Population genetic signatures of diffuse co-evolution between leaf-cutting ants and their cultivar fungi

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Abstract

Switching of symbiotic partners pervades most mutualisms, despite mechanisms that appear to enforce partner fidelity. To investigate the interplay of forces binding and dissolving mutualistic pairings, we investigated partner fidelity at the population level in the attine ant–fungal cultivar mutualism. The ants and their cultivars exhibit both broad-scale co-evolution, as well as cultivar switching, with short-term symbiont fidelity maintained by vertical transmission of maternal garden inoculates via dispersing queens and by the elimination of alien cultivar strains. Using microsatellite markers, we genotyped cultivar fungi associated with five co-occurring Panamanian attine ant species, representing the two most derived genera, leaf-cutters *Atta* and *Acromyrmex*. Despite the presence of mechanisms apparently ensuring the cotransmission of symbiont genotypes, different species and genera of ants sometimes shared identical fungus garden genotypes, indicating widespread cultivar exchange. The cultivar population was largely unstructured with respect to host ant species, with only 10% of the structure in genetic variance being attributable to partitioning among ant species and genera. Furthermore, despite significant genetic and ecological dissimilarity between *Atta* and *Acromyrmex*, generic difference accounted for little, if any, variance in cultivar population structure, suggesting that cultivar exchange dwarfs selective forces that may act to create co-adaptive ant–cultivar combinations. Thus, binding forces that appear to enforce host fidelity are relatively weak and pairwise associations between cultivar lineages and ant species have little opportunity for evolutionary persistence. This implicates that mechanisms other than partner fidelity feedback play important roles in stabilizing the leafcutter ant–fungus mutualism over evolutionary time.

Keywords: attini, leaf-cutting ants, microsatellites, mutualism, switching, symbiosis

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Introduction

Different species engaged in a mutualistic interaction jointly occupy an ecological niche that allows for higher fitness than could be obtained by either symbiont acting alone. The persistence of an obligate mutualism through evolutionary time results from a positive balance between forces that hold the interaction together and other forces that promote selfish behaviours by the symbionts (Frank 1994, 1996). Traditionally, the interplay between selfish

and cooperative forces has been viewed from a perspective of repeated interactions between a pair of mutualistic species (Hoeksema & Bruna 2000; Stanton 2003). However, recent data reveal that virtually all mutualisms involve more than two interacting species with at least occasional, and sometimes ubiquitous, horizontal transfer of symbionts, even when mechanisms ensure general vertical symbiont transfer (Herre *et al*. 1999). In a typical example, fig wasps were previously believed to form strict species pairs with their host figs, vertical cotransmission of symbiont genotypes being ensured by the capture and death of the female wasp upon entering the fig in order to lay eggs. However, recent work revealed the existence of cryptic wasp species that may even produce mixed broods

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consisting of several wasp species within a single fig (Molbo *et al.* 2003; Machado *et al.* 2005). Similarly, although the most widespread yucca moth was initially thought to be a single species, it is now recognized as a species complex with relatively narrow host niches (Pellmyr 1999). Furthermore, the evolution of 'cheater' moths, which over-exploit the yucca seed resources, seems to be linked with host shifts (Pellmyr *et al.* 1996). However, patterns of species sharing, as observed through phylogenetic relationships, could be likewise due to continuous switching between several co-evolving partners, or to periods of long-term pairwise symbiont association, followed by an occasional symbiont switch. Thus, the rate at which symbiont switching happens, and thus, to a large extent, the nature of selection acting to maintain symbioses remains unclear. Only a detailed analysis of the genetic associations within a population of several interacting mutualist species can reveal the relative strength of forces acting to bind vs. dissolve pairings of symbiotic partners.

