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Specificity of the parasite *Escovopsis* among two sympatric species of *Cyphomyrmex* fungus-growing ants

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An abstract of
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Abstract

Specificity of the parasite *Escovopsis* among two sympatric species of *Cyphomyrmex* fungus-growing ants

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The fungus-growing ant system consists of four well-studied symbionts: the ants, their cultivated fungi, mutualistic antifungal-producing actinomycete bacteria, and the obligate parasite, *Escovopsis*. *Escovopsis* strains have evolved to evade the defenses of only a limited range of host cultivars. Thus, *Escovopsis* strains from one type of cultivar often cannot infect a different type of cultivar. There is a broad scale pattern of specificity between different clades of *Escovopsis* and cultivar that suggests specialization of the parasite. In this study, we focus on the microbes associated with two sympatric species of fungus growing ants, *Cyphomyrmex longiscapus* and *C. muelleri*, and their associated *Escovopsis* strains; these species of ants are closely related but have distantly related, morphologically distinct cultivar types. Previous garden infections have shown that *Escovopsis* strains from *C. muelleri* and *C. longiscapus* systems are able to occasionally infect atypical cultivars within the *Cyphomyrmex* system. To determine the potential for host switching between *C. longiscapus* and *C. muelleri* cultivars and their associated *Escovopsis* strains, we performed in vitro bioassays to examine the interactions between the cultivars and their typical *Escovopsis* strains versus their interactions with atypical *Escovopsis* strains from the other colony. Both host specialization and a potential for host-switching were demonstrated both through these in vitro bioassays and through phylogenetic analyses of *Escovopsis* isolates. Extending on previous phylogenetic analyses of *Cyphomyrmex Escovopsis*, additional sampling here supports previous findings of two main *Escovopsis* clades but suggests that one clade is less host-specific than previously assumed. Bioassays support that the two phylogenetically distinct clades of *Cyphomyrmex*-associated *Escovopsis* are generally specialized to utilize different hosts but that likelihood of a given *Escovopsis* strain being able to establish infection of a given host is dependent on genotype-genotype interactions between the host-parasite pair.

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Table of Contents

Introduction

Coevolution

Understanding interspecies interactions is a key feature in understanding evolution. Pioneered by Ehrlich and Raven (1964) in their studies of butterflies and host plants, coevolution has been defined as the "effect of two species exerting selective pressures on each other over a long period" (Combes 2001) or "the process of reciprocal, adaptive genetic change in two or more species" (Woolhouse et. al. 2002). Coevolution can apply to predator-prey, plant-herbivore, competitive, parasitic, or mutualistic interactions. For evolution to occur, these interactions require genetic variation in opposite traits of the interacting species and reciprocal effects of traits impacting fitness of the individuals (Woolhouse et. al. 2002).

The Red Queen Hypothesis, originally proposed by Leigh Van Valen (1973), suggests that there is a relationship between interacting species and each one must change to stay in the race and maintain the same adaptive quality as other competing species; thus, in a constantly changing environment, species must constantly adapt to one another to maximize fitness (Combes 2001). If one species makes an adaptive change to gain fitness over another, the second species must undergo evolutionary advances to avoid extinction. Precipitated by negative frequency-dependent selection, the coevolutionary arms race requires genotype-specific interactions; varying genotypes in species A interact differently to varying genotypes in species B (Kaltz and Shykoff 2002).

Coevolution of species depends greatly on the community context. Contrasting with a pairwise model of coevolution limited to species pairs is diffuse evolution, which takes into account the interaction of multiple species and their effect on the selection of

traits (Janzen 1980, Strauss et. al. 2005). Ecological dynamics such as species proximity, motility, and coinciding life cycles greatly affect opportunities for local adaptation and speciation.

Host-Parasite Coevolution

A parasite is defined as "an organism living in or on another living organism, obtaining from it part or all of its organic nutriment, commonly exhibiting some degree of adaptive structural modification, and causing some degree of real damage to its host" (Price 1977). Effective parasitization is dependent on physical proximity of host and parasite and corresponding behaviors so that parasite and host come into contact. The host must also be able to provide to the parasite adequate nutrition and cannot have effective host defenses against the parasite. Parasites face the challenge of optimizing virulence such that the benefits and costs are balanced; a parasite must infect hosts but also maintain them as a resource (Combes 2001). Parasites can be transmitted horizontally, between unrelated individuals, or vertically, from mother to offspring. Vertical transmission is likely to reduce virulence, compared to horizontal transmission, because survival of the parasite by transmission to the host's offspring is dependent on survival and reproduction of the host. Spatial structuring of host populations, polymorphism in host resistance, host resilience, and population size also impose selection on virulence in pathogens (Galvani 2003).

Generalist parasites infect several host species while specialist parasites infect only one host leading to the evolution of narrow host ranges (Morand et. al. 2002). There are benefits and trade-offs to both of these parasite types: generalists have a broader

chance of infection, but often cannot achieve optimum virulence due to the wide array of host genotypes. In contrast, specialists limit their opportunities for infection specializing on specific host genotypes to achieve optimum virulence. The process of parasite specificity is largely influenced by effective transmission strategies that increase contact between the parasite and new hosts allowing for a greater host range and high genetic variability. Short generation times result in a more effective specialized virulence by increasing the opportunity for beneficial recombination (Pederson et al 2005). Ecological and phenotypic diversity within a parasite taxon are dependent on the diversity of hosts, size of host target, evolutionary time scale, and mobility of hosts (Price 1977).

Although specialization in parasites occurs, it is always advantageous to the parasite to be able to switch hosts. A parasite must live in the same environment as a potential host and overcome this host's defenses for a host switch to occur. Waldenstrom et al. (2002) demonstrated that, contrary to previous beliefs, host sharing between different songbird species is common among various haemospiridian parasitic lineages. Furthermore, transmission of parasites was common between distantly related host bird species. Long-distance host switches between avian and mammalian hosts have also been demonstrated in schistosomes; the driving mechanism of this interaction is attributed to the maintenance of an ancestral trait of nonspecific immune evasion strategy (Brant and Loker, 2005). Thus, for host-switching to occur, a parasite must be within the same ecological context as the novel host and both parasite and host must be intrinsically suitable (i.e.: physiology, biology, and behavior) for parasite infection and transmission (Pearlman and Jaenike 2003).

There are a variety of factors motivating host-parasite coevolution, but the driving influence is selective pressure from a host's immunity (Wilson et. al. 2005). Complex host defenses result in extreme parasite specialization to a specific host and this process drives coevolution and often cospeciation: as a host evolves effective resistance, the parasite must quickly modify its virulence to overcome the host's resistance strategies. The "gene-for-gene" hypothesis states that for every resistance gene in a host there is a corresponding virulence gene in the parasite and different host genotypes react differently to different parasite genotypes (Webster and Woolhouse 1998, Kaltz and Shykoff 2002). This hypothesis helps explain why parasites carrying more virulent alleles are better suited to infection of hosts and why hosts carrying higher resistance alleles are better at avoiding infection (Agrawal and Lively 2002). Thus, in the host-parasite arms race, gene flow is a critical process necessary for introducing novel resistance or virulence alleles into a population (Kaltz and Shykoff 1998). Because host-parasite coevolution is driven by negative frequency-dependent selection, rare genotypes, both parasite and host, are at an advantage (Schulenburg and Ewbank 2004). Gene flow is expected to be low in specialized host-parasite populations because limited gene flow in parasite populations makes them more likely to adapt and speciate rather than attempt a generalist pattern. Additionally, limited gene flow in host populations allows for a lower chance of evolving defenses to overcome the parasite (Tripet et. al. 2002).

Similar to costs of virulence, there are costs to resistance in hosts. In the absence of infection, energy spent on maintaining resistance strategies may result in reduced livelihood, reduced clutches, reduced competitive potential, and increased vulnerability to other diseases (Woolhouse et. al. 2002). The costs associated with both high virulence

and high resistance contribute to the maintenance of variation within and between populations and polymorphism in genes that specify both virulence in parasites and defensive adaptations (Haldane 1949).

Fungus-Growing Ants

Originating about 50 million years ago, fungal cultivation by ants arose only once in evolutionary history, with over 200 known at species in a monophyletic group (tribe Attini). These attine ants have evolved as obligate fungus farmers and propagate their fungal cultivars vertically between nests (Rindos 1984, Mueller 1998). The majority of attine ants cultivate fungi in the family Lepiotaceae as mycelium; the fungus is cultivated with flower parts, insect debris, wood fragments, and other plant debris (Mueller 1998, 2005). Each clade of fungus-growing ants cultivates a specific fungal cultivar. The fungus-growing ant system consists of four well-studied symbionts: the ants, their cultivar fungus, mutualistic antifungal-producing actinomycete bacteria, and the obligate fungal parasite, *Escovopsis*. In addition to these components, ant gardens also contain bacteria-inhibiting black yeast (Little and Currie 2007) and nitrogen-producing bacteria (Currie et, al. 1999). *Escovopsis* hinders the vitality of the ant colony by directly attacking and consuming the ants' fungal cultivar, the colony's primary food source (Reynolds and Currie 2004). Because the ants depend so heavily on their cultivar, infection by *Escovopsis* indirectly diminishes colony survival and reproduction (Currie 2001). Ants manage the actinomycete bacteria specifically for the suppression of infection by the parasitic *Escovopsis*; in addition the ants actively eliminate wastes from their garden to avoid infection (Currie and Stuart 2001).

Phylogenetic studies have shown that specific clades of *Escovopsis* infect specific clades of cultivar; *Escovopsis* tracks the cultivar type rather than the ant species (Figure 1) (Gerardo et al. 2004). *Escovopsis* strains have evolved to evade the defenses of only a limited range of host gardens. Thus, *Escovopsis* strains from one type of cultivar often cannot infect a different type of cultivar. There is a broad scale pattern of specificity between different clades of *Escovopsis* and cultivar that suggests specialization of the parasite (Gerardo et. al. in prep). *Acromyrmex* and *Apterostigma Escovopsis* strains generally infect only their respective host cultivars, but can occasionally infect a cultivar from a different clade (Figure 2).

Phylogenetic congruence has been shown in the *Apterostigma* genus of fungus-growing ants. Specificity in this system is such that *Apterostigma* cultivars are only able to defend themselves against the *Escovopsis* type associated with their colonies in nature; cultivars were unable to defend themselves against novel *Escovopsis* strains isolated from another species of *Apterostigma*. In addition, closely related *Escovopsis* strains infected closely related cultivar hosts and likewise, genetically similar cultivar strains are able to inhibit the same *Escovopsis* strain (Gerardo et al. 2006a, 2006b).

The evolutionary histories of the ants, their cultivar, and the different *Escovopsis* strains associated with each species are highly congruent suggesting that this system has been heavily influenced by coevolution (Taerum et al. 2007). *Escovopsis* can identify chemical signals in the cultivar and are attracted to their hosts by chemotaxis. As a defense mechanism, the cultivar secretes unidentified compounds that can suppress *Escovopsis*. Both of these chemical cues are likely specific between parasite-host pairs. Coupled with this chemical specificity, ant behavior and actinomycetous bacteria

associated with the ants are the driving forces and result of coevolution between *Escovopsis* and the ants' fungal cultivars (Gerardo et al. 2006, Taerum et al. 2007).

