

1 **Biogeography of Leafcutter Ant-Fungus Mutualisms**
2 **Biogeography of Fungi Cultivated by Leafcutter Ants**
3 **Biogeography of Leafcutter-Ant Fungiculture**

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55

56 **Abstract (249 words; limit of 250 words in Mol.Ecol.)**

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

76
77 **Key Words:** *Attamyces bromatificus*, *Leucoagaricus gongylophorus*, *Leucocoprinus gongylophorus*,
78 fungus-growing ant, insect-fungus mutualism, symbiosis

79
80
81 **Introduction**

82 Biogeographic distributions provide clues to infer evolutionary processes, such as ancient dispersal and
83 vicariance events shaping macroevolutionary patterns, or adaptation and gene flow influencing
84 microevolutionary processes (Wallace 1876; Brown & Lemolino 1998; Avise 2009). In mutualistic
85 associations between two partners, similarities or differences in biogeographic distributions between co-
86 dependent partners can facilitate inference of such evolutionary processes (Thompson 2005; Alvarez *et al.*
87 2010; Satler & Carstens 2016; Hembry & Althoff 2016). Co-biogeographic patterns of mutualistic
88 partners require cautious interpretation, however, particularly regarding congruence and incongruence of
89 patterns, because evolutionary forces and demographic histories can differ markedly between partners
90 (Herre *et al.* 1999; Alvarez *et al.* 2010; Espíndola *et al.* 2014; Tian *et al.* 2015; Chomicki *et al.* 2017).
91 For example, population sizes, migration rates, mutation rates, and generation times can differ by orders
92 of magnitude between a host and a symbiotic partner (Lutzoni & Pagel 1997; Moran & Wernegreen 2000;
93 Woolfit & Bromham 2003; Degnan *et al.* 2004; Douglas 2010), and dispersal barriers restricting gene
94 flow for one partner (e.g., pollinating bee) may not impede gene flow for the other partner (e.g.,
95 pollinated plant). Such differences in evolutionary forces are particularly pronounced in mutualistic
96 associations between macro-organisms and fast-evolving microbial symbionts, or microbial symbionts
97 that do not co-migrate with a host, disperse independently of the host, and that are acquired by hosts from
98 local microbial populations (e.g., many plant-endophyte, mycorrhizal plant-fungus, lichen algal-fungus,
99 or host-microbe gut mutualisms) (Wornik & Grube 2010; Dal Grande *et al.* 2012; Silverstein *et al.* 2012;
100 Kaltenpoth *et al.* 2014; Weiblen & Treiber 2015; Palmer *et al.* 2015).

101
102 In many mutualistic host-microbe associations, a greater dispersal ability of the microbial partners results
103 in predictable differences in population-genetic and biogeographic patterns between hosts and microbial
104 symbionts, for example lesser genetic differentiation between populations for the symbiont compared to
105 the host (Nobre *et al.* 2011; Six 2012; Mueller *et al.* 2011a; Kellner *et al.* 2013; Hulcr & Stelinski 2017),
106 or greater potential for a single symbiont lineage to interact with different allopatric host species or with

107 geographically differentiated host populations of the same host species (Thompson 2005; Mueller &
108 Gerardo 2002; Weiblen & Treiber 2015; Palmer *et al.* 2015). In contrast, when symbiont dispersal is
109 limited, populations of symbionts are predicted to differentiate across space, as for example in the
110 symbiotic ectomycorrhizal fungus *Rhizopogon* where limited dispersal by vectoring mammals maintains
111 population-genetic structure between proximate islands (Grubisha *et al.* 2007). As a general rule,
112 however, widely dispersing symbionts are thought to be associated with a greater diversity of hosts than
113 symbionts with limited dispersal (Herre *et al.* 1999; Roy *et al.* 2008; Douglas 2010). Biogeographic
114 analyses of such microbial symbionts are often complicated by insufficient knowledge of species
115 boundaries of microbial symbionts, requiring high-resolution genetic analyses to differentiate species- and
116 population-boundaries across space (e.g., Carriconde *et al.* 2008; Douhana *et al.* 2011; Gazis *et al.* 2011).

117
118 The mutualistic association between leafcutter ants (genera *Atta* and *Acromyrmex*) and their cultivated
119 fungi is one example where dozens of ant-host species are thought to associate across the New World
120 with a widely distributed mutualistic fungal lineage (Silva-Pinhati *et al.* 2004; Mikheyev *et al.* 2006,
121 2007, 2008, 2010; Mikheyev 2008; Mueller *et al.* 2011a; Mueller *et al.* in review). In the leafcutter
122 mutualism, one dominant fungus clade, called Clade-A fungi (Mueller *et al.* in review), is associated with
123 leafcutter ant species across the entire leafcutter range from Argentina to the USA, including also several
124 leafcutter ant species inhabiting Cuba and other Caribbean islands (Mikheyev *et al.* 2006; Mikheyev
125 2008; Mueller *et al.* 2011a; Mueller *et al.* in review). Clade-A fungi sequenced so far were thought to be
126 closely affiliated with the leafcutter fungus *Leucocoprinus gongylophorus* (Heim 1957) that was
127 described from mushrooms (sexual sporocarps) growing from leafcutter gardens of several leafcutter ant
128 species (Möller 1893; Fisher *et al.* 1994; Pagnocca *et al.* 2001; Mueller 2002; Pagnocca *et al.* 2011). [See
129 Supporting Information why the widely-cited placement of these mushrooms into the genus
130 *Leucoagaricus* by Singer (1986) is inaccurate]. No free-living leafcutter fungi have been found so far
131 (i.e., sporocarps or mycelia of leafcutter fungi growing independent of a leafcutter nest are not known),
132 but such free-living mushrooms are known for the cultivars of lower-attine, non-leafcutter ants (Mueller
133 *et al.* 2001; Mueller 2002; Vo *et al.* 2009).

134
135 Although most leafcutter species studied so far cultivate Clade-A fungi, some ecologically prominent
136 leafcutter species from across South America (e.g., *Atta laevigata*, *At. vollenweideri*; Fowler 1983;
137 Fowler *et al.* 1986; Solomon *et al.* 2008; Delabie *et al.* 2011) cultivate Clade-B fungi (Mueller *et al.* in
138 review), a clade of fungi that was thought previously to be associated exclusively with non-leafcutting
139 *Trachymyrmex* and *Sericomyrmex* ants that form sister and outgroup lineages to the two leafcutter ant
140 genera *Atta* and *Acromyrmex*. Moreover, some higher-attine non-leafcutter ant species in the genus
141 *Trachymyrmex* and one lower-attine ant species in the genus *Apterostigma* also cultivate Clade-A fungi
142 (Schultz *et al.* 2015; Mueller *et al.* in review; Fig. 1). These ant-fungus associations indicate that
143 leafcutter and non-leafcutter ants share a pool of fungi belonging principally to two clades of fungi,
144 Clade-A fungi possibly representing a single species of fungus called *Leucocoprinus gongylophorus* (i.e.,
145 formerly called *Attamyces bromatificus* as the vegetative mycelial form; Kreisel 1972), and Clade-B fungi
146 representing at least six well-supported lineages of fungi, each possibly a separate cultivar species
147 (Mueller *et al.* in review; Fig. 1). The so-called higher-attine fungi (Clade-A & Clade-B fungi), cultivated
148 by higher-attine ants in the leafcutter genera *Atta* and *Acromyrmex* and the non-leafcutter ants
149 *Trachymyrmex* and *Sericomyrmex*, therefore co-evolve diffusely with their higher-attine ant hosts, with
150 higher-attine ant lineages occasionally transitioning between Clade-A and Clade-B cultivation. The
151 frequencies of these transitions over evolutionary and ecological time are unknown, but some higher-
152 attine ant species appear to cultivate both Clade-A and Clade-B fungi in some populations (Mueller *et al.*
153 in review; see also Table S10), a kind of local polyculture within an ant population seen also in an asexual
154 lower-attine ant (Himler *et al.* 2009; Kellner *et al.* 2013) but not in all lower-attine ants (Mehdiabadi *et al.*
155 2012).

156

157 Because of vertical inheritance of fungal cultivars from maternal to offspring nests, leafcutter ants and
158 fungi were initially predicted to co-migrate and co-reproduce together, and initially were even thought of
159 as ancient asexual clones (Chapela *et al.* 1994). However, population-genetic and phylogenetic
160 observations are inconsistent with strict vertical inheritance and strict clonal reproduction. These
161 observations include: (a) sharing of fungus-cultivar clones between sympatric leafcutter ant species,
162 indicating frequent exchange of fungal clones between nests of different ant species and possible
163 “sweeps” of cultivars through leafcutter communities through unknown mechanisms of lateral between-
164 nest cultivar transfer, such as garden stealing by ants or cultivar dispersal by unknown vectors (Adams *et*
165 *al.* 2000; Green *et al.* 2002; Mikheyev *et al.* 2007, 2010; Mueller *et al.* 2011a); (b) identity of fast-
166 evolving DNA sequences (e.g., internal transcribed spacer region, ITS) of leafcutter fungi cultivated by
167 different leafcutter ant species across vast geographic distances (e.g., southern to northern Brazil, Silva-
168 Pinhati *et al.* 2004); and (c) genetic admixture between *L. gongylophorus* populations associated with
169 *Atta* and *Acromyrmex* species across North America (Mexico, southern USA, Cuba) implicated by
170 phylogenetic sequence analyses (Mikheyev *et al.* 2006, 2010) and population-genetic microsatellite-
171 marker analyses (Mueller *et al.* 2001a). The observation of genetic admixture between *L. gongylophorus*
172 populations across a significant oceanic barrier (between mainland Mexico and Cuba) that should
173 preclude dispersal of leafcutter ants is significant, because it suggests that *L. gongylophorus* fungi may be
174 able to disperse also independently from the ant hosts (e.g., via spores or non-ant vectors; Möller 1893;
175 Pagnocca *et al.* 2001; Mueller 2002; Mikheyev *et al.* 2006; Mueller *et al.* 2011a). Germination of spores
176 from *L. gongylophorus* mushrooms was documented by Möller (1893), and because this is a relatively
177 inaccessible account written in German, we provide in the Supporting Information a translation of
178 Möller's experiments (see also Mueller 2002, listing all the studies that attempted to germinate spores of
179 mushrooms derived from attine fungi).

180 181 *Biogeography of Leafcutter Ants (Atta, Acromyrmex)*

182 Far more is known about the biogeography of leafcutter ants than about their fungi. The currently
183 recognized 17 *Atta* and 26 *Acromyrmex* leafcutter species (not including parasitic *Acromyrmex* species;
184 Bacci *et al.* 2009; Rabeling *et al.* 2015) form a well-supported monophyletic clade that originated about
185 18-19 million years ago (mya; ranges of 15.6-20.4 mya and 14-24 mya estimated by, respectively,
186 Ješovnik *et al.* 2016 and Branstetter *et al.* 2017). Only six leafcutter species occur in North America (*Atta*
187 *texana*, *At. mexicana*, *At. insularis*, *At. cephalotes*, *Ac. versicolor*, *Ac. octospinosus*) and eight species in
188 Central America (*At. cephalotes*, *At. colombica*, *At. sexdens*; *Ac. octospinosus*, *Ac. echinator*, *Ac.*
189 *coronatus*, *Ac. volcanus*, and the parasitic *Ac. insinator*), whereas about 40 described leafcutter species
190 occur in South America, with the greatest concentration of sympatric leafcutter species in savannah
191 habitat of northern Argentina, Paraguay, Uruguay, and Southern Brazil (Borgmeier 1959; Gonçalves
192 1961; Kusnezov 1963; Fowler 1983; Farji-Brener & Ruggiero 1994; Mayhé-Nunes & Jaffé 1998;
193 Fernández & Sendoya 2004; Mueller & Rabeling 2008; Brandão *et al.* 2011; Delabie *et al.* 2011).

194
195 Because the greatest concentration of leafcutter species diversity occurs in savannahs of southern South
196 America, early biogeographic models (Kusnezov 1963; Fowler 1983) postulated that leafcutter ants
197 originated and diversified in seasonally dry grasslands of South America (i.e., in savannah habitat, not in
198 humid tropical rainforest); from there, leafcutter ants expanded then into diverse habitats across South
199 America, and later into Central and North America once leafcutter ants could disperse across the Central
200 American land bridge. Recently, however, Branstetter *et al.* (2017) were the first to conduct a formal
201 analysis to infer biogeographic history mapped onto a phylogeny of attine ants, and Branstetter *et al.*'s
202 biogeographic modeling is most consistent with an origin of leafcutter ants in seasonally dry habitat in
203 Central America, but their analyses do not rule out a South American origin with confidence. The two
204 conflicting hypotheses of a South American origin (Kusnezov 1963; Fowler 1983) versus a Central
205 American origin (Branstetter *et al.* 2017) make different predictions regarding the biogeographic diversity
206 of leafcutter fungi that should be found in South versus Central America. Assuming that leafcutter ants
207 became specialized to cultivate Clade-A fungi around the time of the origin of the leafcutter clade, as

208 assumed by traditional models of ant-fungus evolution (Stradling & Powell 1986; Chapela *et al.* 1994),
209 and assuming no other factors affect diversity of fungal cultivars (e.g., genetic drift does not affect
210 cultivar genotype diversity differently in different cultivar populations), the hypothesis of a Central
211 American origin predicts that the fungi cultivated by leafcutter ants should be most diverse in Central
212 America, and less diverse in South America colonized secondarily by leafcutter lineages migrating with
213 their cultivars from Central to South America. In contrast, the Kusnezov-Fowler hypothesis of a South
214 American origin predicts the opposite, a greater diversity of leafcutter fungi in South America that
215 accumulated there during the past 19 million years of leafcutter diversification, and less fungal diversity
216 in Central and North America colonized secondarily, and possibly recently (less than 5 mya), by
217 leafcutter lineages migrating out of South America.

218
219 There exists no definitive fossil evidence that indicates a presence of leafcutter ants north of South
220 America prior to the closing of the Central American land bridge 1-5 million years ago (mya), or an
221 earlier presence in South America. Several genera of attine ants, including a species that can be assigned
222 to the higher-attine genus *Trachymyrmex* (Baroni Urbani 1980), have been described from Dominican and
223 Mexican amber dated to about 15-20 mya (de Andrade 2003; Schultz 2007; Brandão *et al.* 2011; LaPolla
224 *et al.* 2013), but no fossil leafcutter ant has been described so far (see additional discussion on fossil attine
225 ants in the Supporting Information). Without leafcutter ant fossils, historical biogeographic distributions
226 of leafcutter ants have to be inferred therefore with the help of current distributions.

227
228 Of four well-supported sub-clades of *Atta* (Bacci *et al.* 2009), representatives from two clades (*Neoatta*,
229 *Atta sensu stricto*) occur in both South America and in Central America, whereas the species-rich *Epiatta*
230 clade occurs exclusively in South America (including dominant pest species such as *bisphaerica*,
231 *capiguara*, *saltensis*, *vollenweideri*, *laevigata*, and *opacipes*), and three species in the *Archeatta* clade
232 occur only in North America (*mexicana*, *texana*, *insularis*; presumably these three species diversified in
233 or near that northernmost region of the *Atta* distribution). The distribution of these *Atta* subclades
234 therefore does not favor either a South or Central American origin of leafcutter ants, except that the far
235 greater diversity of South American *Atta* species appears more consistent with a South American origin.
236 Diversification within species has been analyzed only in three widespread *Atta* species (*cephalotes*,
237 *sexdens*, *laevigata*) for which within-species diversity accumulated in the past 0.5-3 million years
238 (Solomon *et al.* 2008).

239
240 Because no comparable phylogenetic analysis exists for *Acromyrmex*, the biogeography of *Acromyrmex* is
241 less understood than the one for *Atta*. Earlier morphological studies partitioned *Acromyrmex* into two
242 groups (sub-genera *Acromyrmex* and *Moellerius*; Emery 1905; Gonçalves 1961), but molecular-
243 phylogenetic analyses did not confirm these two groups as monophyletic (Cristiano *et al.* 2013; Schultz *et*
244 *al.* 2015; Branstetter *et al.* 2017), and the morphologically unique species *Acromyrmex striatus*,
245 traditionally placed into the *Moellerius* sub-genus (Gonçalves 1961; Fowler 1988), actually represents the
246 most basal leafcutter lineage that is distinct at the molecular level from all other leafcutter ants (Cristiano
247 *et al.* 2013). Because *Ac. striatus* and its likely sister species *Ac. silvestri* occur in savannah habitat of
248 northern Argentina, Paraguay, Uruguay, and southernmost Brazil (Fowler 1983; Farji-Brener & Ruggiero
249 1994; Cristiano *et al.* 2016), the basal position of *Ac. striatus* in the clade of leafcutter ants supports an
250 origin of leafcutter ants in grasslands in southern South America, as postulated by Kusnezov (1963) and
251 Fowler (1983) (see also Brandão *et al.* 2011). The existence of the most basal leafcutter lineage *Ac.*
252 *striatus* and its sister lineage *Ac. silvestri* in southern South America, as well as the main concentration of
253 extant leafcutter species diversity in southern South America, is difficult to reconcile with Branstetter *et*
254 *al.*'s hypothesis of a Central American origin of leafcutter ants.

255 256 *Biogeography of leafcutter fungi*

257 Very little is known about the biogeography of fungi cultivated by leafcutter ants. Population-genetic
258 analyses using microsatellite markers showed that in Panamá, sympatric populations of five leafcutter

259 species (*At. cephalotes*, *At. colombica*, *At. sexdens*, *Ac. octospinosus*, *Ac. echinator*) share a pool of six
260 genotype-clusters of *L. gongylophorus* fungi (Mikheyev *et al.* 2007), with only 10% of the observed
261 genetic variation attributable to differences between leafcutter hosts, indicating local cultivar sharing
262 between and within the leafcutter genera *Atta* and *Acromyrmex*. Likewise, analyses of AFLP markers
263 showed that Panamanian cultivars from sympatric *Ac. octospinosus* and *Ac. echinator* can be grouped
264 into at least 5 distinct clusters (Bot *et al.* 2001), with each cluster containing representatives from either of
265 the two sympatric *Acromyrmex* species. Across North America, five leafcutter species (*At. texana*, *At.*
266 *mexicana*, *At. cephalotes*, *At. insularis*, *Ac. versicolor*) share four genotype-clusters of *L. gongylophorus*
267 (Mueller *et al.* 2011a), with evidence of admixture between these distinct genotype-clusters. No
268 comparable population-genetic analyses involving multiple fungi per leafcutter species exist for South
269 American leafcutter fungi, except Peireira *et al.* (2015) showed that three cultivars from *Ac. heyeri* and
270 three from *Ac. ambiguus* from Ro Grande do Sul in Brazil form two fungal clades grouping by ant
271 species. The population-genetic linkages between South, Central, and North American leafcutter fungi
272 are unknown. Clade-B fungi cultivated by leafcutter ants are known so far only from South America
273 (from Argentina, Brazil, French Guiana, and Venezuela; Mueller *et al.* in review), and Clade-A fungi are
274 cultivated by diverse *Atta* and *Acromyrmex* species ranging from Argentina to the USA (Mueller *et al.* in
275 review).

276
277 In North America, genetically identical clones of *L. gongylophorus*, genotyped at 12 microsatellite loci,
278 can range over large areas. For example, the most widely distributed clones ranged across 50,000-80,000
279 square kilometers in south-central Texas (approximately the area of Costa Rica or Panamá). Comparably
280 detailed population-genetic analyses are currently lacking for leafcutter-fungus populations from Central
281 and South America. Widely-distributed cultivar clones may exist also in South America because fast-
282 evolving sequences (e.g., ITS rDNA) of South American leafcutter fungi can be nearly identical for
283 collections from sites 2600 kilometers distant (Silva-Pinhati *et al.* 2004). On the other hand, genetic
284 admixture between differentiated *L. gongylophorus* populations appears more pronounced in tropical
285 populations in Mexico than in subtropical populations in the USA (Mueller *et al.* 2011a), suggesting that,
286 because of more frequent recombination in the tropics through unknown processes of genetic exchange
287 (e.g., exchange of nuclei between multinucleate mycelium; Mueller *et al.* 2011a; Carlson *et al.* in press),
288 genetically identical cultivar clones may not range as widely in the tropics compared to their ranges
289 observed at the subtropical, northern range limit of the leafcutter distribution.

