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Low variation in ribosomal DNA and internal transcribed spacers of the symbiotic fungi of leaf-cutting ants (Attini: Formicidae)

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Abstract

Leaf-cutting ants of the genera *Atta* and *Acromyrmex* (tribe Attini) are symbiotic with basidiomycete fungi of the genus *Leucoagaricus* (tribe Leucocoprineae), which they cultivate on vegetable matter inside their nests. We determined the variation of the 28S, 18S, and 5.8S ribosomal DNA (rDNA) gene loci and the rapidly evolving internal transcribed spacers 1 and 2 (ITS1 and ITS2) of 15 sympatric and allopatric fungi associated with colonies of 11 species of leafcutter ants living up to 2,600 km apart in Brazil. We found that the fungal rDNA and ITS sequences from different species of ants were identical (or nearly identical) to each other, whereas 10 GenBank *Leucoagaricus* species showed higher ITS variation. Our findings suggest that *Atta* and *Acromyrmex* leafcutters living in geographic sites that are very distant from each other cultivate a single fungal species made up of closely related lineages of *Leucoagaricus gongylophorus*. We discuss the strikingly high similarity in the ITS1 and ITS2 regions of the *Atta* and *Acromyrmex* symbiotic *L. gongylophorus* studied by us, in contrast to the lower similarity displayed by their non-symbiotic counterparts. We suggest that the similarity of our *L. gongylophorus* isolates is an indication of the recent association of the fungus with these ants, and propose that both the intense lateral transmission of fungal material within leafcutter nests and the selection of more adapted fungal strains are involved in the homogenization of the symbiotic fungal stock.

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- *Leucoagaricus*
- *Leucoagaricus gongylophorus*

Introduction

The tribe Attini consists of approximately 200 species of ants living in symbiosis with three distinct groups of microorganisms, in what has been called 'one of the most complex symbiotic association discovered in nature' (1). These microorganisms include symbiotic basidiomycete fungi, which the ants utilize as food source, a parasite fungus of the genus *Escovopsis*, which attacks the symbiotic fungal culture, and actinomycete bacteria, which grow in the ants' body and produce antibiotics that are active against *Escovopsis* (1).

There are 13 genera of attines (2,3), the most derived being the leafcutter ants of the genera *Atta* and *Acromyrmex*, which are the dominant herbivores in the Neotropical region between latitude 12º North and 33º South (4,5). This dominance results from the metabolic integration between the symbionts (6-9), which has been established during 50 million years of co-evolution (10,11).

To understand the processes involved in the association between leaf-cutting ants and symbiotic fungi, the diversity within each member of the symbiont pair needs to be characterized, but although the taxonomy of ants is well advanced (2), the taxonomy, and hence the diversity, of their symbiotic fungi has not yet been completely elucidated. Basidiocarps collected from nests of *Atta* species have been named *Leucocoprinus gongylophorus* (12), *Leucoagaricus gongylophorus* (13) and *Leucoagaricus weberi* (14), but paired tests with mycelia suggest that some *Atta* and *Acromyrmex* ants cultivate a single species of fungus (15).

The multiple fungal species versus single species theories have more than taxonomic implications, being also related to the modes of fungal transmission within leafcutter colonies. Vertical transmission occurs when queen ants leave their parent nest carrying the symbiotic fungus, which is then cultured by the newly founded colony (16). It has been proposed that vertical transmission has selected host-specific lineages of symbiotic fungi in leafcutter nests during the past 23 million years (10), so that the exclusively vertical transmission of fungal cultures within leafcutters is consistent with the existence of multiple symbiotic fungal species. On the other hand, the culturing of the same fungal species by *Atta* and *Acromyrmex* ants may indicate lateral transmission of fungal lineages. In laboratory nests, lateral transmission of symbiotic fungal strains has been induced between and within species of basal attines of the genus *Cyphomyrmex* (17) as well as two *Acromyrmex* species (18) and also seems occasionally to occur in nature (18-21).

