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Phylogeny and evolution of the cryptic fungus-farming ant genus *Myrmicocrypta* F. Smith (Hymenoptera: Formicidae) inferred from multilocus data

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Abstract. Fungus-farming ants (Hymenoptera: Formicidae) have become model systems for exploring questions regarding the evolution of symbiosis. However, robust phylogenetic studies of both the ant agriculturalists and their fungal cultivars are necessary for addressing whether or not observed ant–fungus associations are the result of coevolution and, if so, whether that coevolution has been strict or diffuse. Here we focus on the evolutionary relationships of the species within the ant genus *Myrmicocrypta* and of their fungal cultivars. The fungus-farming ant genus *Myrmicocrypta* was created by Fr. Smith in 1860 based on a single alate queen. Since then, 31 species and subspecies have been described. Until now, the genus has not received any taxonomic treatment and the relationships of the species within the genus have not been tested. Our molecular analyses, using ∼40 putative species and six protein-coding (nuclear and mitochondrial) gene fragments, recover *Myrmicocrypta* as monophyletic and as the sister group of the genus *Mycocepurus* Forel. The species *M. tuberculata* Weber is recovered as the sister to the rest of *Myrmicocrypta*. The time-calibrated phylogeny recovers the age of stem group *Myrmicocrypta* plus its sister group as 45 Ma, whereas the inferred age for the crown group *Myrmicocrypta* is recovered as 27 Ma. Ancestral character-state analyses suggest that the ancestor of *Myrmicocrypta* had scale-like or squamate hairs and that, although such hairs were once considered diagnostic for the genus, the alternative state of erect simple hairs has evolved at least seven independent times. Ancestral-state analyses of observed fungal cultivar associations suggest that the most recent common ancestor of *Myrmicocrypta* cultivated clade 2 fungal species and that switches to clade 1 fungi have occurred at least five times. It is our hope that these results will encourage additional species-level phylogenies of fungus-farming ants and their fungal cultivars, which are necessary for understanding the evolutionary processes that gave rise to agriculture in ants and that produced the current diversity of mutualistic ant–fungus interactions.

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Introduction

Agriculture has arisen at least ten times in humans as early as *c*. 10 000–11 000 years ago (Rindos, 1984; Johns, 1990; Price & Gebauer, 1995; Cavalli-Sforza *et al.*, 1996; Diamond, 1997, 1998; Cavalli-Sforza, 2001; Schultz *et al.*, 2005; Hölldobler & Wilson, 2010). In contrast, agriculture arose a single time in ants *c*. 55–60 Ma in the ancestor of the fungus-farming ants, following or coincident with the K-Pg extinction event (Mueller *et al.*, 2005; Schultz & Brady, 2008; Rabeling *et al.*, 2011; Ward *et al.*, 2015; Nygaard *et al.*, 2016; Branstetter *et al.*, 2017).

The evolutionary history of fungus-farming ants has been the subject of multiple morphologic, molecular phylogenetic, and phylogenomic studies (Schultz & Meier, 1995; Wetterer *et al.*, 1998; Schultz & Brady, 2008; Schultz *et al.*, 2015; Branstetter *et al.*, 2017). However, to date only four genus-level studies have been undertaken (Bacci Jr *et al.*, 2009; Rabeling *et al.*, 2011, 2014; Ješovnik *et al.*, 2017). Of these four studies, two of them focused on the so-called 'higher' fungus-farming ants, which include the conspicuous leaf-cutting ants, and two of them focused on a single genus (*Mycocepurus* Forel) of the so-called 'lower' fungus-farming ants. Higher fungus-farming ants are derived from lower fungus-farming ants and cultivate a clade of 'higher' fungal cultivars that are presumably derived from the group of closely related fungal genera that includes the 'lower' fungal cultivars. Unlike the lower fungal cultivars, which are facultative symbionts, higher fungal cultivars are apparently obligate symbionts that are unable to live apart from their ant hosts and share a suite of derived traits including polyploidy (lower cultivars are diploid), significant shifts (relative to lower cultivars) in decompositional enzyme profiles, and consistent production of gongylidia, swollen hyphal tips preferentially harvested and eaten by the ants (rare in lower cultivars) (de Fine Licht *et al.*, 2010, 2013, 2014; Masiulionis *et al.*, 2014; Nygaard *et al.*, 2016).

In spite of this prior focus on the phylogenetics of higher fungus-farming ants, understanding the evolutionary history of lower fungus-farming ants, especially those belonging to the Paleoattina subclade, the basally diverging genera in the Neoattina subclade, and the nonfungus-farming sister clade to the Attina, is crucial to understanding the transition from hunting-gathering to agricultural behaviour in ants.

Fungus-farming 'attine' ants (Formicidae: Myrmicinae: Attini: Attina) comprise a group of ∼250 exclusively New World species in 17 known genera (Sosa-Calvo *et al.,* 2018) that rely exclusively on basidiomycete fungi {Agaricales: Agaricaceae [specifically *Leucoagaricus* Locq. ex Singer*, Leucocoprinus* Pat.*,* and *Lepiota* (Pers.) Gray] and Pterulaceae} that the ants cultivate for food (Wheeler, 1907; Weber, 1958a, 1966). The ants are so highly dependent on their fungal cultivars that, upon departing their maternal nests, unmated queens carry within their infrabuccal pockets a small portion of the original (natal) nest mycelium. This pellet is then used in the foundation of a new colony (Von Ihering, 1898; Huber, 1905; Weber, 1972; Quinlan & Cherrett, 1978; Mintzer & Vinson, 1985; Fernández-Marín *et al.*, 2004; Seal & Tschinkel, 2007; Mueller *et al.*, 2008; Mueller & Rabeling, 2008). The fungus garden supplies the ant colony with a reliable food source and in return the ants provide the cultivar with nourishment, dispersal, and protection from pathogenic microbes through behavioural, chemical, and biological control (Quinlan & Cherrett, 1977; Currie *et al.*, 1999a; Currie & Stuart, 2001; Mueller *et al.*, 2005; Fernández-Marín *et al.*, 2009). In addition to the ants and their cultivars, the attine agricultural symbiosis also includes an ascomycete fungal crop disease (*Escovopsis,* Hypocreaceae, Hypocreales) that is found, as far as is known, only in attine fungus gardens, where it consumes the fungal cultivar (Currie *et al.*, 1999b; Currie, 2001; Currie & Stuart, 2001; Currie *et al.*, 2003; Reynolds & Currie, 2004), and which is controlled, at least in part, by antibiotics produced by symbiotic actinomycete bacteria (*Pseudonocardia* and *Amycolaptosis*) growing on the ants' integuments (Wilkinson, 1999; Currie *et al.*, 1999a; Kost *et al.*, 2007; Mueller *et al.*, 2008; Sen *et al.*, 2009). Although there has been a debate regarding the roles of attine integumental actinomycetes and the extent to which they have coevolved with the other attine symbionts (Mueller *et al.*, 2008, 2010; Sen *et al.*, 2009; Cafaro *et al.*, 2011; Mattoso *et al.*, 2011; Caldera & Currie, 2012), recent studies largely reconcile these disagreements (Scheuring & Yu, 2012; Andersen *et al.*, 2013, 2015).

Attine agriculture has been divided into four distinct agricultural systems, characterized by consistent associations of ant, cultivar, and crop-disease lineages as: (i) lower agriculture, (ii) coral-fungus agriculture, (iii) yeast agriculture, and (iv) higher agriculture (Schultz & Brady, 2008; Mehdiabadi & Schultz, 2010). Each of these four agricultural systems is characterized by morphologically and phylogenetically distinct attine fungal cultivar groups (clades and grades) associated with phylogenetically distinct subsets (clades and grades) of fungus-farming ants (Mueller, 2002). A fifth agricultural system, leaf-cutting agriculture, is nested within higher agriculture and is characterized by, in the ants, larger colony sizes, strong worker polymorphism, and leaf- and grass-cutting, as well as apparently consistent associations, in most but not all species, with what is thought to be a single higher-attine fungal cultivar species, *Leucocoprinus gongylophorus* (Mueller *et al.*, 2017, 2018). The well-known and conspicuous leaf-cutting ants (*Atta* Fabricius and *Acromyrmex* Mayr, including the social parasite *Pseudoatta* Gallardo), representing approximately 20% of fungus-farming ant diversity, use freshly cut, living plant material as the substrate for their fungus gardens, making them important pests of human agriculture in the New World tropics. Non-leaf-cutting higher-attine ants in the genera *Trachymyrmex* Forel, *Sericomyrmex* Mayr and *Xerolitor* Sosa-Calvo *et al.* take a mixture of fresh vegetation and other organic detritus for substrate and at best may cut tender vegetation and flowers. In contrast, the less derived, lower fungus-farming ants primarily use insect frass and other organic detritus (e.g. seeds, disarticulated flower parts) as garden substrate. These less conspicuous, much more poorly known groups are more likely to provide the biological information necessary for understanding deeper attine evolution. Of these 'lower attines', the genus *Myrmicocrypta*, which is thought to retain many morphological symplesiomorphies for the subtribe, is arguably one of the most important for

understanding the origin and early evolution of agriculture in ants (Schultz & Meier, 1995; Fernández-Marín *et al.*, 2005; Mueller *et al.*, 2005; Schultz, 2007; Schultz & Brady, 2008).

The cryptic fungus-farming ant genus Myrmicocrypta

Myrmicocrypta, one of the largest yet most poorly known fungus-farming ant genera (Schultz & Meier, 1995; Wetterer *et al.*, 1998; Price *et al.*, 2003; Schultz & Brady, 2008), has retained many character states considered plesiomorphic for the group, including characters of wing venation (Kusnezov, 1961, 1962, 1963), male antennae (Kusnezov, 1961), degree of queen/worker polymorphism (Wheeler, 1910), monomorphism of the worker caste (Wheeler, 1910, Emery, 1912), larval morphology, including the form of the galea in some species and straight (rather than curved) body profile (Schultz & Meier, 1995), position on the integument of mutualistic *Pseudonocardia* Henssen (Actinomycetes) bacterial symbionts (Currie *et al.*, 1999a), and the use, by the nest-founding queen, of her shed forewing as a platform for the incipient garden (Fernández-Marín *et al.*, 2004). Based on these characters, some authors have identified *Myrmicocrypta* as the member of the Attina with the most plesiomorphic traits (Wheeler, 1910; Emery, 1912, 1913; Kusnezov, 1955; Schultz & Meier, 1995) or have even concluded that it is paraphyletic with respect to the remainder of the subtribe (Schultz & Meier, 1995). More recent multilocus molecular phylogenetic analyses, including analyses of phylogenomic data, however, have found it to be monophyletic (Schultz & Brady, 2008; Sosa-Calvo *et al.*, 2013; Branstetter *et al.*, 2017).

The genus *Myrmicocrypta* (Formicidae: Myrmicinae: Attini) was established by Fr. Smith (1860) based on an alate gyne collected in São Paulo, Brazil. Distributed in the Neotropics from Mexico through northern Argentina (Kempf, 1972; Fernández & Sendoya, 2004), the genus currently comprises 31 described species and subspecies (Bolton, 1995; Bolton *et al.*, 2006; Sosa-Calvo & Schultz, 2010), but this number will certainly increase (Sosa-Calvo & Schultz, unpublished data). Except for the Republic of Trinidad and Tobago, which is a biotic extension of the mainland, the genus is unknown in the Caribbean (Wheeler, 1922; Weber, 1958a,b, 1968; Wilson, 1988) and from amber inclusions (Wilson, 1988). See Kempf (1972), Mayhé-Nunes & Jaffé (1998), Fernández & Sendoya (2004); Bolton *et al.* (2006) for distributional information.

Colonies of most *Myrmicocrypta* species tend to be small, consisting of as few as 100 individuals (Weber, 1945; Murakami & Higashi, 1997; Price *et al.*, 2003), several hundred individuals (Weber, 1945; Murakami *et al.*, 2000; Sosa-Calvo, 2015), or up to ∼1600 individuals (e.g. *Myrmicocrypta buenzlii* Borgmeier as reported by Weber, 1966), and to possess, as far as is known, a single mated queen per colony (Weber, 1972, 1979; Villesen *et al.*, 1999, 2002). *Myrmicocrypta* workers are cryptic foragers in the leaf litter and are thus rarely hand-collected in the field. *Myrmicocrypta* nests are most commonly excavated in the ground, but at least two species are known to nest in cavities in rotten logs. The nest entrances of the colonies found on the forest floor usually consist of a small mound of soil around an inconspicuous entrance hole (Weber, 1945, 1946, 1947, 1968, 1969; Fernández-Marín *et al.*, 2005). Underground colonies vary in the depth at which the garden chamber is located, usually from ∼2 to ∼100 cm from the surface (Mann, 1916; Weber, 1937, 1941, 1945, 1946, 1947, 1968, 1969; Hölldobler & Wilson, 1990; Murakami & Higashi, 1997; Murakami *et al.*, 2000; Pagnocca *et al.*, 2010; Sosa-Calvo, 2015; Sosa-Calvo *et al.*, 2015; TRS personal observation). Nests usually consist of a single, spherical chamber that contains the fungus garden, which is commonly found suspended from the ceiling of the chamber by rootlets or, during early colony founding, on a platform formed by the queen's forewing (Fernández-Marín *et al.*, 2004). In species that nest in rotten logs, the nest chamber is irregularly shaped and, rather than suspended from the ceiling, the fungus garden rests on the chamber floor. In contrast to the ground-nesting species, which excavate their chambers, the wood-nesting species are thought to occupy abandoned galleries produced by wood-boring insects, especially beetles (Weber, 1937, 1946).

