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Frontier mutualism: coevolutionary patterns at the northern range limit of the leaf-cutter ant–fungus symbiosis

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Tropical leaf-cutter ants cultivate the fungus *Attamyces bromatificus* in a many-to-one, diffuse coevolutionary relationship where ant and fungal partners re-associate frequently over time. To evaluate whether ant–*Attamyces* coevolution is more specific (tighter) in peripheral populations, we characterized the host-specificities of *Attamyces* genotypes at their northern, subtropical range limits (southern USA, Mexico and Cuba). Population-genetic patterns of northern *Attamyces* reveal features that have so far not been observed in the diffusely coevolving, tropical ant–*Attamyces* associations. These unique features include (i) cases of one-to-one ant–*Attamyces* specialization that tighten coevolution at the northern frontier; (ii) distributions of genetically identical *Attamyces* clones over large areas (up to 81 000 km^2, approx. the area of Ireland, Austria or Panama); (iii) admixture rates between *Attamyces* lineages that appear lower in northern than in tropical populations; and (iv) long-distance gene flow of *Attamyces* across a dispersal barrier for leaf-cutter ants (ocean between mainland North America and Cuba). The latter suggests that *Attamyces* fungi may occasionally disperse independently of the ants, contrary to the traditional assumption that *Attamyces* fungi depend entirely on leaf-cutter queens for dispersal. Peripheral populations in Argentina or at mid-elevation sites in the Andes may reveal additional regional variants in ant–*Attamyces* coevolution. Studies of such populations are most likely to inform models of coextinctions of obligate mutualistic partners that are doubly stressed by habitat marginality and by environmental change.

Keywords: *Attamyces*; *Atta texana*; *Acromyrmex versicolor*; coevolution; mutualism; range limit

1. INTRODUCTION

Evolution in peripheral populations can differ markedly from evolution in more central populations within a species range [1–3]. Peripheral populations often exist at lower densities, produce fewer offspring per individual, are more fragmented and are more prone to local extinction [2,3]. These demographic properties leave distinct population-genetic footprints, such as reduced genetic diversity, increased homozygosity and increased levels of unique (private) alleles in peripheral populations compared with central populations [4,5]. A large body of work has confirmed these general population-genetic predictions, although there are exceptions [5–7]. Much less is known about the population-genetic patterns of host–symbiont associations in marginal habitat, yet studies on symbioses near their distributional range limits are needed to inform models of coextinctions of symbiotic partners that are doubly stressed by habitat marginality and by accelerated environmental change [8,9]. To inform such models of host–symbiont coextinction, we elucidate here the genetic diversities and host-specificities of *Attamyces* fungi cultivated by leaf-cutter ants at their northern range limit.

The obligate mutualism between leaf-cutter ants (genera *Atta* and *Acromyrmex*) and their *Attamyces* fungi originated 8–12 Myr ago in the South American tropics but extends today into temperate regions [10–13]. In the Southern Hemisphere, leaf-cutter ants reach 44° S latitude [14]. In the Northern Hemisphere, *Atta texana* reaches 33° N latitude in north-central Texas and northeastern Louisiana [15; this study] and *Acromyrmex versicolor* reaches almost 36° N latitude in northwestern Arizona [16]. It is unknown whether leaf-cutter ants dispersed into the North American continent before or after the closing of the Isthmus of Panama approximately 1–3 Myr ago [17]. However, leaf-cutter ants must have reached their current latitudinal limit in the southern USA sometime during the past 10 000 years following the most recent Pleistocene glaciation. The leaf-cutter ant *At. texana* was abundant in central and east Texas at the time when European settlers arrived [18,19], whereas a historical presence of *Ac. versicolor* in Northern Arizona is unknown, but likely. Because tropical *Attamyces* fungi are cold-intolerant and grow best at the warm, stable temperatures of tropical soils (between 20°C and 30°C) [20–23], the cold-sensitivity of *Attamyces* symbionts is thought to have been one factor that constrained the expansion of the leaf-cutter ant–*Attamyces* symbiosis from tropical into temperate habitats [14,24].

All leaf-cutter ants depend on symbiotic fungi for food, which grow in gardens tended by the ants in excavated

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subterranean cavities or in thatched shelters [10,12,19,25]. The fungi cultivated by leaf-cutter ants belong to the agaric tribe Leucocoprineae (fungal anamorph *Attamyces bromatificus*, teleomorph *Leucocoprinus gongylophorus*, Agaricales, Basidiomycota; [26,27]). All *Attamyces* strains genotyped so far showed polyploid-like allele patterns with more than two alleles per locus [24,28,29], consistent with cytological studies indicating that *Attamyces* cells are multi-nucleate [30]. The polyploid-like allele patterns could derive from a complex heterokaryon (coexistence of genetically differentiated haploid nuclei in the same cell, which is the typical growth form of basidiomycete mycelia), from duplicated genomes within single nuclei or from a combination of both [29]. Because *Attamyces* fungi have not been found so far growing independently of the ants [31,32], *Attamyces* appears to be on obligate symbiont. However, at least some *Attamyces* fungi are fruiting-competent and can produce spore-bearing mushrooms (table 3 in Mueller [26] summarizes records of fruiting leaf-cutter fungi). *Attamyces* fruiting bodies in the field are so far known mostly from *Acromyrmex* leaf-cutter ants that build thatched gardens at ground level [33,34].

Attamyces fungi are clonally propagated by the ants within and between nests [35,36], suggesting the possibility of strict *Attamyces* asexuality imposed by the ant farmers; however, incongruence between the phylogenetic topologies of segregating genes indicates that clonality of *Attamyces* is occasionally punctuated by recombination events [37,38]. In laboratory experiments, co-cultivated cultivar genotypes may recombine in artificially created chimaeric gardens [28,39], possibly through the exchange of nuclei between cultivar mycelia. A separate study testing for chimaeric gardens in natural leaf-cutter nests revealed no evidence for *Attamyces* polyculture in the leaf-cutter ants *At. texana* and *Atta cephalotes*, suggesting that *Attamyces* is grown by the ants in single-strain monoculture throughout the hundreds of gardens within a single mature leaf-cutter nest [36].

Across the leaf-cutter ant range from Argentina to the USA, the approximately 50 described leaf-cutter species are thought to associate with a single cultivar species (*Attamyces bromatificus* Kreisel) in a many-to-one coevolutionary relationship [37,40]. Whereas the leaf-cutter ant clade is estimated to be about 8–12 Myr old, the corresponding clade of *Attamyces* cultivars is significantly younger (about 2–4 Myr old) [40]. *Attamyces* lineages of recent origin, therefore, may have spread by means of horizontal transfer between leaf-cutter ant lineages (i.e. *Attamyces* sweeps through the range of leaf-cutter ants). Comparison of fast-evolving genes of *Attamyces* strains from geographically distant leaf-cutter species across South America [41] and population-genetic patterns of *Attamyces* cultivated by sympatric leaf-cutter species in Panama [38] confirmed the expected sharing of *Attamyces* lineages between tropical leaf-cutter ant species. Because a local community of tropical leaf-cutter ants shares a corresponding local community of cultivar lineages [38,40,41], *Attamyces* cultivars in the tropics are thought to evolve within a continually shifting landscape of the microhabitats occupied by the diverse leafcutter ants between which cultivars are exchanged.

The most detailed population-genetic study of *Attamyces* symbionts to date was conducted by Mikheyev *et al.* [38] for sympatric populations of three *Atta* species and two *Acromyrmex* species from Panama. Despite vertical transmission of *Attamyces* strains from mother to daughter nests, different leaf-cutter ant species and genera sometimes share identical *Attamyces* genotypes (clones), indicating widespread cultivar exchange across Panama. *Attamyces* genotypes group into six distinct *Attamyces* genotype-clusters in Panama, but these clusters do not correspond to the five ant hosts (only about 10% of the structure in genetic variance of *Attamyces* is attributable to species and generic boundaries among ant hosts; [38]). Frequent exchange of *Attamyces* clones between heterospecific ant nests or other forms of *Attamyces* gene flow between nests, therefore, prevents the long-term persistence of specific ant–*Attamyces* combinations, leading to an overall pattern of diffuse coevolution between leaf-cutter ant hosts and *Attamyces* symbionts in this tropical population.

At the northern range limit of leaf-cutter ants (southern USA), *Attamyces* evolution progresses differently than in the tropics, because here leaf-cutter ants do not exist sympatrically with other leaf-cutter species. For example, the Texas leaf-cutter ant *At. texana* is the only leaf-cutter species within its range, except for a contact zone with *Atta mexicana* just south of the USA–Mexico border [19,42]. Likewise, the desert leaf-cutter *Ac. versicolor* overlaps with *At. mexicana* south of the USA–Mexico border, but *Ac. versicolor* is the only leaf-cutter ant in its northern range in the USA. Ant–fungus coevolution in these northernmost leaf-cutter populations, therefore, is expected to be tighter (i.e. involving a single ant species and its *Attamyces* cultivars) compared with the multi-species, diffuse coevolution resulting from sharing of *Attamyces* strains between sympatric leaf-cutter ant species in the tropics. The expectation of possibly tighter ant–fungus coevolution in the northernmost leaf-cutter populations stimulated our investigations into the population genetics of *Attamyces* at the northern range limit of the leaf-cutter ant distribution.

2. MATERIAL AND METHODS

Attamyces fungi were collected from leaf-cutter gardens excavated from nests throughout the US ranges of the northernmost leaf-cutter ants *At. texana* ($n = 165$ *Attamyces* accessions from an equal number of nests) and *Ac. versicolor* ($n = 35$ *Attamyces*). To place these *Attamyces* collections in a larger population-genetic context of North American *Attamyces*, we also collected garden material from five *Atta insularis* nests from Cuba; from seven *At. mexicana* nests from northeastern and southern Mexico; and from eight *At. cephalotes* nests from southeastern Mexico. The electronic supplementary material, table S1 lists information collected for all 220 *Attamyces* accessions collected from the five leaf-cutter ant species from the USA, Mexico and Cuba.

Attamyces accessions were genotyped with a panel of 12 polymorphic microsatellite loci [29]. Population-genetic patterns were analysed in STRUCTURE v. 2.2 [43,44] by clustering individuals into populations on the basis of the multi-locus genotype information. Because *Attamyces* fungi are multinucleate and exhibit genotype profiles of unknown ploidy [29], we did not calculate standard population-genetic parameters (e.g. heterozygosities; *F*-statistics, etc.), but treated all alleles as dominant markers for genotype clustering and inference of population-genetic structure, as recommended for polyploid organisms by Falush *et al.* [44]. STRUCTURE requires that individuals differ by at least one marker, and we therefore included in our population-genetic analyses only one representative per microsatellite genotype. Because

