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The Nature of Parasite Specialization in the Fungus-Growing Ant Symbiosis.

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The Nature of Parasite Specialization in the Fungus-Growing Ant Symbiosis.

by

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This dissertation is dedicated to Julie Zedalis —

my sixth grade biology teacher,

my ninth grade biology teacher,

the woman who inspired me to become a biologist.

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The Nature of Parasite Specialization in the Fungus-Growing Ant Symbiosis.

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Host-parasite coevolution is intricately coupled with parasite specialization. As hosts become resistant, parasites may adapt and overcome that resistance or may become specialized on a narrow range of susceptible hosts. Ultimately, a parasite's host range will dictate ecological host-parasite dynamics and host-parasite coevolution. Here, I use the system of fungus-growing ants and their symbionts to study host-specialization by *Escovopsis*, a parasite of the ants' cultivated fungus. In recent years, the fungus-growing ant symbiosis has emerged as a model system for studying coevolution, speciation, cooperation and conflict between the ants and their fungal cultivars. In chapter one, I outline how this system has also proven to be an easily tractable system for studying the ecological and evolutionary dynamics of hosts and parasites. In chapters two and five, I combine molecular analysis of phylogenetic relationships of host and parasites with finer analysis of population differences within species to identify specialization by parasites on particular host-species and host-genotypes. At the host-species level, *Escovopsis* that attack gardens of *Cyphomyrmex* ants are specific to a narrow range of fungal cultivars propagated by the ants. At the host-genotype level, however, there is little evidence that genotypically similar strains of *Escovopsis* that attack *Apterostigma dentigerum* gardens are specialized on within-species host cultivar genotypes. In chapters three and four, knowledge of such patterns of specialization is used as a foundation for experiments in

which the host fungi and the parasitic fungi are confronted to determine patterns of host resistance and parasite infectivity. I demonstrate that host cultivars can chemically defend themselves against some *Escovopsis* spp., but *Escovopsis* spp. can overcome the defenses of host-species on which they are specialized and can efficiently recognize and be attracted to these susceptible hosts. These host and parasite adaptations are consistent with patterns of parasite specialization and host-switching in the *Apterostigma* ant symbiosis. Thus, this comprehensive approach reveals both process and pattern, demonstrating how mechanisms of resistance and infectivity shape parasite host-specialization and ultimately population dynamics of interacting organisms.

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Chapter 1

Ant crops and their pathogens: what the attine ant-microbe symbiosis may teach us about host-parasite interactions

Abstract: The symbiosis between fungus-growing (attine) ants and their cultivated fungi has emerged in recent years as a model system for studying coevolution, speciation and cooperation between the ants and the fungi that they cultivate as their primary food source. Though a classic example of mutualism, the attine ant-microbe symbiosis is actually a complex association of coevolving mutualists and parasites. *Escovopsis* is a prevalent, potentially virulent parasite that has been attacking the cultivars of fungus-growing ants for millions of years. In order to successively infect a colony, *Escovopsis* must overcome a wide range of colony defenses, including ant behaviors to remove the parasite, as well as antibiotics produced by both bacteria on the ants and the fungi they cultivate. Within this framework, it is therefore possible to study a wide range of evolutionary strategies in defense and resistance. Here, I outline the key features of the fungus-growing ant microbe symbiosis which facilitate research of host-parasite dynamics. I then review recent research in the system in the context of the major themes of host-parasite biology and discuss potential avenues of future research.

1.1 INTRODUCTION

Theoretical and empirical studies of host-parasite interactions have increased our understanding of evolutionary processes in general. Specifically, we have observed species interactions shape complex adaptations (Payne 1977; Clayton *et al.* 2003) and the speed in which populations of hosts and parasites evolve in response to one another (Ebert 1994; Fenner & Fantini 1999; Little & Ebert 2001). Many of these findings have been based on thorough investigation of a few easily tractable plant-pathogen (reviewed in Thompson & Burdon 1992) and animal-parasite systems (Lively 1989; Love & Zuk 1991; Ebert 1995; Fenner & Fantini 1999; Soler & Soler 2000). Research on the ecological and evolutionary dynamics of these systems is beginning to inform agricultural applications (Brown 1996) and medical practices (Stearns *et al.* 1999; Woolhouse *et al.* 2002; Galvani 2003) as concern of global disease transmission and epidemics increases.

There are several attributes of a host-parasite system that facilitate thorough and informative investigation (fig. 1.1). Important system characteristics include feasible sampling, long-term laboratory maintenance and genotyping of both host and parasites. Subsequent experiments can address the outcome of interactions between different symbiont species or genotypes. The fungus-growing ant microbe symbiosis possesses all of these attributes. Colonies of fungus-growing ants are common in most neotropical habitats, are easy to collect and can be maintained long-term in the laboratory. Upon collection, four known key symbionts can be sampled: the ants, their cultivated fungi (the cultivar), the specialized cultivar-attacking parasite *Escovopsis,* and actinomycete bacteria on the ants that inhibit *Escovopsis* growth (fig. 1.2). The fungal and bacterial symbionts can be cultured and maintained under axenic conditions for long-term storage and subsequent revival. A wide spectrum of molecular markers, including newly developed microsatellite and symbiont-specific sequencing primers (Villesen *et al.* 2004;

Adams *et al.*, unpublished data; Gerardo *et al.*, unpublished data), can then be utilized to address a number of broad-scale, species-level and fine-scale, population-level questions (Mueller *et al.* 1998; Green *et al.* 2002; Gerardo *et al.* 2004; chapters 2, 3). Furthermore, these molecular analyses can inform design of cross-infection experiments (fig. 1.1), where different symbiont combinations are evaluated for variation in suseptibility, virulence, persistence and other host-parasite characteristics (Gerardo *et al.* 2004; chapters 2, 4, 5). Having natural history and genotype information inform experimental design systems is a powerful tool in the fungus-growing ant microbe symbiosis, facilitating study of both ecological and evolutionary host-parasite dynamics.

1.2 RECENT FINDINGS

Cospeciation and host-parasite evolution. Cospeciation is the process by which two lineages speciate simultaneously as a consequence of their intimate association with one another. Classic examples of cospeciation involve parasites speciating along with their hosts (see references in Page 2003), though obligate mutualists likely cospeciate as well (Herre *et al.* 1996; Itino *et al.* 2001). If cospeciation occurs successively, it will lead to cocladogenesis, the matching of phylogenies of the two lineages.

In the fungus-growing ant symbiosis, we have a unique case in which, within a single system, we have strong evidence for cospeciation of both mutualists and parasites. Chapela *et al.* (1994) demonstrated the congruency of the phylogenies of the ants and the fungi that they cultivate as their primary food source. Currie *et al.* (2003b) then showed that the phylogeny of *Escovopsis*, the parasite that attacks the ants' cultivars, is congruent with that of the cultivar, and consequently that of the ants themselves, at broad phylogenetic levels.

Phylogenetic dissimilarity and host-switching. If cospeciation was the only diversifying process, then host and parasite phylogenies would match exactly. However, many evolutionary processes lead to dissimilarity of host-parasite and host-mutualist phylogenies. In host-parasite systems, these processes include duplication (parasite

speciation in the absence of host speciation), sorting events (host speciation without commensurate parasite speciation), and host-switching (one parasite switches to a new host) (Johnson *et al.* 2003; Page 2003). These processes apply to coevolving mutualisms as well; in the fungus-growing ant mutualism, though there is a broad scale pattern of phylogenetic concordance in the ants and their cultivars, there is evidence that the ants may either occasionally redomesticate free-living fungal relatives and may steal fungi from other ant colonies (Mueller *et al.* 1998; Green *et al.* 2002). Though these switches seem likely only when the ants encounter fungi closely related to their natal cultivar, they can lead to discordance of the ant and fungal phylogenies at finer scales.

Evidence suggests that host-switching is also possible in relation to *Escovopsis* and its cultivar host. Recent work has shown that *Escovopsis* spp. are generally specific, attacking colonies containing only a narrow range of fungal cultivars (Gerardo *et al.* 2004; chapters 2), but in experimental infection studies, *Escovopsis* spp. can occasionally attack cultivars that they are not associated in the field (Gerardo *et al.* 2004; chapters 2, 3, 4). In these same infection experiments, *Escovopsis* is more likely to successfully infect its typical hosts, which may lead to the long term maintenance of the broad scale pattern of cocladogenesis and may facilitate speciation, but the ability to switch hosts may lead to a lack of cocladogenesis at finer scales (when looking at closely related hosts and their parasites) (chapters 3, 5).

Parasite host-specificity. Thus, *Escovopsis'* ability to switch between some hosts and not others will ultimately dictate historical associations and phylogenetic patterns seen in the symbiosis. What dictates whether *Escovopsis* spp. can successively establish infection on a given host cultivar? In general, a parasite can only infect a potential host if the parasite can 1) co-occur with the host, requiring that the parasite to be able to live under the same general ecological conditions as the host, 2) successively overcome the host's defenses and establish infection, and 3) persist on the host by utilizing the host as a resource (fig. 1.3). In the case of *Escovopsis,* sympatric colonies are not infected by the same *Escovopsis* strain unless they have the same cultivar strain (Gerardo *et al.* 2004;

chapter 2), suggesting that environmental coexistence is not the only factor limiting *Escovopsis* host range. Instead, it appears that the host range of *Escovopsis* is more limited by what hosts it encounters and can successively infect within the shared environment.

Transmission may play a key role in dictating which hosts *Escovopsis* encounters. For example, if the parasites were primarily transmitted vertically when daughter ants fly from their mother's colonies to found new colonies, we would expect that *Escovopsis* rarely would switch hosts because it rarely would pass between colonies with different cultivar genotypes. However, *Escovopsis* infection of young colonies is uncommon and infection rates increase with colony age, suggesting that *Escovopsis* is not in nests upon initiation (Currie 2001). Instead, it is horizontally transmitted into the colonies either by the ants themselves, who may accidentally pick up fungal spores while foraging, or by another vector, such as mites or other invertebrates that are frequently in colonies and could potentially move between colonies. These horizontal vectoring mechanisms may not be colony specific; mites, for example, may readily move between proximate colonies of different ant species. This could widen the range of *Escovopsis* but could also serve to decrease the infection success of the parasite if it is often vectored to colonies of species that it cannot successively infect due to host defense (see more on defense, below). Lack of knowledge of *Escovopsis'* mode of transmission is currently one of the biggest limitations of this system, because it prevents full elucidation of some fundamental ecological dynamics.

What we do know is that these parasites have adaptations to efficiently find and consume susceptible hosts once they are in close proximity. *Escovopsis* spp. are attracted to chemical signals produced by cultivars. More specifically, they are generally attracted to chemical signals produced by susceptible cultivars and are generally not attracted to chemical signals produced by non-susceptible hosts (chapters 4, 5). This attraction may make *Escovopsis* an extremely efficient parasite, moving within the garden matrix to find cultivar to consume. It may also increase the likelihood of establishing and maintaining a

persistent infection and may increase its virulence if it can efficiently spread through the colony before being removed by the ants or being suppressed by the ants' bacteria (see below).

Defense evolution. Ultimately, host susceptibility is dictated by whether the parasite can overcome encountered host defenses. Hosts must adapt to defend themselves against detrimental parasites, and these host defenses may limit a parasite's host range. If the defenses of hosts vary, a parasite may have evolved to overcome the defense of certain hosts and not others.

Escovopsis must overcome a wide range of host defenses mounted by a colony's ants, bacteria and cultivar. First, upon infection, fungus-growing ants mount a rapid and specific response to the presence of pathogens. Ants groom their garden, removing fungal spores, and they weed their garden, removing pieces of infected garden substrate. Their response to *Escovopsis* infection is more intense than their response to infection by generalist pathogens (Currie & Stuart 2001). It is not yet clear whether the ants are adapted to respond more rapidly to those *Escovopsis* spp. that they frequently encounter in their gardens in nature. If these behaviors are highly specific, we would expect *Escovopsis* may track the ants whose defense they can overcome. Gerardo *et al.* (2004), however, demonstrated that the parasites seem to be cultivar, rather than ant, specific (similar parasites attack colonies with similar cultivars rather than colonies with closely related ant species). Thus, it is likely that other defenses are more likely to dictate parasite host-specificity.

One source of antimicrobial defense is actinomycete bacteria found on the cuticle (i.e. outer surface) of fungus-growing ants. These bacteria produce antibiotics that specifically inhibit *Escovopsis* growth (Currie *et al.* 1999b). When the bacterium is removed, infection by *Escovopsis* is much more extensive and garden growth rates subsequently decline (Currie *et al.* 2003a). It is not yet clear whether this antibiotic response is specific to the *Escovopsis* spp. encountered, but future work will elucidate how these two

symbionts, antibiotic-producing bacteria and the targeted fungal parasite, are coevolving defense and resistance in the face of prevalent, virulent infections.

Because *Escovopsis* directly attacks and consumes the cultivar, this host is a likely source of defense. Fungus-growing ant cultivars chemically defend themselves against *Escovopsis* (chapter 4, 5). This chemical defense appears to be relatively specific to the symbiosis; the cultivar is rarely capable of inhibiting fungi other than *Escovopsis* spp. to the extent that it can inhibit *Escovopsis* (Gerardo & Currie, unpublished data). There is also specificity within the symbiosis; *Escovopsis* spp. overcome chemical inhibition by the cultivars that they normally attack and by cultivars closely related to their typical hosts, but *Escovopsis* spp. are generally inhibited by cultivars distantly related to their typical hosts (Chapters 4, 5; Gerardo & Currie unpublished data). This suggests that *Escovopsis* may be adapted to overcome only a narrow range of host defenses, which may in turn facilitate clade-limited parasite colonization, in which the matching of host-parasite phylogenies is maintained by the inability of parasites to make phylogenetically distant host switches. Clade-specific colonization has been shown to maintain phylogenetic congruence between brood parasitic finches (*Vidua* spp.) and their finch hosts (Sorenson *et al.* 2004).

Because *Escovopsis* must overcome behavioral ant defenses as well as antimicrobial bacteria and cultivar defenses, the fungus-growing ant symbiosis can be utilized to gain a further understanding of a wide range of host adaptations. In addition, parasite antibiotic resistance can be explored through the study of *Escovopsis* resistance to both cultivar and actinomycete defenses within the context of a known evolutionary history.

Genetic variation of attack and defense. Host-parasite coevolution is the reciprocal evolution of parasite infectivity and host defense. For evolution in both hosts and parasites to occur, there must be genetic variation in host defense and parasite virulence. Theoretical studies also predict that there would be genotype-genotype interactions; infectivity and resistance would vary depending on what parasite and host genotypes are combined (Frank 1992; Thompson & Burdon 1992; Frank 1996). An understanding of

within-population variation of attack and defense is crucial to applying evolutionary theory to agricultural and medical applications, where there is likely a lot of variation in host susceptibility (Little 2002). Though there is substantial evidence for genetic variation in host susceptibility (Thompson & Burdon 1992; Ebert *et al.* 1998; Webster & Woolhouse 1998; Little & Ebert 1999) and there is also evidence for genetic variation in parasite virulence (Carter *et al.* 2002; Ferguson & Read 2002), there is less information on these traits in relation to the host-parasite genotype interactions. In one of the few studies to explicitly demonstrate host-parasite genotype interactions, Carius *et al.* (2001) showed considerable variation in both host resistance and parasite infectivity and considerable variation for host strain – parasite strain interactions, indicating that parasite strains may be tracking particular host strains.

The fungus-growing ant microbe symbiosis may be an excellent system to explicitly identify the importance of host-parasite genotype interactions. First, there is substantial variation in parasite virulence. In Currie (2001), virulence of *Escovopsis* strains used to infect leaf-cutter ant colonies varied, with one *Escovopsis* isolate having a significantly more detrimental impact than the other experimental strains. This variation seems likely in relation to infection of colonies of other fungus-growing ant genera as well; Gerardo *et al.* (2004) demonstrated that *Escovopsis* isolates from *Cyphomyrmex* spp. colonies had variable success in infecting *Cyphomyrmex* spp. garden pieces. In both of these studies, however, it is unclear whether there is variation in host resistance, in parasite infectivity or in both. It is also unclear whether establishment of infection is dependent on the parasite genotype, the host genotype or the genotype-genotype combination. In chapter 4, however, I have demonstrated that, in fact, 1) there is substantial variation in both host susceptibility and in parasite efficiency and 2) successful establishment of infection depends upon the combination of host and parasite genotype.

1.3 FUTURE TOPICS FOR EXPLORATION

Because of the ease of both molecular characterization and experimental manipulation in the fungus-growing ant symbiosis, there are several promising avenues of research. Here, I address some prospects for future research.

Population-level processes. If either host adaptations to parasites or parasite adaptations to hosts are highly specific, these adaptations will mediate local population-level processes. Parasites are expected to become adapted to overcome the defenses of locally common host genotypes at the possible cost of a loss of ability to infect allopatric hosts (Gandon *et al.* 1996; see Kaltz & Shykoff 1998 for review; Lively 1999). Local parasite adaptation, however, does not always occur. Studies of host-parasite systems indicate local adaptation, local maladaptation or neither (see Lajeunesse & Forbes 2001 for review of studies and results).

Studies of local adaptation are only informative in the context of known host and parasite population structures. Gene flow can swamp out local host-parasite dynamics (Ebert 1994; Gandon *et al.* 1996; Lively 1999; Nuismer *et al.* 1999; Gandon & Michalakis 2002). For example, if host genotypes are more widely distributed across populations than parasite genotypes, in cross-infection studies where hosts from different localities are experimentally infected with different parasites, one would not expect to see local adaptation because the same hosts are being tested across treatments. In reality, most studies of local adaptation have assumed that the tested populations of host and parasites are unique, yet strong population structure is not always the case (see Nadler 1995 for review). Thus, it is critical to have information on the population structures of both host and parasite.

The first population structure analysis of *Escovopsis* and its associated cultivar hosts indicates that there is some concordance between host and parasite population structure. *Escovopsis* and cultivars from colonies of *Apterostigma dentigerum* exhibit similar levels of isolation by distance and have fairly similar migration rate. Pairwise differences

between host and parasite populations, however, are not identical, indicationg that patterns of migration and divergence are not tightly linked between *A. dentigerum's* cultivars and *Escovopsis* (chapter 5). Of course, these results cannot be extrapolated to all *Escovopsis* and cultivar species as many studies in other systems have indicated a wide range of population subdivision in host and parasites (Dydahl & Lively 1998; Delmotte *et al.* 1999; Martinez *et al.* 1999). Thus, future population structure analyses in the fungus-growing ant symbiosis will be necessary to look at genetic variation across the symbiosis.

Virulence evolution. *Escovopsis* is known to be highly virulent under at least some circumstances. Currie (2001) demonstrated that when experimentally infected with *Escovopsis*, colonies of leaf cutter ants in the genus *Atta* had lower garden growth rates, leading to a reduction in worker production. Some colonies were quickly overgrown and died shortly after inoculation. Under field conditions, abandoned colonies have been found overwhelmed with *Escovopsis*. We do not know, however, whether this observed virulence can be extrapolated to *Escovopsis* under all biotic and abiotic conditions. For instance, *Escovopsis* virulence could vary depending on the life stage of a colony; colonies overwhelmed with *Escovopsis* in the field may have already been in decline for other reasons, giving *Escovopsis* the chance to overtake the garden. Or, environmental factors, such as temperature or humidity could dictate virulence (for an excellent example of this, see Blanford *et al.* 2003 where they demonstrate that virulence of a fungal pathogen of aphids depends on temperature). It is also possible that, as in other symbioses, not all *Escovopsis spp.* are virulent parasites. In ectomycorrhizal symbioses, for example, closely related species fall on a continuum from mutualists to parasites (Hibbett *et al.* 2000), and the virulence of nematode parasites of fig wasps depends on transmission rate (increased transmission leads to increased virulence; Herre 1993). Thus, extrapolation of the virulence of one *Escovopsis* sp. to the group of parasites as a whole may not be appropriate. More work is needed on the impact of *Escovopsis* in fungus-growing ant colonies of different genera and at different life stages in both laboratory field experiments and under natural field conditions.

Temporal dynamics. Many theoretical studies of host-parasite interactions predict dynamic genetic interactions (reviewed in Woolhouse *et al.* 2002). In these host-parasite races, parasites are continually adapting to common host genotypes and hosts are simultaneously adapting defenses to common parasite genotypes. In this situation, it is predicted that parasite genotypes efficient at attacking common host genotypes would spread and subsequently those host genotypes then would become less common until those parasite genotypes were disfavored. Despite theoretical expectations of temporal fluctuations, few studies have shown their existence (Dybdahl & Lively 1998; Fenner & Fantini 1999).

The temporal dynamics of infection may be easily studied in relation to disease prevalence in colonies of some species of fungus-growing ant. Species in several attine genera, including *Trachymyrmex*, *Cyphomyrmex* and *Apterostigma*, have small, easily-sampled colonies along streambanks and hillsides. These colonies are often abundant and sometimes only centimeters apart on the same slope. The colonies can be opened and their fungal garden can be sampled with minimal disturbance to the ants or to the survival of the colony (the ants typically repair the damage to their nest structure within 24 hours; Gerardo, personal observation). Thus, over time, the prevalence of host and parasite genotypes, infection rates across host genotypes and fidelity of parasite genotypes to host genotypes can be monitored. The spatial distribution of infection can also be investigated and may give insight into how infection spreads between host colonies over time (see Frank 1997 and Real & McElhany 1996 for discussion on the relevance of spatial processes in disease dynamics). These investigations may be particularly practical if PCR-based methods are used to genotype *Escovopsis* and the cultivar directly from the garden material, facilitating rapid analysis without the need for extensive, time-consuming culture isolation and maintenance. If the fungi are isolated and maintained, however, live fungal cultures can be used for further experimental studies based on the outcomes of molecular analyses (fig. 1.1).

Coinfection. Infection in one host by multiple pathogen genotypes affects both pathogen dynamics and disease evolution (Levin & Bull 1994; Nowak & May 1994; Van Baalen & Sabelis 1995; Taylor *et al.* 1998; Read &Taylor 2001). Multiple infections can lead to within-host competition amongst pathogens, which may increase virulence and thus decrease host growth and survival (Ewald 1987; Bonhoeffer & Nowak 1994; Lenski & May 1994; Hood 2003). Virulence may also be increased as hosts must mount more costly defenses against multiple pathogens (Taylor *et al.* 1998). Multiple infections, however, could also lead to competitive suppression, where competition decreases the spread and thus severity of infection (reviewed in Read & Taylor 2001). Ultimately, pathogen adaptations may arise to attenuate competitive effects. Though there have been numerous theoretical studies on co-infection (Levin & Bull 1994; Nowak & May 1994; Van Baalen & Sabelis 1995 and references therein), empirical studies have been limited to a small number of study systems (e.g. rodent-malaria strains system; Taylor *et al.* 1998), mainly because the dynamics of infection can be difficult to measure.

