Phylogeny of the Attine Ant Fungi Based on Analysis of Small Subunit Ribosomal RNA Gene Sequences

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Complete 16S-like ribosomal RNA coding regions were obtained from the fungal symbiont of five genera of attine (leaf-cutting) ants and two free-living fungi. Phylogenetic analyses with distance matrix, maximum likelihood, and parsimony methods revealed that the attine fungal symbionts are homobasidiomycetes in the order Agaricales. Comparison of the topology of the attine fungal symbiont phylogenetic tree with a tree based on attine ant morphology revealed a congruent branching pattern of the more derived attine ants and their fungal symbionts. The parallel branching pattern suggests a long-term coevolution of derived leaf-cutting attine ants and their fungal symbionts.

Attines (tribe Attini) are unusual among ants in growing symbiotic fungi for food (*1*, *2*). Despite widespread awareness of attine ants and their capacity for defoliation, little is known about the evolution of the fungal symbionts responsible for leaf decomposition (*3*). Unanswered questions include the following: Do different attine lineages cultivate the same fungal symbiont? What free-living fungi share most recent common ancestry with attine fungal symbionts? Did the symbiosis arise multiple times, or was there a single event followed by coevolution? Morphological data were used to resolve the phylogeny of attine ants (*4*). In contrast, the attine fungi possess few morphological characters useful in phylogenetic analysis. Here we examine the complete small subunit ribosomal RNA (16S-like rRNA) coding regions (1810–1821 base pairs) from the fungal symbiont cultivated by five different genera of attines. Our phylogenetic analyses indicate the attine fungi are homobasidiomycetes in the order Agaricales (gilled mushrooms) and that the most derived attine fungi have speciated in parallel with their attendant ants.

The attine ants' mutualism with fungi has allowed the ants to exploit a range of food resources not otherwise available (*2*). The approximately 200 species of attines use a wide variety of material as substrate for growing fungus, for example, insect frass and dry and fresh vegetation (*1*, *2*). The best known attine ants are the "leaf-cutting" ants (*Atta* spp. and *Acromyrmex* spp.). Wilson (*5*) considered the adaptation in leaf-cutting ants that allowed "efficient utilization of almost all forms of fresh vegetation" so unusual and successful "that it can be properly called one of the major breakthroughs in animal evolution."

There has been much disagreement concerning the phylogenetic affinities of the attine fungi. Under natural conditions, attine fungi rarely, if ever, produce sporocarps, the structures traditionally used in taxonomic determinations of higher fungi. Instead, new fungal gardens are propagated asexually from a fragment of the fungus garden carried by each new queen from her natal colony (*1*). On rare occasions, some attine fungi produce sporocarps in culture. Mycologists generally assign these sporocarps to one or more genera within the homobasidiomycete order Agaricales (*6*, *7*). Some have suggested that the attine fungi are a polyphyletic group made up of both ascomycotines and basidiomycotines (*6*, *8*).

