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HOST MICROBE INTERACTIONS

Symbiont-Mediated Host-Parasite Dynamics in a Fungus-Gardening Ant

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Abstract

Group-living can promote the evolution of adaptive strategies to prevent and control disease. Fungus-gardening ants must cope with two sets of pathogens, those that afflict the ants themselves and those of their symbiotic fungal gardens. While much research has demonstrated the impact of specialized fungal pathogens that infect ant fungus gardens, most of these studies focused on the so-called higher attine ants, which are thought to coevolve diffusely with two clades of leucocoprinaceous fungi. Relatively few studies have addressed disease ecology of lower Attini, which are thought to occasionally recruit (domesticate) novel leucocoprinaceous fungi from free-living populations; coevolution between lower-attine ants and their fungi is therefore likely weaker (or even absent) than in the higher Attini, which generally have many derived modifications. Toward understanding the disease ecology of lower-attine ants, this study (a) describes the diversity in the microfungal genus *Escovopsis* that naturally infect fungus gardens of the lower-attine ant *Mycocepurus smithii* and (b) experimentally determines the relative contributions of *Escovopsis* strain (a possible garden disease), *M. smithii* ant genotype, and fungal cultivar lineage to disease susceptibility and colony fitness. In controlled in-vivo infection laboratory experiments, we demonstrate that the susceptibility to *Escovopsis* infection was an outcome of ant-cultivar-*Escovopsis* interaction, rather than solely due to ant genotype or fungal cultivar lineage. The role of complex ant-cultivar-*Escovopsis* interactions suggests that switching *M. smithii* farmers onto novel fungus types might be a strategy to generate novel ant-fungus combinations resistant to most, but perhaps not all, *Escovopsis* strains circulating in a local population of this and other lower-attine ants.

Keywords Parasite-host interactions · Host-pathogen specificity · *Escovopsis* · Mutualism · Coevolution · Attini

Introduction

The evolution of societies, from simple aggregations to eusocial species, has been shaped by benefits and costs of group-living. Close proximity to other members of the same species, for example, can aid in defense against enemies but can also facilitate the spread of diseases and parasites between group members. In response, strategies may evolve in group-living organisms to prevent and to fight disease threats. Social insects have become popular model systems in recent years to study the epidemiology of infectious diseases [1, 2]. Members of insect societies live in close proximity, sharing food and having close body contact. Additionally, social insect societies most often consist of families, which makes them even more vulnerable to pathogens and parasites because individuals are closely related. Similar to human societies, social insects evolved various strategies to prevent and overcome outbreaks of disease, such as sanitary behavior [3, 4], corpse removal [5], exiling moribund colony members [6], early warning systems in response to pathogen exposure [7], self- and allo-grooming to remove pathogens [8], cleaning of offspring [9–11], vaccination-like inoculation [12], and hygienic effects of mutualistic symbionts [13–18].

Fungus-growing ants (tribe Attini) are of special interest for disease ecology because they have to cope with dual disease threats to themselves and also to their fungal garden crops. Attine ants evolved fungiculture of fungi of the family Agaricaceae (formerly Lepiotaceae) (Basidiomycota:

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Agaricales) [19–21]. The fungal cultivars are carefully tended by the ants, which are obligate dependent on their fungus as their primary food source. In return, the fungus receives shelter and protection from ants and is dispersed when the ant colonies reproduce (founding queens carry fungal inocula during nuptial flight to seed gardens in new colonies [22–25]). While most fungal symbionts are cotransmitted vertically within ant lineages, horizontal exchange of fungal symbionts between ant colonies is also possible and differs in frequency between ant lineages [20, 26–28]. The mechanisms underlying horizontal exchange are unknown but could include stealing of garden from neighboring colonies after garden loss [20, 29–32].

While each attine colony appears to grow only one genetic strain of fungal cultivar [33], the ants are essentially managing a consortia of microorganisms because the fungus garden contains also, embedded among the dominant live biomass of cultivated fungus, numerous transitory and resident "auxiliary microbes" [34–40]. In addition to a diverse bacterial community, ant fungus gardens are host to not only many microfungal species, which includes a number of "weedy" competitor species such as *Fusarium*, *Aspergillus*, *Mucor*, *Penicillium*, and *Trichoderma* [41–44], but also specialized garden parasites in the genus *Escovopsis* (Ascomycota: anamorphic Hypocreales) [42, 45]. The fungus *Escovopsis* is thought to consume the garden cultivar [46, 47] and appears to be largely horizontally transmitted between colonies [45, 48, 49]. In some ants in the genus *Acromyrmex*, the ants are thought to protect their gardens against *Escovopsis* with the help of antibiotic-secreting bacteria inhabiting the ant integument, which produce antibiotics with general activity against filamentous fungi, such as entomopathogens [40, 50], but the generality of the hypothesized *Escovopsis*-suppression by integumental bacteria is not known for attine ants at large. While most research has been focused on the so-called higher Attini (the leafcutter ant genera *Atta* and *Acromyrmex* [41, 48, 51] and the non-leaf-cutting higher-attine genus *Trachymyrmex* [42, 49]), relatively few studies have been conducted on the coevolutionary relationships between the so-called lower attine ants and their pathogens [52]. For example, in *Cyphomyrmex longiscapus*, *C. muelleri*, and *C. costatus*, the associated *Escovopsis* parasites are thought to be cultivar-specific because of clade-to-clade congruency between fungal cultivar and the parasite phylogenies [53, 54]. Likewise, in *Apterostigma* ants, closely related *Escovopsis* generally infect closely related fungal hosts, with occasional host-switching by *Escovopsis* or acquisition of new infections from unknown environmental sources [55]. Moreover, to our knowledge, only one in vivo experiment has been reported on the pathogeny of *Escovopsis* in one lower attine species (*Apterostigma pilosum*, [56]).

The lower-attine ant *Mycocepurus smithii* is a widespread clonal species found throughout Central and South America and many Caribbean islands [23, 57–59]. *M. smithii* has been shown to grow a diverse assemblage of fungal cultivars, including some grown by distantly related *Cyphomyrmex* species [20, 26]. *M. smithii* populations in Panamá grow fungal species from two different phylogenetic clades, with occasional horizontal switches between cultivar lineages [26]. The rarity of Actinobacteria found in culture-independent bacterial surveys of *M. smithii* ants and their gardens [37] suggests that Actinobacteria do not play as prominent a role in disease control as is thought to occur in some *Acromyrmex* species [60]. Widespread horizontal transmission and frequent de-novo recruitment from environmental sources of fungal and bacterial symbionts, as well as low specificity of symbionts apparent in phylogenetic analyses, should result in weak and diffuse coevolution among ant hosts, fungal symbionts, pathogenic fungi, and any defensive bacterial symbionts in *M. smithii* [20, 26, 37].

We here examine the disease ecology and coevolutionary relationships found in nature among host ants, fungal cultivar, and *Escovopsis* parasites associated with *M. smithii*. The aim of this study was (1) examine infection rates in the field and test for phylogenetic correspondences between *M. smithii* ants, their fungal cultivars, and associated *Escovopsis* lineages; (2) determine whether *Escovopsis* infects gardens grown by *M. smithii* and if the *Escovopsis* strains infecting *M. smithii* are similar to those infecting gardens grown by *Cyphomyrmex muelleri*, *C. longiscapus*, and *C. costatus* ants, which grow fungal cultivars from the same clades as *M. smithii* [20, 26, 53]; and (3) explore the factors that determine the virulence of *Escovopsis* infection. These results suggest that horizontal switching onto novel fungus types might be a strategy of the ants to escape the effects of pathogens.