In the present paper, we provide new information on the systematics of *Leucoagaricus* symbiotic fungi, which we obtained by sequencing the 28S, 18S and 5.8S rDNA loci and internal transcribed spacers 1 and 2 (ITS1 and ITS2) of two basidiocarp and thirteen mycelial fungal isolates collected from the nests of attine ants located in several geographic regions of Brazil between latitudes 1.52° and 23.43° South. Our results suggest that all the isolates studied belong to the species *Leucoagaricus gongylophorus* and that lateral transmission was a common event through which selected lineages of the symbiotic fungus quickly proliferated in nests of many of the higher attine ants living in the tropical Americas.

Table 1. *Leucoagaricus* isolates obtained from the nests of leaf-cutting ants.

Isolate code	Location in Brazil	Ant	Physical state of the culture
A	Rio Claro	*Atta laevigata*	Mycelial
B	Rio Claro	*Atta sexdens rubropilosa*	Mycelial
C	Rio Claro	*Acromyrmex crassispinus*	Mycelial
D	Rio Claro	*Acromyrmex hispidus fallax*	Mycelial[1]
E	Rio Claro	*Acromyrmex hispidus fallax*	Basidiocarp[1]
F	Botucatu	*Atta capiguara*	Mycelial
G	Botucatu	*Atta bispherica*	Mycelial
H	Botucatu	*Acromyrmex rugosus*	Mycelial
I	Ubatuba	*Acromyrmex disciger*	Mycelial
J	Viçosa	*Atta sexdens rubropilosa*	Mycelial
K	Viçosa	*Acromyrmex subterraneus molestans*	Mycelial
L	Viçosa	*Acromyrmex subterraneus molestans*	Mycelial[2]
M	Viçosa	*Acromyrmex subterraneus molestans*	Basidiocarp[2]
N	Almeirim	*Atta cephalotes*	Mycelial
O	Almeirim	*Acromyrmex laticeps*	Mycelial

[1,2]Basidiocarp and mycelial states collected from the same ant's nest.

Material and Methods

Fungal isolates and culture conditions

The *Leucoagaricus* isolates (Table 1) were collected as basidiocarps or as mycelial states from the nests of *Atta* and *Acromyrmex* ants from July 1985 to December 2001 from

the geographic sites shown in Figure 1. The basidiocarp collected from *Acromyrmex hispidus fallax* was identified as *Leucoagaricus gongylophorus* Heim (22). For the isolation of the mycelial form of the fungus from the fungus garden we used yeast nitrogen base glucose chloramphenicol (YNBGC) agar, which increases the efficiency of isolation of this fungus (Silva-Pinhati ACO, Bacci M, Siqueira CG, Silva A, Pagnocca FC, Bueno OC and Hebling MJA, unpublished results) and consists of yeast nitrogen base medium (YNB, Difco 0392-15-9, Detroit, MI, USA) supplemented with 5 g/l glucose (product number 108342, Merck, Darmstadt, Germany), 17 g/l agar (Merck, product number 1.01614), 0.1 g/l chloramphenicol (product number C-0378, Sigma, St. Louis, MO, USA), and sufficient 2 M NaOH to adjust the pH to 6.0. For maintenance we used YNB-glucose agar (i.e., YNBG without chloramphenicol). The fungus garden material, collected from the underground nests of different ant species, was incubated in the dark at 25ºC and 80% humidity for 10 to 20 days on Petri dishes containing worker ants, which cleaned the garden material by removing soil fragments to a different part of the dish. When white mycelial spots of the fungal symbiont appeared on the leaf material some of the mycelium was collected using an aseptic technique and transferred to YNBGC agar on Petri dishes where it was incubated at 25ºC in the dark for 30 days. Alternatively, the fungus garden material from underground nests was collected and immediately plated onto YNBGC agar and incubated at 25ºC in the dark for 30 days. In both cases, after 30 days of cultivation on YNBGC agar the fungal isolates were subcultured to YNBG agar and cultured for a further 60 days, with the hyphae showing swollen tips (gongylidium), which are a distinctive characteristic of the symbiotic fungi of higher attine ants (5). The experiments described in this paper were carried out after two months to ten years of storage. When needed for DNA extraction, mycelium was transferred to fresh YNBG agar and grown for 30 days, after which approximately 100 mg mycelium was processed as described below.

