B (80% acetonitrile and 0.09% trifluoroacetic acid). Solvent B was maintained at 1% for 10 min and the following gradient was then applied at a flow rate of 200 μl/min: Solvent B was linearly increased from 1 to 10% in 1 min, from 10 to 20% in 30 min, from 20 to 30% in 10 min, from 30 to 40% in 20 min, and from 40 to 100% in 10 min. Most of the fractions denoted 1 to 10 from the DEAE column yielded three peaks by reversed-phase separation. Edman degradation sequencing revealed that the two major peaks, which eluted between 16 and 17% and between 35 and 36% solvent B, corresponded, respectively, to β and α tubulin COOH-terminal peptides. The β tubulin peptide (^{427}DATAEEEGEF^{436}. . .) corresponded to the predicted cleavage site for endoproteinase Asp-N, whereas the major α tubulin peptide (^{424}DLAALE-KDYEEVGIETAEGEG^{444}. . .) resulted from incomplete cleavage in the COOH-terminal domain. The minor peak, which eluted between 20 and 24% solvent B, corresponded to the COOH-terminal peptide of α tubulin generated by complete digestion (^{431}DYEEVGIETAEGEG^{444}. . .). The most acidic fractions that eluted after 34 min from the DEAE column were also purified by reversed-phase HPLC; determination of their amino acid sequences showed that they corresponded to internal acidic sequences of the α and β tubulin subunits.

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9. Amino acid composition was determined with an Applied Biosystems amino acid analyzer (model 420). Derivatization was achieved with phenylisothiocyanate and the hydrolysis was performed on-line. The results confirmed both the presence of the different amino acids contained in the sequences of the tubulin peptides and the variation of the composition in glycyl residues. For example, for the α(424–449) tubulin peptides, the molar ratio of Tyr to Thr (1:1) was constant, whereas the molar ratio of Gly to Thr was significantly higher (8:1 and 13:1 for the peaks denoted 8 and 3, respectively, from the DEAE separation) than the values expected (4:1 or 5:1) for the unmodified peptides (α2 or α1, respectively). For the β(427–442) tubulin peptides, the molar ratio of Asp to Thr (1:1) was constant, whereas the molar ratio of Gly to Thr was significantly higher (8:1 and 14:1 for the peaks denoted 8 and 3, respectively, from the DEAE separation) than the value expected (2:1) for the unmodified peptide.
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12. The sequence (FXE) of the major peptide matched that of ^{436}FEE^{438} of β tubulin (X indicates the presence of a gap in the sequence because at that cycle no derivatized amino acid was identified) (*13*). The protonated molecular ion (MH$^+$) observed by electrospray–mass spectrometry had a mass of 481.0 daltons, corresponding to that of a tetrapeptide composed of one Phe, one Gly, and two Glu residues. Fragmentation of this tetrapeptide confirmed that, according to the amino acid sequence deduced from the corresponding nucleotide sequence, the fragments observed (334.0, 317.0, and 306.0 daltons) could be derived only from the peptide ^{436}FEE^{438} in which Glu^{437} was modified by the addition of one Gly residue. The amino acid analysis also confirmed the addition of one glycyl residue. These results suggest the presence of at least one major glycylation site on Glu^{437} of β tubulin. Thermolysin cleaved the lateral polyglycylated chain, possibly as a result of the broad specificity of this enzyme (*11*), which can cleave peptide bonds at the NH_2-terminal end of glycyl residues. Such peptide bonds present in the polyglycylated chain could be readily cleaved, given that the hydrophobic character of the bulky lateral chain is favorable for recognition by the endoproteinase. After thermolysin digestion, we did not detect any modified α tubulin peptides in the later phase of the DEAE elution profile. Such peptides may have eluted earlier if they were longer and less acidic than those obtained from β tubulin. Molecular mass determination of 50 pmol of peptide was performed on a Trio 2000 mass spectrometer with an electrospray ion source and a quadrupole mass analyzer (VG Biotech, Manchester, UK). The carrier solvent was 50% acetonitrile, 49% water, and 1% formic acid. Fragmentation was obtained by increasing the voltage cone to 70 V.
13. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; D, Asp; E, Glu; F, Phe; G, Gly; I, Ile; K, Lys; L, Leu; M, Met; Q, Gln; R, Arg; T, Thr; V, Val; W, Trp; and Y, Tyr.
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32. Samples were prepared by mixing 1.5 μl of matrix (saturated solution of α-cyano-4-hydroxycinnamic acid in 40% acetonitrile and 0.1% trifluoroacetic acid) with 1 μl of the peptide in 0.1% trifluoroacetic acid (1 to 4 pmol), applying to the stainless steel target, and drying at room temperature. The external standards used for calibration were the hexapeptide LMWRFA and bovine insulin.
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Evolutionary History of the Symbiosis Between Fungus-Growing Ants and Their Fungi

