Natural History Note

The Most Relictual Fungus-Farming Ant Species Cultivates the Most Recently Evolved and Highly Domesticated Fungal Symbiont Species

Ted R. Schultz,[1,*] Jeffrey Sosa-Calvo,[1,2,*,†] Seán G. Brady,[1] Cauê T. Lopes,[3] Ulrich G. Mueller,[4] Mauricio Bacci Jr.,[5] and Heraldo L. Vasconcelos[3]

1. Department of Entomology, National Museum of Natural History, Smithsonian Institution, Washington, DC 20560; 2. Maryland Center for Systematic Entomology, Department of Entomology, University of Maryland, College Park, Maryland 20742; 3. Instituto de Biologia, Universidade Federal de Uberlândia, 38405-320 Uberlândia, Minas Gerais, Brazil; 4. Department of Integrative Biology, University of Texas at Austin, Austin, Texas 78712; 5. Centro de Estudos de Insetos Sociais, Universidade Estadual Paulista, 13506-900 Rio Claro, São Paulo, Brazil

Submitted September 5, 2014; Accepted December 23, 2014; Electronically published March 16, 2015

abstract: Fungus-farming (attine) ant agriculture is made up of five known agricultural systems characterized by remarkable symbiont fidelity in which five phylogenetic groups of ants faithfully cultivate five phylogenetic groups of fungi. Here we describe the first case of a lower-attine ant cultivating a higher-attine fungus based on our discovery of a Brazilian population of the relictual fungus-farming ant *Apterostigma megacephala*, known previously from four stray specimens from Peru and Colombia. We find that *A. megacephala* is the sole surviving representative of an ancient lineage that diverged ~39 million years ago, very early in the ~55-million-year evolution of fungus-farming ants. Contrary to all previously known patterns of ant-fungus symbiont fidelity, *A. megacephala* cultivates *Leucoagaricus gongylophorus*, a highly domesticated fungal cultivar that originated only 2–8 million years ago in the gardens of the highly derived and recently evolved (~12 million years ago) leaf-cutting ants. Because no other lower fungus-farming ant is known to cultivate any of the higher-attine fungi, let alone the leaf-cutter fungus, *A. megacephala* may provide important clues about the biological mechanisms constraining the otherwise seemingly obligate ant-fungus associations that characterize attine ant agriculture.

Keywords: Attini, coevolution, fungus-farming ants, Leucocoprineae, symbiosis.

Introduction and Background

Fungus-farming (attine) ants are a clade of more than 240 described New World species that cultivate fungus gardens on which they obligately depend for food (Weber 1972; Mehdiabadi and Schultz 2009). The first fungus-farming ant evolved in South America from a hunter-gatherer ant ancestor ~50–56 million years ago (Mueller et al. 2001; Schultz and Brady 2008; Ward et al. 2015; fig. 1). This ancestral attine cultivated fungi in the tribe Leucocoprineae (Basidiomycotina: Agaricaceae), the parasol mushrooms, which are still grown today by more than 100 lower-attine ant species in 11 genera (fig. 1, yellow boxes). Such lower-attine fungal cultivars are facultative symbionts that can also be found living freely apart from their ant hosts (Mueller et al. 1998; Vo et al. 2009). However, ~22 million years ago, the association between a particular lower-attine ant and its leucocoprineaceous fungal cultivar underwent a fundamental change, giving rise to higher-attine agriculture (fig. 1, blue boxes). Unlike lower-attine fungi, the multiple extant species of higher-attine fungi are obligate symbionts, unable to live apart from their ant hosts, with significant coevolved modifications such as the increased expression of protein-digesting and detoxifying enzymes and the consistent production of large, nutritious food bodies (gongylidia) that are preferentially harvested and eaten by the ants for food (Möller 1893; Wheeler 1907; Quinlan and Cherrett 1979; de Fine Licht et al. 2010, 2013). Also, for reasons that are poorly understood, all higher-attine fungi—unlike any known lower-attine fungi—are polyploid (Scott et al. 2009; Kooij 2013).

More recently, 2–8 million years ago (Mikheyev et al. 2010), a higher-attine cultivar species gave rise to a new, even more specialized fungal species, *Leucoagaricus gongylophorus*, presumably as the result of prolonged domestication by and/or coevolution with a leaf-cutting ant species. Relative to other higher-attine cultivars, the gongylidia of *L. gongylophorus* contain increased levels of starch-digesting and phenolic-compound-neutralizing enzymes (de Fine Licht et al. 2010, 2013). Today, *L. gongylophorus* is

* Corresponding authors; e-mail: schultzt@si.edu, sossajef@si.edu.
† Present address: Department of Biology, University of Rochester, Rochester, New York 14627.

Am. Nat. 2015. Vol. 185, pp. 693–703.
0003-0147/2015/18505-55746$15.00.
DOI: 10.1086/680501

