JOURNAL OF Evolutionary Biology

doi: 10.1111/jeb.12140

Co-evolutionary patterns and diversification of ant–fungus associations in the asexual fungus-farming ant *Mycocepurus smithii* in Panama

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Keywords:

co-evolution;
host specificity;
host switching;
mutualism;
symbiosis;
thelytoky.

Abstract

Partner fidelity through vertical symbiont transmission is thought to be the primary mechanism stabilizing cooperation in the mutualism between fungus-farming (attine) ants and their cultivated fungal symbionts. An alternate or additional mechanism could be adaptive partner or symbiont choice mediating horizontal cultivar transmission or de novo domestication of free-living fungi. Using microsatellite genotyping for the attine ant *Mycocepurus smithii* and ITS rDNA sequencing for fungal cultivars, we provide the first detailed population genetic analysis of local ant–fungus associations to test for the relative importance of vertical vs. horizontal transmission in a single attine species. *M. smithii* is the only known asexual attine ant, and it is furthermore exceptional because it cultivates a far greater cultivar diversity than any other attine ant. Cultivar switching could permit the ants to re-acquire cultivars after garden loss, to purge inferior cultivars that are locally mal-adapted or that accumulated deleterious mutations under long-term asexuality. Compared to other attine ants, symbiont choice and local adaptation of ant–fungus combinations may play a more important role than partner-fidelity feedback in the co-evolutionary process of *M. smithii* and its fungal symbionts.

Introduction

Mutualisms are ubiquitous cooperative and beneficial interactions among species that can range from diffuse interactions to highly co-evolved associations (Bronstein, 1994; Leigh, 2010). The defining signature of co-evolution is the presence of reciprocal, novel adaptations and novel counteradaptions in co-evolving partners (Mueller, 2012). Tightly interacting species can exhibit phylogenetic congruence (co-phylogenies) (Hafner & Page, 1995; Page, 2003; Light & Hafner, 2007; Refrégier *et al.*, 2008; Schardl *et al.*, 2008) because of co-speciation and co-diversification (Cook & Rasplus, 2003; Quek *et al.*, 2004; Marussich & Machado, 2007).

Fitness of symbionts can be directly linked to host fitness, leading to shared evolutionary interests (Herre *et al.*, 1999), but conflicts can also occur between hosts and symbionts (Mueller, 2002). For example, conflict over the mixing of symbiont types can lead to symbiont–symbiont competition for the same host resources, symbionts may evolve strategies for sole occupancy of a host, and hosts may avoid harbouring more than one symbiont lineage (Frank, 1996). Genetic variation of hosts and symbionts can be further influenced by spatially variable selection and drift (geographic mosaic of co-evolution, Thompson, 1999) leading to geographically varying adaptation or mal-adaptation of host–symbiont combinations (Spoerke *et al.*, 1996; Wilkinson *et al.*, 1996; Bever, 2002).

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Fungus-growing ants (Formicidae: Attini) are prominent systems for research on mutualistic interactions. Some attine ants are dominant herbivores of the Neotropics (Hölldobler & Wilson, 2010), owing their ecological success to mutualistic association with co-evolving fungal cultivars (Chapela *et al.*, 1994). The fungus serves as the ants' main food source and is propagated asexually by the ants (Mueller *et al.*, 2010). This mutualism has been shaped by 40–60 million years of complex ant–fungus re-associations, but specific clades of attine ants associate predominantly with specific clades of fungi in the tribe Leucocoprineae (formerly Lepiotacea, now Agaricaceae, Agaricales, Basidiomycota) (Chapela *et al.*, 1994; Mueller *et al.*, 1998; Munkacsi *et al.*, 2004; Schultz & Brady, 2008; Mikheyev *et al.*, 2010).

Ant–fungus associations are maintained primarily through vertical transmission of fungal symbionts from one generation to the next (Chapela *et al.*, 1994; Mueller *et al.*, 1998; Schultz & Brady, 2008; Caldera *et al.*, 2009). As far as known, young queens start gardens in newly founded nests with fungal inocula collected before dispersal from the maternal nests [vertical symbiont transmission; (Huber, 1905)]. In contrast to predictions from a scenario of strict vertical cultivar inheritance, phylogenetic studies have shown that the cultivars are polyphyletic (indicating multiple domestication events) (Chapela *et al.*, 1994; Mueller *et al.*, 1998; Mikheyev *et al.*, 2010), and that ant and fungal phylogenies are not topologically congruent, implicating horizontal transfer of fungal clones between different attine lineages (Mueller *et al.*, 1998, 2011; Green *et al.*, 2002; Mikheyev & Mueller, 2007), occasional gene flow between free-living and ant-cultivated fungal populations (Mueller *et al.*, 1998; Pagnocca *et al.*, 2001; Dentinger *et al.*, 2009; Vo *et al.*, 2009), or both. Some fungal clades originated more recently than the corresponding clades of host ants (Mikheyev *et al.*, 2010), suggesting *de novo* domestication of free-living fungi.

