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Biogeography of mutualistic fungi cultivated by leafcutter ants

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Abstract

Leafcutter ants propagate co-evolving fungi for food. The nearly 50 species of leafcutter ants (*Atta*, *Acromyrmex*) range from Argentina to the United States, with the greatest species diversity in southern South America. We elucidate the biogeography of fungi cultivated by leafcutter ants using DNA sequence and microsatellite-marker analyses of 474 cultivars collected across the leafcutter range. Fungal cultivars belong to two clades (Clade-A and Clade-B). The dominant and widespread Clade-A cultivars form three genotype clusters, with their relative prevalence corresponding to southern South America, northern South America, Central

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and North America. Admixture between Clade-A populations supports genetic exchange within a single species, *Leucocoprinus gongylophorus*. Some leafcutter species that cut grass as fungicultural substrate are specialized to cultivate Clade-B fungi, whereas leafcutters preferring dicot plants appear specialized on Clade-A fungi. Cultivar sharing between sympatric leafcutter species occurs frequently such that cultivars of *Atta* are not distinct from those of *Acromyrmex*. Leafcutters specialized on Clade-B fungi occur only in South America. Diversity of Clade-A fungi is greatest in South America, but minimal in Central and North America. Maximum cultivar diversity in South America is predicted by the Kusnezov–Fowler hypothesis that leafcutter ants originated in subtropical South America and only dicot-specialized leafcutter ants migrated out of South America, but the cultivar diversity becomes also compatible with a recently proposed hypothesis of a Central American origin by postulating that leafcutter ants acquired novel cultivars many times from other nonleafcutter fungus-growing ants during their migrations from Central America across South America. We evaluate these biogeographic hypotheses in the light of estimated dates for the origins of leafcutter ants and their cultivars.

KEYWORDS

Attamyces bromatificus, insect–fungus mutualism, *Leucoagaricus gongylophorus*, *Leucoagaricus weberi*, *Leucocoprinus gongylophorus*, symbiosis

1 | INTRODUCTION

Biogeographic distributions provide clues about evolutionary processes, such as ancient dispersal and vicariance events that shaped macroevolutionary patterns, or adaptation and gene flow influencing microevolutionary processes (Avise, 2009; Brown & Lomolino, 1998; Wallace, 1876). In mutualistic associations between two partners, similarities or differences in biogeographic distributions between codependent partners can facilitate inference of such evolutionary processes (Alvarez, McKey, Kjellberg, & Hossaert-McKey, 2010; Hembry & Althoff, 2016; Satler & Carstens, 2016, 2017; Thompson, 2005). Cobiogeographic patterns of mutualistic partners require cautious interpretation, however, particularly regarding congruence and incongruence of patterns, because evolutionary forces and demographies can differ markedly between partners (Alvarez et al., 2010; Chomicki, Janda, & Renner, 2017; Espíndola, Carstens, & Alvarez, 2014; Herre, Knowlton, Mueller, & Rehner, 1999; Tian et al., 2015). For example, population sizes, migration rates, mutation rates and generation times can differ by orders of magnitude between a host and a symbiotic partner (Degnan, Lazarus, Brock, & Wernegreen, 2004; Lutzoni & Pagel, 1997; Moran & Wernegreen, 2000; Woolfit & Bromham, 2003), and dispersal barriers restricting gene flow for one partner (e.g., a pollinating bee) may not impede gene flow for the other partner (e.g., the pollinated plant). Such differences in evolutionary forces are particularly pronounced in mutualistic associations between macro-organisms and fast-evolving microbial symbionts, or microbial symbionts that do not comigrate with a host, disperse independently of the host and that are acquired by hosts from local microbial populations (e.g., many plant–endophyte, mycorrhizal plant–fungus, lichen algal–fungus or host–microbe gut mutualisms) (Dal Grande, Widmer, Wagner, & Scheidegger, 2012; Kaltenpoth, Roeser-Mueller, Stubblefield, Seger, & Strohm, 2014; Palmer, Pringle, Stier, & Holt, 2015; Silverstein, Correa, & Baker, 2012; Weiblen & Treiber, 2015; Wornik & Grube, 2010).

In many mutualistic host–microbe associations, a greater dispersal ability of the microbial partners results in predictable differences in population-genetic and biogeographic patterns between hosts and microbial symbionts, for example lesser genetic differentiation between populations for the symbiont compared to the host (Hulcr & Stelinski, 2017; Kellner et al., 2013; Mueller, Mikheyev, Solomon, & Cooper, 2011; Nobre, Koné, Konaté, Linsenmair, & Aanen, 2011; Six, 2012), or greater potential for a single symbiont lineage to interact with different allopatric host species (Mueller & Gerardo, 2002; Palmer et al., 2015; Thompson, 2005; Weiblen & Treiber, 2015). In contrast, when symbiont dispersal is limited, populations of symbionts are predicted to differentiate across space, as, for example, in the symbiotic ectomycorrhizal fungus *Rhizopogon* where limited dispersal by vectoring mammals maintains population-genetic structure between proximate islands (Grubisha, Bergemann, & Bruns, 2007). As a general rule, however, widely dispersing symbionts are thought to be associated with a greater diversity of hosts than symbionts with limited dispersal (Herre et al., 1999; Roy et al., 2008). Biogeographic analyses of such microbial symbionts are often complicated by insufficient knowledge of species boundaries of microbial symbionts, requiring high-resolution genetic analyses to differentiate species and population boundaries (e.g., Douhan, Vincenot, Gryta, &

Selosse, 2011; Gazis, Rehner, & Chaverri, 2011; Lankau & Keymer, 2016).

The mutualistic association between leafcutter ants (genera *Atta* and *Acromyrmex*) and their cultivated fungi is one example where dozens of ant–host species are thought to associate across the New World with a widely distributed mutualistic fungal lineage (Mikheyev, Mueller, & Abbot, 2006; Mikheyev, Mueller, & Abbott, 2010; Mikheyev, Mueller, & Boomsma, 2007; Mikheyev, Vo, & Mueller, 2008; Mueller, Mikheyev, Solomon, et al., 2011; Mueller, et al., in review; Silva-Pinhati et al., 2004). In the leafcutter mutualism, one dominant fungus clade, called Clade-A fungi, is associated with leafcutter ant species across the entire leafcutter range from Argentina to the United States, including several leafcutter ant species inhabiting Cuba and other Caribbean islands (Mikheyev, 2008; Mikheyev et al., 2006; Mueller, Mikheyev, Solomon, et al., 2011; Mueller et al., in review). Clade-A fungi identified so far were called either *Leucocoprinus gongylophorus* (Heim, 1957) or *Leucoagaricus weberi* (Muchovej, Della Lucia, & Muchovej, 1991), two species that were described from mushrooms (basidiomes, a sexual fungal stage) growing from gardens of *Acromyrmex* and *Atta* nests (Fisher, Stradling, & Pegler, 1994; Möller, 1893; Muchovej et al., 1991; Mueller, 2002; Pagnocca, Rodrigues, & Bacci, 2011; Pagnocca et al., 2001). [See Supporting Information why the widely cited placement of these mushrooms into the genus *Leucoagaricus* by Singer (1986) is inaccurate, and why we use here *L. gongylophorus* rather than *L. weberi*.] Mushrooms or mycelia of *L. gongylophorus* cultivar growing independent of a leafcutter nest have so far not been collected, but such free-living mushrooms are known for the cultivars of lower-attine, nonleafcutter ants (Mueller, 2002; Mueller, Gerardo, Aanen, Six, & Schultz, 2005; Mueller, Rehner, & Schultz, 1998; Mueller, Schultz, Currie, Adams, & Malloch, 2001; Solomon et al., 2004; Vo, Mueller, & Mikheyev, 2009).