DNA extraction

Fungal mycelium was disrupted by placing 100 mg mycelium in a 1.5-ml microcentrifuge tube, freezing the tube and contents in liquid nitrogen, adding 0.5 ml ice-cold TE buffer, pH 8.6, containing 0.1% (w/v) SDS and homogenizing the mycelia with a plastic pestle that fitted inside the tube. The nucleic acid was purified using buffered phenol, followed by a buffered phenol:chloroform mixture (1:1, v/v) and finally pure chloroform. Total nucleic acids were precipitated with ethanol and sodium acetate, washed with ethanol and dissolved in 100 µl TE buffer.

Amplification of ribosomal DNA and internal transcribed spacer regions

PCR amplification was carried out in a 100-µl reaction mixture using 100 ng fungal

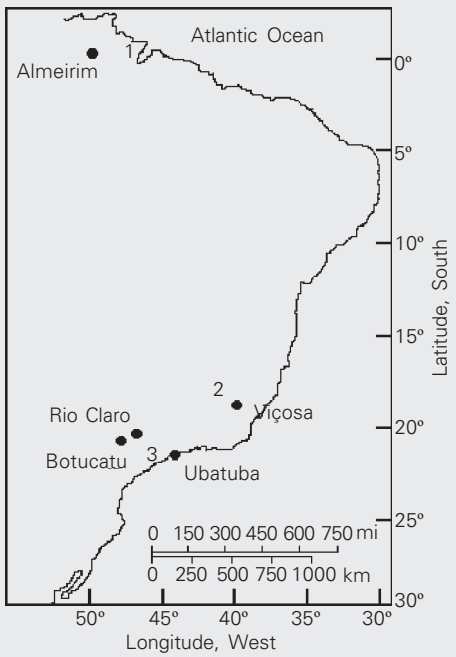

Figure 1. The five geographic sites (Almeirim, Rio Claro, Botucatu, Viçosa, Ubatuba) from which the fungal isolates were collected. The numbers in the map indicate locations of state capitals around each collection site: (1) Macapá (AM); (2) Belo Horizonte (MG); (3) São Paulo (SP).

genomic DNA and 6 pmol each of primers F-5.8S (5'-GGATCACTCGGCTCRTGNRTC GATGAAG-3') and R-635 (5'-GGTCCGT GTTTCAAGACGG-3'). The amplification protocol consisted of initial denaturation at 94ºC for 3 min, followed by 30 PCR amplification cycles of 94ºC for 10 s, 37ºC for 1 min and 72ºC for 3 min. The amplification produced fragments of approximately 1 kb comprising the 5.8S gene, the ITS2 region and 0.6 kb of the 28S gene. The same procedure and protocol were used to amplify the 18S rRNA gene and the ITS1-5.8S-ITS2 region, except that the primers for the 18S rRNA gene were the eukaryotic universal primers (23) A (5'-CCGAATTCGTCGACA ACCTGGTTGATCCTGCCAGT-3') and B (5'-CCCGGGATCCAAGCTTGATCCTTC TGCAGGTTCACCTAC-3') while the ITS4 and ITS5 primers (24) were used to amplify the ITS1-5.8S-ITS2 region. Primers were purchased from Invitrogen/Life Technologies (São Paulo, SP, Brazil).

Cloning

PCR products were cloned in the pGEM-T vector (Promega, Madison, WI, USA), and transformed into competent XL1-BLU *E. coli* cells and recombinant clones selected and purified by a Miniprep procedure (25).

