Presence of Multiparasite Infections Within Individual Colonies of Leaf-Cutter Ants

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ABSTRACT Host–parasite dynamics can be altered when a host is infected by multiple parasite genotypes. The different strains of parasite are expected to compete for the limited host resources, potentially affecting the survival and reproduction of the host as well as the infecting parasites. Fungus-growing ants, including the well-known leaf-cutters, are an emerging model system for studying the evolution and ecology of symbiosis and host-parasite dynamics. We examine whether the fungus gardens of leaf-cutter ants can be simultaneously infected by multiple strains of the fungal pathogen *Escovopsis*. Intensive sampling of *Escovopsis* was conducted from individual gardens, as well as between different garden chambers within individual colonies of leaf-cutting ants. Isolates obtained were genotyped by DNA sequencing. We found that, minimally, 67% of the individual colonies of the leaf-cutter ant genera *Atta* and *Acromyrmex* and 50% of the *At. colombica* garden chambers studied were simultaneously infected by multiple distinct *Escovopsis* strains. Experimental challenges showed that different *Escovopsis* strains do not exhibit obvious antagonism toward each other, suggesting that coinfecting strains of the parasite do not engage in interference competition, although interactions were not studied at the cellular level. Further research is needed to understand interparasite interactions between coinfecting *Escovopsis* strains and to understand the impact of multiparasite infections on the survival of leaf-cutter ant gardens.

KEY WORDS coevolution, parasites, leaf-cutting ants, superinfection

Multiparasite infections within individual hosts, also known as superinfection (Nowak and May 1994), can dramatically influence host–parasite dynamics. Infections by multiple pathogen strains can be more virulent (i.e., detrimental to a host) than single strain infections because of overexploitation (Herre 1993, 1996; Ewald 1994; Nowak and May 1994) or because of cooperation between the parasites to overcome host defenses (Schjørring and Koella 2003, West and Buckling 2003, Massey et al. 2004, Puustinen et al. 2004). Alternatively, multiparasite infections can be less virulent if interference competition occurs between the parasite genotypes (Chao et al. 2000, Massey et al. 2004).

Superinfection may also affect the evolution of parasite virulence. According to models of parasite adaptation (Anderson and May 1979, Ewald 1994, Frank 1996, Lively 2001), a parasite must be adequately virulent to use host resources for growth and reproduction, without inducing early host mortality and sacrificing the parasite's ability to transmit to new hosts. However, competition between parasites may lead to selection for increased parasite virulence as parasites maximize their own resource sequestration (Bremermann and Pickering 1983; Knolle 1989; Frank 1994, 1996; Nowak and May 1994; van Baalen and Sabelis 1995; Davies et al. 2002; Puustinen et al. 2004). Despite the importance of multiparasite infections to the ecology and evolution of parasites and their hosts, the prevalence and impacts of multiparasite infections are unknown in most host-parasite associations (Gower and Webster 2005).

The fungus-growing ant–microbe symbiosis is a model system for studying the ecological and evolutionary dynamics of host-parasite associations (Currie et al. 1999a, b; Currie 2001a, b; Gerardo et al. 2004; Taerum et al. 2007; Gerardo and Caldera 2007). Fungus-growing ants (Hymenoptera: Formicidae: Attini) obligately depend on fungi (Agaricales) they cultivate for food (Weber 1972, Hölldobler and Wilson 1990). Worker ants forage for substrate that they provide their fungal cultivar for growth (Weber 1972), and new queens vertically transmit the fungus from parent to offspring colony. The fungus gardens of attine ants are host to microfungal parasites (Hypocreales, genus *Escovopsis*), which kill and consume the tissue of the ants' fungal mutualist (Currie et al. 1999a, 2003; Reynolds and Currie 2004). Although *Escovopsis* can completely devastate entire fungal gardens (Currie

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1999a), it typically forms persistent infections that significantly reduce the growth rates of fungus gardens (Currie 2001b). The parasite is horizontally transmitted between colonies (Currie et al. 1999a), although the specific mechanism by which this occurs is unknown (Currie 2001a). *Escovopsis* is a prevalent parasite, having been found to infect as many as 75% of colonies in some populations (Currie 2001b).