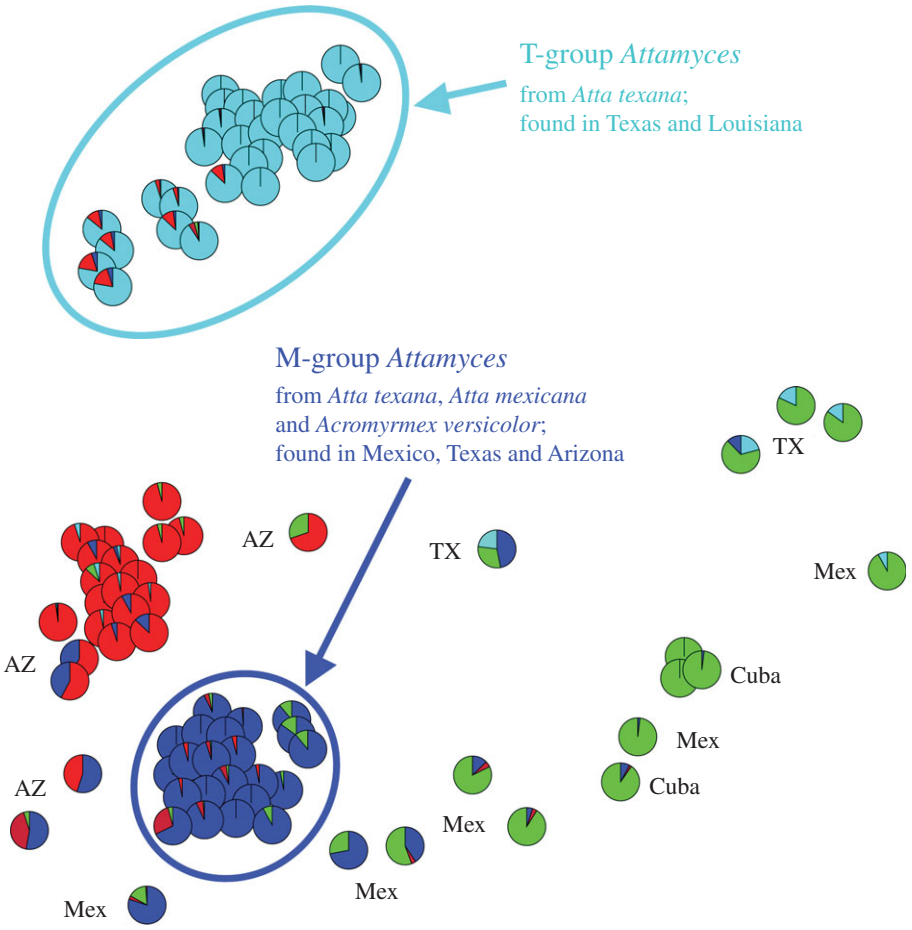

Figure 1. Multi-dimensional-scaling of genetic diversity of *Attamyces* fungi cultivated by five leaf-cutter ant species in North America. STRUCTURE analyses identify four populations of *Attamyces*, shown in different colours. Light blue: *Attamyces* from *At. texana* (T-group *Attamyces*). Dark blue: *Attamyces* from *Ac. versicolor*, *At. texana* and *At. mexicana* in, respectively, Arizona, Texas and Mexico (M-group *Attamyces*). Red: *Attamyces* from *Ac. versicolor* in Arizona and California. Green: diverse *Attamyces* from *At. mexicana* and *At. cephalotes* in Mexico, from *At. insularis* in Cuba and from three unusual collections from *At. texana* (far right); 97.6% of the *Attamyces* from *At. texana* belong to two populations; one population (dark blue) also includes members found in Mexico (hence M-group). The other *Attamyces* population from *At. texana* (light blue) is clearly distinct from the three other populations and is known so far only from Louisiana and Texas (hence T-group). Collection locations: AZ = Arizona, Mex = Mexico, TX = Texas. For most of the genotypes shown, genetically identical duplicates were collected in different leaf-cutter nests; only one representative is shown for each distinct *Attamyces* genotype. The total sample size included 220 *Attamyces* collections, which were grouped by 91 informative microsatellite markers into the 93 unique genotypes shown (listed also in the electronic supplementary material, table S1).

Attamyces is clonally propagated within and between leaf-cutter nests [36], 55.7 per cent of the 220 *Attamyces* accessions possessed a genotype profile that was identical to the profile of at least one other accession (see the electronic supplementary material, table S1). After eliminating duplicates to retain only one representative per genotype, the final dataset analysed in STRUCTURE included 93 unique genotypes (each profiled for 91 variable microsatellite markers). A Markov chain Monte Carlo algorithm implemented in STRUCTURE identified genetically differentiated populations and inferred contributions of ancestry for each allele (likelihood that an allele derived from one of the inferred populations). Admixture (mixed ancestry) was inferred for a particular fungal accession if different alleles of this genotype were assigned with high likelihood to different populations. Such admixture may result from (i) recombination of alleles (as in sexual organisms); or (ii) exchange of nuclei between differentiated *Attamyces* strains, generating re-assortment and novel combinations of nuclei that coexist in the multi-nucleate mycelium of *Attamyces* (see the electronic supplementary material *Admixture* for a detailed description of the diverse mechanism of genetic exchange in multi-nucleate fungi). Separately for the groups of *Attamyces* genotypes from *At. texana* ($n = 165$) and *Ac. versicolor* ($n = 35$), we also estimated the probability of sexual recombination within each group by applying the methods of Bengtsson [45] and De Fine Licht *et al.* [46] in a Bayesian framework implemented in WINBUGS [47]. To visualize the genetic diversity and to validate results obtained from STRUCTURE, we calculated a two-dimensional non-metric multi-dimensional scaling solution of the binary distances between cultivar genotypes (figure 1), which was computed in the programs R and GENALEX (www.anu.edu.au/BoZo/GenAlEx/) [48]. Collection, genotyping and statistical methods are explained in detail in the electronic supplementary material.

3. RESULTS

(a) *Specificity and sharing of* Attamyces *symbionts at the northern range limit*

STRUCTURE analyses inferred four populations of *Attamyces* symbionts cultivated at the northern range limit of

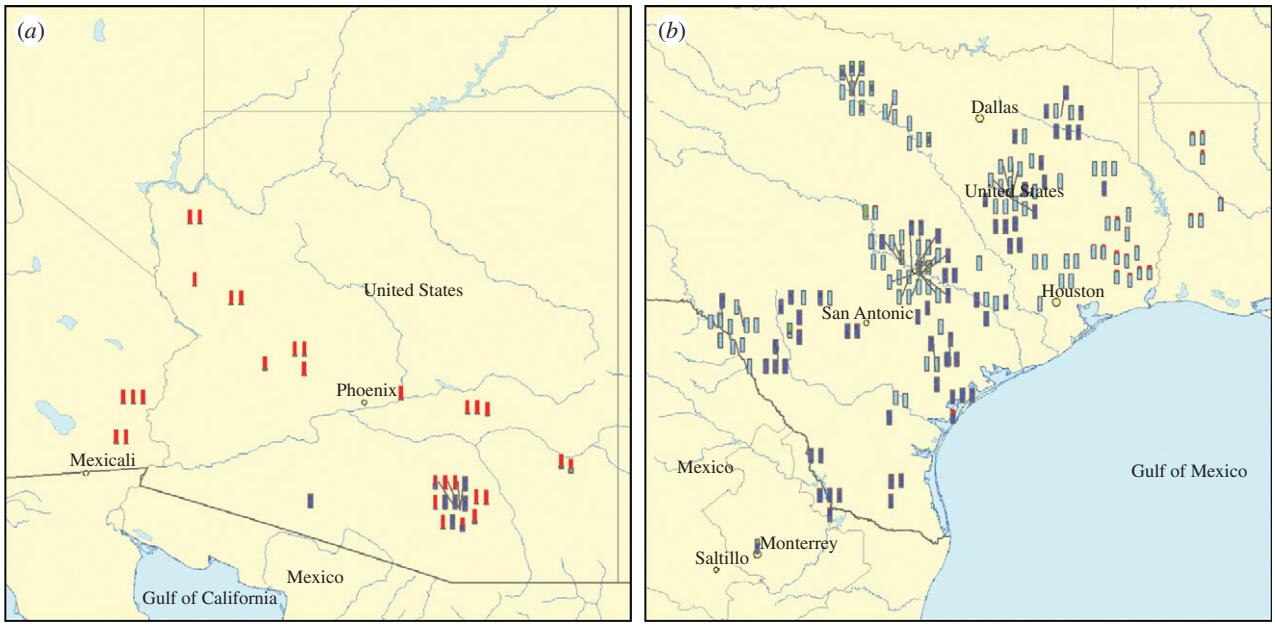

Figure 2. Biogeographic distributions of *Attamyces* groupings inferred in STRUCTURE. (*a*) *Attamyces* from Arizona and California were collected from gardens of the desert leaf-cutter ant, *Ac. versicolor*. (*b*) *Attamyces* from Texas and Louisiana were collected from gardens of the Texas leaf-cutter ant, *At. texana*; the collection from Monterrey, Mexico, was obtained from *At. mexicana*. All other Mexican and Cuban *Attamyces* analysed in figure 1 were collected outside the boundaries of the two maps. Maps in (*a*) and (*b*) are drawn to different scales. Each vertical bar presents the proportional contribution to a particular genotype of markers inferred by STRUCTURE to belong to one of four *Attamyces* populations (light blue, dark blue, red, green). Light blue: *Attamyces* from *At. texana* (T-group *Attamyces*). Dark blue: *Attamyces* from *Ac. versicolor*, *At. texana* and *At. mexicana* in, respectively, Arizona, Texas and Mexico (M-group *Attamyces*). Red: *Attamyces* from *Ac. versicolor* in Arizona and California. Green: rare *Attamyces* from *At. texana* with close affinity to *Attamyces* from *At. insularis* in Cuba and to some *Attomyces* from *At. mexicana* and *At. cephaloes* in Mexico. Ninety four per cent of the *Attamyces* from *At. texana* belong to two populations; one population (dark blue) also includes members found in Mexico (hence M-group *Attamyces*). The other *Attamyces* population from *At. texana* (light blue) is known so far only from Louisiana and Texas (hence T-group *Attamyces*). Only M-group *Attamyces* (dark blue) are currently known from south Texas; only T-group *Attamyces* (light blue) are currently known from east Texas and Louisiana. Admixed genotypes are shown as genotypes that combine significant portions of genetic markers assigned to several *Attamyces* populations (see also the electronic supplementary material, table S1). Because T-group *Attamyces* appear to be distinct types within the greater diversity of North American leaf-cutter fungi (figure 1), and because of the exclusive association of T-group *Attamyces* only with *At. texana*, T-group *Attamyces* may be more tightly coevolved with *At. texana* than M-group *Attamyces* (the latter *Attamyces* are shared also with the leaf-cutter species *At. mexicana*, *At. cephalotes* and *Ac. versicolor*, most likely because M-group *Attamyces* are transferred occasionally in Mexico between sympatric nests of these leaf-cutter species).

the leaf-cutter ant distribution (figure 1). These solutions were convergent between three repeat runs. Modelling more than four populations ($K > 4$) did not significantly improve likelihood scores (see the electronic supplementary material, figure S2). Two of the four inferred populations represent two dominant cultivar types of *At. texana* ('T-group' and 'M-group' *Attamyces*); one of these two *Attamyces* populations from *At. texana* (dark blue, figure 1) also includes *Attamyces* cultivated by other leaf-cutter species in Mexico (hence M-group *Attamyces*) and by *Ac. versicolor* in Arizona. The second *Attamyces* population from *At. texana* (light blue, figure 1) is clearly distinct from the three other populations and was found only in Louisiana and Texas (hence T-group *Attamyces*), but not in Mexico or the western USA. A third population was found only in Arizona and California (cultivated there by *Ac. versicolor*, therefore, called V-group *Attamyces*; red, figure 1). A fourth population includes a more diverse assemblage of *Attamyces* cultivated by *At. insularis* in Cuba (hence C-group *Attamyces*; green, figure 1), by *At. mexicana* and *At. cephalotes* in Mexico and by atypical and rare nests of *At. texana* in Texas (these two rare *Attamyces* genotypes are shown in figure 1 far right centre, and they are listed as genotypes nos. 74 and 75 in the electronic supplementary material, table S1).

Among the 220 collections, therefore, two *Attamyces* populations appear ant–species-specific: V-group cultivars from *Ac. versicolor* and T-group cultivars from *At. texana*. *Attamyces* belonging to the remaining two cultivar populations are shared between several leaf-cutter ant species across North America, paralleling the *Attamyces* sharing observed between sympatric leaf-cutter species in Panama [38]. Additional collections of *Attamyces* from *At. mexicana* in northern Mexico may reveal that V-group and T-group *Attamyces* are less ant–species specific than suggested by the present analysis. This is more likely for the V-group *Attamyces* of *Ac. versicolor* that occur in close proximity to known populations of *At. mexicana* (figure 2*a*), but less likely for T-group *Attamyces* that have so far not been found in south Texas and thus not near the range of *At. mexicana* south of the USA–Mexico border (figure 2*b*).

(b) *Biogeography of* Attamyces *symbionts cultivated by* At. texana

The two dominant *Attamyces* symbiont populations cultivated by *At. texana* together comprise 97.6 per cent of the known *Attamyces* diversity associated with *At. texana* (56.4% T-group *Attamyces* accessions, $n = 93$ of 165 accessions total; 41.2% M-group *Attamyces* accessions,

$n = 68$; see the electronic supplementary material, table S1). The remaining 3.4 per cent of the *Attamyces* accessions ($n = 4$) from *At. texana* includes: (i) one accession (0.6%) that was significantly admixed under the $K = 4$ model (more than 30% of an individual's markers assigned to at least two populations; this single accession is listed as genotype no. 73 in the electronic supplementary material, table S1 and is shown in the centre of figure 1); and (ii) three accessions (1.8%) assigned under the $K = 4$ model to the fourth population of *Attamyces* that is also associated with several *Atta* species in Mexico and Cuba (the two genotypes of these three unusual accessions are listed as genotypes nos 74 and 75 in the electronic supplementary material, table S1 and are shown at the far right of figure 1). Assignment of accessions to either T-group or M-group *Attamyces* does not change when modelling a range of $K = 4$ to $K = 12$ populations (see the electronic supplementary material), and T-group and M-group *Attamyces* populations therefore represent differentiated symbiont types (figure 1) that together dominate the symbiont pool of the leaf-cutter host *At. texana*.