Myrmicocrypta species reportedly use a wide variety of organic matter as substrates for their fungus gardens, including arthropod frass, wood pellets, insect corpses, seeds, flower parts, dry leaves, and other plant debris (Wheeler, 1922; Weber, 1941, 1945, 1946, 1947, 1966, 1968, 1969; Hölldobler & Wilson, 1990; Murakami & Higashi, 1997; Leal & Oliveira, 1998; Mueller *et al.*, 2005; de Fine Licht & Boomsma, 2010). The only thorough study to date dealing with *Myrmicocrypta* biology (Murakami & Higashi, 1997) reports that the garden substrate of the ground-nesting *M. ednaella* Mann consists mainly of wood chips and occasional insect corpses, and that adult workers feed primarily upon plant nectar and sap, which they share with other workers via trophallaxis. Leal & Oliveira (2000) studied the foraging ecology of attine ants in a Brazilian savanna, recording the seasonal use of Cerrado vegetation as fungal substrate by fungus-farming ants. For *Myrmicocrypta*, their results show that, during the rainy season when flowers and fruits can be found near their nests, individuals forage heavily for them, but during the dry season, when those resources are scarce, individuals switch to different substrates, preferring seeds, insect frass (from defoliating caterpillars and wood-boring beetles), or dead insects (Leal & Oliveira, 1998, 2000; Leal *et al.*, 2011).

The aim of this study is to infer, for the first time, the phylogenetic relationships of the species in the poorly known ant genus *Myrmicocrypta* and to infer the relationships of *Myrmicocrypta* to other basally diverging attine species, especially those belonging to the Paleoattina, in order to inform the early evolutionary history of the ant–cultivar symbiosis and to provide the basis for the first taxonomic revision of the genus.

Materials and methods

Ant taxon sampling

Specimens of *Myrmicocrypta* were selected to represent a complete phylogenetic sample of the genus, i.e. to densely

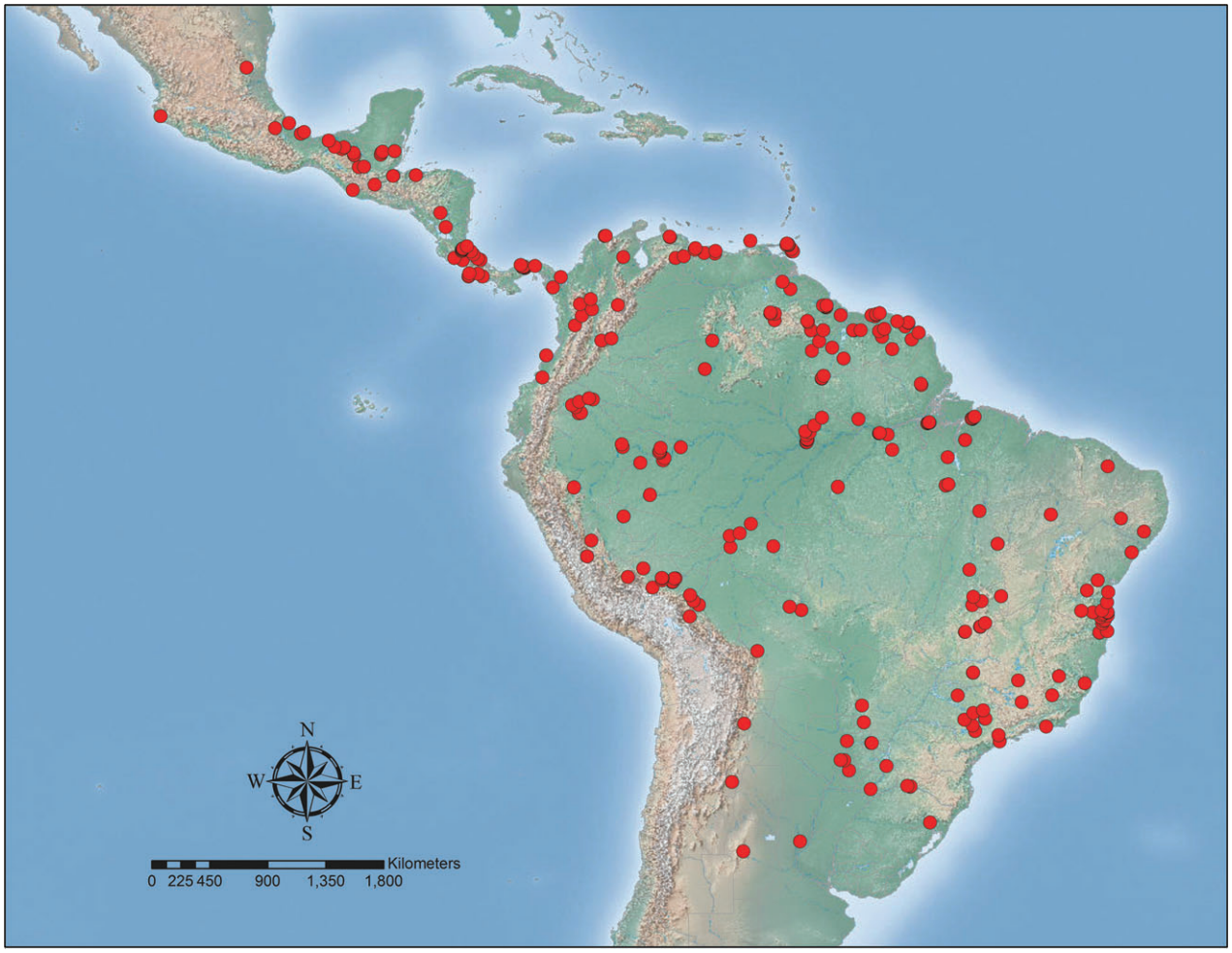

Fig. 1. Distribution map of the fungus-farming ant genus *Myrmicocrypta*. Red dots indicate localities of samples used in this study.

sample *Myrmicocrypta* morphospace (Fig. 1, red dots). Choice of outgroup taxa was based on previous studies based on morphology (Schultz & Meier, 1995), molecular data (Schultz & Brady, 2008; Sosa-Calvo *et al.*, 2013), or a combination of both (Wetterer *et al.*, 1998). Initially, 112 specimens belonging to *Myrmicocrypta* (the ingroup) and 84 taxa (the outgroup, spanning both attine and nonattine species) were included to assess the phylogeny of *Myrmicocrypta* (Fig. 2). In subsequent analyses, the number of taxa was reduced to include only the genera in the Paleoattina (i.e. *Apterostigma* Mayr, *Mycocepurus* Forel, and *Myrmicocrypta*), thus including nine outgroup species and 98 taxa belonging to *Myrmicocrypta* (ingroup) representing 42 species.

Voucher specimens for all extractions (listed in Table S1, Supporting Information) are deposited in the insect collection of the Smithsonian Institution's National Museum of Natural History (USNM). New sequences generated for this study are deposited in GenBank under accession numbers MH342047–MH342612 for the ants, and accession numbers MH373683–MH373735 for the fungal cultivars.

Fungal taxon sampling

Fungal tissue from collected nest series (i.e. entire colonies including brood and fungal garden) were preserved in ethanol. A total of 53 tissue samples were extracted and sequenced, but only those associated directly with a host ant sequence (36 samples, belonging to 20 species of ants) in the phylogeny (Figs 3 and S1, Supporting Information) are employed here for the reconstruction of ancestral traits.

Collection of molecular data

Ants. Fragments from six protein-coding genes were employed in the phylogenetic analyses: the five nuclear genes *elongation factor 1-alpha-F1* (EF1aF1), *elongation factor 1-alpha-F2* (EF1aF2), *wingless* (*wg*), *long-wavelength rhodopsin* (LW Rh), and *Topoisomerase I* (TOPI), and the mitochondrial gene *cytochrome oxidase I* (COI). Primers used to generate the sequence data are listed in Table 1. Gene fragments *EF1α-F1*, *wg*, and *LW Rh* each contain an intragenic region

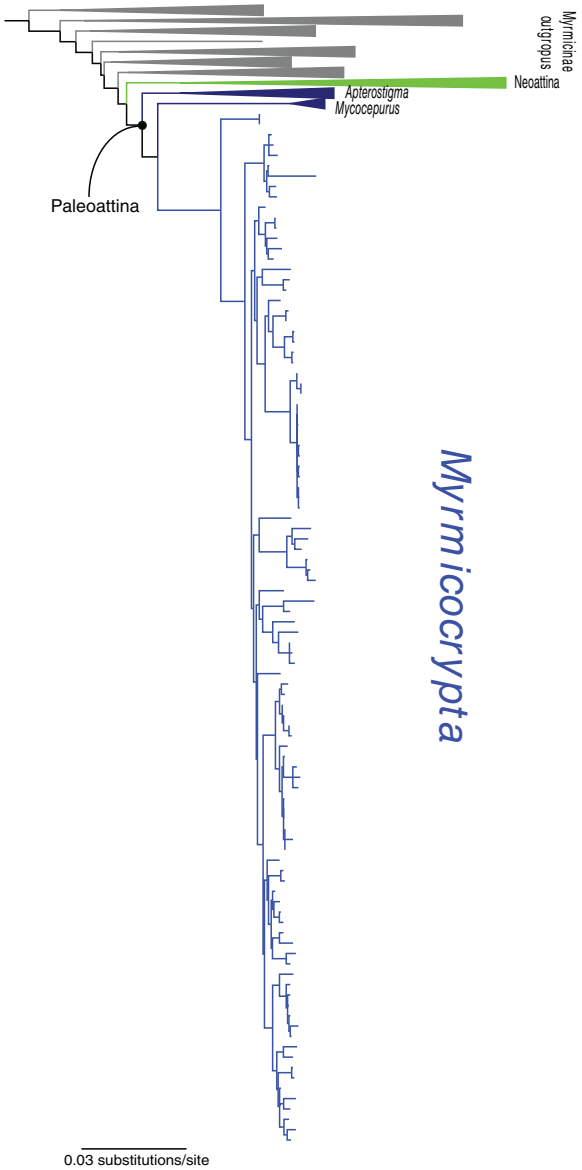

Fig. 2. Fungus-farming ant phylogeny based on four nuclear genes indicating the positions of the genus *Myrmicocrypta* (blue) within the Paleoattina, which also includes the genera *Apterostigma* and *Mycocepurus* (collapsed branches, dark blue), and the position of the Paleoattina with respect to the Neoattina (collapsed branches, green). Distant outgroup taxa belong to the subfamily Myrmicinae (used in Schultz & Brady (2008); collapsed branches, grey).

(intron). These six gene fragments have been successfully used in multiple studies of ant phylogenetics (Brady, 2003; Brady *et al.*, 2006; Jansen & Savolainen, 2010; Ward *et al.*, 2010, 2015; Rabeling *et al.*, 2011, 2014; Blaimer, 2012; Branstetter, 2012; Mehdiabadi *et al.*, 2012; Ward & Sumnicht, 2012; Cristiano *et al.*, 2013; Cardoso *et al.*, 2014a,b; Fernandes, 2017; Sosa-Calvo *et al.*, 2017a, 2018).

Extraction of ant genomic DNA was performed using the Qiagen DNeasy Tissue Kit (Qiagen, Valencia, CA, U.S.A.) following the manufacturer's protocols, with the following exceptions: cell lysis with 20 µL proteinase K was performed over a 24-h period (1–8 h according to the manufacturer's protocol) and the extracted DNA was eluted from the spin mini-column in two steps, each employing 50 µL of nuclease-free water (200 µL of AE buffer according to the manufacturer's protocol). The eluate (~100 µL) was then divided and transferred into two clean, sterile, and properly labelled tubes and stored at −80°C (~50 µL, for long-term storage) and −20°C (~50 µL, for downstream molecular work).