Host–symbiont re-associations are most common in the 'lower attines' (a paraphyletic group including basal attine lineages) because they can import novel strains into the mutualism (Mueller *et al.*, 1998; Vo *et al.*, 2009) and because fungi can be transferred between ant species and lineages through garden stealing and sharing (Adams *et al.*, 2000; Green *et al.*, 2002). Typically, lower-attine ant species tend to associate locally with only one fungal species, but there are exceptions (Mueller *et al.*, 1998; Mikheyev, 2008; Mehdiabadi *et al.*, 2012). The most remarkable exception is the ant *Mycocepurus smithii*, which cultivates at least six different fungal lineages in Central Panama (Mueller *et al.*, 1998), with similar cultivar diversity in Amazonian Brazil (Rabeling, 2004).

Mycocepurus smithii is unique because it reproduces by thelytokous parthenogenesis in most populations across its vast range from northern Argentina to northern Mexico (Fernández-Marín *et al.*, 2005; Himler *et al.*, 2009; Rabeling *et al.*, 2009, 2011). In thelytokous populations, reproducing queens are unmated, and female offspring are genetically identical to their mothers (Himler *et al.*, 2009; Rabeling *et al.*, 2011). The cultivated fungus is likewise clonally propagated by the ants within nests and between ant generations. This 'double asexuality' (Himler *et al.*, 2009) may impose an evolutionary handicap, because adaptive novelty generated by genetic recombination is limited in both mutualistic partners. Additionally, because of clonal propagation, both the clonal ants and the clonal fungi may accumulate deleterious mutations over time [Muller's ratchet, (Maynard Smith, 1983)], which might result in inferior nutritive quality or greater disease susceptibility of the fungi, as well as inferior gardening abilities of the ants, all of which reduces fitness of the overall mutualism. The ants might mitigate this handicap through frequent fungal switching, thus generating novel ant–fungus combinations adapted to varying environmental conditions (Himler *et al.*, 2009).

Here, we use high-resolution molecular markers to characterize phylogeographic patterns of ant–fungus association in *M. smithii* in the Panama Canal Zone, and to evaluate the potential for ant-fungus co-evolution in *M. smithii*. Previous population genetic studies of *M. smithii* investigated either only the ant diversity (Himler *et al.*, 2009; Rabeling *et al.*, 2009, 2011) or only the fungal diversity (Mueller *et al.*, 1998), whereas the present study is the first detailed co-phylogenetic analysis of ant–fungus associations of a single attine species.

Materials and methods

Sample collection

Colonies of *M. smithii* were collected in Central Panama in April 2010 by excavating chambers, as previously described (Mueller *et al.*, 1998; Rabeling *et al.*, 2007). Each chamber was processed separately because nest boundaries were often difficult to discern. Ants and fungus gardens were collected with flame-sterilized forceps and preserved separately in 100% ethanol. Material was collected from 11 nest aggregations: East of the Panama Canal in Gamboa at Building 183, Resort, Greenhouse, Harbor, and Pipeline Road entrance; west of the Panama Canal at Casa Verde, Corozales Afuera, Chorrera, Achiote, Gatun, and Piña Beach (See Table S1, Supporting information). These sampling sites differed in their habitats: Open, grassy, meadow-like and disturbed habitats with light shade were found in Achiote, Chorrera, Casa Verde, Corozales Afuera and at the Pipeline road entrance in Gamboa. In contrast, wooded, heavily shaded and disturbed habitats were found at Building 183, Resort, Greenhouse and the Harbors in Gamboa and Gatun. One habitat very distinct from all others was the Piña Beach, were the ants were found under shrubs in sandy soil close to the tide line.

Molecular methods

Ants

DNA was extracted from three ants per chamber by crushing single, whole ants in liquid nitrogen, incubating in 100-μL 10% Chelex resin (Sigma-Aldrich, St. Louis, MO, USA) for 1.5 h at 60 °C, then 10 min at 99 °C. For microsatellite genotyping, we used 12 specific markers developed by (Rabeling *et al.*, 2011) and one additional marker (A6_2; see Table S1). PCR products were sized on a Capillary Sequencer (ABI PRISM 3100; Applied Biosystems, Foster City, CA, USA) and scored with GeneMarker v1.5 (SoftGenetics, State College, PA, USA).

Fungi

DNA was extracted by incubating hyphal clusters (ca. 0.5 mm) in 100-μL 10% Chelex, following the same protocol as for the ants. The internal transcribed spacer (ITS) rDNA region was amplified with ITS4 and ITS5 primers (White *et al.*, 1990; Mueller *et al.*, 1998). PCR reaction, purification and cycle sequencing (ABI BigDye Terminator Kit; ABI PRISM 3100) followed methods in (Vo *et al.*, 2009). Forward and reverse sequences were assembled in Sequencher 4.6 (GeneCodes, AnnArbor, MI, USA). Sequence information is deposited at NCBI GenBank (JQ405665-JQ405735).

