

Comparative Dating of Attine Ant and Lepiotaceous Cultivar Phylogenies Reveals Coevolutionary Synchrony and Discord

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ABSTRACT: The mutualistic symbiosis between fungus-gardening ants and their cultivars has made fundamental contributions to our understanding of the coevolution of complex species interactions. Reciprocal specialization and vertical symbiont cotransmission are thought to promote a pattern of largely synchronous coevolutionary diversification in attines. Here we test this hypothesis by inferring the first time-calibrated multigene phylogeny of the lepiotaceous attine cultivars and comparing it with the recently published fossil-anchored phylogeny of the attine ants. While this comparison reveals some possible cases of synchronous origins of ant and fungal clades, there were a number of surprising asynchronies. For example, leaf-cutter cultivars appear to be significantly younger than the corresponding ant genera. Similarly, a clade of fungi interacting with primitive fungus-gardening ants—thought to be ancestral to the more derived leaf-cutter symbionts—appears instead to be a more recent acquisition from free-living stock. These macroevolutionary patterns are consistent with recent population-level studies suggesting occasional acquisition of novel cultivar types from environmental sources and horizontal transmission of cultivars between different ant species. Horizontal transmission events, even if rare, appear to form loose ecological connections between diffusely coevolving ant and fungus lineages that permit punctuated changes in the topology of the mutualistic ant-fungus interaction network.

Keywords: *Acromyrmex*, *Atta*, Attini, coevolution, Lepiotaceae, *Leucoagaricus*.

Introduction

Predicting long-term evolutionary processes from present-day ecological interactions remains a key challenge for the study of mutualistic symbioses. So far, analysis of mutualistic coevolution has been easier for physiologically linked coevolving taxa, such as endosymbionts and their

hosts (Clark et al. 2000; Lo et al. 2003; Noda et al. 2007). Extremely specialized interactions often lead to metabolic codependency, vertical transmission, and the loss of traits that would otherwise facilitate horizontal transfer and host shifts (Clark et al. 2000; Moran et al. 2008). Perhaps not surprisingly, given this degree of ecological integration, such systems often exhibit phylogenetic congruence, suggesting codivergence between symbionts (Becerra 2003; Page 2003; Schardl et al. 2008). However, most mutualistic symbioses involve complex patterns of interactions among multiple species pools rather than pairwise specialization (Borowicz and Juliano 1991; Bruns et al. 2002; Clay and Schardl 2002; Thompson 2005; Ollerton 2006; Herre et al. 2008). Less intimate symbioses characterized by host sharing or symbiont switching frequently produce intricate patterns of complete or partial discord between symbiont phylogenies (e.g., termites and their fungal cultivars [Aanen et al. 2002], figs and fig wasps [Machado et al. 2005; Herre et al. 2008; Jackson et al. 2008], yucca moths and yucca [Smith et al. 2008], squid and their bioluminescent symbionts [Dunlap et al. 2007], lichen symbioses [Piercey-Normore and DePriest 2001; Rikkinen et al. 2002], and mycorrhizal symbioses [Bidartondo 2005; Shefferson et al. 2007]). A major goal of current efforts is to understand the coevolutionary dynamics of complex mutualistic interactions (Ollerton 2006; Guimarães et al. 2007; Jousset et al. 2008).

Here we compared the patterns of diversification between lepiotaceous fungi and attine ants. The attines are unique among ants in that they obligately depend on a nutritional symbiosis with basidiomycete fungi, which they cultivate as “gardens” inside their nests. The extent of this integration has been the focus of a series of phylogenetic and population-genetic studies spanning more than a decade (Chapela et al. 1994; Hinkle et al. 1994; Schultz and Meier 1995; Mueller et al. 1998; Wetterer et al. 1998; Silva-Pinhati et al. 2004; Mikheyev et al. 2006, 2007, 2008; Schultz and Brady 2008; Vo et al. 2009). Both ants and cultivars express specialized behavioral and morphological

