Major evolutionary transitions in ant agriculture

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Agriculture is a specialized form of symbiosis that is known to have evolved in only four animal groups: humans, bark beetles, termites, and ants. Here, we reconstruct the major evolutionary transitions that produced the five distinct agricultural systems of the fungus-growing ants, the most well studied of the nonhuman agriculturalists. We do so with reference to the first fossil-calibrated, multiple-gene, molecular phylogeny that incorporates the full range of taxonomic diversity within the fungus-growing ant tribe Attini. Our analyses indicate that the original form of ant agriculture, the cultivation of a diverse subset of fungal species in the tribe Leucocoprineae, evolved ≈50 million years ago in the Neotropics, coincident with the early Eocene climatic optimum. During the past 30 million years, three known ant agricultural systems, each involving a phylogenetically distinct set of derived fungal cultivars, have separately arisen from the original agricultural system. One of these derived systems subsequently gave rise to the fifth known system of agriculture, in which a single fungal species is cultivated by leaf-cutter ants. Leaf-cutter ants evolved remarkably recently (≈8–12 million years ago) to become the dominant herbivores of the New World tropics. Our analyses identify relict, extant attine ant species that occupy phylogenetic positions that are transitional between the agricultural systems. Intensive study of those species holds particular promise for clarifying the sequential accretion of ecological and behavioral characters that produced each of the major ant agricultural systems.

Attini | divergence dating | Formicidae | phylogeny | symbiosis

Attine ants (subfamily Myrmicinae, tribe Attini) comprise a monophyletic group of >230 described species, exclusively New World and primarily Neotropical in distribution (1–4). All attine ants obligately depend on the cultivation of fungus gardens for food. So complete is this dependence that, upon leaving the maternal nest, a daughter queen must carry within her mouth a nucleus of fungus that serves as the starting culture for her new garden (5–7). Attine agriculture achieves its evolutionary apex in the leaf-cutting ants of the genera *Acromyrmex* and *Atta*, the dominant herbivores of the New World tropics (8, 9). Unlike more primitive attine ants that forage for and cultivate their fungus gardens on organic detritus, leaf-cutting ants have acquired the ability to cut and process fresh vegetation (leaves, flowers, and grasses) to serve as the nutritional substrate for their fungal cultivars. This key evolutionary innovation renders a mature *Atta* colony the ecological equivalent of a large mammalian herbivore in terms of collective biomass, lifespan, and quantity of plant material consumed (9).

Attine ant agriculture is the product of an ancient, quadripartite, symbiotic relationship between three mutualists and one parasite. The mutualists include the attine ants, their fungal cultivars (Leucocoprineae and Pterulaceae), and filamentous bacteria in the genus *Pseudonocardia* (Actinomycetes) that grow on the integuments of the ants. The parasite, a fungus in the genus *Escovopsis* (Ascomycetes) known only from attine fungus gardens, infects those gardens as a "crop disease" and is controlled, at least in part, by an antibiotic produced by the *Pseudonocardia* bacterial symbiont (4, 10, 11).

Based on nearly monolithic associations between broad phylogenetic groups of attine ants, cultivars, and *Escovopsis* parasites, attine agriculture has been divided into five biologically distinct agricultural systems, each representing a major transition in the evolution of ant agriculture. These systems are: (*i*) lower agriculture, practiced by species in the majority of attine genera (76 species), including those thought to retain more primitive features, which cultivate a wide range of fungal species in the tribe Leucocoprineae; (*ii*) coral fungus agriculture, practiced by species in the "*pilosum* group" (34 species), a subset of the attine genus *Apterostigma*, which cultivate a clade of fungi in the Pterulaceae; (*iii*) yeast agriculture, practiced by species in the "*rimosus* group" (18 species), a subset of the attine genus *Cyphomyrmex*, which cultivate a distinct clade of leucocoprineaceous fungi derived from the lower attine fungi; (*iv*) generalized higher agriculture, practiced by species in the three genera of non-leaf-cutting "higher attine" ants (63 species), which cultivate another distinct clade of leucocoprineaceous fungi separately derived from the lower attine fungi; and (*v*) leaf-cutter agriculture, a subdivision of higher attine agriculture practiced by species of ecologically dominant ants in the genera *Atta* and *Acromyrmex* (40 species), which cultivate a single highly derived species of higher attine fungus (4, 12–14).

In contrast to important advances in other areas of attine biology, including molecular phylogenies for the other three symbionts (10, 13–25), major features of fungus-growing ant phylogeny remain poorly understood (1, 26, 27). A well supported, resolved phylogeny of the attine ants is necessary for analyzing the coevolution of the ants and their three microbial symbionts as well as for understanding the historical sequence of evolutionary change that produced each of the five attine agricultural systems. To address this problem, we reconstructed the evolution of attine agriculture by inferring the first fossil-calibrated molecular phylogeny for the fungus-growing ants, based on data from four nuclear protein-coding genes and incorporating the full range of attine taxonomic diversity, particularly with regard to poorly understood, rarely collected, and potentially paraphyletic or polyphyletic taxa (1).

Results and Discussion

Origin of Ant Agriculture. Based on the monophyly of the attine ants, on their exclusively New World distribution, and on their apparent center of diversity in the wet Neotropics, some researchers have speculated that ant agriculture arose a single time in the forests of South America after its isolation from Africa