Although most leafcutter species studied so far cultivate Clade-A fungi, some ecologically prominent leafcutter species from across South America (e.g., *Atta laevigata*, *At. vollenweideri*; Delabie, Alves, Reuss-Strenzel, Carmo, & Nascimento, 2011; Solomon, Bacci, Martins, Gonçalves Vinha, & Mueller, 2008) cultivate Clade-B fungi (Mueller et al., in review), a clade of fungi that was thought previously to be associated exclusively with the nonleafcutting *Trachymyrmex* and *Sericomyrmex* ants that, together with the two leafcutter ant genera *Atta* and *Acromyrmex*, comprise the clade of "higher-attine ants." Moreover, some higher-attine nonleafcutter ant species in the genus *Trachymyrmex* and one lower-attine ant species in the genus *Apterostigma* also cultivate Clade-A fungi (Schultz et al., 2015; Sosa-Calvo et al., 2017; Mueller et al., in review; Figure S1). Leafcutter and nonleafcutter higher-attine ants therefore share a pool of fungi belonging to these two fungal clades. Clade-A fungi likely represent a single species of fungus, called *L. gongylophorus* (i.e., formerly called *Attamyces bromatificus* as the vegetative mycelial form; Kreisel, 1972). Clade-B fungi represent at least six well-supported lineages of fungi, each likely a separate cultivar species, and almost all of these Clade-B lineages have been found also in association with leafcutter ants (Figure S1; Mueller et al., in review). The so-called higher-attine fungi (Clade-A & Clade-B fungi) therefore coevolve diffusely with their higher-attine ant hosts (*Atta*, *Acromyrmex*, *Trachymyrmex*, *Sericomyrmex*), and higher-attine ant lineages occasionally transition between Clade-A and Clade-B cultivation. The frequencies of these transitions over evolutionary and ecological times are unknown, but some higher-attine ant species appear to cultivate both Clade-A and Clade-B fungi in some populations (Mueller et al., in review; Table S10), a kind of local polyculture within an ant population seen also in an asexual lower-attine ant (Himler, Caldera, Baer, Fernández-Marín, & Mueller, 2009; Kellner et al., 2013; Rabeling, 2004), but not in all lower-attine ants (Mehdiabadi, Mueller, Brady, Himler, & Schultz, 2012).

Because of vertical inheritance of fungal cultivars from maternal to offspring nests, leafcutter ants and fungi were initially predicted to comigrate and coreproduce together and initially were even thought of as ancient asexual clones (Chapela, Rehner, Schultz, & Mueller, 1994). However, several population-genetic and phylogenetic observations are inconsistent with strict vertical inheritance and strict clonal reproduction. First, different sympatric leafcutter ant species sometimes cultivate genetically identical cultivar clones, suggesting recent exchange of fungal clones between nests of different ant species and possible "sweeps" of cultivars through leafcutter communities through unknown mechanisms of lateral between-nest cultivar transfer, such as garden stealing by ants or cultivar dispersal by unknown vectors (Adams, Mueller, Holloway, Green, & Narozniak, 2000; Green, Adams, & Mueller, 2002; Mikheyev et al., 2007, 2010; Mueller, Mikheyev, Solomon, et al., 2011). Second, molecular-phylogenetic analyses (Mikheyev et al., 2006, 2010) and population-genetic microsatellite-marker analyses (Mueller, Mikheyev, Solomon, et al., 2011) indicate genetic admixture between *L. gongylophorus* populations associated with *Atta* and *Acromyrmex* species across North America (Mexico, southern USA, Cuba). The observation of genetic admixture between *L. gongylophorus* populations across an oceanic barrier (between mainland Mexico and Cuba) that should preclude dispersal of leafcutter ants is significant, because it suggests that *L. gongylophorus* fungi may be able to disperse also independently from the ant hosts (e.g., via spores or nonant vectors; Möller, 1893; Pagnocca et al., 2001; Mueller, 2002; Mikheyev et al., 2006; Mueller, Mikheyev, Solomon, et al., 2011), or were accidentally dispersed by human commerce (e.g., transport in soil of potted plants; Mikheyev, 2008). Germination of spores from *L. gongylophorus* mushrooms has been documented so far only by Möller (1893; details in Supporting Information).

1.1 | Biogeography of leafcutter ants (*Atta*, *Acromyrmex*)

Far more is known about the biogeography of leafcutter ants than about their fungi. The currently recognized 17 *Atta* and 31 *Acromyrmex* leafcutter species (plus at least four social-parasitic *Acromyrmex* species; Rabeling & Bacci, 2010; Rabeling, Schultz, Bacci, & Bollazzi, 2015) form a well-supported monophyletic group that originated 16–19 million years ago (Ma) (Branstetter et al., 2017; Ješovnik,

González, & Schultz, 2016; Nygaard et al., 2016). Two leafcutter species occur at the northern range limit in the United States, five species in Mexico, eight species in Central America (details in Supporting Information), and a parallel gradient in leafcutter species diversity occurs also at the southern range in Argentina (Farji-Brener & Ruggiero, 1994). About 40 described leafcutter species occur in South America, with the greatest concentration of sympatric leafcutter species in grassland habitats of northern Argentina, Paraguay, Uruguay and Southern Brazil (Borgmeier, 1959; Brandão, Mayhé-Nunes, & Sanhudo, 2011; Cristiano, Cardoso, Fernandes-Salomão, & Heinze, 2016; Delabie et al., 2011; Farji-Brener & Ruggiero, 1994; Fernández & Sendoya, 2004; Fowler, 1983; Fowler & Claver, 1991; Gonçalves, 1961; Mayhé-Nunes & Jaffé, 1998; Mueller & Rabeling, 2008; Wild, 2007). Wild (2007), for example, reports 25 leafcutter species for Paraguay.

Because the greatest concentration of leafcutter species diversity occurs in grasslands of southern South America, early biogeographic models (Fowler, 1983; Kusnezov, 1963) postulated that leafcutter ants originated in open habitats of southern South America, specifically in grasslands (Fowler, 1983) and not in humid rainforest (Kusnezov, 1963); from southern South America, leafcutter ants then expanded into diverse habitats across South America and later into Central and North America once leafcutter ants could disperse across the Central American land bridge. Recently, however, Branstetter et al. (2017) inferred the biogeographic history mapped onto a phylogeny of attine ants, and Branstetter et al.'s modelling suggests a possible origin of leafcutter ants in seasonally dry habitats in Central America, but their analyses could not rule out a South American origin with confidence. There exists no definitive fossil evidence that indicates the presence of leafcutter ants outside of South America prior to the closing of the Central American land bridge 1–5 Ma, or an earlier presence in South America (see discussion on attine fossils in the Supporting Information). Without leafcutter fossils, biogeographic histories of leafcutter ants have to be inferred with the help of current distributions.

1.1.1 | *Acromyrmex* biogeography

Because no detailed phylogenetic analyses exist for *Acromyrmex*, the biogeography of *Acromyrmex* is less understood than the one for *Atta*. Earlier morphological studies partitioned *Acromyrmex* into two groups (subgenera *Acromyrmex* and *Moellerius*; Gonçalves, 1961), but molecular-phylogenetic analyses did not recover these two groups as monophyletic (Branstetter et al., 2017; Cristiano, Cardoso, & Fernandes-Salomão, 2013; Schultz et al., 2015), and the morphologically unique species *Acromyrmex striatus*, traditionally placed into the *Moellerius* subgenus (Fowler, 1988; Gonçalves, 1961), actually represents the sister lineage to all other leafcutter ants (Cristiano et al., 2013). Because *Ac. striatus* and its putative sister species *Ac. silvestrii* occur in grassland habitats of northern Argentina, Paraguay, Uruguay and southernmost Brazil (Cristiano et al., 2016; Farji-Brener & Ruggiero, 1994; Fowler, 1983), the sister-group relationship of *Ac. striatus* to the remaining leafcutter ants supports an origin of leafcutter ants in southern South America, as postulated by Kusnezov (1963) and Fowler (1983) (see also Brandão et al., 2011). The existence of *Ac. striatus* and *Ac. silvestrii* in southern South America, as well as the main concentration of extant leafcutter species diversity in southern South America, is difficult to reconcile with Branstetter et al.'s hypothesis of a Central American origin of leafcutter ants.

1.1.2 | *Atta* biogeography

Of four well-supported subclades of *Atta* (Bacci et al., 2009; Borgmeier, 1959), representatives from two clades (*Neoatta*, *Atta sensu stricto*) occur in both South America and in Central America, whereas the species-rich *Epiatta* clade occurs exclusively in South America (including dominant pest species such as *At. bisphaerica*, *At. capiguara*, *At. saltensis*, *At. vollenweideri*, *At. laevigata* and *At. opacipes*), and species in the *Archeatta* clade occur only in North America (*At. mexicana*, *At. texana*, *At. insularis*, *At. cubana*; presumably these species diversified in that northernmost region of the *Atta* distribution). The derived position of the South American *Epiatta* clade within the genus *Atta* and an early-diverging position of the North American *Archeatta* clade within the genus (Bacci et al., 2009; Cristiano et al., 2013) supports an origin of the genus outside of South America. On the other hand, the far greater diversity of South American *Atta* species could suggest a South American origin, but this can also be explained as a radiation of successful *Atta* lineages that spread from Central America across South America. Diversification within species has been analysed only in three widespread *Atta* species (*At. cephalotes*, *At. sexdens*, *At. laevigata*) for which within-species diversity accumulated in the past 0.5–3 million years (Solomon et al., 2008).