The association between fungus-farming ants, their fungal cultivar, and *Escovopsis* is ancient (Currie et al. 2003), likely dating back to the origin of the ant–fungus mutualism, ≈50 million years ago. Over their long shared coevolutionary history, there has been significant diversification in all three symbionts (Chapela et al. 1994, Schultz and Meier 1995, Mueller et al. 2001, Currie et al. 2003, Schultz and Brady 2008). *Escovopsis* infections have a single evolutionary origin within the mutualism, and on the broad scale, the evolution of *Escovopsis* has tracked the evolution of ants and their cultivar, with specific clades of *Escovopsis* infecting the gardens of specific groups of ants (Currie et al. 2003). This, in combination with *Escovopsis* only being known from the gardens of fungus-growing ants, suggests that this parasite is specialized and obligately dependent on the attine-ant–microbe mutualism.

The most phylogenetically derived attine ants, belonging in the genera *Acromyrmex* and *Atta* (Schultz and Meier 1995, Wetterer et al. 1998), are commonly referred to as leaf-cutters because they collect fresh vegetation (i.e., leaves and flowers) to nourish their fungal mutualist (Agaricales: Lepiotaceae: Leucocoprineae). The gardens of leaf-cutting ants are host to a monophyletic but phylogenetically diverse collection of *Escovopsis* parasites (Taerum et al. 2007). In addition, the clade of *Escovopsis* infecting the gardens of leaf-cutter ants are not known to parasitize the gardens of other groups of attine ants. However, *Escovopsis* within the leaf-cutters exhibits broad specificity, with the same strain or very closely related strains infecting species of both *Acromyrmex* and *Atta* (Taerum et al. 2007). More specifically, Taerum et al. (2007) found that *Acromyrmex* and *Atta* gardens can be infected by closely related *Escovopsis* strains, and the gardens of closely related species of leaf-cutter ants can be infected by distantly related *Escovopsis* strains. These findings further support horizontal transmission of the parasite between colonies within populations of leaf-cutter ants. Leaf-cutter ants seem to share the same pool of fungal cultivars, which has been shown to be readily exchanged between colonies of different species and even between the genera *Acromyrmex* and *Atta* (Bot et al. 2001, Poulsen and Boomsma 2005). It is possible that, although leaf-cutter *Escovopsis* specificity does not track the ant phylogeny, there is specificity toward the fungal cultivars. However, Taerum et al. (2007) found that the strains of *Escovopsis* infecting leaf-cutter ant colonies do not vary in their abilities to infect and overgrow diverse cultivars grown by different colonies, species, and genera of leaf-cutter ants. Thus, our current understanding of the dynamics of *Escovopsis* in leaf-cutter ants is that they are specific toward the gardens of leaf-cutter ants, but have low or no specificity toward the gardens of different genera and species of leaf-cutter ants, resulting in horizontal transmission of the parasite between colonies of sympatric leaf-cutter ant species.

In this study, we examined whether different strains of the garden parasite *Escovopsis* co-occur within individual leaf-cutter ant colonies and whether individual fungus gardens within colonies can be infected by multiple strains. Infections by multiple *Escovopsis* strains are likely in leaf-cutter ant colonies because (1) these ants frequently form large and long-lived colonies (Weber 1972, Hölldobler and Wilson 1990); (2) *Escovopsis* is horizontally transmitted (Currie et al. 1999a), allowing colonies to be infected during multiple separate infection events; and (3) *Escovopsis* isolated from leaf-cutter ant gardens shows low host specificity (Taerum et al. 2007). To determine whether leaf-cutter ant colonies are infected by multiple *Escovopsis* strains, we isolated two or more strains each from multiple *Atta* and *Acromyrmex* colonies. We conducted DNA extractions on the isolates and genotyped the strains by sequencing the elongation factor-1α (EF-1α) gene. In addition, the possibility of multiple infections within gardens leads to the prediction that strains of *Escovopsis* engage in antagonistic interactions shaped by inter- and intraspecific competition between strains. To look for evidence of interference competition between parasite strains, we conducted coculture bioassays.

Materials and Methods

Sampling for Garden Parasites. We sampled colonies of different leaf-cutter ants between 2002 and 2004 to determine whether the gardens of leaf-cutter ants are infected by multiple strains of the parasite *Escovopsis.* We sampled single chambers from two *At. cephalotes* colonies located in the forest along the Pipeline Road, in Panamá Province, Panamá, as well as an individual *A. octospinosus* colony from the Pipeline Road, in Panamá Province, Panamá, an *A. echinatior* colony from the Canal Zone in Panamá Province, Panamá, and an *Acromyrmex* sp. A colony from Parque Cruce Caballero, Misiones Province, Argentina. In addition, between August and October, 2004, we intensively sampled five mature colonies of *At. colombica* from the Canal Zone in Panamá Province, Panamá.

