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Comparative analysis of fungal communities in colonies of two leaf-cutting ant species with different substratum preferences

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ABSTRACT

Fungus gardens of leaf-cutting ants harbor diverse alien fungi in addition to their fungal cultivar. Previous work suggested that alien microorganisms are likely derived from the substrata foraged by ant workers and incorporated into the fungus gardens. To test this hypothesis, we sampled 1014 garden fragments from 16 field colonies of *Atta sexdens rubropilosa* (a dicot-cutting ant) and *Atta capiguara* (a grass-cutting ant) in Brazil. From a total of 615 fungal isolates recovered, we observed similar diversity of fungi between colonies of both ant species. However, fungal communities differed in composition of taxa between ant colonies. *Trichoderma spirale*, *Trichosporon chiarellii* and *Penicillium citrinum* were prevalent accounting for 18.5%, 12.2% and 11.7% of the total isolates, respectively. As expected, fungal communities clustered in two major groups supporting the hypothesis that plant substratum has an impact on the composition of the alien fungi found in leaf-cutting ant gardens.

1. Introduction

Leaf-cutting ants are a paramount example of interactions between insects and multiple microbial symbionts. They belong to the tribe Attini (Hymenoptera: Formicidae) which comprises ants that cultivate fungi for food. Depending on the species, ant workers collect either fresh dicot or monocotyledonous leaves as substratum to nourish the mutualistic fungus, *Leucoagaricus gongylophorus* (Basidiomycota: Agaricaceae). In turn, the fungus is the main food source for the brood (Weber, 1972; Silva et al., 2003). Workers accumulate the foraged plant material in the fungus garden, a sponge-like structure composed of fungal mycelium and plant substratum carefully tended by the ants (Weber, 1972). Due to their leaf-cutting habit, these ants are considered pests in agricultural areas causing major economic losses, especially in South America (Della Lucia et al., 2014).

The fungus garden harbors a complex microbiome in addition to the resident fungal mutualist, including filamentous fungi, yeasts and bacteria (Möller, 1893; Fisher et al., 1996; Carreiro et al., 1997; Currie et al., 1999a; Rodrigues et al., 2008; Suen et al., 2010; Montoya et al., 2016). Microbes found in this substratum may be commensals (transients) or may act as (i) auxiliary-microbes in the garden enzymatic metabolism (Suen et al., 2010; Ayward et al., 2012), (ii) as disease-suppressing organisms (Rodrigues et al., 2009; Ishak et al., 2011) or (iii) as pathogens such as the specialized fungal parasite *Escovopsis* (Currie et al., 1999a). Except for the latter fungus which is only found in association with attine ant gardens, most microbes present in the fungus garden are likely derived from the soil adjacent to ant colonies, from the integuments of workers and alates (Little and Currie, 2007; Arcuri et al., 2014; Atilli-Angelis et al., 2014) or from the plant substratum used for nourishing the fungal mutualist (Van Bael et al., 2009, 2012a; Coblentz and Van Bael 2013).

Despite the significant effort dedicated to characterize the microbiome in the fungus garden, little is known about the factors that determine the structure of garden microbial communities. Rodrigues et al. (2011) reported changes in the dynamics of

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filamentous fungal communities in ant gardens over a year-long survey suggesting that the type of substratum may explain variations in fungal diversity. However, the influence of different substrata in alien fungal communities (i.e. fungi not cultured by the ants) of attine gardens has not been systematically studied to date.

Leaf-cutting ant species exhibit diverse preferences for plant substrata to cultivate their fungi. For example, *Atta sexdens rubropilosa*, the most widespread leaf-cutter ant in Brazil, cuts leaves from dicotyledonous plants (Fowler et al., 1986; Andrade et al., 2002). On the other hand, *Atta capiguara* and *Atta bisphaerica* are typical grass-cutting ants mostly found in pastures and grasslands (Fowler et al., 1986; Garcia, 2005). Regardless of the type of substratum, workers process the plant material which decreases the alien fungal loads and the diversity of fungi before incorporating it into the fungus gardens (Andrade et al., 2002; Van Bael et al., 2009, 2012b; Diniz and Bueno, 2010). Exploring the differences between ant species with distinct substratum preference is useful to shed light on whether fungi other than *L. gongylophorus* are either transients or resident components of attine ant gardens. To assess the influence of substratum preferences in fungal communities, we evaluated the dynamics of such fungi in gardens of *A. capiguara* and *A. sexdens rubropilosa* in two consecutive years.

