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ORIGINAL ARTICLE

Microfungal "Weeds" in the Leafcutter Ant Symbiosis

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Abstract Leafcutter ants (Formicidae: tribe Attini) are well-known insects that cultivate basidiomycete fungi (Agaricales: Lepiotaceae) as their principal food. Fungus gardens are monocultures of a single cultivar strain, but they also harbor a diverse assemblage of additional microbes with largely unknown roles in the symbiosis. Cultivar-attacking microfungi in the genus *Escovopsis* are specialized parasites found only in association with attine gardens. Evolutionary theory predicts that the low genetic diversity in monocultures should render ant gardens susceptible to a wide range of diseases, and additional parasites with roles similar to that of *Escovopsis* are expected to exist. We profiled the diversity of cultivable microfungi found in 37 nests from ten *Acromyrmex* species from Southern Brazil and compared this diversity to published surveys. Our study revealed a total of 85 microfungal strains. *Fusarium oxysporum* and *Escovopsis* were the predominant species in the surveyed gardens, infecting 40.5% and 27% of the nests, respectively. No specific relationship existed regarding microfungal species and ant-host species, ant substrate preference (dicot versus grass) or nesting habit. Molecular data indicated high genetic diversity among *Escovopsis* isolates. In contrast to the garden parasite, *F. oxysporum* strains are not specific parasites of the cultivated fungus because strains isolated from attine gardens have similar counterparts found in the environment. Overall, the survey indicates that saprophytic microfungi are prevalent in South American leafcutter ants. We discuss the antagonistic potential of these microorganisms as "weeds" in the ant–fungus symbiosis.

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Introduction

Insect–fungal mutualisms are interspecies associations of great evolutionary success [5, 6, 32]. One such association is the mutualism between the farming ants (Hymenoptera: Formicidae: tribe Attini) and their cultivated fungi, an ancient symbiosis that likely originated about 50 to 65 mya [30]. Within the tribe Attini, the leaf-cutting ants represent one of the most derived groups comprising two ant genera, *Atta* and *Acromyrmex* [43]. In many parts of the New World, leafcutter ants are recognized as highly destructive crop pests [25] because leafcutter nests support millions of individuals, and workers forage for large quantities of fresh leaf material that they cut and bring to their underground nests to use as substrate for fungal cultivation [50].

The cultivated fungus, *Leucoagaricus gongylophorus* (Basidiomycota: Agaricales: Lepiotaceae), together with the plant substrate supplied by the ants to sustain the fungal partner, compose the fungus gardens. The leaf-cutting ants' fungi develop specialized nutritive swellings (gongylidea) that are used by the ants to nourish their brood [50]. The fungus, in turn, benefits from the association because the ants provide a suitable environment for its growth. The ants also disperse the fungus when young queens carry a small fungal inoculum from their natal colony for the foundation of a new nest [31].

According to Poulsen and Boomsma [36] and Scott et al. (in preparation), leaf-cutting ants actively inhibit the growth of multiple strains of fungal cultivars within the nest, thereby maintaining their associated partner as single clones (i.e., monocultures). The resulting lack of genetic diversity in the fungus gardens is expected to render gardens susceptible to diseases and parasites [24]. An analogous problem exists in human monoculture crops [31]. Indeed, Currie et al. [11], sampling for non-mutualistic fungi associated with attine nests, discovered that the attine cultivars are host to a specialized fungal parasite in the genus *Escovopsis* (Ascomycota: anamorphic Hypocreales) that negatively impacts the ant colony. *Escovopsis* infects nests of attine ant species across all genera studied and is the most frequently encountered non-mutualistic fungus found so far in attine gardens of Central America [11, 12]. *Escovopsis* acts as a necrotrophic parasite that destroys the cultivar's hyphae [37] and exhibits a complex pattern of co-evolution with the cultivar. The original claim of ancient *Escovopsis*-cultivar cocladogenesis by Currie et al. [14] suggested parasite–host specificity at broad phylogenetic levels (four *Escovopsis* clades corresponding to four cultivar clades from four ant clades), but more comprehensive sampling [21] revealed occasional switching of *Escovopsis* lineages between cultivar lineages at the finest phylogenetic levels.

In addition to *Escovopsis*, attine ants harbor a community of other microbes in their gardens, including microfungi (filamentous fungi and yeasts) and bacteria [2, 9, 19, 38]. Leafcutter ants can regulate the microbiota in gardens, for example by actively combing out unwanted fungal spores [13] or by application of germination-inhibiting secretions [18]. However, the function of the associated microbiota in the garden matrix is largely unknown. These additional microorganisms could be harmful invaders (or "weeds") when found in high frequency in the ants' gardens [19, 35], neutral and transient commensals (with negligible effects on garden homeostasis), or potentially beneficial ancillary components serving unknown functions such as production of enzymes or antibiotics [2, 32].

Poulsen and Currie [35] suggested that the microfungi other than *Escovopsis* are mere transient guests with no active role in the fungus garden. This view is consistent with studies that documented ubiquitous microfungal species in attine gardens that are commonly found also in many other environmental sources. For instance, Carreiro et al. [9] and Craven et al. [10] reported ubiquitous yeasts species in the fungus gardens of laboratory nests (e.g., *Candida* spp.). However, in a survey of non-mutualistic filamentous fungi, Rodrigues et al. [38] discovered that some microfungi such as *Fusarium oxysporum* and *Trichoderma harzianum* occur in higher frequency in leafcutter gardens than the parasite *Escovopsis* sp. This was observed in nests under stressed conditions (i.e., laboratory nests treated with toxic baits). The same study also documented a high microfungal incidence other than *Escovopsis* sp. in natural *Atta sexdens rubropilosa* colonies. Fungal species such as *Acremonium kiliense*, *Cunninghamella elegans*, *F. oxysporum*, *T. harzianum*, and *Syncephalastrum racemosum* were frequently isolated [38], suggesting that their presence is not casual. In order to further understand the distribution and prevalence of these and other filamentous fungi in gardens of leaf-cutting ants, we conducted a survey of the microfungal species in field nests of leaf-cutting ants from Southern Brazil.

