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 cultivate 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 (14, 15). 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 Leotiaceae (5, 8). The derived (higher) attines, including the leaf-cutting ants, cultivate fungi that belong to two clades of leucocoprineous (Leotiaceae) fungi, which are probably derived from the fungi cultivated by the lower attines (5). One lineage within the lower attine genus Apterotigma has secondarily switched to fungi in the family Tricholomataceae, and ants in this Apterotigma clade thus cultivate fungi that are distantly related to the leotiaceous 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
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 single evolutionary origin (Fig. 1). Four lines of evidence support an ancient origin of *Escovopsis* (11). 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 tri-chomataceous 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 (*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 *Escovopsis* isolate is illustrated 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 + I + I model); numbers below branches indicate bootstrap proportions obtained from 100 ML pseudoreplicates (*TrN + I + 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.
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 stage 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 fungus (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.

References and Notes

17. C. R. Currie, A. N. M. Bot, J. J. Boomser, Oikos, in press.
20. Materials and methods are available as supporting material on Science Online.

Supporting Online Material
www.sciencemag.org/cgi/content/full/299/5605/386/DC1
Materials and Methods
References
5 September 2002; accepted 11 November 2002

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 Leptotaceae (5, 8).