290
291 Three additional expectations about the biogeography of leafcutter fungi derive from biogeographic
292 patterns of widely-distributed *Atta* species in South America (Solomon *et al.* 2008). First, major rivers
293 such as the Amazon or the Orinoco do not represent effective dispersal barriers to *Atta* ants (Solomon *et*
294 *al.* 2008). Because the dispersing female reproductives transport fungal inocula during mating flights,
295 major rivers would therefore also not represent dispersal barriers for leafcutter fungi. In fact, even the
296 oceanic barrier between Cuba and mainland does not appear to be an effective dispersal barrier for
297 leafcutter fungi, because fungi cultivated by *At. insularis* in Cuba have close population-genetic affinities
298 to fungi cultivated by *At. mexicana* and *At. texana* in mainland North America (Mueller *et al.* 2011a),
299 whereas these three ant species are significantly diverged from each other (Bacci *et al.* 2009) and the
300 distance between Cuba and mainland greatly exceeds the dispersal distance of leafcutter ants during a
301 mating flight. This suggested the possibility that leafcutter fungi may disperse independently from the
302 ants, for example through airborne spore dispersal (spore-bearing mushrooms of leafcutter fungi have
303 been observed on rare occasions growing from nests in the field; e.g., Pagnocca *et al.* 2001; Mueller
304 2002), or possibly by dispersal through vectors other than leafcutter ants (Mueller *et al.* 2011a). Second,
305 Pleistocene refugia in South America apparently did not contribute to inter- and intra-species
306 diversification in *Atta* ants (Solomon *et al.* 2008), and presumably therefore also not for the associated
307 fungal cultivars. Third, leafcutter abundance decreases significantly with altitude, and leafcutter ants do
308 not occur at elevations higher than about 2000-2500 meters (Weber 1972; Farji-Brener & Ruggiero 1994;

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

311
312 We build on these previous studies by conducting here the first comprehensive population-genetic and
313 biogeographic analyses of *L. gongylophorus* fungi (i.e., Clade-A fungi *sensu* Mueller *et al.* in review)
314 propagated by leafcutter ants across the ants' entire range from Argentina to the USA (Weber 1972;
315 Solomon *et al.* 2008; Bacci *et al.* 2009). Our study specifically asks whether unknown cultivar types
316 (beyond the known Clade-A and Clade-B cultivars) may be cultivated by leafcutter ants in South
317 America; whether cultivar clones are shared locally between sympatric leafcutter ant species; whether
318 fungal cultivars differ between leafcutter ants that are specialized to cut either dicot or monocot (grass)
319 leaf substrate for fungiculture (Vasconcelos & Fowler 1990); whether the cultivar species *L.*
320 *gongylophorus* is subdivided into many or few sub-populations across the range from Argentina to the
321 USA, with implications for effective migration rates between biogeographic regions; and whether *L.*
322 *gongylophorus* is genetically more diverse in Central and North America compared to South America, as
323 predicted by Branstetter *et al.*'s (2017) hypothesis of a Central American origin of leafcutter ants, or
324 whether genetic diversity is greater in South America, as predicted by Kusnezov's (1963) and Fowler's
325 (1983) hypothesis of a South American origin of leafcutter ants.

326 327 **Materials and methods**

328 *Sample Collection*

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

338
339 The majority of our 474 samples derived from the extensive biogeographic survey of Solomon *et al.*
340 (2008) of three *Atta* species (*At. cephalotes* n=145; *laevigata* n=26; *sexdens* n=43) from South and
341 Central America; from leafcutter-ant surveys of the Bacci and Ortiz Labs in Brazil and Colombia; and
342 from leafcutter-ant surveys of the Mueller Lab in Argentina, French Guiana, Panamá, and the USA. Of
343 200 leafcutter fungi from the USA genotyped in previous analyses (Mueller *et al.* 2011a, 2011b), we
344 included in our survey here 18 representative strains to cover the diversity of leafcutter fungi known from
345 the USA. Our fungus-garden samples from 8 *Atta* and 22 *Acromyrmex* species cover 47% of 17 *Atta*
346 species currently recognized, and 85% of 26 *Acromyrmex* currently recognized (not including parasitic
347 *Acromyrmex* species). Two factors account for the higher proportion of *Acromyrmex* compared to *Atta*
348 species in our survey. First, gardens of *Acromyrmex* nests are typically closer to the surface and thus are
349 easier to excavate than the deeper gardens of *Atta* nests; and second, some *Atta* species have small ranges
350 (Borgmeier 1959; Bacci *et al.* 2009; Delabie *et al.* 2011) that were not covered in surveys of the
351 collaborating labs. We were able to obtain fungus-garden samples from all leafcutter species in Central
352 and North America, and the leafcutter species missed in our survey are therefore all from South America.
353 It is possible that several of the surveyed leafcutter species represent cryptic species complexes that may
354 eventually be split into separate species, for example the *Atta sexdens* complex, but particularly species
355 complexes in the genus *Acromyrmex* (e.g., species *ambiguus*, *balzani*, *coronatus*, *laticeps*, *octospinosus*,
356 *rugosus*, *subterraneus*; Delabie *et al.* 2011), which have received less taxonomic attention than *Atta*
357 species.

358
359 For outgroup rooting in the phylogenetic analyses of leafcutter fungi, we included in our survey also

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

368 *Field Collections*

369 Leafcutter gardens were accessed through excavation with shovels. Two 1 cm³ fragments of healthy,
370 mature garden (substrate suffused with healthy mycelium) were preserved in duplicate vials with 100%
371 ethanol. We typically sampled only one garden per leafcutter nest because leafcutter ants are thought to
372 grow their fungi as monocultures, although only three leafcutter species have been tested so far for
373 monoculture (Poulsen & Boomsma 2005; Mueller *et al.* 2010) and leafcutter ants can co-culture several
374 cultivar strains in experimental chimaeric gardens in the laboratory (Sen *et al.* 2010). Corresponding
375 samples of ants were also preserved in 100% ethanol for species identification of ants. Samples were
376 transported at room temperature to the São Paulo State University Rio Claro or the University of Texas at
377 Austin, then stored at -80°C. Collection information for all samples and locations of permanent storage
378 of vouchers are listed in Table S1.

380
381 *DNA Extraction and Sequencing:* Small (~0.5mm³) mycelial tufts were separated with flame-sterilized
382 forceps under a stereomicroscope from the ethanol-preserved garden material, then immersed in 180 µL
383 of a 20% Chelex buffer (Sigma-Aldrich, St. Louis, Missouri). To release the DNA, samples were
384 vortexed in the Chelex buffer at room temperature for 15 min, incubated at 37°C in a thermal cycler for
385 60 minutes, then heated to 100°C for 15 minutes. EF-1 α , RAD51, and DMC genes were PCR-amplified
386 as described in Mikheyev *et al.* (2006) (see also primer information and annealing temperatures in Table
387 S8). The PCR mix [1µL 10x buffer, 1µL MgCl₂ 25mM, 0.8 µL dNTP mix (2.5mM of each nucleotide),
388 0.6 µL of each primer 10 mM, 0.02 µL Taq polymerase, ddH₂O to a total volume of 10 µL] was heated
389 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
390 min at 72°C; followed by a 10 min extension step at 72°C). All PCR products were cycle-sequenced with
391 the ABI BigDye Terminator Kit (version 3.1) on an ABI PRISM 3100 automated sequencer in the
392 Mueller Lab. Genbank accession numbers for the sequenced fungal cultivars are listed in Table S1
393 [GQ853919–GQ854367 (EF-1 α gene); GQ854817–GQ855186 (RAD gene); HQ391561–HQ391895
394 (DMC gene)].

395 *Phylogenetic analyses*

396 We were able to generate sequence information for 483 fungal accessions (430 fungi from leafcutter ants,
397 40 fungi from *Trachymyrmex* ants, 4 fungi from *Sericomyrmex* ants, and 9 outgroup fungi; Table S1).
398 Because of problems with PCR-amplification of the RAD and DMS genes, we were able to generate
399 RAD and DMS sequence information not for all of our collections (successful sequences for 370
400 accessions for RAD, 335 accessions for DMS; Table S1). Forward and reverse sequences were
401 assembled and edited using Sequencher 4.6 (GeneCodes, Ann Arbor, MI), aligned for each gene using
402 Clustal X (2.0), then manually aligned in MacClade version 4.06 (Maddison & Maddison 2000).

403
404 We initially intended to use information from all three protein-coding genes to resolve phylogenetic
405 structure among Clade-A fungi. However, because preliminary phylogenetic analyses showed that each
406 of the three genes shows insufficient variation to resolve phylogenetic relationships between all Clade-A
407 fungi, we discontinued sequencing of the RAD and DMC genes, and instead relied on information from
408 the EF-1 α gene to classify leafcutter fungi into Clade-A and Clade-B fungi, then resolve genetic
409 differences between Clade-A fungi with a panel of 5 microsatellite markers (below). We present the
410

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

416
417 To place phylogenetic relationships of leafcutter fungi into the context of closely-related cultivars of other
418 fungus-growing ants, we also generated sequence information for representative fungi collected from 40
419 *Trachymyrmex* and 4 *Sericomyrmex* ant nests, as well as fungi from a few representative “lower-attine”
420 ants (two Clade-1 attine cultivars, and one Clade-2 cultivar, as defined by Mueller *et al.* 1998 and Kellner
421 *et al.* 2013); and four representative free-living *Leucocoprinus* species closely related to lower-attine
422 cultivars (Mueller *et al.* 1998). Phylogenetic trees were rooted with one of these free-living
423 *Leucocoprinus* species (accession PA136 collected in Panama), a close free-living relative of Clade-2
424 cultivars of lower-attine ants (Mueller *et al.* 1998). Our final EF-1 α alignment of 475 characters included
425 187 informative, 27 autapomorphic, and 261 invariable characters.

426
427 We evaluated the best-fit model of sequence-evolution using the Bayesian information criterion (BIC) in
428 ModelTest 3.7, which recommended a GTR+I+r model of sequence-evolution. We performed a Bayesian
429 analysis on the EF-1 α alignment using MrBayes (ver. 3.1.2) and the GTR+I+r model of sequence-
430 evolution. We ran seven independent MCMC analyses (each with four chains) for ten million
431 generations, with trees and parameters sampled every 100 generations, and with a burn-in of two million
432 generations. The joint posterior probabilities and parameter estimates of each run were congruent,
433 suggesting the chains were run for a sufficient number of generations. To combine the results from the
434 seven runs, we used MrConverge (as described in Brown & Lemmon 2007) to sub-sample the remaining
435 generations 1/20,000 and construct a consensus tree. We also explored phylogenetic relationships under
436 the maximum-likelihood criterion, using default settings recommended by GARLI (version 0.96b8;
437 Zwickl 2006). We performed ten replicates of heuristic searches in GARLI to identify the phylogenetic
438 reconstruction with the lowest log-likelihood score. We mapped non-parametric bootstrap support values
439 (100 pseudo-replications each) on the corresponding maximum-likelihood trees. Because we use
440 information from the EF-1 α gene here only to identify Clade-A fungi for further analysis with
441 microsatellite markers, we did not explore phylogenetic relationships exhaustively beyond the basic
442 Bayesian and likelihood analyses described above.

443 444 *Microsatellite marker analyses*

445 We generated microsatellite-information for five loci (A1132, C101, C126, C117, B12) developed for the
446 Clade-A fungus *Leucocoprinus gongylophorus* (i.e., formerly *Attamyces bromatificus*; Scott *et al.* 2009).
447 These loci were chosen from among the 23 loci developed by Scott *et al.* (2009) because these loci were
448 among the most polymorphic markers and they could be scored reliably (few scoring errors in previous
449 analyses; Scott *et al.* 2009; Mueller *et al.* 2010). We amplified each locus separately (i.e., not
450 multiplexed) in a 10 μ L reaction using the following thermocycler profile: 95°C for 5 min, then 10 cycles
451 at 94°C for 15 sec, primer-specific annealing temperature for 15 sec as specified in Scott *et al.* (2009),
452 72°C for 25 sec; followed by 25 cycles at 89°C for 10 sec, primer-specific annealing temperature for 15
453 sec, 72°C for 25 sec, and a final extension of 72°C for 30 minutes. Amplification products were
454 visualized under UV light after electrophoresis of 1.5% agarose gel stained with SYBR safe.

455
456 Amplification products were cleaned with Sephadex Centri-Sep 8 spin columns (Princeton Separations
457 Inc, Adelphia, NJ, USA). After cleaning, 1 μ L of product was mixed with 8 μ L of HiDi (Applied
458 Biosystems) and 1.5 μ L of custom-made size standards (CASS; DeWoody *et al.* 2004) using the size-
459 standard ladder ROXF1, ROX 104, ROX 150, ROX 200, ROX 253, ROX 305, and ROX 424. The PCR
460 products, HiDi, and CASS mixture was denatured for 95°C for 2 minutes, then chilled at 10°C for at least

461 2 minutes. Amplified microsatellite markers were analysed on an ABI PRISM 3100 automated sequencer
462 in the Mueller Lab and scored using SoftGenetics GeneMarker v1.5 (State College, PA).

463

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

471

472 Of the 419 fungal samples from Clade-A that we aimed to genotype at each of 5 loci ($5 \times 419 = 2095$ loci
473 amplified total), at the end of the genotyping phase of our study, information was missing for 6 samples
474 for locus A1132; 4 samples for locus C101; 2 samples for locus C126; 8 samples for locus C117; 11
475 samples for locus B12 (Tables S1 & S3). The missing information was because of oversight during the
476 genotyping phase of our study. No fungus was missing information for more than one locus, 31 samples
477 (7%) were genotyped at only 4 loci, 388 samples (93%) were genotyped at all 5 loci. Locus B12 had null
478 alleles (no markers amplified) in 47 samples from north-east South America (mostly in Peru and Ecuador,
479 also in Colombia, Venezuela, and French Guiana; Tables S3 & S4). We did not detect null alleles at any
480 of the other loci (i.e., at least one allele amplified per locus), but null alleles may have been masked by the
481 multi-nucleate, polyploid nature of the genotyped fungi. The total number of alleles per locus, and the
482 averages of these numbers for each locus, are calculated in Table S4. Averages of total alleles scored
483 were 2.34 alleles for locus A1132 (StDev = 0.72; range 1-4 alleles per sample); 2.10 for locus C101
484 (StDev = 0.76; range 1-4); 1.40 for locus C126 (StDev = 0.53; range 1-3); 1.65 for locus C117 (StDev =
485 0.72; range 1-3); and 1.66 for locus B12 (StDev = 0.92; range 0-4).

486

487 *Population- genetic analyses of microsatellite markers*

488 We assessed population structure with STRUCTURE v2.3.4 (Pritchard *et al.* 2000), which clusters
489 individuals into genotype-clusters (i.e., populations) and estimates admixture using multilocus genotypes.
490 Because *L. gongylophorus* fungi are polyploid and multinucleate, we treated each allele as a dominant
491 marker in STRUCTURE, as recommended by Falush *et al.* (2007). Ploidy is also variable between
492 individual strains (Kooij *et al.* 2015a; Carlson *et al.* in press), thus we did not use standard population
493 genetic statistics (e.g., F-statistics, heterozygosity, etc.) to describe inferred populations. We first
494 assessed population structure using the default settings of STRUCTURE, but to reduce bias in prior
495 assumptions in a separate analysis, we also left allele frequencies uncorrelated and chose alpha (α) to be
496 1/10 of the default setting (i.e., $\alpha=0.1$) (Wang 2017). Both the default settings and the modified settings
497 yield identical recommendations of K=3 as the most informative number of clusters, following the
498 method of Evanno *et al.* (2005) (Fig. S4). We processed individual and population matrices from
499 STRUCTURE HARVESTER (Earl *et al.* 2012) in the cluster matching program CLUMPP (Jakobsson &
500 Rosenberg 2007), then processed the q-matrices of CLUMPP in Distruct (Rosenberg 2004) to generate
501 the barplot in Fig. 2 (top) and to map pie charts in Fig. 2 (bottom right) using the open-source geographic
502 information system tools in R (R Development Core Team 2008).

503

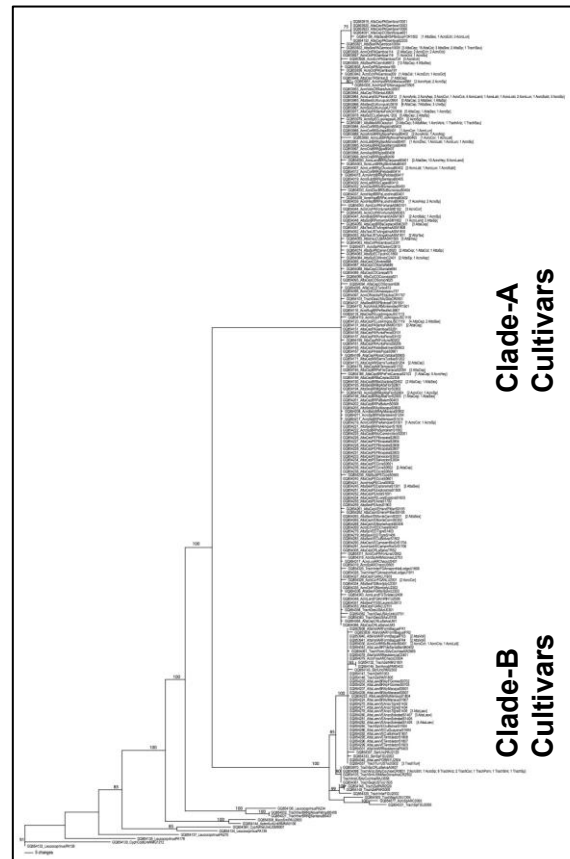
504 **Results**

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

509
510 *Phylogeny of Fungi Cultivated by Leafcutter ants*

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

526
527 **Fig. 1.** Phylogenetic relationships of fungi cultivated by
528 higher-attine ants, based on EF-1 α sequence
529 information. Table S1 summarizes collection
530 information of the 430 fungal cultivars from leafcutter
531 ants included in this analysis, 44 fungal cultivars from
532 *Trachymyrmex* and *Sericomyrmex* ants, and 9 outgroup
533 fungi (lower-attine cultivars and free-living
534 *Leucocoprinus* fungi). We used the phylogenetic
535 information from EF-1 α sequences to classify fungi into
536 Clade-A and Clade-B fungi, and identify possible
537 leafcutter cultivars that fall outside these two clades (we
538 did not find such fungi in our survey). In the
539 phylogenetic tree shown, taxa with identical sequences
540 (excepting sequence ambiguities) are listed next to each
541 other as a string of taxa as the same terminal leaf, and
542 the respective Genbank accessions of the taxa that are
543 united on the same leaf are listed in Table S5. Because
544 we did not recover sufficient phylogenetic structure
545 among Clade-A fungi using EF-1 α sequences, and also
546 using sequence information from two other protein-
547 coding genes (Figs. S2&S3), we genotyped Clade-A
548 fungi by determining allele profiles at 5 microsatellite
549 loci (Table S3; Fig. 2). Because of genetic exchange
550 between Clade-A fungi (Fig. 2), Clade-A fungi form a
551 single evolutionary lineage and represent a single
552 species *Leucocoprinus gongylophorus*.



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

573

574 *Clonal Propagation of Fungal Cultivars*

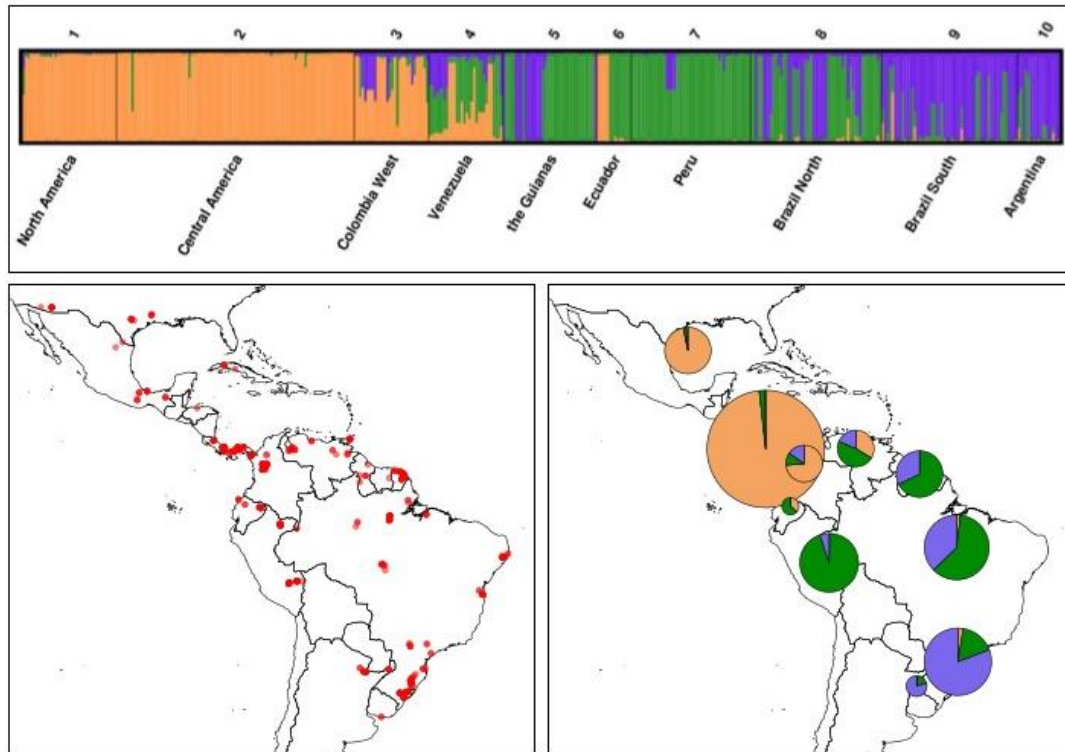
575 The five microsatellite loci (loci A1132, C101, C126, C117, B12; Table S3) identified 241 genotypes
576 (57.5%) among the 419 Clade-A fungi collected from 419 different leafcutter ant nests; that is, 178 fungal
577 genotypes had at least one identical duplicate that had been collected also from a different leafcutter nest.
578 Most of these duplicate cases (75.7%, 56 of 74 cases) of fungus-genotype identity between nests involve
579 nests of the same ant species collected in close geographic proximity (typically within 50 km of each
580 other or less; Table S3). This is consistent with the vertical transmission of cultivar clones within ant
581 lineages, and these fungal genotypes are therefore likely to be identical in different proximate nests of the
582 same ant species because of identity-by-descent of the fungi and limited dispersal per ant generation.
583 Cases of cultivar identity between different ant species and between different leafcutter genera are
584 discussed below.