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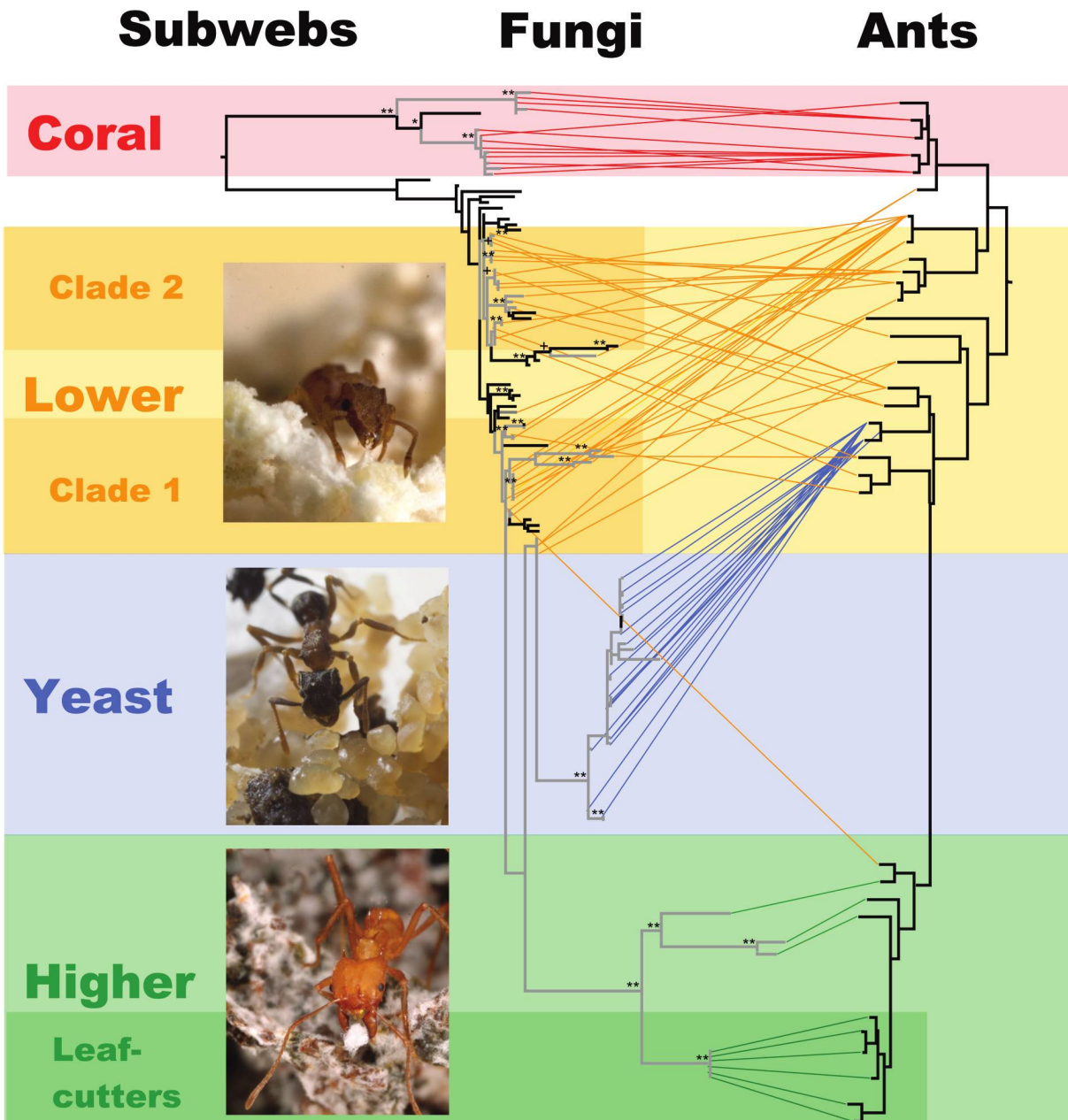