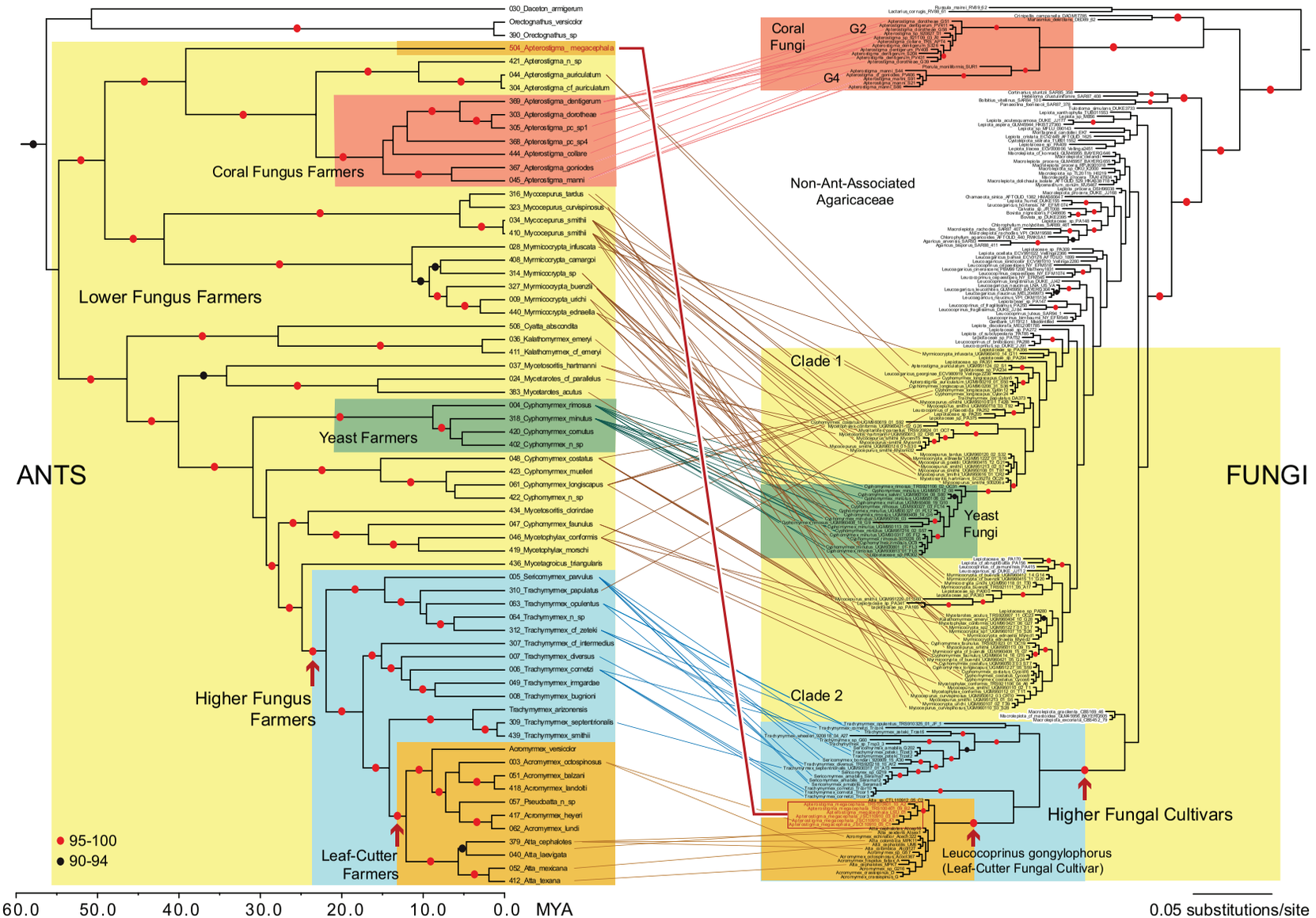

Figure 1: Time-dated chronogram of fungus-farming ants (*left*) and phylogram of ant-associated and free-living fungi (*right*) indicating known ant-fungal associations (connecting lines). The major agricultural systems (colored boxes) are characterized by the nearly monolithic fidelity of their component ants and fungi; that is, within the ancestral "lower" agricultural system, a given lower-attine ant species (*left*, yellow box) may cultivate more than one lower-attine fungal species (*right*, yellow box) but does not cultivate fungi from the other systems. The first exception, reported here, is indicated by the thick red line, in which multiple nests of the recently rediscovered ant species *Apterostigma megacephala* consistently cultivate the most highly derived of all the attine fungi, *Leucoagaricus gongylophorus*. The ant chronogram results from a Bayesian analysis of DNA sequences of four nuclear genes in the program BEAST, version 2.1.2 (Bouckaert et al. 2014), and the fungal phylogram results from a Bayesian analysis of large subunit ribosomal DNA in MrBayes, version 3.2.2 (Ronquist et al. 2013). The identity of the *A. megacephala* cultivar was corroborated by a fungal internal transcribed spacer phylogeny (not shown). Agricultural systems were mapped using maximum likelihood ancestral state reconstruction in Mesquite, version 2.75 (Maddison and Maddison 2011). Red and black circles indicate Bayesian posterior probabilities of 95–100 and 90–94, respectively.

cultivated by most (but not all) leaf-cutting ant species (Silva-Pinhati et al. 2004; Mikheyev et al. 2006, 2010, 2011; U. G. Mueller, unpublished data). Leaf-cutting ants in the genera *Atta* and *Acromyrmex*, which originated ~12 million years ago (fig. 1, orange boxes), have become the major herbivores of the New World tropics. Unlike other fungus-farming ants that cultivate their gardens on organic detritus, leaf cutters have acquired the ability to cut and process live vegetation (leaves, flowers, grasses) to serve as the nutritional substrate for their fungal cultivars. This key evolutionary innovation makes a mature *Atta* colony the ecological equivalent of a large herbivorous mammal in terms of collective biomass, amount of fresh vegetation consumed daily, and life span (10–15 years or more; Fowler et al. 1986; Wirth et al. 2003; Costa et al. 2008; Meyer et al. 2009; Hölldobler and Wilson 2010).

The five attine agricultural systems indicated in figure 1 are characterized by a pattern of symbiont fidelity in which broad phylogenetic groups (paraphyletic or monophyletic) of ants cultivate broad phylogenetic groups of fungi (Chapela et al. 1994; Schultz and Brady 2008). Fungal cultivars are transferred across generations when daughter queens carry within their mouths pellets of fungal cultivar from their maternal nests, producing transgenerational ant-fungus associations with shared reproductive fates. This intergenerational transfer of cultivar clones cannot alone account for the consistent associations that define the agricultural systems, however, because, over evolutionary time spans, reassociations of ant and fungal species within each agricultural system have commonly occurred (Mueller et al. 1998; Mehdiabadi et al. 2012), whereas associations across systems, so far as we know, are restricted to a relatively small number of cases involving higher-attine ants. For example, based on ~700 higher-attine colonies collected throughout the Neotropics and in North America, although most leaf-cutter colonies (fig. 1, orange boxes) cultivate the recently evolved *L. gongylophorus*, a small fraction (<5%) were found cultivating species of more generalized higher-attine fungi commonly associated with the non-leaf-cutting higher-attine ant genera *Trachymyrmex* and *Sericomyrmex* (fig. 1, blue boxes; Silva-Pinhati et al. 2004; Mikheyev et al. 2006, 2010; Mueller et al. 2011; U. G. Mueller, unpublished data). A similarly small fraction of *Trachymyrmex* colonies were found cultivating *L. gongylophorus*, whereas another similarly small fraction of *Trachymyrmex* colonies were found cultivating fungi typically associated with lower-attine ants (U. G. Mueller, unpublished data). An example of the latter is a colony of the higher-attine ant, *Trachymyrmex papulatus*, found with a lower-attine fungus in Argentina (Mueller et al. 1998); a second nest of the same species at the same location was found associated with a typical higher-attine cultivar (fig. 1). In contrast, in more than 1,700 collections of lower-attine colonies from throughout the Neotropics and North America, including >800 *Apterostigma* colonies, no instance of a lower-attine ant species cultivating a higher-attine fungus has so far been observed (Mueller et al. 1998; Gerardo 2004; Villesen et al. 2004; Gerardo et al. 2006; Gerardo and Caldera 2007; Himler 2007; Vo et al. 2009; Mehdiabadi et al. 2012; N. M. Gerardo, personal communication; A. G. Himler, personal communication; U. G. Mueller, T. R. Schultz, J. Sosa-Calvo, unpublished data).

Non-leaf-cutting higher Attini (fig. 1, blue boxes) can be induced to accept the leaf-cutter fungus *L. gongylophorus* (fig. 1, *right*, orange box) under laboratory conditions (Weber 1956; Stradling and Powell 1986; Seal and Tschinkel 2007; Seal et al. 2012; Seal and Mueller 2014). In a recent experiment, colonies of the non-leaf-cutting higher-attine *Trachymyrmex septentrionalis* that were forced to accept *L. gongylophorus* suffered no apparent ill effects for short periods of time (i.e., 6 weeks); over longer periods of time, however, they suffered catastrophic declines in which garden volumes diminished rapidly, workers became inactive, and the majority of nests died (Seal and Mueller 2014). The biological constraints underlying the incompatibility of ants and fungi across agricultural systems remain unknown but may be related to the specialization of ants to control garden diseases specific to their cultivar groups and to within-group interdependencies between ant and fungal microbiomes (Seal and Mueller 2014). Recent evidence from ant genomics suggests that physiological interdependencies may also play a role. For example, some fungus-growing ants appear to have lost the genes required for synthesizing the amino acid arginine, possibly because that role has been assumed by their cultivated fungi (Nygaard et al. 2011; Suen et al. 2011).