1.2 | Biogeography of leafcutter fungi

Very little is known about the biogeography of fungi cultivated by leafcutter ants. Population-genetic analyses using microsatellite markers showed that in Panamá, sympatric populations of five leafcutter species (*At. cephalotes*, *At. colombica*, *At. sexdens*, *Ac. octospinosus*, *Ac. echinatior*) share a pool of six genotype clusters of *L. gongylophorus* fungi (Mikheyev et al., 2007), with only 10% of the observed genetic variation attributable to differences between leafcutter hosts, indicating local cultivar sharing between *Atta* and *Acromyrmex*. Likewise, analyses of AFLP markers showed that Panamanian cultivars from sympatric *Ac. octospinosus* and *Ac. echinatior* can be grouped into at least five distinct clusters (Bot, Rehner, & Boomsma, 2001), with each cluster containing fungi cultivated by the two sympatric *Acromyrmex* species. Across North America, five leafcutter species (*At. texana*, *At. mexicana*, *At. cephalotes*, *At. insularis* and *Ac. versicolor*) share four genotype clusters of *L. gongylophorus* (Mueller, Mikheyev, Solomon, et al., 2011), with evidence of admixture between these distinct clusters. No comparable population-genetic analyses involving multiple fungi per leafcutter species exist for South American leafcutter fungi, except for the study of Pereira et al. (2015) who showed that three cultivars from *Ac. heyeri*

and three from *Ac. ambiguus* from southern Brazil form two closely related fungal clades grouping by ant species. The population-genetic linkages between South, Central and North American leafcutter fungi are unknown. Clade-B fungi cultivated by leafcutter ants are known so far only from South America (from Argentina, Brazil, French Guiana and Venezuela; Mueller et al., in review).

In North America, genetically identical clones of *L. gongylophorus*, genotyped at 12 microsatellite loci, can range over large areas. For example, the most widely distributed clones ranged across 50,000–80,000 km^2 in south-central Texas (approximately the area of Panamá or French Guiana) (Mueller, Mikheyev, Solomon, et al., 2011). Comparably detailed population-genetic analyses are currently lacking for leafcutter fungus populations from Central and South America. Widely distributed cultivar clones may exist also in South America because fast-evolving sequences (e.g., ITS rDNA) of South American leafcutter fungi can be nearly identical for collections from sites 2600 kilometres apart (Silva-Pinhati et al., 2004). On the other hand, genetic admixture between differentiated *L. gongylophorus* populations appears more pronounced in tropical populations in Mexico than in subtropical populations in the United States (Mueller, Mikheyev, Solomon, et al., 2011), suggesting that, because of more frequent recombination in the tropics through unknown processes of genetic exchange (e.g., through spore dispersal, or exchange of nuclei between multinucleate mycelia; Mueller, Mikheyev, Solomon, et al., 2011; Sen, Ishak, Kniffin, & Mueller, 2010; Carlson et al., 2018), genetically identical cultivar clones may not range as widely in the tropics compared to their ranges observed at the subtropical, northern range limit of the leafcutter distribution.

Three additional expectations about the biogeography of leafcutter fungi derive from biogeographic patterns of widely distributed *Atta* species in South America (Solomon et al., 2008). First, major rivers such as the Amazon or the Orinoco do not represent effective dispersal barriers to *Atta* ants (Solomon et al., 2008). Because the dispersing female reproductives transport fungal inocula during mating flights, major rivers would therefore also not represent dispersal barriers for leafcutter fungi. In fact, even the oceanic barrier between Cuba and the mainland does not appear to be an effective dispersal barrier for leafcutter fungi because fungi cultivated by *At. insularis* in Cuba have close population-genetic affinities to fungi cultivated by *At. mexicana* and *At. texana* in mainland North America (Mikheyev et al., 2006; Mueller, Mikheyev, Solomon, et al., 2011), whereas these three ant species are significantly diverged from each other (Bacci et al., 2009) and the current distance between Cuba and mainland greatly exceeds the dispersal distance of leafcutter ants during a mating flight. This suggests the possibility that leafcutter fungi may disperse independently from the ants, for example, through airborne spore dispersal (see above). Second, Pleistocene refugia in South America apparently did not contribute to inter- and intraspecies diversification in *Atta* ants (Solomon et al., 2008) and presumably therefore also not to diversification in the associated fungal cultivars. Third, leafcutter abundance decreases significantly with altitude, and leafcutter ants become rare at elevations of 2,000–2,500 m (Delabie et al., 2011; Farji-Brener & Ruggiero, 1994; Fernández, Castro-Huertas, & Serna, 2015; Weber, 1972; additional discussion in Supporting Information). This suggests that the Andes in northwestern South America (Colombia, Ecuador, Peru) represent a significant, although not insurmountable, dispersal barrier for leafcutter ants and any codispersing fungal cultivars.

Here, we build on these previous studies by conducting the first comprehensive population-genetic and biogeographic analyses of *L. gongylophorus* fungi (i.e., Clade-A fungi *sensu* Mueller et al., in review) propagated by leafcutter ants across the ants' entire range from Argentina to the United States. Our study specifically asks whether cultivar clones are shared locally between sympatric leafcutter ant species; whether fungal cultivars differ between leafcutter ants that are specialized to cut either dicot or monocot (grass) leaf substrate for fungiculture (Vasconcelos & Fowler, 1990), and whether genetic diversity of *L. gongylophorus* changes across its range.

2 | MATERIALS AND METHODS

2.1 | Sample collection and sequencing

Between 1990 and 2008, we collected fungus garden material from 474 leafcutter nests of eight *Atta* species (294 nests) and 22 *Acromyrmex* species (180 nests) from Argentina (n = 29 samples), Uruguay (n = 2), Brazil (n = 123), Peru (n = 48), Ecuador (n = 14), French Guiana (n = 32), Suriname (n = 1), Guyana (n = 6), Venezuela (n = 40), Trinidad and Tobago (n = 8), Colombia (n = 34), Panamá (n = 91), Costa Rica (n = 7), Honduras (n = 1), Mexico (n = 15), Cuba (n = 5) and the United States (n = 18) (Tables S1 and S2). Our garden samples from eight *Atta* and 22 *Acromyrmex* species cover 47% of 17 *Atta* species currently recognized and 71% of 31 *Acromyrmex* species (not including social-parasitic *Acromyrmex* species). Methods of collection, storage and sequencing are described in the Supporting Information. Collection information and GenBank accessions for all garden samples, including samples from nonleafcutter fungus-growing ants used for outgroup analyses, are listed in Table S1.

We obtained sequence information for 483 fungi (430 fungi from leafcutter ants, 40 fungi from *Trachymyrmex* ants, four fungi from *Sericomyrmex* ants and nine outgroup fungi [four lower-attine cultivars, five free-living *Leucocoprinus* fungi]; Table S1). We initially intended to use three intron-spanning genes (EF-1α, RAD and DMC; Mikheyev et al., 2006) to resolve phylogenetic structure among Clade-A fungi. However, because preliminary phylogenetic analyses revealed that each of the three genes shows insufficient variation to resolve phylogenetic relationships between Clade-A fungi, we discontinued sequencing of the RAD and DMC genes and instead relied on information from the EF-1α gene to classify leafcutter fungi into Clade-A and Clade-B fungi, then characterized genetic differences between Clade-A fungi with microsatellite markers. We present the exploratory analyses of the EF-1α, RAD and DMC genes in Figures S1–S4, and we used the information from the most comprehensive EF-1α data set to identify Clade-A fungi to be analysed further with microsatellite genotyping.

2.2 | Microsatellite genotyping

We generated microsatellite information for five loci (A1132, C101, C126, C117 and B12) developed for Clade-A fungi (Scott, Kweskin, Cooper, & Mueller, 2009). We chose these loci because they could be scored reliably with few scoring errors (Mueller, Scott, Ishak, Cooper, & Rodrigues, 2010; Mueller, Mikheyev, Hong, et al., 2011; Mueller, Mikheyev, Solomon, et al., 2011). Details of microsatellite amplification methods and scoring on an ABI PRISM 3100 automated sequencer are in the Supporting Information. All microsatellite chromatograms were scored by a single researcher (HDI) to standardize the allele-calling procedure.

2.3 | Population-genetic analyses of microsatellite markers

We assessed population structure with STRUCTURE v2.3.4 (Pritchard, Stephens, & Donnelly, 2000), which clusters individuals into genotype clusters (i.e., populations) and estimates admixture using multilocus genotypes. Because *L. gongylophorus* fungi are polyploid and multinucleate, we treated each allele as a dominant marker in STRUCTURE, as recommended by Falush, Stephens, and Pritchard (2007). Ploidy appears to be variable between individual strains (Carlson et al., 2018; Kooij, Aanen, Schiøtt, & Boomsma, 2015), so we did not use standard population-genetic statistics (e.g., *F*-statistics, heterozygosity) to describe inferred populations. We first assessed population structure using the default settings of STRUCTURE, but to reduce bias in prior assumptions in a separate analysis, we also left allele frequencies uncorrelated and chose alpha (α) to be 1/10 of the default setting (i.e., $\alpha = 0.1$) (Wang, 2017). Both the default settings and the modified settings yield identical recommendations of $K = 3$ as the most informative number of clusters, following the method of Evanno, Regnaut, and Goudet (2005) (Figure S5). We processed individual and population matrices from STRUCTURE HARVESTER (Earl & vonHoldt, 2012) in the cluster matching program CLUMPP (Jakobsson & Rosenberg, 2007), then processed the q-matrices of CLUMPP in Distruct (Rosenberg, 2004) to generate the barplot in Figure 1 (bottom) and to map pie charts in Figure 1 using the open-source geographic information system tools in R (R Core Team, 2014).