Previous studies on the microfungal diversity in attine nests focused on specific groups of microorganisms under diverse conditions. For example, several studies sampled natural nests of Central American attine species for the presence of *Escovopsis* [11, 12, 20]. Other studies surveyed the yeast diversity in laboratory nests of leaf-cutting ants [9, 10]. Fisher et al. [19] reported changes in the community structure of non-mutualistic filamentous fungi of *Atta cephalotes* laboratory nests when maintained with different types of leaf diets. Lastly, Möeller [29] reported microfungi species, including *Escovopsis* sp., from leafcutter gardens collected in Southern Brazil and maintained in the laboratory.

The present study differs from the above surveys [9–11, 19, 29, 38] of attine gardens in three main aspects: (1) the leaf-cutting ant species surveyed belonged to the genus *Acromyrmex* (*Atta* was largely absent in the surveyed area); (2) the collection sites were located in Southern Brazil (primarily the State of Rio Grande do Sul); and (3) the field nests appeared to be in healthy condition at the time of collection, with no visible signs of disturbance or stress. The survey addresses two primary questions: (1) Are there species-specific relationships among the microfungi and ants? (2) Is *Escovopsis* sp. prevalent in *Acromyrmex* gardens from Southern Brazil, and is its prevalence in Southern Brazil comparable to that of Central America [11, 12]?

Our study confirms previous reports that the gardens of leaf-cutting ants harbor several soil and plant-borne fungi but also shows a comparatively low infection rate by *Escovopsis*. The documented diversity of soil and plant-

borne fungi may function under certain conditions as opportunistic pathogens in leafcutter gardens, constraining the symbiosis by competing with the fungal cultivar for nutrient resources.

Materials and Methods

Fungus Garden Sampling

From 4–17 September 2004, gardens from 37 mature nests of ten *Acromyrmex* species were sampled in different localities of the State of Rio Grande do Sul (RS) in Southern Brazil (see Table 1 for collecting localities). The type of substrate carried by foraging workers at the time of collection was recorded along with the nesting habitat. This information was compared with Gonçalves [22] who provided detailed descriptions of foraging behavior and nest architecture of Brazilian *Acromyrmex* species. When our observations differed from the species-specific characters reported in the literature [22], our own observations were used in the analyses, as summarized in Table 1. The nests were carefully excavated (in the case of soil-dwelling species) or carefully opened (in the case of mound-building species; Table 1) in order to prevent contamination of any accessed garden. Large garden fragments (with workers and brood) were immediately transferred whole (without disrupting the garden) with sterilized forceps to sterile plastic containers (volume capacity=50 ml).

During the 2-week field expedition, all garden containers were kept in a cooler in the dark until transported to the "Centro de Estudos de Insetos Sociais" (CEIS) lab at Rio Claro, where they were maintained for an additional 3 days before fungal isolation.

Microfungi Isolation

We followed two established isolation techniques [11, 39] for profiling the microfungal community in the fungus gardens. From each garden collection, (1) ten fragments (3 mm^3 in diameter) of the gardens were removed and inoculated in potato-dextrose agar plates (PDA, DIFCO®) supplemented with 150 μg ml^{-1} of chloramphenicol (US Biological); (2) six garden fragments (20 mm^3 in diameter) were carefully freed of all the workers and brood (by sorting through each fragment with a sterilized forceps) then placed into a sterile, humidified Petri dish. The dish contained a piece of cotton with sterile distilled water, which provided humidity for continued fungal growth (the so-called "wet chamber"). All plates were incubated at 25°C for 7–14 days in the dark.

PDA plates and wet chambers were checked daily for signs of any filamentous fungal growth. Once a fungus emerged

