Biogeography of mutualistic fungi cultivated by leafcutter ants

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Biogeography of Leafcutter Ant-Fungus Mutualisms
Biogeography of Fungi Cultivated by Leafcutter Ants
Biogeography of Leafcutter-Ant Fungiculture

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Abstract (249 words; limit of 250 words in Mol.Ecol.)
Leafcutter-ants propagate co-evolving fungi for food. The nearly 50 species of leafcutter-ants (*Atta*, *Acromyrmex*) range from Argentina to the USA, with the greatest species diversity in subtropical savannahs of southern South America. To elucidate the biogeography of fungi cultivated by leafcutter-ants, we use sequencing and microsatellite-marker analyses to genotype 474 leafcutter cultivars collected across the leafcutter range. All surveyed cultivars belong to one of two clades (Clade-A, Clade-B). The dominant and widespread Clade-A cultivars group into three genotype-clusters, with their relative prevalence corresponding to southern South America, northern South America, and Central&North America. Admixture between genotype-clusters and gene flow between Clade-A populations support genetic exchange within a single species, *Leucocoprinus gongylophorus*. Leafcutter species preferring grass as fungicultural substrate are more likely to cultivate Clade-B fungi, whereas leafcutter species preferring dicot plants appear specialized on Clade-A fungi. Cultivar sharing between sympatric species occurs frequently within local leafcutter-ant communities, such that cultivars of *Atta* are not distinct from those of *Acromyrmex*. Diversity of Clade-A fungi is greatest in South America, but reduced in Central&North America, and leafcutters specialized on Clade-B fungi occur only in South America. This maximum cultivar diversity in South America is less compatible with a Central American origin of leafcutter ants hypothesized by Branstetter *et al*, but predicted by the Kusnezov-Fowler hypothesis that leafcutter-ants originated in subtropical South America, and only dicot-specialized leafcutter-lineages migrated so far out of South America. We discuss these biogeographic hypotheses in light of estimated dates for the origins of leafcutter-ants and their cultivars.

Key Words: *Attamyces bromatificus*, *Leucoagaricus gongylophorus*, *Leucocoprinus gongylophorus*, fungus-growing ant, insect-fungus mutualism, symbiosis

Introduction

Biogeographic distributions provide clues to infer evolutionary processes, such as ancient dispersal and vicariance events shaping macroevolutionary patterns, or adaptation and gene flow influencing microevolutionary processes (Wallace 1876; Brown & Lemolino 1998; Avise 2009). In mutualistic associations between two partners, similarities or differences in biogeographic distributions between co-dependent partners can facilitate inference of such evolutionary processes (Thompson 2005; Alvarez *et al.* 2010; Satler & Carstens 2016; Hembry & Althoff 2016). Co-biogeographic patterns of mutualistic partners require cautious interpretation, however, particularly regarding congruence and incongruence of patterns, because evolutionary forces and demographic histories can differ markedly between partners (Herre *et al.* 1999; Alvarez *et al.* 2010; Espíndola *et al.* 2014; Tian *et al.* 2015; Chomicki *et al.* 2017). For example, population sizes, migration rates, mutation rates, and generation times can differ by orders of magnitude between a host and a symbiotic partner (Lutzoni & Pagel 1997; Moran & Wernegreen 2000; Woolfit & Bromham 2003; Degnan *et al.* 2004; Douglas 2010), and dispersal barriers restricting gene flow for one partner (e.g., pollinating bee) may not impede gene flow for the other partner (e.g., 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 co-migrate 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) (Wornik & Grube 2010; Dal Grande *et al.* 2012; Silverstein *et al.* 2012; Kaltenpoth *et al.* 2014; Weiblen & Treiber 2015; Palmer *et al.* 2015).

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 (Nobre *et al.* 2011; Six 2012; Mueller *et al.* 2011a; Kellner *et al.* 2013; Hulcr & Stelinski 2017), or greater potential for a single symbiont lineage to interact with different allopatric host species or with

geographically differentiated host populations of the same host species (Thompson 2005; Mueller & Gerardo 2002; Weiblen & Treiber 2015; Palmer *et al.* 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 *et al.* 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; Douglas 2010). 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 across space (e.g., Carriconde *et al.* 2008; Douhana *et al.* 2011; Gazis *et al.* 2011).

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 (Silva-Pinhati *et al.* 2004; Mikheyev *et al.* 2006, 2007, 2008, 2010; Mikheyev 2008; Mueller *et al.* 2011a; Mueller *et al.* in review). In the leafcutter mutualism, one dominant fungus clade, called Clade-A fungi (Mueller *et al.* in review), is associated with leafcutter ant species across the entire leafcutter range from Argentina to the USA, including also several leafcutter ant species inhabiting Cuba and other Caribbean islands (Mikheyev *et al.* 2006; Mikheyev 2008; Mueller *et al.* 2011a; Mueller *et al.* in review). Clade-A fungi sequenced so far were thought to be closely affiliated with the leafcutter fungus *Leucocoprinus gongylophorus* (Heim 1957) that was described from mushrooms (sexual sporocarps) growing from leafcutter gardens of several leafcutter ant species (Möller 1893; Fisher *et al.* 1994; Pagnocca *et al.* 2001; Mueller 2002; Pagnocca *et al.* 2011). [See Supporting Information why the widely-cited placement of these mushrooms into the genus *Leucoagaricus* by Singer (1986) is inaccurate]. No free-living leafcutter fungi have been found so far (i.e., sporocarps or mycelia of leafcutter fungi growing independent of a leafcutter nest are not known), but such free-living mushrooms are known for the cultivars of lower-attine, non-leafcutter ants (Mueller *et al.* 2001; Mueller 2002; Vo *et al.* 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*; Fowler 1983; Fowler *et al.* 1986; Solomon *et al.* 2008; Delabie *et al.* 2011) cultivate Clade-B fungi (Mueller *et al*. in review), a clade of fungi that was thought previously to be associated exclusively with non-leafcutting *Trachymyrmex* and *Sericomyrmex* ants that form sister and outgroup lineages to the two leafcutter ant genera *Atta* and *Acromyrmex*. Moreover, some higher-attine non-leafcutter 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; Mueller *et al*. in review; Fig. 1). These ant-fungus associations indicate that leafcutter and non-leafcutter ants share a pool of fungi belonging principally to two clades of fungi, Clade-A fungi possibly representing a single species of fungus called *Leucocoprinus gongylophorus* (i.e., formerly called *Attamyces bromatificus* as the vegetative mycelial form; Kreisel 1972), and Clade-B fungi representing at least six well-supported lineages of fungi, each possibly a separate cultivar species (Mueller *et al*. in review; Fig. 1). The so-called higher-attine fungi (Clade-A & Clade-B fungi), cultivated by higher-attine ants in the leafcutter genera *Atta* and *Acromyrmex* and the non-leafcutter ants *Trachymyrmex* and *Sericomyrmex*, therefore co-evolve diffusely with their higher-attine ant hosts, with higher-attine ant lineages occasionally transitioning between Clade-A and Clade-B cultivation. The frequencies of these transitions over evolutionary and ecological time 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; see also Table S10), a kind of local polyculture within an ant population seen also in an asexual lower-attine ant (Himler *et al.* 2009; Kellner *et al.* 2013) but not in all lower-attine ants (Mehdiabadi *et al.* 2012).

Because of vertical inheritance of fungal cultivars from maternal to offspring nests, leafcutter ants and fungi were initially predicted to co-migrate and co-reproduce together, and initially were even thought of as ancient asexual clones (Chapela *et al.* 1994). However, population-genetic and phylogenetic observations are inconsistent with strict vertical inheritance and strict clonal reproduction. These observations include: (a) sharing of fungus-cultivar clones between sympatric leafcutter ant species, indicating frequent 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 *et al.* 2000; Green *et al.* 2002; Mikheyev *et al.* 2007, 2010; Mueller *et al.* 2011a); (b) identity of fast-evolving DNA sequences (e.g., internal transcribed spacer region, ITS) of leafcutter fungi cultivated by different leafcutter ant species across vast geographic distances (e.g., southern to northern Brazil, Silva-Pinhati *et al.* 2004); and (c) genetic admixture between *L. gongylophorus* populations associated with *Atta* and *Acromyrmex* species across North America (Mexico, southern USA, Cuba) implicated by phylogenetic sequence analyses (Mikheyev *et al.* 2006, 2010) and population-genetic microsatellite-marker analyses (Mueller *et al.* 2001a). The observation of genetic admixture between *L. gongylophorus* populations across a significant 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 non-ant vectors; Möller 1893; Pagnocca *et al.* 2001; Mueller 2002; Mikheyev *et al.* 2006; Mueller *et al.* 2011a). Germination of spores from *L. gongylophorus* mushrooms was documented by Möller (1893), and because this is a relatively inaccessible account written in German, we provide in the Supporting Information a translation of Möller's experiments (see also Mueller 2002, listing all the studies that attempted to germinate spores of mushrooms derived from attine fungi).

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 26 *Acromyrmex* leafcutter species (not including parasitic *Acromyrmex* species; Bacci *et al.* 2009; Rabeling *et al.* 2015) form a well-supported monophyletic clade that originated 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). Only six leafcutter species occur in North America (*Atta texana*, *At. mexicana*, *At. insularis*, *At. cephalotes*, *Ac. versicolor*, *Ac. octospinosus*) and eight species in Central America (*At. cephalotes*, *At. colombica*, *At. sexdens*; *Ac. octospinosus*, *Ac. echinatior*, *Ac. coronatus*, *Ac. volcanus*, and the parasitic *Ac. insinuator*), whereas about 40 described leafcutter species occur in South America, with the greatest concentration of sympatric leafcutter species in savannah habitat of northern Argentina, Paraguay, Uruguay, and Southern Brazil (Borgmeier 1959; Gonçalves 1961; Kusnezov 1963; Fowler 1983; Farji-Brener & Ruggiero 1994; Mayhé-Nunes & Jaffé 1998; Fernández & Sendoya 2004; Mueller & Rabeling 2008; Brandão *et al.* 2011; Delabie *et al.* 2011).

Because the greatest concentration of leafcutter species diversity occurs in savannahs of southern South America, early biogeographic models (Kusnezov 1963; Fowler 1983) postulated that leafcutter ants originated and diversified in seasonally dry grasslands of South America (i.e., in savannah habitat, not in humid tropical rainforest); from there, leafcutter ants expanded then 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) were the first to conduct a formal analysis to infer biogeographic history mapped onto a phylogeny of attine ants, and Branstetter *et al.*'s biogeographic modeling is most consistent with an origin of leafcutter ants in seasonally dry habitat in Central America, but their analyses do not rule out a South American origin with confidence. The two conflicting hypotheses of a South American origin (Kusnezov 1963; Fowler 1983) versus a Central American origin (Branstetter *et al.* 2017) make different predictions regarding the biogeographic diversity of leafcutter fungi that should be found in South versus Central America. Assuming that leafcutter ants became specialized to cultivate Clade-A fungi around the time of the origin of the leafcutter clade, as

assumed by traditional models of ant-fungus evolution (Stradling & Powell 1986; Chapela *et al.* 1994), and assuming no other factors affect diversity of fungal cultivars (e.g., genetic drift does not affect cultivar genotype diversity differently in different cultivar populations), the hypothesis of a Central American origin predicts that the fungi cultivated by leafcutter ants should be most diverse in Central America, and less diverse in South America colonized secondarily by leafcutter lineages migrating with their cultivars from Central to South America. In contrast, the Kusnezov-Fowler 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 mya), by leafcutter lineages migrating out of South America.

There exists no definitive fossil evidence that indicates a presence of leafcutter ants north of South America prior to the closing of the Central American land bridge 1-5 million years ago (mya), or an earlier presence in South America. Several genera of attine ants, including a species that can be assigned to the higher-attine genus *Trachymyrmex* (Baroni Urbani 1980), have been described from Dominican and Mexican amber dated to about 15-20 mya (de Andrade 2003; Schultz 2007; Brandão *et al.* 2011; LaPolla *et al.* 2013), but no fossil leafcutter ant has been described so far (see additional discussion on fossil attine ants in the Supporting Information). Without leafcutter ant fossils, historical biogeographic distributions of leafcutter ants have to be inferred therefore with the help of current distributions.

Of four well-supported sub-clades of *Atta* (Bacci *et al.* 2009), 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 *bisphaerica*, *capiguara*, *saltensis*, *vollenweideri*, *laevigata*, and *opacipes*), and three species in the *Archeatta* clade occur only in North America (*mexicana*, *texana*, *insularis*; presumably these three species diversified in or near that northernmost region of the *Atta* distribution). The distribution of these *Atta* subclades therefore does not favor either a South or Central American origin of leafcutter ants, except that the far greater diversity of South American *Atta* species appears more consistent with a South American origin. Diversification within species has been analyzed only in three widespread *Atta* species (*cephalotes*, *sexdens*, *laevigata*) for which within-species diversity accumulated in the past 0.5-3 million years (Solomon *et al.* 2008).

Because no comparable phylogenetic analysis exists for *Acromyrmex*, the biogeography of *Acromyrmex* is less understood than the one for *Atta*. Earlier morphological studies partitioned *Acromyrmex* into two groups (sub-genera *Acromyrmex* and *Moellerius*; Emery 1905; Gonçalves 1961), but molecular-phylogenetic analyses did not confirm these two groups as monophyletic (Cristiano *et al.* 2013; Schultz *et al.* 2015; Branstetter *et al.* 2017), and the morphologically unique species *Aromyrmex striatus*, traditionally placed into the *Moellerius* sub-genus (Gonçalves 1961; Fowler 1988), actually represents the most basal leafcutter lineage that is distinct at the molecular level from all other leafcutter ants (Cristiano *et al.* 2013). Because *Ac. striatus* and its likely sister species *Ac. silvestri* occur in savannah habitat of northern Argentina, Paraguay, Uruguay, and southernmost Brazil (Fowler 1983; Farji-Brener & Ruggiero 1994; Cristiano *et al.* 2016), the basal position of *Ac. striatus* in the clade of leafcutter ants supports an origin of leafcutter ants in grasslands in southern South America, as postulated by Kusnezov (1963) and Fowler (1983) (see also Brandão *et al.* 2011). The existence of the most basal leafcutter lineage *Ac. striatus* and its sister lineage *Ac. silvestri* 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.

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 and within the leafcutter genera *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 5 distinct clusters (Bot *et al.* 2001), with each cluster containing representatives from either of the two sympatric *Acromyrmex* species. Across North America, five leafcutter species (*At. texana*, *At. mexicana*, *At. cephalotes*, *At. insularis*, *Ac. versicolor*) share four genotype-clusters of *L. gongylophorus* (Mueller *et al.* 2011a), with evidence of admixture between these distinct genotype-clusters. No comparable population-genetic analyses involving multiple fungi per leafcutter species exit for South American leafcutter fungi, except Peireira *et al.* (2015) showed that three cultivars from *Ac. heyeri* and three from *Ac. ambiguus* from Ro Grande do Sul in Brazil form two 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), and Clade-A fungi are cultivated by diverse *Atta* and *Acromyrmex* species ranging from Argentina to the USA (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 square kilometers in south-central Texas (approximately the area of Costa Rica or Panamá). 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 kilometers distant (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 USA (Mueller *et al.* 2011a), suggesting that, because of more frequent recombination in the tropics through unknown processes of genetic exchange (e.g., exchange of nuclei between multinucleate mycelium; Mueller *et al*. 2011a; Carlson *et al*. in press), 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 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 (Mueller *et al.* 2011a), whereas these three ant species are significantly diverged from each other (Bacci *et al.* 2009) and the distance between Cuba and mainland greatly exceeds the dispersal distance of leafcutter ants during a mating flight. This suggested the possibility that leafcutter fungi may disperse independently from the ants, for example through airborne spore dispersal (spore-bearing mushrooms of leafcutter fungi have been observed on rare occasions growing from nests in the field; e.g., Pagnocca *et al.* 2001; Mueller 2002), or possibly by dispersal through vectors other than leafcutter ants (Mueller *et al.* 2011a). Second, Pleistocene refugia in South America apparently did not contribute to inter- and intra-species diversification in *Atta* ants (Solomon *et al.* 2008), and presumably therefore also not for the associated fungal cultivars. Third, leafcutter abundance decreases significantly with altitude, and leafcutter ants do not occur at elevations higher than about 2000-2500 meters (Weber 1972; Farji-Brener & Ruggiero 1994;

Delabie *et al.* 2011). This suggests that the Andes in north-western South America (Colombia, Ecuador, Peru) may represent a dispersal barrier for leafcutter ants and their co-propagated fungi.

We build on these previous studies by conducting here 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 USA (Weber 1972; Solomon *et al.* 2008; Bacci *et al.* 2009). Our study specifically asks whether unknown cultivar types (beyond the known Clade-A and Clade-B cultivars) may be cultivated by leafcutter ants in South America; 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); whether the cultivar species *L. gongylophorus* is subdivided into many or few sub-populations across the range from Argentina to the USA, with implications for effective migration rates between biogeographic regions; and whether *L. gongylophorus* is genetically more diverse in Central and North America compared to South America, as predicted by Branstetter *et al.*'s (2017) hypothesis of a Central American origin of leafcutter ants, or whether genetic diversity is greater in South America, as predicted by Kusnezov's (1963) and Fowler's (1983) hypothesis of a South American origin of leafcutter ants.

Materials and methods

Sample Collection

We were able to obtain fungal garden material from 474 leafcutter nests, covering 8 *Atta* species (294 nests) and 22 *Acromyrmex* species (180 nests), by combining material from 22 collaborating laboratories (see rightmost column in Table S1). The material was collected between 1990 and 2008 in Argentina (n=29 samples), Uruguay (n=2), Brazil (n=123), Peru (n=46), Ecuador (n=14), French Guiana (n=32), Suriname (n=1), Guyana (n=6), Venezuela (n=40), Trinidad & Tobago (n=8), Colombia (n=34), Panamá (n=91), Costa Rica (n=7), Honduras (n=1), Mexico (n=15), Cuba (n=5), and the USA (n=18) (Table S2). For samples collected after 1998, garden was collected in the field into 100% ethanol, then freeze-stored at -80°C. For samples collected before 1997 (a few samples from Costa Rica, all samples from Guyana and Trinidad&Tobago), cultivar fungi were isolated then stored at -80°C as lyophilized mycelial tissue.

The majority of our 474 samples derived from the extensive biogeographic survey of Solomon *et al.* (2008) of three *Atta* species (*At. cephalotes* n=145; *laevigata* n=26; *sexdens* n=43) from South and Central America; from leafcutter-ant surveys of the Bacci and Ortiz Labs in Brazil and Colombia; and from leafcutter-ant surveys of the Mueller Lab in Argentina, French Guiana, Panamá, and the USA. Of 200 leafcutter fungi from the USA genotyped in previous analyses (Mueller *et al.* 2011a, 2011b), we included in our survey here 18 representative strains to cover the diversity of leafcutter fungi known from the USA. Our fungus-garden samples from 8 *Atta* and 22 *Acromyrmex* species cover 47% of 17 *Atta* species currently recognized, and 85% of 26 *Acromyrmex* currently recognized (not including parasitic *Acromyrmex* species). Two factors account for the higher proportion of *Acromyrmex* compared to *Atta* species in our survey. First, gardens of *Acromyrmex* nests are typically closer to the surface and thus are easier to excavate than the deeper gardens of *Atta* nests; and second, some *Atta* species have small ranges (Borgmeier 1959; Bacci *et al.* 2009; Delabie *et al.* 2011) that were not covered in surveys of the collaborating labs. We were able to obtain fungus-garden samples from all leafcutter species in Central and North America, and the leafcutter species missed in our survey are therefore all from South America. It is possible that several of the surveyed leafcutter species represent cryptic species complexes that may eventually be split into separate species, for example the *Atta sexdens* complex, but particularly species complexes in the genus *Acromyrmex* (e.g., species *ambiguus*, *balzani*, *coronatus*, *laticeps*, *octospinosus*, *rugosus*, *subterraneus*; Delabie *et al.* 2011), which have received less taxonomic attention than *Atta* species.

For outgroup rooting in the phylogenetic analyses of leafcutter fungi, we included in our survey also

fungal garden material from 12 *Trachymyrmex* ant species, 3 *Sericomyrmex* species, two mycelium-cultivating *Cyphomyrmex* species (from the *wheeleri*-group, Mehdiabadi *et al.* 2012), one *Apterostigma* species, and one *Mycocepurus* species (Table S1). The *Trachymyrmex* accessions were chosen to cover the known main clades of higher-attine fungi cultivated by *Trachymyrmex* and *Sericomyrmex* ants (Mueller *et al.* in review; Scott E. Solomon, in preparation). We included five representative free-living *Leucocoprinus* fungi to help root the phylogenetic reconstruction. These *Leucocoprinus* species had been collected in Panamá for a previous phylogenetic analysis of lower-attine fungi (PA136, PA139, PA178, PA234, PA270; see Fig. 1 in Mueller *et al.* 1998).

Field Collections

Leafcutter gardens were accessed through excavation with shovels. Two 1 cm^3 fragments of healthy, mature garden (substrate suffused with healthy mycelium) were preserved in duplicate vials with 100% ethanol. We typically sampled only one garden per leafcutter nest because leafcutter ants are thought to grow their fungi as monocultures, although only three leafcutter species have been tested so far for monoculture (Poulsen & Boomsma 2005; Mueller *et al.* 2010) and leafcutter ants can co-culture several cultivar strains in experimental chimaeric gardens in the laboratory (Sen *et al.* 2010). Corresponding samples of ants were also preserved in 100% ethanol for species identification of ants. Samples were transported at room temperature to the São Paulo State University Rio Claro or the University of Texas at Austin, then stored at -80°C. Collection information for all samples and locations of permanent storage of vouchers are listed in Table S1.

DNA Extraction and Sequencing: Small (~0.5mm^3) mycelial tufts were separated with flame-sterilized forceps under a stereomicroscope from the ethanol-preserved garden material, then immersed in 180 µL of a 20% Chelex buffer (Sigma-Aldrich, St. Louis, Missouri). To release the DNA, samples were vortexed in the Chelex buffer at room temperature for 15 min, incubated at 37°C in a thermal cycler for 60 minutes, then heated to 100°C for 15 minutes. EF-1α, RAD51, and DMC genes were PCR-amplified as described in Mikheyev *et al.* (2006) (see also primer information and annealing temperatures in Table S8). The PCR mix [1µL 10x buffer, 1µL $MgCl_2$ 25mM, 0.8 µL dNTP mix (2.5mM of each nucleotide), 0.6 µL of each primer 10 mM, 0.02 µL Taq polymerase, ddH_2O to a total volume of 10 µL] was heated for 3 min to 94°C to denature the DNA, then amplified (35 cycles of 45 sec at 94°C, 45 sec at 51°C, 1 min at 72°C; followed by a 10 min extension step at 72°C). All PCR products were cycle-sequenced with the ABI BigDye Terminator Kit (version 3.1) on an ABI PRISM 3100 automated sequencer in the Mueller Lab. Genbank accession numbers for the sequenced fungal cultivars are listed in Table S1 [GQ853919–GQ854367 (EF-1α gene); GQ854817–GQ855186 (RAD gene); HQ391561–HQ391895 (DMC gene)].

Phylogenetic analyses

We were able to generate sequence information for 483 fungal accessions (430 fungi from leafcutter ants, 40 fungi from *Trachymyrmex* ants, 4 fungi from *Sericomyrmex* ants, and 9 outgroup fungi; Table S1). Because of problems with PCR-amplification of the RAD and DMS genes, we were able to generate RAD and DMS sequence information not for all of our collections (successful sequences for 370 accessions for RAD, 335 accessions for DMS; Table S1). Forward and reverse sequences were assembled and edited using Sequencher 4.6 (GeneCodes, Ann Arbor, MI), aligned for each gene using Clustal X (2.0), then manually aligned in MacClade version 4.06 (Maddison & Maddison 2000).