To complement the STRUCTURE analysis, we conducted principal component analysis (PCA) and discriminate analysis of principal components (DAPC) using ADEGENET 2.0.1 (Jombart, Devillard, & Balloux, 2010). DAPC transforms genetic data into principal components and then performs a discriminant analysis, which maximizes the variation between samples assigned to *K* clusters and minimizes variation within each cluster. Unlike STRUCTURE, DAPC does not assume a particular population-genetic model (e.g., that markers are in Hardy–Weinberg equilibria and unlinked). As in the STRUCTURE analysis, we specified $K = 3$ clusters prior to implementing DAPC. To reduce overfitting, the number of principal components ($n = 5$) used to calculate the discriminant functions was determined by cross-validation in adegenet, using 10-folds with 20% of the data in each fold. We visualized sample assignments to clusters geographically using GGMAP 2.6.1 (Kahle & Wickham, 2017).

3 | RESULTS

We characterized through sequencing or microsatellite genotyping the cultivar fungi from 474 leafcutter nests from eight *Atta* and 22 *Acromyrmex* species collected in 17 countries ranging from Argentina/Uruguay to the southern United States (Tables S1 and S2).

3.1 | Phylogeny of fungi cultivated by higher-attine ants

Phylogenetic relationships of these fungi (Figures S1 and S2) confirm the pattern already observed in Mikheyev et al. (2008), Ješovnik et al. (2017) and Mueller et al. (in review) that higher-attine fungi fall into two groups, a genetically homogenous group of Clade-A fungi (*L. gongylophorus*) and a more diverse group of Clade-B fungi that is subdivided into at least six distinct subclades (Figure S1). We did not identify any unknown equivalent clades of higher-attine fungi (i.e., no Clade-C or -D fungi).

The three protein-coding genes analysed here (Figures S1–S4), as well as two additional ribosomal genes analysed in Mueller et al. (in review), failed to uncover significant variation within Clade-A fungi across the leafcutter range from Argentina to the United States. This lack of variation in Clade-A fungi contrasts with the substantial generic and species diversity of the Clade-A-cultivating ant hosts, which includes at least seven *Atta* species, 22 *Acromyrmex* species and five *Trachymyrmex* species (Table S1). Because of the minimal genetic diversity found so far among Clade-A fungi (Figure S1–S4; Bich, Castrillo, Villalba, & Zapata, 2016; Lugo, Crespo, Cafaro, & Jofre, 2013; Mikheyev et al., 2006, 2007; Pereira et al., 2015; Silva-Pinhati et al., 2004; Wallace, Asensio, & Tomás, 2014), Clade-A fungi are thought to represent a cohesively evolving lineage (i.e., a single fungal species), confirming the interpretation of Mikheyev et al. (2006) that Clade-A fungiculture (i.e., *L. gongylophorus* fungiculture) is a one-to-many fungus–ant association. Across all higher-attine ants and their known fungi (Figure S1; Mueller et al., in review), however, fungus–ant associations are many-to-many because higher-attine ant lineages switch between Clade-A and Clade-B over evolutionary and ecological time (see below), and long-term ant–fungus co-evolution is therefore less specific than currently believed.

3.2 | Clonal propagation of fungal cultivars

The five microsatellite loci (Table S3) identified 241 genotypes among the 419 Clade-A fungi collected from 419 different leafcutter ant nests; that is, 178 fungal genotypes (42.5%) were collected from more than one leafcutter nest. Most of these duplicate cases (75.7%, 56 of 74 cases) of fungus-genotype identity between nests involve nests of the same ant species collected in close geographic proximity (typically within 50 km of each other or less; Table S3). This is

