Antonie van Leeuwenhoek (2016) 109:1235–1243
DOI 10.1007/s10482-016-0724-3

ORIGINAL PAPER

Prevalence of the genus *Cladosporium* on the integument of leaf-cutting ants characterized by 454 pyrosequencing

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Received: 2 February 2016 / Accepted: 8 June 2016 / Published online: 15 June 2016

Abstract The relationship of attine ants with their mutualistic fungus and other microorganisms has been studied during the last two centuries. However, previous studies about the diversity of fungi in the ants' microenvironment are based mostly on culture-dependent approaches, lacking a broad characterization of the fungal ant-associated community. Here, we analysed the fungal diversity found on the integument of *Atta capiguara* and *Atta laevigata* alate ants using 454 pyrosequencing. We obtained 35,453 ITS reads grouped into 99 molecular operational taxonomic units (MOTUs). Data analysis revealed that *A. capiguara* drones had the highest diversity of MOTUs. Besides the occurrence of several uncultured fungi, the mycobiota analysis revealed that the most abundant taxa were the *Cladosporium*-complex, C*ryptococcus laurentii* and *Epicoccum* sp. Taxa in the genus *Cladosporium* were predominant in all samples, comprising 67.9 % of all reads. The remarkable presence of the genus *Cladosporium* on the integument of leaf-cutting ants alates from distinct ant species suggests that this fungus is favored in this microenvironment.

Keywords Next generation sequencing · ITS · Attini ants · Cuticle · Fungal consortium

Electronic supplementary material The online version of this article (doi:10.1007/s10482-016-0724-3) contains supplementary material, which is available to authorized users.

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Introduction

Colonies of leaf-cutting ants (genera *Atta* and *Acromyrmex*) of the tribe Attini (Formicidae) harbor a wide diversity of microorganisms. The principal member of this complex network of interactions is a basidiomycetous fungus (Agaricaceae, genus *Leucoagaricus*) cultured by these ants for food (Silva et al. 2003). After 50 million years of co-evolution, attine ants and their cultivated fungus are dependent on each other and therefore feature an obligatory mutualistic relationship (Mueller 2012; Schultz and Brady 2008).

Leaf-cutting ants cut large amounts of plant materials as substrate for the cultivation of the mutualistic fungus (Weber 1972) and this is why they are considered pests to agriculture crops causing severe

economical losses. Thus, many studies have been conducted about the relationship of leaf-cutting ants with *Leucoagaricus* and other associated microbial symbionts in order to elucidate their ecology and assist in the search for methods to control these insects (Ortiz and Orduz 2000; Lopez and Orduz 2003; Folgarait et al. 2011).

Once a year, the reproductive caste of leaf-cutting ants, composed by alate individuals, leaves the nest to perform the mating flight. Females (gynes) carry a small portion of *Leucoagaricus* in their infrabuccal pocket and, after the fecundation by males (drones), each gyne tries to establish a new colony (Autuori 1941).

The study of Pagnocca et al. (2008) was the first to analyse the fungal community present in the integument of leaf-cutting gynes from *Atta capiguara* and *Atta laevigata* nests and isolated several fungal species with prevalence of the genus *Cladosporium*. A few years later, Duarte et al. (2014) reported the presence of potentially pathogenic black fungi, such as *Exophiala* and *Ochroconis*, in the gynes' body of the same species. Therefore, the fungi present in the integument of alate ants may be dispersed in the environment and thus compose the fungal community of the newly founded colony.

The first study aiming to evaluate the fungal diversity associated with nests of leaf-cutting ants using a culture-independent method was performed by Rodrigues et al. (2014). Using clone libraries based on the internal transcribed spacer (ITS) region, the authors analysed the fungal community in the chambers' soil and the surrounding soil of *Atta sexdens rubropilosa* and *Atta bisphaerica* nests. The authors found differences between fungal communities in nest and non-nest soils, revealing that ants may influence the mycobiota found in the soil adjacent to their nests.

