RESEARCH ARTICLE

Ecology of microfungal communities in gardens of fungus-growing ants (*Hymenoptera*: *Formicidae*): a year-long survey of three species of attine ants in Central Texas

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Abstract

We profiled the microfungal communities in gardens of fungus-growing ants to evaluate possible species-specific ant–microfungal associations and to assess the potential dependencies of microfungal diversity on ant foraging behavior. In a 1-year survey, we isolated microfungi from nests of *Cyphomyrmex wheeleri*, *Trachymyrmex septentrionalis* and *Atta texana* in Central Texas. Microfungal prevalence was higher in gardens of *C. wheeleri* (57%) than in the gardens of *T. septentrionalis* (46%) and *A. texana* (35%). Culture-dependent methods coupled with a polyphasic approach of species identification revealed diverse and changing microfungal communities in all the sampling periods. Diversity analyses showed no obvious correlations between the number of observed microfungal species, ant species, or the ants' changing foraging behavior across the seasons. However, both correspondence analysis and 5.8S-rRNA gene UNIFRAC analyses suggested structuring of microfungal communities by ant host. These host-specific differences may reflect in part the three different environments where ants were collected. Most interestingly, the specialized fungal parasite *Escovopsis* was not isolated from any attine garden in this study near the northernmost limit of the range of attine ants, contrasting with previous studies that indicated a significant incidence of this parasite in ant gardens from Central and South America. The observed differences of microfungal communities in attine gardens suggest that the ants are continuously in contact with a diverse microfungal species assemblage.

Introduction

Mutualisms are generally viewed as interspecies interactions with reciprocal fitness benefits for both partners. The understanding of mutualisms as dual associations was transformed in the past decade by the realization that the nature of dual interactions can be changed categorically in the presence of additional interactors (Stanton, 2003; Little *et al.*, 2008). Such mutualisms actually need to be understood as an intricate interaction network among diverse hosts and symbionts. For example, insect hosts progress through their life cycle within a changing network composed of a myriad of microorganisms inhabiting their nest environment and their bodies (Ganter, 2006). The task to elucidate the contributions of specific microorganisms to the host life cycle is challenging, and yet such studies of insect-associated microbiota are needed to go beyond the traditional studies of dual mutualistic interactions.

As an example of insect–fungus interactions that involves both specific microbial symbionts as well as non-specialized microorganisms is the fungus-growing ant–microbe mutualism. Ants in the tribe Attini (*Hymenoptera*: *Formicidae*) are social insects that have lived in an obligate symbiosis with fungi for the past 50 million years (Schultz & Brady, 2008). These insects cultivate basidiomycetous fungi as their main source of food and digestive enzymes (Mueller *et al.*, 1998; Silva *et al.*, 2003, 2006a; De Fine Licht *et al.*, 2010; Schiøtt *et al.*, 2010). In order to nourish their symbiotic cultivar, workers forage on different substrate types depending on the ant species. Primitive attine ant species (the 'lower attines') forage for seeds, insect frass, and dry plant debris (e.g. withered flower parts). In contrast, the derived attines (the

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'higher attines') use mostly dry plant substrate in addition to bits of fresh plant material (De Fine Licht & Boomsma, 2010). A subset of species within this group, the well-known leafcutter ants, uses fresh leaves and flower parts to manure their fungal gardens (Weber, 1972). Workers of all attine genera process substrate before they incorporate it into the fungus garden, which the ants build as a three-dimensional structure composed of fungal mycelium and the processed substrate. Substrate processing before incorporation into the garden is thought to help remove unwanted alien microorganisms that could compete with or harm the symbiotic cultivar fungus (Fernández-Marín *et al.*, 2006, 2009).

The understanding of the attine symbiosis has changed dramatically in the past decade (Bacci *et al.*, 1995a, b, 2009; Mueller (2002); Mueller *et al.*, 1998, 2005; Schultz & Brady, 2008; Hölldobler & Wilson, 2010; Mikheyev *et al.*, 2010). Leaf-cutting ants (genera *Atta* and *Acromyrmex*) across the Neotropics cultivate a single, phylogenetically narrow fungal lineage (Silva-Pinhati *et al.*, 2004; Mikheyev *et al.*, 2006, 2010), and all fungus gardens propagated by a single colony of ants appear to be a monoculture of a single cultivar clone (Poulsen & Boomsma, 2005; Mueller *et al.*, 2010). Organisms with reduced genetic diversity can be threatened by fast-evolving pathogens and parasites that readily overcome the defenses of a genetically depauperate host population (Hamilton *et al.*, 1990). This seems to be the case of fungus gardens of attine ants, which are subject to significant parasite pressures (Currie *et al.*, 1999a; Currie, 2001; Currie *et al.*, 2003; Rodrigues *et al.*, 2005, 2008).

Fungal parasites in the genus *Escovopsis* (*Ascomycota*, *Hypocreales*) were previously found in association with attine nests (Currie *et al.*, 1999a; Currie *et al.*, 2003). So far, this parasite has only been found in attine gardens and garden dumps, and it is thought to derive nutrients directly from the fungal cultivar (Reynolds & Currie, 2004), thus negatively impacting garden and colony growth (Currie, 2001). In order to protect gardens from the attack of such parasites, ants try to physically weed out *Escovopsis*-infected garden parts (Currie & Stuart, 2001). Some attine ants also appear to use antibiotic-secreting bacteria (in the genus *Pseudonocardia* or *Streptomyces*) found on their bodies to help suppress *Escovopsis* sp. (Currie *et al.*, 1999b; Cafaro & Currie, 2005; Haeder *et al.*, 2009; Barke *et al.*, 2010; Poulsen & Currie, 2010), but application of actinomycete-derived antibiotics to gardens may also inhibit cultivar growth *in vitro* (Sen *et al.*, 2009; Barke *et al.*, 2010). The cultivars, in turn, can inhibit *Pseudonocardia* growth (Poulsen & Currie, 2010). Despite a decade of research (Currie *et al.*, 1999b), the hypothesized primary importance of *Pseudonocardia* symbionts in *Escovopsis* suppression remains puzzling (Mueller *et al.*, 2008; Boomsma & Aanen, 2009; Poulsen *et al.*, 2009; Sen *et al.*, 2009; Barke *et al.*, 2010; Poulsen & Currie, 2010). It is possible that both coevolved and environmentally acquired actinomycetes help ants to defend their gardens against the parasite *Escovopsis* sp. (Barke *et al.*, 2010 and references within).