Material and Methods

Collections, Infection Prevalence, *Escovopsis* Isolation, and Taxonomic Identification

Fungus garden chambers of colonies of *M. smithii* were collected from 11 locations in the Panama Canal area, Republic of Panamá, by excavating subterranean nests as described in Kellner et al. [26] (see also Table 1). Contents of garden chambers (ants and fungus gardens) from different chambers were collected separately, even if the garden chambers were from the same colony, and transported in a cooler to the laboratory. In total, 36 colonies with ants and gardens from 67 garden chambers were collected. Each colony was housed in the lab in two plastic boxes (7 cm × 7 cm × 2.5 cm), one with sterile plaster bottom as nest chamber and one without plaster bottom as foraging arena [26, 61]. The boxes were connected with a tube. Each of these setups were further enclosed in a covered, plastic shoebox to avoid cross-contamination between

Table 1 Summary of *Escovopsis* strains isolated from *Mycocepurus smithii* fungus gardens. Colony IDs, collection locations, and combination codes of ant lineage × fungus garden cultivars (e.g., G7 is ant—genotype G cultivating fungus garden cultivar 7) correspond to those of Kellner et al. [26]. ch = fungus garden chamber

Escovopsis strain	Spore type (color)	Host colony ID	Collection location of *Mycocepurus* nest	Host colony genotype (ant lineage/fungus garden cultivar)
Esco1	Pink	HF100409-02	Chorrera	G7
Esco2	Pink	UGM100408-02ch1	Corozales Afuera	E6
Esco3	Pink	HF100408-02	Gamboa Harbor	G7
Esco4	Pink	KK100419-01ch1	Gamboa Apartment 183	K4
Esco5	Pink	HF100409-03	Chorrera	G7
Esco6	Yellow	KK100413-01ch3	Gamboa Greenhouse	B5
Esco7	Yellow	KK100411-01ch2	Gamboa Apartment 183	H5
Esco8	Pink	RS100411-06	Gamboa Apartment 183	B5
Esco10	Pink	RS100403-02ch1	Gamboa Greenhouse	B2
Esco12	Pink	RS100412-03ch2	Gamboa Resort	A5
Esco15	Pink	RS100403-01ch2	Gamboa Greenhouse	B2
EscoA	Pink	RS100412-01ch1	Gamboa Resort	A5
EscoX1	Pink	KK100411-02ch3	Gamboa Apartment 183	H5

different colonies. Colonies were fed a standardized diet of sterile polenta and oats, and the plaster bottoms were regularly moistened with sterile water. Feeding and handling of colonies was performed wearing gloves, which were ethanol-sterilized between handling different colonies. After 1-week habituation to laboratory conditions, each garden was screened for the presence of *Escovopsis* (Ascomycota) by pulling small fragments (1–3 mm^3) of clean cultivar from the fungus gardens with flame sterilized forceps, then placing these on PDA (potato dextrose agar, Difco) Petri plates (five pieces on one plate, one plate for each garden; $N = 67$ plates). Plates were sealed with parafilm and incubated at room temperature (about 22 °C). Plates were screened daily for the appearance of *Escovopsis*-like mycelia growing from garden fragments [42]. Possible *Escovopsis* candidates were subcultured by cutting mycelium from the growth-front and transferring the mycelial isolate onto PDA plates. Thirteen candidates with *Escovopsis*-like mycelium and spore-production were sequenced for the EF-1α gene (see below) to confirm the visual identification. As in Gerardo et al. [53], *Escovopsis* isolates were classified into "yellow-spored" and "pink-spored" morphotypes according to spore coloration.

Sequence-Identification of *Escovopsis* Isolates, Phylogenetic Reconstruction, and Parafit Analyses

DNA was extracted by incubating small pieces of mycelia in 100 μl 10% Chelex resin (Sigma-Aldrich) for 1.5 h at 60 °C, then 10 min at 99 °C. Sequencing targeted a 987-bp region spanning one exon of nuclear elongation factor-1 alpha (EF-1α), using primers EF1-2218R and EF1-983F and a touchdown PCR-protocol developed by Rehner and Buckley [62]. PCR products were purified and cycle-sequenced (ABI BigDye Terminator Kit; ABI PRISM 3100) at the UT Austin core facility using standard procedures (https://icmb.utexas.edu/dna-sequencing-facility). Forward and reverse sequences were assembled in Sequencher 4.6 (GeneCodes, AnnArbor, MI, USA). Sequence information is deposited at NCBI GenBank (accession numbers KX259112–KX259124).

To place the new *Escovopsis* isolates into a phylogenetic framework, we obtained from NCBI GenBank sequence-information of *Escovopsis* sp. isolated from other lower-attine ants. Specifically, we downloaded a popset of *Escovopsis* sequences isolated from *Cyphomyrmex* colonies (38 sequences; 1529-bp fragment of EF-1 alpha; GenBank accessions AY629361–AY629398 [53], a popset of *Escovopsis* strains isolated from *Apterostigma* colonies (54 sequences; 987 bp; GenBank accessions DQ848156–DQ848209 [55]), one sequence of *Escovopsis kreiselii* isolated from a *Mycetophylax morschi* colony (KJ808766 [49]), and one *Escovopsis* strain isolated from a *Mycocepurus goeldii* colony (KF033128 [63]). The alignments from Gerardo et al. [53, 55] were merged, and the two additional sequences and our new 13 *Escovopsis* sequences were added using McClade [64], preserving the gaps in Gerardo's original alignments. The final alignment contained 107 sequences and was 992 bp in length. jModelTest 0.1.1 [65, 66] identified the GTR+I+G model as the most suitable model for phylogenetic analyses. A maximum-likelihood tree was computed using GARLI 0.951 [67]. Ten trees were generated from which the tree with the best likelihood score was chosen (likelihood-score of best tree: − 8884.69, 589 constant characters, 314 parsimony informative characters). Bootstrap support was evaluated in 1000 pseudoreplicate analyses.

Because information on genotypes of ants as well as fungus cultivars was available from a previous study [26], it was possible to test for clade-to-clade correspondences between (i) *Escovopsis* strains and fungus cultivars of the host colonies and between (ii) *Escovopsis* strains and ant lineages (asexual ant clones) of the host colonies. We used the *Parafit* function implemented in the R package "ape" [68] to test for phylogenetic congruence between phylogenies of the *M. smithii* ants, fungal cultivars, and *Escovopsis* strains. Parafit performs a test on each single observed link to assess the significance of that particular association. As input files, we used presence/absence matrices of the *Escovopsis* strain—ant genotypes or fungus genotypes (scored as 1 and 0, respectively), a genetic distance matrix from Kimura2P genetic distances among *Escovopsis* strains using Mega [69], a genetic distance matrix obtained from allele-sharing distances among ant genotypes, and a genetic distance matrix from Kimura2P genetic distances among fungus cultivars (both of these matrices were available from [26]). *p* values were obtained through permutations with 9999 pseudoreplicates.

Cultivation of *Escovopsis* and Preparation of Spore Suspensions

Two *Escovopsis* strains isolated from two different *M. smithii* nests were selected for infection experiments (strain *Escovopsis* sp. HF100409-03-Esco5, a pink-spore type, henceforth called strain Esco5; and *Escovopsis* sp. KK100413-01 ch3-Esco6, a yellow-spore type, called Esco6) (see Fig. 1). These two strains were chosen because they were distantly related within the *Escovopsis* diversity known to associate with lower-attine ants, because they differed in spore coloration (pink versus yellow) and because they showed vigorous spore-production on PDA plates. *Escovopsis* strain Esco5 may be more typically associated with *M. smithii*, whereas Esco6 may be less typically associated with *M. smithii* (see "Results" Fig. 1).