The symbiosis between fungus-gardening (attine) ants and their fungal cultivars is a well-studied obligate mutualism, dating back an estimated 60 million years (Mueller *et al.* 2001). Since the origin of this agricultural system, broad–scale co-evolutionary interactions have involved both vertical symbiont transmission and horizontal sharing of lineages (Chapela *et al.* 1994; Hinkle *et al.* 1994; Mueller *et al.* 1998). Association of ant and fungal lineages through time appears to be insured both by the ant queens, which take bits of maternal fungus gardens on mating flights and then use them to start their own gardens (Weber 1972; Mueller 2002), and by the cultivar, which exhibits an aggression response to foreign strains, maintaining a garden monoculture (Bot *et al.* 2001; Poulsen & Boomsma 2005). At the same time, population-level data indicate sharing of cultivar lineages (Bot *et al.* 2001; Green *et al.* 2002), either through garden theft (Adams *et al.* 2000), through cultivar sexual reproduction and recombination (Mikheyev *et al.* 2006), or in the case of the more primitive attine ants, through a free-living fungal intermediate (Mueller *et al.* 1998; Green *et al.* 2002; Vo *et al.* in preparation). Although earlier studies demonstrated the existence of at least occasional exchange of cultivar strains between different host ant species, we know little about the frequency of such exchanges or whether cultivar switching dominates specialization at the population level.

The most derived members of this agricultural symbiosis, the leaf-cutting ants in the sister genera *Atta* and *Acromyrmex*, along with other higher attines in the genera *Trachymyrmex* and *Sericomyrmex* (Schultz & Meier 1995; Wetterer *et al.* 1998) cultivate specialized co-adapted fungi that apparently lack free-living relatives (Mueller 2002; Vo *et al.* in preparation). Although the two leaf-cutter genera consume similar types of vegetation (Weber 1972), they differ substantially in foraging ecology and nesting behaviour

(Hölldobler & Wilson 1990; Wetterer 1995). Mature *Atta* colonies have millions of workers and are housed in enormous subterranean nests that live for decades and occupy a defended territory, while destroying smaller nests of other leaf-cutting ants (Borgmeier 1922; Hölldobler & Wilson 1990; Fowler 1992). By contrast, *Acromyrmex* constructs much smaller, sometimes arboreal, nests that have colony sizes ranging from thousands to hundreds of thousands of workers (Pereira-da-Silva *et al.* 1981; Wetterer 1993, 1995). Fowler (1983) noted that there is a negative correlation in the local abundance of the two genera, suggesting the existence of competition between them. Yet, cultivar sharing has been documented between congeneric species and, likely, even between ant genera (Bot *et al.* 2001; Silva-Pinhati *et al.* 2004; Mikheyev *et al.* 2006). Recent evidence further suggests that most, if not all, of the fungi cultivated by leaf-cutting ants belong to a single biological species (Stradling & Powell 1986; Silva-Pinhati *et al.* 2004; Mikheyev *et al.* 2006) (Fig. 1).

Given the transmission of parental gardens by virgin queens, it seems plausible that some level of association between the ant and fungal lineages exists; certainly co-evolution at the deeper phylogenetic levels occurs (Chapela *et al.* 1994; Hinkle *et al.* 1994). Vertical transmission has been proposed as a dominant mechanism for the ants to control the movement and mixing of their cultivars (Bot *et al.* 2001), as well as an important force in aligning the reproductive interests of the ants and the cultivar (Mueller 2002). Alternatively, vertical transmission may enable a
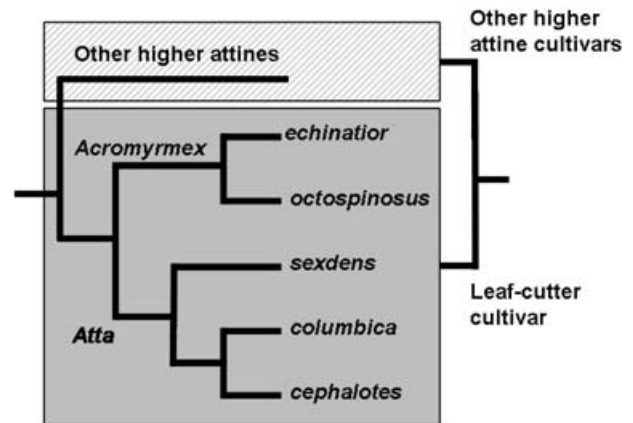