Attine fungus gardens can be viewed as an interacting community of microorganisms (Bacci *et al.*, 1995b; Santos *et al.*, 2004; Mueller *et al.*, 2005). The fungal cultivar is thought to contribute the main biomass to this community, but several other microorganisms such as bacteria (Bacci *et al.*, 1995b), filamentous fungi (Rodrigues *et al.*, 2005), and yeasts (Craven *et al.*, 1970; Carreiro *et al.*, 1997) are also found in this microbiome, including yeast species only associated with attine gardens, as is the case of *Cryptococcus haglerorum* (Middelhoven *et al.*, 2003) and *Trichosporon chiarellii* (Pagnocca *et al.*, 2010). Most noncultivar microorganisms probably enter the garden communities with the plant substrate or when shed from the ant cuticle, but some noncultivar microorganisms are already present in the inoculum carried by queens to start new gardens (Mueller *et al.*, 2008; Pagnocca *et al.*, 2008). The roles of such secondary microorganisms in the attine ant symbiosis are largely unknown. Santos *et al.* (2004) and Rodrigues *et al.* (2009) demonstrated that bacteria and yeasts, respectively, isolated from leafcutter gardens inhibit *Escovopsis* sp., suggesting that diverse microorganisms in the garden matrix may contribute to the protection of the ant garden against diseases.

Although some microorganisms in attine nests may confer benefits to the ants, others may represent antagonists (pathogens) or competitors for nutrients of the cultivated fungus (Bacci *et al.*, 1995a; Silva *et al.*, 2006b). For example, soil microfungi in the nest walls and endophytic fungi in the gardening substrate may thrive in garden chambers that are optimized by the ants for fungal growth (Fisher *et al.*, 1996; Rodrigues *et al.*, 2008; van Bael *et al.*, 2009). Despite the physical and chemical barriers that ants impose to suppress such unwanted microbial invaders, microfungi persist in surprisingly high abundance in gardens of different attine species (Currie *et al.*, 1999a; Rodrigues *et al.*, 2008). For example, the microfungal genera *Cunninghamella*, *Fusarium*, and *Trichoderma* are frequently found in field gardens of *Acromyrmex* and *Atta* leafcutter ants (Rodrigues *et al.*, 2005, 2008). However, little is known regarding how these fungi interact with ants and whether they are in fact parasites or transient commensals. Unlike the specialized *Escovopsis* sp. (Poulsen & Currie, 2006; Rodrigues *et al.*, 2008), frequent invaders such as *Cunninghamella* sp., *Fusarium* sp., and *Trichoderma* sp. appear to be nonspecialized and generalized antagonists ('garden weeds') of the fungal cultivar (Rodrigues *et al.*, 2005, 2008).

To further characterize the associations between attine ants and garden microfungi, and to assess any species-specific associations between microfungi and ants, we conducted a 1-year study of the phenological changes in microfungal diversity in gardens of three fungus-growing

ants in Central Texas. In addition, we investigated whether the diversity of microfungal communities correlates in part with the ant foraging behavior (Weber, 1956, 1972; Mintzer, 1979; Waller, 1986). Our results indicate that the attine species studied are confronted with a diverse assemblage of microfungi that appears to be transient and nonspecific associates in attine gardens.

Materials and methods

Ant species and sampling design

We surveyed the microfungal community of three attine ant species, *Cyphomyrmex wheeleri* (a lower-attine ant), *Trachymyrmex septentrionalis* (a higher-attine ant), and *Atta texana* (a leafcutter ant). These ants represent three of the five main fungicultural systems in the tribe Attini (Schultz & Brady, 2008). Fungus gardens were collected from these species at four sampling periods: (1) winter (December 2005 and January 2006); (2) spring (March–May 2006); (3) summer (July 2006); and (4) fall (September and October 2006).

At each sampling period, four colonies of *C. wheeleri* were excavated at Bull Creek Park (Austin, Travis County, TX – GPS: 30°22′07″N, 97°47′04″W) and four colonies of *T. septentrionalis* at Stengl 'Lost Pines' Biology Station (Smithville, Bastrop County, TX – GPS: 30°05′04″N, 97°10′23″W), totaling 16 nests for each of these two ant species. *Cyphomyrmex wheeleri* nested in the open areas of a cedar-brake forest with dry clay-sand alluvial soils and sparse ground vegetation (e.g. grasses). In such an environment, *C. wheeleri* workers forage for grass seeds, catkins, diverse fibrous plant material, and arthropod frass (U.G. Mueller, pers. commun.). Nests of *T. septentrionalis* nested in the sandy soil of a pine forest, and workers foraged for dry leaves, seeds, and arthropod frass (Seal & Tschinkel, 2008; A. Mikheyev, pers. commun.; J. Seal, pers. commun.). Both ant species build nests with generally two to four garden chambers. In our study, samples were generally collected from the top-most garden accessed through careful excavation.