The intensive sampling of the five *At. colombica* colonies to test for the presence of multiple parasite genotypes involved the excavation of four separate garden chambers from each colony. To ensure that these four garden chambers were spatially separated from each other, we divided each colony mound into four quadrants (in a 2 by 2-m grid), with the center of the grid being placed in approximately the center of the external mound of each colony (estimated from the locations of leaf-cutter ant-excavated mounds; Weber 1972). A single chamber was excavated from each quadrant; within each quadrant, we randomly

selected an excavation site, at which we dug until a garden chamber was exposed.

In the sampling of all leaf-cutter colonies, excavated garden chambers were aseptically transferred to a sterile plastic container, using a flame sterilized metal spoon. To isolate the garden parasite *Escovopsis*, we placed garden pieces, five pieces equal distant apart per plate, on potato dextrose agar (PDA; Difco, Sparks, MD) that contained antibacterial antibiotics (50/mg/liter each of penicillin and streptomycin; MP Biomedicals, Aurora, OH). For each of the four chambers in *At. colombica* and for one chamber in the two *At. cephalotes* colonies sampled, isolations were conducted on the same days as the garden chambers were excavated, and we sampled 100 garden pieces per chamber. In the remaining sampling, 20 pieces of garden were isolated per garden, and colonies were allowed to stabilize on mineral moat islands (to reduce the potential for transfer of infection postcollection) in the laboratory for 2–3 d before sampling. Plates were checked daily for up to 2 wk, and when *Escovopsis* emerged from a piece of fungus garden, it was subcultured on a fresh PDA plate to obtain pure culture of parasite (following the protocol of Currie et al. 1999a).

Once pure cultures of the parasite were obtained, ≈1 ml of *Escovopsis* mycelium from the *Atta* spp. colonies was stored in dimethylsulfoxide (DMSO) solution (20% DMSO, 0.25 M EDTA, NaCl past saturation) at 5°C until DNA extraction. A similar amount (≈1 ml) of *Escovopsis* mycelium from the *Acromyrmex* spp. colonies was stored without preservative at −20°C until DNA extraction.

DNA Extraction, Amplification, and Purification. We performed DNA extractions using a cetyltrimethylammoniumbromide (CTAB) protocol modified from Bender et al. (1983). We diluted *Escovopsis* DNA from its original concentration to a lower concentration (1:100) before polymerase chain reaction (PCR) amplification. Amplifications targeted 873 bp from a single exon of the *EF-1α* gene. This gene was selected based on previous phylogenetic studies on *Escovopsis* (Currie et al. 2003, Gerardo et al. 2004, Taerum et al. 2007). The exon was amplified with the *Escovopsis*-specific primers EF6–20 F (5′-AAGAACATGATCACTGGTACCT-3′) and EF6–1000R (5′-CGCATGTCRCGGACGGC-3′; Taerum et al. 2007). We performed PCR amplifications following Gerardo et al. (2004) temperature profile and amplicon purifications using QIAquick PCR purification kits (Qiagen, Valencia, CA).

Sequencing, Aligning, and Phylogenetic Analysis. We sequenced a total of 67 *Escovopsis* strains from four *At. colombica* colonies collected in the Canal Zone, Panamá (Table 1). No *Escovopsis* strains emerged from the garden pieces of the fifth colony. In addition, we sequenced three strains from one *At. cephalotes* colony, two strains from the second *At. cephalotes* colony, two strains from the *A. echinatior* colony, six strains from the *A. octospinosus* colony, and three strains from the *Acromyrmex* sp. A colony. Sequencing was performed at the Idaho State University Molecular Research Core Facility or at the University of Wisconsin Biotechnology Center. All sequences are deposited in GenBank (accession numbers GQ240691–GQ240770).

Table 1. Number of individual *Escovopsis* cultures isolated from five *At. colombica* colonies

Colony no.	Chamber 1	Chamber 2	Chamber 3	Chamber 4	Total
1	3	3	0	0	6
2	12	6	4	0	22
3	6	5	6	3	20
4	4	5	6	4	19
5	0	0	0	0	0

The isolates are organized by the chamber that they were isolated from in columns 2 through 5.

We aligned the sequences in Sequencher v. 4.2 (Gene Codes, Ann Arbor, MI), using a dirty data assembly algorithm with a 60% minimum match and a 10% minimum overlap. We manually edited the alignments in Maclade v. 4.06 (Maddison and Maddison 2003). We included sequences for 16 strains of *Escovopsis* isolated from leaf-cutting ant gardens (Taerum et al. 2007; GenBank accession numbers EF589910, EF589912, EF589914, EF589915, EF589917–EF589919, EF589922–EF589925, EF589930, EF589931, EF589938, EF589942, and EF589943) in the analyses to provide some phylogenetic structure (Table 2). In addition, we included four previously sequenced strains of *Escovopsis* collected from colonies of *Trachymyrmex*, the sister group to the leaf-cutter ant clade (Schultz and Meier 1995, Schultz and Brady 2008), to serve as an outgroup (Taerum et al. 2007; GenBank accession numbers EF589944–EF589947).