Coinfection in a single garden by multiple morphologically distinct *Escovopsis* spp. is fairly common in the colonies of several fungus-growing ant species (Currie & Gerardo, personal observation), and coinfection by different genotypically-distinct strains of the same *Escovopsis* morphotype also occurs (Gerardo *et al.* 2004; chapter 2). Because colonies can be experimentally infected with these genotypes and then the gardens can be subcultured to examine the establishment of infection by each genotype, this is an excellent system to look at the consequences of multiple infections, as compared to single infection, for disease dynamics. Such experiments, however, must be preceded by more extensive studies on the virulence of infection by single *Escovopsis* strains (as mentioned above). Ultimately, of course, it is possible that infection by *Escovopsis* is also impacted by other, yet unknown parasites and future research addressing the diversity within colonies will elucidate this possibility.

Chemical and molecular coevolution. One of the most fascinating aspects of the fungus-growing ant symbiosis is the potential for complex chemical adaptation on the

part of the various symbionts. For example, *Escovopsis* is attracted to chemical signals produced by the cultivars. Thus, we would hypothesize that it would benefit the cultivars to evolve chemical signatures that could avoid detection. In combining experiment analyses (in-vivo and ex-vivo cross-infections, fig. 1.1) with chemical and molecular analyses, we may be able to elucidate what circumstances facilitate the evolution of novel chemical signatures and what genes underlie these adaptations. We would also expect that it would benefit the cultivars to chemically inhibit all *Escovopsis* types, yet we know that the cultivar defense is only capable of inhibiting novel parasites (i.e. *Escovopsis* strains that the cultivar strains are not normally associated with in nature) rather than those that normally infect the cultivars in nature. How has this antibiotic resistance by *Escovopsis* arisen and been maintained? Is there parallel antibiotic resistance in relation to the interaction between *Escovopsis* and antibiotic producing actinomycete bacteria? Are the underlying genetics a matter of a few genes controlling resistance and susceptibility? Answering these questions will begin to elucidate the mechanisms of host-parasite adaptation and coevolution.

1.4 CONCLUSION

The fungus-growing ant symbiosis shows great promise as a system for future research on the study of host-parasite interactions. Like other well-studied symbioses, infection in ant gardens can be monitored over space and time. Molecular and morphological characteristics can both be used to identify hosts and parasites. Both host and parasites can be maintained long-term for future experiments. Hurdles to future research consist of learning the mode of transmission of the parasite and the nature of virulence of *Escovopsis*. Furthermore, it seems likely that there could be parasites other than *Escovopsis* in the fungus-growing ant symbiosis that are equally relevant and could represent future avenues of comparison.

While the fungus-growing ant symbiosis will be a fruitful system to study general host-parasite theory, it may also be a valuable tool to study the specific nature of agricultural

host-parasite interactions (Shultz *et al.* 2004; Mueller *et al.* 2005). Whereas humans have been farming crops for thousands of years, fungus-growing ants have been cultivating fungi for approximately fifty million years, and *Escovopsis* has likely been attacking this mutualism for much of its history (Currie 2003b). By understanding how ants, bacteria and cultivars combine defenses to suppress infection and how parasite resistance has evolved during this ancient association, we may gain insights into future strategies for agricultural research and activity.

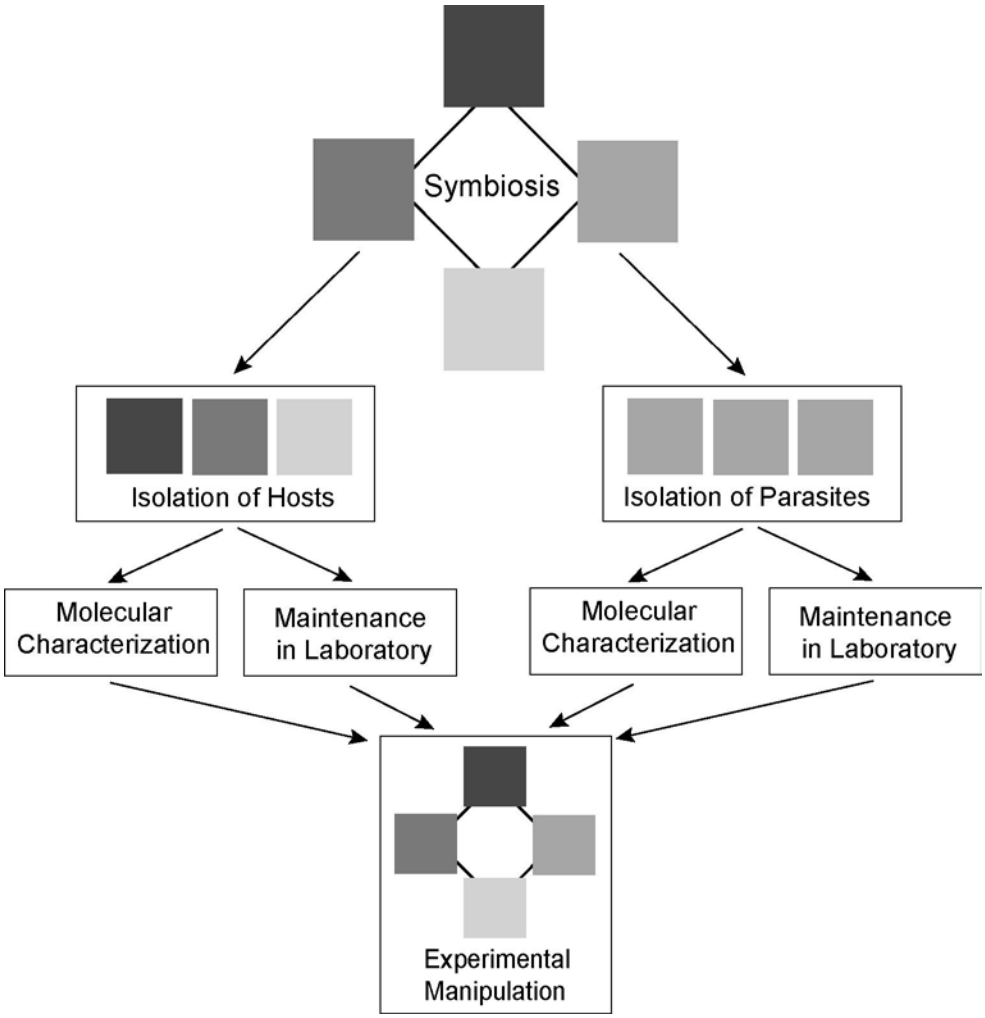

Figure 1.1: Components critical to the study of host-parasite evolution. There are three components of a host-parasite system, and more generally any symbiosis, that make it amenable to evolutionary studies: 1) feasible isolation and maintenance of all symbionts, 2) effective tools for molecular characterization of both symbionts and 3) practical methods for experimental manipulation of the system.

Isolation and Maintenance of Symbionts. First, it is helpful if both players can be isolated and maintained for long-periods of time outside of the symbiotic association. This is limiting in many systems. For example, in vertebrate-parasite systems, vertebrates are often difficult and costly to maintain in the lab, and parasites, such as parasitic wasps

or bird brood parasites, are so dependent on their host that they cannot be maintained away from the host for long periods of time.

Molecular Analyses. If isolation of both players is feasible, this will facilitate the molecular characterization of the players. Molecular analyses elucidate population dynamics and evolutionary history of the association. Feasible molecular analysis is particularly crucial if the host and/or parasite cannot be discriminated through morphological characteristics, as is the case for many microbes. Though the prevalence of molecular studies makes characterization of hosts and parasites seem trivial, there are excellent studies in host-parasite interactions which have been limited by an inability to genotype both symbionts (e.g. Little & Ebert 1999 and Carius *et al.* 2001 could only genotype host *Daphnia* and not their parasites).

Experimental Manipulation. Finally, molecular analyses at the appropriate intra- or interspecific scale, can inform experimental design, using isolated and laboratory-maintained host and parasite strains, to address questions of organismal function (Moran 2002). Such experiments include **cross-infection** (a.k.a. cross-inoculation or switching) experiments in which hosts are faced with typical vs. novel pathogens (either novel pathogen species or interspecific strains) to address questions of adaptation on the part of both host and parasite. For example, Lively & Dybdahl (2000) used such a design to show that parasites track locally common host genotypes. Cross-infection experiments could potentially be in-vivo or ex-vivo. For example, in the fungus-growing ant symbiosis, **in-vivo experiments** would include the inoculation of an attine garden with different parasite strains. Gardens can be easily divided and the sub-gardens can then be treated differently for paired comparison. **Ex-vivo experiments** are comprised mainly of bioassays, in which two fungi (e.g. a cultivar and a parasite strain) are grown on standard media and the result of the interaction is scored (e.g. inhibition of parasite by host, attraction of parasite to host, etc.). The ease of ex-vivo experiments makes them an attractive alternative to intensive in-vivo studies.

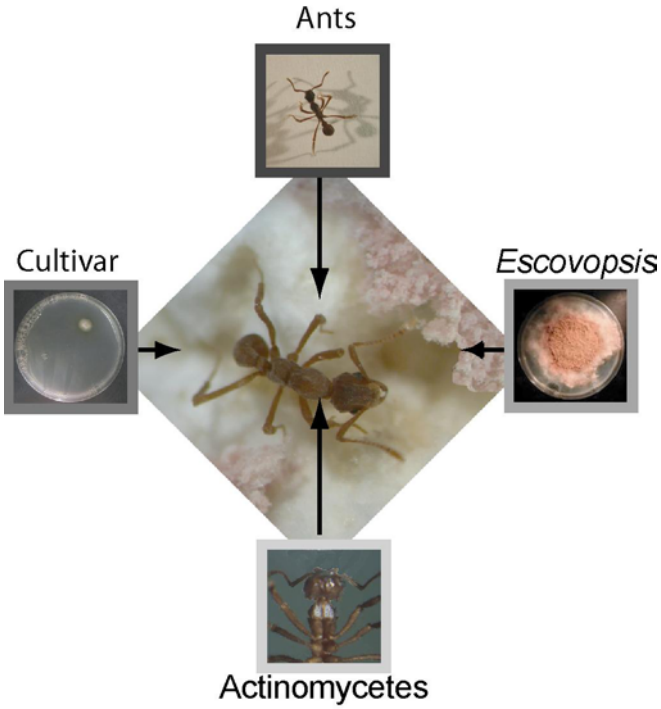

Figure 1.2: Fungus-growing ants and their associated microbes.

Ants. The ability to cultivate fungi for food arose only once in ants, about 50-60 million years ago, and gave rise to roughly 200 known extant species of fungus-growing ants (Tribe Attini). Attine ants are dependent on fungal cultivation; their brood is raised on an exclusively fungal diet. Attines grow their cultivar (their fungi) in subterranean chambers, fertilizing the gardens with dead vegetable debris, or in the case of the leaf-cutter ants, with leaf fragments cut from live plants. Nests of most species number only a few hundred workers, but leaf-cutter colonies may have millions of workers and hundreds of fungal chambers. Leaf-cutter ants are prodigious consumers of leaves and are among the most damaging agricultural pests in South and Central America.

Cultivars. Attine colonies are founded by a mated female who takes fungus from her mother's colony in order to start her new garden. This vertical transmission has led to cultivar specialization by the ants. Most attine species are specialized on growing one of a few strains of fungi in the family Lepiotaceae, though ants in the genus

Apterostigma grow fungi in the family Pteruleaceae (Chapela *et al.* 1994; Munkacsi *et al.* 2004; Villesen *et al.* 2004). All cultivars are grown as asexual mycelia, however some are capable of forming sexual fruiting bodies. The exact rate of sexual recombination is unknown but probably impacts host-parasite evolution (Mueller 2002).

Escovopsis. While ants and their cultivars were long thought to be the only dominant players in this system, recent work indicates that attine gardens are frequently infected by one genus of specialized, highly pathogenic fungi, *Escovopsis* (Ascomycota: Hypocreales), which attacks and consumes the ants' fungal cultivars (Currie *et al.* 1999a; Reynolds & Currie 2004). *Escovopsis* has only been found in nests of fungus-growing ants. It parasitizes gardens of most fungus-growing ant species throughout their geographic range. Though infection rates vary across host species, infections are prevalent in colonies of many attine genera (Currie *et al.* 1999a; Gerardo *et al.* 2004). Different morphotypes are isolated from colonies of different attine genera, and these morphotypes correspond to parasite clades associated with particular cultivar host clades (Currie *et al.* 2003b).

Actinomycete bacteria. All tested species of fungus-growing ants are associated with filamentous bacterium (actinomycetes), which typically cover portions of the ants' cuticles (i.e. body surface). These bacteria are used by the ants to derive antibiotics that specifically inhibit *Escovopsis* growth (Currie *et al.* 1999b). Fungus-growing ants have elaborate structures on their cuticle to house the bacteria and have glands suspected to provide the bacteria necessary nutrients, suggesting an ancient association between these mutualists (Currie *et al.,* unpublished data). Current work will indicate the diversity of actinomycetes and their specialization across the attine symbiosis.

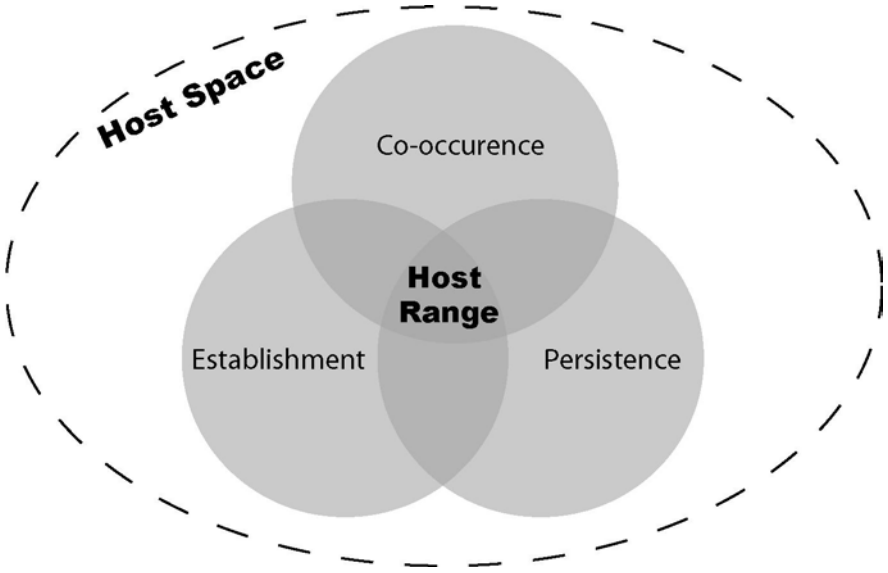

Figure 1.3: Parasite Host-Range. There are three main forces mediating a parasite's host range: 1) whether the parasite can co-occur with the host, 2) whether the parasite can establish infection and 3) whether the parasite can persist on the host. If we think of host space as comprising all possible hosts, then these forces successively narrow the portion of that space that can be utilized by a parasite.

Co-occurence. A parasite must be able to come into contact with a potential host. Such co-occurrence requires two features: 1) a parasite must be able to persist under the same general ecological conditions as a host; and 2) a parasite much not biogeographically isolated from a host. For example, if a parasite has limited dispersal ability, it may be less likely to encounter a host (e.g. chewing lice that parasitize birds and mammals can infect novel hosts in experimental cross-infections but do not do so in nature because of their limited dispersal between hosts; Clayton *et al.* 2004).

Establishment. To establish on a host, a parasite must encounter that host with it habitat and must then overcome initial host defenses. For some parasites, the likelihood of encounter is increased by the parasite's attraction to host-specific signals (e.g. great spotted cuckoos, bird brood parasites of magpies, are attracted to larger host nests. These nests signal that the host is more likely to successfully raise their young; Soler *et al.*

1995). Once a host is encountered, hosts defenses to be overcome by the parasite include behavioral responses (e.g. hosts recognize the eggs of brood parasites and kick them out of the nest, Soler & Soler 2000), chemical responses (e.g. induced chemical defense in plants upon attack, Levin 1976; Maleck & Dietrich 1999) and immune system responses.

Persistence. Once established, a parasite must be able to efficiently utilize the host and maintain infection. Specialist mongeneans, ectoparasites of fish, for example, have attachment organs with anchors specific to host body size. These attachments are expected to be adapted to maintain long-term attachment to the host (Simková *et al.* 2001). Bird lice have similar adaptations that allow them to persist on hosts of only certain sizes that they can attach to efficiently and thus avoid being removed by preening (Clayton *et al.* 2003).

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Chapter 2

Exploiting a mutualism: parasite specialization on cultivars within the fungus-growing ant symbiosis

Abstract: Fungus-growing ants, their cultivated fungi, and the cultivar-attacking parasite *Escovopsis* coevolve as a complex community. Higher-level, phylogenetic congruence of the symbionts suggests specialized, long-term associations of host-parasite clades but reveals little about parasite specificity at finer scales of species-species and genotype-genotype interactions. By coupling sequence and AFLP genotyping analyses with experimental evidence, we examine (a) host specificity of *Escovopsis* strains infecting colonies of three closely related ant species in the genus *Cyphomyrmex*; and (b) potential mechanisms constraining *Escovopsis* host range. Incongruence of cultivar and ant relationships across the three focal *Cyphomyrmex* spp. allows us to test whether *Escovopsis* strains track their cultivar or ant hosts. Phylogenetic analyses demonstrate that the *Escovopsis* phylogeny matches the cultivar phylogeny but not the ant phylogeny, indicating that the parasites are cultivar-specific. Cross-infection experiments establish that ant gardens can be infected by parasite strains with which they are not typically associated in the field, but that infection is more likely when gardens are inoculated with their typical parasite strains. Thus, *Escovopsis* specialization is shaped by the parasite's ability to overcome only a narrow range of garden-specific defenses, but specialization is likely constrained by additional ecological factors, including the other symbionts (i.e. ants and their antibiotic-producing bacteria) within the coevolved fungus-growing ant symbiosis.

2.1 INTRODUCTION

Most parasites are host-specific, specializing on particular host genotypes (Carius *et al.* 2001), on monophyletic host lineages (Herre 1993; Johnson *et al.* 2002), or on unrelated but phenotypically similar hosts (Morand *et al*. 2002; Waldenstrom *et al.* 2002). The extent of a parasite's host range impacts ecological dynamics of host-parasite systems (Woolhouse *et al.* 2001), which in turn influence long-term coevolutionary interactions. Thus, parasite specialization can lead to patterns of congruence in host and parasite phylogenies, suggesting coevolution and cospeciation of both symbionts (Clayton *et al.* 2003a,b). Such associations are known for a wide spectrum of host-parasite associations, including vertebrates and their lice (Hafner *et al.* 1994; Clayton & Johnson 2003), birds and their brood parasites (Sorenson *et al.* 2004), and cultivated fungi of attine ants and their garden parasite *Escovopsis* (Currie *et al.* 2003b).

Specificity arises as a consequence of a parasite's adaptation to environmental and symbiotic forces (Combes 2001). A parasite's host range may be limited by its ability (a) to persist in the habitat of particular hosts (Norton & Carpenter 1998); (b) to recognize and locate susceptible hosts (Sorenson *et al.* 2003); or (c) to overcome defenses of particular hosts (Van der Ackerveken & Bonas 1997). In many parasite systems, it has been possible to determine the host range of a parasite, yet the mechanistic and selective processes determining parasite specificity have remained elusive.

This study elucidates processes shaping fine-scale, species-level parasite specialization in the fungus-growing ant symbiosis. The parasite *Escovopsis* is a morphologically diverse microfungal genus that attacks and consumes fungal cultivars of attine ants (Currie *et al.* 1999a; Reynolds & Currie 2004). *Escovopsis* is horizontally transmitted between colonies and appears to be specialized on the symbiosis; it has only been found associated with fungus-growing ant gardens and dumps (Currie *et al.* 1999a; Currie 2001a; Currie *et al.* 2003b). *Escovopsis* directly attacks and consumes the ants' main cultivated food source, indirectly decreasing ant colony survival and reproduction (Currie *et al.* 1999a; Currie 2001b). Even though the ants use *Escovopsis*-specific sanitary behaviors to

remove the parasite from their colonies (Currie & Stuart 2001), and the ants have filamentous actinomycete bacteria on their exoskeleton that produce *Escovopsis*-inhibiting antibiotics (Currie *et al.* 1999b; Currie *et al.* 2003a), infections are persistent and detrimental (Currie *et al.* 1999a; Currie 2001b).

Because *Escovopsis* is harmful to both ants and their cultivars, *Escovopsis* can be hypothesized to track the evolution of either the ants, which have lower fitness in the face of garden infection, or their cultivars, which are directly attacked. For example, if cultivars can inhibit *Escovopsis*, then the parasite may infect only gardens whose defenses they can overcome, leading to matching of the cultivar and parasite phylogenies (figure 2.1*a*). On the other hand, if ants can recognize and weed only a limited range of *Escovopsis* strains, a particular parasite strain may infect only colonies in which it can overcome the ants' defenses, leading to matching of the parasite and ant phylogenies (figure 2.1*b*). Alternatively, the pattern could be more complicated if it is shaped by an interplay of ant, bacterial and cultivar inhibition.

To determine whether *Escovopsis* is specialized either on particular ant or cultivar hosts, we characterized the association of *Escovopsis* with three sympatric host ant species in the genus *Cyphomyrmex*. *Cyphomyrmex longiscapus* and *C. muelleri* are putative ant sister species with similar habits (Schultz *et al.* 2002). Both species have nests along rainforest stream banks and hillsides, with a single chamber of fungus protected by a mud auricle at the nest entrance (figure 2.2*a*). Despite their similarities in habit, these two closely related ant species are known to cultivate distantly related, morphologically distinct fungal cultivars (Mueller *et al.* 1998; Schultz *et al.* 2002) (figure 2.2*b*). *Cyphomyrmex costatus*, on the other hand, is a more distantly related ant species with larger colonies found under rocks and logs that are rarely in close proximity to *C. longiscapus* and *C. muelleri* colonies. *Cyphomyrmex muelleri* and *C. costatu*s, however, grow morphologically similar and occasionally genotypically identical fungal cultivars (Green *et al.* 2002) (figure 2.2*b*), indicating that these two ant species are specialized on the same narrow clade of cultivar strains. Thus, phylogenetic patterns indicate a

decoupling of ant and cultivar relationships in this system: closely related ants (*C. muelleri* and *C. longiscapus*) grow distantly related cultivars, and distantly related ants (*C. muelleri* and *C. costatus*) grow closely related or identical cultivar strains. Colonies of all three species are infected with the same pink *Escovopsis* morphotype (figure 2.2*c*).

Here, we analyze both amplified fragment length polymorphism (AFLP) and sequence data of *Escovopsis* isolates from *C. longiscapus*, *C. muelleri* and *C. costatus* colonies to examine patterns of association between *Escovopsis* genotypes and their hosts. We then couple these molecular analyses with cross-infection experiments to explore potential mechanisms constraining parasite host range (figure 2.1).