Three nests of *A. texana* were sampled at Hornsby Bend Environmental Research Center (Austin, TX – GPS: 30°13′06″N, 97°38′21″W) and a fourth nest was sampled along Park Road 1C connecting Bastrop State Park and Buescher State Park (Bastrop County, TX – GPS: 30°05′49″N, 97°13′29″W). Both sites have sandy soils, but the forest at Hornsby Bend is mostly riverine pecan and oak trees, whereas the forest at Park Road 1C is dominated by pines (comparable to Stengl Biological Station, where we studied *T. septentrionalis*). Because mature nests of *A. texana* have hundreds of garden chambers, we could repeatedly collect from the same nests at 3-month intervals. Most of the fungus garden sampled from *A. texana* had a white-brown color, which indicates that ants did not recently add much plant substrate. However, gardens for nests UGM060121-01 and UGM060121-02 collected in spring 2006 had a dark brown-green appearance, indicating that workers recently added fresh leaf substrate. In fall 2006, the latter type of garden was found in nest UGM060121-02, but not in the other nests sampled at that time.

All leafcutter nests were excavated according to Rodrigues *et al.* (2009) in the center of the mound, where most fresh excavate had accumulated. A deep hole was dug next to the targeted fungus gardens, and then the soil was carefully shaved off at the side of the hole until a garden chamber was exposed. Gardens were extracted intact, and fragments from the center of the fungus garden were collected with some attending ants into sterile containers without disturbing the natural architecture of the garden, then maintained at environmental and room temperatures until processing in the laboratory (i.e. usually within 4–8 h of collection). Fungus gardens were not collected if any soil accidentally came into contact with a garden during excavation. In such cases, we continued our excavation until we could access a garden without soil contamination. The excavated holes were filled after each collection to prevent colony movements that could be triggered by disturbances. In fall 2006, fungus gardens were found in only three of the four *A. texana* nests; in nest AR060123-01, no fungus garden could be found in 4 days of excavation, but many empty chambers and several chambers filled with decayed garden substrate similar in consistency to attine dumps.

Microfungi isolation

We used culture-dependent methods to study the microfungal diversity in attine gardens. Before microfungal isolation, workers, pupae, and larvae were removed from fungus garden samples using a sterile forceps. Fungus garden fragments of about 3 mm^3 were inoculated in three culture media supplemented with 150 $\mu g\,mL^{-1}$ of chloramphenicol (Sigma): (1) 2% malt agar (MA2% in $g\,L^{-1}$: 20 malt extract and 15 agar; Difco), (2) potato dextrose agar (EMBL – Difco), and (3) synthetic nutrient agar (in $g\,L^{-1}$: 1 KH_2PO_4; 1 KNO_3; 0.5 $MgSO_4 \cdot 7H_2O$; 0.5 KCl; 0.2 glucose and 0.2 sucrose). For each sampling period, 20 fungus garden fragments of *C. wheeleri* and *T. septentrionalis*, and 25 garden fragments of *A. texana* were inoculated per medium. Because *T. septentrionalis* gardens shrink during the winter (Weber, 1956), the little garden that we were able to collect in winter could be separated into only 10 fragments for microfungal isolations.

All plates were incubated at 25 °C for 10 days with a 12 h/12 h light–dark regime. Isolation plates were observed for 4 weeks for any noncultivar fungi sprouting out from the garden fragments. Once a growing fungus was observed; it

was subcultured to a new MA2% plate to obtain a pure culture. Isolates were stored live in 10% glycerol at $-80\,^{\circ}C$ for fungal identification.

Microfungi identification

We followed a polyphasic approach for fungal identification, using both morphological and molecular markers. First, all fungal isolates were grown in oatmeal agar and MA2% for initial morphotyping, and then grouped to the lowest taxonomic level using classical taxonomical keys (Ellis, 1971, 1976; Subramanian, 1971; Domsch *et al.*, 1980; Samson *et al.*, 2000). Following this preliminary morphotyping, isolates were inoculated in taxon-specific media to further study the macroscopic and microscopic characteristics that would allow a more precise taxonomic identification. We used specific taxonomic keys for fungi in the following groups: *Aspergillus* sp. (Klich, 2002), *Cunninghamella* sp. (Liu *et al.*, 2001), *Fusarium* sp. (Nelson *et al.*, 1983), *Penicillium* sp. (Pitt, 1979), *Phoma* sp. (Boerema *et al.*, 2004), *Mucorales* (Zycha, 1935), and insect pathogenic fungi (Samson *et al.*, 1988). Fungal isolates that failed to sporulate in oatmeal agar and MA2% were inoculated in potato carrot agar (Hi-Media) and incubated at 25 °C for several months. These isolates were regularly inspected for any reproductive structures that may allow fungal identification.

Third, for molecular identification of the isolated fungi, the internal transcribed spacer region (ITS1-5.8S-ITS2) was sequenced for representative isolates (these fungi are marked with 'S' in Supporting Information, Tables S2–S4). DNA extraction followed the methods of Mikheyev *et al.* (2006); a small quantity of fresh fungal mycelium was collected from culture plates (grown on MA2% for 7 days at 25 °C), heated to 60 °C for 1.5 h, and then boiled for 10 min in the presence of 100 μL of 5% Chelex resin solution (Sigma). PCR amplifications were carried out using a final volume of 10 μL containing: 10 × KCl buffer, 0.8 μM of each primer (ITS4 and ITS5), 1 mM of each dNTPs, 2.5 mM $MgCl_2$, 1 U of Taq polymerase, and 1 μL of DNA extractions. The PCR profile included an initial denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 1 min, 52 °C for 1 min, and 72 °C for 2 min. Sequences were generated on an ABI 3100 Automated Sequencer (Applied Biosystems).

