PHYLOGENETIC PLACEMENT OF AN UNUSUAL CORAL MUSHROOM CHALLENGES THE CLASSIC HYPOTHESIS OF STRICT COEVOLUTION IN THE *APTEROSTIGMA PILOSUM* GROUP ANT–FUNGUS MUTUALISM

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The ~50 million-year-old fungus-farming ant mutualism is a classic example of coevolution, involving ants that subsist on asexual, fungal biomass, in turn propagating the fungus clonally through nest-to-nest transmission. Most mutualistic ants cultivate two closely related groups of gilled mushrooms, whereas one small group of ants in the genus *Apterostigma* cultivates a distantly related lineage comprised of the G2 and G4 groups. The G2 and G4 fungi were previously shown to form a monophyletic group sister to the thread-like coral mushroom family Pterulaceae. Here, we identify an enigmatic coral mushroom that produces both fertile and sterile fruiting structures as the closest free-living relative of the G4 fungi, challenging the monophyly of the *Apterostigma*-cultivated fungi for the first time. Both nonparametric bootstrap and Bayesian posterior probability support the node leading to the G4 cultivars and a free-living *Pterula* mushroom. These data suggest three scenarios that contradict the hypothesis of strict coevolution: (1) multiple domestications, (2) escape from domestication, (3) selection of single cultivar lineages from an ancestral mixed-fungus garden. These results illustrate how incomplete phylogenies for coevolved symbionts impede our understanding of the patterns and processes of coevolution.

KEY WORDS: Attine, clavarioid fungi, fungus-growing ant, molecular systematics, morphological evolution, parametric bootstrap, Pterulaceae, Symbiosis.

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Until recently, the mutualistic symbiosis between Neotropical fungus-farming ants and their fungal cultivars has been considered an archetype of strict coevolution (Herre et al. 1999; Mueller et al. 2001; Mueller 2002). A complex array of ant foraging and gardening behaviors, unique ant and fungal morphologies, and the ants' adoption of mutualistic bacteria that fend off specialized garden parasites, suggest intricate coadaptation throughout the ~50 million years since the origin of this symbiosis (Weber 1966; Currie et al. 1999a,b; Mueller et al. 2001; Gerardo et al. 2004, 2006). However, phylogenetic analysis of fungus-farming ant cultivars and their free-living relatives have shown that, in contrast to the ants, the cultivars are not monophyletic (Chapela et al. 1994; Mueller et al. 1998). Moreover, recent studies in two genera of attine ants, *Cyphomyrmex* and *Acromyrmex*, have revealed that the evolutionary association between the ants and their fungal cultivars is much more complex than previously thought, involving diffuse associations over ecological and evolutionary time scales (Bot et al. 2001; Green et al. 2002; Mehidiabadi et al. 2006; Mikheyev et al. 2006, 2007). These new findings challenge the widely accepted assumptions of the strict coevolutionary nature of the fungus-farming ant symbiosis and provide new insights into the origin and maintenance of mutualistic coevolution. However, the extent to which these new insights apply to other attine–fungus associations is unknown. Critically, this lack of knowledge stems from the fact that, despite years of attention given to this model of coevolutionary system, the evolutionary histories of the ants, their fungal cultivars, specialized parasites, and symbiotic bacteria are still poorly known. The absence of robust phylogenies for these organisms remains one of the most severe impediments to a thorough understanding of the evolution and ecology of the fungus-farming ant symbiosis.

All attine ants are completely dependent on their asexual fungal cultivars for nutrition (Hölldobler and Wilson 1990; Mueller et al. 2001). Because most of the cultivars never or rarely produce sexual structures (Weber 1957, 1966; Hervey et al. 1977; Bononi et al. 1981; Muchovej et al. 1991; Fisher et al. 1994; Pagnocca et al. 2001), identifying these species has been a major challenge to our understanding of the ant–fungus system. Most of the > 200 species and 13 genera of the monophyletic attine ants (Hymenoptera:Formicidae:Tribe Attini; Schultz and Brady 2008) cultivate two groups of fungi (known as "Group 1" and "Group 3," in reference to the sequence in which these groups were identified), which have been shown to be closely related to free-living gilled mushrooms (agarics) in the family Agaricaceae (sensu Matheny et al. 2006) using DNA sequence data (Chapela et al. 1994). However, it has been proposed that about 10–20 million years ago an ancestor of the "pilosum group" of ants in the genus *Apterostigma* (~34 species) adopted a novel fungal cultivar that now forms two phylogenetic clades of asexual fungi ("Group 2" and "Group 4"), which are only distantly related to the G1 and