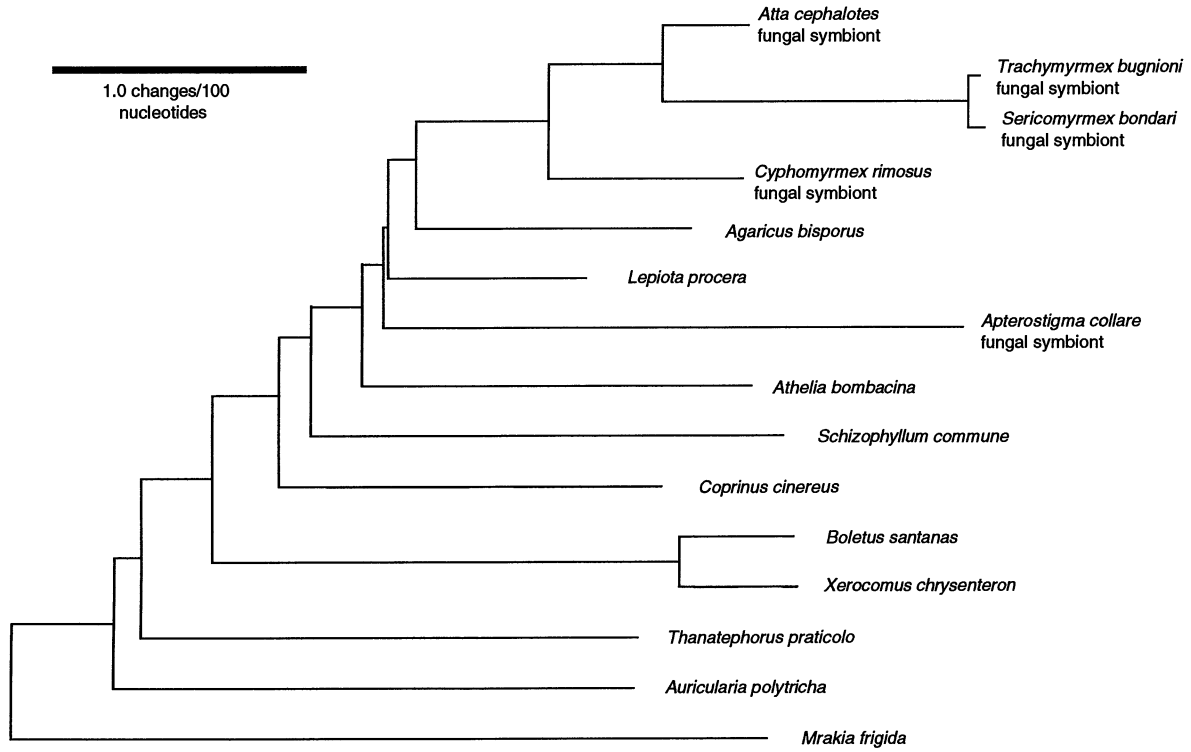

Fig. 1. Distance matrix tree for homobasidiomycete fungi based on 16S-like rRNA gene sequences. The tree was inferred from the comparison of positions that can be unambiguously aligned in all reported full-length homobasidiomycete 16S-like rRNA gene sequences. In this tree, distance matrix methods (*15*) were used to infer relationships for the fungal symbionts of attine ants and representatives of diverse basidiomycete lineages. *Mrakia frigida* was used as the outgroup. The aligned data set and the positions used in the analyses are available upon request from the authors.

Others have proposed that the attine fungi are all closely related or form a cohesive group (9). Because of taxonomic uncertainty, many researchers (9, 10) refer to all strains of attine fungi collectively as *Attamyces bromatificus* and place them in the polyphyletic Fungi Imperfecti.

We examined full-length sequences of 16S-like rRNA coding regions from the fungal symbionts of five different species of attine ants, *Cyphomyrmex rimosus*, *Apterostigma collare*, *Sericomyrmex bondari*, *Trachymyrmex bugnioni*, and *Atta cephalotes*, as well as two free-living Agaricales, *Agaricus bisporus* and *Lepiota procera* (11). The attine fungi we chose to study are morphologically and ecologically dissimilar and seemed least likely to form a monophyletic group. Both *T. bugnioni* and *S. bondari* have "typical" attine fungus gardens that grow as a spongy mycelial mass. *Apterostigma collare* cultivates a fungus with a distinctive veil, unique to this genus. The fungus cultivated by *C. rimosus* grows as balls of unicellular yeast. Finally, the fungus gardens of the leaf-cutting ant *A. cephalotes* are morphologically similar to those of *Trachymyrmex* and *Sericomyrmex* but grow faster and can support colonies of up to several million workers compared with 100 to 5000 workers for colonies of the other attine genera.