Fig. 1 Phylogenetic relationship between leaf-cutting ants in this study and their cultivars (Borgmeier 1959; Chapela *et al.* 1994; Hinkle *et al.* 1994; Schultz & Meier 1995). Two genera of leaf-cutting ants diffusely co-evolve with a widespread cultivar fungus (dark grey box). Most leaf-cutting ants cultivate one cultivar species, distinct from others associated with other higher attine genera *Trachymyrmex* and *Sericomyrmex* (slashed light grey box). However, the association may be even more diffuse than presented here, since leaf-cutters can cultivate *Trachymyrmex* fungus and vice versa in the laboratory (Stradling & Powell 1986; Sánchez-Peña 2005; J. Seal, unpublished).

solitary queen to start a colony without wasting resources on finding a new garden during the critical colony founding stage. Later, the colony may well substitute its cultivar, either through the loss of the original garden or through acquisition of strains better suited to local environmental conditions (e.g. better at processing locally available food sources). At least in the laboratory, *Atta* queens who lose their pellet or incipient garden can raise a brood of workers and potentially acquire a new garden (Fernández-Marín & Wcislo 2005). Finally, sporulation and sexual reproduction by the cultivar may further facilitate genetic exchange between species (Mikheyev *et al*. 2006). Consequently, the ant–cultivar symbiosis could operate under two opposing co-evolutionary models — characterized either by long-term pairwise interaction between an ant species and its cultivar (as traditionally assumed), or by frequent garden exchanges between species.

Prolonged pairwise co-evolution predicts the existence of an association that substantially partitions cultivar genetic variance among the different ant species. By contrast, frequent exchanges should result in relatively unstructured cultivar populations. The unique relationship between the single cultivar species and multiple sympatric species of ants allows for an explicit test of these predictions using powerful population genetic tools. To this end, we used microsatellite markers to survey the genetic structure of cultivars associated with all five species from both leaf-cutting genera (*Atta* and *Acromyrmex*) endemic to the Panama Canal Zone. Furthermore, taking advantage of the nested relationships between ant taxa (multiple ant species in two monophyletic genera), we evaluated the extent to which the genetic structure of the cultivar species tracks that of the ants.

Materials and methods

Samples and collecting

All materials used in the study were collected in the Panama Canal Zone, home to three *Atta* species (*cephalotes*, *columbica* and *sexdens*) and two closely related species of *Acromyrmex* (*echinatior* and *octospinosus*) (Sumner *et al*. 2004). The distributions of all five species overlap in the Panama Canal Zone. Between 2000 and 2004, queenright *Ac. echinatior* and *Ac. octospinosus* colonies were collected around Gamboa (except one *Ac. octospinosus* colony that was collected on Ancon Hill) and were subsequently kept with their original fungus gardens in the laboratory at the University of Copenhagen. Thus, resampling of the same *Acromyrmex* colony in the field was impossible. Having lower population densities, *Atta* were sampled over a slightly larger range, from Pipeline road to Gamboa. However, all of the samples spanned an area with a diameter of only ~25 km, with the vast majority having been collected

within 5 km of Gamboa. Except for 10 live *At. columbica* colonies, which were also collected whole and kept at the University of Copenhagen, most *Atta* samples were collected in 2000 and 2004 and stored in 95% ethanol. The final analysis involved 62 total gardens — 16 from each of the two *Acromyrmex* species and 30 *Atta* gardens (10 *cephalotes*, 15 *columbica* and 5 *sexdens*).