Table 1 General characteristics of the ant species used in this study

Acromyrmex species	City/State	Nest location[b]	Substrate	Nest type
A. ambiguus (2)[a]	Nova Petrópolis/RS	S 29°22′38.2″; W 50°57′18.1″	Dicot	Mound-builder
	Near Pelotas/RS	S 30°50′10.2″; W 51°55′10.4″	Dicot	Mound-builder
A. aspersus (2)	São Marcos/RS	S 28°58′05.6″; W 51°07′58.0″	Dicot	Soil-dweller
A. coronatus (9)	near Registro/RS	(2) S 25°25′50.5″; W 49°04′56.4″	Dicot	Mound-builder
	Itajai/RS	S 25°25′50.5″; W 49°04′56.4″	Dicot	Soil-dweller
	Near Pelotas/RS	S 30°50′10.2″; W 51°55′10.4″	Dicot	Mound-builder
	Vacaria/RS	(2) S 28°27′51.7″; W 50°53′07.0″	Dicot	Mound-builder
	Blumenau/SC	(2) S 26°53′37.8″; W 49°11′29.0″	Dicot	Mound-builder
	Blumenau/SC	S 26°51′48.3″; W 49°16′15.0″	Dicot	Mound-builder
A. crassispinus (1)	Nova Petrópolis/RS	S 29°23′51.4″; W 50°54′27.3″	Dicot	Soil-dweller
A. disciger (2)	Blumenau/SC	S 26°54′04.9″; W 49°10′51.2″	Dicot	Mound-builder
A. hispidus falax (2)	Londrina/PR	S 22°47′22.0″; W 51°36′01.6″	Dicot	Soil-dweller
A. laticeps (5)	Nova Petrópolis/RS	(2) S 29°19.05′9″; W 51°10′13.6″	Dicot	Soil-dweller
	São Marcos/RS	S 28°57′16.5″; W 51°08′20.0″	Dicot	Soil-dweller
	Alto da Serra/RS	(2) S 28°12′26.2″; W 50°45′27.4″	Dicot	Mound-builder
A. lundi (3)	São Marcos/RS	(2) S 28°58′02.8″; W 51°08′08.8″	Dicot	Soil-dweller
	Chuvisca/RS	S 30°50′10.2″; W 51°55′10.4″	Dicot	Soil-dweller
A. heyeri (10)	Sentinela do Sul/RS	(4) S 30°37.57′9.0″; W 51°33′18.2″	Monocot	Soil-dweller
	Chuvisca/RS	S 30°50′10.2″; W 51°55′10.4″	Monocot	Mound-builder
	near Pelotas/RS	(4) S 30°50′10.2″; W 51°55′10.4″	Monocot	Mound-builder
	Santana da Boa Vista/RS	S 30°56′40.0″; W 53°05′10.3″	Monocot	Mound-builder
A. landolti (1)	Taquara/RS	S 29°42′55.7″; W 50°50′21.5″	Monocot	Soil-dweller

PR Paraná; *RS* Rio Grande do Sul; *SC* Santa Catarina

[a] Figures in parentheses (column 1) indicate the number of colonies sampled for each ant species

[b] Figures in parentheses (column 3) indicate the number of colonies found at the same locality

from the garden fragment, an inoculum was transferred to malt agar 2% plates (MA 2%, DIFCO®) in order to obtain pure cultures. When morphologically very similar microfungal colonies were characterized in a single ant garden, a unique representative fungal sample was isolated, and the strains were stored in 10% glycerol at −80°C at CEIS. When insufficient garden material was available to conduct both isolation methods, only one method was used out of necessity, yielding 17 isolations with PDA only, 4 isolations with wet chambers only, and 16 isolations using both methods.

Fungal Identification

Morphological Methods

Colony macromorphology and micromorpholgy were used as main characters to identity the isolates. Species were identified with the help of general taxonomic keys [4, 15, 42] as well as specific taxonomic treatments for some groups of fungi [26, 28, 33].

Molecular Methods

Microfungi were further identified with the help of DNA sequence information. Genomic DNA was extracted using the cetyl trimethylammonium bromide method [20]. Prior to DNA extractions, isolates were grown in aerated liquid cultures (malt extract broth 2%) for 7 days at 25°C, and the mycelia were harvested and lyophilized.

A 25 μl polymerase chain reaction was performed using Ready-to-Go™ beads (GE Healthcare) and 1.0 μl of DNA template (>40 ng). ITS4 and ITS5 primers (6 pmol each) were used to amplify the internal transcribed spacer regions of the ribosomal DNA [51]. For *Escovopsi*s isolates, the primers eafF (5′CATGATCACTGGTACCTCCCAGG3′) and eafR (5′GCATGTCACGGACGGCGAAACGA3′) modified from [14] were used to amplify a fragment spanning the exon 6 of the elongation factor 1-alpha (EF1-a) gene.

The amplification protocol consisted of an initial denaturation step at 94°C for 3 min, followed by 35 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min, followed by a final extension step of 72°C for 15 min. The amplification products were purified with Wizard® Genomic DNA Purification Kit (Promega Corporation) following the manufacturer's protocol.

Cloning was necessary in some cases to obtain good sequence reads. In these cases, the amplicons were inserted in pGEM® T-vector (Promega Corporation) and transformed in competent *Escherichia coli* DH10β cells. DNA from recombinant cells was purified following the miniprep procedure by Sambrook and Russel [41].

The 10 μl cycle sequencing reaction contained 2.5 μl of Big Dye terminator (Applied Biosystems); 2.5 μl of 100 mM Tris and 2.5 mM $MgCl_2$ (pH 9.0), 6 pmol of each primer (the same ones used in the amplification step); and 30–40 ng of the purified polymerase chain reaction products. Reaction conditions included a denaturation step of 96°C for 2 min followed by 28 cycles of 96°C for 45 s, 50°C for 30 s, and 60°C for 4 min. The amplicons were sequenced on an ABI Prism 377 DNA sequencer (Applied Biosystems). For all samples, both forward and reverse sequences were obtained for the internal transcribed spacer (ITS) and EF1-a regions. Sequences from representative isolates are deposited at Genbank as accessions EU082779–EU082803.

Sequence Analysis

Forward and reverse strands were edited using Bioedit v.7.0.5.3 [23], and the consensus sequence was used in BLASTN similarity searches at the National Center for Biotechnology Information-Genbank [1] or at the TrichoKey databases [16] (the latter one just for *Trichoderma* isolates). Sequences presenting 99% similarity with sequences obtained from databases were considered as identified (Table 2).