2.2 MATERIALS AND METHODS

Collection, Natural Infection Rates and Isolation. We collected *Cyphomymrex longiscapus*, *C. muelleri* and *C. costatus* colonies between 2001–2002 at six sites in the hosts' sympatric range in the Republic of Panamá: El Llano–Cartí Suitupo Road (EL), Fort Sherman (FS), Barro Colorado Island (BCI), Gamboa (GA), Ancon Hill (AH), and Pipeline Road (PLR) (see Green *et al.* 2002 for map). To determine natural infection levels in the three host populations, at least ten garden pieces (~8mm^3) from each colony were grown on potato dextrose agar (PDA; Difco, Detroit, MI) with antibiotics (50mg/L each of penicillin and streptomycin). If *Escovopsis* emerged from a garden piece, which typically occurred within 10 days of initial isolation, the colony was scored as infected. *Escovopsis* mycelium was then subcultured, and axenic (pure) cultures were stored at -80^{O}C until DNA extraction, which followed a CTAB extraction protocol modified from Bender *et al.* (1983).

Sequencing Analysis. Sequencing targeted a 1727 nucleotide stretch spanning 4 exons and 2 introns of nuclear elongation factor–1 alpha (EF-1 alpha). A single *Escovopsis* isolate from each of 8 *C. longiscapus* colonies (2 EL, 6 PLR colonies), 14 *C. muelleri* colonies (2 BCI, 2 FS, 10 PLR colonies) and 11 *C. costatus* colonies (1 BCI, 1 GA, 9 PLR colonies) was sequenced. We also sequenced *Escovopsis* isolates from 3

Apterostigma dentigerum colonies and 1 isolate of *Trichoderma* sp. as outgroups. Primers EF1-983F (5' GCY CCY GGH CAY CGT GAY TTY AT 3') and EF1-2218 (5' ATG ACA CCR ACR GCR ACR GTY TG 3') spanned a single exon, while primers EF1-3f (5' CAC GTC GAC TCC GGC AAG TC 3') and EF1-5r1 (5' GTG ATA CCA CGC TCA CGC TC 3') spanned 3 exons and 2 introns. Internal sequencing primers EF1-6mf (5' GTC ACB ACY GAA GTC AAG TC 3') and EF1-6mr (5' GAC TTG ACT TCR GTV GTG AC 3') were used for cycle sequencing in the former case. All sequences have been deposited in GenBank (accession numbers AY629361-AY629398).

Sequences were assembled in SeqMan II (ver 5.05, DNASTAR), aligned using ClustalW WWW (http://www.ebi.ac.uk/clustalw) and edited manually in MacClade (ver 4.06, Maddison & Maddison 2003). The alignment was annotated based on sequences of *Gibberella circinata* (GenBank accession no. AF333930) and *Gongronella butleri* (AF157252). Exon alignments were unambiguous, but intron sequences were unalignable and were excluded.

Aligned sequences were analyzed in PAUP* (ver 4.0b10, Swofford 2002) using maximum-likelihood (ML) and a GTR+Γ+PINVAR sequence evolution model with four Γ-distributed rate classes, which was chosen based on results from Modeltest (Ver 3.06, Posada & Crandall 1998). Tree searches were conducted via TBR-branch swapping on five stepwise-addition trees (assembled in random order). We estimated initial parameters on maximum parsimony trees and then refined the parameters via successive approximation on trees recovered using likelihood. These final parameters were used in all successive analyses and simulations.

We assessed support for each branch using both bootstrap and Bayesian analyses. Nonparametric bootstrap proportions were estimated from 100 pseudo-replication datasets analyzed under the ML criterion. Bayesian posterior probabilities were estimated as the proportion of trees sampled after burn-in that contained each of the observed bipartitions. Bayesian analyses were performed with MrBayes (ver 3.0b4, Huelsenbeck & Ronquist 2001) with GTR +Γ+ PINVAR parameters estimated during the run, using the

default value of four Markov chains and a temperature parameter set to 0.2. We combined trees after burn-in from four Monte Carlo Markov chains (MCMC) (500,000 generations/run, sampled trees every 100 generations, burn-in at 50,000 generations). All trees remaining after burn-in were used to construct a majority rule consensus tree.

We used Analysis of Molecular Variance (AMOVA) in Arlequin (Ver 2.001, Schneider *et al.* 2000) to partition the sequence variation among isolates within and between host species. Population pairwise F_{st} values were then generated to determine the proportion of differences between the parasites associated with each of the three host types. Levels of significance were determined through 100,000 random permutation replicates. A Bonferroni correction was used to correct for multiple, pairwise comparisons.

AFLP Analysis. To investigate phylogenetic relationships within a larger collection of *Escovopsis* isolates, we analyzed the relationships between 126 *Escovopsis* isolates from a total of 42 colonies, using AFLP (amplified fragment length polymorphism) genotyping methods (Mueller & Wolfenbarger 1999). Twenty-three of these 126 isolates were part of the original sequencing analysis (see above). We included multiple *Escovopsis* isolates from single colonies in order to establish whether single gardens could be infected by multiple parasite genotypes. Isolates included *Escovopsis* from 11 *C. longiscapus* colonies (2 EL, 9 PLR colonies; avg. 3.6 isolates/colony), 21 *C. muelleri* colonies (1 EL, 4 BCI, 4 FS, 12 PLR colonies; avg. 3.5 isolates/colony), and 10 *C. costatus* colonies (2 AH, 1 BCI, 1 GA, 6 PLR colonies; avg. 1.4 isolates/colony).

AFLP markers were generated on an ABI Prism 3100 Genetic Analyzer and scored in Genotyper 2.5. Reactions followed the AFLP protocol for small plant genomes (www.appliedbiosystems.com; protocol 4303146), with the modification that preselective products were diluted 2:1 before use in the selective reactions. Five combinations of AFLP-primer extensions were chosen because they generated high levels of polymorphic markers that could be scored reliably: AC/CAT, TC/CAA, TG/CAA, TG/CTA and TC/CAG. AFLP markers were scored blindly by simultaneously comparing all fragments

of a given length across all 126 *Escovopsis* isolates. Only markers that could be scored as unambiguously present/absent across all 126 samples were used in the analysis.

The final AFLP matrix included 299 informative characters that were analyzed in a two-step process under the parsimony criterion in PAUP* (ver 4.0b10, Swofford 2002). In the first step, we completed a heuristic search without saving multiple trees (multrees=off; 50,000 replicates). We then used the best trees from this search as the starting point for a heuristic search (Maxtree=500,000; Multrees=on). Parsimony bootstrap analysis included 500 pseudoreplicates (5 stepwise addition searches per pseudoreplicate; Maxtree=100).

As with the sequence data, we also used AMOVA and comparison of between-host pairwise F_{st} values to partition AFLP variation across *Escovopsis* isolates from the three hosts. To prevent pseudoreplication, we randomly selected only one *Escovopsis* isolate per colony (total of 42 isolates) for AMOVA analysis.

Cross-Infection Experiments. To determine the impact of *Escovopsis* on typical versus atypical hosts, we inoculated garden material with *Escovopsis* isolates from each of the three host types. We used garden pieces from 27 *C. longiscapus* colonies, 38 *C. muelleri* colonies, and 26 *C. costatus* colonies. For each colony, we placed four garden fragments (~100 mg/fragment) without ants onto separate sterile Petri dishes lined with moist cotton and sealed with parafilm. Each garden fragment was then randomly assigned to one of four treatments: (1) inoculation with *Escovopsis* from a *C. longiscapus* colony; (2) inoculation with *Escovopsis* from a *C. muelleri* colony; (3) inoculation with *Escovopsis* from a *C. costatus* colony; (4) or control. We inoculated the garden pieces with a small piece (~6mm^3) of agar with spore-bearing mycelium of an *Escovopsis* culture less than two weeks old. Pieces were cut from media at the leading edge of fungal growth and placed in direct contact with the garden fragment. Controls were "inoculated" with a piece of sterile agar.

For each treatment, garden pieces were randomly assigned *Escovopsis* strains originally isolated from one of three colonies of the appropriate host species. Because we used only

three different *Escovopsis* strains per host, results statistically represent the impact of these particular isolates rather than the population of *Escovopsis* as a whole. These isolates, however, have genotypes common to parasites found in the host populations and thus are representative of the typical parasite population (all experimental *Escovopsis* strains were confirmed via AFLP or sequence analysis to have genotypes frequently isolated from the associated host type). All experimental parasite isolates and *Cyphomyrmex* colonies were from the Panama Canal region.

Over a two-week period, we monitored garden fragments daily for *Escovopsis* growth. Level of growth was recorded as either: suppression (no growth on garden) or overgrowth (*Escovopsis* grew over the entire garden). All colonies for which the control garden fragment was overgrown with *Escovopsis* were considered to have a previously established, natural infection. We thus excluded all garden fragments (both treatment and control) from these previously infected colonies, leaving garden fragments from a total of 26 *C. longiscapus* (4% of colonies excluded), 23 *C. muelleri* (31% of colonies excluded) and 18 *C. costatus* colonies (39% of colonies excluded) for analysis. These prior infection rates parallel the frequency of infection detected in natural field conditions (see results).

We used the GENLOG procedure in SPSS ver 11.5.5 (SPSS Inc., Chicago IL) to determine whether there was an overall interaction between *Escovopsis* type, garden type and infection establishment. This procedure uses a chi-square goodness-of-fit test to determine the independence of three or more categorical variables. We also used individual two-way chi-square analyses to determine whether infection rate varied for a given garden type depending on the treatment.

2.3 RESULTS

Natural Field Infection Rates. *Escovopsis* infection in *Cyphomyrmex* colonies is common. *Escovopsis* emerged in 12% of *C. longiscapus* colonies (n=118 colonies), 29% of *C. muelleri* colonies (n=90 colonies) and 60% of *C. costatus* colonies (n=28 colonies).

Infection rates for *C. muelleri* and *C. costatus* colonies are similar to infection rates reported for colonies of other attine genera (*e.g.,* 33-51% across 5 genera in Currie *et al.* 1999a), but the infection rate for *C. longiscapus* colonies is lower than previously reported for other attines. These and previously reported values likely represent a conservative estimate of the rate of natural infection, because some infections remain undetected when only ten garden pieces per colony are sampled (Gerardo & Currie, unpublished data).

Sequencing Analysis. Of the 1157 positions in our final sequence alignment, 237 sites were variable and 165 of these were parsimony informative. Maximum-likelihood analysis supported a single, best tree. In this tree, *Escovopsis* isolates from *C. longiscapus* colonies formed a well supported clade (figure 2.2). Isolates from *C. muelleri* and *C. costatus* colonies fell into another well supported clade. In several instances, EF-1 alpha sequences of *Escovopsis* isolates from *C. muelleri* and *C. costatus* colonies were identical.

Consistent with these results, analysis of molecular variance (AMOVA) of 35 sequenced samples revealed that 70% of EF1-alpha sequence variation was explained by the host-type from which the parasite was isolated (table 1a). Pairwise comparisons revealed significant differences between *Escovopsis* from all three host-types. There was, however, a much lesser difference between *Escovopsis* from *C. costatus* and *C. muelleri* colonies than between *Escovopsis* from *C. longiscapus* and *C. muelleri* colonies or between *Escovopsis* from *C. longiscapus* and *C. costatus* colonies (table 1*a*).

Thus, the sequence data indicate that *C. longiscapus* and *C. muelleri* (closely related ants that cultivate distantly related fungi) are associated with different pathogens, whereas *C. muelleri* and *C. costatus* (more distantly related ants that grow similar fungal cultivars) are associated with similar pathogens. *Escovopsis* therefore is cultivar-type rather than ant-type specific.

AFLP analysis. AFLP data suggested a similar pattern of cultivar specificity. Parsimony analysis of 299 informative AFLP characters was terminated with 500,000 equally

parsimonious trees. The consensus tree (figure 2.3) contains three main genotype clusters separated by long branches with strong parsimony bootstrap support: one clade with *Escovopsis* isolates from only *C. muelleri* colonies; a second clade primarily comprised of *C. muelleri* and *C. costatus Escovopsis* isolates; and a third clade with mostly *C. longiscapus* isolates. Of the eight isolates from *C. longiscapus* colonies that were included in both the AFLP and sequence studies, all fell within the single '*longiscapus*-type' genotype cluster in the AFLP parsimony consensus tree (bottom right clade, figure 2.3), and of the 16 *C. muelleri* and *C. costatus* isolates included in both studies, all fell within a single AFLP genotype cluster (top right clade, figure 2.3). Thus, the AFLP study, which included more samples, revealed an entire clade of '*muelleri*-specific' *Escovopsis* (top left clade, figure 2.3) not apparent in the more sample-limited sequencing analysis.

Single *Cyphomyrmex* gardens are occasionally infected by multiple *Escovopsis* strains. In the 22 cases in which we were able to genotype multiple *Escovopsis* isolates from the same colony, there were three instances where isolates from a single colony fell into unambiguously distinct genotype clusters, indicating infection by multiple parasite genotypes. In the remaining 19 instances where multiple samples from a single garden were genotyped, the AFLP profile differences were minor (*e.g.,* < 3% of bands differed). Because small AFLP profile differences may be artifacts rather than actual genotypic differences, these 19 colonies were conservatively assumed to have a single infection.

AMOVA analysis of AFLP data revealed a significant proportion of the variation (22%) due to between-host differences. This is lower than the amount of variation explained by between-host differences using sequence information (70%). This disparity may be because AFLP markers evolve at a more rapid rate than sequences or because mutation in AFLP markers is likely to result in autapomorphies that would increase the extent of within-host variation. Despite this, comparison of between-host pairwise F_{ST} values showed the same pattern as sequence data analysis, with more similar *Escovopsis* genotypes infecting similar cultivars (table 1*b*). Thus, both parsimony and AMOVA analysis of the AFLP data suggest *Escovopsis* is cultivar-type specific.

AFLP analyses revealed two parasite isolates from *C. muelleri* colonies that were more similar to isolates from *C. longiscapus* colonies than they were to other *Escovopsis* from *C. muelleri* colonies. Similarly, two isolates from *C. longiscapus* colonies were more similar to isolates from *C. muelleri* and *C. costatus* colonies than they were to other *Escovopsis* from *C. longiscapus* colonies (figure 2.3). These isolates associated with "atypical" hosts represent only 3% of all isolates, but they do indicate that *Escovopsis* can occasionally be associated with atypical hosts. Because we kept colonies separated from one another prior to isolation, these samples associated with atypical hosts are not likely due to post-collection laboratory cross-infection, although this cannot be ruled out entirely. It is interesting to note that one of the '*longiscapus*-type' *Escovopsis* samples from a *C. muelleri* colony was isolated in a colony that was only 3cm away from a *C. longiscapus* colony in the field, suggesting that infection may occasionally spread to neighboring colonies even if the garden is of an atypical cultivar-host type.

Cross-Infection Experiments. We found that *Escovopsis* strains from colonies of the three ant species could infect and overgrow garden pieces from each colony type (figure 2.4). Overall, infection established more frequently on *C. muelleri* and *C. costatus* garden pieces (71% and 85%, respectively) than on *C. longiscapus* gardens pieces (36%), corresponding to lower levels of natural field infections in *C. longiscapus* colonies (see above).

Significant differences in infection establishment are evident across the three colony types (figure 2.4). Chi-square goodness-of-fit analysis indicated a significant interaction between garden-type, *Escovopsis*-type and infection establishment (Pearson chi-square=30.56, df =4, $p < 0.0001$). Analyzing infection in each garden type separately, there was a significant interaction effect between *Escovopsis*-type and infection establishment on both *C. muelleri* (Pearson's chi-square=22.11, df=2, $p<0.0001$) and *C. costatus* gardens (Pearson's chi-square with Yate's continuity correction=8.2174, df=2, p=0.016). For both *C. muelleri* and *C. costatus* gardens, infection establishment was equally likely when inoculated with *Escovopsis* isolates from *C. muelleri* and *C. costatus*

colonies (for *C. muelleri* gardens, chi-square=0.22, df=1, p=0.64; for *C. costatus* gardens, chi-square=0.53, df=1, p=0.47) but was significantly less frequent when inoculated with *Escovopsis* from *C. longiscapus* colonies (for *C. muelleri* gardens, chi-square=19.44, df=1, p<0.0001; for *C. costatus* gardens, chi-square=5.30, df=1, p=0.02). For *C. longiscapus* colonies, a similar, host-specific pattern emerged where infection established more frequently when *C. longiscapus* gardens were inoculated with *Escovopsis* isolates from *C. longiscapus* colonies than from either *C. muelleri* or *C. costatus* colonies, though this result was not statistically significant at the p=0.05 level (Pearson chi-square=5.794, df=2, p=0.055).

2.4 DISCUSSION

The garden parasite *Escovopsis* is host specific, tracking the cultivar in the *Cyphomyrmex* fungus-growing ant system. We found that genotypically similar parasites attack similar cultivars raised by *C. muelleri* and *C. costatus*, whereas more genotypically distant parasites attack the cultivar raised by *C. longiscapus.* In cross-infection experiments, *Escovopsis* strains were more likely to establish infection on typical than on atypical fungal-host species, providing further evidence for host-species specificity.

Moreover, the congruence of cultivar and parasite phylogenetic relationships suggests possible further within-host specificity. Although *Escovopsis* of *C. muelleri* and *C. costatus* are more genetically and phenotypically similar to each other than to *Escovopsis* attacking *C. longiscapus* colonies, and although *C. muelleri* and *C. costatus* colonies are sometimes infected with identical *Escovopsis* strains, AMOVA did reveal significant differences between *Escovopsis* attacking *C. muelleri* and *C. costatus* colonies. Likewise, Green *et al.* (2002) showed that *C. muelleri* and *C. costatus* cultivars are occasionally genotypically identical, yet some cultivar strains are associated with only one of the two ant hosts. Analogous cultivar and *Escovopsis* population structures suggest that the parasite may closely track within-species host genotypes, possibly in a coevolutionary

arms race. Future analyses of cultivars and parasites isolated from the same colonies will determine the extent of parasite host-genotype specificity in the attine system.

What dictates *Escovopsis* specificity? While many parasites are habitat-restricted, either because they themselves can only survive in certain niches or because their vectors function only within certain niches (Norton & Carpenter 1998; Jaenike & Perlman 2002), such habitat specialization does not seem to be the case for *Escovopsis* in the *Cyphomyrmex* system. *Cyphomyrmex longiscapus* and *C. muelleri* colonies are found in similar habitat, are often located within centimeters of each other in the field, and have nearly the same garden architecture and size (figure 2.2a; Schultz *et al.* 2002). Yet, despite their close spatial proximity and relatively open nest architecture, *C. longiscapus* and *C. muelleri* colonies are consistently infected by different *Escovopsis* strains, suggesting that habitat does not constrain *Escovopsis*-host associations. If vector biology maintains *Escovopsis* specificity, the vector itself would have to be cultivar- rather than habitat-specific. Though vector-driven specificity seems somewhat unlikely in the *Cyphomyrmex* system, it is a possibility, and further natural-history observations and experimentation are needed to determine the mechanism by which *Escovopsis* is horizontally transmitted.

Instead, *Escovopsis* specificity is likely due to parasite and host adaptation. For example, parasites may be adapted to efficiently locate and utilize the resources of particular hosts. In localizing hosts, *Escovopsis* is attracted to chemical signals produced by host cultivars (chapters 4, 5). This attraction may allow *Escovopsis* to travel efficiently between neighboring colonies or within infected colonies in order to reach appropriate host cultivar. If *Escovopsis* is adapted to recognize chemical signals produced by specific cultivar types, host-seeking limit *Escovopsis*' to finding a narrow range of chemically similar cultivars. However, when experimentally forced into contact with cultivars from all three *Cyphomyrmex* hosts, *Escovopsis* strains were often unable to infect garden pieces, particularly of atypical hosts. This suggests that even if *Escovopsis* could efficiently seek a wide range of hosts, it may not be able to exploit all hosts. This may be

because *Escovopsis* is adapted to only use certain hosts as a nutritional resource. However, *Escovopsis* strains isolated from all three host types could sometimes successfully infect all three garden types, demonstrating that certain *Escovopsis* isolates were able to consume all host gardens types. Parasite host-seeking and host-use (figure 2.1) are therefore likely coupled with other factors, such as host defense, in maintaining *Escovopsis* specificity.

When potentially virulent infections are common, hosts are selected to adapt defenses targeted against their parasites and parasites are then selected to overcome their host's novel defenses. This perpetual race to adapt is a central theme in host-parasite biology and modern medical evolutionary genetics. In the *Cyphomyrmex* system, we see that natural infection is common, and *Escovopsis* has previously been shown to decrease colony fitness and survival (Currie *et al.* 1999a; Currie 2001b). Thus, tightly coupled host-parasite coevolution is expected. Consistent with this expectation, infection was more likely to establish in cross-infection experiments when hosts were inoculated with parasites isolated from a closely related host rather than from a distantly related host, suggesting that *Escovopsis* strains are adapted to overcome defenses of a limited range of host gardens. Because these gardens are a complex matrix composed of cultivar, soil fungi, endophytic fungi, antibiotic compounds produced by ants, forage material and possibly even remnants of the actinomycete bacteria from the ants' cuticles, further work is needed to determine the precise mechanism by which the host garden defends against *Escovopsis* attack.

None of the three experimentally-infected host types could defend against all atypical parasite strains. This may explain the rare atypical infection seen in nature, where 3% of colonies were infected by a parasite strain with which that host was not normally associated (figure 2.3). All of these atypical infections were in colonies infected with other typical strains, suggesting that, as previously hypothesized (May & Nowak 1995; Read & Taylor 2001), host susceptibility may be affected by the presence of multiple parasites. Further work examining host-parasite genotype interactions and multiple

infection dynamics may explain under what circumstances such atypical infections are able to establish and persist.

Interestingly, *C. longiscapus* gardens were less susceptible to experimental infection and had lower natural infection rates as compared to *C. muelleri* and *C. costatus*, suggesting that some component of the garden matrix is better adapted to inhibiting *Escovopsis* in *C. longiscapus* than in *C. muelleri* and *C. costatus* colonies. The question then arises as to why *C. longiscapus* gardens might maintain higher resistance. Potential explanations include that (a) *Escovopsis* specialized on *C. longiscapus* are more virulent and thus exert greater selective pressure to maintain resistance in cultivars; (b) *C. muelleri* and *C. costatus* gardens are released from maintaining high resistance because of other, effective colony defenses (*e.g.,* actinomycete defenses; see below); or (c) the three cultivar hosts are simply at different stages of the host-parasite arms race cycle.

What other colony defenses could mediate parasite host range? The ants are known to weed and groom *Escovopsis*-infected gardens, contributing to disease suppression (Currie & Stuart 2001). If these ant behaviors are *Escovopsis*-type specific, they could influence *Escovopsis* host range. Additionally, coevolution between actinomycete-produced antibiotics known to specifically suppress *Escovopsis* and antibiotic resistance in *Escovopsis* could play a critical role in shaping *Escovopsis* specificity. Further work is needed to test for behavior- and antibiotic-driven coevolution. Such complexity highlights the novelty of this system, in which three mutualistic symbionts (ants, cultivar, and actinomycete bacteria) are all negatively impacted by the same ubiquitous parasite and thus are expected to simultaneously coevolve adaptations to combat *Escovopsis*. The ease with which these symbionts can be experimentally manipulated and genotyped makes the fungus-growing ant-microbe system ideal for future experimental work on ecological and evolutionary host-parasite dynamics.