We initially intended to use information from all three protein-coding genes to resolve phylogenetic structure among Clade-A fungi. However, because preliminary phylogenetic analyses showed that each of the three genes shows insufficient variation to resolve phylogenetic relationships between all 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 resolve genetic differences between Clade-A fungi with a panel of 5 microsatellite markers (below). We present the

preliminary phylogenetic analyses of the RAD and DMC genes in the Supplemental Information (Figs. S2 & S3), and we present the results of the phylogenetic analyses of the most comprehensive EF-1α dataset in Figs. 1 & S1, but we use the information from the EF-1α dataset here only to identify Clade-A representatives to be analyzed further with microsatellite markers, and to document the limited utility of the sequenced genes to resolve phylogenetic structure among Clade-A fungi (Figs. 1 & S1-S3).

To place phylogenetic relationships of leafcutter fungi into the context of closely-related cultivars of other fungus-growing ants, we also generated sequence information for representative fungi collected from 40 *Trachymyrmex* and 4 *Sericomyrmex* ant nests, as well as fungi from a few representative "lower-attine" ants (two Clade-1 attine cultivars, and one Clade-2 cultivar, as defined by Mueller *et al.* 1998 and Kellner *et al.* 2013); and four representative free-living *Leucocoprinus* species closely related to lower-attine cultivars (Mueller *et al.* 1998). Phylogenetic trees were rooted with one of these free-living *Leucocoprinus* species (accession PA136 collected in Panama), a close free-living relative of Clade-2 cultivars of lower-attine ants (Mueller *et al.* 1998). Our final EF-1α alignment of 475 characters included 187 informative, 27 autapomorphic, and 261 invariable characters.

We evaluated the best-fit model of sequence-evolution using the Bayesian information criterion (BIC) in ModelTest 3.7, which recommended a GTR+I+Γ model of sequence-evolution. We performed a Bayesian analysis on the EF-1α alignment using MrBayes (ver. 3.1.2) and the GTR+I+Γ model of sequence-evolution. We ran seven independent MCMC analyses (each with four chains) for ten million generations, with trees and parameters sampled every 100 generations, and with a burn-in of two million generations. The joint posterior probabilities and parameter estimates of each run were congruent, suggesting the chains were run for a sufficient number of generations. To combine the results from the seven runs, we used MrConverge (as described in Brown & Lemmon 2007) to sub-sample the remaining generations 1/20,000 and construct a consensus tree. We also explored phylogenetic relationships under the maximum-likelihood criterion, using default settings recommended by GARLI (version 0.96b8; Zwickl 2006). We performed ten replicates of heuristic searches in GARLI to identify the phylogenetic reconstruction with the lowest log-likelihood score. We mapped non-parametric bootstrap support values (100 pseudo-replications each) on the corresponding maximum-likelihood trees. Because we use information from the EF-1α gene here only to identify Clade-A fungi for further analysis with microsatellite markers, we did not explore phylogenetic relationships exhaustively beyond the basic Bayesian and likelihood analyses described above.

Microsatellite marker analyses

We generated microsatellite-information for five loci (A1132, C101, C126, C117, B12) developed for the Clade-A fungus *Leucocoprinus gongylophorus* (i.e., formerly *Attamyces bromatificus*; Scott *et al.* 2009). These loci were chosen from among the 23 loci developed by Scott *et al.* (2009) because these loci were among the most polymorphic markers and they could be scored reliably (few scoring errors in previous analyses; Scott *et al.* 2009; Mueller *et al.* 2010). We amplified each locus separately (i.e., not multiplexed) in a 10 µL reaction using the following thermocycler profile: 95°C for 5 min, then 10 cycles at 94°C for 15 sec, primer-specific annealing temperature for 15 sec as specified in Scott *et al.* (2009), 72°C for 25 sec; followed by 25 cycles at 89°C for 10 sec, primer-specific annealing temperature for 15 sec, 72°C for 25 sec, and a final extension of 72°C for 30 minutes. Amplification products were visualized under UV light after electrophoresis of 1.5% agarose gel stained with SYBR safe.

Amplification products were cleaned with Sephadex Centri-Sep 8 spin columns (Princeton Separations Inc, Adelphia, NJ, USA). After cleaning, 1 µL of product was mixed with 8µl of HiDi (Applied Biosystems) and 1.5µl of custom-made size standards (CASS; DeWoody *et al.* 2004) using the size-standard ladder ROXF1, ROX 104, ROX 150, ROX 200, ROX 253, ROX 305, and ROX 424. The PCR products, HiDi, and CASS mixture was denatured for 95°C for 2 minutes, then chilled at 10°C for at least

2 minutes. Amplified mircosatellite markers were analysed on an ABI PRISM 3100 automated sequencer in the Mueller Lab and scored using SoftGenetics GeneMarker v1.5 (State College, PA).

We genotyped only the Clade-A fungi from leafcutter nests because the five microsatellite markers (loci A1132, C101, C126, C117, B12) were specifically developed and optimized for genotyping of Clade-A fungi (Scott *et al.* 2009), and because the few amplification products scored for Clade-B fungi do not follow repeat-patterns indicative of true microsatellite markers (Heather D. Ishak, unpublished). Using the above, standardized molecular methods, several researchers generated genotype information over seven years in the Mueller Lab (2004-2010), but all microsatellite-marker chromatograms were scored at the end by a single researcher (HDI) to standardize the allele-calling procedure.

Of the 419 fungal samples from Clade-A that we aimed to genotype at each of 5 loci (5 x 419 = 2095 loci amplified total), at the end of the genotyping phase of our study, information was missing for 6 samples for locus A1132; 4 samples for locus C101; 2 samples for locus C126; 8 samples for locus C117; 11 samples for locus B12 (Tables S1 & S3). The missing information was because of oversight during the genotyping phase of our study. No fungus was missing information for more than one locus, 31 samples (7%) were genotyped at only 4 loci, 388 samples (93%) were genotyped at all 5 loci. Locus B12 had null alleles (no markers amplified) in 47 samples from north-east South America (mostly in Peru and Ecuador, also in Colombia, Venezuela, and French Guiana; Tables S3 & S4). We did not detect null alleles at any of the other loci (i.e., at least one allele amplified per locus), but null alleles may have been masked by the multi-nucleate, polyploid nature of the genotyped fungi. The total number of alleles per locus, and the averages of these numbers for each locus, are calculated in Table S4. Averages of total alleles scored were 2.34 alleles for locus A1132 (StDev = 0.72; range 1-4 alleles per sample); 2.10 for locus C101 (StDev = 0.76; range 1-4); 1.40 for locus C126 (StDev = 0.53; range 1-3); 1.65 for locus C117 (StDev = 0.72; range 1-3); and 1.66 for locus B12 (StDev = 0.92; range 0-4).

Population- genetic analyses of microsatellite markers

We assessed population structure with STRUCTURE v2.3.4 (Pritchard *et al.* 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 *et al.* (2007). Ploidy is also variable between individual strains (Kooij *et al.* 2015a; Carlson *et al.* in press), thus we did not use standard population genetic statistics (e.g., F-statistics, heterozygosity, etc.) 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 ($\boldsymbol{\alpha}$) to be 1/10 of the default setting (i.e., $\boldsymbol{\alpha}$=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 *et al.* (2005) (Fig. S4). We processed individual and population matrices from STRUCTURE HARVESTER (Earl *et al.* 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 Fig. 2 (top) and to map pie charts in Fig. 2 (bottom right) using the open-source geographic information system tools in R (R Development Core Team 2008).

Results

We characterized through sequencing or microsatellite genotyping the cultivar fungi from 474 leafcutter gardens from 8 *Atta* and 23 *Acromyrmex* species collected in 17 countries ranging from Uruguay and northern Argentina (Misiones, Corrientes, Chaco, Formosa) to the southern USA (Arizona, Texas) (Tables S1 & S2).

Phylogeny of Fungi Cultivated by Leafcutter ants

Phylogenetic relationships of these fungi (Figs. 1 & S1) confirm the pattern already observed in Mueller *et al.* (in review) that higher-attine fungi fall into two groups, a genetically homogenous group of Clade-A fungi (*Leucocoprinus gongylophorus*, formerly *Attamyces bromatificus*) and a more diverse group of Clade-B fungi that is subdivided into at least six distinct subclades of undescribed fungi (Fig. 1). The congruent findings between these two studies is not surprising because the taxa analyzed in Mueller *et al.* (in review) were chosen as representative lineages from the larger collection analyzed here, with the difference that Clade-A fungi, and also the leafcutter-cultivated Clade-B fungi, are covered here more comprehensively [430 Clade-A leafcutter fungi are sequenced here compared to 16 Clade-A fungi in Mueller *et al.* (in review)]. We did not identify any unknown clades of higher-attine fungi in this geographically comprehensive survey of leafcutter fungi (i.e., no Clade-C or -D fungi). However, such additional lineages could emerge in future surveys of regions not covered in our study (e.g., Bolivia, Paraguay, western Brazil, central and western Argentina), in surveys of undersampled, extreme habitats (e.g., leafcutter populations at range limits, such as higher elevations in the Andes, seasonal wetlands of the Pantanal, western cerrado in Brazil), or a survey of the southernmost leafcutter representative *Ac. lobicornis* in Argentina (Farji-Brener & Ruggiero 1994).

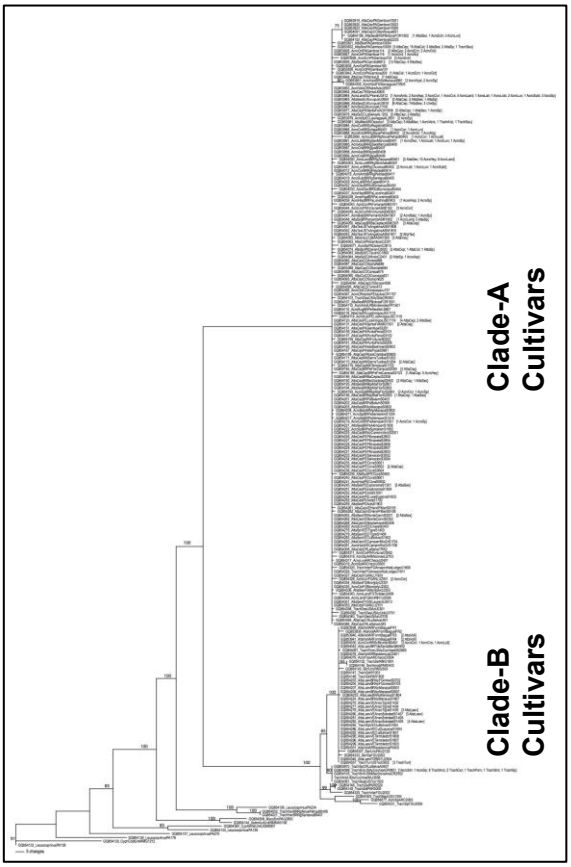

Fig. 1. Phylogenetic relationships of fungi cultivated by higher-attine ants, based on EF-1α sequence information. Table S1 summarizes collection information of the 430 fungal cultivars from leafcutter ants included in this analysis, 44 fungal cultivars from *Trachymyrmex* and *Sericomyrmex* ants, and 9 outgroup fungi (lower-attine cultivars and free-living *Leucocoprinus* fungi). We used the phylogenetic information from EF-1α sequences to classify fungi into Clade-A and Clade-B fungi, and identify possible leafcutter cultivars that fall outside these two clades (we did not find such fungi in our survey). In the phylogenetic tree shown, taxa with identical sequences (excepting sequence ambiguities) are listed next to each other as a string of taxa as the same terminal leaf, and the respective Genbank accessions of the taxa that are united on the same leaf are listed in Table S5. Because we did not recover sufficient phylogenetic structure among Clade-A fungi using EF-1α sequences, and also using sequence information from two other protein-coding genes (Figs. S2&S3), we genotyped Clade-A fungi by determining allele profiles at 5 microsatellite loci (Table S3; Fig. 2). Because of genetic exchange between Clade-A fungi (Fig. 2), Clade-A fungi form a single evolutionary lineage and represent a single species *Leucocoprinus gongylophorus*.

A surprising result is that the three protein-coding genes analyzed here (Fig. 1, Figs. S1-S3), as well as two additional ribosomal genes analyzed in Mueller *et al.* (in review), failed to uncover significant variation within Clade-A fungi across the entire leafcutter range from Argentina to the USA. This lack of variation in Clade-A fungi contrasts with the substantial generic and species diversity of the surveyed ant hosts, which includes at least 7 *Atta* species, 21 *Acromyrmex* species, 5 *Trachymyrmex* species [Table S1 and Mueller *et al.* (in review)], and one *Apterostigma* species (Schultz *et al.* 2015). Because of the minimal genetic diversity found so far among Clade-A fungi (Fig. 1; Silva-Pinhati *et al.* 2004; Mikheyev *et al.* 2006, 2007; Lugo *et al.* 2013; Wallace *et al.* 2014; Pereira *et al.* 2015), Clade-A fungi are thought to represent a cohesively-evolving lineage (i.e., a single species of fungus), confirming the interpretation of Mikheyev *et al.* (2006) that Clade-A fungiculture (i.e., *L. gongylophorus* fungiculture) represents a one-to-many fungus-ant association. Clade-B fungiculture, in contrast, is more difficult to interpret, because Clade-B fungi represent likely at least six fungal species (Fig. 1; Mueller *et al.* in review), with each subclade associated with several or many leafcutter and *Trachymyrmex*/*Sericomyrmex* species, suggesting that Clade-B fungiculture represents overall a many-to-many fungus-ant association (or an alternative view, each Clade-B fungal species appears to form one-to-many fungus-ant associations similar to the single species of Clade-A fungus). Across all higher-attine ants and their known fungi (Fig. 1; Mueller *et al.* in review), however, ant-fungus associations are many-to-many because ant-lineages switch frequently between fungal lineages over evolutionary and apparently also ecological time, and long-term ant-fungus co-evolution is therefore more diffuse than specific.

Clonal Propagation of Fungal Cultivars

The five microsatellite loci (loci A1132, C101, C126, C117, B12; Table S3) identified 241 genotypes (57.5%) among the 419 Clade-A fungi collected from 419 different leafcutter ant nests; that is, 178 fungal genotypes had at least one identical duplicate that had been collected also from a different 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 consistent with the vertical transmission of cultivar clones within ant lineages, and these fungal genotypes are therefore likely to be identical in different proximate nests of the same ant species because of identity-by-descent of the fungi and limited dispersal per ant generation. Cases of cultivar identity between different ant species and between different leafcutter genera are discussed below.

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 (Fig. S4) is the most informative number of genotype clusters for input into STRUCTURE. Fig. 2 plots STRUCTURE assignments of 419 fungal samples to these three genotype-clusters and maps these onto 10 regions defined by country of collection (some adjacent countries are combined, Brazil is divided into north and south): Argentina & Uruguay, southern Brazil, northern Brazil, Peru, Ecuador, the Guianas, Venezuela, Western Colombia, Panama & Costa Rica, Mexico & USA (Table S3). The three genotype clusters correspond approximately to southern South America, northern South America, and North & Central America (Fig. 2), with the largest degree of admixture apparent adjacent to the Isthmus of Panama in north-west South America. Members of Cluster 1 (burnt orange in Fig. 2) are found in North America, Central America, western Colombia (Departamento de Antioquia, west of the Andes), and to a lesser extent in Ecuador and Venezuela. Members of Cluster 2 (green in Fig. 2) and Cluster 3 (purple) occur only in South America. Fungi outside of South America are all assigned by STRUCTURE to Cluster 1 (Fig. 2). If the number of co-occurring genotype-clusters is an indication of local genetic diversity, fungal populations appear 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 genotype-clusters) appears greatest in Colombia and Venezuela (Fig. 2 top), corresponding with the apparent transition zone in genotype-clusters from South America to Central & North America.

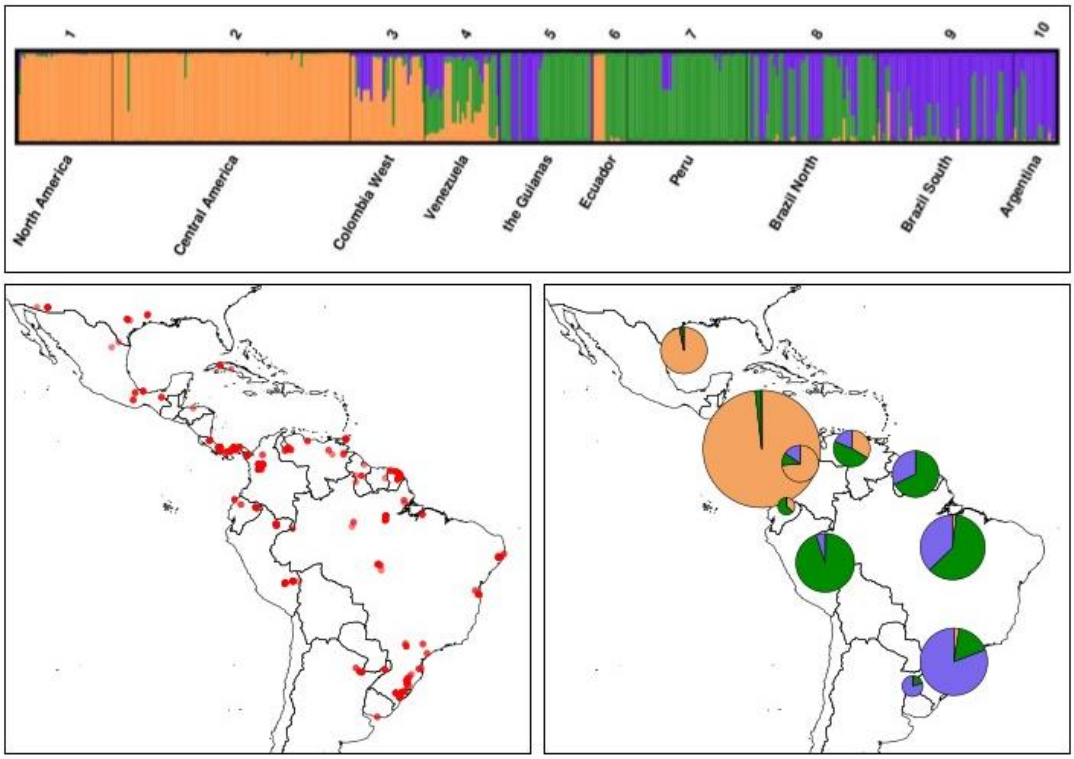

Fig. 2. Biogeographic patterns of 419 *L. gongylophorus* fungi cultivated by leafcutter ants (*Atta*, *Acromyrmex*). Collection locations are shown in the bottom-left panel. Fungi are assigned by STRUCTURE to three genotype-clusters (purple, green, burnt orange), and membership in these three clusters is mapped onto 10 biogeographic regions: 1. North America (Mexico, Cuba, USA). 2. Central America (Panamá, Costa Rica, Honduras). 3. Colombia (west of Andes). 4. Venezuela. 5. The Guianas (Guyana, Suriname, French Guiana). 6. Ecuador. 7. Peru. 8. northern Brazil. 9. southern Brazil. 10. Argentina & Uruguay. Information on exact collection locations, sample sizes, and leafcutter species is summarized in Tables S1 & S2. The size of the pie charts in the bottom-right panel corresponds to the number of leafcutter nests surveyed in each of the 10 biogeographic regions; each pie chart is centered on the centroid of collections from the respective region. The three genotype-clusters map approximately to southern South America, northern South America, and North & Central America. Populations of *L. gongylophorus* fungi in Central and North America appear less diverse than populations in South America.

Biogeographic Patterns of Allele Diversity of L. gongylophorus *Fungi Cultivated by Leafcutter Ants*

In contrast to the strong spatial structure, allele richness of fungi shows no consistent patterns across the entire range of *L. gongylophorus* fungi cultivated by leafcutter ants (Figs. S5A-E). Because *L. gongylophorus* fungi are polyploid, multinucleate fungi and ploidy appears variable between fungal strains (Scott *et al.* 2009; Kooij *et al.* 2015a; Carlson *et al.* in press), we were not able to use standard population-genetic statistics (e.g., heterozygosity, etc.), so we examined the 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 *et al*. 2012), allele richness and heterozygosity are correlated, and allele richness can therefore serve as a proxy of heterozygosity (see Box 1 in Eckert *et al*. 2008). In our survey, allele richness does not change as a function of latitude (Fig. S5); 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 USA and the island population in Cuba do not show reduced allelic diversity (Figs. S5), 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 *et al.* 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; Fig. S5E, 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.

Are there Differences between Fungi Cultivated by Dicot- Versus Grass-Cutting Leafcutter Ants?
Leafcutter ants specialized to forage on grasses, or on both grasses and dicot plants, are more likely to cultivate Clade-B fungi (Table S6), but the association between foraging preferences and cultivar specializations, 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 on grasses or cut both grasses and dicots, 100% of the 23 dicot-specialized leafcutter species cultivate Clade-A fungi (and only two of these sometimes cultivate Clade-B; Table S6) 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 also 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 $p = 0.0051$ (23 counts dicot & Clade-A fungi; 0 counts dicot & Clade-B; 6 counts grass & Clade-B; 4 counts grass & Clade-A), and Barnard's Exact Test statistic is $p = 0.0040$.

Limiting the analysis only to Clade-A fungi and ignoring Clade-B cultivation, our microsatellite marker analyses did not reveal obvious differences between Clade-A fungi cultivated by 22 leafcutter species (both *Acromyrmex* and *Atta*) preferentially foraging on dicots, compared to Clade-A fungi cultivated by three species preferentially foraging on grasses (*Ac. balzani*, *Ac. heyeri*, *Ac. landolti*), or 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 (identity in all alleles across the 5 microsatellite loci), *Ac. landolti* and *At. cephalotes* in Colombia; and *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 cultivate fungi from shared pools of Clade-A fungi circulating locally with a leafcutter ant community, and dicot- and grass-specialized leafcutter species even appear to exchange cultivars on occasion. A more detailed analysis using more loci (e.g., genotyping-by-sequencing), and a larger collection of fungi from multiple sympatric leafcutter species (e.g., in northern Argentina, Uruguay, southern Brazil), may be able to detect genotypic differences between fungi cultivated by grass- versus dicot-specialized leafcutter ant species.

Are there Differences between Clade-A Fungi Cultivated by Atta versus Acromyrmex Ants?
Recent studies argued that the *L. gongylophorus* fungi (= Clade-A) cultivated by *Atta* and *Acromyrmex* leafcutter ants in Panamá could represent separate gene pools (Kooij *et al.* 2015b), and that two *L. gongylophorus* fungi cultivated by *Atta* versus *Acromyrmex* ants in Panamá diverged from each other 7.2 million years ago (confidence interval 5.4-9.0 million years ago; Nygaard *et al.* 2016; Supplementary Methods pages 43&44 lines 744-758 of Nygaard *et al.*). Because we did not find differences between *Atta*-cultivated versus *Acromyrmex*-cultivated *L. gongylophorus* fungi in our phylogenetic analyses (Figs. 1 & S1-S3; also Mueller *et al.* in review), we tested for possible differences using our faster-evolving

microsatellite markers, which should have adequate resolution (ADD REFS) to detect Nygaard *et al.*'s hypothesized ancient diversification dating to 5-9 million years ago. Our analyses do not support genetic isolation between *Atta*-cultivated versus *Acromyrmex*-cultivated *L. gongylophorus* fungi, for two main reasons.