Figure 1: Phylogenies and interaction network structure in the attine ant–cultivar symbiosis. During the 50-million-year history of the symbiosis, there have been repeated domestications of cultivars (*gray lines*), from a pool of free-living fungi (*black lines*), followed by lateral spread within defined ant clades. The most basal attine genera have small cryptic colonies and collect primarily plant detritus or insect frass to nourish their gardens. By contrast, the more derived attine lineages, such as the leaf-cutting ants, have become specialized herbivores, whose large, long-lived colonies may remove as much vegetation as domestic cattle, even competitively displacing these large mammals from grassland (Robinson and Fowler 1982; Fowler and Saes 1986). The network of interactions is compartmentalized into several largely independent subwebs. The lower-attine subweb consists of at least two independently acquired cultivar clades (clades 1 and 2; Mueller et al. 1998). Patterns of host use vary among lower-attine ant species, ranging from specialization to generality (Mueller et al. 1998; Mehdiabadi et al. 2006). The lower-attine cultivars are closely linked to free-living populations either because of repeated domestication or through frequent escape from the symbiosis; lower-attine cultivars have no known modifications for ant cultivation (Mueller et al. 1998; Vo et al. 2009). (Photo depicts *Cyphomyrmex wheeleri* on its garden.) Cultivars in the yeast subweb are grown in single-celled (yeast) form by the ants, unlike the rest of the cultivars, which grow as filamentous mycelia. Despite this

adaptations for symbiosis. For example, ant workers maintain garden health by actively “weeding” out disease fungi, and new queens initiate nests with fungal inocula from parental nests (Currie and Stuart 2001; Mueller et al. 2005; Currie et al. 2006). Fungi are specialized as well. The higher-attine fungi possess, among other metabolic adaptations, unique structures for nutrient provisioning (Weber 1972).

Various studies have suggested broad congruence between ant and fungal phylogenies (Chapela et al. 1994; Mueller et al. 1998; Currie et al. 2003; Schultz and Brady 2008; Caldera et al. 2009). This broad-scale correspondence, resulting from obligate dependence by the ants on the fungi for food and the cotransmission of the symbionts, have led to the general hypothesis of synchronous diversification between ants and fungi (Chapela et al. 1994; Schultz and Brady 2008). However, such diversification scenarios for the attines stand in contrast to a number of studies that indicate gene flow between cultivated and free-living fungal populations, as well as horizontal transfer of cultivars between ant lineages (fig. 1). For example, in the basal ant lineages, different ant species can acquire cultivars from each other or from free-living populations of lepiotaceous fungi (Mueller et al. 1998; Green et al. 2002; Vo et al. 2009). Dispersal among the fungi seems high, as they exhibit little biogeographic structure (Mueller et al. 1998; Mikheyev et al. 2006). In the leaf-cutting ant symbiosis, a single species of highly derived fungal cultivars is shared among divergent ant lineages (Mikheyev et al. 2006, 2007). There are several ways to reconcile the various studies on attine ant–fungus diversification. For example, evidence of specificity in attine-fungal interactions may not derive from a long history of association but rather result when one guild radiates to fill a niche provided by an already existing pool of mutualists (Hafner and Nadler 1988; Page 2003; De Vienne et al. 2007). Uncertainty about large-scale patterns of phylogenetic divergence in the attine mutualism derives from the absence of a robust, time-calibrated fungal phylogeny that can be compared to an

existing phylogeny of attine ants (Schultz and Brady 2008). Here we examine the history of the attine symbiosis by comparing calibrated phylogenies of the ants and corresponding lepiotaceous cultivars.

Material and Methods

Molecular Methods

For the dating analysis, we selected multiple fungi from every subweb in figure 1, as well as representative free-living relatives. Both *bipA* and *ef1-alpha* genes were cloned following polymerase chain reaction (PCR) amplification with degenerate primers designed from alignments of GenBank gene sequences for various basidiomycete fungi. RAD51 primers are described by Mikheyev et al. (2006); table 1 has primer sequences used for the other nuclear genes; ribosomal genes were amplified using primers from White et al. (1990). PCR amplification of *bipA*, *ef1-alpha*, RAD51, and rDNA ITS gene regions was performed on genomic DNA extractions from axenic cultures of attine fungal cultivars, with mycological curation, DNA extraction, and PCR methods as described by Mikheyev et al. (2006). Cloning was performed via a pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Positive clones were sequenced at the University of Texas sequencing facility with Invitrogen vector primers *T7* or *M13*. Sequence identity was confirmed by comparison to homologous GenBank sequences using BLASTn. Alignments were constructed using ClustalX (ver. 1.83, for Mac) and then manually corrected as necessary in MacClade (ver. 4.08). DNA sequences used in this study have been deposited in GenBank under GI numbers 195969607, 110349632, 110349568, and 110349666 and accession numbers GU202421–GU202430.

