Antonie van Leeuwenhoek (2008) 94:517–526
DOI 10.1007/s10482-008-9268-5

ORIGINAL PAPER

# Yeasts and filamentous fungi carried by the gynes of leaf-cutting ants

**Fernando C. Pagnocca · André Rodrigues · Nilson S. Nagamoto · Maurício Bacci Jr.**

Received: 26 May 2008 / Accepted: 9 July 2008 / Published online: 30 July 2008


**Abstract** Insect-associated microbes exhibit a wide range of interactions with their hosts. One example of such interactions is the insect-driven dispersal of microorganisms, which plays an essential role in the ecology of several microbes. To study dispersal of microorganisms by leaf-cutting ants (Formicidae: Attini), we applied culture-dependent methods to identify the filamentous fungi and yeasts found in two different body parts of leaf-cutting ant gynes: the exoskeleton and the infrabuccal pocket. The gynes use the latter structure to store a pellet of the ants' symbiotic fungus during nest founding. Many filamentous fungi ($n = 142$) and yeasts ($n = 19$) were isolated from the gynes' exoskeleton. In contrast, only seven filamentous fungi and three yeasts isolates were recovered from the infrabuccal pellets, suggesting an efficient mechanism utilized by the gynes to prevent contamination of the symbiotic fungus inoculum. The genus *Cladosporium* prevailed (78%) among filamentous fungi whereas *Aureobasidium*, *Candida* and *Cryptococcus* prevailed among yeasts associated with gynes. Interestingly, *Escovopsis*, a specialized fungal pathogen of the leaf-cutting ant-fungus symbiosis, was not isolated from the body parts or from infrabuccal pellets of any gynes sampled. Our results suggest that gynes of the leaf-cutter ants *Atta laevigata* and *A. capiguara* do not vertically transmit any particular species of yeasts or filamentous fungi during the foundation of a new nest. Instead, fungi found in association with gynes have a cosmopolitan distribution, suggesting they are probably acquired from the environment and passively dispersed during nest foundation. The possible role of these fungi for the attine ant–microbial symbiosis is discussed.

**Keywords** *Atta* · Mating flight · *Escovopsis* · Infrabuccal pellet · Attini

F. C. Pagnocca (✉) · A. Rodrigues · M. Bacci Jr.
Center for the Study of Social Insects, UNESP - São Paulo State University, Av. 24-A, 1515, Bela Vista, Rio Claro, SP 13506-900, Brazil
e-mail: pagnocca@rc.unesp.br

F. C. Pagnocca · A. Rodrigues · M. Bacci Jr.
Department of Biochemistry and Microbiology, UNESP - São Paulo State University, Rio Claro, SP, Brazil

N. S. Nagamoto
Department of Vegetal Production, UNESP - São Paulo State University, Botucatu, SP, Brazil

## Introduction

Fungus-growing ants (Hymenoptera, Formicidae, tribe Attini) maintain an obligate symbiosis with basidiomycetous fungi, as occurs in the case of *Leucoagaricus gongylophorus* (Agaricales) which are cultivated for food and supply of essential enzymes to workers (Weber 1972, Silva et al. 2006). In return, the ants provide protection against antagonistic microbes and

parasites to the fungal partner (Mueller 2002, Currie et al. 2003a, Rodrigues et al. 2008) and substrate for fungal growth. This symbiosis has become a model system for the study of coevolution, and many aspects of the evolutionary histories and complex interactions among its participants have been investigated (Chapela et al. 1994, Schultz and Meier 1995, Mueller et al. 1998, Wetterer et al. 1998, Currie 2001a, Pagnocca et al. 2001, Currie et al. 2003c, Cafaro and Currie 2005, Schultz and Brady 2008).

Fungiculture in the tribe Attini (the fungus-growing ants) originated approximately 50 million years ago (Schultz and Brady 2008). Despite significant long-term co-evolution of both organisms, there is evidence that distantly related ant species can cultivate similar fungi and vice-versa. Such "cultivar switches" among ant species have occurred repeatedly throughout the evolutionary history of this symbiosis (Mueller et al. 1998, Villensen et al. 2004).