The populations of T-group and M-group *Attamyces* largely overlap across the range of the host *At. texana*, but with two interesting biogeographic differences. T-group *Attamyces* have so far not been collected in southern Texas (figure 2*b* and electronic supplementary material, table S1; all 11 accessions from *At. texana* south of latitude 28.06° N were M-group *Attamyces*). Second, M-group *Attamyces* have so far not been collected in Louisiana and east Texas (figure 2*b* and electronic supplementary material, table S1; all 19 *Attamyces* accessions collected from far east Texas and Louisiana were T-group *Attamyces*). Although our *Attamyces* collections are somewhat limited in the southern range ($n = 11$ *Attamyces* accessions) and the eastern range ($n = 19$ accessions) of *At. texana*, it appears that T-group *Attamyces* are restricted to more northern latitudes and are prevalent in the eastern range of *At. texana* for unknown ecological or historical reasons (e.g. T-group *Attamyces* are the only types that so far expanded with their host into east Texas and Louisiana).

(c) *Biogeography of* Attamyces *symbionts cultivated by* Ac. versicolor

Within *Ac. versicolor* (35 collections total), most *Attamyces* can be assigned to one of two populations; 69.6 per cent of these ($n = 24$) belonged to the V-group *Attamyces* and 11.1 per cent ($n = 6$) to the M-group *Attamyces* population (which are *Attamyces* lineages shared with the leaf-cutter hosts *At. mexicana* and *At. texana*; figure 1 and electronic supplementary material, table S1). Three *Attamyces* accessions from *Ac. versicolor* were inferred to be significantly admixed (defining admixture, as above, as more than 30% of the markers assigned to at least two *Attamyces* populations). M-group *Attamyces* have been found only in southeast Arizona, and *Ac. versicolor* appears to cultivate only V-group *Attamyces* throughout the rest of its US range (figure 2*a* and electronic supplementary material, table S1). Because of the fewer *Attamyces* collections from *Ac. versicolor* ($n = 35$) compared with *At. texana* ($n = 165$), and because *Ac. versicolor* ranges into Mexico where it is sympatric with *At. mexicana*, the apparent specificity of V-group *Attamyces* on *Ac. versicolor* is provisional and will need to be tested through further collection of *Attamyces* in northwest Mexico.

(d) *Biogeography of* Attamyces *symbionts cultivated by* At. insularis *in Cuba and by* At. mexicana *in Mexico*

Despite the marine dispersal barrier between mainland North America and Cuba, and despite the significant evolutionary divergence between the ant *At. mexicana* and the sister-species pair *At. insularis* and *At. texana* [49], we find no evidence for significant genetic differentiation between *Attamyces* from *At. insularis* and some *Attamyces* from *At. mexicana* (i.e. STRUCTURE assigned Cuban and some mainland *Attamyces* to the same population (C-group); figure 1 and electronic supplementary material, table S1).

(e) *Geographical structure of* Attamyces *clones*

Among the 165 accessions from *At. texana*, 12.7 per cent ($n = 21$) represent unique genotypes (collected in only a single *At. texana* nest), and the remaining 87.3 per cent accessions ($n = 144$) belong to genotypes that were collected in at least two different ant nests. Some of these *Attamyces* cultivars with genetically identical marker profiles were collected from nests at surprisingly distant locations. In *At. texana*, the average distance and the average maximum-distance between genotypically identical *Attamyces* accessions measured, respectively, 68.0 km (± 10.64 s.e.) and 125.0 km (± 11.36 s.e.; see the electronic supplementary material, table S1). In the most extreme case, two genetically identical *Attamyces* genotypes were collected from *At. texana* nests 490 km apart (southern to central Texas). The most wide-ranging M-group *Attamyces* genotype ranged over an area of at least 80 948 km^2 (across south and central Texas), and the most wide-ranging T-group *Attamyces* ranged over an area of at least 22 188 km^2 (across central and east Texas; see the electronic supplementary material, table S1). On average, M-group genotypes ranged over significantly larger areas (average = 23 973 km^2, s.e. = 12 121, $n = 7$) compared with T-group genotypes (average = 2636 km^2, s.e. = 1707, $n = 13$; $t = 2.372$, d.f. = 18, two-tailed $p = 0.029$; see the electronic supplementary material, table S1). Despite the enormous ranges of some *Attamyces* genotypes, the geographical clustering of *Attamyces* by genotype (see the electronic supplementary material, table S1) indicates that gene flow of *Attamyces* is viscous. This population viscosity across the range of the host *At. texana* is consistent with the observation that female *At. texana* disperse less than 20 km per mating flight [50]. Overall, the genotype distributions across space confirm the expectation [35,36] that *Attamyces* cultivars of *At. texana* are primarily clonally propagated by dispersing females over many ant generations (long enough for *Attamyces* to spread as clones across areas of thousands of square kilometres).

Among the 35 accessions from *Ac. versicolor*, 57.1 per cent ($n = 20$) represented unique genotypes. The average distance and average maximum-distance between *Attamyces* accessions belonging to the same genotype measured, respectively, 8.9 km (± 7.13 s.e.) and 17.49 km (± 15.58 s.e.; see the electronic supplementary material, table S1). In the most extreme case, two genetically identical

Attamyces genotypes were collected from *Ac. versicolor* nests 57.1 km apart (in southeast California). The *Attamyces* genotype with the largest and second-largest geographical distribution ranged, respectively, over only 2.61 km^2 (in central Arizona) and 0.56 km^2 (in southeast California; see the electronic supplementary material, table S1). As in *Attamyces* from *At. texana*, these patterns are likewise consistent with clonal propagation of *Attamyces* between *Ac. versicolor* nests; however, the comparatively smaller average range over which genotypically identical *Attamyces* were found in *Ac. versicolor* could indicate that, compared with clonal propagation in *At. texana*, strict clonal propagation of *Attamyces* occurs over fewer ant generations in *Ac. versicolor*. For example, clonal propagation in *Ac. versicolor* may be more frequently punctuated by genetic changes because the polygynous nest-founding of *Ac. versicolor* [16] creates frequent opportunities for co-cultivation of genetically differentiated *Attamyces* strains in incipient gardens (i.e. frequent opportunities for genetic recombination). However, because only 35 *Attamyces* were collected from *Ac. versicolor* (compared with 165 *Attamyces* from *At. texana*), *Attamyces* from *Ac. versicolor* may have been collected at insufficient geographical density relative to the dispersal distances of queens. A larger number of *Ac. versicolor* nests therefore will need to be collected across Arizona and California to rule out sampling biases.

(f) *Admixture*

Under all of the models examined in STRUCTURE, a significant fraction of *Attamyces* accessions was inferred to be admixed (i.e. these accessions combined markers assigned by STRUCTURE to at least two different *Attamyces* populations; see the electronic supplementary material, table S1). In the $K = 4$ model and under a relatively stringent definition of admixture (at least 30% of a genotype's markers were assigned to different populations), admixed *Attamyces* genotypes were found at lower frequency in the host *At. texana* (6.1%, $n = 3$ admixed genotypes of 49 total) compared with the host *Ac. versicolor* (11.5%, $n = 3$ admixed genotypes of 26 total) and compared with the tropical leaf-cutter hosts in Mexico and Cuba (average of 22.2%, $n = 4$ admixed genotypes of 18 total; see the electronic supplementary material, table S1). These proportions are not significantly different (all χ^2 comparisons $p > 0.1$). Under a less stringent definition of admixture (at least 10% of a genotype's markers were assigned to different populations), admixed *Attamyces* occurred at comparable levels in *At. texana* (24.5%, $n =$ 12 admixed genotypes) and *Ac. versicolor* (26.9%, $n = 7$ admixed genotypes), but more frequently in the tropical leaf-cutter hosts (average of 66.7%, $n = 12$ admixed genotypes; see the electronic supplementary material, table S1); this proportion of admixed genotypes among the tropical leaf-cutter hosts is significantly higher (χ^2_1 4.749, $p =$ 0.0293) compared with the two northernmost leaf-cutter hosts. A larger collection of *Attamyces* from tropical leaf-cutter ants will need to be genotyped to corroborate this pattern and rule out sampling artefacts as an explanation for the observed difference in admixture between temperate and tropical *Attamyces*. For the *Attamyces* from *At. texana* ($n = 165$) and *Ac. versicolor* ($n = 35$), fitting equation 8 of Bengtsson [45] within a Bayesian framework to the observed genotype distributions implicated very low frequencies of sexual reproduction per generation (0.3% with a 95% highest posterior density confidence interval (HPD-CI) of 0.01–1% for *At. texana*; 0.6% with a 95% HPD-CI of 0.02–3% for *Ac. versicolor*).

4. DISCUSSION

Population-genetic patterns of *Attamyces* cultivated near the northern range limit reveal some unique features of the ant–fungus mutualism that have so far not been observed for *Attamyces* in tropical habitats [37,38,41], but also some parallels between northern and tropical *Attamyces*.

(a) *Parallels between northern and tropical* Attamyces *populations*

Attamyces is largely clonally propagated between parent and offspring nests in both northern and tropical habitats. Clonality is implicated in our study most strongly by the high frequency (87.3%) of *Attamyces* accessions that were collected as genetically identical genotypes in at least two different *At. texana* nests. The most abundant *Attamyces* genotypes were collected across vast ranges (15 000–80 000 km^2, see the electronic supplementary material, table S1). Because our population-genetic markers mutate at rates typical for microsatellite loci [36], the large ranges of some *Attamyces* clones indicate that the spread of *Attamyces* clones across hundreds of kilometres can occur rapidly relative to the rates of endogenous (mutational) change. A second parallel between northern and tropical *Attamyces* populations is that *Attamyces* lineages are shared between leaf-cutter ant species, exemplified most prominently by the sharing of M-group *Attamyces* between four leaf-cutter species in North America. A third parallel is that *Attamyces* lineages occasionally recombine (implicated by the observation of admixed *Attamyces* genotypes); *Attamyces* propagation is, therefore, not strictly clonal across an endless series of host generations as proposed by initial phylogenetic studies of *Attamyces* [51]. These three basic features of *Attamyces* biology had already emerged in previous population-genetic and phylogenetic analyses of much smaller *Attamyces* datasets from Central and South America ([37,38,41]; see also discussions in [31,40]).

(b) *Differences between northern and tropical* Attamyces *populations*

Several features emerged in the northernmost *Attamyces* populations that have so far not been documented for Central and South American populations: (i) cultivation of specific *Attamyces* lineages by only a single ant host (i.e. ant–fungus specializations that tighten ant–fungus coevolution); (ii) regional or ant-specific differences in admixture between *Attamyces* lineages; and (iii) dispersal by *Attamyces* over significant distances and across ocean barriers, indicated most prominently by the surprisingly close genotypic proximity between *Attamyces* representatives from Cuba and Mexico (figure 1). These three differences are discussed in detail in the following sections.

(i) *Host–symbiont specializations tighten coevolution at the northern frontier*

As expected for peripheral populations, genotypically differentiated cultivar lineages were indeed found in the

northernmost leaf-cutter populations, giving rise to specialized host–symbiont combinations. The evidence for host–symbiont specialization is strongest for T-group *Attamyces*, which emerged as the most distinct symbiont type in the present study (figure 1). This implies that, whereas ant–*Attamyces* coevolution is rather diffuse in the tropics (several leaf-cutter species interact with several *Attamyces* lineages in a complex interaction network [38,40,41]), coevolution is tighter for some ant–fungus combinations at the northern range limit. The most prominent cases of coevolutionary tightening were found, first, in Louisiana where *At. texana* associates with only one narrow subgroup of T-group *Attamyces* (figure 2*b*), and second, in California and northern Arizona where *Ac. versicolor* associates with only a narrow subset of V-group *Attamyces* (figure 2*a*). Moreover, among the *Attamyces* cultivated by *At. texana*, T-group *Attamyces* appear to be more tightly coevolving with the host *At. texana* (because of the exclusive association of this symbiont type only with *At. texana*) compared with M-group *Attamyces* (which have close genetic links to *Attamyces* cultivated by other leaf-cutter species (figure 1). A trendin declining genetic diversity of the cultivated fungi towards the northern range limit and a corresponding tightening of coevolutionary interactions had also been observed in the northernmost fungus-growing ant *Trachymyrmex septentrionalis* (a non-leaf-cutter ant), which spread after the Pleistocene northwards through the eastern and central USA [52].

