EVOLUTION OF ANT-CULTIVAR SPECIALIZATION AND CULTIVAR SWITCHING IN *APTEROSTIGMA* FUNGUS-GROWING ANTS

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Abstract.—Almost all of the more than 200 species of fungus-growing ants (Formicidae: Attini) cultivate litter-decomposing fungi in the family Lepiotaceae (Basidiomycota: Agaricales). The single exception to this rule is a subgroup of ant species within the lower attine genus *Apterostigma*, which cultivate pterulaceous fungi distantly related to the Lepiotaceae. Comparison of cultivar and ant phylogenies suggests that a switch from lepiotaceous to pterulaceous fungiculture occurred only once in the history of the fungus-growing ants. This unique switch occurred after the origin of the genus *Apterostigma*, such that the basal *Apterostigma* lineages retained the ancestral attine condition of lepiotaceous fungiculture, and none of the *Apterostigma* lineages in the monophyletic group of pterulaceous fungiculturists are known to have reverted back to lepiotaceous fungiculture. The origin of pterulaceous fungiculture in attine ants may have involved a unique transition from the ancestral cultivation of litter-decomposing lepiotaceous fungi to the cultivation of wood-decomposing pterulaceous fungi. Phylogenetic analyses further indicate that distantly related *Apterostigma* ant species sometimes cultivate the same cultivar lineage, indicating evolutionarily frequent, and possibly ongoing, exchanges of fungal cultivars between *Apterostigma* ant species. The pterulaceous cultivars form two sister clades, and different *Apterostigma* ant lineages are invariably associated with, and thus specialized on, only one of the two cultivar clades. However, within clades *Apterostigma* ant species are able to switch between fungi. This pattern of broad specialization by attine ants on defined cultivar clades, coupled with flexible switching between fungi within cultivar clades, is also found in other attine lineages and appears to be a general phenomenon of fungicultural evolution in all fungus-growing ants.

Key words.—*Apterostigma*, Attini, fungiculture, fungus-growing ants, Pterulaceae.

Fungus-growing ants (Formicidae, tribe Attini) are well known for their habit of cultivating fungi for food, a behavior that originated in the common ancestor of attine ants about 50–60 million years ago (Wilson 1971; Schultz and Meier 1995; Mueller et al. 2001). Since then, attine ants have undergone an adaptive radiation that has resulted in 13 extant genera containing more than 210 described species, all obligately dependent on the farming of mutualistic fungi, usually in nests excavated in the soil (Schultz and Meier 1995; Wetterer et al. 1998; Brandão and Mayhé-Nunes 2001). Domestication of fungi from free-living populations, as well as cultivar exchanges between ant species in the same and in different genera, have occurred multiple times during the evolutionary history of the attine ants (Chapela et al. 1994; Mueller et al. 1998; Mueller 2002; Mueller and Gerardo 2002).

Distinct modes of fungal cultivation characterize different attine ant lineages. The great majority of species cultivate lepiotaceous fungi (Basidiomycota: Agaricales: Lepiotaceae) from one of two distinct groups, termed G1 and G3 fungi by Chapela et al. (1994). The fungi of the higher attine ants (called G1 fungi, zcultivated by the ant genera *Sericomyrmex*, *Trachymyrmex*, and the leafcutter ants *Atta* and *Acromyrmex*) possess several derived morphological features that probably arose during a long history of coevolution with attine ants.

In contrast, the lower attine ants (eight genera, including the genus *Apterostigma*) cultivate morphologically unspecialized fungi (called G3 fungi) from which the G1 fungi arose (S.A. Rehner, pers. comm.; P. Abbott, N. Gerardo, and U. Mueller, unpubl. data), and which are therefore evolutionarily plesiomorphic with respect to the G1 group (Chapela et al. 1994; Mueller et al. 1998). There is only one known exception to this lepiotaceous cultivation of G1 and G3 fungi: some but not all ant species in the genus *Apterostigma* cultivate fungi that are distantly related to the Lepiotaceae (Fig. 1). These so-called G2 fungi are characterized by a unique micromorphology (Chapela et al. 1994) and are cultivated by their *Apterostigma* hosts in hanging ''veiled gardens'' in which the cultivated fungus also forms a thin mycelial envelope that surrounds the garden proper. G2 fungi were grouped into the family Tricholomataceae (Basidiomycota: Agaricales) in two previous phylogenetic analyses (Chapela et al. 1994; Moncalvo et al. 2000), but recent work suggests a close affinity with the family Pterulaceae (Basidiomycota: Agaricales) (Munkacsi et al. 2004).

This study elucidates the origin and evolution of the cultivation of pterulaceous fungi by ants in the genus *Apterostigma*. Phylogenetic analyses of both fungi and ants support a single origin of pterulaceous fungal cultivation. Our analysis divides the pterulaceous cultivars into two distinct
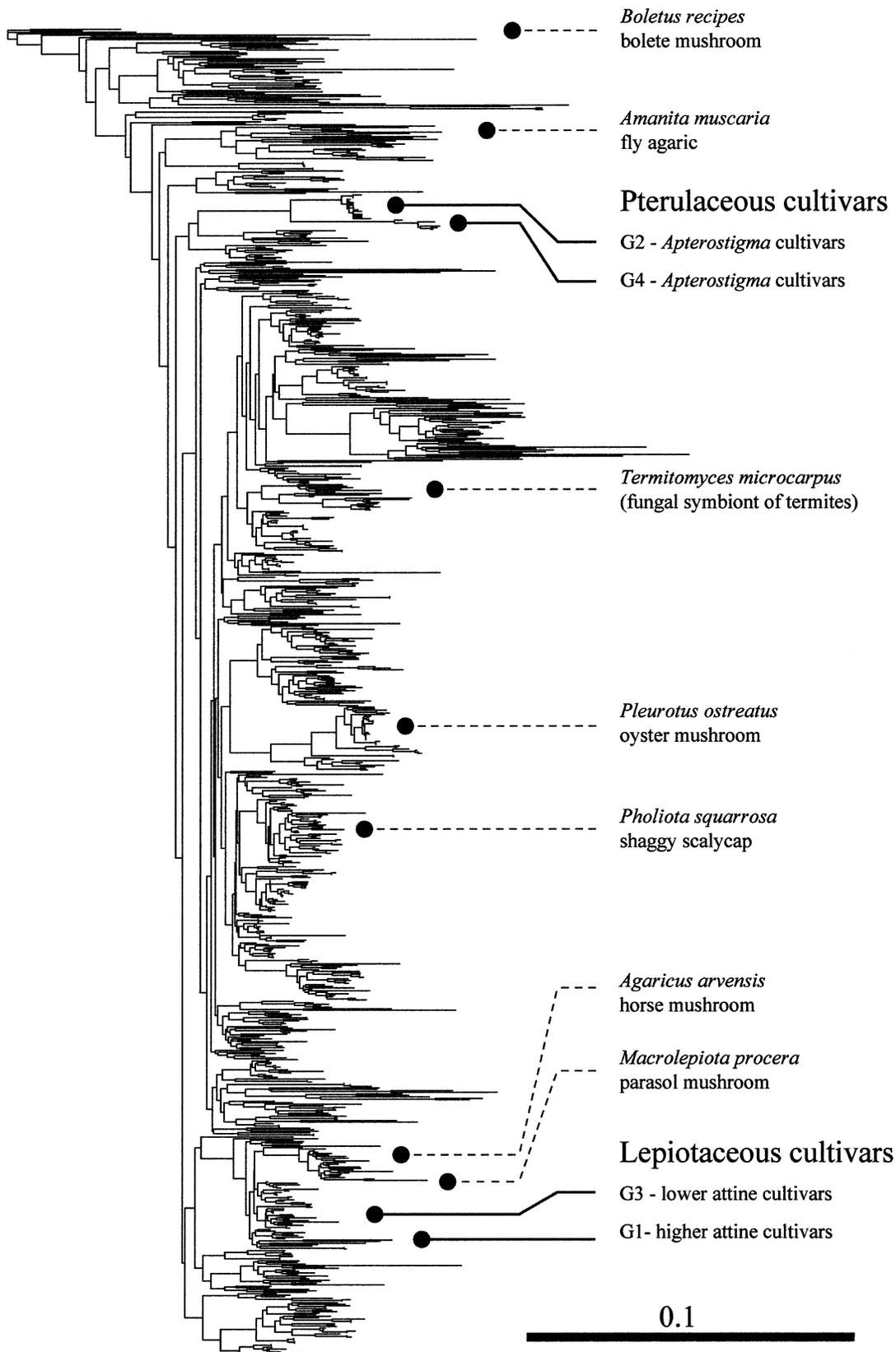
FIG. 1. Relationship between fungi cultivated by fungus-growing ants. ME tree showing the distant phylogenetic position of the fungi cultivated by some *Apterostigma* ants (G2 and G4) relative to the lepiotaceous fungi cultivated by most attine ants (G1 and G3). For comparison, some well-known fungi are indicated. The tree was constructed using 899 fungal sequences from this study and Moncalvo et al. (2002). Sequences were aligned using ClustalW (Thompson et al. 1994), poorly aligned regions were discarded using GBLOCKS (Castresana 2000), and the phylogeny was constructed using FastME (Desper and Gascuel 2002). Figure is provided as an illustration of the position of attine-ant fungi and conveys substantially the same information as figure 1 in Munkacsi et al. (2004).
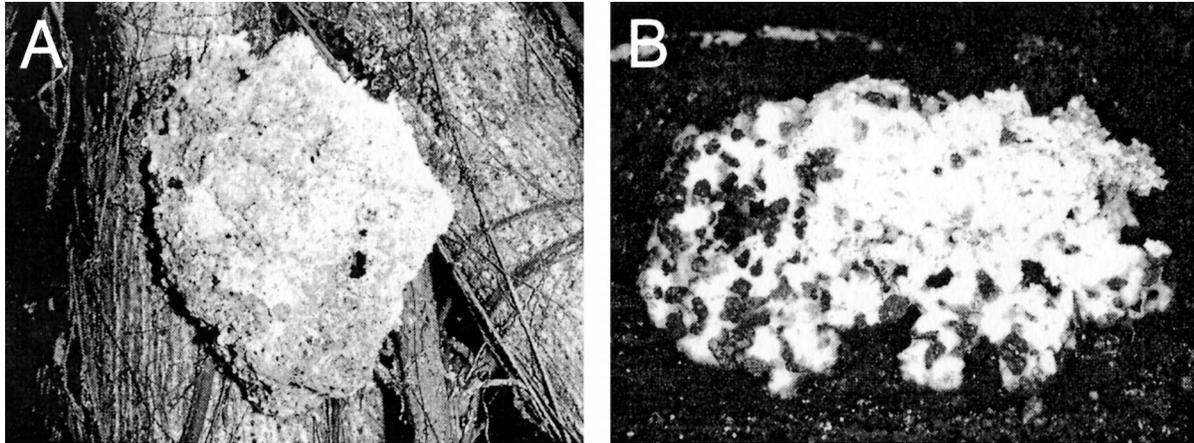
Fig. 2. *Apterostigma* gardens. (A) Garden of the G2-cultivator *Apterostigma collare* hanging in a sheltering crevice formed by tree branches. A mycelial veil surrounds the G2 garden proper. The single nest entrance is visible as a hole in the veil slightly to the lower right of the garden's center. (B) Garden of the G3-cultivator *Apterostigma auriculatum* in a cavity underneath a log on the forest floor. The garden is sessile and is not surrounded by a veil. G4 gardens (not shown) are also unveiled and sessile, and thus resemble the G3 nest architecture shown on the right.

