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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

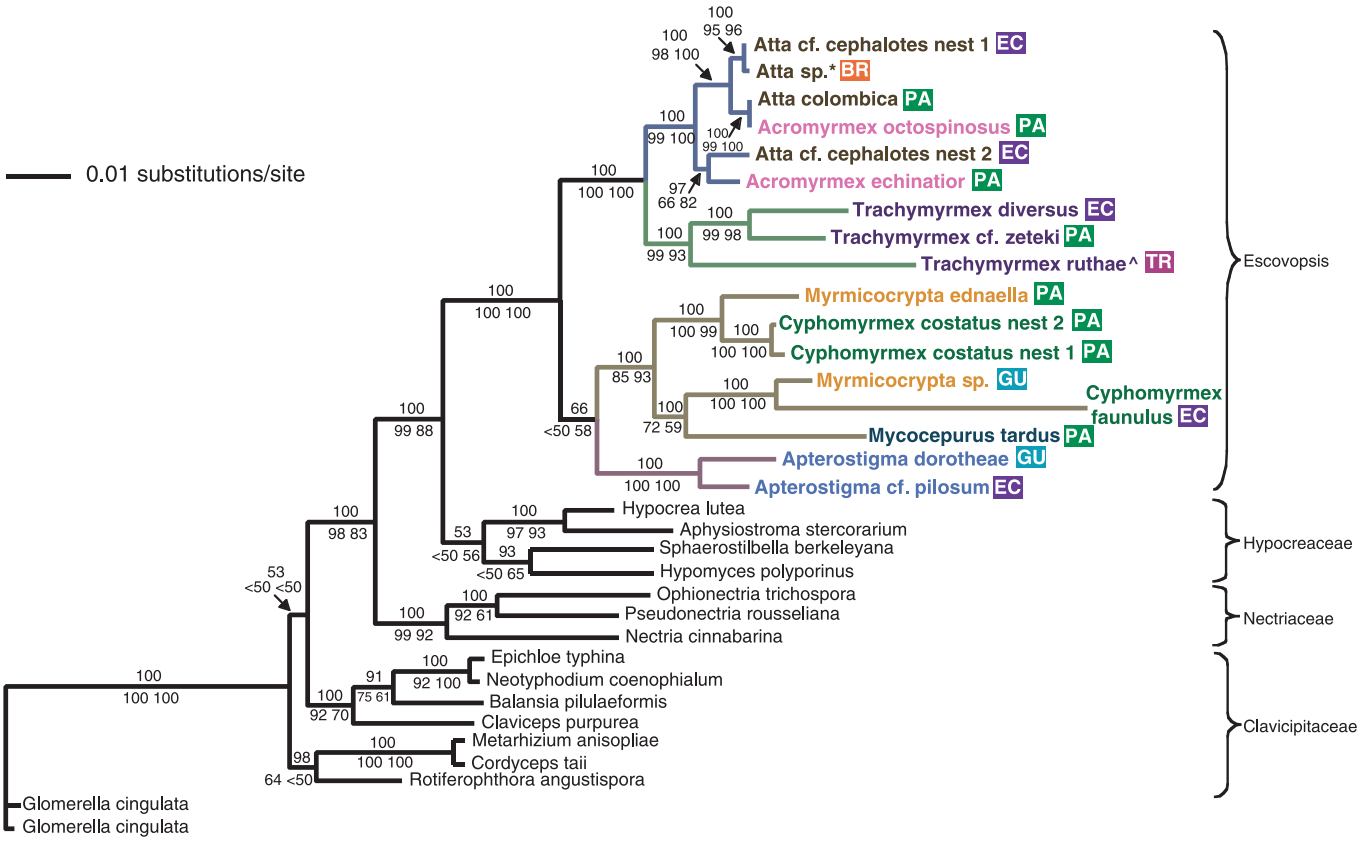


**Fig. 1.** Phylogeny for 17 strains of *Escovopsis* ant garden parasites and 16 ascomycetous fungal outgroups based on 2639 base pairs of DNA sequence data from three nuclear genes (*ssu rDNA*, *lsu rDNA*, and *EF-1 alpha*). Each *Escovopsis* isolate is indicated by the name of the ant species host 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 ∧ and *, respectively.