Methods

Fieldwork

Until very recently, the fungus-growing ant species *Apterostigma megacephala* was known from only four specimens collected in 1988, 1990, and 1992 on the western peripheries of the Amazon basin in Madre de Dios, Peru, and Serrania de La Macarena, Colombia (fig. 2; Lattke 1999). Morphological study of those specimens indicated that *A. megacephala* is the sole remnant of an ancient attine lineage, a relict species that retains a combination of morphological traits more primitive than those found in *Apterostigma* species fossilized in Dominican amber (15–20 million years old; Lattke 1999; Schultz 2007). Based on its hypothesized phylogenetic position, *A. megacephala* was assumed to cultivate a lower-attine fungus (Schultz 2007). Following two failed attempts in 2004–2005 to locate colonies of *A. megacephala* in Peru (the Colombian locality was, unfortunately, inaccessible to research), a single specimen

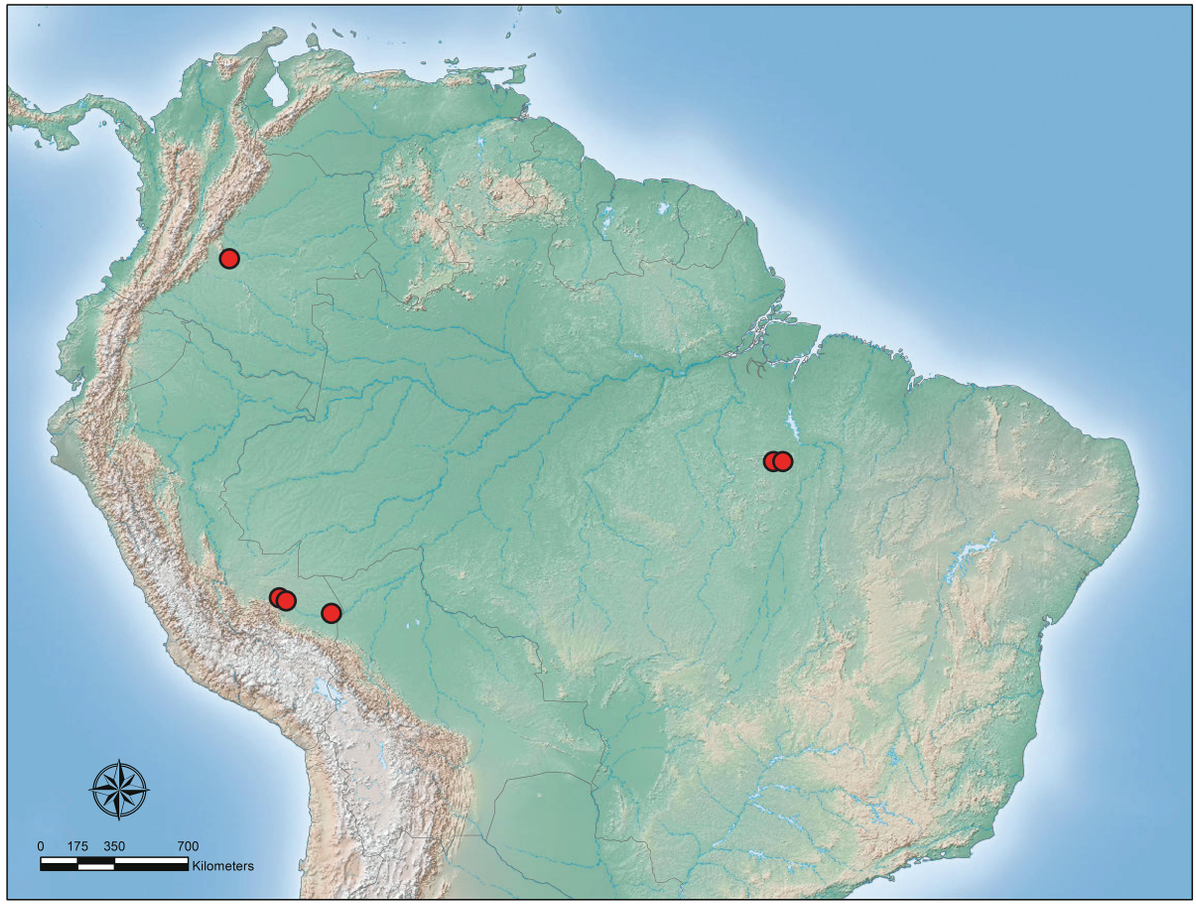

Figure 2: Known distribution of *Apterostigma megacephala* in (circles, counterclockwise from top left) Serrania de La Macarena, Colombia; Madre de Dios, Peru; and Floresta Nacional de Carajás, Pará, Brazil.

of *A. megacephala* turned up in a leaf-litter sample taken at a locality at the opposite end of the Amazon basin, in the Carajás National Forest in Pará, Brazil, ~800 km south of Belém (R. R. da Silva and C. R. F Brandão, personal communication). This led to the discovery of a population of *A. megacephala* by J. Sosa-Calvo and C. T. Lopes in Carajás in 2009. Subsequent field investigations at this location in 2010 and 2011 resulted in the study of 15 living colonies and genetic samples from ants and fungi from 10 colonies (table 1; fig. 3).

Data Generation and Analyses

Fungi. DNA was extracted by placing a small piece of alcohol-preserved tissue, dried and gently squeezed for ~1 min, in 200 μL of 10% Chelex 100 Resin (Bio-Rad, Hercules, CA) solution, incubated in a programmable thermal cycler for 1.5 h at 60°C, and then heated for 15 min at 99°C. The program was paused at 1 h, and samples were mixed by vortexing for 30 s. Samples were then centrifuged at 13,000 rpm for 2 min. To prevent polymerase chain reaction inhibition from the Chelex beads, the supernatant was transferred to new vials and stored at −20°C until needed. Tweezers were flame sterilized between samples to prevent cross-contamination. Amplification and sequencing of the internal transcribed spacer (ITS) and the nuclear large subunit (LSU) ribosomal DNA (rDNA) regions followed the methods of Mueller et al. (1998).

The nuclear LSU rDNA data consist of 219 taxa and 988 aligned nucleotide sites, including indels. Six new sequences were generated for this study (GenBank accession numbers KP406339–KP406344). The ITS data consist of 441 taxa and 1,246 aligned nucleotide sites, including indels. Due to problems associated with polyploidy, only one reasonably clean ITS sequence was generated for this study (GenBank accession number KP406338). The choice of nucleotide substitution model for each region, modeled as a single partition, was determined using the Akaike information criterion (AIC; Posada and Buckley 2004) as implemented in jModelTest, version 2.1.1 (Posada 2008), re-

Table 1: Demographics for 15 colonies of *Apterostigma megacephala* collected in Carajás National Forest, Pará, Brazil, in 2010 and 2011 by T. R. Schultz (TRS), J. Sosa-Calvo (JSC), and C. T. Lopes (CTL)

Nest	Collection code	Collection date	Demography
1	TRS100401-09	April 1, 2010	3L, 29w, 2aq, 1dq
2	TRS100401-10	April 1, 2010	10e, 8L, 31p, 71w, 2aq, 3m, 1dq
3	TRS100402-02	April 2, 2010	11e, 10L, 10p, 50w, 3aq, 4m, 0dq
4	TRS100402-03	April 2, 2010	35e, 41L, 25p, 68w, 7aq, 14m, 0dq
5	JSC100402-11	April 2, 2010	4e, 2L, 1p, 16w, 0dq
6	JSC100402-12	April 2, 2010	19e, 4L, 39w, 4aq, 8m, 1dq
7	JSC110910-01	September 10, 2011	ww, 1dq
8*	JSC110910-03	September 10, 2011	1aq, 2m, 1dq
9	JSC110910-04	September 10, 2011	ww, 1dq
10	JSC110910-05	September 10, 2011	ww, 1dq
11*	TRS110911-15	September 11, 2011	3aq, 4m, 1dq
12	JSC110911-16	September 11, 2011	ww, 1m, 1dq
13	CTL110911-01	September 11, 2011	ww, 0dq
14*	JSC110912-01	September 12, 2011	5aq, 1dq
15	JSC110912-03	September 12, 2011	4m, 1dq

Note: Abbreviations are as follows: e = eggs; L = larvae; p = pupae; w = workers; ww = multiple workers present but not counted; aq = alate queen; m = male; dq = dealate queen. An asterisk indicates colonies in live culture at the time of this writing. 0dq indicates that a queen was not collected but is presumed to have been present.

sulting in the selection of the TIM2+I+G model for LSU and the GTR+I+G model for ITS.