Vertical transmission of the cultivated fungus occurs when gynes leave their parental nest for the mating flight, at which time they transport in their infrabuccal pocket a small fungal pellet which will become the starting inoculum for the gyne's new fungus culture. This culture occurs in underground chambers on a variety of substrates (mostly plant material in leaf-cutting ants); initially, faecal deposits secreted by the gyne are used to grow the cultivar, and later, when workers emerge, they collect the substrate to support the fungus garden (Weber 1972).

Currie et al. (1999a) and Currie (2001b) reported that fungi in the genus *Escovopsis* (Ascomycota: anamorphic Hypocreales) can overrun fungus gardens and the resulting effects are usually dramatic for the ant colony. The ants' response to this threat was to acquire an additional symbiont, actinomycete bacteria which grows on their bodies and is found in all attine genera studied so far (Currie et al. 1999a, b). Initially assigned to the genus *Streptomyces*, these bacteria are now recognized as belonging to the genus *Pseudonocardia* (Currie et al. 2003b) and it was suggested that they can protect attine nests by producing disease-suppressing antibiotic compounds that selectively inhibit *Escovopsis* growth (Currie et al. 1999b). The observations that *Pseudonocardia* are transmitted by gynes from the parental colony to new colonies during nest foundation (i.e. vertical transmission) and exhibit selective antimicrobial activity lead to the conclusion that *Pseudonocardia* have co-evolved with ants and the parasite *Escovopsis* (Cafaro and Currie 2005). However, according to Kost et al. (2007) it is possible that *Pseudonocardia* as well as other actinomycetous symbionts can also be acquired from the environment (i.e. horizontal transmission).

Although the primary participants in the attine symbiosis have been relatively well-studied, comparatively less research has focused on the yeasts and other microfungi associated with this complex system. However, the evidence that the association is tripartite (attine ants-fungal cultivar-actinomycete bacteria) (Currie et al. 2003a) led to additional studies aiming to identify other microbes associated with attine nests. Most such studies have focused on microbes associated with the ants' fungus gardens. For instance, results from previous surveys revealed that bacteria in the genus *Burkholderia* are found in association with *Atta sexdens rubropilosa* and it was suggested that these bacteria can also secret antibiotics targeting *Escovopsis* sp. and other filamentous fungi providing protection for the fungus garden against unwanted microorganisms (Santos et al. 2004). Surprisingly, no systematic microbial surveys have been conducted in soil from the nest mound, the ants' exoskeleton or the fungal pellets carried by foundress queens.

In addition to the cultivar, other filamentous fungi have been found in both field and laboratory nests of *A. sexdens rubropilosa* (Rodrigues et al. 2005) and field nests of *Acromyrmex* ants (Rodrigues et al. 2008). These studies indicate that some fungal species are widespread and prevalent in leafcutter ant nests, suggesting that they could still have some yet unknown role in the symbiosis.

Craven et al. (1970) were the first to reveal that fungus garden of attine species contain yeasts. Carreiro et al. (1997) conducted a survey of yeast diversity using laboratory nests of *A. sexdens rubropilosa*, in which two new yeast species were described (Middelhoven et al. 2003, Carreiro et al. 2004). Little and Currie (2007) described that black yeasts closely related to the genus *Phialophora* could be a new symbiont associated with attine ants. Additional new yeast species have subsequently been found in association with other attine species (Fernando C. Pagnocca, unpublished data). In one year-old nests ($n = 10$) of *A. sexdens rubropilosa* maintained in the laboratory, the number of yeasts per gram ranges from $2.7 \times 10^4$ to $1.3 \times 10^5$ in the fungus garden and $2.2 \times 10^4$ in the waste deposit (Pagnocca et al. 1996), suggesting that various parts of

leafcutter ant nests make suitable environments for yeasts.