clades, the veiled fungi termed the G2 group and a novel unveiled group termed G4. Phylogenetic analyses further reveal a faithfulness of ant lineages to cultivate fungi from only one of the two major clades of pterulaceous cultivars (G2 or G4). Within each of these ant clades, however, single *Apterostigma* species are associated with a wide diversity of cultivars, resulting from evolutionarily recent and possibly ongoing cultivar exchanges between different *Apterostigma* ant species.

MATERIALS AND METHODS

Collection and Sampling Strategy

Collections of *Apterostigma* ants and their gardens were made as part of extensive ant-cultivar surveys in Central and South America (Mueller et al. 1998). Three types of *Apterostigma* gardens could be distinguished during field collection: (1) G2 gardens, comprised of a G2-type fungus and characterized by a hanging, veiled garden architecture (gardens are suspended and surrounded by a mycelial envelope; Chapela et al. 1994; Fig. 2); (2) G3 gardens, comprised of a G3-type fungus and characterized by a sessile, unveiled garden architecture (gardens are spongelike and rest on the bottom of the garden cavity; Chapela et al. 1994; Mueller et al. 1998; Fig. 2); and (3) a heretofore unknown type of garden, termed G4, characterized by a sessile, unveiled garden architecture (thus resembling G3 gardens), but also possessing micromorphological characteristics of G2 fungi (e.g., abundant clamp connections; Chapela et al. 1994; U. Mueller, unpubl.).

A total of 25 *Apterostigma* cultivar isolates from 25 nests of the following nine *Apterostigma* ant species were available for molecular analysis: *A. auriculatum*: Gamboa, Panama (*n* = 1); *A. cf. gonoides*: km 7 El Llano–Cartí Suitupo Road, Panama (*n* = 1); *A. dentigerum*: Gamboa, Panama (*n* = 2); Pipeline Road, Panama (*n* = 1); Tule, Panama (*n* = 1); Fort Sherman Military Reservation, Panama (*n* = 1); *A. dorotheae*: Paramaketoi, Guyana (*n* = 2); Kurupukari; Guyana (*n* = 2);

A. manni: Barro Colorado Island, Panama (*n* = 1); Pipeline Road, Panama (*n* = 2); Gamboa, Panama (*n* = 1); *A. urichii*: Kurupukari, Guyana (*n* = 1); *A. pilosum* sp. 1: Gamboa, Panama (*n* = 4); Kurupukari, Guyana (*n* = 1); *A. pilosum* sp. 2: El Llano, Panama (*n* = 1); and *A. pilosum* sp. 4: Gamboa, Panama (*n* = 3). In all figures presented here, samples are indicated with reference to geographic origin (PA: Panama, GU: Guyana) and sample number followed by the ant species name from which the fungus was obtained. The most recent revision of the genus *Apterostigma* (Lattke 1997) merges several previously recognized species into an unresolved ''*pilosum*-species complex,'' a default group that may be polyphyletic. Three species included in our study fall into the ''*pilosum*-species complex'' and are therefore listed as *A. pilosum* species 1, 2, and 4, respectively. Lattke's (1997) revision splits the genus *Apterostigma* into two broad groups, the *auriculatum* group (represented in our collection only by the single species *A. auriculatum*), and the *pilosum* group (represented in our collection by the remaining eight ant species). (Note that the ''*pilosum* group'' is different from the ''*pilosum* species complex,'' which is a default complex included within the *pilosum* group [Lattke 1997]).

Details of the collection and fungal-isolation methods are described in Mueller et al. (1996, 1998). Isolated fungi were grown in liquid culture, then preserved through lyophilization and cryostorage at −80°C.

DNA Extractions

Fungal mycelium was homogenized under liquid nitrogen, and the homogenate was extracted using a modified phenol-chloroform-CTAB procedure (Doyle and Doyle 1987): 1 hour incubation at 60°C with 2xCTAB (2% hexadecyltrimetyl ammonium bromide, 0.75 M NaCl, 50 mM Tris pH 8.0, and 10 mM EDTA), followed by one extraction with phenol:chloroform, one additional extraction with chloroform:isoamyl-alcohol (24:1), and precipitation with cold 100% ethanol. After resuspension of the DNA in water, the concentration

of DNA was estimated on agarose gels with ethidium bromide, then diluted (5–10 ng/μl) for polymerase chain reaction (PCR) amplification.

Whole ants were extracted using a DNA-binding membrane kit without organic extraction or ethanol precipitation. Either the DNA/RNA isolation kit by Amersham Biosciences (Piscataway, NJ; catalog no. US73750) with the Amersham standard protocol, or the Qiagen DNeasy animal tissue extraction kit (Qiagen, Inc., Valencia, CA; catalog no. 69506) with the Qiagen standard protocol except that 50 μl and 200 μl of extraction buffer were used for the first and second elutions, respectively, and the Proteinase K digestion was carried out at 37°C for 45 min.

RFLP Screen of Cultivated Fungi

Cultivar DNA was analyzed in a two-step process, following the methods in Mueller et al. (1998). Each cultivar isolate was first profiled with internal transcribed spacers-restriction fragment length polymorphism markers, then representative RFLP types were selected for sequencing analysis. ITS-PCR reactions were carried out in a volume of 25 μl. Five μl of ITS-PCR product were digested in a total volume of 20 μl using the following restriction enzymes in separate digestions: Nla III, Hha I, Dra I, RSA, and Hae III. Digested PCR product were electrophoresed in 2% agarose gels and visualized with ethidium bromide.