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The evolutionary history of the symbiosis between fungus-growing ants (Attini) and their fungi was elucidated by comparing phylogenies of both symbionts. The fungal phylogeny based on cladistic analyses of nuclear 28*S* ribosomal DNA indicates that, in contrast with the monophyly of the ants, the attine fungi are polyphyletic. Most cultivated fungi belong to the basidiomycete family Lepiotaceae; however, one ant genus, *Apterostigma*, has acquired a distantly related basidiomycete lineage. Phylogenetic patterns suggest that some primitive attines may have repeatedly acquired lepiotaceous symbionts. In contrast, the most derived attines have clonally propagated the same fungal lineage for at least 23 million years.

Mutualistic symbioses between distantly related organisms have generated major innovations in the evolution of organic complexity, including the endocytotic organelles of the eukaryotic cell, the plant and fungal symbionts in mycorrhizae, and the ubiquitous nitrogen-fixing bacteria present in plants, animals, fungi, and protists (*1*). The reconstruction of the evolutionary history of such symbioses requires explicit phylogenies for both symbionts, a necessity that has limited the number of such studies to date (*2*). Here we report the results of a phylogenetic analysis of the attine ant–fungus symbiosis, an ancient and successful association that has culminated in the leaf-cutting ants, the dominant herbivores of the Neotropics (*3*).

All of the approximately 200 species of ants in the tribe Attini cultivate fungus gardens on which they are obligately depen-

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dent for nourishment (3). The monophyly of the tribe (4) and its exclusively New World and primarily Neotropical distribution (5) argue for a single origin of the fungus-growing behavior about 50 million years ago (6). In spite of a century of research (5, 7, 8), progress toward understanding the origin and subsequent evolution of the ant-fungus symbiosis has been hindered by a lack of informed phylogenetic hypotheses, including the identities of the nearest nonsymbiotic relatives of both ants and fungi.

The phylogeny of the fungi has remained unresolved because of the infrequent production of taxonomically informative fruiting bodies (7–9). When fruiting bodies have been obtained, they have been assigned to the subdivision Basidiomycotina, order Agaricales, and variously classified in the families Cortinareaceae (7), Agaricaceae (10), or Lepiotaceae (9, 11) (see 9, 12, 13 for reviews). However, some investigators have maintained that attine ants form associations with a wide variety of fungal symbionts, including members of the subdivisions Ascomycotina and Deuteromycotina (14).

Thus, it is unclear whether the Attini cultivate a monophyletic group of fungi that has evolved in close association with the ant lineages in a strict coevolutionary sense, or whether the ant fungi represent an array of possibly distantly related species (5, 15). The former hypothesis is supported by the fact that foundress queens of at least some attine species carry a small pellet of fungus from the natal nest with which to start a new garden (5, 16), leading to the expectation of clonally propagated fungal lineages that closely parallel the lineages of their ant hosts. However, this behavior has been reported for only three genera (*Trachymyrmex*, *Acromyrmex*, and *Atta*) within the derived higher attines (5) and in the transitional genus *Cyphomyrmex* (Fig. 1) (17), but it has not been confirmed in any of the more primitive species that are most likely to retain less modified forms of the ancestral fungus-growing behavior.

