Cultivars from *Cyphoymrmex* and *Apterostigma* were isolated from all colonies used for experiments and molecular phylogenetics. These cultivars were cultured on potato dextrose agar (PDA, Difco, Detroit, MI, USA) with antibiotics (50mg/l each of penicillin and streptomycin), serially subcultured, lyophilized and stored at –80 ºC until DNA extraction (following methods in Gerardo *et al.* 2004). The two different cultivars grown by both potential cryptic species complexes in *Cyphoymrmex* and *Apterostigma* display different growth rates and fungal morphotypes when cultured on standard media: a “Slow” type with denser, thick, white, slow mycelial growth, and a “Fast” type with thinner, flat, yellow-white, more rapid growth. The “Slow” and “Fast” morphotypes for each species complex differ in appearance.

***Apterostigma* Molecular Methods**. I conducted a phylogenetic study to determine whether *Apterostigma auriculatum* ants represent two (or more) cryptic species. Genomic

DNA from 81 single workers or gynes was extracted using Qiagen Dneasy™ kits from samples refrigerated in 95% ethanol. Sequencing targeted a mitochondrial gene, cytochrome oxidase 1 (COI), and three nuclear genes: the nuclear elongation factor-1 alpha (EF-1∝), long-wavelength rhodopsin gene (LW *Rh*), and a UV opsin gene. PCR products were sequenced directly in both directions. Primers CI13/14 and CI21/24 (Simon *et al.* 1994) were used in separate PCR reactions to construct the entire COI exon region spanning approximately 1100 nucleotides; primers LR143F/LR639ER amplified the LW *Rh* gene (Ward and Downie 2005); and UV70F (5'-ATTCTRCCAGCAGGACCACCACG-3')/UV464R (5'-TAYGCRATRGCRTTYGTCAT-3') amplified the UV opsin gene (Ward unpublished, S. Brady personal communication). For all reactions, standard PCR conditions (Palumbi 1996) were used with some modifications for optimization. All sequences were run on an ABI 3100 automated sequencer, assembled using Seqman II v.5.05 (DNASTAR), aligned with Clustal X (Thompson *et al.* 1999) and edited manually in MacClade v.4.06 (Maddison and Maddison 2003). Three regions consisting of 125 out of 1118 characters of COI were excluded as unalignable since homology of those sites could not be inferred. Sequences from Genbank were included as additional samples and as outgroups including two *Apterostigma auriculaum* samples, *A. dentigerum*, *A. manni*, *A. pilosum group sp. TRS-2 and -4*, *A. sp. TRS-2003*, *A. cf. Dorotheae* (accession numbers AY398289, AY398292, AY398290, AY398304, AY398299, AY398295, AY398301, AY398294).

In addition, I conducted a phylogenetic analysis of the two types of *A. auriculatum* fungal cultivar, the fast and slow growing types. Fungal cultivar DNA from 87 samples were extracted from lyophilized mycelium using a CTAB protocol modified from Bender *et al.* (1983) and sequenced directly in both directions for rDNA from nuclear internal transcribed spacer region (ITS) using primers ITS4 and ITS5 (White *et al.* 1990). Several regions consisting of 311 out of 824 characters of ITS were excluded as unalignable since homology of those sites could not be inferred. All sequences will be deposited on

Genbank. For analyses several closely related fungi were selected as outgroups based on phylogenetic information in Mueller *et al.* (1998) including *Leucoagaricus hortensis* and *L. naucinus*, *Cyphomyrmex faunulus*, *C. muelleri*, *C. costatus*, and *C. longiscapus*, *Mycocepurus smithii*, *M. goeldii*, and *M. tardus*, *Myrmicocrypta infuscata* and *M. sp.*, *Mycetarotes senticosus* and *M. parallelus*, *Trachymyrmex papulatus,* and *Mycetosoritis hartmanni*.

Parsimony analyses were conducted in PAUP* v.4.0b10 (Swofford 2002) using heuristic searches (all characters weighted equally, gaps scored as missing data) after the ant dataset was pruned of 25 redundant (identical) haplotypes and the cultivar dataset was pruned of 27 redundant haplotypes. Tree searches included 1000 random addition sequence replicates with tree bisection-reconnection (TBR) branch swapping. Non-parametric bootstrapping was performed in order to obtain estimates of clade support, using heuristic searches of 1000 replicate datasets and 10 random addition sequence replicates per dataset. For likelihood and Bayesian analyses of the ants, I used a general time reversible (GTR) model of sequence evolution with a proportion of invariant sites (PINVAR) and four gamma distributed rate classes (Γ) which was the model estimated for the data using MODELTEST v.3.06 (Posada and Crandall 1998). For likelihood and Bayesian analyses of the cultivar, I used the Hasegawa, Kishino, and Yano model (HKY +I+Γ) model of sequence evolution estimated for the cultivar data by MODELTEST v.3.06 (Posada and Crandall 1998). Maximum likelihood searches were performed using the Genetic Algorithm for Rapid Likelihood Inference (GARLI) v.0.95 (Zwickl 2006) using random starting trees and the default settings (GTR+I+Γ estimate parameters) for five separate runs for the ants. For the cultivar, maximum likelihood searches using GARLI were performed for three runs with default settings and compared to two runs using the model and parameters specified by Modeltest since these varied slightly from the default settings in GARLI. There was no difference in tree topology or support values between these cultivar ML searches. Two additional maximum likelihood searches were performed for the ants and cultivar using two different specified starting trees (one

selected Bayesian tree and one star-decomposition tree instead of a neighbor-joining tree to avoid polytomies which GARLI cannot accomodate). Non-parametric bootstrapping was performed with heuristic searches of 500 replicate datasets.

Bayesian analyses were conducted using MRBAYES v.3.1.1 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003) on Phylocluster at UT Austin, estimating the GTR + I + Γ model parameters during four separate Markov Chains Monte Carlo (MCMC) with the default prior distribution for model parameters and the temperature parameter set to 0.2. Runs consisted of 500,000 generations per run, trees sampled every 100 generations. Burn-in was calculated by MRCONVERGE v1.0b1 (http://www.evotutor.org/MrConverge) and all trees after burn-in were combined to construct a majority-rule consensus tree.

To test the hypothesis that *A. auriculatum* ants comprise monophyletic cryptic species groups that each specialize on either fast or slow growing fungi (null hypothesis), I imported the observed Bayesian analysis trees (alternative hypothesis) after burn-in into PAUP* and filtered them with a constraint tree consisting of two monophyletic groups, one group that cultivates fast growing fungus, and one group that cultivates slow growing fungus. The proportion of sample trees consistent with the hypothesized constraint tree topology is the Bayesian posterior probability of that hypothesis (*p* value).

Fungus Switch Experiments: *Cyphomyrmex*. Using nests of *C. longiscapus* and *C. muelleri* collected from the Republic of Panama between 2002-2005 (two nests collected in 2002, nine from 2003, one from 2004, and four from 2005), I performed a no-choice experiment testing whether one ant species (e.g., *C. longiscapus*) would accept fungus of the other species (*C. muelleri*) and vice versa. Fungus was switched within species to compare how readily ants accept fungus of the same species from a different nest (intraspecific switch) to acceptance of fungus from a different ant species (interspecific switch). The two species' acceptance rates were then compared.

For the fungal switches, eight colonies of *C. longiscapus* were randomly assigned to eight colonies of *C. muelleri* (interspecific switch), eight *C. longiscapus* colonies were randomly assigned to each other (intraspecific switch), and eight *C. muelleri* colonies were randomly assigned to each other (intraspecific switch). For each pair of *C. longiscapus/C. muelleri* colonies, two interspecific switch replicates were made, in which *C. longiscapus* ants were given *C. muelleri* fungus, and two interspecific switch replicates were made in the opposite direction, in which *C. muelleri* ants were given *C. longiscapus* fungus. For each pair of *C. longiscapus/C. longiscapus* colonies, two intraspecific switches were made, in which *C. longiscapus ants* from one colony were given *C. longiscapus* fungus from a different colony. Likewise for each pair of *C. muelleri/C. muelleri* colonies, two intraspecific switches were made in which *C. muelleri* ants from one colony were given *C. muelleri* fungus from a different colony. Overall, there were a total of 32 interspecific switches (16 *C. longiscapus* ants on *C. muelleri* fungus and 16 *C. muelleri* ants on *C. longiscapus* fungus), and 32 intraspecific switches (16 *C. longiscapus* ants on *C. longiscapus* fungus and 16 *C. muelleri* ants on *C. muelleri* fungus), for a total of 64 replicates.

A replicate consisted of four randomly selected workers from a colony placed on approximately $20mm^3$ of fresh fungus garden from another colony. Before introduction the fungus garden was examined under a dissecting scope using sterile forceps to ensure no ant brood (eggs, larvae, or pupae) was transferred. Each replicate was housed in a nest chamber consisting of a 6cm diameter plastic Petri dish half-filled with Plaster of Paris and moistened with water to maintain constant humidity. The Petri dish had a three mm hole drilled in one side where ants could exit, and was placed in a 7.4cm x 7.4cm clear plastic chamber (Pioneer Plastics, Inc. Dixon, Kentucky) as an artificial colony chamber adapted from Schultz (1993) to provide space for ants to obtain food and dump waste from the fungus garden.

Ants were allowed to habituate to their new fungus garden for 24 hours, and then the state of the fungus garden was noted (spread out, piled, number of piles, or disposed of)

and position of ants (on garden, tending, off garden, in foraging chamber) was recorded. Each replicate was then supplied with five pieces of dried sterilized polenta cornmeal, a substrate preferred by the ants to feed their gardens (Advani and Mueller 2006) in one corner of the foraging chamber. Addition of substrate to the garden (fungiculture) is a clear indication of garden acceptance by the ants (Mueller *et al.* 2004). Scoring was done blind with respect to ant species. Ant response was measured in the following way: 24 hours after feeding the position of each piece of polenta was recorded per replicate as either incorporated (on the fungus garden), or unincorporated (on the plaster in the chamber or outside in the foraging chamber). The number of the ants on and off the fungus garden was also recorded. Any unincorporated food was then removed. This was repeated four times for a total of five feeding trials, with 24 hours between feedings. Acceptance of a fungus was indicated when the ants fed the fungus garden by placing the substrate (polenta grains) onto the fungus and tended the fungus garden. This is a reasonable criterion because rejection of the fungus is signified by the ants not feeding or tending the garden, or by disposing of the fungus in the external dump chamber (Advani and Mueller 2006).

Acceptance rate was defined as number of acceptances over total number of feeding trials per replicate, which varied between replicates because some replicates lived longer than others and therefore experienced more feeding trials.

***Cyphomyrmex* Experiment Analysis.** A two-tailed, two-sample t-test assuming unequal variances was conducted to compare the inter- and intra-specific fungal acceptance rates for *C. longiscapus*. The same test was used to compare the inter- and intra-specific fungal acceptance rates for *C. muelleri*. To compare the mean difference in acceptance rates between the two ant species, a one-tailed, two-sample t-test assuming unequal variances was conducted. Acceptance rate was defined as number of acceptances over total number of feeding trials per replicate. We previously defined the alternative hypothesis that *C. muelleri* ants accept *C. longiscapus* fungus more readily than *longiscapus* ants accept *muelleri* fungus based on preliminary data (U. G. Mueller personal observation; Mueller

et al. 2004). Therefore a matched pairs design was used to compare the mean difference in acceptance rate of each ant species on the other species' fungus. Analyses were conducted using SAS 8.0.

Fungus Switch Experiments: *Apterostigma*. A preliminary experiment tested whether *A. auriculatum* ants that cultivate two different types of fungus garden, called "Fast" and "Slow" fungal types, will accept the opposite cultivar type from what they normally garden. To assess this preference a no-choice experiment was conducted in which ants that cultivate "Slow" fungus were presented with "Fast" fungus, and ants that cultivate "Fast" fungus were presented with "Slow" type fungus, analogous to potential interspecific switches. Four "Slow" fungus nests (two collected in 2001; two collected in 2002) were paired with two "Fast" nests (from 2002) for reciprocal fungal switches between the two fungal types. Fungal switches consisted of two randomly selected worker ants from a "Slow" fungus nest placed on approximately $20mm^3$ piece of fresh fungus garden from one "Fast" fungus nests, replicated twice. Two randomly selected workers from the same "Slow" nest were placed on a piece of fresh fungus garden from the other "Fast" fungus nest, replicated twice. This was done for all four "Slow" nests, for a total of 16 replicates of "Slow" ants on "Fast" fungus. In the opposite direction of the switch, two randomly selected worker ants from a "Fast" fungus nest were placed on approximately $20mm^3$ piece of fresh fungus garden from each of the four "Slow" fungus nests for a total of four replicates. This was repeated for the other "Fast" fungus nest for a total of eight replicates of "Fast" ants on "Slow" fungus, and a grand total of 24 replicates in the experiment.

Experimental setup was similar to the *Cyphomyrmex* experiment except each switch replicate had two worker ants. For the feeding trials ants were provided with a small pile of dried sterilized polenta cornmeal/oat mix in one corner of the foraging chamber as substrate for the fungus garden. Scoring was done blind to ant species. Twenty-four hours after substrate was provided each replicate was scored for whether the ants added substrate to their fungus garden or not. Acceptance of a fungus was indicated when the

ants fed the fungus garden by placing the substrate onto the fungus and tended the fungus garden (yes/no). This was repeated seven days later, for a total of two feeding trials.

In order increase sample size, a second, larger *auriculatum* switch experiment was conducted. Ants from twelve "Slow" fungus nests (one collected in 2001, one collected in 2002, ten collected in 2003) were switched with six "Fast" fungus nests (from 2003) and vice versa. Due to differences in nest size the limiting factor was number of workers per nest, therefore the number of replicates per particular ant nest/fungus nest pairing varied for a grand total of 50 replicates. No intraspecific fungal switches ("Fast" ants on "Fast fungus or "Slow" ants on "Slow" fungus) were conducted due to a limited number of source nests and worker ants. Experimental set up was as in the first *auriculatum* experiment, except three workers were used in each replicate and five feeding trials were conducted with five or six days between feedings over five weeks. Acceptance of a fungus was indicated when the ants fed the fungus garden by placing the substrate (polenta grains) onto the fungus and tended the fungus garden, which was recorded 24 hours after each feeding trial.

***Apterostigma* Experiment Analysis**. For both *auriculatum* switch experiments, multiple replicates of each particular ant source nest and fungus source nest combination were consolidated to generate one response, Yes or No, so that there was a single response for each ant nest given a particular fungus. For example, if there were two replicates of ants from nest 5 given fungus from nest 17, the responses from these individual replicates were consolidated if the response was unanimous, that is, if both replicates indicated a "yes" (or "no") response. Criteria for consolidation were conservative: the replicate with the most feeding observations was taken, but if two replicates disagreed in their conclusion (one Yes, one No), they were eliminated because a clear response could not be determined. Likewise if there were three replicates of a particular ant nest/fungus nest combination, only cases where a response was clear over two or more feeding trials were used. Any replicate that only lived through one feeding trial was eliminated. Initial analysis revealed no significant difference between the first and second, larger *A.*

auriculatum switch experiments, so data from these experiments were combined. After ant responses were consolidated, there were 22 replicates of slow ants on fast fungus, and 22 replicates of fast ants on slow fungus for analysis. To assess whether there was a difference in acceptance rates between the two types of ants I conducted a Chi-square analysis of a 2x2 contingency table comparing ant type (fast or slow cultivar growing ants) on acceptance (yes or no). Because the data were not normally distributed, and it was not possible to transform the data to restore normality, I used a non-parametric Chi-square analysis of a 2x2 contingency table to examine the effects of cultivar type on ant acceptance.

2.3 RESULTS

Sampling. During my survey, I encountered *C. muelleri* only in central Panama, and *C. longiscapus* only as far north as the northwestern province of Bocas del Toro in Panama. I did not locate either *Cyphomyrmex* species at three collecting sites in Costa Rica: La Selva Biological Station, El Ceibo Biological Station in Braulio Carillo National Park, and Hitoy Cerere reserve in southeastern Costa Rica. Nor did I find *C. longiscapus* or *C. muelleri* at two sites in Ecuador, La Selva and Tiputini Biological Stations. *A. auriculatum* was most abundant in central Panama, but did extend west into Costa Rica. I did not find *auriculatum* at Hitoy Cerere, but I did find it at both La Selva and El Ceibo Biological Stations in Costa Rica, however at much lower abundances than expected given the habitat seemed ideal for this species.

***Apterostigma* Phylogenetic Analyses**. The aligned sequence data for *A. auriculatum* ants consisted of 993bp of mitochondrial COI after excluding unalignable regions. Of the 993 sites, 432 were variable and 362 of these were parsimony informative. Sequence data from the nuclear genes were uninformative. Twenty-two ants from twelve slow and ten fast growing cultivar type colonies were sequenced for the EF-1∝ intron and revealed absolutely no variation. Likewise eight ants, from four slow and four fast growing cultivar type colonies, were sequenced for the nuclear gene LW Rh and were also

completely invariant. I attempted to sequence a third nuclear gene, UV opsin, but after much optimization could only amplify a portion (the first 250 basepairs) of the forward sequence and not the reverse, so this gene was not used.

Results of parsimony, maximum likelihood and Bayesian analyses were concordant in all key features. These analyses revealed that *A. auriculatum* ants do not fall into monophyletic groups corresponding to fungal type (fast or slow) that they cultivate (Figure 2.1). Instead the ants that cultivate either fast or slow fungi were intermixed with one another. The null hypothesis that *A. auriculatum* ants form two monophyletic groups according to the type of fungus they cultivate, one clade comprised of ants that cultivate fast fungus, and one clade comprised of ants that cultivate slow fungus, was rejected at p = 0.0 using a constraints analysis conducted in PAUP* under parsimony. None of the observed 138,480 Bayesian trees matched the null hypothesis topology with or without outgroups in the constraint tree. Little geographic structure was revealed by the gene region used: samples from across central Panama clustered together, often forming polytomies, whereas samples from Costa Rica and Ecuador fell just basal to the main clade from Panama**.** This implies that diversification between the ants does not occur following a switch to a novel cultivar type, and that *A. auriculatum* ant lineages appear to switch frequently between the two cultivar types.

The aligned sequence data for *A. auriculatum* fungal cultivar consisted of 513bp of ITS after excluding 311bp of unalignable regions from 87 taxa. Of the 513bp, 362 characters were variable, and of these 111 were parsimony informative. Bayesian, maximum likelihood, and parsimony analyses of these cultivars agreed in all major aspects and the trees were nearly identical for most taxa. Phylogenetic analyses of *A. auriculatum* cultivars revealed that the cultivars fell into two monophyletic groups, one containing the fast cultivars, and one containing the slow cultivars with little geographic structure (Figure 2.2). Exceptions included four slow type cultivars (S55, S60, S66, S68) that occurred in the fast clade, and two fast type cultivars (S48, S49) that occurred in the slow clade. In addition, three fast type cultivars (S020, S050, and S070) formed their own

group near a free-living sample G11 (reported previously from a *Myrmicocrypta* species from Guyana; Mueller *et al.* 1998), basal to all the other *A. auriculatum* cultivars. Two putatively slow type cultivars (S007 and S101) actually grouped basally to all the other *A. auriculatum* cultivars falling with "clade two" (see Figure 1 in Mueller *et al.* 1998) cultivars near *C. muelleri* and *Mycocepurus tardus*.