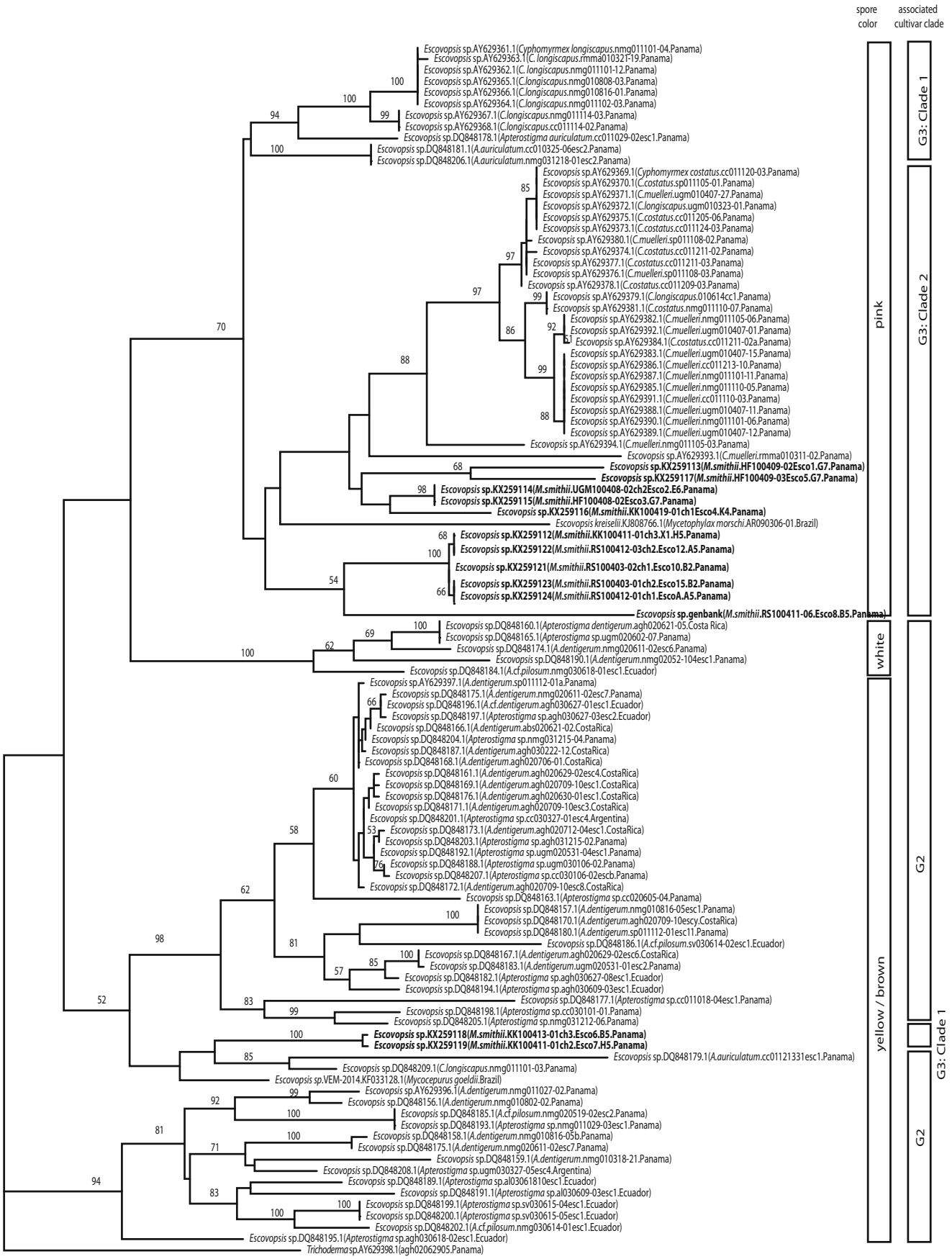
The two strains were kept in long-term storage (sterile water) at − 80 °C. Subcultures were started on sterile PDA plates. After approximately 4 weeks of incubating the plates at room temperature (first spores are produced by mycelia after 2 weeks), spores were harvested and suspended in 10 ml sterile 0.005% Tween80. To check spore viability, 5 μl of a 10× dilution of the spore suspension was applied onto a sterile PDA plate. To determine spore concentration in a suspension, 12 μl of a 100× dilution was evaluated in a spore count chamber (type Neubauer improved). The undiluted spore suspensions contained 11.6 × 10^9 spores/ml for strain HF10040903-Esco5 and 10 × 10^9 spores/ml for KK10041301ch3-Esco6. A 10× dilution of the original spore solution was filled in sterile spray bottles (Esco5 and Esco6 one spray bottle each, 11.6 × 10^8 and 10 × 10^8 spores/ml). One puff out of the spray bottle dispensed approximately 100 μl suspension. Fungus gardens in the experimental series below were inoculated by spraying one puff directly onto the garden surface, resulting in infection doses of 11.6 × 10^7 spores in one puff for Esco5 and 10 × 10^7 spores in one puff for Esco6. Both final infection doses have been shown to be in or above lethal range for attine gardens used in prior infection experiments (6000–100,000 spores [41, 51, 56, 70]). To confirm viability of spores in solution, a single puff of each strain was sprayed onto sterile PDA plates, and growth was confirmed in both cases.

Fig. 1 Phylogenetic tree constructed from EF-1α sequences from *Escovopsis* spp., illustrating the relationships between lower-attine ant species, corresponding fungi cultivated in gardens by these ant species, and *Escovopsis* fungi isolated from these gardens. Each taxon label contains the NCBI GenBank accession number of each *Escovopsis* specimen followed by the host ant species from which the specimen was isolated. Along the y-axis are the corresponding spore color types and ant fungal clades. *Escovopsis* spore color types and fungal clades correspond to Gerardo et al. [55], and Mueller et al. [20], respectively. Bootstrap support values greater than 50% are shown. Sequences from this study are shown in bold

Infection Experiments

Infection experiments were performed in vivo using ants and fungus cultivars from colonies with known genetic backgrounds. Ant lineages (A–J) are based on microsatellite genotyping and fungal cultivars (1–7) are based on ITS sequences (details are described in [26]). A cophylogenetic tree illustrates the relationships between ant and fungal lineages (details in [26] and see Fig. 2a).

1. *Infection of garden fragments not tended by ants*: Fungus-garden fragments of approximately 3 cm^3 were placed in Petri dishes with moistened plaster bottoms (UV sterilized) using flame sterilized forceps. Fungus-garden fragments were obtained from different colonies growing different fungus cultivar genotypes (cultivar types 2, 5, 6, and 7, as defined by [26]). Cultivar types 2 and 5 belong to the lower-attine fungus clade 1 (closely related to fungal cultivars grown by *C. longiscapus*) and cultivar types 6 and 7 to fungus clade 2 (closely related to fungal cultivars grown by *C. muelleri*) (details in [26], and see Fig. 1). Fragments (n = 87) were randomly assigned to treatment and control groups and sprayed-inoculated with either one puff of *Escovopsis* strain Esco5, strain Esco6, or control (sterile 0.005% Tween80). Sample sizes consisted of ten replicates of each cultivar type (5, 6, or 7) sprayed with either Esco5 or Esco6 and five replicates of cultivar type 2 sprayed with either Esco5 or Esco6, respectively. Only five replicates were possible for cultivar 2 because less garden material was available in the source nest. Control groups were five replicates each for cultivars 5, 6, 7, and 2 replicates for cultivar 2. All replicates were spray-


