



Phylogenetic patterns of ant–fungus associations indicate that farming strategies, not only a superior fungal cultivar, explain the ecological success of leafcutter ants

Ulrich G. Mueller¹  | Melissa R. Kardish^{1,2} | Heather D. Ishak^{1,3} | April M. Wright⁴ | Scott E. Solomon^{5,6} | Sofia M. Bruschi^{1,7} | Alexis L. Carlson¹ | Maurício Bacci Jr⁷ 

¹Department of Integrative Biology, University of Texas at Austin, Austin, Texas

²Center for Population Biology, University of California, Davis, California

³Department of Medicine, Stanford University, Stanford, California

⁴Department of Biological Science, Southeastern Louisiana University, Hammond, Louisiana

⁵Department of Ecology and Evolutionary Biology, Rice University, Houston, Texas

⁶Department of Entomology, Smithsonian Institution, Washington, District of Columbia

⁷Centro de Estudos de Insetos Sociais, Universidade Estadual Paulista, Rio Claro, São Paulo, Brazil

Correspondence

Ulrich Mueller, Department of Integrative Biology, University of Texas at Austin, Austin, TX.
Email: umueller@austin.utexas.edu

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Abstract

To elucidate fungicultural specializations contributing to ecological dominance of leafcutter ants, we estimate the phylogeny of fungi cultivated by fungus-growing (attine) ants, including fungal cultivars from (i) the entire leafcutter range from southern South America to southern North America, (ii) all higher-attine ant lineages (leafcutting genera *Atta*, *Acromyrmex*; nonleafcutting genera *Trachymyrmex*, *Sericomyrmex*) and (iii) all lower-attine lineages. Higher-attine fungi form two clades, Clade-A fungi (*Leucocoprinus gongylophorus*, formerly *Attamyces*) previously thought to be cultivated only by leafcutter ants, and a sister clade, Clade-B fungi, previously thought to be cultivated only by *Trachymyrmex* and *Sericomyrmex* ants. Contradicting this traditional view, we find that (i) leafcutter ants are not specialized to cultivate only Clade-A fungi because some leafcutter species ranging across South America cultivate Clade-B fungi; (ii) *Trachymyrmex* ants are not specialized to cultivate only Clade-B fungi because some *Trachymyrmex* species cultivate Clade-A fungi and other *Trachymyrmex* species cultivate fungi known so far only from lower-attine ants; (iii) in some locations, single higher-attine ant species or closely related cryptic species cultivate both Clade-A and Clade-B fungi; and (iv) ant–fungus co-evolution among higher-attine mutualisms is therefore less specialized than previously thought. Sympatric leafcutter ants can be ecologically dominant when cultivating either Clade-A or Clade-B fungi, sustaining with either cultivar-type huge nests that command large foraging territories; conversely, sympatric *Trachymyrmex* ants cultivating either Clade-A or Clade-B fungi can be locally abundant without achieving the ecological dominance of leafcutter ants. Ecological dominance of leafcutter ants therefore does not depend primarily on specialized fungiculture of *L. gongylophorus* (Clade-A), but must derive from ant–fungus synergisms and unique ant adaptations.

KEYWORDS

fungus-growing ant, host–microbe evolution, key innovation, mutualism, symbiosis

1 | INTRODUCTION

Evolutionary innovations allow species to transition into previously unexplored niche-space (Donohue, 2005; Hunter, 1998; Szathmáry, 2015). Charismatic examples of such innovating transitions include the symbiotic origins of eukaryote organelles; multicellularity and sociality; powered flight of insects, birds and bats; as well as language and cultural inheritance in humans (Szathmáry, 2015). Among animals, the symbiosis between leafcutter ants and their cultivated fungi is thought to represent one such innovating “breakthrough” (Wilson, 1986) that enabled leafcutter ants to become one of the most competitive herbivores and thus fundamentally alter the ecology of Neotropical forests and grasslands (Corrêa, Silva, Wirth, Tabarelli, & Leal, 2010, 2016; Costa, Vasconcelos, Vieira-Neto, & Bruna, 2008; Della Lucia, 2011; Farji-Brener & Werenkraut, 2015; Fowler et al., 1989; Hertz, Beyschlag, & Hölldobler, 2007; Leal, Wirth, & Tabarelli, 2014; Meyer, Leal, Tabarelli, & Wirth, 2012; Vasconcelos, Leite, Vilhena, Lima, & Magnusson, 2008; Wirth, Herz, Ryel, Beyschlag, & Hölldobler, 2003).

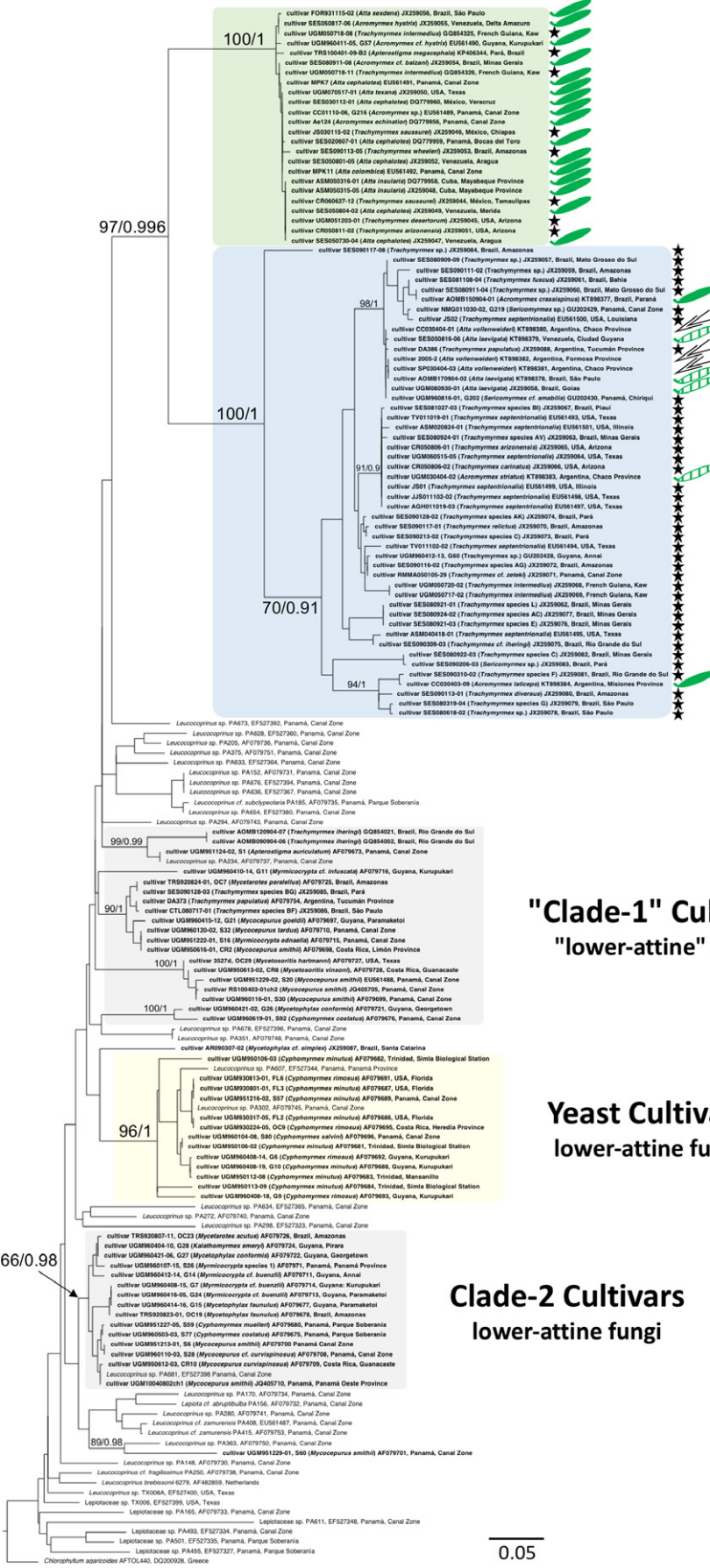
A number of morphological, behavioural, biochemical and genomic traits have been identified that characterize leafcutter ants and their cultivated fungi (Table 1; Aylward, Currie, & Suen, 2012; Bacci et al., 2013; De Fine Licht, Schiøtt, Mueller, & Boomsma, 2010; De Fine Licht et al., 2013; De Fine Licht, Boomsma, & Tunlid, 2014; DeMillo, Rouquette, Mueller, Kellner, & Seal, 2017; Kooij, Aanen, Schiøtt, & Boomsma, 2015; Mehdiabadi & Schultz, 2010; Mueller, 2002; Mueller, 2015; Mueller & Rabeling, 2010; Mikheyev, Mueller, & Abbot, 2006; Nygaard et al., 2011, 2016; Shik et al., 2014; Somera, Lima, Dos Santos-Neto, Lanças, & Bacci, 2015; Suen et al., 2010; Schultz & Brady, 2008), but an analysis of the causes and consequences of the transition from nonleafcutter to leafcutter fungiculture is still lacking. Towards such a synthesis, we use here phylogenetic analyses to (i) classify fungi cultivated by leafcutter ants collected across the entire leafcutter range from Argentina to the United States, including understudied regions in the leafcutter distribution (South America) and (ii) test the prevailing view that the success of leafcutter ants derives from a specialized co-evolutionary transition to a novel lineage of superior, high-yield cultivars (Chapela, Rehner, Schultz, & Mueller, 1994; Cherrett, 1986; Cherrett, Powell, & Stradling, 1989; De Fine Licht et al., 2010, 2013, 2014; Nygaard et al., 2016; Shik et al., 2016; Stradling & Powell, 1986). Some previous studies argued that cultivation of this superior cultivar was *sufficient* to promote colony growth of leafcutter nests and contribute to ecological dominance such that switching of nonleafcutter attines to cultivation of the superior cultivar may also enhance their colony performance. Other studies argued that evolution of a leafcutter-specific superior cultivar was *necessary* to enable ecological dominance of leafcutter ants. We find that (a) some non-leafcutter ants

in the genus *Trachymyrmex* cultivate fungi previously thought to be specific exclusively for leafcutter ants, and (b) some leafcutter ants cultivate fungi previously thought to be specific exclusively for non-leafcutter ants in the genera *Trachymyrmex* and *Sericomyrmex* (Figures 1 and 2, Tables 1 and 2). Combined, these findings argue against the views that cultivation of leafcutter-specific fungi was either necessary or sufficient to explain the ecological success of leafcutter ants.