(ii) *Admixture rates appear lower in temperate than in tropical* Attamyces

Given the biology of United States leaf-cutter ants and their *Attamyces* fungi, it is surprising that our survey revealed few significantly admixed *Attamyces* accessions (three in *At. texana* and three in *Ac. versicolor*; see the electronic supplementary material, table S1) and that Bayesian modelling implicated very low frequencies of sexual reproduction per *Attamyces* generation (0.3% for *At. texana* and 0.6% for *Ac. versicolor*). Higher frequencies could be expected because of (i) the known capacity of *Attamyces* for recombination ([37] and this study; see also the electronic supplementary material *Admixture* for a detailed description of the diverse mechanism of genetic exchange in multi-nucleate fungi); (ii) the extensive overlap between T-group and M-group *Attamyces* across the range of *At. texana* (figure 2*b*) and the overlap of V-group and M-group *Attamyces* in southeast Arizona (figure 2*a*); (iii) the frequent proximity of neighbouring nests with *Attamyces* from distinct *Attamyces*-groups (frequently within 100 m of each other; see the electronic supplementary material, table S1); and (iv) polygynous nest-founding and thus possible cultivar mixing in both ant species (5% polygynous nest-founding in *At. texana* [53]; between 0 and 100% in *Ac. versicolor*, depending on the population [16]).

The low levels of observed recombinants between differentiated *Attamyces* groups in the northern range may have several, non-exclusive explanations. First, when *Attamyces* strains are co-propagated by the ants in a common garden or when a novel strain enters the monoculture of an established nest, recombination between differentiated *Attamyces* strains may be prevented by mycelium–mycelium antagonism (i.e. somatic incompatibility *sensu* [54], preventing anastomosis and migration of nuclei between the two mycelia). Second, *Attamyces* types may be maintained as genotypically differentiated, sympatric lineages because of nucleus–nucleus incompatibilities if nuclei are ever exchanged between two *Attamyces* strains (i.e. the two nuclei do not function together when residing in the same cell, leading to cell death; or nuclei may compete with each other, leading to elimination of competitively inferior nuclei). Third, *Attamyces* recombinants may exhibit hybrid inferiority relative to the unrecombined parental types (i.e. nests with hybrid *Attamyces* therefore may have reduced survivorship, reduced growth rates or reduced fecundity). These three explanations are not mutually exclusive, and they can be tested in laboratory experiments with experimental ant–fungus combinations or with artificially created chimaeric gardens [28].

The possible lower level of recombination (admixture) of *Attamyces* from *At. texana* and *Ac. versicolor* compared with *Attamyces* of tropical leafcutter hosts is intriguing and hopefully will stimulate follow-up work to test for this difference with a larger sample of tropical *Attamyces*. Both *Ac. versicolor* and *At. texana* can found their nests polygynously [16,50,53], whereas *At. mexicana* and *At. cephalotes* are strictly monogynous ([42,55]; U. G. Mueller 1991–2010, personal observations). If co-cultivation of *Attamyces* strains during polygynous nest-founding facilitates genetic exchanges, one would expect that the *Attamyces* of *Ac. versicolor* and *At. texana* actually show higher levels of admixture, whereas we found the opposite pattern. This implicates processes other than co-cultivation as primary factors facilitating genetic exchanges between *Attamyces* lineages.

(iii) Attamyces *fungi can disperse independently of the ant hosts across marine barriers*

Although the link between mainland and Cuban *Attamyces* had already emerged in the phylogenetic analysis of Mikheyev *et al.* [37], our analysis documents the population-genetic proximity between mainland and Cuban *Attamyces* populations with greater resolution and for a more comprehensive sample (220 *Attamyces* strains from North America). Cuba, as part of the proto-Greater Antilles, moved through the gap between North and South America at the end of the Cretaceous (over 60 Myr ago) and may have temporarily abutted what is now Southern Mexico [17,56]. Since the early Tertiary, however, Cuba is thought to have remained separate from the mainland and without temporary land-bridges during changes in sea levels [17,56]. In the absence of gene flow across the ocean between mainland and Cuba, the *Attamyces* populations in Cuba, therefore, should have become genetically differentiated from the mainland populations, a prediction that is not supported by the observed population-genetic patterns (figure 1). Because the host *At. insularis* occurs only in Cuba but not on mainland North America, and because *At. insularis* is significantly derived at both the molecular and morphological levels from the mainland *Atta* lineages ([49], suggesting significant evolutionary time to permit divergence of *At. insularis*), it seems unlikely that the current genetic similarity between mainland and Cuban *Attamyces* was maintained during the divergence process of *At. insularis* without gene flow in *Attamyces*.

Consequently, recent or ongoing gene flow in *Attamyces* between the mainland and Cuba is a more likely explanation for the absence of genetic differentiation between mainland and Cuban *Attamyces*. Such gene flow could occur via three possible avenues.

(i) Leaf-cutter queens occasionally disperse across the ocean barrier from the mainland to Cuba (e.g. across the 250 km wide Yucatan Channel between the Yucatan Peninsula in Mexico and the Guanahacabibes Peninsula in Cuba); these queens vector *Attamyces* to Cuba, establish nests long enough to permit *Attamyces* to be transferred to *At. insularis*, then these temporary colonizers become extinct. Although other poorly dispersing ant species managed to colonize many islands throughout the Caribbean [57], we consider this scenario unlikely, primarily because of the rarity of observed leafcutter dispersal across Caribbean islands [58].

(ii) Similar to (i) but assuming human-mediated dispersal, *Attamyces* fungi were recently introduced to Cuba with leaf-cutter ants brought accidentally by humans from the mainland (e.g. in soil of potted plants); once in Cuba, introduced *Attamyces* replaced pre-existing cultivars in *At. insularis* nests. This scenario would receive support if the only other leaf-cutter ant species in Cuba (*Acromyrmex octospinosus*) can be shown to have colonized Cuba recently, which is not supported by available genetic information (i.e. the Cuban *At. octospinosus* populations appear genetically differentiated from mainland populations [58]).

(iii) *Attamyces* is able to disperse independently of the leaf-cutter hosts, either by airborne spore dispersal (spore-bearing mushrooms of *Attamyces* have been observed on rare occasions; table 3 in Mueller [26] summarizes the relevant literature), or by dispersal with the help of unknown vectors (e.g. by one of the many arthropods frequenting leaf-cutter nests, such as mites, beetles or moths; [10,15,59]).

Irrespective of these unresolved dispersal mechanisms, the close population-genetic links between mainland and Cuban *Attamyces* support the view that tropical *Attamyces* cultivars may be able to survive independently of leaf-cutter ants at least for some time (e.g. as spores, or when dispersed by vectors other than ants). Consequently, *Attamyces* fungi may be more than enslaved domesticates tied inescapably to the fate of their particular leaf-cutter host [26,37,38,40]. If so, long-distance dispersal and genetic mixing of *Attamyces*, including oceanic dispersal independent of leaf-cutter ants, may occur throughout the range of *Attamyces*. Such long-distance dispersal ability of *Attamyces* would contrast with the dispersal ability of *Termitomyces* fungi of the termite–*Termitomyces* symbiosis, in which effective long-distance dispersal of *Termitomyces* across oceanic barriers appears to be rare (e.g. between mainland Africa and Madagascar [60]). However, long-distance dispersal and genetic mixing of *Attamyces* could help explain the much younger coalescence and origin of *Attamyces* fungi (about 2–4 Myr old) compared with the corresponding origin of leaf-cutter ants (about 8–12 Myr old) [40].

5. CONCLUSION

Peripheral populations of the leaf-cutter ant–*Attamyces* symbiosis emerge as particularly interesting test cases of coevolution between ant farmers and their cultivated fungi, for two reasons. First, the documented local reduction of ant–species diversity and *Attamyces* diversity at the northern range limit increases local ant–*Attamyces* specificity; increased specificity, in turn, should lead to tighter, local coevolutionary interactions. Second, because novel *Attamyces* variants can sweep through the range of leaf-cutter ants [40], peripheral populations are least likely to be reached by novel *Attamyces* variants. These demographic processes should affect peripheral and island populations alike; however, because of dispersal across oceanic barriers, marine island populations are no longer the most promising candidates for finding unusual *Attamyces* variants. Specifically, if the Cuban populations of *Attamyces* analysed here are representative, we predict that Caribbean island populations of *Attamyces* known from Trinidad [61], Guadeloupe [58] and Curacao [62] will likewise retain strong population-genetic links to continental populations. Instead, populations at the periphery of the leaf-cutter distribution (in the USA, in Argentina, perhaps at mid-elevation sites in the Andes) are most likely to reveal unique and ant–species-specific leaf-cutter ant–*Attamyces* associations. These peripheral populations therefore contribute the most distinct geographical variants in coevolutionary interactions between leaf-cutter ants and their cultivated fungi. Studies of such marginal populations will inform models of coextinctions of obligate symbiotic partners that are doubly stressed by habitat marginality and by environmental change [8,9].

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Electronic Supplementary Material

Frontier mutualism: Co-evolutionary patterns at the northern range limit of the leafcutter ant-fungus symbiosis

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MATERIALS AND METHODS

The study systems of *Atta texana* and *Acromyrmex versicolor* leafcutter ants in the USA

Atta texana is a soil-nesting leafcutter ant and the northernmost species of its genus (Figure S1). Its two closest relatives, *Atta mexicana* (from Mexico and possibly El Salvador) and *Atta insularis* (exclusively from Cuba), are also North American denizens [1], suggesting either a North American origin of this clade, or a long biogeographic affiliation with North America, or both. A cultivar isolate of *At. insularis* from Cuba was previously described as *Attamyces bromatificus* [2]; because of the population-genetic proximity between cultivar strains of *At. insularis* and cultivar strains of other North American leafcutter ant species (Figure 1 main article), we refer to all cultivars of North American leafcutter ants as *Attamyces*.

In contrast to *At. texana*, the biogeographic context of *Ac. versicolor* is less clear. *Ac. versicolor* is unusual in that it belongs to the subgenus *Acromyrmex (Moellerius)*, and it is the only *Moellerius* species outside of South America, whereas four other *Acromyrmex* species in Central and North America (*Ac. octospinosus*, *echinatior*, *volcanus*, *coronatus*) are all members of the subgenus *Acromyrmex (Acromyrmex)*. Fowler [3] discusses the biogeographic and behavioral uniqueness of *Ac. versicolor* within the subgenus *Moellerius*, and suggests possible biogeographic explanations for the vicariant existence of *Ac. versicolor* in North America.

Attamyces lineages cultivated by the leafcutter ants *At. texana* and *Ac. versicolor* were presumably vectored by dispersing leafcutter queens during their postglacial range expansion northward into the southern USA (Figure S1). Where these two leafcutter species existed during the last Pleistocene glaciation is unclear; refugia in Mexico would seem to be the most likely possibility because the entire southern USA was significantly colder at that time. The northward expansion of leafcutter ants from these putative southern refugia can be dated only broadly (i.e., during the postglacial warming in the past 10,000-15,000 years). *At. texana* was established in central and east Texas at the time when European settlers arrived [4,5]. No detailed records exist documenting a widespread presence of *Ac. versicolor* in Arizona and south-east California, but a pre-Columbian distribution across southern Arizona and south-eastern California seems more likely than a recent introduction or recent range expansion.

Figure S1. Confirmed nest localities of *Atta texana* in Texas and Louisiana (402 records). Figure drawn by Damian Broglie and Christian Rabeling.

Distribution of *Atta texana* ants

Locality information: Information on the occurrence of nests of *At. texana* leafcutter ants (Figure S1) was compiled in 2003-2008 to accumulate a comprehensive list of localities for collection of *Attamyces* material across Texas, Louisiana, and northern Mexico. Locality information of *At. texana* was obtained by (a) examining material in museum collections (Entomology Collection, Brackenridge Field Lab, Austin, TX; Insect Collection, Texas A&M University, College Station, TX; Museum of Texas Tech University, Lubbock, TX; Louisiana State Arthropod Museum, Baton Rouge, LA; National Museum of Natural History, Washington, DC; Museum of Comparative Zoology, Harvard University, Cambridge, MA; Los Angeles County Museum of Natural History, Los Angeles, CA; Bohart Museum of Entomology, University of California at Davis, CA; California Academy of Sciences Collection, San Francisco, CA; American Museum of Natural History, New York, NY); (b) extracting information from the literature (e.g., [6-8] and references in these recent publications); (c) surveying roadsides by car until suitable habitat was located, then inquiring with local residents about the location of *At. texana* nests; (d) networking with naturalists, nature centers, State Park rangers, extension agents, pest-control businesses, and farmers. Because we have found that even experienced naturalists can confuse leafcutter ants with harvester ants (both have large-bodied workers of reddish coloration, have conspicuous mounds, construct foraging trails, and forage on plant material), we included in our dataset only locality information that we could verify by examining museum specimens or by visiting locations to confirm the presence of *At. texana*.