First, at most of the sites for which we obtained adequate samples of *Atta*-cultivated and *Acromyrmex*-cultivated *L. gongylophorus* fungi, we found *Atta*-cultivated versus *Acromyrmex*-cultivated fungal clones that were identical in all alleles across the five microsatellite loci in our study. For example, in locations in Brazil, Peru, Ecuador/Colombia, Colombia, Panama/Colombia, central Panamá, western Panamá, and Mexico/USA, we found instances where fungal clones with identical allele profiles were cultivated by *Atta* and *Acromyrmex* nests (see fungi highlighted in green in Table S3). We also observed 10 cases where identical fungal clones were shared between nests of different species of the same ant genus (highlighted in orange in Table S3), and 56 cases where identical fungal clones were shared between different nests of the same ant species (highlighted in yellow in Table S3), but it is the 8 cases of fungal sharing between leafcutter-ant genera that seems inconsistent with complete separation of gene pools between *Atta*-cultivated versus *Acromyrmex*-cultivated *L. gongylophorus* fungi. The near identical incidence of cultivar sharing between different leafcutter genera (n=8) and between different congeneric species (n=10) could suggest that the same biological processes, such as horizontal transfer of cultivars between nests, may have led to such cultivar identity. I~~n contrast, the far greater incidence of cultivar sharing between nests of the same ant species (n=56) is likely due to genetic identity by descent (i.e., vertical transmission of cultivar clones from mother to offspring nests), as already discussed above.~~

Atta and *Acromyrmex* nests cultivating identical fungal clones were located typically within 50 km of each other (Table S3), but there were also instances of sharing of *Atta* and *Acromyrmex* nests about 1200 km distant (Brazil) and 1900 km distant in Mexico/USA, suggesting that some clonal lineages are widespread geographically and can be shared across that range between *Atta* and *Acromyrmex*. ADD SENTENCE OF DISTANCES OVER WHICH CLONES WERE COLLECTED IN MUELLER 2011, WHERE CLONES WERE GENOTYPED AT 12 LOCI. Because many locations were undersampled in our study (e.g., we were able to obtain collections from only one ant genus from the two leafcutter genera present at a location), sharing of identical cultivar clones is likely more prevalent in nature than indicated in our collection. Lineage sorting of cultivar diversity over 5-9 million years, or convergent evolution of the same microsatellite profile independently in *Atta*- versus *Acromyrmex*-cultivated fungal lineages, both seem implausible explanations for cultivar sharing between *Atta* and *Acromyrmex* nests. Instead, sharing of allele-identical cultivar clones can be explained by horizontal transfer of fungal strains between nests of different leafcutter genera, and possibly also by *de novo* generation of the same fungal genotype through some form of genetic exchange between recombining fungi cultivated by both *Atta* and *Acromyrmex* ants (e.g., exchange of nuclei between differentiated polyploid, heterokaryotic mycelia; Carlson *et al.* in press).

Second, STRUCTURE analyses of fungi from Panamá, the best-sampled region in our survey, indicates that *Atta*- versus *Acromyrmex*-cultivated fungi do not form genetically distinct clusters but are admixed (Fig. S7A-D), regardless of whether we analyze 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 *et al.* (2015b). Our STRUCTURE analyses support the earlier finding by Mikheyev *et al.* (2007) that *Atta* and *Acromyrmex* ants from Gamboa tap locally into the same pool of fungal cultivars, contrary to the findings of Kooij *et al.* (2015b) (see also additional discussion in the Supplemental Information).

If *Atta*-cultivated and *Acromyrmex*-cultivated fungi represent separate gene pools, as hypothesized by Kooij *et al.* (2015b), *Atta*-cultivated and *Acromyrmex*-cultivated fungi may show systematic differences in allele diversity across all sites surveyed. Total number of alleles/fungus does not differ between fungi

from *Atta* and *Acromyrmex* nests (Wilcoxon Signed-Rank Test, W = 90.5, z = 0.181, p = 0.857 two-tailed, n = 19), comparing samples from 19 locations (highlighted in blue in Table S7) for which complete genotype information (all 5 loci) is available for both *Atta*-cultivated and *Acromyrmex*-cultivated fungi. Instead, across these 19 locations, total number of allele/fungus is positively correlated between *Atta*-cultivated and *Acromyrmex*-cultivated fungi (Spearman rank-order correlation t = 2.39, df = 17, p = 0.029, r = 0.502; Table S8). That is, at locations where *Atta*-cultivated fungi show more allele diversity, *Acromyrmex*-cultivated fungi from the same location show likewise more allele diversity. Under the hypothesis of separate gene pools (Kooij *et al.* 2015b), this positive correlation would need to be explained by shared evolutionary forces that determine allele diversity in parallel (i.e., convergently) at different loctions for both *Atta*-cultivated and *Acromyrmex*-cultivated fungi. Alternatively, the positive correlation is predicted by the hypothesis that *Atta* and *Acromyrmex* leafcutter lineages tap at each location into a shared pool of fungal cultivars, mediated either because of local horizontal transfer of fungal cultivars between nests of the two leafcutter genera, because of some form of genetic exchange and hybridization between fungi cultivated by different ant nests, or both.

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 USA, combining collections from 22 collaborating laboratories and surveying leafcutter ants in 17 Neotropical countries (Tables S1&S2). Analyses of 474 fungi cultivated by leafcutter ants revealed (a) no novel cultivar types beyond the known Clade-A and Clade-B cultivars of leafcutter ants (Mueller *et al.* in review) (Fig. 1); (b) 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); (c) extensive cultivar sharing between sympatric leafcutter species within local communities, such that fungi cultivated by *Atta* species are not distinct from those cultivated sympatrically by *Acromyrmex* species; (d) three genotype-clusters of Clade-A fungi across the range from Argentina to the USA (Fig. 2), with local prevalence of these genotype-clusters corresponding approximately to southern South America (Argentina, Uruguay, southern Brazil), northern South America, and Central & North America (Fig. 2); (e) 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 US represent a single species, *Leucocoprinus gongylophorus*; and (f) reduced genetic diversity of leafcutter fungi in Central & North America and greatest genetic diversity of leafcutter fungi concentrated in South America (Fig. 2).

Biogeographic origin of leafcutter fungiculture and leafcutter ants

Kusnezov (1963) and Fowler (1983) hypothesized that leafcutter ants originated in savannah habitat of southern South America (current northern Argentina, Paraguay, Uruguay, sub-Amazonian Brazil) because extant leafcutter ants exhibit the greatest species diversity there, particularly *Acromyrmex* species. In contrast, Branstetter *et al*. (2017) recently conducted a formal analysis to infer biogeographic history mapped onto a phylogeny of attine ants, and their biogeographic modeling suggested an 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 million years ago (mya), and assuming no other factors affect diversity of fungal cultivars (e.g., genetic drift does not affect cultivar genotype diversity differently in different populations across the range of leafcutter ants), the hypothesis of a Central American origin predicts that the 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 mya), by leafcutter lineages migrating out of South America. Our STRUCTURE analyses of genotype diversity of Clade-A fungi (Fig. 2) indicate greater diversity of Clade-A fungi in South America, consistent with the prediction of the Kusnezov-Fowler model of a subtropical South American origin of leafcutter ants.

It is possible to conceive alternative scenarios of leafcutter ant-fungus evolution that assume a Central American origin of the leafcutter ant clade (consistent with Branstetter *et al.* 2017) and a South American origin of Clade-A fungi (as suggested by Fig. 2), and there are no convincing arguments, except perhaps plausibility, that can rule out these alternatives. For example, leafcutter ants may have originated in Central America, but Clade-A cultivars originated in South America in ancestral *Trachymyrmex* lineages; Clade-A cultivars were 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 has spread so far into Central and North America from diverse Clade-A populations in South America (Fig. 1). Other such complex scenarios are also possible, and some of these scenarios, in addition to the Kusnezov-Fowler model of a South American origin of leafcutter ants and leafcutter fungi, can be tested by precise dating of the evolutionary origins of leafcutter fungi relative to the origin of the leafcutter clade.

Dates for crown ages and stem 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). This discrepancy has been interpreted as evidence that Clade-A fungi were acquired secondarily by leafcutter ants, much after the origin of leafcutter fungiculture (Mikheyev *et al.* 2010; Nygaard *et al.* 2016), and Clade-A fungi spread subsequently via nest-to-nest transfer between all leafcutter-ant species across the entire leafcutter range from Argentina to the USA. 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* lineage), the ages are much more in agreement, 22.4-25.0 mya for the stem age of Clade-A fungi, and 17.8-21.0 mya for the stem age of the leafcutter 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* lineages) may have propagated both Clade-A and Clade-B fungi as far back as 22-25 mya, well before the origin of the leafcutter ant lineage, and the propagation 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 by higher-attine ant lineages.

Table 1. Comparison of crown ages and stem ages for Clade-A fungi and for the leafcutter ant clade, estimated in six phylogenetic analyses conducted to date. Mikheyev *et al.* (2010) used a 4-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 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 only a single time-calibrated node in their phylogenetic reconstructions, the last common ancestor of ant-cultivated fungi with *Agaricus*, dated to 73 mya in Mikeyev *et al.* (modeled with more or less conservative distributions around this date), and dated likewise to 73 mya in Nygaard *et al.* (modeled with a 5% minimum age of 55 mya and a 95% maximum age of 91 mya). The ancient time-calibration (i.e., anchor at 73 mya) of the phylogenetic reconstructions is likely to render estimates of the dates of recent

diversifications (e.g., estimate of crown age of Clade-A fungi) more unreliable than estimates for earlier diversifications. mya = million years ago.

CLADE-A FUNGI	LEAFCUTTER ANT CLADE	SOURCE
Crown Age of Clade-A Fungi	**Crown Age of Leafcutter Ant Clade**	
	8 mya (6-15 mya), without the basal *Ac. striatus*	Schultz & Brady 2008
4 mya (0.5-8.0 mya)	(not estimated)	Mikheyev *et al.* 2010
	12.2 mya (9.1-15.3), without *Ac. striatus*	Schultz *et al.* 2015
7.2 mya (5.5-9.0 mya)	16.2 mya (12.6-19.7 mya), without *Ac. striatus*	Nygaard *et al.* 2016
	17.9 mya (15.6-20.4 mya), without *Ac. striatus*	Ješovnik *et al.* 2016
	17.0 mya (13.2-20.8), without *Ac. striatus*	Branstetter *et al.* 2017
	18.2 mya (14.2-22.2), with *Ac. striatus*	Branstetter *et al.* 2017
Stem Age of Clade-A Fungi	**Stem Age of Leafcutter Ant Clade**	
	9 mya (7-15 mya)	Schultz & Brady 2008
25 mya (11-39 mya)	(not estimated)	Mikheyev *et al.* 2010
22.4 mya (16.9-27.9 mya)	17.8 mya (13.7-21.7 mya)	Nygaard *et al.* 2016
	19.9 mya (17.7-22.5 mya)	Ješovnik *et al.* 2016
	19.3 mya (15.2-23.7 mya)	Branstetter *et al.* 2017
Stem Age of Clade-A Fungi	**Stem Age of Higher-Attine Ant Clade**	
	20 mya (17-29 mya)	Schultz & Brady 2008
25 mya (11-39 mya)	(not estimated)	Mikheyev *et al.* 2010
	(not estimated)	Schultz *et al.* 2015
22.4 mya (16.9-27.9 mya)	26.6 mya (19.6-33.8 mya)	Nygaard *et al.* 2016
	33.3 mya (31.3-35.1 mya)	Ješovnik *et al.* 2016
	31.4 mya (25.9-37.2 mya)	Branstetter *et al.* 2017

When focusing on stem-ages rather than crown-ages, there exists no conundrum where the Clade-A ancestors may have existed prior to the hypothesized "secondary acquisition" by leafcutter ants. A conundrum exists only when mis-conceptualizing Clade-A fungi as independently evolving, diversifying lineages, rather than as a single fungal species with a recent coalescent (recent "MRCA"). Comparing crown-ages of Clade-A fungi and the leafcutter clade leads to the misleading conclusion of a phylogenetic discord (Mikheyev *et al.* 2010; Nygaard *et al.* 2016), whereas comparison of stem ages provides a more accurate picture of the time over which Clade-A fungi and leafcutter ants interacted. This misconception can be understood perhaps with a Gedankenexperiment, where we imagine that leafcutter ants evolved 20 million years ago but never diversified into separate species, and only a single leafcutter species existed today, but the Clade-A fungi diversified into 50 independently evolving cultivar species while being propagated by this single species of leafcutter ant, with speciation of Clade-A fungi ongoing continuously during the past 20 million years. The coalescence for this single fictitious leafcutter ant species would be very recent (e.g., 0.5-3 mya based on the estimates for three extant *Atta* species by Solomon *et al.* 2008), whereas the MRCA of Clade-A fungi would be inferred to be much older, leading to the incorrect interpretation that a recently evolved leafcutter ant species acquired recently a diverse set of 50 Clade-A species from other higher-attine ants or from free-living fungal populations, whereas our fictitious leafcutter ant species in fact interacted with Clade-A fungi for the past 20 million years. Comparison of crown-ages in the fictitious leafcutter ant species and its diversified cultivars will reveal a discord and lead to misinterpretation, whereas a comparison between stem-ages provides a more accurate picture of the time over which leafcutter and Clade-A lineages interacted.

To analyze 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 three scenarios seem plausible:

(i) 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., Stradling & Powell 1986; Chapela *et al.* 1994; Hinkle *et al.* 1994).

(ii) A successful lineage of Clade-A fungi entered leafcutter populations from other higher-attine lineages (or even from lower-attine lineages; Schultz *et al.* 2015) after the origin of the leafcutter clade, then spread across most leafcutter lineages through lateral transfer of particularly successful Clade-A cultivar lineages, as assumed by Mikheyev *et al.* (2010) and Nygaard *et al.* (2016).

(iii) Clade-A fungi originated well 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 by Mueller *et al.* (in review). If so, Clade-A and Clade-B fungi may have been shared between the diversifying higher-attine lineages, involving at some later date also the ancestral leafcutter lineages, since the early evolution of higher-attine lineages.

Depending on the biogeographic location of the origin of leafcutter ants, on the biogeographic location of the origin of Clade-A 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 caption of Table 1).

Why Clade-A fungi represent a single species, Leucocoprinus gongylophorus

The three genotype-clusters identified by STRUCTURE among the surveyed 419 Clade-A fungi are not reproductively isolated lineages, because STRUCTURE infers admixed genotypes between these clusters (Fig. 1). The three clusters therefore do not represent separate species. The proportion of admixed genotypes is highest in locations in north-west South America where each of three genotype-clusters occurs sympatrically at appreciable frequencies (Fig. 1), and where admixture is therefore most likely to be detected. Any admixture within each genotype-cluster cannot be documented with the markers generated in this study, but information from additional loci may detect such within-cluster admixture and additional substructure within each of the three genotype-clusters. But even with the limited information from the 5 microsatellite loci, there is evidence that fungal genotypes assigned to different genotype-clusters can potentially exchange genetic material (i.e., they can admix), indicating that all Clade-A fungi are embedded in a shared evolutionary process because of some form of interbreeding. Moreover, gene flow seems to be substantial between different locations, indicated by (a) the distances (hundreds of kilometers) over which genetically identical cultivar clones were detected in this and in previous analyses (Mikheyev *et al.* 2006, 2010; Mueller *et al.* 2011a); (b) the vast distances over which different Clade-A fungi show identity in fast-evolving genes (Silva-Pinhati *et al.* 2004; Mikheyev *et al.* 2006; Mueller *et al.* in review; Figs. 1 & S1-S3 in this study); and (c) the absence of effective dispersal barriers for Clade-A fungi across the entire leafcutter range. Even fungal populations cultivated by leafcutter ants in Cuba, separated by a significant oceanic barrier over which leafcutter ants cannot disperse readily, show very close population-genetic affinities with fungal populations cultivated by leafcutter ants in North and Central America (Mueller *et al.* 2011a; this study). Future breeding experiments documenting absence of effective reproductive boundaries may add to this population-genetic evidence. Because spore-producing sporocarps (mushrooms) of *L. gongylophorus* have not been generated so far from isolated strains under laboratory conditions, and because sporocarps rarely develop in gardens tended by ants in the laboratory (Fisher *et al.* 1994; Mueller 2002; Pagnocca *et al.* 2011), testing for exchange of nuclei between anastomosing mycelia (as in Carlson *et al.* in press) may be the best strategy to test for mechanisms regulating genetic exchange between Clade-A strains.

Extensive cultivar sharing reduces ant-fungus specificity of leafcutter cultivars

Our population-genetic and clonality analyses document extensive 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., *Ac. versicolor* and *T. desertorum* in Arizona; Fig. 1). Clade-A cultivars from *Trachymyrmex* species were unfortunately not included in our microsatellite genotyping analyses because we became aware of the potential population-genetic linkages between leafcutter-cultivated and *Trachymyrmex*-cultivated Clade-A fungi after conclusion of the genotyping phase of our study. Sharing of cultivars between sympatric leafcutter and *Trachymyrmex* ants therefore will need to be evaluated in a future study. 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, there exists now also evidence that single higher-attine species, as currently recognized, can cultivate both Clade-A and Clade-B fungi in some locations (e.g., *At. laevigata* and *Ac. coronatus* in southern Brazil; *T. arizonensis* in Arizona; see discussion of these cases 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 extensive cultivars 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 lineages (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 indeed 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).

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

Our study has several shortcomings, which do not invalidate the conclusions discussed above, but hopefully will be addressed in future research to elucidate the historical biogeography of the leafcutter ant-fungus symbiosis:

(1) Our phylogenetic analyses (Fig. 1; also Mueller *et al.* in review) indicate that some *Trachymyrmex* species can also cultivate Clade-A cultivars, the dominant fungal type cultivated by leafcutter ants. A 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*, *Tr. desertorum*, and *Tr. arizonensis* in Arizona; and the community of Clade-A cultivars of diverse leafcutter species, *T. intermedius*, and *T. opulentus* in north-east South America and in Central America. [*T. opulentus* is labeled *T. wheeleri* in our Fig. 1, but actually synonymized according to Mayhé-Nunes & Brandão 2002]. *T. intermedius* ranges from Mexico to French Guiana, and *T. opulentus* ranges from Honduras to Guyana and 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 species and *Trachymyrmex* species, for example

the Clade-B-cultivating *At. vollenweideri*, *At. laevigata*, *Ac. coronatus*, *Ac. fracticornis*, *Ac. laticeps*, *T. papulatus* (Fig. 1), and likely additional *Trachymyrmex* and possibly *Sericomyrmex* species in southern South America.

(2) Our population-genetic analyses (Fig. 2) rely on information from five highly-polymorphic microsatellite loci of a polyploid fungus (an individual may show more than two alleles/locus), and information from additional microsatellite loci would undoubtedly have increased resolution of population-genetic structure. In fact, prior analyses genotyping leafcutter fungi from the Panamá Canal Zone and from North America with, respectively, 9 and 12 microsatellite loci (Mikheyev *et al.* 2007; Mueller *et al.* 2011a) inferred a larger number of sympatric genotype-clusters (6 clusters in Panamá, 4 clusters in North America; see also Figs. S7A-D identifying 3 clusters when we limit our analyses to Panamanian populations). Identification of three genotype-clusters across the leafcutter range in our 5-locus analysis (Fig. 2) 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 at the same density as the well-surveyed Panamanian population in our study. For example, future studies could use the two multiplex panels (15 microsatellite loci total) of Carlson *et al*. (in press), or consider developing genotyping-by-sequencing methods (e.g., ddRAD) for garden material preserved in ethanol. The latter approach will likely require significant sequencing effort, because ethanol-preserved gardens contain non-cultivar DNA (e.g., from plant substrate, fungal endophytes, fungal pathogens, commensal microorganisms). However, information on non-cultivar DNA in gardens generated in genotyping-by-sequencing analyses could enrich simultaneously the understanding of the plant substrates used by attine ants; identify pathogenic, commensal, and mutualistic microorganisms coexisting in gardens (Mueller *et al.* 2005; Rodrigues *et al.* 2008, 2011; Mueller 2012); and enable tests for interactions between ant hosts, fungal cultivar types, and the diversity and prevalence of any additional microorganisms.

(3) Although our survey covered 17 countries across the leafcutter-ant range, several important countries were not sampled (e.g., Bolivia, Paraguay; much of north-west Central America, including Nicaragua, El Salvador, Guatemala, Belize, and we were able to obtain only one sample from Honduras) (Fig. 2). In addition, several important regions were not surveyed, for example vast regions in western and central Brazil, or a transect sampling across the Andes in Colombia (i.e., the transition zone from cultivation of three genotype-clusters in north-west South America to one genotype-cluster in Panamá; Fig. 2). Most important, the southernmost leafcutter populations in Argentina were not sampled (e.g., *Ac. lobicornis* ranges to ≈44° south, 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. silvestri* (the likely sister species to the Clade-B-cultivating *Ac. striatus*; Fig. 1). Whereas our survey included representative Clade-A genotypes from the well-sampled northern range limit of leafcutter ants in Arizona, California, Texas, and Louisiana (surveyed in Mueller *et al.* 2011a), comparable information on the cultivar diversity is missing for the southern range limit of leafcutter ants.

Conclusion

Most efforts to elucidate leafcutter ant-fungus associations focused so far on leafcutters in Central and North America (Table S6), but these leafcutter symbioses, all of them involving dicot-specialized leafcutter species, are not representative for the more complex leafcutter symbioses existing across South America (Figs. 1 & 2). Leafcutter species specialized on cultivation of Clade-B fungi occur only in South America (ranging from Argentina to Colombia; Fig. 1), the greatest 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 (Fig. 2). 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 savannahs 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 savannahs of southern South America is correct and thus explains the concentrated diversity of leafcutter species there (Borgmeier 1959; Gonçalves 1961; Kuznezov 1963; Mariconi 1970; Fowler 1983; Farji-Brener & Ruggiero 1994; Bacci *et al.* 2009; Delabie *et al.* 2011; Brandão *et al.* 2011; Della Lucia 2011), 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 19 million years ago.

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Fig. 1. Phylogenetic relationships of fungi cultivated by higher-attine ants, based on EF-1α sequence information. Table S1 summarizes collection information of the 430 fungal cultivars from leafcutter ants included in this analysis, 44 fungal cultivars from *Trachymyrmex* and *Sericomyrmex* ants, and 9 outgroup fungi (lower-attine cultivars and free-living *Leucocoprinus* fungi). We used the phylogenetic information from EF-1α sequences to classify fungi into Clade-A and Clade-B fungi, and identify possible leafcutter cultivars that fall outside these two clades (we did not find such fungi in our survey). In the phylogenetic tree shown, taxa with identical sequences (excepting sequence ambiguities) are listed next to each other as a string of taxa as the same terminal leaf, and the respective Genbank accessions of the taxa that are united on the same leaf are listed in Table S5. Because we did not recover sufficient phylogenetic structure among Clade-A fungi using EF-1α sequences, and also using sequence information of two other protein-coding genes (Figs. S2&S3), we genotyped Clade-A fungi by determining allele profiles at 5 microsatellite loci (Table S3; Fig. 2). Because of genetic exchange between Clade-A fungi (Fig. 2), they form a single evolutionary lineage and represent a single species, *Leucocoprinus gongylophorus*.