1.1 | Attine ant–fungus associations

Leafcutter ants include a monophyletic group of about 50 described species in the genera *Atta* and *Acromyrmex*, which together comprise about 15% of the total diversity of nearly 300 described fungus-growing (attine) ant species (Bacci et al., 2009; Ješovnik, González, & Schultz, 2016; Mehdiabadi & Schultz, 2010; Schultz & Brady, 2008). Fungiculture evolved in attine ants about 55–65 million years ago, and leafcutter fungiculture arose from such ancestral ant–fungus associations about 18–19 million years ago (mya; ranges of 15.6–20.4 mya and 14–24 mya estimated by, respectively, Ješovnik et al., 2016 and Branstetter et al., 2017), either in grassland habitats of southern South America (Cristiano, Cardoso, Fernandes-Salomão, & Heinze, 2016; Fowler, 1983; Kusnezov, 1963; Mueller & Rabeling, 2010) or possibly in dry habitat in Central America (Branstetter et al., 2017). Leafcutter and nonleafcutter attine ants appear to cultivate fungal monocultures (Mueller, Scott, Ishak, Cooper, & Rodrigues, 2010; Poulsen & Boomsma, 2005; but see Sen, Ishak, Kniffin, & Mueller, 2010 for long-term leafcutter ant polyculture under laboratory conditions) and depend on obligate fungiculture for food; they do not hunt or scavenge like other ants, and instead plant, manure, weed, shelter and harvest fungal gardens, typically in underground chambers (Weber, 1972).

Despite these fungicultural similarities, ant–fungus interactions are remarkably diverse across all attine lineages, suggesting repeated evolution of novel traits in ant farmers and their fungal crops (De Fine Licht et al., 2014; Mueller, Rehner, & Schultz, 1998; Mehdiabadi & Schultz, 2010; Nygaard et al., 2016; Sosa-Calvo et al., 2013; Schultz et al., 2015; Shik et al., 2016; Weber, 1972; Table 1). As an example of a novel behavioural innovation in the ants, only ants in the monophyletic clade of leafcutter ants habitually use freshly cut vegetation as substrate to grow their fungi, whereas all other attine ant species typically do not cut live vegetation and instead use predominantly nonliving plant substrate for fungal cultivation, such as dried leaves, withered flowers, seeds or arthropod frass (De Fine Licht & Boomsma, 2010; Della Lucia, 2011; Mehdiabadi & Schultz, 2010; Weber, 1972; Wirth et al., 2003; Table 1). As examples of novel fungal innovations, fungi in a phylogenetically derived clade of so-called higher-attine fungi (including the leafcutter fungi) grow



Clade-A Cultivars
Leucocoprinus gonylophorus
(formerly *Attamyces*)

Clade-B Cultivars

'Clade-1' Cultivars
'lower-attine' fungi

Yeast Cultivars
lower-attine fungi

Clade-2 Cultivars
lower-attine fungi

0.05

Chorophyllum azaroides AFTOL40, DC200028, Greece

FIGURE 1 Phylogeny of fungi cultivated by fungus-growing ants. Taxa cover the known diversity of fungi cultivated by each of the main attine ant clades (Figure 2), excluding here only the distantly related pterulaceous fungi cultivated by some *Apterostigma* ants. Clade-A fungi were previously named leafcutter fungi (*Leucocoprinus gongylophorus*; formerly also *Attamyces* fungi) because they were known only from leafcutter ants (ant genera *Atta* and *Acromyrmex*), but this comprehensive phylogeny shows now that both leafcutter and some *Trachymyrmex* ants cultivate Clade-A fungi. Clade-B fungi were sometimes called *Trachymyrmex* fungi in the literature, but this common name *Trachymyrmex* fungi now appears to be a misnomer because many Clade-B fungi are actually cultivated by leafcutter ants, and one subclade of Clade-B fungi is predominantly cultivated by leafcutter ants. Clade-1 and Clade-2 fungi are defined as in Mueller et al. (1998) and are so-called lower-attine fungi, but phylogenetic patterns show here that Clade-1 fungi can also be cultivated by some higher-attine ants in the genus *Trachymyrmex*. As in Mueller et al. (1998), Clade-1 fungi are a phylogenetic grade and are therefore labelled “Clade-1”. Ant-cultivated fungi are labelled in bold “cultivar ...”; these labels include in parentheses the names of the ant species from which fungal gardens were collected. Free-living fungi (collected not associated with attine ants) are labelled with their genus names (*Leucocoprinus*, *Leucoagaricus*, *Lepiota*) and by collection IDs (e.g., PA302). A *Chlorophyllum* fungus was used as outgroup for rooting. Phylogenetic relationships inferred with Bayesian methods corroborate the key features inferred also with likelihood methods (compare likelihood and Bayesian trees in Figure S1). For Clade-A and Clade-B fungi, a symbol to the right of each taxon indicates the gardening substrate preferred by the corresponding ant farmer (see Table S4): asterisk = nonleaf substrate preferred by *Trachymyrmex* and *Sericomyrmex* ants; solid green leaf = dicot-leaf substrate preferred; cross-hatched leaf = both dicot and monocot leaves used as substrate (*Atta laevigata*, *Acromyrmex striatus*); grass bunch = monocot-leaf substrate preferred (*Atta vollenweideri*)

clusters of nutrient-rich, hyphal-tip swellings (gongylidia) that are harvested by the ants for food (De Fine Licht et al., 2010, 2013; De Fine Licht & Boomsma, 2014; Möller, 1893; Mueller, 2002; Weber, 1972; Table 1); and higher-attine fungi are multinucleate (containing more than two nuclei per cell) and polyploid (containing multiplied genomes within a nucleus), whereas lower-attine fungi are dikaryotic (containing two haploid nuclei per cell; i.e., lower-attine fungi are

functional diploids; Carlson et al., 2017; Kooij, Aanen et al., 2015; Scott, Kveskin, Cooper, & Mueller, 2009). Multinucleate cells and polyploid nuclei may increase hyphal growth because of gene-dosage advantages (Kooij, Aanen et al., 2015). The phylogenetically derived fungus thought to be cultivated only by leafcutter ants is called *Leucocoprinus gongylophorus* (Leucocoprini, Agaricales) as sexual morph (Heim, 1957; also sometimes called *Leucoagaricus gongylophorus*,

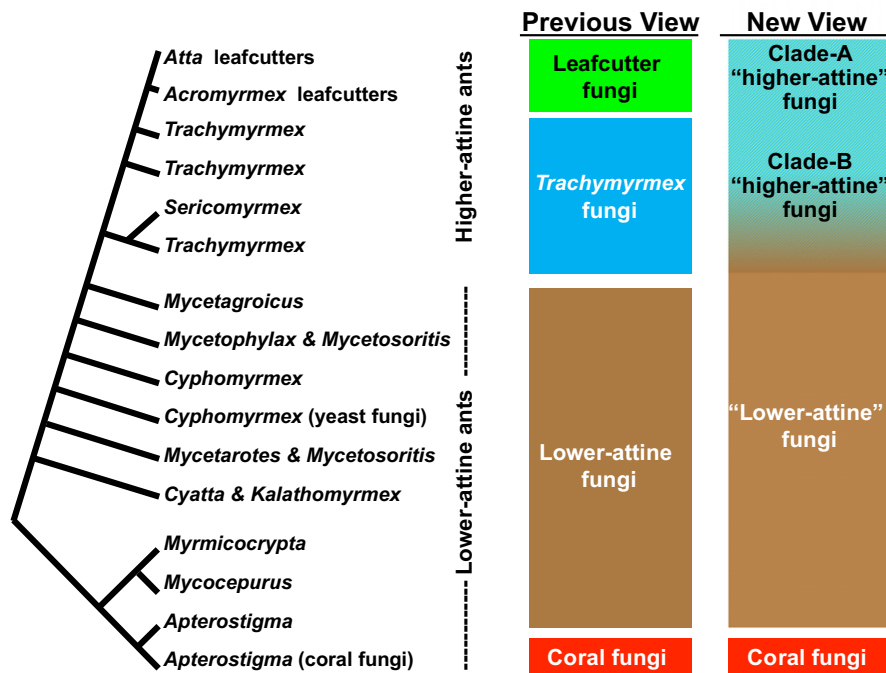


FIGURE 2 Fungicultural systems (right) inferred in previous phylogenetic analyses (Previous View) and the New View derived from our phylogenetic analysis in Figure 1. The fungicultural systems are mapped onto the phylogeny of attine ants (left); this ant phylogeny is from Schultz et al. (2015), but is simplified here for those lower-attine lineages for which generic boundaries remain unresolved (e.g., *Cyphomyrmex*, *Mycetosoritis*). Modifying the Previous View, Figure 1 shows that (i) leafcutter ants (genera *Atta* and *Acromyrmex*) are not strictly specialized to cultivate only leafcutter-specific leafcutter fungi (some leafcutter ants cultivate fungi thought previously to associate only with *Trachymyrmex* ants); (ii) some *Trachymyrmex* ants cultivate fungi thought previously to associate only with leafcutter ants; (iii) leafcutter and *Trachymyrmex* ants therefore can share fungi from a clade of higher-attine fungi (cross-hatched in shades of turquoise colour); and (iv) some *Trachymyrmex* ants cultivate fungi known so far only from lower-attine ants. We do not show here that recently discovered fungi of the lower-attine ant *Apterostigma megacephala* are Clade-A fungi (Schultz et al., 2015; one representative sample is shown in Figure 1 in Clade-A), and this ant–fungus association of *Apt. megacephala* further blurs the distinction between the “higher-attine” versus “lower-attine” fungiculture