Molecular methods

DNA was extracted by incubating a single cluster of fungal gongylidia (structures used to feed ant larvae) in 150 μL of 5% aqueous Chelex resin for 1.5 h at 60 °C and then at 99 °C for 10 min. Cultivars were genotyped using M13-tailed primers at nine microsatellite loci: A0659B, A0815, B0150, B0312, B0358, B0430, B0447, C0625, D0115 (Scott *et al*. in preparation). One microlitre of the extract was used as microlitER template and the reaction was carried out in a total volume of 10 μL. The polymerase chain reaction (PCR) contained $1 \times$ reaction buffer, 1 mM dNTPs, 0.01 μM M13-tailed primers, 0.15 μM other primer, 0.18 μM of fluorescently labelled M13 tail, 5 mM $MgCl_2$ and 0.1 U *Taq* polymerase. PCR conditions involved an initial denaturing step of 94 °C for 2 min, followed by 20 cycles of 94 °C for 10 s, 60 °C for 20 s, and 72 °C for 10 s. Subsequently, 15 more cycles were performed with the annealing temperature at 53 °C in order to assure better annealing of the fluorescently labelled tail. Finally, the reaction was incubated at 72 °C for 1 h. The length of the amplified microsatellite fragments was read using an ABI PRISM 377 genetic analyser.

Statistical analyses

Some of the leaf-cutter cultivars possessed more than two microsatellite alleles (Kweskin 2003), likely a consequence of some polyploidy or genomic duplication. Although the cohabitation of multiple fungal strains in the same garden is also a possible explanation, it is unlikely, since extracts were made of localized garden fragments less than 1 mm in diameter and since ants generally remove alien cultivar strains (Bot *et al*. 2001; Poulsen & Boomsma 2005). In any case, the microsatellites resulted in a potentially not fully codominant data set, since the actual number of the alleles contributing to any one marker-peak could not be exactly determined. Thus, analogous to amplified fragment length polymorphism (AFLP) analysis, we conservatively scored only for the presence or absence of bands of a particular length for each microsatellite primer pair, generating a total of 64 polymorphic dominant markers. The overall data set contained only 3.4% missing data, permitting pairwise distance measures between cultivar genotypes to be carried out in good faith. Linear binary distance computed from the resulting data matrix was analysed using nonmetric multidimensional scaling (NMDS). Additionally,

population genetic structure within the cultivars was analysed using STRUCTURE 2.1 (Falush *et al.* 2003). The analysis was carried out under a no admixture ancestry model (appropriate for dominant data) and correlated allele frequencies, with a burn in of 100 000 generations and a sampling interval of 500 000 additional generations. All other parameters were left as defaults. The most likely number of populations was estimated by increasing the proposed number of populations until the model likelihood peaked. To ensure repeatability of the analysis, each trial was repeated three or more times. Finally, an analysis of molelcular variance (AMOVA) examining genetic differentiation between genera, and between species within genera, was carried out in GENALEX (Peakall & Smouse 2006) using 9999 replicates to generate the null distribution. The probability of encountering genotypes identical at all loci was calculated by bootstrapping the alleles in the original data set and comparing allele profiles of any two randomly chosen individuals within each bootstrap pseudo-replicate. This analysis was carried out in MATLAB using 10^6 pseudo-replicates.

Results

General patterns and NMDS analysis

Although leaf-cutter cultivars do exhibit relatively little diversity at highly variable ITS loci even across great geographical distances (Silva-Pinhati *et al.* 2004), microsatellite markers showed considerable variability at the population level (Table 1). Assuming unlinked markers, the probability of any two genotypes being identical by chance across all 64 dominant loci was only 0.0017. Out of the 62 fungus gardens, 56 had distinct multilocus genotypes. Only two genotypes were detected more than once. One of the shared genotypes occurred in two gardens, each associated with different *Acromyrmex* species. The other shared genotype was found in four gardens, twice with *Acromyrmex echinatior*, once with *Acromyrmex octospinosus* and once with *Atta sexdens*.

Two NMDS components were able to adequately describe the data (stress1 = 0.11), although little ant host-related structure could be observed (Fig. 2).