Culture-independent methods, such as next generation sequencing (NGS), have been widely used in environmental studies of the mycobiome, such as soil fungi (Lim et al. 2010; Lentendu et al. 2011), mycorrhizal fungi (Lumini et al. 2010) and plant-associated fungi (Gillevet et al. 2009; Arfi et al. 2012) but not in mycobiomes found in fungal–insect interactions.

Because almost all studies on the diversity of fungi associated with leaf-cutting ants have used culture-dependent approaches, here we used 454 pyrosequencing to further evaluate the mycobiota diversity on gynes and drones of the ants *A. capiguara* and *A. laevigata*. This study represents the first survey of fungal communities present on the integument of leaf-cutting ants using a NGS method.

Methods

Study site and sampling

One colony of *A. laevigata* and one colony of *A. capiguara* located at Fazenda Santana (Locality: 22°50.6′S; 48°26.1′W; elevation 798 m), Botucatu, São Paulo State, Brazil, were monitored for several months before the mating flight season. This generally occurs once a year from September to November in São Paulo State. On the collection day (November 2012) only these two colonies released alates, thus limiting the number of colonies sampled. All ants were collected on the same day during the beginning of the mating flight, right at the moment when the alate ants appeared at the nest entrance. Ten drones and ten gynes were picked up from the *A. capiguara* colony and ten drones from the *A. laevigata* colony. Gynes of *A. laevigata* were not released at the moment of collection.

Molecular analysis

Each ant was immersed individually in TE buffer (Tris 1 M; EDTA 0.5 M) and sonicated in a Branson 1210 ultrasonic water bath for 10 min. Due to the different sizes, one mL of buffer was used for drones and four mL for gynes. The buffer, containing fungal cells in suspension was evaporated to dryness in a Speed-Vac concentrator (Savant Instruments) and then suspended in 30 μL of TE buffer. The resulting suspension was used for DNA extraction according to the CTAB protocol of Reis et al. (2015). Extracted DNA of each ant group was mixed according to the source, resulting in three samples (*A. capiguara* and *A. laevigata* drones; *A. capiguara* gynes).

The ITS region of the ribosomal DNA was amplified using the primers ITS1 and ITS4 (White et al. 1990). Fifteen ng of template DNA were used for a 25 μL PCR amplification reaction performed as follows: 94 °C for 5 min, followed by 35 cycles consisting of 94 °C for 45 s, 52 °C for 30 s and 72 °C for 1 min, and post elongation step at 72 °C for 7 min. To reduce

PCR biases, six PCR reactions were carried out for each sample and then pooled per sample group. Amplicons were analysed on a 1 % agarose gel and a negative control (DNA free) was also performed. Amplicons were purified using a NucleoSpin® PCR Clean-up (Macherey–Nagel). Pyrosequencing was performed on a Genome Sequencer (GS) FLX+ platform (Roche, Switzerland) at the MACROGEN DNA Synthesis and Sequencing Facility (Seoul, Korea).

Bioinformatics and data analyses

Reads were submitted to the online program PRINSEQ (Schmieder and Edwards 2011) to examine the presence of ambiguities (Ns), quality, average size and GC content. The data have been deposited in the National Center for Biotechnology Information BioProject database, www.ncbi.nlm.nih.gov/bioproject (project ID PRJNA321130). After checking that the reads quality was higher than the 20 PHRED (Ewing and Green 1998; Ewing et al. 1998), sequences shorter than 400 bp were removed using a local script.

The data obtained were analysed using ITScan (Ferro et al. 2014), a recent pipeline dedicated to studies on fungal diversity (available at http://evol.rc.unesp.br:8083/editor/faces/itscan/input.xhtml). Pipeline steps comprise a chimera screening, executed by the Chimera Checker program (Nilsson et al. 2010), removal of chimeric ITS sequences, sequence alignment performed in MAFFT (Katoh and Standley 2013), clustering of aligned sequences into molecular operational taxonomic units (MOTUs) using a sequence similarity of 97 % (Tedersoo et al. 2010) in Mothur (Schloss et al. 2009). Singletons (MOTUs including only one read) were removed from further analysis, as recommended by Tedersoo et al. (2010), in order to improve the precision of diversity estimates.

