COEVOLUTION

# The coevolution of fungus-ant agriculture

Ted R. Schultz[1]*, Jeffrey Sosa-Calvo[1], Matthew P. Kweskin[1], Michael W. Lloyd[1]†, Bryn Dentinger[2], Pepijn W. Kooij[3,4], Else C. Vellinga[5], Stephen A. Rehner[6], Andre Rodrigues[4], Quimi V. Montoya[4], Hermógenes Fernández-Marín[7,8], Ana Ješovnik[1]‡§, Tuula Niskanen[3]¶, Kare Liimatainen[3]¶, Caio A. Leal-Dutra[9], Scott E. Solomon[1]#, Nicole M. Gerardo[10], Cameron R. Currie[11], Mauricio Bacci Jr.[4], Heraldo L. Vasconcelos[12], Christian Rabeling[13, 14], Brant C. Faircloth[15], Vinson P. Doyle[16]

**Fungus-farming ants cultivate multiple lineages of fungi for food, but, because fungal cultivar relationships are largely unresolved, the history of fungus-ant coevolution remains poorly known. We designed probes targeting >2000 gene regions to generate a dated evolutionary tree for 475 fungi and combined it with a similarly generated tree for 276 ants. We found that fungus-ant agriculture originated ~66 million years ago when the end-of-Cretaceous asteroid impact temporarily interrupted photosynthesis, causing global mass extinctions but favoring the proliferation of fungi. Subsequently, ~27 million years ago, one ancestral fungal cultivar population became domesticated, i.e., obligately mutualistic, when seasonally dry habitats expanded in South America, likely isolating the cultivar population from its free-living, wet forest–dwelling conspecifics. By revealing these and other major transitions in fungus-ant coevolution, our results clarify the historical processes that shaped a model system for nonhuman agriculture.**

Agriculture is a particular form of mutualistic symbiosis that has arisen in more than 20 animal lineages, including humans, ants, termites, and ambrosia beetles (*1*–*7*). In fungus-farming ants, agriculture is thought to have originated once (*8*, *9*), leading to their diversification into 247 extant New World, largely Neotropical species (*10*). All are obligate fungus farmers, but they do not cultivate a single fungal species. Rather, ant agriculture comprises four (*11*, *12*) systems in which four phylogenetically related groups of ants cultivate four groups of fungi in the order Agaricales (Fig. 1) (*8*, *9*). These systems include (i) lower agriculture, thought to be the ancestral system, in which a paraphyletic group of 85 ant species cultivates fungi in the family Agaricaceae, tribe Leucocoprineae (Fig. 1, Lower Fungal Cultivars, Clades 1 and 2); (ii) yeast agriculture, in which a clade of 19 ant species (*Cyphomyrmex rimosus* group spp.) cultivates a clade of agaricaceous fungi that grow in a yeast-like phase otherwise unknown in the order Agaricales (Fig. 1, Yeast Cultivars); (iii) coral fungus agriculture, in which a clade of 30 ant species (*Apterostigma pilosum* group spp.) cultivates coral fungi in the family Pterulaceae (Fig. 1, Coral Fungus Cultivars); and (iv) higher agriculture, thought to be the most evolutionarily derived system, in which a clade of 113 ant species cultivates a clade of agaricaceous fungi that are multinucleate or polyploid (*13*) and consistently produce food bodies known as "gongylidia" (Fig. 1, Higher Fungal Cultivars) (*10*, *14*–*16*). Within higher agriculture, an ancestral ant population acquired the ability to harvest fresh vegetation as a fungus-garden nutritional substrate and gave rise to the leaf-cutter ants, 52 species that have become the primary herbivores of the Neotropics, with colonies attaining the highest levels of organizational complexity found in nonhuman animals (*7*, *17*, *18*). Because most leaf-cutter ants cultivate a single higher-cultivar species, *Leucoagaricus gongylophorus,* leaf-cutter agriculture is sometimes regarded as a fifth agricultural system (Fig. 1, *L. gongylophorus*). Rare collections of apparently non–ant-associated Neotropical mu rooms that have subsequently proven to be genetically conspecific with or closely related to ant-cultivated fungi have been interpreted to indicate that lower and yeast cultivars are facultative symbionts (Fig. 2, mushroom icons). By contrast, all collections of coral and higher-cultivar mushrooms have consistently occurred in association with ant nests, strongly suggesting that they are obligate symbionts [*10, 19, 20, 21* (section S3)].