G3 cultivars (Chapela et al. 1994; Mueller et al. 1998). The G2 and G4 fungi are thought to be monophyletic and until recently they were allied with the agaric family Tricholomataceae (Chapela et al. 1994; Mueller et al. 1998; Villesen et al. 2004), but new phylogenetic evidence based on DNA sequence data indicates that they share a recent common ancestor with the thread-like coral mushroom family Pterulaceae (Munkacsi et al. 2004). Assuming the proposed sequence of domestication is correct, this historically unique switch in *Apterostigma* from the cultivation of gilled mushrooms to the cultivation of phylogenetically and phenotypically divergent coral mushrooms occurred for reasons that are still unknown.

Here we report new phylogenetic analyses of *Apterostigma* ants and an expanded sampling of free-living Pterulaceae that reveal *Pterula moniliformis*, an enigmatic coral mushroom that produces both fertile and sterile fruiting structures either separately or as a chimera, to be the closest free-living relative of the G4 group of *Apterostigma* ant cultivars. The simultaneous production of sterile and fertile fruiting structures is anomalous in fungi and rare in biology in general, making *P. moniliformis* easy to recognize in nature and stimulating new hypotheses in the fungus-farming ant mutualism. Our placement of *P. moniliformis* shows that the G2 and G4 cultivars are not monophyletic, supporting the contemporary view in other ant–fungus associations that the coevolutionary history of attine ants and their fungal cultivars is more complex than previously thought. This discovery suggests three scenarios that contradict the hypothesis of strict coevolution between *Apterostigma* and their cultivars: (1) multiple domestications, (2) cultivar escape, or (3) lineage sorting from an ancestral polyculture garden. Although the interpretation of these data provide new insight into the evolutionary history of the ant–fungus mutualism, overall we still rely on weak phylogenetic evidence, a fact that has plagued progress toward fully understanding the evolution of the ant–fungus mutualism. More generally, our results demonstrate how our understanding of coevolution is subject to multiple equally plausible interpretations, none of which can be discounted when phylogenies are weakly supported and incomplete.