TABLE 1 Constellations of traits that define attine ant–fungus mutualisms and that have been hypothesized to characterize innovating transitions in ant–fungus evolution

	Lower-Attine Fungiculture	Pterulaceous Fungiculture	Yeast Fungiculture	Higher-Attine Fungiculture			References
				So-called <i>Trachymyrmex</i> Fungiculture	So-called Leafcutter Fungiculture		
Ant traits							
Ant genera	<i>Myrmicocrypta</i> , <i>Mycocepurus</i> , <i>Apterostigma</i> , <i>Cyatta</i> , <i>Kalathomyrmex</i> , <i>Mycetarotes</i> , <i>Mycetosoritis</i> , <i>Mycetophylax</i> , <i>Paramycetophylax</i> , <i>Cyphomyrmex</i> (<i>wheeleri</i> group), <i>Mycetagroicus</i>	<i>Apterostigma</i> (<i>pilosum</i> group)	<i>Cyphomyrmex</i> (<i>rimosus</i> -group)	<i>Trachymyrmex</i> , <i>Sericomyrmex</i>	<i>Acromyrmex</i>	<i>Atta</i>	Weber (1972), Schultz & Meier (1995), Brandão & Mayhé-Nunes (2008), Schultz & Brady (2008), Klingenberg & Brandão (2009), Mehdiabadi et al. (2012), Schultz et al. (2015), Sosa-Calvo et al. (2013, 2017)
Worker polymorphism	Monomorphic	Monomorphic	Monomorphic	Monomorphic or weakly polymorphic	Polymorphic	Highly polymorphic	Weber (1972), Schultz & Meier (1995)
Number of queens	Typically monogynous, some highly polygynous species (e.g., <i>Mycocepurus</i>)	Monogynous or oligogynous	Monogynous or oligogynous	Monogynous or oligogynous	Monogynous or oligogynous	Mostly monogynous, some oligogynous	Weber (1972), Fernández-Marín, Zimmerman, Wcislo, & Rehner (2007), Himler, Caldera, Baer, Fernández-Marín, & Mueller (2009), Rabeling et al. (2011)
Multiple mating of queens	Single mating	Single mating	Single mating	Single mating	Multiple mating	Multiple mating	Murakami, Higashi, & Windsor (2000), Villesen, Murakami, Schultz, & Boomsma (2002), Villesen & Boomsma (2003)
Garden morphology	Sponge-like, hanging or sessile	Sponge-like, either veiled and hanging (G2) or unveiled and sessile (G4)	Yeast–nodule clusters, sessile	Sponge-like, typically hanging	Sponge-like, sessile or hanging	Sponge-like, sessile	Weber (1972), Villesen et al. (2004), Rabeling et al. (2007), Leal-Dutra (2015), Ješovnik, Chaul, & Schultz (2018)
Substrate collected for fungiculture	Dead vegetable matter (leaf debris, seeds, insect frass), extrafloral nectar?	Dead vegetable matter (leaf debris, seeds, insect frass)	Dead vegetable matter (insect frass, flower debris, seeds), extrafloral nectar?	Dead vegetable matter (leaf debris, seeds, insect frass) some live-cut vegetation	Live-cut vegetation, also some dead plant matter	Mostly live-cut vegetation	Weber (1972), Murakami & Higashi (1997), Wirth et al. (2003), Price et al. (2003),

(Continues)

TABLE 1 (Continued)

	Lower-Attine Fungiculture	Pterulaceous Fungiculture	Yeast Fungiculture	Higher-Attine Fungiculture			References
				So-called <i>Trachymyrmex</i> Fungiculture	So-called Leafcutter Fungiculture		
							Rabeling et al. (2007), De Fine Licht & Boomsma (2010), Ješovnik et al. (2018)
Cutting of live vegetation	Absent	Absent	Absent	Rare	Frequent	Dominant	Weber (1972), Leal et al. (2014)
Biofilm accretions (communities of bacteria and fungi) growing on the ant integument	Bacterial accretions variable; in some ant species, accretions are dominated by integument-colonizing fungi	Bacterial accretions variable	Reduced?	Bacterial accretions variable, sometimes greatly reduced or absent	Bacterial accretions variable	Bacterial accretions greatly reduced or absent	Currie, Scott, Summerbell, & Malloch (1999), Kost et al. (2007), Mueller, Dash, Rabeling, & Rodrigues (2008), Sen et al. (2009), Fernández-Marín, Zimmerman, Nash, Boomsma, & Wcislo (2009), Barke et al. (2010), Schoenian et al. (2011), Ishak et al. (2011), Mattoso, Moreira, & Samuels (2012), Mueller (2012), Andersen, Yek, Nash, & Boomsma (2015), Holmes et al. (2016), Ješovnik & Schultz (2017)
Gut microbiome	Attine-specific bacteria present	Attine-specific bacteria present	Unknown	Attine-specific bacteria present	Attine-specific bacteria present	Attine-specific bacteria present	Meirelles et al. (2016), Sapountzis et al. (2015), Zhukova, Sapountzis, Schiøtt, & Boomsma (2017)
Arginine biosynthesis	Absent	Absent	Unknown	Absent	Absent	Absent	Nygaard et al. (2016), Ješovnik et al. (2016)
Fungal traits							
Taxonomy	Several lineages of leucocoprineaceous Agaricaceae (Basidiomycota)	Two described <i>Pterula</i> species, plus several additional species of Pterulaceae (Ascomycota)	Monophyletic clade within leucocoprineaceous Agaricaceae (Basidiomycota)	Monophyletic clade within leucocoprineaceous Agaricaceae (Basidiomycota)			Chapela et al. (1994), Mueller et al. (1998), Villesen et al. (2004); Munkacsi et al. (2004); Dentinger et al. (2009), Leal-Dutra, (2015), This publication

(Continues)

TABLE 1 (Continued)

				Higher-Attine Fungiculture			References
Lower-Attine Fungiculture	Pterulaceous Fungiculture	Yeast Fungiculture	So-called <i>Trachymyrmex</i> Fungiculture	So-called Leafcutter Fungiculture			
Original view			Monophyletic cultivar clade specific to <i>Trachymyrmex</i> & <i>Sericomyrmex</i> ants (i.e., reciprocal monophyly between ants & fungi)	Monophyletic cultivar clade specific to <i>Atta</i> & <i>Acromyrmex</i> ants (i.e., reciprocal monophyly between ants & fungi)			Chapela et al. (1994), Currie et al. (2003), Price et al. (2003), Schultz & Brady (2008), Kooij, Poulsen et al. (2015), Nygaard et al. (2016)
New view (Figures 1 & 2)			Sharing of cultivars is possible between all genera of higher-attine ants; some fungal clades are cultivated more frequently by leafcutter ants, some more by <i>Trachymyrmex</i> & <i>Sericomyrmex</i> ants; some <i>Trachymyrmex</i> cultivate lower-attine fungi; see Figure 1 and Table 2				This publication, Mueller et al. (2017)
Free-living fungal relatives	Yes	Yes	Yes	Not found so far	Not found so far	Not found so far	Mueller et al. (1998), Mueller (2002), Vo et al. (2009)
Fungi transition in & out of symbiosis	Likely	Likely	Likely	Unlikely	Unlikely	Unlikely	Mueller et al. (1998), Pagnocca et al. (2001), Mueller (2002), Vo et al. (2009)
Fungus growth morph when cultivated	Hyphal	Hyphal	Yeast	Hyphal	Hyphal	Hyphal	Weber (1972), Wang, Mueller, & Clardy (1999), Mueller et al. (1998)
Growth morph in laboratory culture	Hyphal	Hyphal	Yeast or hyphal	Hyphal	Hyphal	Hyphal	Hervey et al. (1977), Wang et al. (1999)
Cultivars can produce fruiting structures	Yes	Yes	Yes	Yes?	Yes	Yes	Hervey et al. (1977), Pagnocca et al. (2001), Mueller et al. (1998), Mueller (2002), Solomon et al. (2004)
Fruiting structures can develop viable spores capable of germination	Yes	Yes	Likely	Unknown	(Yes)	Unknown	Table 3 in Mueller (2002) summarizes literature; Möller (1893), Pagnocca et al. (2001), Dentinger et al. (2009), Mueller et al. (2017)
Number of nuclei per cell	Dikaryotic	Dikaryotic	Dikaryotic	Multinucleate	Multinucleate	Multinucleate	Mohali (1998), Scott et al. (2009), Kooij, Aanen et al. (2015), Carlson et al. (2017)

(Continues)

TABLE 1 (Continued)