Sequence analysis and taxon definition

Forward and reverse sequences were assembled in BIOEDIT v.7.0.5.3 (Hall, 1999) and used for BLASTN at the NCBI-GenBank database (http://www.ncbi.nlm.nih.gov). Sequences found in the database with the highest nucleotide similarities were considered as the closest relative of the query sequence (Tables S2–S4 for GenBank accession numbers of the closest relatives found).

Pairwise-distance comparisons of ITS sequences were computed in MEGA v.4 (Tamura *et al.*, 2007) using default settings. Sequences with $< 3\%$ sequence variability were treated as the same taxon (see Unterseher & Schnittler, 2010). Tables S2–S4 summarize all taxa that were identified by the combination of morphological and molecular markers. Sequences of representative isolates were deposited at NCBI-GenBank under the accession numbers HQ607791–HQ607830, HQ607832–HQ607892, and HQ607894–HQ608158.

Analysis of microfungal communities

To establish the prevalence of microfungi in the sampled fungus gardens, we calculated, separately for each isolation medium and each season, the mean proportion of garden fragments in which microfungi were present. Nonparametric Kruskal–Wallis and Dunn tests implemented in BIOESTAT v.5.0 (Ayres *et al.*, 2007) were used to test at the 0.05 level for statistically significant differences among treatments.

We assessed the actual microfungal species richness through individual-based rarefaction curves computed in ECOSIM v.7.72 (Gotelli & Entsminger, 2011). Such analyses used the respective datasets presented in Tables S2–S4 to evaluate differences in the observed species richness among seasons. Moreover, individual-based rarefaction curves were computed for each ant species using a comprehensive dataset pooled across seasons for each ant species. Chao 1 species richness estimators and diversity indices (Fisher's alpha, Shannon, and Simpson) were calculated for each seasonal dataset and the pooled datasets using ESTIMATES v.8.2 (Colwell, 2009).

To evaluate differences in microfungal community composition between attine gardens, we used the information summarized in Tables S2–S4 for two additional analyses. In the first analysis, we performed a correspondence analysis on the complete dataset in order to investigate the nonapparent relationships among microfungal communities and the three ant species. Such calculations were carried out in JMP® v.4.0.3 (SAS Institute Inc.). In the second analysis, we used just the conserved 5.8S portion of the ITS region for a phylogenetic-based clustering analysis using the program UNIFRAC (Hamady *et al.*, 2010). A sequence alignment was generated using CLUSTAL X (Larkin *et al.*, 2007) and further aligned in MACCLADE 4.08 (Maddison & Maddison, 2005). An approximate maximum likelihood tree was generated using the GTR model in FASTTREE (Price *et al.*, 2009). We performed a UNIFRAC principal coordinate analysis (PCoA) to evaluate the similarities between microfungal communities associated with the different ant species, between collection location, and between seasons.

Results

Prevalence of microfungi in fungus gardens of attine ants

A total of 1435 microfungal isolates were recovered from fungus garden samples of all three ant species studied in our 1-year survey (Table S1). A higher percentage of garden fragments yielded non-cultivar microfungal isolates from *C. wheeleri* (57%) than from *T. septentrionalis* (46%) and *A. texana* (34%); however, these percentages were not significantly different (Kruskal–Wallis, $H = 0.00$, $P = 1.00$, Fig. 1). On the other hand, 43% of the garden fragments of *C. wheeleri*, 54% of *T. septentrionalis*, and 66% of *A. texana* showed growth of the resident cultivar.

There were no significant differences in the proportion of garden fragments with microfungi across all the three culture media used when comparing within ant species: *C. wheeleri* (Kruskal–Wallis, $H = 0.22$, $P = 0.89$), *T. septentrionalis* (Kruskal–Wallis, $H = 0.65$, $P = 0.72$), and *A. texana* (Kruskal–Wallis $H = 1.13$, $P = 0.56$). However, the proportion of fragments with microfungi significantly differed between the four seasons (Fig. S1) for *C. wheeleri* (Kruskal–Wallis, $H = 12.26$, $P = 0.006$), *T. septentrionalis* (Kruskal–Wallis, $H = 11.45$, $P = 0.009$), and *A. texana* (Kruskal–Wallis, $H = 7.91$, $P = 0.04$). In *C. wheeleri*, there were proportionally fewer fragments with microfungi in spring than in winter, summer, and fall (Dunn test, $z = 2.82$, $P < 0.05$). For *T. septentrionalis*, proportionally fewer microfungi were recovered in winter than in summer fungus garden samples (Dunn test, $z = 3.04$, $P < 0.05$). We observed a tendency to recover less microfungal isolates in summer and fall on *A. texana* garden samples; however, the observed differences were not significant (Dunn test, $z = 3.04$, $P > 0.05$). In addition, there was no significant difference in the proportion of garden fragments with microfungi between freshly growing gardens and mature gardens from *A. texana* (Mann–Whitney, $U = 149$, $P = 0.35$).

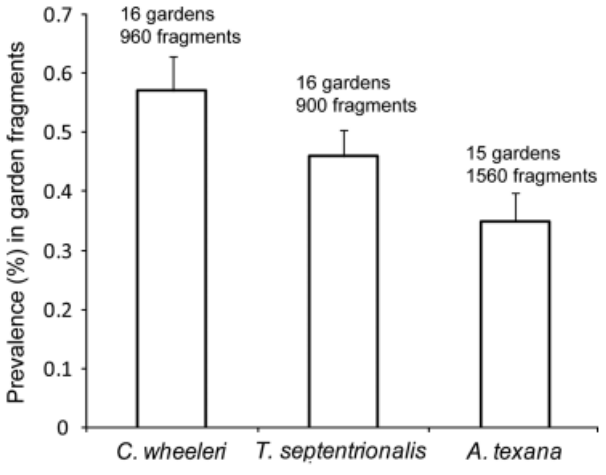

Fig. 1. Proportion of garden fragments from *Cyphomyrmex wheeleri*, *Trachymyrmex septentrionalis*, and *Atta texana* that showed non-cultivar microfungal growth (bars indicate SEs). Sixteen gardens of *C. wheeleri* and *T. septentrionalis* were sampled from individual nests in a year-long survey. For *A. texana*, a total of 15 gardens were sampled from four nests over the same study period.