Sequencing

Both strands of recombinant plasmids or PCR products were individually sequenced with the SequiTherm kit (Epicentre Technologies, Madison, WI, USA) according to manufacturer instructions. Each reaction contained 800 ng of purified DNA and 0.30-0.35 pmol infrared dye-labeled primer (Epicentre Technologies). The 5.8S-ITS2-28S fragments were sequenced using the vector primers M13F (5'-CACGACGTTG TAAAACGAC-3') and M13R (5'-GGATA ACAATTTCACACAGG-3') and the internal primers F-63 (5'-TTCCTCCGCTTAT TGATA-TGC-3') and R-63 (5'-TTCCTCC GCTTATTGATATGC-3') (Invitrogen/Life Technologies). Reaction conditions included denaturation at 94ºC for 3 min followed by 30 amplification cycles of 94º, 50º, and 70ºC for 30 s each. The same procedure was used for sequencing the 18S gene, using the M13F and M13R primers as well as the internal primers 514F (5'-TCTGGTGCCAGCASC CGCGG-3'), 536R (5'-TGGWATTACCGC GGSTGCTG-3'), 1055F (5'-GTGGTGGTGC ATGGCCG-3'), and 1055R (5'-AAGAAC GGCCATGCACCAC-3') (26). Reaction products were sequenced on a Li-Cor 4000L automated sequencing system (Li-Cor Biosciences, Lincoln, NE, USA). The ITS1-5.8S-ITS2 amplicons were sequenced using a 10-µl reaction mixture containing 6 pmol of the same primers as for their amplification, 100 ng template, 2.5 µl Big Dye reactant (product number 4303153, PE Applied Biosystems, Foster City, CA, USA), 2 µl 100 mM Tris and 2.5 mM $MgCl_2$, pH 9.0. Reaction conditions included denaturation at 96ºC for 1.5 min followed by 25 amplification cycles of 96ºC for 12 s, 50ºC for 8 s and 60ºC for 4 min. Reaction products were sequenced on an ABI 377 automated sequence system (Applied Biosystems).

Sequence analysis

DNA sequences were aligned using the ClustalW program (27) and compared to each other using the distance values generated according to Jukes and Cantor's substitution model (28) as implemented by the DNADIST program, version 3.5c, contained in the Phylip package (29). Nucleotide diversity (π), i.e., the average number of differences per site between two homologous sequences, was calculated using the DnaSP software, version 2.0 (30), according to equation 10.5 of Nei (31). Phylogenetic analysis was carried out using the maximum parsimony method as implemented by the PAUP* software 4.0b4a (32). The median-joining

network connecting the sequences was constructed using the program Network 3.1.1.1 (available at www.fluxus-engineering.com) and the median joining algorithm (33). Default settings were chosen ($r = 2$ and $\varepsilon = 0$).

Results

In order to assess heterogeneity within and between isolates we sequenced individual clones containing the 5.8S-ITS2-28S amplicon. At least seven clones were sequenced for each fungal isolate. We were especially interested in the ITS regions, which are known to incorporate changes at relatively high rates (see, e.g., 34,35). The 18S rDNA, which evolves comparatively slowly, was sequenced using pooled clones of each isolate. The 18S and 5.8S-ITS2-28S sequences generated are deposited in GenBank under accession numbers AF076380 to AF076430 and the ITS1-5.8S-ITS2 sequences are deposited under accession numbers AY642807 to AY642816.

Few differences were found between isolates A to F, which were collected from two geographic sites (Table 1) and had identical 1760-bp 18S sequences, the 121-bp 5.8S and 690-bp 28S sequences showing more than 99% similarity. Variation in the ITS2 region (212 to 218 bp) was higher, with the 45 sequenced clones containing 15 distinct ITS2 haplotypes which had 25 variable positions corresponding to 7 micro-satellites (1, 2 or 4 base repeats) and 14 transitions (8 C/T and 6 A/G; Figure 2). Nucleotide diversity in the 45 ITS2 sequenced clones ($\pi = 0.00589 \pm 0.00110$) was too low to allow population