Data analyses

Previous studies showed that *M. smithii* ants and their cultivated fungi are clonal (Rabeling *et al.*, 2011), with no genetic variation within colonies to be expected. Indeed, chambers collected closely to each other (ca. 10 cm) contained identical ant and fungus genotypes, except in two cases where fungi in two single chambers were found to be different and therefore assumed to be from different colonies. The otherwise clonal identity of samples collected from adjacent chambers justified grouping into putative colonies.

Ant population genetics and clone-lineage analyses

We analysed ants from a total of 64 chambers (52 colonies). Analysis of molecular variance (AMOVA) was performed in ARLEQUIN 3.11 (Excoffier *et al.*, 2005): Individual microsatellite genotypes were grouped into colonies within collection sites, and these within the total population. Significance of population structure was tested using a permutation procedure (1000 replicates). Significance for collection site pairwise F_{ST} values was tested using permutation tests (1000 replicates) and adjusted with sequential Bonferroni correction.

Departure from Hardy–Weinberg equilibrium was calculated using GENEPOP 4.0 (Raymond & Rousset, 1995), with a global test [Score (*U*) test] for each population using the Markov chain method (100 batches, 10 000 iterations/batch). Observed and expected heterozygosities were calculated in GDA (Lewis & Zaykin, 2001) for each population and each locus.

Ant genotypes were sorted into clone lineages by 100% allele identity across the 13 microsatellite loci (Table S1). To analyse phylogenetic relationships between clone lineages, we computed a pairwise distance matrix and a neighbour-joining tree based on allele-sharing distances (Chakraborty & Jin, 1993) using POPULATIONS 1.20.30 (Langella, 1999). Support values were obtained by bootstrapping over loci (100 pseudoreplicates). The inferred phylogenetic tree was visualized in MEGA 5 (Tamura *et al.*, 2011).

Fungus population genetics and fungus clone-lineage analyses

We analysed fungi from 64 chambers (52 colonies). Population genetic structure was analysed using AMOVA in ARLEQUIN (Excoffier *et al.*, 2005), testing variation among and within collection sites. Pairwise genetic distances between populations were calculated (Kimura2P distances), and significance of these distances was evaluated with 1000 permutations and sequential Bonferroni correction.

Phylogeny of the fungus cultivars

Lower-attine fungi are grouped into two distinct clades (previously called Clade 1 and Clade 2) (Mueller *et al.*, 1998). To associate our fungal ITS sequences with the existing Clade 1 and Clade 2, we added them into the original lower-attine fungal cultivar alignment from (Vo *et al.*, 2009) (alignment shown in NCBI GenBank PopSet: 140104520), using McClade 4.08.(Maddison & Maddison, 2000). One sequence from each ITS lineage was blasted against NCBI GenBank; sequences with query coverage >94% and maximum identity >85% were added if not already present in the (Vo *et al.*, 2009) alignment. Addition of these new sequences did not cause any changes in the original alignment, that is, gaps and excluded characters were preserved unchanged (complete alignment length 916 bp, 578 bp after excluding ambiguously aligned characters). The total data set consisted of 305 sequences. jModeltest 0.1.1 (Guindon & Gascuel, 2003; Posada, 2008) identified the GTR + I + G model as the most suitable model for phylogenetic analyses. A maximum-likelihood tree was computed using the default settings in GARLI 0.951 (Zwickl, 2006). Ten trees were generated from which the tree with the highest likelihood score was chosen. Bootstrap support was evaluated in 100 pseudoreplicates.

To construct subtrees of Clade 1 and Clade 2, we realigned sequences within each Clade including the new sequences from the *M. smithii* cultivars. For each Clade, a maximum-likelihood tree was generated with GARLI default settings and 1000 pseudoreplicates bootstrap support. *Myrmicocrypta infuscata* G11 (GenBank accession AF07971 6.1) and *Lepiota* cf. *abruptibulba*

PA156 (AF079732.1) were used as outgroups for Clade 1 and Clade 2, respectively.