Fungal DNA Sequencing and Cloning

Target rDNA sequences from nuclear ITS and 25S gene regions were amplified using fungal primers ITS4 and ITS5 for the internal transcribed spacer regions (ITS1 and ITS2, plus the intervening 5.8S region; White et al. 1990), and primers LR0R and LR5 for the first 900 bp of the 25S region (large subunit, LSU; Rehner and Samuels 1994; Mueller et al. 1998). DNA sequencing of the ITS region showed multiple ITS-amplification products (high levels of heterozygosity or within-array ITS diversity), making it necessary to clone PCR products in most cases: 10 random PCR-clones were screened using RFLP and all different RFLP types were subsequently sequenced. This resulted in a varying number of sequences per sample, representing the sequence diversity within that fungal cultivar. Cloned sequences are represented as the sample name followed by an underscore (i.e., GU34_2 corresponds to sample Guyana, sample 34, clone 2). ITS-PCR products were cloned into competent *Escherichia coli* cells, using a TA Cloning Kit (Invitrogen, Carlsbad, CA), then prepared for sequencing. ITS and LSU sequences were generated on an ABI PRISM 377 DNA Sequencer (Applied Biosystems, Foster City, CA) using the BigDye Terminator Cycle Sequencing Kit. Contigs were assembled from forward and reverse sequences using the DNASTAR software package (Madison, WI). Sequences are deposited at Genbank under accessions AY367562–AY367612 (ITS) and AY367613–AY367633 (LSU).

Ant DNA Sequencing

Ant sequence data were generated for a 984 bp fragment (representing 328 amino acid residues) of the mitochondrial

TABLE 1. Primers used to amplify cytochrome c oxidase subunit I (COI) DNA sequences from ants. Numbers in parentheses indicate annealing position of the 5' primer base in the COI coding sequence of the honey bee, *Apis mellifera* (Crozier and Crozier 1993).

Forward primers:	
LCO1490 (17):	5' GGT CAA CAA ATC ATA AAG ATA TTG G 3'
CI13 (187):	5' ATA ATT TTT TTT ATA GTT ATA CC 3'
CI21 (684):	5' CTT TAT CAA CAT TTA TTT TGA TTT TT 3'
Jerry (691):	5' CAA CAT TTA TTT TGA TTT TTT GG 3'
Reverse primers (position indicates annealing position of 5' primer base):	
HCO2198 (728):	5' TAA ACT TCA GGG TGA CCA AAA AAT CA 3'
CI14 (997):	5' GTT TCT TTT TTT CCT CTT TC 3'
CI22 (1259):	5' ACT CCA ATA AAT ATT ATA ATA AAT TGA 3'
Ben (1121):	5' GCW ACW ACR TAA TAK GTA TCA TG 3'
CI24 (1293):	5' TCC TAA AAA ATG TTG AGG AAA 3'

cytochrome c oxidase subunit I (COI) gene, corresponding to positions 211 to 1195 of the *Apis mellifera* COI coding sequence (Crozier and Crozier 1993). Extracted DNA were generally amplified in two steps: In the first step, a fragment of approximately 1277 bp was amplified using the primers LCO1490 (forward) and CI24 (reverse) with a 45°C annealing temperature and 1-min ramping between the annealing and extension steps. Then, using the product of the initial amplification as template, two shorter overlapping fragments were reamplified with a variable (48°C to 52°C) annealing temperature and no ramping, using as primers, for the 5' fragment, either LCO1490/HCO2198 or CI13/CI14, and, for the 3' fragment, primer combinations CI21/CI24, CI21/CI22, Jerry/CI24, or Jerry/Ben3R. Primer sequences are listed in Table 1. Sequences are deposited at Genbank under accessions AY398280–AY398304.

Phylogenetic Analyses

For analyses of fungi, several species closely related to the *Apterostigma* cultivars were selected as outgroups based on a recently published phylogeny of the Agaricales (Petersen and Krisai-Greilhuber 1999, Moncalvo et al. 2000), and respective sequence information was obtained from Genbank. For analyses of ants, species from two genera (*Myrmicocrypta* and *Mycocepurus*) closely related to *Apterostigma* (Schultz and Meier 1995; Lattke 1997, 1999) were used as outgroups. Fungal sequences were initially aligned in Megalign (Dynastar) and further aligned by hand; unalignable regions were excluded from the analysis. Alignment of the 984 bp of ant nucleotide sequence was trivial, as COI is a protein-coding gene and amino acid number is highly conserved across all ants (T. Schultz, unpubl.). DNA sequence alignments can be obtained from http://www.daimi.au.dk/~biopv/cv/apterostigma/.

Four datasets were separately analyzed (1) large subunit (LSU) (25S) for cultivated and free-living related fungi, (2) internal transcribed spacers (ITS) for G2-cultivars, (3) ITS for G4-cultivars, and (4) COI for attine ants (*Mycocepurus*, *Myrmicocrypta*, and *Apterostigma*).

Parsimony (MP) analyses.—Maximum-parsimony analyses were conducted in PAUP 4.0b4a (Swofford 2001) using the heuristic search option with tree bisection-reconnection (TBR) (LSU, G2-ITS, and ants) or branch-and-bound (G4-

Table 2. Cultivar group (G2, G3, and G4), garden architecture, and ITS-RFLP type for 22 *Apterostigma* cultivars isolated from 22 colonies of nine *Apterostigma* species. *Apterostigma auriculatum* is the only *Apterostigma* species that is known to cultivate a lepiotaceous G3-fungus (Mueller et al. 1998); one *A. auriculatum* cultivar was included for comparison with the two other groups (G2 and G4) of pterulaceous *Apterostigma* cultivars. G3 and G4 gardens have a sessile and unveiled architecture, whereas G2 gardens are hanging and veiled (see Fig. 2). Micromorphological similarities between G2 and G4 fungi had suggested a possible close phylogenetic link between G2 and G4 fungi (see Materials and Methods).

Cultivar type (micromorphology)	G3	G4						G2								Total
Garden architecture		sessile, unveiled						hanging, veiled								
RFLP type	A	B	C	D	E	F	G	H	J	K	L	M	N	O	P	
Ant species																
A. auriculatum	1															1
A. pilosum sp. 4			1				2									3
A. cf. gonoides				1												1
A. manni		1	1		1	1										4
A. dentigerum										2	1	1				4
A. dorotheae									1		2		1			4
A. pilosum sp. 1										1					2	3
A. pilosum sp. 2														1		1
A. urichii								1								1
Total	1	1	2	1	1	1	2	1	1	3	3	1	1	1	2	22

ITS) branch swapping and 500 (for fungi) and 1000 (for ants) random-taxon-addition replicates; successive-approximations weighting (SW) analyses used 200 (fungi) and 500 (ants) replicates. Heuristic-search bootstrap analyses (Felsenstein 1985) used TBR branch-swapping and consisted of 1000 (for fungi) and 5000 (for ants) pseudoreplicates, with 10 random-taxon-addition replicates per pseudoreplicate. Maximum-parsimony-based comparisons of unconstrained and constrained MP trees used the K-H test (Kishino and Hasegawa 1989), the Templeton Wilcoxon signed-ranks test (Templeton 1983), and the winning-sites test (Prager and Wilson 1988) in PAUP 4.0b8.

Maximum-likelihood (ML) analyses.—Nucleotide substitution models for ML analyses were evaluated with the data and MP tree(s) using the likelihood-ratio test implemented in ModelTest 3.0 (Posada and Crandall 1998). Maximum-likelihood analyses were conducted in PAUP 4.0b4a (Swofford 2001). For the LSU and ant data, heuristic searches employed the adopted model and an optimal MP tree as the branch-swapping starting tree and consisted of five iterative subsearches, each using updated model parameter values based on the results of the preceding search and each using successively more intensive branch-swapping regimes (Currie et al. 2003; Sallum et al., 2002; Mueller et al., 1998). For the G2-ITS data, heuristic searches employed the adopted model and a series of five TBR branch-swapping subsearches in which model parameters were successively updated. For the G4-ITS data, heuristic searches employed the adopted model and 100 random-taxon-addition subsearches. Heuristic ML bootstrap analyses employed TBR branch-swapping and consisted of 100 (LSU), 1000 (G2-ITS, G4-ITS), and 1500 (ants) pseudoreplicates. Maximum-likelihood-based comparisons of unconstrained and constrained ML trees used the K-H test (Kishino and Hasegawa 1989) and the S-H test (Shimodaira and Hasegawa 1999; Goldman et al. 2000).