AMOVA results	**sequence data *(a)***			**AFLP data *(b)***		
	variance	d.f.	% total	variance	d.f.	% total
Between Hosts	16.61	2	70.46	6.61	2	22.37
Within Hosts	6.96	30	29.54	22.96	39	77.63
	overall F_{st} = 0.70, p<.01			overall F_{st} = 0.22, p<.01		
between-host pairwise comparisons						
	Pairwise F_{ST}			Pairwise F_{ST}		
longiscapus & *muelleri*	0.77			0.24		
longiscapus & *costatus*	0.90			0.35		
muelleri & *costatus*	0.21			0.11		

Table 2.1: AMOVA results and population pairwise comparisons based on sequence and AFLP data. Overall Fst values indicate the proportion of variation seen in sequence data (a) and AFLP data (b) that is attributable to parasite genotype differences between the three hosts. Pairwise comparisons are between *Escovopsis* isolated from host gardens of the three ant species *C. longiscapus*, *C. muelleri* and *C. costatus*. All p-values were calculated by permuting genotypes among samples (100,000 permutations). All p-values for pairwise comparisons are <0.0001.

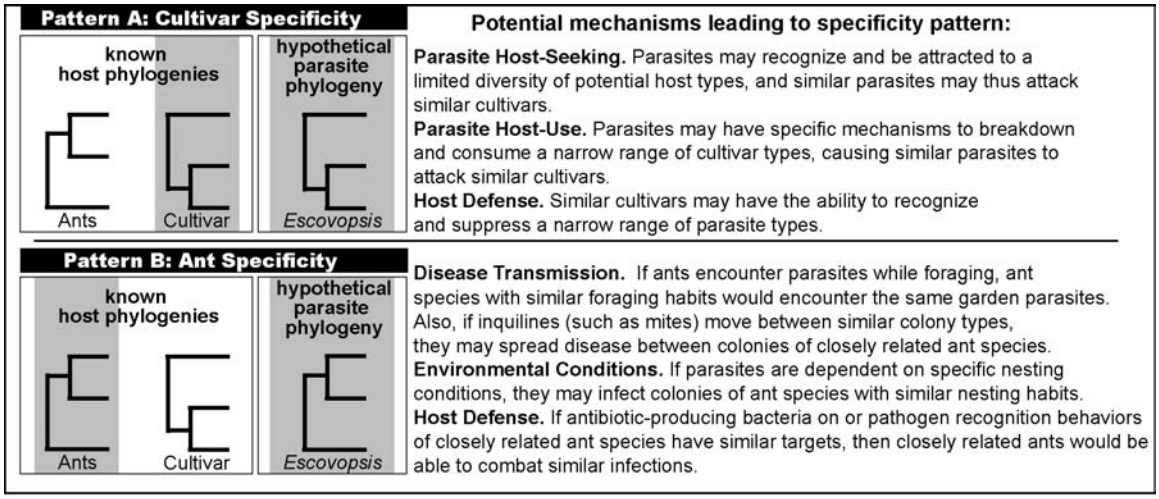

Figure 2.1: Topological relationships between phylogenies predicted by two alternative hypotheses of parasite specialization. *Escovopsis* could be specific to the ant species in whose garden it is found (Pattern A; congruent parasite and ant phylogenies), or *Escovopsis* could be specific to the cultivar that it attacks (Pattern B; congruent parasite and cultivar phylogenies). Gray boxes enclose congruent host and parasite phylogenies in each case. Several mechanisms known to operate in other host-parasite systems are listed on the right, and each mechanism alone could lead to the respective pattern of specificity.

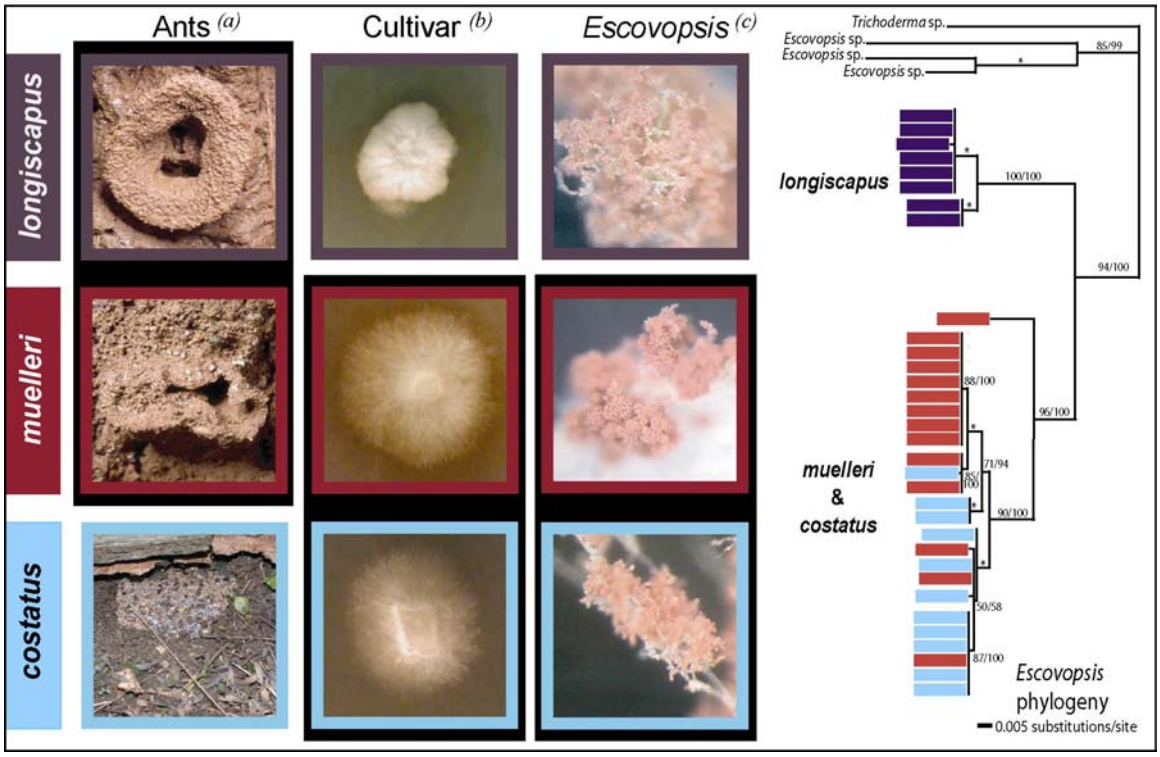

Figure 2.2: Relationships between the symbionts in the *Cyphomyrme*x system. *(a). Cyphomyrmex longiscapus* and *C. muelleri* are closely related ant species with similar nest architecture (nests in black box) while *C. costatus* is a more distantly related ant species with larger colonies. *(b). Cyphomyrmex longiscapus* grows a distantly related, morphologically distinct cultivar to that of *C. muelleri* and *C. costatus*, whose cultivars (linked in black box) are morphologically and genetically similar. *(c). Escovopsis* isolates from all three species are morphologically similar. EF-1 sequence analysis indicates that *Escovopsis* isolates from *C. muelleri* (red) and *C. costatus* (light blue) colonies are more similar to one another than they are to *Escovopsis* isolates from *C. longiscapus* (purple) colonies. Support values are listed above branches (likelihood support/Bayesian posterior probability) for branches with >50% likelihood support. An asterisk indicates branches for which both support values are greater than 95.

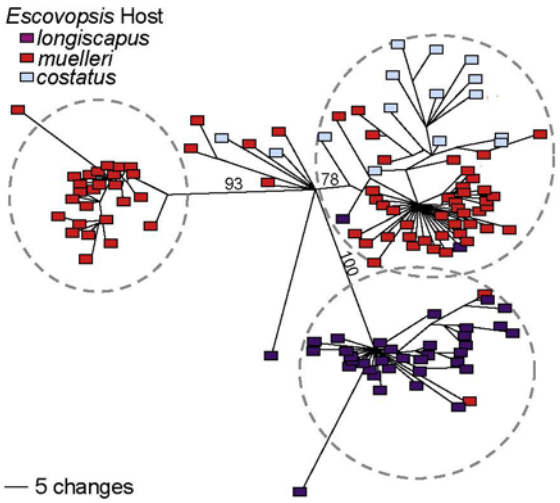

Figure 2.3: AFLP phylogeny of *Escovopsis* isolates from the three host species. Unrooted, strict consensus phylogram based on AFLP data generated through parsimony analysis. Support values are indicated on branches separating three main genotype clusters (identified by dashed circles). One genotype cluster is composed of only *Escovopsis* isolates from *C. muelleri* (red) colonies, a second genotype cluster is composed mostly of isolates from *C. muelleri* and *C. costatus* (light blue) colonies, and a third cluster is composed mostly of isolates from *C. longiscapus* (purple) colonies.

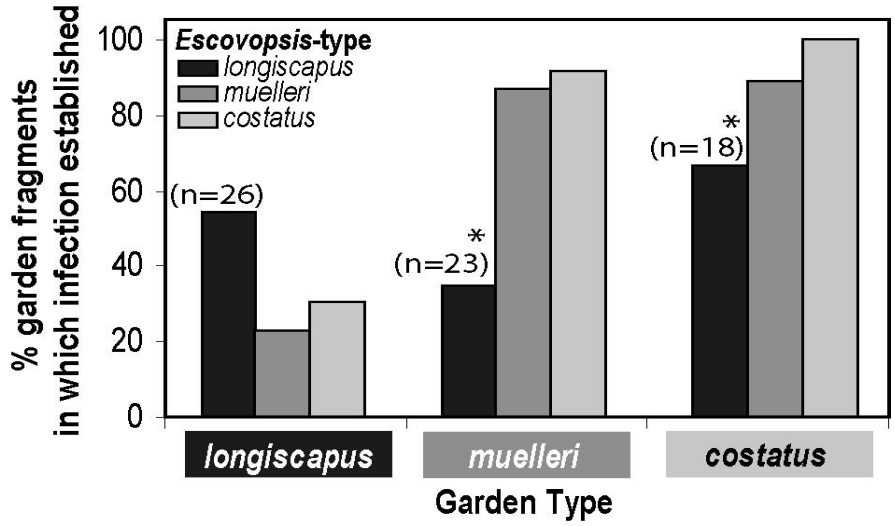

Figure 2.4: *Escovopsis* infection rates in cross-infection experiments. Garden pieces from presumably uninfected *C. longiscapus, C. muelleri* and *C. costatus* colonies were inoculated with either *Escovopsis* isolated from a *C. longiscapus* colony (black), from a *C. muelleri* colony (dark gray) or from a *C. costatus* colony (light gray). The graph indicates the percentage of pieces of a given garden type in which a particular *Escovopsis* type succeeded at establishing infection. On *C. muelleri* and *C. costatus* garden types, ** indicates that infection was significantly less likely to establish with *Escovopsis* from *C. longiscapus* colonies ($p<0.05$). On *C. longiscapus* gardens, * indicates *Escovopsis* from *C. longiscapus* colonies established infection more often than the other *Escovopsis* types, though this difference was not significant at the $p<.05$ level ($p=0.055$).

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Chapter 3

Seeking susceptible host, parasite attraction to cultivated fungi of ants

Abstract: Hosts must adapt to defend against parasites, and parasites must counter-adapt to overcome host defenses. Over evolutionary time this arms race process of host-parasite coevolution can lead to parasite specialization on a narrow range of susceptible hosts. This process has been observed in both natural and experimental populations (Dybdahl & Lively 1998; Buckling & Rainey 2002), and it is postulated to underlie congruence of host and parasite phylogenies (Page 2003). Host-specificity emerges at two levels: at the broad scale, parasite species will be specialized on particular host species (Clayton *et al.* 2004); at finer scales, parasite genotypes of the same species will successfully attack a narrow range of host genotypes (Carius *et al.* 2001). Whereas each of the two specificities has been shown separately for many systems, there are no documented cases where both have been shown to function in the same host-parasite system. I here show such two-tiered specialization functioning in the interaction between the cultivated fungi of fungus-growing ants and *Escovopsis*, a virulent parasite that attacks the ants' fungal cultivars. First, host cultivars can chemically defend themselves against some *Escovopsis* spp., but *Escovopsis* spp. cannot only overcome the defenses of host species on which they are specialized but can efficiently recognize and be attracted to these susceptible hosts. Second, these same adaptive host defense and parasite host-recognition phenotypes are highly variable across within-species host and parasite genotypes, and genetically similar parasites strains are more likely to successfully infect genetically similar cultivar strains. Therefore, switching to a novel host is dependent upon a parasite's genotype and what host genotypes it encounters.

3.1 INTRODUCTION

Fungus-growing ants have coevolved for at least 50 million years with the fungi that they cultivate as their primary food source (Wilson *et al.* 1971; Mueller *et al.* 1998). The virulent parasite *Escovopsis* is a diverse genus of fungi that attacks and consumes the ants' fungal cultivars (Currie *et al.* 1999a). Colonies experimentally infected with *Escovopsis* have decreased worker production and colony survival (Currie 2001). Different *Escovopsis* morphotypes infect colonies of different fungus-growing ant species, and these morphotypes correspond to monophyletic clades that are cultivar-host species specific, leading to long-term coevolution and cocladogenesis of the ants, their cultivars, and *Escovopsis* (Currie *et al.* 2003; Gerardo *et al.* 2004).

There are three main constraints on whether a parasite can utilize a given host: 1) co-occurrence, whether a parasite shares the habitat of a potential host, 2) establishment, whether a parasite can find a host and then overcome the host's initial defenses, and 3) persistence, whether a parasite can then maintain association and utilize the host as resource despite host defenses. Because these constraints successively narrow a parasite's host range, they can lead to the high degree of parasite specificity seen in the fungus-growing ant system.

In the case of *Escovopsis*, colonies within centimeters of each other in the field will be infected by different *Escovopsis* spp. if those colonies propagate different cultivar species; *Escovopsis* spp. are cultivar-specific (chapter 2; Gerardo *et al.* 2004). Thus, though habitat may play some role in constraining the host range of *Escovopsis*, it is not the only factor, and processes of parasite establishment and persistence are likely to a play a large role in dictating *Escovopsis* specificity. These processes are largely shaped by parasite adaptations to find and utilize hosts and by host adaptations to defend against parasites.

3.2 RESULTS AND DISCUSSION

I discovered that *Escovopsis* is attracted to chemical signatures produced by host cultivars. If this attraction is host signature specific, the parasite could more efficiently infect some hosts versus others. In testing for the specificity of the attraction response, using *Escovopsis* isolated from colonies of *Apterostigma dentigerum* ants, I found that in most trials, like the one depicted in fig. 3.1, *Escovopsis* isolates were attracted to both strains of their natural host (cultivar A) and of closely-related cultivars (cultivar B), arriving more rapidly at the ends of these tracks than the control track in most trials (fig. 3.2). Furthermore, most parasites isolates were more rapidly attracted to their host cultivar A than to the related cultivar B (fig. 3.2). This supports the hypothesis of fine-tuned parasite attraction to host species signals. Though I expected that *Escovopsis* isolates would also be attracted to cultivar C, the cultivar distantly related to their natural host, I instead found that in 12 of 17 trials, *Escovopsis* was not attracted to but was inhibited by these cultivars. Even after several months, a zone of inhibition surrounded these cultivars, and *Escovopsis* could not establish infection (fig. 3.1iv). In the five trials in which *Escovopsis* did overgrow the cultivar, there was no evidence for attraction (Wilcoxon rank sum test, control vs. cultivar C, $V = 10$, $p = 0.59$).

Thus, I see a clear case in which parasite efficiency and host defense are coupled to shape parasite host range at the species level. Though the mechanism by which *Escovopsis* is transmitted is not known, one can suppose that if spores of *Escovopsis* are transmitted to a host with which they are typically not associated, the parasite would not be able to overcome the host's defenses. However, upon getting in proximity of a garden with the appropriate cultivar hosts, *Escovopsis* would be able to quickly spread through the garden matrix as it is attracted to portions of the garden with the fungal cultivar. This process would make infection establishment more rapid and may prevent the successful suppression of the parasite by the ants, which have behaviors specific to the removal of *Escovopsis* (Currie & Stuart 2001), and by actinomycete bacteria on the ants, which are known to target and inhibit *Escovopsis* growth (Currie *et al.* 1999b). Such successful

infection of a limited range of hosts is consistent with patterns of long-term coevolution and potential cospeciation of *Escovopsis* parasites and their cultivar hosts, where tight association between particular *Escovopsis* spp. and cultivar clades is maintained (Currie *et al.* 2003).

Though these results clearly indicate that *Escovopsis* is attracted to its natural host and to closely-related cultivar species but is inhibited by atypical host species, there were a few interactions not consistent with this pattern. Specifically, in 5 of 17 trials, the isolates of the typically inhibiting host cultivar C were susceptible to *Escovopsis*. This suggests that under some conditions, *Escovopsis* spp. may be able to successfully switch to novel hosts distantly related to their typical hosts. Such host-switching, if frequent enough, can dramatically impact patterns of cospeciation (Page 2003; Sorenson *et al.* 2004) and coevolutionary dynamics of host-parasite interactions (Antonovics *et al.* 2002).

Because host-parasite coevolution is dependent upon heritable genetic variation in host susceptibility and parasite virulence, I hypothesized that whether *Escovopsis* is able to infect and thus switch to an atypical host (cultivar C) is a consequence of genetic variation in host-susceptibility and parasite-efficiency. In order to examine this, I first paired each of ten *Escovopsis* isolates with ten isolates of cultivar C in a reciprocal cross-inoculation bioassay experiment to look for variation in both host susceptibility and parasite efficiency. I found a wide degree of variation in both. Though some cultivars were able to inhibit all ten *Escovopsis* isolates (gray cells in fig. 3.3), others were susceptible to two to four of the *Escovopsis* isolates (white cells in fig. 3.3), and though some *Escovopsis* isolates were inhibited by all cultivars, some were able to successfully attack up to four of the ten cultivar isolates. Similarly, though parasite attraction to these cultivars was rare, there was also variation in attraction (represented by 'A' in fig. 3.3). In fact, some *Escovopsis* isolates were occasionally attracted to asusceptible hosts (represented by an 'A' in a gray cell in fig. 3.3), suggesting occasional suboptimal host-seeking behavior.

Many theories at the foundation of our current understanding of host-parasite interactions postulate that genotypic interactions drive coevolutionary dynamics (Anderson & May 1982; May & Anderson 1983; Frank 1992; Agrawal & Lively 2002). Each parasite genotype becomes adapted to successfully attack only a narrow range of host genotypes. If this is true, genetically similar parasites (versus genetically dissimilar parasites) might be more likely to successfully attack the same hosts, and genetically similar hosts would be more likely to defend against the same parasites. To test for this, I genotyped the *Escovopsis* and cultivar isolates used in the cross-inoculation bioassays and tested for a correlation between 1) cultivar genotype and cultivar inhibition profile and 2) *Escovopsis* genotype and *Escovopsis* inhibition profile. I found correlations in both cases. Cultivars that are more genetically similar are more likely to inhibit the same *Escovopsis* isolates (Mantel test: $r = 0.43$, $p = 0.04$) and genetically similar *Escovopsis* isolates are able to successfully attack a similar subset of cultivars ($r = 0.35$, $p < 0.01$). Thus, the establishment of infection by *Escovopsis* on these atypical hosts is constrained by what host genotypes a parasite of a given genotype encounters . In the event that *Escovopsis* encounters a susceptible host, it must then spread through the garden to establish a persistent infection. Because these parasites were rarely attracted to susceptible, novel cultivars (attraction without inhibition occurred in only four of the 100 cross-inoculation bioassays), the likelihood of successful establishment by these parasites on these cultivars may be even less likely, and thus host-switching is improbable. This, coupled with *Escovopsis*' preferential attraction to a narrow range of typical hosts, as indicated by the fungal choice bioassays, likely explains the extreme degree of phylogenetic congruence seen in the fungus growing ant-microbe symbiosis.

3.3 METHODS

Collections. All fungi were cultured from *Apterostigma* spp. colonies in Panama and Costa Rica following procedures in Gerardo *et al.* 2004. All *Escovopsis* isolates were from *A. dentigerum* colonies and were of the same yellow morphotype. All cultivar A

isolates were isolated from *A. dentigerum* colonies; these cultivars fall into the 'G2-clade' in Villesen *et al.* 2004. All cultivar B isolates were isolated from *A. cf. manni* colonies; these cultivars fall in the 'G4-clade' in Villesen *et al.* 2004. All cultivar C isolates were from *A. auriculatum* colonies; these cultivars fall in the 'G3-clade' in Chapela *et al.* 1994 and 'Clade-1' in Mueller *et al.* 1998.

Fungal-choice bioassays. Agar in seventeen 14cm Petri dishes filled with 50ml of PDA + antibiotics (Potato Dextrose Agar with 50mg/L each of penicillin and streptomycin) was cut out agar to leave four 4cm-wide tracks. For each plate, each track was then randomly assigned to each of one of four treatments: control (no cultivar), cultivar A, cultivar B or cultivar C. One of eight cultivar A isolates, one of eight cultivar B isolates, and one of four cultivar C isolates was randomly assigned to each plate. Plates were inoculated with ~6mm^3 agar pieces covered with mycelium from cultures of the appropriate cultivar isolate. After one week, the plates were inoculated with a ~6mm^3 agar piece with spores and mycelium of one of twelve randomly assigned *Escovopsis* isolates. Plates were photographed typically every two days. I recorded the number of days (#days) that it took *Escovopsis* to reach the end of each track. I used a random effects analysis of variance (PROC MIXED, SAS Institute Incorporated 1992) to compare #days (log-transformed) to reach cultivars A and B and the end of the control track, treating the plate and the *Escovopsis* strain as random effects and the cultivar as a fixed effect. I used log-likelihood ratio tests to confirm that there was no effect of the random variables and conducted pairwise, Bonferroni-corrected comparisons of the least-squared means of the treatments (cultivar A, cultivar B, control). Because cultivar C inhibited *Escovopsis* growth on 12 of the 17 plates, it was not included in the analysis. Instead, for the five trials in which cultivar C was overgrown, I used a Wilcoxan rank sum test to compare # days to control vs. to cultivar C.

Cross-inoculation bioassays. For each bioassay, I placed a single isolate of fungal cultivar near the edge of a 9cm Petri dish with PDA + antibiotics. After one week, I inoculated the plates with a single *Escovopsis* isolate. The plates were monitored for up

to two months. Interactions were scored for presence/absence of inhibition and presence/absence of attraction. I conducted bioassays for all 100 possible combinations of ten *Escovopsis* and ten cultivar C isolates.