A comprehensive collection of living attine ant (4) and fungal cultures (13, 18), which includes a representative sampling of the primitive attine genera, has provided new information on the evolutionary history of the ant-fungus symbiosis. Cultural, biochemical, and micromorphological characters of the fungal mycelium indicate that the attine fungi are basidiomycetes (13) and that they are subdivided into three major groups (G1, G2, and G3) that are congruent with a phylogeny of the ants based on larval morphology (Fig. 1) (4, 13). Only the G1 group, which includes the fungi of the enormously successful leaf-cutting species in the genera *Atta* and *Acromyrmex*, possesses gongylidia, the nutritious swollen hyphal tips that are harvested by the ants for food (3, 5, 13). Likewise, only the G2 group, cultivated by the species of the morphologically derived genus *Apterostigma*, possesses abundant clamp connections at hyphal septa (a basidiomycete character secondarily lost in many groups, including many Lepiotaceae) and extremely elongate aerial hyphae, which in some species serve to form a protective tent-like veil around the nest (5, 13). The G3 group is a comparatively heterogeneous assemblage that lacks these synapomorphies and, thus, may be paraphyletic (13).

The G3 group fungi, cultivated by the most primitive attine ants, are genetically heterogeneous, as indicated by patterns of pairwise rejection between these strains in vegetative compatibility (VC) studies (13, 18), in some cases even between isolates from different nests of the same ant species. In contrast, the widespread acceptance reactions between the fungi within the G1 group, and between the fungi within the G2 group, indicate strong genetic similarity