In order to provide taxonomic affiliations, comparisons of MOTUs against the NCBI-GenBank were carried out using 1e-03 e-value parameter. The identification of the MOTUs was also manually refined by constructing phylogenetic trees including sequences from reference strains (type) available in the GenBank (neighbor-joining method) using MEGA6 (Tamura et al. 2013).

The taxonomic assignment was determined using the MycoBank (www.MycoBank.org; Robert et al. 2013). Fisher's alpha diversity index, a suitable tool for comparing samples of different sizes (Loro et al. 2012), and rarefaction curves were generated in PAST. Additionally, Sorensen and Jaccard's similarity indices were calculated to compare the communities using EstimateS 8.2.0 software (Colwell et al. 2012).

Results

A total of 142,930 reads were obtained and after filtering out short sequence reads (<400 bp), 99,281 reads (69.5 %) were used in the ITScan pipeline. The number of reads per sample and average read length are shown in Table 1. The PHRED quality score for all samples was higher than 20 and GC content ranged from 46.8 % (*A. capiguara* drones) to 48.1 % (*A. laevigata* drones).

After removing chimeric sequences, 57 singletons were deleted and reads were grouped into 99 distinctive MOTUs: 51 from *A. capiguara* drones, 25 from *A. capiguara* gynes and 46 from *A. laevigata* drones.

Of the 99 MOTUs obtained in the study, 32 were unique to *A. capiguara* drones (including *Cryptococcus laurentti*, *Cunninghamella* and *Periconia* species), 11 were unique to *A. capiguara* gynes (including *Trichosporon chiarellii*) and 37 were unique to *A. laevigata* drones (including *Aureobasidium* and *Epicoccum* species). The mutualistic fungus *Leucoagaricus gongylophorus* was shared by drones (5 reads) and gynes (351 reads) of *A. capiguara*. Additionally, four MOTUs were present in all samples (Table 2). The sequence data of the ITS region are too conserved to distinguish different species in the genera *Aspergillus*, *Cladosporium*, *Penicillium* and *Trichoderma* (Balajee et al. 2007; Visagie et al. 2014; Bensch et al. 2015; Montoya et al. 2016). Therefore these MOTUs were grouped as a species complex. A complete list of the MOTUs and their distribution has been included in Online Resource 1.

According to Jaccard and Sorensen's indices, *A. capiguara* drones and *A. capiguara* gynes shared the highest number of fungal taxa (Jaccard: 0.24; Sorensen: 0.39). This comparison is not possible for *A. laevigata* since gynes were not obtained from this nest.

Most of the MOTUs contained 3–100 reads, however three MOTUs grouped over 1000 reads. These three MOTUs were taxonomically assigned to the *Cladosporium*-complex (n = 24,099), *Cryptococcus laurentii* (n = 2752), and *Epicoccum* sp. (n = 3955) and made up 86.9 % of the reads. Table 3 shows the most representative MOTUs in the samples.

Table 1 Data characteristics before and after ITScan processing

	Raw data		Treated data[a]	
	No. of reads	Average read length	No. of reads	Average read length
A. capiguara drones	44,604	508	22,953	519
A. capiguara gynes	74,966	454	934	512
A. laevigata drones	23,360	504	11,566	505

[a] Reads characteristics after length filtering and removal of chimeric sequences

Table 2 Shared MOTUs for *A. capiguara* drones and gynes, and *A. laevigata* drones

Best hit at NCBI database	Taxonomic assignment	Coverage (%)	Similarity (%)	Accession no.	No. of reads		
					AC drones	AC gynes	AL drones
Ascomycota sp.	Magnaporthales (A)	99	95	JQ692165	17	4	5
Cladosporium-complex	Capnodiales (A)	100	100	KP143685	17,520	377	6202
Fusarium polyphialidicum	Hypocreales (A)	100	97	HQ607880	16	3	67
Fusarium decemcellulare	Hypocreales (A)	100	99	KR534720	5	2	25

A Ascomycota, AC = *A. capiguara*, AL = *A. laevigata*

Rarefaction analysis of the MOTUs showed that the asymptote was approached for all three samples (Fig. 1) suggesting that 454 pyrosequencing recovered most of the fungal species present on the integument of the sampled ants. The diversity index revealed that *A. capiguara* drones had the highest diversity (Fisher's alpha: 6.2) followed by *A. laevigata* drones (Fisher's alpha: 6.1).