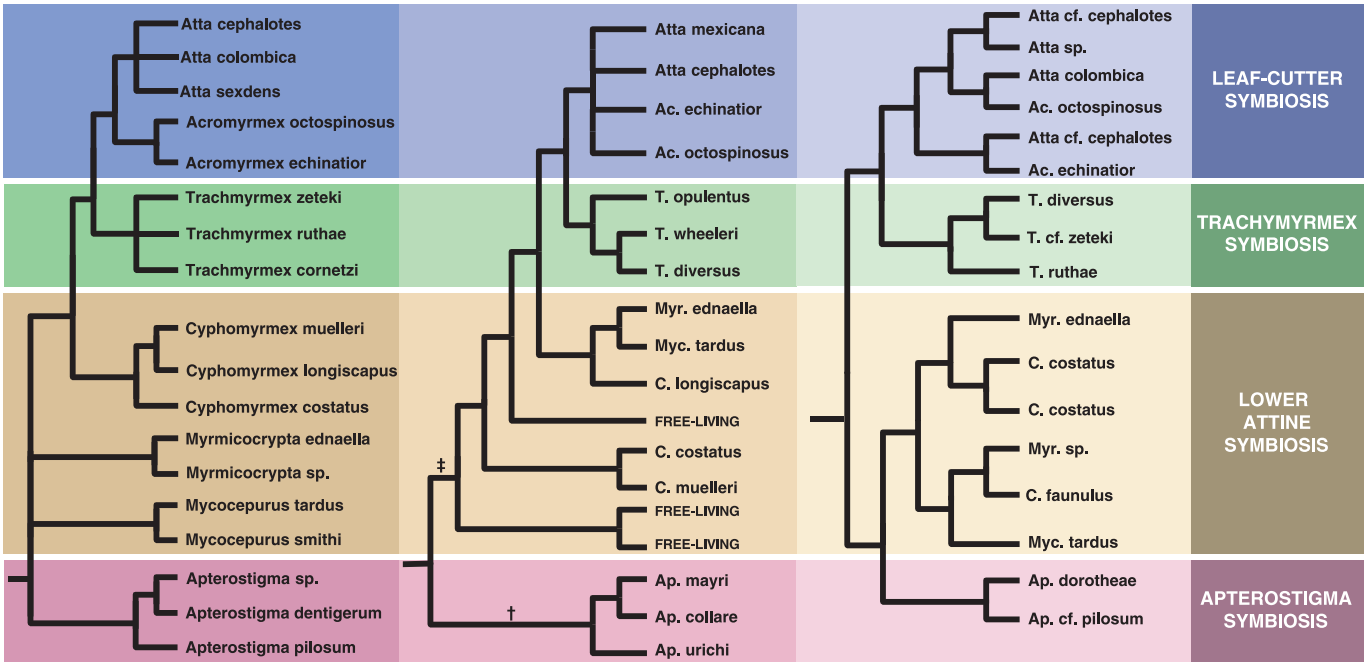


**Fig. 2.** Phylogenetic reconstruction of the ancient tripartite coevolution of fungus-growing ants (left), their fungal cultivars (middle), and the garden pathogen *Escovopsis* (right). The phylogenies of fungus-growing ants and their fungal cultivars are based on previously published work (*5*, *6*, *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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**Supporting Online Material**

www.sciencemag.org/cgi/content/full/299/5605/386/DC1
Materials and Methods
References

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**Supporting Online Material**