Bayesian analyses.—Bayesian analyses were conducted in MrBayes 2.01 (Huelsenbeck and Ronquist 2001). Analyses of the LSU data employed the GTR $+ \Gamma + $ I model (Rod-riguez et al. 1990; general time-reversible model of nucleotide substitution with a proportion of sites invariant and gamma-distributed rates); analyses of the G2-ITS and G4-ITS data employed the HKY model (Hasegawa et al. 1985); and analyses of the ant data employed the SSR $+ \Gamma$ model (Huelsenbeck and Ronquist 2001; site-specific rate classes with gamma-distributed rates) incorporating three codon-position rate classes. All analyses included four separate runs, each consisting of 300,000 Markov chain Monte Carlo (MCMC) generations and four simultaneous MCMC chains (three heated), and each with a ''burn-in'' of 100,000 generations. For each analysis, post-burn-in trees from all four runs were pooled to calculate posterior probabilities. Bayesian topology-based hypothesis tests consisted of enumerating the proportion of pooled post-burn-in trees consistent with each of the competing hypotheses.

RESULTS

Restriction Fragment Length Polymorphisms Typing

The restriction fragment length polymorphisms (RFLP) typing of 25 fungal cultivars from nine different *Apterostigma* species (*A. auriculatum*, *A. cf. gonoides*, *A. manni*, *A. dentigerum*, *A. dorotheae*, *A. pilosum* sp. 1, *A. pilosum* sp. 2, *A. pilosum* sp. 4, and *A. urichii*) is consistent with the three distinct fungal groups (G2, G3, and G4) noted during the field collection (see Materials and Methods; Table 2). Each of the three fungal groups is cultivated by its own distinct, nonoverlapping set of ant species (Table 2). *Apterostigma auriculatum* is the only known *Apterostigma* species to cultivate a G3-fungus; because this fungus was previously shown to be lepiotaceous (Mueller et al. 1998), it is not included in the subsequent phylogenetic analyses of the pterulaceous cultivars. Of the *Apterostigma* ant species cultivating pterulaceous fungi, some ant species, such as *manni*, *dentigerum*, and *dorotheae* (Table 2), cultivate several different RFLP cultivar types *within* their particular cultivar group (G2 vs. G4 fungi, respectively), indicating cultivar diversity with-

in single ant species. Furthermore, the same RFLP cultivar type is shared in four cases between different ant species, suggesting either cultivar exchange between ant species via lateral transfer between nests (e.g., garden stealing), or independent domestication of the same free-living fungal lineage, as has been hypothesized for other lower attine species (Mueller et al. 1998).

Phylogenetic Analyses

Cultivar 25S (LSU) dataset.—Maximum-parsimony analyses of the LSU data produced 16 equally parsimonious trees (MPTs) with parsimony-informative length = 505, CI = 0.485, RI = 0.845; successive approximations weighting favored a subset of two of these trees. For both of these trees, the likelihood ratio test found the TrN + I + Γ model (Tamura and Nei 1993; but with a proportion of sites invariant, and gamma-distributed rates) to be significantly better fitting than the next less complex model (P = 0.000010), with both trees equally likely. ML analysis with this model identified a single tree (Fig. 3) with log likelihood of −4321.41458. A second analysis using the most complex (i.e., most general) model available, GTR + I + Γ (Rodríguez et al. 1990), identified exactly the same topology. Bayesian analyses using this model identified a tree entirely congruent with the ML tree. The *Apterostigma* cultivars are monophyletic and bootstrap proportions and Bayesian posterior probabilities (Fig. 3) strongly support the historical divergence of *Apterostigma* cultivars into two clades. These clades, respectively called G2- and G4-cultivars, are congruent with the G2- and G4-cultivar groups previously revealed by morphological and RFLP data (see above). The genus *Gerronema* is reconstructed as the sister clade to the *Apterostigma* cultivars under both likelihood and parsimony criteria. This relationship was suggested in Moncalvo et al. (2000), although with very low support. Our current taxon sampling fails to recover the hydropoid clade as described in other papers (e.g. Moncalvo et al. 2000), thus the relationships among the outgroup taxa in our phylogeny may be dependent on our particular taxon sampling. This potential criticism of reconstructions among outtgroup taxa does not apply to the interpretation of the phylogenetic relationships among the *Apterostigma* cultivars discussed below.

Cultivar G2-ITS dataset.—MP analyses of the G2-ITS data produced 20 MP trees with parsimony-informative length = 105, C.I. = 0.733, R.I. = 0.927. Successive approximations weighting identified a subset of two of these trees. For both of these trees, the likelihood ratio test found the HKY model (Hasegawa et al. 1985) to be significantly better fitting than the next less-complex model (P < 0.000001), with both trees equally likely. Maximum Likelihood analysis using this model identified a single tree (Fig. 4) with log likelihood of −2139.6617. Bayesian analyses using this model identified a nearly identical tree, differing with regard to the position of one clade (not shown). Single species of *Apterostigma* (e.g., *dentigerum*, *dorotheae*, *pilosum* sp. 1) cultivate diverse sets of fungi drawn from different clades of this phylogeny; thus single *Apterostigma* species appear polymorphic for their cultivars, possibly as a result of cultivar exchanges between different G2-cultivating *Apterostigma* lineages (Fig. 4).

Cultivar G4-ITS dataset.—MP analyses of the G4-ITS da-

taset produced a single MPT with parsimony-informative length = 49, C.I. = 0.898, R.I. = 0.968. Successive approximations weighting identified the same tree. The likelihood ratio test found the HKY model (Hasegawa et al. 1985) to be significantly better fitting to the data and this tree than the next less complex model (P = 0.000006). ML analysis using this model identified a single tree (Fig. 5) with log likelihood of −1274.70873. Bayesian analyses using this model identified the same tree. *Apterostigma pilosum* sp. 4 and *A. manni* each cultivates fungi from the two main clades of this phylogeny; these two *Apterostigma* species thus appear polymorphic for their cultivars, possibly as a result of cultivar exchanges between different G4-cultivating *Apterostigma* lineages (Fig. 5).

Ant dataset.—MP analyses of the ant data set identified two equally parsimonious trees (MPTs) with parsimony-informative length = 1396, C.I. = 0.437, R.I. = 0.658. Successive approximations weighting identified the same two trees. Maximum Parsimony analyses under the constraint that the G4-cultivating ants are monophyletic identified four MPTs with parsimony-informative length = 1404, C.I. = 0.432, R.I. = 0.655. Successive approximations weighting identified two trees (SWTs), a subset of the four MPTs.

Maximum-parsimony comparisons of the unconstrained and constrained trees indicate that the topologies in which the G4-cultivating *Apterostigma* species are constrained to be monophyletic are significantly worse-fitting to the data under both the K-H (P = 0.0454) and Templeton (P = 0.0455) test criteria, and marginally significantly worse fitting under the winning sites test criterion (P = 0.0768). Based on the parsimony criterion, G4-cultivating ants thus do not appear to be monophyletic, but paraphyletic with respect to the G2-cultivating ant clade (Fig. 6).

For both the unconstrained and constrained topologies, the likelihood ratio test found the GTR + I + Γ model (Rodriguez et al. 1990) to be significantly better fitting to the data and the MPTs than the next less complex model (P < 0.000001), with both unconstrained trees equally likely, and both constrained trees equally likely. Unconstrained ML analysis identified a single tree (Fig. 6) with log likelihood of −7105.27198. This tree is identical in ingroup topology to the MPTs. Likelihood analyses under the constraint that the G4-cultivating ants are monophyletic, and using one of the constrained SWTs as a starting tree for branch-swapping, identified a single tree with a log likelihood of −7108.0347. This tree is identical in ingroup topology to the constrained MPTs.

Maximum-likelihood comparisons of the unconstrained and constrained trees indicate that the topologies in which the G4-cultivating *Apterostigma* species are constrained to be monophyletic are not significantly worse fitting to the data, using the K-H test with both normal (P = 0.3167) and 1000 resampling of estimated likelihood (RELL) bootstrap (P = 0.303) approximations, and using the S-H test with 1000 RELL bootstrap approximations (P = 0.150) (Kishino and Hasegawa 1989; Shimodaira and Hasegawa 1999; Goldman et al. 2000). Likelihood tests are thus inconclusive about the paraphyly of G4-cultivating ants.