Bayesian Analyses. Using these models, we conducted Bayesian and maximum likelihood (ML) analyses for LSU and Bayesian analyses for only ITS. Bayesian analyses employed MrBayes, version 3.2.2 (Ronquist et al. 2012), with nucmodel = 4by4, nruns = 2, nchains = 8, and samplefreq = 1,000. The LSU analysis consisted of 10 million generations with a burn-in of 2 million generations; the ITS analysis consisted of 20 million generations with a burn-in of 10 million generations. To address known problems with branch-length estimation in MrBayes (Marshall et al. 2006; Spinks and Shaffer 2009; Brown et al. 2010; Marshall 2010; Ward et al. 2010), we set brlenspr = unconstrained:Exp (100). Burn-in, convergence, and stationarity were assessed using Tracer, version 1.5 (Rambaut and Drummond 2009), by examining potential scale reduction factor values and .stat output files in MrBayes and by using Bayes factor comparisons of harmonic-mean marginal likelihoods of pairs of runs with standard error estimated using 1,000 bootstrap pseudoreplicates in Tracer 1.5 (Rambaut and Drummond 2009), which employs the weighted likelihood bootstrap estimator of Newton and Raftery (1994) as modified by Suchard et al. (2001).

Maximum Likelihood Analyses. Maximum likelihood analyses of the LSU data were carried out with the message passing interface parallel version of GARLI, version 2.0.1019 (Zwickl 2006), on the Smithsonian Hydra supercomputer (Linux-based with AMD processors). Maximum likelihood best-tree analyses consisted of 100 replicate searches and used the default parameter settings. Maximum likelihood bootstrap analyses consisted of 1,000 pseudoreplicates and deviated from default settings as follows: startoptprec = 0.5; minoptprec = 0.01. The data and tree are deposited in TreeBASE at http://purl.org/phylo/treebase/phylows/study/TB2:S16967.

Ants. DNA was extracted from an adult worker of *A. megacephala* using the Qiagen DNeasy Tissue Kit (Qiagen, Valencia, CA) following the manufacturer's protocols. We generated molecular sequence data for four nuclear genes, elongation factor 1-α F1, elongation factor 1-α F2, wingless, and long-wavelength rhodopsin (GenBank accession numbers KP406345–KP406350), using previously published protocols (Brady et al. 2006; Schultz and Brady 2008). These data were added to the phylogenetic data matrix published by Schultz and Brady (2008) and Sosa-Calvo et al. (2013) in order to infer the phylogenetic position and time of divergence for this species. The data partitioning scheme and models of nucleotide evolution were optimized using PartitionFinder, version 1.0.1 (Lanfear et al. 2012). The data matrix was analyzed under a Bayesian framework using BEAST, version 2.1.2 (Bouckaert et al. 2014), with an uncorrelated lognormal relaxed clock model (Drummond et al. 2006). Nucleotide substitution models were unlinked and clock and tree models linked among partitions. A Yule speciation process was used for the tree prior. Three nodes were calibrated using attine Dominican amber fossils (table 2). These fossils are (i) *Apterostigma electropilosum*, a member of the *Apterostigma pilosum* group, and *Apterostigma eowilsoni*, probably a mem-

Figure 3: *Apterostigma megacephala* workers on their fungus garden in Floresta Nacional de Carajás, Pará, Brazil.

ber of the *A. pilosum* group (Schultz 2007); (ii) *Cyphomyrmex maya* and *Cyphomyrmex taino*, both members of the *Cyphomyrmex rimosus* group (de Andrade 2003); and (iii) *Trachymyrmex primaevus*, a fossil of uncertain placement within the genus (Baroni Urbani 1980). The stem-group nodes represented by these fossils were calibrated with the following a priori age distributions (all with zero offset lower bounds of 15 million years ago): *A. pilosum* stem group, lognormal (mean = 2.7, SD = 0.3); *C. rimosus* stem group, lognormal (mean = 2.2, SD = 0.5); *Trachymyrmex* stem group, lognormal (mean = 1.5, SD = 0.5). The node corresponding to the newly defined tribe Attini (s.l., which now includes many more genera in addition to the fungus-growing ants) was given an a priori normal distribution (mean = 67, SD =

Table 2: Chronogram crown-group dates, with 95% confidence intervals, for selected nodes on the ant chronogram (fig. 1)

Crown clade	Age (Ma)	95% HPD (Ma)
Attini s.l.*	66.96	58.45–75.91
Attini s.s. (fungus-farming ants only)	54.80	46.44–63.04
Apterostigma (including *A. megacephala*)	39.19	31.35–47.95
Apterostigma auriculatum + *Apterostigma pilosum* groups (excluding *A. megacephala*)	23.21	19.88–26.99
A. pilosum group only* (fig. 1, pink boxes)	14.99	10.18–19.77
Cyphomyrmex rimosus group* (yeast farmers; fig. 1, green boxes)	8.87	5.24–12.71
Higher fungus-farming ants* (fig. 1, blue boxes)	21.98	17.53–26.41
Leaf-cutter ants (fig. 1, orange boxes)	12.16	9.10–15.32

Note: Asterisks indicate nodes that were assigned prior age distributions; the table reports resulting posteriors. HPD = highest posterior density.

5.1), following Ward et al. (2015). Markov chain Monte Carlo searches were conducted for 100 million generations, with the first 20 million generations discarded as burn-in. Convergence and stationarity were assessed with Tracer, version 1.5 (Rambaut and Drummond 2009), using effective sample size scores and the consistency of results between multiple runs. The post-burn-in generations from three independent runs were manually combined and visualized using FigTree, version 1.4.0 (Rambaut 2012). The data and tree are deposited in TreeBASE at http://purl.org/phylo/treebase/phylows/study/TB2:S16967.

Ancestral State Analyses

Ant taxa were assigned states for a single six-state character representing the four attine agricultural systems and leaf-cutter agriculture (0: no agriculture, 1: lower agriculture, 2: coral-fungus agriculture, 3: yeast agriculture, 4: higher agriculture, 5: leaf-cutter agriculture). Six species (408 *Myrmicocrypta camargoi*, 402 *Cyphomyrmex* n. sp., 419 *Mycetophylax morschi*, 057 *Pseudoatta* n. sp., 417 *Acromyrmex heyeri*, and 040 *Atta laevigata*) received "unknown" (i.e., "?") state assignments, and *Trachymyrmex papulatus* received a "lower agriculture" state assignment (1) based on a single garden collection from Argentina; a second colony from the same locality cultivated a typical higher-attine garden (Mueller et al. 1998; U. G. Mueller and T. R. Schultz, unpublished data). (Alternatively, assigning state 4, higher agriculture, or a missing value to *T. papulatus* had no significant effect on the results, producing probabilities differing less than 0.004 from those reported below.) Character evolution was optimized on the ant Bayesian phylogram under ML ancestral state reconstruction using the Mk1 (est) Markov *k*-state model (Lewis 2001) in the StoChar module (Maddison and Maddison 2006) of the program Mesquite, version 2.75 (Maddison and Maddison 2011), producing the agricultural system mappings indicated by the colored boxes in figure 1. Under the Markov *k*-state model (Lewis 2001), the likelihood that each agricultural system arose in the most recent common ancestor of the corresponding ant clade was, as a proportion of the total probability ($=1.0$) distributed across the six character states, 0.975860198 for lower agriculture, 0.99912027 for yeast agriculture, 0.99011763 for higher agriculture, 0.99361714 for leaf-cutter agriculture, and 0.99847820 for coral-fungus agriculture. The likelihood that the ancestor of the genus *Apterostigma*, including *A. megacephala*, cultivated lower-attine fungi was 0.976423395.