Comparison of the attine fungal symbiont 16S-like RNA genes clearly demonstrates that four of the five taxa sampled represent distinct basidiomycete genera (12). Only *Sericomyrmex* and *Trachymyrmex* are sufficiently similar (two nucleotide changes) to permit assignment within the same genus. Pairwise comparisons between the other attine fungal 16S-like rRNAs range from 26 changes for symbionts of *Atta* and *Cyphomyrmex* to 80 changes between *Apterostigma* and *Sericomyrmex* symbionts. This number of differences is similar to that observed between fungal families, for example, 35 nucleotide differences between the 16S-like rRNAs of *Agaricus bisporus* and *Lepiota procera*. In contrast, the similarity of *Trachymyrmex* and *Sericomyrmex* fungal symbionts suggests a very recent divergence.

To assess the origin of attine ant fungal symbionts, we inferred phylogenetic trees using parsimony (13), maximum likelihood (14), and distance matrix (15) analyses of 16S-like rRNA sequences. All phylogenetic reconstructions show the attine fungal symbionts to be members of the order Agaricales (Fig. 1). The fungal symbionts of "higher" attine ants (*Atta*, *Cyphomyrmex*, *Sericomyrmex*, and *Trachymyrmex*) represent a well-resolved, coherent phylogenetic group that diverged after the separation of the fungal symbiont of *Apterostigma*. More importantly the evolutionary relationships of the higher attine ants and their fungal symbionts are entirely congruent (Fig. 2). Parallel patterns suggest cospeciation and codivergence among higher attine fungi and their associated ants (12, 16). Although we are not able to resolve the branching order of free-living *Agaricus*, *Lepiota*, and the fungal symbiont of *Apterostigma* (17), the congruency of the ant and fungal trees is evidence of stable coevolution for millions of years (18).

The ant tribe Attini is thought to have originated about 50 million years ago (19). The divergence between *Cyphomyrmex* and *Trachymyrmex* is at least 25 to 40 million years old, as evidenced by the occurrence of these two genera in the Dominican Amber (20). The genetic divergence among the fungi of the higher attines (Fig. 1) is consistent with a mutualism of considerable antiquity.

Attine fungi that may not have reproduced sexually for millions of years occasionally produce in culture agaric-like sporocarps. The sporocarps from the fungi of different genera of attines are virtually identical morphologically (7). The lack of phenotypic divergence in sporocarps from taxa that diverged >50 million years ago is striking and suggests that sporocarps may indeed be produced in nature, albeit rarely, or that the genes responsible for sporocarp formation are expressed for alternative functions and hence retained.

Attine ants and the attine fungi are mutually dependent (21). The fungus relies on the ants, not only for substrate and for protection from competing microorganisms but also for propagation. In turn, the fungus is an essential part of the ants' diet. This study suggests that the diversification of the higher attine ants and fungi took place within the symbiosis, and therefore, well-developed symbiont-specific adaptations should be expected. The co-adaptation that led ancestral leaf-cutting ants to use fresh vegetation may have involved a physiological breakthrough on the part of the fungus (10). The yeast of *C. rimosus* evolved from a mycelial ancestor under the care of an ancestral *Cyphomyrmex* species. The adaptive significance of this change in fungal growth form is unknown.

The coevolution of attine ants and their fungi is an excellent example of how symbionts, often unseen or overlooked microorganisms, can underlie the preservation of more obvious and much studied macrobiota. In this example, it is unlikely that any of these attine ants would survive if their fungal partners were exterminated by some environmental insult.