Compared to the evolutionary history of fungus-farming ants, the evolutionary history of their fungal cultivars, including their relationships to noncultivated fungal species, remains largely obscure. Relationships among the fungal cultivar groups are poorly resolved owing to the few reliable phylogenetic markers presently available as well as to inadequate sampling of closely related, non–ant-cultivated fungi. As a result, existing studies disagree about the congruence of ant and cultivar phylogenies, the origins of the four agricultural systems, and the timing of key evolutionary events in ant agriculture (*10*, *13*, *22*–*25*). In this study, we used DNA sequence data from 625 conserved fungal loci to reconstruct a fossil-calibrated chronogram of 475 fungi, including 288 ant cultivars, and we used DNA sequence data from 1934 conserved ant loci to reconstruct a fossil-calibrated chronogram of 276 ants, including 208 fungus-farming ants. We combined the fungal and ant chronograms to clarify the coevolutionary history of ant agriculture.

The fungal chronogram (Fig. 1) indicates that all 288 ant-cultivated fungi belong to two families in the order Agaricales, the Pterulaceae and the Agaricaceae, and that, in the latter family, the ant-cultivated fungi are confined to the tribe Leucocoprineae. Within the Leucocoprineae, two closely related but separate clades of fungi, previously recognized and referred to as "Clade 1" and "Clade 2" (lower cultivars only) (*20*), are cultivated by the paraphyletic lower fungus-farming ants (Fig. 2). Likewise, within the Pterulaceae (coral fungi), two closely related but separate clades of fungi, *Myrmecopterula nudihortorum* and *Myrmecopterula velohortorum,* are cultivated by a subset of ants (the *pilosum* group) in the genus *Apterostigma* (*19*) (Fig. 2, Coral Fungus–Farming Ants).

## The origin of ant agriculture

The ant chronogram indicates that the ancestral fungus-farming ant population arose ~66.65 ± 13.28 million years ago (Ma) at the end of the Cretaceous (Fig. 2, most recent common ancestor (MRCA), Fungus-Farming Ants) [(*21*), section S2.2] and maximum likelihood–based ancestral-state reconstruction of fungal associations on the ant phylogeny indicates that it cultivated fungi in the Leucocoprineae (Fig. 2, Clades 1 and 2 Lower Cultivars) [(*21*), section S3]. Ancestral-state reconstruction on the fungal