Fungus Switch Experiments: ***Cyphomyrmex***. *C. longiscapus* worker ants accepted other *C. longiscapus* fungus much more readily than *C. muelleri* fungus (14.13 ± 5.55 out of 25 possible polenta pieces (56%), versus 1.81± 2.76 polenta pieces out of 25 possible (7.25%); t = 5.32, df = 7, p < 0.001) (Figure 2.3 left two columns). *C. muelleri* worker ants accepted other *C. muelleri* fungus (12.125 ± 2.63 polenta pieces out of 25 possible (48.5%) and *C. longiscapus* fungus at a comparatively higher rate (5.00 ± 3.78 out of 25 possible polenta pieces (20%); t = 5.52, df = 7, $p < 0.001$) (Figure 2.3 right two columns). Thus, each intraspecific switch compared to its interspecific switch was significant for the two-tailed t-test. When the two species' acceptance rates were compared, the mean difference in acceptance rates between *C. longiscapus* intra- and interspecific fungus switches was much greater (12.3125 ± 6.54 polenta pieces; 49.25%), than the difference between *C. muelleri* intra- and interspecific fungus switches (7.125 ± 3.65 polenta pieces; 28.5%) (Figure 2.4). The mean difference in acceptance rates of each ant species on the other species' fungus was significant for the one-tailed t-test (t = 1.99, df = 11, p < 0.038).

Fungus Switch Experiments: ***Apterostigma.*** Both types of ants accepted each other's fungus in 14 out of 22 replicates (N=44; 63%) (Figure 2.5). Acceptance rates were not significantly different based on a Chi-square analysis of a 2x2 contingency table ($\chi^2 = 0.0$, df = 1, p = 1.0).

2.4 DISCUSSION

In this study I explore whether divergence due to host switching occurs in two potential cryptic species complexes of fungus-growing ants. We see different patterns in each case.

In the *C. longiscapus*/*C. muelleri* case we see evidence for genetic and behavioral divergence that corresponds with current patterns of host-cultivar use. In the *A. auriculatum* case genetic and behavioral differences do not correspond with host use, so there is no evidence for cultivar-switch associated divergence. Therefore, it is not clear whether attine ants diversify upon host switching in general. It appears instead that the ants can cultivate a diversity of cultivars, making them generalists, at least on certain clades of cultivar crops, as in the case of *A. auriculatum*. They may only diversify if they switch to sufficiently distantly related host crops, as may have occurred in the *C. longiscapus/C. muelleri* case. Thus, the relationship between host switching and divergence is complex.

The reason for the difference between the *Cyphomyrmex* and *Apterostigma* experiments, and between the two ant species complexes considered here, could lie in the cultivars. The *C. longiscapus* and *C. muelleri* cultivars are quite phylogenetically divergent, occurring in two clades separated by many other attine cultivars as well as other free-living fungi (Figure 1, Mueller *et al.* 1998). Interestingly, although *C. longiscapus* and *C. muelleri* nests occur sympatrically in close proximity in stream embankments, no switch has been recorded in the field after extensive collections (Green *et al.* 2004, Mueller *et al.* 2004). In contrast, the *A. auriculatum* cultivars are much more phylogenetically similar; they occur in distinct clades (Mueller *et al.* 1998 and Figure 2.2), but the two *A. auriculatum* cultivar types are more closely related to each other than the *Cyphomyrmex* fungi are (Mueller *et al.* 1998).

Schultz *et al.* (2002) discussed potential scenarios for divergence associated with host-switching. In the allopatric scenario, they hypothesized that an isolated population of *C. longiscapus sensu lato* (the hypothesized ancestral species) could have switched to a novel cultivar type and become specialized, eventually leading to prezygotic isolation. Alternatively, they suggested a temporal shift in mating flights could be responsible. In the sympatric scenario, reproductive isolation involves some unknown cultivar specific mechanism. One plausible mechanism I suggest involves the cuticular hydrocarbons (CH)

on the ants' exoskeleton that constitute part of their species and nest specific identity (Vander Meer and Morel 1998; Howard and Bloomquist 2005). These profiles could change when the ants change fungal cultivars. Cuticular hydrocarbons are very labile, changing with variation in diet (Liang and Silverman 2000; Richard and Errard 2001) and environment (Boulay *et al.* 2000; Singer 1998). Insects in general and ants in particular have species-specific CH profiles, comprised of a unique mix of queen and nest identifiers, as well as diet, nest site, and nest material components, depending upon the species (Vander Meer and Morel 1998; Lenoir *et al.* 1999; Lenoir 2002). For the attine ants, the fungus garden is both their food and nest, and thus the fungus may play a large role in species and nest identity scent (ie, cuticular hydrocarbons acquired from the fungus by the ants) (Singer and Espelie 1998). Future research should examine the interplay between the behavioral ecology and cuticular hydrocarbon profiles of ants to detect limits to symbiont switching.

The first case examined divergence and fungal preference behavior of ant sister species *Cyphomyrmex longiscapus* and *C. muelleri* fungus-growing ants. *C. longiscapus* is hypothesized to be the ancestral species since it has greater genetic diversity and a much broader geographic range, while *C. muelleri* may be the derived species due to reduced diversity and a limited range, sympatric with *C. longiscapus* in central Panama (Schultz *et al.* 2002; Adams *et al.* unpublished data). (The argument that reduced genetic diversity indicates *C. muelleri* may be the more recently derived species precludes other events such as bottlenecks or selective sweeps that might reduce genetic diversity). The *Cyphomyrmex* fungal switch behavioral experiment showed that two closely related cryptic species *C. longiscapus* and *C. muelleri* each prefer their own cultivar type. Each species accepted fungus from a different nest within that same species more readily than a novel fungus from a different ant species. Furthermore, the results showed that *C. longiscapus*, the putative ancestral sister species, accepted *C. muelleri* fungal cultivar significantly less often than C. *muelleri* accepted C. *longiscapus* cultivar, as hypothesized.

The second case examined divergence and fungal preference behavior between potential cryptic species of *Apterostigma auriculatum* fungus-growing ants. Phylogenetic analyses of *A. auriculatum* ants and their cultivars show that 1) two types of cultivars associated with this species, the fast and a slow growing morphotypes, fall into two distinct phylogenetic clades; and, 2) the ants that raise these cultivars do not separate into distinct clades according to fungal type. This implies that *A. auriculatum* ants comprise one lineage (e.g., species), are not restricted to growing one type of fungal cultivar, and may switch readily between the fast and slow growing cultivar types in the field as both cultivar morphotypes occur sympatrically in most (if not all) populations in central Panama (A. Himler personal observation.). Such nonspecific cultivar reaquisition by *A. auriculatum* ants is supported by behavioral data demonstrating that *auriculatum* ants did not significantly prefer their own cultivar type to the alternate morphotype presented. Together these results imply that *A. auriculatum* ant lineages switch frequently between two cultivar types without diversification between the ants following a cultivar switch. Several possibilities could explain *auriculatum* ants' ease of switching between their two cultivar types, as demonstrated by a lack of significant preference between their cultivars:

a) *A. auriculatum* cultivars are closely related and therefore not divergent enough to prompt the ants to evolve behaviorally strong preferences;
b) *A. auriculatum* ants have not yet diverged significantly after acquiring a novel cultivar type;
c) The consortium of other microbes associated with the ant-fungus mutualism (i.e. other symbiotic garden microbes) of the two *auriculatum* fungal types may be similar enough to permit easy switching between the fast and slow types within the *auriculatum* ant species without destabilizing the mutualism (Mueller, 2002).

It remains unclear why *A. auriculatum* appears limited to switching between only two cultivar types and not other attine fungi. Perhaps the suite of microbes associated with other attine cultivars is too different, and switching with other non-*auriculatum* cultivars

compromises the mutualism by destabilizing the cost-benefit ratio reached by coevolution between *A. auriculatum* ants and their two associated cultivar groups (Mueller 2002).

Phylogenetic analysis showed that *A. auriculatum* ants fell into two very closely related clades (Figure 2.1). These *could* represent two species based not upon fungal cultivar type, but on some other feature, however, verification would require more extensive phylogenetic support at other loci. However, neither the behavior experiment nor the limited data from two nuclear genes (EF-1∝ and LW*Rh*, data not shown) support separate *auriculatum* lineages. At the very least the two ant clades represent two mitochondrial types. Either way, both *auriculatum* ant clades utilize the same pool of cultivars since the fast and slow fungal types are distributed across both ant clades (Figure 2.1). In contrast, *A. auriculatum* fungal cultivars fell into two well-defined separate clades according fast and slow growing fungal morphotypes (Figure 2.2). Exceptions include only two fast types (S048F, S049F) that occur in the slow clade and four slow types (S055S, S060S, S066S, S068S) that occur in the fast clades, suggesting there may be more variability in cultivar types grown by this species, or the fast/slow dichotomy based on plated growth scoring is not always a reliable way to identify fungal morphotype(s), or both. In most cases the difference between fast and slowgrowing *auriculatum* cultivar on PDA plates is clear, but there were a few cases that were ambiguous and it is possible these were scored incorrectly. Future collectors of *A. auriculatum* (and other attine species) should be aware of these possibilities.

One pattern is consistent in both the *A. auriculatum* ant and cultivar phylogenies: three taxa (S020F, S050F, and S071), two from Panama and one from Ecuador, consistently occur in a separate clade. These cultivars fell outside the Clade 1 symbiont group (see Figure 1 in Mueller *et al.* 1998) including the two main types of *auriculatum* cultivars (Figures 2.2). Without intensive sampling such exceptions are likely to be missed. In addition, S007 and S101 cultivars occurred outside the *auriculatum* clade altogether, grouping instead with Clade 2 cultivars (see Figure 1 in Mueller *et al.* 1998) near *C. muelleri* and *Mycocepurus tardus* (Figure 2.2). These cultivars are much faster growing

than the fast type *A. auriculatum* cultivar when growth rates are compared on PDA media. This implies that *auriculatum* ants may very rarely switch to a cultivar similar to that grown by a different genus, *Cyphomyrmex*, which occurs sympatrically and often in very close physical proximity, depending upon the species. For example, *C. costatus* ants cultivate a fungal garden that is sessile on the ground under rocks or logs, like that of *auriculatum*. If *A. auriculatum* ants were to lose their fungus garden, this is the species they would most likely encounter in similar nesting habitats. The other possibility is that *auriculatum* acquired fungus garden from *C. muelleri*, however, these ants nest in the banks of streams and along steep soil banks, a very different microhabitat where *auriculatum* are rarely found.

It would be interesting to compare results of cultivar switching experiments between these two unusual groups, S020F, S050F, S071 and S101, S007, and the main *auriculatum* clade of fast and slow cultivars because they represent different degrees of relatedness to the main *auriculatum* clade. In the first group (S020F, S050F, and S071) both the ants and the cultivars form clades separate from the rest of *auriculatum*, while in the latter case, (S101 and S007) the cultivars are distantly related, occurring outside the *auriculatum* cultivar clade (Figure 2.2), while these ants' mitochondrial types fell within the main *auriculatum* ant clade (in Figure 2.1 of the ants only S101 is shown, S007 was removed due to poor sequence). One explanation for these two unusual cases could be that these two fungal clades represent rare switches to a much more distantly related fungal type: the S101 and S007 ants may have switched very recently and not diverged in mitochondrial type yet, while S071, S050, and S020 ants may have switched in the more distant past with sufficient time for some divergence in the ants to occur. Unfortunately these colonies were not exported live to permit behavior experiments. It is clear that extensive field collections are required to develop a true understanding of any attine system, from detection of rare host-switching events to cultivation of multiple fungal types.

The picture emerging from ongoing work of fungus-growing ants, their fungi and the associated microbes has changed from an example of strict one-to-one specificity (Chapela *et al.* 1994) to a more diffuse assemblage (Green *et al.* 2002; Gerardo *et al.* 2006; Mikheyev *et al.* 2006). While ant allegiance to particular cultivar clades remain true in some cases, as demonstrated here, strict one-to-one specificity between ant species and cultivar species appears to be less and less frequently supported, or altogether untrue (Mueller *et al.* 1998; Bot *et al.*2001; Mueller 2002; Mueller and Gerardo 2002). This has been demonstrated recently for several higher attine species (Mikheyev *et al.* 2006; Bruschi *et al.* in prep.). It was implied for the first time in lower attines when evidence emerged that some of the ants' cultivars closest relatives were free-living fungal species (Mueller *et al.* 1998). A more detailed study by Green *et al.* (2002) showed that *C. muelleri* and *C. costatus* may occasionally switch fungi that are very closely related, or rarely even cultivate the same fungus. Here I show for the first time phylogenetic analyses of both ants and their cultivars complemented with behavioral experiments that fungal specialization by ant species is variable in the lower attines: *C. longiscapus*/*C. muelleri* ants *are* adapted to and prefer their own very divergent cultivars, while *A. auriculatum* ants do not display stringent preferences. These results are contrary to the suggestion in Schultz *et al.* (2002) that the lower attines may speciate when they switch fungal cultivars. It has emerged from recent work on the fungus-growing ant system that while the ants can distinguish between cultivars from different species (Mueller *et al.* 2004; Mehdiabadi *et al.* 2005) as well as detect cultivar quality within species (Advani and Mueller 2006), such preferences do not define strict ant-fungal associations as narrowly as once thought (Mueller 2002). These results demonstrate that fungus-growing ant-cultivar associations are more variable and diffuse than previously thought, revealing greater complexity in the system.

If ants can switch cultivars with a certain amount of latitude, a host of questions about factors governing cultivar choice and movement arise for future study. For example, what factors prompt a fungus switch? These could include garden decline, decimation by

pathogens, invasion by *Megalomyrmex* parasitic ants, or capture of a superior cultivar from elsewhere. Where do ants obtain their new cultivar? From the nearest congeneric ant species or nearest most similar cultivar type? These are not always the same. Cultivar acquisition likely varies by species; some species may obtain it from a nearest neighbor, as is likely for *A. auriculatum*, however this is not the case for switching between *C. muelleri* and *C. costatus* (Green *et al.* 2004). Alternatively, ants may obtain a cultivar through an intermediary population of free-living cultivars (Mueller *et al.* 1998). How does the act of switching occur? Does a single ant worker invade another attine nest and abscond with a piece of cultivar or is it a group effort? It would be interesting to learn whether switching behaviors vary by ant species. What conditions are primary constraints on fungus switching? Do nest microhabitat and proximity govern rates of encounter with new cultivar options? How do ant sensory preferences operate for an attine to identify and select a cultivar, and what role does the consortium of microbes associated with the any-fungus mutualism play? Do the *Actinomycete* bacteria that provide the antibiotics essential for defense against the specialized pathogen *Escovopsis* play a significant role in cultivar choice? How broadly can one type or lineage of *Actinomycete* successfully defend different cultivars that may be attacked by different strains of *Escovopsis?* And finally, could it be in the cultivar's interest for the ant host to switch? The previous questions assume a myrmicocentric view of cultivar switching. However, could symbiont drive (Mueller 2002) *between* nests occur; with selfish cultivars enticing new *A. auriculatum* ant nests to abandon their own, perhaps less productive cultivar, for another type? These and other questions remain to be investigated.

The results of the present study do not universally support a scenario of attine ant speciation following host switching. Perhaps divergence of an ant species may only occur when ants successfully switch to cultivars dissimilar enough from their traditional type, as may have occurred in the *Cyphomyrmex* case, to cause the ants' species-specific scent or some other mate recognition factors to change. Such distant host switches are predicated on the fact that either the *Actinomycete* bacteria that assists the ants' defense

against *Escovopsis* is effective against pathogens of a new distantly related fungal host species, or the ants acquire new *Actinomycetes* from their new cultivars. This remains to be tested. While the factors that govern cultivar switching remain to be elucidated, it is now clear that an increasing number of attine species have flexibility in the cultivars they grow, and that attine ants switch their symbiont fungal crops with greater freedom and frequency than previously thought.

How this newly discovered latitude impacts co-evolution of the ants, their cultivars and associated microbes remains to be investigated, and may prompt revision of this classic coevolutionary paradigm. Future studies exploring the chemical ecology of ant-microbe affiliation will reveal the latitude and limits to fungal switching in this ancient but dynamic symbiosis. Examining attine ant cuticular hydrocarbon profiles (Howard and Bloomquist 2005) and colony fitness (Mehdiabadi *et al.* 2005) after switching fungal cultivars offer a promising starting point. Agricultural flexibility emerges as an integral aspect of these fungus-farming ants, despite the constraining forces of coevolution. Managing microbes, especially in domesticated systems (our crops, even our bodies) has become paramount in modern human agriculture and medicine. Perhaps the attine ants' success in fungus farming, in which they have managed their complex microbial environment for over fifty million years, will provide some insights useful to humans.

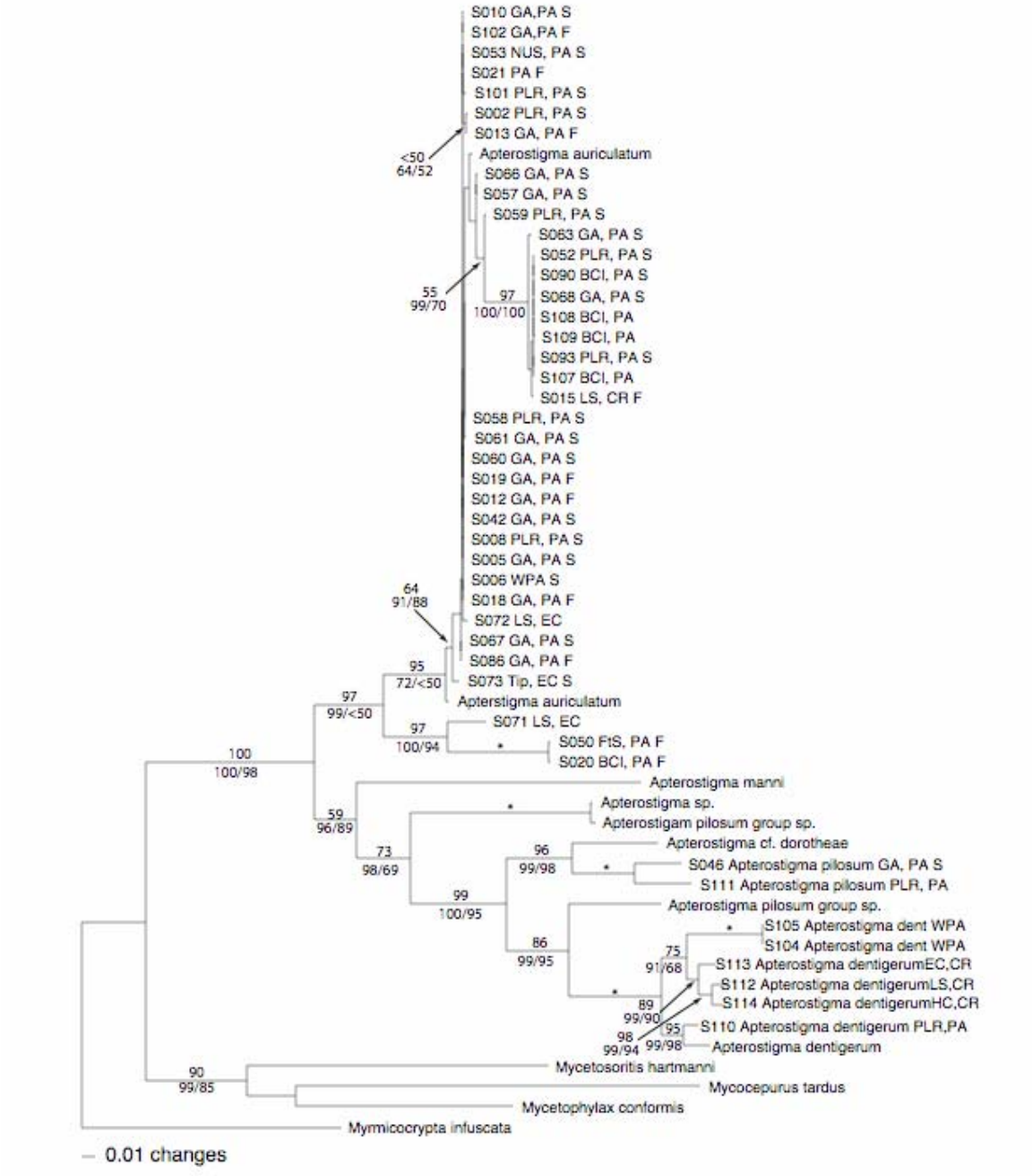

Figure 2.1 Maximum likelihood phylogram of *Apterostigma auriculatum* ants based on COI sequence data. Terminals are labeled with sample codes followed by locality

and country of origin abbreviations (WPA: western panama; GA, PA: Gamboa, Panama; PLR, PA: Pipeline Road, Panama; NUS, PA: Nusagandi, Panama; BCI, PA: Barro Colorado Island, Panama; FtS, PA: Fort Sherman, Panama; HC, CR: Hitory Cerere, Costa Rica; EC, CR: El Ceibo, Costa Rica; LS, CR: La Selva, Costa Rica; Tip, EC: Tiputini, Ecuador; LS, EC: La Selva, Ecuador), and end with cultivar type. Ants that raise the fast growing type fungal cultivar end with "F"; ants that raise the slow growing type cultivar end with "S". Samples not ending in F or S indicate the cultivar growth type is unknown. Branches are labeled with likelihood bootstrap values (above), Bayesian posterior probabilities (below, left) and parsimony bootstrap support values (below, right). Unlabeled branches have values of less than 50 for at least two analyses of support. An asterisk (*) indicates that all three support values are 95 or greater.