The 50% majority-rule consensus of the pooled post-burn-in trees, identified by Bayesian analysis using the SSR + Γ model, is identical in ingroup topology to the trees found in
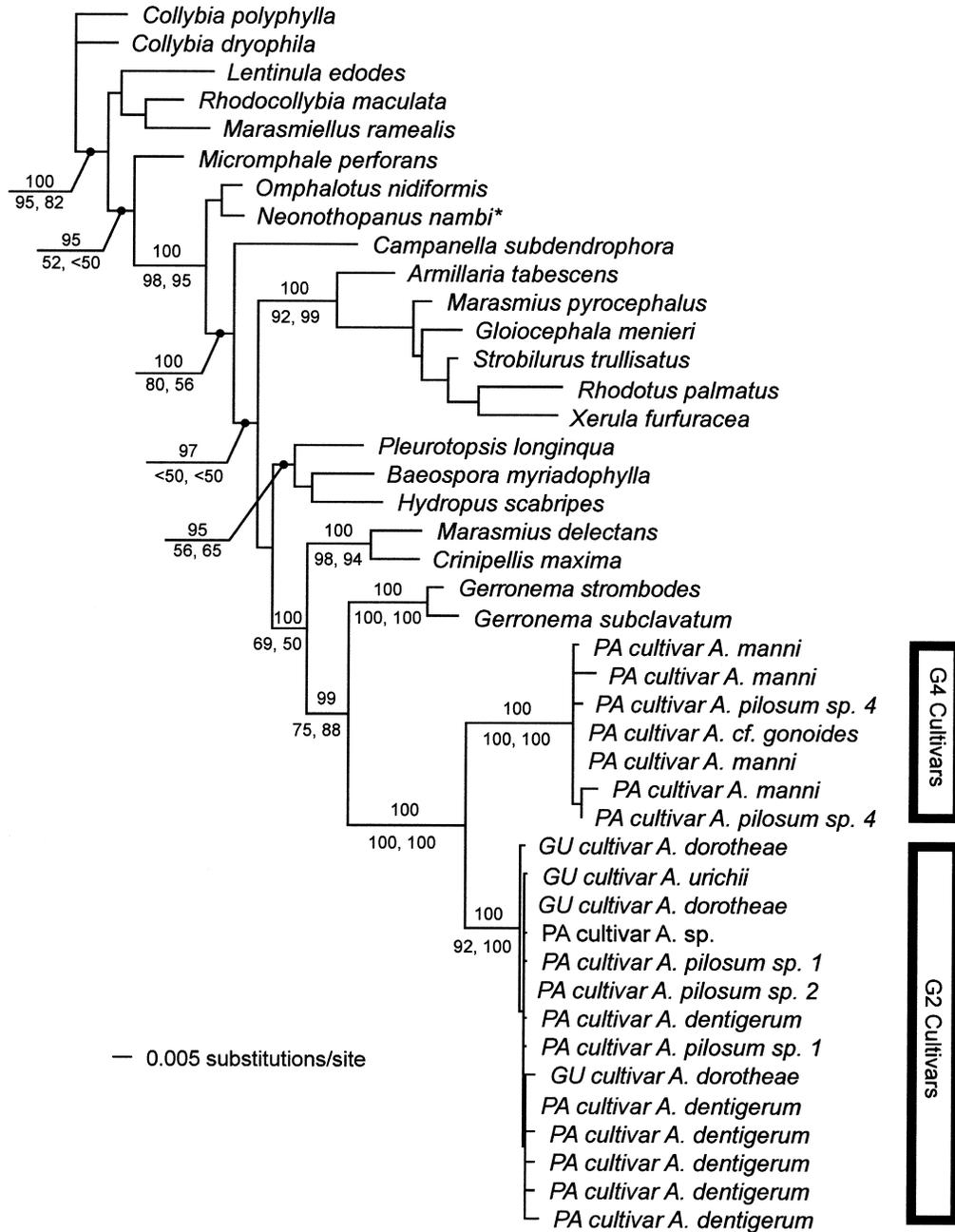
FIG. 3. Maximum-likelihood phylogeny of *Apterostigma* cultivars and free-living fungi currently placed in the family Tricholomataceae (Basidiomycota: Agaricales), based on 25S rDNA sequences. Numbers indicate Bayesian posterior probabilities (above branches), parsimony bootstrap proportions (below branches, left), and likelihood bootstrap proportions (below branches, right). GU (Guyana) and PA (Panama) indicate collection locations of the *Apterostigma* nests. The *Apterostigma* cultivars are monophyletic and divided into two well-supported clades, respectively called G2 and G4 cultivars.

the MP and ML analyses (Fig. 6). Ants in the basal attine genera *Myrmicocrypta* and *Mycocepurus*, as well as the basal *Apterostigma* species *A. auriculatum*, all cultivate lepiotaceous fungi (G3 cultivars), whereas all other *Apterostigma* species in this phylogeny cultivate pterulaceous fungi (G2 and G4 cultivars). The monophyly of the pterulaceous-cultivating *Apterostigma* species indicates that the transition away from lepiotaceous cultivation and to pterulaceous cultivation occurred only once. The basal, paraphyletic position

of G4-cultivating ants among the pterulaceous-cultivating ants further suggests that the direction of this transition was from G3 to G4 fungi, that G2 cultivation evolved subsequent to this original transition, and that G2-cultivating *Apterostigma* ants probably arose from G4-cultivating ancestors.

Examination of the Bayesian post-burn-in trees indicates that the hypothesis that the G4-cultivating *Apterostigma* species are paraphyletic with respect to the G2-cultivating species has a posterior probability of >99.98%. The alternative hypothesis
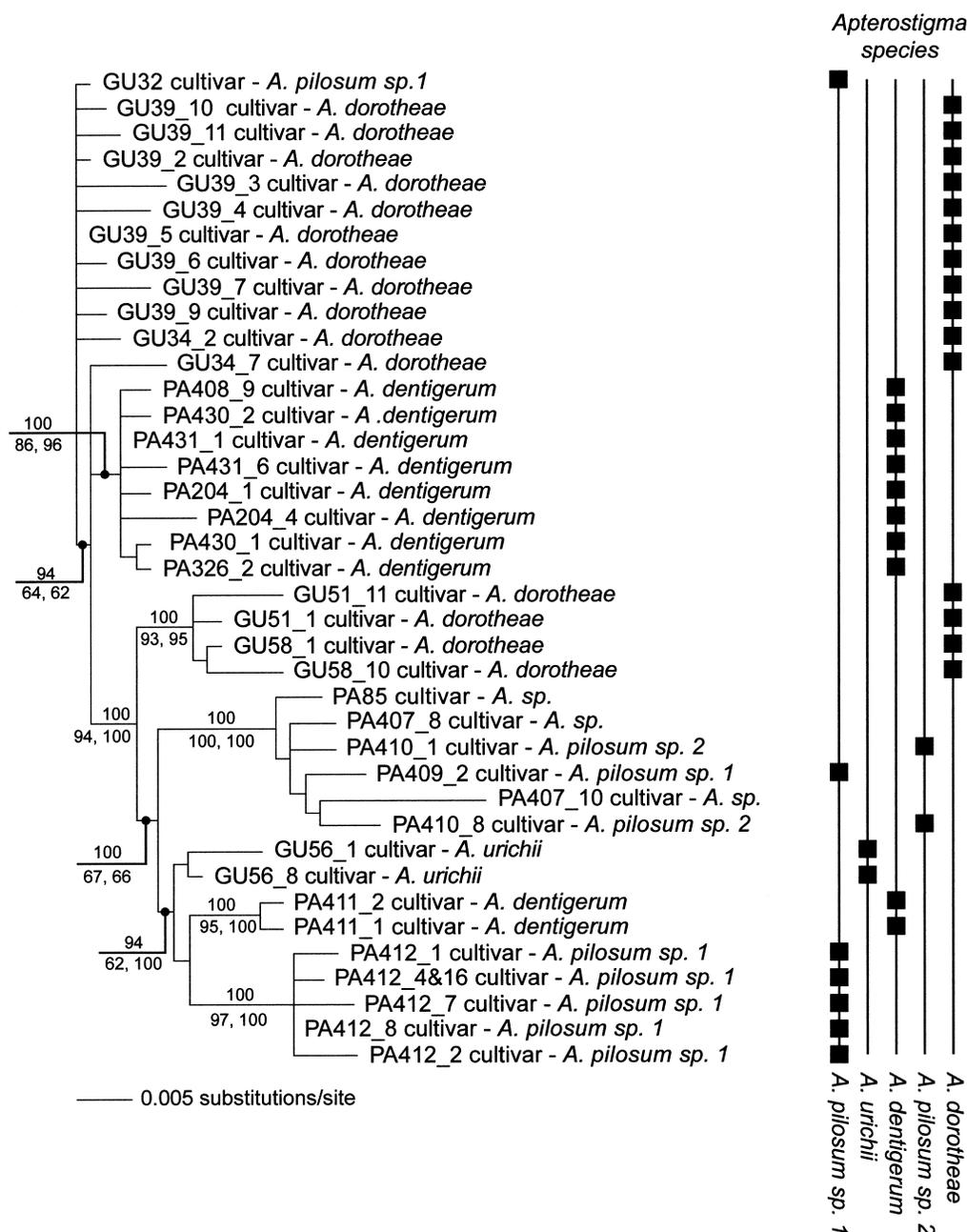
FIG. 4. Maximum-likelihood phylogeny of the G2 *Apterostigma* cultivars, based on ITS sequences. The tree is arbitrarily rooted. Sequences are labeled as collection location of *Apterostigma* nests (GU, Guyana and PA, Panama) followed by sample number. For many cultivars, several ITS sequences were generated after cloning of PCR products (see Methods); these ITS clones are indicated by an underscore (e.g., ''_2'') with the cultivar from which they were derived. Numbers indicate Bayesian posterior probabilities (above branches), parsimony bootstrap proportions (below branches, left), and likelihood bootstrap proportions (below branches, right). Single species of *Apterostigma* appear polymorphic for their cultivars, possibly as a result of cultivar exchange between ant species.

that the G4 cultivators are monophyletic has a posterior probability of <0.02%. Thus, Bayesian MCMC analyses strongly support the conclusion that the G4-cultivating *Apterostigma* species are transitional between the plesiomorphic G3 cultivators and the derived G2-cultivating ant clade.

