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12. The perturbation fields were calculated from the full three-dimensional magnetic field vector and two components of the electric field. The reason for not using the azimuthal (spin-axis) electric field component, E_θ, is that this component is measured with a much shorter boom on the satellite and requires visual inspection in order to guarantee its accuracy. The large-database study presented here prohibits visual inspection. Fortunately, it was shown (*20*) that substorm-related electric fields at altitudes similar to those considered in our study are predominantly polarized in the plane perpendicular to E_θ and, thus, the wave Poynting flux is largely due to the other two electric field components, which lie in the orbital plane, and the azimuthal magnetic field component. As a check, we have performed the data analysis when E_θ is set to zero and when E_θ is unchanged. The qualitative conclusions are identical. We present here the results for $E_\theta = 0$.
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Ancient Tripartite Coevolution in the Attine Ant-Microbe Symbiosis

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The symbiosis between fungus-growing ants and the fungi they cultivate for food has been shaped by 50 million years of coevolution. Phylogenetic analyses indicate that this long coevolutionary history includes a third symbiont lineage: specialized microfungal parasites of the ants' fungus gardens. At ancient levels, the phylogenies of the three symbionts are perfectly congruent, revealing that the ant-microbe symbiosis is the product of tripartite coevolution between the farming ants, their cultivars, and the garden parasites. At recent phylogenetic levels, coevolution has been punctuated by occasional host-switching by the parasite, thus intensifying continuous coadaptation between symbionts in a tripartite arms race.

Symbiosis shapes all levels of biological organization, from individual cells to communities and ecosystems (*1–4*). The attine ant-microbe symbiosis is a paradigmatic example of the generation of organic complexity through symbiotic association (*5–13*). Fungus-growing ants in the tribe Attini maintain an obligate mutualism with the fungi they grow for food. In return, the ants provide the fungus with substrate for growth, a means of dispersal to new locations, and protection from competitors and parasites (*14–16*). Attine fungus gardens are frequently infected by a group of potentially devastating fungal parasite species in the genus *Escovopsis* (*11–13*). A fourth symbiont in the attine symbiosis, a filamentous bacterium (actinomycete), is cultured by the ants on specialized body surfaces to derive antibiotics that inhibit the growth of *Escovopsis* (*10, 12, 17*). The ant-cultivar-parasite-bacterium association thus is a quadripartite symbiosis and one of the most complex symbiotic associations discovered in nature. Although the coevolution of attine ants and their fungal cultivars has been the subject of previous investigations (*5, 6, 8, 18*), nothing is known about the evolution of the *Escovopsis* parasites or the attine bacterial mutualists. Here, we reconstruct the evolutionary history of *Escovopsis* to elucidate its origins and coevolution with fungus-growing ants and their domesticated fungi.

The attine ants, a monophyletic group of 13 genera that includes over 210 described species, have apparently cultivated fungi for over 50 million years (*7*). This mutualism is characterized by ancient evolutionary congruence in which specific groups of attine ants have specialized on specific groups of fungal cultivars. The vast majority of basal (lower) attines exclusively cultivate a group of closely related fungi in the family Lepiotaceae (*5, 8*). The derived (higher) attines, including the leaf-cutting ants, cultivate fungi that belong to two clades of leucocoprineous (Lepiotaceae) fungi, which are probably derived from the fungi cultivated by the lower attines (*5*). One lineage within the lower attine genus *Apterostigma* has secondarily switched to fungi in the family Tricholomataceae, and ants in this *Apterostigma* clade thus cultivate fungi that are distantly related to the lepiotaceous cultivars typical for all other attine ants (*5*). In contrast to the ancient evolutionary congruence between ants and their cultivars, at more recent phylogenetic levels within ant-cultivar groups, cultivars may be transferred laterally between ant nests (*5, 8, 18, 19*), and on multiple occasions free-living leucocoprineous fungi have been domesticated by lower attine ants as novel cultivars (*5, 8, 18*).

The fungus gardens of attine ants are parasitized by microfungi in the genus *Escovopsis*. *Escovopsis* infections cause substantial reductions in garden biomass and indirectly reduce

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the growth rate of colonies (13). In some cases, *Escovopsis* can completely overwhelm and destroy colonies (11, 12). *Escovopsis* species, which occur throughout the ants' geographic distribution, have been isolated exclusively from attine-ant nests, have been found in the gardens of all fungus-growing ant genera examined, and are at present the only known parasite of attine fungus gardens (11–13). Unlike the cultivar, which is vertically transmitted by foundress queens from natal to new nests, *Escovopsis* is horizontally transferred between nests (11).