585

586 *Population Structure of L. gongylophorus fungi cultivated by leafcutter ants*

587 Genetic structure in *L. gongylophorus* is strongly correlated with geography. The methods of Evanno *et al.*
588 (2005) determined that $K=3$ (Fig. S4) is the most informative number of genotype clusters for input
589 into STRUCTURE. Fig. 2 plots STRUCTURE assignments of 419 fungal samples to these three
590 genotype-clusters and maps these onto 10 regions defined by country of collection (some adjacent
591 countries are combined, Brazil is divided into north and south): Argentina & Uruguay, southern Brazil,
592 northern Brazil, Peru, Ecuador, the Guianas, Venezuela, Western Colombia, Panama & Costa Rica,
593 Mexico & USA (Table S3). The three genotype clusters correspond approximately to southern South
594 America, northern South America, and North & Central America (Fig. 2), with the largest degree of
595 admixture apparent adjacent to the Isthmus of Panama in north-west South America. Members of Cluster
596 1 (burnt orange in Fig. 2) are found in North America, Central America, western Colombia
597 (Departamento de Antioquia, west of the Andes), and to a lesser extent in Ecuador and Venezuela.
598 Members of Cluster 2 (green in Fig. 2) and Cluster 3 (purple) occur only in South America. Fungi
599 outside of South America are all assigned by STRUCTURE to Cluster 1 (Fig. 2). If the number of co-
600 occurring genotype-clusters is an indication of local genetic diversity, fungal populations appear less
601 diverse in Central and North America compared to South America. The local proportion of admixed
602 individuals (fungi combining alleles assigned by STRUCTURE to different genotype-clusters) appears
603 greatest in Colombia and Venezuela (Fig. 2 top), corresponding with the apparent transition zone in
604 genotype-clusters from South America to Central & North America.

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

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642

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

644 In contrast to the strong spatial structure, allele richness of fungi shows no consistent patterns across the
645 entire range of *L. gongylophorus* fungi cultivated by leafcutter ants (Figs. S5A-E). Because *L.*
646 *gongylophorus* fungi are polyploid, multinucleate fungi and ploidy appears variable between fungal
647 strains (Scott *et al.* 2009; Kooij *et al.* 2015a; Carlson *et al.* in press), we were not able to use standard
648 population-genetic statistics (e.g., heterozygosity, etc.), so we examined the biogeographic distributions
649 of the maximum number of alleles per locus (allele richness) and private alleles (alleles present only in
650 specific populations). For adequately-sampled populations (i.e., at least 25-30 individuals per population
651 in microsatellite-marker analyses; Hale *et al.* 2012), allele richness and heterozygosity are correlated, and
652 allele richness can therefore serve as a proxy of heterozygosity (see Box 1 in Eckert *et al.* 2008). In our
653 survey, allele richness does not change as a function of latitude (Fig. S5); such latitudinal changes would
654 be expected if migration between biogeographic regions is limited and older populations had more time to
655 accumulate allelic diversity than younger populations founded by recently expanding leafcutter lineages

656 (Eckert *et al.* 2008). Second, populations at the range limit in the USA and the island population in Cuba
657 do not show reduced allelic diversity (Figs. S5), as would be expected for founder populations, for
658 populations with reduced effective population sizes at range limits (Eckert *et al.* 2008), or for populations
659 at an expanding front experiencing allele surfing (Burton & Travis 2008; Peischl *et al.* 2013). Third,
660 there were no private alleles that characterized all individuals in a biogeographic region or in any
661 location. Some alleles occurred only in North America, but only in some, not all, individuals (e.g., alleles
662 212, 215, and 218 at locus A1132); some alleles occurred only in South America [e.g., allele 243 at locus
663 C126, allele 188 at locus A1132]; and a null allele at locus B12 occurred only in northern South America
664 (mostly in Peru and Ecuador, also in Colombia, Venezuela, and French Guiana; Fig. S5E, Table S4).
665 Overall, however, no biogeographic region showed an obviously increased allelic diversity that could
666 indicate a potential location of older populations where leafcutter fungi may have originated and
667 accumulated greater allelic diversity over time, or where evolutionary forces may operate that increase (or
668 decrease) allele diversity.

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

670 Leafcutter ants specialized to forage on grasses, or on both grasses and dicot plants, are more likely to
671 cultivate Clade-B fungi (Table S6), but the association between foraging preferences and cultivar
672 specializations, although statistically significant, is weak. Combining information from *Acromyrmex* and
673 *Atta* (Table S6; additional discussion in Supporting Information), and combining into one group those
674 leafcutter species that are specialized on grasses or cut both grasses and dicots, 100% of the 23 dicot-
675 specialized leafcutter species cultivate Clade-A fungi (and only two of these sometimes cultivate Clade-
676 B; Table S6) and therefore 0% of these 23 dicot-specialized leafcutter species are specialized on Clade-B
677 fungi. In contrast, four (40%) of the 10 species that cut also grasses cultivate Clade-B fungi, but for two
678 of the Clade-B-cultivating species only one single fungus has been identified so far (Table S6). The
679 Fisher's Exact Test statistic for this distribution is $p = 0.0051$ (23 counts dicot & Clade-A fungi; 0 counts
680 dicot & Clade-B; 6 counts grass & Clade-B; 4 counts grass & Clade-A), and Barnard's Exact Test statistic
681 is $p = 0.0040$.

682
683 Limiting the analysis only to Clade-A fungi and ignoring Clade-B cultivation, our microsatellite marker
684 analyses did not reveal obvious differences between Clade-A fungi cultivated by 22 leafcutter species
685 (both *Acromyrmex* and *Atta*) preferentially foraging on dicots, compared to Clade-A fungi cultivated by
686 three species preferentially foraging on grasses (*Ac. balzani*, *Ac. heyeri*, *Ac. landolti*), or one species
687 foraging on both grasses and dicots (*Ac. lobicornis*) (Table S3). In fact, we found two cases where
688 sympatric dicot-specialist and grass-specialist leafcutter species cultivated in the same location the same
689 fungal clone (identity in all alleles across the 5 microsatellite loci), *Ac. landolti* and *At. cephalotes* in
690 Colombia; and *Ac. heyeri*, *Ac. balzani*, and *At. sexdens* in southern Brazil (Table S3). This identity of
691 fungal genotypes suggests that dicot- and grass-specialized leafcutter species cultivate fungi from shared
692 pools of Clade-A fungi circulating locally with a leafcutter ant community, and dicot- and grass-
693 specialized leafcutter species even appear to exchange cultivars on occasion. A more detailed analysis
694 using more loci (e.g., genotyping-by-sequencing), and a larger collection of fungi from multiple
695 sympatric leafcutter species (e.g., in northern Argentina, Uruguay, southern Brazil), may be able to detect
696 genotypic differences between fungi cultivated by grass- versus dicot-specialized leafcutter ant species.

697 *Are there Differences between Clade-A Fungi Cultivated by Atta versus Acromyrmex Ants?*

698
699 Recent studies argued that the *L. gongylophorus* fungi (= Clade-A) cultivated by *Atta* and *Acromyrmex*
700 leafcutter ants in Panamá could represent separate gene pools (Kooij *et al.* 2015b), and that two *L.*
701 *gongylophorus* fungi cultivated by *Atta* versus *Acromyrmex* ants in Panamá diverged from each other 7.2
702 million years ago (confidence interval 5.4-9.0 million years ago; Nygaard *et al.* 2016; Supplementary
703 Methods pages 43&44 lines 744-758 of Nygaard *et al.*). Because we did not find differences between
704 *Atta*-cultivated versus *Acromyrmex*-cultivated *L. gongylophorus* fungi in our phylogenetic analyses (Figs.
705 1 & S1-S3; also Mueller *et al.* in review), we tested for possible differences using our faster-evolving
706

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

711
712 First, at most of the sites for which we obtained adequate samples of *Atta*-cultivated and *Acromyrmex*-
713 cultivated *L. gongylophorus* fungi, we found *Atta*-cultivated versus *Acromyrmex*-cultivated fungal clones
714 that were identical in all alleles across the five microsatellite loci in our study. For example, in locations
715 in Brazil, Peru, Ecuador/Colombia, Colombia, Panama/Colombia, central Panamá, western Panamá, and
716 Mexico/USA, we found instances where fungal clones with identical allele profiles were cultivated by
717 *Atta* and *Acromyrmex* nests (see fungi highlighted in green in Table S3). We also observed 10 cases
718 where identical fungal clones were shared between nests of different species of the same ant genus
719 (highlighted in orange in Table S3), and 56 cases where identical fungal clones were shared between
720 different nests of the same ant species (highlighted in yellow in Table S3), but it is the 8 cases of fungal
721 sharing between leafcutter-ant genera that seems inconsistent with complete separation of gene pools
722 between *Atta*-cultivated versus *Acromyrmex*-cultivated *L. gongylophorus* fungi. The near identical
723 incidence of cultivar sharing between different leafcutter genera (n=8) and between different congeneric
724 species (n=10) could suggest that the same biological processes, such as horizontal transfer of cultivars
725 between nests, may have led to such cultivar identity. ~~In contrast, the far greater incidence of cultivar~~
726 ~~sharing between nests of the same ant species (n=56) is likely due to genetic identity by descent (i.e.,~~
727 ~~vertical transmission of cultivar clones from mother to offspring nests), as already discussed above.~~

728
729 *Atta* and *Acromyrmex* nests cultivating identical fungal clones were located typically within 50 km of
730 each other (Table S3), but there were also instances of sharing of *Atta* and *Acromyrmex* nests about 1200
731 km distant (Brazil) and 1900 km distant in Mexico/USA, suggesting that some clonal lineages are
732 widespread geographically and can be shared across that range between *Atta* and *Acromyrmex*. ADD
733 SENTENCE OF DISTANCES OVER WHICH CLONES WERE COLLECTED IN MUELLER 2011,
734 WHERE CLONES WERE GENOTYPED AT 12 LOCI. Because many locations were undersampled in
735 our study (e.g., we were able to obtain collections from only one ant genus from the two leafcutter genera
736 present at a location), sharing of identical cultivar clones is likely more prevalent in nature than indicated
737 in our collection. Lineage sorting of cultivar diversity over 5-9 million years, or convergent evolution of
738 the same microsatellite profile independently in *Atta*- versus *Acromyrmex*-cultivated fungal lineages, both
739 seem implausible explanations for cultivar sharing between *Atta* and *Acromyrmex* nests. Instead, sharing
740 of allele-identical cultivar clones can be explained by horizontal transfer of fungal strains between nests
741 of different leafcutter genera, and possibly also by *de novo* generation of the same fungal genotype
742 through some form of genetic exchange between recombining fungi cultivated by both *Atta* and
743 *Acromyrmex* ants (e.g., exchange of nuclei between differentiated polyploid, heterokaryotic mycelia;
744 Carlson *et al.* in press).

745
746 Second, STRUCTURE analyses of fungi from Panamá, the best-sampled region in our survey, indicates
747 that *Atta*- versus *Acromyrmex*-cultivated fungi do not form genetically distinct clusters but are admixed
748 (Fig. S7A-D), regardless of whether we analyze regional fungal diversity (Colombia, Panamá, & Costa
749 Rica; n=125 samples), within-country diversity (only Panamá; n=89 samples), provincial diversity
750 (Panamá Canal Zone; n=42 samples), or the local diversity in Gamboa (n=27) also studied by Kooij *et al.*
751 (2015b). Our STRUCTURE analyses support the earlier finding by Mikheyev *et al.* (2007) that *Atta* and
752 *Acromyrmex* ants from Gamboa tap locally into the same pool of fungal cultivars, contrary to the findings
753 of Kooij *et al.* (2015b) (see also additional discussion in the Supplemental Information).

754
755 If *Atta*-cultivated and *Acromyrmex*-cultivated fungi represent separate gene pools, as hypothesized by
756 Kooij *et al.* (2015b), *Atta*-cultivated and *Acromyrmex*-cultivated fungi may show systematic differences
757 in allele diversity across all sites surveyed. Total number of alleles/fungus does not differ between fungi

758 from *Atta* and *Acromyrmex* nests (Wilcoxon Signed-Rank Test, $W = 90.5$, $z = 0.181$, $p = 0.857$ two-tailed,
759 $n = 19$), comparing samples from 19 locations (highlighted in blue in Table S7) for which complete
760 genotype information (all 5 loci) is available for both *Atta*-cultivated and *Acromyrmex*-cultivated fungi.
761 Instead, across these 19 locations, total number of allele/fungus is positively correlated between *Atta*-
762 cultivated and *Acromyrmex*-cultivated fungi (Spearman rank-order correlation $t = 2.39$, $df = 17$, $p =$
763 0.029 , $r = 0.502$; Table S8). That is, at locations where *Atta*-cultivated fungi show more allele diversity,
764 *Acromyrmex*-cultivated fungi from the same location show likewise more allele diversity. Under the
765 hypothesis of separate gene pools (Kooij *et al.* 2015b), this positive correlation would need to be
766 explained by shared evolutionary forces that determine allele diversity in parallel (i.e., convergently) at
767 different locations for both *Atta*-cultivated and *Acromyrmex*-cultivated fungi. Alternatively, the positive
768 correlation is predicted by the hypothesis that *Atta* and *Acromyrmex* leafcutter lineages tap at each
769 location into a shared pool of fungal cultivars, mediated either because of local horizontal transfer of
770 fungal cultivars between nests of the two leafcutter genera, because of some form of genetic exchange
771 and hybridization between fungi cultivated by different ant nests, or both.

772

773 Discussion

774 We aimed to conduct a comprehensive biogeographic and population-genetic analysis of fungi propagated
775 by leafcutter ants across the entire leafcutter range from Argentina to the USA, combining collections
776 from 22 collaborating laboratories and surveying leafcutter ants in 17 Neotropical countries (Tables
777 S1&S2). Analyses of 474 fungi cultivated by leafcutter ants revealed (a) no novel cultivar types beyond
778 the known Clade-A and Clade-B cultivars of leafcutter ants (Mueller *et al.* in review) (Fig. 1); (b)
779 moderate support that those leafcutter species that cut grass as fungicultural substrate show a higher
780 frequency of cultivating Clade-B fungi, whereas all leafcutter species preferring dicot plants as
781 fungicultural substrate seem specialized on cultivation of Clade-A fungi (Table S6); (c) extensive cultivar
782 sharing between sympatric leafcutter species within local communities, such that fungi cultivated by *Atta*
783 species are not distinct from those cultivated sympatrically by *Acromyrmex* species; (d) three genotype-
784 clusters of Clade-A fungi across the range from Argentina to the USA (Fig. 2), with local prevalence of
785 these genotype-clusters corresponding approximately to southern South America (Argentina, Uruguay,
786 southern Brazil), northern South America, and Central & North America (Fig. 2); (e) gene flow among
787 Clade-A fungi cultivated by leafcutter ants in different biogeographic regions, including fungi cultivated
788 by leafcutter species in Cuba, such that all Clade-A fungi from Argentina to the US represent a single
789 species, *Leucocoprinus gongylophorus*; and (f) reduced genetic diversity of leafcutter fungi in Central &
790 North America and greatest genetic diversity of leafcutter fungi concentrated in South America (Fig. 2).

791

792 *Biogeographic origin of leafcutter fungiculture and leafcutter ants*

793 Kusnezov (1963) and Fowler (1983) hypothesized that leafcutter ants originated in savannah habitat of
794 southern South America (current northern Argentina, Paraguay, Uruguay, sub-Amazonian Brazil) because
795 extant leafcutter ants exhibit the greatest species diversity there, particularly *Acromyrmex* species. In
796 contrast, Branstetter *et al.* (2017) recently conducted a formal analysis to infer biogeographic history
797 mapped onto a phylogeny of attine ants, and their biogeographic modeling suggested an origin of
798 leafcutter ants in Central America. These two hypotheses make different predictions regarding the
799 biogeographic region where leafcutter fungi can be expected to be most diverse. Assuming the traditional
800 view that leafcutter ants became specialized to cultivate Clade-A fungi around the time of the origin of
801 the leafcutter clade 19 million years ago (mya), and assuming no other factors affect diversity of fungal
802 cultivars (e.g., genetic drift does not affect cultivar genotype diversity differently in different populations
803 across the range of leafcutter ants), the hypothesis of a Central American origin predicts that the fungi
804 cultivated by leafcutter ants should be most diverse in Central America, and less diverse in South
805 America colonized secondarily by leafcutter lineages dispersing with their cultivars from Central to South
806 America. In contrast, the hypothesis of a South American origin predicts the opposite, a greater diversity
807 of leafcutter fungi in South America that accumulated there during the past 19 million years of leafcutter
808 diversification, and less fungal diversity in Central and North America colonized secondarily, and

809 possibly recently (less than 5 mya), by leafcutter lineages migrating out of South America. Our
810 STRUCTURE analyses of genotype diversity of Clade-A fungi (Fig. 2) indicate greater diversity of
811 Clade-A fungi in South America, consistent with the prediction of the Kusnezov-Fowler model of a
812 subtropical South American origin of leafcutter ants.

813
814 It is possible to conceive alternative scenarios of leafcutter ant-fungus evolution that assume a Central
815 American origin of the leafcutter ant clade (consistent with Branstetter *et al.* 2017) and a South American
816 origin of Clade-A fungi (as suggested by Fig. 2), and there are no convincing arguments, except perhaps
817 plausibility, that can rule out these alternatives. For example, leafcutter ants may have originated in
818 Central America, but Clade-A cultivars originated in South America in ancestral *Trachymyrmex* lineages;
819 Clade-A cultivars were secondarily acquired by leafcutter ants in South America after they dispersed from
820 Central into South America; a successful Clade-A lineage (i.e., *L. gongylophorus*) eventually spread
821 across the entire leafcutter range due to efficient horizontal transmission between leafcutter species, and
822 only a limited genotype diversity of Clade-A cultivars has spread so far into Central and North America
823 from diverse Clade-A populations in South America (Fig. 1). Other such complex scenarios are also
824 possible, and some of these scenarios, in addition to the Kusnezov-Fowler model of a South American
825 origin of leafcutter ants and leafcutter fungi, can be tested by precise dating of the evolutionary origins of
826 leafcutter fungi relative to the origin of the leafcutter clade.

827
828 Dates for crown ages and stem ages for Clade-A fungi and for the leafcutter-ant clade have been
829 estimated in six phylogenetic analyses (Table 1). When comparing crown ages (age of most recent
830 common ancestor, MRCA; coalescence) of Clade-A fungi and the leafcutter ant clade, the MRCA of
831 Clade-A fungi is estimated much younger, by about 10 million years, than the MRCA of leafcutter ants
832 (Table 1). This discrepancy has been interpreted as evidence that Clade-A fungi were acquired
833 secondarily by leafcutter ants, much after the origin of leafcutter fungiculture (Mikheyev *et al.* 2010;
834 Nygaard *et al.* 2016), and Clade-A fungi spread subsequently via nest-to-nest transfer between all
835 leafcutter-ant species across the entire leafcutter range from Argentina to the USA. However, when
836 comparing the stem age of the Clade-A lineage (age of split from Clade-B fungi) with the stem age of the
837 leafcutter ant lineage (age of split from the *Trachymyrmex septentrionalis* lineage), the ages are much
838 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
839 of the leafcutter lineage (Table 1). The somewhat older age of the Clade-A lineage could even suggest
840 that leafcutter ants did not originate coincident with Clade-A fungi as was assumed in the earliest
841 phylogenetic studies (Chapela *et al.* 1994; Hinkle *et al.* 1994), but that the Clade-A lineage may have
842 arisen before the origin of the leafcutter ant lineage, as discussed in Mueller *et al.* (in review). If so,
843 ancestral higher-attine lineages (ancestral to the leafcutter and *T. septentrionalis* lineages) may have
844 propagated both Clade-A and Clade-B fungi as far back as 22-25 mya, well before the origin of the
845 leafcutter ant lineage, and the propagation of both Clade-A and Clade-B fungi observed in extant
846 *Trachymyrmex* species and in extant leafcutter species could therefore be a retention of a plesiomorphic
847 condition of sharing of Clade-A and Clade-B fungi by higher-attine ant lineages.