Methods
TAXON SAMPLING AND DNA SEQUENCE ACQUISITION

New collections and cultures of Pterulaceae used in this study are listed in Supporting information (Table S1). Genomic DNA was extracted and the large subunit of the ribosome (LSU) was amplified as previously reported (Dentinger and McLaughlin 2006; see Supporting information for detailed information on PCR and sequencing). Sequences generated for this study have been submitted to GenBank (accession FJ648328-FJ648343). A file
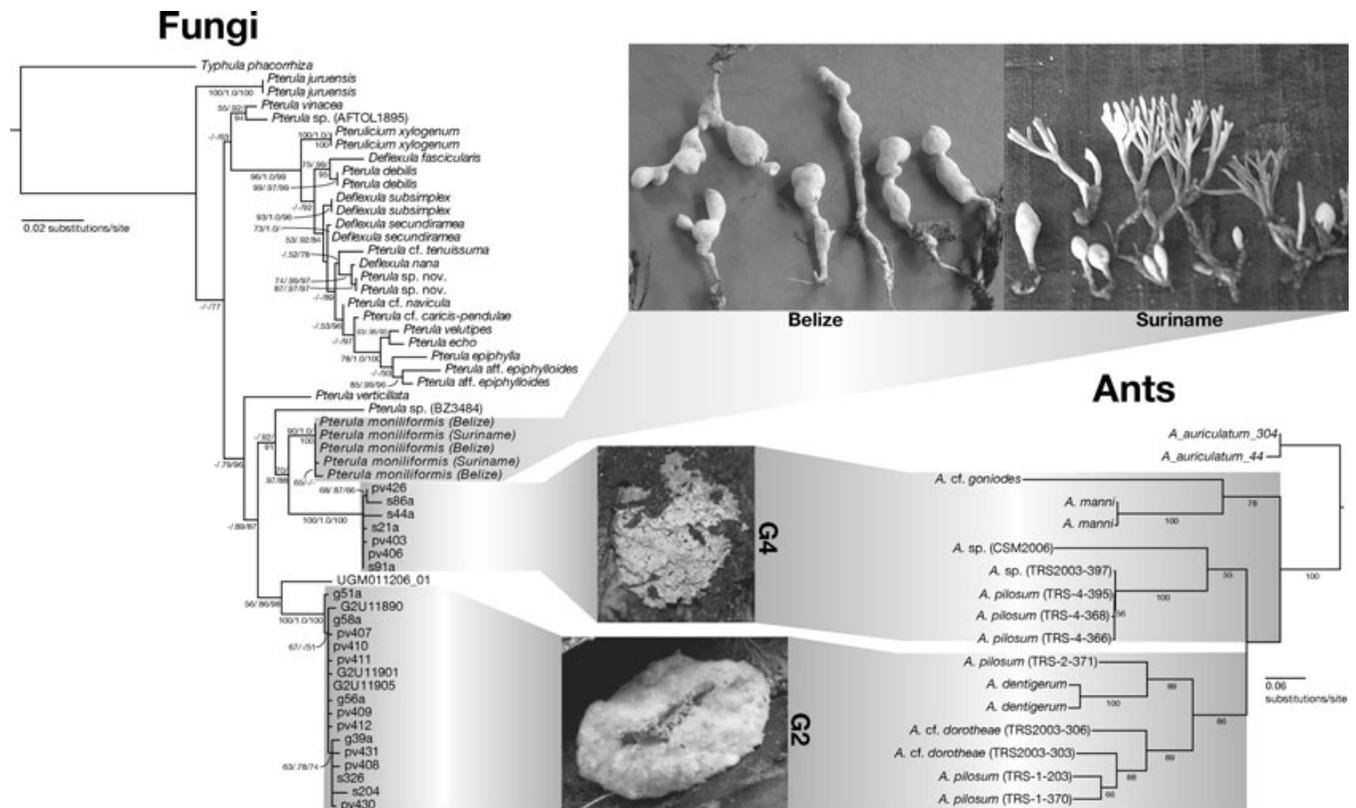
Figure 1. Best maximum-likelihood phylograms of the Pterulaceae and *Apterostigma* ants indicate that the G2/G4 ant cultivars are a paraphyletic group with respect to free-living Pterulaceae. Branch support values on the fungal phylogram are percent nonparametric bootstrap (1000 reps)/Bayesian posterior probability/percent parametric bootstrap (100 reps). Branch support values on the ant phylogram are percent nonparametric bootstrap (100 reps). The G2 fungi, which all produce gardens that have a fungal veil covering them (bottom photo), and the G4 fungi, which all produce unveiled gardens (second photo from bottom), form two monophyletic groups, respectively. The closest free-living relative of the G4 fungi is the extraordinary *Pterula moniliformis*, which produces sterile mushrooms that look like strings of beads (two top photos; from Suriname and Belize) as well as fertile branched coral structures attached to the sterile ones (top right photo; from Suriname) that are more typical of Pterulaceae. UGM011206_01 is a sterile hook-like structure collected from an *Apterostigma* nest (Munkacsi et al. 2004). Photos of *P. moniliformis* by D.J. Lodge. Photos of G2 and G4 gardens by B.T.M. Dentinger.

containing all of the publicly available LSU sequences of Pterulaceae in GenBank was amended with the new sequences that were assembled and edited in Sequencher 3.0 (Gene Codes Corp., Ann Arbor, MI). The *Apterostigma* ant phylogeny was constructed using homologous regions of partial mitochondrial cytochrome oxidase I (mtCOI) DNA sequences from GenBank, corresponding to two previously published studies (Villesen et al. 2004; Moreau et al. 2006).