**Methods**

**Sampling and DNA sequencing.** Seventeen isolates of *Escovopsis* were selected to include all known morphologically distinct strains of the genus and to maximize geographical distribution (i.e., Brazil, Ecuador, Guyana, Panama, and Trinidad). In addition, isolates were selected from a phylogenetically representative collection of fungus-growing ants. Samples of the two described species *Escovopsis weberi* and *E. aspergilloides* were obtained from the Centraalbureau voor Schimmelcultures (CBS 810.71 and CBS 423.93 respectively). Cellular DNA was isolated using the CTAB method. Genes were amplified by PCR with gene specific primers (28S: CLA-F GCATATCAATAAGCGGAGGA, CLA-R GACTCCTTGGTCCGTGTTTCA; 18S: NS1 (Modified) CCAGTAGTCATATGCTTGTCTC, NS4 (Modified) CTTCCGTCAATTCCTTTAAGTT; EF1-Exon 6: EF1-983F GCYCCYGGHCAYCGTGAYTTYAT, EF1-2218R GACTTGACTTCRGTVGTGAC. Internal primers were used for sequencing 18S: NS3 (forward) GCAACTCTGGTGCCAGCAGCC, NS2 (reverse) GGCTGCTGGCACCAGACTTGC; and EF1-Exon 6: EF1-6MF GTCACBACYGAAGTCAAGTC, EF1-6MR GACTTGACTTCRGTVGTGAC). Sequences were generated on an ABI 377 automated DNA sequencer. We obtained partial sequences for the nuclear small (nuc-ssu; 1097 bp) and large (nuc-lsu; 555 bp) subunit ribosomal DNA (rDNA), and elongation factor 1-alpha (EF1-α; 986 bp). To test the phylogenetic placement of *Escovopsis* among the Hypocreales, representatives from three families, Nectriaceae, Hypocreaceae, and Clavicipitaceae, were sampled for a total of 14 hypocrealean taxa. Additionally, sequences from two isolates of

*Glomerella cingulata* of the Phyllachorales were included as outgroup taxa for the purpose of rooting the Hypocreales. Sequences are deposited in Genbank under the following accession numbers: (outgroup: nuc-ssu, AF339579, AF543762–AF543771, AF339584, U32412, U32405, U45942, U48427; nuc-lsu, AF339530, AF543786–AF543793, U00748, U00756, U17396, U17416, U48428, U57681; EF1- α, AF543772–AF543784; *Escovopsis:* nuc-ssu, AY172582–AY172598; nuc-lsu, AY172599–AY172615; EF1- α, AY172616–AY172632).

**Phylogenetic analyses.** Maximum parsimony (MP) and maximum likelihood (ML) analyses were conducted using PAUP 4.0b10 (1); Bayesian analyses used MrBayes 2.01 (2). MP analyses employed the heuristic search option with TBR branch-swapping and 1000 random-taxon-addition replicates, identifying a single most parsimonious tree of length = 1680, C.I. = 0.395, R.I. = 0.652. Heuristic MP bootstrap analysis consisted of 1000 pseudoreplicates (TBR branch swapping), with 10 random-taxon-addition replicates per pseudoreplicate. The likelihood ratio test implemented in ModelTest 3.06 (3, 4) found the TrN+Γ+I model (5; with a proportion of sites invariant, and gamma-distributed rates) to be the best fit for the sequence data and the MP trees, and this model was employed in a heuristic ML analysis. This analysis consisted of five iterative tree searches, each utilizing updated model parameter values based on the results of the preceding search, and converged on a single tree with a log likelihood score of –13566.534. Heuristic ML bootstrap analysis consisted of 100 pseudoreplicates (TBR branch swapping). Because the TrN+Γ+I model is unavailable in MrBayes, Bayesian analyses employed the more general GTR+Γ+I model (6; general time reversible with a proportion of sites invariant and gamma-distributed rates) and included six separate runs, each consisting

of 300K Markov-Chain Monte Carlo (MCMC) generations and each with a "burn-in" of 100K generations. All runs converged on the same topology. Posterior branch probabilities in Figure 1 are calculated from the pooled post-burn-in trees from all six runs. Further details of phylogenetic analyses are available on request.

Phylogenetic analyses using MP, ML, and Bayesian methods all identified the same tree topology, differing only in the position of the clade (*Aphysiostroma* + *Hypocrea*). In the ML tree this clade is the sister group of *Escovopsis*, whereas in the MP and Bayesian trees it is part of a monophyletic Hypocreaceae, comprising the sister group of (*Hypomyces* + *Sphaerostilbella*). However, under no criterion are the data capable of significantly distinguishing between these two alternatives (K-H test for MP: $P = 0.366$; S-H test for ML: $P = 0.384$; Bayesian posterior probabilities: 0.53 for monophyly of Hypocreaceae vs. 0.38 for the alternative).