848
849 **Table 1.** Comparison of crown ages and stem ages for Clade-A fungi and for the leafcutter ant clade,
850 estimated in six phylogenetic analyses conducted to date. Mikheyev *et al.* (2010) used a 4-gene
851 phylogeny to estimate the crown-node date (coalescent) and stem-node date of four Clade-A fungi
852 isolated from two *Acromyrmex* species from Panamá and Guyana and two *Atta* species from Panamá.
853 Nygaard *et al.* (2016) used 1075 orthologous loci from transcriptome-sequencing of two Clade-A fungi
854 from *Ac. echinator* and *Atta colombica* from Panamá. Both Mikheyev *et al.* and Nygaard *et al.* anchored
855 only a single time-calibrated node in their phylogenetic reconstructions, the last common ancestor of ant-
856 cultivated fungi with *Agaricus*, dated to 73 mya in Mikheyev *et al.* (modeled with more or less
857 conservative distributions around this date), and dated likewise to 73 mya in Nygaard *et al.* (modeled with
858 a 5% minimum age of 55 mya and a 95% maximum age of 91 mya). The ancient time-calibration (i.e.,
859 anchor at 73 mya) of the phylogenetic reconstructions is likely to render estimates of the dates of recent

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

862	<u>CLADE-A FUNGI</u>	<u>LEAFCUTTER ANT CLADE</u>	<u>SOURCE</u>
863	Crown Age of Clade-A Fungi	Crown Age of Leafcutter Ant Clade	
864		8 mya (6-15 mya), without the basal <i>Ac. striatus</i>	Schultz & Brady 2008
865	4 mya (0.5-8.0 mya)	(not estimated)	Mikheyev <i>et al.</i> 2010
866		12.2 mya (9.1-15.3), without <i>Ac. striatus</i>	Schultz <i>et al.</i> 2015
867	7.2 mya (5.5-9.0 mya)	16.2 mya (12.6-19.7 mya), without <i>Ac. striatus</i>	Nygaard <i>et al.</i> 2016
868		17.9 mya (15.6-20.4 mya), without <i>Ac. striatus</i>	Ješovnik <i>et al.</i> 2016
869		17.0 mya (13.2-20.8), without <i>Ac. striatus</i>	Branstetter <i>et al.</i> 2017
870		18.2 mya (14.2-22.2), with <i>Ac. striatus</i>	Branstetter <i>et al.</i> 2017
871	Stem Age of Clade-A Fungi	Stem Age of Leafcutter Ant Clade	
872		9 mya (7-15 mya)	Schultz & Brady 2008
873	25 mya (11-39 mya)	(not estimated)	Mikheyev <i>et al.</i> 2010
874	22.4 mya (16.9-27.9 mya)	17.8 mya (13.7-21.7 mya)	Nygaard <i>et al.</i> 2016
875		19.9 mya (17.7-22.5 mya)	Ješovnik <i>et al.</i> 2016
876		19.3 mya (15.2-23.7 mya)	Branstetter <i>et al.</i> 2017
877			
878	Stem Age of Clade-A Fungi	Stem Age of Higher-Attine Ant Clade	
879		20 mya (17-29 mya)	Schultz & Brady 2008
880	25 mya (11-39 mya)	(not estimated)	Mikheyev <i>et al.</i> 2010
881		(not estimated)	Schultz <i>et al.</i> 2015
882	22.4 mya (16.9-27.9 mya)	26.6 mya (19.6-33.8 mya)	Nygaard <i>et al.</i> 2016
883		33.3 mya (31.3-35.1 mya)	Ješovnik <i>et al.</i> 2016
884		31.4 mya (25.9-37.2 mya)	Branstetter <i>et al.</i> 2017
885			
886			

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

907
 908 To analyze evolution of higher-attine fungiculture, therefore, it may be more fruitful to view ant
 909 diversification and fungal-symbiont diversification as separate processes that may be, or may not be,
 910 intimately linked. Specifically, at least three scenarios seem plausible:

- 911 (i) Clade-A fungi originated coincident with the origin of leafcutter ants, and specialization by leafcutter
912 ants on superior Clade-A fungi facilitated the diversification of leafcutter ants, as assumed by earlier
913 studies (e.g., Stradling & Powell 1986; Chapela *et al.* 1994; Hinkle *et al.* 1994).
- 914 (ii) A successful lineage of Clade-A fungi entered leafcutter populations from other higher-attine lineages
915 (or even from lower-attine lineages; Schultz *et al.* 2015) after the origin of the leafcutter clade, then
916 spread across most leafcutter lineages through lateral transfer of particularly successful Clade-A
917 cultivar lineages, as assumed by Mikheyev *et al.* (2010) and Nygaard *et al.* (2016).
- 918 (iii) Clade-A fungi originated well before the origin of the leafcutter clade, such that ancestral Clade-A
919 fungi represented one of several cultivar lineages that circulated in a pool of diverse fungi shared by
920 ancestral higher-attine lineages, as discussed above and by Mueller *et al.* (in review). If so, Clade-A
921 and Clade-B fungi may have been shared between the diversifying higher-attine lineages, involving at
922 some later date also the ancestral leafcutter lineages, since the early evolution of higher-attine
923 lineages.

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

930

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

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

958

959 *Extensive cultivar sharing reduces ant-fungus specificity of leafcutter cultivars*

960 Our population-genetic and clonality analyses document extensive ongoing cultivar sharing between
961 sympatric *Atta* and *Acromyrmex* leafcutter ants, and such cultivar sharing likely involves in some

962 locations also some sympatric *Trachymyrmex* species (e.g., *Ac. versicolor* and *T. desertorum* in Arizona;
963 Fig. 1). Clade-A cultivars from *Trachymyrmex* species were unfortunately not included in our
964 microsatellite genotyping analyses because we became aware of the potential population-genetic linkages
965 between leafcutter-cultivated and *Trachymyrmex*-cultivated Clade-A fungi after conclusion of the
966 genotyping phase of our study. Sharing of cultivars between sympatric leafcutter and *Trachymyrmex* ants
967 therefore will need to be evaluated in a future study. With few exceptions known so far, single leafcutter
968 species seem to be specialized either on Clade-A fungi (e.g., all the dicot-foraging leafcutter species) or
969 on Clade-B fungi (*At. laevigata*, *At. vollenweideri*), which mirrors for leafcutter ants the kind of
970 specialization known also for ant species in the lower-attine *Cyphomyrmex wheeleri*-group, where each
971 *Cyphomyrmex* species cultivates predominantly its own fungal lineage (species), but different
972 *Cyphomyrmex* species are sometimes specialized on the same fungal lineage (i.e., two *Cyphomyrmex*
973 species can share the same kind of fungus; Mehdiabadi *et al.* 2012). Despite such specialization, there
974 exists now also evidence that single higher-attine species, as currently recognized, can cultivate both
975 Clade-A and Clade-B fungi in some locations (e.g., *At. laevigata* and *Ac. coronatus* in southern Brazil; *T.*
976 *arizonensis* in Arizona; see discussion of these cases in Table S10). Such cases of apparent fungal
977 polyculture will need to be elucidated likewise with high-resolution analyses of the respective leafcutter
978 ant hosts, to test for possible cryptic ant species.

979
980 Because of the extensive cultivars sharing of cultivars between sympatric *Acromyrmex*, *Atta*, and likely
981 also some *Trachymyrmex* species, and because of the possibility of genetic exchange between cultivars in
982 different nests, cultivars may not be propagated long enough within a single ant species to evolve
983 adaptations specific to a particular ant species (or ant genus) and its species-specific environment. This is
984 easiest to understand in the well-surveyed Clade-A fungi, where sympatric grass-cutting and dicot-cutting
985 species can cultivate strains of the same clonal lineages (strains that cannot be distinguished with five
986 microsatellite markers; Table S3). This sharing of the same fungal clone-lineages between sympatric
987 grass-cutting and dicot-cutting leafcutter species, as well as between *Atta*, *Acromyrmex*, and possibly also
988 *Trachymyrmex* ants, suggests that Clade-A fungi may indeed have evolved to be “general-purpose
989 genotypes” (Lynch 1984) suited for cultivation by diverse higher-attine species with diverse fungicultural
990 habits, as first suggested by Mikheyev *et al.* (2006).

991
992 *Shortcomings of our study and suggestions for future research on leafcutter fungi*

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

996
997 (1) Our phylogenetic analyses (Fig. 1; also Mueller *et al.* in review) indicate that some *Trachymyrmex*
998 species can also cultivate Clade-A cultivars, the dominant fungal type cultivated by leafcutter ants. A
999 complete population-genetic analyses of Clade-A fungi would therefore include also representative
1000 Clade-A fungi from *Trachymyrmex* species, to test for population-genetic links between leafcutter- and
1001 *Trachymyrmex*-cultivated fungi. Clade-A fungi from *Trachymyrmex* species were unfortunately not
1002 included in our microsatellite analyses because we became aware of Clade-A cultivation by
1003 *Trachymyrmex* ants only after conclusion of the genotyping phase of our study. Sympatric Clade-A
1004 fungus communities that should be evaluated in future studies include, for example, the community of
1005 Clade-A cultivars of *Ac.versicolor*, *Tr. desertorum*, and *Tr. arizonensis* in Arizona; and the community of
1006 Clade-A cultivars of diverse leafcutter species, *T. intermedius*, and *T. opulentus* in north-east South
1007 America and in Central America. [*T. opulentus* is labeled *T. wheeleri* in our Fig. 1, but actually
1008 synonymized according to Mayhé-Nunes & Brandão 2002]. *T. intermedius* ranges from Mexico to
1009 French Guiana, and *T. opulentus* ranges from Honduras to Guyana and northern Brazil, so Clade-A
1010 cultivation by these two *Trachymyrmex* species may occur in sympatry with the well-studied leafcutter
1011 species in Panamá. Lastly, sympatric Clade-B fungus communities likewise need further study, to test for
1012 possible sharing of Clade-B cultivars between leafcutter species and *Trachymyrmex* species, for example

1013 the Clade-B-cultivating *At. vollenweideri*, *At. laevigata*, *Ac. coronatus*, *Ac. fracticornis*, *Ac. laticeps*, *T.*
1014 *papulatus* (Fig. 1), and likely additional *Trachymyrmex* and possibly *Sericomyrmex* species in southern
1015 South America.

1016
1017 (2) Our population-genetic analyses (Fig. 2) rely on information from five highly-polymorphic
1018 microsatellite loci of a polyploid fungus (an individual may show more than two alleles/locus), and
1019 information from additional microsatellite loci would undoubtedly have increased resolution of
1020 population-genetic structure. In fact, prior analyses genotyping leafcutter fungi from the Panamá Canal
1021 Zone and from North America with, respectively, 9 and 12 microsatellite loci (Mikheyev *et al.* 2007;
1022 Mueller *et al.* 2011a) inferred a larger number of sympatric genotype-clusters (6 clusters in Panamá, 4
1023 clusters in North America; see also Figs. S7A-D identifying 3 clusters when we limit our analyses to
1024 Panamanian populations). Identification of three genotype-clusters across the leafcutter range in our 5-
1025 locus analysis (Fig. 2) therefore is a minimum estimate. Information from additional loci, however, is
1026 unlikely to show that fungal populations in Central America are more diverse than those in South
1027 America; rather, it seems likely that far more genotype-clusters will emerge when sampling South
1028 American populations with more loci at the same density as the well-surveyed Panamanian population in
1029 our study. For example, future studies could use the two multiplex panels (15 microsatellite loci total) of
1030 Carlson *et al.* (in press), or consider developing genotyping-by-sequencing methods (e.g., ddRAD) for
1031 garden material preserved in ethanol. The latter approach will likely require significant sequencing effort,
1032 because ethanol-preserved gardens contain non-cultivar DNA (e.g., from plant substrate, fungal
1033 endophytes, fungal pathogens, commensal microorganisms). However, information on non-cultivar DNA
1034 in gardens generated in genotyping-by-sequencing analyses could enrich simultaneously the
1035 understanding of the plant substrates used by attine ants; identify pathogenic, commensal, and mutualistic
1036 microorganisms coexisting in gardens (Mueller *et al.* 2005; Rodrigues *et al.* 2008, 2011; Mueller 2012);
1037 and enable tests for interactions between ant hosts, fungal cultivar types, and the diversity and prevalence
1038 of any additional microorganisms.

1039
1040 (3) Although our survey covered 17 countries across the leafcutter-ant range, several important countries
1041 were not sampled (e.g., Bolivia, Paraguay; much of north-west Central America, including Nicaragua, El
1042 Salvador, Guatemala, Belize, and we were able to obtain only one sample from Honduras) (Fig. 2). In
1043 addition, several important regions were not surveyed, for example vast regions in western and central
1044 Brazil, or a transect sampling across the Andes in Colombia (i.e., the transition zone from cultivation of
1045 three genotype-clusters in north-west South America to one genotype-cluster in Panamá; Fig. 2). Most
1046 important, the southernmost leafcutter populations in Argentina were not sampled (e.g., *Ac. lobicornis*
1047 ranges to $\approx 44^\circ$ south, whereas our southernmost collection was from $\approx 35^\circ$ south in Uruguay), as well as
1048 the western leafcutter populations in Argentina inhabited by unique leafcutter species like *At. saltensis*
1049 and *Ac. silvestri* (the likely sister species to the Clade-B-cultivating *Ac. striatus*; Fig. 1). Whereas our
1050 survey included representative Clade-A genotypes from the well-sampled northern range limit of
1051 leafcutter ants in Arizona, California, Texas, and Louisiana (surveyed in Mueller *et al.* 2011a),
1052 comparable information on the cultivar diversity is missing for the southern range limit of leafcutter ants.

1053 1054 **Conclusion**

1055 Most efforts to elucidate leafcutter ant-fungus associations focused so far on leafcutters in Central and
1056 North America (Table S6), but these leafcutter symbioses, all of them involving dicot-specialized
1057 leafcutter species, are not representative for the more complex leafcutter symbioses existing across South
1058 America (Figs. 1 & 2). Leafcutter species specialized on cultivation of Clade-B fungi occur only in South
1059 America (ranging from Argentina to Colombia; Fig. 1), the greatest concentration of Clade-B-cultivating
1060 leafcutter nests found so far is in southern South America (Table S1), and Clade-A fungi of leafcutter ants
1061 are more diverse in South America than in Central and North America (Fig. 2). This co-occurrence of the
1062 greatest leafcutter ant species diversity and greatest cultivar diversity in southern South America may not
1063 be a coincidence, yet the leafcutter ant-fungus associations in the savannahs of southern South America

1064 are far less understood than those in highly disturbed Central America forests dominated by weedy
1065 leafcutter-ant species. If the Kusnezov-Fowler hypothesis for the origin of leafcutter ants in subtropical
1066 savannahs of southern South America is correct and thus explains the concentrated diversity of leafcutter
1067 species there (Borgmeier 1959; Gonçalves 1961; Kuznezov 1963; Mariconi 1970; Fowler 1983; Farji-
1068 Brener & Ruggiero 1994; Bacci *et al.* 2009; Delabie *et al.* 2011; Brandão *et al.* 2011; Della Lucia 2011),
1069 a comprehensive cultivar survey in Argentina, Uruguay, Paraguay, Bolivia, and sub-Amazonian Brazil is
1070 most likely to uncover unknown types of leafcutter fungi (i.e., "Clade-C" or "Clade-D" cultivars), which
1071 will inform hypotheses on the diversity of cultivars available for cultivation at the origin of leafcutter ants
1072 19 million years ago.

1073
1074

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1082
1083

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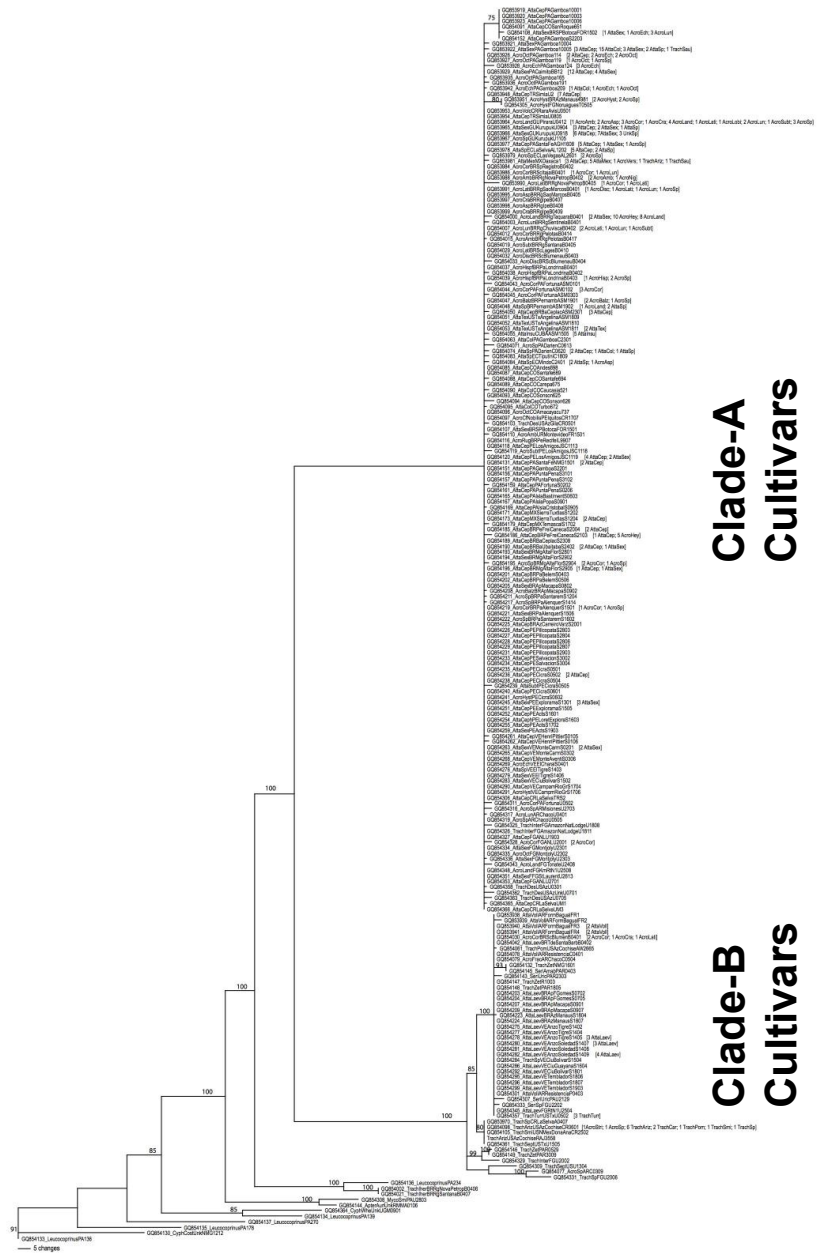
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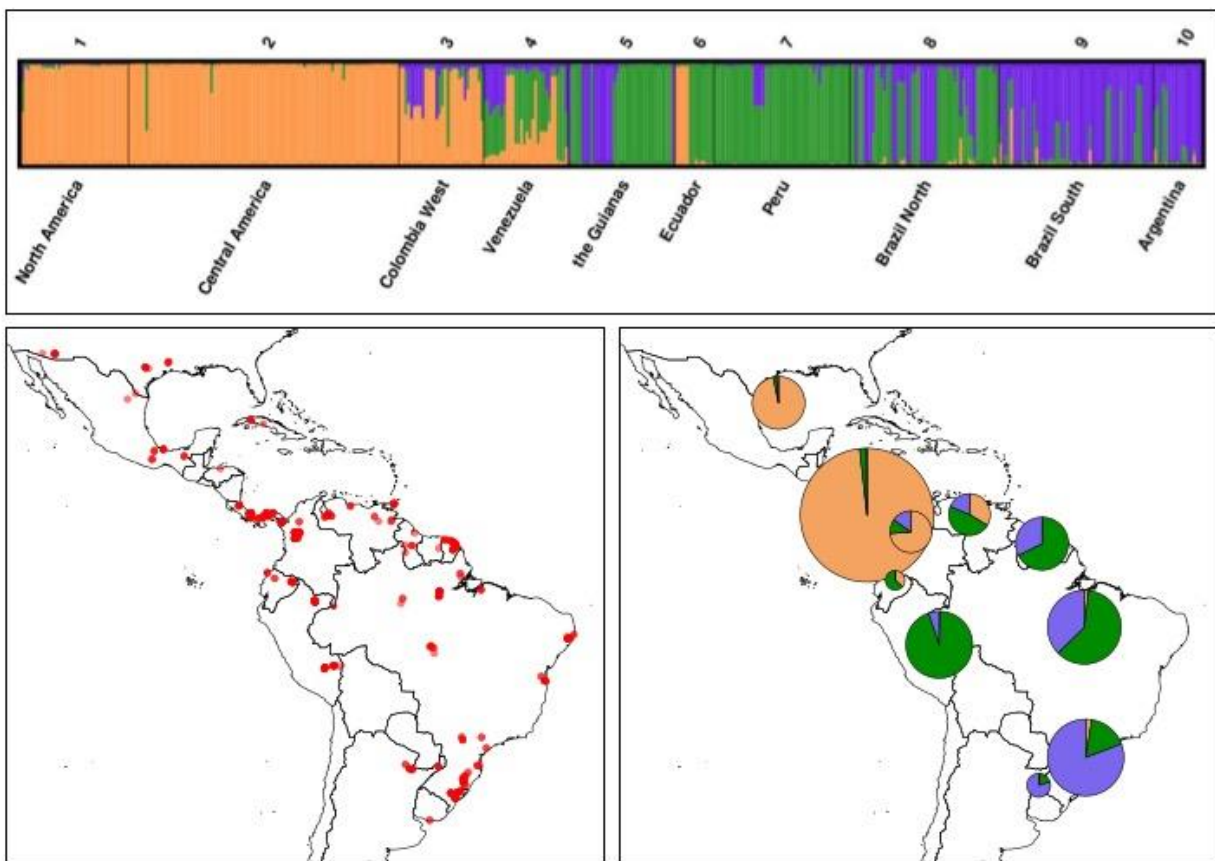
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Fig. 1. Phylogenetic relationships of fungi cultivated by higher-attine ants, based on EF-1 α sequence information. Table S1 summarizes collection information of the 430 fungal cultivars from leafcutter ants included in this analysis, 44 fungal cultivars from *Trachymyrmex* and *Sericomyrmex* ants, and 9 outgroup fungi (lower-attine cultivars and free-living *Leucocoprinus* fungi). We used the phylogenetic information from EF-1 α 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-1 α 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*.