Within-species genetic variation. I used amplified fragment length polymorphisms (AFLPs) to generate neutral genotype fingerprints of cultivar samples used in the reciprocal cross-inoculation study. AFLP analysis followed the protocol outlined in Gerardo *et al.* 2004. For the parasites, to obtain fingerprints of the same level of genetic variability, I sequenced 552 basepairs of elongation factor 1-alpha (ef-1 α) following the protocol in Gerardo *et al.* 2004. Based on these data, I constructed two genetic distances matrices using PAUP* (ver4b10, Swofford 2002): a Nei-Li distance matrix for the ten experimental cultivar isolates and a maximum likelihood distance matrix for the ten experimental *Escovopsis* isolates. For the maximum likelihood distance I used the TrN + I + G model of evolution as determined via Modeltest (Ver 3.06, Posada & Crandall 1998)

Correlation of inhibition and genetic distances. I used ZT (Bonnet & Van de Peer 2002) to conduct Mantel tests to test the correlation between genetic distance (above) and interaction distance. Two interaction distance matrices were constructed. The first consisted of the inhibition distances between each pair of cultivars used in the cross-inoculation bioassays, where each inhibition distance ranged from 0 to 1 and increased 0.1 for each case in which the two cultivars had a different interaction with the same *Escovopsis* isolate. I constructed an *Escovopsis* inhibition matrix similarly; an *Escovopsis* pair had a greater inhibition distance for each of the ten interactions in which the isolates had a different interaction with the same cultivar isolate. In one Mantel test, I tested the correlation between the cultivar genetic distance matrix and the cultivar inhibition distance matrix. In a second Mantel test, I tested for a correlation between *Escovopsis* genetic distance and inhibition distance.

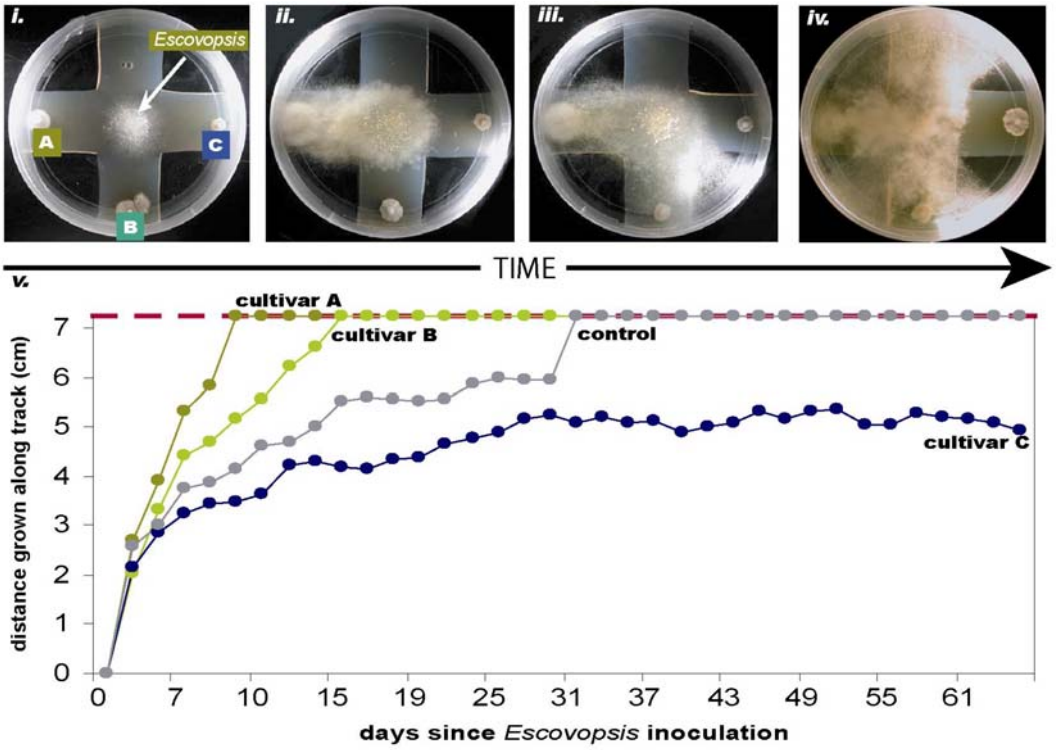

Figure 3.1: Time progression of fungal-choice bioassay. Cultivars A, B and C are placed at the end of each of three tracks and one track is left blank as a control. *Escovopsis* begins to grow concentrically *(i.),* but over time grows more rapidly to cultivar A *(ii.),* then to B *(iii.),* then to the end of the control track *(iv.).* After several months, the parasite has still not overcome the zone of inhibition surrounding cultivar C *(iv.).* The growth trajectory *(v.)* in each direction is, therefore, dramatically different, with *Escovopsis* quickly reaching the track ends (indicated by red dashed line) with cultivars A and B. Distance grown was measured along the center of each track from photographs using Image J (ver 1.24, NIH).

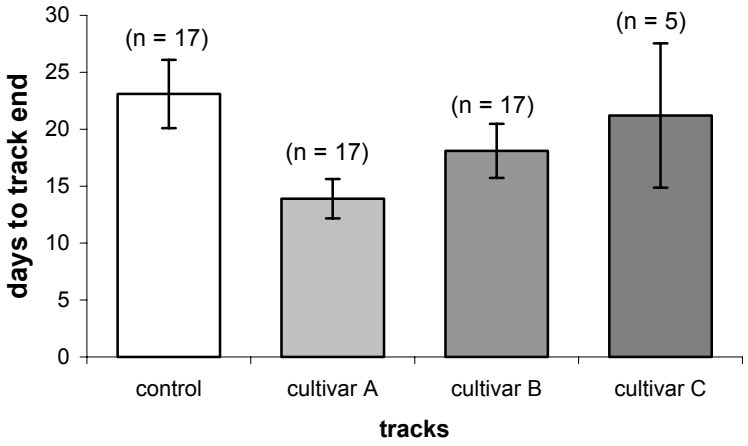

Figure 3.2: Results of fungal-choice bioassays. Attraction to both cultivar A and B is indicated by significantly faster growth to the ends of the tracks with these cultivars as compared to the end of control tracks (PROC MIXED lsmeans: cultivar A vs. control, $p < 0.0001$; cultivar B vs. control, $p = 0.02$). More rapid attraction to natural hosts is represented by significantly more rapid growth to the end of cultivar A tracks than to the end of cultivar B tracks (cultivar A vs. cultivar B, $p = 0.02$). Data for cultivar C is based on the five (of 17) trials in which the parasite successfully reached the typically-inhibiting cultivar. Error bars represent s.e.

		cultivar isolates									
		C1	C2	C3	C4	C5	C6	C7	C8	C9	C10
Escovopsis isolates	E1			A		A	A		A		
	E2										
	E3				A						
	E4					A	A	A			
	E5										
	E6										
	E7		A	A	A	A	A	A	A		A
	E8										
	E9										
	E10										

Figure 3.3: Reciprocal cross-inoculation bioassays. In the 100 cross-inoculation bioassays, the pattern of inhibition varied widely between both parasite and host isolates. Gray-shading represents bioassays in which the cultivar isolate successfully inhibited the parasite isolate, and white represents cases in which the parasite was capable of overcoming defenses and consuming the host. Patterns of attraction varied across both hosts and parasites as well. Cells with 'A' represent trials in which the parasite was attracted to the cultivar isolate.

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Chapter 4

Microevolutionary host-parasite adaptation explains macroevolutionary pattern in the attine ant-microbe symbiosis

Abstract: In chapter 3, I demonstrated that two key adaptations, host defense against parasites and parasite attraction to susceptible hosts, maintain host-specificity of *Escovopsis*, a fungal pathogen that attacks and consumes the cultivated fungi of fungus-growing (attine) ants. Here, I show that *Escovopsis* spp. that exhibit preferential attraction to a narrow host range are less likely to switch hosts than *Escovopsis* spp. that are less specifically attracted to a broader array of hosts. Host-switching by these broadly attracted parasites is still limited by their inability to switch to distantly-related hosts because of host defenses. This leads to a pattern of congruence of host and parasite phylogenies at the broad scale with incongruence due to host-switching at finer scales and suggests that mircoevolutionary adaptive host-parasite dynamics may dictate macroevolutionary patterns of host-parasite association.

4.1 INTRODUCTION

Most parasites are intimately dependent on one or a few hosts. Because of this host fidelity, parasites are expected to track speciating hosts by speciating themselves. This process, known as cospeciation, will lead to cocladogenesis, the matching of symbiont phylogenies. Parasite and host phylogenies are rarely identical, however; forces such as duplication (parasite speciation in the absence of host speciation), sorting events (host speciation without commensurate parasite speciation), and host-switching (parasites begin to use a new host) (Johnson *et al.* 2003; Page 2003) cause their discordance. Despite these complications, congruent symbiont phylogenies are known in host-parasite associations (Hafner *et al.* 1994; Johnson & Clayton 2003; Sorenson *et al.* 2004) and in host-mutualist associations (Herre *et al.* 1996; Itino *et al.* 2001) as well.

The fungus-growing ant symbiosis is a unique case in which, within a single system, cocladogenesis occurs between both mutualists and parasites. Chapela *et al.* (1994) demonstrated congruence of the phylogenies of fungus-growing ants and the fungi that they cultivate as their primary food source. Subsequently, Currie *et al.* (2003) showed that the phylogeny of *Escovopsis*, parasites that attack the ants' fungal cultivars, matches that of the ants' cultivars and consequently that of the ants themselves. The phylogenies, however, are not identical, and Gerardo *et al.* (2004), demonstrated that the parasite *Escovopsis* more closely tracks the cultivars, which it attacks and consumes, than the ants, which maintain the attacked fungal garden and attempt to combat infection.

Macroevolutionary patterns of cocladogenesis, as seen between *Escovopsis* and its hosts, imply that there is some ecological or mechanistic constraint that maintains host fidelity, limiting host-range expansion and host-switching. For example, in some host-parasite systems (e.g. chewing lice of birds and mammals; Clayton *et al.* 2004), parasites with limited dispersal do not come into contact with other hosts, and thus do not switch. In

other systems, microevolutionary adaptive processes increase the success of parasites on their typical host, but parasites switching to novel hosts are unsuccessful and perish. For example, parasite adaptations to efficiently utilize certain hosts can prevent switching to others. This is evident in fish ectoparasites, which have attachment organs with anchors that are adapted to allow for long-term attachment to a host of specific body size, thus limiting their ability to persist on hosts of a different size (Simková *et al.* 2001). Similarly, a parasite's adaptation to overcome one host's defenses may limit its ability to avoid defenses of other hosts. For example, brood parasites have adaptive egg coloration, which mimics that of the host, in order to avoid detection (Langmore *et al.* 2003). This limits the parasites from switching to hosts with eggs of other colors.

There are at least two highly specific parasite and host adaptations that may limit *Escovopsis*' ability to switch hosts. In chapter 3, using an *Escovopsis* sp. commonly isolated from colonies of fungus-growing ants, I demonstrated that *Escovopsis* is attracted to chemical signals produced by cultivars and that this attraction is highly specific; the parasite more rapidly grows towards its own host cultivar than to other cultivar types. Additionally, this preferential attraction to a narrow range of hosts is coupled with inhibition by novel hosts; cultivars with which the *Escovopsis* sp. is not naturally associated can defend against overgrowth by the parasite. This may explain why this particular *Escovopsis* sp. only infects a single monophyletic clade of cultivars in nature. It is yet unclear whether other *Escovopsis* spp. also have narrow recognition and defense responses. If some *Escovopsis* spp. are attracted to a broader array of host signals, or if some *Escovopsis* spp. can overcome the defenses of a broader array of hosts, these parasites may be more likely to switch to novel hosts, which would in turn lead to phylogenetic incongruence.

In order to investigate the association between microevolutionary adaptive processes and macroevolutionary patterns of phylogenetic congruence, I examine here the phylogenetic relationships between *Escovopsis* isolated from colonies of attine ants in the genus *Apterostigma*. I first reconstruct the phylogenetic relationships amongst some of the most

common *Apterostigma*-associated *Escovopsis* types in order to determine the prevalence of host-fidelity and host-switching. I then examine how results of *ex vivo* interactions suggest that *Escovopsis'* attraction to host signals and the cultivars' defense against *Escovopsis* jointly explain patterns of both parasite host-specificity and phylogenetic discordance in the fungus-growing ant symbiosis.

4.2 STUDY SYSTEM

***Apterostigma* ants and their associated microbes.** In ants, the ability to cultivate fungi for food arose only once, about 50-60 million years ago, and gave rise to roughly 200 known extant species of fungus-growing ants (Tribe Attini). Attine ants are dependent on fungal cultivation; their brood is raised on an exclusively fungal diet. As far as is known, each ant species raises a unique, narrow range of cultivars (fungi), most of which are in the family Lepiotaceae. There has been, however, one switch to a distantly related cultivar; most ants in the genus *Apterostigma* now cultivate fungi in the family Pterulaceae (Muncaksi *et al.* 2004), which is distantly related to the Lepiotaceae. These pterulaceous cultivars fall into two monophyletic, morphologically distinct cultivar clades ('G2-cultivars' and 'G4-cultivars' as identified in Villesen *et al.* 2004). Hereafter, I will refer to these pterulaceous cultivar clades and the colonies that contain them as 'clade-A' and 'clade-B' respectively. One *Apterostigma* species, *A. auriculatum,* has retained the ancestral state of growing lepiotaceaous cultivars ('G3-cultivars' as identified in Chapela *et al.* 1994). Hereafter, I will refer to this group of lepiotaceaous cultivars and the colonies that contain them as 'clade-C'. The inset in fig. 4.1 is a schematic representation of the relationship between these three clades.

Attine gardens are frequently infected by one genus of specialized, highly pathogenic fungi, *Escovopsis* (Ascomycota: Hypocreales), which attack and consume the ants' fungal cultivars (Currie *et al.* 1999a; Reynolds & Currie 2004). *Escovopsis* has only been found associated with nests of attine ants. Though infection rates vary across host species, infections are prevalent in colonies of many attine genera throughout their geographic

ranges (Currie *et al.* 1999a; Gerardo *et al.* 2004). Different morphotypes are isolated from colonies of different attine genera, and these morphotypes correspond to parasite clades associated with particular cultivar (host) clades (Currie *et al.* 2003).

There are three *Escovopsis* morphotypes, as identified by spore color, that are commonly isolated from *Apterostigma* colonies: 1) brown *Escovopsis*, which parasitizes both clade-A and clade-B cultivars, 2) yellow *Escovopsis*, which parasitizes clade-A cultivars and 3) pink *Escovopsis*, which parasitizes only clade-C cultivars. These morphotypes may represent different parasite species, though little is known about the reproductive isolation or life history of these fungi. For clarity, I occasionally refer to these *Escovopsis* morphotypes and their morphologically distinct host cultivars as 'species'.

Previous research has focused on biotic interactions between yellow *Escovopsis*, isolated from clade-A colonies, and *Apterostigma* cultivars (chapter 3). This study showed that yellow *Escovopsis* is attracted to chemical signals produced by both clade-A and clade-B cultivars, which cannot defend against yellow *Escovopsis*, but exhibits preferential attraction towards it natural host, clade-A cultivars. Furthermore, yellow *Escovopsis* is not attracted to but is inhibited by clade-C cultivars, which are distantly related to its typical host. It has yet to be shown whether preferential attraction and inhibition are seen in other *Escovopsis*-cultivar associations.

4.3 METHODS

Collections. From 2001-2004, there was an extensive survey and isolation of fungi, bacteria and ants from over 500 *Apterostigma* spp. colonies across Panama (PA), Costa Rica (CR), Ecuador (EC) and Argentina (AR). For the purpose of this study, based on field identification of the ants, garden architecture and growth form of the cultivar, each colony was classified as either a clade-A, clade-B or clade-C colony, which raise respectively clade-A, clade-B and clade-C cultivars (see description of study system).

All fungi were cultured following procedures of Gerardo *et al.* 2004. Experimental samples from Panama, Costa Rica and Argentina were maintained as live cultures on PDA + antibiotics (Potato Dextrose Agar with 50mg/L each of penicillin and streptomycin). For DNA extraction, spores and mycelium of *Escovopsis* isolates were directly frozen at -80 degrees, and mycelium of cultivar isolates was grown in liquid culture before freezing (Mueller *et al.* 1998). Fungal samples from Ecuador were only temporally maintained live after collection and were then stored inviable in 95% alcohol prior to export from the country. DNA extraction followed a CTAB extraction protocol modified from Bender *et al.* (1983).

Samples for phylogenetic reconstruction. To determine the relationship amongst *Escovopsis* strains isolated from *Apterostigma* spp. colonies, samples for phylogenetic reconstruction were selected to include the most commonly occurring *Escovopsis* morphotypes isolated from *Apterostigma* spp. colonies; these *Escovopsis* morphotypes represent approximately 95% of all *Escovopsis* isolates collected from *Apterostigma* spp. colonies. Because colonies with clade-A cultivars are much more commonly found and are more frequently infected with *Escovopsis,* I included more *Escovopsis* strains from clade-A (n = 39) than from clade-B (n = 5) or clade-C (n = 4) colonies. The country of origin of each sample is indicated on the phylogeny in fig. 4.1.

Sequencing targeted a 988 nucleotide stretch spanning 1 exon of nuclear elongation factor–1 alpha (EF-1 α) using primers EF1-983F, EF1-2218, EF1-6mf and EF1-6mr (Gerardo *et al.* 2004). In the final alignment, I included sequences from GenBank for *Trichoderma* sp., *Nectria cinnabarina*, *Pseudonectria rousseliana*, *Ophionectria trichospora*, *Hypomyces polyporinus*, *Sphaerostilbella berkeleyana*, *Aphysiostroma stercorarium*, *Hypocrea lutea*, and *Metarhizium anisopliae* (accession nos. AY629398, AF543774 and AF543779-AF543785) as outgroups. For simplicity, these outgroups are not presented in the phylogeny in fig. 4.1. All sequences were assembled in SeqMan II (ver 5.05, DNASTAR), aligned using Clustal W WWW (http://www.ebi.ac.uk/clustalw) and edited manually in MacClade (ver 4.06, Maddison & Maddison 2003).

Phylogenetic analyses and hypothesis testing. Parsimony analyses were performed in PAUP* (ver 4.0b10, Swofford 2002) using heuristic searches under parsimony with TBR branch swapping and 1000 random addition sequence replicates. In order to obtain estimates of clade support, non-parametric bootstrapping was performed with heuristic searches of 1000 replicate datasets and 50 random addition sequence replicates per dataset.

For maximum likelihood and Bayesian analyses, a model of sequence evolution was estimated for the data set using MODELTEST (Posada & Crandall 1998). The chosen model, TBR + I +G, was used for all maximum likelihood analyses and parametric hypothesis testing. Because it is not possible to set this model in Mr. Bayes, a more complex model of sequence evolution, GTR + I + G, was used in all Bayesian analyses.

For maximum likelihood analysis, I performed a successive approximation search using PAUP* to estimate the topology (Swofford *et al.* 1996). Starting parameter values estimated from a parsimony tree (TBR branch swapping, 100 random addition sequence replicates, multrees=no) were used in an initial maximum-likelihood search. Then parameters were re-estimated from the resulting tree and the search was repeated with these new parameters. This procedure was repeated until the resulting tree was identical in topology to that from the previous iteration.

For Bayesian analyses, using Mr. Bayes (ver 3.0b4, Huelsenbeck & Ronquist 2001), four separate Markov Chain Monte Carlo (MCMC) runs were performed starting from random trees for each of four simultaneous chains. Runs were two million generations with a burn-in of 100,000 generations, default prior distribution for model parameters, and the differential heating parameter set to 0.2. The joint posterior probabilities and parameter estimates of each run were congruent, suggesting the chains were run for a sufficient number of generations to adequately sample the posterior probability landscape.

Phylogenetic analysis with no topological constraints indicated two origins of clade-B *Escovopsis* (fig. 4.1). To test the hypothesis of monophyly of *Escovopsis* isolated from

clade-B colonies, I compared the observed, optimal tree (alternative hypothesis) to trees constrained to represent the null hypothesis of one origin of clade-B *Escovopsis*. Sequence evolution parameters were estimated by using maximum likelihood under the TBR + I + G Model. I used parametric bootstrapping procedures to evaluate 500 simulated datasets generated by using seq-gen 1.2.5. (Rambaut & Grassly 1997).

Cross-phylogeny infection bioassays. To look at patterns of host-parasite interaction across the *Apterostigma* symbiosis, I performed a cross-phylogeny bioassay experiment in which twelve *Escovopsis* strains from clade-A, clade-B and clade-C colonies were interacted with three strains of cultivar from each clade (clade A,B and C) for a total of nine cultivar strains. For this experiment, as well as for the fungal choice experiment described below, all clade-A cultivars were isolated from *A. dentigerum* colonies and all clade-B cultivars were isolated from *A. cf. manni* colonies. All clade-C cultivars were isolated from *A. auriculatum* colonies and fall in 'Clade-1' in Mueller *et al.* 1998. The experimental *Escovopsis* strains included three brown *Escovopsis* strains isolated from clade-A colonies, three yellow *Escovopsis* strains isolated from clade-A colonies, three brown *Escovopsis* strains isolated from clade-B colonies and three pink *Escovopsis* strains isolated from clade-C colonies. All experimental fungal samples were collected in the Republic of Panama.

In a fully factorial design, each of the 12 parasites strains was interacted with three strains of each of the three cultivar types (A, B and C) for a total of 108 bioassays. For each bioassay, I placed a single isolate of cultivar near the edge of a 9cm Petri dish with PDA + antibiotics. After one week, I inoculated the plates with a single *Escovopsis* isolate. The plates were monitored for one month. Interactions were scored for presence/absence of inhibition and presence/absence of attraction.

Fungal-choice bioassays. To determine the relative attraction of brown *Escovopsis* to cultivar strains from different clades, I conducted fungal 'choice' tests (fig. 4.2). Similar to choice experiments in behavioral biology, the fungal choice design allows an

Escovopsis isolate four directions in which to grow. In this study, strains of brown *Escovopsis* from clade-A colonies were presented with the following four tracks along which to grow: a control track with no cultivar at the end, a track with the parasite's natural host (clade-A cultivar), a track with a cultivar closely related to its natural host (clade-B cultivar) and a track with a cultivar distantly related to its natural host (clade-C cultivar). With this design, I can score the time that it takes for the parasite to reach the end of each track. If *Escovopsis* grows more rapidly towards the end of a track with a cultivar than to the end of the control track with no cultivar, it indicates that the parasite is attracted to that cultivar.

Agar in 30 14cm Petri dishes filled with 50ml of PDA + antibiotics was cut to leave four 4cm-wide tracks (fig. 4.2). For each plate, each track was then randomly assigned to one of four treatments: control (no cultivar), clade-A cultivar, clade-B cultivar or clade-C cultivar. One of eight clade-A cultivar isolates, one of two clade-B cultivar isolates, and one of six clade-C cultivar isolates was randomly assigned to each plate. The design was unbalanced due the limited number of clade-B cultivars that have been collected and successfully isolated. Plates were inoculated with ~6mm^3 agar pieces covered with mycelium from cultures of the appropriate cultivar isolate. After one week, the plates were inoculated with a ~6mm^3 agar piece with spores and mycelium of one of ten randomly assigned brown *Escovopsis* strains.