spore color
associated cultivar clade
pink
white
yellow / brown
G3: Clade 1
G3: Clade 2
G2
G2
G3: Clade 1
Escovopsis sp.AY629361.1(Cyphomyrmex longiscapus.nmg011101-04.Panama)
Escovopsis sp.AY629363.1(C. longiscapus.rmma010321-19.Panama)
Escovopsis sp.AY629362.1(C. longiscapus.nmg011101-12.Panama)
Escovopsis sp.AY629365.1(C.longiscapus.nmg010808-03.Panama)
Escovopsis sp.AY629366.1(C.longiscapus.nmg010816-01.Panama)
Escovopsis sp.AY629364.1(C.longiscapus.nmg011102-03.Panama)
Escovopsis sp.AY629367.1(C.longiscapus.nmg011114-03.Panama)
Escovopsis sp.AY629368.1(C.longiscapus.cc011114-02.Panama)
Escovopsis sp.DQ848178.1(Apterostigma auriculatum.cc011029-02esc1.Panama)
Escovopsis sp.DQ848181.1(A.auriculatum.cc010325-06esc2.Panama)
Escovopsis sp.DQ848206.1(A.auriculatum.nmg031218-01esc2.Panama)
Escovopsis sp.AY629369.1(Cyphomyrmex costatus.cc011120-03.Panama)
Escovopsis sp.AY629370.1(C.costatus.sp011105-01.Panama)
Escovopsis sp.AY629371.1(C.muelleri.ugm010407-27.Panama)
Escovopsis sp.AY629372.1(C.longiscapus.ugm010323-01.Panama)
Escovopsis sp.AY629375.1(C.costatus.cc011205-06.Panama)
Escovopsis sp.AY629373.1(C.costatus.cc011124-03.Panama)
Escovopsis sp.AY629380.1(C.muelleri.sp011108-02.Panama)
Escovopsis sp.AY629374.1(C.costatus.cc011211-02.Panama)
Escovopsis sp.AY629377.1(C.costatus.cc011211-03.Panama)
Escovopsis sp.AY629376.1(C.muelleri.sp011108-03.Panama)
Escovopsis sp.AY629378.1(C.costatus.cc011209-03.Panama)
Escovopsis sp.AY629379.1(C.longiscapus.010614cc1.Panama)
Escovopsis sp.AY629381.1(C.costatus.nmg011110-07.Panama)
Escovopsis sp.AY629382.1(C.muelleri.nmg011105-06.Panama)
Escovopsis sp.AY629392.1(C.muelleri.ugm010407-01.Panama)
Escovopsis sp.AY629384.1(C.costatus.cc011211-02a.Panama)
Escovopsis sp.AY629383.1(C.muelleri.ugm010407-15.Panama)
Escovopsis sp.AY629386.1(C.muelleri.cc011213-10.Panama)
Escovopsis sp.AY629387.1(C.muelleri.nmg011101-11.Panama)
Escovopsis sp.AY629385.1(C.muelleri.nmg011110-05.Panama)
Escovopsis sp.AY629391.1(C.muelleri.cc011110-03.Panama)
Escovopsis sp.AY629388.1(C.muelleri.ugm010407-11.Panama)
Escovopsis sp.AY629390.1(C.muelleri.nmg011101-06.Panama)
Escovopsis sp.AY629389.1(C.muelleri.ugm010407-12.Panama)
Escovopsis sp.AY629394.1(C.muelleri.nmg011105-03.Panama)
Escovopsis sp.AY629393.1(C.muelleri.rmma010311-02.Panama)
Escovopsis sp.KX259113(M.smithii.HF100409-02Esco1.G7.Panama)
Escovopsis sp.KX259117(M.smithii.HF100409-03Esco5.G7.Panama)
Escovopsis sp.KX259114(M.smithii.UGM100408-02ch2Esco2.E6.Panama)
Escovopsis sp.KX259115(M.smithii.HF100408-02Esco3.G7.Panama)
Escovopsis sp.KX259116(M.smithii.KK100419-01ch1Esco4.K4.Panama)
Escovopsis kreiselii.KJ808766.1(Mycetophylax morschi.AR090306-01.Brazil)
Escovopsis sp.KX259112(M.smithii.KK100411-01ch3.X1.H5.Panama)
Escovopsis sp.KX259122(M.smithii.RS100412-03ch2.Esco12.A5.Panama)
Escovopsis sp.KX259121(M.smithii.RS100403-02ch1.Esco10.B2.Panama)
Escovopsis sp.KX259123(M.smithii.RS100403-01ch2.Esco15.B2.Panama)
Escovopsis sp.KX259124(M.smithii.RS100412-01ch1.EscoA.A5.Panama)
Escovopsis sp.genbank(M.smithii.RS100411-06.Esco8.B5.Panama)
Escovopsis sp.DQ848160.1(Apterostigma dentigerum.agh020621-05.Costa Rica)
Escovopsis sp.DQ848165.1(Apterostigma sp.ugm020602-07.Panama)
Escovopsis sp.DQ848174.1(A.dentigerum.nmg020611-02esc6.Panama)
Escovopsis sp.DQ848190.1(A.dentigerum.nmg02052-104esc1.Panama)
Escovopsis sp.DQ848184.1(A.cf.pilosum.nmg030618-01esc1.Ecuador)
Escovopsis sp.AY629397.1(A.dentigerum.sp011112-01a.Panama)
Escovopsis sp.DQ848175.1(A.dentigerum.nmg020611-02esc7.Panama)
Escovopsis sp.DQ848196.1(A.cf.dentigerum.agh030627-01esc1.Ecuador)
Escovopsis sp.DQ848197.1(Apterostigma sp.agh030627-03esc2.Ecuador)
Escovopsis sp.DQ848166.1(A.dentigerum.abs020621-02.CostaRica)
Escovopsis sp.DQ848204.1(Apterostigma sp.nmg031215-04.Panama)
Escovopsis sp.DQ848187.1(A.dentigerum.agh030222-12.CostaRica)
Escovopsis sp.DQ848168.1(A.dentigerum.agh020706-01.CostaRica)
Escovopsis sp.DQ848161.1(A.dentigerum.agh020629-02esc4.CostaRica)
Escovopsis sp.DQ848169.1(A.dentigerum.agh020709-10esc1.CostaRica)
Escovopsis sp.DQ848176.1(A.dentigerum.agh020630-01esc1.CostaRica)
Escovopsis sp.DQ848171.1(A.dentigerum.agh020709-10esc3.CostaRica)
Escovopsis sp.DQ848201.1(Apterostigma sp.cc030327-01esc4.Argentina)
Escovopsis sp.DQ848173.1(A.dentigerum.agh020712-04esc1.CostaRica)
Escovopsis sp.DQ848203.1(Apterostigma sp.agh031215-02.Panama)
Escovopsis sp.DQ848192.1(Apterostigma sp.ugm020531-04esc1.Panama)
Escovopsis sp.DQ848188.1(Apterostigma sp.ugm030106-02.Panama)
Escovopsis sp.DQ848207.1(Apterostigma sp.cc030106-02escb.Panama)
Escovopsis sp.DQ848172.1(A.dentigerum.agh020709-10esc8.CostaRica)
Escovopsis sp.DQ848163.1(Apterostigma sp.cc020605-04.Panama)
Escovopsis sp.DQ848157.1(A.dentigerum.nmg010816-05esc1.Panama)
Escovopsis sp.DQ848170.1(A.dentigerum.agh020709-10escy.CostaRica)
Escovopsis sp.DQ848180.1(A.dentigerum.sp011112-01esc11.Panama)
Escovopsis sp.DQ848186.1(A.cf.pilosum.sv030614-02esc1.Ecuador)
Escovopsis sp.DQ848167.1(A.dentigerum.agh020629-02esc6.CostaRica)
Escovopsis sp.DQ848183.1(A.dentigerum.ugm020531-01esc2.Panama)
Escovopsis sp.DQ848182.1(Apterostigma sp.agh030627-08esc1.Ecuador)
Escovopsis sp.DQ848194.1(Apterostigma sp.agh030609-03esc1.Ecuador)
Escovopsis sp.DQ848177.1(Apterostigma sp.cc011018-04esc1.Panama)
Escovopsis sp.DQ848198.1(Apterostigma sp.cc030101-01.Panama)
Escovopsis sp.DQ848205.1(Apterostigma sp.nmg031212-06.Panama)
Escovopsis sp.KX259118(M.smithii.KK100413-01ch3.Esco6.B5.Panama)
Escovopsis sp.KX259119(M.smithii.KK100411-01ch2.Esco7.H5.Panama)
Escovopsis sp.DQ848179.1(A.auriculatum.cc01121331esc1.Panama)
Escovopsis sp.DQ848209.1(C.longiscapus.nmg011101-03.Panama)
Escovopsis sp.VEM-2014.KF033128.1(Mycocepurus goeldii.Brazil)
Escovopsis sp.AY629396.1(A.dentigerum.nmg011027-02.Panama)
Escovopsis sp.DQ848156.1(A.dentigerum.nmg010802-02.Panama)
Escovopsis sp.DQ848185.1(A.cf.pilosum.nmg020519-02esc2.Panama)
Escovopsis sp.DQ848193.1(Apterostigma sp.nmg011029-03esc1.Panama)
Escovopsis sp.DQ848158.1(A.dentigerum.nmg010816-05b.Panama)
Escovopsis sp.DQ848175.1(A.dentigerum.nmg020611-02esc7.Panama)
Escovopsis sp.DQ848159.1(A.dentigerum.nmg010318-21.Panama)
Escovopsis sp.DQ848208.1(Apterostigma sp.ugm030327-05esc4.Argentina)
Escovopsis sp.DQ848189.1(Apterostigma sp.al03061810esc1.Ecuador)
Escovopsis sp.DQ848191.1(Apterostigma sp.al030609-03esc1.Ecuador)
Escovopsis sp.DQ848199.1(Apterostigma sp.sv030615-04esc1.Ecuador)
Escovopsis sp.DQ848200.1(Apterostigma sp.sv030615-05esc1.Ecuador)
Escovopsis sp.DQ848202.1(A.cf.pilosum.nmg030614-01esc1.Ecuador)
Escovopsis sp.DQ848195.1(Apterostigma sp.agh030618-02esc1.Ecuador)
Trichoderma sp.AY629398.1(agh02062905.Panama)
0.005 substitutions/site