DNA was extracted primarily from adult workers and, depending on availability, from adult queens and males. DNA was extracted destructively or nondestructively depending on the number of nest series specimens available. DNA was destructively sampled from individuals that were subsets of nest series (i.e. entire or partially preserved colonies consisting of multiple developmental stages). In such cases, the whole ant specimen was dried for 30 min on a Kimwipe (Kimberly-Clark Roswell, GA, U.S.A.), placed in a 1.5 mL tube with a new or previously sterilized stainless-steel bead, and then finely pulverized at 25.0 Hz for 30 s using a TissueLyser RETSCH MM200 (Qiagen) in order to rapidly disrupt cells and tissues. DNA was nondestructively extracted from individuals that were unique, from very limited nest series, and/or otherwise required for future morphological study. Such individuals were either alcohol-preserved or pin-mounted. Nondestructive DNA extractions followed normal extraction procedures, with the exception that the specimens were left intact. During the nondestructive extraction procedure, the entire individual was first dried for 30 min, then placed directly into a 1.5 mL tube with 20 µL of Proteinase K and 180 µL of ATL buffer for 24 h in a thermomixer dry bath at 55°C. After the 24-h cell-lysis process, the complete ant was removed and stored in a vial in 95% ethanol for subsequent cleaning and mounting. The voucher specimens were then cleaned using several washes of soapy water and vinegar or ethyl acetate before point-mounting.

DNA sequences were amplified by conducting PCR runs in 25 µL solutions containing 1 µL of template DNA, 1 µL of each primer (forward and reverse), 12.2 µL of H_2O, 5 µL of 5× buffer, 2 µL of dNTPs, 2.5 µL of $MgCl_2$, and 0.3 µL of Taq polymerase (Promega, Madison, WI, U.S.A.) or in 20 µL solutions containing 1 µL of template, 0.8 µL of each primer, 5.4 µL of H_2O, and 10 µL of PCR Master Mix (1.5 mM $MgCl_2$, 0.2 mM of dNTPs, and 1 unit of Taq polymerase) (Promega).

Polymerase chain reaction amplifications for all genes, except for *LW Rh*, were performed in a thermal cycler programmed to run the following protocol: 1 min denaturation at 95°C; 34 cycles of 30 s denaturation at 95°C, 1 min annealing at 45–60°C (depending on primer set), and 1.5 min extension at 72°C; 1.5 min final extension at 72°C; and unlimited hold at 4–10°C. For *LW Rh*, a touchdown (TD) PCR program was used. The TD program is similar to the protocol detailed earlier with the exception of the temperature-regime cycle, which begins with a step consisting of five cycles at the highest anneal temperature (55°C), then proceeds through a series of five-cycle steps, each differing from the preceding one by 0.5°C, and ending with a five-cycle step at the lowest temperature (45°C) for a total of 20

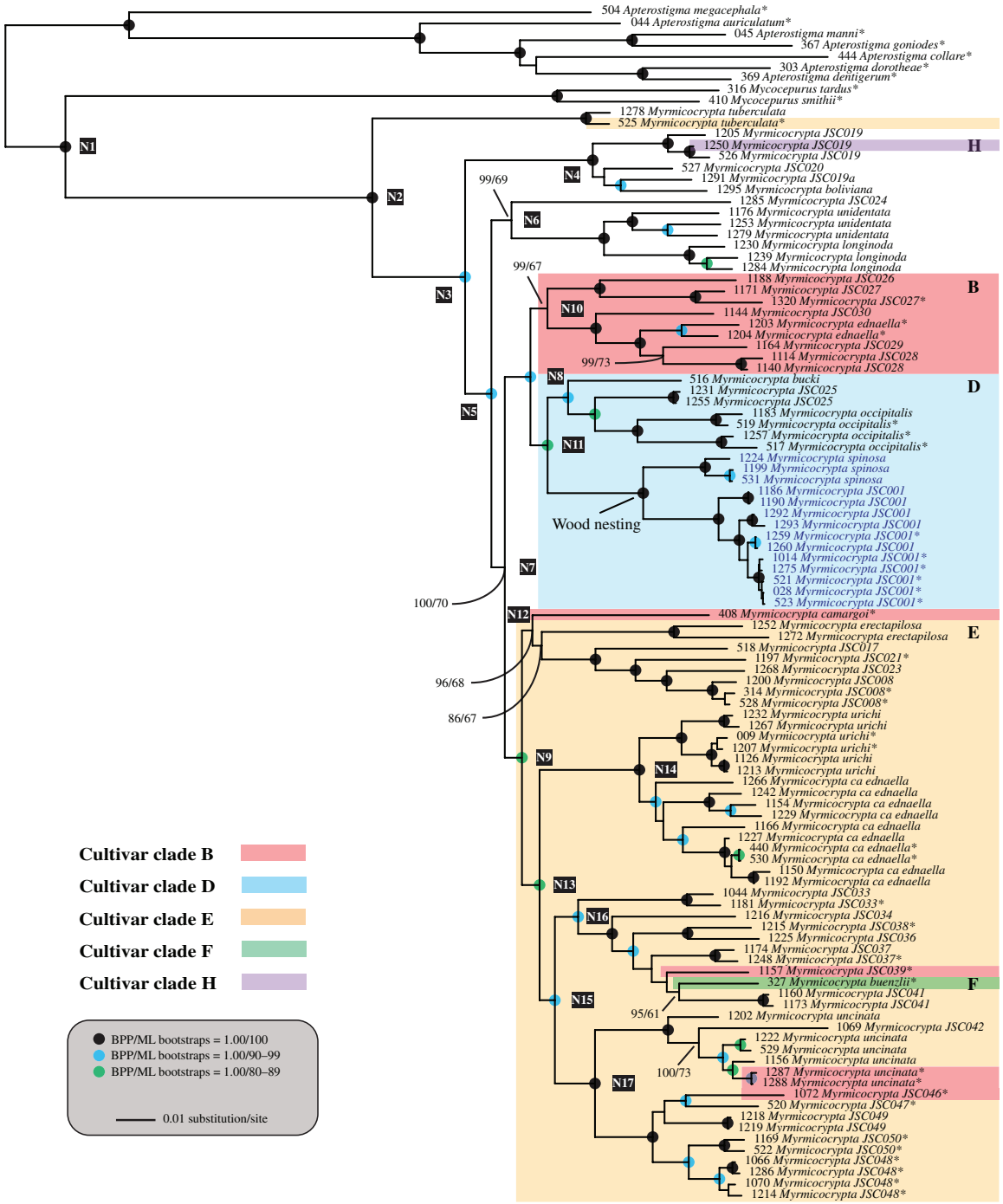

Fig. 3. Phylogeny of the fungus-farming ant genus *Myrmicocrypta* and outgroup species based on a Bayesian analysis of six protein-coding genes. Coloured circles indicate Bayesian posterior probabilities and maximum-likelihood bootstrap proportions (BPP/BS) as follows: black circles 1.00/100; blue circles 1.00/90–99; green circles 1.00/80–89. Other support values are given directly on the tree. Node labels N1–N17 correspond to nodes used in Tables 5 and 8. Coloured boxes indicate ancestral fungal associations of ants as reconstructed under the maximum likelihood Mk-1 (est) Markov k-state 1-parameter ancestral-state-estimation model in StoChar in MESQUITE v2.75. Cultivar subclades (colored boxes) correspond to those identified by Mehdiabadi *et al.* (2012) based on internal transcribed spacer (ITS) sequences of > 440 fungal taxa, including free-living and ant-associated fungi. The likelihood that each cultivar clade association arose in the most recent common ancestor of the corresponding ant clade was: N9 = 0.992 6286; N10 = 0.937 1545; N11 = 0.992 3550; and N13 = 0.9980614. Asterisks (*) after species names indicate taxa from which fungal cultivar DNA was obtained either from collected garden or directly from the ant DNA extraction; an unknown state ('?') was assigned to nonasterisk taxa. Taxon names in blue refer to species that nest in rotten logs (ancestral-state estimation, 0.988 5076), all other species within *Myrmicocrypta* are known to nest underground (ancestral state likelihood N2 = 0.999 8037).

Table 1. Primers used for sequencing mitochondrial (*cytochrome oxidase I* – ***COI***) and nuclear (*elongation factor 1a F1 copy* – ***EF1aF1***; *elongation factor 1a F2 copy* – ***EF1aF2***; *long-wavelength rhodopsin* – ***LW Rh***; *Topoisomerase 1* – ***TOP1***; and *wingless* – ***wg***) gene fragments in ants and the nuclear ribosomal internal transcribed spacer (ITS) region in fungi.

Gene region	Primer	Sequence 5′ to 3′	Source
Ants			
COI	LCO1490	GGT CAA CAA ATC ATA AAG ATA TTG G	Folmer *et al.* (1994)
	HCO2198	TAA ACT TCA GGG TGA CCA AAA AAT CA	Folmer *et al.* (1994)
	CI13	ATA ATT TTT TTT ATA GTT ATA CC	Hasegawa *et al.* (2002)
	CI14	AT TTC TTT TTT TCC TCT TTC	Hasegawa *et al.* (2002)
	Jerry	CAA CAY TTA TTT TGA TTT TTT GG	Simon *et al.* (1994)
	Ben3R	GC WAC WAC RTA ATA KGT ATC ATG	Brady *et al.* (2000)
	MycoGeorge	ATA CCT CGT CGA TAT TCT GA	(Rabeling *et al.*, 2011)
	Pat	ATC CAT TAC ATA TAA TCT GCC ATA	Simon *et al.* (1994)
EF1a-F1	M3 F1-383F	CAT ATW AAC ATT GTS GTS ATY GG	Schultz & Brady, 2008
	10R F1-1887R	ACG GCS ACK GTT TGW CKC ATG TC	Schultz & Brady, 2008
	for2 F1-494F	AAG GAG GCT CAG GAG ATG GG	Schultz & Brady, 2008
	rev1 F1-1044R	CGT CTT ACC ATC GGC ATT GCC	Schultz & Brady, 2008
	U377 F1-792F	TT GGC GTG AAG CAG CTG ATC G	Schultz & Brady, 2008
	TRS1R F1-1189R	ACC TGG TTT YAA GAT RCC GGT	Schultz & Brady, 2008
	U52.1 F1-1109F	CCG CTT CAG GAT GTC TAT AA	Schultz & Brady, 2008
	L53 F1-1551R	CCG CGT CTC AGT TCY TTC AC	Mueller *et al.*, 2008
	TRS4F F1-1424F	GCG CCK GCG GCT CTC ACC ACC GAG G	Brady *et al.* (2006)
	TRS9R F1-1829R	GGA AGG CCT CGA CGC ACA TMG G	Brady *et al.* (2006)
EF1a-F2	515F	GGT TCC TTC AAR TAY GCY TGG GT	P.S. Ward, personal communication
	1371R	CC RAT CTT RTA YAC GTC CTGC	P.S. Ward, personal communication
	557F	GAA CGT GAA CGT GGT ATY ACS AT	Brady *et al.* (2006)
	1118R	TTAC CTG AAG GGG AAG ACG RAG	Brady *et al.* (2006)
LW Rh	LR134F	ACM GTR GTD GAC AAA GTK CCA CC	P.S. Ward, personal communication
	LR143F	GAC AAA GTK CCA CCR GAR ATG CT	Ward & Downie (2005)
	LR639ER	YTTAC CG RTT CCA TCC RAA CA	Ward & Downie (2005)
TOP1	TP1293EF	TKCAG G TGG GAR GAR GAR AAG AA	Ward & Sumnicht (2012)
	TP2266ER3	GTYAC C TAA RAA RTC RAA BAC RAC	Ward & Sumnicht (2012)
	TP1339F	GAR CAY AAR GGA CCK GTR TTY GCA CC	Ward & Sumnicht (2012)
	TP2192R	GA RCA RCA RCC YAC DGT RTC HGC YTG	Ward & Sumnicht (2012)
	TP1901F2	CY AAT GTY ACD TGG CTH GCR TCH TGG AC	Ward & Sumnicht (2012)
wg	wg254F	CGA GAG ACC GCK TTY RTC TAY GC	P.S. Ward, personal communication
	wg1038R	CA CTT NAC YTC RCA RCA CCA RTG	P.S. Ward, personal communication
	wg290F	GCW GTR ACT CAC AGY ATC GC	P.S. Ward, personal communication
	wg645R	CG RTC CTT BAG RTT RTC GCC	P.S. Ward, personal communication
	wg503F	CT CTC TCR TTA CAG CAC GT	(Schultz & Brady, 2008)
	wg524EF	GCA GCA CGT TTC YTC VGA RAT GCG	P.S. Ward, personal communication
	wg578F	TGC ACN GTG AAR ACY TGC TGG ATG CG	Ward & Downie (2005)
	wg1032R	AC YTC GCA GCA CCA RTG GAA	Abouheif & Wray (2002)
Fungi			
ITS	ITS1	TCC GTA GGT GAA CCT GCG G	White *et al.* (1990)
	ITS5	GGA AGT AAA AGT CGT AAC AAG G	White *et al.* (1990)
	5.8SF	TCG ATG AAG AAC GCA GC	Vilgalys & Hester (1990)
	5.8SR	CGC TGC GTT CTT CAT CG	Vilgalys & Hester (1990)
	ITS4	TCC TCC GCT TAT TGA TAT GC	White *et al.* (1990)

five-cycle steps (Korbie & Mattick, 2008). PCR products were visualized on ethidium bromide-stained agarose gels (50 mL of 1.5% TBE gel–Tris/borate/EDTA and 1 μL of ethidium bromide) by running 5 μL of product mixed with 1.5 μL of 6X loading dye and running for ~30 min at 100 V.