Table 1 Number of alleles in each of the nine microsatellite loci used in the study

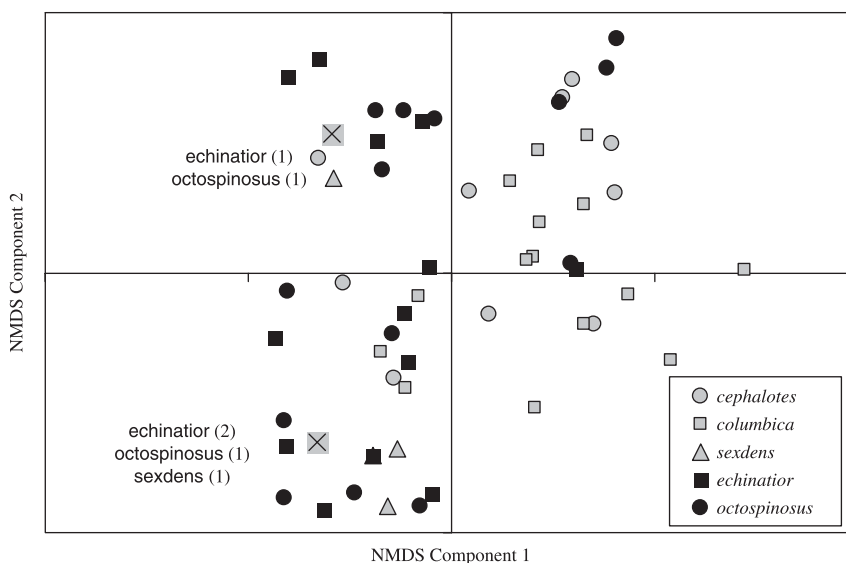| Ant host species	Locus name								
	A0659B	A0815	B0150	B0312	B0358	B0430	B0447	C0625	D0115
At. cephalotes	4	4	8	2	6	7	2	5	4
At. columbica	5	2	5	8	5	6	2	5	2
At. sexdens	3	2	3	5	3	3	2	4	1
Ac. echinatior	3	3	4	8	4	6	2	6	2
Ac. octospinosus	3	3	4	8	4	4	1	5	2

Fig. 2 Plot of the first two components of the nonmetric multidimensional scaling analysis of leaf-cutting ant cultivar genotypes. Cultivars associated with *Atta* and *Acromyrmex* genera are represented by grey and black symbols, respectively. Cultivar genotypes detected across species are indicated by crossed squares and annotations describing host ant species, as well as the numbers of gardens where the cultivar was collected in each species.
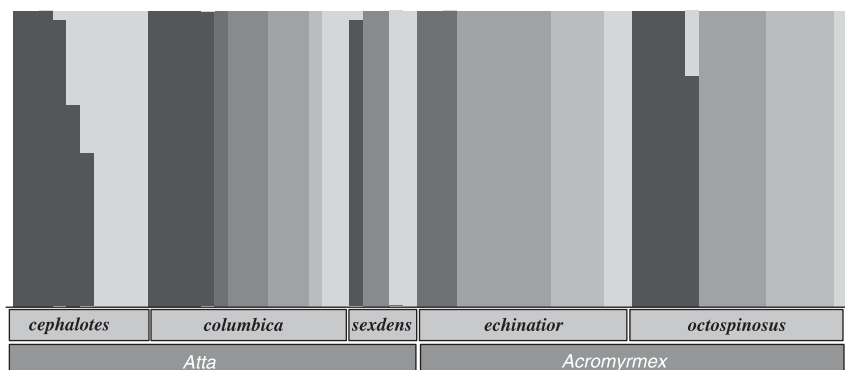
Fig. 3 Genetic structure of cultivar populations with respect to ant host. Six estimated cultivar populations, represented by six shades of grey on a white background, are distributed among five leaf-cutter species.

Atta			Acromyrmex			
cephalotes	columbica	sexdens	echinatior	octospinosus		
	0.158	0.003	0.022	0.001	cephalotes	Atta
0.022		< 0.001	0.006	< 0.001	columbica	
0.187	0.218		0.178	0.333	sexdens	
0.074	0.074	0.049		0.133	echinatior	Acromyrmex
0.108	0.161	0.013	0.029		octospinosus	

Table 2 Pairwise Φ_{PT} distances between cultivar populations (lower triangular matrix) and their uncorrected significance values (upper triangular matrix). Statistically significant Φ_{PT} distances are indicated in italics

Bayesian analysis of population differentiation

The most likely model in the STRUCTURE analysis subdivided cultivars into six populations. The result generally mirrored findings from the NMDS analysis, showing much unstructured variance, with perhaps some slight effect of ant host (Fig. 3). Some members of most STRUCTURE-defined populations were shared between the two genera; sharing of the others cannot be ruled out due to the relatively small number of gardens sampled per species.