Phylogenetic analyses of nuclear DNA sequence data (20) indicate that *Escovopsis* parasitism of attine fungus gardens likely had a single evolutionary origin (Fig. 1). Four lines of evidence support an ancient origin of *Escovopsis*. First, *Escovopsis* is a monophyletic group found in association with the entire attine ant clade (Fig. 1). Second, a comparison of the phylogeny of *Escovopsis* with previously published phylogenies of attine ants and their fungal cultivars (5, 6, 8, 21, 22) indicates that, at the deepest phylogenetic levels, the evolution of

the *Escovopsis* parasites parallels the evolution of both the ants and their fungal cultivars (Fig. 2) (20). Third, *Escovopsis* is phylogenetically (Fig. 1) and morphologically (12) diverse, suggesting a long evolutionary history. Fourth, we found no correlation between *Escovopsis* phylogeny and geographic distribution (Fig. 1), indicating lineage mixing across large geographic areas over extensive time periods. Taken together, these findings suggest that *Escovopsis* originated in the early stages of fungus cultivation by ants (7).

Escovopsis is divided into four major parasite lineages, each of which is exclusively associated with a corresponding group of fungus-growing ants and their domesticated fungi. These groups represent four major evolutionary innovations: (i) the initial "lower attine symbiosis," incorporating leucocoprinaceous fungi and the most primitive attine ant species; (ii) the "*Apterostigma* symbiosis," incorporating tricholomataceous fungi and a clade of ants derived within the genus *Apterostigma*; (iii) the "*Trachymyrmex* symbiosis," incorporating derived leucocoprinaceous fungi and ants in the

genus *Trachymyrmex*; and (iv) the "leaf-cutter symbiosis," incorporating highly derived leucocoprinaceous fungi and the well-known leaf-cutting species (Fig. 2) (20).

Just as recent evidence indicates cultivar switching by lateral cultivar transfer between ant species (8, 18, 19), the *Escovopsis* phylogeny provides evidence for lateral transfer of the parasite between closely related ant species within each of these four broad groupings. Isolates of *Escovopsis* collected from the gardens of the leaf-cutting ants *Acromyrmex* and *Atta* form two well-supported clades, each containing parasite strains that attack the gardens of both ant genera (Figs. 1 and 2). Similarly, strains of *Escovopsis* isolated from nests of *Myrmicocrypta* and *Cyphomyrmex* co-occur within each of two clades (Figs. 1 and 2). This lack of congruence between ant and *Escovopsis* phylogenies at more recent levels may correspond to previously documented switches between ants and cultivars (8, 18, 19), with *Escovopsis* tracking cultivar rather than ant lineages.
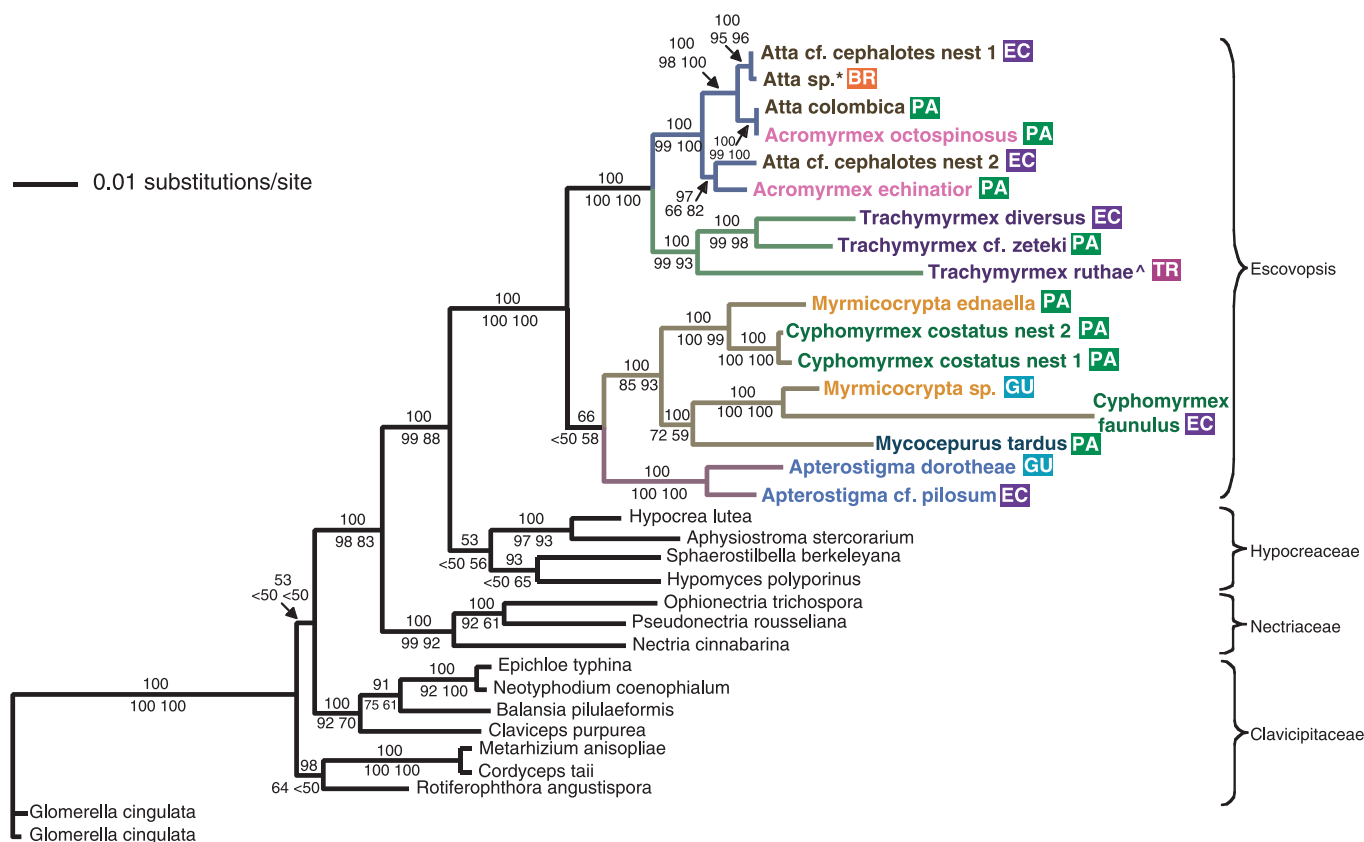
Phylogenetic reconstruction places the