Phylogenetic analyses were performed for two types of microfungi that occurred in high proportions in gardens (*Escovopsis* sp. and *F. oxysporum*). *Escovopsis* sequences were aligned in ClustalW [49] using default parameters and analyzed in PAUP* v. 4.0b10 [47] under the maximum parsimony (MP) criterion. An heuristic search was conducted with 1,000 replicates, random sequence addition, tree bisection-reconnection branch swapping, and the *collapse* and *multrees* options implemented. Maximum likelihood (ML) analysis was conducted in GARLI v. 0.951 [52] using default parameters as recommended in the User's Manual. Branch support for MP and ML analyses was calculated using 1,000 non-parametric bootstrap pseudoreplicates [17] using the same settings as for initial searches. Bayesian analyses were carried out in MrBayes v. 3.1.2 [40]. Four separate runs were conducted, each with four incrementally heated chains and uninformative, default priors; converge and optimal burn-in were assessed as described in [7] using the program MrConverge (Lemmon, in preparation). After discarding burn-in, the posterior samples of tree topologies for each run were combined in PAUP* to obtain the posterior probabilities of each node. Sequences from *Escovopsis* isolates published in other studies [14, 48] were obtained from Genbank (accessions # AY172620, AY172622, EF589910–EF589914, EF589916–EF589919, and EF589921–EF589949).

In order to establish the phylogenetic relationships of *F. oxysporum* isolates from attine gardens and *F. oxysporum* from other environmental sources, a median-joining network [3] was inferred using Network v. 3.1.1.1 (available at www.fluxus-engineering.com). Sequence information for different *F. oxysporum* strains were retrieved from Genbank

Table 2 Microfungi species in fungus gardens of *Acromyrmex* species from Southern Brazil

Fungi classified as	Closest identified relative			*Acromyrmex* spp										% of nests (*n*=37) with the specified microfungus
				A. ambiguus[a]	*A. aspersus*[a]	*A. coronatus*[a]	*A. crassispinus*[a]	*A. disciger*[a]	*A. hispidus*[a]	*A. laticeps*[a]	*A. lundi*[a]	*A. heyeri*[b]	*A. landolti*[b]	
	Fungal species	Accession #	% identity	*n*=2	*n*=2	*n*=9	*n*=1	*n*=2	*n*=2	*n*=5	*n*=3	*n*=10	*n*=1	
Zygomycota														
Cunninghamella binariae	*C. binariae*	AF254935	98		1[c]	1						5		19.0
C. blakesleana	*C. blakesleana*	AF254932	97			1								2.8
C. echinulata	*C. echinulata* var. *antartica*	AF254938	95				1			1	1			8.0
Mucor circinelloides	*M. circinelloides*	DQ118966	99							1				2.8
M. racemosus	*M. racemosus*	AJ271061	99	1	1			1		1	1			10.9
Mucor sp. 1	*Mucor recurvus*	AF412294	92			1								2.8
Mucor sp. 2	n.d.		n.d.			1								2.8
Syncephalastrum racemosum	n.d.	n.d.	n.d.			2								5.4
Ascomycota														
Aspergillus flavus	*A. flavus*	AM745114	100	1					2					8.0
Aspergillus versicolor	*A. versicolor*	AJ937750	99									1		2.8
Escovopsis sp.	See text and Fig. 2 for details			1		2		1		1	2	3		27.0
Eupenicillium javanicum	*E. javanicum*	U18358	100						1					2.8
Chaetomium sp.	*Chaetomium* sp.	AJ279468	89									1		2.8
Cladosporium cladosporioides	*C. cladosporioides*	AF393691	100						1					2.8
Fusarium oxysporum	*F. oxysporum*	AY462580	100	1		1		1	2	3	2	6		40.5
F. equiseti	*F. equiseti*	AY147362	100	1										2.8
F. solani	*F. solani*	AM412639	100									1		2.8
Trichoderma hamatum	*T. hamatum*	DQ286602	100							1				2.8
T. harzianum	*T. harzianum*	AF443922	100			1						1	1	8.0
T. spirale	*T. spirale*	AY154939	99									1		2.8
Trichoderma sp.	*Trichoderma* sp.	AY154942	99		1		1			1		1		5.4
T. virens	*T. virens*	AF099007	100			1								2.8
Lecythophora sp.	*Lecythophora* sp.	AY219880	94									1		2.8
Moniliella-like fungus	*Hypomyces aurantius*	AF055297	82		1						1	1		8.0
Paecilomyces lilacinus	n.d.	n.d.	n.d.			1								2.8
Penicillium citrinum	*P. citrinum*	EF127876	99						1	1				5.4
Penicillium sp. 1	*Penicillium* sp.	DQ092545	99							1				2.8
Penicillium sp. 2	*Penicillium* sp.	DQ279802	100									1		2.8

Table 2 (continued)

Fungi classified as	Closest identified relative			*Acromyrmex* spp										% of nests ($n=37$) with the specified microfungus
				A. ambiguus[a]	*A. aspersus*[a]	*A. coronatus*[a]	*A. crassispinus*[a]	*A. disciger*[a]	*A. hispidus*[a]	*A. laticeps*[a]	*A. lundi*[a]	*A. heyeri*[b]	*A. landolti*[b]	
	Fungal species	Accession #	% identity	$n=2$	$n=2$	$n=9$	$n=1$	$n=2$	$n=2$	$n=5$	$n=3$	$n=10$	$n=1$	
Penicillium sp. 3	*P. miniioluteum*	AY213674	97				1							2.8
P. waksmanii	*P. waksmanii*	AY373940	99			1								2.8
Volutella sp.	*Volutella* sp.	EF029211	99	2						1				5.4
Xylaria sp. 1	*Xylaria* sp.	EF423534	89				1					1		5.4
Xylaria sp. 2	n.d.	n.d.	n.d.									1		2.8
n.i. ascomycetes 1	n.i. ascomycete	AJ279488	100				1							2.8
n.i. ascomycetes 2	uncultured ascomycete	EF027379	89			1								2.8

n Number of nests sampled for each ant species; *n.d.* no data; *n.i.*, not identified

[a] Ant species are dicot-cutting ants (for further details, see Table 1)

[b] Ant species are grass-cutting ants (for further details, see Table 1)

[c] Number of colonies of a particular ant species from which was observed the specified microfungus

as accession # U34571, AJ853769, U34566, U28161, X94173, AF165875, AF069310, and U28159.