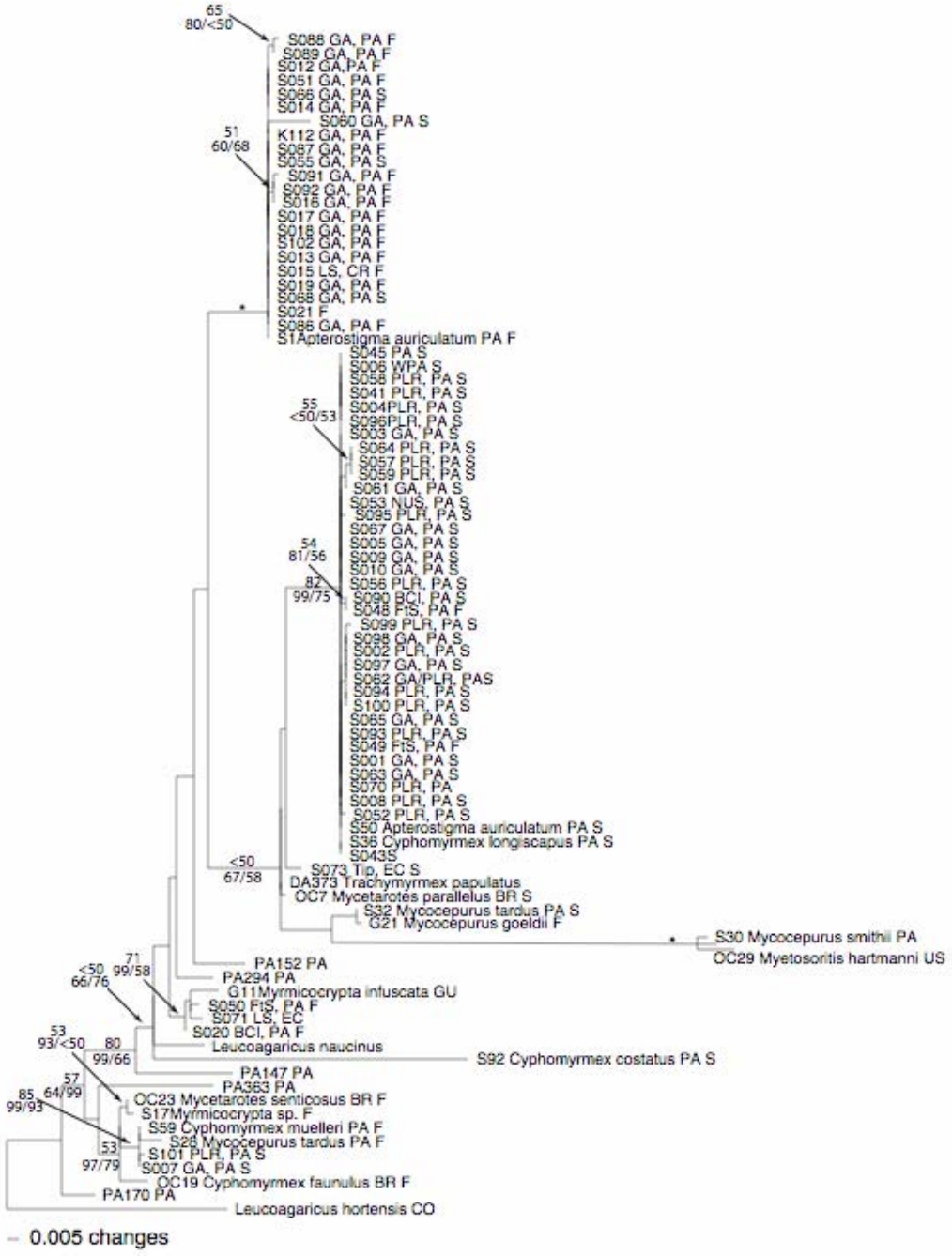
65
80/<50
S088 GA, PA F
S089 GA, PA F
S012 GA, PA F
S051 GA, PA F
S066 GA, PA S
S014 GA, PA F
S060 GA, PA S
51
60/68
K112 GA, PA F
S087 GA, PA F
S055 GA, PA S
S091 GA, PA F
S092 GA, PA F
S016 GA, PA F
S017 GA, PA F
S018 GA, PA F
S102 GA, PA F
S013 GA, PA F
S015 LS, CR F
S019 GA, PA F
S068 GA, PA S
S021 F
S086 GA, PA F
S1Apterostigma auriculatum PA F
S045 PA S
S006 WPA S
S058 PLR, PA S
S041 PLR, PA S
S004PLR, PA S
55
<50/53
S096PLR, PA S
S003 GA, PA S
S064 PLR, PA S
S057 PLR, PA S
S059 PLR, PA S
S061 GA, PA S
S053 NUS, PA S
S095 PLR, PA S
S067 GA, PA S
S005 GA, PA S
54
81/56
S009 GA, PA S
S010 GA, PA S
82
99/75
S056 PLR, PA S
S090 BCI, PA S
S048 FIS, PA F
S099 PLR, PA S
S026 GA, PA S
S002 PLR, PA S
S097 GA, PA S
S062 GA/PLR, PAS
S094 PLR, PA S
S100 PLR, PA S
S065 GA, PA S
S093 PLR, PA S
S049 FIS, PA F
S001 GA, PA S
S063 GA, PA S
S070 PLR, PA
S008 PLR, PA S
S052 PLR, PA S
S50 Apterostigma auriculatum PA S
S36 Cyphomyrmex longiscapus PA S
S043S
<50
67/58
S073 Tip, EC S
DA373 Trachymyrmex papulatus
OC7 Mycetarotes parallelus BR S
S32 Mycocepurus tardus PA S
G21 Mycocepurus goeldii F
S30 Mycocepurus smithii PA
OC29 Myetosoritis hartmanni US
71
99/58
<50
66/76
PA152 PA
PA294 PA
G11Myrmicocrypta infuscata GU
S050 FIS, PA F
S071 LS, EC
S020 BCI, PA F
53
93/<50
80
99/66
Leucoagaricus naucinus
S92 Cyphomyrmex costatus PA S
57
64/99
PA147 PA
PA363 PA
85
99/93
OC23 Mycetarotes senticosus BR F
S17Myrmicocrypta sp. F
S59 Cyphomyrmex muelleri PA F
S28 Mycocepurus tardus PA F
53
97/79
S101 PLR, PA S
S007 GA, PA S
OC19 Cyphomyrmex faunulus BR F
PA170 PA
Leucoagaricus hortensis CO
0.005 changes

Figure 2.2 Maximum likelihood phylogram of *Apterostigma auriculatum* cultivar based on ITS sequence data. Terminals are labeled with sample codes followed by locality and country of origin abbreviations (WPA: western panama; GA, PA: Gamboa, Panama; PLR, PA: Pipeline Road, Panama; NUS, PA: Nusagandi, Panama; BCI, PA: Barro Colorado Island, Panama; FtS, PA: Fort Sherman, Panama; HC, CR: Hitory Cerere, Costa Rica; EC, CR: El Ceibo, Costa Rica; LS, CR: La Selva, Costa Rica; Tip, EC: Tiputini, Ecuador; LS, EC: La Selva, Ecuador; BR: Brazil; GU: Guyana; CO: Colombia), and end with cultivar type. Fast growing type fungal cultivars end with "F"; slow growing type cultivars end with "S". Samples not ending in F or S indicate the cultivar growth type is unknown. Branches are labeled with likelihood bootstrap values (above), Bayesian posterior probabilities (below, left) and parsimony bootstrap support values (below, right). Unlabeled branches have values of less than 50 for at least two analyses of support. An asterisk (*) indicates that all three support values are 95 or greater.

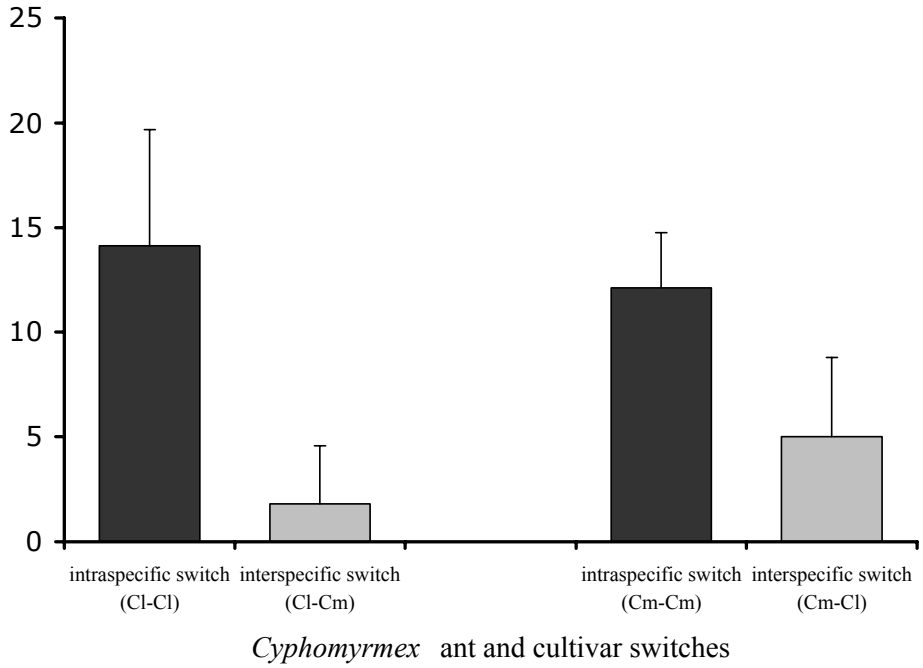

Figure 2.3 Difference in fungus acceptance between the intraspecific and interspecific fungal switches for two *Cyphomyrmex* ant species. Left two columns show the acceptance difference between the intra- and interspecific fungus switches in *Cyphomyrmex longiscapus* ants. Right two columns show the acceptance difference between the intra- and interspecific fungus switches in *Cyphomyrmex muelleri* ants. There was a significant difference between the ants' acceptance of the fungus between the intraspecific switch and the interspecific switch in both species at an α-level of 0.025. In each case, the ants accepted fungus from a different nest of the same species significantly more often than they accepted fungus from a different species of ant. Error bars show 1 standard error.

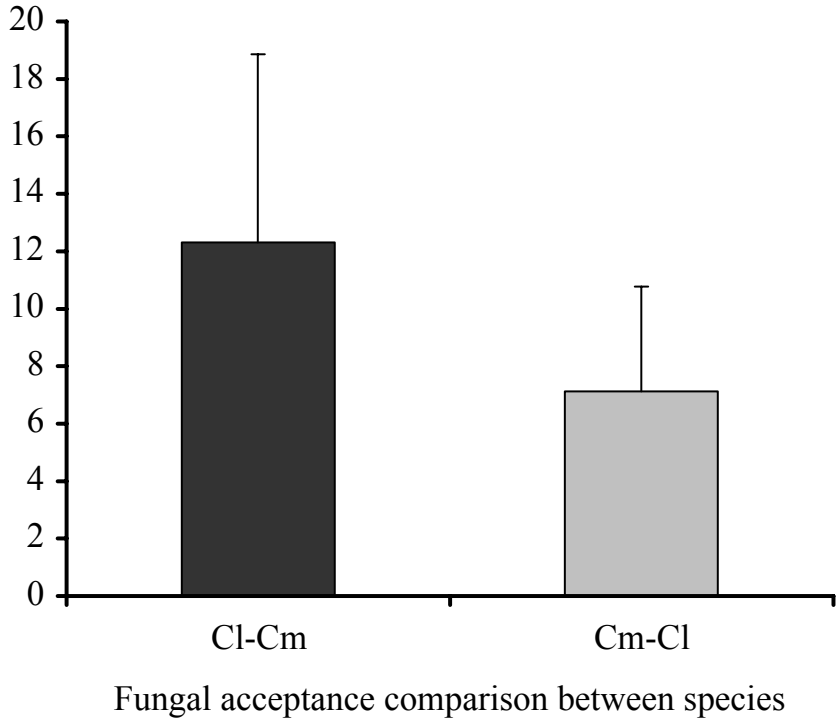

Figure 2.4 Comparison of fungus acceptance rates between two *Cyphomyrmex* ant species. When the two species' acceptance rates were compared, the mean difference in acceptance rates between *C. longiscapus* (Cl) intra- and interspecific fungus switches was greater than the difference between *C. muelleri* (Cm) intra- and interspecific fungus switches. The mean difference in acceptance rates of each ant species on the other species' fungus was significant. Error bars show 1 standard error.

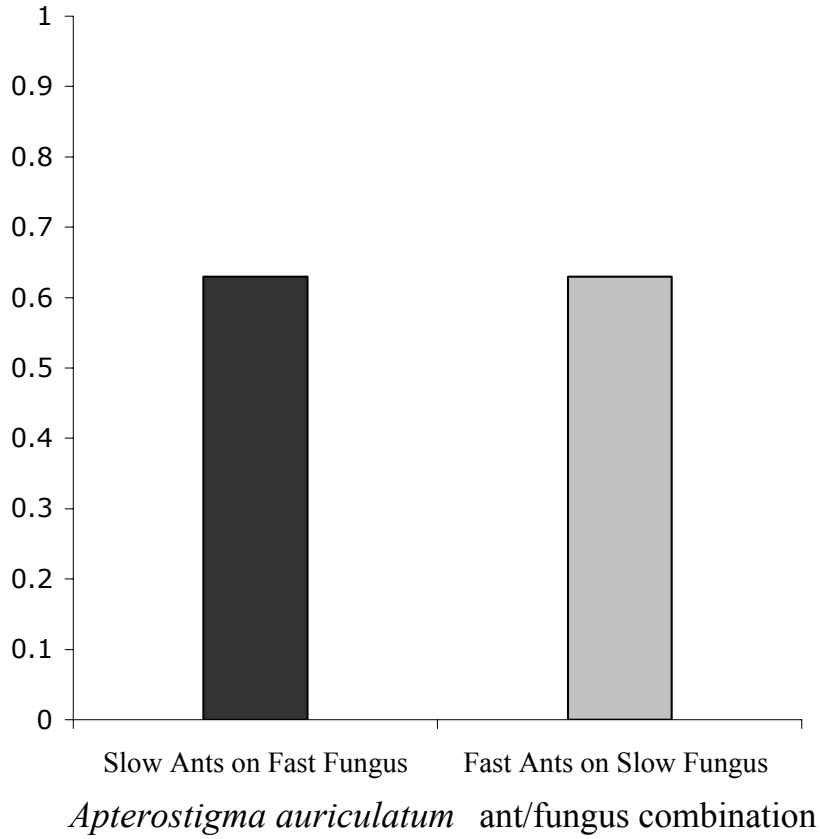

Figure 2.5 Comparison of fungus acceptance rates between *Apterostigma auriculatum* ants on different cultivars. There was no significant difference in acceptance rates of slow fungus type ants on fast fungus or fast fungus type ants on slow fungus at an α-level of 0.025.

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Chapter 3

No sex in fungus-farming ants or their crops

Abstract: Asexual reproduction generates novel genotypes and purges deleterious mutations at lower rates than sexual reproduction, imposing evolutionary handicaps on asexual species and thus rendering them prone to extinction. Here we report the first case of complete asexuality in ants, the fungus-growing ant *Mycocepurus smithii* where queens reproduce asexually but workers are sterile, which is doubly enigmatic because *M. smithii* depends upon asexual propagation of fungi for food. While the fungus may have sex outside the symbiosis, there is good evidence that its propagation within the symbiosis is asexual. Degenerate female mating anatomy, extensive field and laboratory surveys, and DNA fingerprinting implicate complete asexuality in this widespread and ecologically successful ant species. Maternally inherited bacteria (e.g. *Wolbachia*, *Cardinium*) and the fungal cultivars can be ruled out as agents inducing asexuality. The *M. smithii* societies of clonal females provide a unique system to test theories of parent-offspring conflict and reproductive policing in social insects. Asexuality of both ant farmer and fungal crop challenges traditional views proposing that sexual farmer ants outpace coevolving sexual crop pathogens and thus compensate for the vulnerabilities of their asexual crops. Frequent switching between crops (symbiont reassociation) may ameliorate the evolutionary handicap of asexuality in *M. smithii*.

3.1 INTRODUCTION

The vast majority of eukaryotes reproduce sexually. Multicellular asexuals are rare, occur sporadically across the tree of life, and, with few notable exceptions (Judson and Normark 1996; Butlin 2002), are thought to be short-lived descendents derived recently from sexual ancestors (Barton and Charlesworth 1998). Theory predicts asexuality is advantageous because asexual lineages should out-compete sexual ones by circumventing costs of sex (e.g., cost of meiosis, mating effort, producing males), however asexuality is thought to be evolutionarily disadvantageous because it purges deleterious mutations and generates novel genotypes more slowly than sexual reproduction (Butlin 2002). The pervasiveness of sex among multicellular organisms suggests that the advantages outweigh the costs (Barton and Charlesworth 1998). The real evolutionary conundrum, therefore, is not the pervasiveness of sexual lineages, but the persistence of some asexual lineages over extended evolutionary time (Judson and Normark 1996; Herre *et al*. 1999).

Like all other fungus-growing ants in the strictly Neotropical tribe Attini, *Mycocepurus smithii* (Formicidae, Attini) obligately farms basidiomyete fungi for food (Mueller *et al.* 1998), and has one of the widest distributions of any fungus-growing ant, ranging from Mexico and the Caribbean to Argentina (Mackay *et al*. 2004; Fernándéz-Marín *et al*. 2005). Unlike all other fungus-growing ant species, however, no males have been found in extensive collections of *M. smithii* from throughout the Americas (Fernándéz-Marín *et al*. 2005; Rabeling 2004), suggesting *M. smithii* is parthenogenetic (Fernándéz-Marín *et al*. 2005). We tested this hypothesis using genetic, morphological, and experimental analyses.

3.2 MATERIALS AND METHODS

Colony Collections. All *M. smithii* colonies in this study were collected in the Republic of Panama from five populations 50-150km apart (Parque Soberanía, Sherman Forest Reserve, or the Colón Province), in March-April 2001, June 2002, and May 2003. These colonies were maintained in the laboratory up to four years and never produced males. Field surveys in Panama (100 nests), Guyana (5 nests), Ecuador (6 nests), Peru (20 nests), Argentina (7 nests), and Brazil (132 nests) (Rabeling 2004) failed to find any males, complementing Fernández-Marín's survey of 228 male-less *M. smithii* nests in Puerto Rico (Fernándéz-Marín *et al*. 2005). DNA samples were refrigerated in 95% ethanol and extracted using Qiagen Dneasy™ kits.