2. Materials and methods

2.1. Fungus garden sampling

Colonies of *A. capiguara* (a grass-cutting ant) and *A. sexdens rubropilosa* (a dicot-cutting ant) were located on a farm in the municipality of Botucatu, São Paulo state, Brazil (Table S1). At this site *A. capiguara* cuts grasses in a field used to rear cattle, whereas *A. sexdens rubropilosa* nests and forages adjacent to the pasture within stands of *Eucalyptus* trees.

Four colonies of *A. capiguara* and four colonies of *A. sexdens rubropilosa* were excavated in April 2012, and four additional colonies of each ant species were sampled in January and March 2013 ($n = 16$ colonies sampled in total, Table S1). Because these two species have fungus gardens enclosed in underground chambers, colonies were carefully excavated following Rodrigues et al. (2009). Based on field observations *A. sexdens rubropilosa* colonies were apparently more than 4 years-old and *A. capiguara* colonies were more than 2 years-old (except for colony N10 that had the same age then as *A. sexdens* colonies). All ant colonies had multiple garden chambers.

Fungus gardens from the top chambers of each colony, along with tending ants and brood, were collected and kept in UV-sterilized plastic containers with a thin layer of plaster in the bottom which was moistened with 4 ml of sterile distilled water. Samples were transported to the laboratory and kept at 25 °C for up to 4 d after collecting (Table S1). Over this time, no leaves were provided to the ants, and the exhausted fungus garden parts separated by the ants were removed from the containers with a sterile spatula. Ant foragers and minor workers were collected and stored in 96% alcohol as vouchers, and are kept at the Center for the Study of Social Insects (UNESP, Rio Claro).

2.2. Fungal isolation and identification

To check the prevalence of alien fungi in each sample, garden fragments (approximately 3 mm^3) without workers or brood were inoculated on three different culture media: potato dextrose agar (PDA, Acumedia), 2% malt agar (MA2% Acumedia) and synthetic nutrient agar (SNA), all supplemented with 150 $\mu g\ ml^{-1}$ of chloramphenicol (Sigma). A total of 25 garden fragments were inoculated on five Petri plates (five fragments per plate) in each of the three culture media. Thus, a total of 75 garden pieces were plated for each of the 16 samples, totaling 1200 garden pieces examined in the study. The garden pieces were randomly removed from the top and bottom parts of the fungus gardens, which represent the fresh and old garden parts, respectively. Plates were incubated at 25 °C up to 14 d and observed daily for fungal growth. Once a fungus was detected in a garden piece, mycelium fragments (or spores in the case of sporulating fungi) were transferred to a MA2% plate. Then the corresponding garden piece from the isolation plate was removed with an ethanol-flamed spatula and discarded, to prevent overgrowth by fast-growing fungi.

Fungi subcultured in MA2% were initially grouped into morphospecies according to colony morphology and microscopic characteristics of reproductive structures. The latter were observed in wet-mounts and compared to those available in taxonomic keys (Barron, 1968; Carmichael et al., 1980; Domsch et al., 1980; Pitt, 2000; Samson et al., 2000). Fungi that did not sporulate were inoculated on oatmeal agar (OA) containing sterile banana leaves and incubated at 25 °C in the dark up to 2 months. After this procedure, those fungi which did not sporulate were considered sterile mycelia. Representative isolates are kept at UNESP - Microbial Resources Center (CRM - UNESP) in 10% glycerol at - 80 °C, and are available upon request.

The internal transcribed spacer (ITS) region of the rDNA gene was amplified using primers ITS4 and ITS5 (White et al., 1990) for representative isolates from each morphospecies. PCR conditions followed Rodrigues et al. (2014) and amplicons were cleaned up with the Wizard® SV Gel and PCR Clean-Up System kit (Promega). Purified amplicons were quantified with a NanoDrop® (Thermo Scientific) and 20 ng of DNA was prepared for sequencing using BigDye Terminator® v. 3.1 Kit (Life Technologies) according to the manufacturer's protocol. Forward and reverse sequences were generated using the same primers on an ABI 3330xl (Life Technologies) and compiled into contigs in BioEdit v. 7.0.5.3 (Hall, 1999). Sequences generated in the present study were deposited in the NCBI-GenBank database under accession numbers: **KR093827 – KR093967**.

Contigs were queried for homologous sequences at the NCBI-GenBank and the CBS (www.cbs.knaw.nl) databases. Sequences that showed over 97% identity with those deposited in the databases were considered if they were consistent with morphology (Unterseher and Schnittler, 2010; see also Table S3). For isolates belonging to the genus *Trichoderma* we used the *Trich*OKEY barcode database to find the best matches (Druzhinina et al., 2005).