Starting five days after inoculation with *Escovopsis*, plates were photographed regularly (every 1-10 days depending on the stage of growth) to record the progress of growth. From photos, I determined the number of days (#days) that it took *Escovopsis* to reach the end of each track, and, for one trial, used ImageJ (ver 1.24, NIH) to measure the distance that *Escovopsis* had grown along the center of each track for each photographed day (fig. 4.2). Because the design was unbalanced, I used a random effects analysis of variance (PROC MIXED, SAS Institute Incorporated 1992) to compare #days (log-transformed) to reach the clade-A cultivar, the clade-B cultivar and the end of the control track, treating the plate and the *Escovopsis* strain within a treatment as random effects

and the cultivar as a fixed effect. Because the clade-C cultivars inhibited *Escovopsis* growth on 29 of the 30 plates, data on #days to the clade-C cultivars was not included in the PROC MIXED analysis. I used log-likelihood ratio tests to confirm that there was no effect of the random variables and conducted pairwise, Bonferroni-corrected comparisons of the least-squared means of the treatments (A, B, control).

4.4 RESULTS

Phylogenetics and hypothesis testing. The results of parsimony, likelihood and Bayesian analyses were highly concordant, and three well supported clades were identified that correspond to each of the three main *Apterostigma*-associated *Escovopsis* morphotypes: brown, yellow and pink (fig. 4.1). Similar to the relationship between the cultivars, in which clade-C cultivars are basal to the clade-A and clade-B cultivars, *Escovopsis* isolated from clade-C colonies appears basal to *Escovopsis* from clade-A and clade-B colonies, leading to some concordance of the host and parasite phylogenies at the broadest level. However, unlike the cultivar relationships, the clade-A and clade-B associated *Escovopsis* do not form separate, monophyletic clades. Yellow *Escovopsis*, only isolated from clade-A colonies, lies basal to brown *Escovopsis*, and within the brown *Escovopsis*, there are two origins of clade-B associated *Escovopsis*. Parametric-bootstrapping verified the polyphyly of clade-B *Escovopsis* isolates. The null hypothesis of a single origin of clade-B *Escovopsis* was rejected at $p < 0.001$. This implies that there has been at least one event in which clade-A associated *Escovopsis* has switched to a clade-B host.

Cross-phylogeny infection assays. Though there is variation between strains within each *Escovopsis* and cultivar type, an overall pattern emerged in which *Escovopsis* strains are generally attracted to their typical host cultivars and to cultivars closely related to their hosts (cultivars in the same fungal family as their hosts) but are inhibited by cultivars distantly related to their hosts (cultivars in a distant fungal family) (fig. 4.2). Both brown and yellow *Escovopsis* strains isolated from clade-A and clade-B colonies typically were

attracted to both clade-A and clade-B cultivars but inhibited by clade-C cultivars. Moreover, this pattern of attraction to typical hosts and inhibition by distantly-related, novel hosts was maintained in bioassays with *Escovopsis* isolated from clade-C colonies; pink *Escovopsis* strains from clade-C colonies were attracted to clade-C cultivars, their typical hosts, but inhibited by clade-A and clade-B cultivars, which are distantly related to their typical hosts. Cases of deviation from the overall pattern, suggesting within parasite-type variation in infectivity, include one yellow, clade-A *Escovopsis* strain and one brown, clade-B *Escovopsis* strain that were not inhibited by, and were occasionally attracted to, novel clade-C hosts.

Fungal-choice tests. As in the cross-phylogeny infection assays, in fungal-choice tests, strains of brown *Escovopsis* isolated from clade-A colonies were attracted to both their natural hosts (clade-A cultivars) and to closely-related cultivars (clade-B), arriving more rapidly at the ends of these tracks than the control track in most trials (figs. 4.3, 4.4). Thus, overall, it took fewer days for *Escovopsis* growth to reach the ends of the tracks with clade-A and clade-B cultivars than to reach the end of the control tracks ($n = 30$, $p < 0.0001$ in least square means comparison of both A vs. control and B. vs. control; fig 4.3). There was no statistically significant difference in the number of days that it took these parasite isolates to arrive at clade-A vs. clade-B cultivars ($n = 30$, $p = 0.54$; fig. 4.4), suggesting that there was no discrimination between these two hosts' signals. This is in contrast to similar fungal choice tests conducted with yellow *Escovopsis* (chapter 3), which demonstrated that yellow *Escovopsis* is more rapidly attracted to clade-A cultivar cues than clade-B cultivar cues (fig 4.4). Similar to yellow *Escovopsis*, in 29 of 30 trials, brown *Escovopsis* was not attracted to but was inhibited by clade-C cultivars. Even after several months, a zone of inhibition surrounded most clade-C cultivar isolates, and *Escovopsis* could not establish infection (fig. 4.3d).

4.5 DISCUSSION

Phylogenetic analysis of the relationships amongst *Escovopsis* that commonly attack fungus-growing ant gardens reveals two main characteristics: 1) broad-scale congruence of host-parasite phylogenies and 2) incongruence due to host-switching at finer scales. Host-parasite adaptive processes may explain the level of concordance between host and parasite phylogenies and may elucidate why host-switching is more likely by some parasites than by others. Specifically, *Escovopsis* is attracted to chemical signals produced by host cultivars with which it is typically associated in the field and to cultivars closely related to its host but is inhibited by distantly-related cultivar strains. Attraction to typical hosts and inhibition by novel hosts would prevent switching to distantly-related hosts. At the same time, switching between more closely-related cultivars, which leads to phylogenetic incongruence, may be facilitated by non-preferential attraction to closely-related hosts.

Based on phylogenetic analyses and extensive isolation of *Escovopsis* from *Apterostigma* colonies, both yellow and pink *Escovopsis* appear to not switch between the three main *Apterostigma* host cultivar clades. Yellow *Escovopsis* has only been found in colonies with clade-A cultivars. Based on results from chapter 3 (fig 4.4) and on cross-phylogeny infection bioassays (fig 4.2), it appears that switching by yellow *Escovopsis* to distantly-related cultivars, particularly clade-C and other lepiotaceous cultivars, is unlikely because of these hosts' defenses. Switching may be further limited because yellow *Escovopsis* strains grow preferentially towards clade-A versus clade-B cultivar signals (fig. 4.4). If yellow *Escovopsis* does not respond to clade-B cultivar signals aggressively, then if it comes into contact with colonies of this cultivar type, it may not successively establish and maintain infection.

Like yellow *Escovopsis*, the pink *Escovopsis* isolated from *Apterostigma* colonies form a monophyletic, host-specific clade. It is not surprising that this parasite is host-specific in relation to the possible *Apterostigma* hosts given that these parasites are inhibited by both

alternative hosts, the clade-A and clade-B cultivars. Fungus-growing ants in other attine genera, however, do grow clade-C cultivars and are in fact parasitized by morphologically-similar, pink *Escovopsis*. Though previous studies have shown that pink *Escovopsis* isolated from colonies of other attine genera are specific to particular cultivars within clade-C (Gerardo *et al.* 2004, chapter 2), it is possible that pink *Apterostigma*-associated *Escovopsis* could infect colonies of other attine genera. Further phylogenetic analyses coupled with laboratory and field experimental infection will elucidate the degree to which these parasites are specific within the range of possible clade-C hosts, and how host defense and parasite host-attraction mediate this specificity.

Unlike yellow and pink *Escovopsis*, the non-monophyly of brown *Escovopsis* suggests that there has been at least one switch between clade-A and clade-B hosts. There are two clades of brown *Escovopsis* isolated from clade-B colonies; one clade is basal to all the other brown *Escovopsis*, while the other is subsumed within a derived clade of parasites that show little divergence and that mostly attack clade-A colonies. One possible explanation is that historically there was a single monophyletic clade of *Escovopsis* associated with clade-B cultivars (the more basal clade-B *Escovopsis* in fig. 4.1), and then there was a switch of some clade-A *Escovopsis* to clade-B cultivars. This switch may not be complete; in the first stage of a host-switch, a parasite species expands its range to a novel host but remains on its original host, while in the second stage, the parasite remains on its novel host and goes extinct on its former host (Page 2003). Both processes can lead to discordance of host and parasite phylogenies, and it is difficult to detect whether there has been a host range expansion or a full host switch from clade-A to clade-B cultivars by some *Escovopsis* genotypes.

Results of both cross-phylogeny infection and fungal choice bioassays indicate that either host range expansion or complete host switch by clade-A brown *Escovopsis* strains is possible because the clade-B cultivars would be susceptible to the 'switching' parasite, and this switch (or range expansion) would be facilitated by the parasite's attraction to chemical signals produced by the novel, clade-B host. This is in contrast to yellow

Escovopsis, for which there is no phylogenetic evidence of host-switching (i.e. it has only been isolated from clade-A colonies). Yellow *Escovopsis* may be less likely to switch to clade-B cultivars than brown *Escovopsis* because, while brown *Escovopsis* is not preferentially attracted to clade-A vs. clade-B cultivar cures, yellow *Escovopsis* is preferentially attracted. Thus, though yellow *Escovopsis* is not strongly inhibited by clade-B cultivars, it may not be able to as efficiently establish and maintain infection in clade-B colonies because it is not rapidly attracted to theses cultivars' cues. Brown *Escovopsis*, however, may be equally likely to persist in both clade-A and clade-B colonies and thus more likely to switch between these two host types. In sum, host defensive adaptations and parasite attraction to hosts maintain host fidelity in some cases and facilitate occasional host-switching in others.

Parasite attraction to hosts plays a critical role in maintaining host-specificity in other host-parasite systems as well. In fact, many diverse parasites are attracted to host-specific signals. These parasites include salmon lice, which travel towards salmon-specific chemical cues (Devine *et al.* 2000), and trematode worm larvae, which use a variety of host-specific cues, including chemical gradients, to orient towards their hosts (Hass 2003). Lack of specificity in recognition, as seen with the brown *Escovopsis* here, is hypothesized to underlie the host switch of at least one other parasite, *Schistosoma mansoni*, to a novel snail host after introduction to South America (Kalbe *et al.* 2004). Parasite host-seeking is likely critical in both maintaining host fidelity and in promoting host-switching in other host-parasite systems as well.

Host defense is also common and highly variable across host-parasite systems. Defenses include behavioral responses (e.g. hosts recognize the eggs of brood parasites and remove them from the nest, Soler & Soler 2000), chemical responses (e.g. induced chemical defense in plants upon attack, Levin 1976; Maleck & Dietrich 1999), and immune system responses. Here, it appears that the cultivar is producing antibiotics that inhibit parasite growth. This, along with results from chapter 3, is the first evidence that the cultivar can play a role in its own defense against *Escovopsis.*

Cultivar defenses are coupled with ant behavioral defenses and bacterial antibiotics in a three-pronged approach to combat *Escovopsis* infection. Though *Escovopsis* attacks and consumes the fungal cultivars, the ants themselves are impacted because the cultivar is their primary food source. The ants, therefore, weed and groom *Escovopsis*-infected gardens, contributing to disease suppression (Currie & Stuart 2001). Additionally, the ants have actinomycete bacteria on their exoskeleton that produce *Escovopsis*-suppressing antibiotics (Currie *et al.* 1999b). Coevolution between actinomycete-produced antibiotics and antibiotic resistance in *Escovopsis* could play a critical role in shaping *Escovopsis* specificity, and other coevolving microbes that are closely associated with the cultivars may facilitate parasite suppression as well. Further work will elucidate how these defenses complement one another and how they chemically and behaviorally vary across the fungus-growing ant symbiosis.

It is assumed that the cultivar, ants and bacteria are all defending against a highly virulent parasite, *Escovopsis*. Though some *Escovopsis* strains are highly virulent under some circumstances (Currie *et al.* 2001), it is not clear whether all *Escovopsis* strains are detrimental or under what conditions infection has significant negative consequences for host fitness and survival. Colonies of some species can survive for years with persistent infections (per. obs.), suggesting that not all infections lead to rapid mortality. In fact, it is even possible that *Escovopsis* may play some beneficial role for a colony (e.g. if it can consume or otherwise suppress other fungi in the garden that are competing for the cultivars' resources). More work on the epidemiology of this parasite clearly is needed.

The microevolutionary patterns seen here do not, however, require that *Escovopsis* be a virulent parasite in order to explain either their origin or maintenance. There are, in fact, several evolutionary scenarios for the maintenance of both parasite host-seeking and host defense. In the first, 'parasite' scenario, host cultivars, in the face of a virulent parasite, have adapted parasite-specific defenses. *Escovopsis* strains have adapted to overcome these defenses, but each is limited to overcoming the defenses of only a narrow range of hosts. Once constrained to a narrow host range, through recognition of host-specific

signals, *Escovopsis* strains may be able to rapidly spread through a colony to establish infection on the cultivar and may use host signals to move continuously from depleted garden material to fresh, healthy cultivar, making it difficult for the ants to suppress or remove the parasite. Alternatively, in a second, 'mutualist' scenario, *Escovopsis* strains may have some, unknown benefit to a narrow range of hosts and thus the cultivars may have adapted to facilitate infection by a narrow range of 'parasites', explaining why cultivars do not defend against their typical *Escovopsis* associates. Under this scenario, the cultivars would benefit if they could facilitate infection by signaling to *Escovopsis*, and it would benefit the 'parasite' to quickly establish infection through recognition of host signals.

Regardless of the nature of *Escovopsis* (obligate parasite, mutualist-parasite switcher), microevolutionary adaptive processes can explain historical patterns of symbiont association in the fungus-growing ant symbiosis. Though both parasite (Devine *et al.* 2000; Hass 2003; Clayton *et al.* 2004) and host (Clayton et al. 2003b) adaptations have been suggested to shape patterns of parasite host-specificity in other systems, few studies tie both processes into the framework of known host and parasite phylogenies. This is feasible in the fungus-growing ant symbiosis, because both hosts and parasites can be easily sampled and characterized molecularly and because interactions between novel host and parasite combinations can be easily experimentally set-up using biological assays. Further work may elucidate the molecular and chemical basis for these host and parasite adaptations, providing yet another avenue for investigating the evolutionary ecology of this complex symbiosis.

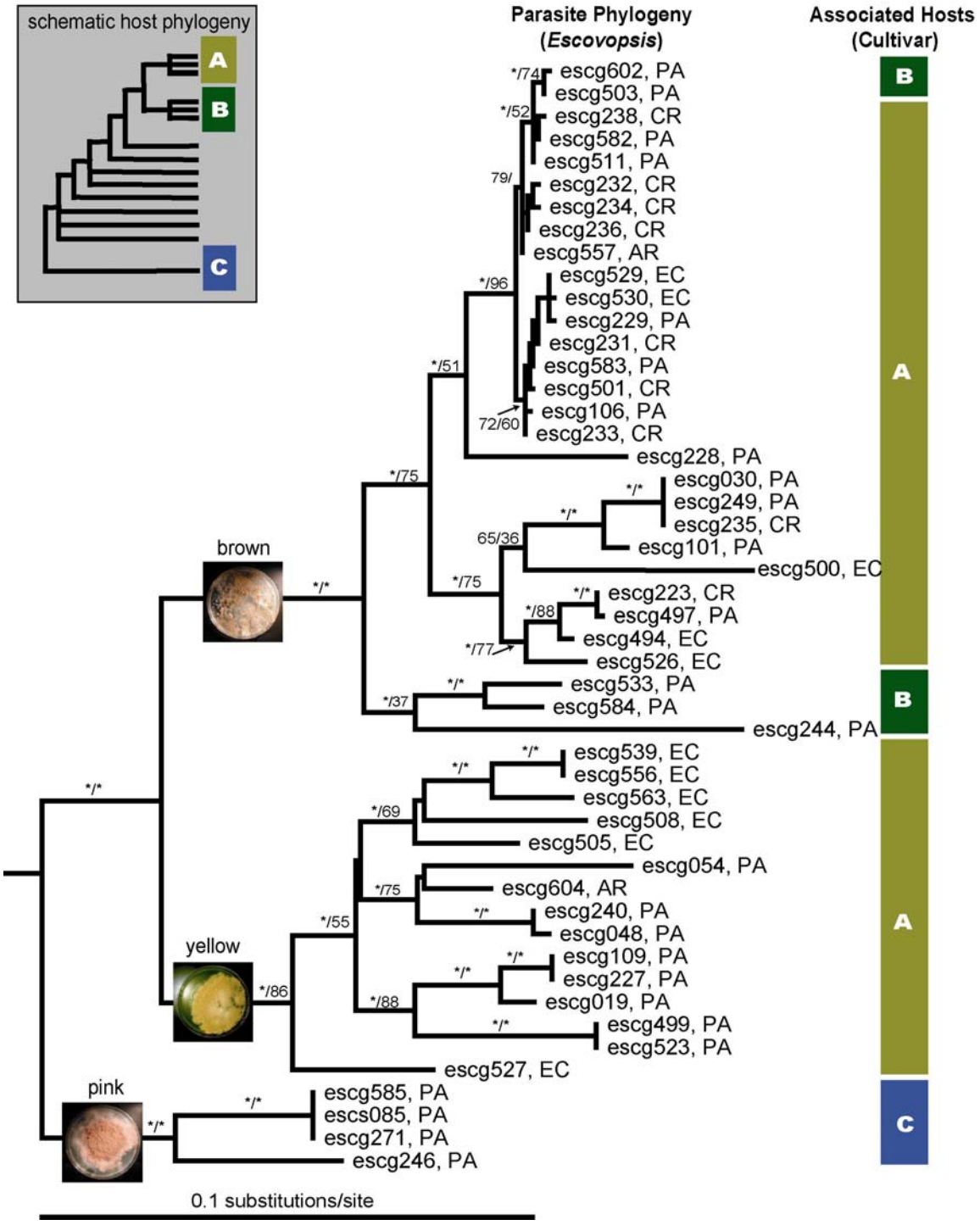
schematic host phylogeny
A
B
C
Parasite Phylogeny
(Escovopsis)
Associated Hosts
(Cultivar)
escg602, PA
escg503, PA
escg238, CR
escg582, PA
escg511, PA
escg232, CR
escg234, CR
escg236, CR
escg557, AR
escg529, EC
escg530, EC
escg229, PA
escg231, CR
escg583, PA
escg501, CR
escg106, PA
escg233, CR
escg228, PA
escg030, PA
escg249, PA
escg235, CR
escg101, PA
escg500, EC
escg223, CR
escg497, PA
escg494, EC
escg526, EC
escg533, PA
escg584, PA
escg244, PA
escg539, EC
escg556, EC
escg563, EC
escg508, EC
escg505, EC
escg054, PA
escg604, AR
escg240, PA
escg048, PA
escg109, PA
escg227, PA
escg019, PA
escg499, PA
escg523, PA
escg527, EC
escg585, PA
escs085, PA
escg271, PA
escg246, PA
brown
yellow
pink
*/74
*/52
79/
*/96
*/51
72/60
*/75
/
65/36
*/75
*/88
*/77
*/37
*/69
*/55
*/86
0.1 substitutions/site

Figure 4.1: Maximum likelihood phylogram of *Apterostigma*-associated *Escovopsis*. The topology of the likelihood tree is almost identical to that of Bayesian and parsimony analyses. Terminals are labeled with sample codes proceeded by the country of origin. The morphotype of each of the three main clades is indicated along the branch leading to each clade. Bayesian posterior probabilities and non-parametric, parsimony bootstrap values over 50 are above branches, except for short branches in the brown clade-A *Escovopsis*, where values above 50 have been left off for simplicity. * represents a support value of ≥ 95. In the top left corner, the schematic phylogeny represents the relationship between the three main cultivar host clades. The hosts corresponding to each parasite-clade are indicated down the right side of the *Escovopsis* phylogeny.

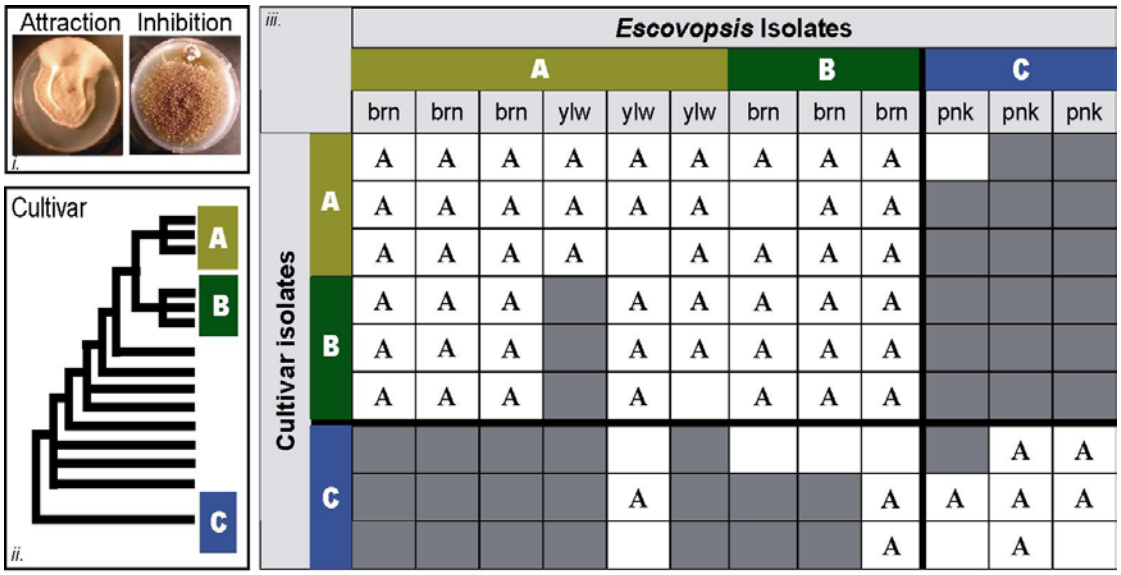

Figure 4.2: Cross-phylogeny bioassays. *i.* Representative plates indicating attraction (left) and inhibition (right). *ii.*. Schematic phylogeny of the cultivars, emphasizing that clade-A and clade-B cultivars are closely related while clade-C cultivars are distantly related. *iii.* Each cell represents the outcome of the interaction between one cultivar and one *Escovopsis* isolate. Gray cells indicate inhibition; white cells indicate no inhibition. 'A' indicates cases in which *Escovopsis* was attracted to the cultivar isolate. There were no cases in which there was both attraction and inhibition. The horizontal line marks the division between the closely-related pterulaceous cultivars (clades A and B) and the distantly-related lepiotaceous cultivars (clade C). Similarly, the division between the clade-A and clade-B associated *Escovopsis* and the clade-C associated *Escovopsis* is denoted by the thick vertical line. *Escovopsis* morphotypes are indicated: brown = brn, yellow = ylw, pink = pnk.