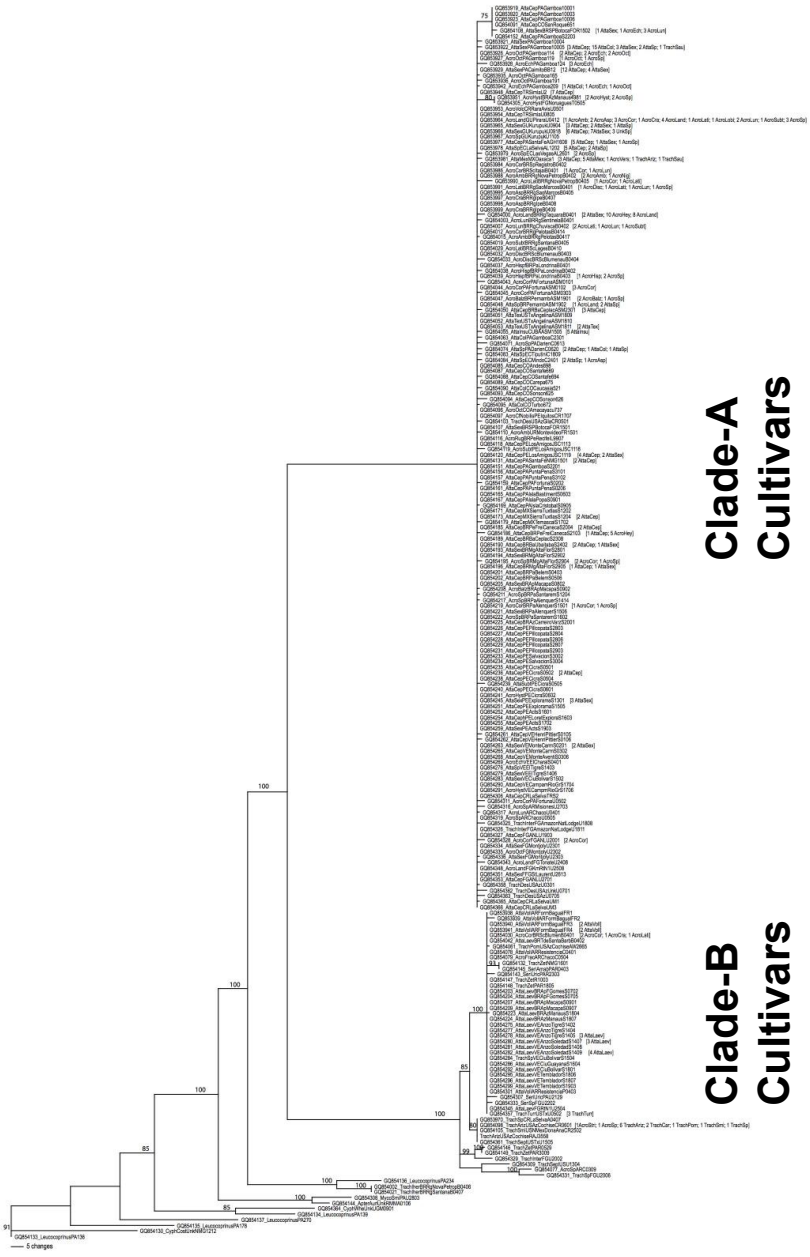

Fig. 2. Biogeographic patterns of 419 *L. gongylophorus* fungi cultivated by leafcutter ants (*Atta*, *Acromyrmex*). Collection locations are shown in the bottom-left panel. Fungi are assigned by STRUCTURE to three genotype clusters (purple, green, burnt orange), and membership in these three clusters is mapped onto 10 biogeographic regions: 1. North America (Mexico, Cuba, USA). 2. Central America (Panamá, Costa Rica, Honduras). 3. Colombia (west of Andes). 4. Venezuela. 5. The Guianas (Guyana, Suriname, French Guiana). 6. Ecuador. 7. Peru. 8. northern Brazil. 9. southern Brazil. 10. Argentina & Uruguay. Information on exact collection locations, sample sizes, and leafcutter ant-hosts is summarized in Tables S1 & S2. The sizes of the pie charts in the bottom-right panel corresponds to the number of leafcutter nests surveyed in each of the 10 biogeographic regions; each pie chart is centered on the centroid of collection locations from the respective region. The three genotype clusters map roughly to southern South America, northern South America, and North & Central America. Populations of *L. gongylophorus* fungi in Central and North America appear less diverse than populations in South America.

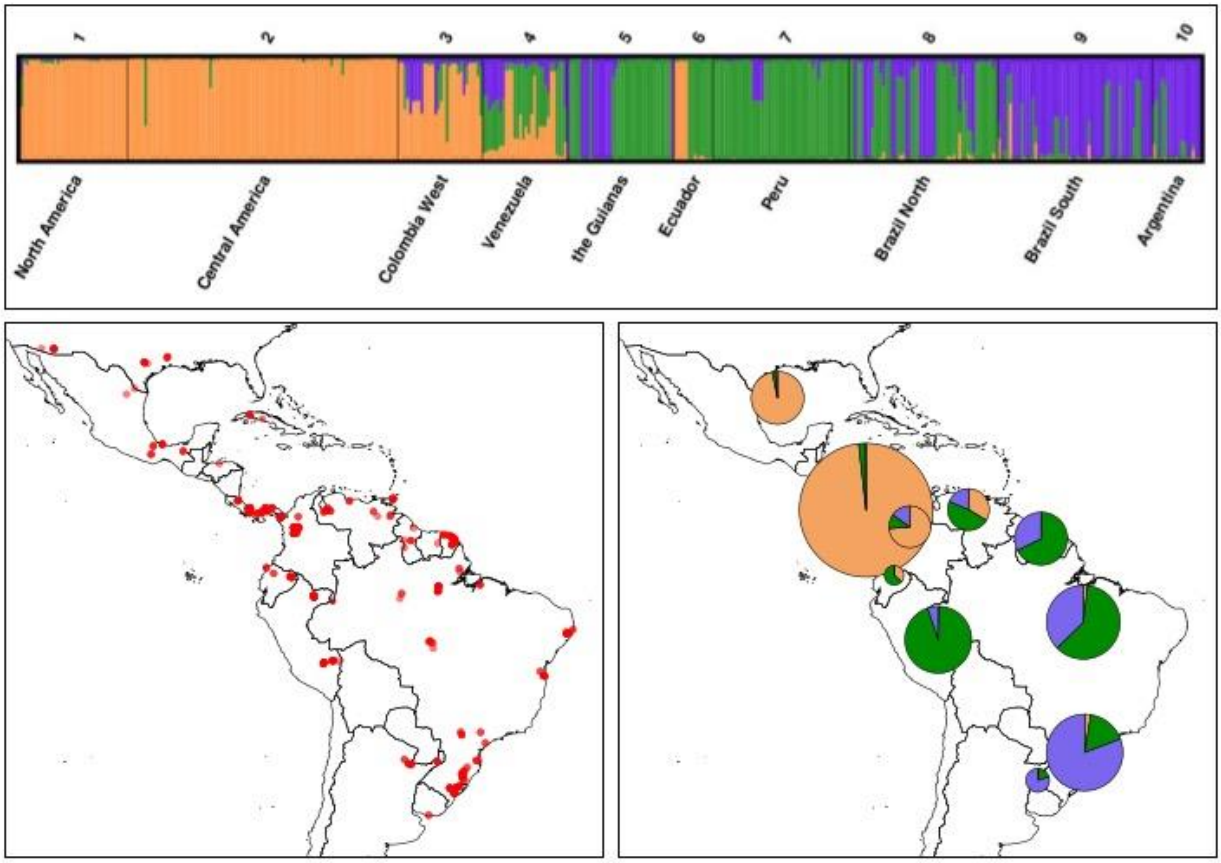

Table 1. Comparison of crown ages and stem ages for Clade-A fungi and for the leafcutter ant clade, estimated in six phylogenetic analyses conducted to date. Mikheyev *et al.* (2010) used a 4-gene phylogeny to estimate the crown-node date (coalescent) and stem-node date of four Clade-A fungi isolated from two *Acromyrmex* species from Panamá and Guyana and 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 only a single time-calibrated node in their phylogenetic reconstructions, the last common ancestor of ant-cultivated fungi with *Agaricus*, dated to 73 mya in Mikeyev *et al.* (modeled with more or less conservative distributions around this date), and dated likewise to 73 mya in Nygaard *et al.* (modeled also with a 5% minimum age of 55 mya and a 95% maximum age of 91 mya). The ancient time-calibration (i.e., anchor at 73 mya) of the phylogenetic reconstructions is likely to render estimates of the dates of recent diversifications (e.g., estimate of crown age of Clade-A fungi) more unreliable than estimates for earlier diversifications. mya = million years ago.

CLADE-A FUNGI	LEAFCUTTER ANT CLADE	SOURCE
Crown Age of Clade-A Fungi	**Crown Age of Leafcutter Ant Clade**	
	8 mya (6-15 mya), without the basal *Ac. striatus*	Schultz & Brady 2008
4 mya (0.5-8.0 mya)	(not estimated)	Mikheyev *et al.* 2010
	12.2 mya (9.1-15.3), without *Ac. striatus*	Schultz *et al.* 2015
7.2 mya (5.5-9.0 mya)	16.2 mya (12.6-19.7 mya), without *Ac. striatus*	Nygaard *et al.* 2016
	17.9 mya (15.6-20.4 mya), without *Ac. striatus*	Ješovnik *et al.* 2016
	17.0 mya (13.2-20.8), without *Ac. striatus*	Branstetter *et al.* 2017
	18.2 mya (14.2-22.2), with *Ac. striatus*	Branstetter *et al.* 2017
Stem Age of Clade-A Fungi	**Stem Age of Leafcutter Ant Clade**	
	9 mya (7-15 mya)	Schultz & Brady 2008
25 mya (11-39 mya)	(not estimated)	Mikheyev *et al.* 2010
22.4 mya (16.9-27.9 mya)	17.8 mya (13.7-21.7 mya)	Nygaard *et al.* 2016
	19.9 mya (17.7-22.5 mya)	Ješovnik *et al.* 2016
	19.3 mya (15.2-23.7 mya)	Branstetter *et al.* 2017
Stem Age of Clade-A Fungi	**Stem Age of Higher-Attine Ant Clade**	
	20 mya (17-29 mya)	Schultz & Brady 2008
25 mya (11-39 mya)	(not estimated)	Mikheyev *et al.* 2010
	(not estimated)	Schultz *et al.* 2015
22.4 mya (16.9-27.9 mya)	26.6 mya (19.6-33.8 mya)	Nygaard *et al.* 2016
	33.3 mya (31.3-35.1 mya)	Ješovnik *et al.* 2016
	31.4 mya (25.9-37.2 mya)	Branstetter *et al.* 2017

Supporting Information

Biogeography of Leafcutter Ant-Fungus Mutualisms

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BACKGROUND INFORMATION

Fossil Record of Attine Ants from Dominican and Chiapas Amber (15-20 Million Years Ago)

In a description of a new species of *Apterostigma* from Dominican amber, Schultz (2007) summarizes the prior literature: "To date, three attine ant species have been described from Dominican amber: *Trachymyrmex primaevus* (Baroni Urbani 1980), *Cyphomyrmex maya*, and *Cyphomyrmex taino* (de Andrade 2003). In addition, Brown (1973) refers to possible *Mycetosoritis* males in Chiapas amber (Oligo-Miocene, ~20 mya), Wilson (1985) refers to *Cyphomyrmex* in Dominican amber, and Baroni Urbani (1995) refers to *Apterostigma* and *Cyphomyrmex* in Dominican amber." These publications represent the complete literature to date on described fossil attine species. The ages of Dominican and Mexican (Chiapas) amber are typically cited as ~20 million years old (mya), dating to the early Miocene. LaPolla *et al.* (2013) list ages of 16-19 mya for Dominican amber and 15-20 mya for Mexican amber.

There exist two published photographs labeled "*Acromyrmex*" fossils in Dominican amber (page 446 in Grimaldi & Engel 2005; page 246 in Nudds & Selden 2013), but an unambiguous assignment of these fossils to the genus *Acromyrmex* is not possible from the spinulation, head, and integumental features visible in these photographs. The specimen shown in Nudds & Selden (2013) measures about 2mm length, which would be an unusually small caste size for extant *Acromyrmex* species. The "chewed leaf fragments" embedded with an attine ant in the same amber fossil shown in Grimaldi & Engel (2005) reveals leaf-damage that is atypical for leafcutter ants (the damaged edges are serrated in the fossilized leaf fragments, unlike the smooth cuts made in leaves by extant leafcutter species), and the presence of both damaged leaf fragments and an attine ant in the same amber fossil could be coincidental. The photographed attine ant specimens could represent higher-attine lineages predating the origin of leafcutter ants, or higher-attine lineages outside of the leafcutter clade, such as lineages near *Trachymyrmex primaevus* described also from Dominican amber (Baroni Urbani 1980; *T. primaevus* measures 3-4 mm in length, somewhat larger than the specimen shown in Nudds & Selden 2013). The fossil specimen appearing on page 446 in Grimaldi & Engel (2005) appears to be lost, per communication by David Grimaldi with Ted Schultz.

Fossilized gardens and garden chambers dating to 5.7–10 mya, possibly from *Acromyrmex* or *Trachymyrmex* ants, have been described from La Pampa Province in Argentina (Genise *et al.* 2013; see also Laza 1982). Fossilized gardens are not known from Central or North America.

The name *Leucocoprinus gongylophorus*, not *Leucoagaricus gongylophorus*, is the proper name for the sporocarp described by Möller (1893) from an *Acromyrmex* garden in southern Brazil

The widely-cited placement by R. Singer (1986) of sporocarps (mushrooms) of fungi cultivated by leafcutter ants into the genus *Leucoagaricus* is based on inaccurate reporting of prior literature. To discuss these inaccuracies, we first provide the exact writing from Singer's book (*The Agaricales in Modern Taxonomy*. 4th Edition. Koeltz Scientific Books, Koenigstein, Germany, 1986):

> "It has been indicated by me (1951) [= Singer, *Lilloa Revista de Botánica*, vol. 22, p. 429: "Judging from the original account which includes a photograph, the often cited *Rozites gonlylophora* Moeller is not a *Rozites* but an *Agaricus* sp."] that the fungus (or one of the fungi) living in symbiosis with ants (Attini) - "cultivated" by the ants according to some zoologists - is an agaricaceous fungus. N.A. Weber (in several papers 1955-57) has shown that the fungi appearing in his cultures actually belong

in *Leucoagaricus*, and so do some of the isolates from various ants indicated by Hervey, Rogerson & Long (*Brittonia* 29: 226-236. 1977). According to Heim (*Rev. Mycol.* 22: 299. 1957) and Singer (*Agaricales in Modern Taxonomy* 3rd ed. p. 455. 1975) both Weber's and Hervey's agarics are specifically identical and congeneric with a *Leucoagaricus* which should be known as *Leucoagaricus gongylophorus* (Möller) Sing. (c.n. *Rozites gongylophorus* Möller, *Bot. Mitt. Trop.* 6: 70, pl. I-II. 1893). A.H. Smith (in Weber) was the first to identify Weber's agaric correctly as *Leucoagaricus*."

The preceding paragraph represents the entire treatment of ant-cultivated fungi in Singer's book (1986).

The paragraph from Singer (1986) contains several inaccuracies:

(1) Heim (*Rev. Mycol.* 22: 293-299. 1957) re-described Möller's original description of *Rozites gonlylophora* as *Leucocoprinus gongylophorus* [page 299: "dénomination nouvelle de *Leucocoprinus gongylophorus* (Möller)"], not *Leucoagaricus*, as incorrectly stated in the above writing by Singer.

(2) Hervey, Rogerson & Long (*Brittonia* 29: 226-236. 1977) do not identify sporocarps of leafcutter fungi, but sporocarps grown from pure cultures of lower-attine fungi (fungi from the ants *Myrmycocrypta buenzlii, Mycetophylax conformis, Apetostigma auriculatum, Cyphomyrmex costatus*), and they identify these as "*Lepiota* sp. (*Leucocoprinus* sp.)" (page 234), not *Leucoagaricus*, as incorrectly stated in the above writing by Singer.

(3) Weber's publications from 1955-1957 identify the fungi cultivated by attine ants as (a) unidentified "pure cultures" (Weber 1955, *Science* 121: 109; cultures isolated from gardens of *Apterostigma*, *Cyphomyrmex*, *Trachymyrmex*, *Atta*), (b) unidentified "pure culture" (Weber 1956, *Ecology* 37: 197-199; culture of the fungus isolated from garden of *Trachymyrmex septentrionalis*), and (c) "*Lepiota*" (Weber 1957, *Ecology* 38: 480-494; sporocarp from pure culture of *Cyphomyrmex costatus*). That is, Weber did not succeed at growing, and did not examine, a sprorocarp of a leafcutter fungus, but a sprorocarp from a lower-attine ant (*C. costatus*), and Weber reported this to be a *Lepiota*. The sprorocarp identified by "A.H. Smith (in Weber)" (quote from Singer's above paragraph) is therefore a sporocarp produced by a culture of fungus from *C. costatus* (a lower-attine fungus), and Smith believed this to be a *Lepiota* (Weber 1957). Therefore, Singer's above claim is incorrect that " N.A. Weber (in several papers 1955-57) has shown that the fungi appearing in his cultures actually belong in *Leucoagaricus*", and Singer's above claim is incorrect that "H. Smith (in Weber) was the first to identify Weber's agaric correctly as *Leucoagaricus*" (instead, Smith identified one lower-attine fungus as a *Lepiota*).

(4) Singer's reference to his earlier writing (1975; *Agaricales in Modern Taxonomy* 3rd ed. p. 454; not p. 455 as stated in Singer's above writing from 1986) refers to a single sentence in Singer (1975) discussing the placement of ant-cultivated fungi (page 454): "N.A. Weber (in several papers 1955-57) has shown that the fungi appearing in his cultures actually belong in *Leucoagaricus*." Singer (1975) therefore makes the same mistake as Singer (1986) in incorrectly stating that Weber identified the leafcutter-cultivated fungi as *Leucoagaricus* (instead, Weber reports a *Lepiota* sporocarp grown from a culture of a lower-attine fungus).

Because of the inaccuracies in Singer (1986), we therefore follow here Heim (1957) and Hervey *et al.* (1977) in placing all ant-cultivated fungi into the genus *Leucocoprinus*. Moreover, Else Vellinga (UC Berkeley) communicated to us (19. May 2017) that she will submit shortly a revision of leucocorpinaceous fungi, including all *Leucoagaricus* and *Leucocoprinus*. Because described species of *Leucoagaricus* are not monophyletic, and because described species of *Leucocoprinus* are likewise not monophyletic (Vellinga 2004), E. Vellinga will reassign all *Leucoagaricus* to *Leucocoprinus* (the name *Leucocoprinus* has priority over *Leucoagaricus*), to generate a monophyletic genus (*Leucocoprinus sensu lato*), with monophyly supported by several r-DNA genes.

Spore Germination of *Leucocoprinus gongylophorus*

Mueller (2002) lists published studies that attempted to germinate spores of *L. gongylophorus,* but to our knowledge only Möller (1893) succeeded so far at germinating spores of *L. gongylophorus* and obtain from these spores mycelium that developed staphylae (clusters of gongylidia) typical for *L.*

gongylophorus. Because Möller's work is not readily available and was written in German, we provide here an English translation of Möller's experiments describing germination of spores produced by *Leucocoprinus gongylophorus* mushrooms (called *Rozites gongylophora* by Möller). Our translation eliminates a few peripheral details, but the translation faithfully presents the essence of the experiments. Phrases appearing in our translation in square brackets [....] are inserted to improve clarity. We translate both "Kohlrabihäufchen" (literally: small aggregate of kohlrabi, a kind of turniplike cabbage) and "Futterkörperchen" (literally: food corpuscle) as "staphylae" (= aggregate of gongylidia).

Pages 78-79 in Alfred Möller (1893) *Die Pilzgärten einiger südamerikanischer Ameisen* (Verlag Gustav Fisher, Jena, Germany) describe Möller's spore germination experiments, starting on page 78 with "Die Sporen keimen..." and ending on page 79 with "... sogar überlegen waren".

"The spores germinate in water or nutrient broth two days after inoculation, with only one germ tube, which emanates from a small opening opposite to the original attachment site of the spore [attachment at the basidum] ... and the germ tube expands to a thickness of 7-8 µm (Fig. 8). Spore germination progressed irregularly, and invariably only a limited number of spores germinated. Frequently the germ tube develops a side branch shortly after exiting the spore. The subsequent growth of the mycelia is very slow. Only on the ninth day does the germinating mycelium form a whitish speck, which shows aerial hyphae, that is visible to the unaided eye. The individual hyphae contained granule- and vacuole-rich protoplam, the hyphae show early a tendency for irregular thickness and swellings, and the hyphae were characterized by extraordinary variable thickness; not infrequently one could see a side branch of 3 µm diameter branching from hyphae of 10 µm diameter. I made the same kind of observation in cultures that I obtained from staphylae [Kohlrabihäufchen] taken from [natural gardens in] nests. Subsequently, the mycelia derived from the basidiospores became also similar to the mycelia derived from staphylae in that the aerial hyphae exhibited a winding and corkscrew-like growth. Finally, after five weeks of careful maintenance (20. February until 8. April 1892), the mycelia derived from germinating spores developed staphylae. The development of these staphylae [derived from germinating spores] began in exactly the same way as described for the cultures obtained from staphylae from gardens, as shown in Fig. 33 (Plate VIII). The staphylae increased gradually in abundance, and staphylae [Futterkörperchen] materialized that were of equal size to, or sometimes larger than, the staphylae in [natural gardens in] nests."

ADDITIONAL DISCUSSION OF RESULTS

Are there Differences between Fungi Cultivated by Dicot- Versus Grass-Cutting Leafcutter Ants?

Acromyrmex: For 23 *Acromyrmex* species for which fungicultural information is known, all of the 17 dicot specialists cultivate Clade-A fungi, and only two of these 17 (*Ac. coronatus*, *Ac. crassispinus*) also cultivate sometimes Clade-B fungi (Table S6). Of the four *Acromyrmex* species specialized to cut grasses, one species (*Ac. fracticornis*) cultivates a Clade-B fungus, but only a single fungus was identified for this species. Of the two *Acromyrmex* species cutting both dicots and grasses, one species (*Ac. striatus*) cultivates a Clade-B fungus, but again, only a single fungus was identified for this species.

Atta: For 10 *Atta* species for which fungicultural information is known, all of the 6 dicot specialists cultivate Clade-A fungi. Of the three *Atta* species specialized to cut grasses, one species, *Atta vollenweideri*, cultivates a Clade-B fungus (8 fungi from two sites were surveyed for *At. vollenweideri*), whereas the two other grass-cutting species *At. capiguara* and *At. bisphaerica* cultivate a Clade-A fungus, but only one fungus was identified for each of these two species (Silva Pinhati *et al.* 2004; Table S6). *Atta laevigata* forages on both grasses and dicots (Nagamoto *et al.* 2009), and cultivates a Clade-B fungus throughout much of its range from Venezuela to southern Brazil (28 nests from multiple sites were surveyed; Table S6), but we observed also two cases of Clade-A cultivation by *At. laevigata*, known for this species so far only from southern Brazil (details in Table S10).

Apparent cultivar specialization of *Atta* vs *Acromyrmex* ants reported in a previous analysis

Kooij *et al.* (2015b, page 13) write that "fungal symbionts of *Atta* and *Acromyrmex* colonies showed that they were completely separated … consistent with earlier findings by Mikheyev *et al.* (2007) for the same sampling site". This statement is misleading because Mikheyev *et al.* (2007) actually documented that *Atta* and *Acromyrmex* ants "shared identical fungus garden genotypes, indicating wide-spread cultivar exchange" (Abstract in Mikheyev *et al.* 2007). The leafcutter cultivar lineages studied by Mikheyev *et al.* (2007, Abstract) were "largely unstructured with respect to host ant species, with only 10% of the structure in genetic variance being attributable to partitioning among ant species and genera". Specifically, Fig. 3 in Mikheyev *et al.* (2007) shows that fungal diversity associated with Panamanian leafcutter ants is structured into 6 fungal genotype-clusters, and that there exists very little correlation between these fungal clusters and leafcutter ant genera (i.e., each of the leafcutter ant species essentially cultivates representatives from each or most of these 6 fungal genotype-clusters). The same conclusion of absence of cultivar specialization of *Atta* vs *Acromyrmex* ants emerged also in our much larger analysis of leafcutter cultivars from Panamá (Fig. S7). Therefore, the fungi examined by Kooij *et al.* (2015b) were unfortunately selectively sampled from the true diversity of fungi cultivated by each leafcutter species in Panamá, such that Kooij *et al.* (2015b) oversampled one fungus lineage for *Atta* ants (n=9 fungi) and oversampled another fungus lineage for *Acromyrmex* ants (n=9 fungi).

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List of Tables in Supporting Information

Table S1. Collection information of fungal samples (collection ID, host-ant species, collection location, GPS, voucher storage, etc), summary of sequence information generated (EF-1α, RAD, DMS genes), Genbank accessions, and summary of microsatellite-marker information generated (loci A1132, C101, C126, C117, B12).