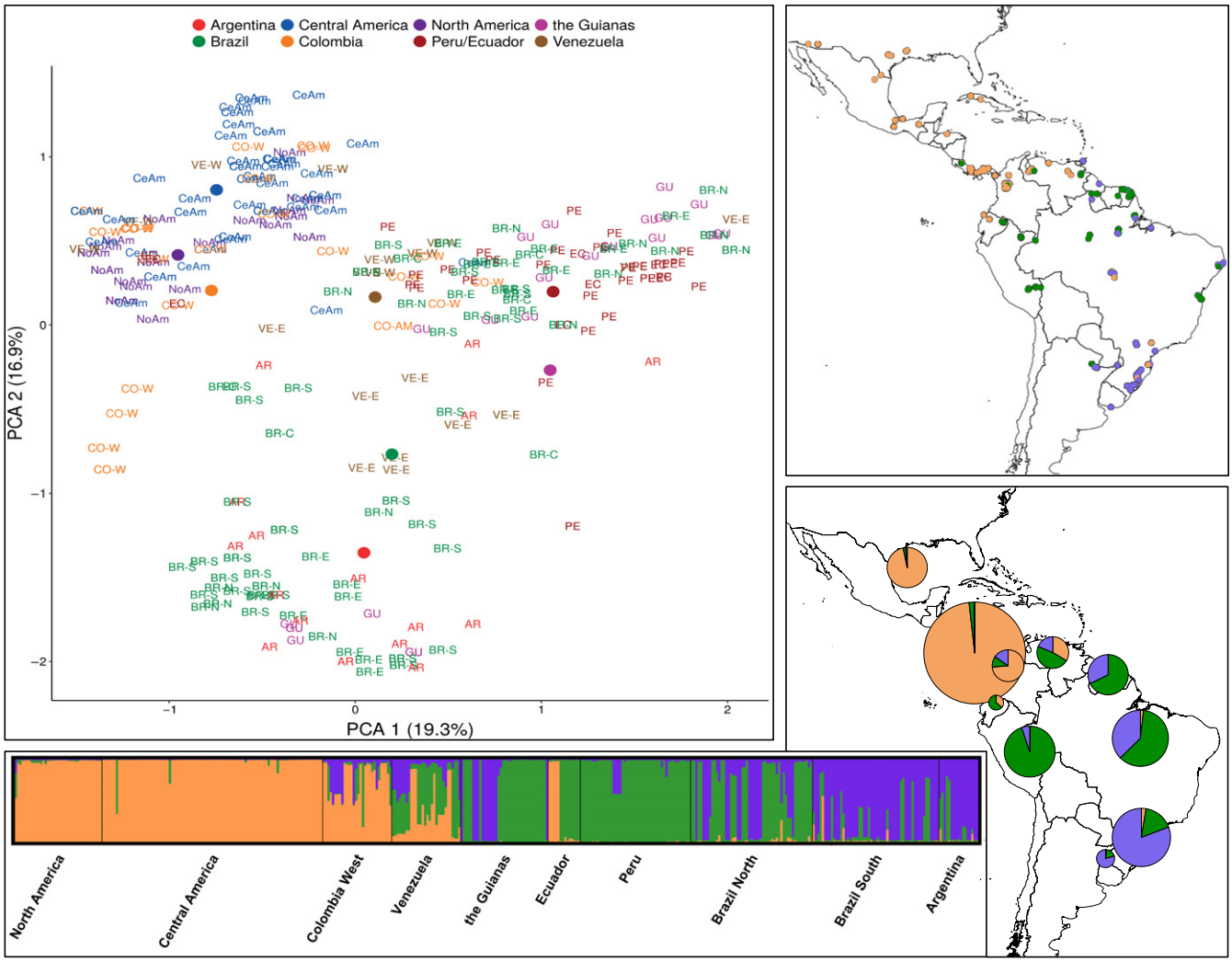

FIGURE 1 Biogeographic patterns of 419 *Leucocoprinus gongylophorus* fungi cultivated by leafcutter ants. Top-left: A principal component analysis (PCA) of microsatellite-marker profiles. The first two principal components, representing 38.3% of the genetic variation, group the leafcutter fungi into three clusters, with PCA axis 2 corresponding to latitude south to north. Fungi from northwest of the Andes cluster as a cohesive group at the top-left in the PCA plot (i.e., collections from western Colombia = CO-W, western Venezuela = VE-W, Central America = CeAm, North America = NoAm). Fungi from northern South America cluster mostly at the top-right [Ecuador = EC, Peru = PE, eastern Venezuela = VE-E, the Guyanas (Guyana, Suriname, French Guiana) = GU, northern Brazil = BR-N, CO-AM = Amazonian Colombia], and fungi from southern South America cluster mostly at the bottom-right (central Brazil = BR-C, eastern Brazil = BR-E, southern Brazil = BR-S, Argentina and Uruguay = AR). Solid dots mark the centroids of the main collection regions. Top-right: Discriminant analysis of principal components (DAPC). The first 10 principal components, representing 78.0% of the genetic variation, resulted in assignments of leafcutter fungi to three clusters similar to the geographic distribution of clusters in a STRUCTURE analysis (bottom panels), with clusters coded purple, green and burnt orange. The geographic visualization of these sample assignments also identifies collection locations ranging from Uruguay to the southern United States. Table S1 lists exact collection locations. Bottom panels: As in the PCA and the DAPC, STRUCTURE analysis of microsatellite profiles assigns the fungi to three clusters (purple, green and burnt orange). To visualize biogeographic patterns, membership in these three clusters is mapped onto ten biogeographic regions: Argentina and Uruguay, southern Brazil, northern Brazil, Peru, Ecuador, the Guianas (Guyana, Suriname, French Guiana), Venezuela, Colombia, Central America (Panamá, Costa Rica, Honduras) and North America (Mexico, Cuba, United States). The size of each pie chart corresponds to the number of leafcutter nests surveyed in each region; each pie chart is centred on the centroid of the collections from a region. In both the PCA and the STRUCTURE analysis, populations of *L. gongylophorus* fungi in Central and North America are less diverse than populations in South America

consistent with vertical transmission of cultivar clones within ant lineages, and these fungal genotypes are likely identical in proximate nests of the same ant species because of limited dispersal per ant generation and vertical inheritance of fungal clones. Cases of cultivar identity between different ant species and between different leafcutter genera are discussed below.

3.3 | Population structure of *L. gongylophorus* fungi cultivated by leafcutter ants

Genetic structure in *L. gongylophorus* is strongly correlated with geography. The methods of Evanno et al. (2005) determined that $K = 3$ (Figure S5) is the most informative number of genetic sources

(populations) for modelling in STRUCTURE. Figure 1 plots STRUCTURE assignments of 419 fungal samples to these populations and maps these onto ten regions defined by country of collection (some adjacent countries are combined, and Brazil is divided into north and south) (Table S3). The three populations correspond approximately to southern South America, northern South America and North and Central America (Figure 1). Fungi from outside of South America and most samples from west of the Andes in Colombia and Venezuela are assigned by STRUCTURE to the "orange" population (Figure 1). Members of the "green" population (Figure 1) and the "purple" population occur almost exclusively in South America. If the number of co-occurring genetic sources (populations) inferred by STRUCTURE is an indication of local genetic diversity, fungal populations are less diverse in Central and North America compared to South America. The local proportion of admixed individuals (fungi combining alleles assigned by STRUCTURE to different genetic sources) appears greatest in Colombia and Venezuela (Figure 1 bottom).

Analysis of the principal components and their discriminant functions using DAPC yielded similar population subdivision as in STRUCTURE. The first two principal components (representing 38.3% of the genetic variation) group the Clade-A leafcutter fungi into three clusters, with PCA axis 2 corresponding to latitude south to north (Figure 1 top-left). All fungi from North and Central America, plus almost all fungi from west of the Andes in Colombia and Venezuela, cluster as a cohesive group, which have less diversity than the fungi belonging to two clusters from South America. A second cluster includes predominately fungi from Peru, Ecuador, Venezuela, the Guianas and northern Brazil and a third cluster mostly fungi from Argentina and southern Brazil (Figure 1). Discriminant analysis of the first 10 principal components, which contain 78.0% of the genetic variation, resulted in assignments of leafcutter fungi to clusters (Figure 1 top-right) also similar to the geographic distribution of clusters in the STRUCTURE analysis. In both the DAPC and STRUCTURE analyses, therefore, populations of *L. gongylophorus* fungi in Central and North America are less diverse than populations in South America.

Estimating admixture using DAPC requires an a priori assignment of samples to populations. We did not have an a priori hypothesis regarding population structure and thus did not attempt an admixture analysis using DAPC.

3.4 | Biogeographic patterns of allele diversity of *L. gongylophorus* fungi cultivated by leafcutter ants

In contrast to the strong spatial structure, allele richness (total number of alleles) of fungi shows no consistent patterns across the entire range of *L. gongylophorus* fungi cultivated by leafcutter ants (Figure S6a–e). Because *L. gongylophorus* fungi are polyploid, multinucleate fungi and ploidy appears variable between fungal strains (Carlson et al., 2018; Kooij, Aanen, et al., 2015; Scott et al., 2009), we were not able to use standard population-genetic statistics (e.g., heterozygosity), so we examined biogeographic distributions of the maximum number of alleles per locus (allele richness) and private alleles (alleles present only in specific populations). For adequately sampled populations (i.e., at least 25–30 individuals per population in microsatellite-marker analyses; Hale, Burg, & Steeves, 2012), allele richness and heterozygosity are correlated, and allele richness can therefore serve as a proxy of heterozygosity (Eckert, Samis, & Lougheed, 2008). In our survey, allele richness does not change as a function of latitude (Figure S6); such latitudinal changes would be expected if migration between biogeographic regions is limited and older populations had more time to accumulate allelic diversity than younger populations founded by recently expanding leafcutter lineages (Eckert et al., 2008). Second, populations at the range limit in the United States and the island population in Cuba do not show reduced allelic diversity (Figure S6), as would be expected for founder populations, for populations with reduced effective population sizes at range limits (Eckert et al., 2008), or for populations at an expanding front experiencing allele surfing (Burton & Travis, 2008; Peischl, Dupanloup, Kirkpatrick, & Excoffier, 2013). Third, there were no private alleles that characterized all individuals in a biogeographic region or in any location. Some alleles occurred only in North America, but only in some, not all, individuals (e.g., alleles 212, 215, and 218 at locus A1132); some alleles occurred only in South America (e.g., allele 243 at locus C126, allele 188 at locus A1132); and a null allele at locus B12 occurred only in northern South America (mostly in Peru and Ecuador, also in Colombia, Venezuela and French Guiana; Figure S6e, Table S4). Overall, however, no biogeographic region showed an obviously increased allelic diversity that could indicate a potential location of older populations where leafcutter fungi may have originated and accumulated greater allelic diversity over time, or where evolutionary forces may operate that increase (or decrease) allele diversity.

3.5 | Are there differences between fungi cultivated by dicot- vs. monocot-cutting leafcutter ants?

Leafcutter ants specialized to forage on monocotyledonous plants (grasses), or on both grasses and dicotyledonous (dicot) plants, are more likely to cultivate Clade-B fungi (Table S6), but the association between foraging preference and cultivar specialization, although statistically significant, is weak. Combining information from *Acromyrmex* and *Atta* (Table S6; additional discussion in Supporting Information), and combining into one group those leafcutter species that are specialized to cut grasses or cut both grasses and dicots, 100% of the 23 dicot-specialized leafcutter species cultivate Clade-A fungi (and for only two of these leafcutter species, there is evidence that they also cultivate Clade-B fungi at some locations; Tables S6 and S10), and therefore, 0% of these 23 dicot-specialized leafcutter species are specialized on Clade-B fungi. In contrast, four (40%) of the 10 species that cut grasses cultivate Clade-B fungi, but for two of the Clade-B-cultivating species, only one single fungus has been identified so far (Table S6). The Fisher's exact test statistic for this distribution is $p = .0051$ (23 counts dicot and Clade-A fungi; 0 counts dicot and Clade-B; 4 counts grass and Clade-B; 6 counts grass and Clade-A), and Barnard's exact test statistic is $p = .0040$.

Limiting the analysis to only Clade-A fungi and ignoring Clade-B cultivation, our microsatellite-marker analyses did not reveal obvious differences between Clade-A fungi cultivated by the 22 leafcutter species in our survey (both *Acromyrmex* and *Atta*) that preferentially forage on dicots compared to Clade-A fungi cultivated by three species preferentially foraging on grasses (*Ac. balzani*, *Ac. heyeri*, *Ac. landolti*) or compared to one species foraging on both grasses and dicots (*Ac. lobicornis*) (Table S3). In fact, we found two cases where sympatric dicot-specialist and grass-specialist leafcutter species cultivated in the same location the same fungal clone (defined as identity in all alleles across the five microsatellite loci), that of *Ac. landolti* and *At. cephalotes* in Colombia and that of *Ac. heyeri*, *Ac. balzani* and *At. sexdens* in southern Brazil (Table S3). This identity of fungal genotypes suggests that dicot- and grass-specialized leafcutter species may cultivate fungi from shared pools of Clade-A fungi circulating locally with a leafcutter ant community, and even dicot- and grass-specialized leafcutter species may exchange cultivars on occasion.