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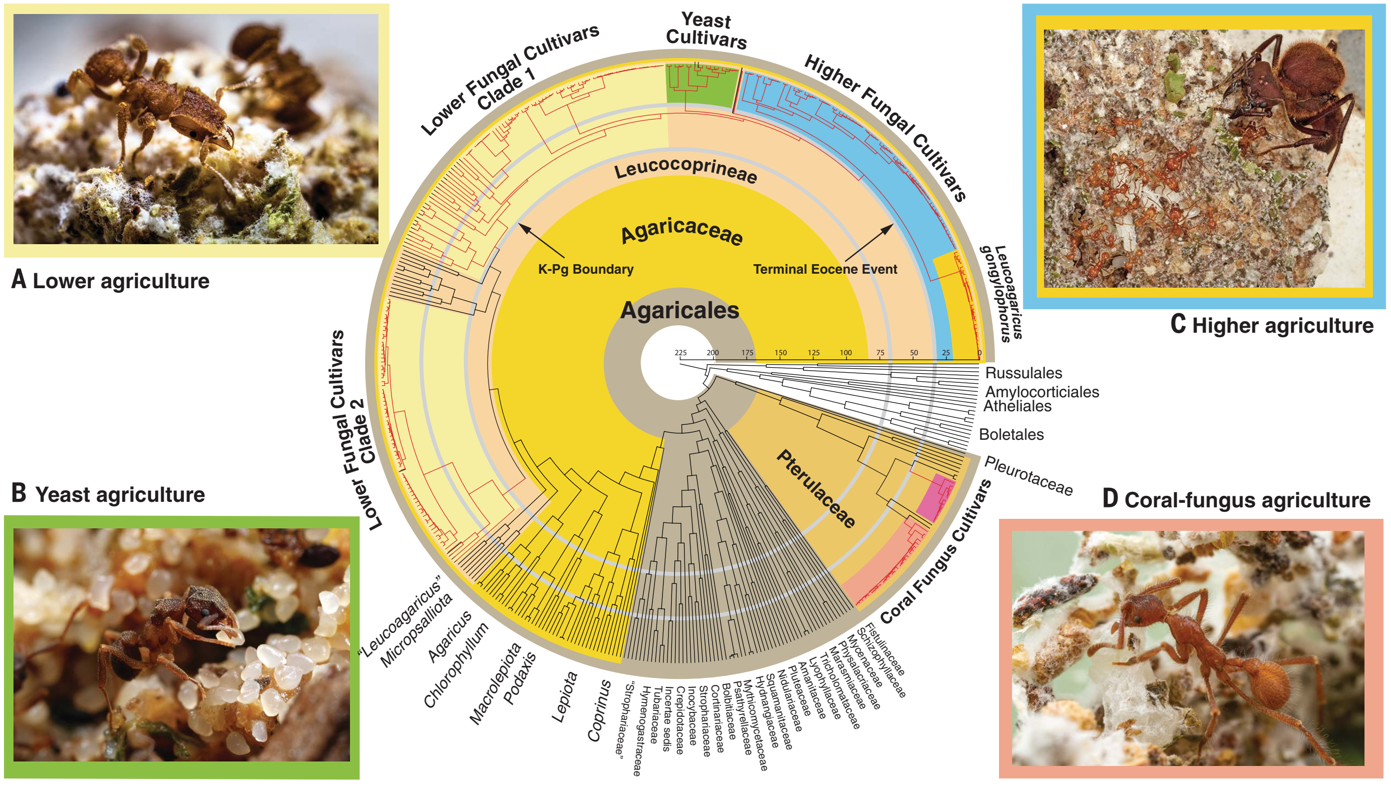
[1]National Museum of Natural History, Smithsonian Institution, Washington, DC, USA. [2]School of Biological Sciences, University of Utah, Salt Lake City, UT, USA. [3]Jodrell Laboratory, Royal Botanic Gardens, Kew, Surrey, UK. [4]Institute of Biosciences, Department of General and Applied Biology, São Paulo State University (UNESP), Rio Claro, São Paulo, Brazil. [5]University Herbarium, University of California at Berkeley, Berkeley, CA, USA. [6]Mycology and Nematology Genetic Diversity and Biology Laboratory, US Department of Agriculture Agricultural Research Center, Beltsville, MD, USA. [7]Centro de Biodiversidad y Descubrimiento de Drogas, Instituto de Investigaciones Científicas y Servicios de Alta Tecnología (INDICASAT AIP), Clayton, Panama. [8]Smithsonian Tropical Research Institute (STRI), Ancon, Panama. [9]Section for Ecology and Evolution, Department of Biology, University of Copenhagen, Copenhagen, Denmark. [10]Department of Biology, Emory University, Atlanta, Georgia, USA. [11]Faculty of Health Sciences, McMaster University, Hamilton, ON, Canada. [12]Instituto de Biologia, Universidade Federal de Uberlândia, Uberlândia, Minas Gerais, Brazil. [13]Social Insect Research Group, School of Life Sciences, Arizona State University, Tempe, AZ, USA. [14]Department of Integrative Taxonomy of Insects, Institute of Biology, and KomBioTa – Center for Biodiversity and Integrative Taxonomy; University of Hohenheim, Stuttgart, Germany. [15]Department of Biological Sciences and Museum of Natural Science, Louisiana State University, Baton Rouge, LA, USA. [16]Department of Plant Pathology and Crop Physiology, Louisiana State University AgCenter, Baton Rouge, LA, USA.
***Corresponding author. Email: schultzt@si.edu**
†Present address: The Jackson Laboratory, Bar Harbor, ME, USA.
‡Present address: Croatian Myrmecological Society, Zagreb, Croatia.
§Present address: Department of Biology, University of Zagreb, Zagreb, Croatia.
¶Present address: Botany Unit, Finnish Museum of Natural History, University of Helsinki, Helsinki, Finland.
#Present address: Department of Biosciences, Rice University, Houston, TX, USA.