Particular effort was spent to locate nests and collect garden material at the limits of the reported distribution of *At. texana*, including the westernmost (Del Rio, Val Verde County, TX), northernmost (Fort Belknap, Young County, TX; Ogburn, Wood County, TX; Minden, Webster Parish, LA), and easternmost populations (Catahoula Parish, LA; Pineville, Rapides Parish, LA; Oberlin, Allen Parish, LA) (Figure S1). We concentrated on the northernmost populations to elucidate the ecology and evolution of *At. texana* and its cultivated fungi under the environmental conditions at the northern limit of the entire leafcutter distribution. The southern populations of *At. texana* in the USA along the lower Rio Grande River (Cameron, Hidalgo, Starr, Zapata, and Webb Counties, TX) were less extensively surveyed; however, garden from populations near Salineno (Starr County) and Raymondsville (Cameron County) were collected as the southernmost representatives in our population-genetic and mycological studies of *Attamyces*. We could not confirm the reported extensive presence of *At. texana* in Mexico, except for locations near the Rio Grande River between Ciudad Acuña and Piedras Negras in the State of Cuahuila, Mexico. It is likely that *At. texana* extends further south into Coahuila and perhaps the neighboring state of Nuevo Leon. However, a recent survey of leafcutter ants in Nuevo Leon and the adjoining Tamaulipas failed to find *At. texana* in these Mexican border states [9]. Instead, the closely related *Atta mexicana* [1] occurs abundantly in both Nuevo Leon and Tamaulipas, and it appears that this Mexican *Atta* species replaces *At. texana* somewhat south of the US-Mexico border [9]. Likewise, we could not confirm the presence of *At. texana* mentioned in the literature for Foard, Knox, Denton, and Grayson Counties in north Texas [6] and for Bowie, Red River, and Cass Counties [10,11] in northeast Texas, despite considerable effort to find nests in these counties using the strategies mentioned above. Such unconfirmed county records were not included in our database. Our final dataset included 402 confirmed locality records of *At. texana* (Figure S1).

Distribution of *Acromyrmex versicolor* ants

Locality information: Information on the occurrence of nests of *Ac. versicolor* leafcutter ants was compiled in 2005-2008 to accumulate a comprehensive list of localities for collection of *Attamyces* material across Arizona and California. Locality information for *Ac. versicolor* was obtained by (a) compiling information from private collectors (Robert E. Johnson Ant Collection, Arizona State University, Tempe, AZ; Lloyd R. Davis Ant Collection, Gainesville, FL; Phil Ward Ant Collection, University of California, Davis, CA; Gordon C. and Roy R. Snelling Ant Collection, Los Angeles, CA; Ken Helms Ant Collection, University of Vermont, Burlington, VT); (b) examining material in museum collections (Entomology Collection, University of Arizona, Tucson, AZ; National Museum of Natural History, Washington, DC; Museum of Comparative Zoology, Harvard University, Cambridge, MA; Los Angeles County Museum of Natural History, Los Angeles, CA; Bohart Museum of Entomology, University of California at Davis, CA; California Academy of Sciences Collection, San Francisco, CA; American Museum of Natural History, New York, NY); and (c) extracting information from the literature [12-23]. As for *At. texana*, we included in our dataset only locality information that was identified by an ant specialist or that we could verify by either examining museum specimens or by visiting putative collection sites.

Collection of *Attamyces* from garden material

Gardens of *At. texana* were collected by digging into the center of leafcutter mounds with a shovel to reach the topmost gardens. Because *At. texana* cultivates a monoculture of the same fungal strain throughout its hundreds of gardens [24], a fragment from a single garden was sufficient to obtain the resident *Attamyces* strain in a particular nest. All garden collections from *At. texana* nests were preserved in 100% ethanol and stored at -80°C for population-genetic analyses [24-26].

Nests were chosen for excavation principally because of ease of access (e.g., permission by landowner; location along roadside or on public land), rather than ease of excavation in sand versus alluvial clay. In a few cases, no clean garden could be collected because too much soil collapsed onto the garden and compressed it; in such cases, a garden sample could still be ethanol-preserved for genotyping. Two excavation attempts in alluvial clay soil failed because no garden could be found within the top two meters, and one attempt in sandy soil failed because garden could not be accessed between the roots of a large oak tree. Vouchers of collections are stored at -80°C in 100% ethanol at the Attine Collection in the Mueller Lab, Integrative Biology, University of Texas at Austin.

Gardens from the other four leafcutter ant species (*Ac. versicolor, At. mexicana, At. cephalotes, At. insularis*) were likewise collected by digging into the center of leafcutter mounds and by preserving garden fragments in 100% ethanol, then stored at -80°C, as described in [27].

DNA fingerprinting with microsatellite DNA markers

Attamyces fungi were genotyped using twelve microsatellite markers [25]. Because *At. texana* cultivates a monoculture of the same fungal strain throughout all gardens of a single nest [24], it is sufficient to genotype a fragment from a single garden to profile the resident *Attamyces* strain cultivated by a given nest. For DNA extraction, a small fragment of pure mycelium (free of

garden substrate) or fungal staphylae (aggregation of hyphal-tip swellings typical for *Attamyces*) was picked under the microscope with flame-sterilized forceps. DNA was extracted from mycelium by placing it in 100μl of 10% Chelex buffer (Sigma-Aldrich) at 60ºC for 1.5 hrs, followed by 10 minutes at 99ºC. One microliter of this extract was used as template in a 10 µl PCR amplification volume. Amplification products were characterized on an ABI-3100 Capillary Genotyper. Seven of the twelve markers were multiplexed as follows: loci A1030, B12, and C625 (common annealing temperature T_m = 60°C); loci B150, C101, C126, and C117 (common annealing temperature T_m = 58°C). Five additional loci (A1132, A1151, B319, B430, A128) were amplified and analyzed individually (not multiplexed), as specified in [25].

The multiplex PCR reaction contained 1X PCR buffer, 0.3125 mM of each dNTP, 5mM $MgCl_2$, 10 µg BSA, 2 nmol of each primer, and 0.25 units of Taq polymerase. For the five markers analyzed individually, the ingredients for the 10μl PCR reaction were the same as for the multiplex reactions, except 0.2 mM of each dNTP and 2.5 mM $MgCl_2$ were used. For all amplifications, the temperature profile involved an initial denaturing step of 94ºC for 5 minutes, followed by 35 cycles of 10 seconds denaturation, 15 seconds at the annealing temperature (see above), and 25 seconds of extension at 72ºC. The first 10 cycles used a denaturation step at 94ºC, the remaining denaturation steps were at 89ºC. A final extension step at 72ºC was run for 45 minutes. One microliter of the PCR product was added to 1.5 µl of size-standard (lab-made, following the methods of [26]) and to 7.5 µl of HiDi formamide (Applied Biosystems). The mix was heated to 95ºC for 5 minutes, then cooled to 10ºC and separated by electrophoresis on an ABI-3100 Genotyper. Microsatellite marker sizes were scored using GeneMarker v1.5 (Softgenetics, State College, PA).

Fungi of leafcutter ants are multinucleate, yielding up to 5 alleles per locus per individual [25]. Screening of twelve loci yielded information on the presence/absence of 91 variable markers in the sample of 220 *Attamyces* fungi isolated from North American leafcutter ants.

Sample selection

Because *Attamyces* clones can be exchanged between leafcutter species [27-29], we believed that a population-genetic analysis of the *Attamyces* cultivated by *At. texana* and *Ac. versicolor* would be best conducted within the context of the *Attamyces* cultivated by other North American leafcutter species, including *Atta mexicana* (throughout Mexico and extreme southern Arizona), *Atta cephalotes* (south-eastern Mexico), and *Atta insularis* (Cuba). We included in our population-genetic analysis all *Attamyces* available to us from North American leafcutter species (165 *Attamyces* from *At. texana* from Texas and Louisiana; 35 from *Acromyrmex versicolor* from Arizona and California; 5 from *Atta insularis* from Cuba; 7 from *Atta mexicana* from the States of Nuevo León, Oaxaca, and Chiapas in Mexico; 8 from *Atta cephalotes* from the States of Veracruz and Oaxaca in Mexico). These additional collections had been amassed as part of a larger survey of the fungi cultivated by leafcutter ants throughout North, Central, and South America. The additional collections were made between 2003-2007 by excavation (as described above) and by preserving garden material in 100% ethanol.

Population-genetic analysis

Population-genetic patterns were analyzed in Structure Version 2.2 ([30,31]; freeware at http://pritch.bsd.uchicago.edu/software), which uses a Markov chain Monte Carlo (MCMC)

algorithm to cluster individuals into populations on the basis of multilocus genotype data [30-32]. For each value of K (number of populations modeled to partition the overall genotype variation), we conducted three independent runs and checked results for convergence. Each run involved a burnin of 50,000 generations and an additional 100,000 generations of MCMC sampling. Structure assumes that all of the genetic material of the sampled individuals comes from one or more of K unobserved populations. Structure characterizes each population by a set of allele frequencies at each locus. Individuals may have pure ancestry (possessing alleles assigned to only one population) or mixed ancestry in more than one of the K populations (possessing alleles assigned to more than one population; termed admixed genotypes). Structure is commonly used to detect migrants or admixed individuals, to infer historical population admixture, and to identify cryptic population structure [30-32].

Because *Attamyces* are multinucleate and exhibit complex (polyploid-like) genotype profiles [25], we treated all alleles as dominant markers, as recommended by [31] for polyploid species. Marker information that was uncertain was scored as "?" (e.g., because of conflicting scoring in repeat genotyping, or because of possible stutter amplification), but presence/absence of only 6 markers (0.07%) of 8463 markers total remained uncertain in the final data-matrix. Structure requires that individuals included in an analysis differ by at least one marker, and we included in our analysis therefore only one representative per genotype. Because *Attamyces* is clonally propagated within and between leafcutter nests, more than 50% of the collections possessed a genotype profile that was identical to the profile of another individual. After eliminating duplicates to retain only one representative per genotype, the final dataset included 93 unique genotypes (each profiled for 91 variable microsatellite markers). To visualize the genetic diversity and to validate results obtained from Structure, we also calculated a two-dimensional non-metric multidimensional scaling solution of the binary distances between cultivar genotypes (computed respectively in R and GenAlEx; www.anu.edu.au/BoZo/GenAlEx/ [33]) (Figure 1).

Admixture

A Markov chain Monte Carlo (MCMC) algorithm implemented in Structure identified genetically differentiated populations and inferred contributions of ancestry for each allele carried by an individual *Attamyces* genotype (i.e., the MCMC algorithm computed the likelihood that an allele derived from one the inferred populations). Admixture (mixed ancestry) was inferred for a particular fungal accession if different alleles of this genotype were assigned with high likelihood to different populations.

In mushroom-forming basidiomycete fungi, admixture can be the result of several mechanisms of genetic exchange [34-36]. First, two monokaryotic (one nucleus per cell) fungal strains may fuse to form a dikaryotic mycelium (two nuclei per cell). Such monokaryotic strains typically germinate from uni-nucleate spores in basidiomycete fungi. To our knowledge, the number of nuclei per spore was never determined in the few instances where *Attamyces* spores have been observed [37], and the existence of monokaryotic mycelium of *Attamyces* has yet to be documented. Second, admixture may occur through the movement of a nucleus from a germinating spore into a monokaryotic mycelium. Third, admixture may occur through the movement of nuclei from multinucleate (polykaryotic) *Attamyces* strains into monokaryotic strains. Fourth, nuclei may be exchanged between dikaryotic or multinucleate *Attamyces* strains. Such nucleus exchange occurs in dikaryotic or multinucleate basidiomycete fungi typically

between two homokaryotic mycelia (the multiple nuclei in each mycelial cell are genetically identical) if the exchanging mycelia are genetically different from each other at their mating loci. Lastly, it is not possible to rule out for *Attamyces* that nucleus exchange may sometimes also occur between heterokaryotic *Attamyces* mycelia (each mycelial cell carries a genetically diverse population of nuclei, and nuclei are therefore exchanged between multinucleate, heterokaryotic strains).

Another possibility of genetic admixture in leafcutter gardens is a mixture of several, comingled fungal strains, but this possibility is not supported by the available DNA fingerprinting and population-genetic evidence: (a) we have failed previously to find more than a single *Attamyces* genotype in single *Atta* nest ([24]; 5 and 6 nests screened from *Atta texana* and *At. cephalotes*, respectively); (b) in single *Atta* nests, each *Attamyces* genotype was stable over at least five years ([24]; 3 *At. texana* nests sampled longitudinally over six years, 4 *At. cephalotes* nests sampled longitudinally for five years); (c) identical genotypes can be found in distant *At. texana* nests distributed across large areas (as large as 80,000 km^2; this study; Table S1). This constancy of *Attamyces* genotypes within nests and between nests is predicted by monoculture and clonal propagation, but the genotypic constancy is much more difficult to reconcile with co-growth of separate, genetically-differentiated *Attamyces* mycelia co-existing in the same garden. This is because, under co-growth of separate mycelia, sometimes only one of the mycelia would be isolated or genotyped from a nest, which we did not observe in our monoculture study [24]. Under co-growth of separate mycelia, the observed DNA fingerprinting patterns and genotype constancy across large areas can only be explained if the two co-growing mycelia are tied together intimately by some unknown mechanism (i.e., the two mycelia do not separate readily, and the two co-growing mycelia therefore behave like a multinucleate entity). The hypothesis of co-growing mycelia can be tested further by histological analyses that track individual nuclei, but such a study has not yet been performed for *Attamyces*.