PHYLOGENETIC ANALYSIS

Sequences were aligned and analyzed using Bayesian (BA; fungi) and maximum-likelihood (ML; ants and fungi) methods. Branch support was evaluated using nonparametric (npbs; ants and fungi) and parametric (pbs; fungi) bootstrapping for ML analyses and posterior probabilities (pp; fungi) in BA. One sequence of *Typhula phacorrhiza* (AF261374) was used to root the Pterulaceae phylogeny based on the results of Matheny et al. (2006) and the ant dataset was rooted using *Apterostigma auriculatum* (Villesen

et al. 2004; Schultz and Brady 2008). Alternative topologies for the fungal phylogeny were evaluated using the Shimodaira–Hasegawa (SH) test and a parametric bootstrap test (Shimodaira and Hasegawa 1999). Further detail on the phylogenetic analyses can be found in the Supporting information.

Results

The LSU Pterulaceae dataset consisted of 56 sequences (including 24 *Apterostigma* cultivars), and one outgroup. Of the 937 aligned positions, 104 were parsimony informative and 177 were constant. ModelTest selected a general-time reversible model with six substitution rate parameters, a gamma distribution, and a proportion of invariant sites (GTR + G + I). All base frequencies were estimated from the dataset. The ML analysis converged on a single topology ($-\ln L = 2667.58141$) after 27,100 generations (Fig. 1) and no stochastic effects between runs were detected. The *Apterostigma* cultivars were recovered as paraphyletic. The node

leading to the G4 cultivars plus the free-living *P. moniliformis* is well supported by both nonparametric bootstrap (npbs = 70%) and posterior probability (pp = 0.97), but the parametric bootstrap was not significant (pbs = 88%).

Although we recovered strong support for the branch leading to *P. moniliformis* plus the G4 cultivars, we could not reject the alternative hypothesis that the cultivars are monophyletic using several tests. The difference in −lnL between unconstrained searches and searches constrained to recover the ant cultivars as monophyletic was 10.60021. SH test could not reject that a monophyletic cultivar clade was an equally plausible hypothesis for the data (*P* = 0.183). The parametric bootstrap test did not support the node subtending the G4 cultivars + *P. moniliformis* (*P* = 0.35).

To conduct phylogenetic analysis of *Apterostigma* ants, we assembled a dataset of 17 taxa. Of the 982 aligned positions, 278 were parsimony informative and 642 were constant. ModelTest selected a general-time reversible model with six substitution rate parameters, a gamma distribution, and a proportion of invariant sites (GTR + G + I). All base frequencies were estimated from the dataset. The ML analysis supports the G2-cultivating ants as monophyletic (npbs = 86%), whereas the G4-cultivating ants make two clades (npbs = 55% and 78%) that are unresolved with respect to each other (Fig. 1). Topology tests conducted by Villesen et al. (2004) on a nearly identical dataset could not reject the alternative hypothesis that the G4-cultivating ants are monophyletic, which is consistent with the weak branch support we recovered for the node subtending the clade containing both G2- and G4-cultivating ants.

Discussion
COEVOLUTION OF PTERULACEAE AND APTEROSTIGMA
The classic hypothesis of strict coevolution in the ant–fungus association predicts a pattern of complete congruence between the ant and fungal phylogenies, a view that was reinforced in several earlier molecular phylogenetic studies with limited taxonomic sampling (Chapela et al. 1994; Currie et al. 2003; Villesen et al. 2004; Munkacsi et al. 2004). However, here we report the discovery of a previously unsampled free-living species (*P. moniliformis*) that challenges this assumption because its inferred phylogenetic position renders the domesticated G2/G4 fungal cultivars paraphyletic (Fig. 1). The pattern of relationships within the *Apterostigma pilosum* group cultivars we present here suggests three hypotheses: (1) there have been multiple, independent domestications of coral mushrooms, (2) there has been reversion to a free-living lifestyle since the original domestication event, or (3) the ancestral cultivar was a mixture of two lineages of free-living Pterulaceae that have now sorted among the different species of *Apterostigma* ants. These three hypotheses are discussed below.