**A** Lower agriculture

**B** Yeast agriculture

**C** Higher agriculture

**D** Coral-fungus agriculture

**Fig. 1. Chronogram resulting from Bayesian divergence-dating analyses of phylogenomic data for 288 ant-cultivated and 187 non–ant-cultivated fungi.** Orders and important families and genera are labeled. Red branches indicate ancestral-state reconstruction of the trait "cultivation by ants" under, in the Leucocoprineae, the relaxed ancestral-state reconstruction, earliest-possible origin scenario dicussed in the text. All fungal cultivars arise in two families in the order Agaricales, the Pterulaceae and the Agaricaceae, and, in the latter, in the tribe Leucocoprineae. In the Leucocoprineae, cultivation by ants arose in two separate cultivar clades (Clade 1 and Clade 2) coincident with the origin of fungus-farming ants and the K-Pg mass-extinction event. From within the Clade 1 cultivars, two highly specialized cultivar groups (the Yeast Cultivars and the Higher Cultivars) subsequently arose following the TEE. More recently, two separate cultivar clades originated in the Pterulaceae. (**A**) *Mycetophylax asper* worker on Clade 2 fungus garden; (**B**) *C. cf. rimosus* worker on yeast garden; (**C**) *Atta cephalotes* queen and workers on *L. gongylophorus* fungus garden; (**D**) *Apterostigma collare* worker on *M. velohortorum* coral fungus garden. [Photo credits: (A) Don Parsons; (B and D) Alex Wild; (C) Karolyn Darrow]

chronogram indicates that the trait "cultivation by ants" arose separately in Clades 1 and 2, and ancestral-state reconstruction under "relaxed" coding further indicates that branches 2.1 in Clade 2 and 1.3 in Clade 1 are the earliest on which this trait could have originated (Fig. 2, red branches) [(*21*), section S3]. Under this "earliest possible" reconstruction, ant and fungal node-age posterior probability distributions strongly overlap (Fig. 3) [(*21*), section S2.4], consistent with a near-simultaneous origin of agriculture in the ants and in both Clade 1 and 2 fungi. Of the possible branches of origin in Clades 2 (branches 2.1 to 2.3) and 1 (branches 1.3 to 1.7) (Fig. 2) [(*21*), section S3], the highest rates of positive selection in ultraconserved element (UCE) protein-coding regions also occurred on branches 2.1 and 1.3 (Fig. 2 and tables S2.4.02 and S4.3) [(*21*), section S4]. This is consistent with a hypothesis of directed evolutionary change favoring ant-cultivation traits because the gene functions of many of the positively selected UCE loci match those of genes identified in prior studies of the fungus-ant mutualism [(*21*), section S4; (*26*–*28*)]. It remains possible that, rather than on branches 2.1 and 1.3, cultivation by ants arose later in Clade 2 or Clade 1 on branches 2.2 and 1.4, respectively (Figs. 2 and 3), although the rates of positive selection on those branches are much lower, and their age distributions overlap much less with those of fungus-farming ants (Fig. 3, figs. S2.4.04 and S2.4.07, and table S2.4.02). Alternatively, "strict" ancestral-state reconstruction indicates four origins of cultivation by ants that do not temporally overlap with the origin of the ants (Fig. 2, red asterisks, and figs. S2.4.02 and S3.17) [(*21*), section S3].