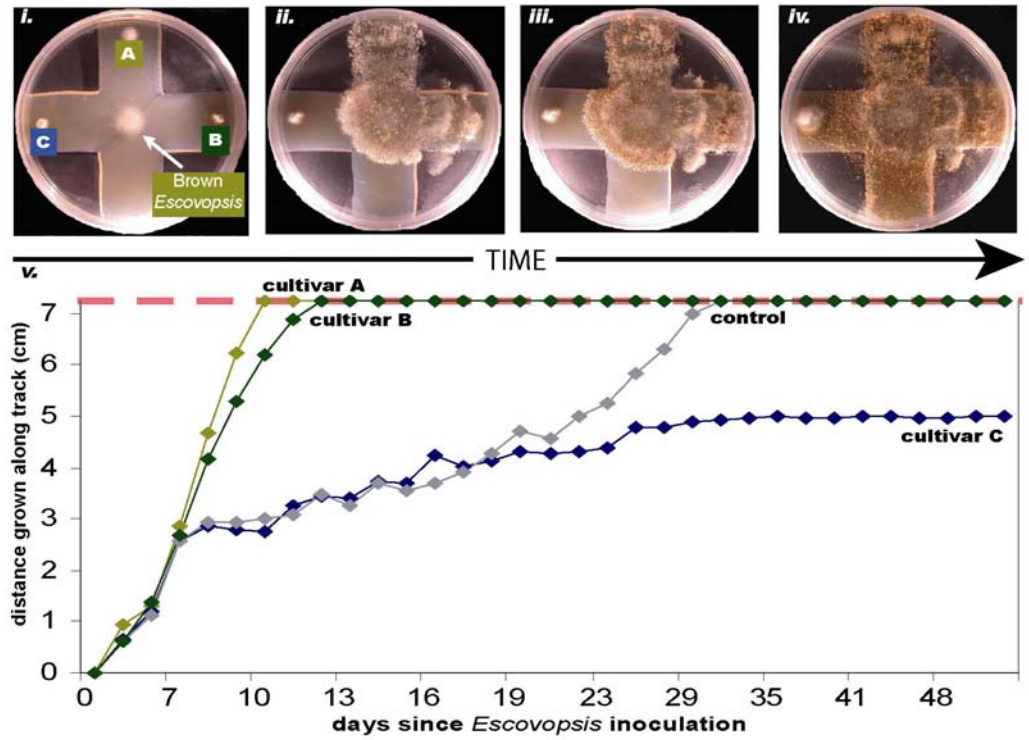

Figure 4.3: Time progression of fungal-choice bioassay with brown *Escovopsis*. *i.* Isolates of clade-A, clade-B and clade-C cultivars are placed at the end of each of three tracks and one track is left blank as a control. After inoculation, *Escovopsis* begins to grow concentrically. *ii.* The parasite then reaches the end of the track with the clade-A cultivar, and is close to reaching the end of the track with clade-B cultivar. *iii. Escovopsis* has reached the clade-B cultivar. *iv. Escovopsis* has reached the end of the control track, but the parasite has still not overcome the clade-C cultivar. In this trial, *Escovopsis* did not overgrow the clade-C cultivar during the three months in which the plates were maintained. *iv.* Number of days since inoculation with *Escovopsis* vs. the distance (cm) grown along each track. The red, dashed line indicates the total track length from center of the plate to the track end.

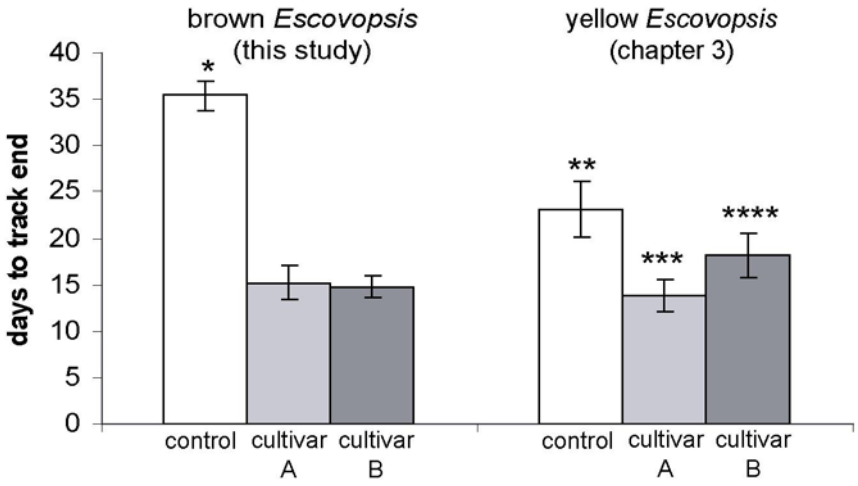

Figure 4.4: Average number of days to reach each cultivar type in fungal-choice bioassays with brown *Escovopsis* (this study) and with yellow *Escovopsis* (chapter 3). For brown *Escovopsis*, * indicates that the mean number of days that it took *Escovopsis* to reach the end of the control lanes was significantly greater than the number of days that it took the parasite to reach the clade A and B cultivars, suggesting that *Escovopsis* is attracted to these cultivars. There was no statistical difference between the time that it took to reach the two cultivars. By comparison, when fungal-choice bioassays were conducted with yellow *Escovopsis* (chapter 3), there was a significant difference between all three treatments (indicated by # of *s). Data for #days to overcome clade-C cultivars are not included because inhibition by these cultivars prevented *Escovopsis* from reaching the end of the tracks in most trials. Errors bars represent s.e..

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Chapter 5

Host-parasite associations in structured populations: comparing genetic diversity of fungus-growing ant cultivars and their parasites

Abstract: Adaptation and counter-adaptation by hosts and parasites in sympatry can lead to local adaptation, whereby parasites have higher mean fitness on sympatric than on allopatric hosts. Theoretical studies of host-parasite coevolution suggest that the relative migration rate and population structure of hosts and parasites conditions the evolution of local adaptation. To determine the likelihood of local parasite adaptation, I here compare gene flow between populations of the cultivated fungi of the fungus-growing ant *Apterostigma dentigerum*, and the cultivar-attacking parasite *Escovopsis.* I utilize amplified fragment length polymorphism markers (AFLPs), to genotype host cultivars and parasitic *Escovopsis* from seventy-seven colonies throughout the geographic range of *A. dentigerum*. Lower overall genetic differentiation for parasites than for hosts suggests that the parasites migrate slightly more than their hosts, which theory predicts to favor local parasite adaptation. Limitation of parasite genotypes to a narrow range of host genotypes would be evidence for local adaptation. Host and parasite genetic distances, however, are uncorrelated, suggesting that genetically similar hosts are not parasitized by genetically similar parasites. Thus, there is little evidence for local adaptation in the fungus-growing ant microbe symbiosis.

5.1 INTRODUCTION

Parasites are specialized at numerous scales. At the broad level, most parasites attack particular taxonomic host groups (e.g. birds, annual plants, bacteria); at the finest level, some parasites track locally abundant host genotypes. Specialization to locally common host genotypes is a consequence of adaptation and counter-adaptation of sympatric hosts and parasites leading to higher parasite fitness on their local hosts (Kaltz & Shykoff 1998). Once parasites become locally adapted, they may be precluded from switching to novel host genotypes.

The ratio of host and parasite migration rates strongly affects local adaptation: if parasite migration is greater than host migration, coevolutionary models predict local adaptation (Gandon 1996; Lively 1999; Gandon & Michalakis 2002). Thus, to understand the coevolutionary dynamics of a host-parasite system, it is necessary to estimate the population structure and geneflow of both host and parasite.

While researchers have recognized the importance of elucidating parasite population structure in order to understand disease dynamics (Anderson *et al.* 2000; McCoy *et al.* 2003; Schriefer *et al.* 2004), only a handful of studies have compared population structures of hosts and parasites across the same geographic scale in order to elucidate the relative distribution of host and parasite genetic diversity (Mulvey *et al.* 1991; Davies *et al.* 1999; Martinez *et al.* 1999; Sire *et al.* 2001; Jerome & Ford 2002; Johannesen & Seitz 2003). Results of these studies have varied. Jobet *et al.* (2000), for example, found similar differentiation between populations of the urban cockroach (*Blatella germanica*) and its nematode parasite (*Blatticola blattae*). Delmotte *et al.* (1999), however, found that populations of the fungal pathogen *Mircobotryum violaceum* were much more strongly differentiated than were populations of the host plant, *Silene latifolia,* and Dybdahl and Lively (1996) found that populations of trematode parasites (*Microphallus* sp.) were

much less differentiated than those of their host snails (*Potamopyrgus antipodarum*). In light of this variation between systems, little can be generalized to host-parasite population dynamics as a whole.

To date, there have been no population-level studies of host-parasite interactions in the fungus-growing ant-microbe symbiosis. In this insect agricultural system, approximately 210 ant species are known to cultivate fungus as their primary food source. When new colonies are formed, founding queens take a piece of fungus from their mother's colony to start new colonies, leading to long-term association between and facilitating coevolution of the ants and their fungal cultivars (Chapela *et al.* 1994). While this mutualism between ants and fungus has been established as a system to study coevolution, cospeciation, cooperation and conflict, it has also emerges as a tractable system in which to study the dynamics of hosts and parasites (Currie 2001; Currie *et al.* 2003b; Gerardo *et al.* 2004). The cultivars of fungus-growing ants are attacked by *Escovopsis*, a genus of ascomycete fungi only found in association with fungus-growing ant colonies (Currie *et al.* 1999a; Reynolds & Currie 2004). Work at the interspecific level indicates that *Escovopsis* spp. are highly specific: particular *Escovopsis* clades only attack specific clades of fungal cultivars (Currie *et al.* 2003b; Gerardo *et al.* 2004). This interspecific specificity is driven by *Escovopsis'* attraction to host-specific cues as well as by the ability of cultivars to inhibit some *Escovopsis spp*. but not others (Gerardo *et al.,* in prep). It is possible that these mechanisms function to maintain intraspecific specificity of parasite genotypes to a narrow range of host genotypes as well. Highly specific parasites are likely to be locally adapted to common host genotypes (Gandon 2002).

Here, I investigate the population structure of cultivars and parasitic *Escovopsis* isolated from colonies of *Apterostigma dentigerum* ants. I then examine patterns of association between host and parasite genotypes to determine whether there is evidence for intraspecific parasite specificity and thus for local adaptation.

5.2 STUDY SYSTEM

***Apterostigma dentigerum* colonies and their associated microbes.** While most studies of fungus-growing ants have concentrated on the leaf-cutter ants (*Atta* spp. and *Acromyrmex* spp.) because of their large, conspicuous colonies and prodigious consumption of fresh vegetation used to feed their fungus, many other species of lesser studied fungus-growers are of equal interest because their colonies are abundant and easily sampled. Colonies of *Apterostigma dentigerum* are common along stream banks and under logs throughout much of Central and South America. Unlike subterranean ant colonies, *A. dentigerum* colonies can be easily detected and identified because of a conspicuous white veil of fungus that protects their internal fungal garden (Villesen *et al.* 2004; fig 5.1). Garden material and ants can be easily collected, and microbial cultures and whole colonies can be maintained in the laboratory, facilitating both molecular analyses and experimental manipulations.

Unlike most fungus-growing ant species, which cultivate fungi in the family Lepiotaceae (Chapela *et al.* 1994; Mueller *et al.* 1998), most *Apterostigma* spp., including *A. dentigerum*, grow fungus in the family Pterulaceae (Munkacsi *et al.* 2004; Villesen *et al.* 2004). Pterulaceous cultivars are attacked by only a few, specialized *Escovopsis* spp. (Currie *et al.* 2003b; Chapter 4); two *Escovopsis* morphotypes, a yellow and a brown are common. These parasites have been shown to be attracted to chemical cues produced by pterulaceous, but not lepiotaceous, cultivars, and pterulaceous cultivars have been shown to be unable to defend against these parasites (chapters 3 and 4). These host and parasite adaptations (attraction and defense) likely restrict the host range of *Escovopsis*.

5.3 METHODS

Collection and Isolation. *Apterostigma dentigerum* colonies were collected between 2001–2003 across their range in Central and South America. Localities included three sites in Costa Rica: La Selva Biological Station (LSC), El Ceibo Biological Station

(ELC), Hitoy Cerere Biological Reserve (HCE); and eight sites in Panama: approximately 25km north of Fortuna Biological Station (FOR), Fort Sherman (FTS), Barro Colorado Island (BCI), Gamboa (GAM), Pipeline Road (PLR), near Coclecito (COC) , El Llano–Cartí Suitupo Road (ELL), and Rancho Frío in Darien Province (DAR) (fig. 5.1). Collections were also made at La Selva Lodge and Biological Station in Ecuador (LSE) and Parque Pícal in Argentina (ARG). At least ten garden pieces (~8mm^3) from each colony were grown on potato dextrose agar (PDA; Difco, Detroit, MI) with antibiotics (50mg/L each of penicillin and streptomycin), and pieces identified as either cultivar or as *Escovopsis* were subsequently subcultured to obtain axenic (pure) cultures before storage at -80^{O}C.

For this study, there were seventy-seven colonies from which both cultivar and brown-morphotype *Escovopsis* were isolated; *Escovopsis* isolates of other morphotypes were not included. Using only samples isolated in tandem (e.g. cultivar and *Escovopsis* from the same colony) assured that both hosts and parasites were sampled similarly across space and time. Sample sizes for each population are listed in fig. 5.1.

AFLP techniques. To examine the distribution of host and parasite genotypes both across populations and relative to one another, I used amplified fragment length polymorphisms, AFLPs, to fingerprint cultivars and *Escovopsis* isolated from the same 77 colonies. Preceding amplification, DNA from a single cultivar and a single parasite isolate from each colony was extracted following a CTAB protocol modified from Bender *et al.* (1983). Cultivar and parasite isolates from two randomly selected colonies were extracted twice and genotyped to detect the amount of noise (unreliable marker differences) generated during the amplification and scoring process. These duplicates were not included in graphical or statistical analyses.

For all cultivar and *Escovopsis* isolates, AFLP markers were generated on an ABI Prism 3100 Genetic Analyzer and scored in Genotyper 2.5. Reactions followed the AFLP protocol for small plant genomes (www.appliedbiosystems.com; protocol 4303146), with

the modification that preselective products were diluted 2:1 before use in the selective reactions. Six combinations of AFLP-primer extensions were chosen because they generated high levels of polymorphic markers that could be scored reliably: AC/CAA, AC/CTG, AC/CTC, TG/CAG, TG/CTC and TC/CAG. All cultivar and parasite samples were run at the same time in 96-well plates and using the same reagents to minimize differences in host and parasites being caused by noise rather than by real variation in population structure. AFLP markers were scored blindly by simultaneously comparing all fragments of a given length across all 77 *Escovopsis* isolates and, in a separate analysis, across all 77 cultivar isolates. Only markers that could be scored as unambiguously present/absent across all parasite or host samples were used in analyses.

Host and parasite population differentiation. For analysis of population structure and genetic diversity, I performed parallel analyses of the cultivar (host) and *Escovopsis* (parasite) datasets and then compared results between the two. To compare host and parasite population structure, I conducted two Analyses of Molecular Variance (AMOVA) in Arlequin (Ver 2.001, Schneider *et al.* 2000) to partition the AFLP variation both among host and among parasite isolates within and between localities. The AMOVA module in Arlequin generates Φ statistics, equivalent to Weir and Cockerham's (1984) θ statistics, which are a molecular analog to Fisher's F_{st} (Excoffier 2001). Population pairwise Φ_{st} values were also generated to determine the proportion of differences between hosts, and separately between parasites, associated with each locality. Levels of significance were determined through 100,000 random permutation replicates. For all population analyses, I excluded the three localities at which only a single sample was collected (LSE, ARG, ELL), because no within-locality variation could be determined.

For hosts, and separately for parasites, I conducted Mantel tests in ZT (Bonnet & Van de Peer 2002) to determine correspondence between each pairwise Φ_{st} (genetic distance) matrix and a pairwise geographical distance matrix. A significant, positive correlation would indicate the effects of isolation by distance. All Mantel tests mentioned hereafter were also conducted using ZT and were performed with 10,000 permutations. Pairwise,

linear geographical distances between localities were calculated using the program Range (Luetgert, USGS). I also plotted the relationship between pairwise Φ_{st} and geographical distance for both pairs of host populations and pairs of parasite populations.

To visualize the relationship among cultivar populations and among parasite populations, I used ARLEQUIN to construct two matrices: 1) the Nei's corrected average pairwise cultivar population differences; and 2) the Nei's corrected average pairwise *Escovopsis* population differences (Nei & Li 1979). These matrices were used to generate two separate non-metric multidimensional scaling (NMDS) plots (one for host populations, one for parasite populations) using NCSS (ver. 2000, Hintze 2001). NMDS is an ordination technique that detects nonhierarchical structure by reducing the multidimensional relationship between entities to a smaller number of dimensions.

Genotypic associations of host and parasites. To visualize the relationships between the seventy-seven cultivar isolates, I used mean character distances (i.e. the sum of loci differences between two samples / total no. of loci), generated in PAUP* (ver4.b10, Swofford 2002), to construct a non-metric multidimensional scaling (NMDS) plot using NCSS. A similar plot was created for the parasite isolates. Mean character distances were used for these and all subsequent analyses, because though Nei-Li (1979) restriction distances are often selected for AFLP data analysis, many *Escovopsis* pair distances were undefined using this method. The Nei-Li and mean character distances for the cultivars were highly correlated (Mantel test, $r = 0.78$, $p < 0.0001$), however, and the results of no analysis were changed if the cultivar Nei-Li distances were used in place of the mean character differences. To verify the clustering produced through NMDS, I also used PAUP* to construct UPGMA dendrograms; UPGMA is a clustering algorithm often used with AFLP data.

In addition to the visual inspection above, to evaluate whether genetically similar parasites are attacking genetically similar hosts, I conducted three separate statistical analyses. First, to determine whether host and parasite populations exhibited a similar

spatial pattern of divergence, I used a Mantel test to assess correspondence between the matrix of cultivar pairwise Φ_{st} values and parasite pairwise Φ_{st} values. A significant correlation would indicate that parasite populations show similar relative divergence to the host populations that they are attacking. This is a common method used in analyses of host-parasite population structure.

Correlation between host and parasite population pairwise differences would indicate that more genetically similar populations of hosts and more genetically similar populations of parasites are associated, but this would not reveal parasite specialization at finer levels (i.e. whether each parasite genotype within a population is attacking a narrow range of host genotypes within a population). Therefore, for my second analysis of host-parasite association, I used a Mantel test to determine the correspondence between the host and parasite mean character difference matrices. Significance would indicate that more genetically similar parasite isolates (those with smaller mean character differences) attack more genetically similar host isolates, both between and within populations.

Third, in both the NMDS plot and the UPGMA dendrogram, cultivar isolates fell into the same six visually distinct clusters. To verify the genetic distinctiveness of these clusters, I assigned each cultivar isolate to a cluster (cluster 1–6) and then used AMOVA to determine whether the clustering explained a significant and substantial proportion of the genetic variation among cultivar isolates. I then conducted pairwise comparisons to confirm that each cluster was significantly distinct from all other clusters. Then, to determine whether the host-cluster with which an *Escovopsis* isolate is associated could explain genetic variation among parasite isolates, I assigned each *Escovopsis* isolate to the cluster in which its host belonged and then used these groups as a basis for AMOVA. A significant overall Φ_{st} would indicate the proportion of *Escovopsis* variation attributable to their association with genotypically distinct host clusters. I also conducted pairwise comparisons to determine which parasite groups, as defined based on host genotype cluster, were genetically differentiable. Significant pairwise difference would

indicate cases in which genotypically differentiable parasite groups are attacking genotypically differentiable hosts.

5.4 RESULTS

AFLP diversity. For the host cultivars, a total of 804 AFLP loci were identified using the six primer systems; all were polymorphic and 208 (26%) were autapomorphic. For parasitic *Escovopsis*, a total of 933 AFLP loci were identified; all were polymorphic and 334 (36%) were autapomorphic. Both cultivars and *Escovopsis* samples were diverse; mean character differences between cultivar isolates ranged from 0.02 to 0.29 (mean = 0.16, s.d. = 0.05), and mean character differences between *Escovopsis* isolates ranged from 0.04 to 0.22 (mean = 0.14, s.d. = 0.04). Of the four duplicated samples (two cultivar isolates and two parasite isolates), the mean character difference between duplicates was low, ranging from 0.02 to 0.07 (mean = 0.04, s.d. = 0.02), and the difference between cultivar duplicates and between parasite duplicates was similar. This suggests that the majority of variation between samples was due to real genotypic differences rather than AFLP artifacts, though small differences between samples should be interpreted with caution because they do not necessarily reflect genetic differences.

Host and parasite population differentiation. Population differentiation of host cultivars and the fungal parasite *Escovopsis* are similar in magnitude. Eleven percent of the variation among cultivar isolates is attributable to between population differences (Table 5.1a), while seven percent of the variation among *Escovopsis* isolates is attributable to locality (Table 5.1b). This suggests that there is slightly more migration by parasites than by hosts. NMDS solutions of cultivars and *Escovopsis* suggest some degree of geographic isolation for both (fig. 5.2). For cultivars, dimension one in the NMDS plot accounts for 64% of the total variation and dimension two accounts for an additional 8%. Indications of host geographic structure include: 1) Costa Rican populations all fall near the lower right-hand quadrant of the dimension space, and 2) most populations along the Panama Canal (PLR, BCI, GAM, FTS) lie in a similar portion of dimension space. For

Escovopsis, dimension one in the NMDS analysis accounts for 50% of the total variation and dimension two accounts for an additional 19%. Indications of host geographic structure include: 1) Costa Rican populations all fall near the upper right-hand quadrant of the dimension space, and 2) three populations along the Panama Canal (PLR, GAM, FTS) lie in a similar portion of dimension space.

A Mantel test of the correspondence between cultivar pairwise Φ_{st} values and pairwise geographic distances confirms the effect of geographic isolation by distance ($r = 0.34$, $p = 0.04$). Similarly, *Escovopsis* exhibits similar correlation between genetic and geographic distances ($r = 0.38$, $p = 0.03$). This positive relationship between genetic and geographic distances is represented in fig. 5.2.

Genotypic associations of hosts and parasites. Cultivars exhibit substantial genetic structure, as seen through the clustering of isolates in both the NMDS plot and the UPGMA dendrogram (fig 5.4 a,c). Both clustering algorithms group isolates into 6 main clusters, and all isolates fall into the same cluster in both analyses. For the NMDS plot, dimension one captures 29% of the cultivar variation and dimension two captures an additional 22%. Upon aposteriori assignment of each of the cultivars to one of the six genotypic clusters, the resulting clusters account for 54% of the variation among isolates (Table 5.2a, AMOVA, overall $\Phi_{st} = 0.54$), substantially more than when the cultivars are assigned to populations rather than to genotypic clusters (Table 5.1a, AMOVA, overall $\Phi_{st} = 0.11$).