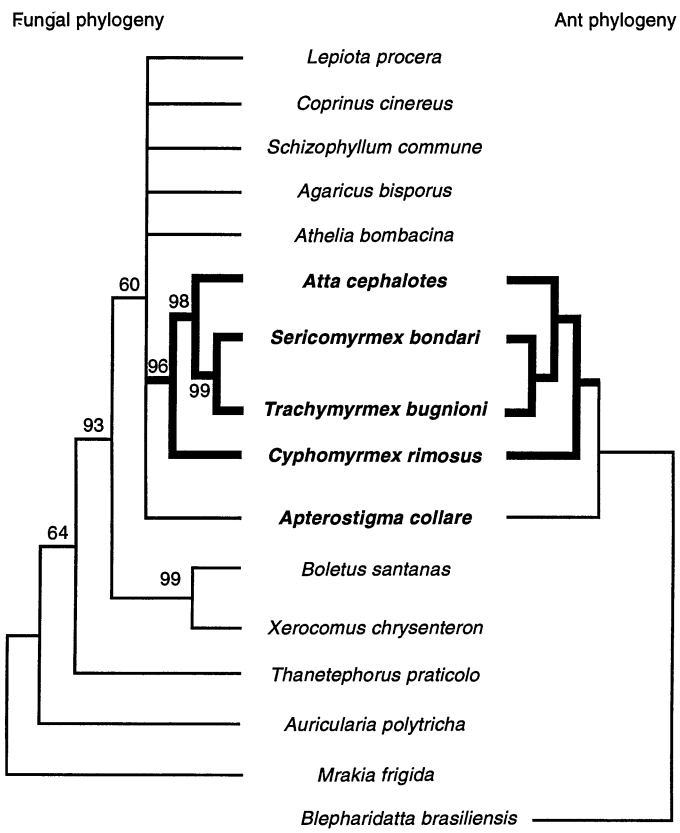

Fig. 2. Phylogenetic tree of symbiotic attine ants and fungi. The attine fungal tree was inferred by comparison of unambiguously aligned positions of small subunit rRNA sequences with maximum parsimony (13). The positions used in the analysis are identical to those used in Fig. 1. The percentage of 100 bootstrap resamplings and subsequent heuristic searches that support identical clades above the 50% level is indicated. The topology of the attine ant phylogeny is derived from a cladistic analysis of 44 prepupal worker larva morphological characters from 51 attine species (4). The topology of the corresponding higher attine ant and fungal symbionts are delineated with a thick line.

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11. Axenic attine fungus cultures were supplied by J. Cazin (symbiont of *T. bugnioni*) and I. Chapela (symbionts of *Cyphomyrmex rimosus*, *Apterostigma collare*, *Sericomyrmex bondari*, and *Atta cephalotes*). Preparation of genomic DNA [S. B. Lee and J. L. Taylor, in *PCR Protocols: A Guide to Methods and Applications*, M. A. Innis, D. H. Gelfand, J. J. Sninsky, T. J. White, Eds. (Academic Press, San Diego, CA, 1990), pp. 282–287] and the polymerase chain reaction (PCR) amplification [L. Medlin, H. J. Elwood, S. K. Stickel, M. L. Sogin, *Gene* **71**, 491 (1988)] and sequencing of 16*S*-like rRNA [H. J. Elwood, G. J. Olsen, M. L. Sogin, *Mol. Biol. Evol.* **2**, 399 (1985)] were previously described. GenBank accession numbers are as follows: *Apterostigma collare* fungal symbiont (U09535), *Atta cephalotes* fungal symbiont (U09538), *Cyphomyrmex rimosus* fungal symbiont (U09536), *Sericomyrmex bondari* fungal symbiont (U09539), *Trachymyrmex bugnioni* fungal symbiont (U09537), *Agaricus bisporus* (L36658), and *Lepiota procera* (L36659).
12. Nucleotide differences between 16*S*-like rRNAs of the attine fungal symbionts are consistent with a phylogenetic analyses based on partial 28*S*-like rRNA sequence determinations from genera corresponding to those included in this study [I. H. Chapela, S. A. Rehner, T. R. Schultz, U. G. Mueller, *Science* **266**, 1691 (1994)].
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17. Maximum parsimony analysis (with branch and bound search strategies) produced two trees of 426 steps. Both trees contain the five attine fungal symbionts analyzed in a coherent phylogenetic assemblage. However, bootstrap parsimony (*14*), maximum likelihood (*15*), and distance matrix (*16*) methods do not identify a preferred branching order among *Agaricus*, *Lepiota*, and the fungal symbiont of *Apterostigma*.
18. If it had been possible to demonstrate that the fungal symbiont of *Apterostigma* branched before the divergence of *Lepiota* and *Agaricus*, two competing interpretations would have had to have been considered. In the first, two independent origins of symbiotic fungi with attine ants may have occurred. In the alternative interpretation, *Lepiota* and *Agaricus* may have escaped the symbioses in favor of a free-living life-style.
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22. We thank J. Cazin and I. Chapela for the attine fungus cultures; M. Wetterer, I. Chapela, D. Hibbett, D. Miller, and D. Yu for comments on the manuscript; David Bermudes for samples of *Lepiota procera*; NSF, the National Geographic Society (J.K.W.), the G. Unger Vetleson Foundation, and NIH (grant number GM32964 to M.L.S.) for financial support.