Nested analysis of molecular genetic variance

An AMOVA allowed an explicit evaluation of the extent to which different levels of ant phylogeny (genera and species nested within them) contributed to the structuring of fungal molecular variance. In fact, confirming conclusions of exploratory NMDS and Bayesian data analysis methods, fungal molecular variance was largely unstructured with respect to ant phylogeny — garden to garden variation within ant species accounted for 90% of the total genetic variance ($\Phi_{PT} = 0.098$, $P < 0.001$). Differences between species accounted for 8% ($\Phi_{PR} = 0.077$, $P < 0.001$). Genus-level differences accounted for only 2% of the total variance and had only marginal significance ($\Phi_{PR} = 0.022$, $P = 0.053$) (Table 2). Thus, deeper levels of ant phylogeny contribute little, if anything, to the structure of cultivar genetic variance.

Discussion

Our results are consistent with those of earlier studies of leaf-cutting ant cultivars, which found low genetic variability and evidence of genetic exchange between different leaf-cutting ant genera across large geographical scales (Silva-Pinhati *et al.* 2004; Mikheyev *et al.* 2006). Our analyses also show that such garden exchange likely happens continuously at the population level, as has previously been shown for congeneric species of both lower attines and leaf-cutting ants (Bot *et al.* 2001; Green *et al.* 2002). In total, these studies suggest ubiquitous cultivar sharing throughout the attine ant–fungus symbiosis, although the limits to such exchanges between different host ant lineages remain to a large degree unknown. It does appear that higher attines, such as *Trachymyrmex*, cultivate lower attine cultivars in nature on rare occasion (Mueller *et al.* 1998), while, at least in the laboratory, cultivars may be reciprocally exchanged between leaf-cutters and *Trachymyrmex* (Stradling & Powell 1986; Sánchez-Peña 2005; J. Seal, unpublished). The existence of widespread exchange parallels that in the fungus-gardening termites, where horizontal transmission through fungal fruiting normally is the default (Aanen *et al.* 2002; De Fine Licht *et al.* 2006). Thus, it seems that, in both the ants and the termites, co-evolution occurs between suites of symbiotic species, rather than between species pairs.

The presence of the same genotype across different species and genera of ants indicates that fungus gardens

routinely undergo replacement with strains acquired horizontally, as clones, from other species. We observed two repeatedly sampled genotypes that were shared between two or three species, indicating at least three horizontal interspecific transfers of clonal garden material. Thus, at least 5% (3/62) of colonies appeared to cultivate garden clones whose genotypes have originated in other species. Relatively limited sampling at the species level, which reduced the likelihood that a shared cultivar strain would also be collected in another species, likely made us underestimate the horizontal exchange of cultivars. Whatever the actual number of genotypes shared among species, these results argue against complex pairwise genetic integration between cultivar strains and particular ant species, because an identical genotype of the cultivar can service both leaf-cutter genera.