Table S2. Number of leafcutter-fungus collections characterized by DNA sequencing and/or microsatellite marker analyses, listed by ant-host species and by country of collection.

Table S3. Microsatellite allele profiles of 419 fungal cultivars from gardens of leafcutter nests, collection information of fungal samples (collection ID, host-ant species, country of collection, GPS), and information on foraging preferences of the respective ant-host (preference to foraged on grass, dicot plants, or both as main fungicultural substrate; see also Table S6). Additional collection information is in Table S1 for all samples. To identify samples that are identical in all alleles across the 5 microsatellite loci screened (i.e., samples assigned to the same fungal genotype or "clone", as defined by the 5 loci), the samples are sorted in the spreadsheet by allele and locus. A total of 241 fungal genotypes are among the 419 cultivars screened. Samples that are identical in all alleles are color-coded as follows: Yellow identifies cultivar samples of identical genotype (same fungal "clone") collected in different nests of the same ant-host species. Orange identifies genotypes ("clones") for which at least some samples were collected in different nests of different ant-host species of the *same* ant genus. Green identifies genotypes ("clones") for which at least some samples were collected in different nests of *different* ant-host genera (*Atta* or *Acromyrmex*). Highlighting in other colors (pale yellow, pale orange) identifies genotypes for which allele information was missing at one locus, so it was not possible to determine for these genotypes whether they had identical allele profiles across all 5 microsatellite loci (i.e., these genotypes were defined by allele identity across the 4 loci for which information was available).

Table S4. Total number of alleles scored at each locus for each of the 419 cultivars from gardens of leafcutter nests, and the averages of these total numbers across the 419 cultivars for each of the 5 loci screened. Table S4 is identical to Table S3, except for the addition of columns for calculating of total number alleles and averages. The averages across all individuals are at the bottom of Table S4.

Table S5. Taxa with identical sequences (except sequence ambiguities) that are listed next to each other as a string of taxa as the same terminal leaf in Fig. 1.

Table S6. Foraging preferences of leafcutter ant species and their fungicultural specializations on Clade-A fungi, Clade-B fungi, or both types of fungi.

Table S7. Total number of alleles at 5 microsatellite loci for fungi from *Atta* and *Acromyrmex* nests, summarized for narrow geographic region from within which both *Atta* and *Acromyrmex* were collected and genotyped at all 5 loci. Total number of alleles/fungus does not differ between fungi from *Atta* and *Acromyrmex* nests (Wilcoxon Signed-Rank Test, $W = 90.5$, $z = 0.181$, $p = 0.857$ two-tailed, $n = 19$), comparing samples from 19 locations (highlighted in blue in Table S7) for which complete genotype information (all 5 loci) is available for both *Atta*-cultivated and *Acromyrmex*-cultivated fungi. The raw data summarized in Table S7 appear in Table S8.

Table S8. Raw data used to generate statistics summarized in Table S7.

Table S9. Primer sequences developed by Mikheyev *et al.* (2006) for EF-1α, DMC1, and RAD51 genes, as well as corresponding annealing temperatures (Tm).

Table S10. Summary of higher-attine ant species found so far to cultivate both Clade-A and Clade-B fungi.

Tables S1-S6 & S8 are attached as separate spreadsheets. Tables S7, S9 & S10 are inserted below in this document.

Table S1. Collection information of samples, summaries of sequencing (EF-1α, RAD, DMS genes; Genbank accessions) and microsatellite-marker genotyping (loci A1132, C101, C126, C117, B12).

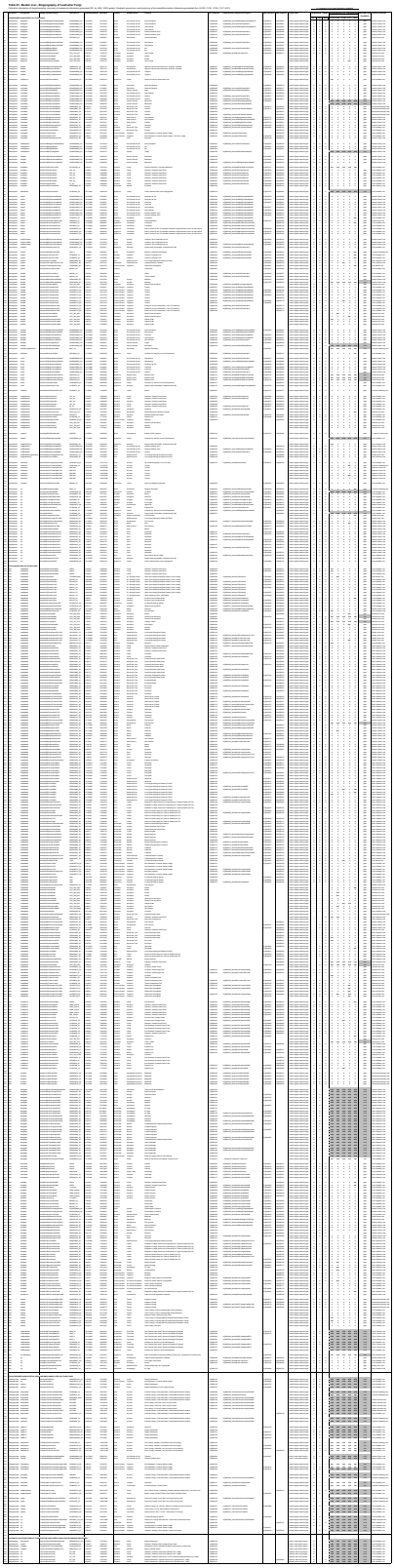

Table S2. Number of leafcutter-fungus collections characterized by DNA sequencing or microsatellite-marker genotyping, listed by ant-host species and by country of collection.

Table S2. Number of attine fungus-cultivar collections characterized by DNA sequencing and/or microsatellite marker analyses. Each sample was collected from a different nest (180 *Acromyrmex* nests, 294 *Atta* nests). Only one fungus sample was characterized per nest because leafcutter nests are thought to cultivate their fungi in monocultures, and because fungal genotypes are identical in different chambers of individual *Atta texana* and *Atta cephalotes* nests surveyed in the field (Mueller *et al.* 2010) and in *Acromyrmex echinatior* nests surveyed in the lab (Poulsen & Boomsma 2005).

		Country of Collection																	
Genus of Ant Host	**Species of Ant Host**	**Argentina**	**Uruguay**	**Brazil**	**Peru**	**Ecuador**	**French Guiana**	**Suriname**	**Guyana**	**Venezuela**	**Trinidad & Tobago**	**Colombia**	**Panamá**	**Costa Rica**	**Honduras**	**Mexico**	**Cuba**	**USA**	**Total**
Acromyrmex	*ambiguus*		1	6															7
	aspersus			4								4							8
	balzani			4															4
	coronatus			13			2						6						21
	crassispinus			4															4
	disciger			3															3
	echinatior									1			7						8
	fracticornis	1																	1
	heyeri	6	1	8															15
	hispidus	1		3															4
	hystrix			1	1	2	1		1	1									7
	landolti			2			10		2			6							20
	laticeps			8															8
	lobicornis	1																	1
	lundii	5		6															11
	nobilis				1														1
	octospinosus						1					4	8						13
	rugosus			1															1
	striatus	1																	1
	subterraneus	1		3	2														6
	versicolor																	7	7
	volcanus													1					1
	sp. (species unknown)	4		15	2	3		1					2	1					28
Atta	*cephalotes*			21	32	7	10			15	8	17	37	5	1	9			162
	colombica											3	23						26
	insularis																5		5
	laevigata			8			1			18									27
	mexicana															6			6
	sexdens			12	9		7		3	4			8						43
	texana																	11	11
	vollenweideri	8																	8
	sp. (species unknown)	1		1	1	2				1									6
	Total by Country =	**29**	**2**	**123**	**46**	**14**	**32**	**1**	**6**	**40**	**8**	**34**	**91**	**7**	**1**	**15**	**5**	**18**	
																		Total =	**474**

Table S3. Microsatellite allele profiles of 419 fungal cultivars from gardens of leafcutter nests, collection information of samples, and information on foraging preferences of the respective ant-host (preference to foraged on grass, dicot plants, or both as main fungicultural substrate; see also Table S6). Additional collection information is in Table S1 for all samples. Samples that are identical in all alleles across the 5 microsatellite loci screened are assigned to the same fungal genotype (i.e., same "clone"). A total of 241 fungal genotypes are among the 419 cultivars screened. Yellow identifies cultivar samples of the same fungal clone collected in different nests of the same ant-host species. Orange identifies the same fungal clones for which at least some samples were collected in different nests of different ant-host species of the *same* ant genus. Green identifies the same fungal clone for which at least some samples were collected in different nests of *different* ant-host genera (*Atta* or *Acromyrmex*). Highlighting in other colors (pale yellow, pale orange) identifies genotypes for which allele information was missing at one locus, so it was not possible to determine for these genotypes whether they had identical allele profiles across all 5 loci.

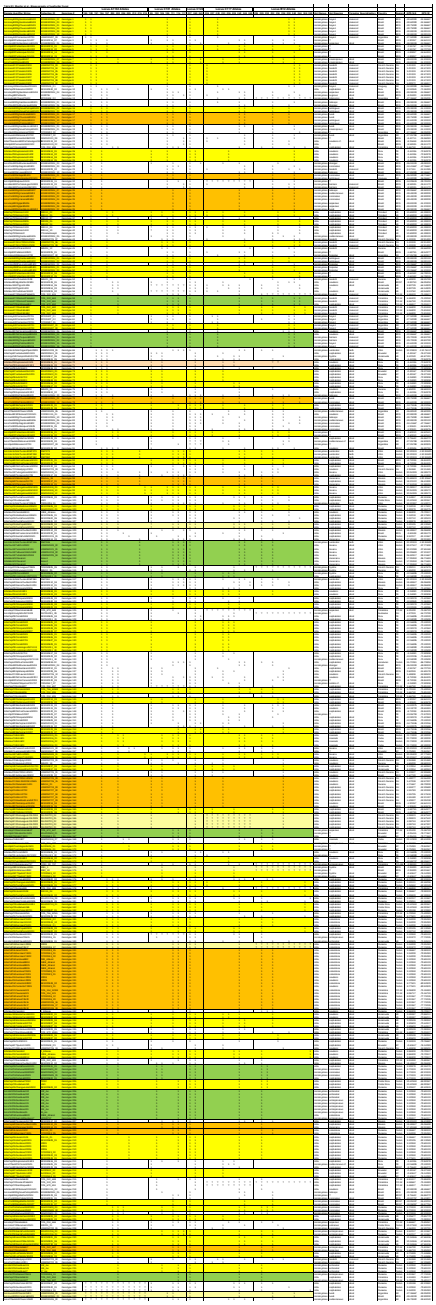

Table S4. Total number of alleles scored at each locus for each of the 419 cultivars from gardens of leafcutter nests, and the averages of these total numbers across the 419 cultivars for each of the 5 loci screened. Table S4 is identical to Table S3, except for the addition of columns for calculating of total number of alleles. Averages across all individuals are at the bottom of Table S4.

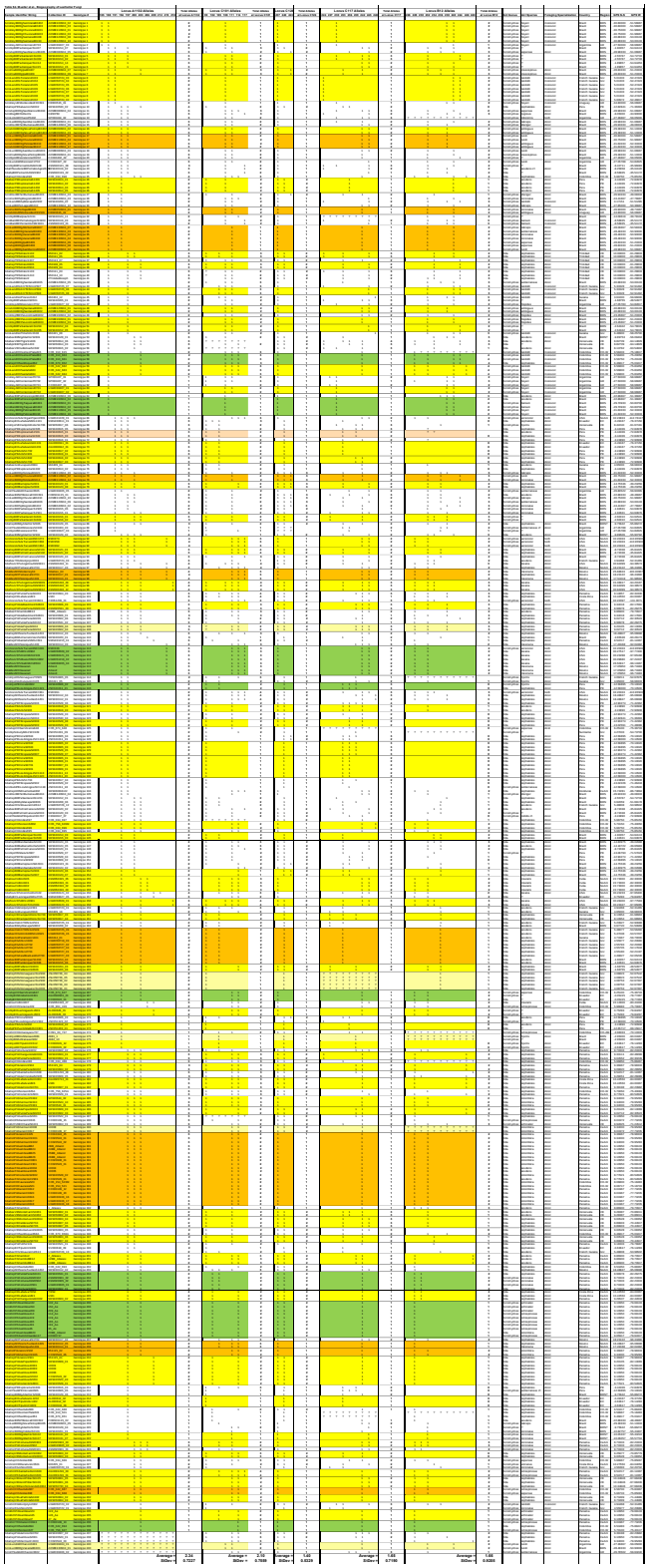

Table S5. Taxa with identical EF-1α sequences (except sequence ambiguities) and listed next to each other as a string of taxa on the same terminal leaf in the phylogenetic tree in Fig. 1.

Table S5. Mueller et al., Biogeography of Leafcutter Fungi

For all Genbank accessions listed in Table S5, the corresponding sample IDs and collection records are listed in Table S1