Microfungal diversity and composition in attine gardens

In the 1-year survey of garden-associated microfungi from *C. wheeleri*, a total of 524 isolates were recovered (Table S1), belonging to 111 observed taxa. These taxa comprise 52 genera, 72 species, and 13 unidentified ascomycetes as determined by our polyphasic approach of fungal identification (Table S2). According to rarefaction analysis of the pooled dataset across seasons (Fig. 2a, Table S5), the Chao 1 richness estimator predicted a total of 292 ± 74.7 taxa (mean $\pm$ SD) in gardens of *C. wheeleri*, which indicates that more sampling effort is necessary to cover the full diversity of microfungi in fungus gardens of *C. wheeleri*.

Microfungi that were consistently present in all seasons in *C. wheeleri* gardens were *Cladosporium cladosporioides*, *Cochliobolus* sp. 1, *Fusarium solani*, and *Nigrospora sphaerica*. However, we detected significant differences in the observed taxon richness across seasons for *C. wheeleri* (Fig. S2a). Microfungi richness was higher during spring and winter when compared with the richness found in summer and fall (Fig. S2a). The prevalent microfungal taxa in each season were (relative to the total number of isolates in each season) *Alysidium* sp. 1 (13%), *Mucorales* sp. 1 (8%), and *N. sphaerica* (8%) in winter; *Verticillium* sp. 1 (12%), *C. cladosporioides* (11%), and *Aspergillus oryzae* (9%) in spring; an unidentified ascomycetes strain (30%), *Cochliobolus* sp. (14%), and an unidentified *Dothideales* sp. 1 (11%) in summer; and *N. sphaerica* (23%), an unidentified *Dothideales* sp. 1 (8%), and *Trichoderma koningii* (7%) in fall (Table S2).

Fungus garden fragments of *T. septentrionalis* rendered a total of 386 isolates (Table S1) belonging to 117 taxa. The observed taxa richness comprises 47 genera, 66 species, 30 unidentified ascomycetes, and one unidentified basidiomycetes fungus (Table S3). The Chao 1 richness estimator predicted a total of 218.16 ± 36.05 taxa (mean $\pm$ SD) in gardens of *T. septentrionalis*, which also indicates that more sampling effort is necessary to cover the full diversity of microfungal taxa in the fungus gardens of such ant species (Fig. 2b, Table S5). In *T. septentrionalis* gardens, microfungi occurring in all seasons were *Mortierella* sp. 1, *Penicillium verruculosum*, *Penicillium* sp. 3, and *Trichoderma harzianum*. When analyzed by season, the observed species richness was not significantly different among the spring, summer, and

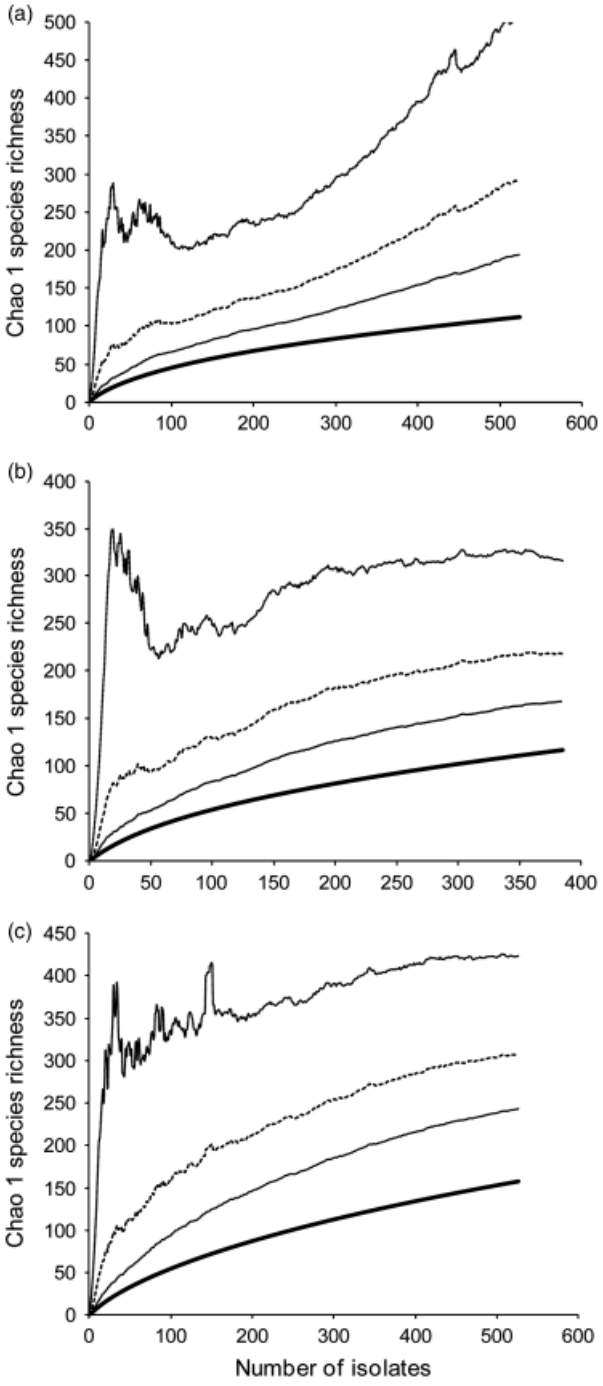

Fig. 2. Individual-based rarefaction curves (dark solid lines) and Chao 1 richness estimators (dashed lines) of microfungal communities in gardens of three attine ants from Central Texas: (a) *Cyphomyrmex wheeleri*, (b) *Trachymyrmex septentrionalis*, (c) *Atta texana*. Curves were generated by pooling samples across seasons for each ant species. Light solid lines represent Chao 1 95% confidence intervals.