Polymerase chain reaction product was purified by adding 3 μL of the enzymatic cleanup reagent ExoSAP-IT® (Affymetrix Inc., Santa Clara, CA, U.S.A.; exonuclease I and shrimp alkaline phosphatase), previously diluted in nuclease-free water (9:1), into the remaining 15–20 μL of PCR product. The solution was placed in a thermal cycler for 30 min at 37°C in order for the enzyme to remove unincorporated nucleotides and primers, followed by 15 min at 80°C, for enzyme deactivation. The primers employed in both amplification and sequencing are listed in Table 1. Sequencing reactions used 1 μL of the cleaned PCR product.

Sequencing was performed in the Laboratories for Analytical Biology (LAB) of the Smithsonian Institution on an ABI

3100 automated sequencer using an ABI BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems Inc., Foster City, CA, U.S.A.). Sequence data were assembled and edited using the program SEQUENCHER v.4.10.1 (Gene Codes Corp., Ann Arbor, MI, U.S.A.).

Fungal cultivars. Fungal DNA was extracted by immersing a small piece of dried, ethanol-preserved tissue in approximately 200 μL of 10% Chelex® 100 Resin (Bio-Rad, Hercules, CA, U.S.A.) solution. Samples were incubated in a programmable thermal cycler for 1.5 h at 60°C and heated for 15 min at 99°C. The programme was paused at 1 h and samples were mixed by vortexing for 30 s. DNA was then centrifuged at 15 700 *g* for 2 min. To prevent PCR inhibition from the Chelex beads, the supernatant was removed and placed in new vials and stored at −20°C until needed. Forceps were flamed between samples to prevent cross-contamination.

Amplification and sequencing of the internal transcribed spacer (ITS) region followed the methods of Mueller *et al.* (1998). Primers used for ITS amplification are listed in Table 1. In addition, we tested amplification and sequencing of ITS directly from the ant DNA extraction with a success rate of 48%. Fifteen ant DNA extraction templates were used to generate ITS sequences, of which seven were successful and in agreement with the reference cultivar extraction. In these cases amplification and sequencing of the ITS gene fragment were conducted in a similar fashion as described earlier.

Alignment and phylogenetics

The phylogenetic position of *Myrmicocrypta* within the Attina was tested by adding new taxa to the alignment of Schultz & Brady (2008). Initially, all six genes were aligned using the program MAFFT v.7 (Katoh *et al.*, 2002, 2009; Katoh & Toh, 2010; Katoh & Standley, 2013) as implemented in GENEIOUS v.6.1.6 (Biomatters Ltd., Auckland, New Zealand). Prior to alignment, noncoding (intron) regions were removed from the outgroup taxa in order to reduce indels and thus maximize the number of informative sites for the ingroup taxa. Translation to amino acids in MACCLADE v.4.08 OS X (Maddison & Maddison, 2005) was performed for all genes to make sure nucleotide sequences were not out of frame.

Each noncoding region (*EF1-aF1* intron, *LW Rh* intron, and *wg* intron) was aligned separately using the online version of the program MAFFT v.7 (Katoh *et al.*, 2002; Katoh *et al.*, 2009; Katoh & Toh, 2010; Katoh & Standley, 2013) maintained by the Computational Biology Research Center of the National Institute of Advanced Industrial Science and Technology (AIST, Japan: http://mafft.cbrc.jp/alignment/server/). The alignment was performed using the AUTO strategy and the *scoring matrix for nucleotide sequences* set to 1PAM/K = 2, which is suggested for closely related DNA sequences. For other parameters (*gap opening penalty* and *offset value*) the default settings were used (1.53 and 0.0, respectively). Alignment of noncoding regions was performed under the iterative refinement method (*FFT-NS-i*).

Each MAFFT alignment of the noncoding regions was then submitted to the guide-tree-based GUIDANCE alignment web server (Penn *et al.*, 2010a; http://guidance.tau.ac.il/), which uses the GUIDANCE algorithm to calculate confidence values for each column in the alignment (Penn *et al.*, 2010b; Privman *et al.*, 2012). The GUIDANCE algorithm uses a set of bootstrapped guide trees (in our analyses based on 100 pseudoreplicates), generates an alignment for each tree, then assigns a confidence score for each site in the alignment of the observed data based on its consistency across the bootstrap-based alignments. This confidence score was used to determine which positions in the variable noncoding regions to exclude (mask) from further analyses. We used a rigorous 95% confidence value as a cutoff for identifying sites that were poorly aligned, which were excluded from the alignment. Masked alignments were further inspected by eye using MACCLADE v.4.08 OS X (Maddison & Maddison, 2005). Sites determined to be poorly aligned (in addition to those sites previously identified by the GUIDANCE algorithm) were also excluded from further analyses.

Gene fragments were concatenated in GENEIOUS v.6.1.6 OS X (Biomatters Ltd., Auckland, New Zealand) and inspected by eye in MACCLADE v.4.08 OS X (Maddison & Maddison, 2005). Partitions were identified using two approaches: (i) a user-chosen (subjective) partitioning scheme; and (ii) an algorithm-driven (objective) partitioning scheme.

In the user-chosen (subjective) approach, the concatenated alignment was partitioned according to the following schemes: (1) *partition1*, the unpartitioned, entire alignment; (2) *partition5*, consisting of five partitions: (i) COI first and second positions, (ii) COI third positions, (iii) combined introns, (iv) combined nuclear-gene first and second codon positions, and (v) combined nuclear-gene third codon positions; (3) *partition7*, in which each gene is a separate partition and the three introns are combined into a single, seventh subset (*COI*, *EF1α–F1*, *EF1α–F2*, *LWRh*, *TOP1*, *wg*, introns); (4) *partition9*, similar to *partition7*, except that each intron comprises a separate partition; and (5) *partition16*, in which each nuclear gene is divided into codon positions 1 + 2 and codon position 3, each intron comprises a separate partition, and in which *COI* is divided into three partitions corresponding to each codon position (Table 2). For each data partition in each scheme, the most appropriate evolutionary model was selected using the corrected Akaike information criterion (AICc) (Posada & Buckley, 2004) calculated with JMODELTEST v2.1.4 (Posada, 2008, 2009; Darriba *et al.*, 2012) (Table 2).

In the algorithm-driven (objective) approach, partition schemes were identified using the program PARTITIONFINDER v1.1.1 (Lanfear *et al.*, 2012) to identify the partition scheme and substitution models that best fit the data and to avoid overparameterization, which has been shown to cause strong bias in posterior probability estimation (Lemmon & Moriarty, 2004). PARTITIONFINDER analyses were performed by comparing all 56 models of evolution ('*models = all*') under the Bayesian information criterion ('*model_selection = BIC*'), as suggested in the manual (Lanfear *et al.*, 2012). Due to the relatively large size of this dataset, the parameter '*Search*' was set to the '*greedy*' algorithm, which performs heuristic searches

Table 2. Sequence characteristics, user-selected partitions, and models chosen for each data partition. Models of evolution suggested by JMODELTEST v2.1.4.

		All taxa	Ingroup only	Model		User-selected partitions				
Gene fragment	No. of sites	PIS	PIS	AICc	BIC	Part1	Part5	Part7	Part9	Part16
EF1a F1	1507	272	229	TrN + I + G	K80 + G					
EF1a F1 (exc intron)	1075	179	136	TrN + I + G	TrNef+I + G			**x**	**x**	
EF1a F1 exon Pos 1 + 2	716	16	13	TrN + I	TrN + I					**x**
EF1a F1 exon Pos 3	359	163	123	HKY + G	HKY + G					**x**
EF1a F1 intron (exc 90%)	395	90	90	HKY + G	TPM1uf + G				**x**	**x**
wg	1048	180	134	TrN + G	TrNef+G					
wg exon (exc intron)	703	105	59	TrNef+G	TrNef+G			**x**	**x**	
wg exon Pos 1 + 2	468	7	2	K80	K80					**x**
wg exon Pos 3	235	98	57	JC	HKY + G					**x**
wg intron (inc 90%)	319	75	75	HKY	HKY				**x**	**x**
LW Rh	549	98	68	HKY + G	K80 + G					
LW Rh exon	458	87	57	K80 + G	HKY + G			**x**	**x**	
LW Rh exon Pos 1 + 2	305	32	25	K80 + I	K80 + I + G					**x**
LW Rh exon Pos 3	153	55	32	TrNef+I	HKY + I					**x**
LW Rh intron (exc 90%)	89	11	11	TIM2ef + I	K80 + G				**x**	**x**
EF1a F2 exon	517	99	58	TrNef+G	TrNef+G			**x**	**x**	
EF1a F2 exon Pos 1 + 2	344	12	8	JC + I	JC + I					**x**
EF1a F2 exon Pos 3	173	87	50	K80 + G	TrNef+G					**x**
TOP1 exon	883	214	153	TrN + I + G	TrN + G			**x**	**x**	
TOP1 exon Pos 1 + 2	588	41	25	TrN + G	TrN + G					**x**
TOP1 exon Pos 3	295	173	128	K80	HKY + G					**x**
COI exon	1042	520	495	TIM2 + I + G	TIM2 + I + G			**x**	**x**	
COI exon Pos 1	347	137	123	GTR + I + G	GTR + I + G					**x**
COI exon Pos 2	347	50	43	TIM3 + I + G	TIM3 + I + G					**x**
COI exon Pos 1 + 2	694	109	166	TIM3 + I + G	TIM3 + I + G		**x**			
COI exon Pos 3	348	333	329	TIM2 + G	TIM2 + I + G		**x**			**x**
All introns (exc 90%)	803	176	176	TPM3uf + I + G	HKY + I + G		**x**	**x**		
All genes (inc introns)	5546	1585	1339	GTR + I + G	GTR + I + G	**x**				
Nuclear exon	3635	684	462	TrN + I + G	TrN + I + G					
Nuclear exon Pos 1 + 2	2421	109	73	TIM3 + I + G	TIM3 + I + G		**x**			
Nuclear exon Pos 3	1214	575	389	TIM2 + G	TPM2uf + G		**x**			

PIS, parsimony informative sites; AICc, corrected Akaike information criterion; BIC, Bayesian information criterion; *COI*, *cytochrome oxidase I*; *EF1aF1*, *elongation factor 1a F1 copy*; *EF1aF2*, *elongation factor 1a F2 copy*; *LW Rh*, *long-wavelength rhodopsin*; *TOP1*, *Topoisomerase I*; *wg*, *wingless*; exc 90%, refers to exclusion of ambiguously aligned sites according to Guidance algorithm; PartN, partitions including the number of gene fragments used, selected based on the information given.

to find the best partitioning scheme. Rather than the default neighbour-joining tree, a tree resulting from the Bayesian analysis (MRBAYES v3.2.2; Ronquist & Huelsenbeck (2003); Ronquist *et al.* (2012)) of the Partition16 (see earlier) was submitted to PARTITIONFINDER as '*user_tree_topology*'. Data blocks submitted to PARTITIONFINDER included codon positions 1, 2, and 3 for each of the six genes and three noncoding (intron) regions (for *EF1aF1*, *wg*, *LW Rh*) for a total of 21 data blocks. PARTITIONFINDER identified ten partitions (henceforth 10PS scheme) (Table 3), which were employed in both Bayesian and maximum likelihood (ML) analyses. When the model identified by PARTITIONFINDER was not implemented in MRBAYES, the next most complex available model was used. Additional analyses were conducted for reduced datasets, including datasets from which the introns and/or the mitochondrial gene were removed, resulting in 6PS (no introns and no COI), 7PS (no COI), and 9PS (no introns) schemes (Table 3).

Bayesian analyses were performed on the different datasets using the program MRBAYES v.3.2.2 (Ronquist & Huelsenbeck, 2003; Ronquist *et al.*, 2012) with the following settings consistently applied across runs: *nucmodel = 4by4*, *nchains = 8* (seven heated and one cold; temperature parameter set at 0.05), and *nruns = 2*. To avoid known problems with branch-length estimation (Marshall *et al.*, 2006; Spinks & Shaffer, 2009; Brown *et al.*, 2010; Marshall, 2010), branch length priors were modified as follows: *prset applyto = (all)*, *brlenspr = unconstrained:exponential* (*100*). Parameters *samplefreq*, *ngen*, and *burn-in* varied according to run. All parameters except topology and branch length were unlinked across data subsets by using the command *prset applyto = (all)*, *ratepr = variable*. Burn-in and stationarity were assessed in TRACER v1.6 (Rambaut & Drummond, 2007), by examination of the potential scale reduction factor values (close to 1.00); by examination of effective sample size values (> 200); and by examination of the

Table 3. The ten partitioning schemes (10PS) and best-fitting models identified by PARTITIONFINDER v1.1.1 (Lanfear *et al.*, 2012). The Bayesian models are the models available in MRBAYES that are closest to the models identified by PARTITIONFINDER.