derived single-celled growth form, yeast-cultivars are nonetheless capable of a free-living filamentous existence (Mueller et al. 1998). (Photo depicts *Cyphomyrmex rimosus* attending yellowish nodules of yeast garden.) Cultivars in the higher-attine subweb appear to lack a free-living existence. Grapelike clusters of swollen cells (gongylidia) produced by higher-attine fungi act as the primary food source of the ant larvae (Quinlan and Cherrett 1979). The leaf-cutting ants form a monophyletic group in the higher attines and primarily associate with a single species of cultivar fungus (Mikheyev et al. 2006). (Photo depicts a minor worker of *Atta cephalotes* harvesting gongylidia.) In addition to the major lepiotaceous fungi associated with the fungus-gardening ants, distantly related pterulaceous coral fungi have also been domesticated by a lineage of phylogenetically primitive fungus-gardening ants in the genus *Apterostigma* (Munkacsi et al. 2004; Dentinger et al. 2009). Although rare, connections between subwebs do exist, and more will likely be uncovered with increased sampling. The cultivar phylogeny was generated using GARLI (Zwickl 2006) from published data sets of large ribosomal subunit sequences (Chapela et al. 1994; Hinkle et al. 1994; Mueller et al. 1998; Munkacsi et al. 2004; Silva-Pinhati et al. 2004; Dentinger et al. 2009). The figure shown is the best maximum likelihood estimation tree with support values computed from 100 bootstrap pseudoreplicates (*plus sign*, $\geq 80\%$; *one asterisk*, $\geq 90\%$; *two asterisks*, $\geq 95\%$). Named subwebs are strongly supported in previous analyses based on multiple genes (Chapela et al. 1994; Mueller et al. 1998; Mikheyev et al. 2008). The ant tree is from a recent comprehensive phylogeny of the ants by Schultz and Brady (2008). See appendix for taxa labels.

Table 1: Primer sequences used to amplify nuclear genes and taxa that they were used to amplify

Primer	Sequence	Taxa amplified
bip1F	AACGG(G/T)TTCGGTCG(C/T)AT(C/T)GG	All free-living fungi, except PA408; all lower-attine cultivars, except S77
bip1R	GTGACCTC(G/C)AT(C/T)TGAGGAAC	All free-living fungi, except PA408; all lower-attine cultivars, except S77
bip2F	GGTGACACTCA(C/T)CTTGGTGG	<i>Cyphomyrmex costatus</i> cultivar (S77)
bip2R	GCTCGAA(C/T)TTGCCGAG(G/C)AGG	<i>Cyphomyrmex costatus</i> cultivar (S77)
bip3F	GATGT(C/T)AGCAAGAACCT(C/T)CG	All higher-attine fungi and PA408
bip4R	TTGTCCTTGGTGAG(G/C)G(G/C)ACG	All higher-attine fungi and PA408
ef1a1F	ACAAGTG(T/C)GGTGGTATCGAC	Free-living fungi, lower-attine fungi, some <i>Trachymyrmex</i> and <i>Sericomyrmex</i> fungi
ef1a1R	G(G/C)GGGGTCGTTCTTGAGTC	Free-living fungi, lower-attine fungi, some <i>Trachymyrmex</i> and <i>Sericomyrmex</i> fungi
ef1a2F	AGCTCGGCAAGGGTTCCTTC	Rest of <i>Trachymyrmex</i> and <i>Sericomyrmex</i> fungi
ef1a2R	CCCTTGTACCARGGCATGC	Rest of <i>Trachymyrmex</i> and <i>Sericomyrmex</i> fungi
ef1a3F	TTCATCAAGAACATGATCAC	Leaf-cutter fungi
ef1a3R	TGCAT(T/C)TC(A/G)ACGGACTTGAC	Leaf-cutter fungi

Phylogenetic Reconstruction

We also reconstructed a cultivar phylogeny using representative sets of taxa from within each of the lepiotaceous subwebs pictured in figure 1, using MRBAYES (Huelsenbeck and Ronquist 2001). The analysis was conducted on a partitioned 3,733-bp four-gene data set with GTR + G model parameters independently estimated for each gene. The Bayesian analysis was run using four chains until the standard deviation of split frequencies dropped below 0.001. Convergence, length of burn-in, and adequate sampling were confirmed using Tracer (ver. 1.4; Rambaut and Drummond 2007).