fall seasons (Fig. S2b). In the winter season, fewer taxa were recovered from gardens of *T. septentrionalis*, but this was not significantly different from the observed richness of the spring season. The prevalent microfungal taxa in each season were *Penicillium restrictum* (22%) and *Ceratocystis fimbriata* (8%) in winter; *Trichoderma hamatum* (11%) and *Penicillium* sp. 1 (9%) in spring; *Gloeotinia* sp. anamorph (19%), *P. verruculosum* (12.5%), and *Penicillium* sp. 3 (11%) in summer; and *Verticillium* sp. complex (8%) and *Penicillium decumbens* (7%) in fall (Table S3).

Fungus garden fragments of *A. texana* yielded a total of 525 isolates (Table S1) with an observed richness of 153 taxa. A total of 53 genera, 106 species, and 23 unidentified ascomycetes fungi were observed on *A. texana* samples (Tables S4). The Chao 1 richness estimator predicted a total of 308 ± 44.53 taxa (mean $\pm$ SD) in the gardens of *A. texana*, which again indicated that more sampling effort is necessary to cover the full microfungal diversity in the gardens of *A. texana* (Fig. S2c, Table S5). Microfungi occurring in all seasons were *Alternaria alternata*, *Aspergillus ustus*, *Beauveria* sp. 1, and one unidentified ascomycetes. Comparisons across seasons revealed that the observed species richness was significantly higher in spring and summer than for the other seasons (Fig. S2c). The most prevalent microfungal species in such gardens were: *C. cladosporioides* (16%), *A. ustus* (11%), and *Aspergillus ochraceus* (10.8%) in winter; *C. cladosporioides* (8%) and *A. alternata* (7.4%) in spring; an unidentified *Chaetothyriales* sp. 1 (7.6%) and *Penicillium* sp. (7.6%) in summer; and *Cladosporium* sp. (14%), *Cochliobolus australiensis* (10%), and *Cochliobolus lunatus* (9%) in fall (Table S4).

Overall, Fisher's alpha, Shannon, and Simpson diversity indices suggest that *A. texana* and *T. septentrionalis* have similar species richness when compared with fungus gardens of *C. wheeleri* (Table S5). Fungus gardens of *C. wheeleri* harbored a more diverse community in winter and spring than summer and fall (Table S5). In the fungus gardens of *T. septentrionalis*, the microfungal community appeared to be more diverse in spring and fall. In addition, the diversity indices indicate that microfungal communities in *A. texana* gardens were diverse in spring and summer. Interestingly, the specialized parasite *Escovopsis* sp. was not isolated from any of the nests sampled in this study over the 1-year long period.

Garden microfungal community structure

Cluster analyses suggest structuring of microfungal communities in fungus gardens of the three studied ants. Correspondence analysis using taxa identified by a combination of morphological and molecular markers (Tables S2–S4) revealed that microfungal communities clustered by ant genera (Fig. 3). The two main components in the correspondence analysis explain 76.5% of the total variation observed. The analysis also suggests that grouping by ant genera is further resolved by collecting sites as microfungal

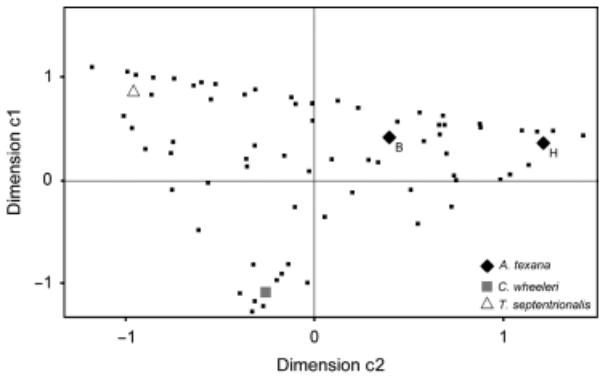

Fig. 3. Correspondence analysis of microfungal community diversity from the three attine ant species *Cyphomyrmex wheeleri*, *Trachymyrmex septentrionalis*, and *Atta texana*. Black squares represent microfungal observations from any of the three ant host (recorded as presence–absence data in a particular ant host). Microfungal communities separate by ant species. The biplot suggests further separation by collection site, as the *A. texana* samples collected at different sites separate in two groups: Hornsby Bend Environmental Research Center (H) and Buescher State Park (B).

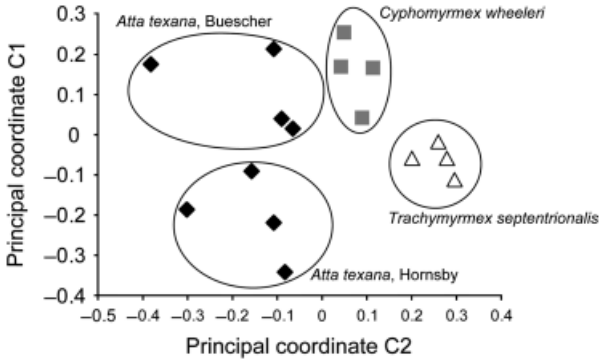

Fig. 4. PCoA of the unweighted UNIFRAC distance matrix based on the 5.8S rRNA gene-region of microfungi associated with fungus gardens of attine ants. Microfungal communities cluster by genus of ant host. The *Atta texana* samples separate out into two distinct clusters, each collected at a different location: Hornsby Bend Environmental Research Center and Buescher State Park.

communities of *A. texana* were sampled in two distinct sites: Bastrop and Hornsby Bend (Fig. 3).