Phylogenetic analyses of fungal LSU sequence data under MP, ML, and Bayesian criteria reconstruct two distinct clades of symbionts cultivated by *Apterostigma* ants (Fig. 3): the G2

group, previously recognized by Chapela et al. (1994), and a novel G4 group, which is the sister clade to the G2 fungi and is recognized here for the first time. Any given *Apterostigma* ant species cultivates fungi exclusively in either the G2 or the G4 group, indicating broad specialization of *Apterostigma* species on one of the two cultivar groups. The closest free-living (i.e., nonsymbiotic) relatives of the G2 and G4 *Apterostigma* cultivars in our analysis are the fungi
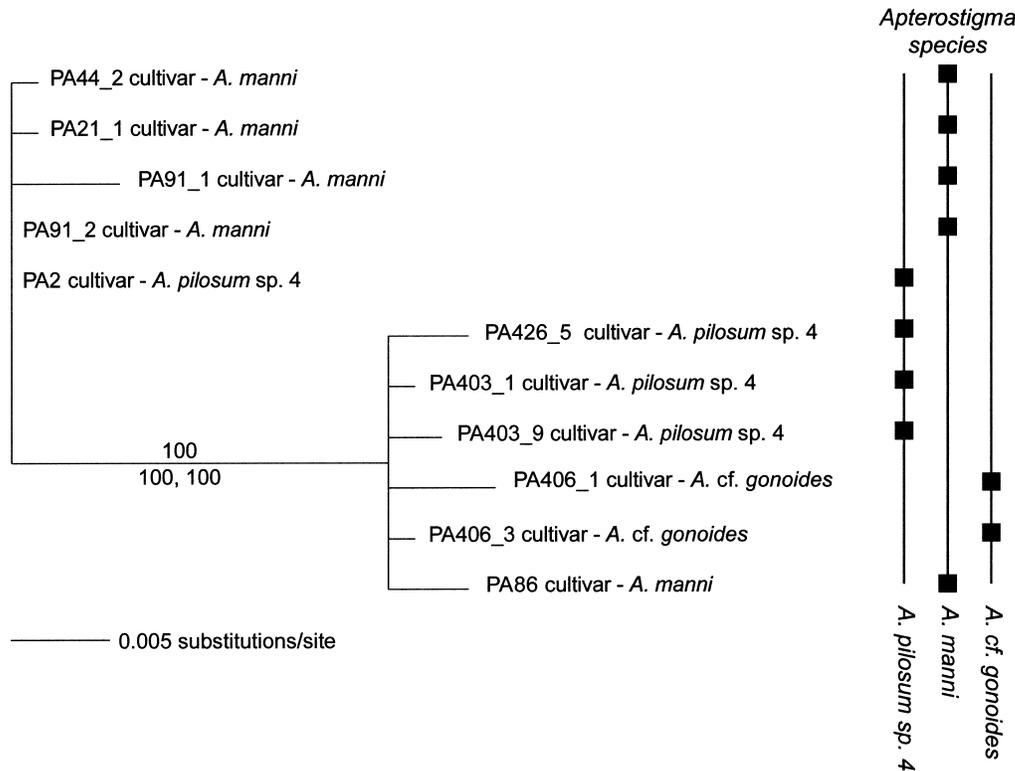
FIG. 5. Maximum-likelihood phylogeny of the G4 *Apterostigma* cultivars, based on ITS sequences. The tree is arbitrarily rooted. Sequences are labeled as collection location of *Apterostigma* nests (GU, Guyana and PA, Panama) followed by sample number. For many cultivars, several ITS sequences were generated after cloning of PCR products (see Methods); these ITS clones are indicated by an underscore (e.g., ''_2'') with the cultivar from which they were derived. Numbers indicate Bayesian posterior probabilities (above branches), parsimony bootstrap proportions (below branches, left), and likelihood bootstrap proportions (below branches, right).

in the genus *Gerronema*. This relationship to *Gerronema* is supported in phylogenetic analyses with bootstrap proportions of 88% under MP and 75% under ML, and by a Bayesian posterior probability of 99%.

Because phylogenetic analyses of LSU sequences unambiguously support the grouping of ant-cultivated pterulaceous fungi into two distinct clades, ITS sequences were aligned separately for the G2 and G4 fungi. This subalignment of the ITS sequences reduced the alignment ambiguities (and thus reduced the number of excluded characters) that would have resulted from a global alignment of ITS sequences for all *Apterostigma* cultivars. The separate phylogenies for both the G2 and G4 clades reveal that (1) single *Apterostigma* species cultivate a diversity of sometimes distantly related cultivars; and (2) different *Apterostigma* species sometimes cultivate fungi from the same fungal lineage (Figs. 4, 5).

Phylogenetic analyses of *Apterostigma* ants agree with the taxonomic conclusions of Lattke (1997, pers. comm.), including the division of the genus into an ''*auriculatum*'' subgroup (probably plesiomorphic and possibly paraphyletic, represented in this study by two specimens of *A. auriculatum*, one from Panama and one from Bahia, Brazil) and a derived, monophyletic ''*pilosum*'' subgroup. Our results indicate that the G2-cultivating *Apterostigma* species represent a well-supported, derived, monophyletic group within the genus. The precise relationship of the G4-cultivating *Apterostigma* species is less clear, but, under the well-supported assumption

that G3 cultivation (represented by *A. auriculatum*) is plesiomorphic for the genus (Chapela et al. 1994; Mueller et al. 1998; Mueller et al. 2001), the G4 cultivators represent either (1) a paraphyletic group that is transitional between the G3 and G2 cultivators (the hypothesis favored by both the most parsimonious, most likely, and Bayesian trees, Fig. 6); or else (2) the G4 cultivators represent a monophyletic group that is the sister group to the G2 cultivators. The latter hypothesis, that the G4-cultivating *Apterostigma* ant species are not necessarily transitional between the plesiomorphic G3-cultivators and the derived G2 cultivators, was found to be (1) significantly worse fitting to the data than the alternative in two parsimony-based statistical tests; (2) marginally significantly worse fitting in a third parsimony-based test; and (3) significantly improbable ($<0.02\%$) in Bayesian analyses. However, this hypothesis was not found to be significantly worse fitting to the data than the alternative in three likelihood-based tests. The data thus support a sequential transition from G3 to G4 to G2 cultivation in the evolution of *Apterostigma*, but the question remains open to further investigation. It bears noting that under all criteria the data strongly support the monophyly of the G2 cultivators.

DISCUSSION

Origin and Historical Ecology of Apterostigma Fungiculture

Our phylogenetic reconstructions place the nonlepiotaceous *Apterostigma* cultivars (Chapela et al. 1994) into the

vicinity of the genus *Gerronema*, a group of mostly wood-decomposing fungi that are particularly abundant in the tropics and subtropics (Singer 1970). This placement is consistent with the recent findings by Munkacsi et al. (2004) who were able to place the non-lepiotaceous *Apterostigma* cultivars more precisely into the family Pterulaceae (hence our use of the term ''pterulaceous *Apterostigma* cultivars'' for these nonlepiotaceous attine cultivars). The reciprocal monophyly between the pterulaceous *Apterostigma* cultivars and the ants that cultivate them indicates that this shift to pterulaceous fungiculture most probably occurred only once and thus represents a historically unique event in attine ant evolution. This rarity in major cultivar shifts is consistent with the view that faithfulness of ant lineages to the broad symbiont groups (G1, G2, G3, and G4) is highly constrained, most likely because of coevolutionary processes leading to adaptation of ant lineages on particular cultivar groups, and vice versa (Mueller et al. 2001; Mueller 2002). Transitions between these cultivar groups, or to other distantly related fungi, therefore are evolutionarily unlikely (Mueller et al. 2001; Mueller 2002).