From the 99 fungal taxa identified, 78.8 % belonged to Ascomycota, 16.2 % to Basidiomycota and 5 % to Mucoromycotina. The orders Pleosporales, Capnodiales and Hypocreales encompassed the highest number of MOTUs for ascomycetous fungi (Fig. 2). Basidiomycetous fungi were mostly classified in the order Tremellales. On the other hand, Mucoromycotina is represented exclusively by the order Mucorales and encompasses only *Cunninghamella* and *Mucor* species. Also, 13.1 % of the MOTUs represent uncultured fungi.

Discussion

Studies on the mycobiota present on the integument of leaf-cutting ants are relatively recent and only four studies are published on this subject (Pagnocca et al. 2008; Guedes et al. 2012; Arcuri et al. 2014; Duarte et al. 2014). The relationship of these cuticular fungi with ants is still unknown but Attili-Angelis et al. (2014) suggested that the presence of melanised fungi (order Chaetothyriales) may be favored by the hydrocarbon composition in the cuticle of these insects.

Although these works contributed to the knowledge of fungi present on the integument of attine ants, the sole use of culture-dependent techniques limited the study of fungal diversity and the finding of putative unknown species that fail to grow on artificial media. In this context, the present study evaluated the fungal diversity associated with the integument of alate leaf-cutting ants using pyrosequencing.

As expected, we found the mutualistic fungus on gynes of *A. capiguara*. *L. gongylophorus* was also found on drones of *A. capiguara*, even though in a lower number of reads. As the reproductive caste is raised in the parental fungus garden, the mutualistic fungal hyphae could be attached to the integument of gynes and drones, however *L. gongylophorus* was not detected on drones of *A. laevigata*. Besides that, because ant female alates carry mycelium pellets in internal infrabucal pockets (Autuori 1941) it is likely that our method to recover DNA from the integument

Table 3 The ten top abundant MOTUs found in each sample based on closest match in NCBI database

	Best hit at NCBI database	Taxonomic assignment	Coverage (%)	Similarity (%)	Accession #	No. of reads	% total
A. capiguara drones	*Cladosporium*-complex	Capnodiales (A)	100	100	GU395509	17,520	76.2
	Cryptococcus laurentii	Tremellales (B)	81	98	KP131909	2754	12
	Dothideomycetes sp.	Pleosporales (A)	100	99	KM519276	970	4.2
	Cunninghamella sp.	Mucorales (M)	88	90	KR812209	339	1.5
	Phoma draconis	Pleosporales (A)	88	100	KP276621	322	1.4
	Teratosphaeriaceae sp. 2	Capnodiales (A)	100	94	KT833169	213	0.93
	Coniothyrium sp.	Pleosporales (A)	87	98	DQ092505	145	0.63
	Cunninghamella bainieri	Mucorales (M)	99	93	KF201293	145	0.63
	Periconia macrospinosa	Pleosporales (A)	100	99	JQ781723	66	0.29
	Pseudocercospora norchiensis	Capnodiales (A)	100	100	EF394859	52	0.23
A. capiguara gynes	*Cladosporium*-complex	Capnodiales (A)	99	99	HQ608074	377	39.8
	Leucoagaricus gongylophorus	Agaricales (B)	100	99	KJ531208	351	37.5
	Teratosphaeriaceae sp. 4	Capnodiales (A)	100	99	KT833162	58	6.1
	Fusarium oxysporum	Hypocreales (A)	100	100	KT794176	35	3.7
	Teratosphaeriaceae sp. 2	Capnodiales (A)	100	98	KT833169	32	3.4
	Teratosphaeriaceae sp. 1	Capnodiales (A)	100	100	KT833155	11	1.2
	Phanerochaete tuberculata	Polyporales (B)	98	94	AY219356	10	1.1
	Clonostachys byssicola	Hypocreales (A)	100	100	KC806270	6	0.63
	Uncultured fungus 4	Pleosporales (A)	100	99	FJ213550	6	0.63
	Pseudocercospora fuligena	Capnodiales (A)	100	88	GU214675	5	0.53
	Trichosporon chiarellii	Tremellales (B)	99	99	GQ338074	5	0.53
A. laevigata drones	*Cladosporium*-complex	Capnodiales (A)	100	99	GU395509	6202	53.5
	Epicoccum sp.	Pleosporales (A)	100	99	KR024724	3955	34
	Aureobasidium leucospermi	Dothideales (A)	100	99	JN712487	399	3.4
	Bionectria sp.	Hypocreales (A)	100	99	HM849058	111	0.96
	Teratosphaeriaceae sp. 3	Capnodiales (A)	98	97	KT833168	107	0.92
	Fusarium incarnatum-equiseti complex	Hypocreales (A)	100	100	KJ562367	96	0.83
	Uncultured fungus 1	Pleosporales (A)	100	99	GU053794	95	0.82
	Alternaria alternata	Pleosporales (A)	100	100	KU866390	74	0.64
	Fusarium polyphialidicum	Hypocreales (A)	100	97	HQ607880	67	0.58
	Trichosporon asahii	Tremellales (B)	100	99	KU095859	63	0.54