We generated a phylogeny using PAUP* v. 4.0b10 (Swofford 2002), under the criteria of maximum parsimony (MP). We ran the MP analyses using the TBR

Table 2. *Escovopsis* strains included in this study from Taerum et al. (2007)

Collection location (state/province, country)	Species of leaf-cutter ant	Figure 1 (no.)
Coclé, Panamá	*At. sexdens*	1
Bocas del Toro, Panamá	*At. cephalotes*	2
Orellana, Ecuador	*A. coronatus*	3
Panamá, Panamá	*A. octospinosus*	4
Misiones, Argentina	*At. sexdens*	5
Panamá, Panamá	*At. colombica*	6
Panamá, Panamá	*At. cephalotes*	7
Misiones, Argentina	*Acromyrmex sp. A*	8
Darién, Panamá	*A. octospinosus*	9
Panamá, Panamá	*A. echinatior*	10
Veracruz, Mexico	*At. mexicana*	11
Chiriquí, Panamá	*At. sexdens*	12
Panamá, Panamá	*At. cephalotes*	13
Orellana, Ecuador	*At. cephalotes*	14
Orellana, Ecuador	*A. hystrix*	15
Orellana, Ecuador	*A. hystrix*	16
Ecuador	*T. diversus*	17
Ecuador	*T. cornetzi*	18
Panamá	*T. zeteki*	19
Trinidad	*T. ruthae*	20

Numbers in the third column correspond to values listed in Figure 1.

branch swapping option and 1,000 random-taxon-addition replicates. We obtained bootstrap support using 100 maximum parsimony pseudoreplicates. Bayesian analysis was performed with MrBayes v3.1.2 (Huelsenbeck and Ronquist 2001). All of the analyses used one cold chain and three incrementally heated chains (T = 0.2). Four independent Markov Chain Monte Carlo searches, with two million generations each, were conducted, with the initial 50,000 generations from each run discarded as burn-in, and 1 in every 100 generations sampled to calculate posterior probabilities for each branch. The general time reversible (GTR + I + G) model was used. Priors for the substitution rates were set to a flat distribution.

***Escovopsis* Bioassay Challenges.** We conducted bioassays between different strains of *Escovopsis* to look for evidence of interference competition. We conducted each experimental challenge using 100 by 15-mm petri dishes with potato dextrose agar (Difco). To obtain single spore inoculations, we placed ≈8-mm^3 pieces of PDA with mature parasite spores in microcentrifuge tubes containing 2 ml of sterile deionized water. We vortexed the tubes briefly and poured them on fresh petri dishes containing PDA. After individual "islands" of *Escovopsis* hyphae appeared (usually after 2 d), these islands were immediately cut from the petri dish and placed opposite from other *Escovopsis* strains on fresh PDA plates.

For the experimental challenges, we selected 19 *Escovopsis* strains, which represented much of the phylogenetic diversity of leaf-cutter associated *Escovopsis* (see Results and Fig. 1). The *Escovopsis* strains selected were all isolated from the colonies of *At. colombica* collected in Gamboa, Panamá. A total of 43 different combinations of *Escovopsis* strains were conducted, with at least two replicates of each. Fifteen of the combinations tested a strain from one clade (Fig. 1) against a strain from a second clade. In addition, we conducted 13 bioassay challenges competing individual strains from within the same clade (8 for one clade, 5 for the other clade). Finally, we ran 15 control challenges (9 for one clade, 6 for the second clade), with both sides of a petri dish being inoculated with the same strain of *Escovopsis.*

We observed the plates twice a day until they were entirely covered by *Escovopsis* (typically 7–8 d after inoculation). Before and during contact, the interface between the two strains was observed under ×35 magnification using a stereomicroscope (Accu-Scope, Sea Cliff, NY). Any evidence of antagonistic interactions between the hyphae of the two strains was noted (Tuininga 2005).

Results

Multiple Infections Within Colonies and Chambers. Of the 873 positions in the final alignment, 230 were variable, and 117 were parsimony informative. In our phylogenetic analyses, leaf-cutter ant–associated *Escovopsis* grouped into three distinct and well-supported clades (Fig. 1). As has been found in other studies (Currie et al. 2003, Taerum et al. 2007), *Escovopsis* isolated from leaf-cutter ant colonies form a monophyletic group.