Within cultivar groups, the following factors may play a role in forcing colonies to acquire new cultivars: (1) loss of a fungus garden due to parasites and pathogens (Currie et al. 1999a; Currie et al. 2003); (2) loss of a fungus garden due to garden stealing or raiding by other ants (Adams et al. 2000a, b); (3) semiotic recruitment by other members of an ant-attractant guild of free-living fungi (Mueller et al. 2001); (4) accidental introduction of alien fungi (e.g., on gardening substrate brought into the nest by workers), which subsequently outcompeted the resident cultivar strains (Mueller et al. 2001; Mueller 2002); or (5) unknown abiotic factors. It is possible that one or more of these factors may have played a role in the switch away from ancestral G3-cultivation within *Apterostigma*.

Extant *Apterostigma* ants, including G3-cultivators, manure their fungi with a mixture of insect feces, dead plant debris, and wood fragments (Weber 1972; Murakami 1998; N. Gerardo and U. Mueller, pers. obs.). These vegetable materials also represent suitable substrates for *Gerronema*-like and pterulaceous fungi (Singer 1970; Munkacsi et al. 2004). Moreover, many extant *Apterostigma* species, including the basal G3-cultivating *Apterostigma* species, construct their nests under or inside of decomposing logs on the rainforest floor (Weber 1941; Weber 1972; Lattke 1997), and it is possible that occupation of this microhabitat preceded the shift from the ancestral lepiotaceous, litter-decomposing fungal cultivars to a putative wood-decomposing fungal cultivar. There exist many examples of similar mutualistic associations arising out of ancestrally accidental interactions between wood-saprophytic fungi and rotting-wood inhabiting insects (Batra 1979; Gilbertson 1984; Mueller and Gerardo 2002). Although nest construction in or under rotting wood may have generated the close physical proximity necessary for frequent ant-saprophyte interaction, and were as evolution within this unique niche may have modified ant behavior to ''preadapt'' an ancestral *Apterostigma* for an association with a wood-decomposing pterulaceous cultivar, some additional unknown factor (possibly chance alone) must also have played a role in the origin of this association, because many

other attine ant species (e.g., species in the diverse genera *Myrmicocrypta*, *Cyphomyrmex*, *Trachymyrmex*, and *Acromyrmex*) also occupy niches in rotten wood, yet have not made a switch to cultivars outside the litter-decomposing Lepiotaceae.

The phylogenies in Figures 3 and 6 are consistent with a single domestication of a free-living pterulaceous fungus by an ancestral *Apterostigma* ant, and provide no evidence for multiple domestications, such as have occurred in the G3-cultivating lower attines (Mueller et al. 1998). However, testing for multiple domestications requires a far more comprehensive collection of free-living, putative relatives than is represented in our analysis. Fortunately, the robust placement of the non lepiotaceous *Apterostigma* cultivars into the Pterulaceae (Munkacsi et al. 2004) should facilitate the search for free-living fungi that may occupy phylogenetic positions within the G2 or G4 clades. Recent phylogenetic analysis of free-living fungi in the genera *Pterula* and *Deflexula* (currently still incorrectly placed in the family Clavariaceae, order Aphyllophorales; but see Pine et al. 1999; Munkacsi et al. 2004) revealed the possible placement of *Pterula* lineages nested within the group of *Apterostigma* cultivars (Munkacsi et al. 2004). The latter scenario would support a hypothesis of multiple domestications of *Apterostigma* cultivars and possibly ongoing import of novel pterulaceous cultivars from free-living populations into the *Apterostigma* symbiosis. One case of a possible escape of a pterulaceous *Apterostigma* cultivar from a garden was reported by Mueller (2002), suggesting links between cultivated pterulaceous strains in the symbiosis and free-living strains outside the symbiosis. Such links to free-living fungal populations have been documented for some lower-attine G3 cultivars (Mueller et al. 1998).

Transitions between Cultivar Groups

The phylogeny of *Apterostigma* ants (Fig. 6) indicates that the transition from lepiotaceous cultivation to pterulaceous cultivation occurred only once. The most parsimonious, most likely, and Bayesian trees (which are essentially identical), as well as a subset of the statistical tree-comparison tests, suggest that the direction of this transition was from G3 to G4 to G2 cultivation (Fig. 6). This sequence of transitions is indicated by the basal position of G3-cultivating *Apterostigma* species and the paraphyly of G4-cultivating ant species with respect to the monophyletic G2-cultivating species (i.e., the G2-cultivating *Apterostigma* lineages appear to have been derived from a G4-cultivating ancestor). The hypothesis of a G3 > G4 > G2 sequence is strongly supported by the Bayesian analysis (>99.98 % posterior probability), were as the alternative topology, in which the G4 and G2 cultivators are sister groups, has very low support (<0.02 % posterior probability).

Extended phylogenetic analyses of *Apterostigma* ant species and their fungal cultivars will directly test whether cultivation of lepiotaceous cultivars (G3 cultivation) is plesiomorphic for the genus *Apterostigma*, a scenario favored by Mueller et al. (1998, 2001). The current taxonomy of the ant genus (Lattke 1997) indicates only a division into two broad groups, the *auriculatum* group and the *pilosum* group (the *pilosum* group is different from the ''*pilosum* species com-
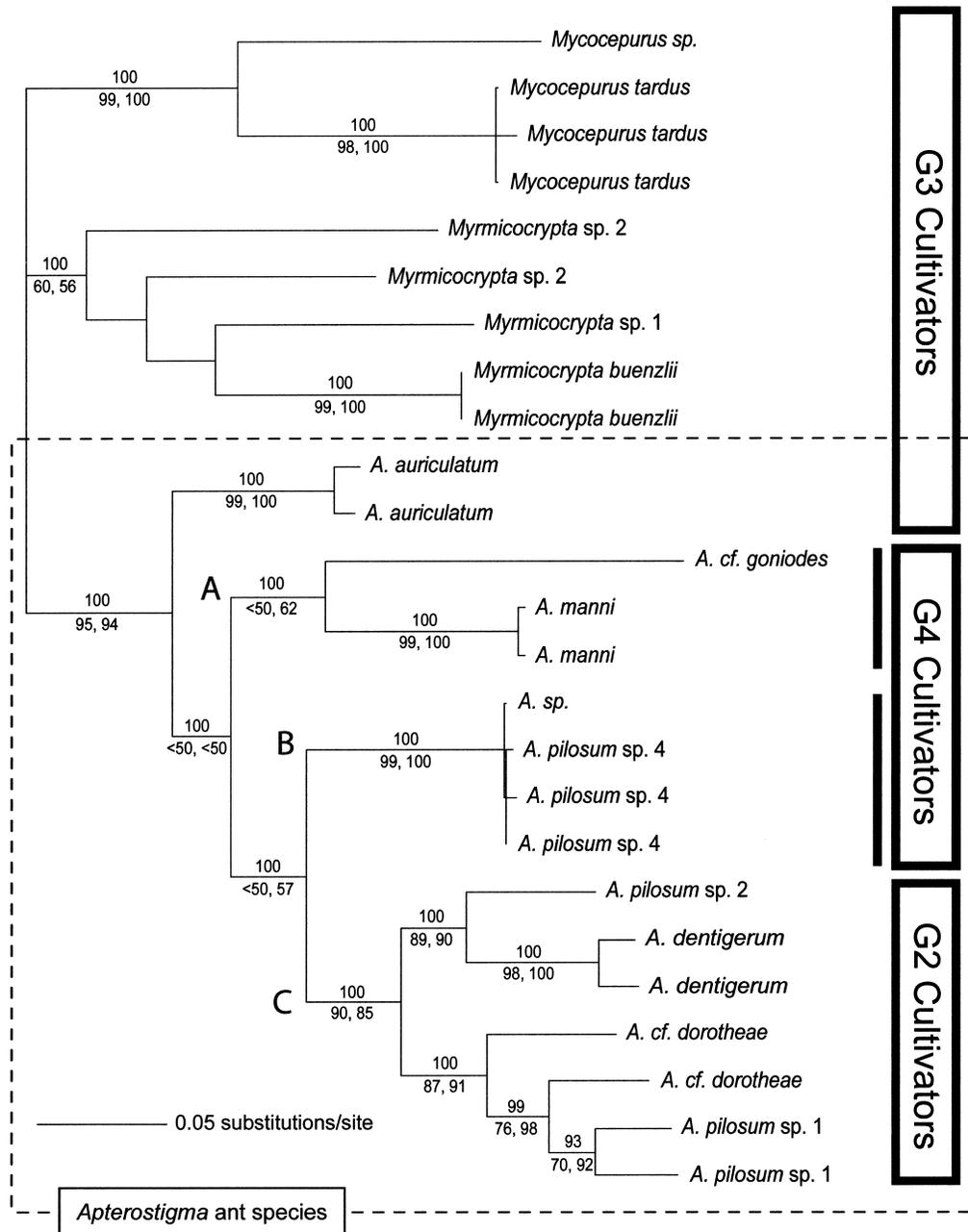
FIG. 6. Maximum-likelihood phylogeny of *Myrmicocrypta*, *Mycocepurus*, and *Apterostigma* ant species, based on COI sequences. Numbers indicate Bayesian posterior probabilities (above branches), parsimony bootstrap proportions (below branches, left), and likelihood bootstrap proportions (below branches, right). Monophyly of the combined G2- and G4-cultivating ant species indicates that the transition occurred only once. The basal position of the G4-cultivating species indicates that G2 cultivation evolved subsequent to G4 cultivation.