To further ensure correct taxonomic affiliation of fungi, phylogenetic trees were inferred using homologous sequences retrieved from the databases. Sequences were aligned using MAFFT v. 7.110 (Katoh and Standley, 2013). The trees were generated in MEGA v. 5.2 (Tamura et al., 2011), using the neighbor-joining algorithm, Kimura 2-parameter as nucleotide substitution model and 1000 bootstrap pseudoreplicates. Such phylogenies were generated for each major group of fungi found in the present study and are available upon request.

2.3. Analyses of fungal communities

The prevalence of fungi in gardens of *A. capiguara* and *A. sexdens rubropilosa* was compared using the proportion of garden fragments with alien fungi. A test for equality of proportions was used to compare the proportions of garden fragments positive for alien fungi between ant species and between culture media. This comparison was carried out in R v. 3.0.1 (R Development Core Team, 2013). In addition, rarefaction curves were built for comparison between communities (Colwell, 2013), and the estimated richness of fungal taxa was calculated using the Chao 1 (Magurran and Gill,

2011). For fungal diversity comparisons between samples, the inverse Simpson and Shannon indices were used. The Jaccard, Sorensen and Bray-Curtis indices were also calculated to determine the shared taxa between the communities. All the analyses were done in EstimateS v. 9.1.0 (Colwell, 2013), and a Mann-Whitney test was employed in R v. 3.0.1 to compare the indices. In addition, the number of taxa obtained from each community was used for the construction of a Venn diagram in R v. 3.0.1.

To investigate the structure from all 16 fungal communities we carried out a principal coordinate analysis (PCoA) using the Bray-Curtis similarity index. Additionally, we employed SIMPER test to verify the contribution of each species to the overall differences in fungal composition among ant species (Clarke, 1993). Both PCoA and SIMPER analyses were carried out in Past v. 2.17c (Hammer et al., 2001).

3. Results

3.1. Prevalence of alien fungi in gardens of leaf-cutting ants

A total of 1200 fungus garden fragments were sampled from 16 colonies of two leaf-cutting ant species. Of these, 186 (15.5%) were contaminated with bacteria and no fungi grew on these fragments. From the remaining fragments (n = 1014), in 391 (38.5%) only the mutualistic fungus was detected, and in 623 fragments (61.5%) alien fungi were observed (Table 1).

Using culture-dependent techniques we recovered a total of 624 fungal isolates from gardens of both leaf-cutting ant species, and in only one garden fragment were two different species detected. Thus, we recovered a total of 323 and 301 fungal isolates from *A. capiguara* and *A. sexdens rubropilosa* gardens, respectively. We observed a high prevalence of alien fungi in the garden matrix for both ant species. The overall proportion of garden fragments positive for alien fungi significantly differed for *A. capiguara* (72%) and for *A. sexdens rubropilosa* (53%, Chi-squared = 37.683, df = 1, $p < 0.05$, Fig. 1a). The isolation procedure did not show differences in the proportion of fungi recovered between the three different culture media (Chi-squared = 3.8505, df = 2, $p > 0.05$, Fig. 1b).

3.2. Composition of alien fungal communities in gardens of leaf-cutting ants

After purification of fungal cultures, a total of 615 isolates were considered axenic and identification attempted. Our morphotyping approach rendered 365 morphospecies from which 353 were successfully sequenced. The remaining 12 morphospecies were identified only by morphological markers due to unsuccessful amplification of the ITS region (Table S3).

Overall, our polyphasic approach for fungal identification recovered 61 genera, 135 species and 10 non-identified isolates (Table S3). *Trichoderma spirale* (18.5% from 615 isolates), *Trichosporon chiarellii* (12.2%) and *Penicillium citrinum* (11.7%) were the most abundant fungi. All other fungi occurred below 4%, including *Escovopsis* (Table 2).

In the fungus gardens of *A. capiguara*, 47 genera, 70 species and five unidentified fungi were obtained. *T. spirale* was the prevalent species represented by 23.8% of isolates, followed by *P. citrinum* representing 23.2% of the isolates. *Escovopsis* sp. corresponded to 8% of the isolates in *A. capiguara* gardens. Taxa that occurred in less frequency in such gardens include unidentified Dothideomycetes isolates (4.5%), *Xylaria* sp. 4 (3.5%) and *Bipolaris micropus* (2.9%); the remaining isolates occurred in proportions below 2% (Table 2).

In the fungus gardens of *A. sexdens rubropilosa*, 57 genera, 84 species and 5 unidentified fungi were obtained. The yeast *T. chiarellii* was prevalent in those gardens, represented by 25.4% of the isolates, followed by *T. spirale* with 13.5% of the isolates. Taxa such as *Preussia* sp. 1, *Mycosphaerella* sp. 1 and isolates from the Chaetothyriales order represented 4.4%, 3.4% and 2.4%, respectively. The remaining isolates occurred in proportions below 2% (Table 2).