A Ascomycota, *B* Basidiomycota, *M* Mucoromycotina

also extracted DNA from the mutualistic fungus present in the pellets.

Besides the ant mutualistic fungus, our results revealed a diverse fungal community associated with both ant species examined. Moreover, it has become more apparent that the ants' integument represents a microenvironment for several microorganisms that interact among themselves and may interfere in the complex attine ant-microbe interaction (Little and Currie 2007; Ishak et al. 2011).

As gynes are at least twice the size than drones (Online Resource 2), and for this reason they have a higher contact surface with surrounding environment, we expected a higher number of reads obtained from these insects; however, we observed that *A. capiguara* gynes had the lowest number of reads (n = 934) and MOTUs (n = 25). Using a culture-dependent technique that favored the growth of melanised fungi, Duarte et al. (2014) isolated 17 fungal taxa from 100 *A. capiguara* gynes'

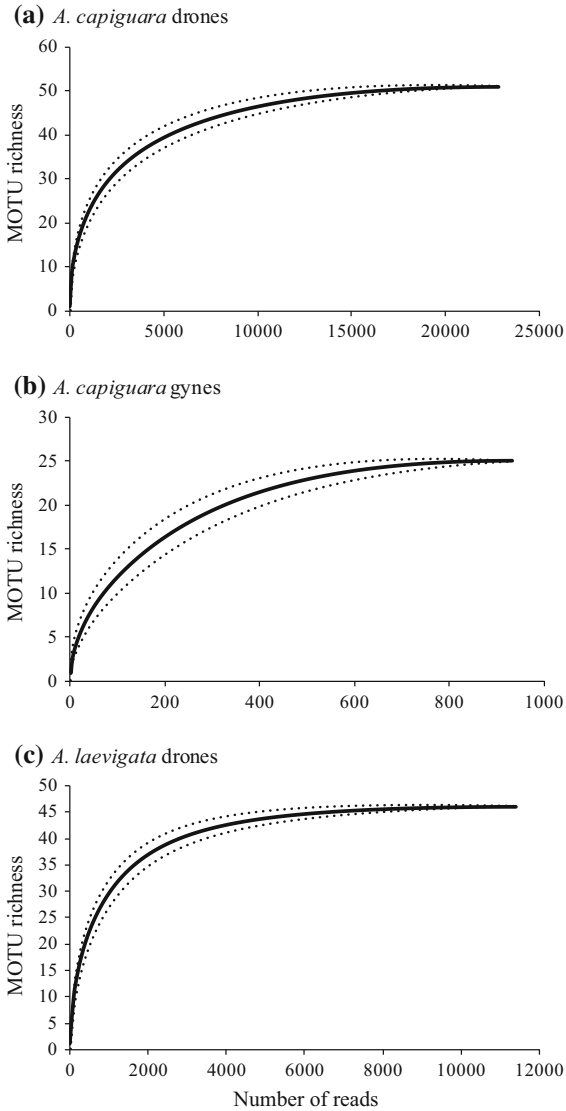

Fig. 1 Rarefaction curves (*solid lines*) of observed MOTU richness at 97 % sequence similarity along with the 95 % confidence intervals (*dotted lines*) on the integument of *A. capiguara* drones (**a**), *A. capiguara* gynes (**b**) and *A. laevigata* drones (**c**)