In order to assess the relative likelihoods of independent transitions by different attine species to clade 2 cultivars (fig. 1) versus a single origin and subsequent sharing, we conducted several Bayesian analyses of the two clades, using sequences and methodology from Vo et al. (2009). One analysis was unconstrained, representing a single origin of the novel agricultural system and sharing between ant taxa, while the other analysis was constrained by ant species, representing multiple independent acquisitions of different cultivar strains by each ant species. The relative likelihoods of the two models were compared using Bayes factors computed in Tracer (Rambaut and Drummond 2007).

Divergence Dating

To determine divergence dates, we used the Bayesian relaxed-clock uncorrelated exponential approach implemented in BEAST, version 1.4.6 (Drummond and Rambaut 2007), using two independent sets of assumptions. First, we used an independent estimate for the age of the *Agaricus* node by Geml et al. (2004). The root was given

a prior with a normal age distribution (mean = 73, SD = 9). In order to confirm the robustness of our results with respect to the choice of prior, we also repeated the above dating analysis with a less informative prior (SD = 15), obtaining the same results. Three Markov chain Monte Carlo searches were run for 50,000,000 generations each, with the first 5,000,000 discarded as burn-in and the results being pooled. The searches achieved adequate mixing as assessed by the high evolutionarily stable strategy values for all parameters, plateaus for divergence time estimates over generations after burn-in, and repeatability of results over multiple independent runs. Additionally, in order to account for sampling bias, we used BEAST to conduct separate coalescent analyses of clade 2 cultivars and the leaf-cutter cultivar, using substitution rate priors inferred from the overall phylogenetic analysis and a Bayesian skyline plot reconstruction to estimate the demographic history of the cultivars. For this analysis, we used ITS and large subunit ribosomal data for 20 clade 2 cultivar species used by Mueller et al. (1998) and EF1a and RAD sequences (exons only) from 190 leaf-cutter cultivar fungi sampled throughout their range from Argentina to the United States (U. G. Mueller et al., unpublished manuscript; accession nos. GQ853919–GQ855186).

Results

Figure 2 shows the attine cultivar phylogeny inferred with the four-gene data set. Consistent with de novo acquisition of free-living fungi, some fungal clades are substantially younger than their ant cultivators (table 2), as first hypothesized by Mueller et al. (1998). For example, clade 2 cultivars (fig. 1), which form part of the lower-attine sym-

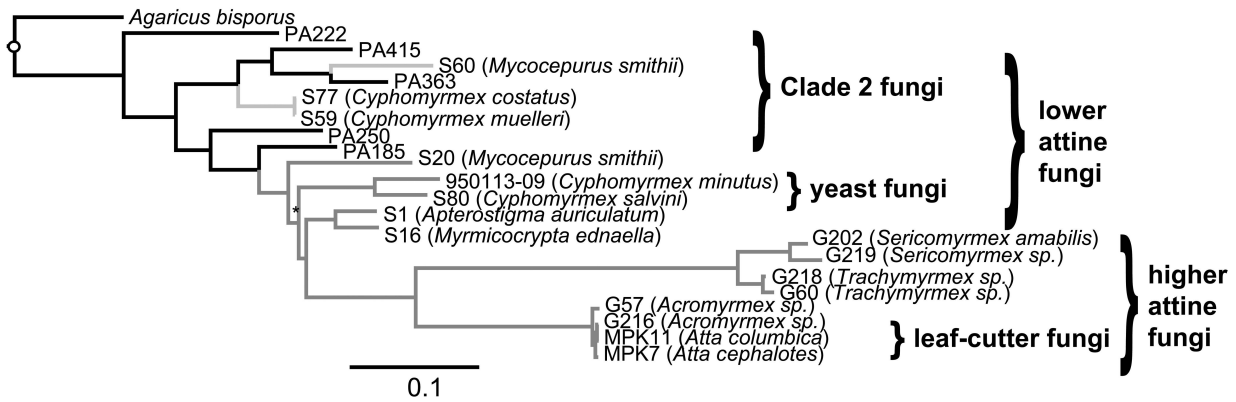


Figure 2: Phylogeny of the cultivars and their free-living relatives. As in figure 1, free-living taxa are shown in black, while cultivars are shown in gray, with hosts ant species listed in parentheses. The phylogeny of the cultivars is well resolved: all nodes have $\geq 99\%$ posterior probability, except one indicated by the asterisk, which has 95%. A circle indicates the calibration point.