Analyses of ant–fungus associations

Genetic information for both ant and cultivar was available for a total of 58 ant–fungus chambers. Fungal genetic variation between and within ant host genotypes was analysed with AMOVA. We used ParaFit (Legendre *et al.*, 2002) to test for significant phylogenetic codivergence between ant lineages and fungus types. ParaFit was developed originally for analysis of parasite–host relationships where parasites chose to associate with a particular host or chose to switch between hosts. Because attine ants are the choosing partner in the ant–fungus symbiosis, we defined the ants as 'parasites' (or 'the ones who choose the partner') in the ParaFit input files, and the fungi as 'hosts' (or 'the ones who get chosen by the partner') [Defining the choosing and the chosen partners in ParaFit correctly is most critical for evaluating statistical significance of associations; P. Legendre, personal communication]. ParaFit performs two tests: The null hypothesis of the ParaFitGlobal test is that the evolution of the two groups has been independent, as inferred from the observed association links between the two trees. Additionally, ParaFit performs a test on each single observed link to assess the significance of that particular association. As input files we used, (i) a presence/absence matrix of the 21 observed host–cultivar associations (scored as 1 and 0, respectively), (ii) a principal coordinate matrix obtained from allele-sharing distances among ant genotypes and (iii) a transposed matrix of principal coordinates obtained from Kimura2P genetic distances among fungus cultivars. To verify the significance of the ParaFitGlobal results, and to apply a method without preassumption on who the choosing and who the chosen part in the mutualism is, we performed a second permutation test with a different test statistic (Hommola *et al.*, 2009). To run this analysis, we used the R code available under http://www1.maths.leeds.ac.uk/~kerstin/ with 10 000 iterations on R.2.13.0 (2011). Because ParaFit tests are performed irrespective of geographic structure, to evaluate the influence of geographic structure on ant–fungus associations and population genetic differentiation, we performed Mantel and partial Mantel tests to partition the effects of geographic distance and ant host occurrence (occurrence of ant genotypes at sample sites), using the program IBD 1.52 (Bohonak, 2002). Specifically, we tested for correlations between fungus population F_{ST}, ant population F_{ST} (as obtained from ARLEQUIN, see above) and geographic distances between sample sites. Ant host occurrence was also used as an additional influencing factor, which was coded into a presence/absence matrix of ant genotype occurrence at the sample sites.

To investigate possible influences of sampling effort, we used EstimateS 8.2.0 (Colwell, 2006) to construct sample-based rarefaction curves (Fig. S1, Supporting information).

Results

Population genetics of ant hosts

Eleven of the 13 microsatellite loci were polymorphic (2–5 alleles per locus). Two loci (B4, C2) were monomorphic and therefore excluded from subsequent analyses. As expected for a clonal organism, all populations and all loci showed significant deviation from Hardy–Weinberg equilibrium (averaged over all populations: $P < 0.01$, over all loci: $P < 0.001$). Observed heterozygosities (*Ho*) were higher than expected (*He*) (averaged over loci: *Ho*: 0.55, *He*: 0.48; averaged over colonies *Ho*: 0.55, *He*: 0.33), leading to negative values for inbreeding coefficients (over loci: $f = -0.14$, over colonies $f = -1.00$) (Table S2). Genetic variation of the ants was partitioned among collecting sites (32.4%) and among colonies within collecting sites (67.6%). Collecting sites were genetically differentiated from each other ($F_{ST} = 0.32$, $P < 0.001$; see also Table S3).

We found 11 different clone lineages among the ants (genotypes A–K; Fig. 1; Fig. S2a,b). Genotype A in our study exactly matches genotype GamboaB and ShermanC found by (Rabeling *et al.*, 2011) in a survey across the entire range of *M. smithii*. Genotype K was most closely related (two allele differences) to genotypes found in Venezuela, Nicaragua and southern Mexico. Genotype G was nearly identical (one allele difference) to genotypes GamboaA and ShermanB reported in (Rabeling *et al.*, 2011). Genotypes F and G are closely related to genotypes from Costa Rica and Venezuela (Rabeling *et al.*, 2011). We found only one ant genotype at each of the seven sites (Gamboa Pipeline Road, Gamboa Harbor, Gatun, Corozales Afuera, Piña Beach, Chorrera, Achiote) (Fig. 1), but more than one ant genotype at four collection sites (six ant genotypes at Gamboa Building 183; two each at Gamboa Resort, Gamboa Greenhouse and Casa Verde). Seven ant genotypes were exclusive to one site, the other four types were found at several sites (Fig. 1).

The number of nests excavated at a site was not significantly correlated with the number of ant lineages found at a site (Spearman rank-order correlation $P = 0.519$), nor with the number of fungus types found ($P = 0.506$). There was no correlation between the number of ant clones and the number of fungus types found at a site ($P = 0.285$). Ant and fungal diversities at a site therefore were not closely dependent on sampling effort per site.