Subset	Gene fragment block	ML Best model	Bayesian Best model
ps1	*EF1aF1*pos3, *TOP1*pos3	TIM + G	GTR + G
ps2	*EF1aF1*pos1, *EF1aF2*pos1	TrN + I	GTR + I
ps3	*EF1aF1*pos2, *wg*pos1, *wg*pos2	K80	K80
ps4	*EF1aF1* Intron, *wg* intron	K81uf + G	GTR + G
ps5	*EF1aF2*pos3, *LW Rh*pos3, *wg*pos3	TrNef+G	SYM + G
ps6	*LW Rh* intron, *LW Rh*pos1, *LW Rh*pos2, *TOP1*pos1	K80 + I + G	K80 + I + G
ps7	*EF1aF2*pos2, *TOP1*pos2	F81 + I + G	HKY + I + G
ps8	*COI*pos1	GTR + I + G	GTR + I + G
ps9	*COI*pos2	GTR + I + G	GTR + I + G
ps10	*COI*pos3	GTR + I + G	GTR + I + G

ML, maximum likelihood.

split-frequencies diagnostic (close to 0.00). Based on this information, burn-in was set at 1 million generations. Clade support was assessed by combining the post-burn-in trees in MRBAYES and used to generate a 50% majority-rule consensus tree with posterior probabilities (PPs) in PAUP* v4.0a128 (Swofford, 2003) or in FIGTREE v1.2.3 (Rambaut, 2009).

Maximum likelihood tree inference was performed using the program GARLI 2.0 (Zwickl, 2006, 2011) using parallel processing. The partitioning scheme (10PS scheme) identified by PARTITIONFINDER was used in the ML analysis, and each subset was assigned the model suggested by PARTITIONFINDER. Unconstrained ML best-tree analyses consisted of 200 replicate searches and were conducted using default settings with the exception of *linkmodels = 0*; *subsetspecificrates = 1*; *topoweight = 0.01*; *brlenweight = 0.002*; and *modweight* = modified according to the number of partitions as 0.0005 × (number of subsets + 1) (as suggested by the author). Branch support was estimated by conducting 1000 bootstrap pseudoreplicates differing from default settings as follows: *genthreshfortopoterm = 10 000*; *scorethreshforterm = 0.10*; *startoptprec = 0.5*; *minoptprec = 0.01*; *brlenweight = 0.002*; *numberofprecreductions = 1*; *topoweight = 0.01*; *treerejectionthreshold = 20.0*; and *modweight = 0.0005* × (*number of subsets + 1*). These settings increase the speed of the analysis by reducing the rigorousness of each bootstrap run (D.J. Zwickl, personal communication).

Additional analyses were conducted to explore the effects of using different data sets. First, two data sets were analyzed in MRBAYES v3.2.2 (Ronquist *et al.*, 2012) and Garli (Zwickl, 2011) using the same parameter settings described above. One data set did not include *COI* and the other did not include *COI* and intron regions. These analyses explored the effects of possible noise (oversaturation in *COI* and poorly aligned regions in the introns) (Branstetter, 2012). Both datasets were partitioned based on results from PARTITIONFINDER for the entire dataset.

Table 4. The partitioning schemes for each single gene and best model identified by PARTITIONFINDER v1.1.1 (Lanfear *et al.,* 2012) used in single-gene Bayesian analyses.

Gene fragment	Subset	Partition scheme	Bayesian Best model
EF1aF1	1	*EF1aF1*pos1	TrN + I
	2	*EF1aF1*pos2	JC
	3	*EF1aF1*pos3	TIM + G
	4	*EF1aF1* intron	TIM + G
wg	1	*wg*pos12	GTR
	2	*wg*pos3	GTR + G
	3	*wg* intron	GTR + I
LW Rh	1	*LW Rh*pos12 + *LW Rh* intron	K80 + I + G
	2	*LW Rh*pos3	TrNef+G
EF1aF2	1	*EF1aF2*pos12	K81 + I
	2	*EF1aF2*pos3	TrNef+G
TOP1	1	*TOP1*pos12	TrN + I
	2	*TOP1*pos3	HKY + G
COI	1	*COI*pos1	GTR + I + G
	2	*COI*pos2	GTR + I + G
	3	*COI*pos3	GTR + I + G

COI, *cytochrome oxidase I*; *EF1aF1*, *elongation factor 1a F1 copy*; *EF1aF2*, *elongation factor 1a F2 copy*; *LW Rh*, *long-wavelength rhodopsin*; *TOP1*, *Topoisomerase I*; *wg*, *wingless*.

Single-gene analyses were performed using Bayesian inference in MRBAYES v3.2.2 (Ronquist & Huelsenbeck, 2003; Ronquist *et al.*, 2012) in order to assess the possibility of gene-tree conflict and to compare clade support for each gene. Each run consisted of 10 million generations with a burn-in of 0.5 million generations (except for *wg*, for which each run consisted of 20 million generations with a burn-in of 1 million generations). Partitions for each gene were identified by PARTITIONFINDER v1.1.1 (Lanfear *et al.*, 2012) and summarized in Table 4. A summary of the performance of each gene and concatenated datasets are presented in Table 5, and 50% majority-rule consensus trees for each gene are presented in Fig. S2, Supporting Information.

DNA sequences of the *Myrmicocrypta* fungal cultivars were incorporated into a large alignment (> 400 sequences) of attine fungal cultivars and free-living Agaricaceae assembled during the past ten years (Mueller *et al.*, 1998; Vo *et al.*, 2009; Mehdiabadi *et al.*, 2012; J. Sosa-Calvo & T.R. Schultz, unpublished data). The ITS alignment consisted of 1078 aligned nucleotide sites, including indels. The alignment was analysed as a single-partition set with the GTR + I + G nucleotide substitution model based on an analysis in PARTITIONFINDER v1.1.1 (Lanfear *et al.*, 2012).

Model-testing in JMODELTEST and PARTITIONFINDER and phylogenetic and divergence-dating analyses were conducted using the Smithsonian Hydra High Performance Computing Cluster (Herndon, VA, U.S.A.), the Smithsonian NMNH LAB Topaz Computer Cluster (Washington, DC, U.S.A.), and the CIPRES Science Gateway (Miller *et al.*, 2010; http://www.phylo.org/).

Analysis of the ITS dataset was conducted using the ML criterion in RAXML v8.2 (Stamatakis, 2014) using a simultaneous best-tree search and rapid bootstrap analysis (*−f* option) with 1000 bootstrap replicates, employing the GTR + G + I model

Table 5. Support values for nodes inferred from analyses of single genes and from three concatenated data sets. For the single-gene analyses, the support values are Bayesian posterior probabilities (BPPs). For the concatenated analyses the support values are reported as BPPs/maximum likelihood bootstraps. Concatenated analyses employed three different partitioning schemes based on separate analyses in PARTITIONFINDER (Lanfear *et al.*, 2012). 6PS included all nuclear genes but excluded *COI* and introns; 7PS included all nuclear genes and introns but excluded *COI*; 9PS included all genes but excluded introns.

			Single genes						Concatenated		
Node	Description	No. genes	*EF1aF1*	*EF1aF2*	*wg*	*LW Rh*	*TOP1*	*COI*	6PS	7PS	9PS
N1	*Myrmicocrypta* + sister (*Mycocepurus*)	6	96	95	100	100	100	100	100/100	100/100	100/100
N2	Crown *Myrmicocrypta*	6	100	100	100	100	100	100	100/100	100/100	100/100
N3	*M. boliviana* + JSC019 clade + rest of *Myrmicocrypta*	4	100	100	–	100	–	99	100/99	100/100	100/99
N4	*M.* JSC019 + *M. boliviana* clade	2	–	–	–	100	–	100	100/100	100/100	100/100
N5	*M. unidentata* and *longinoda* clade + rest of *Myrmicocrypta*	2	96	–	–	–	–	96	100/84	100/89	100/91
N6	*M.* JSC024 + *longinoda-unidentata* clade	1	–	–	–	–	–	100	100/78	100/68	100/81
N7	*M. ednaella*-JSC001 + *camargoi-uncinata* clades	1	–	–	–	–	97	96	98/58	100/59	100/72
N8	*M. ednaella* + *M.* JSC001 clades	3	–	–	–	94	98	97	100/92	100/93	100/98
N9	*M. camargoi* + *uncinata*	1	–	–	61	–	–	–	88//61	100/75	100/81
N10	*M. ednaella* group	1	–	–	–	–	99	–	91/58	51/54	100/69
N11	*M.* JSC001 group	1	–	–	–	–	86	–	100/96	100/91	100/93
N12	*M. camargoi* + *erectapilosa* clade	1	–	–	–	–	94	–	– / –	94/ –	94/61
N13	*M. urichi* + *M. uncinata* clade	1	–	–	–	–	–	97	100/89	100/73	100/98
N14	*M. urichi* group	5	99	–	75	100	100	100	100/100	100/100	100/100
N15	*M. buenzlii* + *M. uncinata* clades	3	–	–	93	–	90	99	99/81	100/94	98/71
N16	*M. buenzlii* group	3	–	–	100	–	93	100	100/90	100/96	100/99
N17	*M. uncinata* group	5	100	–	95	75	58	97	100/99	100/100	100/100

of evolution. The resulting phylogeny is depicted in Fig. S1, Supporting Information.

Divergence dating (ants)

Estimation of species divergence times was inferred using a node-dating approach implemented in the program MCMCTREE, as part of the PAML package v4.9e (Yang, 2007). To calibrate the analysis, we used two calibration points: (i) the *Apterostigma pilosum* stem group set at 15 Ma (see later); and (ii) the age of the root, the ancestor of Paleoattina, set at 49 Ma, which was the mean bound of the 95% highest posterior density (HPD) interval for that split in a recent study of the evolution of fungus-farming ants (Branstetter *et al.*, 2017). To reduce computation time, we performed the divergence-dating analyses using an unpartitioned dataset (*ndata* = 1) under the HKY + G4 substitution model. We conducted two independent MCMCTREE runs, each with 50 million generations and a burn-in of 5 million generations (*sampfreq* = 5000, *nsample* = 10 000, and *burnin* = 5 000 000). We assessed run convergence and performance by examining mcmc.txt files in TRACER v1.6 (Rambaut & Drummond, 2007).

As *Myrmicocrypta* is not known from the fossil record, we calibrated the *Apterostigma pilosum* stem group using information from the only two fossils currently known for the Paleoattina, *Apterostigma eowilsoni* Schultz and *A. electropilosum* Schultz, both described from Dominican amber inclusions (Schultz, 2007) . The age of Dominican amber has been estimated to be 15–20 Ma in the Miocene (Grimaldi, 1995; Iturralde-Vinent & MacPhee, 1996; LaPolla *et al.*, 2013). As there has been some disagreement about the age (see Poinar, 2010), we used 15 Ma as a conservative estimate, as did Schultz & Brady (2008) and Branstetter *et al.* (2017).

Ancestral-state estimation (ants)

Ant taxa were assigned single states in three characters, including:

1. Nesting behaviour: 0, underground; 1, in rotten log; and 2, under leaves.
2. Fungal cultivar group: 0, subclade B; 1, subclade D; 2, subclade E; 3, subclade F; 4, subclade G; 5, subclade H; 6, group G1; 7, group G2; 8, group G4 (information from Villesen *et al.* (2004); Mehdiabadi *et al.* (2012)).
3. General agricultural systems: 0, higher attine (G1); 1, pterulaceous group 1 (G2); 2, pterulaceous group 2 (G4); 3, lower attine (G3) clade 1; 4, lower attine (G3) clade 2 [information from Chapela *et al.* (1994); Villesen *et al.* (2004); Schultz & Brady (2008); Schultz *et al.* (2015); Sosa-Calvo *et al.* (2017b)].

Species for which information was not available received 'unknown' (i.e. '?') state assignments. Species and

character-state assignments are listed in Table 6. Optimization of character evolution was performed on the phylogram of the genus *Myrmicocrypta* and outgroups resulting from the Bayesian analysis employing the 10PS partitioning scheme, under ML ancestral-state estimation using the Markov k-state 1 parameter model (Mk1-est; Lewis, 2001) included in the STOCHCHAR v.1.1 package (Maddison & Maddison, 2006) of the program MESQUITE v.2.75 (Maddison & Maddison, 2011).