Taxon listed leftmost as terminal leaf in Fig. 1	Taxa with identical sequences (except sequence ambiguities) and identical to the sequence of the taxon listed leftmost as terminal leaf in Fig. 1
Clade-A	
GQ854108_AttaSexBRSPBotocaFOR1502	*Atta sexdens* (n=1) GQ854108, FOR931115_02, Brazil *Acromyrmex echinatior* (n=1) GQ854126, LDL020518_01, Panamá *Acromyrmex lundii* (n=3) GQ854111, FR931101_01, Argentina GQ854112, FR931101_02, Argentina GQ854113, FR931101_03, Argentina
GQ853922_AttaSexPAGamboa10005	*Atta cephalotes* (n=3) GQ854172, SES030112_03, Mexico GQ854175, SES030113_01, Mexico GQ854260, SES050730_04, Venezuela *Atta colombica* (n=15) GQ853924, 10008, Panamá GQ853944, 24BB_Attacol, Panamá GQ853945, 25BB_Attacol, Panamá GQ853946, 26BB_Attacol, Panamá GQ853947, 2BB_Attacol, Panamá GQ853962, 951115_02, Panamá GQ854062, CC010324_25, Panamá GQ854064, CC020524_01, Panamá GQ854065, CC020524_02, Panamá GQ854067, CC020524_05, Panamá GQ854069, CC020608_01, Panamá GQ854073, CC030106_17, Panamá GQ854154, SES020528_02, Panamá GQ854312, UGM030106_16, Panamá GQ854314, UGM030106_18, Panamá *Atta sexdens* (n=3) GQ853922, 10005, Panamá GQ853925, 1_Attasex, Panamá GQ854068, CC020529_01, Panamá *Atta* sp. (n=3) GQ854070, CC030106_12, Panamá GQ854176, SES030114_01, Mexico GQ854177, SES030114_03, Mexico *Acromyrmex octospinosus* (n=1) GQ853976, AGH031216_03, Panamá *Acromyrmex* sp. (n=2) GQ853974, AGH030624_01, Ecuador GQ853975, AGH031216_01, Panamá *Trachymyrmex saussueri* (n=1) GQ854117, JS030115_02, Mexico
GQ853926_AcroOctPAGamboa114	*Atta cephalotes* (n=2) GQ853961, 951115_01, Panamá GQ854158, SES020602_01, Panamá *Acromyrmex echinator* (n=2) GQ853930, 132_Ae, Panamá GQ853934, 150_Ae, Panamá *Acromyrmex octospinosus* (n=2) GQ853926, 114_Ao, Panamá GQ853949, 35_Ao, Panamá
GQ853927_AcroOctPAGamboa119	*Acromyrmex octospinosus* (n=1) GQ853927, 119_Ao, Panamá *Acromyrmex* sp. (n=1) GQ854072, CC030106_15, Panamá
GQ853928_AcroEchPAGamboa124	*Acromyrmex echinator* (n=3) GQ853928, 124_Ae, Panamá GQ853932, 145_Ae, Panamá GQ853950, 47_Ae, Panamá
GQ853929_AttaSexPACaimitoBB12	*Atta cephalotes* (n=12) GQ853963, 951115_04, Panamá GQ853972, AGH020713_01, Costa Rica GQ854066, CC020524_03, Panamá GQ854155, SES020529_01, Panamá GQ854160, SES020602_04, Panamá GQ854162, SES020603_01, Panamá GQ854163, SES020604_01, Panamá GQ854164, SES020606_01, Panamá GQ854166, SES020607_01, Panamá GQ854168, SES020609_04, Panamá GQ854170, SES030112_01, Mexico GQ854367, UM6, Costa Rica *Atta sexdens* (n=4) GQ853929, 12BB_Attasex, Panamá GQ853931, 13BB_Attasex, Panamá GQ853933, 14BB_Attasex, Panamá GQ853937, 2_Attasex, Panamá
GQ853942_AcroEchPAGamboa209	*Atta colombica* (n=1) GQ853943, 20BB_Attacol, Panamá *Acromyrmex echinator* (n=1) GQ853942, 209_Ae, Panamá *Acromyrmex octospinosus* (n=1) GQ854115, HF030717, Panamá
GQ853948_AttaCepTRSimlaU2	*Atta cephalotes* (n=7) GQ853948, 2TrinidadAceph, Trinidad GQ853955, 950109_04, Trinidad GQ853956, 950111_02, Trinidad GQ853957, 950111_03, Trinidad GQ853958, 950111_05, Trinidad GQ853959, 950113_07, Trinidad GQ853960, 950114_10, Trinidad
GQ853951_AcroHystBRAzManaus4981	*Acromyrmex hystrix* (n=2) GQ853951, 4981_1, Brazil GQ854082, CC030615_12, Equador *Acromyrmex* sp. (n=2) GQ853952, 4982_10, Brazil GQ854081, CC030615_02, Ecuador
GQ853964_AcroLandGUPiraraU0412	*Acromyrmex ambiguus* (n=3) GQ853989, AOMB060904_03, Brazil GQ854017, AOMB120904_01, Brazil GQ854018, AOMB120904_02, Brazil *Acromyrmex aspersus* (n=2) GQ853994, AOMB080904_04, Brazil GQ853996, AOMB080904_06, Brazil *Acromyrmex coronatus* (n=3) GQ853985, AOMB040904_03, Brazil GQ854025, AOMB130904_02, Brazil GQ854027, AOMB130904_04, Brazil *Acromyrmex crassispinus* (n=1) GQ853987, AOMB060904_01, Brazil *Acromyrmex landolti* (n=4) GQ853964, 960404_12, French Guiana GQ853969, 960421_08, French Guiana GQ854346, UGM050725_06, French Guiana GQ854347, UGM050725_07, French Guiana *Acromyrmex laticeps* (n=1) GQ854028, AOMB130904_07, Brazil *Acromyrmex lobicornis* (n=1) GQ854300, SP030404_02, Argentina *Acromyrmex lundii* (n=2) GQ853992, AOMB080904_02, Brazil GQ854024, AOMB130904_01, Brazil *Acromyrmex subterraneus* (n=1) GQ854026, AOMB130904_03, Brazil *Acromyrmex* sp. (n=3) GQ854212, SES040212_05, Brazil GQ854218, SES040214_15, Brazil GQ854315, UGM030327_02, Argentina
GQ853965_AttaSexGUKurupukU0904	*Atta cephalotes* (n=3) GQ854246, SES040613_02, Peru GQ854253, SES040616_02, Peru GQ854258, SES040619_01, Peru *Atta sexdens* (n=2) GQ853965, 960409_04, Guyana GQ854249, SES040615_01, Peru *Atta* sp. (n=1) GQ853973, AGH030612_01, Ecuador
GQ853966_AttaSexGUKurupukU0918	*Atta cephalotes* (n=6) GQ854206, SES040208_06, Brazil GQ854210, SES040212_01, Brazil GQ854289, SES050817_03, Venezuela GQ854354, UGM050727_02, French Guiana GQ854355, UGM050727_03, French Guiana GQ854356, UGM050727_04, French Guiana GQ854123, JSL050729_02, French Guiana GQ854124, JSL050729_03, French Guiana GQ854125, JSL050729_05, French Guiana *Atta sexdens* (n=7) GQ853966, 960409_18, Guyana GQ853968, 960414_01, Guyana GQ854216, SES040214_04, Brazil GQ854344, UGM050725_01, French Guiana GQ854349, UGM050725_09, French Guiana GQ854350, UGM050726_12, French Guiana GQ854352, UGM050726_15, French Guiana
GQ853977_AttaCepPASantaFeAGH1608	*Atta cephalotes* (n=5) GQ854150, SES020521_01, Panamá GQ854267, SES050803_04, Venezuela GQ854272, SES050807_02, Venezuela GQ854273, SES050807_04, Venezuela GQ853977, AGH031216_08, Panamá *Atta sexdens* (n=1) GQ854183, SES040120_02, Brazil *Acromyrmex* sp. (n=1) GQ854184, SES040120_03, Brazil
GQ853978_AttaSpECLaSelvaAL1202	*Atta cephalotes* (n=5) GQ854121, JSC041011_20, Peru GQ854122, JSC041011_21, Peru GQ854197, SES040129_06, Brazil GQ854243, SES040607_01, Peru GQ854244, SES040609_01, Peru *Atta* sp. (n=2) GQ853978, AL030612_02, Equador GQ854304, SV030618_09, Equador
GQ853979_AcroSpECLasVegasAL2601	*Acromyrmex* sp. (n=2) GQ853979, AL030626_01, Equador GQ853980, AL030626_03, Equador
GQ853981_AttaMexMXOaxaca1	*Atta cephalotes* (n=3) GQ854174, SES030112_05, Mexico GQ854178, SES030117_01, Mexico GQ854180, SES030117_03, Mexico *Atta mexicana* (n=5) GQ853981, Amex1, Mexico GQ853982, Amex2, Mexico GQ853983, Amex3, Mexico GQ854060, Attamex04, Mexico *Acromyrmex versicolor* (n=1) GQ854323, UGM041228_01, US *Trachymyrmex arizonensis* (n=1) GQ854102, CR050811_02_Tarizonensis, USA *Trachymyrmex saussurei* (n=1) GQ854104, CR060627_12_Tsaussurei, Mexico
GQ853986_AcroCorBRScItajaiB0401	*Acromyrmex coronatus* (n=1) GQ853986, AOMB050904_01, Brazil *Acromyrmex lundii* (n=1) GQ854009, AOMB110904_04, Brazil
GQ853988_AcroAmbBRRgNovaPetropB0402	*Acromyrmex ambiguus* (n=2) GQ853988, AOMB060904_02, Brazil GQ854011, AOMB110904_13, Brazil *Acromyrmex niger* (n=1) GQ854075, CC030327_02, Argentina
GQ853990_AcroLatiBRRgNovaPetropB0405	*Acromyrmex coronatus* (n=1) GQ854199, SES040131_07, Brazil *Acromyrmex laticeps* (n=1) GQ853990, AOMB060904_05, Brazil
GQ853991_AcroLatiBRRgSaoMarcosB0401	*Acromyrmex disciger* (n=1) GQ854035, AOMB140904_06, Brazil *Acromyrmex laticeps* (n=1) GQ853991, AOMB080904_01, Brazil *Acromyrmex lundii* (n=1) GQ853993, AOMB080904_03, Brazil *Acromyrmex* sp. (n=1) GQ854076, CC030402_02, Argentina
GQ854000_AcroBalzBRRgTaquaraB0401	*Atta sexdens* (n=2) GQ854040, AOMB160904_04, Brazil GQ854041, AOMB160904_06, Brazil *Acromyrmex heyeri* (n=10) GQ854109, FR000515_01, Uruguay GQ854080, CC030407_01, Argentina GQ854004, AOMB100904_02, Brazil GQ854005, AOMB100904_03, Brazil GQ854006, AOMB110904_01, Brazil GQ854008, AOMB110904_03, Brazil GQ854013, AOMB110904_15, Brazil GQ854014, AOMB110904_16, Brazil GQ854022, AOMB120904_08, Brazil GQ854023, AOMB120904_10, Brazil *Acromyrmex landolti* (n=6) GQ854337, UGM050724_02, French Guiana GQ854338, UGM050724_03, French Guiana GQ854339, UGM050724_04, French Guiana GQ854340, UGM050724_05, French Guiana GQ854341, UGM050724_06, French Guiana GQ854342, UGM050724_07, French Guiana *Acromyrmex balzani* (n=2) GQ854000, AOMB090904_01, Brazil GQ854001, AOMB090904_02, Brazil
GQ854007_AcroLunBRRgChuviscaB0402	*Acromyrmex laticeps* (n=2) GQ854010, AOMB110904_10, Brazil GQ854016, AOMB110904_20, Brazil *Acromyrmex lundii* (n=1) GQ854007, AOMB110904_02, Brazil *Acromyrmex subterraneus* (n=1) GQ854020, AOMB120904_06, Brazil
GQ854039_AcroHispfBRPaLondrinaB0403	*Acromyrmex hispidus fallax* (n=1) GQ854039, AOMB160904_03, Brazil *Acromyrmex* sp. (n=2) GQ854213, SES040213_01, Brazil GQ854214, SES040213_02, Brazil
GQ854044_AcroCorPAFortunaASM0102	*Acromyrmex coronatus* (n=3) GQ854044, ASM020601_02, Panamá GQ854046, ASM020605_02, Panamá GQ854310, UGM020605_01, Panamá
GQ854047_AcroBalzBRPernambASM1901	*Acromyrmex balzani* (n=2) GQ854047, ASM040119_01, Brazil GQ854182, SES040119_03, Brazil *Acromyrmex* sp. (n=1) GQ854049, ASM040121_08, Brazil
GQ854048_AttaSpBRPernambASM1902	*Atta* sp. (n=2) GQ854048, ASM040119_02, Brazil GQ854181, SES040119_02, Brazil *Acromyrmex landolti* (n=1) GQ854086, CO5_042_684, Colombia
GQ854050_AttaCepBRBaCeplacASM2301	*Atta cephalotes* (n=3) GQ854050, ASM040123_01, Brazil GQ854187, SES040123_06, Brazil GQ854188, SES040123_07, Brazil
GQ854053_AttaTexUSTxAngelinaASM1811	*Atta texana* (n=2) GQ854053, ASM040418_11, USA GQ854054, ASM040418_12, USA
GQ854055_AttaInsuCUBAASM1505	*Atta insularis* (n=5) GQ854055, ASM050315_05, Cuba GQ854056, ASM050316_01, Cuba GQ854057, ASM050316_02, Cuba GQ854058, ASM050316_03, Cuba GQ854059, ASM050318_07, Cuba
GQ854074_AttaSpPADarienC0620	*Atta cephalotes* (n=2) GQ854270, SES050804_02, Venezuela GQ854271, SES050804_03, Venezuela *Atta colombica* (n=1) GQ854313, UGM030106_17, Panamá *Atta* sp. (n=1) GQ854074, CC030106_20, Panamá
GQ854084_AttaSpECMindoC2401	*Atta* sp. (n=2) GQ854129, NMG030627_05, Equador GQ854084, CC030624_01, Equador *Acromyrmex aspersus* (n=1) GQ854092, CO5_674_647, Colombia
GQ854120_AttaCepPELosAmigosJSC1119	*Atta cephalotes* (n=4) GQ854120, JSC041011_19, Peru GQ854215, SES040214_03, Brazil GQ854220, SES040215_03, Brazil GQ854230, SES040529_02, Peru *Atta sexdens* (n=2) GQ854242, SES040606_03, Peru GQ854257, SES040618_02, Peru
GQ854131_AttaCepPASantaFeNMG1501	*Atta cephalotes* (n=2) GQ854131, NMG031215_01, Panamá GQ854153, SES020528_01, Panamá
GQ854173_AttaCepMXSierraTuxtlasS1204	*Atta cephalotes* (n=2) GQ854173, SES030112_04, Mexico GQ854266, SES050803_03, Venezuela
GQ854185_AttaCepBRPeFreiCanecaS2004	*Atta cephalotes* (n=2) GQ854185, SES040120_04, Brazil GQ854274, SES050807_05, Venezuela
GQ854186_AttaCepBRPeFreiCanecaS2103	*Atta cephalotes* (n=1) GQ854186, SES040121_03, Brazil *Acromyrmex heyeri* (n=5) GQ854302, SP030407_01, Argentina GQ854303, SP030407_02, Argentina GQ854320, UGM030407_01, Argentina GQ854321, UGM030407_02, Argentina GQ854322, UGM030407_03, Argentina
GQ854190_AttaCepBRBaUbaitabaS2402	*Atta cephalotes* (n=2) GQ854190, SES040124_02, Brazil GQ854191, SES040124_03, Brazil *Atta sexdens* (n=1) GQ854192, SES040124_05, Brazil
GQ854195_AcroSpBRMgAltaFlorS2904	*Acromyrmex coronatus* (n=2) GQ854198, SES040131_02, Brazil GQ854200, SES040201_01, Brazil *Acromyrmex* sp. (n=1) GQ854195, SES040129_04, Brazil
GQ854196_AttaCepBRMgAltaFlorS2905	*Atta cephalotes* (n=1) GQ854196, SES040129_05, Brazil *Atta sexdens* (n=1) GQ854256, SES040618_01, Peru
GQ854219_AcroCorBRPaAlenquerS1501	*Acromyrmex coronatus* (n=1) GQ854219, SES040215_01, Brazil *Acromyrmex* sp. (n=1) GQ854232, SES040529_07, Peru
GQ854236_AttaCepPECicraS0502	*Atta cephalotes* (n=2) GQ854236, SES040605_02, Peru GQ854237, SES040605_03, Peru
GQ854245_AttaSexPEExploramaS1301	*Atta sexdens* (n=3) GQ854245, SES040613_01, Peru GQ854247, SES040614_02, Peru GQ854248, SES040614_04, Peru
GQ854263_AttaSexVEMonteCarmS0201	*Atta sexdens* (n=2) GQ854263, SES050802_01, Venezuela GQ854264, SES050802_02, Venezuela
GQ854328_AcroCorFGANLU2001	*Acromyrmex coronatus* (n=2) GQ854328, UGM050720_01, French Guiana GQ854332, UGM050722_01, French Guiana
CLADE-B	
GQ853940_AttaVollARFormBagualFR3	*Atta vollenweideri* (n=2) GQ853940, 2005_3, Argentina GQ854114, FR961001_01, Argentina
GQ853941_AttaVollARFormBagualFR4	*Atta vollenweideri* (n=2) GQ853941, 2005_4, Argentina GQ854127, MB020401_01, Argentina
GQ854030_AcroCorBRScBlumenB0401	*Acromyrmex coronatus* (n=2) GQ854030, AOMB140904_01, Brazil GQ854031, AOMB140904_02, Brazil *Acromyrmex crassispinus* (n=1) GQ854036, AOMB150904_01, Brazil *Acromyrmex laticeps* (n=1) GQ854034, AOMB140904_05, Brazil
GQ854278_AttaLaevVEAnzoTigreS1405	*Atta laevigata* (n=3) GQ854278, SES050814_05, Venezuela GQ854285, SES050816_01, Venezuela GQ854294, SES050818_05, Venezuela
GQ854280_AttaLaevVEAnzoSoledadS1407	*Atta laevigata* (n=3) GQ854280, SES050814_07, Venezuela GQ854297, SES050819_01, Venezuela GQ854298, SES050819_02, Venezuela
GQ854282_AttaLaevVEAnzoSoledadS1409	*Atta laevigata* (n=4) GQ854282, SES050814_09, Venezuela GQ854287, SES050816_06, Venezuela GQ854288, SES050816_07, Venezuela GQ854293, SES050818_03, Venezuela
GQ854357_TrachTurrUSTxU0502	*Trachymyrmex turrifex* (n=3) GQ854357, UGM051105_02, USA GQ854359, UGM060305_02, USA GQ854360, UGM060403_08, USA
GQ854098_TrachArizUSAzCochiseCR0601	*Acromyrmex striatus* (n=1) GQ854318, UGM030404_02, Argentina *Acromyrmex? sp.* (n=1) GQ853971, AGH020708_01, Costa Rica *Trachymyrmex arizonensis* (n=6) GQ854098, CR050806_01_Tarizonensis, USA GQ854128, ML050806_1023_Tarizonensis, USA GQ854138, RAJ3560_Tarizonensis, USA GQ854139, RAJ3565_Tarizonensis, USA GQ854140, RAJ3566_Tarizonensis, USA GQ854142, RAJ3599_Tarizonensis, USA *Trachymyrmex carinatus* (n=2) GQ854099, CR050806_02_Tcarinatus, USA GQ854100, CR050806_03_Tcarinatus, USA *Trachymyrmex pomonae* (n=1) GQ854101, CR050810_02_Tpomonae, USA *Trachymyrmex smithi* (n=1) GQ854105, CR070425_01_Tsmithi, USA *Trachymyrmex* sp. (n=1) GQ854141, RAJ3595_Tsp_nov_SWRS, USA

1709 **Table S6.** Foraging preferences of leafcutter ant species and their fungicultural specializations on Clade-
1710 A fungi, Clade-B fungi, or both types of fungi.
1711

Table S6. Foraging preferences of leafcutter ant species and their fungicultural specializations on Clade-A or Clade-B fungi (or cultivation of both types of fungi; see details in Table S10). Preferences to forage primarily on dicot plants, monocot plants (grasses, sedges), or both (dicots & grass) are taken from Gonçalves (1961), Mariconi (1970), Fowler *et al.* (1986), Herrera (2008), Nagamoto *et al.* (2009), Lopes (2005), Wetterer 1995, Wetterer *et al.* 2001, and observations by Flavio Roces and Ulrich Mueller.

Ant Genus	Ant Species	Plant Substrate Typically Cut	Fungiculture Clade-A or Clade-B	Fungus Identification s = sequencing ITS, LSU, or EF m = microsatellite genotyping	Sample Size Total Cultivars Identified Between All Studies	References for Fungus Identification
Acromyrmex	*ambiguus*	dicot	A	s, m	10	this study; Peirera *et al.* 2015
	aspersus	dicot	A	s, m	8	this study
	coronatus	dicot	mostly A, some B	s, m	21	this study
	crassispinus	dicot	both A & B	s, m	5	this study; Silva-Pinhati *et al.* 2004
	disciger	dicot	A	s, m	4	this study; Silva-Pinhati *et al.* 2004
	echinatior	dicot	A	s, m	43	this study; Kooij *et al.* 2015a&b; Wallace *et al.* 2014; Poulsen *et al.* 2009; Mikheyev *et al.* 2006, 2007
	hispidus	dicot	A	s, m	6	this study; Silva-Pinhati *et al.* 2004
	hystrix	dicot	A	s, m	7	this study
	laticeps	dicot	A	s, m	9	this study; Silva-Pinhati *et al.* 2004
	lundii	dicot	A	s, m	11	this study
	nobilis	dicot	A	s, m	1	this study
	octospinosus	dicot	A	s, m	54	this study; Kooij *et al.* 2015a; Wallace *et al.* 2014; Poulsen *et al.* 2009; Mikheyev *et al.* 2006, 2007
	pubescens	dicot	A	s	1	Bich *et al.* 2016
	rugosus	dicot	A	s, m	2	this study; Silva-Pinhati *et al.* 2004
	subterraneus	dicot	A	s, m	7	this study; Silva-Pinhati *et al.* 2004
	versicolor	dicot, some grass	A	s, m	35	this study; Mueller *et al.* 2011
	volcanus	dicot	A	s, m	1	this study
	balzani	grass	A	s, m	4	this study
	fracticornis	grass	B	s	1	this study
	heyeri	grass	A	s, m	18	this study; Peirera *et al.* 2015
	landolti	grass	A	s, m	20	this study
	lobicornis	both	A	s	9	this study; Lugo *et al.* 2013
	striatus	both	B	s	1	this study
Atta	*cephalotes*	dicot	A	s, m	194	this study; Kooij *et al.* 2015a; Wallace *et al.* 2014; Mueller *et al.* 2011; Mikheyev *et al.* 2006, 2007; Silva-Pinhati *et al.* 2004; Chapela *et al.* 1994
	colombica	dicot	A	s, m	39	this study; Kooij *et al.* 2015b; Wallace *et al.* 2014; Mikheyev *et al.* 2006, 2007
	insularis	dicot	A	s, m	5	this study; Mueller *et al.* 2011
	mexicana	dicot	A	s, m	7	this study; Mueller *et al.* 2011; Chapela *et al.* 1994
	sexdens	dicot	A	s, m	51	this study; Kooij *et al.* 2015a; Mikheyev *et al.* 2007; Silva-Pinhati *et al.* 2004
	texana	dicot	A	s, m	165	this study; Mueller *et al.* 2011; Mikheyev *et al.* 2006, 2008
	bisphaerica	grass	A	s	1	Silva-Pinhati *et al.* 2004
	capiguara	grass	A	s	1	Silva-Pinhati *et al.* 2004
	vollenweideri	grass, some dicot	B	s	8	this study
	laevigata	both	mostly B, few A	s	28	this study; Silva-Pinhati *et al.* 2004

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Table S7. Total number of alleles/fungus at 5 microsatellite loci for fungi from *Atta* and *Acromyrmex* nests, summarized for narrow geographic region from within which both *Atta* and *Acromyrmex* were collected and genotyped at all 5 loci. Total number of alleles/fungus does not differ between fungi from *Atta* and *Acromyrmex* nests (Wilcoxon Signed-Rank Test, W = 90.5, z = 0.181, p = 0.857 two-tailed, n = 19), comparing samples from 19 locations (highlighted in blue in Table S7) for which complete genotype information (at all 5 loci) is available for both *Atta*-cultivated and *Acromyrmex*-cultivated fungi from that location. Table S7 summarizes the raw data and statistics in Table S8. Across the 19 locations, total number of alleles/fungus is correlated between *Atta*- and *Acro*-cultivated fungi (Fig. S6), i.e., at locations where *Atta*-cultivated fungi show more alleles, the sympatric *Acro*-cultivated fungi show likewise more alleles (Spearman rank-order correlation t = 2.39, df = 17, p = 0.029, r = 0.502; Fig. S6), indicating that, at each location, *Atta* and *Acromyrmex* recruit cultivars from the same shared community of fungi.

Country, Province/State	**Average Total Number Alleles/Fungus Across All 5 Loci Screened**	
	Fungi from *Atta* nests	**Fungi from *Acromyrmex* nests**
USA	**13.6** (n=11 nests, 1 ant species)	**12.6** (n=7 nests, 1 ant species)
Mexico	**11.2** (n=15 nests, 2 ant species)	no fungus from *Acromyrmex* collected
Cuba	**10.8** (n=5 nests, 1 ant species)	no fungus from *Acromyrmex* collected
Honduras	**10.0** (n=1 nest, 1 ant species)	no fungus from *Acromyrmex* collected
Costa Rica, Heredia Province	**10.6** (n=5 nests, 1 ant species)	**9.0** (n=1 nest, 1 ant species)
Panamá All	**9.9** (n=61 nests, 1 ant species)	**8.3** (n=22 nests, 3 ant species)
Panamá West, Bocas del Toro Province	**11.4** (n=20 nests, 1 ant species)	**9.8** (n=6 nests, 1 ant species)
Panamá Central Coclé Province	**9.7** (n=6 nests, 3 ant species)	**10.2** (n=2 nests, 1 ant species)
Panamá Canal Zone	**9.1** (n=26 nests, 3 ant species)	**7.5** (n=12 nests, 2 ant species)
Panamá El Llano	**9.3** (n=4 nests, 2 ant species)	no fungus from *Acromyrmex* collected
Panamá East, Darien Province	**9.0** (n=5 nests, 1 ant species)	**7.0** (n=2 nests, 1 ant species)
Colombia West, Antiochia Province	**9.3** (n=12 nests, 2 ant species)	**9.4** (n=11 nests, 3 ant species)
Trinidad & Tobago	**12.1** (n=8 nests, 1 ant species)	no fungus from *Acromyrmex* collected
Venezuela	**9.3** (n=18 nests, 2 ant species)	**11.0** (n=2 nests, 2 ant species)
Guyana	**8.0** (n=3 nests, 1 ant species)	**9.0** (n=3 nests, 2 ant species)
French Guiana	**7.5** (n=12 nests, 2 ant species)	**7.8** (n=13 nests, 2 ant species)
Ecuador	**6.2** (n=9 nests, 1 ant species)	**6.4** (n=5 nests, 2 ant species)
Peru All	**7.4** (n=45 nests, 2 ant species)	**6.8** (n=6 nests, 3 ant species)
Peru North	**7.6** (n=16 nests, 2 ant species)	**9.0** (n=1 nest, 1 ant species)
Peru South	**7.2** (n=25 nests, 2 ant species)	**6.4** (n=5 nests, 2 ant species)
Brazil North All	**9.8** (n=29 nests, 2 ant species)	**9.6** (n=22 nests, 4 ant species)
Brazil North 1, Amapá State	**8.0** (n=2 nests, 2 ant species)	**10.0** (n=1 nest, 1 ant species)
Brazil North 2, Pará State	**8.3** (n=7 nests, 2 ant species)	**10.5** (n=12 nests, 2 ant species)
Brazil North 3, Amazonas State	**8.0** (n=1 nest1, 1 ant species)	no fungus from *Acromyrmex* genotyped at all 5 loci
Brazil North 4, Pernambuco State	**11.6** (n=7 nests, 2 ant species)	**10.6** (n=5 nests, 2 ant species)
Brazil North 5, Mato Grosso State north	**9.8** (n=4 nests, 2 ant species)	**5.3** (n=4 nests, 1 ant species)
Brazil North-East, Bahia State	**10.3** (n=8 nests, 2 ant species)	no fungus from *Acromyrmex* genotyped at all 5 loci
Brazil South All	**7.8** (n=4 nests, 1 ant species)	**8.9** (n=49 nests, 11 ant species)
Brazil South, Sao Paulo State	**9.0** (n=2 nests, 1 ant species)	**10.5** (n=2 nests, 1 ant species)
Brazil South, Paraná State	**6.5** (n=2 nests, 1 ant species)	**8.3** (n=3 nests, 1 ant species)
Brazil South, Santa Catarina State	no fungus from *Atta* collected	**8.6** (n=5 nests, 3 ant species)
Brazil South, Rio Grande do Sul State	no fungus from *Atta* collected	**9.1** (n=39 nests, 9 ant species)
Argentina	no fungus from *Atta* genotyped	**8.2** (n=10 nests, 4 ant species)
Uruguay	no fungus from *Atta* collected	**7.5** (n=2 nests, 2 ant species)

Table S8. Raw data used to generate the statistics summarized in Table S7.

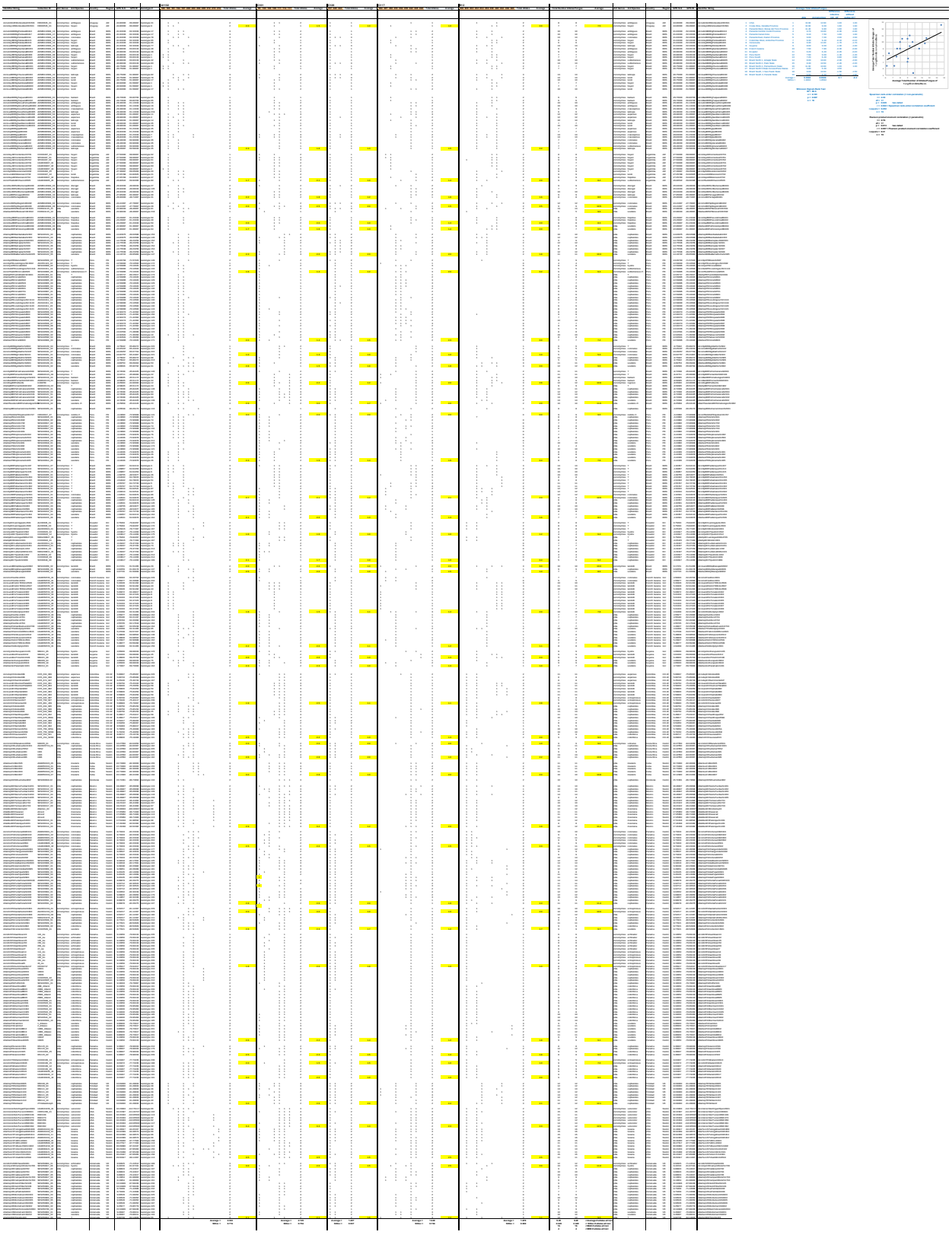


Table S9. Primer sequences developed by Mikheyev *et al.* (2006) for EF-1α, DMC1, and RAD51 genes, as well as corresponding annealing temperatures (Tm).