3.6 | Are there differences between Clade-A fungi cultivated by *Atta* vs. *Acromyrmex* ants?

Recent studies argued that *L. gongylophorus* fungi (i.e., Clade-A fungi) cultivated by *Atta* and *Acromyrmex* ants in Panamá represent separate gene pools (Kooij, Poulsen, Schiøtt, & Boomsma, 2015) and that two *L. gongylophorus* fungi cultivated by *Atta* vs. *Acromyrmex* ants in Panamá diverged from each other 7.2 Ma (confidence interval 5.4–9.0 Ma; Nygaard et al., 2016; pages 43 & 44 in the Supplementary Methods of Nygaard et al.). Because we did not find differences between *Atta*-cultivated vs. *Acromyrmex*-cultivated *L. gongylophorus* fungi in our phylogenetic analyses (Figures S1–S4), we tested for possible differences using our faster-evolving microsatellite markers, which should have adequate resolution to detect Nygaard et al.'s hypothesized ancient diversification dating to 5–9 Ma. Our analyses do not support genetic isolation between *Atta*-cultivated vs. *Acromyrmex*-cultivated *L. gongylophorus* fungi, for two main reasons.

First, at most of the locations at which we obtained adequate samples of *L. gongylophorus* fungi from both *Atta* and *Acromyrmex* nests, we found *Atta*-cultivated and *Acromyrmex*-cultivated fungal clones that were identical in all alleles across the five microsatellite loci (Table S3). *Atta* and *Acromyrmex* nests cultivating identical fungal clones (as defined by our five markers) were located typically within 50 km of each other, but there were also instances of apparent cultivar identity between *Atta* and *Acromyrmex* nests about 1,200 km distant (Brazil) and 1,900 km distant in Mexico/United States (Table S3). Because many locations were undersampled in our study (e.g., we were able to obtain collections from only one genus from the two leafcutter genera present at a location; Table S3), sharing of identical cultivar clones is likely more prevalent in nature than indicated in our collection. Overall, we found eight cases of sharing of fungal clones between different leafcutter genera and 10 cases of sharing of cultivar clones between different congeneric species (Table S3). The near-identical incidence of cultivar sharing (8 vs. 10 cases) could suggest that the same biological processes led to such cultivar identity (e.g., horizontal transmission of cultivars between nests) and that cultivars may transfer almost as readily between nests of different leafcutter genera as between nests of the same leafcutter genus.

Second, STRUCTURE analyses of fungi from Panamá, the best-sampled region in our survey, indicates that *Atta*- vs. *Acromyrmex*-cultivated fungi do not form genetically distinct clusters, but are admixed (Figure S8a–d), regardless of whether we analyse regional fungal diversity (Colombia, Panamá, Costa Rica; $n = 125$ samples), within-country diversity (only Panamá; $n = 89$ samples), provincial diversity (Panamá Canal Zone; $n = 42$ samples) or the local diversity in Gamboa ($n = 27$) also studied by Kooij, Poulsen, et al. (2015) (Figure S8a–d; see additional discussion in the Supporting Information). Our STRUCTURE analyses therefore agree with the findings of three previous studies: Mikheyev et al.'s (2007) STRUCTURE analysis showing that *Atta* and *Acromyrmex* ants from Gamboa share a pool of fungal cultivars; Kooij, Aanen, et al.'s (2015) sequence analysis showing that Panamanian leafcutter fungi do not group into separate clades of *Atta*-cultivated and *Acromyrmex*-cultivated fungi; and Mueller, Mikheyev, Solomon, et al.'s (2011) STRUCTURE analysis showing that *Atta* and *Acromyrmex* ants share cultivars from the same genotype cluster (so-called M-fungi) in North America.

4 | DISCUSSION

We aimed to conduct a comprehensive biogeographic and population-genetic analysis of fungi propagated by leafcutter ants across the entire leafcutter range from Argentina to the United States, combining collections from 22 collaborating laboratories and surveying leafcutter ants in 17 Neotropical countries (Tables S1 and S2). Analyses of 474 fungi cultivated by leafcutter ants revealed (i) no novel cultivar types beyond the known Clade-A and Clade-B cultivars of leafcutter ants (Figure S1; see also Mueller et al., in review); (ii) moderate support that those leafcutter species that cut grass as fungicultural substrate show a higher frequency of cultivating Clade-B fungi, whereas all leafcutter species preferring dicot plants as fungicultural substrate seem specialized on cultivation of Clade-A fungi (Table S6); (iii) cultivar sharing between sympatric leafcutter species within local communities such that fungi cultivated by *Atta* species are overall not distinct from those cultivated sympatrically by *Acromyrmex* species; (iv) three genotype clusters of Clade-A fungi across the range from Argentina to the United States (Figure 1), with local prevalence of these genotype clusters corresponding approximately to southern South America, northern South America and Central and North America (Figure 1); (v) gene flow among Clade-A fungi cultivated by leafcutter ants in different biogeographic regions, including fungi cultivated by leafcutter species in Cuba such that all Clade-A fungi from Argentina to the United States represent a single species, *L. gongylophorus* (additional discussion in Supporting Information); and (vi) reduced genetic diversity of leafcutter fungi in Central and North America and greatest genetic diversity of leafcutter fungi concentrated in South America (Figure 1, Table S1).

4.1 | Biogeographic origin of leafcutter fungiculture and leafcutter ants

Kusnezov (1963) and Fowler (1983) hypothesized that leafcutter ants originated in southern South America, because extant leafcutter ants exhibit the greatest species diversity there, particularly *Acromyrmex* species. In contrast, Branstetter et al. (2017) recently inferred biogeographic history mapped onto a phylogeny of attine ants, and their biogeographic modelling suggested a possible origin of leafcutter ants in Central America. These two hypotheses make different predictions regarding the biogeographic region where leafcutter fungi can be expected to be most diverse. Assuming the traditional view that leafcutter ants became specialized to cultivate Clade-A fungi around the time of the origin of the leafcutter clade 19 Ma, and assuming no other factors affect diversity of fungal cultivars (e.g., genetic drift, gene flow and horizontal cultivar transfer do not affect cultivar diversity differently in different populations across the range of leafcutter ants), the hypothesis of a Central American origin predicts that fungi cultivated by leafcutter ants should be most diverse in Central America and less diverse in South America colonized secondarily by leafcutter lineages dispersing with their cultivars from Central to South America. In contrast, the hypothesis of a South American origin predicts the opposite, a greater diversity of leafcutter fungi in South America that accumulated there during the past 19 million years of leafcutter diversification, and less fungal diversity in Central and North America colonized secondarily, and possibly recently (less than 5 Ma), by leafcutter lineages migrating out of South America. Our phylogenetic analyses indicate a mix of Clade-B and Clade-A cultivation by leafcutter ants in southern South America (and apparent absence of Clade-B cultivation by leafcutter ants in the well-surveyed Central American populations; Table S1, Figure S1), and our principal component and STRUCTURE analyses indicate greatest diversity of Clade-A fungi in South America (Figure 1). Both phylogenetic and population-genetic patterns of cultivar diversity are consistent with the prediction of the Kusnezov–Fowler model of a South American origin of leafcutter ants and a secondary expansion into Central and North America.

It is possible to conceive alternative scenarios of leafcutter ant–fungus evolution that assume a Central American origin of the leafcutter ant clade and a South American origin of Clade-A fungi. For example, the origin of leafcutter ants may have been decoupled from the origin of Clade-A fungi. Specifically, leafcutter ants may have originated in Central America, but Clade-A cultivars originated in South America in ancestral *Trachymyrmex* lineages, as discussed by Mueller et al. (in review); Clade-A cultivars were then secondarily acquired by leafcutter ants in South America after they dispersed from Central into South America, a successful Clade-A lineage (i.e., *L. gongylophorus*) eventually spread across the entire leafcutter range due to efficient horizontal transmission between leafcutter species, and only a limited genotype diversity of Clade-A cultivars spread so far into Central and North America from diverse Clade-A populations in South America (Figure 1). Other such *ad hoc* scenarios are also possible, and some of these scenarios can be tested by precise dating of the evolutionary origins of leafcutter fungi relative to the origin of the leafcutter clade.