T. spirale was isolated from gardens of both *A. capiguara* and *A. sexdens rubropilosa* (9 out of the 16 colonies) and in all sampling periods. On the other hand, *P. citrinum* occurred only in colonies of *A. capiguara* (4 out of 8 colonies) in the 2012 sampling period. *T. chiarellii* was only found in colonies of *A. sexdens rubropilosa* (3 out of 8 colonies) in all sampling periods. *Escovopsis* sp. was isolated from gardens of both ant species and occurred in 19% of the colonies sampled.

3.3. Alien fungal communities in the gardens varies with ant substratum preferences

The Simpson and Shannon indices indicate that there was no significant difference in the diversity of alien fungi between gardens of *A. sexdens rubropilosa* and *A. capiguara* (Mann-Whitney, $p > 0.05$, Figure S1). According to the Chao-1 index, there were no significant differences in estimated richness of fungi between gardens of *A. sexdens rubropilosa* and *A. capiguara* (Mann-Whitney, $p > 0.05$, Figure S1).

Rarefaction curves suggest that gardens of both ant species have similar richness of fungal taxa (Figure S2a). However, differences in richness were observed between sampling periods (Figure S2b), showing that gardens of *A. sexdens rubropilosa* have higher richness of fungal taxa than gardens of *A. capiguara*, particularly in the samples collected in 2013. Comparing richness between sampling periods both gardens of *A. sexdens rubropilosa* and *A. capiguara* showed similar richness in the 2012 and 2013 sampling periods (Figure S2b). No rarefaction curve reached an asymptote, suggesting that more sampling effort is necessary to unravel the total richness of fungi in fungus gardens of leaf-cutting ants.

The Jaccard (0.03 ± 0.01), Sorensen (0.06 ± 0.02) and Bray-Curtis (0.05 ± 0.02) indices revealed that gardens of both ant species shared few fungal taxa. Different collection periods for each individual ant species also showed a low number of shared fungal species revealing a dynamic composition in the alien fungal community (Table S2). Although the number of fungal taxa shared between both *Atta* species was low (n = 19), these taxa accounted for a total of 198 isolates, which is a figure close to the number of isolates from the taxa found exclusively in gardens of *A. capiguara* (n = 188 isolates) and *A. sexdens rubropilosa* (n = 229 isolates, Figure S3). This result suggests that the shared species are

Table 1
Incidence of alien fungi in garden fragments from two leaf-cutting ant species in two consecutive years.

Ant colonies								
Garden fragments	*Atta capiguara* 2012				*Atta sexdens rubropilosa* 2012			
	N1	N2	N3	N4	N5	N6	N7	N8
A	39	28	37	66	75	42	23	60
T	42	46	44	72	75	67	72	60
Garden fragments	*Atta capiguara* 2013				*Atta sexdens rubropilosa* 2013			
	N9	N10	N11	N12	N13	N14	N15	N16
A	30	73	32	18	43	14	4	39
T	59	73	59	53	73	72	75	72

A: number of garden fragments with alien fungi.
T: total number of sampled fragments.
All the other garden fragments (the difference between T-A) only had the mutualistic fungus (*Leucoagaricus gongylophorus*).

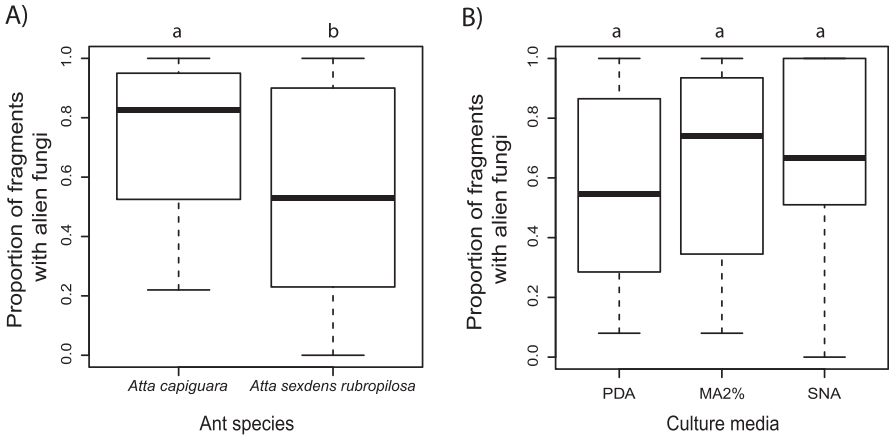

Fig. 1. Proportion of fungus garden fragments with the presence of alien fungi in gardens of the leaf-cutting ants (A) *Atta capiguara* and *Atta sexdens rubropilosa* considering the eight colonies sampled for each ant species; and (B) in relation to the culture media used for fungal isolation (PDA - potato dextrose agar, MA 2%–2% malt agar and SNA - synthetic nutrient agar). Box-plots with different letters at the top show significant differences ($p < 0.05$) in the overall proportions. Bars indicate the maximum and the minimum values of proportions of garden fragments with alien fungi.

abundant and common taxa in gardens of these ants.