Fig. 1. Phylogeny for 17 strains of *Escovopsis* ant garden parasites and 16 ascomycetous fungal outgroups based on 2639 base pairs of DNA sequence data from three nuclear genes (*ssu rDNA*, *lsu rDNA*, and *EF-1 alpha*). Each *Escovopsis* isolate is indicated by the name of the ant species host garden from which the parasite was isolated. This Bayesian consensus tree is topologically identical to trees obtained from maximum parsimony (MP) and maximum likelihood (ML) analyses, with the exception of the statistically nonsignificant position of a group in the Hypocreaceae in the ML analysis (20). The numbers above the branches indicate posterior probabilities obtained from six independent Bayesian analyses encompassing 1.8 million markov chain Monte Carlo generations (GTR + Γ + I model); numbers below branches indicate bootstrap proportions obtained from 100 ML pseudoreplicates (TrN + Γ + I base-substitution model) (left number) and bootstrap proportions obtained from 1000 parsimony pseudoreplicates (right number). Color branches indicate three distinct *Escovopsis* clades that correspond exactly to the major clades of fungus-growing ants and their fungal cultivars (Fig. 2). Colored boxes indicate the country of origin for each *Escovopsis* strain (EC, Ecuador; BR, Brazil; PA, Panama; TR, Trinidad; GU, Guyana). The two previously described species, *Escovopsis aspergilloides* and *E. weberi*, are identified by the symbols $\wedge$ and *, respectively.
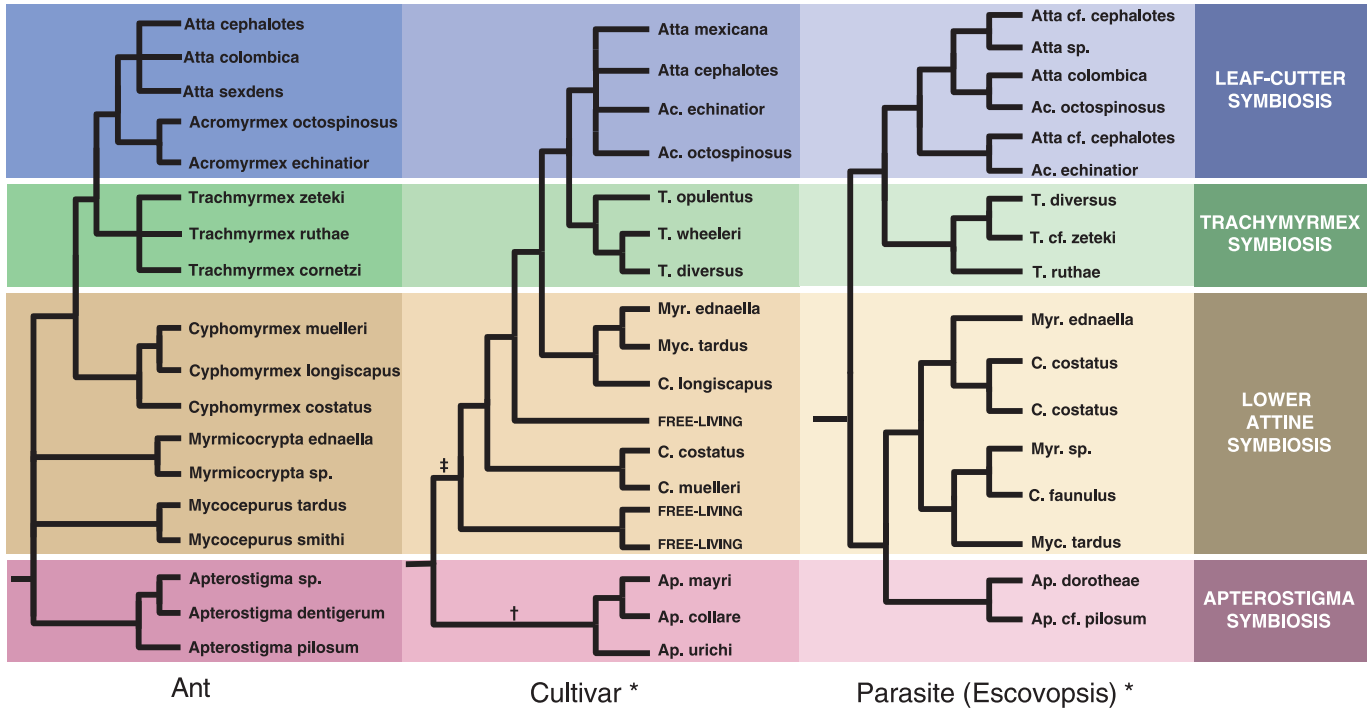
Fig. 2. Phylogenetic reconstruction of the ancient tripartite coevolution of fungus-growing ants (left), their fungal cultivars (middle), and the garden pathogen *Escovopsis* (right). The phylogenies of fungus-growing ants and their fungal cultivars are based on previously published work (*5, 6, 8, 21, 22*). Colors indicate congruent phylogenetic groups of the three symbionts. *Cultivar and *Escovopsis* strains are indicated by the name of the ant species' host garden from which they were isolated. The symbol † indicates that the derived members of the attine ant genus *Apterostigma* secondarily switched from lepiotaceous fungiculture to fungi in the family Tricholomataceae (*5*). The symbol ‡ indicates that cultivars associated with the lower attine ants are not monophyletic but instead are part of a group that also includes free-living species of Lepiotaceae (*5, 8*).