MULTIPLE DOMESTICATIONS
The paraphyletic pattern of ant–cultivated fungi we show in Figure 1 could be explained by two domestication events by two different lineages of ants. This hypothesis implies that attine ants occasionally reacquire their fungal cultivars from free-living fungi. In fact, the most ancient lineage of *A. auriculatum*, which is assumed to have cultivated the ancestral ("primitive") attine cultivar since its origin (Schultz and Brady 2008), recently domesticated a G3 fungal cultivar from an unidentified free-living lepiotaceous mushroom, with which it shares an identical internal transcribed spacer sequence (Mueller et al. 1998). If this kind of foraging behavior is symplesiomorphic in *Apterostigma*, then new horizontal acquisition of fungal symbionts may not be uncommon and the acquisition of independent lineages of a free-living *Pterula* spp. by the G2- and G4-cultivating ants could be explained as an accidental consequence of their occasional interaction with free-living fungi. Alternatively, multiple domestications might indicate that there was selective pressure to seek out new cultivars, in response to parasite pressure from specialized pathogens (Currie et al. 2003) or inadequate cultivar performance. In either case, it is worth noting that if there were multiple domestications of free-living fungi, the ants domesticated two closely related species. This could have happened by chance, or it could indicate that the ants were incapable of distinguishing between two closely related species of *Pterula*.

ESCAPE FROM DOMESTICATION
A second hypothesis that could explain the paraphyletic pattern of ant-cultivated fungi shown in Figure 1 is that there was a single domestication event followed by divergence of the ancestral cultivar into two extant clades. This is the hypothesis supported by previous studies (Munkacsi et al. 2004; Villesen et al. 2004). However, if this hypothesis is correct then, given our new results, *P. moniliformis* represents a lineage that escaped from cultivation and now exists only as a free-living form. This may also be true of *Pterula* sp. BZ3484, although branch support is weak (Fig. 1). Escape from domestication is a possible strategy for the fungal cultivars to alleviate conflicts of interest that may arise in the fungus-farming ant symbiosis (Mueller 2002). Some evidence that such conflicts of interest may have shaped the symbiosis between ants and fungi comes from the discovery that horizontal transfer of fungal cultivars and maintenance of gene flow with free-living fungi through sexual reproduction occurs frequently in Agaricaceae-cultivating (G1 and G3) attines (Green et al. 2002; Mikheyev et al. 2006). This occurs despite the fact that the highly regulated architecture of attine nests is thought to be an adaptation to impede cultivar escape by intentionally limiting the contact between the garden and surrounding substrate (Mueller 2002). The only potential documentation of vegetative escape comes from a single G2-cultivating *Apterostigma* nest, where the hyphal veil

that covered the garden was observed to extend outwards into the surrounding leaf litter (Mueller 2002). Our data suggest that *P. moniliformis* may be a representative of a lineage of domesticated fungi that escaped from cultivation early on, perhaps after suffering genetic degradation under clonal propagation that reduced its ability to produce fertile fruiting structures. The contemporary individuals of *P. moniliformis* that produce sterile fruiting structures may in fact be suffering from a genetic hangover of ancestral domestication that resulted in loss-of-function mutations in fruiting genes in the absence of recombination (e.g., Normark et al. 2003).

It is noteworthy that, to date, no sexual structures have been documented from the Pterulaceae-cultivating *Apterostigma* nests, although a single 1 mm sterile hook-like structure found in one nest (UGM011206_01) is reminiscent of an immature coral mushroom and is phylogenetically grouped in the Pterulaceae sister to the G2 fungi (Fig. 1; Munkacsi et al. 2004). The lack of reproductive structures from G2 and G4 nests may indicate that these cultivars are not capable of fruiting or that the ants actively suppress mushroom production in the garden. Active suppression of fruiting body formation is known to occur in other attines (Mueller 2002), but has not been documented in the G2 and G4 nests.