Structuring analysis (PCoA) showed that fungal communities clustered according to leaf-cutting ant species for most colonies, indicating that distinct substratum preferences are involved in the fungal community structure. Colonies of *A. sexdens rubropilosa* formed a tight cluster in comparison to the colonies of *A. capiguara* (Fig. 2). Also, fungal communities from gardens of *A. capiguara* clustered according to the collection period (coordinate 2 in Fig. 2). Conversely, fungal communities from gardens of *A. sexdens rubropilosa* gardens did not cluster by collection periods (Fig. 2).

A similarity analysis (SIMPER) revealed that *T. spirale*, *P. citrinum*, *T. chiarellii* and *Escovopsis* sp. were the taxa that most contributed to the observed differences in composition between fungal communities of *A. capiguara* and *A. sexdens rubropilosa* gardens. These fungi contributed approximately 40% of the overall differences in composition between communities of the two ant species.

4. Discussion

Here, we characterized the fungal communities in gardens of two leaf-cutting ant species that use different plant substrata to cultivate their mutualistic fungus. Reports suggest that prevalence of alien fungi in fungus gardens differ between attine ant genera (Currie et al., 1999a; Rodrigues et al., 2011). Using a similar method for fungal isolation, both studies recovered lower proportions (<40%) of alien fungi in gardens of leaf-cutters (either from Central America and North America) compared to gardens of the lower attine genera (>70%, Currie et al., 1999a; Rodrigues et al., 2011). Our results showed that *A. sexdens rubropilosa* and *A. capiguara* gardens had higher percentages of alien fungi (53 and 72%) in comparison to the previous reports. Together, these data demonstrate that the prevalence of fungi is variable in gardens when different culture media are used for fungal isolation. In addition, as indicated in Table 2 fungi are derived from different sources and may occur mainly as spores (for example conidia) in the attine fungus gardens. However, the occurrence of other types of fungal structures in the garden matrix (such as mycelium fragments and resting spores) cannot be ruled out.

Differences in the proportion of fungi between garden fragments of the two leaf-cutting ant species are likely related to variations in substratum processing, since *A. sexdens rubropilosa* workers groom the entire substratum surface, then they chew the material into small pieces (less than 1 mm) of substratum that are incorporated in the fungus garden (Fowler et al., 1986; Andrade et al., 2002). On the other hand, *A. capiguara* workers groom certain regions of the substratum and do not chew the material after cleaning, so that large plant fragments are incorporated in the garden (Garcia, 2005). Because *A. capiguara* does not chew the entire plant material, this likely allows higher proportions of alien fungi compared with *A. sexdens rubropilosa* (Yek et al., 2012).

In this context, we expected differences in richness and diversity of fungi between gardens of the two ant species. Instead, our findings indicated that richness and diversity of fungi between gardens were similar (Figures S1 and S2a). Although substratum processing differs between ant species, both insects need to protect the fungus gardens from the growth of undesirable fungi that compete with the mutualistic cultivar (Silva et al., 2006; Folgarait et al., 2011). In fact, ants and their microbial symbionts control alien fungal populations by secreting antimicrobial compounds that inhibit antagonistic microbes (Currie et al., 1999b; Mueller, 2012), potentially explaining why the richness and diversity are similar. Another aspect that may explain this result is the bias in the isolation method. It is possible that the culture media used in this study recovered certain fungal species which exhibit fast-growing behavior. For example, *T. spirale* was found in high proportions possibly due to its fast-growing behavior in artificial media, which might prevent the isolation of slow-growing fungi and thereby affect the estimation of richness and diversity of fungi in gardens of both ant species.

Despite the similarities in richness and diversity of fungi, gardens of *A. sexdens rubropilosa* and *A. capiguara* showed unique fungal composition with few shared fungal taxa. Most interesting, *P. citrinum* and *T. chiarellii* were found as the most abundant taxa in gardens of *A. capiguara* and *A. sexdens rubropilosa*, respectively. Since *P. citrinum* is a cosmopolitan fungus found in several environments and has been observed in association with monocotyledonous plants (Domsch et al., 1980; Samson et al., 2000), it is certainly plausible that it has a transient nature in attine gardens. On the other hand, *T. chiarellii* has so far only been isolated from attine ant nests (Pagnocca et al., 2010; Arcuri et al., 2014) although a specific relationship between this yeast and ants has not been investigated. Although *T. chiarellii* was isolated from three nests of *A. sexdens rubropilosa*, in one of them this yeast represented 80% of the isolates, suggesting a possible symbiotic role in the ant gardens. The fact that we did not find this yeast in gardens of *A. capiguara*

Table 2
Identification, abundance and putative origin of fungi isolated from fungus gardens of two leaf-cutting ant species examined in the present study.