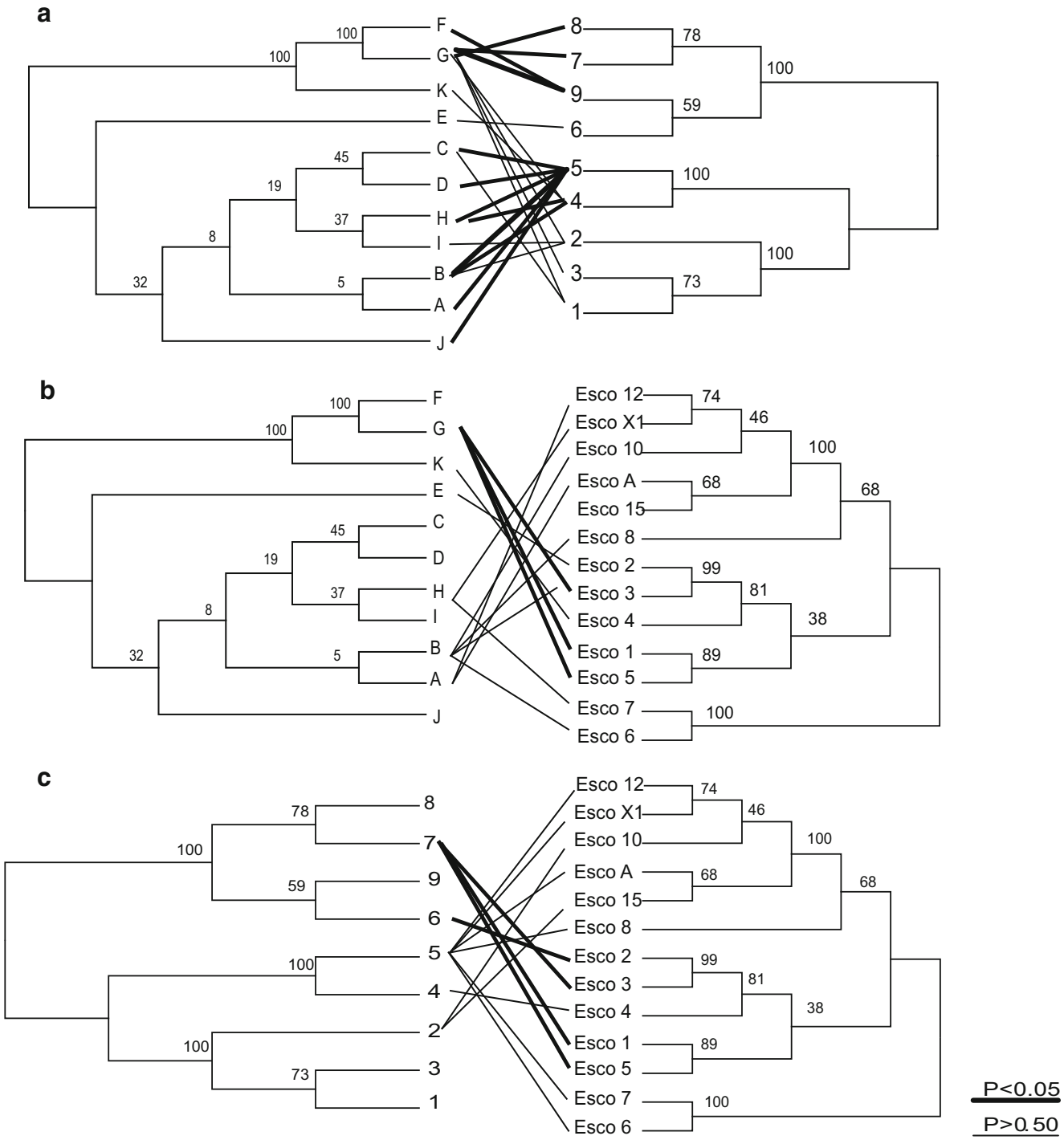


Fig. 2 Cophylogenetic trees illustrating the symbiotic relationships between **a** lineages of the clonal fungus-farming ant *Mycocepurus smithii* and its fungal cultivars; **b** lineages of *M. smithii*; and **c** fungal cultivars and the different isolated *Escovopsis* strains. Lines connecting the cophylogenies illustrate associations observed in the field; line thickness corresponds to statistical significance of the association as inferred in Parafit analysis. Phylogenetic trees are based on microsatellite genotypes for the ant lineages (A–K), ITS sequences for the fungal cultivar lineages (1–9) (details in [26]), and EF1-α sequences for the *Escovopsis* lineages (Esco1–EscoA). Ant and fungal cophylogenies have been published previously [26]. The linkages suggest that ants from lineage G, fungus from cultivar lineage 7 and strain *Escovopsis* 5 have a coevolutionary history

inoculated on the same day and monitored daily for 10 consecutive days. *Escovopsis* infection was determined by visual identification of *Escovopsis* mycelia growing from the sprayed garden fragment (see Fig. 3a and Table 2). All scoring was performed blindly and without knowledge of treatment or fungus genotype [71].

2. *Infection of garden fragments tended by different numbers of ants*: Fungus-garden fragments from two different colonies: A5 colonies (ants from ant lineage "A" and fungus from fungal cultivar lineage 5) and E6 colonies (lineage E ants growing fungus from fungal cultivar lineage 6) the same size (3 cm^3) were placed in Petri dishes with moistened plaster bottoms (UV sterilized) using sterile forceps. Ants were added onto the garden fragments in three experimental groups: ten ants, five ants, and two ants per garden fragment. The three groups were sprayed with one puff spore solution with either Esco5 (five replicates each for cohorts of ten ants; ten replicates each for cohorts of five and two ants), Esco6 (five replicates each for cohorts of ten ants; ten replicates for cohorts of five and two ants), or with one puff sterile 0.005% Tween80 only (control groups) (five replicates each for cohorts of ten, five, and two ants). All replicates were sprayed on the same day and monitored daily for 10 consecutive days. After 10 days, small pieces of each experimental colony were transferred onto sterile PDA plates with sterile forceps and scored for *Escovopsis* mycelia growing from garden fragments (Fig. 3b). All scoring was performed blindly and without knowledge of treatment [71].
3. *Effect of infection on garden mass loss*: Subcolonies were obtained from taking fungus garden fragments of approximately the same size (3 cm^3) along with five worker ants from five different colonies (composed of the following ant-fungal combinations G7, E6, J5, A5, B5, see [26]) were placed in Petri dishes with moistened plaster bottoms (UV sterilized) using sterile forceps. Subcolonies received either one of three treatments: Spray-inoculation with Esco5 (5 replicates each), with Esco6 (5 replicates each), and control spraying with sterile 0.005% Tween80 (3 replicates each). Thus, there were five ant-fungal combinations, two manipulations (Escovopsis lineage), and a control. All replicates were monitored daily for 10 consecutive days. After 10 days, small pieces of each experimental garden were transferred onto PDA plates with sterile forceps and scored for the appearance of *Escovopsis* mycelia. Fresh weights of gardens were measured before and after the experiment, and the loss of garden mass was calculated as percentage of the original weight. Data analysis was conducted using two-way ANOVA with ant-fungal combination and *Escovopsis* lineage each as main effects. Post hoc comparisons were conducted with Tukey's test. All measurements and scoring were performed blindly and without knowledge of treatment or ant × fungus combination [71]. To meet parametric assumptions, data were arc sine transformed because they were percentages. All statistical analyses were performed in R Version 3.1.3 [72].

Results

Infection Rate in the Field

A total of 27 isolates were sequenced, and 13 of these were identified by NCBI-BLAST search as *Escovopsis* sp. and the remaining isolates as *Fusarium* (n = 6) and *Trichoderma* (n = 3) or unspecified (n = 5). The 13 *Escovopsis* sp. had been isolated from 13 different gardens from 13 colonies (i.e., we did not find any double-infection with two different *Escovopsis* isolates from the same garden) (see Table 1). Because we had sampled 67 gardens, the estimated minimum infection rate of field colonies by *Escovopsis* is 36% (13 out of 67 gardens). More than one *Escovopsis* strain was found at several collection locations, with the Gamboa/Apartment 183 location yielding four different strains (see Table 1). Other collection locations in the Panama Canal region (e.g., Beach, Pipeline Road, Achiote, and Gatun, see details in [26]) did not yield any *Escovopsis*, but it is unclear whether *M. smithii* gardens at these sites were free of *Escovopsis*, whether these sites were undersampled, or whether *Escovopsis* strains prevalent at these sites are more difficult to isolate. For details on all sample locations see [26].