Results and Discussion

Nests of *Apterostigma megacephala* at the Carajás location consisted of a single, shallow, subspherical chamber 5–16 cm in horizontal diameter and 4–10 cm in height, located 0–16.5 cm below the surface. The shallowest chambers partly occupied the lower levels of the leaf litter. Chamber floors were lined with small, uncut leaflets, probably those of *Newtonia suaveolens* (Fabaceae), that the ants were observed to carry into the nest, but the leaflets were never observed to be incorporated into the garden. As in other lower-attine ants, garden substrate consisted largely of arthropod frass. Colony demographics are summarized in table 1.

Molecular phylogenetic and diversification dating analyses indicate that *A. megacephala* is the sole surviving representative of an ancient lineage that diverged ~39 million years ago from the ancestor of all other known extant and fossil *Apterostigma* species (fig. 1), making it the oldest known single-species—that is, relict—fungus-farming ant lineage. The most primitive *Apterostigma* species known previously, members of the *Apterostigma auriculatum* group (13 described species), cultivate, in all known cases, lower-attine fungi (fig. 1, yellow boxes; Mueller et al. 1998; Gerardo 2004; Villesen et al. 2004; Gerardo et al. 2006; Himler 2007; Schultz and Brady 2008; Vo et al. 2009; Mehdiabadi et al. 2012; U. G. Mueller, T. R. Schultz, J. Sosa-Calvo, unpublished data; N. M. Gerardo, personal communication; A. G. Himler, personal communication). A derived clade of >30 described species within *Apterostigma*, the *A. pilosum* group, cultivates coral fungi (Pterulaceae), presumably as the result of an ancestral cultivar switch unique within the Attini (fig. 1, pink boxes; Munkacsi et al. 2004; Villesen et al. 2004; Dentinger et al. 2009). In direct contrast to both of these previously known *Apterostigma* fungal associations, DNA sequences from two nuclear genes, LSU (fig. 1) and ITS, of fungal cultivars from 10 nests of *A. megacephala*, as well as the consistent presence of gongylidia in all 15 sampled colonies, indicate that *A. megacephala* is the only lower-attine ant so far discovered to cultivate a higher-attine fungus. Even more unexpectedly, not only does *A. megacephala* cultivate a higher-attine fungus, it cultivates the most highly derived and most recently evolved higher-attine cultivar, *Leucoagaricus gongylophorus*, the species cultivated by most leaf-cutter ants (fig. 1, thick red line). This cultivation of *L. gongylophorus* by *A. megacephala* in eastern Amazonian Brazil can only be due to a horizontal transfer event in which *L. gongylophorus* replaced the fungus (a lower-attine cultivar) previously grown by *A. megacephala* because (i) ancestral state analyses clearly indicate that the ancestor of *A. megacephala* cultivated lower-attine fungi (likelihood = 0.976; see "Methods"); (ii) the ancestor of *A. megacephala* diverged from the rest of the Attini ~39 million years ago (fig. 1); and (iii) *L. gongylophorus* originated, presumably as the product of coevolution between a higher-attine ant and a higher-attine cultivar, ~2–8 million years ago, at least 30 million years after the origin of the *A. megacephala* lineage (Mikheyev et al. 2010).

More nests of *A. megacephala* from additional locations must be discovered and studied to determine whether all *A. megacephala* populations consistently cultivate *L. gongylophorus* across its geographic range or whether some populations also cultivate lower-attine fungi. The consistent cultivation of *L. gongylophorus* by *A. megacephala* at all localities would suggest that the association may be obligate and that the transition from a lower-attine fungal cultivar to *L. gongylophorus* occurred prior to the geographic separation of extant *A. megacephala* populations, which are currently found at opposite ends of the Amazon basin. Obviously, however, that association cannot predate the origin of *L. gongylophorus* 2–8 million years ago. Alternatively, variation in cultivar association across populations or even between nests within a population would suggest that, with regard to fungal association, *A. megacephala* is the most symbiotically labile attine ant so far discovered. In either case, it is clear for a number of reasons that the cultivation of *L. gongylophorus* by *A. megacephala* in the Carajás population represents an evolutionarily stable state that has endured for multiple generations. First, every collected colony ($n = 15$) was associated with *L. gongylophorus*. Second, most of the collected colonies were sexually mature and in good reproductive health; at least 10 colonies contained alate male and female reproductive adults, and all colonies collected contained workers and brood (table 1). Third, the fungi in the six *A. megacephala* colonies from which sequence data were obtained form a clade (fig. 1, red boxes; Bayesian posterior probability = 1.00, ML bootstrap proportion = 0.86) that consistently differs at two LSU nucleotide sites from the remainder of *L. gongylophorus* strains, including a strain isolated from an *Atta* sp. nest at the same Carajás locality (fig. 1, *Atta* sp. CTL110912 05 C2, orange box). The phylogeny of the more variable ITS region further indicates with strong support (posterior probability = 0.99) that the strain of *L. gongylophorus* cultivated by *A. megacephala* in Carajás is very closely related to a cultivar strain collected in Manaus over 900 km away. These phylogenetic results indicate that the strain of *L. gongylophorus* associated with *A. megacephala* in Carajás has been transferred intergenerationally over multiple colony-founding events. Fourth, three colonies of *A. megacephala* have been successfully maintained in the lab for 2.5 years (table 1) without any of the adverse effects observed in the forced association of *Trachymyrmex septentrionalis* and *L. gongylophorus* (Seal and Mueller 2014) discussed previously.

The ability of *A. megacephala* to cultivate *L. gongylophorus* is likely to be important for understanding the currently unknown biological constraints enforcing symbiont fidelity at the level of the five attine agricultural systems, in particular the constraints preventing lower-attine ants from cultivating higher-attine fungi. As discussed previously, in higher-attine ants, occasional across-system switches have been encountered in nature at low frequencies (<5%, $n > 700$), whereas, except for *A. megacephala*, no other case of a lower-attine ant cultivating a higher-attine fungus has ever been encountered ($n > 1{,}500$), suggesting that the fitness costs of such an association may be particularly severe. Although the biological constraints underlying ant-fungus fidelity at the level of the five agricultural systems are entirely unknown and may be different in each system, two broad, nonmutually exclusive categories of constraints have been proposed: (i) physiological and (ii) microbial symbiotic.

An example of a physiological constraint is the previously mentioned loss of two genes in the arginine synthesis pathway in leaf-cutting ants, presumably because that amino acid is reliably supplied by the leaf-cutter fungal symbionts (Nygaard et al. 2011; Suen et al. 2011). Whether this loss occurred in the ancestor of all fungus-farming ants or more recently in a sublineage containing the leaf-cutting ants remains unknown, but comparative transcriptomics and genomics of attine fungi indicate that higher-attine fungi produce increased quantities, relative to lower-attine fungi, of multiple amino acids (although, curiously, not arginine) as well as of detoxifying and plant-degrading enzymes (de Fine Licht et al. 2013, 2014).