Carreiro (2000) hypothesized that yeasts, while taking advantage of the nutrients available in the fungus garden, might also be beneficial to the ants. By secreting enzymes into the fungus garden, these yeasts might contribute to the digestion of the plant substrate collected by the ants, making more nutrients available to the ants, to the symbiotic fungus and to themselves as well. These enzymes might also contribute to the degradation of plant tissue by the fungal cultivar by breaking down structural plant polysaccharides such as pectin and hemi-cellulose (Carreiro 2000).

The present study aims to investigate the yeasts and filamentous microfungi carried by gynes of the leaf-cutting ants *Atta laevigata* Smith (1858) and *Atta capiguara* Gonçalves (1944). To accomplish this objective we monitored field nests for nuptial flight preparation in 2006 and 2007 and collected gynes just before their flight. Using culture-dependent methods coupled with molecular methods we identified the yeasts and other microfungi associated with these insects.

## Material and methods

### Nest location and gyne collection

Two adult nests of *A. capiguara* and one of *A. laevigata* were located at Fazenda Santana, Botucatu, São Paulo State, Brazil (GPS location: 22°50.6′ S; 48°26.1′ W; elevation 798 m) in a field containing almost exclusively *Paspalum* sp. grass. At the beginning of the rainy season (September), the nests were periodically monitored in order to predict the day of the mating flight. In the state of São Paulo, flights usually occur in October following significant rainfall (enough to soak the soil surface) (Autuori 1941, Moser 1967, Nilson S. Nagamoto, personal observation).

Two collections were performed in the course of this study (Table 1): (i) in October 6th, 2006 a total of 147 gynes (84 of *A. laevigata*, 45 and 18 of *A. capiguara* nests #1 and #2, respectively) were picked up with sterile forceps at the moment they appeared at the nest entrance (Fig. 1b); (ii) in October 27th 2007, 60 gynes were collected from the same nest of *A. laevigata* and 60 from nest #2 of *A. capiguara*. In 2007, alates were not observed in nest #1 of *A. capiguara* on the day of collection. All gynes were put into individual sterile paper bags and transported to the laboratory in the next four hours.

### Yeast and filamentous fungi isolation

#### *Isolation from the exoskeleton (E1 and E2)*

In order to isolate the yeasts associated with gynes' exoskeleton, two different procedures were conducted, each with multiple trials (Table 1). In the E1 method, winged females collected in 2006 ($n = 147$) and 2007

**Table 1** Number of gynes, isolation trials and isolates in nests of leaf-cutting ants

| Year | Parameter | Ant nest | | | | | | | | | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *A. laevigata* | | | *A. capiguara* 1[a] | | | *A. capiguara* 2 | | | |
| | | E1 | E2 | P | E1 | E2 | P | E1 | E2 | P | |
| 2006 | Gynes collected | | 84 | | | 45 | | | 18 | | 147 |
| | Isolation trials | 84 | 23 | 84 | 45 | – | 43 | 18 | – | – | 297 |
| | Yeast isolates | 4 | 5 | 3 | 7 | – | 0 | 0 | – | – | 19 |
| 2007 | Gynes collected | | 60 | | | – | | | 60 | | 120 |
| | Isolation trials | 60 | 50 | 30 | – | – | – | 60 | 50 | 30 | 280 |
| | Yeast isolates | 2 | 0 | 0 | – | – | – | 1 | 0 | 0 | 3 |
| | Filamentous fungus isolates | 77 | – | 4 | – | – | – | 65 | – | 3 | 149 |

Filamentous fungi and yeasts isolated by: E1 = gynes walked in culture medium for four hours; E2 = gynes were immersed whole in liquid medium and, P = fungal pellet were dissected from gynes

[a] This nest was not sampled in 2007 (see methods for details)