Population genetics of fungal symbionts

We identified nine different cultivar ITS-sequence types (labelled ITS-types 1–9). Only one ITS-type was found

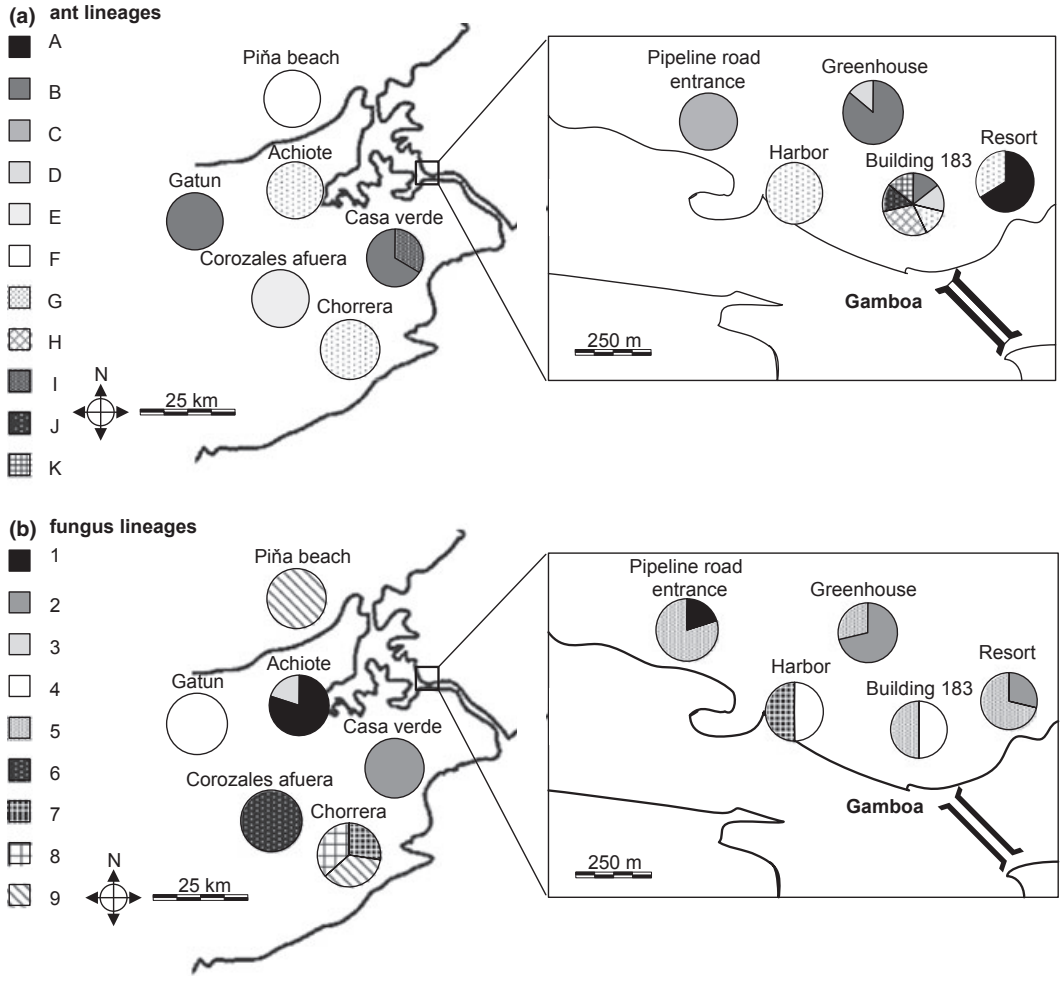

Fig. 1 Map of collection localities of the lower-attine ant *Mycocepurus smithii* in Central Panama. Pie charts represent the frequencies of ant microsatellite genotypes (a) and fungus cultivar ITS-types (b) found at each collection locality. Number of chambers collected: Achiote: 9, Piña Beach: 3, Casa Verde: 3 (four fungus), Chorrera: 10 (11 fungus), Gatun: 3, Corozales Afuera: 5, Gamboa Harbor: 2, Pipeline road entrance: 5, Greenhouse:7, Resort: 10, Building 183: 7.

at four of the eleven collection sites (Piña Beach, Corozales Afuera, Casa Verde, Gatun), but more than one fungal ITS-type was found at each of the other seven sites (Resort, Greenhouse, Pipeline Road, Gamboa Building 183, Harbor and Achiote: 2 ITS-types each; Chorrera: 3). Only one of the nine ITS-types was exclusive to one collection site (type 6: Corozales Afuera) (Fig. 1). AMOVA results revealed more genetic variation between sites (78.4%) than within sites (21.6%) for the fungi, with significant genetic differentiation between sites (F_{ST} = 0.77, $P < 0.001$; see also Table S3).

Fungal symbiont phylogeny

The placement of our nine ITS-types within the lower-attine cultivar phylogeny is consistent with results from previous analyses (Mueller *et al.*, 1998) (Fig. S3). Fungus types 1–5 were placed into Clade 1 (types 1–3 in Clade 1A, and types 4 & 5 in Clade 1B) and fungus types 6, 7, 8 and 9 cluster together in Clade 2 (Fig. S4a,b).