LINEAGE SORTING OF ANCESTRAL POLYCULTURE
A third hypothesis that would explain the paraphyly between free-living and domesticated fungi (see Fig. 1) is a single domestication event of two (or more) species of free-living *Pterula* by an ancestral *Apterostigma* ant (perhaps due to misidentification, see above), resulting in a mixed-strain garden, followed by loss of one of the strains in each of the two ant lineages ("lineage sorting"). Multiple domestications of many different fungi and a loss of some could also lead to this pattern. Mixed-strain fungal gardens had never been reported in attine nests until a recent account (Sanhudo et al. 2008) that indicates this is a realistic scenario. Our results are consistent with this scenario if the fungal strains sorted among the ant lineages over time, either by a neutral process or because of conflict between the two cultivar lineages, making mixed-strain gardens unsustainable.

PHYLOGENETIC UNCERTAINTY
Our results show that the G2 and G4 cultivars do not form a monophyletic group according to analyses using both nonparametric bootstrapping and Bayesian posterior probabilities. Nonetheless, a degree of caution in interpreting these data is warranted considering that we only have data from a single gene and our parametric bootstrap results were inconclusive. Therefore, it is possible that the paraphyletic pattern we observed is an artifact of phylogenetic error, which can only be addressed with more data. Similarly, Villesen et al. (2004) were also unable to reject the alternative hypothesis that the G2- and G4-cultivating ants formed a mono-

phyletic group based on their single gene dataset. Both of these reports illustrate the need for more extensive sampling of genes and taxa for phylogenetic studies in the *Apterostigma* ant–fungus mutualism.

ASSUMPTIONS IN THE FUNGUS-FARMING ANT MUTUALISM
The three scenarios above, all equally plausible given our results, illustrate how our present knowledge of coevolution between fungus-farming ants and their cultivars still relies on a set of assumptions, phylogenetic and otherwise, that are largely unsubstantiated. For example, several of the prevailing hypotheses explaining the adoption of a new cultivar by one lineage of *Apterostigma* ants are based on the assumption that the ancestor(s) of the cultivars was wood-dwelling. Consequently, the ants and their nests, which are often in logs or associated with other woody materials, may have been in frequent, direct contact with the ancestral *Pterula* sp. (Mueller 2002; Villesen et al. 2004; Munkacsi et al. 2004; Schultz 2007; Schultz and Brady 2008). However, these are at best speculative hypotheses, for it is clear that not all Pterulaceae are wood-dwelling. In addition to at least one pathogen and one endophyte (Munkacsi et al. 2004; Harsh et al. 2005), many species of Pterulaceae are found in leaf litter. In fact, the closest relatives of the fungal cultivars (e.g., *P. moniliformis*, *P. verticillata*, *P. vinacea*, and *Pterula* sp. BZ3484) produce mushrooms that are terrestrial and emerge from soil rather than wood or detritus. Similarly, although phylogenies of *Apterostigma* consistently show the "basal" G3-cultivating lineages sister to the Pterulaceae-cultivating lineages, and thus the most parsimonious interpretation is that the ancestral cultivar must have been a G3 fungus, this interpretation assumes that all cultivar transitions are correlated with cladogenetic events. Alternatively, the long branch leading to *A. auriculatum* in phylogenies (Villesen et al. 2004; Schultz and Brady 2008) indicates a substantial amount of time susceptible to anagenetic evolution. Thus, "basal" does not necessarily mean "ancestral" and the interpretation that the ancestral cultivar in *Apterostigma* was a G3 fungus might be premature.

CONCLUSION
Our knowledge of the evolutionary history of the free-living relatives of ant cultivars is so incomplete that our phylogenetic placement of a single free-living *Pterula* species requires the reinterpretation of the fungus-farming ant mutualism in this group of ants from an assumption of strict coevolution to one of loose and independent associations. Consequently, a premium must be placed on exhaustively sampled, multigene phylogenies for both ants and fungi in future studies and this will require a concerted effort to collect new samples and to conduct rigorous systematics research. Unless a greater amount of scrutiny is applied to

the phylogenetic histories of coevolved symbioses such as the fungus-farming ant mutualism, our understanding of the patterns and processes of coevolution will remain incomplete.

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Supporting Information

The following supporting information is available for this article:

Appendix S1.   Additional details on materials and methods used in this study.

Table S1.   Taxon and voucher information for specimens used for DNA sequences generated in this study.

Supporting Information may be found in the online version of this article.

(This link will take you to the article abstract).

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