–, no data


**Fig. 1** (**a**) Collection site and nest mound of *Atta laevigata* indicating the enlarged openings (arrow) just before the beginning of the mating flight. (**b**) The same nest showing gynes ready to leave the nest. (**c**) Fungal pellet of *Atta capiguara*; (**d**) Microfungi growing in yeast-malt extract medium isolated from gynes walking on plates (arrow indicates a single cream-coloured yeast colony)

($n = 120$) were allowed to walk during four hours on yeast-malt extract agar (YMA) plates (3 g yeast extract, 3 g malt extract, 5 g peptone, 10 g D-glucose and 15 g agar per litre at pH 5.5) supplemented with 0.1 mg $ml^{-1}$ of chloramphenicol (US Biological) (Yarrow 1998). Most of the culture medium surface was scraped by the ants, so additional molten YMA (10 ml) was added to each plate to reconstitute the medium's surface before incubation. In the second method, called E2, an additional set of gynes ($n = 23$, Table 1) of *A. laevigata* had their whole body immersed in 10 ml YMA—chloramphenicol broth in test tubes (one queen per test tube).

*Isolation from the infrabuccal pellet (P)*

Gynes which had been subjected to the E1 method (Table 1) were picked up from the YMA plates and the pellet containing the symbiotic fungus inoculum was aseptically removed from their infrabuccal pocket (Fig. 1c) and transferred to test tubes containing 3.0 ml YM broth supplemented with 0.1 mg $ml^{-1}$ of chloramphenicol in the 2006 collection or plated in potato dextrose agar in the 2007 collection.

All plates and test tubes were incubated at 22°C for 10 days and monitored daily. Samples of the test tubes showing turbidity and/or sediment were streaked on YMA medium and yeast colonies growing on the surface of this medium were isolated. Special attention was paid to filamentous fungi from the 2007 mating flight (see Results below).

Yeast identification

The yeasts isolated from leaf-cutting ants gynes were maintained in GYMP medium (Rosa et al. 1995) at 4°C and at −85°C in 30% glycerol. Isolates were initially grouped according to their colonial morphology (e.g., color, size, shape, texture) and physiological characteristics (ability to assimilate carbohydrates), staining reaction with diazonium blue-B (DBB) and urease reaction. The methods and keys for identification were used according to Kurtzman and Fell (1998) and Barnett et al. (2000). Three main groups of isolates were recognized; the D1/D2 domains of the nuclear large subunit (26S) ribosomal DNA were sequenced (O'Donnel 1993, Kurtzman and Robnett 1998) for representative strains of each group as well as strain R3 (single strain). Thus, a total of 16 strains were sequenced (Table 2). DNA extraction followed the protocol described in Almeida (2005). DNA was amplified with primers NL1 (5′GCATATCAATAA GCGGAGGAAAAG3′) and NL4 (5′GGTCCGTGTT TCAAGACGG3′). Twenty-five µl polymerase chain reactions were carried out using 1µl of 50 mM $MgCl_2$, 4 µl of 1.25 mM of dNTP Mix, 2.5 µl of 10× PCR

**Table 2** Yeast species isolated from leaf-cutting ants gynes prior to their mating flight. Unless otherwise indicated, isolation from the exoskeleton used the E1 method