Ant–fungus associations

We did not find a pattern of strict one-to-one association between ant hosts and fungal symbionts (Fig. 2). Seven of the eleven ant genotypes were found in association with only a single fungus type, whereas four ant genotypes (B, C, H, G) were found in association with 2–6 different fungi. ParaFit analyses showed an overall barely significant association between ant and fungus phylogenetic relationships (ParaFitGlobal = 0.027, P = 0.048), rejecting therefore the null hypothesis of independence between ant and fungus phylogenies. The permutation test after Hommola *et al.*, 2009

supported this result (overall pattern of coevolution between the ant and the fungus phylogenies was confirmed; $P = 0.0047$). Twelve of 21 association links emerged as significant links in ParaFit ($P = 0.014$–0.043), whereas nine links were nonsignificant ($P > 0.05$) (Fig. 2). Significant association links between ant clones and fungal types presumably resulted from vertical cultivar transmission between ant generations, whereas the nonsignificant links likely resulted from recent cultivar switching. Some ant clones appear to be specialized on specific fungus Clades: whereas ant genotypes F and G are found in significant association with Clade 2 cultivars, ant genotypes A, B, C, D, H, I and J are found in significant association with Clade 1 fungus. In contrast, associations between ant lineage G and various fungus cultivars from Clade 1 (1, 2, 3, and 4) were nonsignificant, and these associations therefore derived most likely from recent between-Clade switches.

Fungi were significantly differentiated between ant hosts ($F_{\text{among ant hosts}} = 0.52$, $P < 0.001$); however, overall genetic variation of fungi was partitioned similarly among (51.9%) and within ant hosts (48.2%).

Genetic distance of fungus populations was correlated with geographic distance (Mantel test, $r = 0.34$, $P = 0.034$), even when controlling for genetic distance of ant populations (partial Mantel test, $r = 0.34$, $P = 0.040$) and when controlling for ant host occurrence (partial Mantel test, $r = 0.34$, $P = 0.042$). In contrast, no correlations between genetic and geographic distance were found in the ant populations ($r = -0.11$, $P = 0.19$). Genetic distances between fungus populations were independent of genetic distances between ant populations (Mantel test, $r = -0.06$, $P = 0.317$).

Discussion

Ant–fungus associations between the thelytokous fungus-farming ant *M. smithii* and its asexual fungal cultivars would seem to be more transient than in other fungus-farming ants. For example, ants in the *Cyphomyrmex wheeleri*-complex exhibit cultivar fidelity across millions of years (Mehdiabadi *et al.*, 2012) and *Mycocepurus goeldii* appears to specialize on one of two fungal lineages either north or south of the Amazon River (Rabeling, 2004). Thus, vertical transmission appears to be frequently disrupted in *M. smithii* either by *de novo* domestication of fungi from free-living sources or by horizontal cultivar transmission between nests of *M. smithii* or other sympatric, lower-attine ant species. Compared to other attine ants, therefore, partner-fidelity feedback is probably relatively less important, and symbiont choice relatively more important, in stabilizing the mutualism (preventing the invasion of inferior self-serving symbionts that are of lesser benefit to the ants).

The observed large number of fungal ITS-types cultivated locally by a single attine ant species (nine fungal types from two different clades in Central Panama) is so far unknown for any lower-attine ant and extends the elevated cultivar diversity of *M. smithii* noted in a previous analysis (Mueller *et al.*, 1998). The different cultivar types found in association with *M. smithii* could have been acquired through transfer from other sympatric ant hosts, or through *de novo* domestication of free-living lepiotaceaeus fungi belonging to Clade 1 and Clade 2 cultivars. For example, cultivar type 3 of *M. smithii* in our study was found to be sequence-identical in its ITS sequence to cultivars of *Mycocepurus tardus* (GenBank accession number AF079710) and *Myrmicocrypta ednaella* (AF079715) collected previously in Panama. The sister-cultivar types 7 and 8 are most closely related to a known cultivar of *Mycocepurus curvispinosus* from Costa Rica (AF079709). Cultivar type 6 is nearly sequence-identical in ITS (except for two nucleotides) to a sympatric free-living lepiotaceous fungus from Panama (EF527398; see Fig. S3).