Phylogenetic Affinities of *Escovopsis* Isolates

A maximum likelihood analysis (Fig. 1) shows that the *Escovopsis* strains isolated from *M. smithii* gardens are closely related to strains from *C. longiscapus* and *C. muelleri* ants [53], with the *Escovopsis* strains from the clade 1 and clade 2 fungus groupings corresponding to the respective groupings known for the two *Cyphomyrmex* species (Fig. 1; see also [20, 26, 27]). Most of the *Escovopsis* strains (85%, 11 of 13 total) belonged to the pink-spored morphotype, which was also the most frequent morphotype isolated by Gerardo et al. [53] from *Cyphomyrmex* gardens. Only two of the isolates were visually categorized as yellow-spored morphotype (strains Esco6 and -7), and phylogenetic analyses placed these two into the yellow-spored clade of *Escovopsis* previously described for *Apterostigma* gardens ([53, 55]; these *Apterostigma* grow a phylogenetically very distinct fungal cultivar than *M. smithii*). As illustrated in Fig. 1, pink-spored *Escovopsis* strains were isolated from *M. smithii* gardens of both fungal cultivar clades 1 and 2.

The ParaFitGlobal test, with ant lineages defined as hosts and *Escovopsis* strains as parasites, was overall significant (ParaFitGlobal = 0.0052, p = 0.016), indicating that observed clade-to-clade correspondences between ant and *Escovopsis*

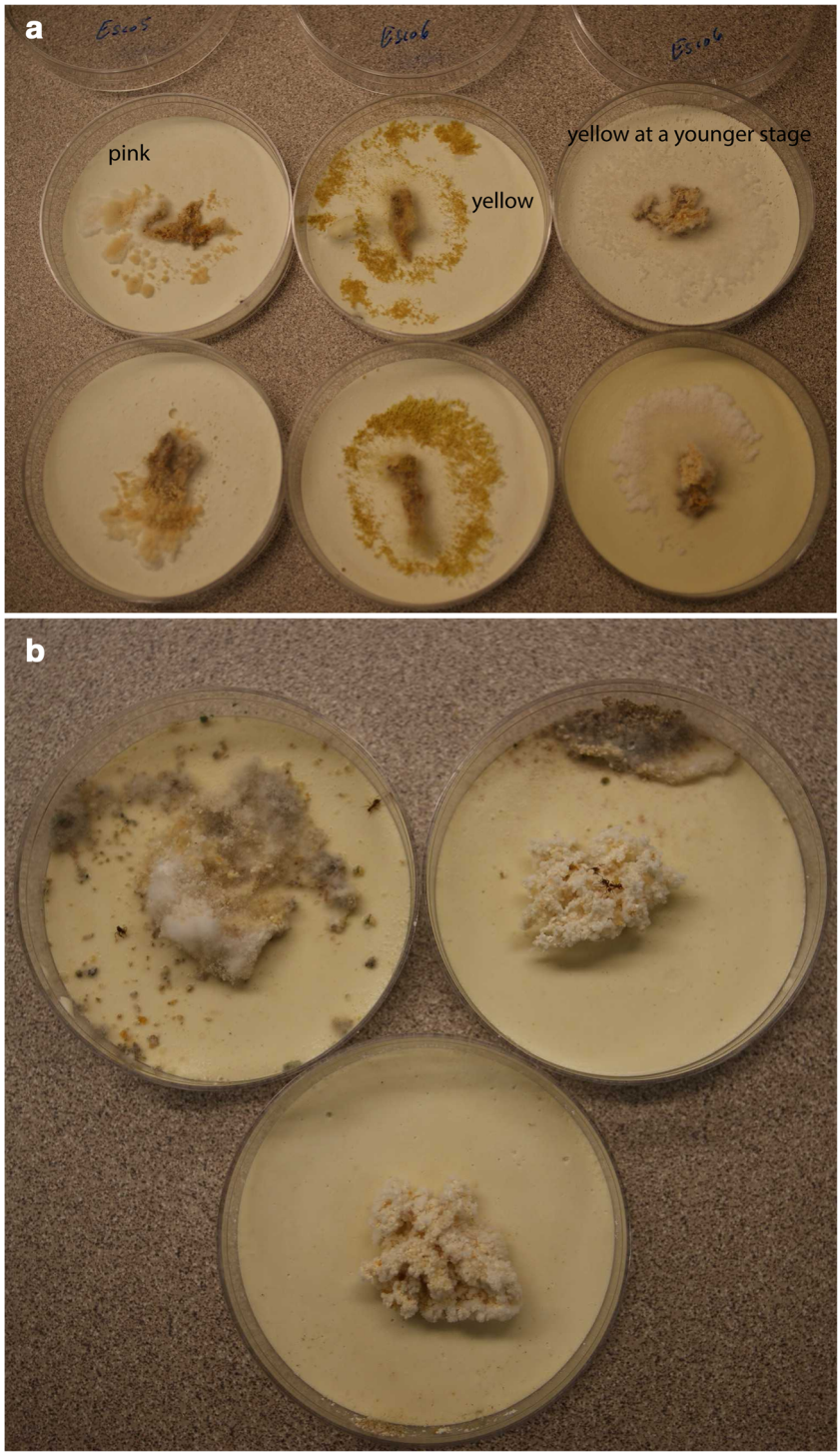


Fig. 3 **a** *Escovopsis* mycelial growth and spore production of experimentally inoculated garden fragments that are not tended by ants. Left: *Escovopsis* strain Esco5 pink spore type. Middle: *Escovopsis* strain Esco6 yellow spore type. Right: *Escovopsis* strain Esco6 yellow spore type at a younger stage (spores still white and not yet yellow). **b** *Escovopsis* infection of garden fragments tended by ants. Top row, left: Diseased garden tended by two ants, overgrown with *Escovopsis* strain Esco5 mycelia. Top row, right: Fungus garden tended by five ants, *Escovopsis* strain Esco5 mycelia emerge from refuse pile at top edge of dish, while the garden itself is maintained healthy by the ants. Bottom: Healthy garden tended by ten ants, spray-inoculated with spore solution of *Escovopsis* strain Esco5, no visible signs of infection

phylogenies are either due to a coevolutionary history or clade-specific de-novo acquisition and/or persistence of ant-*Escovopsis* associations. However, only three out of 13 individual association links were significant (Fig. 2b: G ants—Esco5, $p = 0.005$, G ants—Esco1, $p = 0.001$, G ants—Esco3, $p = 0.021$), suggesting that these links represent a co-evolutionary history. In contrast, the ParaFitGlobal test for fungus lineages defined as hosts and *Escovopsis* strains as parasites was overall not significant (ParaFitGlobal = 0.00044, $p = 0.0683$), indicating no overall coevolutionary history between fungal cultivar lineages and *Escovopsis* strains. However, four of the 13 host-parasite links gave significant results (Fig. 2c: fungus 6—Esco2, $p = 0.035$; fungus 7—Esco5, $p = 0.006$; fungus 7—Esco1, $p = 0.003$;

Table 2 Infection rates of different fungus garden cultivars after spray-inoculation with *Escovopsis* in the absence of garden-tending ants. Fungus garden fragments were spray-inoculated with either *Escovopsis* strains Esco5 or Esco6, or sprayed with sterile 0.005% Tween80 as control. Infection was scored when *Escovopsis* growth was visible on a fungus garden fragment. Numbers of replicates are listed in parenthesis for each treatment

Fungus cultivar type	Spray treatment	Infection rate after 10 days in %
2	Esco5 (5)	100
	Esco6 (5)	100
	Control (2)	0
5	Esco5 (10)	100
	Esco6 (10)	100
	Control (5)	0
6	Esco5 (10)	80
	Esco6 (10)	100
	Control (5)	0
7	Esco5 (10)	100
	Esco6 (10)	100
	Control (5)	0

fungus 7—Esco3, $p = 0.030$), indicating that these links could be coevolutionary associations in the otherwise overall random association between the fungal cultivar phylogeny and the parasite *Escovopsis*. Significant association patterns between ants × fungus, ants × *Escovopsis*, and fungus × *Escovopsis* (Fig. 2) indicate coevolutionary history between G ants, fungus 7, and *Escovopsis* strain Esco1, -3, and -5.