	Lower-Attine Fungiculture	Pterulaceous Fungiculture	Yeast Fungiculture	Higher-Attine Fungiculture			References
				So-called <i>Trachymyrmex</i> Fungiculture	So-called Leafcutter Fungiculture		
Gongyldia	Absent, one known exception	Absent	Absent	Present	Present	Present	Hervey et al. (1977), De Fine Licht et al. (2014), Masiulionis et al. (2014)
Clamp connections	Absent, or very rare	Present	Absent, or very rare	Absent, or very rare	Absent, or very rare	Absent	Hervey et al. (1977), Mohali (1998), Carlson et al. (2017)
Dolipore septum	Present	Absent	Unknown	Present	Present	Present	Hervey et al. (1977), Mohali (1998), Carlson et al. (2017)
Detox-enzyme activity (e.g., laccases)	Low	Low	Low	Low	High	High	De Fine Licht et al. (2013, 2014), Nygaard et al. (2016)
Carbohydrate degradation genes, plant fibre digestion	Comparable to free-living agaric fungi	Unknown	Unknown	Reduced	Reduced / variable	Reduced	Nygaard et al. (2016), DeMolto et al. (2017)
Garden infections by specialized & unspecialized diseases	Yes	Yes	Unknown	Yes	Yes	Yes	Currie et al. (2003), Gerardo, Jacobs et al. (2006), Gerardo, Mueller et al. (2006), Taerum et al. (2007), Rodrigues, Bacci, Mueller, Ortiz, & Pagnocca (2008), Rodrigues, Mueller, Ishak, Bacci, & Pagnocca (2011), Augustin et al. (2013), Meirelles et al. (2015), de Man et al. (2016), Birnbaum & Gerardo (2016), Barcoto, Pedrosa, Bueno, & Rodrigues (2017), Varanda-Haifig et al. (2017), Kellner, Kardish, Seal, Linksvayer, & Mueller (2018), Dhodary, Schilg, Wirth, & Spitteller (2018)

The genus *Pseudoatta* is subsumed here as a specialized social parasite arising within the genus *Acromyrmex*. Future research is likely to reveal important fungicultural differences between attine ant lineages subsumed here under Lower-Attine Fungiculture (e.g., *Apterostigma megacephala* exhibits traits typical for lower-attine ants, but cultivates a Clade-A fungus; Schultz et al. (2015)). Many genomic changes likely occurred in the ants at the origin of attine fungiculture (De Fine Licht et al., 2014; Ješovnik et al., 2016; Mueller et al., 2001; Nygaard et al., 2016); such genomic changes are not listed here because only one lower-attine ant and one lower-attine fungus have been whole-genome-sequenced so far, and no closely related free-living leucocoprineaceous fungi have been whole-genome-sequenced for comparison. Higher-Attine Fungiculture is listed here subdivided into so-called Leafcutter Fungiculture and *Trachymyrmex* Fungiculture discussed in previous literature, but the phylogenetic analysis in Figure 1 shows that this subdivision within Higher-Attine Fungiculture is not a strict partition (Figure 2). Because leafcutter species can cultivate also Clade-B fungi, and because some leafcutter species appear specialized to cultivate Clade-B fungi, the label *Trachymyrmex* Fungiculture for Clade-B cultivation is a misnomer.

TABLE 2 Prevalence of Clade-A-cultivating, Clade-B-cultivating and Clade-1-cultivating leafcutter ants and *Trachymyrmex/Sericomyrmex* ants represented in Figure 1, listed separately for (a) southern South America, (b) northern South America, (c) Central America and (d) North America

	Clade-A higher-attine fungi	Clade-B higher-attine fungi	Clade-1 lower-attine fungi	Total sample size
(a) Southern South America				
Leafcutter Ants <i>Atta/Acromyrmex</i>	20% (2)	80% (8)	0% (0)	10
<i>Trachymyrmex/Sericomyrmex</i>	0% (0)	75% (12)	25% (4)	16
(b) Northern South America				
Leafcutter Ants <i>Atta/Acromyrmex</i>	83% (5)	17% (1)	0% (0)	6
<i>Trachymyrmex/Sericomyrmex</i>	18% (3)	76% (13)	6% (1)	17
(c) Central America				
Leafcutter Ants <i>Atta/Acromyrmex</i>	100% (4)	0% (0)	0% (0)	4
<i>Trachymyrmex/Sericomyrmex</i>	0% (0)	100% (3)	0% (0)	3
(d) North America				
Leafcutter Ants <i>Atta/Acromyrmex</i>	100% (5)	0% (0)	0% (0)	5
<i>Trachymyrmex/Sericomyrmex</i>	22% (4)	78% (11)	0% (0)	15

Sample sizes are listed in parentheses. Because the samples included in this Table and in Figure 1 were not selected to be biogeographically representative, the percentages shown for the different fungal groups in different biogeographic regions need to be interpreted cautiously. See text (Section 3.7).

following Singer, 1986; or formerly called *Attamyces bromatificus* as asexual morph, following Kreisel, 1972; the valid name is *Leucocoprinus gongylophorus*, Mueller et al., 2017). With very few documented exceptions explained below, all other ant-cultivated fungi grow as unmodified mycelia, which resembles mycelium of closely related free-living fungi (Hervey, Rogerson, & Leong, 1977; Mueller et al., 1998; Vo, Mueller, & Mikheyev, 2009).

Previous phylogenetic analyses led to the view that the phylogenetically derived higher-attine ants (genera *Trachymyrmex*, *Sericomyrmex*, and the leafcutter genera *Atta* and *Acromyrmex*) cultivate derived gongylidia-bearing higher-attine fungi that belong to a monophyletic clade of leucocoprineaceous fungi (Agaricaceae, formerly Lepiotaceae; Chapela et al., 1994; Hervey et al., 1977; Mikheyev, Mueller, & Abbott, 2010; Mikheyev et al., 2006; Schultz et al., 2015). In contrast, the early-branching, lower-attine ants are thought to cultivate morphologically unmodified, leucocoprineaceous fungi (Hervey et al., 1977; Kellner et al., 2013; Mueller et al., 1998), with the exception of one subclade of ants (*pilosum* group in the ant genus *Apterostigma*) that cultivate fungi in the distantly related basidiomycete family Pterulaceae (coral fungi; Dentinger, Lodge, Munkacsı, Desjardin, & McLaughlin, 2009; Leal-Dutra, 2015; Munkacsı et al., 2004; Villesen, Mueller, Schultz, Adams, & Bouck, 2004). Most phylogenetic information published to date for lower- and higher-attine fungi derived from surveys in Central America, primarily Panamá (De Fine Licht & Boomsma, 2014; Green, Adams, & Mueller, 2002; Kellner et al., 2013; Mehdiabadi, Mueller, Brady, Himler, & Schultz, 2012; Mikheyev, Mueller, & Boomsma, 2007; Mueller et al., 1998), and from surveys focusing on specific locations or specific attine ant species in Brazil, Argentina, and the United States (Lugo, Crespo, Cafaro, & Jofre, 2013; Mikheyev, Vo, & Mueller, 2008; Mueller, Mikheyev, Solomon, & Cooper, 2011; Pereira et al., 2015;

Silva-Pinhati et al., 2004). Phylogenetic patterns emerging from these previous surveys conformed to this tidy ant–fungus correspondence between higher- versus lower-attine fungiculture (Mueller, Schultz, Currie, Adams, & Malloch, 2001; Price, Murakami, Mueller, Schultz, & Currie, 2003; Schultz & Brady, 2008; Mehdiabadi & Schultz, 2010; Figure 2).

Three observations have been reported, however, that are inconsistent with a strict distinction between this traditional understanding of higher- versus lower-attine fungiculture. First, one nest of the higher-attine ant *Trachymyrmex papulatus* from Tucumán, Argentina, was found to cultivate a lower-attine fungus (collection DA373 shown in Figure 1 of Mueller et al., 1998), whereas a second nest of that same ant species and collected at the same site cultivated a typical gongylidia-bearing higher-attine fungus (see footnote 10 in Mueller et al., 1998). These two fungi are included as DA373 and DA386 in the below analyses, and these collections were the first to suggest the possibility that different nests of the same attine ant species may cultivate both lower- and higher-attine fungi; that is, a population of a single higher-attine ant species (or closely related cryptic species) may be polymorphic for its fungi, cultivating a fungal diversity more polymorphic than known for some lower-attine ants (Kellner et al., 2013; Mehdiabadi et al., 2012; Mueller et al., 1998). Second, a lower-attine fungus cultivated by the lower-attine *Mycoprepurus smithii* from Brazil was recently found to grow gongylidia-like structures (Masiulionis et al., 2014). It is unknown whether these structures are homologous or convergent to gongylidia of higher-attine fungi, and gongylidia may therefore not be a synapomorphy unique to higher-attine fungi. Third, and most unexpected, although ants in the genus *Apterostigma* were long believed to cultivate only lower-attine leucocoprineaceous fungi or pterulaceous fungi (Dentinger et al., 2009; Mueller et al., 1998; Munkacsı et al., 2004; Villesen

et al., 2004), a population of the lower-attine ant *Apterostigma megacephala* in Brazil cultivates a type of fungus previously known only from leafcutter ants (Schultz et al., 2015).