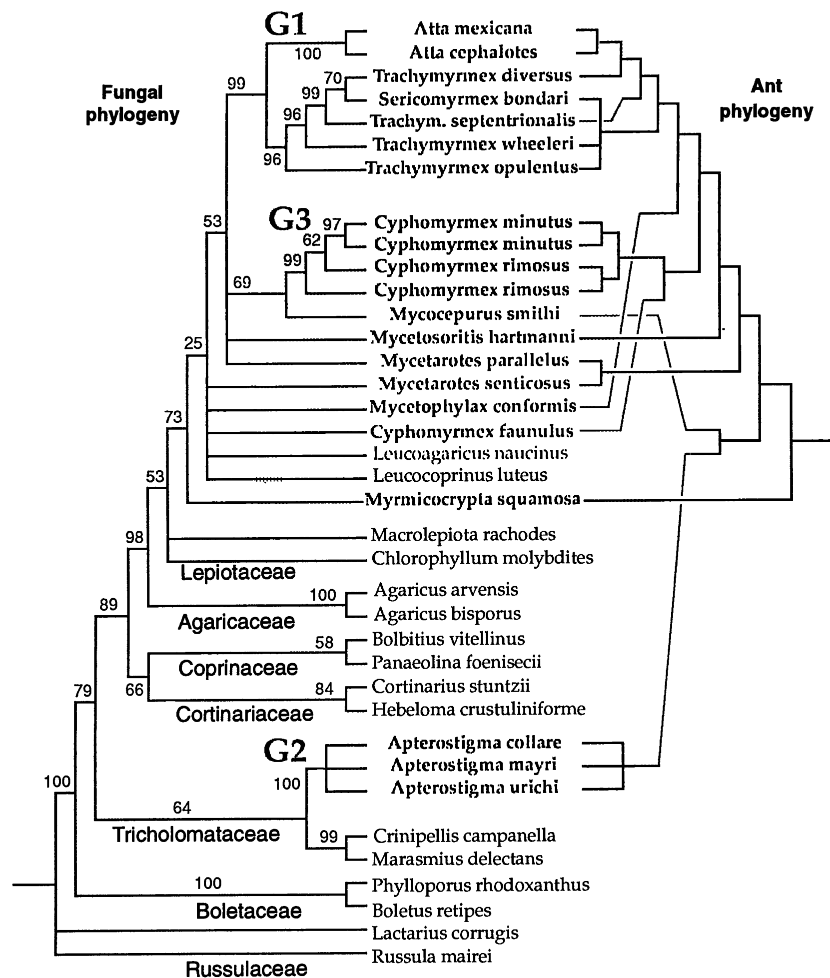

Fig. 1. A comparison of the attine fungal phylogeny obtained from 28*S* nuclear ribosomal DNA (*20*) and the attine ant phylogeny obtained from larval morphological characters (*4*). All unshaded names refer to free-living fungal species; shaded names refer both to ant species and their fungal symbionts. The monophyletic group consisting of the ant genera *Sericomyrmex*, *Trachymyrmex*, *Acromyrmex*, and *Atta* is traditionally called the higher attines and the remaining genera the lower (primitive) attines (*5*). The fungal tree is the strict consensus of 22 equally parsimonious trees and is identical to the 50% majority-rule bootstrap consensus tree, except for the presence of the group containing *Leucoagaricus naucinus*, *Leucocoprinus luteus*, and the lepiotaceous attine fungi exclusive of that cultivated by *Myrmicocrypta squamosa*. Bootstrap proportions for branches are provided as a crude indicator of character support but should not be interpreted as confidence levels (*32*). The phylogeny of the ants is congruent with the three fungal symbiont morphogroups G1, G2, and G3 (*13*). Both the G1 fungi, cultivated by the derived higher attines, and the G2 fungi, cultivated by ants of the primitive genus *Apterostigma*, are monophyletic. The G2 fungal group represents a shift by one lineage of ants to a distantly related fungal symbiont; in contrast, all other attine fungi belong to the Lepiotaceae (Basidiomycotina). Within the G3 group, some fungi are more closely related to those in the G1 group, whereas others are more closely related to two free-living lepiotaceous species (*Leucoagaricus naucinus* and *Leucocoprinus luteus*, indicated by the white box) than they are to other attine fungi. Thus, the G3 fungal group is paraphyletic.

within each of these groups. These observations have led to the hypothesis (*18*) that the G3 group fungi are genetically diverse because some of them have been repeatedly acquired by the ants from free-living forms, whereas the fungi within the G1 and G2 groups are genetically similar because they are the products of clonal reproduction, consistent with behavioral evidence that G1 group fungi are clonally propagated by their ant hosts (*5*, *19*). This "acquisition-clonality" hypothesis predicts that (i) the two clonally reproduced lineages G1 and G2 are monophyletic entities and (ii) some G3 fungi are more closely related to free-living fungi than to other attine fungi.

To (i) determine the taxonomic position of the attine fungi, (ii) detail the phylogenetic relationships among attine fungi vis-à-vis the existing phylogeny of the ants (*4*), and (iii) test the two predictions of the acquisition-clonality hypothesis, we carried out a phylogenetic analysis of a 964–base pair region of the nuclear large subunit (28S) ribosomal DNA (*20*). Sequences were obtained for 21 attine fungi isolated from the nests of 19 attine ant species (*21*), as well as for 16 free-living basidiomycetes in the order Agaricales (*22*). The phylogenetic tree generated by parsimony analysis (Fig. 1) (*23*) is the strict consensus of 22 equally parsimonious trees of length 556, with a consistency index of 0.502 and a retention index of 0.742 (*24*). Additional analyses that used a variety of phylogenetic methods also produced trees with the topological features emphasized here (*25*).

Most attine fungi are members of a monophyletic group in the Lepiotaceae (Fig. 1), which corroborates the conclusions of some previous investigators (*9*, *11*) and contradicts others (*7*, *14*). However, the fungi cultivated by *Apterostigma* species arise in a position on the Agaricales tree well removed from the lepiotaceous attine fungi. The shortest tree in which the *Apterostigma* fungi are constrained to group with the other attine fungi is considerably longer (27 steps) than the most parsimonious trees. Furthermore, a test of the a priori hypothesis that the attine fungi (including the *Apterostigma* fungi but not the two free-living lepiotaceous species; see Fig. 1) are monophyletic was rejected by both the T-PTP ($P < 0.05$) (*26*) and maximum likelihood (*27*) methods. These results support a polyphyletic origin of the attine fungi and a nonlepiotaceous origin of the fungi cultivated by *Apterostigma*.

As predicted by the acquisition-clonality hypothesis (*18*), the G1 and G2 groups are monophyletic (Fig. 1). In contrast, the consensus tree (Fig. 1) indicates that the G3 group is paraphyletic because some G3 fungi are more closely related to the excluded G1 group, and some G3 fungal isolates are more closely related to two free-living lepiotaceous fungi, *Leucoagaricus naucinus* and *Leucocoprinus luteus*, than they are to other G3 fungi. In each of the 22 most parsimonious trees, these two free-living species are invariably nested at least four nodes within the attine fungal clade (Fig. 2). These results are consistent with the second prediction of the acquisition-clonality hypothesis that some attine fungi in the G3 group are more closely related to free-living fungi than they are to other attine fungi and therefore must have been secondarily acquired by the ants. However, the alternative hypothesis, that these two free-living fungi are the sister group to the lepiotaceous attine clade, requires a tree only one step longer than the most parsimonious tree.