Results

Microfungal Distribution in *Acromyrmex* Nests

Aiming to improve our assessment of the microfungal diversity in the fungus gardens, we have carried out two different isolation techniques. The effect of this strategy can be evaluated by the results obtained from the 16 nests which had enough material to be used in both techniques. These 16 nests were found to contain 22 fungal species, but only four of these species (*Cunninghamella binariae*, *Escovopsis*, *F. oxysporum*, and *T. harzianum*) were isolated by both technical procedures; eight species (*Fusarium solani*, *Mucor circineloides*, *Penicillium* sp. 2, *Penicillium waksmanii*, *S. racemosum*, *Trichoderma* sp., *Xylaria* sp. 1, and *Xylaria* sp. 2) were isolated uniquely through the wet-chamber method; and ten species (*Chaetomium* sp., *Lecithophora* sp., *Moniliella*-like fungi, *Mucor* sp. 1, *Mucor* sp. 2, *Mucor racemosus*, *Trichoderma spirale*, *Volutella* sp., and two isolates of non-identified fungi) were recovered only by the PDA method. These results suggest that the two isolation methods worked complimentary to each other in order to depict the microfungal diversity in *Acromyrmex* gardens.

Application of these two isolation techniques to the gardens of *Acromyrmex* ants resulted in the recovering of 85 microfungal strains. This pool of isolates comprised 33 fungal species from 16 genera that were identified either by morphological or sequencing analyses. In addition, two non-sporulating, morphologically unidentifiable fungal isolates could only be classified based on ITS sequence information (Table 2).

Among the 16 fungal genera found, *Cunninghamella*, *Escovopsis*, *Fusarium*, *Mucor*, *Penicillium*, and *Trichoderma* were the most prevalent, occurring at least in 18% of the gardens (Table 2). *Fusarium* and *Cunninghamella* were isolated in 26% and 19% of grass-cutting ant's gardens, respectively, whereas *Fusarium*, *Mucor*, and *Escovopsis* were found in 18.5%, 14.8%, and 13% in dicot-cutting ant's gardens, respectively (Fig. 1a). Ten out of 16 microfungal genera were observed in monocot-cutting ants, and 14 out of 16 microfungal genera were found in gardens of dicot-cutting ants. Only eight genera (*Aspergillus*, *Cunninghamella*, *Escovopsis*, *Fusarium*, *Moniliella*-like, *Penicillium*, *Trichoderma*, and *Xylaria*) were common in gardens of both monocot and dicot-cutting ants (Fig. 1a).

When comparing the microfungi profile between nest-type, *Fusarium* and *Escovopsis* were the most prevalent, occurring in 24% and 15% of mound-building ant species,