These accumulating exceptions suggest that additional undescribed ant–fungus associations could emerge in careful and comprehensive surveys of attine symbioses. Of particular interest are surveys covering South America, the presumed location where attine ants originated about 55–65 mya (Branstetter et al., 2017; Ješovnik et al., 2016; Schultz et al., 2015) and where extant attine ants exhibit the greatest species and generic diversity (Mehdiabadi & Schultz, 2010; Sosa-Calvo et al., 2013; Weber, 1972). Previous phylogenetic analyses of attine fungi focused on samples with limited geographic distribution, whereas our analysis here includes the most comprehensive sampling to date, covering representative diversity of attine fungi across South, Central and North America, including undersampled regions in southern South America where leafcutter ants are most diverse (Cristiano et al., 2016; Fowler, 1983; Kusnezov, 1963; Weber, 1972). We also include a cultivar of *Acromyrmex striatus*, the most basal (earliest-branching) of all leafcutter ant lineages (Branstetter et al., 2017; Cristiano, Cardoso, & Fernandes-Salomão, 2013; Cristiano et al., 2016), for which no cultivar has been characterized to date. Phylogenetic relationships of these accumulated collections (Figure 1) reveal previously undetected patterns of ant–fungus associations in higher-attine fungiculture and prompt rethinking of prevailing beliefs about a strict distinction between leafcutter versus nonleafcutter fungiculture among higher-attine ants.

2 | MATERIALS AND METHODS

2.1 | Sample selection and sequencing strategy

We selected phylogenetically representative cultivar samples from the entire diversity of more than 2,500 samples of ethanol-preserved garden collections stored frozen at -80°C in the Attine Collections of the Bacci Lab (UNESP Rio Claro) and the Mueller Lab (University of Texas at Austin). We selected samples to complement sequence information on attine cultivars generated in previous surveys of cultivars of lower-attine ants (Kellner et al., 2013; Mehdiabadi et al., 2012; Mueller et al., 1998), nonleafcutter higher-attine ants (Solomon et al., in preparation) and leafcutter ants (Mueller et al., 2017) and thus enable a global phylogenetic reconstruction of attine-cultivated leucocoprineaceous fungi. We included representatives from all known lepiotaceous fungus clades cultivated by lower-attine ants, such as Clade 1A & 1B and Clade 2 as defined by Kellner et al., 2013; plus representatives from the two known fungal clades and their known subclades cultivated by higher-attine ant genera *Atta*, *Acromyrmex*, *Trachymyrmex* and *Sericomyrmex*). We selected phylogenetically representative samples for which either the ITS rDNA gene, the 25S Large Subunit (LSU) rDNA gene or the EF1- α gene had already been sequenced in previous analyses and then generated new sequence information (Table S1) such that a total of 153 samples were sequenced for both the faster-evolving ITS and EF1 genes.

The only four taxa for which we had information only for the EF1 gene were the important samples AOMB090904-06 (labelled TrachylherBRAOMB09090406, GQ854002, in Figure 1), AOMB120904-07 (TrachylherBRAOMB12090407, GQ854021), UGM050718-08 (TrachylInterUGM05071808, GQ854325) and UGM050718-11 (TrachylInterUGM05071811, GQ854326), which we chose not to exclude from analyses because of their significance in elucidating ant–fungus associations (Figure 1). We also included one LSU sequence (KP406344) available for a cultivar of *Apterostigma megacephala* to indicate the close phylogenetic relationship of *megacephala* cultivar to fungi cultivated by some leafcutter ant species, as already reported by Schultz et al. (2015); no EF1 sequence was available for any cultivar of *Apt. megacephala*, and the single ITS sequence (KP406338) available for a cultivar of *Apt. megacephala* was poorly sequenced (as already noted by Schultz et al., 2015), so this ITS sequence did not permit reliable alignment. We did not sequence the slower-evolving LSU gene for all samples in Clade-A and Clade-B because that gene provides less resolution for recent diversifications (e.g., within the group of Clade-A fungi, the LSU gene is mostly invariable, likewise within some subclades of Clade-B), but we made sure to sequence the LSU gene for multiple samples from within each main clade across the phylogeny (e.g., Clade-A, the yeast–fungus clade, each known subclade within Clade-B) and for most free-living leucocoprineaceous fungi (fungi not cultivated by attine ants), such that information from the LSU gene could help differentiate between the main clades and help stabilize the phylogenetic backbone subtending the main clades.

2.2 | Sequencing methods and phylogenetic analyses

To generate new sequence information from garden collections, we separated hyphal mycelium or clusters of gongylidia (if present) under a microscope from garden substrate and then extracted the fungal material using a standard Chelex protocol (Sigma-Aldrich; Kellner et al., 2013). We generated sequence information for the Internal Transcribed Spacer (ITS) region using primers ITS4 and ITS5 (Mueller et al., 1998; White, Bruns, Lee, & Taylor, 1990); for a segment of the 25S Large Subunit (LSU) rDNA gene using primers LR0R and LR3 (Mueller et al., 1998; Vilgalys & Hester, 1990); and a segment of the EF1- α gene using primers EF1F and EF1R (Mikheyev et al., 2006). Sequences were generated via Sanger sequencing on an ABI 3100 DNA Analyzer (Applied Biosystems) in the Mueller Lab, or an ABI 3730 DNA Analyzer at the ICBM Core Sequencing Facility of the University of Texas at Austin (icbm.utexas.edu/dna-sequencing-facility). We assembled forward and reverse sequences, and edited contigs, in Sequencher version 4.6 or GENEIOUS v.6.1, then aligned sequences using the Clustal function implemented in MACCLADE version 4.06 (Maddison & Maddison, 2003). Two authors inspected edited sequences and alignments to correct obvious sequencing errors and confirm unusual characters by reinspection of sequencing chromatograms. In some regions of the alignments (e.g., hypervariable portions of ITS1 and ITS2; introns of the EF-gene), character

homologies could not be determined with absolute certainty across the diversity of all taxa; these regions of uncertain alignment were excluded from phylogenetic analyses (see alignment available in the Appendix S1). Regions included in analyses therefore included the beginning and end regions of ITS1 and ITS2; the 5.8 gene embedded in the ITS gene and adjacent regions of the 5.8 gene in ITS1 and ITS2 that could be aligned with confidence; the portion of the LSU (25S) gene between primers LR0R and LR3; and the exon portions of the EF1 gene between primers EF1F & EF1R. All sequences in this alignment are listed with their respective GenBank accessions and collection information in Table S1. New sequences are deposited at NCBI GenBank under Accession nos KT898377-KT898391. The concatenated alignment (ITS, characters 1-217; LSU, characters 218-861; EF1- α , characters 862-1204), showing all the alignable characters used in our phylogenetic analyses, is available in the Appendix S1.

We analysed the alignment (ITS, LSU, EF1) with both maximum-likelihood and Bayesian phylogenetic methods. We used PARTITIONFINDER 2.0.0 (Lanfear, Frandsen, Wright, Senfeld, & Calcott, 2016) to test for the number of partitions and the model of sequence evolution that best describes the data. PARTITIONFINDER also fits a model of sequence evolution to each partition. We tested three biologically realistic partitioning schemes, and three algorithmically derived schemes. The partitions tested were as follows: (i) all three genes in one single partition; (ii) each gene in its own partition (3 total partitions); (iii) each gene and each codon in its own partition (five total partitions); (iv) an algorithmically generated partition scheme created by running PartitionFinder's exhaustive partition search (search = kmeans); and (v) two best-fitting partition schemes found by searching the three biologically inspired partition schemes (search = all, performed for each of schemes 1–3). We evaluated the partitioning schemes using the AIC_c criterion (scores listed in Table S2). The best-supported partitioning scheme was scheme (iii).

We conducted a likelihood analysis in RAXML 8.2.11 (Stamatakis, 2014), with 100 bootstrap replicates to assess support for nodes in the tree, and a Bayesian phylogenetic analysis in REVBayes 1.0.5 (Höhna et al., 2014, 2016). Both programs have fewer nucleotide sequence models available than PARTITIONFINDER. If the best-fit model was not available, a simpler model encapsulating the important facets of the model was chosen (typically GTR + I + G). Bayesian model fitting differs from likelihood-based model fitting because it incorporates the effect of the prior. Therefore, we used Bayes factor model fitting as described in Xie, Lewis, Fan, Kuo, and Chen (2011) to estimate the best-fit model in a Bayesian context. Convergence of the Bayesian estimation was checked with TRACER 1.6.0 (Rambaut, Suchard, Xie, & Drummond, 2014). Bayesian analyses produce a sample of trees, rather than a point estimate. We then summarized the sample in REVBayes as a consensus tree to maintain comparability to the RAXML results. The resulting best likelihood and Bayesian consensus trees are juxtaposed in Figure S1; only the likelihood tree is shown in Figure 1, because the main features emphasized in the discussion (monophyly of Clade-A, monophyly of Clade-B) are well supported in both likelihood and Bayesian reconstructions (Figure S1).