Despite apparent cotransmission of mutualist genotypes during colony reproduction (Weber 1972) and exclusion of alien garden lineages (Bot *et al.* 2001; Poulsen *et al.* 2005), cultivar switching dominates the symbiosis. Thus, associations between ant genotypes, be it at the level of species or genus, do not persist through evolutionary time. Yet, some significant differentiation of the cultivar population nonetheless exists at the species level. Several mechanisms, both adaptive and nonadaptive, may create such structure in the cultivar population. Adaptive forces may associate particular ant–cultivar lineage combinations, either through differential fitness between host-symbiont pairings or through symbiont choice, whereby ants select fungi offering higher fitness (Mueller *et al.* 2004). Nonadaptive explanations include shared genealogy, fine-scale habitat partitioning between ant species (making intraspecific encounters and garden exchanges less frequent), and likely many others. The phylogenetic nesting of the five leaf-cutter species within two genera permits some discrimination between these two classes of explanations. If co-adaptation between ants and fungi occurs, substantial amounts of cultivar genetic variance should be partitioned with respect to ant genus, given the different evolutionary histories and ecological niches of *Acromyrmex* and *Atta* (Fowler 1983; Hölldobler & Wilson 1990; Wetterer 1995). Our data do not support this prediction, exhibiting marginally non-significant amounts of intergeneric structure; far less than even intraspecific genetic structure. Alternatively, selection could act on fungi with respect to host habitat preference. *Atta cephalotes* and *Acromyrmex octospinosus* both prefer relatively undisturbed forest habitats, whereas *Atta sexdens* and *Acromyrmex echinatior* often occur at relatively more disturbed, drier sites and *Atta columbica* being an intermediate between these two extremes. However, our data do not support that expectation either, since both *Acromyrmex*-associated fungi being more similar to those cultivated by *Atta sexdens*, as evidenced by clustering in the NMDS analysis in Fig. 2, the Φ_{PT} values

in Table 2 and by the observation that *Acromyrmex* and *Atta sexdens* may share identical cultivar genotypes.

The diffuse nature of the ant–fungus interaction may require adjustments in the conceptual model of co-evolution used to explain many features of the attine symbiosis, which is based on pairwise species interactions (Frank 1994). Our present results show that while avoidance of host exchange plays an important role in the mutualism (Bot *et al.* 2001; Mueller 2002; Poulsen & Boomsma 2005), it does not ensure interaction specificity in the longer term. At that level, leaf-cutting ant–fungus interactions are characterized by: (i) a widespread cultivar undergoing frequent exchange by a group of phylogenetically diverse ant hosts, and (ii) independent dispersal by the cultivar, possibly much farther than its ant hosts (Mikheyev *et al.* 2006).

Prospects for co-adaptation

Frequent host switching may diminish the extent to which fungi are able to adapt to their ant hosts. The fate of a mutation arising in the fungus garden, and changing the fitness consequences of the cultivar's interaction with its current ant host, is less predictable than in a pairwise species-interaction model. When a fungus becomes transferred to a different host, the fitness value of such a mutation depends both on the frequencies of other ant species to which a fungus may be transferred and the way this mutation affects pairwise interactions with these other hosts. For example, a mutation enhancing fungal fitness in one ant species may be detrimental in others, thus making its spread through the general leaf-cutter community unlikely. Furthermore, even if a mutation affects one ant host, with zero effect on the others, the fitness consequences of any mutation affecting a particular ant host, even while having no effect on the others, may still become attenuated due to the selection-drift balance if host shifts occur commonly enough.

Alternatively, reshuffling of the cultivar genome through recombination (Mikheyev *et al.* 2006) may create favourable gene-host combinations. In this case, a single gene mutation in a fungus garden, although still vulnerable to selection-drift balance, may be maintained indefinitely in association with a particular ant host, though the nonspecific remainder of the fungal genome continues to be freely exchanged among ant species. This scenario is likewise consistent with our data, based on presumably neutral microsatellite loci, which would not be expected to show any species–specific associations. However, this scenario appears unlikely, since genetically identical clones were observed shared between the different species and genera of ants.

Although co-evolution characterizes the attine ant–fungus symbiosis at the deepest phylogenetic levels

(Chapela *et al.* 1994; Hinkle *et al.* 1994), its origins and maintenance appear mysterious in the face of frequent host switches observed at the global and population levels. However, in many other nonobligate mutualists, regionally co-adapted pairings may still exist if selection acts across a geographical mosaic of mutualist populations (Thompson 2005). Thus, a closer look at differences across populations of the same species may provide a key to elucidating the forces binding ant and cultivar lineages. Such investigations will be made easier by the realization that relatively frequent fungal cultivar switching occurs across the genera of leaf-cutting ants, creating intriguing new possibilities for exploring such co-evolutionary trajectories.

Acknowledgements

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