Because the above mechanisms underlying admixture in *Attamyces* are complex, and because several of these mechanisms may contribute to admixture, the most cautious interpretation of *Attamyces* genotypes carrying alleles assigned to more than one population is that these genotypes are of "uncertain population affiliation", where the uncertainty of affiliation likely derives from the recombination of genetic material derived from differentiated populations.

Estimating the Probability of Sexual Reproduction in *Attamyces*

Equation 8 in [38] calculates the expected distribution of clones in a sample drawn from a predominantly clonal population at equilibrium. This calculation depends on the population size, and the frequency of sexual events per generation. Using the observed distribution of clones in our *Attamyces* samples from *At. texana* (n=165) and *Ac. versicolor* (n=35), and assuming a uniform prior for population size, we inferred the frequency of sexual reproduction in a Bayesian framework implemented in WinBUGS (version 1.4) [39]. The respective population sizes of *Attamyces* from *A. texana* and *A. versicolor* are not known, however, they are likely to be large (well over 10,000 individuals, most likely exceeding 100,000 individuals). Using a uniform prior ranging from 10^3-10^6 *Attamyces* individuals per ant host, estimates of sexual reproduction did not change substantially within the modeled range of population sizes, and estimates therefore appear to be robust.

RESULTS

Population-genetic analysis of North American *Attamyces* fungi

Structure analyses inferred four populations of *Attamyces* among the 93 unique genotypes (Figure 1). These solutions were convergent between MCMC runs. Modeling more than four populations (K>4) did not significantly improve likelihood scores [Figure S2]. Two of the four inferred populations represent two dominant cultivar types of *At. texana* (T-group and M-group *Attamyces*); one of these two *Attamyces* populations from *At. texana* (dark blue, Figure 1) also includes *Attamyces* cultivated by other leafcutter species in Mexico (hence M-group *Attamyces*) and *Ac. versicolor* in Arizona. The second *Attamyces* population from *At. texana* (light blue, Figure 1) is clearly distinct from the three other populations and is known so far only from Louisiana and Texas (hence T-group *Attamyces*), but not from Mexico or the western USA. A third population is known so far only from Arizona and California (cultivated there by *Ac. versicolor*, therefore called V-group *Attamyces*; red, Figure 1), and a fourth population (green, Figure 1) includes a more diverse assemblage of *Attamyces* cultivated by *At. mexicana* and *At. cephalotes* in Mexico, by *At. insularis* in Cuba (hence C-group *Attamyces*), and by two unusual *Attamyces* genotypes from nests of *At. texana* (Figure 1).

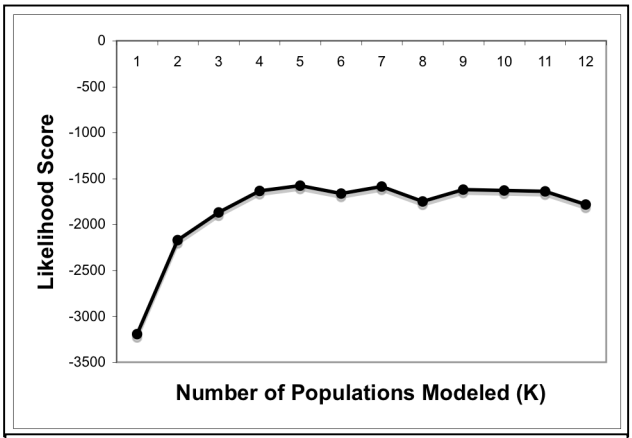

Figure S2. Ln likelihood scores from Structure runs that modeled K number of populations within the sample of 93 unique *Attamyces* genotypes. Likelihood scores do not change significantly if more than four populations are modeled.

The two dominant *Attamyces* symbiont populations cultivated by *At. texana* together comprise 97.6% of the known *Attamyces* diversity associated with *At. texana* (56.4% T-group *Attamyces* accessions, n=93 of 165 accessions total; 41% M-group *Attamyces* accessions, n=68). The remaining 3.4% of *Attamyces* accessions (n=4) from *At. texana* included (a) one accession (0.6%) that was significantly admixed under the K=4 model (more than 30% of an individual's markers assigned to at least two populations; this single accession is listed as Genotype #73 in Table S1 and is shown in the center of Figure 1), and (b) three accessions (1.8%) assigned under the K=4 model to the fourth population of *Attamyces* that is associated also with several *Atta* species in Mexico and Cuba (the two genotypes of these three unusual accessions are listed as Genotypes #74 and #75 in Table S1 and they are shown at the far right of Figure 1). Assignment of accessions to either T-group or M-group *Attamyces* does not change when modeling a range of K=4 to K=12 populations, and these two *Attamyces* populations therefore represent well-differentiated symbiont types (Figure 1) that together dominate the symbiont pool of the leafcutter host *At. texana*. Overall, therefore, for values of $4 \leq K \leq 12$, assignment of *Attamyces* from *At. texana* to either the T-group or the M-group population is consistent and robust.

For values of K=2 and K=3, assignment of *Attamyces* from *At. texana* to the T-group population was identical to the assignments for values of $K \geq 4$ (as is expected, because the T-group *Attamyces* are most differentiated from the rest of the *Attamyces* analyzed; Figure 1). Also for values of K=2 and K=3, M-group *Attamyces* from *At. texana* were grouped with *Attamyces* from

Ac. versicolor and from Mexican *Atta* species (as is expected, because of the proximity of the M-group *Attamyces* from *At. texana* to the *Attamyces* from the Mexican leafcutter species depicted in Figure S1). In summary, therefore, under all models examined (K=2 to K=12), T-group and M-group *Attamyces* collections were identified consistently, justifying biogeographic comparisons (see main article) and phenotype comparisons [40] of these two distinct types of *Attamyces* cultivated by the leafcutter ant *At. texana*.

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Table S1. *Attamyces* accessions collected from five leafcutter ant host-species in North America. Global positioning system (GPS) coordinates are in decimal degrees. *Attamyces* accessions listed under the same genotype number showed identical microsatellite profiles across all of the 91 variable markers scored. Accessions are grouped according to the four *Attamyces* genotype-clusters identified in the Structure Analysis (V-, M-, T-, and C-group) or identified as *admixed* (genotype profile that combined markers assigned to at least two of the four *Attamyces* genotype-clusters; see text). Admixture is listed for different minimum admixture values: at least 10%, 20%, or 30% of the markers of a particular genotype profile were assigned by Structure to different *Attamyces* genotype-clusters. For genotypes collected at multiple locations, the within-genotype average pairwise distance between collection locations was calculated in kilometers. For genotypes collected at more than two locations, the area of distribution was calculated from the GPS polygon. *Incipient nests* were founded in spring of the collection year; *1-year-old nests* were founded in the year preceding the collection year; *small nests* were estimated to be 2-4 years old, *large nests* were older than 4 years. AZ=Arizona, CA=California, LA=Louisiana, TX=Texas, MX=Mexico. *Ac.* = *Acromyrmex*. The ending "…R" of an Accession ID indicates that the microsatellite genotyping was repeated blindly to confirm or correct unusual marker scorings in the first genotyping.

***Attamyces* Accession ID**	**Ant Host**	**Location & Location ID**	**GPS North**	**GPS West**	**Source (garden or pellet; field or lab nest)**	**Genotype number**	**Group Assignment & Admixture (clustering by Structure Analysis into V-, M-, T-, or C-group *Attamyces*)**			**Within-genotype average pairwise location distance in km (maximum distance in parentheses)**	**Area of genotype distribution in km²**
							10% minimum admixture	**20% minimum admixture**	**30% minimum admixture**		
UGM070324-01R	Ac.versicolor	AZ DolanSprings1	35.65	114.20	field garden, large nest	1	V	V	V		
UGM070324-03R	Ac.versicolor	AZ DolanSprings2	35.65	114.20	field garden, large nest	2	V	V	V		
UGM070325-07R	Ac.versicolor	AZ Wikieup1	34.68	113.70	field garden, large nest	3	V	V	V		
UGM070325-08	Ac.versicolor	AZ Wikieup2	34.68	113.70	field garden, large nest	4	V	V	V		
UGM070327-07R	Ac.versicolor	AZ Safford2	32.66	109.70	field garden, large nest	5	admixed	admixed	admixed		
JNJ061105-01	Ac.versicolor	CA WileyWell1	33.49	114.88	field garden, large nest	6	V	V	V	26.6 km (57.1 km)	2.61 km²
UGM061203-01	Ac.versicolor	CA WileyWell2	33.49	114.88	field garden, large nest	6	V	V	V		
JNJ061204-01	Ac.versicolor	CA WileyWell3	33.49	114.88	field garden, large nest	6	V	V	V		
JNJ061205-01	Ac.versicolor	CA AlgodonesDunes1	33.01	115.09	field garden, large nest	6	V	V	V		
UGM061205-02R	Ac.versicolor	CA AlgodonesDunes2	33.01	115.09	field garden, large nest	7	admixed	V	V		
UGM070330-01	Ac.versicolor	AZ Gladden	33.90	113.30	field garden, large nest	8	admixed	V	V		
RDAZ06R	Ac.versicolor	AZ SahuaritaPark	31.95	110.91	lab garden, started by mated females	9	admixed	admixed	admixed		
UGM061206-02	Ac.versicolor	AZ Tucson TumamocHill3	32.22	111.00	field garden, large nest	10	admixed	admixed	admixed		
UGM070328-01	Ac.versicolor	AZ Tucson TanqueVerde1	32.28	110.75	field garden, large nest	11	V	V	V	0.78 km (0.78 km)	
UGM070328-02	Ac.versicolor	AZ Tucson TanqueVerde2	32.29	110.75	field garden, large nest	11	V	V	V		
UGM051206-01R	Ac.versicolor	AZ Tucson TumamocHill1	32.22	111.00	field garden, 1-year-old nest	12	V	V	V		
RMC361Type2a	Ac.versicolor	AZ SouthTucson361	32.20	110.95	lab garden, started by mated females	13	V	V	V	caught at same mating swarm	
RMC101Type2a	Ac.versicolor	AZ SouthTucson101	32.20	110.95	lab garden, started by mated females	13	V	V	V		
RMC99Type2b	Ac.versicolor	AZ SouthTucson99	32.20	110.95	lab garden, started by mated females	14	V	V	V		
RMC281Type2c	Ac.versicolor	AZ SouthTucson281	32.20	110.95	lab garden, started by mated females	15	admixed	V	V		
UGM070327-06R	Ac.versicolor	AZ Safford1	32.71	109.72	field garden, large nest	16	admixed	V	V		
UGM070330-02	Ac.versicolor	AZ Wickenburg1	33.96	112.82	field garden, large nest	17	V	V	V	8.0 km (11.9 km)	0.56 km²
UGM070330-03	Ac.versicolor	AZ Wickenburg2	33.96	112.82	field garden, large nest	17	V	V	V		
UGM070330-04R	Ac.versicolor	AZ WickenburgNorth	34.07	112.82	field garden, large nest	17	V	V	V		
UGM070327-02	Ac.versicolor	AZ Cutter	33.34	110.65	field garden, large nest	18	V	V	V		
UGM070327-03R	Ac.versicolor	AZ Globe1	33.37	110.73	field garden, large nest	19	V	V	V	0.05 km (0.05 km)	
UGM070327-04	Ac.versicolor	AZ Globe2	33.37	110.73	field garden, large nest	19	V	V	V		
UGM070325-01R	Ac.versicolor	AZ Yucca	34.90	114.14	field garden, large nest	20	V	V	V		
UGM070331-02R	Ac.versicolor	AZ PhonDSutton	33.53	111.66	field garden, very large nest	21	V	V	V		
UGM041228-01R	Ac.versicolor	AZ Why BLMCampground	32.23	112.76	field garden, very large nest	22	M	M	M		
RMC273Type1a	Ac.versicolor	AZ SouthTucson273	32.20	110.95	lab garden, started by mated females	23	M	M	M	caught at same mating swarm	
RMC358Type1a	Ac.versicolor	AZ SouthTucson358	32.20	110.95	lab garden, started by mated females	23	M	M	M		
RMC362Type1b	Ac.versicolor	AZ SouthTucson362	32.20	110.95	lab garden, started by mated females	24	M	M	M		
RMC246Type1c	Ac.versicolor	AZ SouthTucson246	32.20	110.95	lab garden, started by mated females	25	M	M	M		
UGM061206-01R	Ac.versicolor	AZ Tucson TumamocHill2	32.22	111.00	field garden, 1-year-old nest	26	M	M	M		
							26.9% of genotypes admixed	**11.5% of genotypes admixed**	**11.5% of genotypes admixed**		