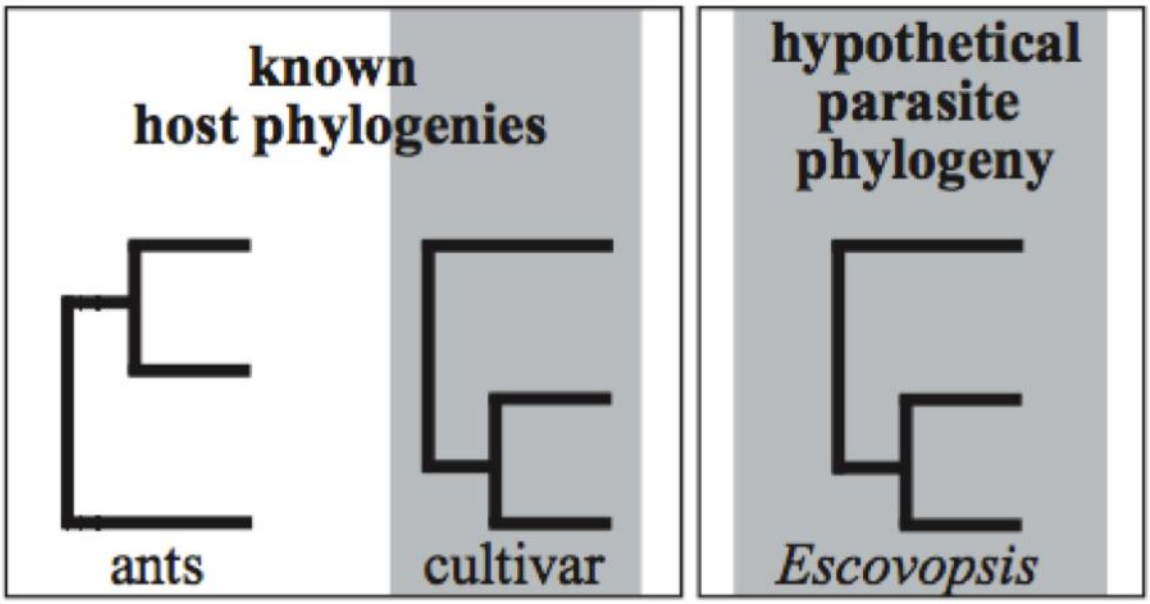

Figure 1: The parasite *Escovopsis* is specialized to the ant's fungal cultivar, not the ants. This specialization may be motivated by parasite recognition and attraction to a limited range of host cultivars or the ability to breakdown a narrow range of cultivars for food. Alternatively, specialization may be driven by the host's ability to recognize and suppress a narrow range of *Escovopsis* types (Adapted from Gerardo et. al. 2004).

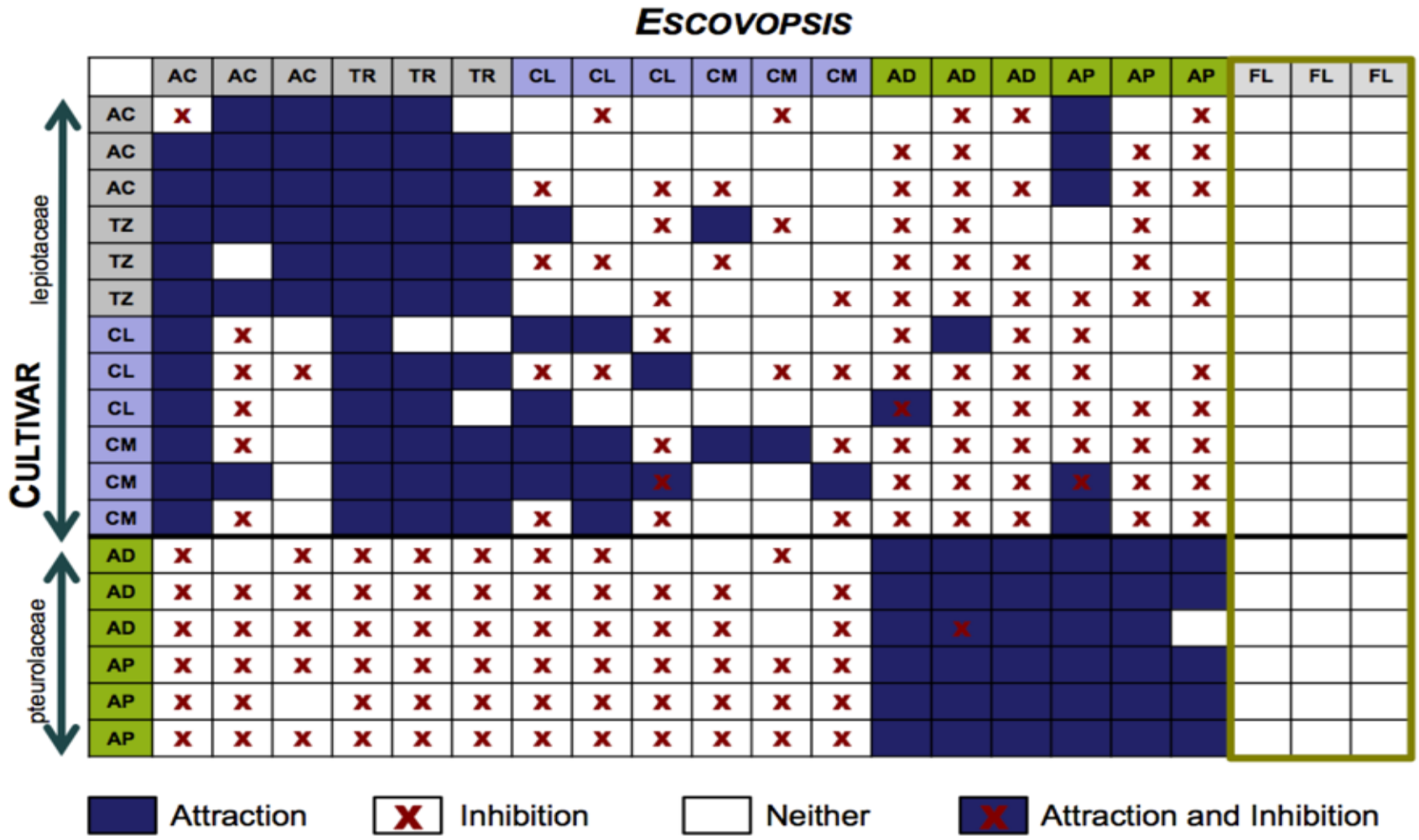

Figure 2: This figure shows interactions between various clades of *Escovopsis* and cultivar (AC=*Acromyrmex*, TR=*Trachymyrmex*, CL=*C. longiscapus*, CM=*C. muelleri*, AD=*Apterostigma dentigerum*, AP=*Apterostigma pilosum*. There is broad specificity between associated clades of *Escovopsis* and cultivar, but occasional infection of novel cultivars occurs. There is much more variation between the interactions of *C. muelleri* and *C. longiscapus Escovopsis* and cultivars (taken from Gerardo et al. in prep).

The Cyphomyrmex System

In this study, we focus on the microbes associated with two sympatric species of fungus growing ants, *Cyphomyrmex longiscapus* and *C. muelleri*, and their associated *Escovopsis* strains (Fig. 3). These species of ants are closely related but have distantly related, morphologically distinct cultivar types (Mueller et al. 1998); *C. longiscapus* and *C. muelleri* colonies are also associated with genetically distant *Escovopsis* strains (Gerardo et al. 2004). Gerardo et al. (2004) examined cultivar-*Escovopsis* specificity between *C. longiscapus*, *C. muelleri*, and *C. costatus*. The last is a more distantly related ant to the other *Cyphomyrmex* species that grows a cultivar closely related to *C. muelleri* cultivar. Additionally, an *Escovopsis* similar to that of *C. muelleri* infects *C. costatus* cultivar (Fig. 3). *C. longiscapus* and *C. muelleri* colonies are found on steep, clay riverbanks, often less than a meter apart, while *C. costatus* colonies are found under rocks and logs; *C. longiscapus* and *C. muelleri* colonies have wide niche overlap, but their niches never overlap with that of *C. costatus* (Green et. al. 2002).

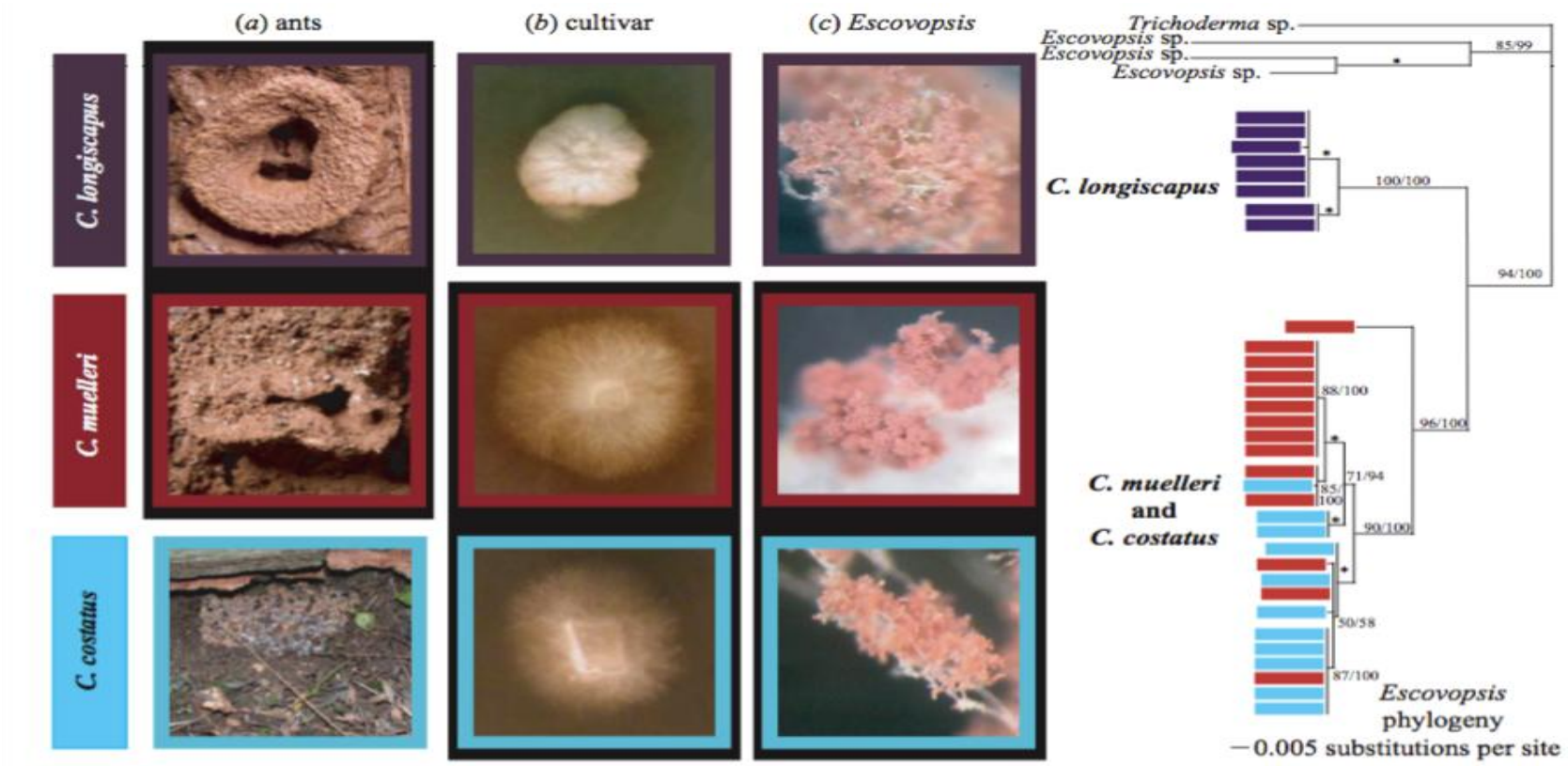

Figure 3: "Relationships between the symbionts in the *Cyphomyrmex* system. (a) Cyphomyrmex longiscapus and C. muelleri are closely related ant species with similar nest architectures (nests in black box) whereas C. costatus is a more distantly related ant species with larger colonies. (b) Cyphomyrmex 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, but EF-1 alpha 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. The support values are listed above the branches (likelihood support/Bayesian posterior probability) for branches with more than 50% likelihood support. An asterisk indicates branches for which both support values are greater than 95" (Taken from Gerardo et. al. 2004).