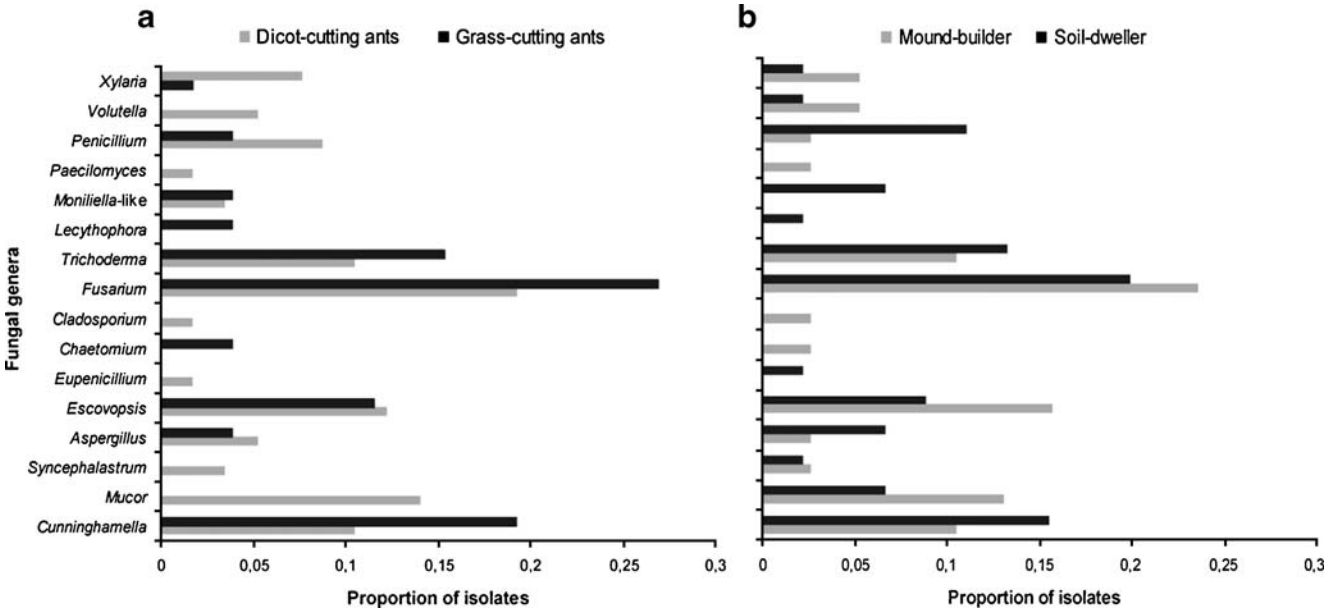

Figure 1 Relative abundance of fungal isolates (grouped by genus) found in *Acromyrmex* leafcutter ant nests in Southern Brazil. Ant species are grouped according to: **a** The type of plant material used by the workers to nourish their cultivars, dicot-cutting ants (n=57 fungal isolates) and grass-cutting ants (n=26 fungal isolates). **b** Nesting habit, mound-builder (n=38 fungal isolates) and soil-dweller (n=45 fungal isolates). Unidentified ascomycete fungi are not included

respectively. *Fusarium* and *Cunninghamella* were the two predominant genera in soil-dwelling ant species (Fig. 1b). Ten out of 16 microfungal species were associated with both mound-building and soil-dwelling ant species (Fig. 1b).

While ascomycete fungi comprised the majority of the isolates from both monocot and dicot-cutting ants, zygomycetes were not found in high frequency in monocot-cutting ants, with the exception of *C. binariae* which was found in *Acromyrmex heyeri* nests (Table 2). On the other hand, zygomycete fungi were found in association with seven out of eight dicot-cutting ant species studied (Table 2).

The most frequent fungal species in the present survey were: *F. oxysporum* from 40.5% of the colonies across seven ant species; *E. weberi* from 27% of the colonies from six ant species; *C. binariae* from 19% of the colonies of three ant species; and *M. racemosus* from 10.9% of the colonies of five ant species (Table 2). The remaining microfungal species were present in less than 10% of the total nests sampled.

Phylogenetic Analyses

Because *F. oxysporum* was the most prevalent species in our survey, we evaluated whether any particular *F. oxysporum* strains were specialized in infecting *Acromyrmex* gardens. This was accomplished by assessing the phylogenetic relationship between ITS haplotypes from our *F. oxysporum* isolates with published ITS haplotypes from *F. oxysporum* strains commonly found in soil or plant substrates. Because ITS2 is known to have paralogous copies in *Fusarium* [34], we confirmed first that the major ITS2 type present in our isolates were the ITS2-type I described by O'Donnell and Cigelnik [34]. There was a low polymorphism of the ITS1 and ITS2 regions within the analyzed strains, with one nucleotide difference on average. The median-joining network (not shown) suggested a scenario which is not compatible with specialized infection of *Acromyrmex* by *F. oxysporum*, since no genetic group containing only closely related isolates from leafcutter nests was characterized. In addition, 12 haplotypes were shared by leafcutter isolates and other isolates from several environmental sources, including soil and plant substrates.

We also investigated species-specificity regarding *Escovopsis* strains and leafcutter species by inferring the phylogenetic relationships among our isolates as well as other previously studied *Escovopsis* strains [14, 48]. The phylogeny inferred from the EF1-a marker (Fig. 2) showed that all of the *Escovopsis* isolates in our survey fall within *Escovopsis* group A, as defined by Taerum et al. [48]. No species-specificity was detected between *Acromyrmex* ants and *Escovopsis* strains from Southern Brazil, since (1) closely related *Escovopsis* strains were associated with different ant species, and (2) gardens of the same ant species were associated with more distantly related *Escovopsis* strains (Fig. 2). Although *Escovopsis* isolates from group B did not form a monophyletic clade in our phylogenetic analyses, as they do in previous studies [48], this discrepancy is a result of the shorter EF1-a fragments used in our analyses (697 base pairs versus >1,400 base pairs in Taerum's et al. study [48]).