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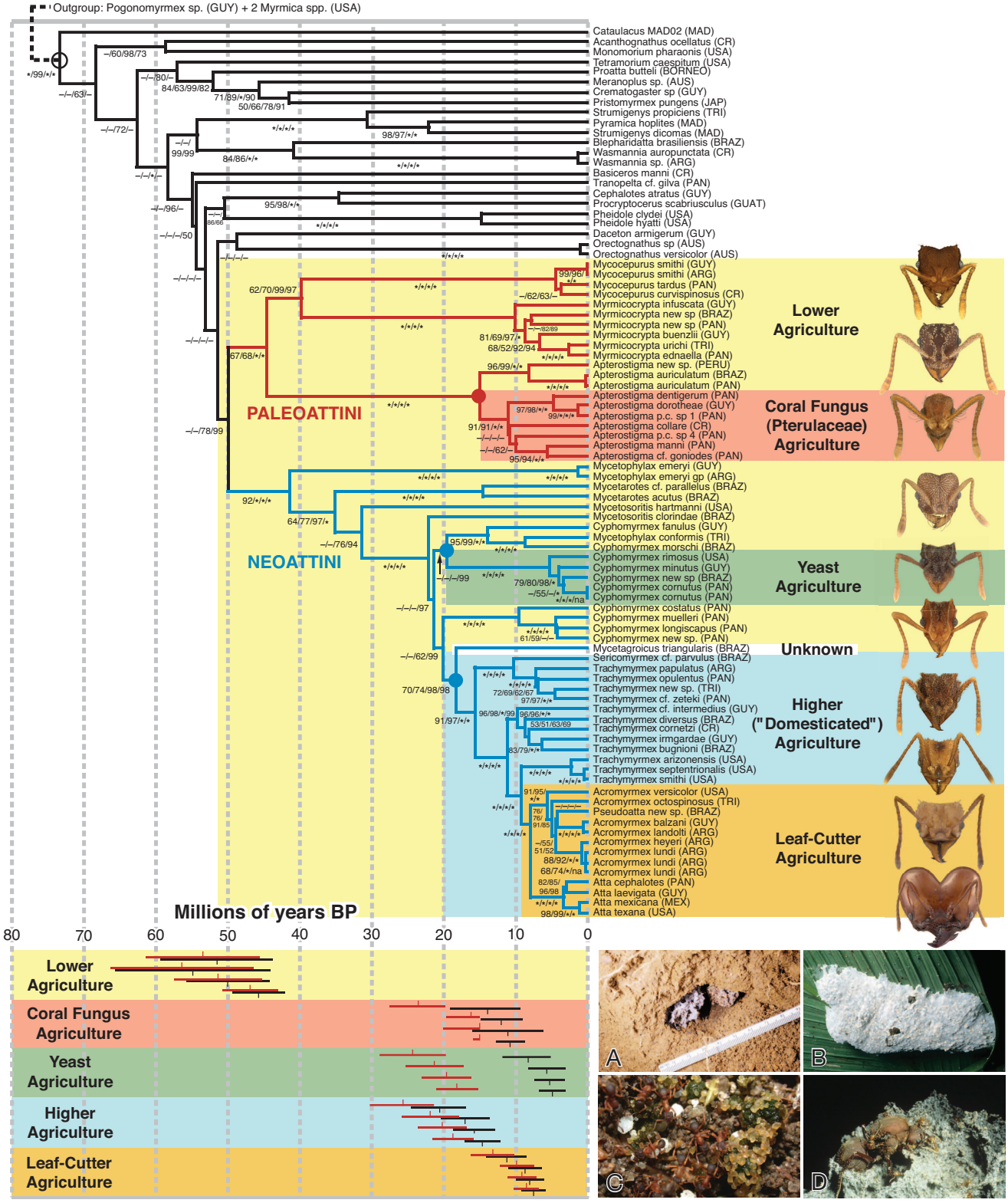

Fig. 1. A time-calibrated phylogeny of the attine fungus-growing ants with age estimates for the origins of the five known ant agricultural systems. Agricultural systems, indicated by colored rectangles, are defined by phylogenetically distinct groups of associated fungal cultivars and were reconstructed under likelihood and parsimony methods with identical results. Tree topology is the maximum-likelihood reconstruction, identical with regard to attine phylogeny to the Bayesian codon-model result. Numbers on branches indicate support values from four analyses: parsimony bootstraps, ML bootstraps, Bayesian nucleotide-model posterior probabilities, and Bayesian codon-model posterior probabilities (''–,'' < 50; ''*,'' 100). The three solid circles represent node assignments for Dominican amber fossil calibrations, and the open circle marks the root of the dating-analysis tree. Bars below the time scale summarize four separate relaxed-molecular-clock analyses dating the origin of the five agricultural systems. Black bars represent the most recent node containing all members of the system (''crown-group'') and red bars additionally include the branch leading to that node (''stem-group''). For each system, pairs of red and black bars from top to bottom correspond to (*i*) Bayesian uncorrelated lognormal, root age prior to 73.5 ± 4.5 mya; (*ii*) penalized likelihood, root age 81 mya; (*iii*) penalized likelihood, root age 73.5 mya;

(1–3, 28–31). The results of our Bayesian codon-model and molecular-dating analyses (Fig. 1) provide strong corroboration for this view, indicating that ant agriculture had a single origin ≈50 million years ago and, because this date is far more recent than the last connection between South America and Africa ≈90 mya, indicating that ant agriculture originated on the South American continent. Significantly, the origin of fungus-growing coincides with the early Eocene climatic optimum (50–55 mya), a period of global warming in which an extraordinary diversity of plants with tropical affinities occurred at middle and high latitudes in South America (32). Unfortunately, our data are insufficient to identify the closest relative (i.e., sister group) of the Attini. Although in our phylogeny (Fig. 1) a clade consisting of *Daceton* and *Orectognathus* species is reconstructed as that sister group, this result is not significantly supported by any method of analysis, and we strongly caution against drawing any inferences based on it. Indeed, with few exceptions, the relationships of most nonattine myrmicines remain unresolved in this and in a previous study of ant relationships (33), indicating the critical need for additional data for resolving the profoundly important question of what group of ants is the closest non-fungus-growing relative of the Attini (1).

Lower Agriculture. Our results (Fig. 1) indicate that the first fungus-growing ant practiced lower agriculture and that all extant members of a series of basally diverging lineages continue to practice this form of agriculture. This corroborates the hypothesis of some researchers that lower agriculture was the first attine agricultural system (31) but contradicts a long-standing hypothesis that yeast agriculture was the first system (9, 29, 30, 34) and a recently proposed hypothesis that coral-fungus agriculture was the first (35). Lower attine fungal cultivars all belong to a paraphyletic grade within the tribe Leucocoprineae (''parasol mushrooms'') and are, so far as is known, entirely capable of a feral, free-living existence outside of the attine symbiosis (17, 36). Current data indicate that a corresponding paraphyletic grade of *Escovopsis* (24, 37) infects lower attine fungal cultivars. It remains unknown whether *Escovopsis* infects cultivars while they are in the free-living phase.