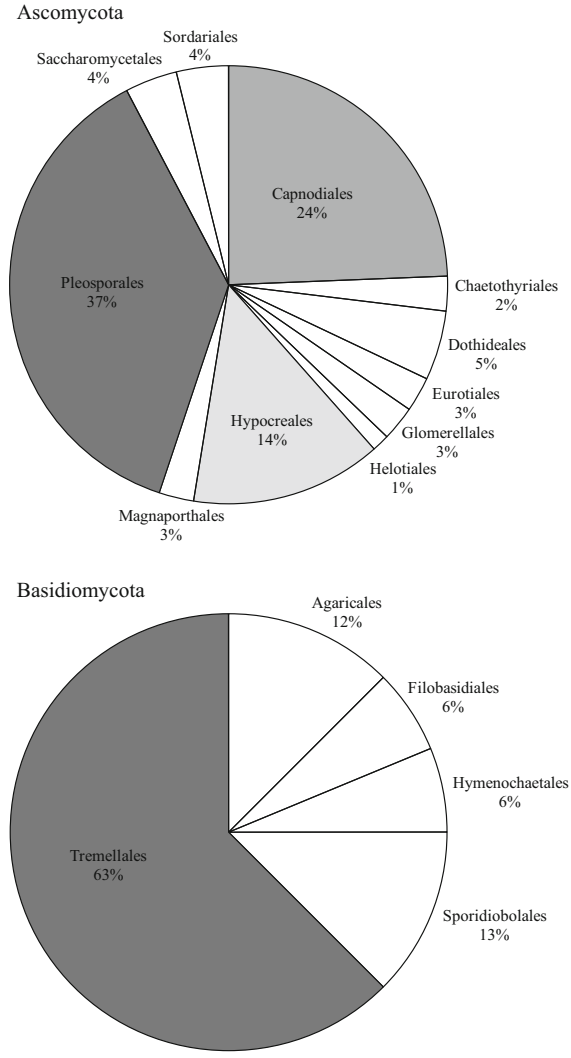

Fig. 2 Distribution of the 99 fungal MOTUs obtained from the integument of *A. capiguara* drones, *A. capiguara* gynes and *A. laevigata* drones, according to the phylogenetic assignment

integuments, a lower fungal richness compared to the present study.

Fisher's alpha diversity index showed that drones of *A. capiguara* and *A. laevigata* had the highest fungal richness. In fact, these samples also exhibited the highest number of MOTUs (51 and 46, respectively). The study on the mycobiota associated with drones is recent and Arcuri et al. (2014) showed a wide diversity of yeasts on the integument of *A. sexdens rubropilosa* drones, with predominance of *Aureobasidium* and *Cryptococcus* species.

According to the Jaccard and Sorensen's similarity indices, drones and gynes of *A. capiguara* are more similar to each other than to drones of *A. laevigata* regarding the fungal diversity. In addition, the number of shared MOTUs among the three samples was very low (n = 4). These results support the hypothesis of Duarte et al. (2014) that the fungal community associated with *A. capiguara* and *A. laevigata* alate ants are distinct. However, this conclusion should be carefully considered as it is based in a limited number of samples.