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Naturally Occurring Variation in Bristle Number and DNA Polymorphisms at the *scabrous* Locus of *Drosophila melanogaster*

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The association between quantitative genetic variation in bristle number and molecular variation at a candidate neurogenic locus, *scabrous*, was examined in *Drosophila melanogaster*. Approximately 32 percent of the genetic variation in abdominal bristle number (21 percent for sternopleural bristle number) among 47 second chromosomes from a natural population was correlated with DNA sequence polymorphisms at this locus. Several polymorphic sites associated with large phenotypic effects occurred at intermediate frequency. Quantitative genetic variation in natural populations caused by alleles that have large effects at a few loci and that segregate at intermediate frequencies conflicts with the classical infinitesimal model of the genetic basis of quantitative variation.

Knowledge of the genetic basis of quantitative characters is important with regard to medicine, the improvement of domestic species, and our understanding of evolution, yet little is known about the particular Mendelian variants that give rise to the heritable component of these traits. One hypothesis is that allelic variation at loci important in the development of a particular trait is a major source of quantitative differences in that trait (*1*). The numbers of abdominal and sternopleural bristles of *Drosophila melanogaster* are typical quantitative characters (*2*). Because *Drosophila* bristles are sensilla (sensory organs) of the peripheral nervous system, candidate genes for bristle number traits are the 10 to 20 proneural and neurogenic loci that determine the presence or absence of sensory hairs (*3*). Alleles of large effect at some of these loci may contribute to the response to artificial selection for high and low bristle numbers (*4*), and insertional polymorphisms in the proneural *achaete-scute* complex (ASC) are associated with naturally occurring genetic variation in bristle number (*1*). The *scabrous* (*sca*) locus encodes a signal protein important in lateral inhibition of the developing nervous system, and mutant *sca* alleles have large effects on bristle number and eye morphology (*5*). We have now tested the hypothesis that allelic variation at *sca* contributes to quantitative genetic variation in natural populations of *D. melanogaster* by associating molecular polymorphisms at this locus with genetic variation in bristle number.

We determined the mean abdominal and sternopleural bristle numbers for each sex from 47 independent second chromosome lines extracted from a natural population and placed in an isogenic genetic background (*6*) (Table 1). The overall means (±SE) were 16.30 ± 0.42 abdominal bristles and 16.15 ± 0.22 sternopleural bristles. Assuming additivity, the total additive genetic variance (σ^2_A) of the second chromosome was estimated as 1.23 for abdominal bristle number and 0.64 for sternopleural bristle number (*7*). The additive genetic covariance between both characters was 0.39, with a genetic correlation coefficient of 0.43 (*7*). These estimates are consistent with previous observations (*8*).

Restriction map variation of a 45-kb region including the *sca* locus was quantified among the 47 chromosomes (Fig. 1 and Table 1). There were 18 restriction site polymorphisms and 25 length polymorphisms (insertions and deletions). Single-stranded conformation polymorphism (SSCP) was determined (*9*) for three fragments that encompass the last intron and

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