1369 **Fig. 2.** Biogeographic patterns of 419 *L. gongylophorus* fungi cultivated by leafcutter ants (*Atta*,
 1370 *Acromyrmex*). Collection locations are shown in the bottom-left panel. Fungi are assigned by
 1371 STRUCTURE to three genotype clusters (purple, green, burnt orange), and membership in these three
 1372 clusters is mapped onto 10 biogeographic regions: 1. North America (Mexico, Cuba, USA). 2. Central
 1373 America (Panamá, Costa Rica, Honduras). 3. Colombia (west of Andes). 4. Venezuela. 5. The Guianas
 1374 (Guyana, Suriname, French Guiana). 6. Ecuador. 7. Peru. 8. northern Brazil. 9. southern Brazil.
 1375 Argentina & Uruguay. Information on exact collection locations, sample sizes, and leafcutter ant-hosts is
 1376 summarized in Tables S1 & S2. The sizes of the pie charts in the bottom-right panel corresponds to the
 1377 number of leafcutter nests surveyed in each of the 10 biogeographic regions; each pie chart is centered
 1378 on the centroid of collection locations from the respective region. The three genotype clusters map roughly
 1379 to southern South America, northern South America, and North & Central America. Populations of *L.*
 1380 *gongylophorus* fungi in Central and North America appear less diverse than populations in South
 1381 America.



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1384 **Table 1.** Comparison of crown ages and stem ages for Clade-A fungi and for the leafcutter ant clade,
 1385 estimated in six phylogenetic analyses conducted to date. Mikheyev *et al.* (2010) used a 4-gene
 1386 phylogeny to estimate the crown-node date (coalescent) and stem-node date of four Clade-A fungi
 1387 isolated from two *Acromyrmex* species from Panamá and Guyana and two *Atta* species from Panamá.
 1388 Nygaard *et al.* (2016) used 1075 orthologous loci from transcriptome-sequencing of two Clade-A fungi
 1389 from *Ac. echinator* and *Atta colombica* from Panamá. Both Mikheyev *et al.* and Nygaard *et al.* anchored
 1390 only a single time-calibrated node in their phylogenetic reconstructions, the last common ancestor of ant-
 1391 cultivated fungi with *Agaricus*, dated to 73 mya in Mikeyev *et al.* (modeled with more or less
 1392 conservative distributions around this date), and dated likewise to 73 mya in Nygaard *et al.* (modeled also
 1393 with a 5% minimum age of 55 mya and a 95% maximum age of 91 mya). The ancient time-calibration
 1394 (i.e., anchor at 73 mya) of the phylogenetic reconstructions is likely to render estimates of the dates of
 1395 recent diversifications (e.g., estimate of crown age of Clade-A fungi) more unreliable than estimates for
 1396 earlier diversifications. mya = million years ago.

1397	<u>CLADE-A FUNGI</u>	<u>LEAFCUTTER ANT CLADE</u>	<u>SOURCE</u>
1398	Crown Age of Clade-A Fungi	Crown Age of Leafcutter Ant Clade	
1399		8 mya (6-15 mya), without the basal <i>Ac. striatus</i>	Schultz & Brady 2008
1400	4 mya (0.5-8.0 mya)	(not estimated)	Mikheyev <i>et al.</i> 2010
1401		12.2 mya (9.1-15.3), without <i>Ac. striatus</i>	Schultz <i>et al.</i> 2015
1402	7.2 mya (5.5-9.0 mya)	16.2 mya (12.6-19.7 mya), without <i>Ac. striatus</i>	Nygaard <i>et al.</i> 2016
1403		17.9 mya (15.6-20.4 mya), without <i>Ac. striatus</i>	Ješovnik <i>et al.</i> 2016
1404		17.0 mya (13.2-20.8), without <i>Ac. striatus</i>	Branstetter <i>et al.</i> 2017
1405		18.2 mya (14.2-22.2), with <i>Ac. striatus</i>	Branstetter <i>et al.</i> 2017
1406	Stem Age of Clade-A Fungi	Stem Age of Leafcutter Ant Clade	
1407		9 mya (7-15 mya)	Schultz & Brady 2008
1408	25 mya (11-39 mya)	(not estimated)	Mikheyev <i>et al.</i> 2010
1409	22.4 mya (16.9-27.9 mya)	17.8 mya (13.7-21.7 mya)	Nygaard <i>et al.</i> 2016
1410		19.9 mya (17.7-22.5 mya)	Ješovnik <i>et al.</i> 2016
1411		19.3 mya (15.2-23.7 mya)	Branstetter <i>et al.</i> 2017
1412			
1413	Stem Age of Clade-A Fungi	Stem Age of Higher-Attine Ant Clade	
1414		20 mya (17-29 mya)	Schultz & Brady 2008
1415	25 mya (11-39 mya)	(not estimated)	Mikheyev <i>et al.</i> 2010
1416		(not estimated)	Schultz <i>et al.</i> 2015
1417	22.4 mya (16.9-27.9 mya)	26.6 mya (19.6-33.8 mya)	Nygaard <i>et al.</i> 2016
1418		33.3 mya (31.3-35.1 mya)	Ješovnik <i>et al.</i> 2016
1419		31.4 mya (25.9-37.2 mya)	Branstetter <i>et al.</i> 2017
1420			
1421			
1422			

1423 **Supporting Information**

1424

1425 **Biogeography of Leafcutter Ant-Fungus Mutualisms**

1426 Ulrich G. Mueller, Heather D. Ishak, Sofia M. Brushi, Scott E. Solomon, Chad C. Smith, Jacob J.
1427 Herman, Alexander S. Mikheyev, Jarrod J. Scott, Michael Cooper, Henrik H. De Fine Licht, Adriana
1428 Ortiz, Heraldo L. Vasconcelos, Ted. R. Schultz, The Leafcutter-Ant Consortium, and Mauricio Bacci Jr

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1431 **BACKGROUND INFORMATION**

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1433 **Fossil Record of Attine Ants from Dominican and Chiapas Amber (15-20 Million Years Ago)**

1434 In a description of a new species of *Apterostigma* from Dominican amber, Schultz (2007) summarizes the
1435 prior literature: "To date, three attine ant species have been described from Dominican amber:

1436 *Trachymyrmex primaevus* (Baroni Urbani 1980), *Cyphomyrmex maya*, and *Cyphomyrmex taino* (de

1437 Andrade 2003). In addition, Brown (1973) refers to possible *Mycetosoritis* males in Chiapas amber

1438 (Oligo-Miocene, ~20 mya), Wilson (1985) refers to *Cyphomyrmex* in Dominican amber, and Baroni

1439 Urbani (1995) refers to *Apterostigma* and *Cyphomyrmex* in Dominican amber." These publications

1440 represent the complete literature to date on described fossil attine species. The ages of Dominican and

1441 Mexican (Chiapas) amber are typically cited as ~20 million years old (mya), dating to the early Miocene.

1442 LaPolla *et al.* (2013) list ages of 16-19 mya for Dominican amber and 15-20 mya for Mexican amber.

1443 There exist two published photographs labeled "*Acromyrmex*" fossils in Dominican amber (page 446 in

1444 Grimaldi & Engel 2005; page 246 in Nudds & Selden 2013), but an unambiguous assignment of these

1445 fossils to the genus *Acromyrmex* is not possible from the spinulation, head, and integumental features

1446 visible in these photographs. The specimen shown in Nudds & Selden (2013) measures about 2mm

1447 length, which would be an unusually small caste size for extant *Acromyrmex* species. The "chewed leaf

1448 fragments" embedded with an attine ant in the same amber fossil shown in Grimaldi & Engel (2005)

1449 reveals leaf-damage that is atypical for leafcutter ants (the damaged edges are serrated in the fossilized

1450 leaf fragments, unlike the smooth cuts made in leaves by extant leafcutter species), and the presence of

1451 both damaged leaf fragments and an attine ant in the same amber fossil could be coincidental. The

1452 photographed attine ant specimens could represent higher-attine lineages predating the origin of leafcutter

1453 ants, or higher-attine lineages outside of the leafcutter clade, such as lineages near *Trachymyrmex*

1454 *primaevus* described also from Dominican amber (Baroni Urbani 1980; *T. primaevus* measures 3-4 mm in

1455 length, somewhat larger than the specimen shown in Nudds & Selden 2013). The fossil specimen

1456 appearing on page 446 in Grimaldi & Engel (2005) appears to be lost, per communication by David

1457 Grimaldi with Ted Schultz.

1458 Fossilized gardens and garden chambers dating to 5.7–10 mya, possibly from *Acromyrmex* or

1459 *Trachymyrmex* ants, have been described from La Pampa Province in Argentina (Genise *et al.* 2013; see

1460 also Laza 1982). Fossilized gardens are not known from Central or North America.

1461

1462 **The name *Leucocoprinus gongylophorus*, not *Leucoagaricus gongylophorus*, is the proper name for**
1463 **the sporocarp described by Möller (1893) from an *Acromyrmex* garden in southern Brazil**

1464 The widely-cited placement by R. Singer (1986) of sporocarps (mushrooms) of fungi cultivated by

1465 leafcutter ants into the genus *Leucoagaricus* is based on inaccurate reporting of prior literature. To

1466 discuss these inaccuracies, we first provide the exact writing from Singer's book (*The Agaricales in*

1467 *Modern Taxonomy*. 4th Edition. Koeltz Scientific Books, Koenigstein, Germany, 1986):

1468 "It has been indicated by me (1951) [= Singer, *Lilloa Revista de Botánica*, vol. 22, p. 429: "Judging
1469 from the original account which includes a photograph, the often cited *Rozites gongylophora* Moeller is

1470 not a *Rozites* but an *Agaricus* sp.]" that the fungus (or one of the fungi) living in symbiosis with ants

1471 (Attini) - "cultivated" by the ants according to some zoologists - is an agaricaceous fungus. N.A.

1472 Weber (in several papers 1955-57) has shown that the fungi appearing in his cultures actually belong

1473 in *Leucoagaricus*, and so do some of the isolates from various ants indicated by Hervey, Rogerson &
1474 Long (*Brittonia* 29: 226-236. 1977). According to Heim (*Rev. Mycol.* 22: 299. 1957) and Singer
1475 (*Agaricales in Modern Taxonomy* 3rd ed. p. 455. 1975) both Weber's and Hervey's agarics are
1476 specifically identical and congeneric with a *Leucoagaricus* which should be known as *Leucoagaricus*
1477 *gongylophorus* (Möller) Sing. (c.n. *Rozites gongylophorus* Möller, *Bot. Mitt. Trop.* 6: 70, pl. I-II.
1478 1893). A.H. Smith (in Weber) was the first to identify Weber's agaric correctly as *Leucoagaricus*.
1479 The preceding paragraph represents the entire treatment of ant-cultivated fungi in Singer's book (1986).

1480 The paragraph from Singer (1986) contains several inaccuracies:

- 1481 (1) Heim (*Rev. Mycol.* 22: 293-299. 1957) re-described Möller's original description of *Rozites*
1482 *gongylophora* as *Leucocoprinus gongylophorus* [page 299: "dénomination nouvelle de *Leucocoprinus*
1483 *gongylophorus* (Möller)"], not *Leucoagaricus*, as incorrectly stated in the above writing by Singer.
- 1484 (2) Hervey, Rogerson & Long (*Brittonia* 29: 226-236. 1977) do not identify sporocarps of leafcutter
1485 fungi, but sporocarps grown from pure cultures of lower-attine fungi (fungi from the ants
1486 *Myrmecocrypta buenzlii*, *Mycetophylax conformis*, *Apetostigma auriculatum*, *Cyphomyrmex*
1487 *costatus*), and they identify these as "*Lepiota* sp. (*Leucocoprinus* sp.)" (page 234), not *Leucoagaricus*,
1488 as incorrectly stated in the above writing by Singer.
- 1489 (3) Weber's publications from 1955-1957 identify the fungi cultivated by attine ants as (a) unidentified
1490 "pure cultures" (Weber 1955, *Science* 121: 109; cultures isolated from gardens of *Apterostigma*,
1491 *Cyphomyrmex*, *Trachymyrmex*, *Atta*), (b) unidentified "pure culture" (Weber 1956, *Ecology* 37: 197-
1492 199; culture of the fungus isolated from garden of *Trachymyrmex septentrionalis*), and (c) "*Lepiota*"
1493 (Weber 1957, *Ecology* 38: 480-494; sporocarp from pure culture of *Cyphomyrmex costatus*). That is,
1494 Weber did not succeed at growing, and did not examine, a sporocarp of a leafcutter fungus, but a
1495 sporocarp from a lower-attine ant (*C. costatus*), and Weber reported this to be a *Lepiota*. The
1496 sporocarp identified by "A.H. Smith (in Weber)" (quote from Singer's above paragraph) is therefore
1497 a sporocarp produced by a culture of fungus from *C. costatus* (a lower-attine fungus), and Smith
1498 believed this to be a *Lepiota* (Weber 1957). Therefore, Singer's above claim is incorrect that "N.A.
1499 Weber (in several papers 1955-57) has shown that the fungi appearing in his cultures actually belong
1500 in *Leucoagaricus*", and Singer's above claim is incorrect that "H. Smith (in Weber) was the first to
1501 identify Weber's agaric correctly as *Leucoagaricus*" (instead, Smith identified one lower-attine
1502 fungus as a *Lepiota*).
- 1503 (4) Singer's reference to his earlier writing (1975; *Agaricales in Modern Taxonomy* 3rd ed. p. 454; not p.
1504 455 as stated in Singer's above writing from 1986) refers to a single sentence in Singer (1975)
1505 discussing the placement of ant-cultivated fungi (page 454): "N.A. Weber (in several papers 1955-57)
1506 has shown that the fungi appearing in his cultures actually belong in *Leucoagaricus*." Singer (1975)
1507 therefore makes the same mistake as Singer (1986) in incorrectly stating that Weber identified the
1508 leafcutter-cultivated fungi as *Leucoagaricus* (instead, Weber reports a *Lepiota* sporocarp grown from
1509 a culture of a lower-attine fungus).

1510 Because of the inaccuracies in Singer (1986), we therefore follow here Heim (1957) and Hervey *et al.*
1511 (1977) in placing all ant-cultivated fungi into the genus *Leucocoprinus*. Moreover, Else Vellinga (UC
1512 Berkeley) communicated to us (19. May 2017) that she will submit shortly a revision of
1513 leucocoprinaceous fungi, including all *Leucoagaricus* and *Leucocoprinus*. Because described species of
1514 *Leucoagaricus* are not monophyletic, and because described species of *Leucocoprinus* are likewise not
1515 monophyletic (Vellinga 2004), E. Vellinga will reassign all *Leucoagaricus* to *Leucocoprinus* (the name
1516 *Leucocoprinus* has priority over *Leucoagaricus*), to generate a monophyletic genus (*Leucocoprinus sensu*
1517 *lato*), with monophyly supported by several r-DNA genes.

1519 **Spore Germination of *Leucocoprinus gongylophorus***

1520 Mueller (2002) lists published studies that attempted to germinate spores of *L. gongylophorus*, but to our
1521 knowledge only Möller (1893) succeeded so far at germinating spores of *L. gongylophorus* and obtain
1522 from these spores mycelium that developed staphylae (clusters of gongylidia) typical for *L.*

1523 *gongylophorus*. Because Möller's work is not readily available and was written in German, we provide
1524 here an English translation of Möller's experiments describing germination of spores produced by
1525 *Leucocoprinus gongylophorus* mushrooms (called *Rozites gongylophora* by Möller). Our translation
1526 eliminates a few peripheral details, but the translation faithfully presents the essence of the experiments.
1527 Phrases appearing in our translation in square brackets [...] are inserted to improve clarity. We translate
1528 both "Kohlrabihäufchen" (literally: small aggregate of kohlrabi, a kind of turniplike cabbage) and
1529 "Futterkörperchen" (literally: food corpuscle) as "staphylae" (= aggregate of gongylidia).

1530 Pages 78-79 in Alfred Möller (1893) *Die Pilzgärten einiger südamerikanischer Ameisen* (Verlag Gustav
1531 Fisher, Jena, Germany) describe Möller's spore germination experiments, starting on page 78 with "Die
1532 Sporen keimen..." and ending on page 79 with "... sogar überlegen waren".
1533 "The spores germinate in water or nutrient broth two days after inoculation, with only one germ tube,
1534 which emanates from a small opening opposite to the original attachment site of the spore [attachment at
1535 the basidium] ... and the germ tube expands to a thickness of 7-8 μm (Fig. 8). Spore germination
1536 progressed irregularly, and invariably only a limited number of spores germinated. Frequently the germ
1537 tube develops a side branch shortly after exiting the spore. The subsequent growth of the mycelia is very
1538 slow. Only on the ninth day does the germinating mycelium form a whitish speck, which shows aerial
1539 hyphae, that is visible to the unaided eye. The individual hyphae contained granule- and vacuole-rich
1540 protoplasm, the hyphae show early a tendency for irregular thickness and swellings, and the hyphae were
1541 characterized by extraordinary variable thickness; not infrequently one could see a side branch of 3 μm
1542 diameter branching from hyphae of 10 μm diameter. I made the same kind of observation in cultures that
1543 I obtained from staphylae [Kohlrabihäufchen] taken from [natural gardens in] nests. Subsequently, the
1544 mycelia derived from the basidiospores became also similar to the mycelia derived from staphylae in that
1545 the aerial hyphae exhibited a winding and corkscrew-like growth. Finally, after five weeks of careful
1546 maintenance (20. February until 8. April 1892), the mycelia derived from germinating spores developed
1547 staphylae. The development of these staphylae [derived from germinating spores] began in exactly the
1548 same way as described for the cultures obtained from staphylae from gardens, as shown in Fig. 33 (Plate
1549 VIII). The staphylae increased gradually in abundance, and staphylae [Futterkörperchen] materialized
1550 that were of equal size to, or sometimes larger than, the staphylae in [natural gardens in] nests."

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1553 **ADDITIONAL DISCUSSION OF RESULTS**

1554

1555 **Are there Differences between Fungi Cultivated by Dicot- Versus Grass-Cutting Leafcutter Ants?**

1556 *Acromyrmex*: For 23 *Acromyrmex* species for which fungicultural information is known, all of the 17
1557 dicot specialists cultivate Clade-A fungi, and only two of these 17 (*Ac. coronatus*, *Ac. crassispinus*) also
1558 cultivate sometimes Clade-B fungi (Table S6). Of the four *Acromyrmex* species specialized to cut
1559 grasses, one species (*Ac. fracticornis*) cultivates a Clade-B fungus, but only a single fungus was identified
1560 for this species. Of the two *Acromyrmex* species cutting both dicots and grasses, one species (*Ac.*
1561 *striatus*) cultivates a Clade-B fungus, but again, only a single fungus was identified for this species.
1562 *Atta*: For 10 *Atta* species for which fungicultural information is known, all of the 6 dicot specialists
1563 cultivate Clade-A fungi. Of the three *Atta* species specialized to cut grasses, one species, *Atta*
1564 *vollenweideri*, cultivates a Clade-B fungus (8 fungi from two sites were surveyed for *At. vollenweideri*),
1565 whereas the two other grass-cutting species *At. capiguara* and *At. bisphaerica* cultivate a Clade-A fungus,
1566 but only one fungus was identified for each of these two species (Silva Pinhati *et al.* 2004; Table S6).
1567 *Atta laevigata* forages on both grasses and dicots (Nagamoto *et al.* 2009), and cultivates a Clade-B fungus
1568 throughout much of its range from Venezuela to southern Brazil (28 nests from multiple sites were
1569 surveyed; Table S6), but we observed also two cases of Clade-A cultivation by *At. laevigata*, known for
1570 this species so far only from southern Brazil (details in Table S10).