The three markers for phylogenetic analyses (ITS, LSU and EF1) had been chosen to resolve with confidence the main clades of attine-cultivated leucocoprineaceous fungi, indicated by the high support values supporting monophyly of Clade-A, Clade-B, the clade of higher-attine fungi (=Clade-A&B) and the clade of yeast fungi in the analyses using information from all three genes (Figure 1; Figure S1). Clade-A and Clade-B also emerged in exploratory analyses using information only from the EF1 gene (Figure S2) and only from the ITS gene (Figure S3), corroborating phylogenetic relationships that emerged in previous analyses using these same genes (Mueller et al., 1998, 2017). Also consistent with previous findings (Mueller et al., 1998), because the LSU is the slowest-evolving gene in our analysis of all three genes, an analysis using information only from the LSU gene did not resolve phylogenetic relationships within the group of leucocoprineaceous fungi (Figure S4), but the information from the LSU gene helped with reconstruction of the phylogenetic "backbone" (the more ancient diversifications) and with the rooting with the outgroup fungus *Chlorophyllum* (Figure S4). As expected, therefore, no single gene contained sufficient signal to resolve diversifications with adequate certainty, but the different genes added to each other in the partitioned analyses combining information from all three genes (Figure 1; Figure S1). While Clade-A, Clade-B and the sister relationship between Clade-A and Clade-B are well supported in the partitioned analyses using information from the three genes, Clades 1 & 2 of lower-attine fungi and the more ancient diversifications are less well resolved (see the lower support values of the backbone of the phylogenetic trees in Figure 1; Figure S1), and Clade-1 emerges as a phylogenetic grade rather than a monophyletic group. This inadequate resolution of these ancient diversifications and the phylogenetic grade of "Clade-1" fungi was already seen in the 2-gene phylogeny (ITS & LSU) of lower-attine fungi by Mueller et al. (1998). While the addition of new sequence information from the EF1-gene for all fungi in our analysis improved support for the main clades compared to previous phylogenetic analyses, addition of the EF1-gene provided only moderately better resolution of the phylogenetic backbone across the leucocoprineaceous fungi. Addition of sequence information from slower-evolving genes (e.g., 18S rDNA) or ultraconserved genes will therefore be necessary to help elucidate these ancient diversifications across the leucocoprineaceous fungi. The insufficient resolution of the ancient diversifications does not affect the main conclusions emphasized in the main text, which depend entirely on the strong support of the monophyly of Clade-A (likelihood bootstrap support 100%; Bayesian posterior probability 1.0), the monophyly of Clade-B (likelihood 100%, Bayesian 1.0) and the monophyly the so-called higher-attine fungi in Clades A&B, (likelihood 97%, Bayesian 0.99; Figure 1).

3 | RESULTS AND DISCUSSION

3.1 | Leafcutter ants are not strictly specialized to cultivate only leafcutter-specific fungi

Consistent with earlier, less comprehensive phylogenetic analyses (Chapela et al., 1994; Ješovnik et al., 2016; Mikheyev et al., 2006,

2008, 2010; Nygaard et al., 2016), fungi cultivated by higher-attine ants group into two well-supported clades, called here Clade-A and Clade-B fungi (Figure 1). Clade-A fungi are traditionally called leafcutter fungi (*Leucocoprinus gongylophorus* according to Heim, 1957), or previously also *Attamyces* fungi (based on the description of the original *Attamyces* isolate from a garden of *Atta insularis* from Cuba; Kreisel, 1972), cultivated by ants said to practice leafcutter fungiculture. In contrast, Clade-B fungi are thought to be cultivated only by the two other higher-attine ant genera *Trachymyrmex* and *Sericomyrmex* that are said to practice *Trachymyrmex* fungiculture (Currie et al., 2003; Mikheyev et al., 2008; Price et al., 2003; Schultz, Mueller, Currie, & Rehner, 2005). Our increased sampling reveals previously undetected patterns of ant–fungus associations (Figure 1; Table 2) that are inconsistent with this clade-to-clade ant–fungus correspondence among higher-attine ants. While most fungi characterized so far for leafcutter ants are Clade-A fungi, and while most fungi characterized so far for *Trachymyrmex* and *Sericomyrmex* ants are Clade-B fungi, Figure 1 shows that there exists no absolute phylogenetic difference between the fungi cultivated by these two groups of ants: some leafcutter ants cultivate Clade-B fungi, some *Trachymyrmex* ants cultivate Clade-A fungi, and the frequencies of these previously unknown ant–fungus associations appear to vary biogeographically (Table 2). Consequently, the traditional view of *Trachymyrmex*-specific versus leafcutter-specific fungiculture needs to be modified (Figure 2), especially because some widely distributed *Atta* species in South America (*Atta laevigata* ranging across most of South America from southern Brazil to Colombia/Venezuela, and *Atta vollenweideri* ranging across northern Argentina, Paraguay and southern Brazil) appear to be specialized on one well-supported subclade of Clade-B fungi that also includes closely related cultivars from *Trachymyrmex*, *Sericomyrmex* and *Acromyrmex* ants (Figure 1). Because a number of leafcutter species cultivate Clade-B fungi (Figure 1), and because future surveys in South America may document additional such leafcutter species, it seems inaccurate to continue to refer to Clade-B fungi as “*Trachymyrmex* fungi.”

3.2 | Leafcutter and *Trachymyrmex* ants share fungi; *Atta* and *Acromyrmex* ants share fungi

The two leafcutter genera *Atta* and *Acromyrmex* do not cultivate fungi specific to ant genus (Figures 1 and 2), and there exists no evidence that *Atta* ants co-evolve only with *Atta*-specific fungi while *Acromyrmex* ants co-evolve only with *Acromyrmex*-specific fungi. Nygaard et al. (2016) concluded, for example, that *Atta*-specific and *Acromyrmex*-specific fungi diverged about 7 mya, but this date is uncertain because of poor time calibration of the phylogenetic tree (a single time anchor at 73 mya), as discussed in Mueller et al. (2017). Moreover, a large population-genetic survey of leafcutter fungi analysed with microsatellite markers (Mueller et al., 2017) suggested frequent horizontal cultivar transfer between *Atta* and *Acromyrmex* nests at multiple locations across the entire leafcutter range, as well as genetic admixture between *Atta*-cultivated and *Acromyrmex*-cultivated fungi, as already reported by Mikheyev et al. (2006)

and Mueller, Mikheyev, Solomon et al. (2011). The apparent phylogenetic difference between *Atta* and *Acromyrmex* fungi reported in Kooij, Poulsen, Schiøtt, and Boomsma (2015) therefore emerges as a sampling artefact of regional fungal diversity. In fact, Mikheyev et al. (2007), studying the same leafcutter population as Kooij, Poulsen et al. (2015), had already shown population-genetic patterns indicating that *Atta* and *Acromyrmex* ants can exchange cultivar clones in central Panamá, or fungi of *Atta* and *Acromyrmex* exchange genetic material on occasion, or some combination of both processes. (See also the discussion in the Supporting Information of Mueller et al., 2017; explaining the different sampling strategies of Mikheyev et al., 2007 and Kooij, Poulsen et al. 2015). Leafcutter ants therefore co-evolve with fungal cultivars (Aylward et al., 2012; Mueller, 2002, 2012; Nygaard et al., 2016), but such co-evolution appears to be far less specialized than previously thought, involving regionally multiple leafcutter and multiple *Trachymyrmex*/*Sericomyrmex* species that interact with multiple fungal lineages from both Clade-A and Clade-B (Figure 1). Association specificities and potential for highly specialized versus less-specialized co-evolution may vary between locations (De Fine Licht & Boomsma, 2014; Mueller, Mikheyev, Solomon et al. 2011; Mueller Mikheyev, Hong, et al., 2011), depending on (i) the pool of cultivars circulating among ant species in a location; (ii) habitat segregation between ant species that precludes frequent cultivar exchange between ant species specialized on different habitats (i.e., ant species in the same habitat are more likely to exchange cultivars because of spatial proximity); and (iii) the strength of selection favouring specific ant–fungus combinations (Kellner et al., 2013; Mueller et al., 1998; Mueller, Mikheyev, Solomon et al. 2011; Mueller Mikheyev, Hong, et al., 2011; Seal & Mueller, 2014; Smith et al., in review).

3.3 | Fungiculture at the origin of leafcutter ants

Because our sample of only 76 higher-attine fungi already implicates multiple transitions between Clade-A and Clade-B cultivation during the evolutionary history of higher-attine ants (Figure 1), a question is whether association with Clade-A fungi, with Clade-B fungi, or with both types of fungi was ancestral at the origin of leafcutter ants 18–19 million years ago (mya; ranges of 15.6–20.4 mya and 14–24 mya estimated by, respectively, Ješovnik et al., 2016 and Branstetter et al., 2017). As sister clades, both Clade-A and Clade-B fungi originated at the same time, and Mikheyev et al. (2010) and Nygaard et al. (2016) dated the last common ancestor of these two clades to, respectively, 25 mya (range: 11–39 mya) and 22.4 mya (16.9–27.9 mya), suggesting that Clade-A fungi may have originated before the origin of leafcutter fungiculture (see Mueller et al., 2017 for further discussion). The cultivation of a Clade-B fungus by *Acromyrmex striatus* (Figure 1), the most basal (earliest-diverging) lineage in the leafcutter clade (Cristiano et al., 2013; Branstetter et al., 2017; Pereira, Reis, Cardoso, & Cristiano, 2018), would support a possible ancestral leafcutter fungiculture that included Clade-B fungi. However, the cultivation of a mix of Clade-A and Clade-B fungi by species in the leafcutter clade and especially in the *septentrionalis*-clade

of *Trachymyrmex* (sister clade to the leafcutter ant clade; Rabeling, Cover, Johnson, & Mueller, 2007; Schultz & Brady, 2008; Schultz et al., 2015) suggests the possibility of a mix of Clade-A and Clade-B cultivation that preceded the origin (most recent common ancestor) of leafcutter ants and the *septentrionalis*-clade. Specifically, of the *Trachymyrmex* species shown in Figure 1 for the *septentrionalis*-clade, (i) *T. saussurei* (Central America & North America) and *T. desertorum* (North America) have been found so far only in association with Clade-A fungi; (ii) different nests of the North American *T. arizonensis* (or cryptic species currently classified as *T. arizonensis*) occur in association with either Clade-A or Clade-B fungi; and (iii) the North American *T. septentrionalis*, *T. smithi*, *T. pomonae* and *T. carinatus* have been found so far only in association with Clade-B fungi (Figure 1; Mueller et al., 2017). Combining this information of (i) mixed Clade-A and Clade-B cultivation by species in the *septentrionalis*-clade with (ii) Clade-B cultivation by the earliest-branching *Ac. striatus* and with (iii) predominant Clade-A cultivation but also some Clade-B cultivation among all other leafcutter ants, any of the following three scenarios seem viable hypotheses for fungicultural specialization by the ancestral leafcutters: either an exclusive specialization on Clade-A fungi, or exclusive specialization on Clade-B fungi, or mixed Clade-A & Clade-B cultivation by the ancestral leafcutter ants. A comprehensive reconstruction of ant–fungus association throughout the phylogenetic history of higher-attine ants, using a more representative sampling of fungal cultivars from all *Trachymyrmex/Sericomyrmex* lineages than in Figure 1, will be necessary to test these different hypotheses (Solomon et al., in preparation).