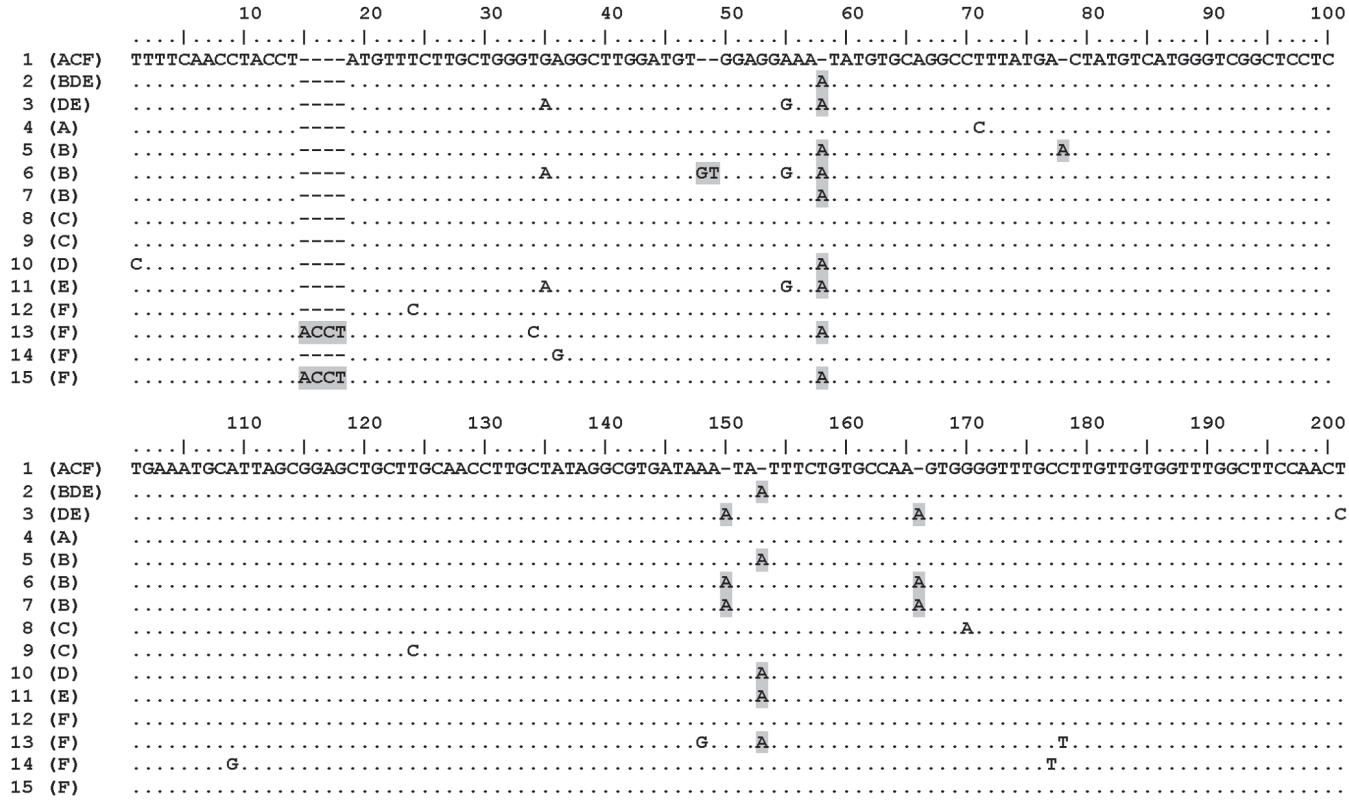

Figure 2. Polymorphic sites of the 15 ITS2 haplotypes of six symbiotic fungal isolates. Fungal isolates were collected from nests of *Atta laevigata* (A), *Atta sexdens rubropilosa* (B), *Acromyrmex crassispinus* (C), *Acromyrmex hispidus fallax* (D = mycelium, E = basidiocarp), and *Atta capiguara* (F). The ITS2 region of these isolates contained 212 to 218 bp, of which a 201-bp alignment containing the ITS2 polymorphic sites is represented. Dots indicate conserved sites and letters polymorphic sites, micro-satellite repeats are shaded and transitions are unshaded. Haplotypes are numbered from 1 to 15 and isolates in which a known ITS2 haplotype was found are given within parentheses.

studies and, similarly, the 2 parsimony-informative characters present in the 14 haplotypes (haplotype 11 was not considered in the parsimony analysis, since isolated D and E are from the same source nest) resulted in a highly polytomic tree (data not shown).

Although the information available was insufficient to reveal detailed relationships between our isolates in a phylogenetic tree or to support a population study of these isolates, it was possible to group them by considering the three ITS2 haplotypes which were shared by several isolates (Table 2). The distribution of the shared haplotypes suggests that leafcutter fungal isolates A to F belong to two genetic groups, one group being composed of isolates A (from *Atta laevigata*), C (from *Acromyrmex crassispinus*) and F (from *Atta capiguara*), all containing ITS2 haplotype 1 but not haplotype 2, and the other group containing isolate B (from *Atta sexdens rubropilosa*) and isolates D and E (both from *Acromyrmex hispidus fallax*), all containing ITS2 haplotype 2 but not haplotype 1. Fungal isolates D and E also shared ITS2 haplotype 3. A phylogenetic network derived by the median-joining method showed that haplotypes 1 and 2 are likely centers of radiation/convergence of the ACF and BDE groups, respectively, so that some haplotypes are more closely related within the ACF (haplotypes 1, 4, 8, 9, 12, 14) or BDE (haplotypes 2, 5, 10, 11) groups than between groups, although haplotypes 3, 6, 7, 13, and 15 were equally related to both ACF and BDE groups (Figure 3). These results suggest that the ACF and BDE genetic groups are in fact two distinct lineages of symbiotic fungi.