With regard to microbial symbiotic constraints, with the possible exception of yeast agriculture, all attine gardens are parasitized by fungal species in the genus *Escovopsis* (Currie et al. 1999*a*; Gerardo et al. 2006), which are thought to be controlled in part by antibiotics produced by bacteria that occur on the integuments of fungus-farming ants, in some species in specialized integumental crypts, and in attine gardens (Currie et al. 1999*b*, 2006; Mueller et al. 2005, 2008; Schultz et al. 2005; Kost et al. 2007; Sen et al. 2009; Cafaro et al. 2011; Yek et al. 2012; Andersen et al. 2013). It has been suggested that particular complements of bacterial and possibly fungal species—that is, coadapted microbial consortia—are necessary for maintaining long-term garden health, including the control of *Escovopsis* and other ant and garden parasites, and that these consortia may fundamentally differ across agricultural systems (Currie 2001; Mueller et al. 2005; Boomsma and Aanen 2009; Sen et al. 2009; Rodrigues et al. 2010, 2013; Schoenian et al. 2011; Aylward et al. 2012; Mattoso et al. 2012; Mendes et al. 2012; Pagnocca et al. 2012; Andersen et al. 2013; Seal and Mueller 2014). Coadapted microbial consortia optimized for particular attine agricultural systems imply fitness costs for across-system ant-cultivar associations.

It is plausible that in an early stage of attine ant evolution, there existed a common ancestor that was committed to fungivory and farming but that had not yet evolved any biological constraints, physiological or microbial, that limited it to any particular attine agricultural system. Rather, it may have been able to cultivate and benefit nutritionally

from a wide variety of fungi, for example, multiple families within the Agaricales or multiple genera within the Agaricaceae. If so, then perhaps *A. megacephala* has retained this symbiotic plasticity. If, further, the recently evolved *L. gongylophorus* provides *A. megacephala* with a fitness advantage relative to other fungi, then perhaps *A. megacephala* has been able to take advantage of *L. gongylophorus* in a way that all other lower-attine ants, as well as many higher-attine ants, cannot. A potential problem with this scenario is that, because *A. megacephala* is not the sister to all other attine ants, the primitive condition of symbiotic plasticity must have been lost multiple times in fungus-farming ants. Put another way, the scenario minimally requires that the most recent common ancestors (MRCAs) of three lineages of fungus-farming ants convergently specialized on leucocoprineaceous fungi: (i) the MRCA of the *A. auriculatum* species group; (ii) the MRCA of *Mycocepurus* and *Myrmicocrypta;* and (iii) the MRCA of all the remaining attine ants (fig. 1). Since it has been suggested that leucocoprineaceous fungi may actively recruit ants as dispersal agents (Mueller et al. 2001; Schultz et al. 2005), perhaps three episodes of convergent evolution are not as improbable as might first be supposed. Alternatively, perhaps during the 39 million years that separate it from all other lower-attine ants, *A. megacephala* alone evolved a biological feature that uniquely preadapted it to take advantage of *L. gongylophorus* following that cultivar's origin sometime between 2 million and 8 million years ago. Distinguishing between these largely speculative scenarios will require comparative study of the genomes of *A. megacephala* and other carefully chosen fungus-farming ants as well as the characterization of the microbial symbionts associated with *A. megacephala*. Fortunately, the discovery of a population of *A. megacephala* in Carajás, Brazil, makes such research possible.

Acknowledgments

We are grateful to R. C. F. Brandão and R. Rosa da Silva for alerting us to the occurrence of *Apterostigma megacephala* in Carajás; to F. Drumond Martins and E. Esteves of Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio) at Floresta Nacional de Carajás for their help and guidance; and to E. Okonski for collections and research support. For permission to conduct field research, we thank the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq; Processo EXC 039/07; Portarias 267, 359), the Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (IBAMA), and the ICMBio (permits 14789-1, 14789-2). T.R.S. and J.S.-C. were supported by National Science Foundation (NSF) grant DEB-0949689 and the National Museum of Natural History (NMNH) Small Grants program; T.R.S. by the Smithsonian Institution Scholarly Studies Program; J.S.-C. by an NMNH Peter Buck Predoctoral Fellowship and a Max and Vera Britton Environmental Science Award (Cosmos Club Foundation); U.G.M. by NSF DEB-0919519; and M.B. by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP; 2011/50226-0) and CNPq (311562/2012-4 and 487639/2012-0).

Literature Cited

Andersen, S. B., L. H. Hansen, P. Sapountzis, S. J. Sørensen, and J. J. Boomsma. 2013. Specificity and stability of the *Acromyrmex-Pseudonocardia* symbiosis. Molecular Ecology 22:4307–4321.

Aylward, F. O., K. E. Burnum, J. J. Scott, G. Suen, S. G. Tringe, S. M. Adams, K. W. Barry, et al. 2012. Metagenomic and metaproteomic insights into bacterial communities in leaf-cutter ant fungus gardens. ISME Journal 6:1688–1701.

Baroni Urbani, C. 1980. First description of fossil gardening ants (Amber Collection Stuttgart and Natural History Museum Basel; Hymenoptera: Formicidae. I: Attini). Stuttgarter Beiträge zur Naturkunde B: Geologie und Paläontologie 54:1–13.

Boomsma, J. J., and D. K. Aanen. 2009. Rethinking crop-disease management in fungus-growing ants. Proceedings of the National Academy of Sciences of the USA 106:17611–17612.

Bouckaert, R., J. Heled, D. Kuknert, T. Vaughan, C.-H. Wu, D. Xie, M. A. Suchard, A. Rambaut, and A. J. Drummond. 2014. BEAST 2: a software platform for Bayesian evolutionary analysis. PLoS Computational Biology 10:e1003537.

Brady, S. G., T. R. Schultz, B. L. Fisher, and P. S. Ward. 2006. Evaluating alternative hypotheses for the early evolution and diversification of ants. Proceedings of the National Academy of Sciences of the USA 103:18172–18177.

Brown, J. M., S. M. Hedtke, A. R. Lemmon, and E. M. Lemmon. 2010. When trees grow too long: investigating the causes of highly inaccurate Bayesian branch-length estimates. Systematic Biology 59:145–161.

Cafaro, M. J., M. Poulsen, A. E. F. Little, S. L. Price, N. M. Gerardo, B. Wong, A. E. Stuart, B. Larget, P. Abbot, and C. R. Currie. 2011. Specificity in the symbiotic association between fungus-growing ants and protective *Pseudonocardia* bacteria. Proceedings of the Royal Society B: Biological Sciences 278:1814–1822.

Chapela, I. H., S. A. Rehner, T. R. Schultz, and U. G. Mueller. 1994. Evolutionary history of the symbiosis between fungus-growing ants and their fungi. Science 266:1691–1694.

Costa, A. N., H. L. Vasconcelos, E. H. M. Vieira-Neto, and E. M. Bruna. 2008. Do herbivores exert top-down effects in Neotropical savannas? estimates of biomass consumption by leaf-cutter ants. Journal of Vegetation Science 19:849–854.

Currie, C. R. 2001. A community of ants, fungi, and bacteria: a multilateral approach to studying symbiosis. Annual Review of Microbiology 55:357–380.

Currie, C. R., U. G. Mueller, and D. Malloch. 1999*a*. The agricultural pathology of ant fungus gardens. Proceedings of the National Academy of Sciences of the USA 96:7998–8002.

Currie, C. R., M. Poulsen, J. Mendenhall, J. J. Boomsma, and J. Billen. 2006. Coevolved crypts and exocrine glands support mutualistic bacteria in fungus-growing ants. Science 311:81–83.

Currie, C. R., J. A. Scott, R. C. Summerbell, and D. Malloch. 1999*b*. Fungus-growing ants use antibiotic-producing bacteria to control garden parasites. Nature 398:701–704.

de Andrade, M. L. 2003. First descriptions of two new amber species of *Cyphomyrmex* from Mexico and the Dominican Republic (Hymenoptera: Formicidae). Beiträge zur Entomologie 53:131–139.

de Fine Licht, H. H., J. J. Boomsma, and A. Tunlid. 2014. Symbiotic adaptations in the fungal cultivar of leaf-cutting ants. Nature Communications 5:1–10.

de Fine Licht, H. H., M. Schiøtt, U. G. Mueller, and J. J. Boomsma. 2010. Evolutionary transitions in enzyme activity of ant fungus gardens. Evolution 64:2055–2069.

de Fine Licht, H. H., M. Schiøtt, A. Rogowska-Wrzesinska, S. Nygaard, P. Roepstorff, and J. J. Boomsma. 2013. Laccase detoxification mediates the nutritional alliance between leaf-cutting ants and fungus-garden symbionts. Proceedings of the National Academy of Sciences of the USA 110:583–587.