Very early in their evolution, the Attini diverged into two lineages that would subsequently diversify into what Kusnezov (38) first recognized as the two major clades of attines, the ''Paleoattini'' and the ''Neoattini'' (Fig. 1). The three paleoattine genera are remarkably different from one another morphologically, a difference attributable to the span of time (≈40–45 mya) since they diverged from a common ancestor. Despite their morphological differences, these genera share a number of biologically important features (26, 38–40), the most striking of which is the consistent occurrence of a unique clear spot of unknown biological function on the wings of gynes (41). Early in the evolution of the Neoattini (50–30 mya) a temporal series of three successive divergences generated a grade of primitive lineages. These lineages are currently represented by, in order of oldest to youngest, the *Mycetophylax emeryi* species group, the genus *Mycetarotes*, and the species *Mycetosoritis hartmanni* (occurring in the southern U.S., with a sister species or conspecific in Central America) (42) (Fig. 1). Biological study of these extant, poorly known remnants of primitively diverged neoattine lineages may clarify the early evolution of ant agriculture.

Coral Fungus Agriculture. During the 50-million-year evolution of the fungus-growing ants, there occurred only one known transition to a nonleucocoprineaceous fungal cultivar. Although the majority of paleoattine species, including one of the basally diverging clades within *Apterostigma*, practices lower attine (leucocoprineaceous) agriculture, all known species in the ''*pilosum* group'' clade of the genus *Apterostigma* cultivate a clade of coral fungi (Pterulaceae) closely related to the genera *Pterula* and *Deflexula* (21, 22). Our results clearly indicate that the earliest *Apterostigma* species cultivated leucoprineaceous fungi, but between 10 and 20 mya, an *Apterostigma* species acquired a radically different fungal cultivar in the Pterulaceae that all its descendant species continue to cultivate. Recent research indicates that coral fungus agriculture is infected by a specialized grade of *Escovopsis* that is derived from a lower attine *Escovopsis* species and, further, that this grade subsequently gave rise to a clade that infects higher agricultural cultivars (24). This pattern most likely indicates that, after the origin of coral fungus agriculture, a coral-fungus-infecting *Escovopsis* switched hosts and began infecting higher attine cultivars. The broad overlap in dates of origin of coral fungus and higher attine agriculture (Fig. 1) is consistent with this hypothesis.

Yeast Agriculture. Another remarkable shift in cultivar type occurs in yeast-growing ants. Unlike typical attine mycelial gardens, yeast gardens consist of clusters of small, irregularly shaped nodules ≈0.5 mm in diameter (Fig. 1*C*) composed of fungal cultivars growing in a single-celled yeast phase rather than in the mycelial phase common to all other attine cultivars. Yeast agriculture is confined to the *Cyphomyrmex* ''*rimosus* group,'' which our results (Fig. 1) and prior work (1, 43, 44) indicate is monophyletic. The branch of the phylogeny subtending the *C. rimosus* group is remarkably long, indicating extensive evolutionary change and bracketing a broad potential time interval of 5–25 mya for the origin of yeast agriculture (Fig. 1). Significantly, this long branch in the ant phylogeny parallels a similarly long branch in the cultivar phylogeny (17) that subtends the attine yeast cultivars, members of a highly derived clade of leucocoprineaceous fungi that grow as yeast morphs when associated with attine ants. Like the lower attine cultivars from which they are derived, yeast cultivars are capable of a free-living, feral existence independent of the attine symbiosis (17) in which they grow on leaf litter in the mycelial phase typical for the rest of the tribe. Because yeast-phase growth is otherwise unknown in the order Agaricales, and because the attine yeast cultivars grow as yeasts only when associated with ants (or, depending on conditions, in artificial culture), yeast agriculture has been cited as a case of coadaptation and/or domestication (4). The parasite *Escovopsis* is unknown from yeast agriculture, suggesting that there may be some feature of the yeast morph that resists or prevents *Escovopsis* infection.

Higher Agriculture, Including Leaf-Cutter Agriculture. The transition to higher agriculture and the subsequent origin of leaf cutting are arguably the two most ecologically significant events in the evolutionary history of the Attini. The cultivars of higher attine ants are descended from lower agricultural cultivars (4, 15) but are derived in two features that suggest a significant degree of ''domestication,'' i.e., modification for life with ants. First, higher attine fungi do not appear capable of a free-living existence separate from their ant hosts, and, second, only higher attine

(*iv*) penalized likelihood, root age 66 mya. The tree shown here is the result of dating analysis (*iii*). Ant head photos (top to bottom): *Mycocepurus tardus*, *Myrmicocrypta infuscata*, *Apterostigma collare*, *Mycetophylax emeryi*, *Cyphomyrmex rimosus*, *Cyphomyrmex longiscapus*, *Trachymyrmex opulentus*, *Trachymyrmex cornetzi*, *Acromyrmex octospinosus*, *Atta laevigata*. Fungus gardens: (*A*) Lower attine agriculture. (*B*) Coral fungus agriculture. (*C*) Yeast agriculture. (*D*) Higher leaf-cutter agriculture. Country abbreviations: ARG, Argentina; AUS, Australia; BRAZ, Brazil; CR, Costa Rica; MAD, Madagascar; CR, Costa Rica; JAP, Japan; PAN, Panama; GUAT, Guatemala; GUY, Guayana; TRI, Trinidad; MEX, Mexico. Photo credits are given in *Acknowledgments*.

fungi produce "gongylidia," nutritious swollen hyphal tips produced by the fungus and harvested by the ants for food.