UGM060318-08R	Atta texana	TX DelRio1	29.36	100.88	field garden, large nest	27	M	M	M		
UGM061122-05	Atta texana	TX Lamar1	28.15	96.98	field garden, large nest	27	M	M	M		
UGM061122-06	Atta texana	TX Lamar2	28.14	96.98	field garden, large nest	27	M	M	M		
UGM070224-06	Atta texana	TX Goliad2	28.66	97.39	field garden, large nest	27	M	M	M		
UGM070224-08	Atta texana	TX Goliad3	28.65	97.35	field garden, large nest	27	M	M	M		
UGM070225-05	Atta texana	TX Alice	27.78	98.14	field garden, large nest	27	M	M	M		
UGM061124-09R	Atta texana	TX Linn2	26.7	98.10	field garden, large nest	27	M	M	M	227.6 km (490.1 km)	80948 km^2
UGM060517-01R	Atta texana	TX Attwater	29.66	96.24	field garden, large nest	27	M	M	M		
UGM070310-03R	Atta texana	TX CarrizoSprings1	28.62	100.00	field garden, large nest	27	M	M	M		
UGM070315-04	Atta texana	TX ColetoCreek2	28.73	97.13	field garden, large nest	27	M	M	M		
UGM070309-02	Atta texana	TX Luling	29.64	97.58	field garden, large nest	27	M	M	M		
UGM070422-01	Atta texana	TX RoansPrairie1	30.59	96.00	field garden, large nest	27	M	M	M		
UGM070422-02	Atta texana	TX RoansPrairie2	30.59	95.99	field garden, large nest	27	M	M	M		
UGM070310-04R	Atta texana	TX CarrizoSprings2	28.61	100.00	field garden, large nest	28	M	M	M	0.08 km	
UGM070310-06	Atta texana	TX CarrizoSprings4	28.61	100.00	field garden, large nest	28	M	M	M	(0.08 km)	
UGM060518-07	Atta texana	TX EagleLake7	29.64	96.42	field garden, large nest	29	M	M	M		
UGM060518-09R	Atta texana	TX Columbus	29.70	96.53	field garden, large nest	29	M	M	M	86.1 km (123.1 km)	782 km^2
UGM070315-03	Atta texana	TX ColetoCreek1	28.73	97.13	field garden, large nest	29	M	M	M		
UGM070412-03	Atta texana	TX GarnerStatePark2	29.58	99.73	field garden, large nest	30	M	M	M		
UGM070411-02	Atta texana	TX Bandera2	29.73	99.11	field garden, large nest	30	M	M	M	74.4 km (108.6 km)	2563 km^2
UGM070412-05R	Atta texana	TX Leakey	29.74	99.75	field garden, large nest	30	M	M	M		
UGM070127-02	Atta texana	TX Lytle2	29.19	98.82	field garden, large nest	30	M	M	M		
UGM070127-01R	Atta texana	TX Lytle1	29.19	98.82	field garden, large nest	31	M	M	M		
UGM070311-12	Atta texana	TX Uvalde2	29.20	99.77	field garden, large nest	32	M	M	M		
UGM070412-02R	Atta texana	TX GarnerStatePark1	29.58	99.73	field garden, large nest	32	M	M	M		
UGM070422-03	Atta texana	TX Bastrop2	30.15	97.23	field garden, large nest	32	M	M	M		
UGM051218-02R	Atta texana	TX Bastrop1	30.09	97.22	field garden, large nest	32	M	M	M	132.1 km (267.4 km)	10217 km^2
UGM060121-01R	Atta texana	TX Austin HornsbyBend1	30.23	97.65	field garden, large nest	32	M	M	M		
UGM061029-01	Atta texana	TX Austin HornsbyBendJN	30.23	97.65	field garden, large nest	32	M	M	M		
Travis-5	Atta texana	TX HudsonBend5	30.39	97.93	pellet from a mated female	32	M	M	M		
UGM070420-01	Atta texana	TX Austin WalnutCreek	30.39	97.67	field garden, large nest	33	M	M	M	15.6 km	
UGM050509-02R	Atta texana	TX Austin BFL2	30.28	97.77	pellets from dispersing females	33	M	M	M	(15.6 km)	
UGM061122-04R	Atta texana	TX Rockport	28.01	97.09	field garden, large nest	34	M	M	M		
UGM061121-01R	Atta texana	TX PortAransas	27.80	97.09	field garden, large nest	35	admixed	admixed	admixed		
UGM061124-06	Atta texana	TX Linn1	26.65	98.11	field garden, large nest	36	M	M	M		
UGM061124-10R	Atta texana	TX Linn3	26.74	98.10	field garden, large nest	36	M	M	M		
UGM070310-05	Atta texana	TX CarrizoSprings3	28.61	100.00	field garden, large nest	36	M	M	M		
UGM061125-06	Atta texana	TX SanYgnacio1	27.16	99.42	field garden, large nest	36	M	M	M		
UGM061125-07	Atta texana	TX SanYgnacio2	27.16	99.42	field garden, large nest	36	M	M	M		
UGM060205-04	Atta texana	TX Salineno1	26.51	99.11	field garden, large nest	36	M	M	M	187.0 km (435.5 km)	57192 km^2
UGM061125-02	Atta texana	TX Salineno2	26.51	99.11	field garden, large nest	36	M	M	M		
UGM061125-04R	Atta texana	TX Salineno3	26.51	99.11	field garden, large nest	36	M	M	M		
UGM061125-05	Atta texana	TX Salineno4	26.51	99.11	field garden, large nest	36	M	M	M		
UGM070315-05	Atta texana	TX Victoria	28.73	97.02	field garden, large nest	36	M	M	M		
UGM060524-02R	Atta texana	TX Austin HornsbyBend11	30.21	97.64	field garden, large nest	36	M	M	M		
UGM070315-01	Atta texana	TX Gonzalez	29.56	97.50	field garden, large nest	37	M	M	M		
UGM070421-03	Atta texana	TX Kosse	31.32	96.56	field garden, large nest	38	M	M	M		
UGM070421-05	Atta texana	TX Flynn1	31.23	96.13	field garden, large nest	38	M	M	M		
UGM070421-06	Atta texana	TX Flynn2	31.22	96.13	field garden, large nest	38	M	M	M		
UGM070421-07	Atta texana	TX Flynn3	31.22	96.12	field garden, large nest	38	M	M	M		
UGM070609-02	Atta texana	TX Donie SpringSeat2	31.46	96.14	field garden, incipient nest	38	M	M	M		
UGM070505-02	Atta texana	TX Buffalo	31.50	95.98	field garden, large nest	38	M	M	M		
UGM070506-07	Atta texana	TX CampTonkawa4	31.82	94.62	field garden, large nest	38	M	M	M		
UGM070506-09	Atta texana	TX Montalba	31.92	95.73	field garden, large nest	38	M	M	M	105.2 km (207.3 km)	16105 km^2
UGM070506-10	Atta texana	TX BoisDArc	31.93	95.74	field garden, large nest	38	M	M	M		
UGM070509-06	Atta texana	TX Eustace2	32.36	95.91	field garden, large nest	38	M	M	M		
UGM070510-01	Atta texana	TX Rhonesboro3	32.73	95.19	field garden, large nest	38	M	M	M		
UGM070510-02	Atta texana	TX Rhonesboro4	32.74	95.18	field garden, large nest	38	M	M	M		
UGM070510-04	Atta texana	TX Quitman3	32.77	95.39	field garden, large nest	38	M	M	M		
UGM070510-05	Atta texana	TX Quitman4	32.76	95.38	field garden, large nest	38	M	M	M		
UGM060401-05R	Atta texana	TX Rhonesboro1	32.75	95.14	field garden, large nest	38	M	M	M		
UGM061222-01	Atta texana	TX Quitman1	32.79	95.32	field garden, large nest	38	M	M	M		
UGM060514-03	Atta texana	TX FortBelknap3	33.14	98.75	field garden, incipient nest	39	admixed	admixed	admixed		
UGM060514-07	Atta texana	TX FortBelknap7	33.15	98.75	field garden, incipient nest	39	admixed	admixed	admixed		
UGM060514-09	Atta texana	TX FortBelknap9	33.15	98.75	field garden, incipient nest	39	admixed	admixed	admixed	45.4 km (135.7 km)	11 km^2
UGM060514-10	Atta texana	TX FortBelknap10	33.15	98.75	field garden, incipient nest	39	admixed	admixed	admixed		
UGM070518-02	Atta texana	TX CoxBend2	32.30	97.70	field garden, large nest	39	admixed	admixed	admixed		
UGM070519-02	Atta texana	TX FortBelknap2	33.15	98.75	field garden, large nest	39	admixed	admixed	admixed		
UGM060319-01R	Atta texana	TX DelRio2	29.34	100.94	field garden, large nest	40	T	T	T		
UGM070311-02	Atta texana	TX SycamoreCreek1	29.25	100.75	field garden, large nest	41	T	T	T	23.2 km	152 km^2
UGM060319-05R	Atta texana	TX Brackettville1	29.33	100.53	field garden, large nest	41	T	T	T	(48.6 km)	
UGM070415-01	Atta texana	TX Brackettville3	29.28	100.42	field garden, large nest	41	T	T	T		