Gene	Forward-F (5' → 3')	Reverse-R (5' → 3')	Tm
EF-1α	GTT GCT GTC AAC AAG ATG GAC ACT AC	GCC TTG ATG ATA CCA GTC TCG ACA CG	55 °C
DMC1	AAG CTG CAC ACA AAA TCT TGG TTA G	GTC AAT GTC AAG AGA TCG GAT ACA C	51 °C
RAD51	GGC AAA TGT TTG TAT ATA GAT ACT G	CAC CGA TAG GTT TCT TCT CAT TAC C	51 °C

Table S10. Summary of higher-attine ant species found so far to cultivate both Clade-A and Clade-B fungi. Each of these ant species could potentially represent different cryptic species, and the different cryptic species may be specialized on different fungal clades. However, cryptic ant species have not been found in the sequencing analyses of *Atta laevigata* (Fig. 4 in Solomon *et al.* 2008) and *Trachymyrmex arizonensis* (Fig. 21 in Rabeling *et al.* 2007). The below information is summarized from Table S1 (this study), Table S1 of Mueller *et al.* (in review), and one unpublished collection (UGM080928-02).

Atta laevigata

Of 28 cultivars from 28 *Acromyrmex laevigata* nests characterized so far, 26 are Clade-B fungi (Genbank sequences GQ854042, GQ854203, GQ854204, GQ854207, GQ854209, GQ854223, GQ854224, GQ854345, GQ854275, GQ854277, GQ854278, GQ854280, GQ854281, GQ854282, GQ854285, GQ854286, GQ854287, GQ854288, GQ854292, GQ854293, GQ854294, GQ854295, GQ854296, GQ854297, GQ854298, GQ854299) and 2 are Clade-A fungi (Genbank sequences AF076409-AF076416 from six cloned ITS sequences from mycelium from a single garden of *At. laevigata* reported in Silva-Pinhati *et al.* 2004; ADD GENBANK # from this study here). The 26 Clade-B-cultivating nests were collected in Venezuela, French Guyana, and throughout Brazil (States of Amapá, Amazonas, São Paulo; Table S1). The Clade-A-cultivating nests were collected in Rio Claro, São Paulo, Brazil (Silva-Pinhati *et al.* 2004), and near Piracanjuba, Goiás, Brazil (collection UGM080928-02, a young nest with a single garden of about 3 cm diameter at 18 cm depth, with garden substrate of "only grass", characteristic for *At. laevigata*). The corresponding ant hosts were identified from morphological characters and sequencing by Solomon *et al.* (2008), and from morphological characters by Silva-Pinhati *et al.* (2004). A worker of the Clade-A-cultivating nest UGM080928-02 was identified by sequencing of the mitochondrial cytochrome-oxidase 1 (CO1) gene (see below; GENBANK #), and the fungus of that same nest UGM080928-02 was identified by sequencing the EF-1α gene (see below; GENBANK #). Locations of vouchers are listed in Table S1. Clade-B- and Clade-A-cultivating nests of *At. laevigata* therefore appear to be sympatric in southern Brazil.

Genbank accessions for nest UGM080928-02 are not yet released, so we list here actual sequences:
EF-1α sequence of fungus (Clade-A cultivar) from nest UGM080928-02:
ATTCCGATTATACTGATCATGTCAGTGTTGATGTCCGTTTCAGTGGAGTGAGGACCGTTTCAATGAAATCATTAAGGAAACTTCCGTTTTCAT
CAAGAAGGTCGGTTACAACCCGAAGGCCGTTGCCTTCGTTCCCATTTCTGGATGGTTGGGTGACAACATGTTGGAGGAATCTCCCAAGTAC
TTCAATTTCTTATTACGAGAATTCTATTTGGTTCTAATTGATTCATTTCAGCATGTCCTGGTACAAGGGTTGGCAGAAAGAGACTAAGGCTGG
TGTCGTCAAAGGCAAGACTCTCCTCGATGCTATTGACGCTATTGAACCTCCCGTACGTCCGTCTGAGAAGCCTCTCCGTCTCCCCCTCCAG
GATGTCTACAAAATCGGTGGTATTGGTACAGTGCCGGTTGGTCGTGTCGAGACTGGTATCATCAAGGCA
This EF-1α sequence of fungus UGM080928-02 is identical to other EF-1α sequences known from Clade-A cultivars from nests of other leafcutter species across South America (appearing in our Fig. 1).

CO1 sequence of worker ant (*Atta laevigata*) from nest UGM080928-02:
GATTTTTTGGTCATCCAGAAGTTTATATTTTAATCCTACCAGGATTTGGTTTAATCTCACAAATTATTATAAGAGAAAGAGGAAAAAAAGAAAC
TTTCGGAACCTTAGGAATAATTTATGCTATAATAGCTATTGGTCTTTTAGGTTTTATCGTTTGAGCTCATCATATATTTACTATTGGTCTTGAT
GTTGACACCCGAGCTTATTTTACTTCTGCTACTTTAATCATTGCTATCCCAACTGGAATTAAAGTTTTTAGATGATTAGCAACACTTCACGGT
ATAAAAATTAATTATAATCCTGCCTTATGATGATCATTGGGATTTATCTTTTTATTTTCAATAGGAGGACTCACAGGAATTATACTATCTAACTC
TTCTATTGATATTGTTCTCCATGATACCTAT
This CO1 sequence of ant UGM080928-02 is, with one nucleotide difference, identical to the CO1 sequence of *At. laevigata* SES040201-02, collected by Scott Solomon in 2004 in Itaúba, Mato Grosso, Brazil, about 900km distant from collection UGM080928-02 from Piracanjuba, Goiás, Brazil. No garden was collected for nest SES040201-02, and information on Clade-A/B cultivation is therefore not available for that nest. The phylogenetic position of SES040201-02 is shown in Fig. 4 of Solomon *et al.* (2008) as part of *laevigata*-subgroup B of the within-species diversity of *At. laevigata*, suggesting that the *Atta laevigata* collection UGM080928-02 from Piracanjuba, Goiás, also belongs to *laevigata*-subgroup B.

Acromyrmex coronatus

Of 21 cultivars from 21 *Acromyrmex coronatus* nests characterized so far, 19 are Clade-A fungi (Genbank sequences GQ853984, GQ854012, GQ854043, GQ854045, GQ854311, GQ853985, GQ853986,

GQ854025, GQ854027, GQ854198, GQ854199, GQ854200, GQ854219, GQ854328, GQ854332, GQ854044, GQ854046, GQ854310, GQ855074) and 2 are Clade-B fungi (GQ854030, GQ854031). The Clade-A-cultivating nests were collected in Panamá, French Guyana, northern Brazil, and southern Brazil. The two Clade-B-cultivating nests were collected in Blumenau, Santa Catarina, Brazil, and the closest Clade-A-cultivating nests were collected 40 km distant from Blumenau in Itajai, Santa Catarina, Brazil, and in various locations in the neighboring State of Rio Grande do Sul, Brazil. The corresponding ant hosts were identified from morphological characters by researchers in the Bacci Lab. Locations of vouchers are listed in Table S1. Clade-A- and Clade-B-cultivating nests of *Ac. coronatus* appear to be sympatric in the Blumenau area in Santa Catarina State, Brazil.

Acromyrmex crassispinus

Of 4 cultivars from 21 *Acromyrmex crassispinus* nests characterized so far, 3 are Clade-A fungi (Genbank sequences GQ853997, GQ853999, GQ853987) and 1 is a Clade-B fungus (GQ854036). The 3 Clade-A-cultivating nests were collected in Ipê and in Nova Petrópolis, Rio Grande do Sul, Brazil. The Clade-B cultivating nest was collected in Tibagi, Paraná, Brazil, about 500 km north of the collection sites in Rio Grande do Sul. The corresponding ant hosts were identified from morphological characters by researchers in the Bacci Lab. Locations of vouchers are listed in Table S1. Because of the distance of 500 km between the Clade-B- and Clade-A-cultivating nests of *Ac. crassispinus*, additional collections from the States of Paraná and Rio Grande do Sul are needed to establish sympatry of Clade-A- and Clade-B-cultivating nests of *Ac. crassispinus*.

Acromyrmex laticeps

Of 8 cultivars from 8 *Acromyrmex laticeps* nests characterized so far, 7 are Clade-A fungi (Genbank sequences GQ854029, GQ853990, GQ853991, GQ854010, GQ854016, GQ854028) and 1 is a Clade-B fungus (GQ854034). The 7 Clade-A-cultivating nests were collected in the States of Santa Catarina and Rio Grande do Sul, Brazil. The Clade-B-cultivating nest was collected in Blumenau, Santa Catarina, about 150 km distant from the closest collection sites of a Clade-A-cultivating nest in Lages, Santa Catarina. The corresponding ant hosts were identified from morphological characters by researchers in the Bacci Lab. Locations of vouchers are listed in Table S1. Because of the distance of 150 km between the Clade-B- and Clade-A-cultivating nests of *Ac. laticeps*, additional collections are needed to establish sympatry of Clade-A- and Clade-B-cultivating nests of *Ac. laticeps*.

Trachymyrmex arizonensis

Of 8 cultivars from 8 *Trachymyrmex arizonensis* nests characterized so far, 7 are Clade-B fungi (Genbank sequences GQ854098, GQ854128, GQ854138, GQ854139, GQ854140, GQ854142, GQ855143) and 1 is a Clade-A fungus (Genbank sequence GQ854102). The Clade-A-cultivating nest was collected by Christian Rabeling (CR050811- 02) near the South-West Research Station, Chiricahua Mountains, Cochise County, Arizona, within 1 km of a Clade-B-cultivating nest also collected by Christian Rabeling (CR050806-01), and close to two other Clade-B-cultivating nests collected by Robert Johnson and Ulrich Mueller near the South-West Research Station. The corresponding ant hosts were identified from morphological characters and by sequencing of the mitochondrial cytochrome-oxidase 1 (CO1) gene by Christian Rabeling. Ant-sequences from the Clade-A-cultivating nest CR050811- 02 and the Clade-B-cultivating nest CR050806-01 appear in Fig. 21 of Rabeling *et al.* 2007, indicating no sequence divergence at the CO1 gene that would support separate species. Locations of vouchers are listed in Table S1. In the Chiricahua Mountains of Arizona, therefore, Clade-B- and Clade-A-cultivating nests of *T. arizonensis* appear to be sympatric.

Trachymyrmex intermedius

Of 4 cultivars from 4 *Trachymyrmex intermedius* nests characterized so far, 2 are Clade-B fungi (Genbank sequences GQ854329, JX258959) and 2 are Clade-A fungi (Genbank sequences GQ854325, GQ854326). The sequence JX258959 is part of the phylogenetic analysis of Mueller *et al.* (in review; see

Table S1 in that study), but is not part of our study here. All four nests were collected by Ulrich Mueller within about 200 meters of each other at the Amazon Nature Lodge, Kaw Mountains, French Guiana. The corresponding ant hosts were identified from morphological characters by Scott Solomon. Locations of vouchers are listed in Table S1. In the Kaw Mountains of French Guiana, therefore, Clade-B- and Clade-A-cultivating nests of *T. intermedius* appear to be sympatric.

Fig. S1. Phylogenetic trees inferred in Bayesian (left) and likelihood (right) analyses of EF-1α sequence information. Methods are described in the main text. The EF-1α alignment (475 bases length) will be deposited at DRYAD. Both trees show a lack of resolution among Clade-A fungi cultivated by higher-attine ants, therefore requiring genotyping of Clade-A fungi with microsatellite markers (Fig. 2).

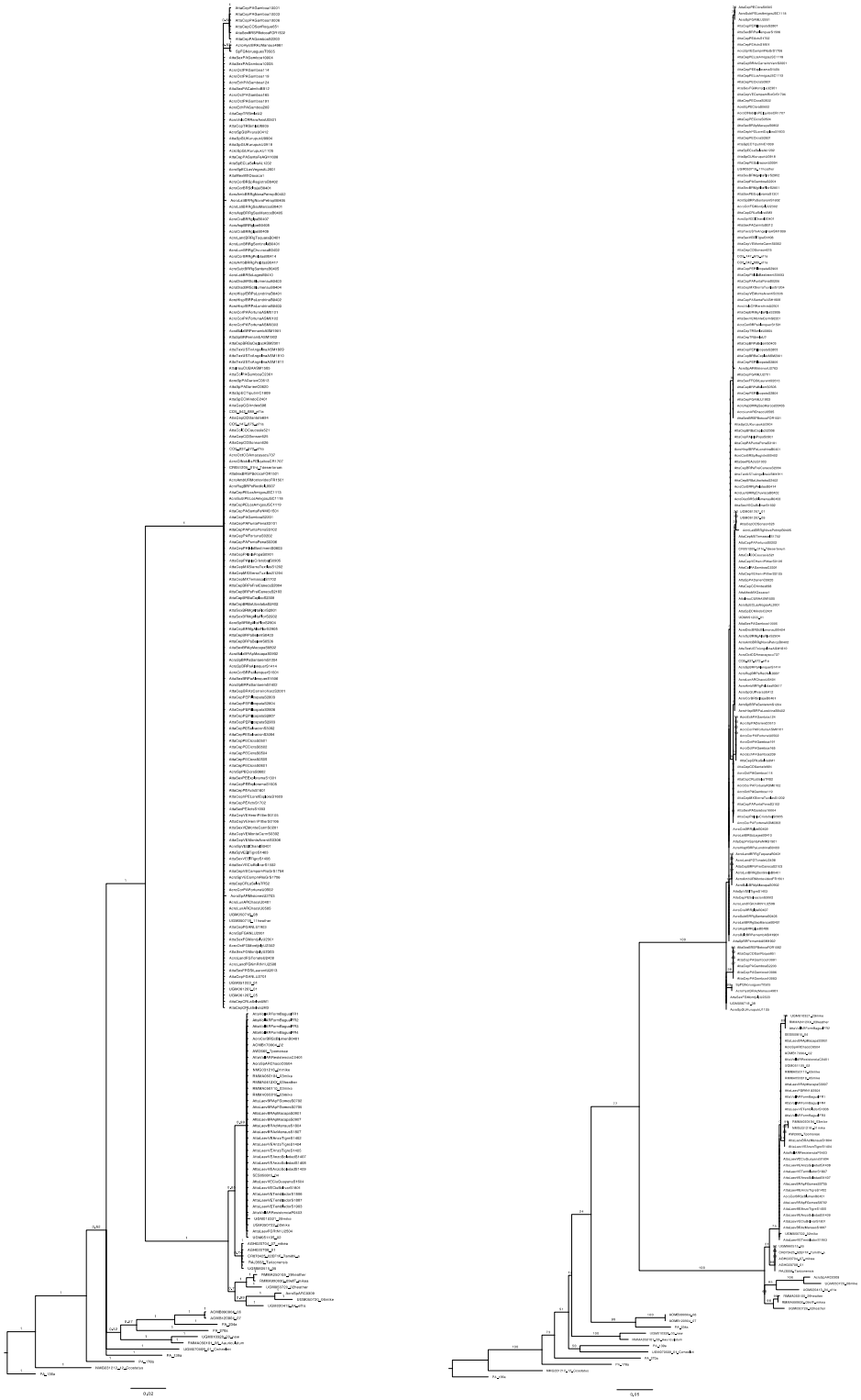


Fig. S2. Phylogenetic tree inferred in preliminary analyses under the likelihood criterion using available sequence information from the RAD gene. As in the EF-1α and DMC trees (Figs. S1 & S3), the RAD tree lack of resolution among Clade-A fungi cultivated by higher-attine ants. Because this analysis was preliminary to test the utility of the RAD gene, and because our results and conclusions do not use information from the RAD gene, we have not deposited the preliminary RAD alignment (515 bases length) at DRYAD.

Fig. S3. Phylogenetic tree inferred in preliminary analyses under the likelihood criterion using available sequence information from the DMC gene. Apart from a basal split, the DMC tree shows a lack of resolution among Clade-A fungi cultivated by higher-attine ants. Because this analysis was preliminary to test the utility of the DMC gene, and because our results and conclusions do not use information from the DMC gene, we have not deposited the preliminary DMC alignment (425 bases length) at DRYAD.

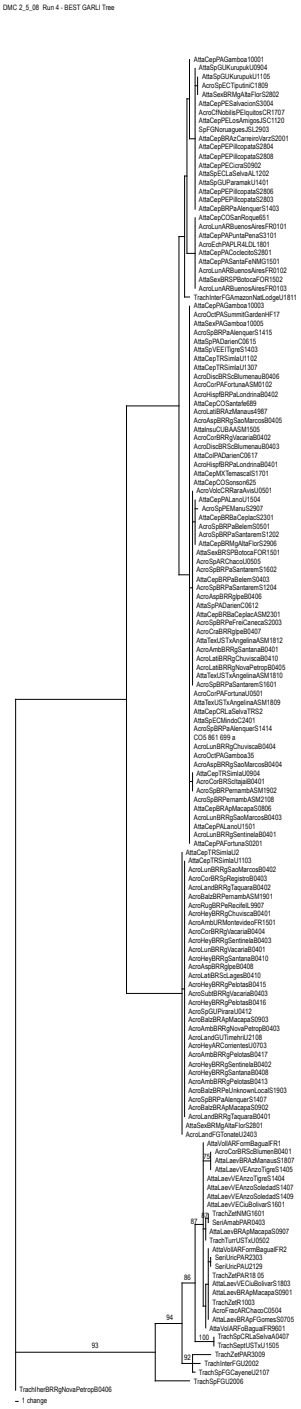
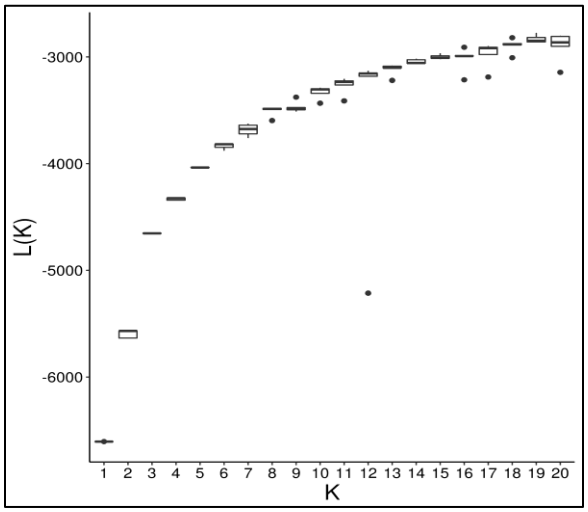


Fig. S4. Estimation of K from STRUCTURE analysis of fungal microsatellite marker profiles.
A. & B. Using the default settings in STRUCTURE, we ran STRUCTURE using K=1 to K=20 clusters (n=5 replicates each, 100,000 iterations for parameter estimation after a burn-in of 100,000 iterations), calculated the difference in the posterior probability of K and K-1, and selected the value of K with the greatest relative increase in log-likelihood, as recommended by Evanno *et al.* (2005).
C & D. 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., **α**=0.1) (Wang 2017).

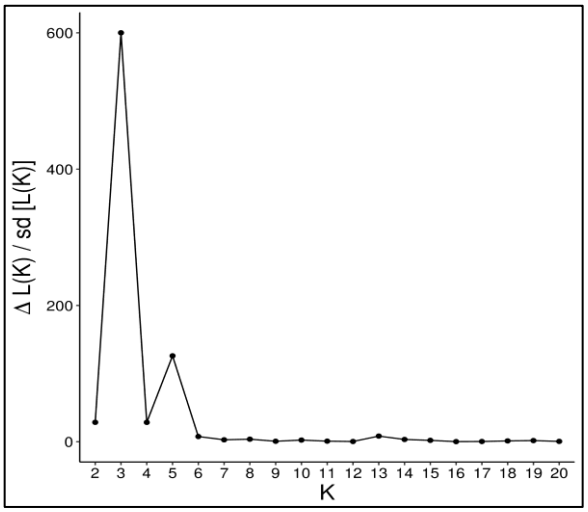
Both the default settings and the modified settings yield identical recommendations of K=3.

A. Default settings: Boxplots of the log-likelihoods of K ranging from 1 to 20.
B. Default settings: Change in the average log-likelihood of K [L(K) – L(K-1)] divided by the standard deviation of K.
C. Modified settings: Rate of change of likelihood distribution [calculated as L′(K) = L(K) – L(K – 1)].
D. Modified settings: Absolute values of the second order rate of change of the likelihood distribution (mean ± SD) calculated according to the formula: |L″(K)| = |L′(K + 1) – L′(K)|.

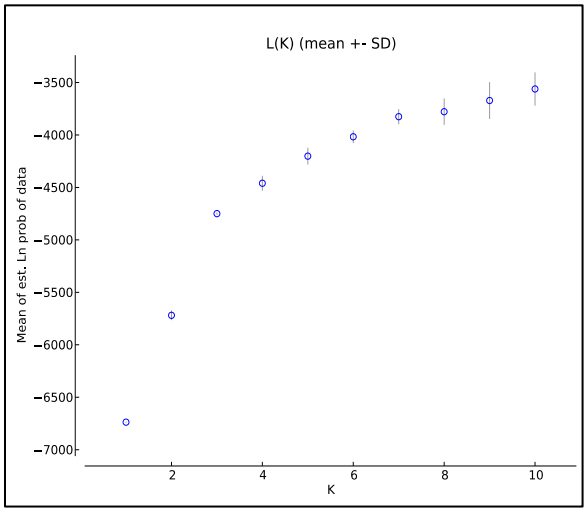
A.



B.



C.



D.

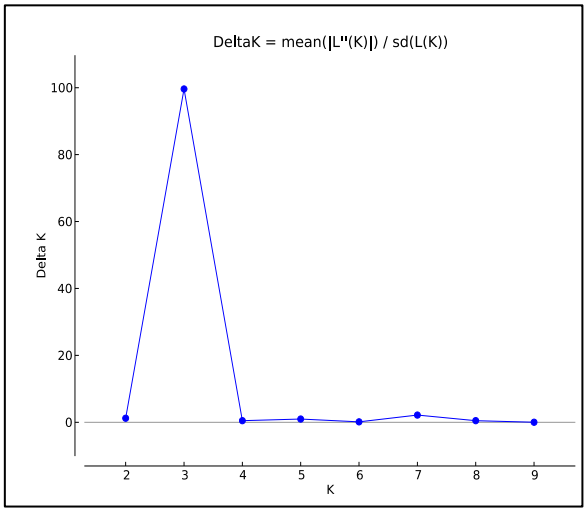
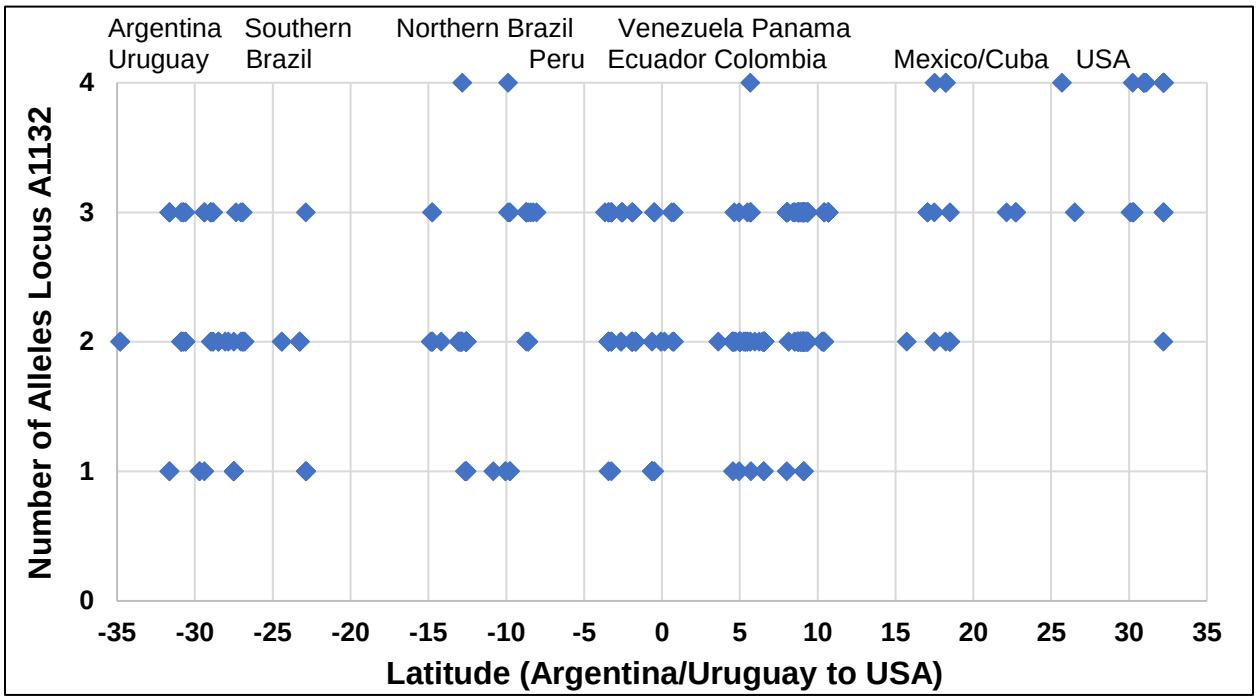


Fig. S5 A-E. Number of alleles per fungus at each individual locus (A1132, C126, C101, B12, C117) as a function of latitude. These analyses include only the 388 samples for which allele information is available for all five loci (i.e., any sample for which allele information is missing for at least one locus is excluded here; including these additional samples does not change overall patterns; data in Table S8).