Fungal taxa	N. of isolates	Abundance	Putative origin	References[a]
Mucoromycotina				
Absidia cylindrospora	1	0.16	soil	20
Cunninghamella sp.	1	0.16	soil	20
Gongronella butleri	2	0.33	soil; root	71
Mortierella sp.	1	0.16	soil	27
Mucor spp.	5	0.82	soil	20; 71
Umbelopsis nana	1	0.16	root; soil; wood	71
Ascomycota				
Acremonium sp.	1	0.16	endophytic	39
Alternaria sp.	1	0.16	endophytic	37
Aspergillus fumigatus	2	0.33	mushroom substrate cultivation	60
Aspergillus sp.	1	0.16	marine sponge	59
Beltrania pseudorhombica	1	0.16	plant: *Pinus tabulaeformis*	16
Bipolaris micropus	9	1.46	clinical isolated	17
Chaetothyriales	7	1.14	ant: *Cladomyrma petalae*	70
Chloridium sp.	1	0.16	decomposing grasses	62
Cladophialophora sp.	4	0.65	plant: *Phyllostachys bambusoides*	12
Cladosporium cladosporioides	4	0.65	soil; air; plants	7
Cladosporium flabelliforme	1	0.16	plant: *Melaleuca cajuputi*	7
Cladosporium perangustum	11	1.79	plants associated	7
Cladosporium spp.	9	1.47	soil; air; plants	7
Clonostachys rosea	2	0.33	fungus garden	56
Colletotrichum spp.	6	0.97	phytopathogen; endophytic	21; 69
Curvularia trifolii	3	0.49	sorghum	22
Curvularia spp.	6	0.98	sorghum	22
Cytospora variostromatica	2	0.33	isolated from eucalyptus	2
Cytospora eucalypticola	1	0.16	isolated from eucalyptus	2
Cytospora sp.	1	0.16	skin	1
Discosia sp.	2	0.33	leaves	15
Dothideomycetes	14	2.28	lichen fungus associated	67
Epicoccum nigrum	2	0.33	fungus garden	56
Epicoccum sorghinum	2	0.33	maize	25
Escovopsioides nivea	2	0.33	fungus garden	4
Escovopsis sp.	26	4.23	fungus garden	4
Eutypella spp.	2	0.33	endophytic	37
Fusarium equiseti	3	0.49	clinical isolated	43
Fusarium cf. *oxysporum*	2	0.33	soil	33
Fusarium cf. *solani*	4	0.65	plants	64
Geomyces sp.	4	0.65	endophytic	29
Guignardia sp.	2	0.33	plant: *Citrus maxima*	72
Hypocreales	1	0.16	termite: *Odontotermes formosanus*	61
Metarhizium carneum	1	0.16	soil	32
Mycoleptodiscus indicus	1	0.16	fungus garden	56
Mycosphaerella sp. 1	26	4.23	plant: *Eucalyptus* spp.	13; 14; 48
Neofusicoccum eucalyptorum	1	0.16	plant: *Blepharocalyx salicifolius*	49
Neofusicoccum parvum	3	0.49	plant: *Populus nigra*	58
Nigrospora oryzae	2	0.33	plant: *Arundo donax*	73
Nigrospora sp.	2	0.33	fungus garden	56
Ochroconis sp.	3	0.49	dead leaf	19
Oidiodendron sp.	1	0.16	house dust	26
Paecilomyces sp.	5	0.81	clinical isolated	38
Paraphaeosphaeria michotii	1	0.16	plant: *Miscanthus giganteus*	62
Penicillifer diparietisporus	4	0.65	mangrove soil	35
Penicillium citrinum	72	11.71	endophytic	29
Penicillium spp.	23	1.15	root, soil, wood, endophytic	6; 9; 28; 34; 40; 53; 54; 55; 56; 57; 62
Pestalotiopsis spp.	3	0.49	rock, fungus garden	5; 56
Phaeomoniella sp.	1	0.16	leaves	75 (unpublished)
Phaeosphaeria herpotrichoides	2	0.33	plant: *Musa* sp.	50
Phaeosphaeria spp.	2	0.33	soil, leaves, stalks; seeds	18; 47
Phaeosphaeriopsis sp.	1	0.16	house dust	52
Phoma sp.	3	0.49	plant: *Juniperus virginiana*	31
Phomopsis spp.	12	1.95	phytopathogens; endophytic; saprobic	24; 66
Podospora sp.	1	0.16	fungus garden	56
Preussia spp.	17	2.76	fungus garden	56
Pseudoplagiostoma sp.	1	0.16	leaves	15
Purpureocillium lilacinum	5	0.81	water fountains	45
Robillarda sp.	2	0.33	coarse, outer fur of *Bradypus variegatus*	30
Scedosporium boydii	1	0.16	clinical isolate	76
Scytalidium spp.	3	0.49	fungus garden	56
Setophoma chromolaena	2	0.33	plant: *Chromolaena odorata*	50
Sordariomycetes	3	0.49	lichen fungus associated, endophytic	39; 67
Spegazzinia sp.	1	0.16	endophytic	41
Tetraplosphaeria sp.	1	0.16	plant: *Sasa senanensis*	63
Trichoderma deliquescens	2	0.33	root	74