Genotyping: DNA Extraction and Microsatellite Fingerprinting. To test whether *M. smithii* offspring were clones of their mothers, we screened fourteen microsatellite primer pairs developed for other fungus-growing ant genera (Villesen *et al.* 2002). Thirteen of these loci either did not amplify or were monomorphic and thus uninformative. Using the single informative locus Cypho15-16 (two alleles; 150bp, 152bp), we genotyped 66 specimens of *M. smithii*, from 12 colonies collected in Panama for which both queen and workers were available (queen and 4-10 offspring per colony). DNA was extracted from single whole workers and queens' abdomens. Microsatellite PCR products were run on an ABI 3100 automated sequencer and analyzed using Genescan v3.7 and Genotyper v3.6. Microsatellite primer Cypho 15-16 amplified ant DNA under the following PCR conditions: 1 cycle of 94°C for 3 minutes; 35 cycles of 94°C for 40s, 59°C for 40s, and 72°C for 30s; and 1 final extension cycle at 72° for 15 minutes. Each 10ul PCR reaction contained: 1X enzyme buffer (Promega), 3.75mM $MgCl_2$ (Promega), 0.25mM of each

dNTP (Promega), 0.25U Taq DNA polymerase (Promega), 1µM of each primer, and 1 µl DNA template at approximately 50ng/µl.

Female Reproductive Tract. Colonies of *M. smithii* and *M. tardus* were excavated and six resident non-winged queens of each species were dissected to inspect their reproductive organs and determine reproductive status. Mated, reproductively active queens are characterized by (1) presence of sperm in the spermatheca (empty spermatheca appears translucent grey; sperm-filled spermatheca appears opaque white) (Fig 1), (2) fully developed ovaries containing mature eggs in the ovarioles, and (3) the presence of yellow bodies in the ovarioles. Yellow bodies, remnants of follicular epithelium, indicate the ant had laid eggs.

Molecular Screen for Endosymbiotic Bacteria. We tested *M. smithii* for presence of *Wolbachia*, *Cardinium* and other endosymbiotic bacteria by PCR (Zchori-Fein and Perlman 2004; Holden *et al*. 1993; Zhou *et al.* 1998; Jeyaprakash and Hoy 2000). DNA was extracted from individual workers or gyne abdomens for the survey with three *Wolbachia*-specific primers (Wenseleers and Billen 2000; Pearcy *et al.* 2005; Fournier *et al.* 2005), and from gyne larvae or adult ovaries for screens with *Cardinium*-specific and universal bacterial primers (Zchori-Fein and Perlman 2004; Jeyaprakash and Hoy 2000). One to three workers, gynes, or queens per colony were sampled from 15 colonies for a total of 31 samples; in only two colonies was a single ant tested. To verify PCR amplification, *Formica truncorum* ants and *Drosophila simulans* infected with *Wolbachia* served as positive controls and uninfected *Formica sanguinae* as the negative control. Twelve *M. smithii* individuals were tested using primers Ch-F/Ch-R that amplify *Cardinium* and other related *Bacteroidetes* symbionts (Zchori-Fein and Perlman 2004), with the wasp *Encarsia pergandiella* as a positive control. Two universal bacterial primer pairs, U519F/U1406R and 338-356F/16S-8R, were used to detect bacterial symbionts

other than *Wolbachia* or *Cardinium*, with *Escherichia coli* as a positive control and sterile water as a negative control (Jeyaprakash and Hoy 2000). DNA quality of all samples was verified by successful amplification of the mitochondrial cytochrome oxidase I (COI) gene (Simon *et al*. 1994).

Antibiotic Experiment. Unmated virgin female *M. smithii* gynes were treated with antibiotics to test whether curing infection by potentially parthenogenizing microbes would permit male production. Thirty-five subcolonies were split from three source colonies. Subcolony replicates from the same source colony were blocked in groups of five for a total of seven groups. Each replicate subcolony contained a $20mm^3$ garden fragment, 20 winged queens and approximately 75 workers, except one quintuplet set with six queens per subcolony. Within each group, replicates were randomly assigned to one of four antibiotic treatments: 10% streptomycin, 5% penicillin, 5% tetracycline, 0.5% rifampicin or a sucrose solution control. Antibiotic concentrations were selected based on pilot experiments in which females readily imbibed antibiotic sugar solutions of maximal concentrations that did not cause significant mortality (less than 10%) during a 7-day treatment, compared to control females kept without sugar solution that showed near 100% mortality. This difference in mortality demonstrated indirectly that the treated females were ingesting the antibiotic solutions. Replicate subcolonies were habituated for eight weeks in a two-chamber system before antibiotic treatment (Schultz 1993). During this time gardens doubled in size, and virgin females eclosed from pupae hidden in the transferred fungus garden, increasing the number of females per subcolony to an average of 36.5 (±7.6) that were all then treated with the assigned antibiotic. For treatment, all gynes were removed from each subcolony, and supplied with a drop of either 10% (weight/volume) sucrose solution (control) or a 10% sucrose solution laced with one of four antibiotics. Fresh sucrose solutions were provided daily for seven days, and treated

gynes were then returned to their subcolony. Subcolonies were maintained for 16 months until after the next round of annual gyne production was complete. Number of new queens produced after treatment was calculated per subcolony as number of live queens plus dead queens collected during the experiment, minus queens present at start of experiment.

Fungus Switch Experiment. Ninety laboratory-reared, unmated gynes were randomly chosen from six *Mycocepurus smithii* nests and placed individually on a brood-free fungus garden fragment ($20mm^3$) in a 6 cm Petri dish with a moistened plaster of Paris base. These ninety gynes were randomly assigned to one of three garden types: native cultivar (from *M. smithii*), closely related cultivar (from *Cyphomyrmex costatus*), or distantly related cultivar (from *Cyphomyrmex longsicapus*). Replicates were maintained for 15 months over three generations and were fed weekly with a sterilized mix of polenta and ground oats as substrate for the fungus. Because of nonnormality, we examined the effect of fungal type on the number of workers and gynes produced using a Kruskal-Wallis test, and the effect of fungal type on queen survival using a Pearson's Chi-square test. All tests were conducted using a statistical significance level of 0.05.

***M. smithii* Worker Reproduction Experiment.** To determine whether *M. smithii* workers were sterile or could reproduce, workers were isolated without queens on their native fungus and maintained until senescence (8 months). Five adult workers randomly selected from a colony [excluding newly emerged (callow) workers] were isolated on a $20mm^3$ garden fragment from their natal nest, and maintained in 60x15mm diameter Petri dish, the bottom filled with plaster moistened to maintain humidity. Three replicate subnests from six different source nests comprised 18 replicates. These were supplied with sterilized polenta to feed their garden and watered weekly.

Putative *M. smithii* Male. Three males originally assigned to *Mycocepurus reconditus* by W. Kerr (1961) were tentatively assigned to *M. smithii* by Kempf (1963). The three males were collected in 1961 in a mating swarm of about 500 *M. goeldii* males in Rio Claro, São Paulo, Brazil, and were recognized by both Kerr and Kempf as distinct from *M. goeldii* because of their slightly smaller size. Because *M. goeldii* and *smithii* were the only two *Mycocepurus* species known in the region at that time, Kempf suggested a possible affiliation of the three small males with *M. smithii*. However, since no unambiguous *smithii* males have ever been collected (e.g., from nest excavations), the three small males may belong to other species of *Mycocepurus* discovered later in that region, indicating Kempf's tentative, circumstantial assignment may be invalid. To place the three small males within the genus *Mycocepurus* with molecular techniques, we attempted to amplify DNA from one of the males using both non-destructive and destructive DNA extraction methods.

3.3 RESULTS

Colony Collections. One hundred colonies of *M. smithii* collected from five populations in Panama produced over 10,000 new queens (gynes) during five years in the laboratory, an estimated 10-20 times that number of workers, but no males. In contrast, 30 different species of fungus-growing ants maintained in the same laboratory produced males. Unmated *M. smithii* queens born in the laboratory produced both worker and gyne offspring, starting clonal female lineages that can be propagated without mating over several generations.

Microsatellite Fingerprinting and Analysis. Microsatellite DNA fingerprinting revealed that workers and gynes had the identical genotype of their mother in twelve *M.*

smithii colonies for which both queens and offspring were available. After screening 14 microsatellite loci, we genotyped nine heterozygous and three homozygous colonies with the single informative locus (Villesen 2002). Within heterozygous queen colonies, the probability across all nine colonies of producing only heterozygous offspring was $(1/2)^{54} = 5.55 \times 10^{-17}$, indicating sexual reproduction was absent (Table 3.1). Another potential explanation for the observed genotype distributions (e.g. absence of homozygous offspring in nests with heterozygous queens) is very strong selection against homozygotes at some pre-adult stage, but calculation shows that the strength of selection required is so extreme (selection coefficients > 0.94) that homozygotes should be very rare in the population (<2%). However, 25 % (3/12 colonies) of the genotyped colonies were homozygous. Therefore the alternative explanation that homozygotes produced by a heterozygous queen mating are lethal can be ruled out. Offspring genotypes were always identical to their mothers' in both heterozygous and homozygous colonies, supporting clonal reproduction. Although the cytological mechanism underlying asexual propagation is unknown, these data support asexual reproduction by apomixes for *M. smithii* (mitotic parthenogenesis), rather than automictic reproduction (meiotic parthenogenesis) (Normark *et al*. 2003).

Female Reproductive Tract. Dissections of the female reproductive tracts of *M. smithii* queens collected from mature nests confirmed that they were never inseminated (empty spermathecae), although they had fully developed ovaries containing mature eggs and yellow bodies, indicating that they were active egg layers (Figure 3.1). In contrast, *M. tardus* queens, a closely related species, had sperm-filled spermathecae. *M. smithii* queens also lack the "mussel organ", a female reproductive structure found in other attine species into which the male's sclerotized genitalia lock during mating (Figure 3.1) (Baer and Boomsma 2005). The substantial degeneration of the mating apparatus renders

cryptic sex unlikely for *M. smithii* (Judson and Normark 1996; Normark *et al*. 2003). Combined with the widespread Neotropical distribution of *M. smithii* from northern Argentina to northern Mexico, this suggests that the evolutionary origin of *M. smithii* from a sexual ancestor was not recent.

Molecular Screen for Endosymbiotic Bacteria. As in other Hymenoptera (Werren and Windsor 2000), asexuality in *M. smithii* could be caused by infection of endosymbionts such as *Wolbachia* (Stouthamer *et al*. 1999), *Cardinium* (Zchori-Fein and Perlman 2004), or other microbes (Zchori-Fein and Perlman 2004) that can manipulate reproduction, or by a vertically transmitted exosymbiont (e.g. the fungal cultivar) (Mueller 2002). *Wolbachia* has been found in several sexually reproducing ant species (Wenseleers *et al*. 1998) including fungus-growing ants (Van Borm *et al*. 2003), but is absent in six partially asexual ant species (Wenseleers and Billen 2000; Pearcy *et al*. 2005; Fournier *et al*. 2005). PCR screens for endosymbiotic *Wolbachia* (Holden *et al*. 1993; Zhou *et al*. 1998; Jeyaprakash and Hoy 2000), *Cardinium* (Zchori-Fein and Perlman 2004) and other bacteria (Baker *et al*. 2004) in *M. smithii* were negative.

Antibiotic Experiment. As an additional test that bacteria might cause parthenogenesis in *M. smithii*, we treated 1320 gynes from 28 experimental colonies with four different classes of antibiotics. Antibiotic purging of parthenogenizing bacterial symbionts reinstates male production in some asexual arthropods (Stouthamer and Mak 2002; Weeks *et al*. 2001). However, the antibiotic-treated *M. smithii* queens produced 7,488 daughter queens but no males during 16 months post treatment (Table 3.2). The combined molecular and antibiotic evidence therefore indicates absence of a male-eliminating bacterial endosymbiont in *M. smithii*.

Fungus Switch Experiment. Another proximate cause for asexuality in *M. smithii* could be the fungal cultivar, which, because of its maternal inheritance, could maximize its transmission between ant generations by biasing the sex ratio towards females (Mueller 2002). To test this hypothesis, we conducted a fungal-switch experiment in which *M. smithii*'s normal fungus garden was replaced with a different fungus. Ninety newly emerged, unmated *M. smithii* queens were isolated either on (1) their own fungus, (2) a closely related fungus, or (3) on a distantly related fungus. Surviving colonies were raised for two successive generations during which 21 unmated queens produced exclusively female offspring (213 F1 workers and 15 F1 gynes). These 15 gynes later produced 17 F2 workers, but no males (Table 3.3). Cultivar type (N=3) did not affect queen survival (N = 75) (Pearson's Chi-square test $\chi^2 = 3.99$, df = 2, P = 0.1363). Mortality rates ranged from approximately 70% on fungus garden from *M. smithii* and on fungus garden from *C. longiscapus* (fungus distantly related to the typical *M. smithii* fungus), to 90% on fungus garden from *C. costatus* (fungus closely related to the typical *M. smithii* fungus). Asexual reproduction by queens was independent of cultivar type (Table 3.3), and cultivar substitution did not induce male production. Most significantly, queens always produced workers before gynes, suggesting queen control over offspring caste, rather than extrinsic factors such as sex-ratio biasing microbes.

***M. smithii* Worker Reproduction Experiment.** Workers survived for up to seven months, well over the estimated eight weeks necessary for egg to adult development (Weber 1972). However, unmated workers did not produce haploid males as they can in other ants, nor diploid females, if capable of asexual reproduction like *M. smithii* queens. This confirmed that *M. smithii* workers are functionally sterile, corroborating dissections of field-collected workers by M. Djikstra which revealed that *M. smithii* worker ovaries

are undeveloped like those in the closely related *M. tardus* and many other attine species whose workers also do not reproduce (Dijkstra and Boomsma, In prep.)

Putative *M. smithii* Male. Despite multiple attempts to amplify various small segments of mtDNA (cytochrome oxidase I), the DNA appeared to be too degraded and could not be amplified. Molecular identification of Kerr's (1961) small *Mycocepurus* males therefore failed. We conclude there is no unambiguous evidence for*M. smithii* males in existing collections.

In sum, six lines of evidence support complete and endogenous asexuality in *M. smithii*: absence of males, significant degeneration of the female mating apparatus, DNA fingerprint identity between mothers and daughters, absence of sex-ratio-biasing endosymbiotic bacteria, and the inability to induce male production by antibiotic treatment or fungal substitution. *M. smithii* represents, to our knowledge, the first case of a completely male-less species of ant.

3.4 DISCUSSION

Whereas asexual reproduction is part of normal hymenopteran reproduction where haploid males develop from unfertilized eggs (arrhenotokous parthenogenesis), asexual reproduction of diploid females from unfertilized eggs (thelytokous parthenogenesis) is exceedingly rare in ants. Only six distantly related ant species produce females asexually (Wenseleers and Billen 2000; Pearcy *et al*. 2005; Fournier *et al*. 2005) primarily by unmated workers, ranging from facultative asexual reproduction after queen death to nearly obligate asexuality in which the queen caste is absent or morphologically reduced (Schilder *et al*. 1999b; Itow *et al*. 1984). However, males occur in all six of these ant

species, contrasting with the complete absence of males in *M. smithii*. Three distinct reproductive strategies of asexuality therefore appear to exist in ants: (1) worker reproduction of females with a trend toward queen loss [*Messor capitatus, Platytherea punctata, Cerapachys biroi, Pristomyrmex pungens* (Pearcy *et al*. 2005)]; (2) a mixed strategy where mated queens produce workers sexually but new queens asexually [*Cataglyphis cursor*, *Wasmannia auropunctata* (Pearcy *et al*. 2005; Fournier *et al*. 2005)]; and (3) strict queen thelytoky with sterile workers (*Mycocepurus smithii*). *Pristomyrmex pungens* was proposed as an obligate asexual since it reproduces by worker thelytoky; however, males with functional genitalia and normal spermatogenesis occur, indicating the potential for sex (Itow *et al*. 1984; Tsuji 1988). In contrast, *M. smithii* has evolved a unique strategy in which queens produce both sterile workers and new queens asexually, while workers are completely sterile. Strict clonal reproduction eliminates queen-worker conflicts that plague sexual insect societies, providing a unique model system to test theories of parent-offspring conflict and reproductive policing in *M. smithii* (Ratnieks *et al*. 2006).

Theory predicts that long-term asexuality increases extinction potential because absence of recombination constrains a species' ability to evolve, eventually rendering asexual lineages inferior to competing sexual lineages (Barton and Charlesworth 1998). This cost of asexuality is exacerbated in *M. smithii* through its dependence on asexual cultivar propagation over many ant generations (Mueller *et al*. 1998; Mueller *et al*. 2005). While these cultivars are not ancient asexuals (Mueller 2002), they are cultivated clonally within a nest and between nests over many years, making them vulnerable to rapidly coevolving pathogens (Currie *et al*. 2003).

It is unclear how *M. smithii* copes with this double asexuality handicap, yet sustains one of the most widespread distributions and the greatest local abundances of all attine

species. One possibility is that *M. smithii* can colonize a diversity of habitats because it represents a "general purpose genotype" (GPG) able to tolerate broad environmental conditions (Lynch 1984), an explanation recently applied to the ancient asexual darwinulid ostracods (Van Donick *et al.* 2002). If so, *M. smithii* would be the first case of a symbiosis GPG and support views suggesting that clonality tends towards greater ecological generalization rather than specialization. Unlike all other fungus-growing ants that typically specialize on a narrow clade of fungi, *M. smithii* is the only attine species known to cultivate a great diversity of fungi between different nests because it frequently switches between distantly related fungal crops (AG Himler and UG Mueller unpublished data) (Herre *et al*. 1999). Frequent switching (symbiont reassociation) may mitigate the double asexuality handicap because switching generates novel combinations of ant farmer and crop genomes, potentially creating sufficient variation in ant-crop synergisms to outpace coevolution by crop pathogens (Mueller 2002; Van Doninck *et al*. 2002) and cope with environmental fluctuations.

The widespread distribution and ecological abundance of *M. smithii* despite its double asexual handicap challenge traditional views proposing that sexuality enables fungus-growing ants to assume the coevolutionary arms races of their asexual cultivars, effectively converting crop-pathogen arms races into races between ant farmers and crop pathogens (Herre *et al*. 1999). Asexuality of *M. smithii* precludes such hypothesized arms race transfer, and the ecological success of the dual asexual symbiosis between clonal ant farmers and their clonal crops therefore defies current theoretical expectations, adding a novel form of asexual scandal to a growing list of long-term asexuals (Judson and Normark 1996; Normark *et al.* 2003). We predict that *M. smithii* will emerge not only as a new empirical system to test theories of parent-offspring conflict and policing in eusocial insects (Ratnieks *et al*. 2006), but also as a model permitting easy controlled

symbiont-switch experiments in order to understand the evolutionary persistence of asexual lineages within a network of coevolving sexual pathogens and asexual mutualists.