Dentinger, B. T. M., D. J. Lodge, A. B. Munkacsi, D. E. Desjardin, and D. J. McLaughlin. 2009. Phylogenetic placement of an unusual coral mushroom challenges the classic hypothesis of strict coevolution in the *Apterostigma pilosum* group ant-fungus mutualism. Evolution 63:2172–2178.

Drummond, A. J., S. Y. W. Ho, M. J. Phillips, and A. Rambaut. 2006. Relaxed phylogenetics and dating with confidence. PLoS Biology 4:e88.

Fowler, H. G., V. Pereira-da-Silva, L. C. Forti, and N. B. Saes. 1986. Population dynamics of leaf-cutting ants: a brief review. Pages 123–145 *in* C. S. Lofgren and R. K. Vander Meer, eds. Fire ants and leaf-cutting ants: biology and management. Westview, Boulder, CO.

Gerardo, N. M. 2004. The nature of parasite specialization in the fungus-growing ant symbiosis. PhD diss. University of Texas, Austin.

Gerardo, N. M., and E. J. Caldera. 2007. Labile associations between fungus-growing ant cultivars and their garden pathogens. ISME Journal 1:373–384.

Gerardo, N. M., U. G. Mueller, and C. R. Currie. 2006. Complex host-pathogen coevolution in the *Apterostigma* fungus-growing ant-microbe symbiosis. BioMed Central Evolutionary Biology 6:1–9.

Himler, A. G. 2007. Evolutionary ecology and natural history of fungus-growing ants: host-switching, divergence, and asexuality. PhD diss. University of Texas, Austin.

Hölldobler, B., and E. O. Wilson. 2010. The leafcutter ants: civilization by instinct. W. W. Norton, New York.

Kooij, P. W. 2013. Fungal adaptations to mutualistic life with ants. PhD diss. University of Copenhagen, Denmark.

Kost, C., T. Lakatos, I. Boettcher, W.-R. Arendholz, M. Redenbach, and R. Wirth. 2007. Non-specific association between filamentous bacteria and fungus-growing ants. Naturwissenschaften 94:821–828.

Lanfear, R., B. Calcott, S. Y. W. Ho, and S. Guindon. 2012. PartitionFinder: combined selection of partitioning schemes and substitution models for phylogenetic analyses. Molecular Biology and Evolution 29:1695–1701.

Lattke, J. E. 1999. A new species of fungus-growing ant and its implications for attine phylogeny (Hymenoptera: Formicidae). Systematic Entomology 24:1–6.

Lewis, P. O. 2001. A likelihood approach to estimating phylogeny from discrete morphological character data. Systematic Biology 50:913–925.

Maddison, W. P., and D. R. Maddison. 2006. StochChar: a package of Mesquite modules for stochastic models of character evolution. Version 1.1. http://mesquiteproject.org/.

———. 2011. Mesquite: a modular system for evolutionary analysis. Version 2.75. http://mesquiteproject.org/.

Marshall, D. C. 2010. Cryptic failure of partitioned Bayesian phylogenetic analyses: lost in the land of long trees. Systematic Biology 59:108–117.

Marshall, D. C., C. Simon, and T. R. Buckley. 2006. Accurate branch length estimation in partitioned Bayesian analyses requires accommodation of among-partition rate variation and attention to branch length priors. Systematic Biology 55:993–1003.

Mattoso, T. C., D. D. O. Moreira, and R. I. Samuels. 2012. Symbiotic bacteria on the cuticle of the leaf-cutting ant *Acromyrmex subterraneus subterraneus* protect workers from attack by entomopathogenic fungi. Biology Letters 8:461–464.

Mehdiabadi, N. J., U. G. Mueller, S. G. Brady, A. G. Himler, and T. R. Schultz. 2012. Symbiont fidelity and the origin of species in fungus-growing ants. Nature Communications 3:840.

Mehdiabadi, N. J., and T. R. Schultz. 2009. Natural history and phylogeny of the fungus-farming ants (Hymenoptera: Formicidae: Myrmicinae: Attini). Myrmecological News 13:37–55.

Mendes, T. D., A. Rodrigues, I. Dayo-Owoyemi, F. A. L. Marson, and F. C. Pagnocca. 2012. Generation of nutrients and detoxification: possible roles of yeasts in leaf-cutting ant nests. Insects 3:228–245.

Meyer, S. T., I. R. Leal, and R. Wirth. 2009. Persisting hyper-abundance of leaf-cutting ants (*Atta* spp.) at the edge of an old Atlantic forest fragment. Biotropica 41:711–716.

Mikheyev, A. S., U. G. Mueller, and P. Abbot. 2006. Cryptic sex and many-to-one coevolution in the fungus-growing ant symbiosis. Proceedings of the National Academy of Sciences of the USA 103:10702–10706.

———. 2010. Comparative dating of attine ant and lepiotaceous cultivar phylogenies reveals coevolutionary synchrony and discord. American Naturalist 175:E126–E133.

Möller, A. 1893. Die Pilzgärten einiger Südamerikansicher Ameisen. Botanische Mitheilungen aus den Tropen 6:1–142.

Mueller, U. G., D. Dash, C. Rabeling, and A. Rodrigues. 2008. Coevolution between attine ants and actinomycete bacteria: a reevaluation. Evolution 62:2894–2912.

Mueller, U. G., N. M. Gerardo, D. K. Aanen, D. L. Six, and T. R. Schultz. 2005. The evolution of agriculture in insects. Annual Review of Ecology, Evolution, and Systematics 36:563–595.

Mueller, U. G., A. S. Mikheyev, S. E. Solomon, and M. Cooper. 2011. Frontier mutualism: coevolutionary patterns at the northern range limit of the leaf-cutter ant-fungus symbiosis. Proceedings of the Royal Society B: Biological Sciences 278:3050–3059.

Mueller, U. G., S. A. Rehner, and T. R. Schultz. 1998. The evolution of agriculture in ants. Science 281:2034–2038.

Mueller, U. G., T. R. Schultz, C. R. Currie, R. M. M. Adams, and D. Malloch. 2001. The origin of the attine ant-fungus mutualism. Quarterly Review of Biology 76:169–197.

Munkacsi, A. B., J. J. Pan, P. Villesen, U. G. Mueller, M. Blackwell, and D. J. McLaughlin. 2004. Convergent coevolution in the domestication of coral mushrooms by fungus-growing ants. Proceedings of the Royal Society B: Biological Sciences 271:1777–1782.

Newton, M. A., and A. E. Raftery. 1994. Approximate Bayesian inference with the weighted likelihood bootstrap. Journal of the Royal Statistical Society B: Statistical Methodology 56:3–48.

Nygaard, S., G. Zhang, M. Schiøtt, C. Li, Y. Wurm, H. Hu, J. Zhou, et al. 2011. The genome of the leaf-cutting ant *Acromyrmex echinatior* suggests key adaptations to advanced social life and fungus farming. Genome Research 21:1339–1348.

Pagnocca, F. C., V. E. Masiulionis, and A. Rodrigues. 2012. Specialized fungal parasites and opportunistic fungi in gardens of attine ants. Psyche: A Journal of Entomology 2012:1–9.