These results suggest that ant agriculture arose coincidently, or nearly so, with the Cretaceous-Paleogene (K-Pg) boundary (Figs. 2 and 3)—when a bolide collided with Earth, caused firestorms for days to weeks, and shut down photosynthesis for several or more months—during which fungi proliferated and global mass extinctions occurred (*29*–*31*). The types of animals most likely to survive such conditions were small, semisubterranean detritivores or their predators, including, among the former, invertebrates specialized on locating and consuming decaying organic matter or scattered dormant seeds and insects (*32*, *33*). Under such conditions, preadapted (see "Proto-agriculture" below) fungus-farming ants and leucocoprineaceous fungi would have constituted a formidable mutualism in which the ants located organic materials and the fungi digested them, sharing the nutrition with the ants. This would have allowed both partners to persist and coevolve through a period of mass extinctions and, ultimately, to radiate into an entirely restructured Neotropical rain forest ecosystem ~59 to 59.5 Ma (*34*) (Fig. 2, PO). Corroborating this scenario, the ancestor of the ant subtribe Dacetina, the sister group that arose simultaneously with the fungus-farming ants (*9*) (Fig. 2) [(*21*), section S2.2], was likely a specialized predator of Collembola (*35*), another detritivorous food source that was also likely abundant during the post–K-Pg period.

## Proto-agriculture

In an evolutionarily convergent pattern, cultivation by ants arose twice within the Leucocoprineae at the origin of ant agriculture and,