Table 2 (*continued*)

Fungal taxa	N. of isolates	Abundance	Putative origin	References[a]
Trichoderma hamatum	5	0.81	root	74
Trichoderma cf. *harzianum*	3	0.49	water fountains	45
Trichoderma spirale	114	18.54	seed	68
Trichoderma spp.	10	1.63	Soil, sugarcane bagasse, fungus garden	3; 30; 36; 56
Xylaria spp.	16	2.59	rotten wood, insects, soil	8; 44; 56
unidentified Ascomycota	7	1.14	–	–
Basidiomycota				
Agaricus fiardii	2	0.33	fungus garden	56
Agaricus sp.	1	0.16	fungus garden	56
Ceriporiopsis sp.	1	0.16	plant: *Populus tremuloides*	10
Grammothele sp.	1	0.16	endophytic	11
Hyphodermella sp.	1	0.16	basidiome on *Castanea sativa*	65
Oudemansiella canarii	1	0.16	endophytic	23
Peniophora crassitunicata	1	0.16	air (indoor)	52
Phanerochaete sp.	6	0.98	fungus garden	56
Phlebia sp.	3	0.49	leaf	42
Phlebia subserialis	1	0.16	air (indoor)	52
Polyporales	2	0.33	decomposing seagrass leaves, air (indoor)	51; 52
Trametes hirsuta	2	0.33	soil	27
Trichosporon chiarellii	75	12.2	fungus garden	46
unidentified Basidiomycota	2	0.33	–	–
Insertae sedis				
unidentified fungi	3	0.49	–	–
Total	615	100		

[a] Information on the origin (substratum) of each fungus was retrieved from GenBank after running BLAST with the ITS contigs (see Table S3 for details and references in the supplementary material).

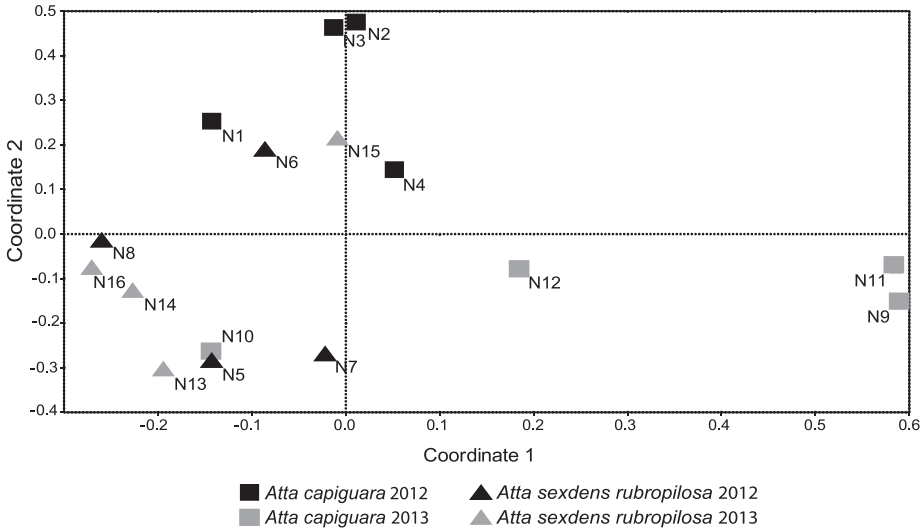

Fig. 2. Community structure of fungi found in the fungus gardens of *Atta capiguara* (n = 8 colonies) and *Atta sexdens rubropilosa* (n = 8 colonies) as demonstrated by principal coordinates analysis. The data suggest that fungal communities separated by ant species (coordinate 1) and by sampling periods (coordinate 2).

argues against a putative role as symbiont, but more surveys in other attine ant genera will reveal whether *T. chiarellii* takes part in the attine ant-microbe symbiosis.