Cultivar switching by ants between Clade-A and Clade-B fungi may have occurred frequently during the early evolution of leafcutter ants, at a time when Clade-A and Clade-B fungi were presumably not as diverged from each other as extant higher-attine fungi, and when transitioning between Clade-A and Clade-B fungi may therefore have been less constrained. This complicates inferences of ancient evolutionary transitions in higher-attine fungiculture from patterns of extant ant–fungus associations, as already noted by Mikheyev et al. (2010). Moreover, current sampling is too sparse for *Ac. striatus* (e.g., the fungus of only one nest of *Ac. striatus* is known so far; Figure 1), and additional sampling of early-branching leafcutter lineages and sampling across all *Trachymyrmex/Sericomyrmex* subclades are necessary, as well as more precise dating of the origins of Clade-A and Clade-B fungi, to permit any useful discussion of ancestral fungicultural states at the transition to leafcutter fungiculture.

3.4 | Some *Trachymyrmex* ants cultivate fungi known primarily from lower-attine ants

Because some *Trachymyrmex* ants cultivate fungi known so far only from lower-attine ants (Figure 1), fungiculture by higher-attine ants is more nonspecific than postulated by previous studies (Currie et al., 2003; Ješovnik et al., 2017; Mehdiabadi & Schultz, 2010; Price et al., 2003; Schultz & Brady, 2008; Schultz et al., 2015). This possibility already emerged in an earlier phylogenetic analysis (Mueller et al., 1998) reporting a single case of cultivation of a so-called Clade-1

lower-attine fungus by a nest of *Trachymyrmex papulatus* from Argentina. It appears now that such associations between Clade-1 fungi and some *Trachymyrmex* ants, previously considered aberrant, could represent the norm for some *Trachymyrmex* species.

Cultivation of lower-attine fungi by *Trachymyrmex* ants is known so far only for Clade-1 fungi (Figure 1), but not for the Clade-2 fungi cultivated by many lower-attine ants (Mueller et al., 1998), which are thought to be more recently domesticated fungi than Clade-1 fungi (Mikheyev et al., 2010). Because higher-attine fungi originated from Clade-1 fungi or from closely related free-living leucocoprineaceous species (Figure 1), this suggests the possibility that cultivation of Clade-1 fungi by extant *Trachymyrmex* species could represent retention of the ancestral (plesiomorphic) state of Clade-1 cultivation practiced by the ancestral ant lineage that gave rise to higher-attine ants (Solomon et al., 2011). More comprehensive collection of *Trachymyrmex* ants and lower-attine ants could further address this hypothesis, particularly more collections from South America (Solomon et al., in preparation). Ant–fungus associations reported so far for the well-studied Panamanian *Trachymyrmex/Sericomyrmex* ants and leafcutter ants therefore represent only a subset of the far greater diversity of ant–fungus associations found in each of these same host clades elsewhere in the Neotropics. Consequently, future research on attine fungiculture will need to be more cautious in interpreting ant–fungus associations found at a single site (e.g., a single forest in Panamá), and survey regions more exhaustively, to prevent premature conclusions based on insufficiently sampled biodiversity.

3.5 | Single species of higher-attine ants cultivate both Clade-A and Clade-B fungi

Some populations of single *Trachymyrmex* species appear to cultivate both Clade-A and Clade-B fungi. Two such cases are included in our analysis (Figure 1), *T. intermedius* from the Kaw Mountains in French Guyana and *T. arizonensis* from Arizona in the United States (details in Table S3). The case of *T. intermedius* is particularly interesting because, for four *T. intermedius* nests, two nests cultivating Clade-A fungi and two nests cultivating Clade-B fungi were collected by UGM in the same population within about 200 m of each other (Table S3). In addition, some leafcutter ant species appear to cultivate both Clade-A and Clade-B fungi, such as *Acromyrmex crassispinus*, *Ac. coronatus* and *Atta laevigata* in Brazil (Figure 1, Table S3; Mueller et al., 2017). For example, *At. laevigata* collected in Venezuela, French Guyana and throughout much of Brazil was found so far to cultivate Clade-B fungi (Figure 1); however, in southern Brazil, different nests of *A. laevigata* cultivate either Clade-A or Clade-B fungi (Table S3), and Silva-Pinhati et al. (2004) had already reported a Clade-A fungus cultivated by a nest of *A. laevigata* from Rio Claro, São Paulo State (8 cloned sequences of ITS2 of this Clade-A fungus are available at GenBank as Accession nos AF076408–AF076416).

It is possible that some of these fungi–polymorphic ant species could represent cryptic ant species, each specialized on different fungi. Precedents of such fungal specialization by cryptic ant hosts were described for *Cyphomyrmex* ants (Mehdiabadi et al., 2012;

Schultz et al., 2002) and *Trachymyrmex* ants (e.g., different cryptic *Trachymyrmex* species may be associated with different subclades of Clade-B fungi in Panamá; De Fine Licht & Boomsma, 2014). In contrast, local polymorphisms of both Clade-A and Clade-B fungiculture by the same higher-attine ant species (e.g., *T. arizonensis*, *Atta laevigata*; Table S3) are an unexpected finding. Specifically, phylogeographic studies of *Atta laevigata* did not reveal phylogenetically distinct *laevigata* lineages coexisting in sympatry in Brazil (Solomon, Bacci, Martins, Gonçalves Vinha, & Mueller, 2008; Table S3), making it less likely that cryptic species explain the polyculture of both Clade-A and Clade-B fungi by different sympatric nests of *A. laevigata*. Likewise, phylogenetic analysis of *T. arizonensis* ants, including some from the same nests that are shown in Figure 1 to cultivate either Clade-A or Clade-B fungi, does not reveal any cryptic species within the currently recognized *T. arizonensis* (Rabeling et al., 2007; Table S3). Available sequence information for *At. laevigata* and *T. arizonensis* ants therefore supports the hypothesis that, in some locations, single populations of the same *Atta* species, and of the same *Trachymyrmex* species, cultivate both Clade-A and Clade-B fungi. Larger sample sizes covering additional populations of these ant species, as well as analyses with additional high-resolution markers, will be necessary to rigorously test this hypothesis of within-species fungal polymorphism.

3.6 | Key innovations in leafcutter fungiculture

The emerging picture of attine ant–fungus associations (Figures 1 and 2) includes fewer clade-to-clade correspondences than previously thought (Currie et al., 2003; Mehdiabadi & Schultz, 2010; Mueller, Gerardo, Aanen, Six, & Schultz, 2005; Schultz & Brady, 2008; Taerum, Cafaro, Little, Schultz, & Currie, 2007). Lineages of higher-attine ants, including leafcutter ants, appear capable of transition between Clade-A and Clade-B fungi, with some species of leafcutter ants found so far only in association with Clade-A fungi (e.g., *Atta cephalotes*, *A. colombica*, *A. sexdens*); other leafcutter species found so far primarily with one subclade of Clade-B fungi (e.g., *Atta vollenweideri*, *A. laevigata*); and many *Trachymyrmex* and *Sericomyrmex* species, but not all *Trachymyrmex* species, found so far primarily in association with Clade-B fungi (some *Trachymyrmex* species, such as *T. desertorum* and *T. saussurei*, have been found so far only in association with Clade-A fungi). The ecological success of leafcutter ants, and the presumed ecological inferiority of *Trachymyrmex* ants, therefore does not derive primarily from an innovating association between superior Clade-A cultivars and leafcutter farmers (Cherrett et al., 1989; De Fine Licht et al., 2014; Nygaard et al., 2016; Shik et al., 2016; Stradling & Powell, 1986). The hypothesis that Clade-A cultivation is sufficient to enhance fitness of higher-attine ant colonies had already been questioned by Seal and Tschinkel (2007), who showed that *Trachymyrmex septentrionalis* ants can be experimentally switched to grow Clade-A cultivar without improving ant fitness and garden productivity. Subsequent experiments demonstrated significant variability in *Trachymyrmex* ants to adopt Clade-A fungi, with some *Trachymyrmex* species reluctant to adopt Clade-A fungi or

incapable of Clade-A cultivation, some *Trachymyrmex* species capable of transient Clade-A cultivation before failing, and others (e.g., *Trachymyrmex arizonensis*) successful at Clade-A cultivation in the laboratory for more than 5 years without apparent fitness detriment (Seal & Mueller, 2014; Seal, Schiøtt, & Mueller, 2014; J. Seal, personal communication, December 4, 2016). Interestingly, *T. arizonensis* is one of the higher-attine ant species in which local populations appear to cultivate both Clade-A and Clade-B cultivars in sympatry (Figure 1; Table S3).