To extend our observations to a wider geographic region and other ant species, we investigated fungal isolates G to O (Table 1), collected from the nests of seven leafcutter species living in five different geographic sites. Both ITS1 and ITS2 regions were analyzed by sequencing the amplified ITS1-5.8S-ITS2 fragments, but few polymorphic positions were found in the first clones obtained from isolates K and N. Because of this we decided to directly sequence the PCR products of isolates E and G to O and found them to be identical or to differ by only a maximum distance of 1.5%, in contrast to the 6.7 to 26% distance found between the 10 most closely related sequences of the GenBank *Leucoagaricus* species (see spe-

Table 2. Distribution and number of hits of each of the 15 ITS2 haplotypes found in the 45 sequenced clones of six sympatric or allopatric fungal isolates from *Atta* and *Acromyrmex* leafcutter ants.

Isolate[1]	ITS2 haplotype															Total
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	
A	7	–	–	1	–	–	–	–	–	–	–	–	–	–	–	8
B	–	2	–	–	3	1	1	–	–	–	–	–	–	–	–	7
C	5	–	–	–	–	–	–	1	2	–	–	–	–	–	–	8
D	–	4	3	–	–	–	–	–	–	1	–	–	–	–	–	8
E	–	4	2	–	–	–	–	–	–	–	1	–	–	–	–	7
F	2	–	–	–	–	–	–	–	–	–	–	2	1	1	1	7
Total	14	10	5	1	3	1	1	1	1	1	1	2	1	1	1	45

[1]Fungus symbiotic with *Atta laevigata* (A), *Atta sexdens rubropilosa* (B), *Acromyrmex crassispinus* (C), *Acromyrmex hispidus fallax* (D = mycelium, E = basidiocarp), and *Atta capiguara* (F). Shaded numbers indicate the number of hits for haplotypes that were shared by distinct isolates.

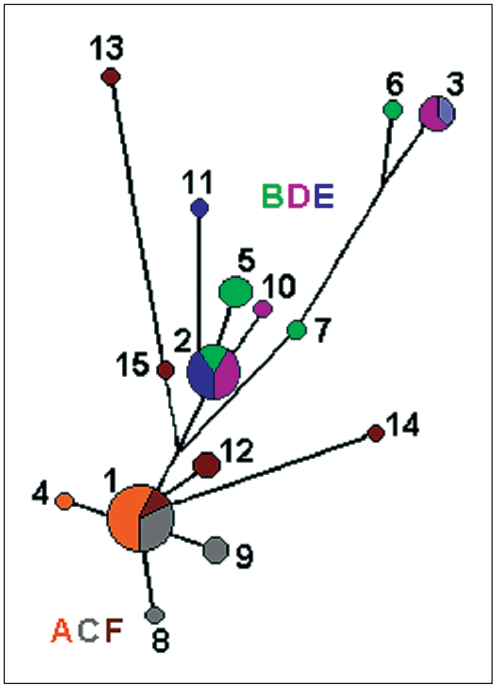

Figure 3. Phylogenetic relationship between the 15 ITS2 haplotypes of six symbiotic fungal isolates. Network nodes (circles) indicate haplotypes, which are numbered in bold and proportionally sized to the number of sequences they represent, i.e., 1, 2, 3, 5, 10, and 14 sequences. The areas of the colored parts of the circles are proportional to the number of sequence copies from each of the fungal isolates A to F (see also Table 2). Note that haplotypes from fungal isolates A, C and F are, in general, more closely related to each other than to haplotypes from isolates B, D and E.

cies names in Figure 4).