To test whether the perfect association of *Escovopsis* strains with the four major attine ant/fungus symbiotic groups (lower attine, *Apterostigma, Trachymyrmex,* and leaf-cutter) is correlated with *Escovopsis* phylogeny to a degree that significantly departs from chance expectation; we employed a version of the permutation test of Kelley and Farrell (7). Using the unrooted phylogenetic network of 17 *Escovopsis* taxa (Fig. 1) with non-*Escovopsis* taxa excluded, we compared the parsimony tree length of the observed 4-state association character (length=3) with the length distribution of 1000 characters created by randomizing the taxon-state assignments with the “Shuffle” command in MacClade 4.01 (8). The results confirm that the observed distribution significantly differs from that expected due to chance at the level of $P < 0.001$.

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# On Ant Farm, a Threesome Coevolves

One of nature's oddest partnerships is that between certain ants and the fungi they cultivate. The two have evolved in synchrony for millions of years. But there is a third wheel in this relationship—a pathogen that infects the fungi. And now Cameron Currie of the University of Kansas, Lawrence, and his colleagues report on page 386 that, in terms of evolutionary history, this pathogen is as tightly entwined with the other two as they are with each other.

The data show that "almost immediately after this unique and beautiful cooperative system [between ants and cultivated fungi] evolved, the fungal parasites were there, and they've never gone away," says Koos Boomsma, an evolutionary ecologist at the University of Copenhagen, Denmark.

Attine ants, which include leaf-cutter ants that can defoliate a tree in one night, can't digest plant matter themselves. But they retrieve leaves and other detritus from their surroundings and heap them up in their nests as offerings for hungry fungi. Thus nourished, the fungi send out nutrient-filled threads that are eaten by their faithful keepers.

Six years ago, researchers demonstrated that ant farming of fungi developed 50 million years ago. Since then, the ants and fungi have maintained their intimate symbiosis even as new species of both arose. Other research has shown that early on in evolutionary history, it's likely that the ant species weren't that picky about which fungal species they grew. But today, many of the 210 attine ants are faithful to a particular fungus.

This happy relationship can be wrecked by the pathogen *Escovopsis*. Infections of this microfungus can reduce both the size of the "farm" and the ant workforce; some have destroyed entire colonies. The ants fight back by weeding out infected bits of fungi and removing the pathogen's spores.

To better understand the pathogen, Currie and his colleagues analyzed DNA from 17 strains, focusing on 2600 bases from several genes. Using the differences in the bases, they built an evolutionary tree. It pointed to a common ancestor that dated back to the days of the first cultivation of fungi by ants.

The researchers are not sure how *Escovopsis* initially got involved with this pair. Currie and his colleagues at first suspected that it was once an insect pathogen and switched hosts when the ants started cultivating fungi. But now they think *Escovopsis* started out as a pathogen of the free-living ancestors of the fungi currently farmed.

The evolutionary history also revealed that different branches of *Escovopsis* appeared in parallel with new branches of ants and fungi. "It looks to me as if the pathogen was locked into the relationship" early on, notes Daniel Janzen, an evolutionary biologist at the University of Pennsylvania in Philadelphia. Today, there are four lineages of the microfungus, and each is associated with a particular ant-fungi system. "It's a nice, clean example" of coevolution, Janzen adds.

The social circle isn't complete, however. Currie showed previously that there's a fourth partner that has yet to be studied. Many of the ants host bacteria on their bodies that produce antibiotics targeted against the pathogens. These too are likely to show some signs of coevolution, and DNA studies should help reveal their relationship to the ant and the fungi, he predicts.

**Bountiful harvest.** A queen ant presides over her workers as they tend their fungal garden.

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Rod Page, a theoretical systematist at the University of Glasgow, U.K., knows of only one other instance in which researchers have attempted to understand a three-way partnership: that between a fig, a fig wasp, and a nematode that infects the wasp. Now, he adds, the ant-fungus-microfungus threesome "might encourage people to think about how many layers are in these associations and what [species] they are tracking" as these organisms evolve.

—ELIZABETH PENNISI