We detected the presence of at least two distinct genotypes (using four or more base pair differences as a conservative cut-off for classifying different genotypes) of *Escovopsis* in six of eight of the colonies of leaf-cutter ants found to be infected with the parasite in our study (Fig. 1). More specifically, three distinct *Escovopsis* strains were found in two infected *At. colombica* colonies, and two distinct *Escovopsis* strains were found in two infected *At. colombica* colonies, one of the two *At. cephalotes* colonies, and the *Acromyrmex* sp. A colony. Also, we found that individual garden chambers can be infected by genetically distinct strains of *Escovopsis.* Of the 20 intensively sampled *At. colombica* chambers, one chamber was infected by three distinct *Escovopsis* strains, and nine were infected by two distinct strains. In addition, we found that seven of the eight colonies and 12 of 20 of the individual *At. colombica* chambers were simultaneously infected by *Escovopsis* from two different clades of the pathogen.

Bioassay Challenges. In each of the bioassay challenges, the two tested strains physically encountered each other between 2 and 5 d after inoculation (Fig. 2). After the strains came in close proximity, they continued to grow to cover the remainder of the petri dish. There was no evidence of one strain of *Escovopsis* inhibiting another, and no strain of *Escovopsis* was observed to overgrow another *Escovopsis* (Fig. 2). In addition, there were no obvious signs of vegetative incompatibility, although vegetative intermixing and sexual reproduction between interacting strains did not occur.

Discussion

Individual leaf-cutter ant colonies are often simultaneously infected by multiple genotypes of the fungal pathogen *Escovopsis.* Of the nine colonies studied, at least 67% were infected by multiple *Escovopsis* strains. This probably represents an underestimate, because it is likely that, despite the significant time required to sample 100 garden pieces, it still represents sampling from only a small proportion of the fungus garden biomass of a leaf-cutter ant colony (see Gerardo et al. 2004). In addition, of the 20 individual *At. colombica* chambers examined, 50% were infected by multiple *Escovopsis* strains. Infections of single colonies by multiple strains of *Escovopsis* were observed in the colonies of both leaf-cutting genera, *Atta* and *Acromyrmex.* In addition, we found evidence that individual colonies and chambers were simultaneously infected by *Escovopsis* strains representing multiple clades of the pathogen. Our results indicate that distantly related *Escovopsis* strains frequently encounter each other within individual colonies and garden chambers in leaf-cutter colonies ant gardens.

Infections of individual leaf-cutter ant colonies by multiple *Escovopsis* strains have important ecological and evolutionary implications. According to theory on multiparasite infections (Read and Taylor 2001), coin-