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1573 **Apparent cultivar specialization of *Atta* vs *Acromyrmex* ants reported in a previous analysis**
1574 Kooij *et al.* (2015b, page 13) write that “fungal symbionts of *Atta* and *Acromyrmex* colonies showed that
1575 they were completely separated ... consistent with earlier findings by Mikheyev *et al.* (2007) for the same
1576 sampling site”. This statement is misleading because Mikheyev *et al.* (2007) actually documented that
1577 *Atta* and *Acromyrmex* ants “shared identical fungus garden genotypes, indicating wide-spread cultivar
1578 exchange” (Abstract in Mikheyev *et al.* 2007). The leafcutter cultivar lineages studied by Mikheyev *et al.*
1579 (2007, Abstract) were “largely unstructured with respect to host ant species, with only 10% of the
1580 structure in genetic variance being attributable to partitioning among ant species and genera”.
1581 Specifically, Fig. 3 in Mikheyev *et al.* (2007) shows that fungal diversity associated with Panamanian
1582 leafcutter ants is structured into 6 fungal genotype-clusters, and that there exists very little correlation
1583 between these fungal clusters and leafcutter ant genera (i.e., each of the leafcutter ant species essentially
1584 cultivates representatives from each or most of these 6 fungal genotype-clusters). The same conclusion of
1585 absence of cultivar specialization of *Atta* vs *Acromyrmex* ants emerged also in our much larger analysis of
1586 leafcutter cultivars from Panamá (Fig. S7). Therefore, the fungi examined by Kooij *et al.* (2015b) were
1587 unfortunately selectively sampled from the true diversity of fungi cultivated by each leafcutter species in
1588 Panamá, such that Kooij *et al.* (2015b) oversampled one fungus lineage for *Atta* ants (n=9 fungi) and
1589 oversampled another fungus lineage for *Acromyrmex* ants (n=9 fungi).

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1636 **List of Tables in Supporting Information**

1637 **Table S1.** Collection information of fungal samples (collection ID, host-ant species, collection location,
1638 GPS, voucher storage, etc), summary of sequence information generated (EF-1 α , RAD, DMS genes),
1639 Genbank accessions, and summary of microsatellite-marker information generated (loci A1132, C101,
1640 C126, C117, B12).

1641 **Table S2.** Number of leafcutter-fungus collections characterized by DNA sequencing and/or
1642 microsatellite marker analyses, listed by ant-host species and by country of collection.

1643 **Table S3.** Microsatellite allele profiles of 419 fungal cultivars from gardens of leafcutter nests, collection
1644 information of fungal samples (collection ID, host-ant species, country of collection, GPS), and
1645 information on foraging preferences of the respective ant-host (preference to foraged on grass, dicot
1646 plants, or both as main fungicultural substrate; see also Table S6). Additional collection information is in
1647 Table S1 for all samples. To identify samples that are identical in all alleles across the 5 microsatellite
1648 loci screened (i.e., samples assigned to the same fungal genotype or "clone", as defined by the 5 loci), the
1649 samples are sorted in the spreadsheet by allele and locus. A total of 241 fungal genotypes are among the
1650 419 cultivars screened. Samples that are identical in all alleles are color-coded as follows: Yellow
1651 identifies cultivar samples of identical genotype (same fungal "clone") collected in different nests of the
1652 same ant-host species. Orange identifies genotypes ("clones") for which at least some samples were
1653 collected in different nests of different ant-host species of the *same* ant genus. Green identifies genotypes
1654 ("clones") for which at least some samples were collected in different nests of *different* ant-host genera
1655 (*Atta* or *Acromyrmex*). Highlighting in other colors (pale yellow, pale orange) identifies genotypes for
1656 which allele information was missing at one locus, so it was not possible to determine for these genotypes
1657 whether they had identical allele profiles across all 5 microsatellite loci (i.e., these genotypes were
1658 defined by allele identity across the 4 loci for which information was available).

1659 **Table S4.** Total number of alleles scored at each locus for each of the 419 cultivars from gardens of
1660 leafcutter nests, and the averages of these total numbers across the 419 cultivars for each of the 5 loci
1661 screened. Table S4 is identical to Table S3, except for the addition of columns for calculating of total
1662 number alleles and averages. The averages across all individuals are at the bottom of Table S4.

1663 **Table S5.** Taxa with identical sequences (except sequence ambiguities) that are listed next to each other
1664 as a string of taxa as the same terminal leaf in Fig. 1.

1665 **Table S6.** Foraging preferences of leafcutter ant species and their fungicultural specializations on Clade-
1666 A fungi, Clade-B fungi, or both types of fungi.

1667 **Table S7.** Total number of alleles at 5 microsatellite loci for fungi from *Atta* and *Acromyrmex* nests,
1668 summarized for narrow geographic region from within which both *Atta* and *Acromyrmex* were collected
1669 and genotyped at all 5 loci. Total number of alleles/fungus does not differ between fungi from *Atta* and
1670 *Acromyrmex* nests (Wilcoxon Signed-Rank Test, $W = 90.5$, $z = 0.181$, $p = 0.857$ two-tailed, $n = 19$),
1671 comparing samples from 19 locations (highlighted in blue in Table S7) for which complete genotype
1672 information (all 5 loci) is available for both *Atta*-cultivated and *Acromyrmex*-cultivated fungi. The raw
1673 data summarized in Table S7 appear in Table S8.

1674 **Table S8.** Raw data used to generate statistics summarized in Table S7.

1675 **Table S9.** Primer sequences developed by Mikheyev *et al.* (2006) for EF-1 α , DMC1, and RAD51 genes,
1676 as well as corresponding annealing temperatures (T_m).

1677 **Table S10.** Summary of higher-attine ant species found so far to cultivate both Clade-A and Clade-B
1678 fungi.

1679 **Tables S1-S6 & S8 are attached as separate spreadsheets. Tables S7, S9 & S10 are inserted below in**
1680 **this document.**

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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).

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

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1688 **Table S3.** Microsatellite allele profiles of 419 fungal cultivars from gardens of leafcutter nests, collection
1689 information of samples, and information on foraging preferences of the respective ant-host (preference to
1690 foraged on grass, dicot plants, or both as main fungicultural substrate; see also Table S6). Additional
1691 collection information is in Table S1 for all samples. Samples that are identical in all alleles across the 5
1692 microsatellite loci screened are assigned to the same fungal genotype (i.e., same "clone"). A total of 241
1693 fungal genotypes are among the 419 cultivars screened. Yellow identifies cultivar samples of the same
1694 fungal clone collected in different nests of the same ant-host species. Orange identifies the same fungal
1695 clones for which at least some samples were collected in different nests of different ant-host species of the
1696 *same* ant genus. Green identifies the same fungal clone for which at least some samples were collected in
1697 different nests of *different* ant-host genera (*Atta* or *Acromyrmex*). Highlighting in other colors (pale
1698 yellow, pale orange) identifies genotypes for which allele information was missing at one locus, so it was
1699 not possible to determine for these genotypes whether they had identical allele profiles across all 5 loci.
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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.

The image displays a large, multi-column heatmap table. The columns represent different loci and cultivars, while the rows represent individual data points. The cells are color-coded, with yellow and orange indicating higher values and green indicating lower values. The table is organized into several vertical sections, with the rightmost section containing summary statistics for each locus. The overall layout is dense and detailed, typical of a large-scale genomic or genetic data analysis.

1707 **Table S5.** Taxa with identical EF-1 α sequences (except sequence ambiguities) and listed next to each
 1708 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 accession listed in Table S5, the corresponding sample IDs and collection records are listed in Table S1

Table with columns for taxon IDs and their corresponding EF-1 α sequences. The table is organized into sections: Clade A, Clade B, and Clade C. Each section contains multiple rows of taxon IDs and their associated sequences.

Clade A

Clade B

Clade C

Clade D

Clade E

Clade F

Clade G

Clade H

Clade I

Clade J

Clade K

Clade L

Clade M

Clade N

Clade O

Clade P

Clade Q

Clade R

Clade S

Clade T

Clade U

Clade V

Clade W

Clade X

Clade Y

Clade Z

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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 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 EFM = microsatellite genotyping	Sample Size Total Cultivars Identified Between All Studies	References for Fungus Identification
<i>Acromyrmex</i>	<i>ambiguus</i>	dicot	A	s, m	10	this study; Peirera <i>et al.</i> 2015
	<i>asperus</i>	dicot	A	s, m	8	this study
	<i>coronatus</i>	dicot	mostly A, some B	s, m	21	this study
	<i>crassispinus</i>	dicot	both A & B	s, m	5	this study; Silva-Pinhati <i>et al.</i> 2004
	<i>dfasciger</i>	dicot	A	s, m	4	this study; Silva-Pinhati <i>et al.</i> 2004
	<i>echinator</i>	dicot	A	s, m	43	this study; Kooij <i>et al.</i> 2015a,b; Wallace <i>et al.</i> 2014; Poulsen <i>et al.</i> 2009; Mikheyev <i>et al.</i> 2006, 2007
	<i>hispidus</i>	dicot	A	s, m	6	this study; Silva-Pinhati <i>et al.</i> 2004
	<i>hydris</i>	dicot	A	s, m	7	this study
	<i>laticeps</i>	dicot	A	s, m	9	this study; Silva-Pinhati <i>et al.</i> 2004
	<i>lundii</i>	dicot	A	s, m	11	this study
	<i>nobilis</i>	dicot	A	s, m	1	this study
	<i>octospinosus</i>	dicot	A	s, m	54	this study; Kooij <i>et al.</i> 2015a; Wallace <i>et al.</i> 2014; Poulsen <i>et al.</i> 2009; Mikheyev <i>et al.</i> 2006, 2007
	<i>pubescens</i>	dicot	A	s	1	Bich <i>et al.</i> 2016
	<i>rugosus</i>	dicot	A	s, m	2	this study; Silva-Pinhati <i>et al.</i> 2004
	<i>subterraneus</i>	dicot	A	s, m	7	this study; Silva-Pinhati <i>et al.</i> 2004
	<i>versicolor</i>	dicot, some grass	A	s, m	35	this study; Mueller <i>et al.</i> 2011
	<i>volcanus</i>	dicot	A	s, m	1	this study
	<i>balzani</i>	grass	A	s, m	4	this study
	<i>fracticornis</i>	grass	B	s	1	this study
	<i>heyeri</i>	grass	A	s, m	18	this study; Peirera <i>et al.</i> 2015
	<i>landolti</i>	grass	A	s, m	20	this study
	<i>lobicornis</i>	both	A	s	9	this study; Lugo <i>et al.</i> 2013
	<i>striatus</i>	both	B	s	1	this study
<i>Atta</i>	<i>cephalotes</i>	dicot	A	s, m	194	this study; Kooij <i>et al.</i> 2015a; Wallace <i>et al.</i> 2014; Mueller <i>et al.</i> 2011; Mikheyev <i>et al.</i> 2006, 2007; Silva-Pinhati <i>et al.</i> 2004; Chapela <i>et al.</i> 1994
	<i>colombica</i>	dicot	A	s, m	39	this study; Kooij <i>et al.</i> 2015b; Wallace <i>et al.</i> 2014; Mikheyev <i>et al.</i> 2006, 2007
	<i>insularis</i>	dicot	A	s, m	5	this study; Mueller <i>et al.</i> 2011
	<i>mexicana</i>	dicot	A	s, m	7	this study; Mueller <i>et al.</i> 2011; Chapela <i>et al.</i> 1994
	<i>sexdens</i>	dicot	A	s, m	51	this study; Kooij <i>et al.</i> 2015a; Mikheyev <i>et al.</i> 2007; Silva-Pinhati <i>et al.</i> 2004
	<i>texana</i>	dicot	A	s, m	165	this study; Mueller <i>et al.</i> 2011; Mikheyev <i>et al.</i> 2006, 2008
	<i>bisphaerica</i>	grass	A	s	1	Silva-Pinhati <i>et al.</i> 2004
	<i>capiguara</i>	grass	A	s	1	Silva-Pinhati <i>et al.</i> 2004
	<i>vollenweideri</i>	grass, some dicot	B	s	8	this study
	<i>laevigata</i>	both	mostly B, few A	s	28	this study; Silva-Pinhati <i>et al.</i> 2004

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1713 **Table S7.** Total number of alleles/fungus at 5 microsatellite loci for fungi from *Atta* and *Acromyrmex*
 1714 nests, summarized for narrow geographic region from within which both *Atta* and *Acromyrmex* were
 1715 collected and genotyped at all 5 loci. Total number of alleles/fungus does not differ between fungi from
 1716 *Atta* and *Acromyrmex* nests (Wilcoxon Signed-Rank Test, $W = 90.5$, $z = 0.181$, $p = 0.857$ two-tailed, $n =$
 1717 19), comparing samples from 19 locations (highlighted in blue in Table S7) for which complete genotype
 1718 information (at all 5 loci) is available for both *Atta*-cultivated and *Acromyrmex*-cultivated fungi from that
 1719 location. Table S7 summarizes the raw data and statistics in Table S8. Across the 19 locations, total
 1720 number of alleles/fungus is correlated between *Atta*- and *Acro*-cultivated fungi (Fig. S6), i.e., at locations
 1721 where *Atta*-cultivated fungi show more alleles, the sympatric *Acro*-cultivated fungi show likewise more
 1722 alleles (Spearman rank-order correlation $t = 2.39$, $df = 17$, $p = 0.029$, $r = 0.502$; Fig. S6), indicating that,
 1723 at each location, *Atta* and *Acromyrmex* recruit cultivars from the same shared community of fungi.
 1724

Country, Province/State	Average Total Number Alleles/Fungus Across All 5 Loci Screened	
	Fungi from <i>Atta</i> nests	Fungi from <i>Acromyrmex</i> 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 <i>Acromyrmex</i> collected
Cuba	10.8 (n=5 nests, 1 ant species)	no fungus from <i>Acromyrmex</i> collected
Honduras	10.0 (n=1 nest, 1 ant species)	no fungus from <i>Acromyrmex</i> 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 <i>Acromyrmex</i> 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 <i>Acromyrmex</i> 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 nest, 1 ant species)	no fungus from <i>Acromyrmex</i> 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 <i>Acromyrmex</i> 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 <i>Atta</i> collected	8.6 (n=5 nests, 3 ant species)
Brazil South, Rio Grande do Sul State	no fungus from <i>Atta</i> collected	9.1 (n=39 nests, 9 ant species)
Argentina	no fungus from <i>Atta</i> genotyped	8.2 (n=10 nests, 4 ant species)
Uruguay	no fungus from <i>Atta</i> collected	7.5 (n=2 nests, 2 ant species)

1725

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

The image displays a large, multi-column table of raw data. The table is oriented vertically on the page. It contains numerous columns of data, with some cells highlighted in yellow. In the top right corner of the table area, there is a small inset plot showing a scatter plot with a regression line. The plot has a y-axis labeled 'Number of ...' and an x-axis labeled 'Number of ...'. The regression line shows a positive correlation between the two variables. The table itself is very dense with text, likely representing individual data points or observations across different categories or time points.

1728 **Table S9.** Primer sequences developed by Mikheyev *et al.* (2006) for EF-1 α , DMC1, and RAD51 genes,
1729 as well as corresponding annealing temperatures (T_m).
1730

Gene	Forward-F (5' → 3')	Reverse-R (5' → 3')	T_m
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

1731

1732 **Table S10.** Summary of higher-attine ant species found so far to cultivate both Clade-A and Clade-B
1733 fungi. Each of these ant species could potentially represent different cryptic species, and the different
1734 cryptic species may be specialized on different fungal clades. However, cryptic ant species have not been
1735 found in the sequencing analyses of *Atta laevigata* (Fig. 4 in Solomon *et al.* 2008) and *Trachymyrmex*
1736 *arizonensis* (Fig. 21 in Rabeling *et al.* 2007). The below information is summarized from Table S1 (this
1737 study), Table S1 of Mueller *et al.* (in review), and one unpublished collection (UGM080928-02).
1738

1739 *Atta laevigata*

1740 Of 28 cultivars from 28 *Acromyrmex laevigata* nests characterized so far, 26 are Clade-B fungi (Genbank
1741 sequences GQ854042, GQ854203, GQ854204, GQ854207, GQ854209, GQ854223, GQ854224,
1742 GQ854345, GQ854275, GQ854277, GQ854278, GQ854280, GQ854281, GQ854282, GQ854285,
1743 GQ854286, GQ854287, GQ854288, GQ854292, GQ854293, GQ854294, GQ854295, GQ854296,
1744 GQ854297, GQ854298, GQ854299) and 2 are Clade-A fungi (Genbank sequences AF076409-AF076416
1745 from six cloned ITS sequences from mycelium from a single garden of *At. laevigata* reported in Silva-
1746 Pinhati *et al.* 2004; **ADD GENBANK #** from this study here). The 26 Clade-B-cultivating nests were
1747 collected in Venezuela, French Guyana, and throughout Brazil (States of Amapá, Amazonas, São Paulo;
1748 Table S1). The Clade-A-cultivating nests were collected in Rio Claro, São Paulo, Brazil (Silva-Pinhati *et al.*
1749 *et al.* 2004), and near Piracanjuba, Goiás, Brazil (collection UGM080928-02, a young nest with a single
1750 garden of about 3 cm diameter at 18 cm depth, with garden substrate of "only grass", characteristic for *At.*
1751 *laevigata*). The corresponding ant hosts were identified from morphological characters and sequencing
1752 by Solomon *et al.* (2008), and from morphological characters by Silva-Pinhati *et al.* (2004). A worker of
1753 the Clade-A-cultivating nest UGM080928-02 was identified by sequencing of the mitochondrial
1754 cytochrome-oxidase 1 (CO1) gene (see below; **GENBANK #**), and the fungus of that same nest
1755 UGM080928-02 was identified by sequencing the EF-1 α gene (see below; **GENBANK #**). Locations of
1756 vouchers are listed in Table S1. Clade-B- and Clade-A-cultivating nests of *At. laevigata* therefore appear
1757 to be sympatric in southern Brazil.

1758 Genbank accessions for nest UGM080928-02 are not yet released, so we list here actual sequences:

1759 EF-1 α sequence of fungus (Clade-A cultivar) from nest UGM080928-02:

1760 ATTCGATTATACTGATCATGTCCAGTGTGATGTCCGTTTCAGTGGAGTGAGGACCGTTTCAATGAAATCATTAAAGGAACTCCGTTTTCAT
1761 CAAGAAGGTCGGTTACAACCCGAAGGCCGTTGCCCTTCGTTCCATTCTGGATGGTTGGGTGACAACATGTTGGAGGAATCTCCCAAGTAC
1762 TTCAATTTCTATTACGAGAATTTCTATTGGTTCTAATTGATTCAATTCAGCATGTCCTGGTACAAGGGTTGGCAGAAAGAGACTAAGGCTGG
1763 TGTCGTCAAAGGCAAGACTCTCCTCGATGCTATTGACGCTATTGAACCTCCCGTACGTCCTGCTGAGAAGCCTCTCCGCTCCCCCTCCAG
1764 GATGTCTACAAAATCGTGGTATTGGTACAGTGCCGGTTGGTCGTGTCGAGACTGGTATCATCAAGGCA

1765 This EF-1 α sequence of fungus UGM080928-02 is identical to other EF-1 α sequences known from Clade-
1766 A cultivars from nests of other leafcutter species across South America (appearing in our Fig. 1).

1767 CO1 sequence of worker ant (*Atta laevigata*) from nest UGM080928-02:

1768 GATTTTTGGTCATCCAGAAGTTTATATTTAATCCTACCAGGATTTGGTTAATCTCACAAATTATTATAAGAGAAAGAGGAAAAAAGAAAC
1769 TTTCCGAACCTTAGGAATAATTTATGCTATAATAGCTATTGGTCTTTTAGGTTTATCGTTGAGCTCATATATTTACTATTGGTCTTGAT
1770 GTTGACACCCGAGCTTATTTACTTCTGCTACTTTAATCATTGCTATCCCAACTGGAATTAAGTTTTAGATGATTAGCAACACTTCACGGT
1771 ATAAAAATTAATTATAATCCTGCCTTATGATGATCATTGGGATTTATCTTTTATTTTCAATAGGAGGACTCACAGGAATTATACTATCTAACTC
1772 TTCTATTGATATTGTTCTCCATGATACCTAT

1773 This CO1 sequence of ant UGM080928-02 is, with one nucleotide difference, identical to the CO1
1774 sequence of *At. laevigata* SES040201-02, collected by Scott Solomon in 2004 in Itaúba, Mato Grosso,
1775 Brazil, about 900km distant from collection UGM080928-02 from Piracanjuba, Goiás, Brazil. No garden
1776 was collected for nest SES040201-02, and information on Clade-A/B cultivation is therefore not available
1777 for that nest. The phylogenetic position of SES040201-02 is shown in Fig. 4 of Solomon *et al.* (2008) as
1778 part of *laevigata*-subgroup B of the within-species diversity of *At. laevigata*, suggesting that the *Atta*
1779 *laevigata* collection UGM080928-02 from Piracanjuba, Goiás, also belongs to *laevigata*-subgroup B.