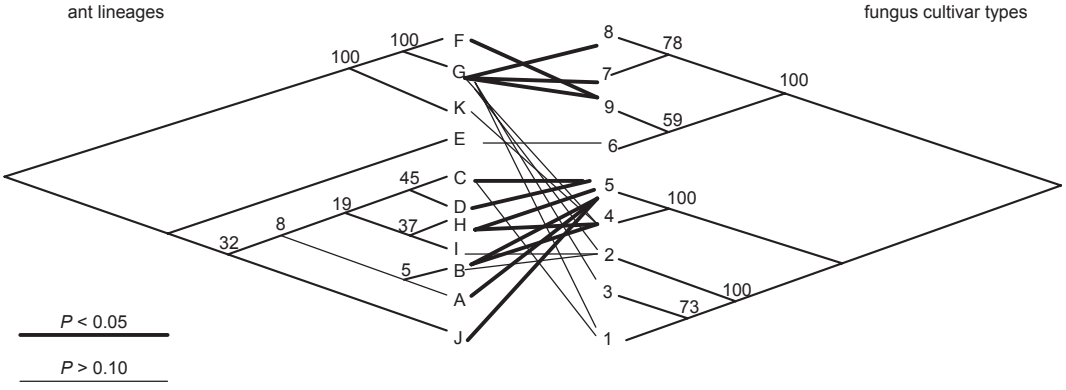

Fig. 2 Tanglegram phylogenies of *Mycocepurus smithii* ant clones (microsatellite genotypes) and their corresponding fungal cultivars (ITS-types) from Central Panama. Ant–fungus associations observed in natural nests are indicated through lines linking the two trees. The fungal diversity is split into two main clades, corresponding to Clade 1 Cultivar (top clade) and Clade 2 Cultivar (bottom clade). Significance of each link was inferred in ParaFit (Legendre *et al.* 2002). For complete ParaFit results, see Table S4.

We confirmed thelytokous parthenogenesis (Fernández-Marín *et al.*, 2005; Himler *et al.*, 2009; Rabeling *et al.*, 2009, 2011) as the primary mode of reproduction in Panamanian populations, with a total of eleven sympatric ant genotypes from three distinct groups; Fig. S2). Some ant genotypes in our survey correspond to types that have been found previously in the Panama Canal Zone (Rabeling *et al.*, 2011), and some ant genotypes are closely related to lineages known only from neighbouring countries in Central America, but not from more distant regions across the range of *M. smithii*. This indicates that the ant genotypes included in our study represent genotypes typical for Panama, that the ant populations in our study are embedded in a viscous population structure across Latin America, and that the ant–fungus associations studied here represent local or regional associations.

We did not find strict one-to-one association patterns between ants and their cultivated fungi. A few fungal types were only associated with one ant lineage (Fig. 2), but such apparent one-to-one associations could be an artefact of insufficient sampling (see rarefaction analysis of ant–fungus combinations in Fig. S1b). More typically, we found ant lineages associated with more than one fungus type, and likewise, fungus types associated with more than one ant genotype (Fig. 2). This finding agrees with our AMOVA analyses, which revealed that genetic variation of fungi was equally partitioned among and within ant hosts, indicating only weak host-cultivar specialization. Permutation tests for co-speciation revealed evidence of statistically significant, global co-phylogenetic associations between ant and fungal types in Panama. This suggests that fungus cultivars are inherited vertically across a limited number of ant generations, but that such inheritance is not indefinite; rather, novel ant–fungus combinations may be frequently generated by (i) horizontal cultivar transmission of fungal cultivars between ant nests, (ii) *de novo* recruitment of free-living fungal clones into the mutualism, (iii) gene flow between different symbiotic fungi, or between symbiotic and nonsymbiotic fungi, or (iv) some combination of these processes. The lower F_{ST} values and lower isolation-by-distance effect in the ants than in the fungi indicate that gene flow is stronger between ant populations than between fungus populations, possibly because of a higher effective dispersal-potential in the ants than in the fungus (queens might regularly substitute their native cultivar and re-acquire a local fungus after dispersal). The finding that ant genetic variance among colonies within sites is higher than variance between sites, whereas fungal genetic variance is lower within sites than between sites, further supports the conclusion of greater gene flow in the ants than in the fungus.

Horizontal fungal transfer between ant lineages could occur through several mechanisms in *M. smithii*. First, it is possible that, compared with other attine ants, incipient fungus gardens during colony founding are more frequently lost in *M. smithii* [many founding queens often lack fungus gardens; (Fernández-Marín *et al.*, 2005)]. Second, colonies may lose their gardens during catastrophes (e.g. flooding, garden diseases), which forces the ants to recruit free-living fungi or steal garden from neighbouring colonies, as has been shown for *Cyphomyrmex* ants in laboratory experiments (Adams *et al.*, 2000). At sites where diverse ant and fungal types co-occur, such re-association could generate novel ant–fungus combinations. Third, pleometrotic (multiple foundress) nest-founding, which occurs in *M. smithii* and a few other attine ants (Rissing *et al.*, 1989; Mintzer, 1990; Bekkevold *et al.*, 1999; Fernández-Marín *et al.*, 2005), also facilitates generation of novel combinations whenever fungi are transferred between co-founding queens. In such multiple-foundress nests, gardens may be chimaeric during the early nest-founding stage. Coexistence of different cultivars of the same fungal species has been observed in chimaeric gardens in laboratory experiments of the more derived higher Attini (Sen *et al.*, 2010). In *M. smithii* it is likely that different fungal species will be vegetatively incompatible and thus compete with each other if they were co-cultivated by *M. smithii* in a chimaeric garden, and co-founding queens may even bias cultivation in favour of a specific strain in a chimaeric garden. This predicts absence or rapid transience of chimaeric gardens in *M. smithii*, and indeed, we never observed more than one fungus type in a single chamber in our field survey.