Our analyses produced a series of unexpected results that hold the potential for reconstructing the origin and subsequent evolution of higher agriculture with a high degree of resolution. First, the *Cyphomyrmex costatus* species group is the sister group of the combined higher Attini and *Mycetagroicus.* The four described species in the *C. costatus* group have always been regarded as aberrant members of the genus (43–45), but a phylogenetic position entirely removed from *Cyphomyrmex* as the sister group to the higher attines is unexpected. Second, the most recently discovered attine genus, *Mycetagroicus*, is the sister group of the higher attines. Described in only 2001 (3), nothing is known of the biology of the three *Mycetagroicus* species, including the form of agriculture they practice. Given that both the *C. costatus* species group and *Mycetagroicus* belong to lineages that successively diverged during the transition from lower to higher agriculture, biological study of these groups promises to elucidate the sequence of evolutionary change that generated this transition. Third, ants formerly placed in two major groups of *Trachymyrmex*, including the *T. opulentus* and *T. urichi* groups (46, 47), form a well supported clade that includes the genus *Sericomyrmex* and that is the sister group to the remainder of the higher attines. Fourth, the *Trachymyrmex septentrionalis* species group, which includes *T. diversus* and allied species (48), is closely related to the leaf-cutting ants. In fact, a clade of North American species (including *T. septentrionalis*) is the sister group of the leaf-cutting ants. This surprising result suggests that renewed biological study of the *T. septentrionalis* group, broadly defined, is likely to yield new information about the transition from generalized higher agriculture to leaf-cutter agriculture, one of the most successful evolutionary transitions in the animal kingdom (8, 9). Importantly, members of this group (*T. cornetzi* and *T. diversus*) have been observed to cut leaves (1) (T.R.S., personal observation), and *T. intermedius* is morphologically one of the most "*Acromyrmex*-like" of all *Trachymyrmex* species. Finally, leaf-cutting ants are remarkably young, originating between 8 and 12 mya. Such a recent origin for this ecologically dominant group explains their conspicuous absence from Dominican amber (15–20 mya) and may help to explain why, so far as is known, most leaf-cutting ants cultivate the same cultivar species (12–14).

Concluding Remarks. Agriculture is a specialized form of symbiosis that has evolved in only four known animal groups: humans, bark beetles, termites, and ants (11). Some researchers have hypothesized that similar evolutionary mechanisms may have driven the early evolution of agriculture in all of these groups (4, 49). Identifying those common mechanisms requires an understanding of the historical sequence of events that generated each system. Our results confirm that, like termites (50) but unlike humans (51, 52) and bark beetles (53), ants discovered agriculture a single time and discovered each of their derived agricultural systems a single time. We cannot know how many agricultural systems may have evolved during the 50-million-year-long evolutionary history of the Attini. Indeed, the attine ants are so poorly known (2) that it is possible that additional extant systems await discovery. Lineages that diverged at the critical evolutionary junctures that produced the five known attine agricultural systems are, fortunately, still represented by extant ant species that are available for biological study. Such study offers the most promising route for reconstructing the sequential accretion of ecological and behavioral characters that produced each ant agricultural system. Understanding the sequential evolution of the attine agricultural systems will, in turn, inform general hypotheses about the evolution of agricultural symbioses.

Methods

Data. Our data, obtained by using standard PCR techniques, consist of 2,459 aligned nucleotide sites from the coding regions of four nuclear genes: elongation factor 1-α F1 (EF1αF1) (1,075 bp), elongation factor 1-α F2 (EF1αF2) (517 bp), wingless (409 bp), and long-wavelength rhodopsin (opsin) (458 bp). All data in this study represent protein-coding (exon) sequences; intervening introns in opsin and EF1αF1 were not used because they could not be aligned confidently. We sampled 65 attine taxa and 26 nonattine outgroups. All sequences generated are new to this study except for previously published fragments from 4 attine and 10 nonattine outgroup species (33). Primers used for PCR amplification and sequencing are found in supporting information (SI) Table S1. Of the total 2,459 included nucleotide positions from all genes, 952 were variable and 847 parsimony informative. Sequences are deposited in GenBank; taxa and accession numbers are listed in Table S2.

Phylogenetic Analyses. Phylogenetic analyses used four methods: (*i*) parsimony, (*ii*) maximum likelihood, (*iii*) Bayesian nucleotide-model Markov Chain Monte Carlo (MCMC), and (*iv*) Bayesian codon-model MCMC.

Parsimony. Maximum parsimony (MP) analyses were conducted in PAUP* v4.0b10 (54) using heuristic searches with tree bisection–reconnection (TBR) and 1,000 random-taxon-addition replicates. Nonparametric bootstrap analyses (55) used TBR branch-swapping and consisted of 1,000 pseudoreplicates, with 10 random-taxon-addition replicates per pseudoreplicate. Analyses identified 12 most-parsimonious trees (MPTs) of length = 4,383, CI = 0.270, RI = 0.704. Successive-approximations-weighting analyses identified a single tree, one of the MPTs.

Maximum Likelihood (ML). The data and the MPT identified by successive-approximations weighting were evaluated under the Akaike information criterion (AIC) (56) as calculated in ModelTest v3.06 (57), identifying the GTR+I+Γ model of evolution. ML analyses consisted of four separate searches conducted in GARLI v0.951 (58) using the GTR+I+Γ model (with six Γ rate categories) and resulted in the topology presented in Fig. 1, with a log likelihood of $-$24,868.84927. A subsequent heuristic search in PAUP* using the most likely tree identified by the GARLI searches as the starting tree and employing TBR branch-swapping and the GTR+I+Γ model (with six Γ rate categories) resulted in exactly the same topology and likelihood score. Nonparametric bootstrap analyses consisted of 500 pseudoreplicates in GARLI under the same conditions as the ML search.

Bayesian MCMC. Bayesian analyses were conducted in MrBayes v3.1.2 (59). Burn-in and run convergence were assessed by comparing the mean and variance of log likelihoods, both by eye and by using the program Tracer v1.3 (available at http://beast.bio.ed.ac.uk/Tracer) (60); by examination of the MrBayes ''.stat'' output file; and by examination of the split frequencies diagnostic. For the nucleotide-model analyses, sequence data were divided into eight character partitions, four partitions consisting of the combined first and second codon positions for each of the four genes and four partitions consisting of the third codon position for each of the four genes. Based on ModelTest results, the wingless third-position character partition was assigned the GTR+Γ model; opsin and EF1αF2 third positions were separately assigned the HKY+I+Γ model; and all other character partitions were separately assigned the GTR+I+Γ model. Nucleotide-model analyses consisted of two independent runs of 5 million generations, each distributed over eight chains (seven heated and one cold; temperature parameter 0.05) with trees sampled every 100 generations and with a burn-in of 4.2 million generations. Codon-model analyses used a 2,454-bp dataset, from which incomplete codon triplets were excluded, and 88 taxa, in which multiple exemplars representing two species (*Cyphomyrmex cornutus* and *Acromyrmex lundi*) were reduced to a single exemplar. Sequence data were divided into four character partitions, one for each gene. Each partition was separately assigned the codon model. Codon-model analyses consisted of two independent runs of 10 million generations, each distributed over eight chains (seven heated and one cold; temperature parameter 0.05) with trees sampled every 100 generations and with a burn-in of 9 million generations.