Results

Molecular data

After the exclusion of ambiguously aligned regions (547 bp), the concatenated dataset for all six genes consisted of 5481 bp (1470 bp for *EF1aF1*, 1022 bp for *wg*, 547 bp for *LW Rh*, 517 bp for *EF1aF2*, 883 bp for *TOP1*, 1042 bp for *COI*, including introns) (Table 2). The data contained 1585 parsimony-informative sites (PIS) for all taxa and 1339 PIS for ingroup-only taxa, with the majority concentrated in third-codon positions (74% sites for all taxa and 58% sites for ingroup-only taxa). Of these 1585 PIS for all taxa, exon regions of nuclear and mitochondrial genes contained 684 and 520 PIS, respectively; whereas for the 1339 PIS for the ingroup-only taxa 462 and 495 PIS were contained in nuclear and mitochondrial regions, respectively. Noncoding regions (introns) contained 176 PIS within the ingroup-only taxa.

Ant phylogeny based on concatenated data

Maximum likelihood and Bayesian phylogenetic analyses of the concatenated six-gene dataset, under the 10PS partitioning scheme, yielded similar topologies (Fig. 3, Bayesian reconstruction, with support from both analyses indicated). Regardless of the partitioning scheme employed, all ML and Bayesian analyses resulted in nearly identical topologies, with minor changes in branch lengths and topology at weakly supported nodes. Analyses from which *COI* was removed differed mostly with regard to support for a sister-group relationship between *Myrmicocrypta* JSC042 and *M. uncinata*, whereas in analyses in which *COI* was included, *M*. JSC042 rendered *M. uncinata* paraphyletic. Summary statistics for Bayesian analyses of different datasets are given in Table 7.

The description of relationships given in the following refers to the 10PS scheme analyses unless otherwise noted. In all analyses (including those with different partitioning schemes), strong support was found for the monophyly of the genus *Myrmicocrypta* [Bayesian posterior probability (BPP)/ML bootstraps: 1.00/100] and for a sister-group relationship between *Myrmicocrypta* and the genus *Mycocepurus*. The relict species *Apterostigma megacephala* was reconstructed as the sister taxon to the rest of *Apterostigma* with high support (1.00/100). Both the sister-group relationship of *Myrmicocrypta* and *Mycocepurus* and the sister-group relationship of *A. megacephala* and all other *Apterostigma* species have been proposed before based on morphological and molecular data (Lattke, 1999; Schultz, 2007; Schultz & Brady, 2008; Rabeling *et al.*, 2011, 2014; Sosa-Calvo *et al.*, 2013; Ward *et al.*, 2015; Schultz *et al.*, 2015; Branstetter *et al.*, 2017).

Within *Myrmicocrypta*, the species *M. tuberculata* was recovered in all analyses as the sister taxon to the rest of *Myrmicocrypta* with strong support (1.00/100). The next most recent divergence (N3) is recovered as a sister-group relationship between the clade containing *M*. JSC019, *M*. JSC019a, and *M. boliviana* and the rest of *Myrmicocrypta* with high support (1.00/99). Most of the deep nodes within *Myrmicocrypta* are strongly supported (N5, 1.00/91; N8, 1.00/99; N13, 1.00/91; N15, 1.00/93; N16, 1.00/99; N17, 1.00/100); with a few nodes moderately to weakly supported (N6, 0.99/69; N7, 1.00/70; N9, 1.00/85; N10, 0.99/67; N11, 1.00/88; N12, 0.96/68) (Fig. 3).

The recently described species *M. bucki*, *M. camargoi*, and *M. erectapilosa* (Sosa-Calvo & Schultz, 2010), which are characterized by having erect simple to spatulate hairs, depart from the ancestral state of having scale-like hairs that characterize the majority of *Myrmicocrypta* species. It was expected that the unusual condition of erect hairs had appeared a single time in *Myrmicocrypta*, i.e. that the species with erect simple hairs would form a monophyletic group. Instead, ancestral-state reconstruction under ML in MESQUITE v.2.75 (Maddison & Maddison, 2011) suggests that this condition has independently appeared at least seven times in *Myrmicocrypta*, once in the common ancestor of *M*. JSC026 and *M*. JSC027; once in JSC028; once in *M. bucki*; once in *M. camargoi*; once in *M. erectapilosa*; once in *M*. JSC023; and once in the common ancestor of *M*. JSC046 and *M*. JSC048. In addition, new species with erect simple hairs have been recently discovered (Sosa-Calvo & Schultz, unpublished data), some of which may not belong to any of the groups listed earlier, and for which we did not have recently collected material to be included in these analyses.

Fungal cultivar phylogeny based on ITS data

Internal transcribed spacer (ITS) sequences for 53 *Myrmicocrypta*-associated fungal cultivars were generated and incorporated into a larger dataset of ant-associated and free-living fungi and analyzed with ML, indicating that species in the genus *Myrmicocrypta* grow fungi from both of the lower-attine fungal clades 1 and 2 (Fig. S1, Supporting Information). Comparison with the results of Mehdiabadi *et al.* (2012) indicates that *Myrmicocrypta* grows at least five subclades of fungi, two belonging to clade 1 [Figs 3 and S1, B and D (Supporting Information); red and blue boxes, respectively] and three in clade 2 [Figs 3 and S1, E, F, (Supporting Information) and a newly identified subclade, referred to here as H; yellow, green, and purple boxes, respectively].

Ant divergence dating

The time-calibrated phylogeny derived from the MCMCTREE analysis (Fig. 4) recovered the stem-group age of

Table 6. Taxa, characters, and character states used in the ancestral-state estimation in MESQUITE v2.75 (Maddison & Maddison, 2011).

Taxa	A	B	C	Source
504 Apterostigma megacephala	0	6	0	Schultz *et al.* (2015); Sosa-Calvo *et al.*, (2017b)
044 Apterostigma auriculatum	1	0	3	Schultz & Brady, 2008
045 Apterostigma manni	0	8	2	Villesen *et al.* (2004)
367 Apterostigma goniodes	0	8	2	Villesen *et al.* (2004)
444 Apterostigma collare	2	7	1	Schultz & Brady, 2008
303 Apterostigma dorotheae	0	7	1	Villesen *et al.* (2004)
369 Apterostigma dentigerum	2	7	1	Villesen *et al.*, 2004
316 Mycocepurus tardus	0	0	3	Schultz & Brady (2008)
410 Mycocepurus smithii	0	4	3	Schultz & Brady (2008)
1278 Myrmicocrypta tuberculata	0	?	?	
525 Myrmicocrypta tuberculata	0	2	4	
1250 Myrmicocrypta JSC019	0	5	?	
1205 Myrmicocrypta JSC019	0	?	?	
526 Myrmicocrypta JSC019	0	?	?	
527 Myrmicocrypta JSC020	0	?	?	
1291 Myrmicocrypta JSC019a	0	?	?	
1295 Myrmicocrypta boliviana	0	?	?	
1285 Myrmicocrypta JSC024	?	?	?	
1176 Myrmicocrypta unidentata	0	?	?	
1253 Myrmicocrypta unidentata	0	?	?	
1279 Myrmicocrypta unidentata	0	?	?	
1230 Myrmicocrypta longinoda	0	?	?	
1239 Myrmicocrypta longinoda	0	?	?	
1284 Myrmicocrypta longinoda	0	?	?	
1188 Myrmicocrypta JSC026	?	?	?	
1171 Myrmicocrypta JSC027	0	?	?	
1320 Myrmicocrypta JSC027	0	0	3	
1144 Myrmicocrypta JSC030	0	?	?	
1203 Myrmicocrypta ednaella	0	0	3	
1204 Myrmicocrypta ednaella	0	0	3	
1164 Myrmicocrypta JSC029	?	?	?	
1114 Myrmicocrypta JSC028	0	?	?	
1140 Myrmicocrypta JSC028	0	?	?	
516 Myrmicocrypta bucki	0	?	?	
1231 Myrmicocrypta JSC025	?	?	?	
1255 Myrmicocrypta JSC025	?	?	?	
1183 Myrmicocrypta occipitalis	0	?	?	
519 Myrmicocrypta occipitalis	0	1	3	
1257 Myrmicocrypta occipitalis	0	1	3	
517 Myrmicocrypta occipitalis	0	1	3	
1224 Myrmicocrypta spinosa	1	?	?	
1199 Myrmicocrypta spinosa	1	?	?	
531 Myrmicocrypta spinosa	1	?	?	
1186 Myrmicocrypta JSC001	1	?	?	
1190 Myrmicocrypta JSC001	1	?	?	
1292 Myrmicocrypta JSC001	1	?	?	
1293 Myrmicocrypta JSC001	1	?	?	
1259 Myrmicocrypta JSC001	1	1	3	
1260 Myrmicocrypta JSC001	1	?	?	
1014 Myrmicocrypta JSC001	1	1	3	
1275 Myrmicocrypta JSC001	1	1	3	
521 Myrmicocrypta JSC001	1	1	3	
028 Myrmicocrypta JSC001	1	1	3	Schultz & Brady (2008)
523 Myrmicocrypta JSC001	1	1	3	
408 Myrmicocrypta camargoi	0	0	3	
1252 Myrmicocrypta erectapilosa	?	?	?	
1272 Myrmicocrypta erectapilosa	?	?	?	
518 Myrmicocrypta JSC017	0	?	?	
1197 Myrmicocrypta JSC021	0	2	4	

Table 6. Continued.

Taxa	A	B	C	Source
1268 Myrmicocrypta JSC023	?	?	?	
1200 Myrmicocrypta JSC008	0	?	?	
314 Myrmicocrypta JSC008	0	2	4	Schultz & Brady (2008)
528 Myrmicocrypta JSC008	0	2	4	
1232 Myrmicocrypta urichi	0	?	?	
1267 Myrmicocrypta urichi	0	?	?	
009 Myrmicocrypta urichi	0	2	4	Schultz & Brady (2008)
1207 Myrmicocrypta urichi	0	2	4	
1126 Myrmicocrypta urichi	0	?	?	
1213 Myrmicocrypta urichi	0	?	?	
1266 Myrmicocrypta ca ednaella	0	?	?	
1242 Myrmicocrypta ca ednaella	0	?	?	
1154 Myrmicocrypta ca ednaella	0	?	?	
1229 Myrmicocrypta ca ednaella	0	?	?	
1166 Myrmicocrypta ca ednaella	0	?	?	
1227 Myrmicocrypta ca ednaella	0	?	?	
440 Myrmicocrypta ca ednaella	0	2	4	Schultz & Brady (2008)
530 Myrmicocrypta ca ednaella	0	2	4	Schultz & Brady (2008)
1150 Myrmicocrypta ca ednaella	0	?	?	
1192 Myrmicocrypta ca ednaella	0	?	?	
1044 Myrmicocrypta JSC033	0	?	?	
1181 Myrmicocrypta JSC033	0	2	4	
1216 Myrmicocrypta JSC034	0	?	?	
1215 Myrmicocrypta JSC038	0	2	4	
1225 Myrmicocrypta JSC036	?	?	?	
1174 Myrmicocrypta JSC037	0	?	?	
1248 Myrmicocrypta JSC037	0	2	4	
1157 Myrmicocrypta JSC039	0	0	3	
327 Myrmicocrypta buenzlii	0	3	4	Schultz & Brady (2008)
1160 Myrmicocrypta JSC041	0	?	?	
1173 Myrmicocrypta JSC041	0	?	?	
1202 Myrmicocrypta uncinata	0	?	?	
1069 Myrmicocrypta JSC042	0	?	?	
1222 Myrmicocrypta uncinata	0	?	?	
529 Myrmicocrypta uncinata	0	?	?	
1156 Myrmicocrypta uncinata	0	?	?	
1287 Myrmicocrypta uncinata	0	0	3	
1288 Myrmicocrypta uncinata	0	0	3	
1072 Myrmicocrypta JSC046	0	0	3	
520 Myrmicocrypta JSC047	0	2	4	
1218 Myrmicocrypta JSC049	0	?	?	
1219 Myrmicocrypta JSC049	0	?	?	
1169 Myrmicocrypta JSC050	0	2	4	
522 Myrmicocrypta JSC050	0	2	4	
1066 Myrmicocrypta JSC048	0	2	4	
1286 Myrmicocrypta JSC048	0	2	4	
1070 Myrmicocrypta JSC048	0	2	4	
1214 Myrmicocrypta JSC048	0	2	4	

Characters are as follows: (A) nest: 0, underground; 1, rotten wood; 2, under leaves. (B) fungal cultivar (states indicate predefined subclades): 0, subclade B; 1, subclade D; 2, subclade E; 3, subclade F; 4, subclade G; 5, subclade H; 6, subclade G1; 7, subclade G2; 8, G4 (from Mehdiabadi *et al.*, 2012); (C) fungal cultivar 2 (refers to major groups): 0, G1; 1, G2; 2, G4; 3, G3 clade 1; 4, G3 clade 2 (from Chapela *et al.*, 1994; Villesen *et al.*, 2004; Schultz & Brady (2008)).