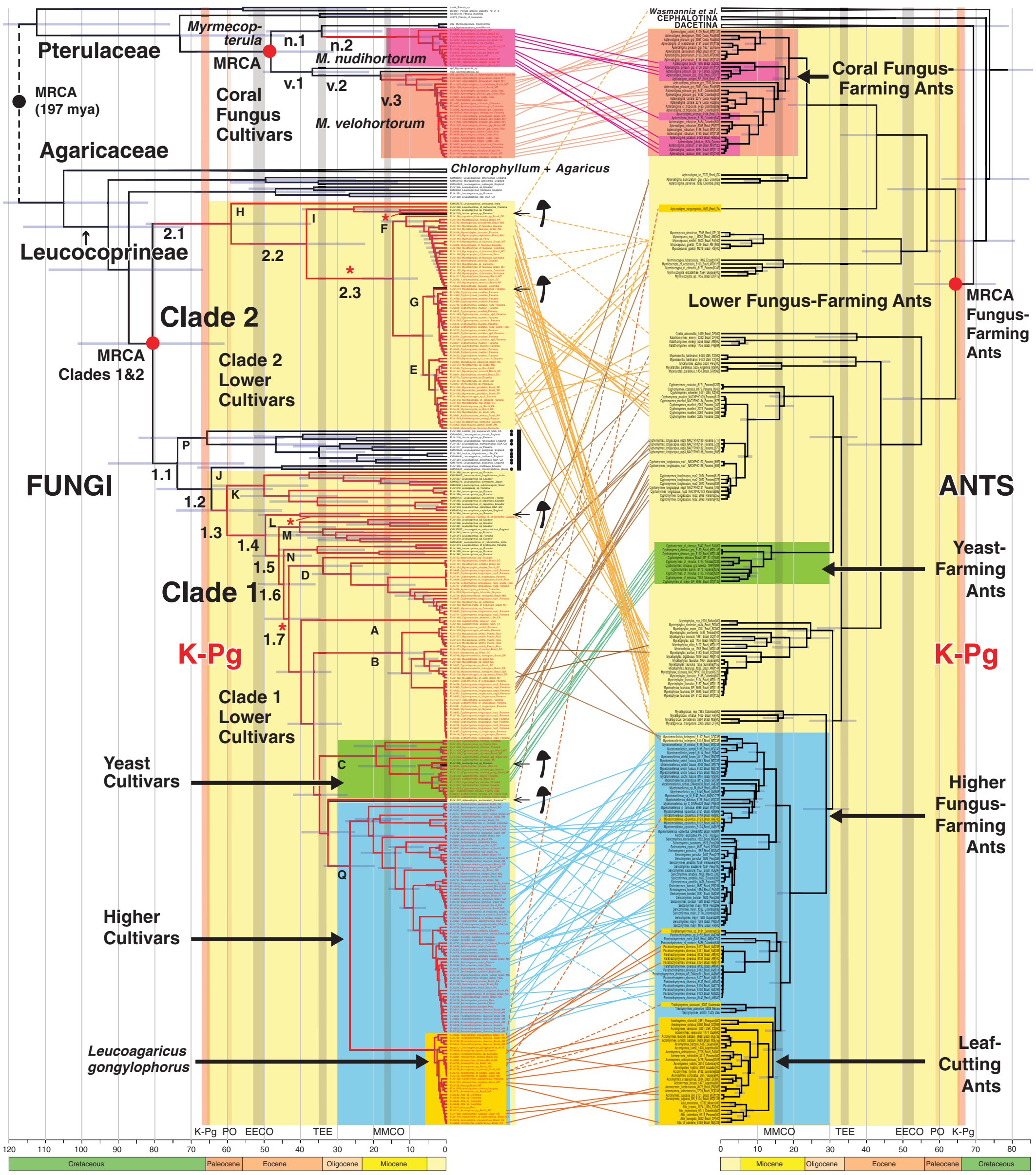


**Fig. 2. Fungus and ant chronograms resulting from Bayesian divergence-dating analyses of phylogenomic data.** Chronograms (fungus, left; ant, right) are excerpted from larger chronograms (Fig. 1) [(*21*), section S2.2] and presented at the same chronological scale to visualize fungus-ant coevolution. Fungi named in red were collected from ant gardens, and fungi named in black were collected as free-living mushrooms. Mushroom icons indicate fungal species known from both ant gardens and free-living mushrooms [(*21*), section S3]. Black circles and the vertical black bar to the right of subclades P and J indicate fungal species with distributions not known to overlap with those of fungus-farming ants. Solid lines connecting fungi and ants (center) indicate pairs collected from the same 137 nests, and dashed lines indicate fungus-ant associations known from prior observations. Red branches indicate ancestral-state reconstruction of the trait "cultivation, by ants" under the relaxed ancestral-state reconstruction, earliest-possible origin scenario discussed in the text; red asterisks indicate branches of origin inferred under strict ancestral-state reconstruction. Fungal subclade labels A to Q reference prior naming conventions (*22*, *60*); numbered branches are referenced in the text. PO, post–K-Pg peak of origin of neotropical plant species (*34*); EECO, Early Eocene Climatic Optimum; MMCO, Mid-Miocene Climatic Optimum.

more recently, twice within the Pterulaceae ~21 Ma (Fig. 2). The alternative hypothesis that cultivation by ants had single origins in the Leucocoprineae and the Pterulaceae (Fig. 2; MRCA, Clades 1 and 2; and MRCA, *Myrmecopterula*) is strongly contradicted by ancestral-state reconstruction owing to the separation of ant-cultivated clades in both groups by interpolated, non–ant-cultivated fungi (Fig. 2, black branches) [(*21*), section S3]. Natural history data suggest a common explanation for these convergent dual origins: non–ant-cultivated ancestors in *Myrmecopterula* and in the Leucocoprineae were likely involved in proto-agricultural symbiotic associations with ants and were thus preadapted for cultivation (*14*, *36*–*38*). Basidiomes of non–ant-cultivated *Myrmecopterula* species have been found emerging from inactive and active fungus-farming ant nests, including those of Leucocoprineae cultivators, where they are thought to obtain nourishment from fungus gardens (*19*). The origin of the *Myrmecopterula* MRCA around 48.16 ± 18.75 Ma (crown node age) (Fig. 2) [(*21*), section S2] is consistent with this ecological relationship and suggests that proto-agricultural coral fungi may have been associating with leucocoprineaceous cultivars as parasites or commensals since shortly after the origin of ant agriculture. As in *Myrmecopterula*, some extant, non–ant-cultivated Leucocoprineae species thrive in nutrient-rich, disturbed habitats, including abandoned ant and termite nests (*39*). Facultative fungivory and unidentified fungi growing in nests have been reported in the non–fungus-farming ant genera *Blepharidatta* and *Wasmannia*, close relatives of fungus-farming ants (*40*–*42*). In this work, we corroborate those prior observations by identifying one such fungus, collected as hyphae in the debris wall of a nest of *Wasmannia auropunctata* in Brazil, as belonging to leucocoprineaceous cultivar Clade 2 (Fig. 2, Clade 2 Lower Cultivars, subclade F, associated with "*Wasmannia* et al.," right). Prior to the origin of ant agriculture, the ancestors of fungal cultivars were likely transported by ants to refuse piles and debris walls in or near their nests, as in the case of *W. auropunctata*, and the fungi likely evolved to encourage such transport, most likely through food rewards, leading to increased fungivory in the ants (*12*, *14*, *38*). Such proto-agricultural symbioses are evolutionarily convergent with the origins of many human domesticates, including chenopods, cucurbits, and tomatoes, that, prior to cultivation, thrived as "camp followers" in human-disturbed habitats, such as clearings, kitchen middens, and refuse dumps, into which they were similarly transported by humans (*1*, *37*, *43*–*45*).