Comparison of the ant and fungal phylogenies reveals topological incongruence, indicating the absence of strictly parallel evolution, particularly between the primitive attine ants and their fungi (*28*). For example, within each of the genera *Mycetarotes* and *Cyphomyrmex*, closely related ants cultivate distantly related G3 fungi (Fig. 1). Likewise, *Mycocepurus smithi*, one of the most primitive attine ants, cultivates a fungus that is most closely related to those cultivated by the transitional species *Cyphomyrmex rimosus* and *Cyphomyrmex minutus*. These incongruences may be explained either by (i) inaccuracies in the phylogenetic reconstructions, (ii) lineage sorting (*29*), (iii) horizontal transfer of fungal clones across ant species (*15*), or (iv) secondary acquisition of fungi from a pool of free-living forms (*30*). The latter hypothesis is supported by vegetative compatibility patterns (*18*) and the nested position of the two free-living lepiotaceous species within the G3 group (Fig. 2).

From the time of its origin around 50 million years ago (*6*), the ant-fungus symbiosis has resulted in the evolution of complex behavioral (*5*) and physiological (*31*) modifications in the ants and in corresponding morphological and biochemical modifications in at least some of the fungal symbionts (*5*, *13*, *31*). It is now clear that the origin of the fungus-growing behavior was an extremely rare event, having occurred only once in the evolutionary history of the ants. Switching from the original host lineage was also rare: the first ant-fungus association probably involved a species in the Lepiotaceae, and only one switch to a fungal symbiont outside of this family has subsequently occurred, in the case of *Apterostigma*. On the other hand, switching by ants between fungi within the lepiotaceous lineage may have occurred frequently over evolutionary time, at least in the nonclonal G3-cultivating ant species. Precise identification of the fungal species within this group, as well as study of the biology of the most primitive attine ants and their closest non–fungus-growing relatives, is the most promising route to a complete understanding of the origin and evolution of the attine ant-fungus symbiosis.

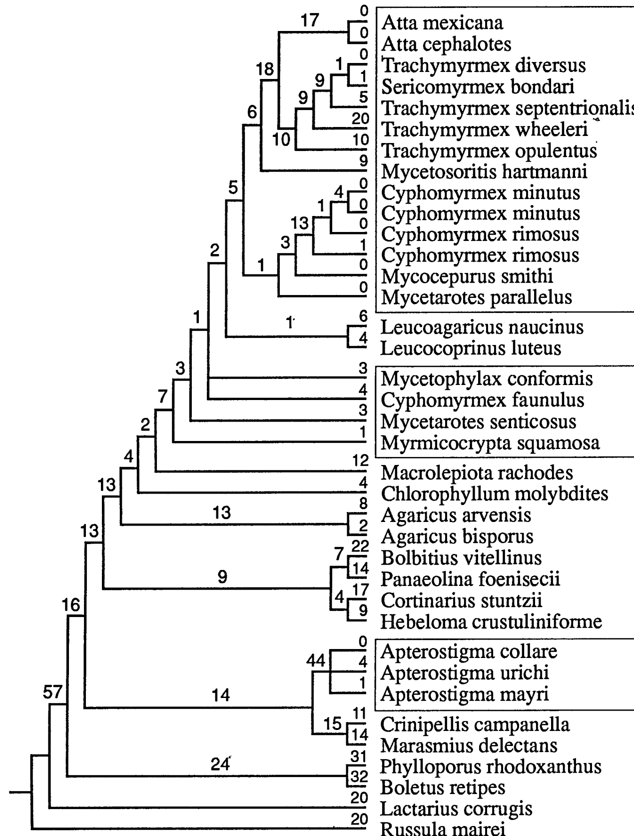

Fig. 2. The resolved fungal phylogeny as it appears in one of the 22 equally parsimonious trees obtained from the unweighted parsimony analyses (*25*). Names of attine fungal symbionts appear in boxes; free-living fungi are unboxed. Numbers correspond to branch lengths. This tree is identical to the 64% majority-rule consensus tree of the 22 equally parsimonious trees (*33*). In all of the 22 trees, the free-living species invariably arise at a level of either four or five nodes within the attine clade, consistent with the hypothesis that free-living fungi have been independently acquired by separate ant lineages during the evolutionary history of the ant-fungus symbiosis.