Phylogenetic Mapping of Agricultural Systems. Terminal taxa were assigned states for a single six-state character representing the four attine agricultural systems and leaf-cutter agriculture (i.e., no agriculture, lower agriculture, yeast agriculture, higher agriculture, leaf-cutter agriculture, coral-fungus agriculture). Five species (*Myrmicocrypta* n. sp. Brazil, *Mycetagroicus triangularis*, *Cyphomyrmex* n. sp., *Cyphomyrmex morschi*, *Trachymyrmex irmgardae*, and *Pseudoatta* n. sp.) received ''unknown'' (i.e., ''?'') state assignments, and

Trachymyrmex papulatus received a ''lower agriculture'' state assignment based on a single garden collection from Argentina (a second colony from the same locality cultivated a typical higher attine garden). Character evolution was optimized onto the Bayesian codon-model consensus tree (with branch lengths) under both parsimony using MacClade (61) and maximum likelihood using the StochChar module provided in the Mesquite package (available at http://mesquiteproject.org) (62). Both methods produced the mappings shown in Fig. 1. Under parsimony, ancestral-state optimizations were unambiguous. Under the Markov k-state 1-parameter model (63), 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, 0.9831 for lower agriculture, 0.9995 for yeast agriculture, 0.9905 for higher agriculture, 0.9924 for leaf-cutter agriculture, and 0.9998 for coral-fungus agriculture.

Divergence Dating. We inferred divergence dates using both semiparametric and Bayesian relaxed clock methods. The first method used was the semiparametric penalized likelihood approach implemented in r8s v1.7 (64, 65). Branch lengths were first estimated on the ML topology using PAUP* under a GTR+I+Γ model. The *Pogonomyrmex* and two *Myrmica* species were used to root the tree during branch length estimation and were subsequently removed from all dating analyses. Thus, the root of the tree for all dating analyses represents the origin of the ''core myrmicines,'' a well supported clade established by previous work (33). Smoothing parameters were estimated by using the cross-validation feature in r8s. Confidence intervals were calculated by using 100 nonparametric bootstrap replicates of the dataset generated by Mesquite, followed by reestimation of branch lengths and divergence times for each replicate.

We calibrated three nodes with minimum-age constraints using attine Dominican amber fossils. These fossils are (*i*) *Apterostigma electropilosum*, a member of the *A. pilosum* group (40); (*ii*) *Cyphomyrmex maya* and *Cyphomyrmex taino*, both members of the *C. rimosus* group (66); and (*iii*) *Trachymyrmex primaevus*, a fossil of uncertain placement within the genus (67) (but see below). The fossils were used to calibrate stem-group nodes in the phylogeny (68). Because Dominican amber is dated between 15 and 20 mya (69), we calibrated these three nodes using a minimum age constraint of 15 mya. The r8s program requires that at least one node in the tree be either fixed or constrained with a maximum age. Using a maximum-age constraint for the root node proved unsatisfactory, because the program simply inferred the age of that node to be identical to the chosen maximum age, a common phenomenon in r8s that is underappreciated in many studies. We therefore conducted separate analyses in which the root node (i.e., ''core myrmicines'') was fixed with ages representing the range of plausible dates for that node obtained from a separate study (33). The root ages were 81, 73.5, and 66 mya.

The second method used was the Bayesian relaxed clock uncorrelated lognormal approach implemented in BEAST v1.4.6 (70, 71) with the SRD06 two-partition codon-specific rates model of sequence evolution (72) and a Yule process for the tree prior. The root node was given a normal (mean = 73.5; SD = 4.5) age prior distribution. The stem-group nodes represented by the three attine fossils described above were given the following age prior distributions (all with zero offset lower bounds of 15 mya): *Apterostigma 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). MCMC searches were run for 10,000,000 generations, with the first 2,000,000 discarded as burn-in. The searches achieved adequate mixing as assessed by the high ESS values for all parameters, plateaus for divergence time estimates over generations after burn-in, and repeatability of results over multiple independent runs.

Based on direct examination of a fossil specimen of *T. primaevus*, we find the placement of this species within the genus uncertain. Because Mayhé-Nunes and Brandão (47, 48) suggest that *T. primaevus* belongs to the *T. septentrionalis* group, we additionally tested the effects of this placement on age estimates for the origins of higher agriculture and leaf-cutter agriculture. In analyses with the *T. primaevus* calibration assigned to the *T. septentrionalis* group (*sensu lato*) branch, we obtained ages 2–4 million years older for the origins of higher agriculture and leaf-cutter agriculture. With the *T. primaevus* calibration excluded entirely, age estimates are 0–2 million years older than those reported.

Numerical values of all divergence dates are listed in Table S3 and Table S4. For more information, see the *SI Text*.

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Supporting Information

Schultz and Brady 10.1073/pnas.0711024105

Supporting Text

Hypothesis Testing. Both *Cyphomyrmex* and *Trachymyrmex* were found to be nonmonophyletic in all phylogenetic analyses. We conducted two parametric bootstrapping tests under maximum likelihood to test the alternative hypotheses of a monophyletic *Cyphomyrmex* (including *Mycetophylax conformis*) and a monophyletic *Trachymyrmex*. We used the SOWH test, which is valid on trees derived *a posteriori* without enumeration of all possible phylogenies (1, 2). For each test, the tree with the highest likelihood under the constraint of monophyly was inferred by using GARLI. One hundred replicate datasets were evolved by using the ML model parameters from the optimal tree over both the unconstrained and constrained phylogenies using Mesquite v.1.12 (3). These simulated data were then analyzed with GARLI using a simple batch script to infer and compare the likelihoods of the unconstrained and constrained hypotheses for each replicate. Statistical significance was assessed by determining the position of the difference between the constrained and unconstrained likelihoods for the observed data in the distribution of those differences for the simulated datasets. The results of both tests were highly significant ($P < 0.01$), indicating rejection of the hypotheses of *Cyphomyrmex* and *Trachymyrmex* monophyly given the data and model of molecular evolution used for the tests.