Posada, D. 2008. jModelTest: phylogenetic model averaging. Molecular Biology and Evolution 25:1253–1256.

Posada, D., and T. R. Buckley. 2004. Model selection and model averaging in phylogenetics: advantages of Akaike information criterion and Bayesian approaches over likelihood ratio tests. Systematic Biology 53:793–808.

Quinlan, R. J., and J. M. Cherrett. 1979. The role of fungus in the diet of the leaf-cutting ant *Atta cephalotes* (L.). Ecological Entomology 4:151–160.

Rambaut, A. 2012. FigTree: tree figure drawing tool. Version 1.4.0. Institute of Evolutionary Biology, University of Edinburgh.

Rambaut, A., and A. J. Drummond. 2009. Tracer. Version 1.5. http://tree.bio.ed.ac.uk/software/tracer/.

Rodrigues, A., M. R. Z. Passarini, M. Ferro, N. S. Nagamoto, L. C. Forti, M. Bacci, L. D. Sette, and F. C. Pagnocca. 2013. Fungal communities in the garden chamber soils of leaf-cutting ants. Journal of Basic Microbiology 54:1186–1196.

Rodrigues, A., A. Silva, M. Bacci, L. C. Forti, and F. C. Pagnocca. 2010. Filamentous fungi found on foundress queens of leaf-cutting ants (Hymenoptera: Formicidae). Journal of Applied Entomology 134:342–345.

Ronquist, F., J. P. Huelsenbeck, and M. Teslenko. 2013. MrBayes. Version 3.2.2. http://mrbayes.sourceforge.net/download.php.

Ronquist, F., M. Teslenko, P. van der Mark, D. L. Ayres, A. Darling, S. Höhna, B. Larget, L. Liu, M. A. Suchard, and J. P. Huelsenbeck. 2012. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Systematic Biology 61: 539–542.

Schoenian, I., M. Spiteller, M. Ghaste, R. Wirth, H. Herz, and D. Spiteller. 2011. Chemical basis of the synergism and antagonism in microbial communities in the nests of leaf-cutting ants. Proceedings of the National Academy of Sciences of the USA 108:1955–1960.

Schultz, T. R. 2007. The fungus-growing ant genus *Apterostigma* in Dominican amber. Memoirs of the American Entomological Institute 80:425–436.

Schultz, T. R., and S. G. Brady. 2008. Major evolutionary transitions in ant agriculture. Proceedings of the National Academy of Sciences of the USA 105:5435–5440.

Schultz, T. R., U. G. Mueller, C. R. Currie, and S. A. Rehner. 2005. Reciprocal illumination: a comparison of agriculture in humans and in fungus-growing ants. Pages 149–190 *in* F. E. Vega and M. Blackwell, eds. Insect-fungal associations: ecology and evolution. Oxford University Press, New York.

Scott, J. J., M. K. Kweskin, M. Cooper, and U. G. Mueller. 2009. Polymorphic microsatellite markers for the symbiotic fungi cultivated by leaf cutter ants (Attini, Formicidae). Molecular Ecology Resources 9:1391–1394.

Seal, J. N., J. Gus, and U. G. Mueller. 2012. Fungus-gardening ants prefer native fungal species: do ants control their crops? Behavioral Ecology 23:1250–1256.

Seal, J. N., and U. G. Mueller. 2014. Instability of novel ant-fungal associations constrains horizontal exchange of fungal symbionts. Evolutionary Ecology 28:157–176.

Seal, J. N., and W. R. Tschinkel. 2007. Co-evolution and the superorganism: switching cultivars does not alter the performance of fungus-gardening ant colonies. Functional Ecology 21:988–997.

Sen, R., H. D. Ishak, D. Estrada, S. E. Dowd, E. Hong, and U. G. Mueller. 2009. Generalized antifungal activity and 454-quantification of *Pseudonocardia* and *Amycolatopsis* bacteria in nests of fungus-growing ants. Proceedings of the National Academy of Sciences of the USA 106:17805–17810.

Silva-Pinhati, A. C. O., M. Bacci Jr., G. Hinkle, M. L. Sogin, F. C. Pagnocca, V. G. Martins, O. C. Bueno, and M. J. A. Hebling. 2004. Low variation in ribosomal DNA and internal transcribed spacers of the symbiotic fungi of leaf-cutting ants (Attini: Formicidae). Brazilian Journal of Medical and Biological Research 37: 1463–1472.

Sosa-Calvo, J., T. R. Schultz, C. R. F. Brandão, C. Klingenberg, R. M. Feitosa, C. Rabeling, M. Bacci, C. T. Lopes, and H. L. Vasconcelos. 2013. *Cyatta abscondita*: taxonomy, evolution, and natural history of a new fungus-farming ant genus from Brazil. PLoS ONE 8: e80498.

Spinks, P. Q., and H. B. Shaffer. 2009. Conflicting mitochondrial and nuclear phylogenies for the widely disjunct *Emys* (Testudines: Emydidae) species complex, and what they tell us about biogeography and hybridization. Systematic Biology 58:1–20.

Stradling, D. J., and R. J. Powell. 1986. The cloning of more highly productive fungal strains: a factor in the speciation of fungus-growing ants. Experientia 42:962–964.

Suchard, M. A., R. E. Weiss, and J. S. Sinsheimer. 2001. Bayesian selection of continuous-time Markov chain evolutionary models. Molecular Biology and Evolution 18:1001–1013.

Suen, G., C. Teiling, L. Li, C. Holt, E. Abouheif, E. Bornberg-Bauer, P. Bouffard, et al. 2011. The genome sequence of the leaf-cutter ant *Atta cephalotes* reveals insights into its obligate symbiotic lifestyle. PLoS Genetics 7:e1002007.

Villesen, P., U. G. Mueller, T. R. Schultz, R. M. M. Adams, and A. C. Bouck. 2004. Evolution of ant-cultivar specialization and cultivar switching in *Apterostigma* fungus-growing ants. Evolution 58: 2252–2265.

Vo, T. L., U. G. Mueller, and A. S. Mikheyev. 2009. Free-living fungal symbionts (Lepiotaceae) of fungus-growing ants (Attini: Formicidae). Mycologia 101:206–210.

Ward, P. S., S. G. Brady, B. L. Fisher, and T. R. Schultz. 2010. Phylogeny and biogeography of dolichoderine ants: effects of data partitioning and relict taxa on historical inference. Systematic Biology 59:342–362.

———. 2015. The evolution of myrmicine ants: phylogeny and biogeography of a hyperdiverse ant clade (Hymenoptera: Formicidae). Systematic Entomology 40:61–81.

Weber, N. A. 1956. Fungus-growing ants and their fungi: *Trachymyrmex septentrionalis seminole*. Ecology 37:197–199.

———. 1972. Gardening ants: the attines. American Philosophical Society, Philadelphia.

Wheeler, W. M. 1907. The fungus-growing ants of North America. Bulletin of the American Museum of Natural History 23:669–807.

Wirth, R., H. Herz, R. J. Ryel, W. Beyschlag, and B. Hölldolber. 2003. Herbivory of leaf-cutting ants: a case study on *Atta colombica* in the tropical rainforest of Panama. Ecological Studies. Vol. 164. Springer, New York.

Yek, S. H., J. J. Boomsma, and M. Poulsen. 2012. Towards a better understanding of the evolution of specialized parasites of fungus-growing ant crops. Psyche: A Journal of Entomology 2012:1–10.

Zwickl, D. J. 2006. Genetic algorithm approaches for the phylogenetic analysis of large biological sequence datasets under the maximum likelihood criterion. PhD diss. University of Texas, Austin.

Natural History Editor: Mark A. McPeek