1781 *Acromyrmex coronatus*

1782 Of 21 cultivars from 21 *Acromyrmex coronatus* nests characterized so far, 19 are Clade-A fungi (Genbank
1783 sequences GQ853984, GQ854012, GQ854043, GQ854045, GQ854311, GQ853985, GQ853986,

1784 GQ854025, GQ854027, GQ854198, GQ854199, GQ854200, GQ854219, GQ854328, GQ854332,
1785 GQ854044, GQ854046, GQ854310, GQ855074) and 2 are Clade-B fungi (GQ854030, GQ854031). The
1786 Clade-A-cultivating nests were collected in Panamá, French Guyana, northern Brazil, and southern Brazil.
1787 The two Clade-B-cultivating nests were collected in Blumenau, Santa Catarina, Brazil, and the closest
1788 Clade-A-cultivating nests were collected 40 km distant from Blumenau in Itajai, Santa Catarina, Brazil,
1789 and in various locations in the neighboring State of Rio Grande do Sul, Brazil. The corresponding ant
1790 hosts were identified from morphological characters by researchers in the Bacci Lab. Locations of
1791 vouchers are listed in Table S1. Clade-A- and Clade-B-cultivating nests of *Ac. coronatus* appear to be
1792 sympatric in the Blumenau area in Santa Catarina State, Brazil.
1793

1794 ***Acromyrmex crassispinus***

1795 Of 4 cultivars from 21 *Acromyrmex crassispinus* nests characterized so far, 3 are Clade-A fungi (Genbank
1796 sequences GQ853997, GQ853999, GQ853987) and 1 is a Clade-B fungus (GQ854036). The 3 Clade-A-
1797 cultivating nests were collected in Ipê and in Nova Petrópolis, Rio Grande do Sul, Brazil. The Clade-B
1798 cultivating nest was collected in Tibagi, Paraná, Brazil, about 500 km north of the collection sites in Rio
1799 Grande do Sul. The corresponding ant hosts were identified from morphological characters by
1800 researchers in the Bacci Lab. Locations of vouchers are listed in Table S1. Because of the distance of
1801 500 km between the Clade-B- and Clade-A-cultivating nests of *Ac. crassispinus*, additional collections
1802 from the States of Paraná and Rio Grande do Sul are needed to establish sympatry of Clade-A- and Clade-
1803 B-cultivating nests of *Ac. crassispinus*.
1804

1805 ***Acromyrmex laticeps***

1806 Of 8 cultivars from 8 *Acromyrmex laticeps* nests characterized so far, 7 are Clade-A fungi (Genbank
1807 sequences GQ854029, GQ853990, GQ853991, GQ854010, GQ854016, GQ854028) and 1 is a Clade-B
1808 fungus (GQ854034). The 7 Clade-A-cultivating nests were collected in the States of Santa Catarina and
1809 Rio Grande do Sul, Brazil. The Clade-B-cultivating nest was collected in Blumenau, Santa Catarina,
1810 about 150 km distant from the closest collection sites of a Clade-A-cultivating nest in Lages, Santa
1811 Catarina. The corresponding ant hosts were identified from morphological characters by researchers in
1812 the Bacci Lab. Locations of vouchers are listed in Table S1. Because of the distance of 150 km between
1813 the Clade-B- and Clade-A-cultivating nests of *Ac. laticeps*, additional collections are needed to establish
1814 sympatry of Clade-A- and Clade-B-cultivating nests of *Ac. laticeps*.
1815

1816 ***Trachymyrmex arizonensis***

1817 Of 8 cultivars from 8 *Trachymyrmex arizonensis* nests characterized so far, 7 are Clade-B fungi (Genbank
1818 sequences GQ854098, GQ854128, GQ854138, GQ854139, GQ854140, GQ854142, GQ855143) and 1 is
1819 a Clade-A fungus (Genbank sequence GQ854102). The Clade-A-cultivating nest was collected by
1820 Christian Rabeling (CR050811- 02) near the South-West Research Station, Chiricahua Mountains,
1821 Cochise County, Arizona, within 1 km of a Clade-B-cultivating nest also collected by Christian Rabeling
1822 (CR050806-01), and close to two other Clade-B-cultivating nests collected by Robert Johnson and Ulrich
1823 Mueller near the South-West Research Station. The corresponding ant hosts were identified from
1824 morphological characters and by sequencing of the mitochondrial cytochrome-oxidase 1 (CO1) gene by
1825 Christian Rabeling. Ant-sequences from the Clade-A-cultivating nest CR050811- 02 and the Clade-B-
1826 cultivating nest CR050806-01 appear in Fig. 21 of Rabeling *et al.* 2007, indicating no sequence
1827 divergence at the CO1 gene that would support separate species. Locations of vouchers are listed in
1828 Table S1. In the Chiricahua Mountains of Arizona, therefore, Clade-B- and Clade-A-cultivating nests of
1829 *T. arizonensis* appear to be sympatric.
1830

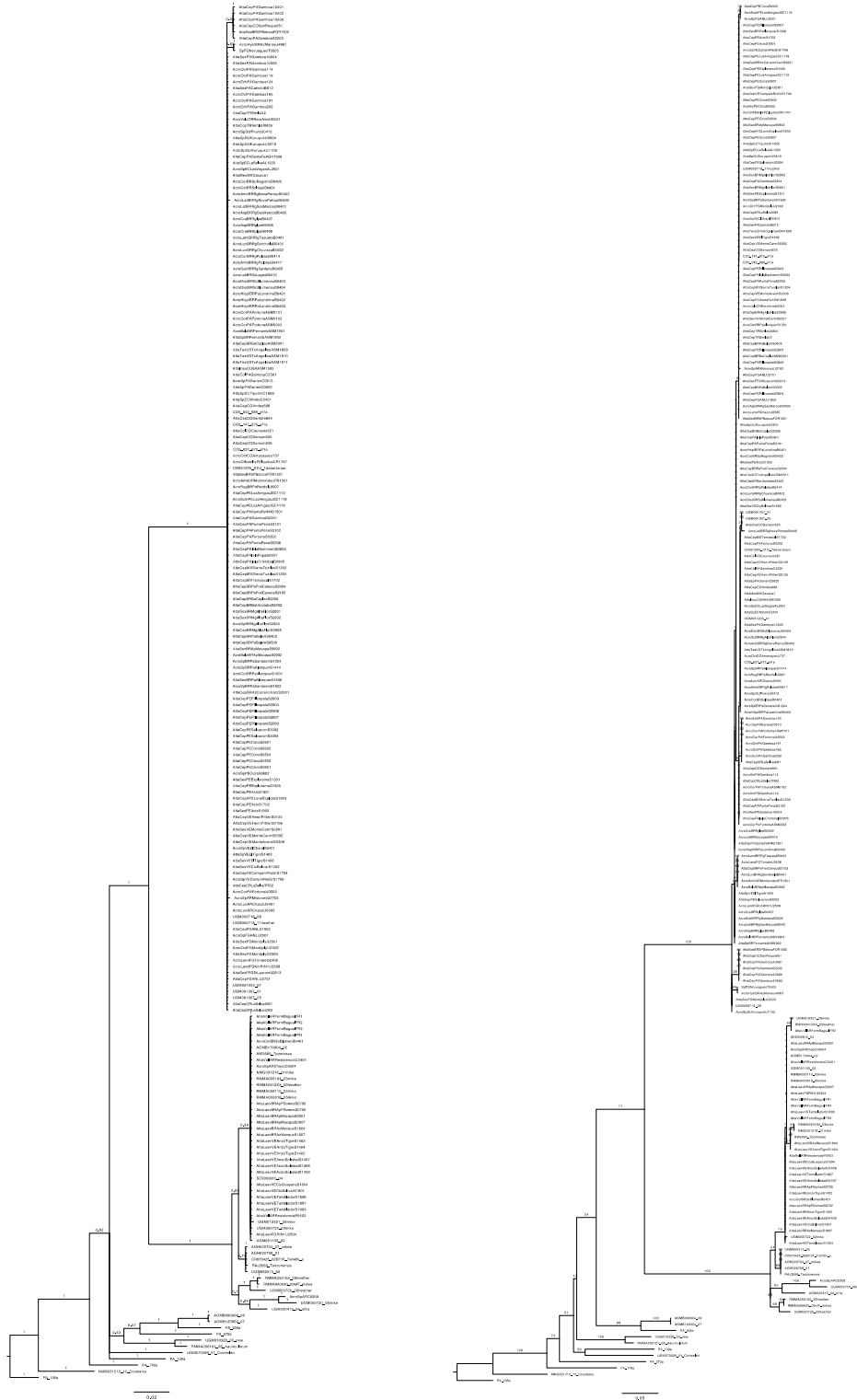
1831 ***Trachymyrmex intermedius***

1832 Of 4 cultivars from 4 *Trachymyrmex intermedius* nests characterized so far, 2 are Clade-B fungi
1833 (Genbank sequences GQ854329, JX258959) and 2 are Clade-A fungi (Genbank sequences GQ854325,
1834 GQ854326). The sequence JX258959 is part of the phylogenetic analysis of Mueller *et al.* (in review; see

1835 Table S1 in that study), but is not part of our study here. All four nests were collected by Ulrich Mueller
1836 within about 200 meters of each other at the Amazon Nature Lodge, Kaw Mountains, French Guiana.
1837 The corresponding ant hosts were identified from morphological characters by Scott Solomon. Locations
1838 of vouchers are listed in Table S1. In the Kaw Mountains of French Guiana, therefore, Clade-B- and
1839 Clade-A-cultivating nests of *T. intermedius* appear to be sympatric.
1840

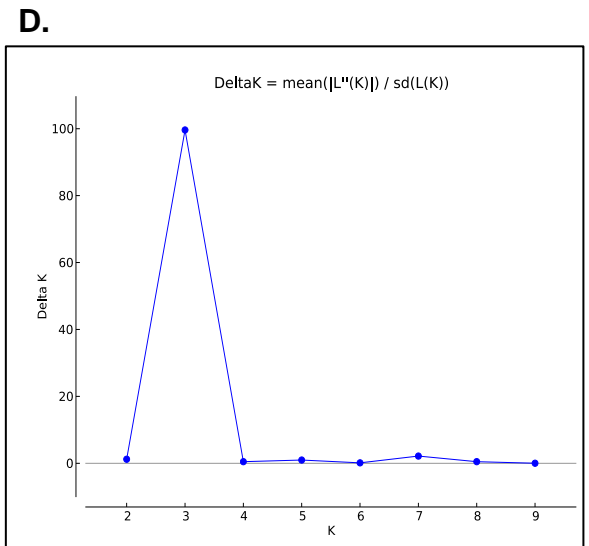
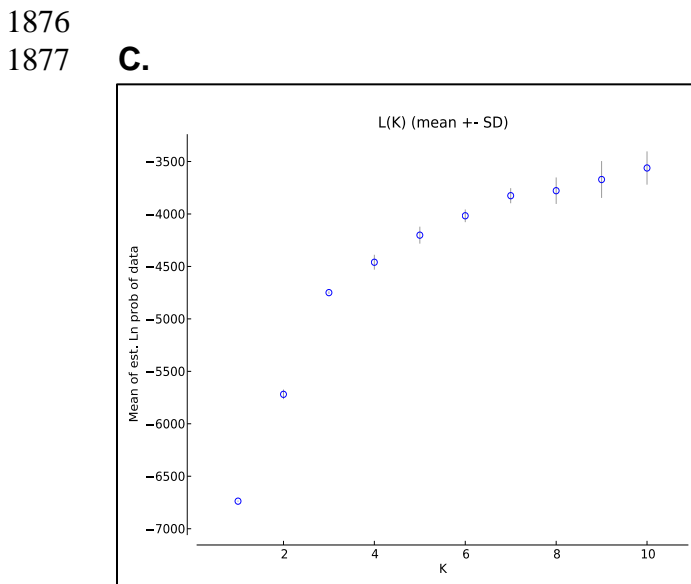
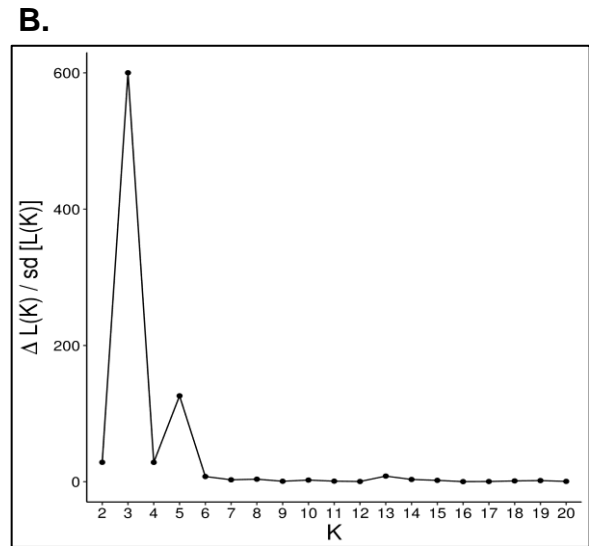
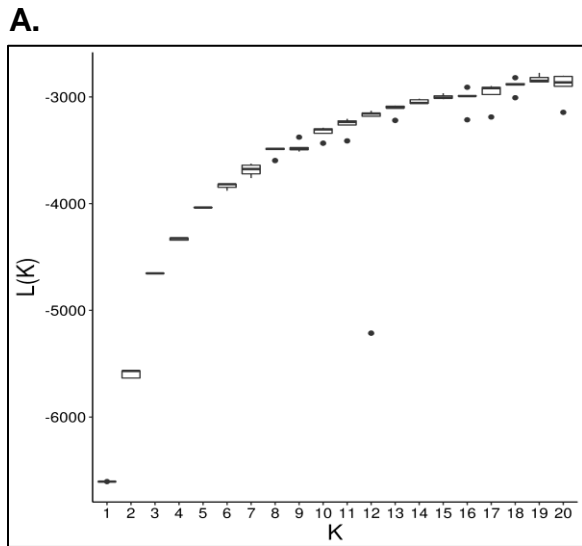
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1845

Fig. S1. Phylogenetic trees inferred in Bayesian (left) and likelihood (right) analyses of EF-1 α sequence information. Methods are described in the main text. The EF-1 α 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).

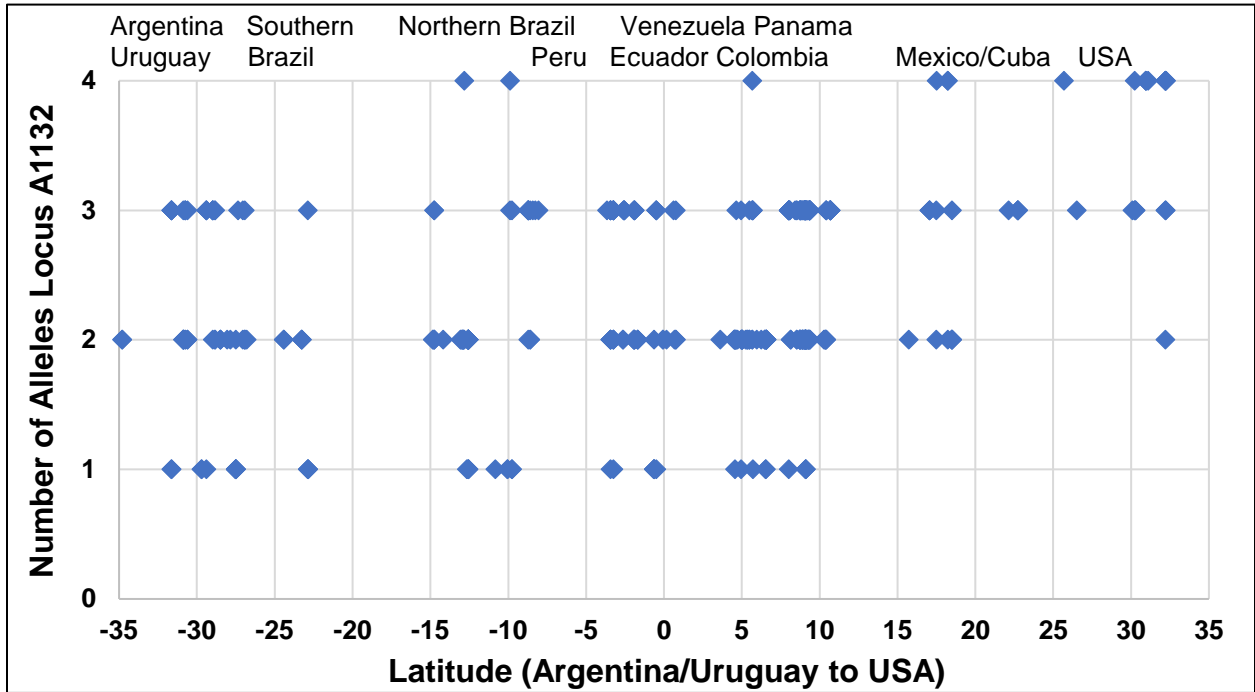


1860 **Fig. S4.** Estimation of K from STRUCTURE analysis of fungal microsatellite marker profiles.
 1861 **A. & B.** Using the default settings in STRUCTURE, we ran STRUCTURE using K=1 to K=20 clusters
 1862 (n=5 replicates each, 100,000 iterations for parameter estimation after a burn-in of 100,000 iterations),
 1863 calculated the difference in the posterior probability of K and K-1, and selected the value of K with the
 1864 greatest relative increase in log-likelihood, as recommended by Evanno *et al.* (2005).
 1865 **C & D.** To reduce bias in prior assumptions in a separate analysis, we also left allele frequencies
 1866 uncorrelated and chose alpha (α) to be 1/10 of the default setting (i.e., $\alpha=0.1$) (Wang 2017).
 1867 Both the default settings and the modified settings yield identical recommendations of K=3.