Leafcutter ants can therefore have dominant ecological impacts when cultivating either Clade-A fungi (e.g., *Atta cephalotes*, *At. colombica*, *At. sexdens*) or Clade-B fungi (e.g., *Atta vollenweideri*, *At. laevigata*; Figure 1). Regardless of cultivar type, these leafcutter species reach comparable nest sizes of millions of workers that command huge foraging territories covering several hectares (Costa et al., 2008; Della Lucia, 2011; Hertz et al., 2007; Leal et al., 2014; Vasconcelos et al., 2008; Wirth et al., 2003). In contrast, *Trachymyrmex* ants cultivating either Clade-A or Clade-B fungi can be locally abundant without achieving the ecological dominance of sympatric leafcutter ants. Cultivation of Clade-A fungi per se therefore may have been less innovating during leafcutter evolution than currently believed (Cherrett et al., 1989; De Fine Licht et al., 2014; Nygaard et al., 2016; Shik et al., 2016; Stradling & Powell, 1986), particularly if the origin of Clade-A fungi preceded the origin of leafcutter ants (see discussion above, and Mueller et al., 2017). Instead, ecological success of leafcutter ants derives either from innovating ant–fungus synergisms that emerged from modifications of both ants and fungi (DeMillo et al., 2017), or from key adaptations of leafcutter ants, such as unique foraging and leaf-processing strategies, or innovating communication between workers and colony organization enhancing colony efficiencies (Della Lucia, 2011; Hölldobler & Wilson, 2010; Kleineidam, Ernst, & Roces, 2001; Roces, Tautz, & Hölldobler, 1993; Wirth et al., 2003).

A dichotomy in attine biology is the distinction between higher- versus lower-attine fungiculture, suggesting that higher-attine fungiculture could represent a key innovation involving a constellation of several innovating features in the fungi, such as gongylidia-bearing hyphae, multinucleate cells that can sustain more efficient metabolism, higher proteinase activity or diversification of detoxifying enzymes through gene duplication in the fungi (Table 1; Carlson et al., 2017; De Fine Licht et al., 2010, 2013, 2014; Hervey et al., 1977; Kooij, Aanen et al., 2015; Nygaard et al., 2016; Semenova, Hughes, Boomsma, & Morten Schiøtt, 2011). However, there already exist exceptions that are inconsistent with this tidy dichotomy between lower- and higher-attine fungiculture. Specifically, several *Trachymyrmex* species cultivate lower-attine fungi (Figure 1) and one lower-attine fungus is known to grow gongylidia-like structures when cultivated by a lower-attine ant (Masiulionis et al., 2014), suggesting that gongylidia may not be a unique synapomorphy of higher-attine fungi, and one lower-attine ant cultivates a Clade-A fungus (Schultz et al., 2015). The distinction between lower- and higher-attine fungiculture is therefore a statistical preponderance (e.g., most higher-attine ants cultivate gongylidia-bearing attine fungi

from Clades A or -B, most lower-attine ants do not), and it seems no longer tenable to view higher- versus lower-attine fungiculture as a key distinction between ecologically inferior lower-attine ants cultivating primitive fungal strains using primitive fungicultural techniques, whereas ecologically dominant higher-attine ants gain ecological prominence primarily from cultivation of gongylidia-producing superior fungi. In fact, nests of many lower-attine ant species cultivating allegedly inferior lower-attine cultivars can be numerically extremely abundant, as for example nests of *Mycocrepus* or the yeast-cultivating *Cyphomyrmex* species (Kellner et al., 2013; Rabeling et al., 2011; Weber, 1972). Rather than focusing on revolutionary adaptations in either fungi or ant farmers, therefore, future research may make more rapid progress in understanding evolutionary success and ecological prominence of attine lineages by analysing ant–fungus synergisms (DeMillo et al., 2017), particularly for single ant species or cryptic species-pairs that transition frequently (or transitioned very recently) between Clade-A and Clade-B fungi and that therefore offer opportunities to study populations or recently diverged species-pairs that are polymorphic for both of these types of fungi in the same environment (e.g., *Trachymyrmex arizonensis* in Arizona, USA; *T. intermedius* in French Guyana; *Atta laevigata* in southern Brazil; Table S3).

3.7 | Implications for the biogeography of higher-attine cultivars

Because the primary aim of our phylogenetic analysis was to elucidate previously unknown diversity of higher-attine ant–fungus associations (Figure 1), and because we reserved comprehensive biogeographic studies for separate analyses of the biogeography of Clade-A fungi (Mueller et al., 2017) and Clade-B fungi (Solomon et al., in preparation), the biogeographic coverage of the samples in Figure 1 is incomplete. The relative prevalence of Clade-A versus Clade-B cultivation summarized in Table 2 for different biogeographic regions therefore need to be interpreted cautiously. However, in conjunction with the previously unknown ant–fungus associations found in our survey, all available biogeographic information (Mueller et al., 2017; Solomon et al., in preparation; Table S4) shows that (i) Clade-B cultivating leafcutter ants have been found so far only in South America; (ii) Clade-A cultivating *Trachymyrmex* ants have been found so far in northern South America and in North America and therefore such Clade-A cultivating *Trachymyrmex* likely occur also in Central America, possibly also in southern South America; and (iii) *Trachymyrmex* ants cultivating so-called lower-attine Clade-1 fungi have been found so far only in South America, but not in Central or North America. All fungi characterized so far for *Sericomyrmex* ants were Clade-B fungi (Figure 1; Chapela et al., 1994; Mikheyev et al., 2010; Ješovnik et al., 2016). The Clade-A-cultivating *Apterostigma megacephala* occurs in northern and central South America (Schultz et al., 2015; Sosa-Calvo, Ješovnik, Vasconcelos, Bacci, & Schultz, 2017), whereas all other fungi from *Apterostigma* characterized to date are either pterulaceous fungi or, less frequently, lower-attine Clade-1 fungi (Gerardo, Jacobs, Currie, &

Mueller, 2006; Gerardo, Mueller, & Currie, 2006; Mueller et al., 1998; Villesen et al., 2004). Because most higher-attine fungi characterized to date were collected in Central and North America (Mueller et al., 2017; Table S4), and because ant–fungus associations in these two biogeographic region are not representative for ant–fungus associations across South America (Table 2), the proportions reported in previous surveys overestimated the true prevalence of Clade-A-cultivation across all leafcutter ants and overestimated the true prevalence of Clade-B-cultivation across all *Trachymyrmex* ants. South American populations will have to be sampled at the same densities as those already sampled in Central and North America to generate a complete picture of the biogeography of higher-attine ant–fungus associations across their entire range.

4 | CONCLUSION

The current conception of cultivar specialization by leafcutter ants, *Trachymyrmex/Sericomyrmex* ants, and lower-attine ants—each thought to be clade-to-clade specialized on its own clade of fungal cultivars with its own specialized *Escovopsis* parasites—derives largely from ant–fungus associations found in focused collections from central Panamá and from scattered collections from northern South America. New phylogenetic information (Figures 1 and 2) incorporating collections from understudied regions in South America where higher-attine fungiculture originated (Branstetter et al., 2017; Fowler, 1983; Kusnezov, 1963; Mueller et al., 2017) now shows that it was premature to generalize from the early, geographically limited studies to attine diversity across their entire range. It is even possible that the prevailing view of clade-to-clade ant–fungus specializations may have led occasionally to dismissal of “unexpected” ant–fungus associations, but hopefully future studies will now heed unusual attine ant–fungus associations, and verify these through replicated sampling (as in Schultz et al., 2015). More sampling is needed across South America to capture representatives from the entire spectrum of attine ant–fungus associations, but also in Central America outside of central Panamá. For example, the observation that some *Trachymyrmex* species from North America and northern South America cultivate Clade-A fungi (Figure 1), as well as the observation that at least one *Atta* species in Colombia and Venezuela cultivates Clade-B fungi (Figure 1), suggests that other such nontraditional associations may exist in Central American populations.

Most importantly, arguments assuming that the ecological dominance of leafcutter ants derives from an innovating association with one specific superior fungus, or assuming that specialized cultivation of a Clade-A fungus by leafcutter ants was either sufficient or necessary to cause ecological dominance of leafcutter ants, now need to be re-evaluated. On the other hand, the observations that leafcutter ant species have transitioned multiple times between Clade-A and Clade-B fungi during their evolutionary history, that some *Trachymyrmex* ants appear to transition between higher-attine and lower-attine fungi and that there exist populations where a single ant species (or recently diverged cryptic species) appears to cultivate both Clade-A

and Clade-B fungi (Table S3) offer new possibilities to elucidate ant–fungus interactions through (i) comparative analyses (e.g., Are Clade-B fungi cultivated by leafcutter ants as highly polyploid as Clade-A fungi?), and (ii) experimentation exploring ant–fungus synergy in cultivar switch experiments (DeMilto et al., 2017; Mehdiabadi, Hughes, & Mueller, 2006; Seal, Gus, & Mueller, 2012; Seal & Tschinkel, 2007; Seal et al., 2014).

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DATA ACCESSIBILITY

All sequences used in the alignment are listed with respective GenBank and collection information in Table S1 in the Supporting Information. New sequences are deposited at NCBI GenBank under Accession nos KT898377–KT898391. The concatenated alignment (ITS, LSU, EF1- α) is available in the Supporting Information.

AUTHOR CONTRIBUTIONS

U.G.M., M.B. and S.E.S. planned and coordinated research. H.D.I., M.R.K. and S.B. generated sequence information. A.M.W., A.L.C., M.R.K. and U.G.M. conducted phylogenetic analyses. U.G.M., A.L.C., M.R.K. and A.M.W. wrote the manuscript. All authors revised the manuscript and approved the final manuscript.

ORCID

Ulrich G. Mueller  <http://orcid.org/0000-0003-2677-8323>

Maurício Bacci Jr  <http://orcid.org/0000-0002-5619-1411>

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