In the *M. smithii* mutualism, both partners reproduce asexually; in the absence of sexual recombination, creation of novel genotypes in the ants and the fungal symbiont is therefore limited to mutation. Moreover, as in endosymbionts of other insects (Moran, 1996; Pettersson & Berg, 2007), both of the asexual partners might be prone to the inevitable accumulation of deleterious mutations, a process known as Muller's ratchet operating in all asexual lineages of finite population size (Maynard Smith, 1983). Ant–fungus re-association would alter the combined number of deleterious mutations accumulating within an ant–fungus combination, and such re-associations therefore could function as a recombinatory analogue operating on the level of ant–fungus combinations, potentially purging the ants of degenerate fungi and purging the fungi of degenerate ants.

Symbiont choice could enable *M. smithii* to choose between cultivar types that are superior or inferior in different habitats (habitats offering different garden substrate, or different environmental conditions such as moisture or temperature, or different biotic challenges such as garden diseases). Symbiont choice could be particularly important if the fungi are adapted to local conditions, and the ants disperse widely between habitats and across ecological gradients. Consistent with this scenario, we found distinct cultivar types and distinct

cultivar prevalence between the varying habitats surveyed in Panama (Fig. 1); in addition, isolation-by-distance tests showed that genetic variation (about 30%) correlates significantly with geographic distance in the fungal cultivars, but not in the ants. This suggests that the ants disperse regionally, but they tend to grow local crops because of frequent cultivar replacement after dispersal. Dispersing queens might try to propagate their natal cultivar in newly colonized habitats, which could lead occasionally to 'local mal-adaptations', as predicted by the geographic mosaic theory of co-evolution (Thompson, 1999). Alternatively, dispersing queens might lose cultivars and then be forced to switch to new, locally adapted fungal strains. Whether *M. smithii* indeed exerts symbiont choice by selecting specific cultivars that best matches their own genotype to optimize fitness synergisms, or whether *M. smithii* selects cultivars optimally adapted to local conditions, is the topic of a future investigation.

Acknowledgments

We thank the Autoridad Nacional del Ambiente de Panamá, the Smithsonian Tropical Research Institute and Orelis Arosemena and Annette Aiello for collecting and export permits; Jon Seal, Nancy Lowe and Nicole Gerardo for help with collections; Pierre Legendre for advice on ParaFit, and Jon Seal, Christian Rabeling and Quinn McFrederick and two anonymous reviewers for helpful comments on the manuscript. The work was supported by a National Science Foundation award (DEB-0919519) to TAL and UGM, and by the W.M. Wheeler Lost Pines Endowment.

Data accessibility

DNA sequences used in this study are deposited at NCBI GenBank (Accessions JQ405665-JQ405735 for the ITS fungus sequences, and JX000236 for microsatellite A6-2).

Microsatellite loci A5, A6, A9, B1, B4, C2, C6, C104, C119, D8, D11, D117 were developed by Rabeling *et al.*, 2011 and are deposited at GenBank (Accessions JN054745-JN055353).

Fungus ITS sequences from Mueller *et al.*, 1998 are deposited at GenBank (Accessions AF079591-AF079754) and from Vo *et al.*, 2009 at GenBank (EF527400-EF527280), and the original alignment is shown in GenBank PopSet 140104520.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1 Multilocus genotypes of *Mycocepurus smithii* samples, GPS data information for sample locations, primer sequence for locus A6_2.

Table S2 Expected (H_e) and observed (H_o) heterozygosities of *M. smithii* ants.

Table S3 Pairwise genetic differentiation (F_{ST} values) between eleven populations of *Mycocepurus smithii* ants and fungus.

Table S4 Statistics for the significance of ant–fungus associations analysed in ParaFit.

Figure S1 Sample-based rarefaction curves (lineage-accumulation curves) of (a) fungus types; (b) ant lineages; and (c) ant/fungus combination of *M. smithii* collected in the Republic of Panamá in 2010.

Figure S2 Phylogenetic relationships (neighbour-joining tree) of eleven ant lineages of *M. smithii* in Central Panama.

Figure S3 Maximum-likelihood phylogeny of 131 lower-attine cultivars and 174 free-living lepiotaceous fungi.

Figure S4 Maximum-likelihood phylogeny inferred from ITS rDNA sequence information for (a) Clade 1 and (b) Clade 2 fungi cultivated by lower-attine ants.

Data deposited at Dryad: doi:10.5061/dryad.cb850

Received 29 October 2012; revised 30 January 2013; accepted 2 February 2013