plex,'' which is a default complex included within the *pilosum* group; see Materials and Methods). The *auriculatum* group is probably paraphyletic (Lattke 1997, 1999, pers. comm.), whereas the *pilosum* group appears to be monophyletic (Fig. 6) and contains all the ant species cultivating the fungi (G2, G4) investigated in this study. Current, vastly incomplete knowledge suggests that most species of the *auriculatum* group cultivate lepiotaceous fungi (G3), whereas all species of the *pilosum* group from which cultivars were obtained so far cultivate pterulaceous fungi (G2 and G4). The

pilosum group therefore appears to have originated coincident with the switch to pterulaceous fungiculture (Fig. 6), and at present there is no indication that any of the *Apterostigma* lineages derived from this switched ancestor ever reverted back to lepiotaceous fungiculture.

Cultivar Exchange between Apterostigma Ant Species

At the level of cultivar groupings (G2, G3, and G4), the ant phylogeny (Fig. 6) strongly suggests that ant lineages are

faithful to cultivar groups; within these groups, however, ant and cultivar lineages are not consistently associated (i.e., ant and cultivar phylogenies are topologically incongruent *within* each cultivar group). The discovery of closely related cultivar strains that are shared by several *Apterostigma* species (Figs. 4, 5) is consistent with an evolutionary history of cultivar exchange between ant species. In some cases these exchanges may have occurred recently, because different species share very closely related fungal cultivars (*A. dentigerum* from Panama and *A. dorotheae* from Guyana, Fig. 4; *A. manni* and *A. pilosum* sp. 4 in Panama, Fig. 5) were was both species locally cultivate a broad diversity of cultivars (Figs. 4, 5). The emerging picture therefore suggests that pterulaceous fungiculture parallels lepiotaceous fungiculture in other lower-attine ants and also in the leafcutter ants, wherein cultivars are exchanged between different ant lineages with appreciable frequency over evolutionary time (Mueller et al. 1998; Bot et al. 2001; Green et al. 2002).

Diversity of Cultivar Use by Single Apterostigma Species

Single species of *Apterostigma* cultivate several distantly related fungal cultivars, a pattern similar to that found for G3-cultivating lower attine ant species (Mueller et al. 1998). Most *Apterostigma* species conform to this pattern, in which associations with particular ant species occur at several distinctly separated positions across the cultivar phylogeny (Figs. 4, 5). Single *Apterostigma* species, as currently defined in Lattke's (1999) recent revision, therefore appear to be ''polymorphic'' for their cultivars. However, each ant species may subsume several unrecognized cryptic species, each specialized on its own, distinct cultivar type. Schultz et al. (2002) recently recognized such a case in the lower attine *Cyphomyrmex longiscapus* complex and decomposed the species *Cyphomyrmex longiscapus s.l.,* previously thought to be as a single ant species cultivating two distinct kinds of fungi (Mueller et al. 1998), into two cryptic sibling species each specialized on its own cultivar type. The ''polymorphic'' *Apterostigma* species apparent in Figures 4 and 5 may represent similar cases of unrecognized cryptic species, and future taxonomic work should focus on testing this hypothesis by delineating species boundaries with morphological and molecular techniques (Schultz et al. 2002).

G2 versus G4 Cultivation and Apterostigma Nest Architecture

The G2-cultivating *Apterostigma* ant species have an unusual garden architecture quite unlike that of other attine species (see Materials and Methods, and Fig. 2). G2 gardens are pendant, and the associated ants hang the gardens underneath logs, inside cavities in logs, or, more rarely, in shallow cavities in the ground (Fig. 2). These gardens are surrounded by a mycelial veil that is apparently woven by the ants and that shelters the garden, presumably by serving as a protective barrier (against parasites, predators, and contaminant spores) and preventing desiccation of the garden suspended inside the mycelial envelope. In contrast, the G4-cultivating *Apterostigma* species have sessile gardens like those of most other lower attines that are either constructed under logs or in excavated cavities in the ground, and these

gardens are not surrounded by a veil. G2 gardens therefore appear more derived than G4 gardens, suggesting (as do the phylogenetic reconstructions; Fig. 6) that the transition from lepiotaceous to pterulaceous fungi may have initially involved a G4 cultivar (unveiled, sessile gardens), and that G2 cultivation (veiled, hanging gardens) arose only later in *Apterostigma* diversification.

Specialization on G2 versus G4 Fungi

The absence of switches by any *Apterostigma* species between the G2- and G4-cultivar sister clades (Fig. 3) indicates coevolutionary specialization, at least at a more ancient phylogenetic level. If one of the groups is derived from the other (e.g., G2 fungi from the G4 fungi, as hypothesized above), then a highly evolved set of coadaptations between ants and fungi that arose early in the evolution of the derived group could have served to isolate the two systems and to prevent cultivar switches across groups. Possible candidates for such coadaptations include the unusual G2-nest architecture and the putative ant behaviors involved in weaving the mycelial veil. Additional coadaptations between ants and specific cultivars, including physiological and biochemical ones, might only be observed through mechanistic investigations and experimentation.

ITS Variation Within the rDNA Array and Allele Sequence Divergence

The multiple PCR products amplified from the ITS rDNA locus (Figs. 4, 5) suggest that rDNA arrays of many *Apterostigma* cultivars are polymorphic. Alternatively, allele sequence divergence (ASD; Judson and Normark 1996; Birky 1996) under long-term asexual cultivar propagation could have contributed to diversification of homologous ITS sequences, thus also generating multiple amplification products. Significant levels of ASD are predicted for attine cultivars if they indeed represent ''ancient asexuals'' (Judson and Normark 1996), as hypothesized by Chapela et al. (1994). However, assuming a simple diploid (dikaryotic) state in the cultivated fungi, ASD under cultivar asexuality would generate at most two alleles per cultivar, not the large number of ITS sequences revealed in Figures 4 and 5 for single cultivar isolates. Thus, although ASD may well be accumulating in *Apterostigma* cultivars under long-term asexual cultivar propagation by the ants, ASD cannot be a complete explanation for the observed ITS diversity in single cultivars. Either the *Apterostigma* cultivars are highly polyploid, or, more likely, ITS polymorphisms exist within the rDNA and explain the multiple (up to nine) distinct ITS sequences observed in single *Apterostigma* cultivars (Fig. 4). Testing for ASD in attine cultivars (i.e., testing for potential ancient asexuality under strict clonal propagation by attine ants; Judson and Normark 1996; Birky 1996; Mueller et al. 1998) therefore will be best accomplished by sequencing of fast-evolving, single-copy nuclear genes (Normark 1999; Welch and Meselson 2000).

Conclusion

Since the first domestication of fungal cultivars by the attine ants around 50–60 million years ago (Wilson 1971;

Mueller et al. 2001), the attine ant-fungus symbiosis has evolved in a complex mosaic of coevolutionary interactions. These interactions include the attine ants and their fungal cultivars (Weber 1972), as well as mutualistic actinomycete bacteria that produce antibiotics specifically targeted against specialized attine garden parasites in the fungal genus *Escovopsis* (Currie et al. 1999a, b; Currie et al. 2003). The vast majority of attine ants cultivate fungi drawn from two lepiotaceous genera, *Leucocoprinus* and *Leucoagaricus*. A switch away from these original cultivar lineages seems to have occurred only once in the entire evolutionary history of the Attini (Chapela et al. 1994; Mueller et al. 1998), when an ancestral *Apterostigma* species began cultivating a pterulaceous fungus (Munkacsi et al. 2004). This shift led to a subsequent diversification into two cultivar groups (G2 and G4 cultivars), each of which is currently cultivated by a discrete group of *Apterostigma* ants. Additional study of the pterulaceous-cultivating *Apterostigma* may shed light on the factors that produced this unique historical event. Such analyses may also illuminate the stabilizing factors that have otherwise maintained the remarkable conservatism demonstrated by the majority of attine ants with regard to their fungal cultivars. A better understanding of attine historical ecology may, in turn, inform current models of the general mechanisms that govern stasis and change in the evolution of symbioses.

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