The PCoA results indicate that the plant substratum foraged by leaf-cutting ants is one factor involved in the community structure in the gardens of these insects (Fig. 2). This suggests that different plant substrata foraged by ants (containing distinct communities), resulted in different fungal communities in gardens. Rodrigues et al. (2011) suggested that the plant substratum could influence fungal communities present in gardens of attine ants studied in Texas, USA. However, the impact of substratum on fungal community structure had not been evaluated. The present findings on two different ant species from the same locality indicate that the type of plant substratum plays a part in influencing the differences in fungal communities. However, whether this or additional factors significantly contributes to the observed fungal community structure needs to be tested.

Moreover, our analysis demonstrated that the sampling period (2012 and 2013) and colony age may also contribute to community structure. Gardens of *A. capiguara* clustered according to sampling periods, but the same pattern was not observed for gardens of *A. sexdens rubropilosa*. Regarding colony age, fungus garden from colony N10 of *A. capiguara* (the only colony of this ant species that was 4 yr old) also grouped with gardens of *A. sexdens rubropilosa*, which were also 4 yr old (Fig. 2). Thus, to determine the magnitude of the contribution of such factors in the community structure, further studies should systematically address multiple sampling periods and also different stages of ant colony development. In

addition to these two factors, the delay between garden collection and fungal isolation from colony N10 may also account for the massive presence of *Trichoderma* species (see Table S1).

T. spirale was the prevalent species in the fungus gardens of *A. capiguara* and was also found in gardens of *A. sexdens rubropilosa*. Rodrigues et al. (2014), studying soils next to the garden chambers of the same leaf-cutting ant species, also noted *T. spirale* as the prevalent fungus, however, this species was also prevalent in soils distant to the ant nest, demonstrating that soil is the likely natural source of this fungus. Some species of this genus have been reported from the fungus gardens (Rodrigues et al., 2005, 2008), from the waste material from the colonies (Rodrigues et al., 2005; Lacerda et al., 2013), as well as in the exoskeleton of winged females of *A. capiguara* and *Atta laevigata* (Pagnocca et al., 2008). Although they are not considered specific parasites of the ant fungal cultivar, Ortiz and Orduz (2000) and Silva et al. (2006) demonstrated in *in vitro* experiments that *Trichoderma* acts as an antagonist of the cultivar.

The specialized fungal parasite *Escovopsis* was found in 19% of the gardens sampled. According to Rodrigues et al. (2005) the frequency of occurrence of this fungus in colonies of leaf-cutting ants in São Paulo, Brazil, was 15% and 21% in field and laboratory colonies of *A. sexdens rubropilosa*, respectively; and up to 27% of *Acromyrmex* colonies collected in Southern Brazil (Rodrigues et al., 2008). Thus, the proportion of parasites found in the present study is similar to that found in previous works. However, in colonies sampled in Central America, *Escovopsis* was found in up to 75% of leaf-cutter ant colonies (Currie et al., 1999a; Currie, 2001). As discussed by Rodrigues et al. (2011), *Escovopsis* may have a low frequency in North American ant populations, moderate frequency in ant populations in South America (Brazil) and high frequency of the parasite in ant populations in Central America. This scenario was based on the work by Rodrigues et al. (2005, 2008) that used different isolation methods to those used in the studies of Currie et al. (1999a) and Currie (2001). In addition, the present work and the studies carried out by Rodrigues et al. (2005, 2008) were conducted in a more temperate region when compared with the studies by Currie et al. (1999a) and Currie (2001) which were carried out in tropical regions. Although the present study provides additional evidence of lower *Escovopsis* occurrence in leaf-cutting ants in Brazil and its epidemiological scenario, the broad-scale pattern described in Rodrigues et al. (2011) should be tested considering a systematic survey of *Escovopsis* across the full occurrence range of attine ants.

Using culture-dependent methods to characterize the diversity and the fungal community structure, we found that gardens of both leaf-cutting ant species comprise a high diversity of alien fungi other than the mutualist *L. gongylophorus*. Overall, our findings are indicative of a transient nature of the majority of alien fungi found in attine ant gardens. Furthermore, our analyses show that community structure and fungal composition differ between gardens of the ant species and suggest that they are strongly influenced by the plant substratum foraged by workers, likely along with other ecological factors. Future studies should consider further leaf-cutting ant species in order to evaluate if substratum preferences also influence garden communities in other dicot and grass-cutting attine ants.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.funeco.2016.03.004.

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