Similarly, the distance between the ITS2 regions of isolates A to O was zero to 2.5%, while the distance between the ITS2 regions of the 10 most closely related GenBank *Leucoagaricus* isolates was 9.4 to 26%. In addition, the ITS1 locus presented a highly variable portion that had a base composition and sequence length characteristic for each of the GenBank 10 *Leucoagaricus* species investigated. Conversely, this region was identical for our leafcutter isolates E and G to O (Figure 4). These results indicate that the leafcutters studied by us cultivate very closely related fungal material, probably the same species, over a wide geographic region.

Discussion

In the past it has been difficult to demonstrate that a basidiocarp found in *Atta* or *Acromyrmex* nests is that of the ant's symbiotic fungus and not one of the many contaminant fungi existing in the nests (36). This situation has led to uncertainties in the identification of ant fungi, which have received over a dozen names (4,37,38).

In our investigation we found that the *Leucoagaricus gongylophorus* basidiocarp (E in Table 1) and the mycelial isolate (D in Table 1), both collected from the same *Acromyrmex hispidus fallax* nest, had two identical (and some nearly identical) ITS2 sequences (Table 2). Since ITS regions have been used to distinguish species of fungi (e.g., 39) our results suggest that this *Leucoagaricus gongylophorus* basidiocarp represents the sexual stage of the symbiotic fungus of *Acromyrmex hispidus fallax*, supporting our previous results showing identical RAPD fingerprints for these two isolates (22).

The *Leucoagaricus gongylophorus* basidiocarp was also found to have ITS2 sequences which were identical, or nearly identical, to those of mycelial isolates from the nests of *Atta laevigata* (isolate A), *Atta sexdens rubropilosa* (isolate B) and *Acromyrmex crassispinus* (isolate C), all sympatric leafcutter species collected near the town of Rio Claro, SP, as well as to fungal isolate F collected from an *Atta capiguara* nest near the town of Botucatu, SP, 105 km from Rio Claro, and other fungal symbionts (isolates G to O) from sites near the towns of Botucatu, Ubatuba, Viçosa, and Almeirim, which are up to 2,600 km apart from each other. The variations found in these ITS2 sequences were very low compared to those of GenBank ITS2 sequences belonging to 10 distinct *Leucoagaricus* species. In addition, the highly variable portion of ITS1, which contains a characteristic signature for each *Leucoagaricus* species, was identical in 10 of our fungal symbionts (isolates E and G to O) living in each of the investigated sites.

Taken together, these results indicate that the sympatric and allopatric leafcutter species studied in the present investigation are associated with closely similar fungal material, which may represent a single *Leucoagaricus* species, suggesting that leaf-cutting ants cultivate the same fungal species over a wide geographic region of South America and indicating that the 'single species theory' regarding fungal culture by

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                                    10        20
                                ....|....|....|....|....|.
L. americanus                   GTCTTTTT--GGGGGCTGACAAGCGA
L. bresadolae                   ......CGGG.TCT.GGA.GCG..--
L. crystallifer                 --G.C...GA...CTGA..GCG..TG
L. hortensis                    ......G.GG..TT.AGA.GTG..CG
L. marriagei                    .....C.-...........TG...A.
L. naucinus                     ..A..CG.GT.A.C.TC..GGGAA.-
L. purpureolilacinus            ......G-..-A.......G..CA.T
L. sereneus                     ......GGAGAAT.AGATGG..CA.T
L. sericifer                    ......C.CTT........GG.AA.T
L. wychanskyi                   --GC....GA...CTGA..GCG.A.T
L. gongylophorus E, G - O       .....C..TCAA..T.GA.GTG..TT
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Figure 4. Characteristic signature for each *Leucoagaricus* species. Representation of the portion of the ITS1 sequence that was highly variable in *Leucoagaricus* species but identical in ten fungal isolates collected from eight leafcutter species living in five geographic sites. Dots represent the conserved sites and letters the polymorphic sites of the ITS1 sequences compared to *Leucoagaricus americanus*. The accession numbers of the GenBank *Leucoagaricus* sequences were LAU85317, AF295929, AF482863, AF482843, AF482866, LNU85315, AF482869, AF482871, AF482872, AF482874 (in order from the top to the bottom of the figure).

leafcutters is more likely than the 'multiple species theory'. Therefore, it is likely that the *Leucoagaricus gongylophorus* basidiocarp (E in Table 1) represents the sexual stage of all the mycelial state isolates studied in the present investigation.