biosis, were acquired by lower attines within the past 11 million years (table 2; fig. 3), significantly younger than the common ancestor of the corresponding ant lineages (50 million years). The more recent origin of clade 2 cultivars is also implicated by substantially lower within-clade diversity relative to that of clade 1 (2.7% vs. 9.8% average pairwise sequence divergence, not including the derived higher-attine cultivars). Because independent acquisitions of clade 2 fungi by different ant species can be confidently ruled out (Bayes factor = 388), the incorporation of clade 2 into the lower-attine symbiosis most likely occurred by lateral sharing of novel fungal lineages initially acquired by one or a few ant populations and then was shared across all lower-attine lineages.

Discussion

An apparent topological congruence between ant and fungal lineages led previous studies to conclude that macroevolutionary patterns reflect a history of strict vertical transmission and coevolution between ant and fungal lineages (Chapela et al. 1994). Our analysis revealed more complex coevolutionary dynamics stemming from repeated episodes of de novo domestication of cultivars and horizontal transmission of cultivar lineages. In the lower attines, the clade 2 symbiosis was previously thought to be ancient and possibly basal to the entire symbiosis (Chapela et al. 1994; Hinkle et al. 1994; Schultz and Brady 2008). However, the substantially younger age of clade 2 fungi suggests a recent evolutionary acquisition of the novel fungal group. The broad association of these fungi with phylogenetically primitive attines presumably resulted from lateral dissemination of cultivars across divergent ant lineages. Once enmeshed within a symbiotic network,

these cultivars continued to interact with the lower-attine ants over the course of millions of years while maintaining a capacity for free-living existence (Vo et al. 2009). Thus, lateral symbiont spread has affected the coevolutionary topology of the attine symbiosis.

Another remarkable example of lateral symbiont spread can be seen in the leaf-cutting ants, which acquired a novel cultivar lineage within the past 2–4 million years (fig. 2). This fungal lineage then spread across the much older (8–12-million-year-old) leaf-cutting ant genera, engaging in an asymmetric interaction with two host genera. The relatively recent sweep appears to have eliminated any phylogenetic signal of the ancestral cultivar(s), spreading

Table 2: Crown node dates for the origins of cultivar clades and their interacting ant clades

Clade	Mean	95% HPD	
		Low	High
Cultivar clade 1	34	16	51
Cultivar clade 2	27	6	50
Cultivar clade 2 (coalescent)	11	1	31
Lower-attine ants	52	44	59
Yeast cultivars	13	2	28
Yeast-cultivating ants	8	5	12
Higher-attine cultivars	25	11	39
Higher-attine ants	21	17	25
Leaf-cutter cultivar	2	.2	7
Leaf-cutter cultivar (coalescent)	4	.5	8
Leaf-cutting ants	13	10	16

Note: The dates and highest posterior densities (HPDs) for ant clades are from Schultz and Brady (2008). These dates are represented graphically in figure 3. Ages of ant clades are shown in bold.