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Table S1. Primers used for PCR amplification and sequencing

Gene	Primer	Sequence (5′ to 3′)	Position	Source
EF1αF1	F1-383F	CATATWAACATTGTSGTSATYGG	*Apis* 383-405	This study
	F1-1887R	ACGGCSACKGTTTGWCKCATGTC	*Apis* 1887-1865	This study
	F1-494F	AAGGAGGCTCAGGAGATGGG	*Apis* 494-513	This study
	F1-1044R	CGTCTTACCATCGGCATTGCC	*Apis* 1044-1019	This study
	F1-792F	TTGGCGTGAAGCAGCTGATCG	*Apis* 792-812	This study
	F1-1189R	ACCTGGTTTYAAGATRCCGGT	*Apis* 1189-1169	This study
	F1-1109F	CCGCTTCAGGATGTCTATAA	*Apis* 1109-1128	This study
	F1-1551R	CCGCGTCTCAGTTCYTTTAC	*Apis* 1551-1532	This study
	F1-1424F	GCGCCKGCGGCTCTCACCACCGAGG	*Apis* 1424-1448	Ref. 1
	F1-1829R	GGAAGGCCTCGACGCACATMGG	*Apis* 1829-1808	Ref. 1
EF1αF2	F2-557F	GAACGTGAACGTGGTATYACSAT	*Apis* 557-579	Modified from ref. 2
	F2-1118R	TTACCTGAAGGGGAAGACGRAG	*Apis* 1118-1097	Ref. 1
wingless	Wg503F	CTCTCTCRTTACAGCACGT	*Pheidole* 503-521	This study
	Wg578F	TGCACNGTGAARACYTGCTGGATGCG	*Pheidole* 578-603	Ref. 3
	Wg1032R	ACYTCGCAGCACCARTGGAA	*Pheidole* 1032-1013	Ref. 1
opsin	LR143F	GACAAAGTKCCACCRGARATGCT	*Apis* 143-165	Ref. 3
	LR639ER	YTTACCGRTTCCATCCRAACA	*Apis* ≈639-624	Ref. 3

Position numbers correspond to: *Apis mellifera* GenBank X52884 (EF1αF1); *Apis mellifera* GenBank AF015267 (EF1αF2); *Pheidole morrisi* GenBank AY101369.1 (wingless); and *Apis mellifera* GenBank U26026 (opsin). For EF1αF1, initial PCR amplifications were conducted by using F1–383F and F1–1887R. This PCR product was used for nested reamplifications using the following primer pairs: F1–494F and F1–1044R; F1–792F and F1–1189R; F1–1109F and F1–1551R; F1–1424F and F1–1829R. For wingless, in some cases an initial amplification was conducted using Wg503F and Wg1032R, followed by heminested reamplification using Wg578F and Wg1032FR.

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3. Ward PS, Downie DA (2005) The ant subfamily Pseudomyrmecinae (Hymenoptera: Formicidae): phylogeny and evolution of big-eyed arboreal ants. *Syst Entomol* 30:310–335.