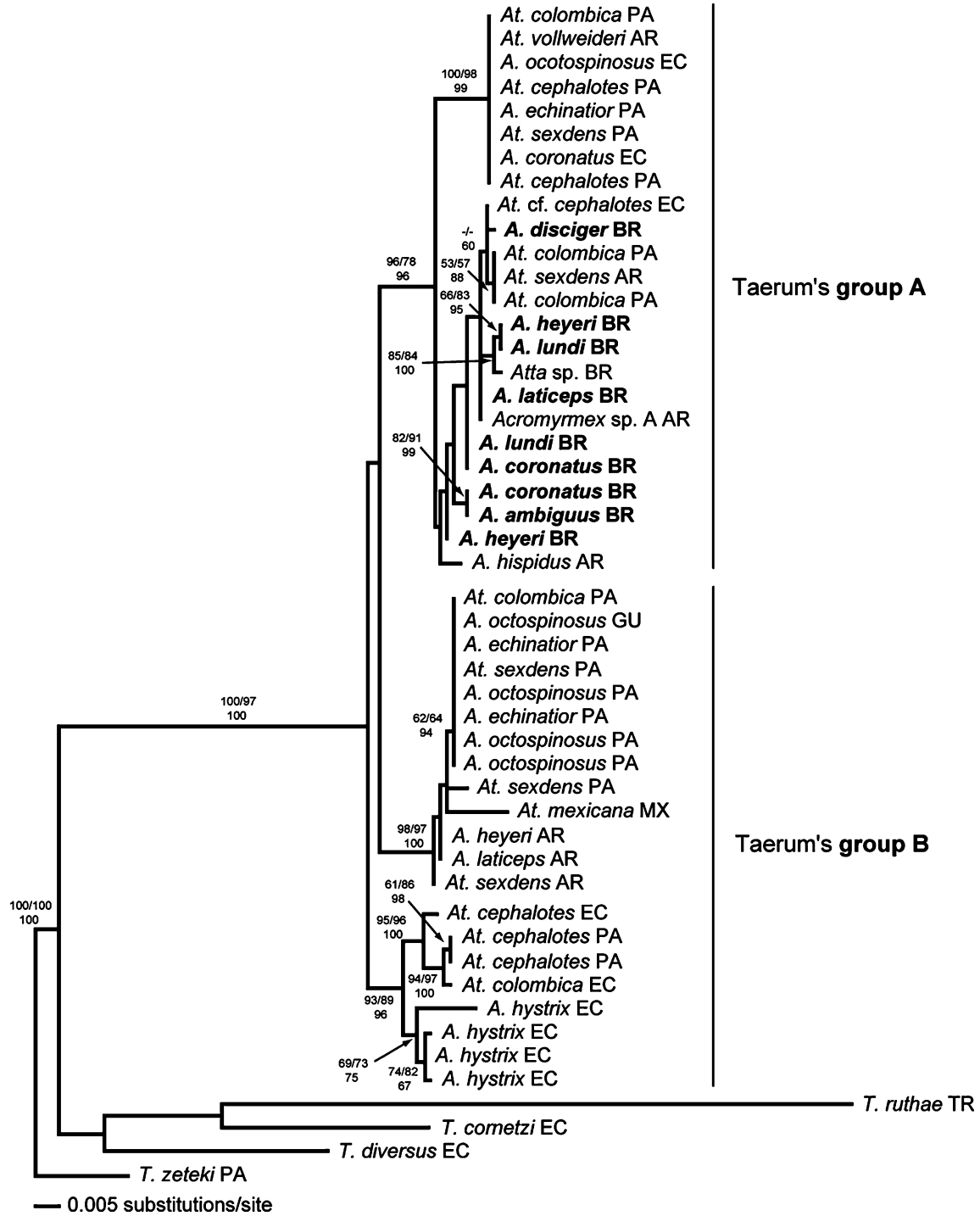

Figure 2 Maximum likelihood tree of *Escovopsis* isolates inferred from a 697-base-pair fragment of the elongation factor 1-alpha gene. *Numbers on branches* are bootstrap support under maximum parsimony (*top*, *left*) and maximum likelihood (*top*, *right*) criteria (values under 50% are not shown) and Bayesian posterior probabilities (*below*). The fungi are named after the ant species from which they were isolated (*At.*, *Atta* sp.; *A.*, *Acromyrmex* sp.; *T.*, *Trachymyrmex* sp.) followed by the country of origin (*AR*, Argentina; *BR*, Brazil; *EC*, Ecuador; *GU*, Guadeloupe; *MX*, Mexico; *PA*, Panama; *TR*, Trinidad). *Escovopsis* isolates from the present study are shown in *bold face* and cluster in the group A as described by Taerum et al. [48]

Discussion

Microfungi in *Acromyrmex* Nests

Earlier work has shown that attine gardens harbor a complex microbiota, including soil-inhabiting microfungi [11, 38, 39] as well as epiphytic and endophytic fungi [19]. In the present study, we profiled and characterized the cultivable microfungi in the fungus gardens of *Acromyrmex* spp. from Southern Brazil by using two distinct and complimentary isolation techniques. This allowed the assessment of the microfungal diversity in these gardens through the recovering of several microfungal isolates.

Gardens of monocot or dicot-cutting and mound-builder or soil-dweller leafcutter ants harbored slightly distinct microfungal communities, as would be expected if garden substrate and nest-type influenced microfungal contamination (Table 2, Fig. 1). Eight genera of microfungi occurred in gardens of both monocot- and dicot-cutting ants (Fig. 1a) and 10 out of 16 genera occurred in both mound-building and soil-dwelling species (Fig. 1b). No microfungal lineage was clearly specialized on either garden substrate or nest type. Other factors such as specific plant species harvested by the ants, the age of the colony, and infestation by arthropod garden commensals that may vector contaminants into the garden, or interactions between some of these factors, may have determined the microfungal garden community. As a classical example of factors influencing the garden microbiota, Fisher et al. [19] concluded that changes in microfungi species composition associated with *A. cephalotes* nests reared in the laboratory were due to changes in the plant substrate offered to the ants.

Microfungal profiles also revealed no ant–fungal species specificity with fungi having instead a rather diffuse association with *Acromyrmex* spp. (Table 2). For example, fungi such as *Aspergillus* sp., *Cladosporium* sp., *Penicillium* sp., and others were isolated from different ant species independent of nest-type (mound versus soil) or the leaf substrate (monocot versus dicot). Also, most microfungi in *Acromyrmex* gardens from this study are species commonly found in the environment [15], suggesting no apparent specialization to the symbiosis. This ubiquity is generally due to the strategy as saprophytes, i.e., they are important due to its general role in the nutrient cycling in ecosystems. For instance, fungi in the genus *Xylaria* are well-known saprophytes, decaying wood of living or dead plants [27].

Despite the lack of specificity between ant and fungal species found in our survey, there are some interesting fungi that deserve closer consideration. First, the soil-borne fungal genus *Cunninghamella* was found in 19% of the leafcutter gardens, a figure rather comparable to the levels of infection by *Escovopsis* observed in this study (27%) and previous studies [11, 38, 39] (see further discussion below). Like *Escovopsis*, *Cunninghamella* species can have drastic effects on leafcutter gardens, overrunning gardens of laboratory nests within a few days after insecticide treatment [38], thus suggesting these fungi may have an important role within the attine ant–microbe symbiosis. However, parsimony analysis (data not shown) indicated that *Cunninghamella* isolates from leafcutter gardens clustered with several isolates of the same genus found in other plant substrates such as nuts [28], indicating that our *Cunninghamella* strains isolated from gardens are not specialized on the ant–fungus symbiosis.