Such findings were also corroborated when the microfungal community was analyzed with just the sequenced fungal isolates (Tables S2-S4). Using a phylogenetic approach, the UNIFRAC PCoA indicates that fungal isolates of each community clustered by ant genera (Fig. 4, the two principal coordinate axes explained 34.5% of the total variation). On the other hand, no clear clustering by season was observed (data not shown). Similar to the correspondence analysis, UNIFRAC grouping revealed that clustering by ant genera is further explained by collection sites because *A. texana* samples collected in Bastrop and Hornsby Bend formed two distinct communities.

Discussion

Fungus gardens of attine ants are a miniature ecosystem of diverse microbial consortia (Bacci *et al.*, 1995b; Mueller *et al.*, 2005; Gerardo *et al.*, 2006; Youngsteadt, 2008). Microfungi are usually thought to be largely transient components of the microbiome in an attine nest (Poulsen & Currie, 2006). This view is supported by the observation that most microfungi associated with attine nests are ubiquitous species that ants may accidentally introduce into their nests. On the other hand, microfungi are usually found in high frequencies in gardens of attine ants in the tropics (e.g. 40.5% and 54%, in field and laboratory nests, respectively; Rodrigues *et al.*, 2005, 2008). These high frequencies suggest a predictable rather than an accidental presence in attine gardens. Our study showed that fungus gardens of three North American attine species harbor as a diverse community of microfungi as gardens of tropical attine ants.

Fungus gardens of *C. wheeleri* had proportionally more microfungi than gardens of *T. septentrionalis* and *A. texana* (Fig. 1). Currie *et al.* (1999a), working with attine ants from Panama, observed a similar pattern of microfungal prevalence across attine genera where gardens of lower-attine ants, such as *Cyphomyrmex* sp., exhibited a higher prevalence of microfungi than gardens of higher-attine ants. In fact, Currie (2004) observed that, even in the presence of garden-tending workers, laboratory nests of lower-attine ants were occasionally overgrown by fungi other than *Escovopsis* sp.; such severe garden infection by generalist microfungi was assumed to be rare in laboratory gardens of higher-attine ants (Currie, 2004), but additional data are needed to support this view.

The observed proportions of microfungi in attine gardens are likely associated with (1) the mycobiota found in soils next to ant nests (Hughes *et al.*, 2004), (2) the type of plant substrate collected by the ants, or (3) both. Soil adjacent to ant nests may be a source of microfungi (Hughes *et al.*, 2004) and ants at different sites may be in contact with different soil microfungal communities (see the discussion below). Another explanation is that lower- and higher-attine ants may treat microfungi in different ways. For example, lower-attine ants engage in a less elaborative substrate preparation process when compared with higher-attine ants (Mangone & Currie, 2007), and lower-attine ants utilize on average more diversified gardening substrate than higher-attine ants (De Fine Licht & Boomsma, 2010). Specifically, lower-attine ants remove alien contaminants after the substrate becomes incorporated into the fungus gardens. In contrast, higher-attines engage in a more elaborative substrate preparation by removing contaminants from plant substrate before incorporation into the fungus garden (Magone & Currie, 2007). Additionally, higher-attine ants have special behaviors and adaptations like weeding and

grooming of fungus gardens in order to remove *Escovopsis* sp. (Currie & Stuart, 2001). Perhaps, these adaptations may also increase the removal of generalist microfungi in the garden matrix, contributing to the observed low proportions of microfungi in *A. texana* gardens in comparison with other attine species. In support of this view, Rodrigues *et al.* (2004) observed that workers of *Atta sexdens rubropilosa* intensively removed fungus garden fragments after inoculation with spores of microfungi (e.g. *Syncephalastrum racemosum*) other than *Escovopsis* sp.

Our culture-dependent approach revealed that the most abundant microfungal species from gardens of the three target ant species were *A. ustus* (2.4%, relative to the total number of isolates), *A. ochraceus* (1.8%), *C. cladosporioides* (6%), *Cochliobolus* sp. 1 (2.7%), and *N. sphaerica* (4.2%), well-known cosmopolitan representatives that are common in soil and plant substrates (Tables S2–S4). Rodrigues *et al.* (2008) likewise found soil microfungi such as *Cunninghamella* sp., *Fusarium* sp., and *Trichoderma* sp. prevalent in gardens of leaf-cutting ants in south Brazil. These microfungal genera were also recovered in the present study, but they were represented by only a few isolates (Tables S2–S4). Although most microfungal species were not consistently present throughout all four sampling periods, a few species were persistent. For example, microfungal taxa such as *C. cladosporioides*, *Cochliobolus* sp. 1, *F. solani*, and *N. sphaerica* were found in all seasons in *C. wheeleri* gardens (Table S2). Again, these are ubiquitous taxa found in diverse environments such as soil, plant material, or even air samples (Samson *et al.*, 2000; Crous *et al.*, 2009).

Because no particular set of microfungi was associated with only one of the three ant species, our survey revealed no clear species-specific associations between microfungal species and the three ant species studied. Similarly, Rodrigues *et al.* (2008) found a diffuse association of microfungal species and several *Acromyrmex* species from southern Brazil. If more subtle species-specific interactions between microfungi and attine ants exist, these were not detected in the present study. A survey of a large number of gardens will be necessary to evaluate such subtle specificities.