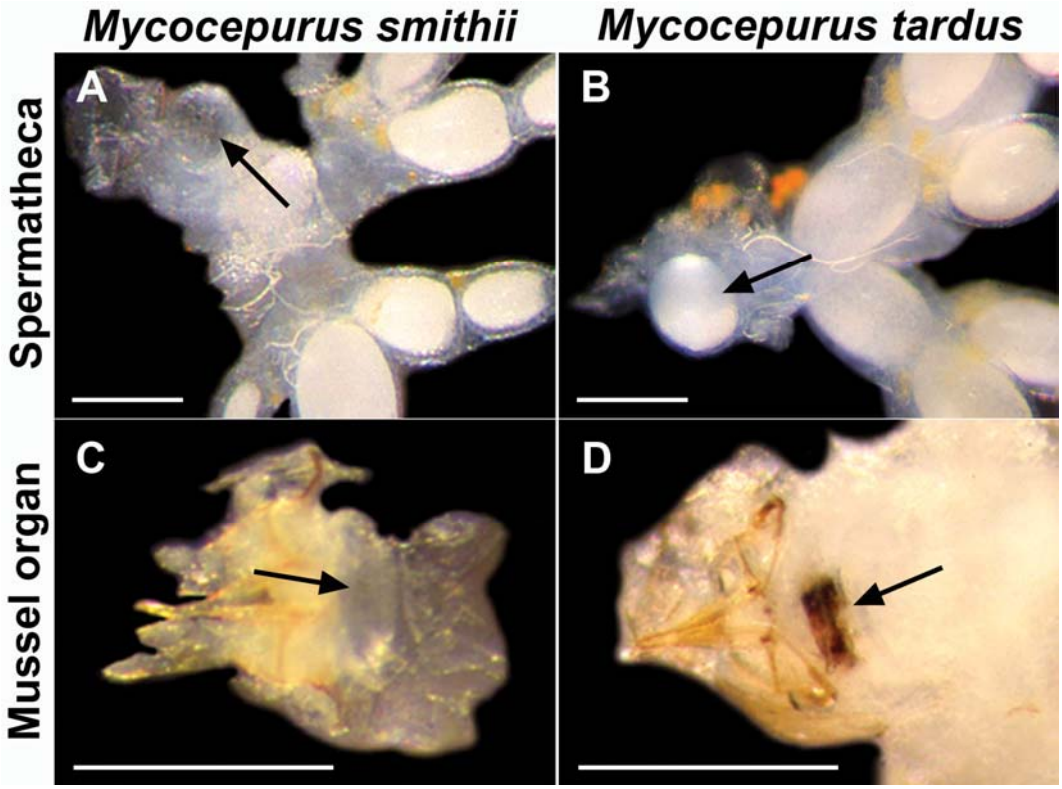

Figure 3.1. Reproductive tracts of *Mycocepurus smithii* and *Mycocepurus tardus* queens. The spermatheca of *M. smithii* is translucent (A), indicating that it is empty. In contrast, a sperm-filled spermatheca appears opaque, as shown for the congener *M. tardus* (B). Fully developed ovaries shown in both species contain mature eggs and several yellow bodies indicating the queens were active egg layers. The mussel organ, an internal lock structure of the female mating apparatus, is degenerate and unsclerotized in *M. smithii* (C) compared to the sclerotized functional mussel organ of *M. tardus* (D). Scale bar, 0.25mm

Heterozygous queen	# offspring genotyped per heterozygous queen	Pr (all offspring heterozygous / queen mated)	Probability all offspring heterozygous
Colony 1	7	$(1/2)^{7}$	0.0078
Colony 2	8	$(1/2)^{8}$	0.0039
Colony 3	8	$(1/2)^{8}$	0.0039
Colony 4	5	$(1/2)^{5}$	0.0313
Colony 5	7	$(1/2)^{7}$	0.0078
Colony 6	6	$(1/2)^{8}$	0.0039
Colony 7	5	$(1/2)^{5}$	0.0313
Colony 8	4	$(1/2)^{4}$	0.0625
Colony 9	4	$(1/2)^{4}$	0.0625
Total	54	$(1/2)^{54}$	5.55×10^{-17}

Table 3.1. Probability of all heterozygous female offspring from nine heterozygous *Mycocepurus smithii* nests.

The probability that each heterozygous queen produced all heterozygous offspring, assuming sexual reproduction, was calculated based on the number of female offspring genotyped per colony. Values ranged from 0.0625 to 0.0039. Summing over all 54 offspring, the probability is vanishingly small at 5.55 x 10-17. Under Mendelian segregation with haplo-diploid mating, the expectation is 50% heterozygous offspring from a heterozygous mother.

Antibiotic	% Antibiotic solution	# Subcolonies	# Queens treated per subcolony	Total # Queens treated	Queens produced	Males produced
Streptomycin	10%	7	37±17	264	931	0
Rifampicin	0.5%	7	38±16	263	2560	0
Penicillin	5%	7	34±16	236	1666	0
Tetracycline	5%	7	36±12	249	1558	0
Control (sucrose)	10%	7	47±16	332	1129	0
Total				1344	7844	0

Table 3.2. Antibiotic treatment of unmated queens

Total number of queens produced (7,844) over 16 months after antibiotic treatment. No males were produced. Concentrations were derived from similar experiments with wasps, and further tested in pilot experiments to maximize dose administered without causing significant mortality (up to 10%). Number of queens treated per subcolony represents the average ± 1 standard deviation.

	Fungus Garden Source			
Offspring (F1+F2)	*M. smithii* fungus (control fungus)	*C. costatus* fungus (closely related fungus)	*C. longiscapus* fungus (distantly related fungus)	Total
# Workers produced	77	8	128	213
# Gynes produced	7	1	7	15
% Queens reproducing	6/30 = 20%	3/30 = 10%	6/30 = 20%	15/90=17%

Table 3.3. Fungus switch experiment

Ninety unmated *M. smithii* queens were placed either on their own fungus (control), a closely related, or a distantly related fungus. No males were produced on any fungus type while female workers and new queens (gynes) were produced over three generations. There was no effect of fungus type on number of workers produced (Kruskal-Wallis test $\chi^2 = 1.69$, df = 2, $P = 0.4289$), on the number of gynes produced (Kruskal-Wallis test $\chi^2 = 0.0728$, df = 2, $P = 0.9643$) or on queen survival (Pearson's chi-square test $\chi^2 = 3.99$, df = 2, P=0.1363).

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Chapter 4

New species of *Cyphomyrmex* in the *longiscapus* species group: phylogenetic characterization and nesting biology.

Abstract. The *Cyphomyrmex cf. longiscapus* species group has become a model system for a diverse array of studies in behavior, ecology and evolution in the context of the fungus-growing ant mutualism (Mueller and Wcislo 1998; Adams *et al.* 2000; Schultz *et al.* 2002; Green et al. 2002; Mueller et al. 2002; Mehdiabadi et al. 2005). Despite these recent findings, novel aspects of the system continue to emerge from taxonomic and phylogenetic revisions (Schultz and Brady, unpublished; Schultz and Meier 1995, Schultz *et al.* 2002; Mikheyev *et al.* 2006) to behavior: [(Mueller *et al.* 2004; Mehdiabadi *et al.* 2005; Advani and Mueller 2006; Himler and Mueller unpublished (Chapter 2)], host switching (Adams *et al.* 2000; Green *et al.* 2002; Mehdiabadi *et al.* 2005; Himler and Mueller unpublished (Chapter 2)), to complex microbial interactions (Gerardo *et al.* 2004). The *Cyphomyrmex* genus occupies a unique phylogenetic position, intermediate between the basal 'lower' attine genera and the phylogenetically derived 'higher' attine genera including the leaf cutter ants. Thus insights into this genus may identify the key differences that permitted evolution of the highly derived leaf cutter genera from their basal ancestors. Here I reconstruct the phylogeny of the mycelium-cultivating *Cyphomyrmex cf. longiscapus* species group in order place in context newly discovered species of *Cyphomyrmex*. Results show that the *C. muelleri* type from Costa Rica and Western Panama is a new species in the monophyletic *longiscapus*-group and provide novel information on their nesting ecology and habitat.

4.1 INTRODUCTION

The Genus. The *Cyphomyrmex* genus consists of 38 (Kempf 1968; Snelling and Longino 1992; Schultz *et al.* 2002) known species ranging from southern United States to Argentina, with most species occurring in Neotropical wet forests (Kempf 1964; 1966). *Cyphomyrmex* are distinguished from other attine genera by their laterally expanded frontal lobes (J.T. Longino http://www.evergreen.edu/ants/genera/cyphomyrmex/home.html). All *Cyphomyrmex* species are small inconspicuous ants, with relatively small nests (ranging from tens to hundreds of workers, rarely exceeding 1000 workers per nest in some species). Their nests are primarily terrestrial, occurring in protected alcoves in or under clay stream embankments, rocks, or logs. Nest architecture varies widely in the genus from little to no nest structure (*C. costatus*, *C. rimosus*, *C. salvini*) (Wheeler 1907) to highly specialized (*C. longiscsapus*, *C. muelleri*, *C. sp. 1*), and even arboreal nest structures (*C. cornutus*) (Mueller and Wcislo 1998; Schultz *et al.* 2002; Adams and Longino, *in press*). The genus is divided into two groups: the *strigatus*-group and the *rimosus*-group, each containing an estimated 16-20 species (Snelling and Longino 1992), although more undoubtedly remain to be discovered and described. The *strigatus*-group contains only mycelium growing *Cyphomyrmex* that occur in South America (Kempf 1964). The *rimosus*-group contains all yeast-cultivating *Cyphomyrmex* species and a small group of mycelium growing species that occur in North, Central and South America (Kempf 1964, 1966, 1968). This small group of mycelial growers, termed the *longiscapus*-group (Schultz and Brady, in prep) include *C. longiscapus, muelleri, wheeleri*, and *costatus*, and may comprise a separate third group in the genus, as these species do not conform fully with the rest of the *rimosus*-group (Kempf 1968). Within this *longiscapus*-group I concentrated on the Neotropical *C. longiscapus* and *C. muelleri* model system. A potential new species discussed here from Panama and Costa Rica has affinities with *C. longiscapus sensu stricto* from central Panama, and may therefore be part of this third species group. I provide the first phylogenetic treatment of this potentially new third

longiscapus-group within the *Cyphomyrmex* genus, and place the newly discovered Central American species within this group.

Recent work revealed that this third *longiscapus*-group is sister to the monophyletic clade of the higher attines, the leaf-cutting ants (Schultz and Brady, in prep.) The *Cyphomyrmex* genus contains a unique evolutionary transition in the attine tribe from the more typical mycelium-growing *Cyphomyrmex* species (in the *strigatus*-group of *Cyphomyrmex*) and all other lower attines, to the more derived species in the *C. salvini* and *C. rimosus* complexes (in the *rimosus*-group of *Cyphomyrmex*) that cultivate fungi in the form of single celled yeast (Schultz and Meier 1995, Mueller *et al.* 1998). Thus the *longiscapus*-group species occupy a significant phylogenetic position to study the evolutionary transition from mycelium to yeast cultivation (Schultz *et al.* 2002). In addition, this *longiscapus*-group also shares several morphological features with the higher attines, unlike all other lower attines, including harboring actinomycete bacteria on the anterior-ventral pleural plates of the thorax, and having modifications of their integument underlying the cuticular bacteria they carry (Currie *et al.* 1999a). Thus the *longiscapus*-group contains key species with unique characteristics that may elucidate the evolutionary transition from the lower attine cryptic ants with relatively small nests to the notably successful tropical leaf-cutting ants.

C. sp. nov. **Nest descriptions**. Outside central Panama, *C. longiscapus s.s.* and *C. muelleri s.s.* are known from only a very few collections in Costa Rica, Ecuador and Colombia (Kempf 1968; Snelling and Longino 1992). In order to expand collections and delineate species ranges of *C. longiscapus* and *C. muelleri*, I surveyed at or near previous reported locations where possible, as well as new localities in Western Panama, Costa Rica, and Ecuador. All specimens were collected in tropical wet forest at several sites in Costa Rica, Panama and Ecuador, with the exception of Colombia, where a few specimens of *longiscapus* were previously reported, which was not safe to access at the time (Snelling and Longino 1992; Mueller and Wcislo 1998; Schultz *et al.* 2002). During these surveys, I encountered a new species of *Cyphomyrmex*, referred to as *C. sp. nov.*,

with two different nest morphologies, generating the hypothesis that either they represent two different species of ant with distinctive nest architectures, or they are the same species of ant (as the fungus garden and worker ants appear very similar), and unknown local ecological factors dictate the nest type built. Both nesting morphologies occurred in generally similar habitat in primary and secondary tropical wet forest and were often microsympatric: both nest types in Panama and Costa Rica occurred on a protected underside of a vertical face of rock, fallen tree, or wet clay bank, usually in close proximity to streams or rivers. Due to their specialized nest structures and microhabitat, these relatively common attines were only recently discovered because the solitary foragers elude common sampling techniques.

One nest type was dubbed the "funnel" type, similar to the "swallow's nest form" of *C. longiscapus* (Schultz *et al.* 2002) due to its distinctive nest entrance constructed of a thin layer of accreted soil in the shape of a downward projecting tube expanding upwards to a single chamber housing a relatively large fungus garden (for lower attines) affixed to rootlets or the surface of a rock/tree/claybank, with a soil envelope covering the entire garden (Figure 4.1). The funnel varied in length and diameter, perhaps a function of local habitat conditions (table 1), but was always projecting downwards. This may discourage predators and/or maintain humidity of the fungus garden, although some funnels were quite shallow in length and very wide, exposing the fungus garden. The fungus garden was usually visible from below when looking up the funnel. The soil envelope of the funnel entrance and covering the nest was extremely moist or completely saturated with water to the point of dripping. The second nest type was termed "cryptic" due to the dome or envelope of accreted soil completely covering a single relatively large fungus garden affixed to the sheltered side or underside of a tree, log or rock, rendering the nest well hidden and difficult to detect. The nest entrance consisted either of a tiny hole with no structure around it, or was not visible (Figure 4.2). These cryptic nests seemed less constrained to occur directly near water than the swallows' nest type; while some were on the underside of a log directly over a small stream, others occurred several meters further

from water, but still in proximity to water (no distance measurements from streams were recorded). The *C. sp. nov.* nest architecture in Ecuador generally consisted of a single fungus garden in a shallow depression beneath a log, or within a cavity in a rotten log (Figure 4.3). Interestingly, these nests occasionally had beetle elytra on/embedded in the soil not touching the fungus garden, similar to *C. muelleri* in central Panama.

The mycelial fungus garden of both the funnel and cryptic nest types consisted of a spongiform mass composed in a dense lattice of fine pellets, granular in texture, and very fragile and light crumbling easily when disturbed. The granular texture is likely due to the insect frass that appears the favored substrate. Other substrates appeared to be some filamentous plant debris and occasional dead arthropod parts such as beetle elytra. The fungus was light tan in color, but color may vary depending upon substrate provided. In both cases the fungus garden was attached at the top of the funnel or cryptic nest by rootlets when available, or directly to the rock, log or clay ceiling or vertical surface against which the soil covering the nest was constructed. Both the funnel and cryptic nest cultivars appeared very consistent in color and texture. Differences in microhabitat and substrate type available contribute to local variation, and molecular characterization of these cultivars would be the best way to determine their relatedness as well as to other lepiotacious G3 cultivars predominantly raised by the lower attine species. The Ecuadorian *C. sp. nov.* fungus gardens are terrestrial in or under logs, attached at the top or side to the log, are larger and slightly more solid fungal mass, perhaps due to the substrate fed to the fungus. Some nests also had beetle elytra and insect exoskeleton parts embedded in soil around it.

4.2 MATERIALS AND METHODS

Collection. Specimens were collected in 2002 and 2003 on a biogeographic transect across western Panama and the eastern drainage of Costa Rica. Panamanian sites include Santa Fe (two sites: Café el Rio (CER) (N 08° 31.548'; W 81° 7.807 +/- 37 ft) and Tercero Brazo (TB) (N 08° 32.333'; W 81° 05.038' acc 98 ft, elevation 435m)), and

Bocas del Toro province, between Chiriqui and Chirique Grande (N 08° 46.744'; W 082° 11.352'). Costa Rican sites include Hitoy Cerere (HC) (N 09º 40.361'; W 083º 01.465' Alt402 feet) and La Selva Biological Station (LS, CR) (N 10°26'; W 84°00', 50m). Specimens from Ecuador were collected from La Selva (LS,EC) (S 00° 29.851; W x76° 22.483 +/- 22ft) and Tiputini (Tip, EC) (S 00° 38.295'; W 76° 8.959) field stations.

Colonies were collected or sampled by hand using sterile forceps. When possible, whole intact queenright colonies were exported live from Panama and Costa Rica. Ecuador did not permit export of live material so DNA vouchers were exported. Many *C. sp. nov.* colonies were too large to permit complete colony collection for maintenance and transport in the field. Some of these nests were subsampled in which a small portion of workers and fungus garden were collected. Live colonies and subcolonies exported to the USA were maintained at room temperature in multi-chamber artificial colonies following Schultz (1993) at the University of Texas at Austin. DNA vouchers of all ants and cultivars were preserved in 95% ethanol. Nest dimensions were recorded from a subset of nests including width and length across the nest entrance and at the widest point across the center of the nest (Table 1). Some nests were also excavated in their entirety and all colony members counted (Table 1).

Molecular Methods. A phylogenetic study was conducted to determine whether the newly discovered *Cyphomyrmex* species represent new species, and reveal their position in the genus. Genomic DNA from single workers or gynes was extracted using Qiagen Dneasy™ kits from samples refrigerated in 95% ethanol. Sequencing targeted the F1 paralog of nuclear elongation factor –1 alpha (EF-1∝) which spans an intron flanked by exons. Nested PCR was performed using primers M3trs2 (5' – CAT ATW AAC ATT GTS GTS ATY GG – 3')/trs10R (ACG GCS ACK GTT TGW CKC ATG TC – 3') to amplify approximately 1.5kb region, and then primers U52.1 (5' – CCG CTT CAG GAT GTC TAT AA- 3')/L53 (CCG CGT CTC AGT TCY TTC AC – 3') were used to amplify the smaller "I" region of approximately 700bp using the first PCR as template (T.R.

Schultz, unpublished). Standard PCR conditions (Palumbi 1996) were used with some modifications for optimization.

All sequences were run on an ABI 3100 automated sequencer, assembled using Seqman II v.5.05 (DNASTAR), aligned with Clustal X (Thompson, Plewniak, and Poch 1999) and edited manually in MacClade v.4.06 (Maddison and Maddison 2003). Three regions consisting of 195 out of 945 characters were excluded as unalignable since homology of those sites could not be inferred. The final matrix consisted of 750 characters for 59 taxa including outgroups after removing three redundant taxa. All sequences will be deposited on Genbank. A model of sequence evolution was estimated for the data using MODELTEST v.3.06 (Posada and Crandall 1998) for maximum likelihood analysis. MODELTEST selected the Hasegawa, Kishino, and Yano model of sequence evolution with four gamma distributed rate classes (HKY+Γ). Maximum likelihood analyses were conducted using the Genetic Algorithm for Rapid Likelihood Inference (GARLI) v.0.942 (Zwickl 2006) using random starting trees and default settings for four separate runs. An additional maximum likelihood search was performed using a specified starting tree (neighbor-joining tree). Estimates of clade support were obtained by performing non-parametric bootstrapping with heuristic searches of 500 replicate datasets and 10 random addition sequences replicates per dataset.

4.3 RESULTS

Collection. Number of *C. sp. nov*. nests collected per site per year: Bocas del Toro, Panama, 2002 (24); CER and TB sites near Santa Fe, Panama , 2003 (21); Hitoy Cerere, Costa Rica, 2002 (10); La Selva Biological Station, Costa Rica, 2002 (15); El Ceibo, Costa Rica, 2003 (38); Tiputini, Ecuador, 2003 (15) and La Selva, Ecuador, 2003 (14).

At collecting localities CER and TB near Santa Fe, Panama, some colonies had nest entrances very reminiscent of *C. longiscapus s.s.* with small earlike auricles above streams, except that they were pointing downwards instead of the generally outward

orientation of *C. longiscapus s.s.* nest entrances. This led to the hypothesis that either this was a zone of sympatry between *C. sp. nov.* and *C. longiscapus s.s.* from central Panama, or these were incipient colonies typical of the new species. The molecular genetic results showed that these samples from TB and CER, Panama, were *C. sp.1* even though this species generally has a more pronounced downward pointing funnel. These CER and TB sites near Santa Fe, Panama were the closest on the transect to the Panama canal region where *C. longiscapus s.s.* and *C. muelleri s.s*. occur. It would be interesting to examine the range just east and west of this site to determine how far west *C. longiscapus s.s*. extends and how far east *C. sp.1* reaches, to determine if their distributions ever overlap or if they remain parapatric. If they are strictly parapatric, it would be interesting to know factors that determine such species displacement. Unlike Panama, The two nest types were more abundant and sympatric at La Selva and El Ceibo sites in Costa Rica, but this may be due to more suitable habitat at the sites surveyed.