Myrmicocrypta as 45 Ma (95% HPD = 37–52 Ma). The inferred age of 45 Ma for the most-recent common ancestor of *Myrmicocrypta* + *Mycocepurus* is somewhat older than estimates from recent studies, which are in the range of 37–43 Ma (Schultz & Brady, 2008; Rabeling *et al.*, 2011; Ward *et al.*, 2015; Branstetter *et al.*, 2017), although the 95% posterior density values overlap with the estimates of Ward *et al.* (2015). The root of the tree (Fig. 4), the origin of the informal group Paleoattina (Kusnezov, 1963), was recovered at an inferred age of 48 Ma (HPD 40–53 Ma), which is consistent with the dates

Table 7. Summary of Bayesian analyses.

Partition name	Subsets	Run	Chains	Generations[a]	Burn-in[a]	TL	Marginal likelihood
Full dataset							
10PS	10	1	8	20	1	2.86	−45 220.786 ± 0.361
10PS	10	2	8	20	1	2.86	−45 220.803 ± 0.330
10PS	10	3	8	20	1	2.86	−45 220.846 ± 0.321
10PS	10	4	8	20	1	2.86	−45 220.864 ± 0.346
16Part	16	1	8	20	1	3.38	−53 105.496 ± 0.408
16Part	16	2	8	20	1	3.38	−53 104.775 ± 0.381
No COI and introns							
6PS	6	1	8	20	1	2.03	−15 952.668 ± 0.336
6PS	6	2	8	20	1	2.05	−15 950.961 ± 0.343
No COI							
7PS	7	1	8	20	1	1.63	−18 651.660 ± 0.104
7PS	7	2	8	20	1	1.99	−18 653.927 ± 0.095
No introns							
9PS	9	1	8	20	1	3.13	−41 089.042 ± 0.332
9PS	9	2	8	20	1	3.13	−41 089.451 ± 0.290
Individual genes							
EF1aF1	4	1	8	10	0.5	0.85	−6442.567 ± 0.506
EF1aF1	4	2	8	10	0.5	0.85	−6443.539 ± 0.500
EF1aF2	2	1	8	10	0.5	1.49	−2433.916 ± 0.501
EF1aF2	2	2	8	10	0.5	1.49	−2433.818 ± 0.527
wg	3	1	8	20	1	1.32	−4206.051 ± 0.514
wg	3	2	8	20	1	1.31	−4206.777 ± 0.600
LW Rh	2	1	8	10	0.5	1.90	−2417.433 ± 0.439
LW Rh	2	1	8	10	0.5	1.91	−2418.068 ± 0.465
TOP1	2	1	8	10	0.5	1.01	−4377.264 ± 0.446
TOP1	2	1	8	10	0.5	1.01	−4378.818 ± 0.539
COI	3	1	8	10	0.5	6.13	−24 941.606 ± 0.687
COI	3	1	8	10	0.5	6.13	−24 939.565 ± 0.683

COI, *cytochrome oxidase I*; *EF1aF1*, *elongation factor 1a F1 copy*; *EF1aF2*, *elongation factor 1a F2 copy*; *LW Rh*, *long-wavelength rhodopsin*; *TOP1*, *Topoisomerase I*; *wg*, *wingless*.

Values for the branch-length prior (λ) were set to 100; temperature for all independent runs was set to 0.05; burn-in was set after run completion based on output in TRACER; the estimated marginal likelihood was calculated, for each run, in TRACER (see text for description).

[a]In millions of generations.

recovered for this clade by Schultz & Brady (2008), Ward *et al.* (2015), and Branstetter *et al.* (2017).

The inferred age for the crown group *Myrmicocrypta* was recovered at 27 Ma (HPD 22–33 Ma), which contrasts with the younger date of 10 Ma obtained in the study by Schultz & Brady (2008) and Branstetter *et al.* (2017). Their sample size, however, was smaller than the one presented here and, significantly, did not include the early-diverging species *Myrmicocrypta tuberculata* Weber. Divergence time estimates and 95% HPD ranges for each node, as numbered in Fig. 4, are summarized in Table 8.

Ant ancestral state estimation

Several characters and character states were estimated under the Markov k-state 1-parameter model (Lewis, 2001) in MESQUITE 2.75. For nesting behaviour, the likelihood that the most common ancestor of *Myrmicocrypta* nested underground was estimated as 0.999 8037, whereas the switch to nesting in rotten logs by the undescribed species *M*. JSC001 and *M. spinosa* is reconstructed as having occurred once in the common ancestor of these two sister species (0.988 5076) (see Fig. 3; taxon names in blue). As mentioned previously, attine agriculture has been divided into four agricultural systems (Schultz & Brady, 2008). (In a fifth system, leafcutter agriculture, associations have been recently shown to be less consistent (Mueller *et al.*, 2017, 2018).) One of those, lower agriculture (which includes *Myrmicocrypta*), has been subdivided into two clades, clade 1, containing subclades A–D, and clade 2, containing subclades E –G (Mehdiabadi *et al.*, 2012), and a new subclade, H, described here. Based on ancestral character-state reconstruction, the most recent common ancestor of *Myrmicocrypta* cultivated clade 2 fungal species (0.9910619). Switches to clade 1 fungi have occurred multiple times, once in the most recent common ancestor of *Myrmicocrypta* JSC001 and *M. ednaella* (Fig. 3, N8: 0.990 0897) and once in each of the species *M. camargoi*, *M*. JSC039, *M*. JSC046, and *M. uncinata*. The rest of *Myrmicocrypta* species for which fungal association data were available cultivate fungi in clade 2. Although we conducted ancestral character-state analyses using the subclades A–H, no subclade could be assigned to the most recent common

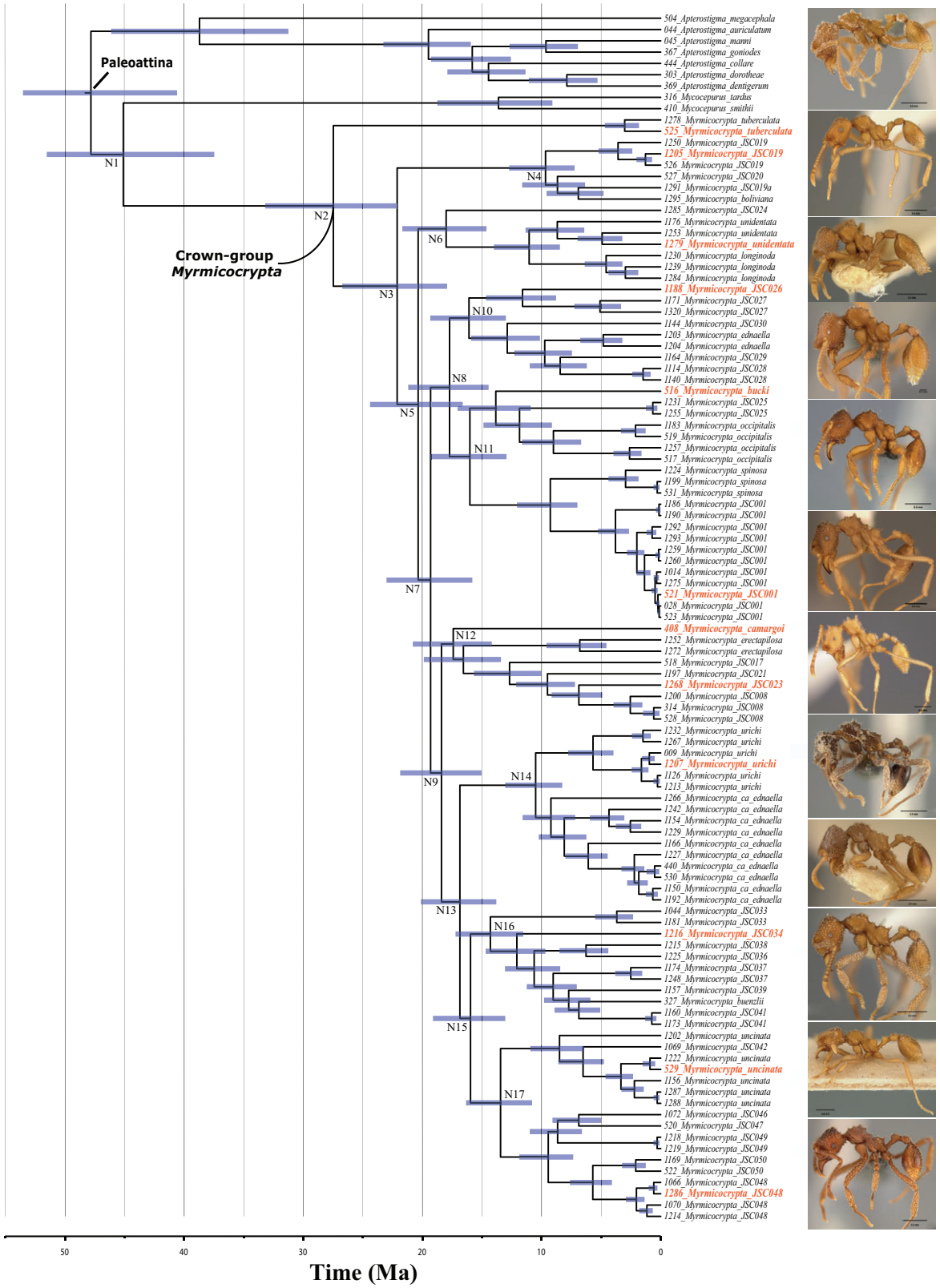

Fig. 4. Time-calibrated phylogeny of *Myrmicocrypta*, inferred from an MCMCTREE analysis. Blue bars indicate the 95% highest probability density (HPD). Automontage images of *Myrmicocrypta* in lateral view correspond to the species in the chronogram with names in orange.

Table 8. Divergence time estimates and 95% highest posterior density (HPD) output from MCMCTREE are given Ma.

Node	Description	Age	95% HPD (Ma)
N1	*Myrmicocrypta* + sister (*Mycocepurus*)	45.1	37.5–51.5
N2	Crown *Myrmicocrypta*	27.5	22.1–33.2
N3	*Myrmicocrypta boliviana* + JSC019 clade + rest of *Myrmicocrypta*	22.1	17.9–25.7
N4	*M.* JSC019 + *M. boliviana* clade	9.7	7.2–12.7
N5	*M. unidentata* and *longinoda* clade + rest of *Myrmicocrypta*	20.4	16.6–24.4
N6	*M.* JSC024 + *longinoda-unidentata* clade	18.0	14.6–1.7
N7	*M. ednaella*-JSC001 + *camargoi-uncinata* clades	19.3	15.8–23.0
N8	*M. ednaella* + *M.* JSC001 clades	17.7	14.4–21.2
N9	*M. camargoi* + *uncinata*	18.4	15.0–21.9
N10	*M. ednaella* clade	16.1	13.0–19.3
N11	*M.* JSC001 clade	16.0	12.9–19.3
N12	*M. camargoi* + *erectapilosa* clade	17.4	14.2–20.8
N13	*M. urichi* + *M. uncinata* clade	16.9	13.8–20.1
N14	*M. urichi* clade	10.5	8.3–13.1
N15	*M. buenzlii* + *M. uncinata* clades	16.0	13.0–19.1
N16	*M. buenzlii* clade	14.3	11.5–17.0
N17	*M. uncinata* clade	13.4	10.8–16.3

ancestor of *Myrmicocrypta* with high probability. However, the likelihood that one of those subclades was associated with the most recent common ancestor of particular ant clades was in some cases significant: 0.992 6286 for group E (N9); 0.992 3550 for group D (N11); and 0.937 1545 for group B (N10). Several species within *Myrmicocrypta* that are not closely related have independently switched to the same fungal cultivar subclades (Fig. 3, red boxes).

Discussion

Origin and evolution of Myrmicocrypta

Here, we present, for the first time, a phylogeny of the cryptic fungus-farming ant genus *Myrmicocrypta*, reconstructing the relationships of its species, estimating the time of origin of the genus, and reconstructing patterns of association with fungal cultivar species.