## Domestication

“Domestication” has been variously defined (*46*) but has perhaps been defined most stringently as requiring genetic modification of the domesticate that benefits the farmer, but that would reduce the fitness of the domesticate in its original niche (*1*). By this definition, the higher fungal cultivars (Fig. 2, Higher Cultivars), which are multinucleate or polyploid and unable to live apart from their ant farmers (*13*), are clearly domesticated and, as such, may represent the most notable evolutionary transition in ant agriculture (*12*). Our results indicate that the ancestor of the higher cultivars evolved from a Clade 1 lower cultivar (Fig. 2, subclade Q) [(*21*), sections S2.4 and S3] and that its origin was accompanied by the highest rate of positive selection of any branch that we measured (fig. S4.3 and table S2.4.02) [(*21*), section S4]. Combined data from the fungus and ant chronograms indicate that higher ant agriculture originated ~27 Ma (Fig. 2) [(*21*), section S2.4] following the Terminal Eocene Event (TEE, also known as the Eocene-Oligocene Transition, ~34 Ma; Fig. 2, vertical gray lines), a period of abrupt global cooling and decreased atmospheric carbon dioxide that in South America precipitated the expansion of seasonally dry habitats, particularly woody savannahs and grasslands, resulting in the fragmentation of previously continuous wet tropical forests (*47*–*51*). A post-TEE origin of higher agriculture is consistent with the hypothesis that, as some species of wet forest–dwelling, fungus-farming ants adapted to dry or seasonally dry habitats and transported their forest-adapted fungal cultivars into those habitats, cultivar species became isolated from their extended, free-living ancestral populations (*9*). As in many examples from human agriculture in which cultivars were carried beyond their ancestral ranges (*52*–*56*), transport into seasonally dry habitats by ants would have facilitated the process of fungal cultivar domestication and the observed dependence of extant higher cultivars on their ant farmers for propagation and survival. Like the higher cultivars, the yeast cultivars also arose from a Clade 1 lower cultivar and also have a post-TEE origin (~32 Ma) (Fig. 2) [(*21*), section S2.4], so it is plausible that they are likewise the products of prolonged ant-fungus coevolution in a seasonally dry habitat, especially in light of the recent discovery that the sister species of the yeast-farming ants is *Paramycetophylax bruchi,* known only from xeric habitats in Argentina (*57*).