Dates for crown-group and stem-group ages for Clade-A fungi and for the leafcutter ant clade have been estimated in six phylogenetic analyses (Table 1). When comparing crown ages (age of most recent common ancestor, MRCA; coalescence) of Clade-A fungi and the leafcutter ant clade, the MRCA of Clade-A fungi is estimated much younger, by about 10 million years, than the MRCA of leafcutter ants (Table 1), generating a time discord (Mikheyev et al., 2010) rather than the synchrony expected if leafcutter ants and leafcutter-specific cultivars originated at the same time (Chapela et al., 1994; Hinkle, Wetterer, Schultz, & Sogin, 1994; Stradling & Powell, 1986). However, when comparing the stem age of the Clade-A lineage (age of split from Clade-B fungi) with the stem age of the leafcutter ant lineage (age of split from the *Trachymyrmex septentrionalis* species group), the ages are much more in agreement, 22.4–25.0 Ma for the stem age of Clade-A fungi and 17.8–21.0 Ma for the stem age of the leafcutter ant lineage (Table 1). The somewhat older age of the Clade-A lineage could even suggest that leafcutter ants did not originate coincident with Clade-A fungi as was assumed in the earliest phylogenetic studies (Chapela et al., 1994; Hinkle et al., 1994), but that the Clade-A lineage may have arisen before the origin of the leafcutter ant lineage, as discussed in Mueller et al. (in review). If so, ancestral higher-attine lineages (ancestral to the leafcutter and *T. septentrionalis*-group lineages) may have cultivated both Clade-A and Clade-B fungi as far back as 22–25 Ma (Table 1), well before the origin of the leafcutter ant lineage, and the apparent cultivation of both Clade-A and Clade-B fungi observed in extant *Trachymyrmex* species and in extant leafcutter species could therefore be a retention of a plesiomorphic condition of sharing of Clade-A and Clade-B fungi between higher-attine ant lineages.

To analyse evolution of higher-attine fungiculture, therefore, it may be more fruitful to view ant diversification and fungal–symbiont diversification as separate processes that may be, or may not be, intimately linked. Specifically, at least four scenarios have been discussed in the literature:

1. *Coincident-Scenario*: Clade-A fungi originated coincident with the origin of leafcutter ants, and specialization by leafcutter ants on superior Clade-A fungi facilitated the diversification of leafcutter ants, as assumed by earlier studies (e.g., Chapela et al., 1994; Hinkle et al., 1994; Stradling & Powell, 1986). Under this scenario, the documented cases of Clade-A cultivation by *Trachymyrmex* ants (Figure S1; Mueller et al., in review) would be the result of later horizontal transfer of Clade-A cultivars from leafcutter ants to *Trachymyrmex* ants.
2. *After-Scenario*: Clade-A fungi cultivated by leafcutter ants "originated subsequent to the origin of [leafcutter ants] from a fungal lineage cultivated by *Trachymyrmex* ants" and "leafcutting ants horizontally acquired a replacement cultivar after *Atta* and *Acromyrmex* had diverged" (Nygaard et al., 2016, page 2). Under this scenario, the cultivar types propagated by leafcutter ants during their early diversification are unspecified (maybe Clade-B

TABLE 1 Comparison of crown ages and stem ages for Clade-A fungi, for the leafcutter ant clade and for the higher-attine ant clade, estimated in six published phylogenetic analyses

Clade-A fungi	Leafcutter ant clade	Source
Crown age of Clade-A Fungi	*Crown age of leafcutter ant clade*	
	8 Ma (6–15), without *Ac. striatus*	Schultz and Brady (2008)
4 Ma (0.5–8.0)	(not estimated)	Mikheyev et al. (2010)
	12.2 Ma (9.1–15.3), without *Ac. striatus*	Schultz et al. (2015)
7.2 Ma (5.4–9.0)	16.2 Ma (12.6–19.7), without *Ac. striatus*	Nygaard et al. (2016)
	17.9 Ma (15.6–20.4), without *Ac. striatus*	Ješovnik et al. (2016)
	17.0 Ma (13.2–20.8), without *Ac. striatus*	Branstetter et al. (2017)
	18.2 Ma (14.2–22.2), with *Ac. striatus*	Branstetter et al. (2017)
Stem age of Clade-A fungi	*Stem age of leafcutter ant clade*	
	9 Ma (7–15)	Schultz and Brady (2008)
25 Ma (11–39)	(not estimated)	Mikheyev et al. (2010)
	≈14 Ma (from figure 1 in Schultz et al., 2015)	Schultz et al. (2015)
22.4 Ma (16.9–27.9)	17.8 Ma (13.7–21.7)	Nygaard et al. (2016)
	19.9 Ma (17.7–22.5)	Ješovnik et al. (2016)
	19.3 Ma (15.2–23.7)	Branstetter et al. (2017)
Stem age of Clade-A fungi	*Stem age of higher-attine ant clade*	
	20 Ma (17–29)	Schultz and Brady (2008)
25 Ma (11–39)	(not estimated)	Mikheyev et al. (2010)
	≈25 Ma (from figure 1 in Schultz et al., 2015)	Schultz et al. (2015)
22.4 Ma (16.9–27.9)	26.6 Ma (19.6–33.8)	Nygaard et al. (2016)
	33.3 Ma (31.3–35.1)	Ješovnik et al. (2016)
	31.4 Ma (25.9–37.2)	Branstetter et al. (2017)

Mikheyev et al. (2010) used a four-gene phylogeny to estimate the crown-node date (coalescent) and stem-node date of four Clade-A fungi isolated from two *Acromyrmex* species from Panamá and Guyana and from two *Atta* species from Panamá. Nygaard et al. (2016) used 1075 orthologous loci from transcriptome-sequencing of two Clade-A fungi from *Ac. echinatior* and *Atta colombica* from Panamá. Both Mikheyev et al. and Nygaard et al. anchored a single time-calibrated node in their phylogenetic reconstructions, the last common ancestor of ant-cultivated fungi with *Agaricus*, dated to 73 Ma in Mikheyev et al. (modelled with more or less conservative distributions around this date) and dated likewise to 73 Ma in Nygaard et al. (modelled with a 5% minimum age of 55 Ma and a 95% maximum age of 91 Ma). The ancient time calibration (anchor at 73 Ma) of the fungal phylogeny likely generates estimates for the dates of recent diversifications (e.g., crown age of Clade-A fungi) that are more unreliable than estimates for earlier diversifications. Ma = million years ago.

cultivars or some unknown cultivar lineage), and these early-associated cultivar types were substituted in leafcutter lineages by "horizontally acquired . . . replacement" of Clade-A fungi.

3. *Before-Scenario*: Clade-A fungi originated before the origin of the leafcutter clade such that ancestral Clade-A fungi represented one of several cultivar lineages that circulated in a pool of diverse fungi shared by ancestral higher-attine lineages, as discussed above and in Mueller et al. (in review). If so, Clade-A and Clade-B fungi may have been shared between the diversifying higher-attine lineages since the early evolution of higher-attine ants, and this sharing included also the ancestral leafcutter ant lineages.
4. *Recent Cultivar* Sweeps: Frequent horizontal sweeps of novel, successful Clade-A cultivars between leafcutter nests, coupled with gene flow and hybridization between all Clade-A cultivars across the entire leafcutter range, generated a recent coalescence of all extant Clade-A cultivars, as discussed by Mikheyev et al. (2010). Variants of such cultivar exchange and hybridization are also possible under the *Coincident-*, *After-* and *Before-Scenarios*.

Depending on the biogeographic location of the origin of leafcutter ants, on the biogeographic location of the origin of Clade-A and Clade-B fungi, and on the relative dates of the origins of leafcutter ants and Clade-A fungi, it may be possible to derive testable predictions of biogeographic distribution of ant and fungal diversities. As a first step towards these analyses, it will be important to improve estimates of stem and crown ages for Clade-A and Clade-B fungi by improving the time calibration of phylogenetic histories of the ant-cultivated fungi (see footnote of Table 1).

4.2 | Cultivar sharing reduces ant–fungus specificity of leafcutter cultivars

Our population-genetic and clonality analyses document ongoing cultivar sharing between sympatric *Atta* and *Acromyrmex* leafcutter ants, and such cultivar sharing likely involves in some locations also some sympatric *Trachymyrmex* species (e.g., cultivar exchange between *Ac. versicolor* and *T. desertorum* in Arizona; Figure S1). With

few exceptions known so far, single leafcutter species seem to be specialized either on Clade-A fungi (e.g., all the dicot-foraging leafcutter species) or on Clade-B fungi (*At. laevigata*, *At. vollenweideri*), which mirrors for leafcutter ants the kind of specialization known also for ant species in the lower-attine *Cyphomyrmex wheeleri* group, where each *Cyphomyrmex* species cultivates predominantly its own fungal lineage (species), but different *Cyphomyrmex* species are sometimes specialized on the same fungal lineage (i.e., two *Cyphomyrmex* species can share the same kind of fungus; Mehdiabadi et al., 2012). Despite such specialization, single higher-attine species, as currently recognized, can cultivate both Clade-A and Clade-B fungi in some locations (e.g., *At. laevigata* in southern Brazil; *T. arizonensis* in Arizona; details in Table S10). Such cases of apparent fungal polyculture will need to be elucidated likewise with high-resolution analyses of the respective leafcutter ant hosts, to test for possible cryptic ant species.