Second, *F. oxysporum* was isolated in 40.5% of the nests, a percentage somewhat higher than the 23% found in field colonies of *A. sexdens rubropilosa* in southeast Brazil [38]. Apparently, *F. oxysporum* is a soil-borne fungus that has a high prevalence in attine gardens [38] (this study) but there is currently no evidence that *F. oxysporum* plays a detrimental role in ant gardens. Because *F. oxysporum* varieties are disease-causing fungi on plants such as cotton wilt [46] and soil is a natural reservoir for this fungus, we tested whether the strains associated with attine gardens form a specific group. A haplotype analysis revealed that all attine-associated *F. oxysporum* strains have plant-associated counterparts (including identical-sequence strains) which can exist either in soil or in plants, thus outside of the association with leaf-cutting ants. Although the analysis is based on few strains, it suggests that *F. oxysporum* can enter nests from the surrounding environment and not via nest-to-nest transmission as hypothesized for *Escovopsis* [11].

Escovopsis Natural Infection Rates and Diversity

Since 1999, our knowledge on the microfungus genus *Escovopsis* has been growing [11, 14, 20, 21, 48]. The main reason for the advances on *Escovopsis* biology and ecology is due to several studies that have specifically surveyed for *Escovopsis* diversity, revealing that this fungus is present in gardens of most attine species. This fungus is currently the best well-known pathogen in the attine ant–microbe symbiosis.

Currie et al. [11] studied *Escovopsis* distribution in a variety of attine genera, mostly from Central America and adjacent areas, and established that *Escovopsis* could be isolated in 33–51% of the nests, depending on the ant genus. With respect to just the leaf-cutting ants (*Atta* and *Acromyrmex*), Currie et al. [11] and Currie [12] reported *Escovopsis* infection rates ranging from 51% in some *Atta* species to as high as 68.4% in *Acromyrmex octospinosus* and 75% in *Acromyrmex echinatior*. Our study found comparatively lower *Escovopsis* infection rates (27%). It is unclear whether the levels of *Escovopsis* infection of Brazilian subtropical *Acromyrmex* sp. are naturally lower

compared to Central American attines and whether these differences are due to (1) different feeding habits exhibited by the ants, (2) nest density differences, (3) microhabitat variations, (4) nest age, or even (5) antagonism by other alien microorganism. The fact that the present study made use of two types of isolation methods (in contrast to other studies [11, 12] that used one method) could be another factor contributing to the observed differences.

Our phylogenetic analyses (Fig. 2) corroborate results by Taerum et al. [48] in which Brazilian isolates all fell into a single group named clade A, which also contained isolates from Argentina, Ecuador, and Panama. However, our survey in southern Brazil did not discover any isolates in clade B of Taerum et al., which contained *Escovopsis* from Argentina, Ecuador, and Central America. Thus, our results suggest that ants in the geographic region in Rio Grande do Sul are exclusively infected by *Escovopsis* strains in clade A, which is compatible with some geographic structure in this parasite's distribution. More extensive sampling is needed, especially in other regions of Brazil, to investigate whether other cases of *Escovopsis* geographic structuring exist. Furthermore, the antibiotic-producing bacteria that the ants carry on their cuticle as a defense against *Escovopsis* are predicted to having corresponding geographic structure [8].

Microfungi as Antagonists in the Ant Farm

According to Poulsen and Boomsma [36] and Scott et al. (in preparation), leafcutter cultivars are maintained by the ants as single-genotype fungus garden (monoculture), conditions that are predicted to facilitate the spread and coevolution of pathogens [24]. Our results indicate that other non-mutualistic fungi in *Acromyrmex* nests are indeed prevalent in leafcutter gardens but also that these potential pathogens do not appear to be as specialized as *Escovopsis* [14, 21].

Some of the garden weeds appear to act as antagonists of the ant-cultivated fungi, as already documented by Silva et al. [45]. For example, attine gardens can be overgrown by several microfungi, all causing garden death [38, 39] similar to garden destruction by *Escovopsis* sp. [11]. Fungi such as *Cunninghamella* species are considered sugar-free fungi and can readily assimilate simple sugars, quickly building up a large biomass of mycelia [15]. It is known that fungus gardens of attine ants contain high levels of simple sugars (i.e., glucose) [44], and for most fungi, ant gardens are therefore a suitable environment for growth. Future studies should address whether the sugars available in the fungus gardens help non-cultivar fungi to outgrow the ants' defense mechanisms of constant weeding. Within this nutritional milieu of attine gardens, microfungal weeds therefore can critically impact garden health.

Considering the ecological roles of *F. oxysporum* and *Cunninghamella* sp., we hypothesize that these microorganisms act as antagonists in the attine–microbe symbiosis. However, the negative impact of *F. oxysporum* and *Cunninghamella* sp. appears to be due to nutritional competition and is not as specific as the impact of the cultivar-infecting parasite *Escovopsis*, yet some degree of adaptation and pathogenicity of *F. oxysporum* and *Cunninghamella* species is implied. Future studies should evaluate the extent of negative impacts of these fungi on the leafcutter ants' fitness and their usefulness in the biological control of these agricultural pests.

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