Infection Experiments

1. *Infection of garden fragment not tended by ants*: The earliest signs of infection were observed on day 2 in replicates of the cultivar 2 × Esco5 combination. Signs of infection were scored on day 7 in the cultivar 6 × Esco6 and cultivar 6 × Esco5 combinations in five and seven replicates, respectively. One hundred percent infection rate was reached after 7 days in cultivar 7 × Esco5 and Esco6 combinations and after 8 days in cultivar 5 × Esco5 and Esco6 combinations. The slowest rate of infection was recorded in cultivar 6 × Esco5 combination, where two replicates remained uninfected after 10 days. All control replicates (sprayed with sterile 0.005% Tween80 only) showed no sign of infection (see Fig. 3a and Table 2).
2. *Effect of worker number on Escovopsis infection of fungal garden fragments*: (i) Cohort of ten ants: On day 1, all replicates except the controls started to accumulate refuse piles. Ant workers were observed grooming the gardens, removing pieces of fungus, and adding them onto refuse piles located at the edges of the Petri dishes (Fig. 3b). While refuse-piles sizes increased and garden sizes decreased, no visible signs of infection or illness were observed during the experiment. *Escovopsis* growth appeared on PDA plates from all replicates except in the ant × fungus × *Escovopsis* combination A × 5 × Esco5. (ii) Cohort of five ants: Similar to the ten-ant cohorts, all replicates except the controls started to make refuse depots. While infection of fungus gardens was observed only in a few cases, *Escovopsis* mycelia was observed to grow on all refuse piles. *Escovopsis* mycelia growth appeared on PDA plates from all replicates except controls. (iii) Cohort of two ants: All replicates except the controls started to accumulate refuse piles. Infection of fungus gardens was observed in all replicates of all ant × fungus × *Escovopsis* combinations on days 2 and 3. By day 10, *Escovopsis* mycelium had taken over each garden fragment and ant workers had abandoned the gardens. *Escovopsis* mycelium appeared on PDA plates from all replicates except the controls (see Fig. 3b and Table 3).
3. *Effect of ant-fungus-Escovopsis combinations on garden mass loss:* Similar to the outcome of the five-ant cohort experiment, refuse piles accumulated in all *Escovopsis*-treated replicates, but none of the *Escovopsis*-treated and control replicates had any visible signs of *Escovopsis* mycelia growth or disease outbreak on the fungus gardens or on refuse piles. *Escovopsis* growth was found on all PDA subculture tests except for the controls plates (experimental gardens were subcultured on day 10), confirming presence of viable spores in the *Escovopsis*-treated gardens during the experiment. Although no visible signs of infection were found, *Escovopsis* treatment led to significant losses in garden weights. *Escovopsis* strain Esco5 appeared to be the more virulent strain because experimental replicates treated with *Escovopsis* strain Esco5 lost significantly more garden weight than replicates treated with strain Esco6 or the control group ($F_{2,50} = 9.76$, $p < 0.0001$, Fig. 4). The main effect of ant-fungal combination was marginally significant with J5 colonies experiencing more weight loss than B5 colonies ($F_{4,50} = 2.74$, $p = 0.04$) (Fig. 5). Additionally, we found evidence of synergistic (interaction) effects: *Escovopsis* treatments interacted significantly with ant-fungal combination of *M. smithii* colonies, such that G7 colonies exposed to *Escovopsis* 5 lost more garden weight ($F_{8,50} = 2.2$, $p = 0.04$, Fig. 5) than G7 colonies exposed to *Escovopsis* 6 or the control. G7 colonies exposed to *Escovopsis* 5 also lost more weight than E6 colonies exposed to *Escovopsis* 6 and B5 colonies exposed to *Escovopsis* 5.

Table 3 Infection rate of ant lineage × fungus garden cultivar combinations as a function of the number of ants tending the gardens. Ant-fungus combinations were either spray-inoculated with *Escovopsis* strains Esco5 or Esco6, or sprayed with sterile 0.005% Tween80 as control. While the sizes of fungus garden fragments were identical between all replicates at the beginning of each experiment, the number of worker ants tending garden fragments varied (2, 5, or 10 ants). Sample sizes (number of replicates) are given in parenthesis

Ant/fungus combination	Treatment	Infection rate after 10 days		
		Ten-ant cohort	Five-ant cohort	Two-ant cohort
A5	Esco5	0 (5)	60 (10)	100 (10)
	Esco6	0 (5)	70 (10)	100 (10)
	Control	0 (5)	0 (5)	0 (5)
E6	Esco5	0 (5)	0 (10)	100 (10)
	Esco6	0 (5)	0 (10)	100 (10)
	Control	0 (5)	(5)	0 (5)

Discussion

The goals of this study were to (a) describe the *Escovopsis* lineages that naturally infect *M. smithii* gardens and (b) experimentally determine the relative contributions of experimental *Escovopsis* infection, *M. smithii* ants, and fungal cultivar lineages to disease susceptibility and ant colony fitness. We did not find evidence for universally *Escovopsis*-resistant or universally *Escovopsis*-susceptible cultivar lineages or ant genotypes. Rather, colony fitness reduction after *Escovopsis* infection (i.e., *Escovopsis* virulence) was dependent on unique ant-cultivar-*Escovopsis* interactions, such that specific ant-cultivar combinations were susceptible to specific *Escovopsis* infection.

Within the larger phylogenetic context of *Escovopsis* strains isolated from other lower-attine ant species, we found no *Escovopsis* clade specifically associated only with *M. smithii* colonies (Fig. 1). The *Escovopsis* types isolated in our survey are known to associate generally with typical lower-attine clade 1 and clade 2 cultivars, such as the fungi grown by mycelium-cultivating *Cyphomyrmex* ants, among diverse other lower-attine ants (Fig. 1) [20, 26, 42, 53, 55, 73, 74]. For example, two of the *Escovopsis* strains isolated here were found to be similar to those isolated from *Apterostigma* ants (Fig. 1, [55]), and another *Escovopsis* strain is related to an isolate from a garden of Brazilian *M. goeldii* [63], a species closely related to *M. smithii* (see Fig. S1 in [58]). The fungus-cultivar and *Escovopsis* types associated with *M. smithii* are therefore closely related to those known from other lower-attine ants. This suggests that *Escovopsis* might be more specialized on certain fungus-cultivars rather than the ant species which tend these gardens. A similar result has been found in *Cyphomyrmex* ants, where an *Escovopsis* phylogeny was shown to match with a cultivar phylogeny but not with the corresponding ant-host phylogeny [53].

Notably, the infection rate of *Escovopsis* in the Panamá Canal area was lower in the field (35%) than the rates reported for other lower-attine ants (up to 60% in *Cyphomyrmex* [53],

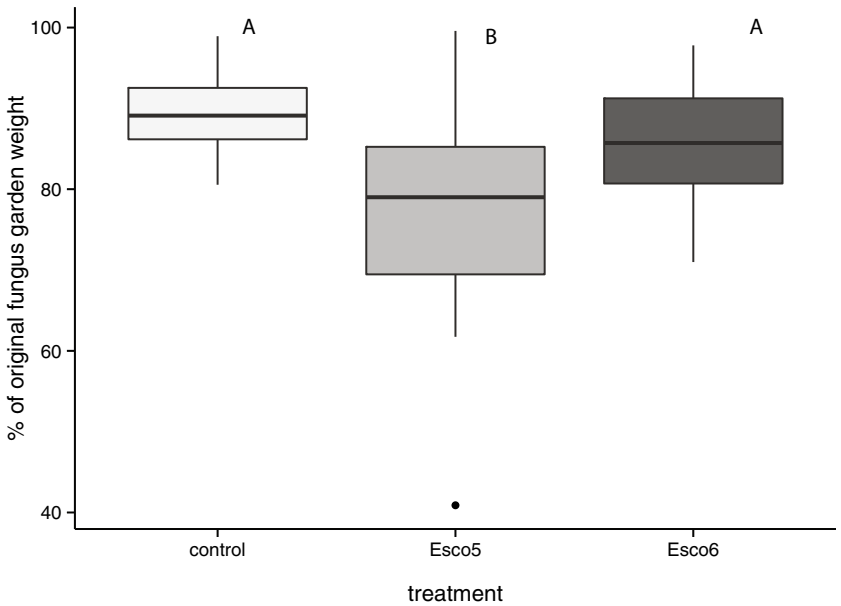


Fig. 4 Median weight loss of fungus gardens as a function of *Escovopsis* treatment. Garden weight loss is measured as a percentage of the start weight (the weight at the end of the experiment relative to the original weight). Treatment with *Escovopsis* strain Esco5 caused significantly greater loss than treatment with strain Esco6 or the control treatment (sterile 0.005% Tween80) (one-way ANOVA, $F_{2,62} = 7.66$, $p = 0.001$, Tukey HSD test, $p < 0.05$). Different letters correspond to significant differences ($\alpha = 0.05$). Garden weight loss is measured as a percentage of the start weight (the weight at the end of the experiment relative to the original weight). Boxplots correspond to first and third quartiles; data beyond the whiskers are outliers and plotted as points