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21. Fungal isolates were obtained from ant gardens collected at the following locations: Manaus, Brazil: *Sericomyrmex bondari* and *Mycetarotes senticosus*; São Gabriel, Brazil: *Mycetarotes parallelus* and *Cyphomyrmex faunulus*; km 44, BR174, Amazonas, Brazil: *Trachymyrmex wheeleri* and *T. diversus*; Simla Biological Station, Trinidad: *Apterostigma urichi*; Morne La Croix, Trinidad: *Apterostigma mayri*; km 44, Manzanilla-Mayaro Road, Trinidad: *C. rimosus* and *Mycetophylax conformis*; Arima, Trinidad: *Myrmicocrypta squamosa*; La Selva, Costa Rica: *Apterostigma collare, C. minutus, Trachymyrmex opulentus*, and *Atta cephalotes*; Limon, Costa Rica: *Mycocepurus smithi*; Oaxaca, Mexico: *Atta mexicana*; Sam Houston National Forest, TX, USA: *Mycetosoritis hartmanni*; Archbold Biological Station, FL, USA: *Trachymyrmex septentrionalis* and *C. minutus*; Darien, GA, USA: *C. rimosus*. Two isolates from *C. rimosus* and two from *C. minutus* were included because ants presently categorized as *C. rimosus* may represent multiple species (J. T. Longino, personal communication), and the taxonomic distinction between *C. rimosus* and *C. minutus* is unclear (T. R. Schultz and U. G. Mueller, unpublished data).
22. Collection localities and herbarium numbers for free-living fungal species are the following: King County, WA: *Agaricus bisporus*, SAR 88/411; *Bolbitius vitellinus*, SAR 84/100; *Hebeloma crustuliniforme*, SAR 87/408; *Leucoagaricus naucinus*, SAR 87/396; *Macrolepiota rachodes*, SAR 87/407; *Panaeolina foenisecii*, SAR 87/378. Durham County, NC: *Boletus retipes*, SAR 91/1; *Chlorophyllum molybdites*, SAR 89/461; *Lactarius corrugis*, RV 82/61; *Phylloporus rhodoxanthus*, SAR 91/2. Prince Georges County, MD: *Agaricus arvensis*, SAR 93/1. Grant County, WA: *Cortinarius stuntzii*, SAR 85/358. Knox County, TN: *Marasmius delectans*, DED 4518. Orange County, NC: *Russula mairei*, RV 89/62. Washington, DC: *Leucocoprinus luteus*, SAR 94/1. Ontario, Canada: *Crinipellis campanella*, DAOM 17785.
23. PAUP (phylogenetic analysis using parsimony), 3.1; D. L. Swofford, Illinois Natural History Survey, Champaign, IL. The sequenced region contained 193 informative sites after four highly variable regions of uncertain alignment, containing a total of 49 sites, were removed from consideration.
24. The expected consistency index for a study of this size is 0.378, considerably worse than the value reported here [M. J. Sanderson and M. J. Donaghue, *Evolution* **43**, 1781 (1989)]. A permutation tail probability (PTP) test [D. P. Faith and P. S. Cranston, *Cladistics* **7**, 1 (1991)], as implemented by the Random Cladistics computer program (version 2.1, M. E. Siddall, University of Toronto, Ontario, Canada), found significant cladistic character covariation in the data ($P < 0.01$) when 99 randomized matrices were generated, analyzed, and compared with an analysis of the actual data by using the mh command of Hennig86 (version 1.5, J. S. Farris; A. Kluge, University of Michigan, Ann Arbor, MI).
25. Multiple unweighted parsimony analyses with the computer programs PAUP (*23*) and Hennig86 (*24*) produced trees identical to that in Fig. 1 when (i) all gaps were treated as missing, (ii) all gaps were treated as a fifth character state, or (iii) only some gaps were treated as a fifth state, whereas those that were not clearly the product of independent evolutionary events were treated as missing. A parsimony analysis in which transversions were given twice the weight of transitions produced a tree with the same topological features relevant to the arguments presented, including the monophyly of the G1 and G2 fungi and a monophyletic group containing the lepiotaceous attine fungi and the two free-living lepiotaceous species. These results were also obtained with implied-weight parsimony [P. A. Goloboff, *Cladistics* **9**, 83 (1993)] by using the PeeWee computer program (version 2.0, P. A. Goloboff, The American Museum of Natural History, New York) under both strong (concavity = 2) and weak (concavity = 6) differential weighting. Neighbor-joining [implemented in PHYLIP (Phylogeny Inference Package), version 3.4, J. Felsenstein, University of Washington, Seattle, WA], though less reliable for a study with this number of nucleotides [D. M. Hillis, J. P. Huelsenbeck, C. W. Cunningham, *Science* **264**, 671 (1994)], and maximum likelihood analyses (implemented in PHYLIP) also produced trees supporting these features.