I did not find evidence of *C. muelleri*-like nests at Esmeraldas, Ecuador where the single previous *C. muelleri*-like ant was reported (Schultz *et al.* 2002). Primary forest was difficult to find near this area, indicating human development had likely decreased forest habitat since the specimen was found. At Tiputini and La Selva, Ecuador, two new collecting localities, I encountered not the typical *C. longiscapus* or *C. muelleri* nests in embankments, but rather terrestrial nests primarily in or under logs as described in the introduction.

Nest architecture. The average width of cryptic nests was 8.0cm (± 2.6) and length was 9.5cm (± 5.3) from a subset measured from El Ceibo, Costa Rica (N= 15 nests) (Table 4.1). The average width of funnel nests was 6.8cm (± 3.4), and length was 7.0cm (± 3.5), the average funnel length was 4.3cm (± 2.1), and funnel entrance dimensions averaged 3.0cm (± 1.2) in width and 2.5cm (± 1.1) in length (N= 11 nests) (Table 4.2). The average number of workers per nest was 201 (N=17) for nests from western Panama and two from Hitoy Cerere, Costa Rica (Table 4.3). Almost all funnel nests encountered had a single downward pointing soil funnel entrance, however there were two exceptions, both

in western Panama, in which a single nest had two separate funnel entrances, constructed with the openings pointed away from each other. One large funnel nest in Costa Rica had a primary funnel entrance, but folds of soil composing smaller partial funnels were built into the soil above the main entrance. These were closed, but they could potentially be used as alternative entrances. In June 2002 in western Panama I found several empty soil envelopes that appeared characteristic of *C. sp. nov.*; one contained a small round mass overgrown with a white fungus, potentially an attine fungus garden destroyed by pathogenic fungi. This was not subcultured to identify the pathogen. All nests sampled were monogynous when the entire nest was collected and counted, however, many nests were so large that only a small portion of garden and workers were collected for vouchers and a live subcolony for the laboratory, so queen number was not always established. It appeared that the queen tried to escape the nest as soon as the soil envelope was disturbed (A. Himler and U. Mueller, personal observation). No polygynous nests were discovered during this survey with the exception of one from Hitoy Cerere that had two queens, however it was unclear whether both were actively reproducing; one could have been a dealate daughter that did not disperse but may not reproduce.

For both the funnel and cryptic nest types, the soil envelope over the garden was thicker at the edges where the nest attached to a substrate surface (1.5cm in a cryptic nest), thinning towards the center over the nest (3mm in a cryptic nest). My general impression was that the cryptic nest covering was thicker than the funnel type (although this was not measured) and not as saturated with water, coincident with its perceived greater distance from steams. These ants may have limited dispersal as large established funnel nests often had several incipient colonies flanking them within 3-12 centimeters, or daughter nests are denser in optimal habitat. Incipient nests have a small rounded bulge around the fungus garden chamber with a tiny fluted funnel entrance, reminiscent of *C. longiscapus s.s.* auricles from central Panama. Whether the cryptic and funnel nest ants have the same incipient nest architecture is unclear; only observation over time will establish this. Small

incipient nests surrounding larger mature nests appeared less common for the cryptic nest type, so they may indeed have tiny cryptic incipient nests that are more difficult to find.

Phylogenetic results. The aligned sequence data for *C. sp. nov.* ants consisted of 750bp of nuclear gene EF-1∝ after excluding unalignable regions. Of the 750 sites, 359 were variable, and 319 of these were parsimony informative. Results of maximum likelihood analyses run under a variety of conditions: a) default model GTR+I+Γ estimating parameters, b) HKY+Γ model using parameter values estimated by ModelTest, and c) default and specified model using a specified start tree (neighbor joining) were nearly identical except for placement of a few sister taxa. All major clades were identical. Maximum likelihood analysis revealed that the *C. sp. nov.* ants sampled fell into two monophyletic groups (Figure 4.4) somewhat consistent with geographic region. *C. sp. nov.* ants from Western Panama and all Costa Rican sites clustered together forming a near polytomy. Within this clade, a subset of samples from Western Panama formed a small separate clade very close to the Western Panama and Costa Rican clade, but two samples (202 and 206) from the same region fell within the larger clade with the rest of the Costa Rican and Panamanian samples. Samples from Ecuador clustered together outside the *longiscapus*-group, likely representing a separate species distantly related to the central Panama and Costa Rican specimens in the *longiscapus* group. This was not unexpected given a) the significantly different nest habitat and structure, b) ant morphometrics (Johnson and Schultz unpublished data), and c) the greater geographic distance from the Panamanian and Costa Rican specimens. It was surprising that specimens from any of the Central American localities did not sort according to nest type, as expected if they were separate species. Interestingly, a putative *C. muelleri*-like sample from Costa Rica (280) grouped with Panamanian representatives of *C. longiscapus s.s.* and *C. muelleri s.s.* with strong bootstrap support, rather than with all other samples from Western Panama and Costa Rica.

4.4 DISCUSSION

Analysis revealed that the *Cyphomyrmex sp. nov.* samples comprise a new phylogenetic species in the *longiscapus*-group, sister to *C. longiscapus s.s.* and *C. muelleri s.s.* species (Figure 4). This is not surprising given *C. sp. nov.*'s distinctive nest architecture, cultivar, larger colony size (several hundred up to a thousand workers) and larger ant size. In addition, results showed that the samples from Ecuador comprise a separate species basal to *C. sp. nov.* from Central America. This also is not surprising considering their nesting habitat in and under logs is very different from the Central American *C. sp. nov*. nest types examined here. Hereafter I will refer to *C. sp. nov*. ants from far western Panama (Bocas del Toro and Veraguas Provinces) and Costa Rica (La Selva and El Ceibo Biological Stations, and Hitoy Cerere Biological Reserve) as *C. sp. 1*, and *C. sp. nov*. ants from Ecuador as *C. sp. 2*. Further molecular and taxonomic comparisons to other South American *Cyphomyrmex* species are required to determine whether *C. sp. 2* comprises a new species within the *strigatus*-group of this genus (T.R. Schultz, personal communication). Surprisingly, the Central American *C. sp. 1* ants did not sort phylogenetically by nest type (funnel and cryptic types), despite their strikingly different nest morphology. This could be due to the fact that the EF-1∝ intron region sequenced was not evolving fast enough to reveal recent divergence, however the data harbored substantial variation, and this region was recommended for distinguishing cryptic species (T. R. Schultz, personal communication.). Additional loci to explore include the long-wavelength rhodopsin gene (LW *Rh*) (Ward & Downie 2005) that has been useful in other ants and attines (S. Brady personal communication), and the UV opsin gene that has worked less successfully in attines (Ward unpublished, S. Brady, personal communication.). However, both these loci are reportedly slower evolving than EF-1∝ (S. Brady, personal communication.) This research provides the first in depth phylogenetic treatment of the *Cyphomyrmex longiscapus*-group locating it within the genus, complemented by new information on their biology. Incorporating the few older

previously collected specimens by taxonomic comparison will help clarify uncertainty about their species identity.

Previous work showed that *C. longiscapus sensu stricto* and *C. muelleri* occur in central to southeastern Panama, nesting in stream embankments with species-specific soil auricle nest entrances (Mueller and Wcislo 1998, Schultz *et al.* 2002). *C. muelleri* has a limited distribution sympatric within the *C. longiscapus* range, restricted to Central Panama (see Chapter 2), while *C. longiscapus s.s.* occurs from central Panama to the Darien Province in the southeast. Morphometric comparison of *C. longiscapus s.s.* with *C. longiscapus* from the Darien region in Panama found no significant difference between them (Johnson and Schultz, unpublished data). A few specimens thought closely affiliated with the *C. longiscapus sensu lato* group have been collected in Central and South America and have been loosely categorized as either *longiscapus*-like or *muelleri*-like (Mueller and Wcislo 1998; Schultz *et al.* 2002). However, this classification was often based on few specimens, with no or limited information about their nesting biology (Snelling and Longino 1992; Mueller and Wcislo 1998; Schultz *et al.* 2002). These specimens include *C. muelleri*-like specimens from the Osa Peninsula and from La Selva Biological Station, Costa Rica (Schultz, T. R., Longino, J.T., and R. M. M. Adams, personal communication), a *C. muelleri*-like specimen from Ecuador (Schultz *et al.* 2002; R. M.M. Adams personal communication) and *C. longiscapus*-like specimens from Colombia (Weber 1940; Kempf 1966; Snelling and Longino 1992; see Schultz *et al.* 2002 for summary of specimen and locality details). Morphometric measurements confirmed that the Colombian *C. longiscapus*-like specimen is consistently larger than *C. longiscapus s.s.* and *C. muelleri s.s.* from central Panama (Schultz *et al.* 2002) and similar in size to the Costa Rican *C. sp. 1* and previous Costa Rican specimens (Johnson and Schultz, unpublished data). Thus there is ambiguity and potential cryptic species in the *C. longiscapus*-group.

The *C. sp.1* specimens from western Panama and Costa Rica are dissimilar to *C. longiscapus s.s.* in the following ways: 1) the ants are larger in size, 2) the colony size is

much larger containing up to 600 workers, 3) the nest architecture is significantly different consisting of up to 15cm accreted soil envelope fixed to the side of a rock, clay bank, or tree log covering a single large fungus garden, and, 4) the cultivar is phylogenetically distinct, similar to Clade 1 cultivars in Mueller *et al.* (1998), or a unique type. In contrast, the *C. sp. 2* samples from Ecuador nest in or under logs, lack a soil envelope covering the nest, and the mycelium is a different texture, potentially representing a Clade 2 cultivar (Mueller *et al.* 1998). Features in common between the Central American and Ecuadorian *C. sp. nov*. samples are larger ant size and larger nest size (up to several hundred workers). However, morphometric measurements comparing workers found a significant difference between the Costa Rica type and *C. longiscapus s.s.* (Johnson and Schultz, unpublished data).

Previously, taxonomy of the single putative *C. muelleri*-like specimen from Ecuador placed it very closely related to the Central American (Central Panama) *C. muelleri*, more closely than to *C. longiscapus*, representing perhaps an intermediate between *C. muelleri* and the Costa Rican *C. sp. 1* specimens (Schultz, personal communication.). This is based on the fact that the Central American *C. muelleri s.s.*, the Costa Rican *C. sp. 1* considered here, and the single *C. muelleri*-like Ecuadorian specimen share more derived morphological features: reduced metanotal groove, smoother mesosomal dorsum without tubercles, and the hind femur with a ventral lobe and groove. In fact, the evenly curved back (smooth mesosomal profile) is "carrying [the] morphological trend that separated *C. muelleri* from *C. longiscapus* to an even greater extreme" (Schultz, pers. comm.). The three derived character states listed above are uncommon in other attines, and unite these three groups (T. R. Schultz, personal communication). This generates two hypotheses: 1) they evolved in parallel, from a common widespread ancestral *C. longiscapus sensu lato* type, each converging on these characteristics, or 2) they evolved once, forming a monophyletic group, and secondarily experienced range contractions or some other ecological factors that divided their current distribution. The phylogenetic results presented here refute the second more parsimonious hypothesis, placing the Costa Rican

and western Panama *C. sp. 1* specimens together, sister to the *C. longiscapus* and *C. muelleri* clade, while the Ecuadorian species group lies basal outside the *C. longiscapus*-group altogether. These results conflict slightly with a recent analysis of the entire attine tribe that placed the Costa Rican *C. sp. 1* sister to *C. longiscapus s.s.* instead of *C. muelleri* (Schultz 2006). However, my study utilized only one fast evolving nuclear gene that harbored substantial variation and focused more on the population level of *C. sp. 1* and its relationship to close allies, while the Schultz and Brady (unpublished) study combined five genes and examined species relationships between genera for the tribe as a whole using only a few *C. sp. nov.* specimens.

The *C. longiscapus s.s.*, *C. muelleri*, and *C. sp. 1*, and *C. sp. 2* lineages occur in a geographic mosaic of parapatric forms, occasionally broadly sympatric (*C. longiscapus s.s.* and *C. muelleri s.s.* in central Panama). This mirrors an apparently common pattern in nature seen, for example, in *Rana pipiens*, *Heliconius* butterflies, and army ants, (Coyne and Orr 2004). At this point, every species in this *longiscapus*-group within the *C. rimosus* group of mycelium cultivating fungus-growers has its own distinct fungus, but the degree of cultivar sharing in the lower attines is only now being elucidated. For example, it has been shown that *C. muelleri* and *C. costatus* routinely trade cultivars, and very rarely even cultivate identical strains of fungus (Green *et al.* 2002, Gerardo *et al.* 2004), while *A. auriculatum* and *C. longiscapus* cultivate the same Clade 1 fungus, at least on one occasion (Mueller *et al.* 1998). Future work mapping the cultivar types from the *C. sp. 1* and *C. sp. 2* specimens onto the ant phylogeny will demonstrate whether and to what extent cultivar exchange might occur in this species group.

Regarding the Ecuadorian (South American) *C. sp. 2*, it has been suggested that it would make an excellent lower attine model species to compare to the *C. longiscapus/C. muelleri* model system in Central America (T.R. Schultz, personal communication.). While another attine genus, *Mycetarotes*, was recently proposed as a potential South American model species (Solomon *et al.* 2006), *C. sp. 2* and relatives in South America may be more appropriate candidates as they are widespread in South American wet

forests, contains a diverse number of species for comparative studies, and importantly, unlike many fungus-growing ants with subterranean nests (including *Mycetarotes*), are easy to collect from beneath or within rotting logs, requiring no digging for excavation. However, first phylogenetic and taxonomic comparison of *C. sp. 2* to other South American *Cyphomyrmex* ants in the *strigatus*-group are necessary to establish the whether this is indeed a new South American species, and place it in proper context within the genus. While the work presented here clarifies this important transitional *Cyphomyrmex longiscapus*-species group further, clearly this group offers a rich resource worth additional investigation to address a host of questions pertaining to fungus-growing ant diversification, host switching, habitat specialization, and evolution of unique nest architectures, as well as their associated microbial community interactions that have not yet been examined.

Figure 4.1: *C. sp. nov.* funnel nest type. Funnel nest (left), open funnel nest (right)

Figure 4.2: *C. sp. nov.* cryptic nest type. Cryptic nest (left), open cryptic nest (right)

Figure 4.3: *C. sp. nov.* Terrestrial nest type from Ecuador.

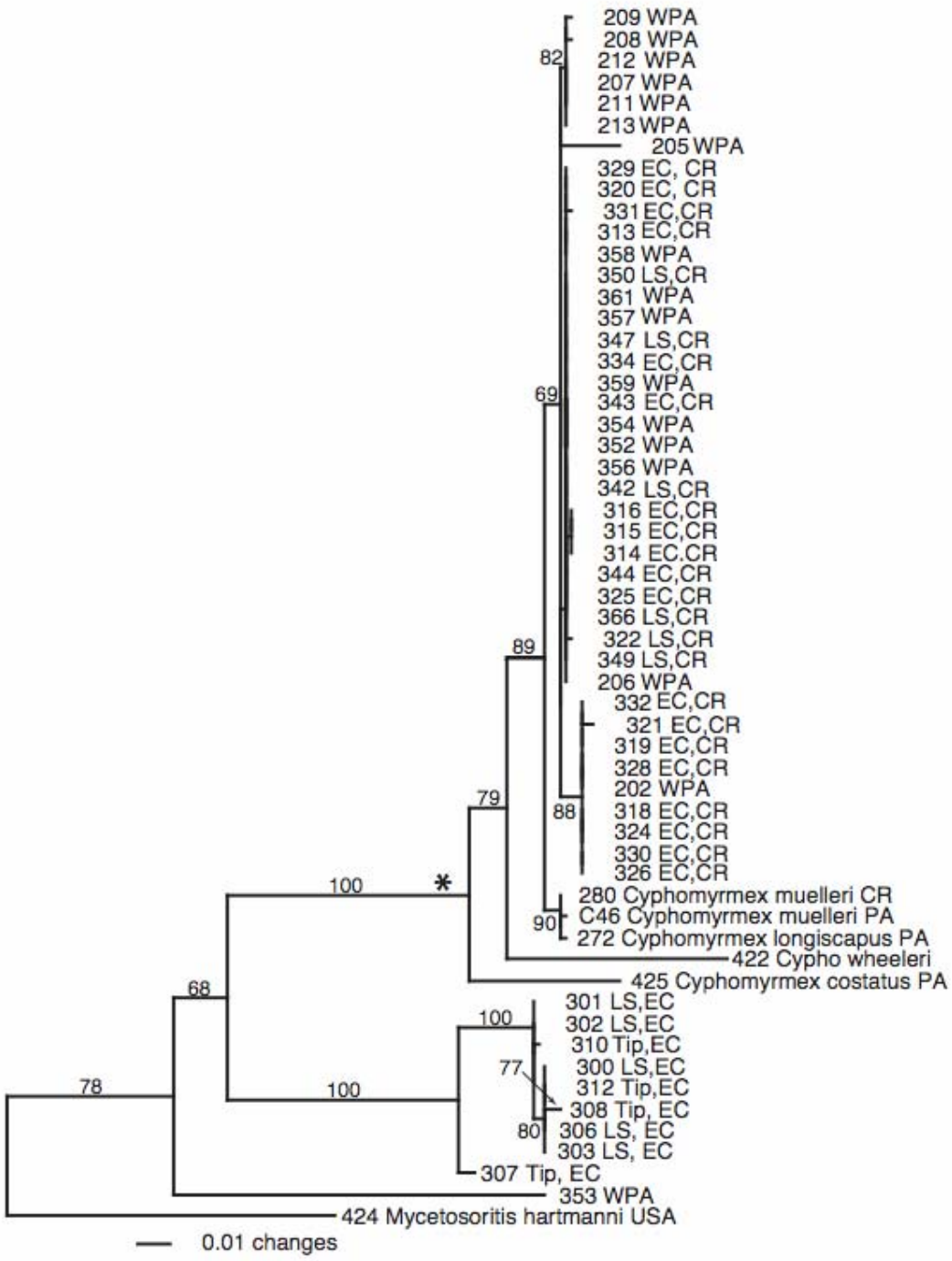
209 WPA
208 WPA
82
212 WPA
207 WPA
211 WPA
213 WPA
205 WPA
329 EC, CR
320 EC, CR
331 EC,CR
313 EC,CR
358 WPA
350 LS,CR
361 WPA
357 WPA
347 LS,CR
334 EC,CR
359 WPA
69
343 EC,CR
354 WPA
352 WPA
356 WPA
342 LS,CR
316 EC,CR
315 EC,CR
314 EC.CR
344 EC,CR
325 EC,CR
366 LS,CR
89
322 LS,CR
349 LS,CR
206 WPA
332 EC,CR
321 EC,CR
319 EC,CR
328 EC,CR
79
202 WPA
88
318 EC,CR
324 EC,CR
330 EC,CR
326 EC,CR
100
*
280 Cyphomyrmex muelleri CR
90
C46 Cyphomyrmex muelleri PA
272 Cyphomyrmex longiscapus PA
422 Cypho wheeleri
425 Cyphomyrmex costatus PA
68
301 LS,EC
100
302 LS,EC
310 Tip,EC
77
300 LS,EC
78
100
312 Tip,EC
308 Tip, EC
80
306 LS, EC
303 LS, EC
307 Tip, EC
353 WPA
424 Mycetosoritis hartmanni USA
0.01 changes

Figure 4.4: Maximum likelihood phylogram of *Cyphomyrmex sp. nov.* based on EF-1α sequence data. Terminals are labeled with a sample code, followed by the ant species name for outgroups, or no name for *C. sp. nov.* samples, and end with the collection locality and country of origin (WPA: western Panama; HC, CR: Hitory Cerere, Costa Rica; EC, CR: El Ceibo, Costa Rica; LS, CR: La Selva, Costa Rica; Tip, EC: Tiputini, Ecuador; LS, EC: La Selva, Ecuador). Branches are labeled with likelihood bootstrap values. Unlabeled branches have values less than 50. An asterisk (*) indicates the monophyletic *longiscapus*-group. Not all outgroups are shown for clarity.

Table 4.1: *Cyphomyrmex sp. nov*. nest architecture measurements. Nest measurements of *C. sp. 1* cryptic nest types. W = width in cm; L = length in cm.