garden parasite *Escovopsis* in the fungal order Hypocreales, as a close relative of the family Hypocreaceae (Fig. 1) (*20*). Many species in the hypocreaceous genera *Hypocrea* and *Hypomyces* are parasites of the vegetative and fruiting structures of mushrooms. For example, *Trichoderma harzianum,* an anamorphic state of *Hypocrea,* is a virulent parasite of the commercially cultivated mushroom *Agaricus bisporus* (*23*). Thus, *Escovopsis* parasitism of the attine ant-microbe symbiosis likely originated with a parasite of free-living leucocoprineous fungi that invaded the symbiosis along with the domestication of these free-living fungi.

Known host-pathogen arms races involve two symbiont lineages engaged in an escalating series of adaptations and counter-adaptations (*24*). In contrast, the attine ant-microbe system involves three mutualists—the ant, mutualistic bacterium, and cultivar—that all depend on successful fungal cultivation and are therefore aligned in their opposition to *Escovopsis*. The fungus garden is defended by the ants, which use specialized behaviors to remove the *Escovopsis* (*16*), and by the bacterium, which produces antibiotics that specifically inhibit *Escovopsis* (*10, 17*). The direct involvement of three diverse mutualists in defending the fungus garden against *Escovopsis*, in conjunction with our finding that *Escovopsis* has a long coevolutionary history within this symbiosis, indicates that this mutualism has been shaped by an arms race

involving four symbiont lineages. Empirical and theoretical investigations into the evolutionary dynamics of this multi-symbiont arms race will inform a general model of the evolution of host-pathogen associations and parasite virulence.

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