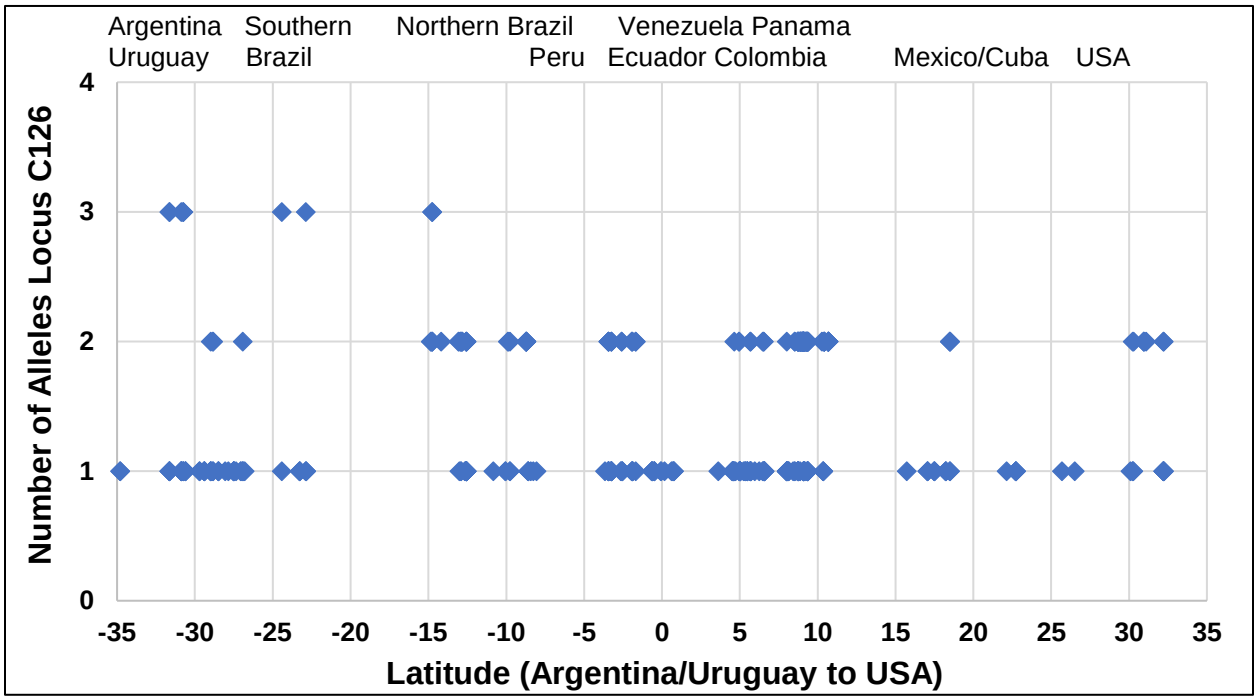
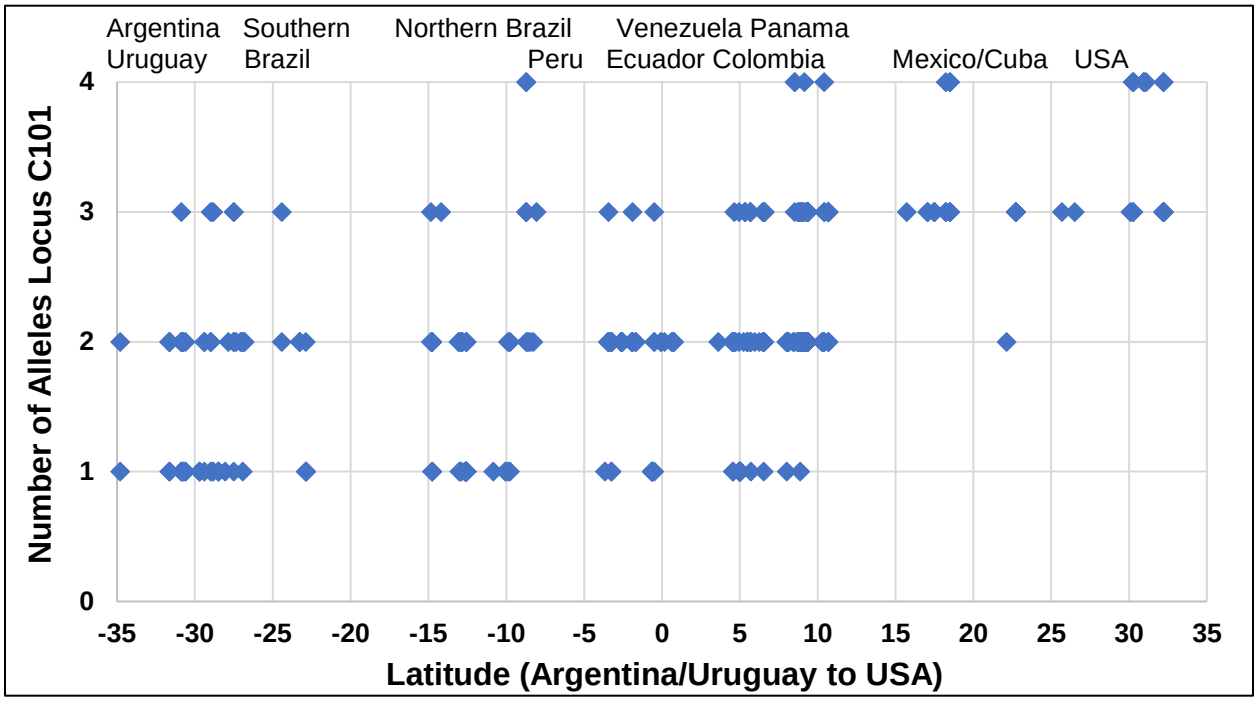


Fig. S5 (continued). Note that, at locus B12, some individuals show null alleles in some populations in northern South America (allele number is zero for these individuals at locus B12).



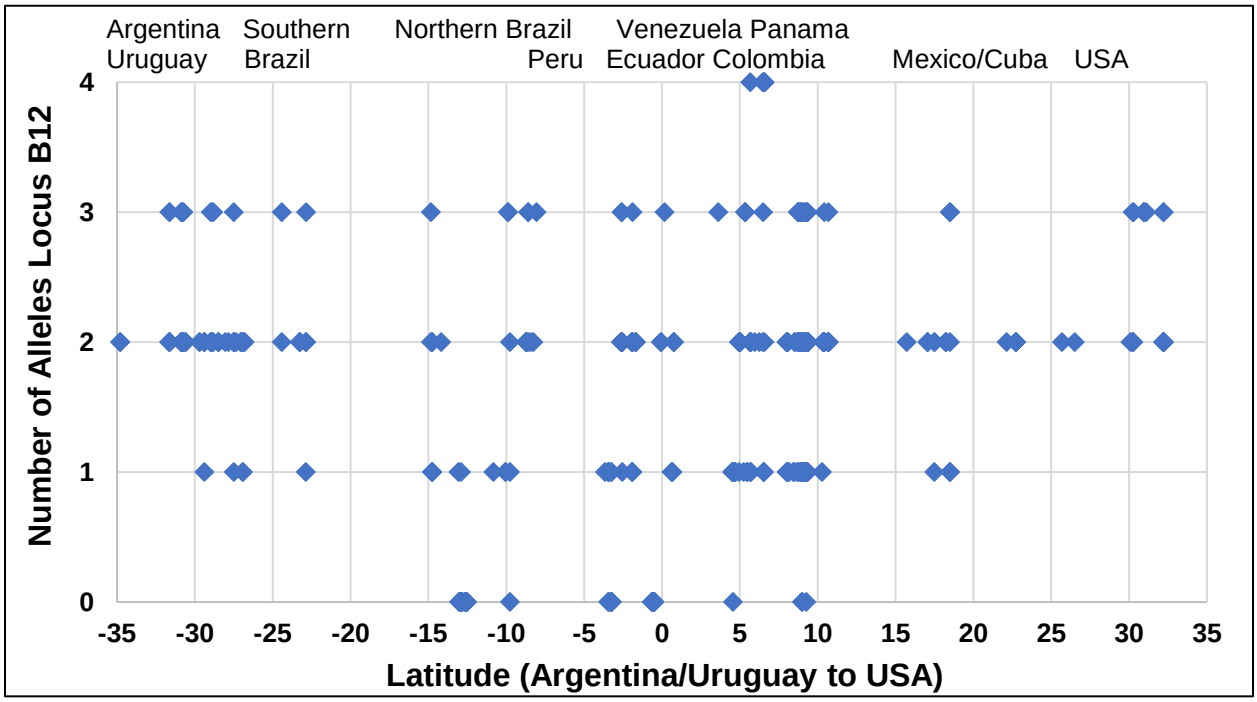


Fig. S5 (continued).

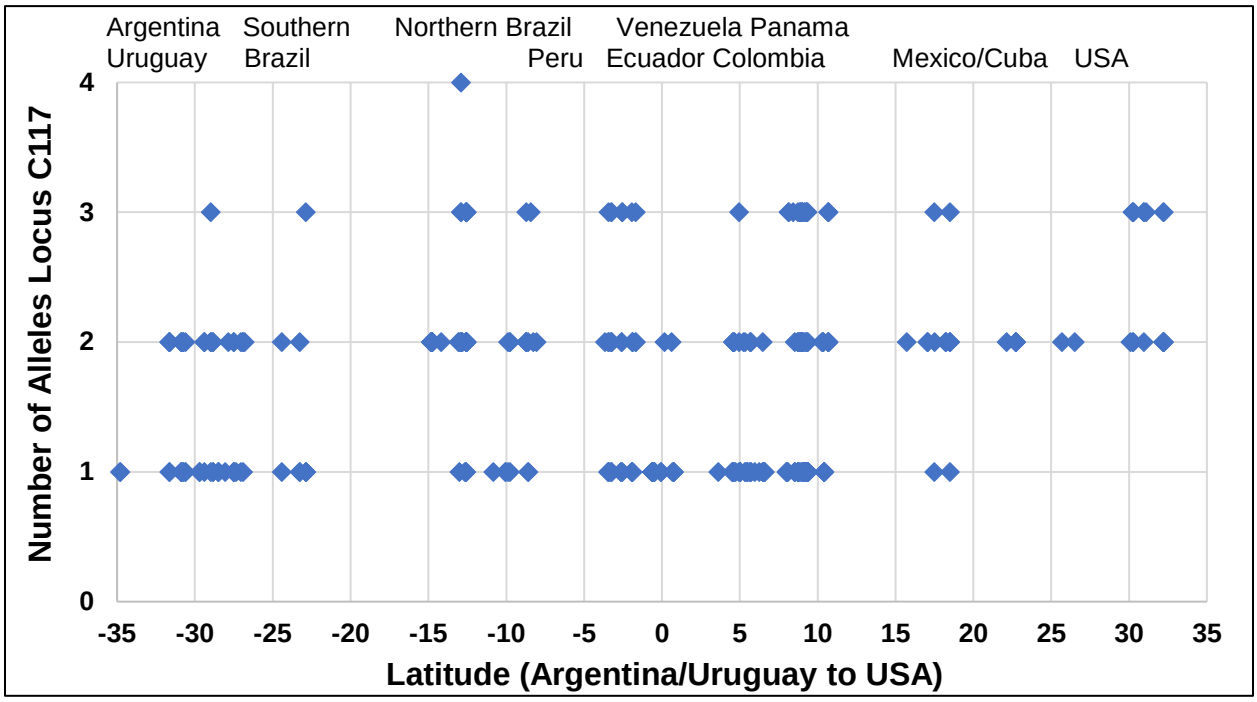


Fig. S6. Correlation between total allele diversity (allele richness) of *Atta*-cultivated and *Acromyrmex*-cultivated fungi, comparing samples from 19 locations (highlighted in blue in Table S7) for which genotype information is available for both *Atta*-cultivated and *Acromyrmex*-cultivated fungi. The graph plots for each of the 19 locations the average total number of alleles observed in fungi at 5 microsatellite loci (all alleles/fungus summed across the 5 loci, then averaged across all samples from a genus of ant collected at a location). The data plotted in the graph are listed also in Table S7, which summarizes information from the raw data in the Excel sheet in Table S8. Statistics of the Spearman rank-order correlation are $t = 2.39$, $df = 17$, $p = 0.029$, $r = 0.502$, $r^2 = 0.252$, $n = 19$. If *Atta*-cultivated and *Acromyrmex*-cultivated fungi represent separate gene pools, as hypothesized by Kooij *et al.* (2015b), this positive correlation can be explained by parallel evolutionary forces that determine allele diversity independently (convergently) at different sites for both *Atta*-cultivated and *Acromyrmex*-cultivated fungi. Alternatively, the positive correlation can be explained by shared local pools of cultivars into which both *Atta* and *Acromyrmex* species tap at each location, for example because of local horizontal transfer of fungal cultivars between nests from the two leafcutter genera, because of some form of genetic exchange and hybridization locally between fungi cultivated by different ant nests, or both.

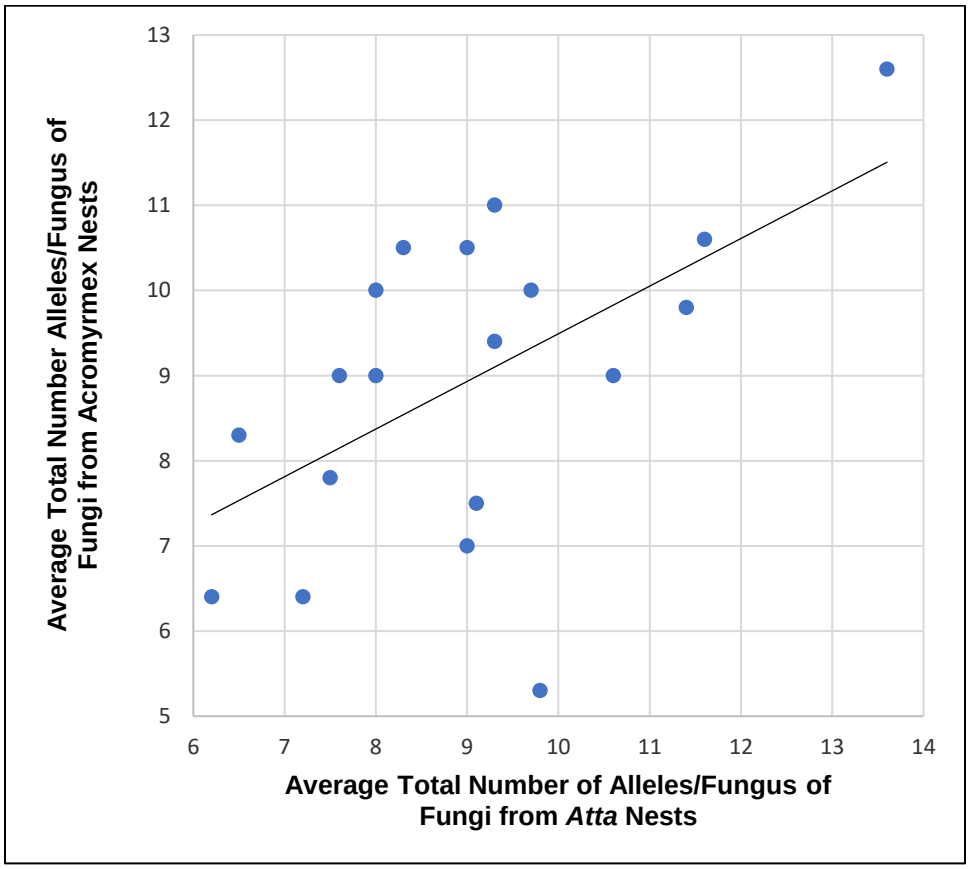
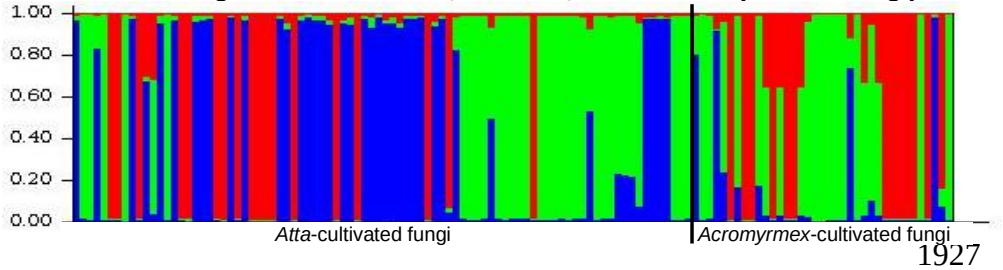
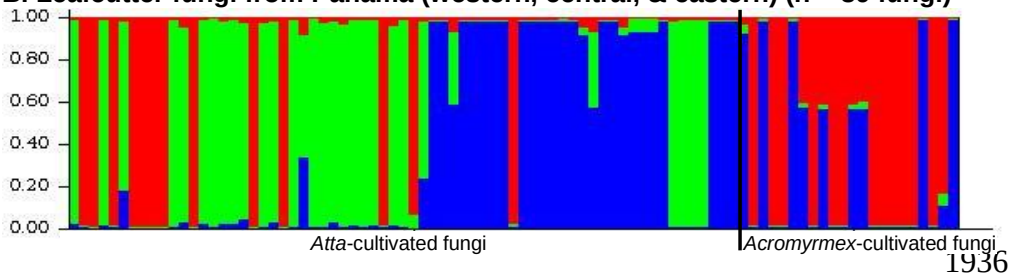


Fig. S7 A-D. Results of STRUCTURE analyses testing whether fungi are partitioned into *Atta*- versus *Acromyrmex*-cultivated fungi, using samples from Panamá and neighboring countries that were most comprehensively surveyed in our study. STRUCTURE analyses indicate K=3 fungal genotype-clusters for each of four datasets that were sub-sampled at increasingly more local scale: **A.** regional fungal diversity (Colombia, Panamá, & Costa Rica); **B.** within-country diversity (all Panamá); **C.** provincial diversity (Panamá Canal Zone); or **D.** the local diversity in Gamboa (town within Panamá Canal Zone) studied earlier also by Mikheyev *et al.* (2007) and Kooij *et al.* (2015b).

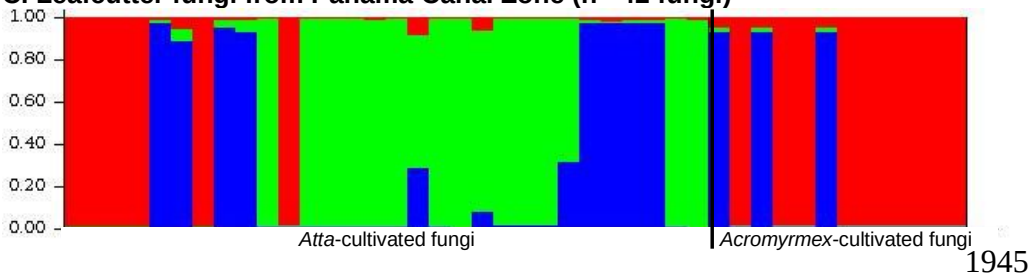
A. Leafcutter fungi from Costa Rica, Panamá, & Colombia (n = 125 fungi)



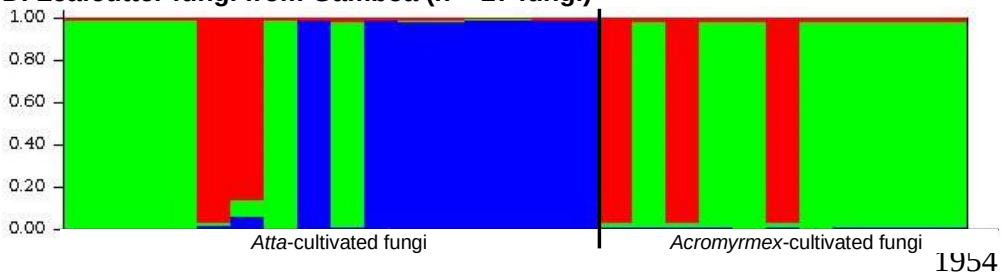
B. Leafcutter fungi from Panamá (western, central, & eastern) (n = 89 fungi)



C. Leafcutter fungi from Panamá Canal Zone (n= 42 fungi)



D. Leafcutter fungi from Gamboa (n = 27 fungi)



Our STRUCTURE analyses confirm the earlier finding by Mikheyev *et al.* (2007) that *Atta* and *Acromyrmex* ants from Gamboa tap locally into the same pool of fungal cultivars derived from several genotype-clusters, contrary to the conclusions of Kooij *et al.* (2015b). Specifically, Kooij *et al.* (2015b, page 13) write that "fungal symbionts of *Atta* and *Acromyrmex* colonies showed that they were completely separated … consistent with earlier findings by Mikheyev *et al.* (2007) for the same sampling site". Mikheyev *et al.* (2007) actually documented that *Atta* and *Acromyrmex* ants "shared identical fungus garden genotypes, indicating wide-spread cultivar exchange" (Abstract in Mikheyev *et al.* 2007).

The leafcutter cultivar lineages studied by Mikheyev *et al.* (2007, Abstract) were "largely unstructured with respect to host ant species, with only 10% of the structure in genetic variance being attributable to partitioning among ant species and genera". Using more microsatellite loci than in our study (and thus having greater resolution to differentiate between fungal genotypes), Figure 3 in Mikheyev *et al.* (2007) shows that fungal diversity associated with Panamanian leafcutter ants can be structured into 6 genotype-clusters, and that there exists little correlation between these fungal lineages and leafcutter ant genera (i.e., each of the leafcutter ant species cultivates locally representatives from most of these 6 fungal lineages). Therefore, the fungi examined by Kooij *et al.* (2015b) appear to have been selectively sampled from the true diversity of fungi cultivated by each leafcutter species in central Panamá and in Gamboa, such that Kooij *et al.* (2015b) oversampled one fungus lineage for the *Atta* ants studied (fungi from 9 nests) and oversampled another fungus lineage for *Acromyrmex* ants studied (fungi from 9 nests) (e.g., Kooij *et al.* 2015b may have oversampled the blue genotype-cluster for *Atta* ants and the green genotype-cluster for *Acromyrmex* ants shown in our Fig. S7 D). Our analyses of Panamanian leafcutter-fungus diversity show that *Atta* and *Acromyrmex* ants are not specialized on different cultivar lineages, but that the two ant genera cultivate in different localities representatives from different genotype-clusters at different frequencies.