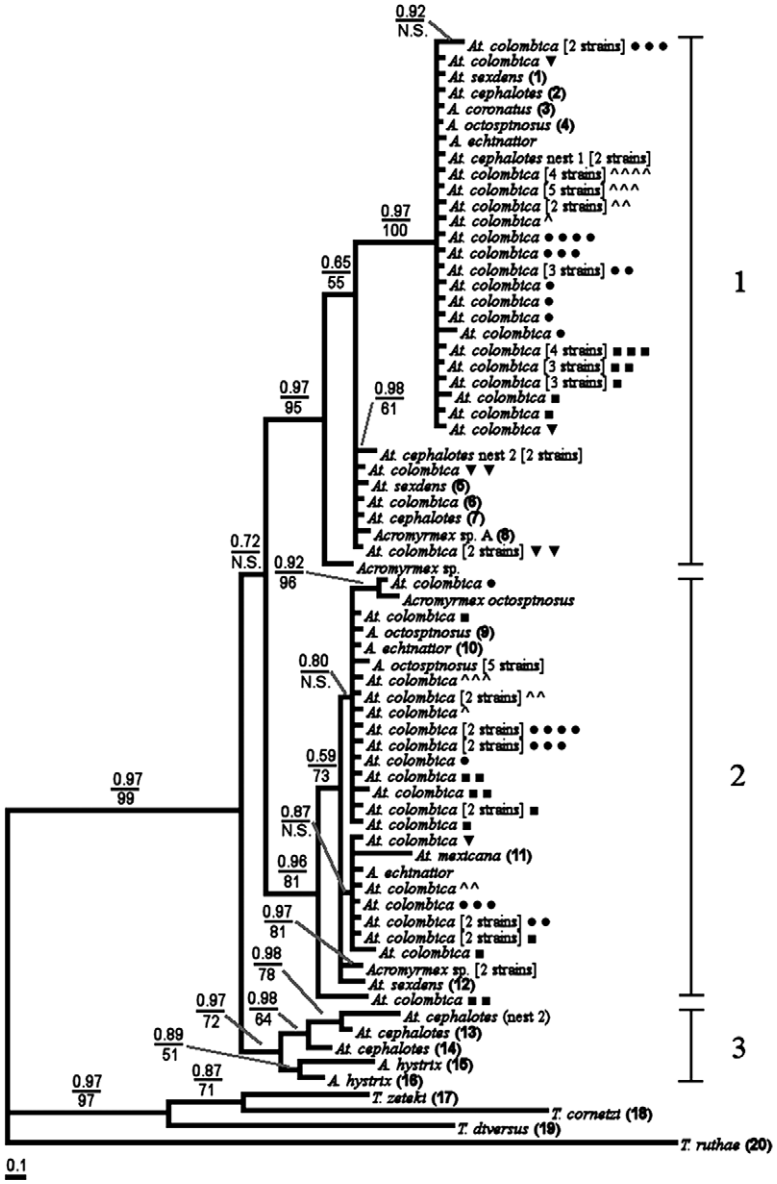

Fig. 1. Elongation factor 1-alpha phylogeny of 99 *Escovopsis* isolates from leaf-cutter ant colonies and four *Escovopsis* isolates from *Trachymyrmex* colonies. Each *Escovopsis* isolate from the *At. colombica* nests is followed by symbols that differentiate between nests (nest 1 = ●, 2 = ■, 3 = ▼, 4 = ^), whereas the chambers within each nest are differentiated by the numbers of symbols (● = chamber 1 from nest 1, ●=● = chamber 2 from nest 1, etc.). If multiple *Escovopsis* isolates from the same chamber are identical (based on 100% sequence match), the number of identical isolates is shown behind it in square parentheses. Numbers in parentheses after names indicate previously sequenced *Escovopsis* strains (see Table 2). At the nodes, the upper values correspond to Bayesian PP, whereas the lower values correspond to maximum parsimony bootstrap proportions. Three distinct clades of *Escovopsis* (labeled 1, 2, and 3) are demarcated.

fecting parasites are expected to compete for host resources. Competitive interactions between parasites with different genotypes have been shown in other multiparasite interactions, including helminths and protozoans that occupy mammal guts (Christensen et al. 1987, Lello et al. 2004), microsporidia that infect *Daphnia* (Vizoso and Ebert 2005), and malaria-causing plasmodia that infect animals (Taylor et al. 1997). Different parasites infecting an individual host may compete by exploitation competition (i.e., an individual parasite strain is limited by what host resources other parasite strains have exploited), interference competition (i.e., a parasite strain chemically or mechanically suppresses other parasite strains), or apparent competition (i.e., the infection of a host by one parasite strain induces a host immune response that