26. D. P. Faith, *Syst. Zool.* **40**, 366 (1991); J. W. O. Ballard *et al.*, *Science* **258**, 1345 (1992).
27. To test whether the fit of the data to the set of trees constraining the attine fungi to be monophyletic (583 steps) was significantly worse than the fit of the data to the most parsimonious trees (556 steps), we determined the difference in log likelihoods between each of 55 fully resolved constraint trees and each of the 66 fully resolved trees implied by the 22 most parsimonious trees [H. Kishino and M. Hasegawa, *J. Mol. Evol.* **29**, 170 (1989)]. The difference in log likelihoods was significant at the 0.05 level in comparisons with all but 2 of the 66 trees; these 2 trees showed marginal significance levels (0.05 to 0.1) in less than half of the otherwise significant comparisons with the 55 constraint trees.
28. Within the G3 group, the only evidence for possible clonal propagation is found in the case of *C. rimosus* and *C. minutus*, in which both the ants and their fungi form monophyletic groups. Interestingly, the fungi of these ants occur as a morphologically unique yeast phase, unlike the mycelial forms cultivated by all other attines, including the congener *C. faunulus*. On the basis of the relationships of eight of the known yeast-growing ant species, yeast growing probably had a single origin in the attine ants (*4*). If the yeast-phase attine fungi are also monophyletic, then it is possible that yeast growing by some species in the transitional genus *Cyphomyrmex* is a third example of strict clonal propagation of fungi. Alternatively, if the yeast-growing *Cyphomyrmex* species occasionally acquire fungi from free-living stocks, they may preferentially take only particular lepiotaceous species, some of which are known to show a strong tendency to produce the yeast morph (I. H. Chapela, unpublished data).
29. P. Pamilo and M. Nei, *Mol. Biol. Evol.* **5**, 568 (1988); C.-I. Wu, *Genetics* **127**, 429 (1991).
30. Secondary acquisition of free-living fungi may have occurred by means of at least two different scenarios: (i) Clonal propagation of fungi may be the general rule in all attine species, but repeated acquisitions (for example, by workers after loss of a fungus garden) may have occurred frequently enough over evolutionary time to produce an interwoven pattern of relationships between attine fungi and free-living forms (Fig. 1). (ii) Acquisition of free-living fungi by each new generation of nest-founding queens may be the rule, at least in some species. The latter scenario predicts that ant-associated fungi found within a given area are more closely related to one or more local free-living species than they are to fungi cultivated by the same species of ant in different areas. Tests of this prediction require greater knowledge of the pool of neotropical lepiotaceous fungi that may be sampled by the attine ants, as well as detailed information on population structure within and across ant and fungal species.
31. M. Martin, *Science* **169**, 16 (1970); *Invertebrate-Microbial Interactions* (Cornell Univ. Press, Ithaca, NY, 1987).
32. D. M. Hillis and J. J. Bull, *Syst. Biol.* **42**, 182 (1993). We generated and analyzed 1000 bootstrap replicates using the computer program PAUP (*23*).
33. D. L. Swofford, in *Phylogenetic Analysis of DNA Sequences*, M. M. Miyamoto and J. Cracraft, Eds. (Oxford Univ. Press, New York, 1991).

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