Because of the sharing of cultivars between sympatric *Acromyrmex*, *Atta*, and likely also some *Trachymyrmex* species, and because of the possibility of genetic exchange between cultivars in different nests, cultivars may not be propagated long enough within a single ant species to evolve adaptations specific to a particular ant species (or ant genus) and its species-specific environment. This is easiest to understand in the well-surveyed Clade-A fungi, where sympatric grass-cutting and dicot-cutting species can cultivate strains of the same clonal lineage (strains that cannot be distinguished with five microsatellite markers; Table S3). This sharing of the same fungal clone lineages between sympatric grass-cutting and dicot-cutting leafcutter species, as well as between *Atta*, *Acromyrmex*, and possibly also *Trachymyrmex* ants, suggests that Clade-A fungi may have evolved to be "general-purpose genotypes" (Lynch, 1984) suited for cultivation by diverse higher-attine species with diverse fungicultural habits, as first suggested by Mikheyev et al. (2006).

4.3 | Shortcomings of our study and suggestions for future research on leafcutter fungi

Our study has several shortcomings, which do not invalidate the above conclusions, but hopefully will be addressed in future research:

1. Our phylogenetic analyses (Figure S1; Mueller et al., in review) indicate that some *Trachymyrmex* species can also cultivate Clade-A cultivars, the dominant fungal type cultivated by leafcutter ants. Complete population-genetic analyses of Clade-A fungi would therefore include also representative Clade-A fungi from *Trachymyrmex* species to test for population-genetic links between leafcutter- and *Trachymyrmex*-cultivated fungi. Clade-A fungi from *Trachymyrmex* species were unfortunately not included in our microsatellite analyses because we became aware of Clade-A cultivation by *Trachymyrmex* ants only after conclusion of the genotyping phase of our study. Sympatric Clade-A fungus communities that should be evaluated in future studies include, for example, the community of Clade-A cultivars of *Ac. versicolor*, *T. desertorum* and *T. arizonensis* in Arizona, and the community of Clade-A cultivars of diverse leafcutter species, *T. intermedius* and *T. opulentus* in northeast South America and in Central America. (*T. opulentus* is labelled *T. wheeleri* in our Figure S1, but was synonymized by Mayhé-Nunes & Brandão, 2002.) *Trachymyrmex intermedius* ranges from Mexico to French Guiana, and *T. opulentus* ranges from Honduras to northern Brazil, so Clade-A cultivation by these two *Trachymyrmex* species may occur in sympatry with the well-studied leafcutter species in Panamá. Lastly, sympatric Clade-B fungus communities likewise need further study, to test for possible sharing of Clade-B cultivars between leafcutter, *Trachymyrmex* and *Sericomyrmex* species.
2. Our analyses (Figure 1) rely on information from five highly polymorphic microsatellite loci of a polyploid, multinucleate fungus (an individual fungus may show more than two alleles per locus), and information from additional microsatellite loci would undoubtedly have increased resolution of population-genetic structure. In fact, prior analyses that genotyped leafcutter fungi from Panamá and North America with, respectively, 9 and 12 microsatellite loci (Mikheyev et al., 2007; Mueller, Mikheyev, Solomon, et al., 2011) inferred a larger number of sympatric genotype clusters (six clusters in Panamá, four clusters in North America). Identification of three genotype clusters across the leafcutter range in our five-locus analysis (Figure 1) therefore is a minimum estimate. Information from additional loci, however, is unlikely to show that fungal populations in Central America are more diverse than those in South America; rather, it seems likely that far more genotype clusters will emerge when sampling South American populations with more loci, and when sampling at the same density as the well-surveyed Panamanian population in our study. Future studies could use, for example, the two multiplex panels (15 microsatellite loci total) of Carlson et al. (2018) or consider developing genotyping-by-sequencing methods for preserved garden material.
3. Although our survey covered 17 countries across the leafcutter-ant range, several important regions were not sampled, for example Bolivia, Paraguay and parts of Central America; vast regions in western and central Brazil; extreme habitats (e.g., higher elevations in the Andes, seasonal wetlands of the Pantanal, western cerrado in Brazil); or a densely sampled transect across the Andes in Colombia, the transition zone from cultivation of three genotype clusters in northwest South America to one genotype cluster in Panamá (Figure 1). Most important, the southernmost leafcutter populations in Argentina remain to be surveyed [e.g., *Ac. lobicornis* ranges to ≈44° south (Farji-Brener & Ruggiero, 1994), whereas our southernmost collection was from ≈35° south in Uruguay], as well as the western leafcutter populations in Argentina inhabited by unique leafcutter species like *At. saltensis* and *Ac. silvestrii* (the putative sister species to the Clade-B-cultivating *Ac. striatus*). Future surveys in subtropical and temperate South America should ideally also include behavioural studies of *Ac. striatus* and *Ac. silvestrii* to inform hypotheses on whether the ancestral leafcutter lineage may have been

specialized to cut grass or dicot leaves, or utilized both types of leaves for fungiculture. *Ac. striatus* and *Ac. silvestrii* reportedly cut both grass and dicots, with foraging preferences possibly changing seasonally between grass and dicots (Bucher & Montenegro, 1974; Fowler & Claver, 1991; Fowler, Forti, Pereira-da-Silva, & Saes, 1986; Gonçalves, 1961).

5 | CONCLUSION

Most efforts to elucidate leafcutter ant–fungus associations focused so far on leafcutter ants in Central and North America (Table S6), but these leafcutter symbioses, all of them involving dicot-specialized leafcutter species, are not representative of the more complex leafcutter symbioses existing across South America (Figures 1 and S1). Leafcutter species specialized on cultivation of Clade-B fungi occur only in South America (ranging from Argentina to Colombia; Figure S1), the highest concentration of Clade-B-cultivating leafcutter nests found so far is in southern South America (Table S1), and Clade-A fungi of leafcutter ants are more diverse in South America than in Central and North America (Figure 1). This co-occurrence of the greatest leafcutter ant species diversity and greatest cultivar diversity in southern South America may not be a coincidence, yet the leafcutter ant–fungus associations in the grasslands of southern South America are far less understood than those in highly disturbed Central America forests dominated by weedy leafcutter ant species. If the Kusnezov–Fowler hypothesis for the origin of leafcutter ants in subtropical southern South America is correct and accounts for the concentrated diversity of leafcutter species there (Bacci et al., 2009; Borgmeier, 1959; Brandão et al., 2011; Delabie et al., 2011; Della Lucia, 2011; Farji-Brener & Ruggiero, 1994; Fowler, 1983; Gonçalves, 1961; Kusnezov, 1963; Mariconi, 1970; Mueller & Rabeling, 2008; Wild, 2007), a comprehensive cultivar survey in Argentina, Uruguay, Paraguay, Bolivia and sub-Amazonian Brazil is most likely to uncover unknown types of leafcutter fungi (i.e., "Clade-C" or "Clade-D" cultivars), which will inform hypotheses on the diversity of cultivars available for cultivation at the origin of leafcutter ants.

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DATA ACCESSIBILITY

DNA sequences: GenBank accessions GQ853919–GQ854367, GQ854817–GQ855186, HQ391561–HQ391895.

Sample information, microsatellite genotypes, analyses: Tables S1–S10 in Supporting Information.

AUTHOR CONTRIBUTIONS

Project coordination: M.Ba. Jr, U.G.M. Coordination of regional collections: M.Ba. Jr, M.Bo., C.R.F.B., A.G.H., J.E.L., I.R.L., A.S.M., U.G.M., A.O., F.C.P., C.R., F.R., S.A.R., S.E.S., T.R.S., R.W., H.L.V. Field work, sample vouchering: R.M.A., M.Ba. Jr, M.Bo., R.M.C., A.G.H., J.E.L., J.S.L., I.R.L., R.A.J., A.S.M., U.G.M., A.O., F.R., C.R., S.A.R., A.R., T.R.S., J.J.S., S.E.S., J.S.-C., H.L.V., R.W. Fungal isolations: A.O., A.R., U.G.M., S.A.R. Molecular analyses: S.M.B., H.D.I., M.C., A.S.M., J.J.S. Microsatellite marker development: J.J.S., M.C., H.D.I. Microsatellite marker scoring, phylogenetic analyses: H.D.I. Population genetic analyses: C.C.S., J.J.H. Writing of manuscript: U.G.M., H.D.I., T.R.S., C.R., S.A.R., F.R. All authors edited, commented on, and approved submission of the manuscript.

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SUPPORTING INFORMATION

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