Escovopsis exhibits less genetic structure, with little clustering in the NMDS plot or UPGMA dendrogram (fig 5.4 b,d). For the NMDS plot, dimension one captures 37% of the isolate variation and dimension two captures an additional 15%. The relative lack of parasite clustering in fig. 5.4 as compared to host clustering suggests that there may be little concordance between host genetic similarity and parasite genetic similarity. Such concordance would be expected if particular parasite genotypes were evolving in tandem with their particular hosts. Absence of substantial parasite tracking of hosts genotypes

was confirmed: there is not significant correspondence between 1) cultivar population pairwise Φ_{st} values and *Escovopsis* population pairwise Φ_{st} values ($r = 0.22$. $p = 0.139$); nor between 2) mean host differences and their respective mean parasite differences ($r = 0.04$, $p = 0.065$). However, when *Escovopsis* isolates were assigned to groups based on the genotypic cluster with which their host cultivar was associated, AMOVA did show that this clustering explained a small, though significant portion of the variation among parasite isolates (Table 5.2b, overall $\Phi_{st} = 0.025$). Pairwise comparisons between groups of *Escovopsis* isolates that attack the different host genotype clusters indicated that parasites attacking cultivars in cluster one were genotypically significantly distinct from parasites attacking cultivars in cluster three (Table 5.2). Thus, in this case, there is some evidence that more genotypically similar parasites are coming into contact with or preferentially attack more genotypically similar hosts.

5.5 DISCUSSION

Similar to several other studies comparing host and parasite population structure (Dybdahl & Lively 1996; Martinez *et al.* 1999; Mutikainen & Koskela 2002), I found slightly stronger differentiation between host cultivar than between parasitic *Escovopsis* populations (table 5.1), which is consistent with higher rates of parasite migration than host migration. Higher relative parasite migration is predicted to lead to local adaptation (Gandon 1996; Gandon 2002; Gandon & Michalakis 2002), and in several systems in which local adaptation has been tested, this has been verified. Dybdahl and Lively (1996) found much higher levels of gene flow in trematode parasites relative to their snail hosts, and it has been demonstrated that these parasites are locally adapted to common host genotypes (Lively 1989; Lively & Dybdahl 2000). Similarly, Mutikainen and Koskela (2002) found higher parasite gene flow in parasitic plants than their perennial hosts, and these parasites had been previously reported to be locally adapted to their hosts (Koskela *et al.* 2000). In these cases, however, host populations were respectively 10 and 3 times more differentiated than their parasites, whereas here, with cultivar and *Escovopsis*,

overall Φ_{st} values of host and parasite populations are less than twofold different. Moreover, results of Mantel tests between genetic and geographic distance indicate that the effects of isolation by distance in cultivars and *Escovopsis* are similar (fig. 5.3), suggesting that though there may be higher parasite migration between proximate populations, over larger spatial scales host and parasites are migrating similarly. Therefore, though there may be some tendency for higher *Escovopsis* migration to facilitate local adaptation to cultivars, it may be less likely than in other host-parasite associations.

If local adaptation is occurring, then we would expect that genotypically similar parasites would be attacking genotypically similar hosts because selection on genes controlling traits involved in parasite virulence and host defense will follow different coevolutionary trajectories in each population. In this case, genotypically similarity or divergence would be specifically associated with loci directly involved in host-parasite interaction traits (i.e. genes controlling resistance and infectivity). However, if loci under selection are linked to neutral markers (e.g. AFLPs), then hosts with similar neutral marker fingerprints would be attacked by parasites with similar neutral marker fingerprints if local adaptation is leading to strict parasite host-specificity. There are two reasons to believe that selectively adaptive parasite and host loci would be linked to neutral markers in the cultivar-*Escovopsis* system. First, as Little and Ebert (1999) argued, in predominantly asexual organisms, multi-locus gene complexes are preserved during reproduction, and thus hosts which differ at resistance loci and parasites which differ at infectivity loci may also differ at neutral marker loci. Both the cultivars and *Escovopsis* are presumed to be predominantly asexual. Second, I have shown for other cultivar-*Escovopsis* species (chapter 3) that genetically similar parasite strains are more likely to successfully infect genetically similar cultivar strains. The genetic similarity in this case was not in genes under selection but in neutrally evolving DNA sequence and AFLP fingerprints. This indicates a correlation between host defense, parasite infectivity and neutral markers, and suggests that neutral markers can be used to verify the extent of local adaptation.

Based on AFLP analysis, there is little evidence that genetically similar *Escovopsis* are attacking genetically similar hosts. First, there is no correspondence between host and parasite population pairwise Φ_{st} values and little similarity between NMDS plots of host and parasite populations (fig. 5.4), suggesting that hosts and parasites in the same populations are not similarly diverged. Second, there is also no correspondence between a matrix of host mean character differences and a matrix of parasite mean character differences. Finally, whereas cluster analyses suggest several genetically distinct host clusters, there is no corresponding divergence in parasite isolates (fig. 5.4). Grouping of parasites according to associated host cluster did account for a small, but significant proportion of the variation between *Escovopsis* isolates. Pairwise Φ_{st} values between parasites grouped according to host cluster, however, found only one significant pairwise difference, suggesting that only in this one case were parasites attacking hosts within one genotype cluster more similar to each other than they were to parasites attacking hosts within another genotype cluster. Because both hosts and parasites are similarly affected by isolation by distance, this slight association of similar parasites to similar hosts may be more an effect of geographic isolation than local adaptation (i.e. at large scales, some populations of parasites will be restricted to only the hosts which reach that population).

Thus, while population structure analyses suggest similar overall divergence between cultivar and *Escovopsis* populations, there is little evidence that this similarity is driven by tight tracking of parasites genotypes on host genotypes. The capacity for a given *Escovopsis* genotype to attack multiple cultivar genotypes has broad implications for host-parasite dynamics. The ability to use multiple host species is expected to affect the ability of parasites to establish in communities (Holt *et al.* 2003) as well as parasite virulence and epidemiology (Woolhouse *et al.* 2001). These same issues, to some degree are likely affected by whether a parasite utilizes one versus many within-host species genotypes.

The brown morphotype of *Escovopsis* on which I focus here has been previously shown to be specific to attacking only the pterulaceous cultivars raised by *Apterostigma* spp.

(chapter 4). A lack of evidence for intraspecific *Escovopsis*-cultivar specificity suggests, however, that the mechanisms maintaining this interspecific specificity may not function to maintain tight association of within-species cultivar and *Escovopsis* genotypes. This is not to say that intraspecific specificity and local adaptation are not occurring within the symbiosis as a whole. *Escovopsis* could in fact be adapting with other symbionts. In attacking the cultivars of fungus-growing ants, *Escovopsis* must overcome antibiotics produced by actinomycete bacteria found on the ant's bodies (Currie *et al.* 1999b; Currie *et al.* 2003a). Though *Escovopsis*' host range may be broadly limited to only a narrow range of cultivar species, maybe it is narrowly limited to overcoming only a narrow range of actinomycete genotypes. Future population-level studies of the fungus-growing ant symbiosis should include detailed analyses of the genotypic interaction of all four players: the ants, their cultivars, the parasite *Escovopsis*, and the parasite-inhibiting actinomycete bacteria. Further studies should also identify genes involved directly in host-parasite interactions (e.g. genes controlling parasite virulence, parasite host-recognition and host defense) to determine whether there is local selection upon them, and in addition, cross infection studies involving switching of all four players at different spatial scales will verify whether local selection is important in the coevolutionary dynamics of this host-parasite association.

AMOVA results **Population differences**			**Cultivar (host)** ***(a)***			**Escovopsis (parasite)** ***(b)***				
			variance	d.f.	% total	variance	d.f.	% total		
Between populations			7.3	9	11.1	4.8	9	7.4		
Within populations			58.9	64	88.9	60.5	64	92.6		
			overall Φ_{st} = 0.11, p < 0.01			**overall Φ_{st} = 0.07, p < 0.01**				
between-population pairwise Φ_{st} values **Cultivar (below diagonal) & *Escovopsis* (above diagonal)**										
	ELC	LSC	HIT	FOR	COC	FTS	BCI	PLR	GAM	DAR
ELC		0.01	0.06	0.04	0.05	**0.06**	**0.28**	**0.13**	0.06	0.06
LSC	**0.13**		0.03	0	0	0.01	**0.2**	0.05	0.01	0.01
HIT	**0.32**	0.02		0.01	0.04	0.05	**0.31**	**0.11**	0.06	**0.23**
FOR	**0.26**	0.002	0		0	0	**0.24**	0	0	**0.30**
COC	0.11	0.09	**0.24**	0.16		0.06	0.12	0.04	0.14	0.01
FTS	0.06	0	0.12	0.10	0		0.02	0	0	0.02
BCI	**0.10**	**0.17**	**0.27**	**0.15**	0.07	**0.13**		0.03	**0.11**	**0.30**
PLR	**0.09**	**0.08**	**0.15**	**0.14**	0	0	0		0.01	0
GAM	**0.12**	**0.21**	**0.32**	**0.29**	0	0.06	0	0		0.01
DAR	0.02	0.17	**0.36**	0.15	0.03	0.10	0.02	0.15	0.08	

Table 5.1: AMOVA results and pairwise comparisons for host and parasite localities. Overall Φ_{st} values indicate the proportion of variation attributable to host *(a)* and parasite *(b)* genotype differences between populations. Pairwise comparisons are between populations, with pairwise Φ_{st} values for cultivar below and for parasite above the diagonal. All p-values were derived by permuting genotypes among samples (100,000 permutations). Signficant pairwise Φ_{st} values ($p < 0.05$) are in bold.

AMOVA results Host Clusters	**Cultivar (host) *(a)***			***Escovopsis* (parasite) *(b)***		
	variance	d.f.	% total	variance	d.f.	% total
Between host clusters	40.4	5	54.1	1.64	5	2.53
Within host clusters	34.3	71	45.9	63.13	71	97.47
	overall Φ_{st} = 0.54, p < 0.001			**overall Φ_{st} = 0.025, p = 0.048**		

between-host cluster pairwise Φ_{st} values
Cultivar (below diagonal) & *Escovopsis* (above diagonal)

	cluster1	cluster 2	cluster 3	cluster 4	cluster 5	cluster 6
cluster1		0.04	**0.08**	0	0	0
cluster2	**0.66**		0.02	0	0.01	0.06
cluster3	**0.54**	**0.46**		0	0.03	0.08
cluster4	**0.77**	**0.71**	**0.42**		0	0
cluster5	**0.61**	**0.59**	**0.50**	**0.52**		0
cluster6	**0.60**	**0.49**	**0.43**	**0.61**	**0.49**	

Table 5.2: AMOVA results and pairwise comparisons for host genotype clusters and their associated parasites. Overall Φ_{st} values indicate the proportion of cultivar genotypic variation that is captured by assigning each host to a genotype cluster *(a)* and the proportion of *Escovopsis* genotypic variation that is captured by assigning parasites to their respective host clusters *(b)*. Pairwise comparisons below the diagonal are between each groups of cultivars assigned aposteriori to clusters, and pairwise comparisons above the diagonal are between groups of *Escovopsis* isolates assigned to their hosts' clusters. All p-values were calculated by permuting genotypes among samples (100,000 permutations). Significant pairwise Φ_{st} values ($p < 0.05$) are in bold.

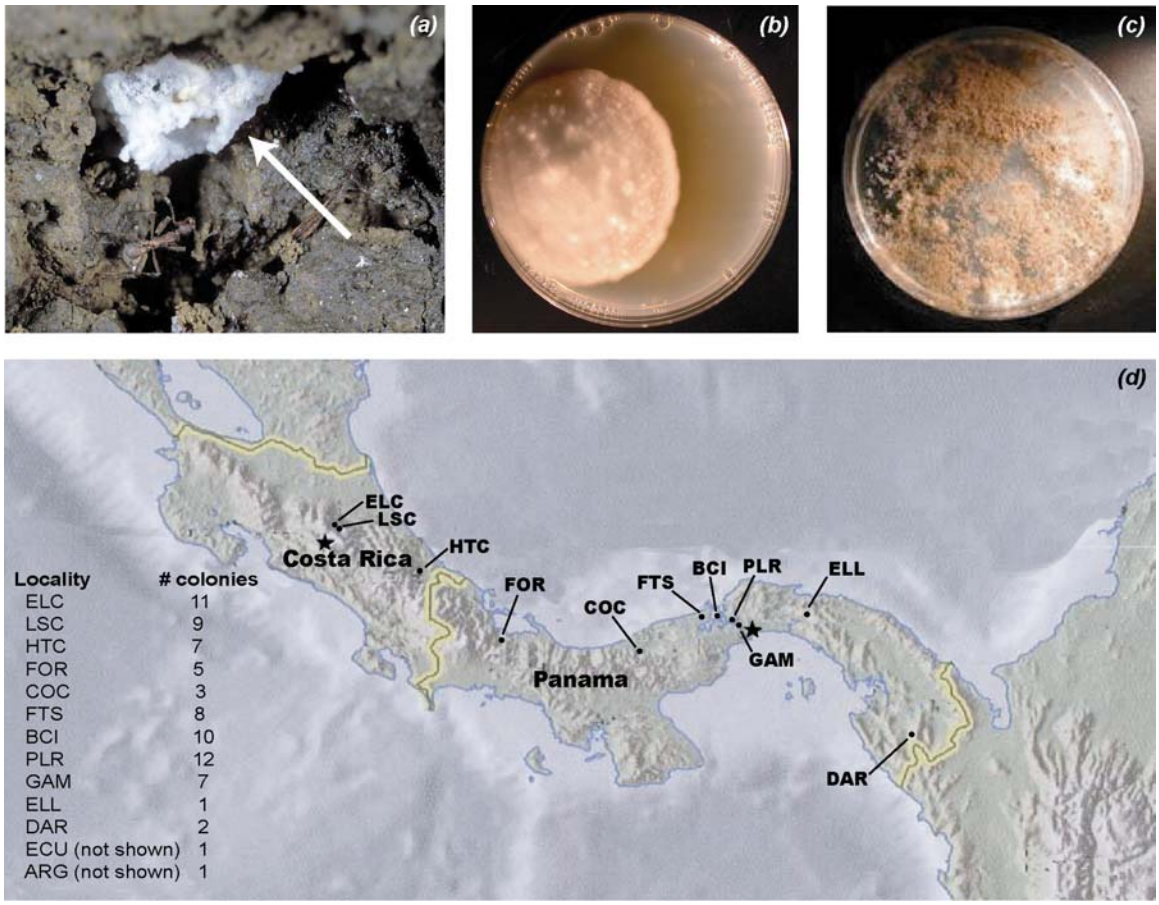

Figure 5.1: Sampling of *Apterostigma dentigerum* colonies. *(a) A. dentigerum* colonies are easily located in the field because of the conspicuous, white fungal veil (see arrow) that protects their garden. After collection for this study, gardens were sampled to obtain pure isolates of the ants cultivated fungi *(b)* and the parasitic fungus *Escovopsis (c). (d)*. Map of collecting sites. Cultivar and *Escovopsis* were collected from 77 colonies throughout Costa Rica, Panama, Ecuador and Argentina (the latter two are not shown). Stars mark the country capitals. Full locality names are in the main text.

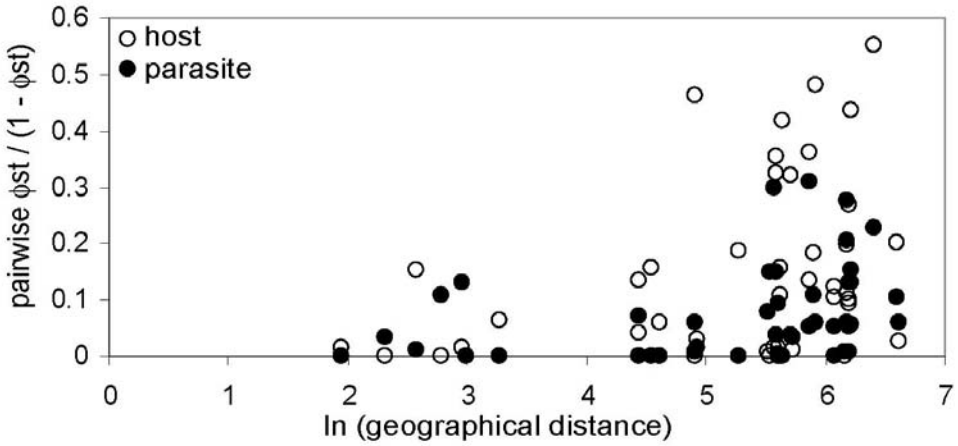

Figure 5.2: Isolation by distance. Plot of pairwise Φst / (1 – Φst) against pairwise geographical distance between each of 10 populations of hosts (open circles) and parasites (filled circles). The relationship between genetic and spatial distances was assessed using Mantel tests and is significant for both cultivars (r = 0.34, p = 0.04) and *Escovopsis* (r = 0.38, p = 0.03).

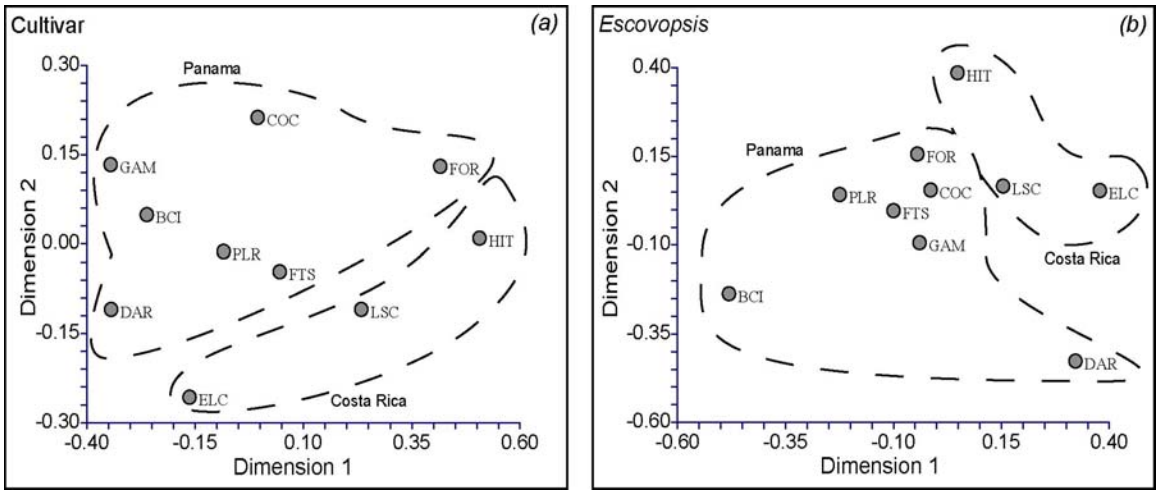

Figure 5.3: NMDS solution for localities of cultivars *(a)* and *Escovopsis (b).* For clarity, dashed lines demarcate populations in Panama and Costa Rica.

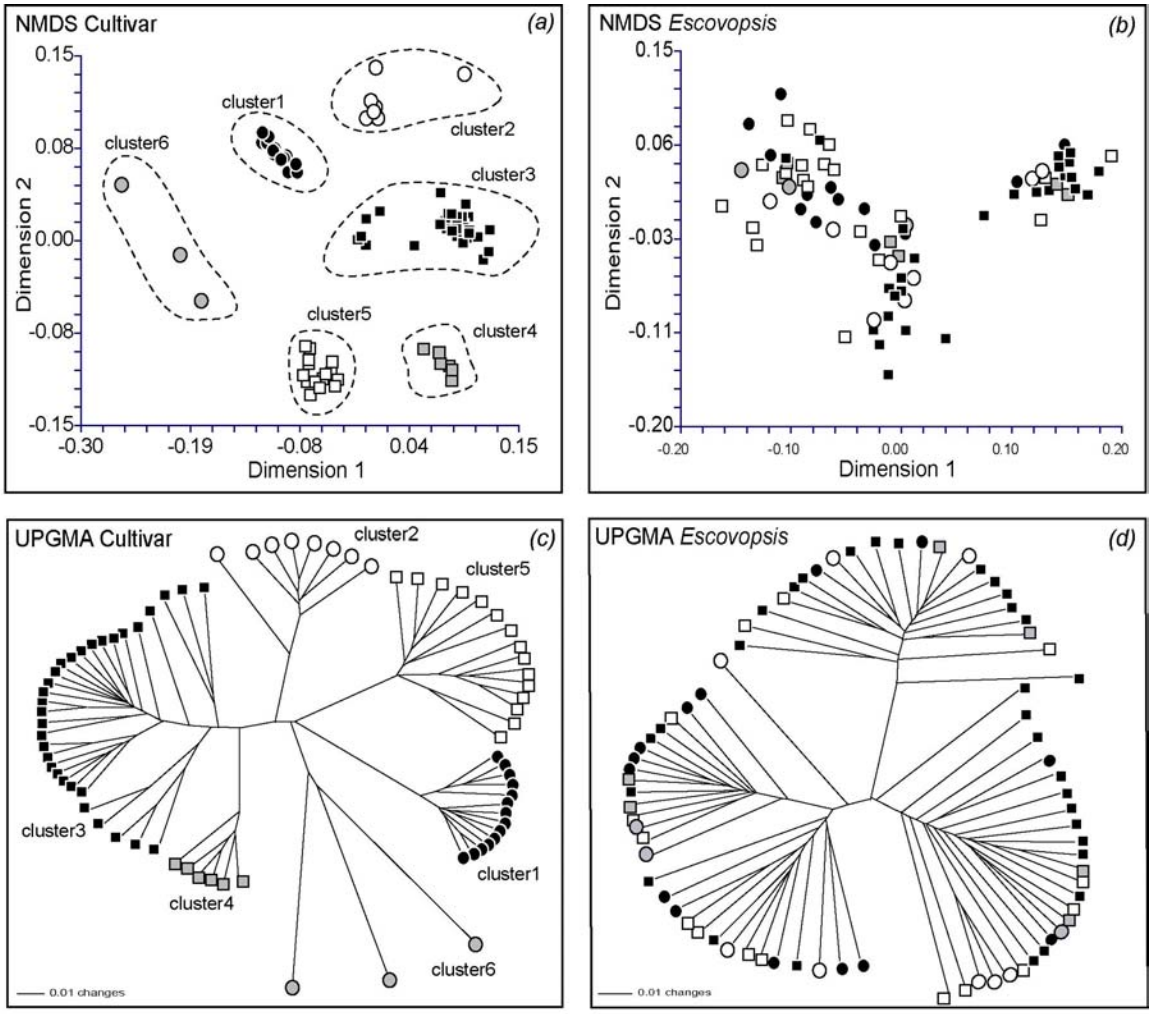

Figure 5.4: Clustering solutions for all host and parasite isolates. *(a).* NMDS solution for all cultivar isolates. Dashed lines demarcate six main host genotype clusters. Pairwise Φst comparisons indicate that all clusters are genetically differentiable (Table 5.2). *(b).* NMDS solution for all *Escovopsis* isolates. Isolates are coded by the cluster (1-6) of their associated host. *(c)*,*(d).* UPGMA dendrograms indicate similar relationships as the NMDS solutions; the same six host genotype clusters identified in *(a)* are apparent in *(c).*

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