Using fungal garden piece infection assays, Gerardo et al. (2004) demonstrated that between cultivars from *C. longiscapus*, *C. muelleri*, and *C. costatus*, none could defend themselves against all introduced *Escovopsis* types. In this study, *C. longiscapus* cultivar was infected less often by *C. muelleri* and *C. costatus Escovopsis* strains than *C. muelleri* and *C. costatus* cultivars by *C. longiscapus* strains. This suggests that the *Escovopsis* associated with *C. longiscapus* colonies may be more virulent or that *C. muelleri* and *C. costatus* colonies have other defenses against parasitism in addition to antibiotics secreted by the cultivar itself. Alternatively, all three cultivars could be at different stages in the host-parasite arms-race cycle. Unlike the *Apterostigma* system, host switching seems to be an active process between these *Cyphomyrmex* species.

If host switching is indeed occurring between these two sympatric *Cyphomyrmex* species, there will be variation in the infection ability of each *Escovopsis* strain: some *C. longiscapus Escovopsis* strains will be able to attack some *C. longiscapus* and *C. muelleri* cultivars but not others, and some C. *muelleri Escovopsis* will be able to attack some *C. muelleri* and *C. longiscapus* cultivars but not others. To determine the potential for host switching between *C. longiscapus* and *C. muelleri* cultivars and their associated *Escovopsis* strains, we performed in vitro bioassays to examine the interactions between the cultivars and their typical *Escovopsis* strains versus their interactions with atypical *Escovopsis* strains from the other colony. We also infected *C. longiscapus* and *C. muelleri* garden pieces with *C. longiscapus, C. muelleri, Apterostigma dentigerum*, and *Acromyrmex octospinosus Escovopsis* strains. *A. dentigerum* and *Acro. octospinosus Escovopsis* strains are not found in *C. longiscapus* and *C. muelleri* colonies in nature and

should not successfully infect these garden pieces. In addition, we analyzed sequence data from *Escovopsis* strains used in the bioassays to determine the relationship between a strain's ability to infect a novel cultivar and its genetic similarity to other strains associated with the novel cultivar.

Methods

Collections

C. muelleri and *C. longiscapus* colonies are widely found on steep clay banks of rivers and streams in the tropics. We collected *C. muelleri* and *C. longiscapus* colonies in late March and early April of 2009 from the Pipeline Road area of Gamboa, Panama. Cultivars and *Escovopsis* strains were isolated from these collections and maintained as live cultures on potato dextrose agar (PDA) with 50mg/L each of penicillin and streptomycin. Cultivar and *Escovopsis* strains from collections at the Smithsonian Institute, the University of Texas at Austin, and the University of Wisconsin were also used in bioassays; these were provided as samples stored in glycerol or on PDA plates. These samples were collected from March to November 2001, May and June 2002, and January to April 2003 (Appendix A, B). Collections used for our bioassays are representative of the genetic diversity present in the microbes associated with both species of *Cyphomyrmex*.

Cross-Infection Bioassays

To examine the interaction between *Escovopsis* on natural and novel hosts, we grew each cultivar [*C. longiscapus* (22 strains), *C. muelleri* (13), and *C. costatus* (5)] on

the edge of 100mm petri dishes of PDA with the antibiotics described above. Cultivars were allowed to grow 7-14 days and then each *Escovopsis* strain [*C. muelleri* (9 strains) and *C. longiscapus* (8)] was grown in the middle of each plate (Fig. 4). Plates were monitored daily and scored for attraction if the *Escovopsis* grew towards and overgrew the cultivar or inhibition if the *Escovopsis* grew around but not over the cultivar (Fig. 5). If the *Escovopsis* did not grow directly towards the cultivar but still overgrew it, the bioassay was scored as neither attraction nor inhibition. Additionally, bioassays were scored if there appeared to be attraction that later resulted in inhibition or vice versa. We switched the orientation of cultivar and *Escovopsis*, cultivar on the edge and *Escovopsis* in the middle, to ensure that our bioassay results were repeatable. To determine if there were significant differences between *Escovopsis* and cultivar interactions based on colony-type of origin (e.g., are *Escovopsis* from *C. muelleri* colonies different from *Escovopsis* from *C. longiscapus* colonies in their interaction with cultivars from each colony type?), we performed analyses in R version 2.4.0 using generalized linear models (GLM). Proportions of attraction and inhibition between each cultivar-*Escovopsis* combination were analyzed separately using two generalized linear models with quasibinomial error distributions. Models were checked for normality of error distributions and homogeneity of variance.

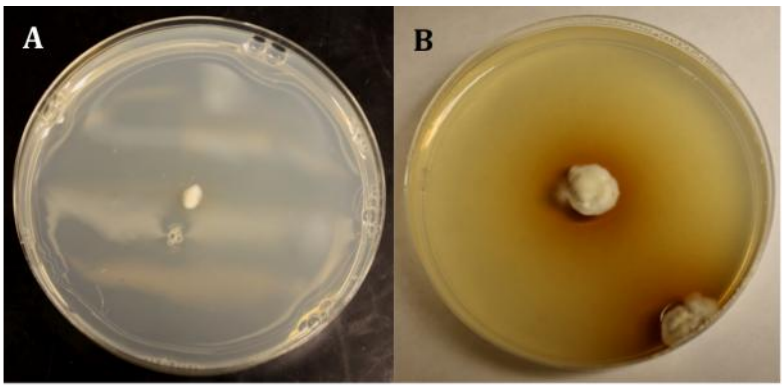

Figure 4: Bioassay setup: A) cultivar piece in middle of PDA B) *Escovopsis* on the edge of the plate.

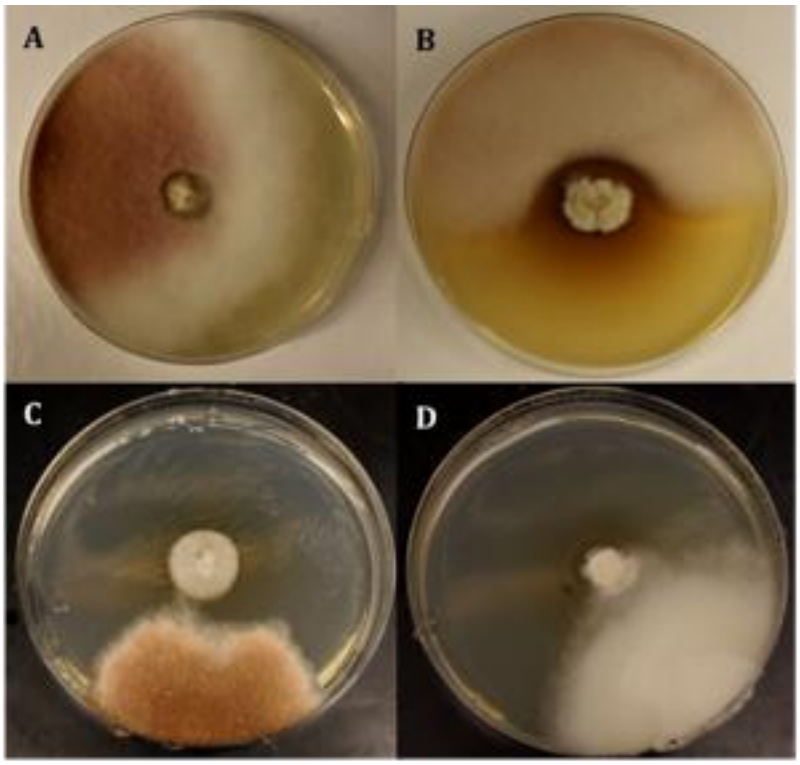

Figure 5: A, B) Inhibition of *Escovopsis* (growth from edge) by cultivar (middle). C, D) Attraction of *Escovopsis* (growth from edge) by cultivar (middle).

Garden Infections

We traveled to the Smithsonian Tropical Research Institute in Gamboa, Panama in December 2009-January 2010. Ant colonies were collected from the Pipeline Road area in Gamboa. Garden infections were performed for each *C. longiscapus* and *C. muelleri* cultivars crossed with *Apterostigma dentigerum*, *Acromyrmex octospinosus*, *C. longiscapus*, and *C. muelleri* associated *Escovopsis* strains. We performed garden infections by placing a 1-2mm piece of *Escovopsis* on a 6cm petri dish filled with dampened plaster of paris; a 5mm-1cm fragment of cultivar garden was placed on top of the *Escovopsis*. A 5mm-1cm fragment of cultivar garden without *Escovopsis* served as the control. Ten *C. longiscapus* colonies were infected with *A. dentigerum* and *A. octospinosus Escovopsis* strains. We also randomly assigned three different *C. longiscapus* and *C. muelleri Escovopsis* strains to infect 16 *C. longiscapus* and 8 *C. muelleri* colonies (Appendix C. D). Dishes were monitored each day and *Escovopsis* growth over the cultivar was recorded.

DNA Extractions and Sequencing

The animal tissue spin-column protocol in the Qiagen DNA Extraction kit was used to extract DNA from the mycelium and spores of 21 *Escovopsis* strains used in bioassays. PCR was performed with primers EF1-983F (50 – GCYCCYGGHCAYCGTGAYTTYAT-30) and EF1-2218 (50 - ATGACACCRACRGCRACRGTYTG-30) spanning a single exon and primers EF1-3f (50 -CACGTCGACTCCGGCAAGTC-30) and EF1-5r1 (50 - GTGATACCACGCTCACGCTC-30) spanning three exons and two introns. Four exons

and two introns of nuclear elongation factor-1 alpha (EF-1 alpha) were sequenced from each of the following *Escovopsis* strains used in the in vitro bioassays described above: SB090329-14, SB090401-18, SB090402-08, SB090402-17, UGM010407-05, SB090327-07, SB090401-17, CC030402-06, CC010324-01, UGM010407-02, CC030105-13, FS4, UGM010407-16. Sequences for the following strains were downloaded from genbank (accession numbers AY629361–AY629398): NMG011105-03, NMG011105-06, UGM010407-11, UGM010407-12. The following strains could not be sequenced and thus not included in our results: UGM010407-15, UGM010407-27, CC030403-01.

Sequences were assembled using SEQMAN II v. 5.05 (DNASTAR), aligned using CLUSTALW WWW (http://www.ebi.ac.uk/clustalw) and edited manually in Maclade v. 4.06 (Maddison and Maddison 2003). The aligned sequences were analyzed with Mr. Bayes using the general time reversible (GTR) sequence-evolution model with four Γ-distributed rate classes and a proportion of invariant sites (PINVAR). The analysis combined two runs of 1,000,000 generations each, with sampling every 500 generations (burnin = 250,000 generations).