Recently (Schultz & Brady, 2008; Ward *et al.*, 2015; Ješovnik *et al.*, 2016; Nygaard *et al.*, 2016; Branstetter *et al.*, 2017) the origin of the fungus-farming ants has been estimated at ∼55–65 Ma, following the K-Pg extinction event at the end of the Cretaceous and preceding a period of warm global temperatures known as the Eocene Climatic Optimum (Zachos *et al.*, 2001; Graham, 2011). The time-calibrated phylogeny of *Myrmicocrypta* (Fig. 4) supports an origin of the stem group *Myrmicocrypta* (plus the stem group *Mycocepurus*, its sister group) during the late Eocene (45 Ma, CI = 37–52) towards the end of the Mid-Eocene Climatic Optimum, whereas for crown group *Myrmicocrypta* the origin is estimated to be in the early-mid-Oligocene (27 Ma, CI = 22–33), a transition period of stable conditions between the Eocene and the mid-Miocene climatic optimums that potentially favoured adaptation to and colonization of newly available ecosystems (Graham, 2011). Lineage-through-time plots show an increase in the diversification of *Myrmicocrypta*, with the majority of the species appearing from 15 Ma to the present, which coincides with the Mid-Miocene Climatic Optimum (15–17 Ma), a period of global warmth in which ecosystems like Caatinga and Cerrado appeared (Graham, 2011), now mostly restricted to Brazil and with small fragments in Paraguay and Bolivia (Oliveira & Marquis, 2002).

Relationships within the fungus-farming ant genera

The results from the concatenated four-gene and six-gene datasets (Figs 2 and 3) support the position of *Myrmicocrypta* within the informal clade Paleoattina, which also includes the genera *Apterostigma* and *Mycocepurus*, the latter the sister group of *Myrmicocrypta* (Fig. 3). This result is not surprising, as historically the genus *Myrmicocrypta* has been grouped within the Paleoattina based on morphological characters of the worker and wing venation (Emery, 1913; Kusnezov, 1963) and previous molecular analyses have corroborated this finding (Schultz & Brady, 2008; Rabeling *et al.*, 2011; Sosa-Calvo *et al.*, 2013; Ward *et al.*, 2015; Branstetter *et al.*, 2017). Morphologically, the paleoattine genera share: (i) the presence of a fenestra (clear spot) on the forewing of queens (secondarily lost in the forewing of the social parasite *Mycocepurus castrator*, absent in the paleoattine male forewing, and absent in nonfungus-farming and neoattine ants) (Emery, 1913, 1922; Fernández-Marín *et al.*, 2005; Rabeling and Bacci Jr, 2010); (ii) the antennal funicular segment I (pedicel) in males much shorter (∼2× shorter) than the funicular segment II (Sosa-Calvo & Schultz, 2010); and (iii) the presence of *Pseudonocardia* on basisternum II under the forelegs (Currie *et al.*, 1999a).

The ant genus Myrmicocrypta

It is our hope that the phylogeny presented here (Fig. 3) will facilitate future research into the evolution of fungus-farming behaviour in ants. Although the sample used in the phylogenetic analyses is relatively large (98 taxa for the ingroup alone, encompassing 42 putative species; Sosa-Calvo & Schultz, unpublished data), it is far from complete. Several known species (including *M. collaris* Emery, *M. corniculata* Emery, *M. dilacerata* (Forel), *M. godmani* Forel, *M. rudiscapa* Emery, and *M. triangulata* Forel, to mention some) were not included in the analyses because their DNA was unusable due to specimen age or preservation or because the species are only known from the type specimens, many of which were described in the late 1800s or early 1900s. In spite of these shortcomings, this represents the first attempt to reconstruct the relationships within the genus.

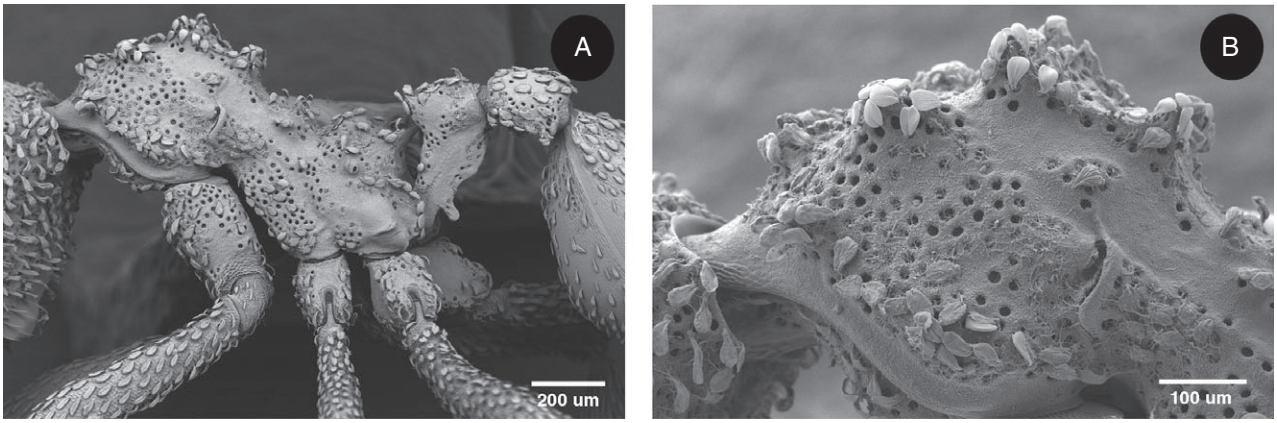

Fig. 5. *Myrmicocrypta tuberculata* worker with integumental crypts. (A) Lateral view of mesosoma, petiole, and postpetiole. (B) Lateral view of promesonotum.

The relationships of the species of *Myrmicocrypta* presented here are in agreement with the relationships obtained by Schultz & Brady (2008) and Branstetter *et al.* (2017). However, the sample in the present study is larger. In the studies by Schultz & Brady (2008) and Branstetter *et al.* (2017), the branches subtending each of the three paleoattine genera and the branch subtending *Kalathomyrmex* are long. Subsequent discoveries of the new genus *Cyatta* and of the relict species *Apterostigma megacephala* break the long branches leading to *Kalathomyrmex* and *Apterostigma*, respectively (Sosa-Calvo *et al.*, 2013, 2017b; Schultz *et al.*, 2015). Here, the branch subtending *Myrmicocrypta* is only slightly shortened by the addition of new taxa. The still-long branch subtending *Myrmicocrypta* could indicate that more undescribed species remain to be discovered or that all early-diverging lineages are now extinct.

One interesting result is the recovery of the species *M. tuberculata* Weber as the sister taxon to the rest of the species in the genus. This species was described from several workers collected by Mann in Bolivia during the Mulford expedition (MacCreagh, 1985). Several specimens, including some fungal cultivars, were later collected from the Amazonian forests of Ecuador and Colombia. This species is interesting because it is the only species in the genus in which the integument of the workers, queens, and males are covered with crypts (i.e. small pits) (Fig. 5). It has been shown (Currie *et al.*, 2006) that certain species of fungus-farming ants evolved cuticular crypts, which are lined with exocrine glands, which host and nourish antibiotic-producing bacteria used by the ants to protect their fungus gardens from parasitic fungi in the genus *Escovopsis*. Based on the dating analyses of the genus, *M. tuberculata* represents the earliest diverging fungus-farming ant lineage in which the crypts are present.

The presence of scale-like or squamate hairs was long considered a consistent character for distinguishing *Myrmicocrypta* species from those of other genera in the fungus-farming ants (Hölldobler & Wilson, 1990; Bolton, 1994). However, *Myrmicocrypta* species have been recently described that lack this character state and instead have erect simple or spatulate hairs (Sosa-Calvo & Schultz, 2010). Nonetheless, ancestral character-state analyses suggest that squamate or scale-like hairs are the ancestral condition in *Myrmicocrypta*, whereas the presence of erect simple or spatulate hairs is a derived condition that has arisen separately multiple times in the genus. Another derived character in *Myrmicocrypta* is the switch from underground nesting to rotten-wood nesting. This seems to have evolved a single time in the ancestor of the species *M. spinosa* and *M*. JSC001. The ability to occupy possibly abandoned pre-existing galleries in rotten wood (probably created by wood-boring beetles) has allowed these two species to spread throughout the Amazonian and Guiana Shield forests of South America. Of course, these galleries in rotten wood represent a less isolated environment for their cultivated fungi than underground chambers. For example, a colony of *Myrmicocrypta* has been observed to share the same fungus garden with an unrelated species, *Cyphomyrmex faunulus*, also nesting in the same rotten wood (J. Sosa-Calvo, personal observation). This behaviour, *parabiosis*, has also been observed between *Apterostigma urichii* and *C. faunulus* (Sanhudo *et al.*, 2008).

Fungal cultivars associated with Myrmicocrypta

As fungus-farming ants depend on their cultivated gardens for food, it was long assumed that both the ants and the fungi evolved by strict coevolution (Weber, 1972), a view consistent with the observation that daughter queens, prior to departing from the maternal nest, store a nucleus of fungal mycelium within their infrabuccal pockets. After mating, the foundress queen establishes a new nest, in which she regurgitates the fungal pellet and uses it to start her own garden (Huber, 1905; Mehdiabadi & Schultz, 2010). However, studies during the past two decades have shown that lower attines occasionally acquire new fungi from the environment and from each other, i.e. by horizontal transmission (Chapela *et al.*, 1994; Hinkle *et al.*, 1994; Mueller *et al.*, 1998; Vo *et al.*, 2009). *Myrmicocrypta* is known to practise lower agaricaceous agriculture (Chapela

et al., 1994), which involves the cultivation of fungi in two distantly related clades (clades 1 and 2) in the genera *Lepiota*, *Leucoagaricus*, and *Leucocoprinus*. Recently, Mehdiabadi *et al.* (2012) showed that fungus-farming ant species in the *Cyphomyrmex wheeleri* species group have each been exclusively associated with a single species of fungal cultivar for 5 million or more years, possibly creating conditions favouring strict ant–fungus coevolution, and that rare shifts to new cultivars were associated with ant speciation events.

Although we were not able to obtain DNA sequences for all of the fungi associated with the ant species included in this study, the available 53 sequences from fungi associated with 20 ant species indicate that: (i) species of *Myrmicocrypta* are associated with both fungal clades 1 and 2; (ii) ancestral-state reconstruction, in which associated fungal subclades (A–H) are treated as characters of ant species, suggests that the most recent common ancestor of *Myrmicocrypta* cultivated clade 2 fungi and that switches to clade 1 fungi have occurred at least five times; and (iii) *Myrmicocrypta* species grow fungi in at least four of the seven known cultivar subclades (B, D, E, and F) identified by Mehdiabadi *et al.* (2012) as well as in a new cultivar subclade, H, identified here (Figs 3, S1, Supporting Information). Although our cultivar sampling is sparse, it significantly increases the data available for future research on the fidelity of ant–cultivar associations in *Myrmicocrypta*. One such project could address the widely distributed species *Myrmicocrypta* JSC001, which is commonly found inhabiting rotten logs in the wet forests of the Amazon Basin and the Guiana Shield. Due to its habit of nesting in rotten logs, the opportunity for acquiring new cultivars is arguably higher in this species than in species with underground, more restricted, nesting habits. Such a project will benefit from current advances in phylogenomics, which facilitate reliable species delimitation by employing large amounts of genetic data for both the ants and their fungal cultivars.

Future studies should test the phylogenetic hypotheses proposed here and more thoroughly reconstruct the evolutionary history of *Myrmicocrypta* using newly developed phylogenomic markers (ultraconserved elements) for the ants and including all the species in the genus (Sosa-Calvo & Schultz, unpublished data). Ultraconserved elements have been successfully amplified from specimens aged 100 years and older (Blaimer *et al.*, 2016). Phylogenomic data for both the ants and their cultivars will hopefully produce a much clearer picture of patterns of ant–fungus association in *Myrmicocrypta* and enable tests of coevolution in this speciose and important group of fungus-farming ants.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Species identifications, extraction codes, collection codes, voucher specimen depository, collection data, and collector(s) of specimens used in this study.

Figure S1. Results of maximum likelihood (ML) analysis of 53 *Myrmicocrypta* fungal cultivar ITS sequences combined with > 400 pre-existing ant-associated and free-living fungal ITS sequences. (I) Fungal phylogeny excluding higher-attine cultivars. Major clades are represented in coloured boxes (blue = clade 2, red = clade 1) and the names of *Myrmicocrypta* cultivars are likewise colour-coded. Names of other fungi are in black. Free-living fungi are represented by grey collapsed branches and grey names. (II) Detail showing clade 2 with *Myrmicocrypta* cultivars in blue. Three fungal subclades are identified: subclade F (green box), subclade H (purple box), and subclade E (yellow box). Clade 1 is represented by collapsed red branch. (III) Detail showing clade 1 with *Myrmicocrypta* cultivars in red. Two fungal subclades are identified: subclade B (red box) and subclade D (blue box). Clade 2 is represented by collapsed blue branch.

Figure S2. Majority-rule consensus tree resulting from a Bayesian analysis of each gene used in this study, including five nuclear genes, *EF1aF1* (S2-A), *EF1aF2* (S2-B), *LW Rh* (S2-C), *wg* (S2-D), *TOP1* (S2-E); and one mitochondrial gene, *COI* (S2-F).

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