The degree of polymorphism that we found in the ITS sequences was too low to reveal detailed relationships between our isolates in a phylogenetic tree. However, distribution of ITS sequences within our fungal isolates collected from the Rio Claro or Botucatu areas suggests that these isolates belong to distinct genetic groups characterized by a specific ITS2 haplotype (Table 2), haplotype 1 only being found in isolates A, C and F (14 hits in 23 sequenced clones) and haplotype 2 only in isolates B, D and E (10 hits in 22 sequenced clones). These results, as well as the phylogenetic relationship between haplotypes derived by median-joining analysis (Figure 3), suggest that these two genetic groups are in fact two distinct fungal lineages. Thus, it seems that the fungal strains cultured by *Atta laevigata*, *Acromyrmex crassispinus* and *Atta capiguara* (belonging to the ACF lineage) are more similar to each other than they are to the fungal strains cultured by *Atta sexdens rubropilosa* and *Acromyrmex hispidus fallax* (BDE lineage). Chapela et al. (10) hypothesized that there has been restricted vertical transmission of symbiotic fungi in the higher attines for the last 23 million years, which suggests that the symbiotic fungi of *Atta* ants should be more similar to each other than they are to the symbiotic fungi of *Acromyrmex* ants. However, our findings do not support the exclusively vertical transmission of symbiotic fungi in higher attines, suggesting instead that lateral transmission may occur under certain circumstances.

Our findings are supported in part by those of Bot et al. (18), who found that, in Panama, lateral transmission of fungal material may have occurred between the sympatric species *Acromyrmex octospinosus* and *Acromyrmex echinatior*. However, these investigators also mention that lateral transfer of symbiotic fungi between *Atta* and *Acromyrmex* ants does not occur, although our results show that some *Atta* and *Acromyrmex* species do indeed cultivate the same lineage of *Leucoagaricus gongylophorus* and hence have probably recently shared their symbiotic fungi.

Markers may exist, which are evolving faster than the ITS regions, and their sequences would provide more polymorphic sites and suitable information for population studies, so that detailed information on the propagation of *Leucoagaricus gongylophorus* lineages throughout distinct leafcutter nests could be assessed. Such propagation seems to be a general characteristic within leafcutters, considering the evidence that we found for lateral transmission of fungal material within the ants from Rio Claro and Botucatu, in Brazil, as well as similar evidence described for some Panamanian *Acromyrmex* (18). Thus, it is conceivable that during the symbiosis evolution a continuous sharing of fungal strains has often occurred and has led to the homogenization of the symbiotic fungal stock through the selection of the *Leucoagaricus gongylophorus* lineages found in both the sympatric and allopatric leafcutters sampled in our study. This scenario of intense lateral transmission of fungal material between leafcutters is consistent with the low variation in both ITS1 and ITS2 found in our isolates and resembles the 'mycocentric' view of the ant-fungus symbiosis that was recently pointed out by Mueller (40), who suggested that symbiotic fungi may play a dominant part in the ant-fungus association. This point of view stands in contrast with the traditional 'myrmicocentric' view of the ant-fungus symbiosis in which the fungus has the passive role of a mere cultivated crop that is manipulated by the ants according to their needs. The symbiotic fungus, *Leucoagaricus gongylophorus*, may be transferred by leafcutters directly

from one nest to another or, conversely, this transmission may involve an intermediary free-living fungal stage so that many clones of the original symbiotic fungus could have been spread over the period of time during which these ants have been living in America. Thus, it is possible that free-living close relatives (or even free-living forms) of *Leucoagaricus gongylophorus* exist.

In addition to intensive lateral transmission, some features of symbiotic fungal lineages may have shaped the population structure of *Leucoagaricus gongylophorus*, such as resistance to antibiotics produced either by the ants (40) or the actinomycete symbiont or to the parasite *Escovopsis*, so that more adapted fungal strains may have been selected. Thus, the complete elucidation of the factors that have led to the low variation in *Leucoagaricus gongylophorus* ITS sequences requires an intensive sampling and characterization of the two groups of symbiotic microorganisms of many leafcutters from distinct geographic sites in America.

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