Comparison of Bioassay Results and DNA Sequences

To determine whether the variation in interactions from our bioassays was associated with genetic differences of the *Escovopsis* strains, we conducted a Mantel test to look for a correlation between various distance matrices. Correlations with genetic distance would indicate parasite specialization on a particular host genotype is reflected in neutral genetic differentiation between strains. An interaction distance matrix was constructed to quantify the relationship between 17 *Escovopsis* strains and their

interactions with 40 cultivars. Each bioassay difference ranged from 0 to 1 and increased .5 for each case in which the two *Escovopsis* strains had a different inhibition result with the same cultivar strain. A second matrix comparing mean genetic distances was constructed using PAUP*. We then used ZT (http://ideas.repec.org/a/jss/jstsof/07i10.html) to conduct a simple Mantel test with 1,000,000 randomizations to examine the correlation between the *Escovopsis* bioassay distances and *Escovopsis* genetic distances.

Results

Cross-Infection Bioassays

Bioassay results demonstrated variation between cultivar isolates in their susceptibility to *Escovopsis*, and variation in *Escovopsis* in the infectivity of cultivars. Both *Escovopsis* strains from *C. longiscapus* and *C. muelleri* colonies occasionally were able to use cultivar strains from each colony type. Bioassay results were highly repeatable regardless of the orientation of the cultivar and parasite strains on the plate; of 57 cultivar-Escovopsis strains combinations that were repeated with the cultivar both in the middle of the plate (and *Escovopsis* on edge) and *Escovopsis* in the middle of the plate (and cultivar on edge), 48 were scored the same in both trials.

C. muelleri Escovopsis strains were generally attracted to *C. muelleri* and *C. costatus* cultivars and rarely inhibited by these cultivars; these strains were also generally inhibited by *C. longiscapus* cultivars, but there were occasional occurrences of attraction. Each of the *C. muelleri Escovopsis* strains showed attraction towards at least one *C. longiscapus* cultivar (Fig. 6). Two *C. longiscapus* cultivars were infected by every *C. muelleri Escovopsis*. In addition, *C. muelleri Escovopsis* was able to infect all of the *C. costatus* cultivars. Four *C. muelleri Escovopsis* strains (SB090329-14, SB090401-18, SB090402-08, SB090402-17) were better able to infect novel *C. longiscapus* cultivars than the others: 62 non-inhibition interactions, i.e. attraction, both inhibition and attraction, and neither inhibition nor attraction, out of 82 total interactions (75.6%) as compared to 23 non-inhibition interactions out of 110 total (20.9%). Most of the variation within *Escovopsis* strains was driven by the type of cultivar, *C. muelleri* vs. *C. longiscapus*, but there was some variation within these groups as well.

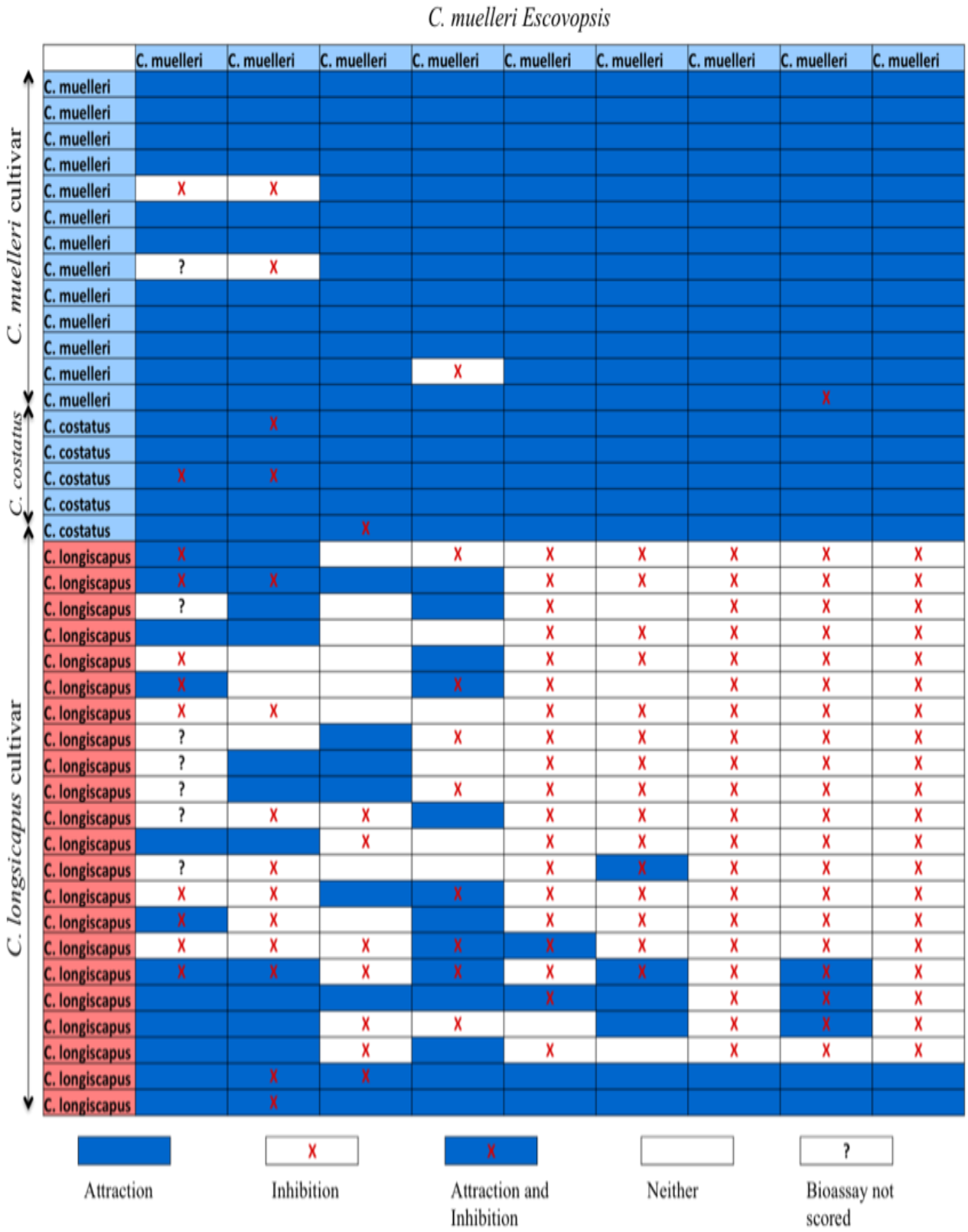

Figure 6: Interactions between *C. muelleri Escovopsis* and each type of cultivar (top to bottom): *C. muelleri*, *C. costatus*, and *C. longiscapus*.

There was more variation in bioassay results among *C. longiscapus Escovopsis* strains (Figure 7). Five of the 8 *C. longiscapus Escovopsis* strains (CC030402-06, CC010324-01, UGM010407-02, FS4, UGM010407-16) showed interactions with the three different cultivars more characteristic of *C. muelleri Escovopsis* with attraction of *C. muelleri* and *C. costatus* cultivars and vast inhibition of *C. longiscapus* cultivars. Two *C. longiscapus Escovopsis* strains (SB090401-17, CC030105-13) demonstrated inhibition from *C. muelleri* cultivars with a few instances of attraction or both inhibition and attraction. These two strains also exhibited all four interactions characteristics toward *C. longiscapus* cultivars: attraction (17 out of 43 total interactions, 39.5%), neither attraction nor inhibition (20, 46.5%), both attraction and inhibition (2, 4.6%), inhibition 5, 11.6%). While both of these *C. longiscapus Escovopsis* strains were inhibited by *C. muelleri* and *C. costatus* cultivars, strain CC030105-13 vastly demonstrated neither inhibition nor attraction by the *Escovopsis* and but simply grew over *C. longiscapus* cultivars instead of being attracted towards them. One *C. longiscapus Escovopsis* strain (SB090327-07) showed wide attraction among all cultivar types with inhibition (5 out of 40 total interactions, 12.5%) only from *C. longiscapus* cultivars.

Overall, *C. muelleri Escovopsis* strains infected more *C. muelleri* and *C. costatus* cultivar than *C. longsicapus Escovopsis* strains (Fig. 8). Additionally, *C. muelleri Escovopsis* was less effective at infecting *C. longiscapus* cultivars than its typical *C. muelleri* and *C. costatus* hosts. The interactions of *C. longiscapus Escovopsis* strains showed more variation in that this strain had near equal infection of all cultivar types.

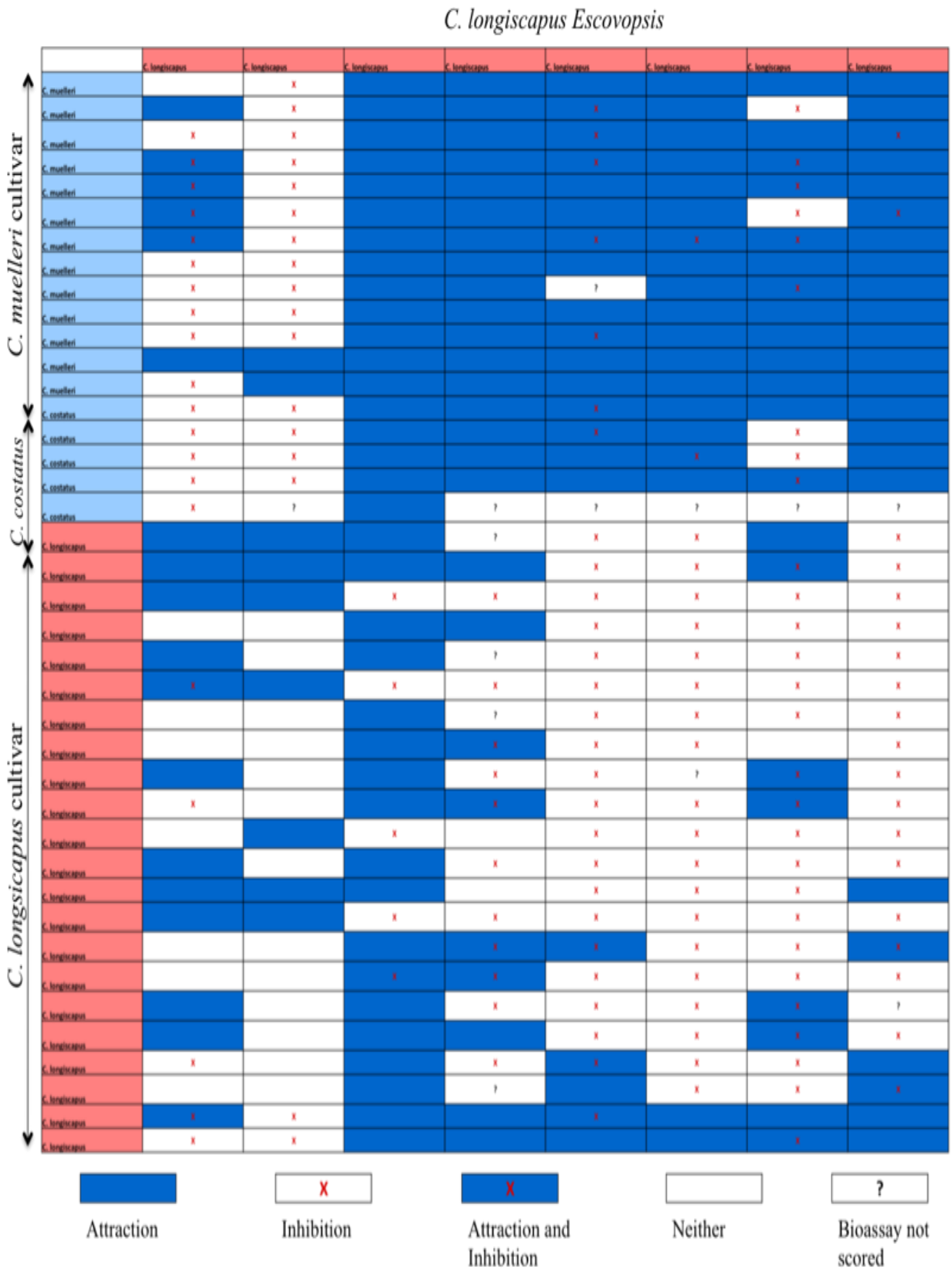

Figure 7: Interactions between *C. longiscapus Escovopsis* and each type of cultivar (top to bottom): *C. muelleri*, *C. costatus*, and *C. longiscapus*.