Table S2. Taxa with GenBank accession numbers

Genus	Species	Collector's no.	EF1aF1 exon 1	EF1aF1 exon 2	EF1aF2	opsin exon 1	opsin exon 2	wingless
Acanthognathus	*ocellatus*	McGlynn 297775	EU204345	EU204436	EU204586	EU204511	EU204268	EU204192
Acromyrmex	*octospinosus*	TRS921112-09	EU204298	EU204389	EU204541	EU204465	EU204222	EU204145
Acromyrmex	*versicolor*	PSW15404	EU204378	EF013211	EF013373	EF013534	EF013534	EF013662
Acromyrmex	*heyeri*	TRS030324-01	EU204363	EU204453	EU204604	EU204529	EU204286	EU204210
Acromyrmex	*landolti*	TRS030323-20	EU204364	EU204454	EU204605	EU204530	EU204287	EU204211
Acromyrmex	*lundi*	Roces 931103	EU204331	EU204422	EU204573	EU204497	EU204254	EU204178
Acromyrmex	*lundi*	UGM030404-01	EU204360	EU204450	EU204601	EU204526	EU204283	EU204207
Acromyrmex	*lundi*	TRS030409-01	EU204361	EU204451	EU204602	EU204527	EU204284	EU204208
Acromyrmex	*balzani*	TRS960404-04	EU204323	EU204414	EU204565	EU204490	EU204247	EU204170
Apterostigma	*auriculatum*	TRS 960824-10	EU204377	EF013230	EF013392	EF013549	EF013549	EF013677
Apterostigma	*pilosum complex sp. 4*	UGM980619-05	EU204348	EU204439	EU204589	EU204514	EU204271	EU204195
Apterostigma	*auriculatum*	UGM951208-01	EU204317	EU204408	EU204559	EU204484	EU204241	EU204164
Apterostigma	*cf. goniodes*	UGM980607-29	EU204347	EU204438	EU204588	EU204513	EU204270	EU204194
Apterostigma	*collare*	APT3/SIANTDB3568	EU204374	EU204464	EU204615	EU204540	EU204297	EU204221
Apterostigma	*dentigerum*	UGM980613-01	EU204349	EU204440	EU204590	EU204515	EU204272	EU204196
Apterostigma	*dorotheae*	TRS960416-09	EU204334	EU204425	EU204576	EU204500	EU204257	EU204181
Apterostigma	*manni*	TRS960429-06	EU204318	EU204409	EU204560	EU204485	EU204242	EU204165
Apterostigma	*new sp.*	AL030614-01	EU204367	EU204457	EU204608	EU204533	EU204290	EU204214
Apterostigma	*pilosum complex sp. 1*	UGM951208-02	EU204335	EU204426	EU204577	EU204501	EU204258	EU204182
Atta	*cephalotes*	UGM 960808-01	EU204350	EU204441	EU204591	EU204516	EU204273	EU204197
Atta	*laevigata*	TRS960417-01	EU204314	EU204405	EU204556	EU204481	EU204238	EU204161
Atta	*mexicana*	Chapela1/SIANTDB3588	EU204324	EU204415	EU204566	EU204491	EU204248	EU204171
Atta	*texana*	SES031122-02	EU204359	EU204449	EU204600	EU204525	EU204282	EU204206
Basiceros	*manni*	BLF10423	EU204379	EF013232	EF013394	EF013551	EF013551	EF013679
Blepharidatta	*brasiliensis*	TRS920825-14	EU204315	EU204406	EU204557	EU204482	EU204239	EU204162
Cataulacus	*sp. MAD02*	BLF10344	EU204380	EF013240	EF013402	EF013558	EF013558	EF013686
Cephalotes	*atratus*	TRS960407-10	EU204313	EU204404	EU204555	EU204480	EU204237	EU204160
Cyphomyrmex	*rimosus*	UGM940324-02	EU204299	EU204390	no seq	EU204466	EU204223	EU204146
Crematogaster	*sp.*	TRS960407-18	EU204328	EU204419	EU204570	EU204494	EU204251	EU204175
Cyphomyrmex	*cornutus*	UGM020604-07	EU204355	EU204445	EU204596	EU204521	EU204278	EU204202
Cyphomyrmex	*cornutus*	UGM020604-07	EU204366	EU204456	EU204607	EU204532	EU204289	EU204213
Cyphomyrmex	*costatus*	TRS960429-09	EU204321	EU204412	EU204563	EU204488	EU204245	EU204168
Cyphomyrmex	*faunulus*	TRS960407-20	EU204320	EU204411	EU204562	EU204487	EU204244	EU204167
Cyphomyrmex	*longiscapus*	UGM951211-05	EU204330	EU204421	EU204572	EU204496	EU204253	EU204177
Cyphomyrmex	*minutus*	TRS960408-19	EU204342	EU204433	EU204583	EU204508	EU204265	EU204189
Cyphomyrmex	*muelleri*	UGM960214-05	EU204369	EU204459	EU204610	EU204535	EU204292	EU204216
Cyphomyrmex	*new sp.*	TRS920818-01	EU204354	no seq	EU204595	EU204520	EU204277	EU204201
Cyphomyrmex	*new sp.*	UGM020603-13	EU204368	EU204458	EU204609	EU204534	EU204291	EU204215
Cyphomyrmex	*morschi*	NEST5/SIANTDB3672	EU204365	EU204455	EU204606	EU204531	EU204288	EU204212
Daceton	*armigerum*	TRS960410-11	EU204376	EF013251	EF013414	EF013565	EF013565	EF013693
Meranoplus	*sp.*	TRS990104-01	EU204346	EU204437	EU204587	EU204512	EU204269	EU204193
Monomorium	*pharaonis*	TRS960714-01	EU204326	EU204417	EU204568	no seq	no seq	EU204173
Mycetarotes	*acutus*	TRS000227-06	EU204351	EU204442	EU204592	EU204517	EU204274	EU204198
Mycetarotes	*cf. parallelus*	TRS920824-01	EU204307	EU204398	EU204549	EU204474	EU204231	EU204154
Mycetoagroicus	*triangularis*	TRS920729-03	EU204371	EU204461	EU204612	EU204537	EU204294	EU204218
Mycetophylax	*conformis*	TRS921106-06	EU204319	EU204410	EU204561	EU204486	EU204243	EU204166
Mycetophylax	*cf. emeryi*	TRS030323-19	EU204358	EU204448	EU204599	EU204524	EU204281	EU204205
Mycetophylax	*emeryi*	TRS960405-03	EU204311	EU204402	EU204553	EU204478	EU204235	EU204158
Mycetosoritis	*clorindae*	UGM040909-01	EU204370	EU204460	EU204611	EU204536	EU204293	EU204217
Mycetosoritis	*hartmanni*	SPC3527d	EU204312	EU204403	EU204554	EU204479	EU204236	EU204159
Mycocepurus	*tardus*	UGM960120-02	EU204341	EU204432	EU204582	EU204507	EU204264	EU204188
Mycocepurus	*smithi*	TRS960417-06	EU204310	EU204401	EU204552	EU204477	EU204234	EU204157
Mycocepurus	*smithi*	TRS030323-09	EU204357	EU204447	EU204598	EU204523	EU204280	EU204204
Mycocepurus	*curvispinosus*	UGM950612-03	EU204343	EU204434	EU204584	EU204509	EU204266	EU204190
Myrmica	*sp.*	TRS960207-01	EU204305	EU204396	EU204547	EU204472	EU204229	EU204152
Myrmica	*striolagaster*	PSW14963	EU204381	EF013296	EF013458	EF013598	EF013598	EF013726
Myrmicocrypta	*infuscata*	TRS960410-14	EU204375	EF013299	EF013461	EF013600	EF013600	EF013728
Myrmicocrypta	*buenzlii*	TRS960416-03	EU204344	EU204435	EU204585	EU204510	EU204267	EU204191
Myrmicocrypta	*ednaella*	UGM960121-02	EU204373	EU204463	EU204614	EU204539	EU204296	EU204220