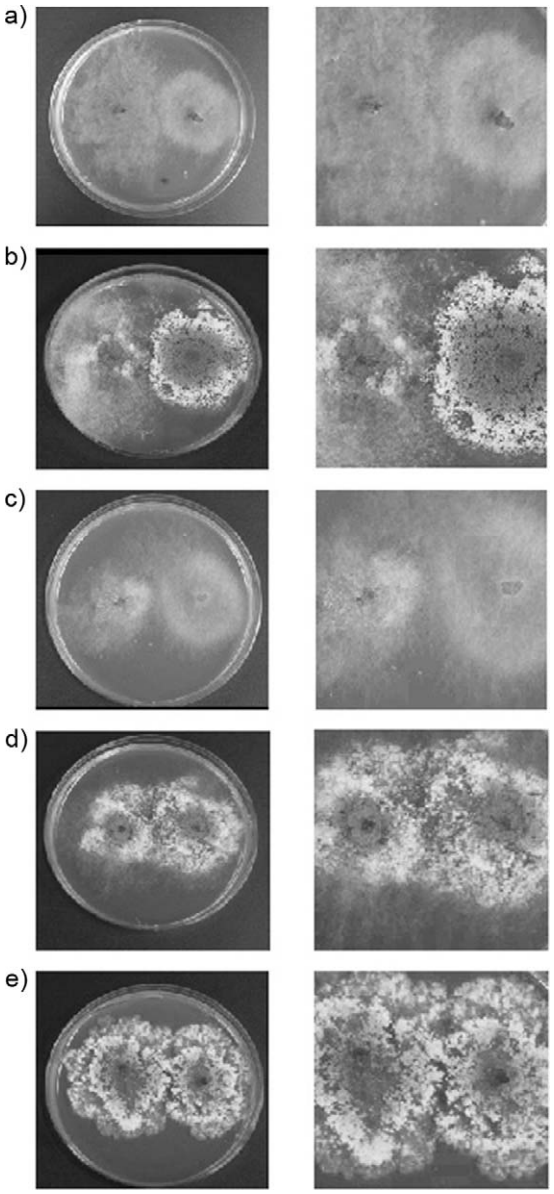

Fig. 2. Images of bioassay challenges between different combinations of *Escovopsis* strains after 4–7 d. Photographs were taken when the two strains visibly came in contact with each other. Photos on the right are 200% enlargements of photos on the left, focusing on the interface between the two strains. Challenge photos are as follows: (a–c) two genetically distinct (>4 bp different) strains and (d and e) two identical strains (control) after 6 d.

affects other parasite strains; Read and Taylor 2001). Based on its life history and taxonomic placement, individual *Escovopsis* strains might use modes of interference competition to suppress other *Escovopsis* strains. *Escovopsis* secretes enzymes to attack and breakdown the fungal cultivar of attine ant colonies (Reynolds and Currie 2004), suggesting that *Escovopsis* may similarly produce biomolecules to inhibit other strains of the parasite. In addition, *Escovopsis* is allied to the order Hypocreales (Currie et al. 2003), a group of mycoparasitic fungi whose members frequently use interference competition to attack other fungi (Jeffries and Young 1994). For example, strains of *Trichoderma,* a genus of fungi allied with the order Hypocreales, use both hyphal penetration (Jeffries and Young 1994) and allelopathy (Dennis and Webster 1971) during antagonistic interactions with other fungi. Contrary to predictions, our experimental challenges between different *Escovopsis* strains indicated no inhibition or overgrowth between strains. These results suggest that *Escovopsis* is unable to exclude other strains of the parasite from leaf-cutter ant chambers or that negative interactions occurred between the different strains but were unobservable, because interactions between the different isolates were not observed at a cellular level.

The lack of antagonism, even between *Escovopsis* strains isolated from the gardens of different leaf-cutter ant species, is surprising, considering that fungal cultivars from different leaf-cutter ant species often show strong antagonism toward each other (Poulsen and Boomsma 2005). A possible explanation for the lack of observable antagonism between *Escovopsis* strains is that the different strains, although diverse, are sufficiently related to preclude interference competition between them. Under the kin selection hypothesis (Hamilton 1964), closely related parasites are expected to exhibit less antagonism and competition than more distantly related parasites (Frank 1994). This has been shown in other host–parasite associations (Gardner et al. 2004, Puustinen et al. 2004) and could explain why different *Escovopsis* strains do not exhibit obvious antagonism when they co-occur on a plate. This hypothesis should be tested with challenges between *Escovopsis* isolated from leaf-cutter ant colonies and more distantly related strains of *Escovopsis* (i.e., isolated from the nests of other fungus-farming ants, such as *Trachymyrmex* spp.), or other genera of fungi, where we would predict to observe strong antagonism. Alternatively, individual strains could be adapted to occupy different microniches within leaf-cutter ant chambers and therefore not exhibit antagonism when encountering other *Escovopsis* strains.

An additional potential explanation for the experimental results is that *Escovopsis* strains may exhibit inhibitory responses to each other if grown on leaf-cutter ant cultivar instead of artificial medium. *Escovopsis* strains do exhibit different growth patterns when grown on artificial medium instead of leaf-cutter ant cultivar (unpublished data). Bioassay challenges between different *Escovopsis* strains using leaf-cutter ant cultivar as the substrate would determine whether the parasite strains respond differently to each other in situ than in vitro. If *Escovopsis* strains do exhibit interference competition when grown on leaf-cutter ant cultivar, the findings that individual leaf-cutter ant chambers are frequently infected by multiple *Escovopsis* strains might indicate that the different strains often infect leaf-cutter ant chambers at different sites

and are prevented from encountering each other (possibly because of inhibition by the ants and other symbionts).

If multiple *Escovopsis* strains do not exhibit interference competition when simultaneously infecting individual chambers, a different form of competition likely occurs between the *Escovopsis* strains. One possibility is that *Escovopsis* strains compete by increasing spore production, as has been observed with microsporidia that infect *Daphnia* (Vizoso and Ebert 2005). Alternatively, apparent competition (Read and Taylor 2001) could occur between strains; infection by one *Escovopsis* strain could induce responses by the leaf-cutter ants, their cultivar, or the antibiotic-producing bacteria that would be detrimental to other *Escovopsis* strains. Finally, competition between *Escovopsis* strains could be limited to resource competition, where individual *Escovopsis* strains compete by using colony resources more quickly.