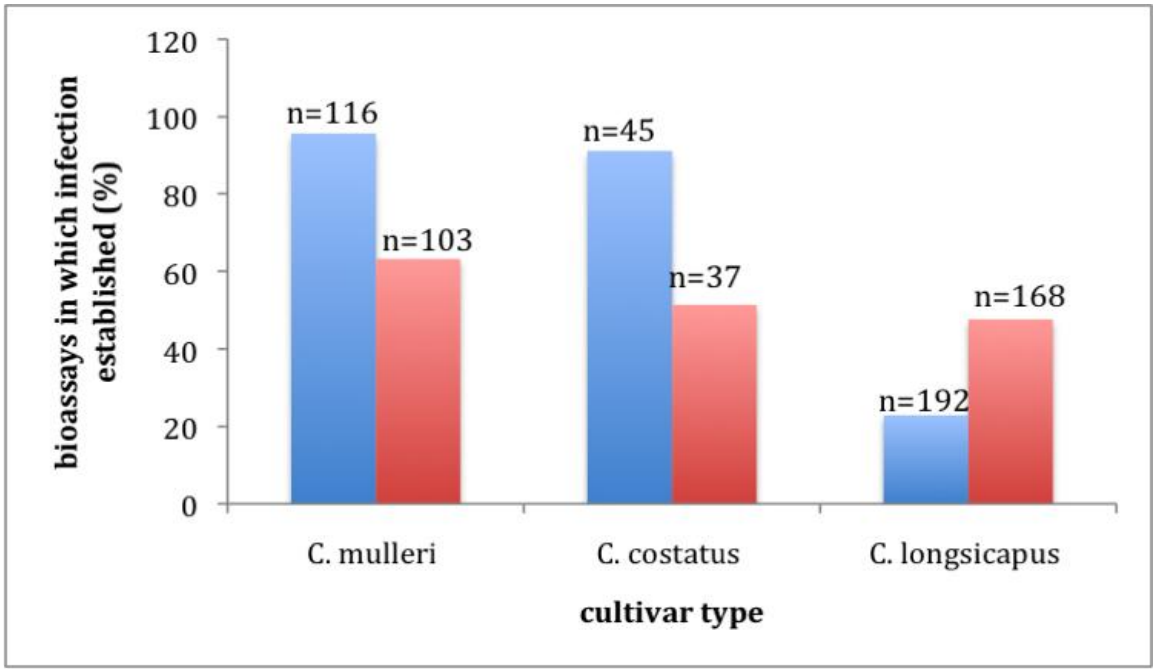

Figure 8: *Escovopsis* infection rates in cross-infection bioassays. The graph indicates the percentage of cultivars of a given type in which *C. muelleri Escovopsis* (blue) and *C. longiscapus Escovopsis* (red) succeeded in infection.

For attraction, cultivar type (i.e., *C. muelleri* vs. *C. longiscapus*) but not *Escovopsis* type had a significant effect (cultivar type: p = .005; Escovopsis type: p = 0.72) on overall bioassay results. Comparison of reduced models suggested that there was a significant cultivar type by *Escovopsis* type interaction ((F (1,30) p = .04). Both *C. muelleri* and *C. longiscapus Escovopsis* were more attracted to *C. muelleri* than *C. longiscapus* colonies (Fig. 9). For inhibition, there was neither a significant effect of cultivar type (p = 0.14) nor *Escovopsis* type (p = 0.59), but there was a significant interaction between cultivar type and *Escovopsis* type (F(1,30), p = 0.02). Both *C. muelleri* and *C. longiscapus* derived *Escovopsis* were more likely to be inhibited by *C.*

longiscapus cultivars, but the cultivar type effect was much stronger for *C. muelleri Escovopsis* (Fig. 10).

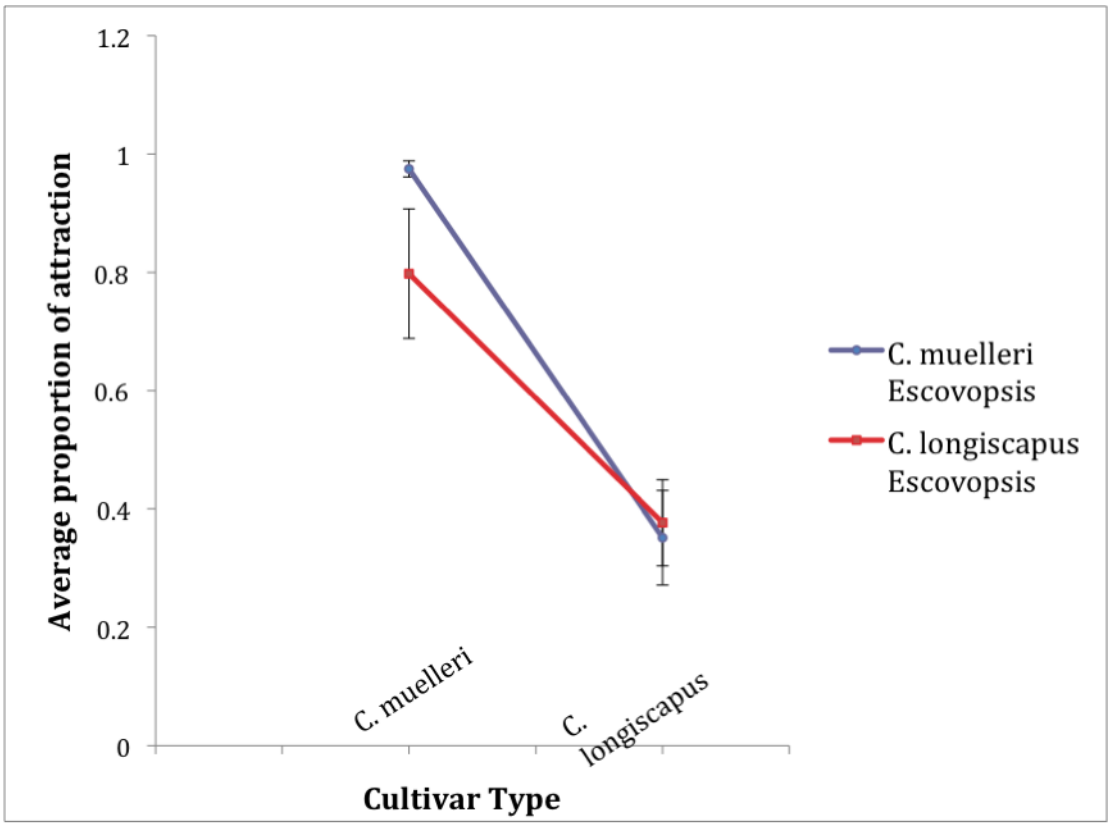

Figure 9: This chart demonstrates a significant interaction between cultivar type and proportion of attraction for each *Escovopsis* type. Both *C. muelleri* and *C. longiscapus Escovopsis* strains were more effective at infecting *C. muelleri* cultivars, but there was not a significant interaction demonstrating a difference between attraction proportions for each *Escovopsis* type. Error bars represent SEM.

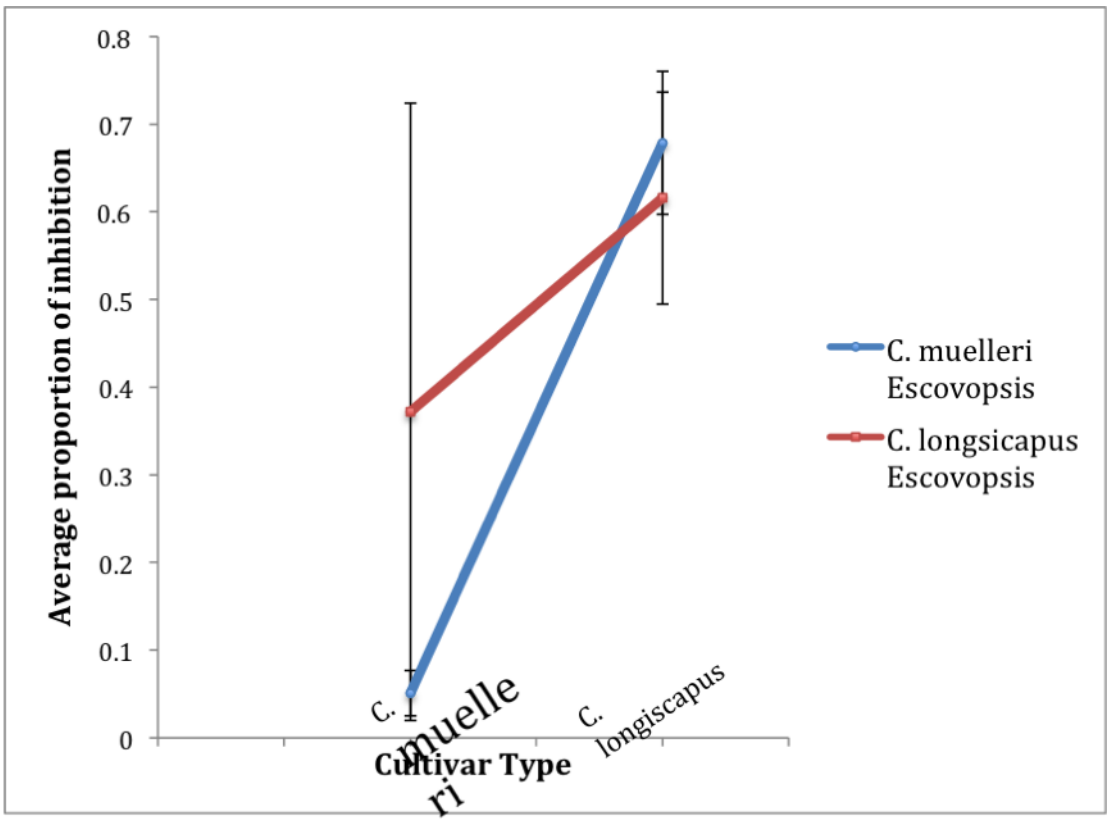

Figure 10: This chart demonstrates a significant interaction between cultivar type and proportion of inhibition for each *Escovopsis* type. Both *C. muelleri* and *C. longiscapus Escovopsis* strains were inhibited most by *C.* longiscapus cultivars, but there was not a significant interaction demonstrating a difference between inhibition proportions for each *Escovopsis* type. Error bars represent SEM.