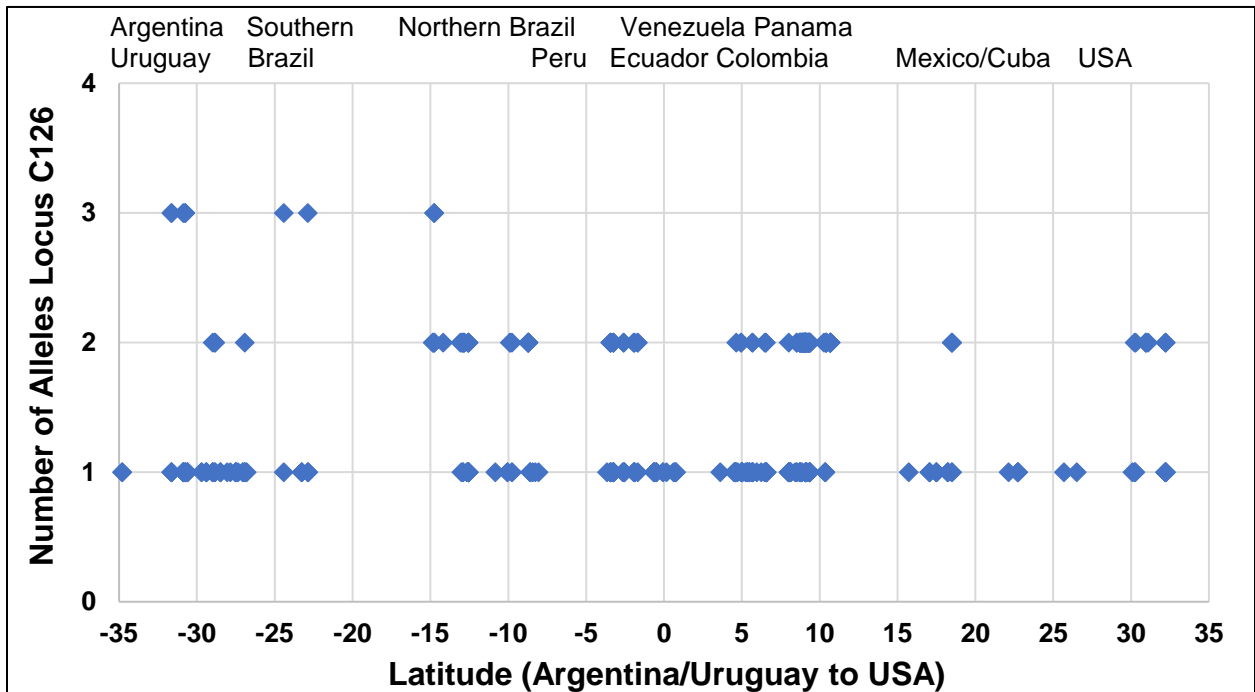
1868 **A.** Default settings: Boxplots of the log-likelihoods of K ranging from 1 to 20.
 1869 **B.** Default settings: Change in the average log-likelihood of K [$L(K) - L(K-1)$] divided by the standard
 1870 deviation of K.
 1871 **C.** Modified settings: Rate of change of likelihood distribution [calculated as $L'(K) = L(K) - L(K - 1)$].
 1872 **D.** Modified settings: Absolute values of the second order rate of change of the likelihood distribution
 1873 (mean \pm SD) calculated according to the formula: $|L''(K)| = |L'(K + 1) - L'(K)|$.
 1874
 1875



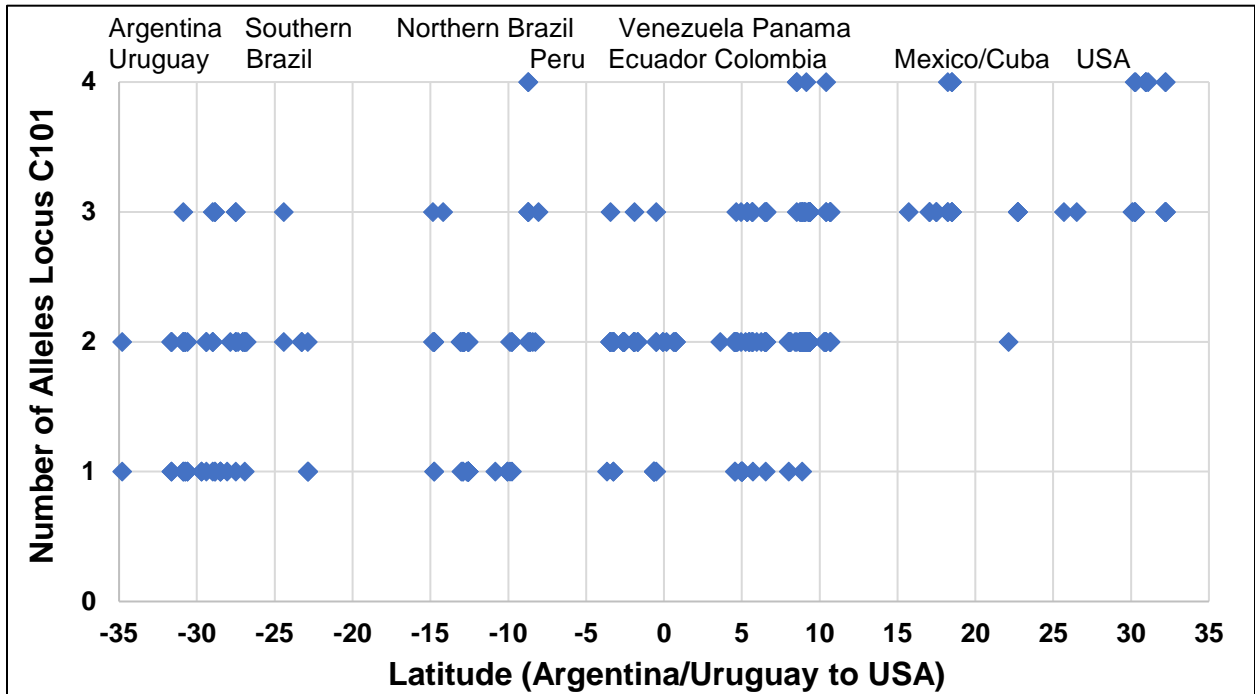
1879 **Fig. S5 A-E.** Number of alleles per fungus at each individual locus (A1132, C126, C101, B12, C117) as a
 1880 function of latitude. These analyses include only the 388 samples for which allele information is
 1881 available for all five loci (i.e., any sample for which allele information is missing for at least one locus is
 1882 excluded here; including these additional samples does not change overall patterns; data in Table S8).
 1883



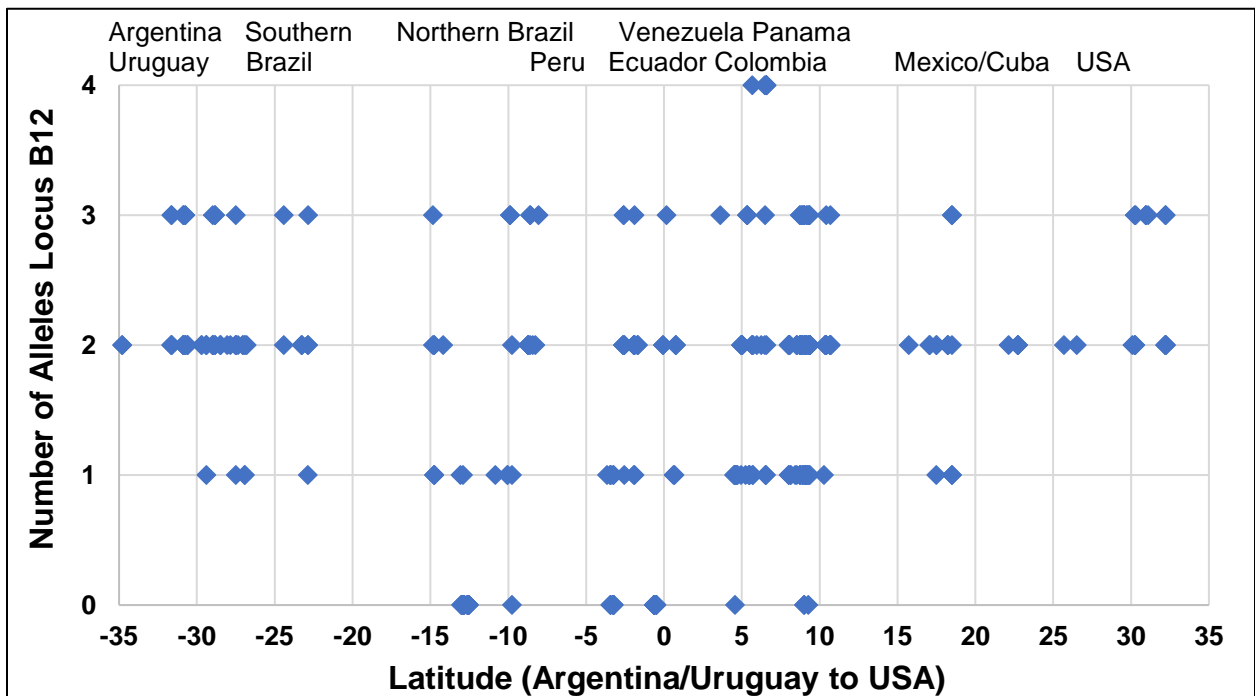
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 1885



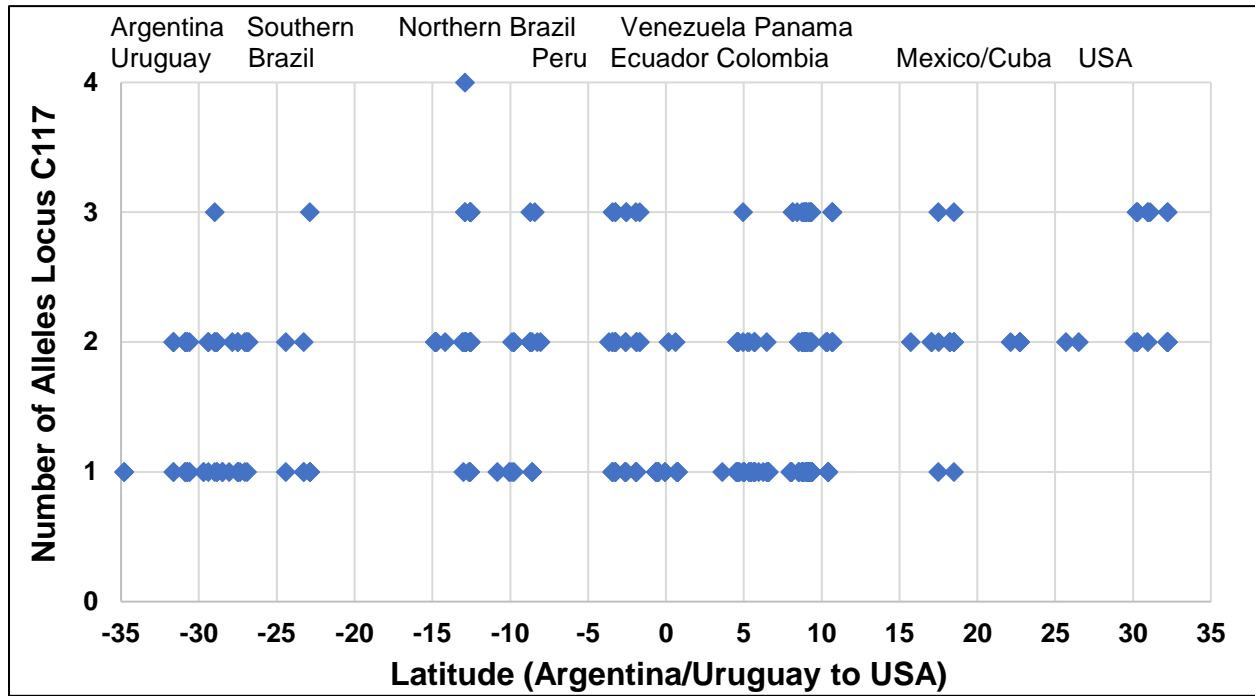
1886 **Fig. S5 (continued).** Note that, at locus B12, some individuals show null alleles in some populations
1887 in northern South America (allele number is zero for these individuals at locus B12).
1888



1889

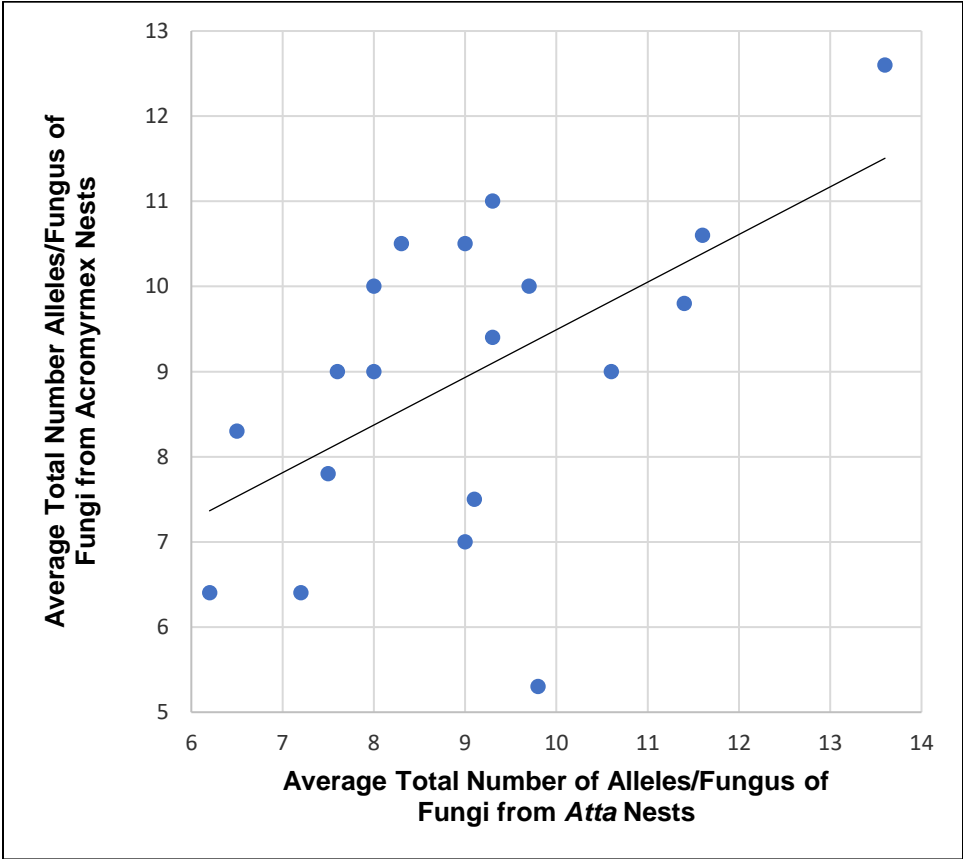


1890 Fig. S5 (continued).



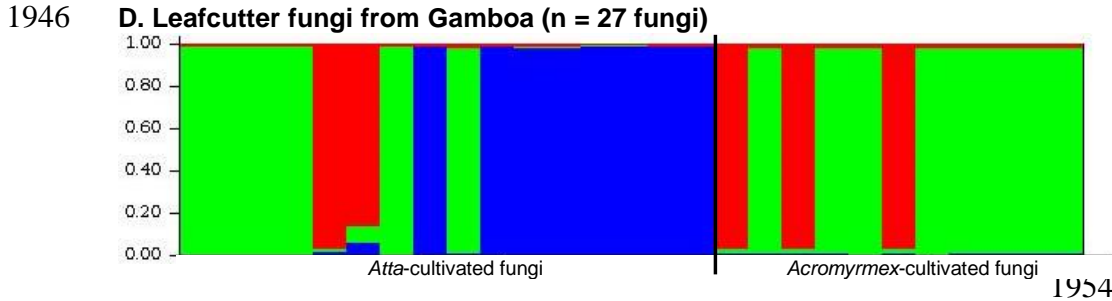
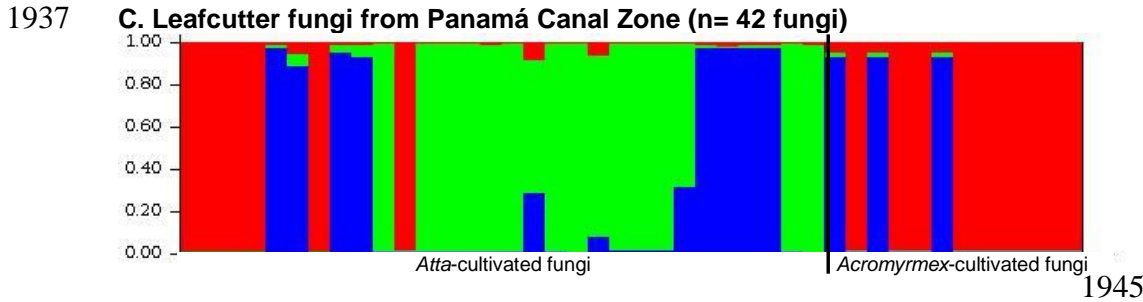
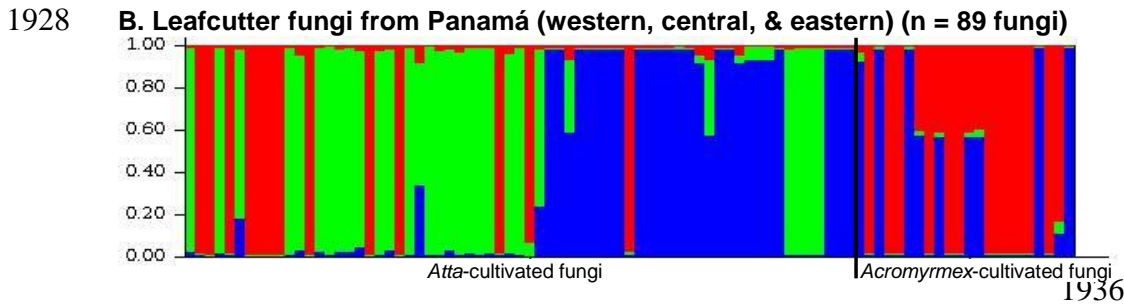
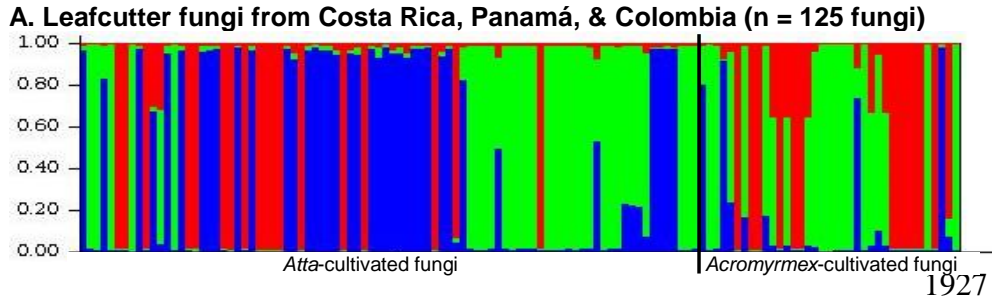
1891

1892 **Fig. S6.** Correlation between total allele diversity (allele richness) of *Atta*-cultivated and *Acromyrmex*-
 1893 cultivated fungi, comparing samples from 19 locations (highlighted in blue in Table S7) for which
 1894 genotype information is available for both *Atta*-cultivated and *Acromyrmex*-cultivated fungi. The graph
 1895 plots for each of the 19 locations the average total number of alleles observed in fungi at 5 microsatellite
 1896 loci (all alleles/fungus summed across the 5 loci, then averaged across all samples from a genus of ant
 1897 collected at a location). The data plotted in the graph are listed also in Table S7, which summarizes
 1898 information from the raw data in the Excel sheet in Table S8. Statistics of the Spearman rank-order
 1899 correlation are $t = 2.39$, $df = 17$, $p = 0.029$, $r = 0.502$, $r^2 = 0.252$, $n = 19$. If *Atta*-cultivated and
 1900 *Acromyrmex*-cultivated fungi represent separate gene pools, as hypothesized by Kooij *et al.* (2015b), this
 1901 positive correlation can be explained by parallel evolutionary forces that determine allele diversity
 1902 independently (convergently) at different sites for both *Atta*-cultivated and *Acromyrmex*-cultivated fungi.
 1903 Alternatively, the positive correlation can be explained by shared local pools of cultivars into which both
 1904 *Atta* and *Acromyrmex* species tap at each location, for example because of local horizontal transfer of
 1905 fungal cultivars between nests from the two leafcutter genera, because of some form of genetic exchange
 1906 and hybridization locally between fungi cultivated by different ant nests, or both.
 1907



1908
 1909
 1910

1911 **Fig. S7 A-D.** Results of STRUCTURE analyses testing whether fungi are partitioned into *Atta*- versus
 1912 *Acromyrmex*-cultivated fungi, using samples from Panamá and neighboring countries that were most
 1913 comprehensively surveyed in our study. STRUCTURE analyses indicate K=3 fungal genotype-clusters
 1914 for each of four datasets that were sub-sampled at increasingly more local scale: **A.** regional fungal
 1915 diversity (Colombia, Panamá, & Costa Rica); **B.** within-country diversity (all Panamá); **C.** provincial
 1916 diversity (Panamá Canal Zone); or **D.** the local diversity in Gamboa (town within Panamá Canal Zone)
 1917 studied earlier also by Mikheyev *et al.* (2007) and Kooij *et al.* (2015b).
 1918
 1919



1955 Our STRUCTURE analyses confirm the earlier finding by Mikheyev *et al.* (2007) that *Atta* and
 1956 *Acromyrmex* ants from Gamboa tap locally into the same pool of fungal cultivars derived from several
 1957 genotype-clusters, contrary to the conclusions of Kooij *et al.* (2015b). Specifically, Kooij *et al.* (2015b,
 1958 page 13) write that “fungal symbionts of *Atta* and *Acromyrmex* colonies showed that they were
 1959 completely separated ... consistent with earlier findings by Mikheyev *et al.* (2007) for the same sampling
 1960 site”. Mikheyev *et al.* (2007) actually documented that *Atta* and *Acromyrmex* ants “shared identical
 1961 fungus garden genotypes, indicating wide-spread cultivar exchange” (Abstract in Mikheyev *et al.* 2007).

1962 The leafcutter cultivar lineages studied by Mikheyev *et al.* (2007, Abstract) were “largely unstructured
1963 with respect to host ant species, with only 10% of the structure in genetic variance being attributable to
1964 partitioning among ant species and genera”. Using more microsatellite loci than in our study (and thus
1965 having greater resolution to differentiate between fungal genotypes), Figure 3 in Mikheyev *et al.* (2007)
1966 shows that fungal diversity associated with Panamanian leafcutter ants can be structured into 6 genotype-
1967 clusters, and that there exists little correlation between these fungal lineages and leafcutter ant genera
1968 (i.e., each of the leafcutter ant species cultivates locally representatives from most of these 6 fungal
1969 lineages). Therefore, the fungi examined by Kooij *et al.* (2015b) appear to have been selectively sampled
1970 from the true diversity of fungi cultivated by each leafcutter species in central Panamá and in Gamboa,
1971 such that Kooij *et al.* (2015b) oversampled one fungus lineage for the *Atta* ants studied (fungi from 9
1972 nests) and oversampled another fungus lineage for *Acromyrmex* ants studied (fungi from 9 nests) (e.g.,
1973 Kooij *et al.* 2015b may have oversampled the blue genotype-cluster for *Atta* ants and the green genotype-
1974 cluster for *Acromyrmex* ants shown in our Fig. S7 D). Our analyses of Panamanian leafcutter-fungus
1975 diversity show that *Atta* and *Acromyrmex* ants are not specialized on different cultivar lineages, but that
1976 the two ant genera cultivate in different localities representatives from different genotype-clusters at
1977 different frequencies.