Table S2. Continued

Genus	Species	Collector's no.	EF1aF1 exon 1	EF1aF1 exon 2	EF1aF2	opsin exon 1	opsin exon 2	wingless
Myrmicocrypta	*sp.*	UGM951227-01	EU204340	EU204431	EU204581	EU204506	EU204263	EU204187
Myrmicocrypta	*urichi*	UGM950118-01	EU204304	EU204395	EU204546	EU204471	EU204228	EU204151
Myrmicocrypta	*new sp.*	SIANTDB2655 (coll. Camargo)	EU204356	EU204446	EU204597	EU204522	EU204279	EU204203
Orectognathus	*versicolor*	PSW15299	EU204382	EF013312	EF013474	EF013611	EF013611	EF013739
Orectognathus	*sp.*	RS130/99	EU204352	EU204443	EU204593	EU204518	EU204275	EU204199
Pheidole	*clydei*	PSW14991	EU204383	EF013317	EF013479	EF013615	EF013615	EF013743
Pheidole	*hyatti*	PSW15214	EU204384	EF013318	EF013480	EF013616	EF013616	EF013744
Pogonomyrmex	*sp.*	TRS960405-06	EU204325	EU204416	EU204567	EU204492	EU204249	EU204172
Pristomyrmex	*pungens*	TRS020804-01	EU204353	EU204444	EU204594	EU204519	EU204276	EU204200
Proatta	*butteli*	SIANTDB4114	EU204329	EU204420	EU204571	EU204495	EU204252	EU204176
Procryptocerus	*scabriusculus*	PSW15064	EU204385	EF013336	EF013498	EF013632	EF013632	EF013760
Pseudoatta	*new sp.*	SIANTDB3579 (coll. Delabie)	EU204327	EU204418	EU204569	EU204493	EU204250	EU204174
Pyramica	*hoplites*	BLF5138	EU204386	EF013341	EF013503	EF013636	EF013636	EF013764
Sericomyrmex	*cf. parvulus*	TRS920823-02	EU204300	EU204391	EU204542	EU204467	EU204224	EU204147
Strumigenys	*dicomas*	BLF9176	EU204387	EF013352	EF013514	EF013645	EF013645	EF013773
Strumigenys	*propiciens*	TRS921112-07	EU204306	EU204397	EU204548	EU204473	EU204230	EU204153
Tetramorium	*caespitum*	TRS960526-01	EU204308	EU204399	EU204550	EU204475	EU204232	EU204155
Trachymyrmex	*arizonensis*	PSW15219	EU204388	EF013364	EF013526	EF013655	EF013655	EF013783
Trachymyrmex	*bugnioni*	TRS920825-05	EU204303	EU204394	EU204545	EU204470	EU204227	EU204150
Trachymyrmex	*cf. intermedius*	TRS960410-16	EU204336	EU204427	no seq	EU204502	EU204259	EU204183
Trachymyrmex	*cf. zeteki*	UGM951118-02	EU204339	EU204430	EU204580	EU204505	EU204262	EU204186
Trachymyrmex	*cornetzi*	TRS910324-02	EU204301	EU204392	EU204543	EU204468	EU204225	EU204148
Trachymyrmex	*diversus*	TRS920825-01	EU204302	EU204393	EU204544	EU204469	EU204226	EU204149
Trachymyrmex	*new sp.*	UGM950108-02	EU204333	EU204424	EU204575	EU204499	EU204256	EU204180
Trachymyrmex	*smithi*	UGM051208-01	EU204372	EU204462	EU204613	EU204538	EU204295	EU204219
Trachymyrmex	*irmgardae*	TRS960412-11	EU204322	EU204413	EU204564	EU204489	EU204246	EU204169
Trachymyrmex	*opulentus*	UGM951211-10	EU204332	EU204423	EU204574	EU204498	EU204255	EU204179
Trachymyrmex	*papulatus*	Agosti 373 no. 2 Nov 1994	EU204338	EU204429	EU204579	EU204504	EU204261	EU204185
Trachymyrmex	*septentrionalis*	UGM930313-01	EU204337	EU204428	EU204578	EU204503	EU204260	EU204184
Tranopelta	*cf. gilva*	TRS960424-13	EU204309	EU204400	EU204551	EU204476	EU204233	EU204156
Wasmannia	*auropunctata*	TRS920630-09	EU204316	EU204407	EU204558	EU204483	EU204240	EU204163
Wasmannia	*sp.*	TRS030324-02	EU204362	EU204452	EU204603	EU204528	EU204285	EU204209

Vouchers are accessible by request to T. R. Schultz, USNM.

Table S3. Inferred dates of origin for the major fungus-growing agricultural types

	Root = 66	Root = 73.5	Root = 81	Root prior = mean 73.5; SD 4.5
Lower agriculture				
Crown	46 (42, 49)	50 (44, 56)	55 (44, 66)	52 (44, 59)
Stem	47 (43, 51)	51 (45, 57)	56 (46, 66)	53 (46, 61)
Yeast agriculture				
Crown	5 (3, 7)	5 (3, 7)	6 (3, 8)	8 (5, 12)
Stem	18 (15, 21)	20 (16, 23)	21 (17, 25)	24 (20, 29)
Coral fungus agriculture				
Crown	11 (9, 13)	11 (6, 16)	12 (9, 15)	14 (9, 19)
Stem	15 (15, 16)	15 (15, 16)	16 (15, 20)	24 (20, 29)
Higher agriculture				
Crown	15 (12, 17)	16 (13, 19)	17 (14, 20)	21 (17, 25)
Stem	19 (16, 22)	20 (17, 24)	22 (18, 26)	26 (21, 30)
Leaf-cutter agriculture				
Crown	8 (6, 9)	8 (6, 10)	9 (8, 12)	11 (9, 14)
Stem	9 (7, 10)	9 (7, 11)	10 (8, 12)	13 (10, 16)

For each type, dates are given for the crown-group (i.e., earliest possible origin within the group) and the stem-group (i.e., latest possible origin). Relaxed-clock-divergence dating was conducted by using semiparametric penalized likelihood with the program r8s under a range of fixed values for the root node (root = 66, 73.5, 81) as well as a Bayesian uncorrelated lognormal approach using the program BEAST with a normal prior on the root node (mean 73.5; SD 4.5). Age estimates are in units of millions of years ago, with the lower and upper 95% confidence bounds shown in parentheses.

Table S4. The effect of the *Trachymyrmex primaevus* fossil calibration on the inferred ages of higher attine lineages

	r8 s			BEAST		
	A	B	C	A	B	C
Higher agriculture						
Crown	16(13,19)	19(17,21)	16(13,19)	21(17,25)	23(20,27)	22(18,27)
Stem	20(17,24)	24(20,27)	20(17,24)	26(21,30)	29(24,33)	28(23,33)
Leaf-cutter agriculture						
Crown	8 (6,10)	10 (8,12)	8 (6,10)	11 (9,14)	13(10,15)	12 (9,15)
Stem	9 (7,11)	12 (9,13)	9 (7,11)	13(10,16)	15(12,18)	14(11,17)

In all dating analyses reported in the main text, placement of this fossil within the genus is regarded as uncertain, and this fossil is used therefore to calibrate the stem lineage of the entire genus (treatment A in the table above). As discussed in *Materials and Methods*, it has been suggested that this fossil may be a member of the *T. septentrionalis* group. To examine the effect of this possibility on the inferred ages of the two higher attine agricultural systems, we conducted additional analyses in which this fossil instead calibrates the stem lineage of the *T. septentrionalis* group (*sensu lato*) (treatment B above). We also conducted analyses in which this fossil calibration was entirely removed (treatment C above). These comparisons were conducted by using both semiparametric penalized likelihood with the program r8 s (root = 73.5) and a Bayesian uncorrelated lognormal approach using the program BEAST with a normal prior on the root node (mean 73.5; SD 4.5). Age estimates are in units of millions of years ago, with the lower and upper 95% confidence bounds shown in parentheses.