Garden Infections

Of the 10 *C. longiscapus* gardens used for infection with *A. dentigerum* and *A. octospinosus Escovopsis* strains, only one garden showed a positive control indicating that this colony was already infected with *Escovopsis* (Fig. 11). Excluding this colony, two gardens were infected by *A. dentigerum Escovopsis* and two different gardens were infected by *A. octospinosus Escovopsis* (Fig. 13).

Colony	Control	AD Escovopsis	AO Escovopsis
CL1	N	N	N
CL2	N		N
CL3	N	N	N
CL4	N	N	N
CL5	N	N	
CL6	N		N
CL7			
CL8	N	N	
CL9	N	N	N
CL10	N	N	N

Escovopsis growth over garden

N

No *Escovopsis* growth

Figure 11: Infection of *C. longiscapus* (CL) gardens with *A. dentigerum* (AD) and *A. octospinosus* (AO) *Escovopsis*.

None of the *C. longiscapus* or *C. muelleri* gardens used for infection with *C. longiscapus* and *C. muelleri Escovopsis* strains showed initial infection with *Escovopsis* (Fig. 12). Four of the 16 *C. longiscapus* gardens demonstrated infection with *C. longiscapus Escovopsis* while another four gardens demonstrated infection with *C. muelleri Escovopsis*; none of the gardens were infected by both *Escovopsis* types. Five of the 8 *C. muelleri* gardens were infected by *C. muelleri Escovopsis* while only three gardens were infected by *C. longiscapus Escovopsis* (Fig. 13). Only one garden was infected by both *Escovopsis* types .

Colony	Control	CL Escovopsis	CM Escovopsis
CL1	N	N	N
CL2	N	N	
CL3	N	N	
CL4	N	N	N
CL5	N	N	
CL6	N		N
CL7	N	N	N
CL8	N	N	N
CL9	N		N
CL10	N	N	N
CL11	N	N	
CL12	N	N	N
CL13	N		N
CL14	N	N	N
CL15	N	N	N
CL16	N		N
CM1	N		
CM2	N	N	
CM3	N		N
CM4	N	N	
CM5	N	N	
CM6	N		N
CM7	N	N	N
CM8	N	N	

Escovopsis growth over garden	N: No *Escovopsis* growth

Figure 12: Infection of *C. longiscapus* (CL) and *C. muelleri* (CM) gardens with *C. longiscapus* and *C. muelleri Escovopsis*.

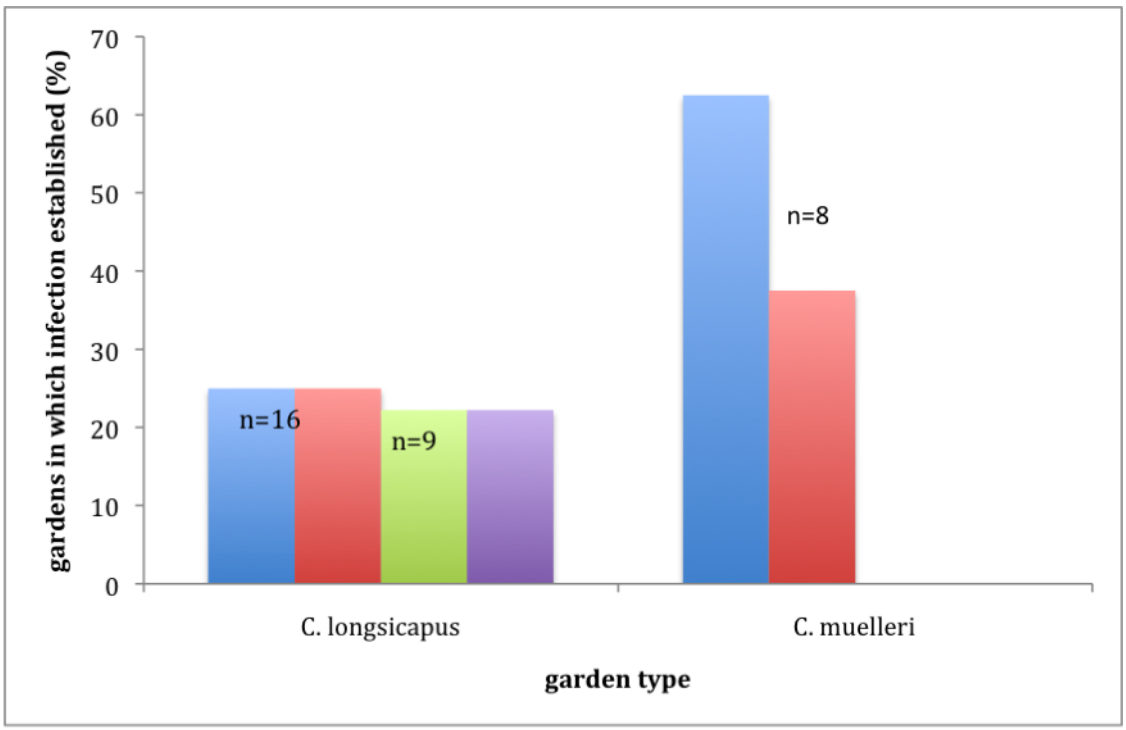

Figure 13: *Escovopsis* infection rates in garden infections. The graph indicates the percentage of gardens of a given type in which *C. muelleri Escovopsis* (blue), *C. longiscapus Escovopsis* (red), *A. dentigerum Escovopsis* (green), and *A. octospinosus Escovopsis* (purple) succeeded in infection.

DNA Extractions and Sequencing

Three clades were identified through Bayesian analysis of our *Escovopsis* sequences (Fig. 14). Most *C. muelleri Escovopsis* strains sequenced belong to a single clade on our phylogeny (Clade II). There is one *C. muelleri* outlier (SP011108-04) in a separate clade (Clade III), but this strain was not successfully bioassayed and thus is not included in our in vitro bioassay or Mantel test results. One *C. longiscapus* strain belongs to Clade III with the outlying *C. muelleri* strain. Only two *C. longiscapus* strains are in a separate clade (Clade I) from the *C. muelleri* strains; all other *C. longiscapus* strains were more closely related to *C. muelleri* strains and are included in Clade II.

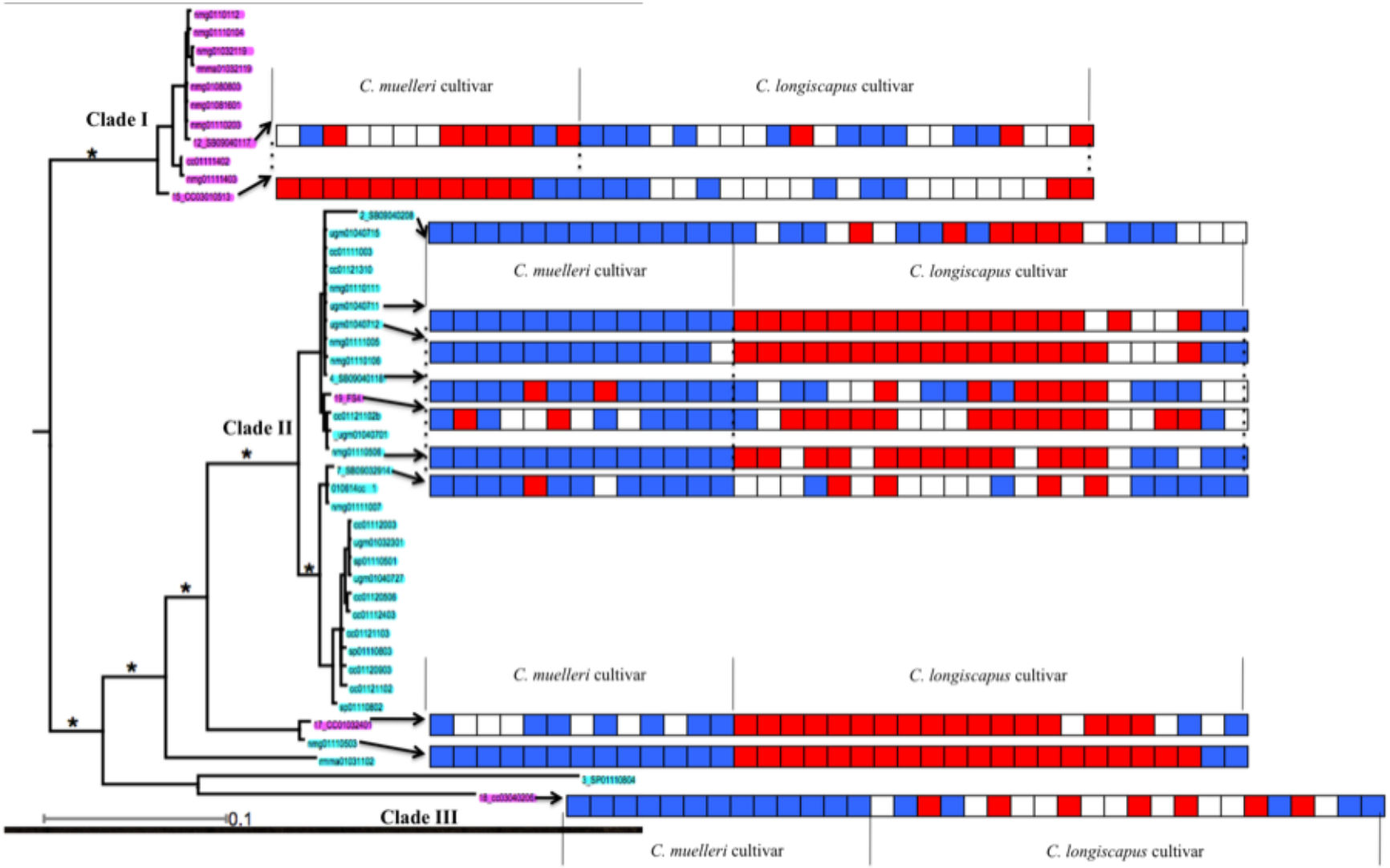

Figure 14: EF-1 alpha Bayesian phylogeny showing relationship of *C. longsicapus Escovopsis* (pink) and *C. muelleri Escovopsis* (blue) strains. Tables to the right of the phylogeny represent the outcomes of bioassays for each *Escovopsis* (blue: attraction towards the cultivar, red: inhibition). An asterisk represents greater than 95 posterior probability.

Correlation of Bioassay Interactions and Genetic Differences

The relationship between interaction differences and genetic differences of the *Escovopsis* strains used in bioassays was not statistically significant ($r = -.2$, $p = .09$).

Discussion

Experimental Test for Specificity

Both host specialization and a potential for host-switching were demonstrated both through in vitro bioassays and through phylogenetics. Bioassays support that two phylogenetically distinct clades of *Cyphomyrmex*-associated *Escovopsis* are generally specialized to utilize different hosts. One clade of *Escovopsis* (clade I) has only been isolated from *C. longiscapus* colonies, and bioassays of isolates from this clade show a propensity for these fungi to be attracted to *C. longiscapus* cultivars and inhibited by *C. muelleri* and C. *costatus* cultivars, suggesting that they are unlikely to be able to switch hosts. A second clade of *Escovopsis* (clade II), however, shows a broader host range, with most strains being isolated from *C. muelleri* and *C. costatus* colonies, but occasionally isolation from *C. longiscapus* colonies. All strains, regardless of host origin, appear to be more likely to be attracted to *C. muelleri* and *C. costatus* cultivars and inhibited by *C. longiscapus* cultivars. Presence in *C. longiscapus* colonies, however, may be facilitated by the ability of more of these strains to not be inhibited by *C. longiscapus* cultivars.