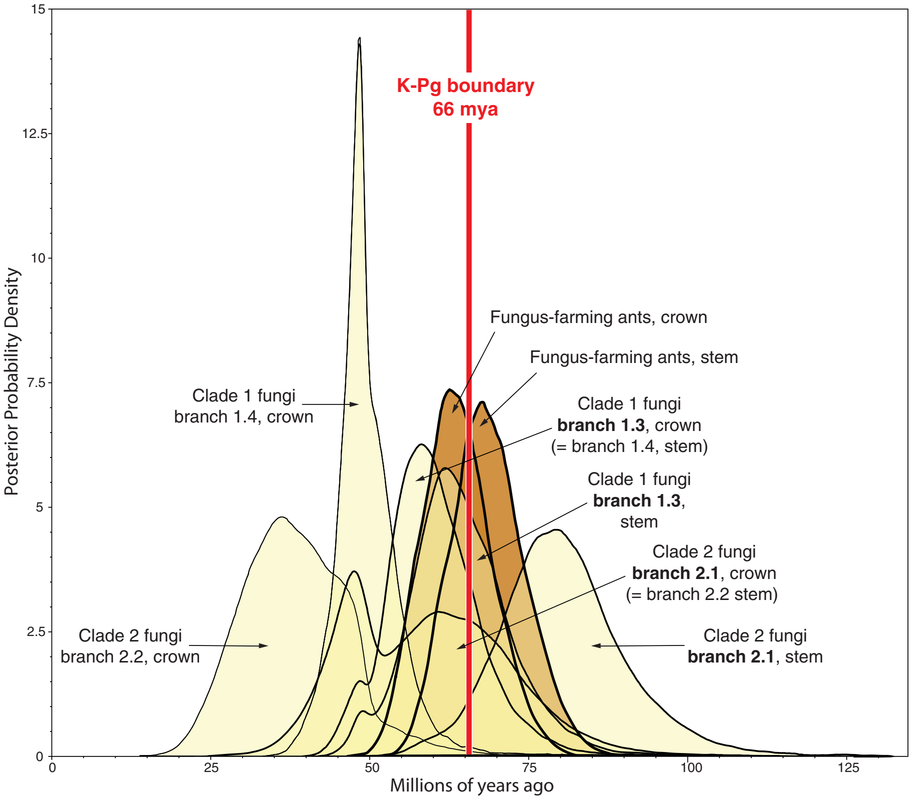


**Fig. 3. Posterior-probability distributions for stem and crown node ages of the ancestral branch of fungus-farming ants (brown) on the ant phylogeny and for four branches on the fungal phylogeny (yellow) on which “cultivation by ants” may have originated.** See Fig. 2 for the ancestral ant branch (MRCA Fungus-Farming Ants) and for branches 2.1 and 2.2 in Clade 2 and 1.3 and 1.4 in Clade 1 on the fungal phylogeny. The vertical red line (K-Pg) indicates the end-of-Cretaceous extinction event. Based on relaxed ancestral-state reconstruction, branches 1.3 and 2.1 (bold) are the earliest possible branches on which cultivation by ants could have originated, but branches 1.4 and/or 2.2 are also candidates, although their crown-node ages only slightly overlap with the ant ages of origin [(*21*), section S3].

## Conclusions

We have mapped major transitions in fungus-ant coevolution to corresponding synchronous branches of the fungal and ant chronograms. In the fungi, significant levels of positive selection occurred in protein-coding regions of UCE loci on those branches, but, although the functions of many of these loci have been previously implicated in fungus-ant coevolution (*26*–*28*), they involve basic cellular processes with poorly understood implications for the evolution of ant agriculture [(*21*), section S4]. Reconstructing the reciprocal fungus-ant genomic coevolution that presumably coincided with the origins of proto-agriculture and agriculture—as well as with those of yeast agriculture, coral fungus agriculture, and higher agriculture—will require the comparative study of genes directly involved in the ant-fungus mutualism, including, in the fungi, laccases, hemicellulases, chitinases, lignin-modifying enzymes, and other plant-degrading enzymes and, in the ants, genes associated with chemoreception, behavior, and energy metabolism, among others (*26*–*28*, *58*, *59*). Our results will serve to guide those future efforts because they identify the branches of origin on the fungal and ant chronograms and, thus, the extant fungal and ant species most critical for comparative genomic study.

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## SUPPLEMENTARY MATERIALS

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Supplementary Text
Figs. S1.4.01 to S4.15
Tables S1.1 to S4.3
References (*62–157*)
MDAR Reproducibility Checklist

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