The clustered MOTUs suggest that the fungal populations present on the integument of leaf-cutting ants belong to the phyla Ascomycota, Basiodiomycota and Mucoromycotina. The two ascomycetous orders with the highest number of MOTUs, Pleosporales and Capnodiales, comprise epiphytes, endophytes, saprobes and plant parasites (Crous et al. 2009; Zhang et al. 2012). Hypocreales was another ascomycetous order frequently obtained in this study and includes plant pathogens and biocontrol agents (Samuels et al. 2006). Thus, the most abundant orders comprise plant-related fungi that may enter into the nests through different plants harvested by these leaf-cutting ants and, once incorporated in the fungus gardens, they can contaminate the integument of worker ants and alates as well.

Reads assigned to the genus *Cladosporium* (Capnodiales, Ascomycota) were prevalent in all samples and accounted for 67.9 % of all reads obtained. Pagnocca et al. (2008) reported massive isolation of this genus from the integument of *A. capiguara* and *A. laevigata* gynes, suggesting that leaf-cutting ants may be an important carrier of *Cladosporium* species.

Species of *Cladosporium* are ubiquitous and, therefore, they are found in a wide range of environments (Bensch et al. 2012). Besides, *Cladosporium* is frequently isolated from soil (Domsch et al. 1980), air (O'Gorman and Fuller 2008) and is a common endophyte (Kumaresan and Suryanarayanan 2002) and plant pathogen (Thomma et al. 2005). Although their role in the attine symbiosis is unknown, the remarkable presence of *Cladosporium* in the integument of these ants suggests that this fungus is favored in this microenvironment and might use the ants as dispersal vehicle.

Contrary to the results obtained by Duarte et al. (2014), we found a lower diversity of chaetothyrialean fungi that could be explained by the small number of ants evaluated (n = 30) as both studies sampled the same nests of alate ants species. The melanised fungi *Cladophialophora*, *Ochroconis* and *Phialophora* isolated by Duarte et al. (2014) were not observed in the pyrosequencing. Furthermore, *Phialophora*-related species, suggested to be symbiont of attine ants by Little and Currie (2007), were also not found.

From the Basidiomycota phylum, C*ryptococcus laurentii* (order Tremellales) was the most abundant taxon and observed only in *A. capiguara* drones. This basidiomycetous yeast has been shown to be prevalent in the attine ants microenvironment and it has been reported in the fungus garden (Middelhoven et al. 2003) and integument of gynes (Pagnocca et al. 2008) and drones (Arcuri et al. 2014). Mendes et al. (2012) showed that, besides *C. laurentii*, many other yeasts present in the fungus garden of leaf-cutting ants, are able to assimilate galacturonic acid, a compound derived from the hydrolysis of pectin (Siqueira et al. 1998) by *Leucoagaricus* and considered harmful to ants' survival.

Interestingly, *Trichosporon chiarellii* has been frequently reported in association with attine ants (Pagnocca et al. 2010) and so far was not found in another environment. Here, we found this yeast species on the integuments of *A. capiguara* gynes (five reads). Arcuri et al. (2014) isolated *T. chiarellii* from the integument of *A. sexdens rubropilosa* drones before the mating flight. Because alate ants might be responsible for the vertical transmission of some microorganisms, the present report supports the hypothesis that *T. chiarellii* could be an autochtonous species in attine ant colonies.

Thirteen MOTUs were identified as uncultured fungi but all were classified to at least the order level. These results show that members of the fungal community associated with the integument of leaf-cutting ants remain poorly described.

Our study indicates that the integument of leaf-cutting ant alates harbor a wide diversity of fungi and many have not been recovered by culture-dependent methods. The complex relationship among fungi and other associated organisms in this microenvironment has been insufficiently researched and may contain the fundamental key to elucidate the mechanisms used by the ant in nurturing the mutualistic fungus and defence against natural enemies.

Acknowledgments This study was supported by "Fundação de Amparo a Pesquisa do Estado de São Paulo" (FAPESP—Proc. 2013/08540–4) and CNPq (National Council for Scientific and Technological Development; Grant Number: 560.682/2010–7 and 448941/2014–7). Collections were conducted under collecting Permit Number 36333–1 issued by the "Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis" (IBAMA) and the "Instituto Chico Mendes de Conservação da Biodiversidade" (ICMBio).We also thank José Luiz Novelli, for allowing the ants collections in the Fazenda Santana, São Paulo State, Brazil.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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