C. longiscapus and *C. muelleri Escovopsis* types were able to infect all three cultivar types: *C. longiscapus, C. muelleri,* and *C. costatus*. All *C. costatus* cultivars were attracted by all of the *C. muelleri Escovopsis* strains, owing to the fact that *C. costatus* and *C. muelleri* colonies cultivate an identical fungal cultivar (Green et al.

2002). *C. longiscapus Escovopsis* strains that were able to infect *C. muelleri* cultivars were also able to infect *C. costatus* cultivars, but those strains that could not infect *C. muelleri* cultivars were also not able to infect *C. costatus* cultivars, further supporting the conclusion that *C. muelleri* and *C. costatus* are infected by similar pathogens. This evidence also suggests a gene-for-gene interaction between *Escovopsis* and cultivars.

C. longiscapus and *C. muelleri Escovopsis* types were able to infect the novel cultivars of each *C. longiscapus* and *C. muelleri* species demonstrating host switching. More *C. muelleri Escovopsis* strains demonstrated attraction to novel *C. longiscapus* cultivars than *C. longiscapus Escovopsis* to novel *C. muelleri* cultivars. Overall, there was much more variation in infection of *C. longiscapus* cultivars. This may be due to differences in *Escovopsis* host specificity in that *C. muelleri Escovopsis* strains are less specialized. Alternatively, differences in novel cultivar infection may be due to differing host defense tactics: *C. muelleri* cultivars may exhibit stronger antibiotic defenses.

Of the eight *C. longiscapus Escovopsis* strains used in bioassays, only two strains (SB090401-17, CC030105-13) behaved as we hypothesized with attraction to their host cultivar, *C. longiscapus*, and inhibition with some instances of attraction by the novel cultivar, *C. muelleri*. All other *C. longiscapus Escovopsis* strains were widely inhibited by *C. longiscapus* cultivars and attracted to *C. muelleri* cultivars demonstrating behavior more characteristic of *C. muelleri Escovopsis* types. Upon inspection of the phylogeny constructed for *Escovopsis* strains, the two *C. longiscapus Escovopsis* strains exhibiting the expected behavior are isolated in Clade I, separate from all other *Escovopsis* strains (Clades II and III). Those *C. longiscapus Escovopsis* strains demonstrating behaviors more similar to *C. muelleri Escovopsis* strains are within Clade II with *C. muelleri*

Escovopsis strains. The detection of *Escovopsis* strains in *C. longiscapus* colonies that are more genetically related to *C. muelleri Escovopsis* types is evidence that *C. muelleri*-type *Escovopsis* strains sometimes infect *C. longiscapus* colonies in nature and is indicative of the potential for host switching in nature.

In statistical comparison of both attraction and inhibition profiles, *Escovopsis* are significantly better at infecting *C. muelleri* cultivars. There was a significant difference between infection based on cultivar type but not a significant difference of infection based on *Escovopsis* type. This is likely due to the fact that only two *C. longiscapus Escovopsis* strains were isolated in Clade I while all other *Escovopsis, C. muelleri* and *C. longiscapus*, strains were grouped in Clade II. Clade differences within *C. longiscapus* strains would also explain why both *Escovopsis* strains were more effective at infecting *C. muelleri* cultivars. Inclusion of more isolates from Clade I will be beneficial in confirming that *Escovopsis* from the two clades have different, specialized infection profiles. Unfortunately, Clade I *Escovopsis* strains are more rare and few are in culture at this time.

Based on our phylogeny, Clade II appears to be far less specialized than Clade I. AFLP data from Gerardo et al. (2004) also demonstrates variation within clades of *C. longiscapus, C. muelleri*, and *C. costatus Escovopsis* strains. Additionally, variation in infection abilities of *C. longiscapus* and *C. muelleri Escovopsis* may arise from differences in host stages in the host-parasite arms-race cycle. More sampling, including temporal sampling, would provide better insight into the variations and trends present in the *Cyphomyrmex* system, but large sample numbers inherently produces more variation in both types of *Escovopsis* and cultivars isolated and also in the results these bioassays

produce. Because only two of our *C. longiscapus Escovopsis* strains were isolated in a separate clade from the *C. muelleri Escovopsis,* additional bioassays should be performed with *C. longiscapus Escovopsis* strains also in Clade I.

Garden Infections

Previous bioassay results indicate that *Cyphomyrmex* gardens should be least likely to be infected with *A. dentigerum* and *A. octospinosus Escovopsis*, as each appear to be highly specialized on *Apterostigma* and *Acromyrmex* gardens, respectively. However, in some cases, the bioassays indicate that even *Apterostigma Escovopsis* are occasionally not inhibited by *Cyphomyrmex* cultivars and thus may be able to infect these gardens (Fig. 2, Gerardo et al. in prep.) Our garden infections also demonstrated infection of *C. longiscapus* by the novel *A. dentigerum* and *A. octospinosus Escovopsis* types demonstrating the potential for infection of *Cyphomyrmex* gardens by novel *Escovopsis* types. However, it should be noted that natural infections of *Cyphomyrmex* colonies with *Apterostigma* or *Acromyrmex*-associated *Escovopsis* have never been found in nature (Gerardo, personal comment). Ant behaviors to remove pathogens or bacteria-derived antibiotics utilized by the ants may prevent such host-switching.

Our garden infections showed infection of *C. muelleri* and *C. longiscapus* gardens by both *C. muelleri* and *C. longiscapus Escovopsis*. Only one garden, a *C. muelleri* one, demonstrated infection by both *Escovopsis* types. *C. longiscapus* gardens were equally infected by each type of *Escovopsis* while *C. muelleri* gardens were infected more by *C. muelleri Escovopsis* (five gardens out of 8) than *C. longiscapus Escovopsis* (three gardens out of 8). Because a single colony was not often infected by both types of

Escovopsis, this may indicate a difference in specialized defenses of each colony type. An ant colony and its associated garden may only be able to defend themselves against a narrow range of *Escovopsis*. More likely, particular *Escovopsis* types may only be specialized to overcome a narrow range of hosts.

In addition, the equal infection of *C. longiscapus* gardens by both types of *Escovopsis* is also demonstrated by our bioassays where host-switching occurred more often to novel *C. longiscapus* cultivars than to novel *C. muelleri* cultivars. *C. longiscapus* cultivars were more often infected by the novel *C. muelleri Escovopsis* than vice versa. These results suggest that *C. longiscapus* cultivars are more susceptible to infection than *C. muelleri* cultivars, but these findings are contrary to previous findings of Gerardo et al. (2004). They reported that *C. longiscapus* cultivars were less susceptible to experimental infection and had lower natural infection rates as compared to *C. muelleri* and *C. costatus* colonies.

Gerardo et al. (2004) suggest that the higher resistance indicated by their results could be attributed to differences in virulence between the *Escovopsis* strains specialized on the different species of ant's fungal gardens. Specifically, they suggest that *C. longiscapus Escovopsis* is more virulent, but our results suggest the opposite: *C. muelleri Escovopsis* appeared to be more virulent in our studies as this *Escovopsis* type was better able to infect novel cultivar types. This conclusion is confounded by the overwhelming presence of *Escovopsis* strains isolated from *C. longiscapus* colonies that are more closely related to *C. muelleri Escovopsis* strains. Variation in virulence of *Escovopsis* strains found within a single population has been demonstrated and this variation in such

a small spatial context likely results in competition among *Escovopsis* strains within populations and colonies (Currie 2001).

Correlation of Bioassay Interactions and Genetic Differences

Inhibition differences between *Escovopsis* strains are provided in Appendix E and mean genetic differences are provided in Appendix F. The relationship between interaction differences and genetic differences of the *Escovopsis* strains used in bioassays was not statistically significant. We expected a significant correlation between *C. muelleri* and *C. longiscapus Escovopsis* cultivar interactions and their genetic differences, but there were only two samples in the *C. longiscapus* Clade I. No correlation in our analysis likely reflects cultivar switching in the larger Clade II. In addition, genetic differences between *Escovopsis* strains are based on a neutral gene, EF-1, found widely in fungal populations. If the specific genes underlying infection abilities were identified and compared between strains, there likely may be a stronger correlation within our samples.

Future Directions

Further research should target identifying the specific chemotaxis signals secreted by the cultivar and distinguish any differences in the chemicals secreted by *C. longiscapus* and *C. muelleri* cultivars. Additionally, differences in the actinomycetous bacteria found on the ants may help explain infection of the isolated cultivar without the context of the whole colony. Studies investigating the evolution of virulence in *Escovopsis* would also help discern the interactions between the parasite and its host

cultivar. Finally, it has yet to be demonstrated whether the ants can recognize differences between parasite strains and act accordingly if infection is likely (parasite is common among their gardens and specialized to their clade) or unlikely to establish (parasite is novel and not specialized).

Appendix A: List of cultivars used for bioassays.

Colony Code	Genus	Species	Date of Collection	Type	Location	Mantel Code
SB090327-01	Cyphomyrmex	muelleri	03.27.09	cultivar	Pipeline Road	cul1
SB090327-07	Cyphomyrmex	muelleri	03.27.09	cultivar	Pipeline Road	cul2
SB090329-03	Cyphomyrmex	muelleri	03.29.09	cultivar	Pipeline Road	cul3
SB090329-11	Cyphomyrmex	muelleri	03.29.09	cultivar	Pipeline Road	cul4
SB090329-14	Cyphomyrmex	muelleri	03.29.09	cultivar	Pipeline Road	cul5
SB090401-03	Cyphomyrmex	muelleri	04.01.09	cultivar	Pipeline Road	cul6
SB090401-08	Cyphomyrmex	muelleri	04.01.09	cultivar	Pipeline Road	cul7
SB090402-12	Cyphomyrmex	muelleri	04.02.09	cultivar	Pipeline Road	cul8
SB090402-17	Cyphomyrmex	muelleri	04.02.09	cultivar	Pipeline Road	cul9
NMG010811-01	Cyphomyrmex	muelleri	08.11.01	cultivar	Pipeline Road	cul10
SP011108-04	Cyphomyrmex	muelleri	11.08.01	cultivar	Ft. Sherman	cul11
NMG011114-02	Cyphomyrmex	muelleri	11.12.01	cultivar	El Llano	cul12
SP011108-02	Cyphomyrmex	muelleri	11.08.01	cultivar	Ft. Sherman	cul13
CC020602-04	Cyphomyrmex	costatus	06.02.04	cultivar	Gamboa	cul37
CC030106-04	Cyphomyrmex	costatus	01.06.03	cultivar	not specified	cul38
CC020605-06	Cyphomyrmex	costatus	06.05.02	cultivar	Gigante	cul39
AS020605-01	Cyphomyrmex	costatus	06.05.02	cultivar	Gigante	cul40
SP011105-01	Cyphomyrmex	costatus	11.05.01	cultivar	Barro Colorado Island	cul41
SB090326-10	Cyphomyrmex	longsicapus	03.26.09	cultivar	Pipeline Road	cul15
SB090327-03	Cyphomyrmex	longsicapus	03.27.09	cultivar	Pipeline Road	cul16
SB090327-05	Cyphomyrmex	longsicapus	03.27.09	cultivar	Pipeline Road	cul17
SB090327-12	Cyphomyrmex	longsicapus	03.27.09	cultivar	Pipeline Road	cul18
SB090329-07	Cyphomyrmex	longsicapus	03.29.09	cultivar	Pipeline Road	cul19
SB090329-13	Cyphomyrmex	longsicapus	03.29.09	cultivar	Pipeline Road	cul20
SB090331-?	Cyphomyrmex	longsicapus	03.31.09	cultivar	Pipeline Road	cul21
SB090331-01	Cyphomyrmex	longsicapus	03.31.09	cultivar	Pipeline Road	cul22
SB090331-02	Cyphomyrmex	longsicapus	03.31.09	cultivar	Pipeline Road	cul23
SB090331-07	Cyphomyrmex	longsicapus	03.31.09	cultivar	Pipeline Road	cul24
SB090331-10	Cyphomyrmex	longsicapus	03.31.09	cultivar	Pipeline Road	cul25
SB090331-12	Cyphomyrmex	longsicapus	03.31.09	cultivar	Pipeline Road	cul26
SB090331-14	Cyphomyrmex	longsicapus	03.31.09	cultivar	Pipeline Road	cul27
SB090401-06	Cyphomyrmex	longsicapus	04.01.09	cultivar	Pipeline Road	cul28
SB090402-16	Cyphomyrmex	longsicapus	04.02.09	cultivar	Pipeline Road	cul29
NMG011114-03	Cyphomyrmex	longsicapus	11.14.01	cultivar	Pipeline Road	cul30
SP020529-03	Cyphomyrmex	longsicapus	05.29.02	cultivar	Pintada	cul31
NMG011101-12	Cyphomyrmex	longsicapus	11.01.01	cultivar	Pipeline Road	cul32
RMMA010321-19	Cyphomyrmex	longsicapus	03.21.01	cultivar	Pipeline Road	cul33
CC011213-17	Cyphomyrmex	longsicapus	12.13.01	cultivar	not specified	cul34
I(J)S020526-05	Cyphomyrmex	longsicapus	05.26.02	cultivar	Ft. Sherman	cul35
UGM010407-15	Cyphomyrmex	longsicapus	04.07.01	cultivar	unknown	cul36