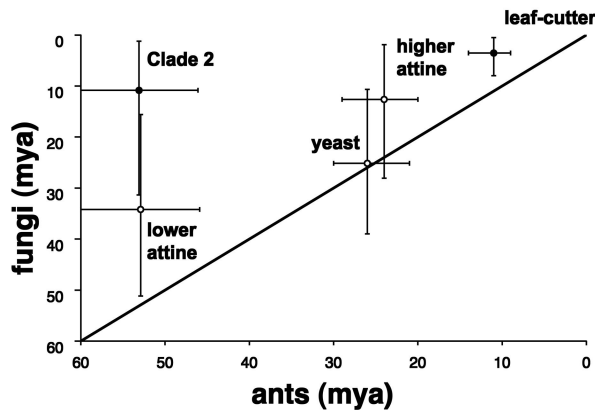


Figure 3: Comparative dating of major evolutionary events in the ant and cultivar phylogenies. Error bars correspond to 95% highest posterior density intervals around the age estimates. Ant and fungal taxa in the lower-attine, yeast, and higher-attine subwebs originated at approximately the same time. The fungi of clade 2 were a more recent addition to the 50-million-year-old lower-attine subweb, having been incorporated within the past 11 million years. There was also a more recent sweep of the leaf-cutting ant cultivar, which coalesces to just 2–4 millions of years ago (mya), much more recent than the ~10-million-year-old origin of leaf-cutting ants. Associations that reject the hypothesis of simultaneous origins are highlighted in black.

across the entire leaf-cutter range (Argentina to United States). The complete replacement of ancestral leaf-cutter cultivars stands in contrast to the acquisition of cultivar clade 2 by the lower fungus-gardening ants, incorporated into the symbiosis “core” without entirely replacing the more ancestral clade 1 cultivars.

These results have a number of implications for our understanding of the evolutionary dynamics of the fungus-growing ant mutualism. Most important, distinct lineages of attine ants and their fungal symbionts are not necessarily independent of one another, since different ant clades can ultimately converge on a single symbiont type via lateral acquisition, potentially erasing the evolutionary history of earlier interactions. Also, such symbiont sweeps, both ancient and ongoing, may produce patterns of apparent phylogenetic correspondence between symbiont lineages, even without a history of long-term pairwise coevolutionary interactions. This is in fact exactly what we see in the genus *Apterostigma*, which has independently acquired at least two lineages of pterulaceous coral fungi unrelated to all other lepiotaceous attine cultivars (Munkacsı et al. 2004; Dentinger et al. 2009). More important, studies comparing the relationships between fungi and other symbionts have generally treated lower-attine agriculture as a single evolutionary unit (e.g., Schultz and Brady 2008). However, unlike those of cultivar clade 1, the association of cultivar clade 2 with its symbionts (including mutualists and pre-

sumably their parasites; Currie et al. 2003) did not arise solely from long-term reciprocal natural selection acting on ants and fungi continuously associated over millennial scales. Rather, the mutualistic association with fungal clade 2 emerged from the independent association with different ant lineages. Consequently, clade 1 and clade 2 symbioses have had vastly different evolutionary histories and should be considered separately in ecological and evolutionary studies of this symbiosis.

Given the dynamics operating in the attine symbiosis between ants and fungi, we expect asynchronous origins of interacting clades to be widespread in other symbioses exhibiting high rates of host switching. For example, the fungus-gardening termites share many evolutionary and natural history similarities with the fungus-gardening ants (e.g., single origin of the symbiosis, a mixture of vertical and horizontal cultivar transmission, etc.) and exhibit a range of specificity and asymmetry in termite/fungus interactions (Aanen et al. 2002, 2007; de Fine Licht et al. 2007). Thus, we predict asynchronous origins of some of the interacting guilds, even in cases where they appear to be tightly linked, as, for example, in the genus *Microtermes*, which interacts with a restricted clade of closely related cultivars (Aanen et al. 2002).

Studies of highly intimate symbioses, such as the intracellular symbiosis between aphids and their primary bacterial symbionts, have shown high levels of specificity and synchronous origins of interacting clades, which are predictable outcomes of the natural history of these symbioses (e.g., the constraints by transovarial inheritance; Moran et al. 2008). However, most coevolutionary interactions occur between diffusely interacting guilds rather than pairs of species. This study illustrates that the coevolutionary dynamics of mutualistic symbioses may combine elements of synchronous diversification with ecological events that confound easy interpretation of coevolutionary processes from phylogenetic patterns. We are just beginning to understand how the present-day topologies of ecological interactions influence resilience to ecological change or affect the future evolution of a symbiosis (Memmott et al. 2004; Bascompte et al. 2006; Bascompte and Jordano 2007; Guimarães et al. 2007; Rezende et al. 2007). Examples, such as that of the attine symbiosis, that show how networks of interactions actually change over long timescales will contribute to our understanding of how stable communities are assembled and, ultimately, how they can be conserved (Montoya et al. 2006).

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