The actual ecological and evolutionary consequences of infections by multiple *Escovopsis* strains are unknown. According to most theoretical expectations (Ewald 1994, Nowak and May 1994, Massey et al. 2004), infections by multiple strains of parasites should result in more serve infections and select for evolution of increased virulence (i.e., competition between strains). This leads to the prediction that morbidity of leaf-cutter ant fungus gardens should be greater if a colony is infected by several *Escovopsis* strains, because the fungal gardens should be overexploited, and competition between *Escovopsis* strains potentially select for greater virulent.

An alternate consequence of infections by multiple *Escovopsis* strains could be that the different strains cooperate to overcome host defenses (Schjørring and Koella 2003, West and Buckling 2003, Massey et al. 2004, Puustinen et al. 2004), because the ant, cultivar, and bacterial mutualists would be less able to defend against a wide diversity of *Escovopsis* strains than against an individual strain (see below). In addition, a diversity of *Escovopsis* strains might be better able to outcompete more distantly related parasites that can infect leaf-cutter ant gardens (Currie 2001b) than individual *Escovopsis* strains.

A third potential outcome of multiparasite infections is that the interparasite competition in fact benefits the gardens. Studies on other host–parasite symbioses have shown that multiparasite interactions sometimes results in selection for different modes of interference in the parasites, resulting in the underexploitation of the host (Chao et al. 2000, Massey et al. 2004). If this occurs in leaf-cutter ant colonies, *Escovopsis* would be less virulent if competing with other parasite strains and would be potentially more easily inhibited by the ants and their mutualists. Future studies on the effects of infections by diverse *Escovopsis* strains on leaf-cutter ant colonies (e.g., experimentally infecting leaf-cutter ant chambers with single or multiple *Escovopsis* strains and comparing results) are needed to determine whether interparasite interactions are detrimental to the different *Escovopsis* strains and whether infections by multiple diverse *Escovopsis* strains are more or less virulent to leaf-cutter ant colonies than infections by individual strains.

Competitive interactions between *Escovopsis* and other genera of fungi within the gardens of leaf-cutter ant colonies may also be important. Although *Escovopsis* is the only fungus that has been experimentally shown to be a parasite of the ant fungus garden, other fungi have been isolated from this niche (Rodrigues et al. 2005, 2008), which could represent pathogens or opportunistic "weeds" that alter the dynamics of the system. Because different *Escovopsis* strains are more closely related to each other than they are to potential opportunistic fungal pathogens, *Escovopsis* strains are predicted to show more antagonism to co-occurring opportunistic pathogens than to other *Escovopsis* strains (c.f., Frank 1994). In addition to exhibiting resource competition, *Escovopsis* may exhibit interference competition with these opportunistic fungi. One possible explanation for the apparent absence of other significant fungal pathogens of leaf-cutter ant gardens is that *Escovopsis*, in securing its position in the fungus garden, actively preventing other more transient fungi from infecting the garden. The interactions between *Escovopsis* and other fungi that may occur in the ants' fungus garden should be further studied.

The discovery that individual leaf-cutter ant colonies and chambers are infected by multiple *Escovopsis* strains has major implications in the leaf-cutter ant–microbe symbiosis. Leaf-cutter ants vertically transmit their cultivars and antibiotic-producing bacteria. However, *Escovopsis* is horizontally transmitted (Currie et al. 1999a), a fact further supported by this study because the presence of different *Escovopsis* strains in individual colonies shows that multiple infection events occurred. Because leaf-cutter ant colonies are long-lived, large, and frequently multichambered (especially *Atta* colonies), this study supports the expectation that leaf-cutter ant colonies are repeatedly exposed to different *Escovopsis* strains, including those from the colonies of other leaf-cutter ant species. Because *Escovopsis* is highly virulent and occasionally overgrows entire colonies, attacks from diverse *Escovopsis* strains may present added challenges to the ant fungal cultivar and bacterial mutualists. The ants may be required to alter their *Escovopsis*-removal behaviors, such as weeding and grooming fungal gardens, and using the antibiotic-producing bacteria in the presence of diverse parasites. The combination of the horizontal transmission of *Escovopsis*, the high virulence of the parasite, and the ability of multiple *Escovopsis* strains to infect leaf-cutter ant gardens suggest that the ant, fungal cultivar, and bacterial mutualists are under constant attack from the parasite and therefore need robust defenses to survive and reproduce.

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