Despite the apparent lack of specific ant–microfungus associations in our study, correspondence analysis revealed distinct microfungal communities between the three ant species studied (Fig. 3). These differences could be because ants foraged on different substrates, which may have contained different microfungal species. A second factor explaining the structuring of microfungal communities is habitat (collection location). For example, the *A. texana* samples collected in different sites cluster in two separate groups (samples collected at Horsnby Bend and at Buescher State Park) in the correspondence and UNIFRAC analyses (Figs 3 and 4). Thus, ants collected in different sites may be exposed to different microfungal communities present in the soil and in the gardening substrates utilized, which could account for the observed differences. Indeed, the soil mycobiota next to ant colonies may be a source of microfungi that may potentially infects the fungus gardens (Hughes *et al.*, 2004).

In contrast to our expectations, differences in microfungal diversity across seasons were not correlated with ant foraging behavior. For example, in the northern hemisphere, attine ants decrease their foraging activity during the colder periods of the year (Mintzer, 1979), and in extreme cases, such as *T. septentrionalis*, workers completely cease their activities during the winter (Weber, 1956; Seal & Tschinkel, 2008). Thus, if fungal diversity in gardens is mostly driven by the foraging behavior of ants, we expected that, compared with warmer seasons, we would find reduced diversity of microfungal species in months with low temperatures and little addition of substrate (Weber, 1972). However, this was not the case; rarefaction curves and diversity indices predicted the same number of species in gardens of *C. wheeleri* and *T. septentrionalis* for the winter and summer seasons as for the winter and fall (Fig. S2, Table S5). Moreover, microfungi prevalence in garden fragments was not significantly different between winter and summer for *C. wheeleri* as for spring and fall for *T. septentrionalis* (Fig. S1). On the other hand, the results from *A. texana* gardens seem to support our expectation because the diversity of microfungi species in spring and summer was higher than that for winter (Fig. S2). In fact, Chao 1 estimated microfungal species richness for *A. texana* gardens as 123 (spring) and 271 (summer), respectively, compared with 88 (winter) and 85 (fall) (Table S5). Our results indicate that additional factors other than the ant foraging behavior (see the above discussion on microfungal community in soils next to ant nests) contributed to the observed differences in microfungi prevalence (Fig. S1) and species richness (Fig. S2) over the seasons.

A surprising finding was the complete absence of the parasite *Escovopsis* sp. in the gardens of all three surveyed attine ant species. Using the same isolation methods described above, additional sampling of gardens of 12 young *A. texana* nests (~6 months old) from east Texas revealed no *Escovopsis* sp. (A. Rodrigues, unpublished data). Finally, a nest of *A. texana* from which hundreds of garden pieces were sampled by C.R. Currie from dozens of gardens in February 2001 did not yield any *Escovopsis* isolate (U.G. Mueller & C.R. Currie, unpublished data). According to Currie *et al* (1999a), *Escovopsis* sp. is found at high frequencies in gardens of attine ants from Central America, with as many as 50% of the colonies infected with *Escovopsis*, depending on the ant genus. Gerardo *et al.* (2006) found that ants in the genus *Apterostigma* (a lower-attine ant) from Central America may have up to 52% of gardens infected by *Escovopsis* sp. In south Brazil (State of Rio Grande do Sul), *Escovopsis* sp.

prevalence in gardens was estimated at 27% for *Acromyrmex* gardens (Rodrigues *et al*., 2008). In contrast to these tropical populations, our study indicates that *Escovopsis* appears to be rare or absent in attine gardens in Central Texas.

The observation that *Escovopsis* sp. may be locally infrequent should stimulate further studies on the geographic variation of *Escovopsis* sp. prevalence in attine gardens. Such studies would benefit from intensive and systematic sampling from different localities, especially in other northern temperate areas as well as southern temperate regions and high-altitude habitats. Determining *Escovopsis* sp. incidence in these localities might reveal additional aspects of the attine ant–microorganism symbiosis as observed in the cold-tolerant fungal symbionts cultivated by *A. texana* in the northern range of occurrence of this ant species (Mueller *et al*., 2011).

Using culture-dependent methods, the present study shows that the attine garden microbiome harbors diverse microfungal species. The absence of clear species-specific associations of garden microfungi or a clear correlation with ant foraging preferences suggests that most garden microfungi are transient components in such an environment. Although microfungi may act as transient antagonists of the ant cultivar (Silva *et al*., 2006b); this contrasts with the roles reported for *Burkholderia* sp. bacteria (Santos *et al*., 2004), actinobacteria (Sen *et al*., 2009), nitrogen-fixing bacteria (Pinto-Tomás *et al*., 2009), and plant biomass-degrading bacteria (Bacci *et al*., 1995b; Suen *et al*., 2010), which appear to be resident symbionts in attine gardens.

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Supporting Information

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

Fig. S1. Seasonal changes in the proportion of garden fragments from *Cyphomyrmex wheeleri*, *Trachymyrmex septentrionalis* and *Atta texana* that yielded some microfungal growth.
Fig. S2. Individual-based rarefaction curves of microfungal communities associated with fungus-growing ants from a year-long survey in Central Texas.
Table S1. Number of microfungal isolates recovered from three attine ant species during a year-long survey in Central Texas (2005–2006).
Table S2. Microfungi associated with fungus gardens of *Cyphomyrmex wheeleri* surveyed in Winter, Spring, Summer, and Fall 2005/2006 in Central Texas.
Table S3. Microfungi associated with fungus gardens of *Trachymyrmex septentrionalis* surveyed in Winter, Spring, Summer, and Fall 2005/2006 in Central Texas.
Table S4. Microfungi associated with fungus gardens of *Atta texana* surveyed in Winter, Spring, Summer, and Fall 2005/2006 in Central Texas.
Table S5. Observed species richness, richness estimators, and diversity indices of microfungal communities associated with fungus gardens of attine ants in Central Texas.

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