UGM060319-06R	Atta texana	TX Brackettville2	29.28	100.32	field garden, large nest	41	T	T	T		
UGM070311-09R	Atta texana	TX Quemado1	28.97	100.63	field garden, large nest	42	T	T	T		
UGM070311-01R	Atta texana	TX DelRio3	29.36	100.88	field garden, large nest	43	T	T	T		
UGM070310-07	Atta texana	TX EaglePass	28.61	100.43	field garden, large nest	44	T	T	T	53.0 km (77.8 km)	419 km^2
UGM070311-03	Atta texana	TX SycamoreCreek2	29.26	100.75	field garden, large nest	44	T	T	T		
UGM070311-10	Atta texana	TX Quemado2	29.09	100.55	field garden, large nest	44	T	T	T		
UGM070411-03R	Atta texana	TX Vanderpool	29.73	99.55	field garden, large nest	45	T	T	T		
UGM070411-01R	Atta texana	TX Bandera1	29.73	99.11	field garden, large nest	46	T	T	T		
UGM070225-02	Atta texana	TX LakeCorpusStatePark1	28.06	97.87	field garden, large nest	47	T	T	T	0.69 km (0.69 km)	
UGM070225-03R	Atta texana	TX LakeCorpusStatePark2	28.06	97.87	field garden, large nest	47	T	T	T		
UGM060512-03R	Atta texana	TX Kingsland	30.65	98.43	field garden, large nest	48	T	T	T	138.7 km (245.9 km)	6807 km^2
UGM050509-01R	Atta texana	TX Austin BFL1	30.28	97.78	pellets from dispersing females	48	T	T	T		
UGM070224-02	Atta texana	TX Goliad1	28.66	97.38	field garden, 1-year-old nest	48	T	T	T		
UGM070224-09	Atta texana	TX Goliad4	28.64	97.35	field garden, large nest	48	T	T	T		
UGM070315-02R	Atta texana	TX Cuero	29.26	97.29	field garden, large nest	48	T	T	T		
UGM060521-05R	Atta texana	TX PerdenalesStatePark1	30.32	98.23	field garden, large nest	49	T	T	T	28.2 km (58.0 km)	100 km^2
UGM060521-07	Atta texana	TX PerdenalesStatePark2	30.31	98.23	field garden, large nest	49	T	T	T		
UGM060523-01	Atta texana	TX Austin KempRoad	30.24	97.69	field garden, large nest	49	T	T	T		
AR060123-01	Atta texana	TX Austin HornsbyBend6	30.23	97.65	field garden, large nest	49	T	T	T		
UGM060524-01	Atta texana	TX Austin HornsbyBend10	30.21	97.64	field garden, large nest	49	T	T	T		
UGM060524-03	Atta texana	TX Austin HornsbyBend12	30.21	97.64	field garden, large nest	49	T	T	T		
UGM070504-02	Atta texana	TX Austin HornsbyBend1Y	30.22	97.65	field garden, 1-year-old nest	49	T	T	T		
UGM070426-03R	Atta texana	TX Bend1	31.13	98.52	field garden, large nest	50	admixed	T	T		
Travis-1	Atta texana	TX HudsonBend1	30.39	97.93	pellet from a mated female	51	T	T	T	122.9 km (242.5 km)	1538 km^2
Travis-2	Atta texana	TX HudsonBend2	30.39	97.93	pellet from a mated female	51	T	T	T		
Travis-4	Atta texana	TX HudsonBend4	30.39	97.93	pellet from a mated female	51	T	T	T		
UGM070518-01	Atta texana	TX Rainbow	32.26	97.69	field garden, incipient nest	51	T	T	T		
UGM070518-04	Atta texana	TX CoxBend	32.30	97.70	field garden, large nest	51	T	T	T		
UGM060304-04	Atta texana	TX Wheatherford	32.57	97.81	field garden, large nest	51	T	T	T		
Travis-3	Atta texana	TX HudsonBend3	30.39	97.93	pellet from a mated female	52	T	T	T		
UGM061103-02R	Atta texana	TX Austin HornsbyBendNorth	30.23	97.63	field garden, large nest	53	T	T	T		
UGM060511-01R	Atta texana	TX Austin HornsbyBend8	30.22	97.65	field garden, large nest	54	T	T	T	0.26 km (0.26 km)	
UGM060511-02	Atta texana	TX Austin HornsbyBend9	30.22	97.65	field garden, large nest	54	T	T	T		
UGM070420-02	Atta texana	TX Austin HergotzRoad	30.24	97.68	field garden, large nest	55	T	T	T		
UGM070609-01	Atta texana	TX Donie SpringSeat1	31.46	96.14	field garden, incipient nest	56	T	T	T	118.5 km (357.3 km)	22188 km^2
UGM070609-03	Atta texana	TX Donie SpringSeat3	31.46	96.14	field garden, incipient nest	56	T	T	T		
UGM070609-04	Atta texana	TX Donie SpringSeat4	31.46	96.14	field garden, incipient nest	56	T	T	T		
UGM070609-05	Atta texana	TX Donie SpringSeat5	31.46	96.14	field garden, incipient nest	56	T	T	T		
UGM070609-06	Atta texana	TX Donie SpringSeat6	31.46	96.14	field garden, incipient nest	56	T	T	T		
UGM070609-07	Atta texana	TX Donie SpringSeat7	31.46	96.14	field garden, incipient nest	56	T	T	T		
UGM070609-09	Atta texana	TX Donie SpringSeat9	31.46	96.14	field garden, incipient nest	56	T	T	T		
UGM060518-11R	Atta texana	TX Columbus	29.71	96.54	field garden, large nest	56	T	T	T		
UGM070409-01	Atta texana	TX LakeHouston1	30.09	95.15	field garden, large nest	56	T	T	T		
UGM070316-05	Atta texana	TX Montgomery1	30.32	95.58	field garden, large nest	56	T	T	T		
UGM070316-06	Atta texana	TX Montgomery2	30.32	95.58	field garden, large nest	56	T	T	T		
UGM070421-08	Atta texana	TX Flynn4	31.21	96.12	field garden, large nest	56	T	T	T		
UGM070421-09	Atta texana	TX Centerville	31.26	96.06	field garden, large nest	56	T	T	T		
UGM070506-03	Atta texana	TX CampTonkawa1	31.83	94.60	field garden, large nest	56	T	T	T		
UGM070506-05	Atta texana	TX CampTonkawa3	31.83	94.60	field garden, large nest	56	T	T	T		
UGM070506-08	Atta texana	TX Slocum	31.63	95.33	field garden, large nest	56	T	T	T		
UGM070509-05	Atta texana	TX Eustace1	32.36	95.91	field garden, large nest	56	T	T	T		
UGM070510-03	Atta texana	TX Quitman2	32.77	95.39	field garden, large nest	56	T	T	T		
UGM070609-08	Atta texana	TX Donie SpringSeat8	31.46	96.14	field garden, incipient nest	57	T	T	T	135.7 km (203.6 km)	3 km^2
UGM070505-01R	Atta texana	TX Donie SpringSeat	31.46	96.14	field garden, large nest	57	T	T	T		
UGM060518-04	Atta texana	TX EagleLake4	29.64	96.42	field garden, large nest	57	T	T	T		
UGM070409-02	Atta texana	TX LakeHouston2	30.08	95.15	field garden, large nest	58	T	T	T	79.7 km (146.7 km)	1856 km^2
UGM070408-02	Atta texana	TX LakeSomerville	30.29	96.65	field garden, large nest	58	T	T	T		
UGM070409-04	Atta texana	TX Fostoria1	30.32	95.21	field garden, large nest	58	T	T	T		
UGM070409-05	Atta texana	TX Fostoria2	30.32	95.22	field garden, large nest	58	T	T	T		
UGM070316-01R	Atta texana	TX Sugarland	29.63	95.66	field garden, large nest	59	T	T	T		
UGM070506-04R	Atta texana	TX CampTonkawa2	31.83	94.60	field garden, large nest	60	T	T	T		
UGM060401-06R	Atta texana	TX Rhonesboro2	32.75	95.14	field garden, large nest	61	T	T	T		
UGM070506-12R	Atta texana	TX FoxMeadow	31.96	95.79	field garden, large nest	62	T	T	T		
UGM060513-03	Atta texana	TX MineralWells1	32.73	98.17	field garden, large nest	63	T	T	T	10.2 km (14.0 km)	22 km^2
UGM061217-02	Atta texana	TX MineralWells2	32.76	98.15	field garden, large nest	63	T	T	T		
UGM061216-02R	Atta texana	TX SodaSprings	32.68	98.04	field garden, large nest	63	T	T	T		
UGM070519-03	Atta texana	TX Graham1	33.08	98.73	field garden, small nest	64	T	T	T	5.5 km (8.1 km)	9 km^2
UGM070519-04	Atta texana	TX Graham2	33.08	98.73	field garden, small nest	64	T	T	T		
UGM060514-02	Atta texana	TX FortBelknap2	33.14	98.72	field garden, large nest	64	T	T	T		
UGM060514-08	Atta texana	TX FortBelknap8	33.15	98.75	field garden, incipient nest	64	T	T	T		
UGM070317-03R	Atta texana	TX Kountze3	30.39	94.25	field garden, large nest	65	admixed	T	T	35.7 km (35.7 km)	
UGM070317-06	Atta texana	TX Vidor3	30.14	94.02	field garden, large nest	65	admixed	T	T		

UGM070409-06R	Atta texana	TX Rye1	30.48	94.77	field garden, large nest	66	admixed	T	T		
UGM070409-07R	Atta texana	TX Rye2	30.48	94.78	field garden, large nest	67	T	T	T		
UGM070317-01R	Atta texana	TX Kountze1	30.39	94.26	field garden, large nest	68	T	T	T		
ASM040418-09	Atta texana	TX SandyCreek Angelina1	31.08	94.20	field garden, 1-year-old nest	68	T	T	T	41.5 km	630 km^2
ASM040418-10	Atta texana	TX Colmesneil Angelina2 *	30.94	94.38	field garden, 1-year-old nest	68	T	T	T	(77.2 km)	
ASM040418-12	Atta texana	TX Colmesneil Angelina3 *	30.94	94.38	field garden, 1-year-old nest	68	T	T	T		
ASM040418-11R	Atta texana	TX Colmesneil Angelina4 *	30.94	94.38	field garden, 1-year-old nest	69	admixed	admixed	T		
UGM070317-02R	Atta texana	TX Kountze2	30.39	94.25	field garden, large nest	69	admixed	admixed	T	54.1 km	540 km^2
UGM070317-04	Atta texana	TX Vidor1	30.14	94.01	field garden, large nest	69	admixed	admixed	T	(95.1 km)	
UGM070317-05	Atta texana	TX Vidor2	30.14	94.02	field garden, large nest	69	admixed	admixed	T		
UGM060404-03	Atta texana	TX Silsbee	30.34	94.23	field garden, large nest	70	admixed	T	T	124.7 km	
UGM060403-06	Atta texana	LA FortPolk1	30.99	93.17	field garden, large nest	70	admixed	T	T	(124.7 km)	
UGM060403-10R	Atta texana	LA FortPolk2	30.99	93.17	field garden, 1-year-old nest	71	admixed	T	T	55.2 km	
JM2005-08	Atta texana	LA Gardner	31.26	92.69	lab garden, large nest	71	admixed	T	T	(55.2 km)	
UGM061223-01R	Atta texana	LA Bienville1	32.31	93.07	field garden, large nest	72	admixed	admixed	T		
UGM061223-03	Atta texana	LA Bienville2	32.34	93.05	field garden, large nest	72	admixed	admixed	T	7.2 km (9.2 km)	18 km^2
UGM060402-04	Atta texana	LA Jamestown	32.33	93.15	field garden, large nest	72	admixed	admixed	T		
UGM061111-01R	Atta texana	TX Uvalde1	29.20	99.77	field garden, large nest	73	admixed	admixed	admixed		
UGM060121-02R	Atta texana	TX Austin HornsbyBendFord1	30.23	97.65	field garden, large nest	74	admixed	admixed	C	0.21 km	
UGM070303-01	Atta texana	TX Austin HornsbyBendFord2	30.23	97.65	field garden, large nest	74	admixed	admixed	C	(0.21 km)	
UGM070426-04R	Atta texana	TX Bend2	31.12	98.52	field garden, large nest	75	admixed	C	C		
							24.5% of genotypes admixed	**12.29% of genotypes admixed**	**6.1% of genotypes admixed**		
SES030114-01	Atta mexicana	MX Chiapas Palenque2	17.49	92.02	field garden, large nest	76	C	C	C		
SES030112-05	Atta cephalotes	MX Veracruz SierraTuxtlas5	18.48	95.06	field garden, large nest	77	admixed	C	C		
SES030117-02	Atta cephalotes	MX Oaxaca Temascal2	18.23	96.41	field garden, large nest	78	admixed	admixed	C		
Amex1	Atta mexicana	MX Oaxaca Oaxaca1 *	17.05	96.71	lab garden, started by a mated female	79	M	M	M	0.1 km	
Amex3	Atta mexicana	MX Oaxaca Oaxaca3 *	17.05	96.71	lab garden, started by a mated female	79	M	M	M	(0.1 km)	
Amex2R	Atta mexicana	MX Oaxaca Oaxaca2 *	17.05	96.71	lab garden, started by a mated female	80	M	M	M		
AttaMex04R	Atta mexicana	MX NuevoLeon Monterrey	25.68	100.30	field garden, large nest	81	admixed	admixed	admixed		
SES030112-03	Atta cephalotes	MX Veracruz SierraTuxtlas3	18.48	95.06	field garden, large nest	82	admixed	admixed	admixed		
SES030117-03	Atta cephalotes	MX Oaxaca Temascal3	18.23	96.41	field garden, large nest	83	admixed	M	M		
SES030113-01	Atta mexicana	MX Chiapas Palenque1	17.51	91.98	field garden, large nest	84	admixed	M	M		
SES030117-01	Atta cephalotes	MX Oaxaca Temascal1	18.23	96.41	field garden, large nest	85	admixed	M	M		
SES030112-01	Atta cephalotes	MX Veracruz SierraTuxtlas1	18.48	95.06	field garden, large nest	86	admixed	admixed	admixed		
SES030112-04R	Atta cephalotes	MX Veracruz SierraTuxtlas4	18.48	95.06	field garden, large nest	87	admixed	admixed	admixed		
SES030114-03R	Atta mexicana	MX Chiapas Palenque3	17.49	92.02	field garden, large nest	88	admixed	admixed	M		
SES030112-02	Atta cephalotes	MX Veracruz SierraTuxtlas2	18.48	95.06	field garden, large nest	89	admixed	C	C		
ASM050315-05	Atta insularis	CUBA Batabano1 *	22.73	82.30	field garden, large nest	90	C	C	C		
ASM050316-01	Atta insularis	CUBA Batabano2 *	22.73	82.30	field garden, large nest	91	C	C	C		
ASM050316-02	Atta insularis	CUBA Batabano3 *	22.73	82.30	field garden, large nest	92	C	C	C	<0.5 km*	
ASM050316-03	Atta insularis	CUBA Batabano4 *	22.73	82.30	field garden, large nest	92	C	C	C		
ASM050318-07	Atta insularis	CUBA Cienfuegos	22.12	80.41	field garden, large nest	93	admixed	C	C		
							66.7% of genotypes admixed	**33.3% of genotypes admixed**	**22.2% of genotypes admixed**		

* GPS coordinates were not recorded for individual nests at these particular locations; distances between nest sites are estimated from field notes.