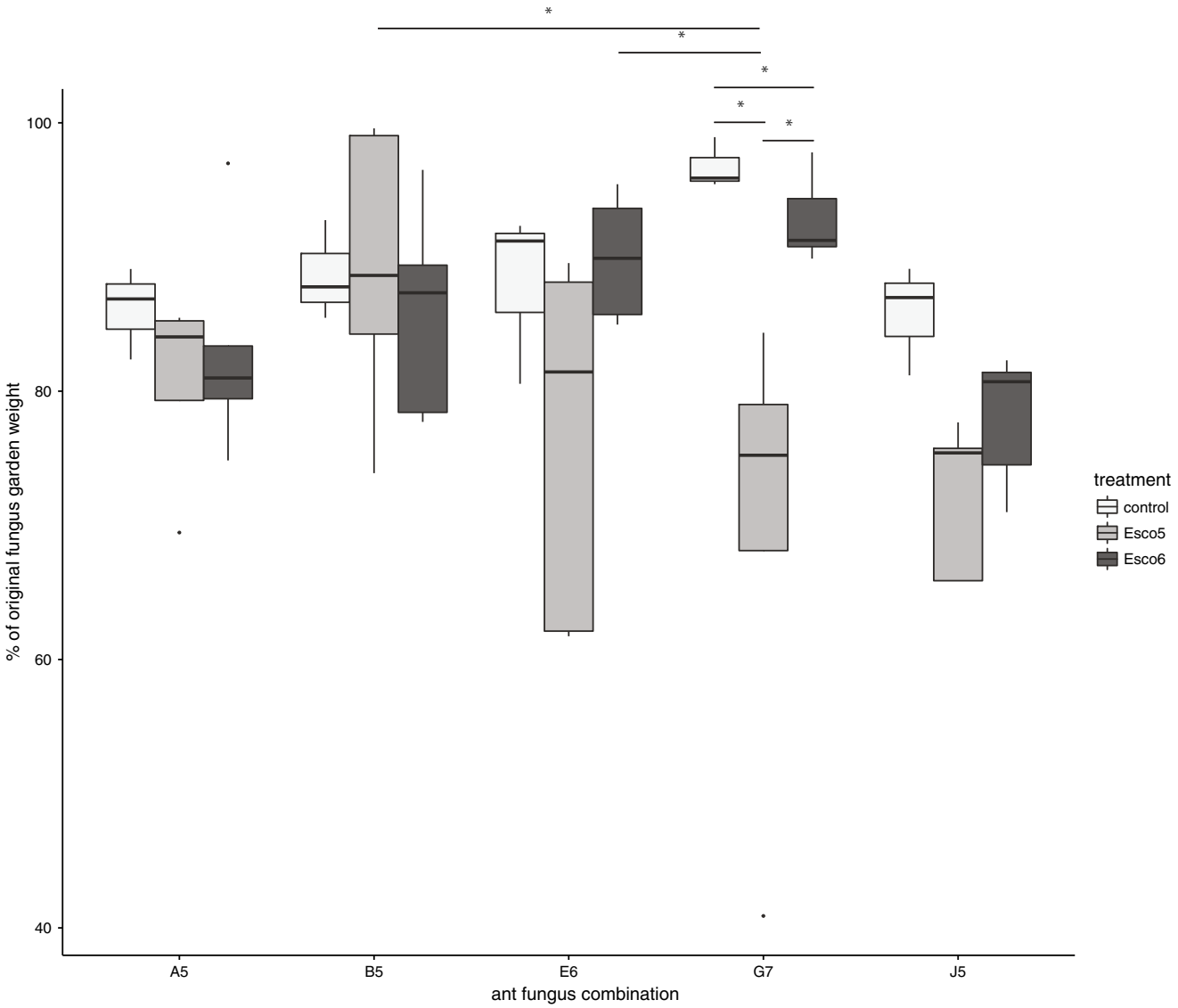


Fig. 5 Median weight loss of fungus gardens as a function of synergistic interactions between **a** *Escovopsis* lineages and ant fungal combinations (subcolonies composed of naturally occurring combinations of ants and fungi). The significant interaction term appeared to be driven by in that G7 ants exposed to *Escovopsis* lineage 5 lost more weight than G7 colonies exposed to *Escovopsis* 6 and E6 colonies growing *Escovopsis* 6 or B5 colonies growing *Escovopsis* 5 ($F_{8,50}$ = 2.2, Tukey's HSD test, p = 0.04*). Garden weight loss is measured as the percentage of the weight at the end of the experiment relative to the original weight. Boxplots correspond to first and third quartiles; data beyond the boxplot-whiskers are plotted as points

up to 67% in *Apterostigma* [55], and in higher-attine ants: 33–51% across five genera [75]). Although we collected in total 67 colony chambers and isolated *Escovopsis* from 13 of these, we did not find any gardens that were visually diseased (i.e., overgrown with *Escovopsis*). Other parasitic fungi, like *Trichoderma* and *Fusarium*, were also easily isolated from garden fragments (KK unpublished data), which suggests that *Escovopsis* is one of many weedy or competitor fungi that can be present as spores or mycelium in gardens, as reported previously for attine gardens [44, 76, 77].

Our Parafit analyses revealed significant association patterns between ant genotype G and three *Escovopsis* lineages (Fig. 2). While most host-parasite association links seem to be not supported statistically, non-random associations were found among *Escovopsis* strains 1, 3, and 5 and colonies with ant/fungus combinations G7, which therefore could reflect more specific coevolutionary interactions. *Escovopsis* strain Esco5 used in our infection experiments was isolated from a colony with ant-fungal combination G7, which is also the most common combination found in our field population (not influenced by sampling efforts, see [26] for rarefaction analysis). Interestingly, Esco5 also appeared to be more virulent toward G7 colonies than Esco6. It has been shown that *Escovopsis* strains isolated from fungus gardens of higher attines differ in their virulence, with certain strains being more aggressive than others [78, 79].

While other investigations used in vitro bioassays to study the pathology of *Escovopsis* in lower-attine ants (e.g., testing growth and infection behavior on culture plates [53, 73]), to our knowledge, our study is the first using in vivo whole-colony experiments to investigate pathology of *Escovopsis* within controlled ant genotype and cultivar genotype backgrounds of lower-attine ants. We found significant interaction effects on garden mass loss among ant-fungal combinations and *Escovopsis* lineages. The overall pattern suggests that colonies are more susceptible to a parasite with a longer co-evolutionary history than a more distantly related (foreign) parasite, which is similar to what Gerardo et al. found in *Cyphomyrmex* ants and their cultivars [53, 54]. Our experiments illustrated that certain combinations (e.g., G7 colonies × Esco5) cause the severest virulence as measured as the most significant garden weight loss. Additionally, our experiments illustrated that, in the absence of the ants, the fungus garden itself has little defense against viable *Escovopsis* spore infection. On the other hand, in the presence of ants, the infection can be contained. Interestingly, the ratio between worker number and garden size plays an important role: while two ants were insufficient to protect a garden fragment against *Escovopsis* in our experiment, ten ants were adequate to prevent a similarly sized garden fragment from becoming overgrown with *Escovopsis* mycelia (Table 3). Five ants were sufficient to physically remove spores and infected garden parts to prevent outbreak of infection on the garden, even though we observed growth of *Escovopsis* mycelia in the refuse piles. This suggests that *M. smithii* ants can remove pathogenic spores and mycelia from the gardens by grooming and weeding and in addition might suppress *Escovopsis* growth with chemical substances from the ants or ant-associated microbial symbionts. Consequently, stress from a garden size: worker number ratio imbalance may be the main reason why workers sometimes cannot control disease outbreaks, as has been hypothesized previously [80–82]. As a result, catastrophic infection by *Escovopsis* might be rare in natural gardens that are attended by an adequate number of workers. *Escovopsis* outbreaks may be more likely in nests that experience drastic worker loss, for example, as result of an entomopathogen epidemic or attack by nest-raiding ants or when maintained under stressed conditions in the laboratory.

Occasional horizontal exchange of fungal cultivars reported by Kellner et al. [26], as well as de novo domestication from free-living populations of potential cultivars, may generate novel ant-fungal combinations that are less susceptible to *Escovopsis* attack. Horizontal switching of ants onto new fungal cultivars (i.e., dependence on a new crop) might therefore be a mechanism to evade diseases through an analog of crop rotation in human agriculture. This symbiont-reassociation mechanism could be very important in a symbiosis that is built on low genetic diversity resulting from clonal ant hosts growing clonal fungal cultivars, perhaps making this symbiosis more vulnerable to diseases than the typical, sexually-reproducing attine host.

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