Appendix B: List of *Escovopsis* strains used for bioassays and sequencing.

Colony Code	Genus	Species	Date of Collection	Type	Location	Mantel Code	Sequencing Code
SB090329-14	Cyphomyrmex	muelleri	03.29.09	Escovopsis	Pipeline Road	esc1	SEQesc7
SB090401-18	Cyphomyrmex	muelleri	04.01.09	Escovopsis	Pipeline Road	esc2	SEQesc4
SB090402-08	Cyphomyrmex	muelleri	04.02.09	Escovopsis	Pipeline Road	esc3	SEQesc2
SB090402-17	Cyphomyrmex	muelleri	04.02.09	Escovopsis	Pipeline Road	esc4	SEQesc5
UGM010407-11	Cyphomyrmex	muelleri	04.07.01	Escovopsis	unknown	esc5	SEQesc8
NMG011105-06	Cyphomyrmex	muelleri	11.05.01	Escovopsis	Barro Colorado Island	esc6	SEQesc14
UGM010407-05	Cyphomyrmex	muelleri	04.07.01	Escovopsis	unknown	esc7	SEQesc13
UGM010407-12	Cyphomyrmex	muelleri	04.07.01	Escovopsis	unknown	esc8	SEQesc10
NMG011105-03	Cyphomyrmex	muelleri	11.05.01	Escovopsis	Barro Colorado Island	esc10	SEQesc9
SP011108-04	Cyphomyrmex	muelleri	11.08.01	Escovopsis	Ft. Sherman	esc11	SEQesc3
UGM010427-07	Cyphomyrmex	muelleri	04.27.01	Escovopsis	unknown	esc9	SEQesc11
SB090327-07	Cyphomyrmex	longsicapus	03.27.09	Escovopsis	Pipeline Road	esc12	SEQesc6
SB090401-17	Cyphomyrmex	longsicapus	04.01.09	Escovopsis	Pipeline Road	esc13	SEQesc12
CC030402-06	Cyphomyrmex	longsicapus	04.02.03	Escovopsis	not specified	esc15	SEQesc18
CC010324-01	Cyphomyrmex	longsicapus	03.24.01	Escovopsis	not specified	esc16	SEQesc17
UGM010407-02	Cyphomyrmex	longsicapus	04.07.01	Escovopsis	unknown	esc17	SECesc20
CC030105-13	Cyphomyrmex	longsicapus	01.05.03	Escovopsis	not specified	esc18	SEQesc15
FS4	Cyphomyrmex	longsicapus		Escovopsis		esc19	SECesc19
UGM010407-16	Cyphomyrmex	longsicapus	04.07.01	Escovopsis	unknown	esc20	SEQesc16
UGM010407-15	Cyphomyrmex	longsicapus	04.07.01	Escovopsis	unknown	esc14	SEQesc1
CC030403-01	Cyphomyrmex	longsicapus	04.03.03	Escovopsis	Argentina	esc16	SECesc21

Appendix C: List of gardens used for garden infection experiments.

Colony Code	Genus	Species	Date of Collection	Type	Location	Garden Infection Code
SS100102-10	Cyphomyrmex	longsicapus	01.02.10	garden	Pipeline Road	CL1
SS100102-04	Cyphomyrmex	longsicapus	01.02.10	garden	Pipeline Road	CL2
SB100102-02	Cyphomyrmex	longsicapus	01.02.10	garden	Pipeline Road	CL3
SB100102-12	Cyphomyrmex	longsicapus	01.02.10	garden	Pipeline Road	CL4
SB100102-06	Cyphomyrmex	longsicapus	01.02.10	garden	Pipeline Road	CL5
SC100102-12	Cyphomyrmex	longsicapus	01.02.10	garden	Pipeline Road	CL6
RB100102-02	Cyphomyrmex	longsicapus	01.02.10	garden	Pipeline Road	CL7
SB100102-01	Cyphomyrmex	longsicapus	01.02.10	garden	Pipeline Road	CL8
SC100102-14	Cyphomyrmex	longsicapus	01.02.10	garden	Pipeline Road	CL9
RB100102-03	Cyphomyrmex	longsicapus	01.02.10	garden	Pipeline Road	CL10
SC100102-06	Cyphomyrmex	longsicapus	01.02.10	garden	Pipeline Road	CL11
SB100103-07	Cyphomyrmex	longsicapus	01.03.10	garden	Pipeline Road	CL12
SB100103-10	Cyphomyrmex	longsicapus	01.03.10	garden	Pipeline Road	CL13
SS100102-11	Cyphomyrmex	longsicapus	01.02.10	garden	Pipeline Road	CL14
RB100102-01	Cyphomyrmex	longsicapus	01.02.10	garden	Pipeline Road	CL15
SS100102-01	Cyphomyrmex	longsicapus	01.02.10	garden	Pipeline Road	CL16
SC100102-15	Cyphomyrmex	muelleri	01.02.10	garden	Pipeline Road	CM1
SB100102-18	Cyphomyrmex	muelleri	01.02.10	garden	Pipeline Road	CM2
SB100102-14	Cyphomyrmex	muelleri	01.02.10	garden	Pipeline Road	CM3
SB100103-12	Cyphomyrmex	muelleri	01.03.10	garden	Pipeline Road	CM4
SB100103-09	Cyphomyrmex	muelleri	01.03.10	garden	Pipeline Road	CM5
SB100103-01	Cyphomyrmex	muelleri	01.03.10	garden	Pipeline Road	CM6
SB100103-13	Cyphomyrmex	muelleri	01.03.10	garden	Pipeline Road	CM7
SC100102-10	Cyphomyrmex	muelleri	01.02.10	garden	Pipeline Road	CM8

Appendix D: List of *Escovopsis* strains used for garden infection experiments.

Colony Code	Genus	Species
UGM010407-15	Cyphomyrmex	longsicapus
SB090327-07	Cyphomyrmex	longsicapus
SB090401-17	Cyphomyrmex	longsicapus
NMG011105-06	Cyphomyrmex	muelleri
UGM010407-11	Cyphomyrmex	muelleri
NMG011114-02	Cyphomyrmex	muelleri
ED5	Apterostigma	dentigerum
EC2	Acromyrmex	octospinosus

Appendix E: Matrix of *Escovopsis* inhibition distances with each cultivar.

	esc 1	**esc 2**	**esc 3**	**esc 4**	**esc 5**	**esc 6**	**esc 8**	**esc 10**	**esc 12**	**esc 13**	**esc 15**	**esc 17**	**esc 18**	**esc 19**	**esc 20**	**esc 21**
esc1	1															
esc2	0.84	1.00														
esc3	0.64	0.68	1.00													
esc4	0.70	0.59	0.72	1.00												
esc5	0.73	0.62	0.72	0.69	1.00											
esc6	0.81	0.69	0.72	0.69	0.90	1.00										
esc8	0.75	0.62	0.71	0.68	0.92	0.91	1.00									
esc10	0.72	0.60	0.73	0.68	0.96	0.88	0.95	1.00								
esc12	0.78	0.72	0.74	0.72	0.64	0.67	0.63	0.60	1.00							
esc13	0.36	0.45	0.46	0.49	0.31	0.35	0.35	0.32	0.46	1.00						
esc15	0.79	0.65	0.85	0.82	0.86	0.79	0.91	0.85	0.83	0.38	1.00					
esc17	0.66	0.58	0.61	0.57	0.82	0.78	0.79	0.82	0.57	0.29	0.72	1.00				
esc18	0.72	0.62	0.72	0.66	0.93	0.86	0.92	0.87	0.59	0.30	0.81	0.80	1.00			
esc19	0.28	0.42	0.38	0.36	0.24	0.26	0.21	0.22	0.29	0.72	0.24	0.33	0.24	1.00		
esc20	0.56	0.59	0.62	0.49	0.67	0.64	0.71	0.71	0.51	0.38	0.64	0.62	0.74	0.46	1.00	
esc21	0.76	0.58	0.66	0.66	0.88	0.88	0.88	0.89	0.67	0.33	0.80	0.79	0.86	0.26	0.64	1.00

Appendix F: Matrix of genetic differences between *Escovopsis* strains.

	esc 1	**esc 2**	**esc 3**	**esc 4**	**esc 5**	**esc 6**	**esc 8**	**esc 10**	**esc 12**	**esc 13**	**esc 15**	**esc 17**	**esc 18**	**esc 19**	**esc 20**	**esc 21**
esc1	1															
esc2	0.03	1														
esc3	0.05	0.02	1													
esc4	0.01	0		1												
esc5	0.02	0	0.06	0	1											
esc6	0.04	0.01	0.04	0	0.03	1										
esc8	0.05	0.01	0.05	0	0.03	0	1									
esc10	0.11	0.08	0.14	0.05	0.1	0.09	0.09	1								
esc12	0.03	0	0.01	0	0	0.01	0.01	0.08	1							
esc13	0.11	0.13	0.14	0.07	0.13	0.13	0.13	0.13	0.12	1						
esc15	0.18	0.18	0.22	0.1	0.19	0.18	0.18	0.18	0.17	0.17	1					
esc17	0.07	0.08	0.1	0.05	0.09	0.08	0.08	0	0.08	0.13	0.17	1				
esc18	0.11	0.01	0.02	0	0.02	0	0	0.17	0	0.26	0.33	0.17	1			
esc19	0.13	0.13	0.17	0.06	0.13	0.14	0.14	0.13	0.12	0.02	0.17	0.12	0.26	1		
esc20	0.02	0.01	0	0	0.01	0	0	0.1	0	0.13	0.2	0.08	0	0.15	1	
esc21	0.12	0.07	0.08	0	0.07	0.07	0.07	0.21	0	0.3	0.37	0.17	0.07	0.29	0.08	1

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