Colony ID	Site Name, Country	Nest W	Nest L	Entrance W	Entrance L	Comments
AGH030212-05	El Ceibo, Costa Rica	12.5	18.0	0.4	0.4	underside dead tree; very large nest
AGH030212-07	El Ceibo, Costa Rica	3.5	4.0			under bark on ground; very large nest
AGH030214-01	El Ceibo, Costa Rica	7.3	20.0			against large dead stump, amorphous shape
AGH030214-06	El Ceibo, Costa Rica	10.0	8.5			against large dead stump, amorphous shape
AGH030214-08	El Ceibo, Costa Rica	5.5	10.0			inside tree trunk
AGH030215-01	El Ceibo, Costa Rica	8.0	4.5			built into crevice flush with underside small log
AGH030215-08	El Ceibo, Costa Rica	7.5	14.0			underside log
AGH030215-11	El Ceibo, Costa Rica	6.0	5.0			built into indentation in underside of log
AGH030215-12	El Ceibo, Costa Rica	6.5	6.0			built beneath protected shelf in log across small stream
AGH030216-02	El Ceibo, Costa Rica	7.0	7.0			under large log across small stream
AGH030217-01	El Ceibo, Costa Rica	13.0	6.5			in log near stream, no visible entrance
AGH030218-12	El Ceibo, Costa Rica	11.0	4.5			under large dead tree, no visible entrance
AGH030221-06	El Ceibo, Costa Rica	7.5	8.5			jutting out 2.5cm from end of log
AGH030222-03	El Ceibo, Costa Rica	6.5	17.3			on underside of leaning fence post
AGH030222-04	El Ceibo, Costa Rica	8.5	9.0			underside large dead tree in crevice
AVERAGE		**8.0**	**9.5**			
Stand Dev.		**2.6**	**5.3**			
N=15						

Table 4.2: *Cyphomyrmex sp. nov*. nest architecture measurements. Nest measurements of *C. sp. 1* funnel nest types. W = width in cm; L = length in cm.

Colony ID	Site Name, Country	Nest W	Nest L	Funnel L	Entrance W	Entrance L	Comments
AGH030212-12	El Ceibo, Costa Rica	5.0	2.5	2.5	N/A	N/A	Medium-large nest on bank above stream, garden on rootlets
AGH030213-01	El Ceibo, Costa Rica	7.9	7.7	7.0	4.5	3.2	large nest, in soil of treefall, funnel entrance in curving triangular shape
AGH030213-05	El Ceibo, Costa Rica	5.4	8.5	8.5	2.7	1.8	in crook where 2 large dead branches meet, large triangular nest, funnel smoothly grades into nest chamber
AGH030216-06	El Ceibo, Costa Rica	12.0	10.0	partial	0.6	1.0	against rock above stream, partly defined soil entrance
AGH030218-05	El Ceibo, Costa Rica	8.5	12.0	5.0	3.5	3.1	against rock, downward funnel
AGH030219-01	El Ceibo, Costa Rica	9.0	9.5	4.5	3.8	3.5	in bank, funnel entrance imperfect "wrinkled" edges
AGH030221-07	El Ceibo, Costa Rica	9.5	5.0	4.0	4.0	4.2	large nest in bank under rock, soil folds around entrance, many active workers collecting grass
AGH030222-02	El Ceibo, Costa Rica	2.2	2.5	2.5	2.2	1.7	medium nest, downward funnel in protected hollow of soil bank
AGH030222-06	El Ceibo, Costa Rica	3.0	5.0	1.5	1.9	1.2	medium nest, downward pointing funnel
AGH030222-07	El Ceibo, Costa Rica	2.4	3.5	3.5	2.3	2.5	very straight funnel, not curving, in small indentation in clay bank
AGH030222-08	El Ceibo, Costa Rica	10.0	11.0	4.0	4.0	3.0	large nest, downward pointing funnel, in bank along stream
AVERAGE		**6.8**	**7.0**	**4.3**	**3.0**	**2.5**	
Stand Dev.		**3.4**	**3.5**	**2.1**	**1.2**	**1.1**	
N=11							

Table 4.3: *Cyphomyrmex sp. nov*. nest architecture measurements. Nest measurements and colony counts for *C. sp. 1* from La Selva and Hitoy Cerere, Costa Rica, and Bocas del Toro Province, Panama. Empty cells indicte data not collected.

Colony ID	**Site Name, Country**	**Nest W**	**Nest L**	**Funnel Length**	**Entrance W**	**Entrance L**	**# Workers**	**Comments**
UGM020531-07	Bocas del Toro Province, Panama	12.0	9.0				429	Depth protruding from rock 4-6cm
UGM020601-01	Bocas del Toro Province, Panama						398	
AGH020601-01	Bocas del Toro Province, Panama						161	

Colony ID	Site Name, Country	Nest W	Nest L	Funnel Length	Entrance W	Entrance L	# Workers	Comments
UGM020602-04	Bocas del Toro Province, Panama						372	
AGH020602-01	Bocas del Toro Province, Panama						212	
UGM020603-05	Bocas del Toro Province, Panama						439	Alates: 88 males,1 female
UGM020603-06	Bocas del Toro Province, Panama						24	
UGM020603-07	Bocas del Toro Province, Panama						77	
UGM020603-08	Bocas del Toro Province, Panama						29	
UGM020603-09	Bocas del Toro Province, Panama	11.0	13.3		6.7	2.1	180	fungus suspended from rootlets
UGM020603-10	Bocas del Toro Province, Panama	6.0	43.7		3.3	2.8	103	
UGM020603-11	Bocas del Toro Province, Panama						17	
UGM020603-12	Bocas del Toro Province, Panama	13.2	15.5		8.7	8.0	628	
UGM020603-13	Bocas del Toro Province, Panama						486	
AGH020604-01	Bocas del Toro Province, Panama						138	2 female alates, 2 entrance funnels, in bank over stream
UGM020604-01	Bocas del Toro Province, Panama				2.0	2.0	9	In clay embankment
UGM020604-03	Bocas del Toro Province, Panama						88	In clay between tree roots, 2 entrance funnels pointing opposite directions
AGH020621-08	Hitoy Cerere, Costa Rica						6	
ABS020622-03	Hitoy Cerere, Costa Rica						233	2 queens
AGH020706-02	La Selva, Costa Rica				1.2	1.2		Long entrance tube, approx 4inches; in clay bank under root *looks like C. sp nov from HC, CR w/narrow funnel
AGH020709-09	La Selva, Costa Rica	11.3			7.9	4.5		Huge nest, wide funnel entrance, Q, w, male + female alates
AGH020709-11	La Selva, Costa Rica						7	Small nest, no alates
AGH020710-04	La Selva, Costa Rica				2.0	5.8	15	No alates
AGH020712-01	La Selva, Costa Rica	6.7			5.7	5.3		Broad funnel (2cm long) protrudes down from underside rock
AGH020712-02	La Selva, Costa Rica	0.4	0.4		1.5			1 Queen, 0 w, incipient nest
AGH020712-05	La Selva, Costa Rica	2.5	3.3		4.7			Funnel entrance 5.7cm

Colony ID	Site Name, Country	Nest W	Nest L	Funnel Length	Entrance W	Entrance L	# Workers	Comments
AGH020712-11	La Selva, Costa Rica				7.0	3.5		Huge nest underside log
AGH020712-12	La Selva, Costa Rica			1.4	0.8	0.8		1 Queen, 0 w, incipient nest
AGH020712-13	La Selva, Costa Rica				3.5	3.0		Medium size nest, funnel 1.4cm long
AGH020712-14	La Selva, Costa Rica				1.7	1.6		Medium size nest, funnel 4.1cm long
AVERAGE		**9.0**	**17.0**		**4.5**	**3.6**		
Stand Dev.		**3.9**	**15.7**		**2.6**	**2.1**		
N=28								

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Chapter 5

Nesting biology of the North American fungus-growing ant, *Mycetosoritis hartmanni* (Formicidae: Attini)

Abstract. The fungus-growing ant *Mycetosoritis hartmanni* (tribe Attini, Formicidae) is among the least studied attine ants of North America. Here I present the first detailed population study of this little known fungus-growing ant examining nest architecture and sex ratio changes during the reproductive season. *M. hartmanni* has a consistent nest architecture of up to four chambers arranged in a vertical series. The species is active in Central Texas between April and October. After winter dormancy between November and February, egg laying commences in April, the first worker larvae are found in May, and the first worker pupae are found in early June. Reproductives are produced primarily in July and August with proportionally more females earlier in the summer and proportionally more males later in the summer. This population maintained a male-biased sex ratio throughout the reproductive season. Mating flights and initiation of new nests occur primarily in late June and July, but can extend through September. The abundance of *M. hartmanni* appears to be relatively unaffected by the invading fire ant, *Solenopsis invicta*, because *S. invicta* does not thrive in the sandy, sun-exposed habitat preferred by *M. hartmanni*.

5.1 INTRODUCTION

Fungus-growing ants in the tribe Attini (Formicidae: Myrmecinae) are partners in an obligate symbiosis with the fungus they cultivate for food. These ants occur exclusively in the New World (Hölldobler and Wilson 1990), and consist of a monophyletic group of the well known leaf-cutter ants, and their less prominent ancestors the primitive genera of non-leaf-cutting ants, the "lower attines" (Chapela *et al.* 1994; Mueller *et al.* 1998). Due to the tribe's primarily Neotropical distribution, most attine studies focus on tropical species, particularly leaf-cutting species, considered agricultural pests in the Neotropics (Leal and Oliveira 2000) and the model lower attine system *Cyphomyrmex longiscapus* species group (Schultz *et al.* 2002). Little is known about the biology of the few species of North American non-leafcutting fungus-growing ants (Wheeler 1907; Weber 1940). Indeed, relatively little natural history information exists about most species of attines, and none about *M. hartmanni* besides records appearing in sporadic ant surveys in Texas and Louisiana (Weber 1972; Wheeler and Wheeler 1985; Dash 2004). Here I present the first detailed study of *Mycetosoritis hartmanni*, an exclusively North American fungus-growing ant, providing novel in depth information on nest architecture and seasonal sex ratio changes of a population in Central Texas, USA.

M. hartmanni has a strictly North American distribution as far as is known, based on a few records of collections in Central and Eastern Texas and Western Louisiana, although MacKay *et al.* (1998) speculates it may occur in Mexico. Mexican populations have not yet been documented, but the presence of *M. hartmanni* in areas just north of the Rio Grande (Cameron County; Wheeler and Wheeler 1985; UG Mueller, personal observation) suggests that it may extend into Mexico. *M. hartmanni* nests in open areas, invariably in sandy soils (Wheeler 1907; Dash 2004; U. G. Mueller, personal observation). Records on ecological regions include collections in Blackland Prairie and the Edwards Plateau in Travis and Brazos counties respectively, in Texas (Wheeler and Wheeler 1985; O'Keefe *et al.* 2000), and in the west gulf coastal plain longleaf pine forest ecoregion, in Rapides Parish, Louisiana (Smith 1979; Dash 2004).

Here we add a new collecting locality: Stengl "Lost Pines" Biological Station, Bastrop County, Texas, GPS coordinates 0° 04' 51" North and 97° 10' 23" West, occurring in Post Oak Savannah (http://www.sbs.utexas.edu/philjs/Stengl/about.html). The study population occurred in a patch of sandy soil containing a dense aggregation of nests at Stengl Biological Station of the University of Texas at Austin. Based on this population and the few existing records (Wheeler 1907; Dash 2004, U. G. Mueller, personal communication), sandy soil appears to be its preferred nesting substrate. It has been suggested that the encroachment of the red important fire ant, *Solenopsis invicta*, may limit *M. hartmanni*'s distribution or disperal (S. Cover, personal communication), so an alternative explanation is that perhaps *M. hartmanni*'s nest aggregations in sandy soil are refuges, but this remains to be confirmed. Nests consisted of a vertical series of subterranean chambers with a single nest entrance, surrounded in spring and early summer by a 2-4cm high, tumulus of soil indicating active excavation by the ants. *M. hartmanni* workers are terrestrial solitary foragers (personal observation), and they do not appear to follow foraging trails. Dash (2004) reports floral anthers as the garden substrate for *M. hartmanni* in Louisiana.

The genus *Mycetosoritis* contains five species (Bolton 1994) with a disjunct distribution (MacKay 1998, Dash 2004). *M. hartmanni* is the sole nearctic resident in the genus (Dash 2004); *M. vinsoni* was recently described from Costa Rica (MacKay 1998; but see comment by J. T. Longino questioning its species status on Ants of Costa Rica: http://www.evergreen.edu/ants/genera/mycetosoritis/species/vinsoni/vinsoni.html); the remaining three species, *M. asper*, *M. clorindae*, and *M. explicata*, occur in Northern Argentina and Southern Brazil (Weber 1972 p.19; Kempf 1968; MacKay 1998). *M. hartmanni* has been referred to "either as a degenerate and simplified *Trachymyrmex* or as an aberrant *Cyphomyrmex*", similar in "form and pilosity" to the former genus, but closer in size and certain morphological characteristics to the latter genus (Wheeler 1907; Dash 2004). A new phylogenetic analysis of the tribe Attini places *M. hartmanni* in close proximity to the genus *Cyphomyrmex* (S. Brady and T. R. Schultz, in preparation). A detailed taxonomic description of *M. hartmanni* appears in Wheeler (1907).

Characteristics distinguishing the species *hartmanni* include (taken from Dash 2004): frontal lobes flattened to cover antennal insertions, but not as broad as in *Cyphomyrmex*; eleven segmented antennae with terminal segment elongate, and the "occipital border of the head with an angular emargination" (Dash 2004 p.78, from Smith 1947); hairs on the scapes are straight and semierect, median pronotal denticles and petiolar teeth are well developed (MacKay 1998).

The sex ratio, the ratio of reproductive males to females (alates) produced by a social insect colony, of this population was studied over one reproductive season, from spring to fall in 2000. In all social Hymenoptera, there is an inherent conflict over the sex ratio between the queen and her worker daughters because of the difference in their relatedness to reproductives due to haplodiploid sex determination (Trivers and Hare 1976). Theory predicts at the population level that total investment in males and females should be equal (Fisher 1930; Crozier and Pamilo 1996), but the numerical and investment sex ratios can vary over the reproductive season based on a number of factors, such as resource availability or sex-ratio biasing by workers (Crozier and Pamilo 1996). An additional complication for the fungus-growing ants is the reproductive interest of their fungal symbiont, which is solely dispersed by reproductive females. Therefore, both the workers and the cultivar could be selected to induce female-biased sex ratios. Due to the dearth of knowledge about the phenology, demographics, and seasonal sex ratio variation in lower attines I investigated nest architecture and sex ratio over one annual reproductive season in *Mycetosoritis hartmanni*.

5.2 MATERIALS AND METHODS

Collection. Colonies of *Mycetosoritis hartmanni* were collected at Stengl Biological Station (30° 04' 51" N and 97° 10' 23") from April – October 2000. Nests were located by fresh soil mounds surrounded the nest entrance as evidence of recent ant excavation, and by observing active foragers return to their nest. Nest architecture was studied by careful excavation of complete colonies in the following manner. A hole was dug

adjacent to the nest entrance approximately 25-35cm from the entrance to avoid accidental destruction of the nest. Once the hole reached desired depth of approximately 30cm or more, soil was carefully excavated laterally towards the nest entrance from the top down until the first chamber was encountered. Ants and any fungus garden present in a chamber were collected into a vial using forceps and an aspirator to maximize the proportion of the nest collected. If a tunnel leading further down could be located, it was followed down to the next chamber, until all chambers were reached and colony collection was complete. When possible, chamber dimensions were recorded: chamber depth (from soil surface to chamber floor), chamber dimensions (width x height at greatest points), and chamber contents (Table 5.1). An estimate of colony size was obtained by counting workers, queens, reproductives (male and female alates) and brood for a subset of nests. Voucher specimens will be deposited at the National Museum of Natural History (Smithsonian Institution) in Washington, D.C. and at the Insect Collection at the Brackenridge Field Laboratory of the University of Texas at Austin. Cultivar from several colonies was isolated using sterile technique and grown on potato dextrose agar (PDA; Difco, Detroit, MI) with antibiotics (50mg/L each of penicillin and streptomycin). Cultivar mycelium was then subcultured and axenic (pure) cultures were stored at -80^{O}C for future DNA extraction.

Sex Ratio. The numerical sex ratio (percent male) was calculated per month or half-month as total number of adult male reproductives divided by the total number of adult male and female reproductives. The investment sex ratio was calculated per month or half month as the total adult male reproductive weight divided by the total adult male and female reproductive weight (Table 5.2, Figure 5.1).

5.3 RESULTS

Collection. A total of 67 *M. hartmanni* nests were collected at Stengl Biological Station, University of Texas at Austin, between April and October 2000. The mean number of workers collected per nest was 48.03 (± 32.34, N=34 nests), representing an estimate of

colony size (Table 5.1). Previous estimates of nest size were 60-70 workers (Wheeler 1907). Usually a single dealate queen was collected in 44 of 45 (98%) queenright nests collected, although one nest was found with two queens. This suggests this species is primarily monogynous. The second dealate queen could be a daughter that did not disperse the previous year; alternatively, she could be a cofoundress. It is unknown whether unrelated queens occasionally cofound in this species, but all four incipient nests found in this study were monogynous (Table 5.1). As spring progressed gardens contained brood and reproductives (alates) (Table 5.1). The fungus garden of *M hartmanni* hangs from the ceiling of subterranean chambers excavated by the ants from the soil where it is protected from predation. Because the top chambers are rather shallow (15-20 cm deep) and because *M. hartmanni* nests in open, sun-exposed habitat, the fungus gardens must experience significant heat during the peak summer months. The fungus hangs in delicate curtains of mycelium. Brood and queens were found within the fungus garden. Substrate used to feed the fungus garden appeared to be floral parts, fine vegetative matter, and perhaps grass seeds.

Nest architecture. All nests had a single entrance, often surrounded by a steep mound of soil recently excavated by the ants known as a "turriform crater" (Dash 2004), leading straight down to a series of garden chambers. Colonies contained up to four chambers, including a foundress chamber, arranged in vertical linear fashion with a single tunnel connecting between them. This connecting tunnel was sometimes apparent in the chamber floor as the tunnel entrance was surrounded by a small, elevated cone of soil built up from the chamber floor. Chamber depth, width, and height measurements were recorded per nest and per chamber (Table 5.1). The first chamber was the foundress chamber, a small chamber at shallow depth, on average 10.74 cm deep (± 3.33cm, N=14 nests), where the queen first establishes her nest after mating on a nuptial flight. The foundress chamber appears to contain garden in newly founded nests, but is rarely used for gardening in mature nests. Below this was the first garden chamber on average 18.16 cm deep (± 3.66 cm, N=37 nests), followed by the second chamber on average 34.96 cm deep (± 6.36 cm, N=36 nests), and for some nests followed by a third chamber, on

average 50.65 cm deep (± 9.92 cm, N=16 nests). In some lower attine species, colonies may abandon the foundress chamber as they mature and deeper large chambers are constructed (Weber 1972; U. G. Mueller, personal observation). In addition, data from four incipient (new) colonies are reported at the bottom of Table 5.1. Incipient colonies consisted of a single chamber, with one queen and up to four workers, as some queens had not yet hatched their first workers. Average incipient colony chamber depth was 15.0 cm (± 2.6 cm, N= 4), average width was 1.0 cm (± 0.1 cm, N= 3), and average height was 1.0 (± 0.2 cm, N= 3) (Table 5.2).

Sex ratio. Over the collection period from April to October 2000, the number of reproductives (alates) increased with peak production in the second half of July through August (Figure 5.1). The numerical sex ratio shifted from more female production early in the season to predominantly male production later in the season (July-August) (Figure 5.1 and Table 5.2). However, the number of males was greater than the number of females per nest from April through September, with the exception of one colony in early July (AGH 000707-02) that had 36 reproductive female alates to one male alate (Table 5.2).

The investment sex ratio (percent investment in males by adult weight) peaked in the second half of July at 90.65%, primarily by the increase in the number of males produced, not an increase in their individual weight (Figure 5.1, Table 5.2). Numerical and investment sex ratios (in males, by definition) remained high in July to August after which colony production of alates abruptly declined. Only one of three colonies excavated in September contained some alates (four male alates and one female alate; UGM000930-02), and one very unusual nest (UGM001028-03) out of the four colonies excavated in October that contained 96 dealate females (Table 5.2). This is extremely anomalous; perhaps these queens all missed the mating flight during drought conditions in summer 2000. According to local measurements, Stengl experienced drought receiving only 26.06 inches of rain in 2000, 11.38 inches below the annual average of 37.44 inches, based on the past 71 years of annual rainfall data. In addition, in 1999 this region

experienced even less rainfall receiving only 21.98 inches of rain that year, 15.46 below the annual average. It appears based on other nests that alate production stopped for most nests at the end of August. Like other lower attines in Central Texas, mating flights probably occur at dawn on days with stable weather, after a day with significant rainfall. Mating flights most likely occur regularly in late June or July, less frequently in August with the cessation of rains, and increase with higher frequency in September and possibly early October.

5.4 DISCUSSION

This is the first detailed population study on the primitive fungus-growing ant, *Mycetosoritis hartmanni*. The accumulated information reveals that its nest architecture is remarkably consistent, with most nests containing up to four subterranean chambers arranged in a vertical series. The sex ratio (% males) shifted over the summer reproductive season, with females more abundant in June (protogyny) and 40-50% males in June and early July (Figure 5.1), to males more abundant approaching near100% later in the summer at the end of the reproductive season. Male alate production peaked in late July and remained at approximately 80% for late July through August, before declining abruptly. Possible reasons for the shift in the sex ratio over the season include a mating flight midsummer after which it is less costly for the colonies to produce the cheaper (lower investment) sex, the males (Crozier and Pamilo 1996).

One very unusual finding was a nest excavated near the end of October that contained 96 dealate females. These queens either skipped the mating flight if conditions were suboptimal (ie. too dry given the droughts in 1999 and 2000), or, less probable, they were produced very late in the season. Indeed, only one out of four nests excavated in September had a few reproductives, and none of the other three nests excavated in late October on the same date contained any reproductives. It is known that some dealate females can survive the winter (U. G. Mueller, personal communication) but it is very unlikely that this great a number (96) were all from the previous year.

The *Mycetosoritis* genus occupies an interesting phylogenetic position: the genus is basal and sister to *Cyphomyrmex*, the transitional genus between the lower and higher attines (Schultz and Meier 1995, Schultz and Brady, unpublished data). Investigations into the biology of *Mycetosoritis* and its sole North American species *M. hartmanni* may give insight into lower attine characteristics that separate them from the leaf cutter higher attines. As an "aberrant" *Cyphomyrmex*, *Mycetosoritis hartmanni* may reveal features that unite it with the lower attines, elucidating features that changed so drastically to permit the leaf cutter ants' highly derived evolution. The fungus cultivated by *M. hartmanni* appears to be most closely related *Mycocepurus smithii* cultivar, and occupies a relatively long branch separated from other slow growing 'Clade 1 ' cultivars (Mueller *et al.* 1998).

Nest construction consisted of a vertical series of chambers approaching up to over half meter depth (deepest chamber was 72cm). This nest architecture is consistent with a few other lower attine genera including North American species such as *Trachymyrmex septentrionalis*, *T. turrifex*, *T. desertorum*, and *Cyphomyrmx wheeleri*, as well as Neotropical *Mycocepurus* species. Other lower attines typically have one to two relatively shallow larger subterranean chambers, particularly the Neotropical genera *Mycetarotes* (Solomon *et al.* 2006) and *Myrmicocrypta*. The North American fungus-growing ants represent the northern distribution of this primarily Neotropical tribe. The present study contributes the first detailed information on nest architecture and seasonal reproduction of a little studied species. The reason that *M. hartmanni*'s current distribution is restricted to Texas and Louisiana is unknown. MacKay (1998) and J. T. Longino (Ants of Costa Rica website) suggest it may occur in Mexico and dry habitats from Texas to northern Costa Rica, however this remains to be shown. It is likely *M. hartmanni* occurs in the border states of Mexico. I hope this work will prompt further study of the biology of this and other neglected North American fungus-growing ants. Suggestions for future work on *M. hartmanni* and its fungus garden include investigating over-wintering tactics, sex ratio changes spanning years, examining whether *M. hartmanni* occurs in habitats other than sandy soil, testing whether the red imported fire

ant is affecting its growth or survival, and molecular characterization of these ants and their cultivar.

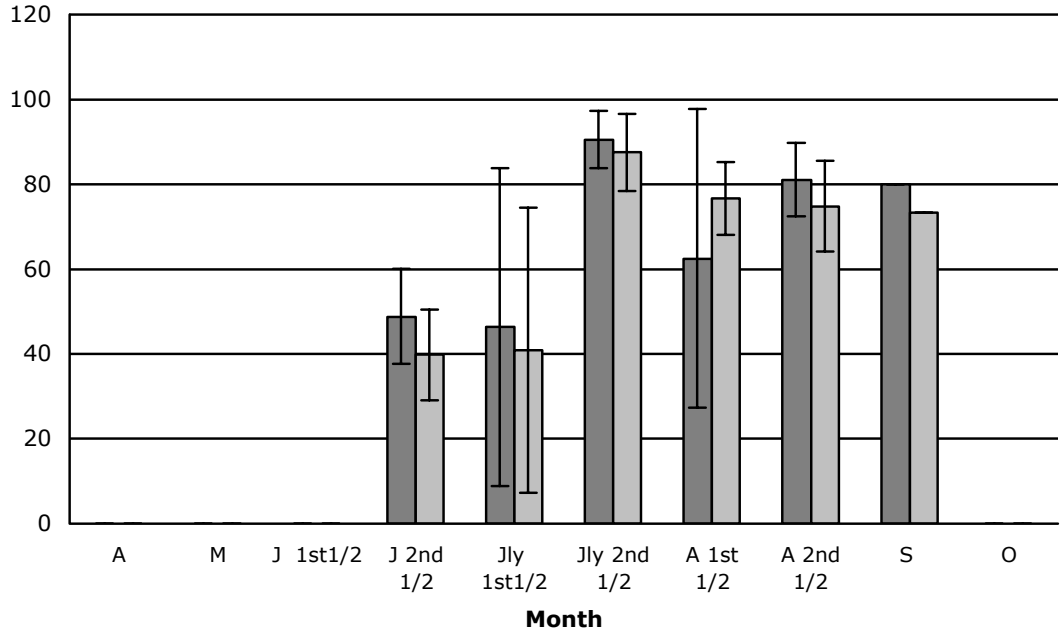

Figure 5.1: Sex ratio data for *Mycetosoritis* colonies over one reproductive season. Numerical sex ratio (dark grey) and investment sex ratio (light grey) of *Mycetosoritis hartmanni* colonies shown per month or half month from April to October 2000. Error bars are one stand deviation. Columns without error bars contain only a single measurement.

Table 5.1: Nest architecture data for *Mycetosoritis* colonies. Summary of *M. hartmanni* nest measurements for colonies excavated from April to October 2000. All measurements are given in centimeters. D= Chamber depth in cm; W = Chamber Width in cm; H = Chamber height in cm. N = total count for that measurement. Empty cells indicate data not taken; "-" indicates data not applicable (no chamber to measure).

Nest ID	# Queens	# Workers	Foundress Chamber			First Chamber			Second Chamber			Third Chamber		
			D	W	H	D	W	H	D	W	H	D	W	H
UGM000413-02	0					15	2.0	2.0	34.0	3.5	3.5	49.0	3.5	3.5
UGM000413-03		7												
UGM000420-01	0					20			27.0	3.5	3.5	43.0	2.0	2.0
UGM000420-02	2	148				16	3.5	3.5	32.0	5.5	4.0	39.0	2.0	1.5
UGM000420-03	0					16	2.0	2.0	32.0	3.0	3.0	43.0	3.0	3.0
UGM000420-04	0								35.0	3.5	3.5	45.0	3.0	3.0
AGH000420-05	1	48	11.5			20.5								
AGH000420-06	1	74	9.0									-	-	-
UGM000512-01	1	74				19	3.0	2.5	34.0	4.0	3.5	72.0	1.5	1.0
UGM000512-02	1	21				13	2.0	2.0	28.0	2.0	2.0	51.0	3.0	2.0
UGM000512-03	1	19				14	2.0	3.0	35.0	3.0	2.5	48.0	3.0	3.0
UGM000512-04		54				15			32.0					
UGM000606-01	1	12	12.5			19.5	1.5	1.5	43.0	3.5	2.5	-	-	-
UGM000606-02	1	20				13	1.5	2.0	34.0	5.3	4.2	-	-	-
AGH000606-01	1	76				15.3	3.5		26.6			61.4		
AGH000606-02	1	61				20.5	3.8		42.2			-	-	-
UGM000620-01	1	19				16	2.0	2.0	27.0	3.5	2.7	-	-	-
UGM000620-03	1	28	7.0	1.2	0.9	14.5	4.2	3.5	24.0	5.3	4.1	-	-	-
AGH000620-01	1	65	15.3			27.3			38.1					
AGH000628-01	0		15.0											

Nest ID	# Queens	# Workers	Foundress Chamber			First Chamber			Second Chamber			Third Chamber		
			D	W	H	D	W	H	D	W	H	D	W	H
AGH000707-01						17.5			41.0	3.5	2.2	-	-	-
AGH000707-02	1	29				20	2.0	2.0	42.0			-	-	-
AGH000709-01						19	1.3	2.5	-	-	-	-	-	-
AGH000709-02						15			26.5	3.0				
AGH000709-03	1					16	1.5		30.0	1.0		-	-	-
AGH000709-04									34.5	3.0	5.0			
AGH000714-01	1		13.0	1.2		18	1.0					51.0	3.0	3.0
AGH000714-02	1	43	11.5	0.7		19	4.0	3.0	46.0	5.0	6.0			
CC000714-01	0					25	3.0		32.0	2.0	3.0	-	-	-
UGM000721-01	0	72	4.5	1.2	1.5	17.5	4.1	2.7	39.5	5.5	3.8	-	-	-
UGM000721-03		39	11.0	2.2	2.2	17.5	3.6	2.7	35.0	4.2	3.9	-	-	-
AGH000721-01	1	38	10.5	1.0	1.0	26.5	2.0	3.0	45.5	2.0	3.0	-	-	-
AGH000721-02	1													
UGM000731-01	1	63				15.5	2.2	1.6	28.0	3.2	2.7	42.0	2.0	1.8
AGH000731-01	0													
AGH000731-02	1	49				23	1.5	1.5	35.5	2.0	2.0	44.0	4.0	4.0
AGH000731-04	1	49												
AGH000814-01	1	59				15	2.0	1.5	34.0	3.0	3.0	-	-	-
AGH000814-03	1	24				21	4.0	3.0	36.5	3.0	3.0	-	-	-
AGH000814-04	1	17				15	1.5	2.0	40.0			43.0		
UGM000831-02	1	89	14.5	2.1	2.1	21	3.4	2.9	34.0	3.3	2.7	49.0	2.5	2.5
CC000731-03	1	97				19			31.5	3.0	2.5			
AGH000831-01	1	23	9.0	1.3	1.5	24	4.3	1.3	53.0	3.0	2.5	62.0		
UGM000930-01	1	14	6.0			14	2.0	2.0	31.0	1.6	2.4	-	-	-
UGM000930-02	1	43				19	2.7	3.1	39.0	4.6	3.9	68.0	4.1	3.4
AGH001028-01	1	15												
AGH001028-02	1	26												

Nest ID	# Queens	# Workers	Foundress Chamber			First Chamber			Second Chamber			Third Chamber		
			D	W	H	D	W	H	D	W	H	D	W	H
UGM001028-03	1	118												
N	**35**	**34**	**14**	**8**	**6**	**37**	**30**	**25**	**36**	**30**	**28**	**16**	**13**	**13**
Mean		**48.03**	**10.74**	**1.36**	**1.53**	**18.16**	**2.57**	**2.35**	**34.96**	**3.38**	**3.24**	**50.65**	**2.82**	**2.59**
Range		**7-148**	**6-15.3**	**0.7-2.2**	**0.9-2.2**	**13-27.3**	**1-4.3**	**1.3-3.5**	**24-53**	**1-5.5**	**2-6**	**42-72**	**1.5-4.1**	**1-4**
Standard Dev		**32.38**	**3.33**	**0.52**	**0.54**	**3.66**	**1.02**	**0.66**	**6.36**	**1.16**	**0.92**	**9.92**	**0.79**	**0.88**
INCIPIENT NESTS														
UGM000620-02	1	1	12.0	1.1	0.8	-	-	-	-	-	-	-	-	-
UGM000831-01	1	0	14.0	1.0	1.2	-	-	-	-	-	-	-	-	-
UGM000930-03	1	0	16.0	1.0	1.0	-	-	-	-	-	-	-	-	-
AGH000707-03	1	4	18			-	-	-	-	-	-	-	-	-
N	**4**	**4**	**4**	**3**	**3**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
Mean		**1.25**	**15.0**	**1.0**	**1.0**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
Range		**0-4**	**12-18**	**1-1.1**	**0.8-1.2**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
Standard Dev		**1.9**	**2.6**	**0.1**	**0.2**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**

Table 5.2: Sex ratio data for *Mycetosoritis* colonies over one reproductive season. Summary of reproductive and colony counts for *M. hartmanni* nest measurements for colonies excavated from April to October 2000. Data include counts of all reproductives, workers, and worker brood, for a subset of colonies counted. Blank cells indicate data not taken.

Nest ID	# Queen	#Male Alates	#Male Pupae	#Fem Alates	#Fem Pupae	# Dealate Fem	NumSex Ratio (% male)	InvSex Ratio (% male)	# Sexuals	# Workers	#Work Pupae	#Total Pupae	# Larvae	# Eggs
April 2000														
UGM000413-01	0													
UGM000413-03	0									7				
UGM000420-02	2									148				
AGH000420-05	1									48				
AGH000420-06	1									74				
RMA000420-07	1	0	0	0	0	0			0	32				
RMA000420-08	1									62				
N														
Mean														
Standard Dev														
May 2000														
UGM000512-01	1									74	1	1	very few	30-40
UGM000512-02	1									21		0	0	1
UGM000512-03	1									19		0	0	1
UGM000512-04	0									54		0	~10	7
N														
Mean														
Standard Dev														

Nest ID	# Queen	#Male Alates	#Male Pupae	#Fem Alates	#Fem Pupae	# Dealate Fem	NumSex Ratio (% male)	InvSex Ratio (% male)	# Sexuals	# Workers	#Work Pupae	#Total Pupae	# Larvae	# Eggs
June 1st half 2000														
UGM000606-01	1	0		0		0			0	12	6	6	9	
UGM000606-02	1	0		0		0			0	20	2	2	9	
AGH000606-01	1	0		0		0			0	76	1		4	
AGH000606-02	1	0		0		0			0	61	12		20	
N														
Mean														
Standard Dev														
June 2nd half 2000														
UGM000620-01	1	0	21	0	16	0	56.80%	47.40%	0	19	0	37	22	2
UGM000620-02	1	0	0	0	0	0			0	1	0	0	few	few
UGM000620-03	1	0	18	0	26	0	40.90%	32.20%	0	28	0	44	49	7
AGH000620-01	1	0		7			0.00%	0.00%	7	65	19	19	10	
AGH000628-02	1									1				
AGH000628-03	1									4				
N		**0**	**39**	**7**	**42**	**0**								
Mean							**48.85%**	**39.80%**						
Standard Dev							**(±11.24)**	**(±10.75)**						
July 1st half 2000														
AGH000707-01														
AGH000707-02	1	1	1	36	27	0	3.08%	2.10%	37	29			8	19
AGH000707-03		0	0	0	0	0			0	4	0	0	0	0
CC000707-01	1	22	8	10	3	0	69.76%	61.30%	33	36			2	9
CC000707-03	1	0	0	0	0	0			0	10	0	0	0	8
AGH000709-03	1													

Nest ID	# Queen	#Male Alates	#Male Pupae	#Fem Alates	#Fem Pupae	# Dealate Fem	NumSex Ratio (% male)	InvSex Ratio (% male)	# Sexuals	# Workers	#Work Pupae	#Total Pupae	# Larvae	# Eggs
AGH000714-01	1													
AGH000714-02	1	42	11	17	8	2	66.25%	59.20%	59	43			11	12
N		**65**	**20**	**63**	**38**	**2**								
Mean							**46.36%**	**40.87%**						
Standard Dev							**(±37.53)**	**(±33.59)**						
July 2nd half 2000														
UGM000721-01	0	117	46	0	0	0	100%	100%	117	72	0	48	33	5
CC000721-02	1	26	1?	3	0	0	90.00%	86.00%	29	22	2		3	14
UGM000721-03		29	14	11	0	0	79.60%	72.80%	40	39	10	14	19	5
AGH000721-01	1	28	0	5	0	0	84.84%	79.90%	33	38	0	0	7	4
AGH000721-02	1	21	?	3	0	0	87.50%	82.70%	24					
CC000721-02	1	27	1	3			90.32%	89.70%	30	22			3	14
UGM000731-01	1	0	0	0	0	0			0	63	21	21	0	3
AGH000731-02	1	42		0	0	0	100%	100%	42	49	5	5	3	4
CC000731-03	1	109		9	0	5	88.62%	84.20%	118	97	14		0	15
AGH000731-04	1	38		1	0	1	95.00%	92.90%	39	49	2		0	8
N		**437**	**61**	**35**	**0**	**6**								
Mean							**90.65%**	**87.58%**						
Standard Dev							**(±6.74)**	**(±9.06)**						
August 1st half 2000														
AGH000814-01	1	0	0	0	0	0			0	59	8	8	1	4
CC000814-02	0	7	0	1	0	0	87.50%	82.70%	8	6	0		1	0
AGH000814-03	1	2	0	0	0	7	22.22%		9	24	0	0	1	6
AGH000814-04	1	28	0	8	0	0	77.78%	70.60%	36	17				

Nest ID	# Queen	#Male Alates	#Male Pupae	#Fem Alates	#Fem Pupae	# Dealate Fem	NumSex Ratio (% male)	InvSex Ratio (% male)	# Sexuals	# Workers	#Work Pupae	#Total Pupae	# Larvae	# Eggs
N		**37**	**0**	**9**	**0**	**7**								
Mean							**62.50%**	**76.65%**						
Standard Dev							**(±35.22)**	**(±8.56)**						
August 2nd half 2000														
UGM000831-01	1	0	0	0	0	0			0	0	0	0	3	0
UGM000831-02	1	54	0	14	0	4	75.00%	67.30%	72	89	18	0	6	3
AGH000831-01	1	41	0	3	0	3	87.20%	82.40%	47	23				
N		**95**	**0**	**17**	**0**	**7**								
Mean							**81.10%**	**74.85%**						
Standard Dev							**(±8.63)**	**(±10.68)**						
September 2000														
UGM000930-01	1	0	0	0	0	0			0	14	0	0	2	0
UGM000930-02	1	4	0	1	0	0	80.00%	73.30%	5	43	3	0	2	0
UGM000930-03	1	0	0	0	0	0								
N		**4**	**0**	**1**	**0**	**0**								
Mean							**80.00%**	**73.30%**						
Standard Dev														
October 2000														
AGH001028-01	1	0	0	0	0	0			0	15	1	1	0	0
AGH001028-02	1	0	0	0	0	0			0	26	0	0	0	1
UGM001028-03	1	0	0	0	0	96	0.00%		0	118	0	0	0	0
RMA001028-04	1	0	0	0	0	0			0	9	0	0	0	0
N						**96**								
Mean														
Standard Dev														

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