| Isolate ID[a] | Nest | Body part | GenBank best hit (Accession no.) |
|---|---|---|---|
| R01 | *A. capiguara* 1 | Exoskeleton | *Aureobasidium pullulans* (nd) |
| R02 | *A. capiguara* 1 | Exoskeleton | *Aureobasidium pullulans* (nd) |
| R03 | *A. laevigata* | Pellet | *Rhodotorula glutinis* (AF335985) |
| R04 | *A. laevigata* | Exoskeleton[b] | *Aureobasidium pullulans* (AY213693) |
| R05 | *A. capiguara* 1 | Exoskeleton | *Cryptococcus laurentii* (AJ876597) |
| R06 | *A. capiguara* 1 | Exoskeleton | *Cryptococcus laurentii* (nd) |
| R07 | *A. capiguara* 1 | Exoskeleton | *Aureobasidium pullulans* (EU560881) |
| R08 | *A. laevigata* | Exoskeleton[b] | *Candida parapsilosis* (AY894827) |
| R09 | *A. laevigata* | Exoskeleton[b] | *Candida parapsilosis* (AY894827) |
| R10 | *A. laevigata* | Exoskeleton | *Aureobasidium pullulans* (AY213693) |
| R11 | *A. laevigata* | Exoskeleton | *Cryptococcus laurentii* (AJ876597) |
| R12 | *A. laevigata* | Exoskeleton | *Aureobasidium pullulans* (nd) |
| R13 | *A. capiguara* 1 | Exoskeleton | *Cryptococcus laurentii* (EF068207) |
| R14 | *A. laevigata* | Exoskeleton[b] | *Candida parapsilosis* (AY894827) |
| R15 | *A. capiguara* 1 | Exoskeleton | *Cryptococcus laurentii* (AJ876597) |
| R16 | *A. laevigata* | Exoskeleton | *Candida parapsilosis* (EF694608) |
| R17 | *A. laevigata* | Pellet | *Aureobasidium pullulans* (nd) |
| R18 | *A. laevigata* | Exoskeleton[b] | *Candida parapsilosis* (AJ749821) |
| R19 | *A. laevigata* | Pellet | *Cryptococcus laurentii* (nd) |
| R20 | *A. laevigata* | Exoskeleton | *Cryptococcus laurentii* (EU427437) |
| R21 | *A. laevigata* | Exoskeleton | *Cryptococcus laurentii* (EU427437) |
| R22 | *A. capiguara* 2 | Exoskeleton | *Cryptococcus laurentii* (EU427437) |

[a] Yeasts strains R1 through R19 were isolated from the 2006 collection; the remaining isolates are from the 2007 collection

[b] The E2 method was used for isolation of these strains

nd, no molecular data; yeasts were identified only by phenotypic similarity

Molecular identification was based on BLASTn results (at least 99%-similarity) of rDNA D1/D2 regions of isolates regarding GenBank homologous sequences

buffer, 2 µl of 10 µM of each primer, 0.2 µl of 5 U/µl of Taq polymerase and 5 µl of DNA template (diluted 1:750) and 8.3 µl PCR water. The amplification protocol consisted in an initial step of 96°C for 3 min, followed by 35 cycles: 96°C for 30s, 61°C for 45s and 72°C for 1 min. Amplicons were cleaned up with GFX DNA and Gel Band Purification Kit (Amersham Biosciences, Piscataway, NJ, USA) and the same set of primers were used in cycle sequencing reactions. Both DNA strands were generated in an ABI Prism 377 DNA automated sequencer (Applied Biosystems Inc., Foster City, CA, USA), manually resolved and aligned through the software BioEdit Sequence Alignment Editor v. 7.0.5.3 (Hall 1999).

In order to find the closest relatives of our isolates, contigs were compared with their homologues retrieved from National Center for Biotechnology Information (NCBI)—GenBank (http://www.ncbi.nlm.nih.gov) using BLASTn (Altschul et al. 1997). Yeasts strains exhibiting 99% similarity in the D1/D2 domains with sequences from the GenBank were considered conspecific (see Kurtzman and Robnett 1998, Berkov et al. 2007).

### Filamentous fungi identification

After initial incubation, morphological types of filamentous fungi growing in YMA plates (used in E1 isolation method) and in PDA plates (used in the P method in the 2007 collection) were selected and subcultured in 2% malt agar for identification. Colony macromorphology and micromorphology were used as characters to identify the isolates with the help of general taxonomic keys (Domsch et al. 1980, Samson et al. 2000), as well as specific taxonomic treatments for some groups of fungi such as *Cunninghamella* and *Cladosporium* (Liu et al. 2001, Schubert et al. 2007).

## Results

On October 6th, 2006 on a warm and moist afternoon, gynes of *A. laevigata* and of *A. capiguara* were leaving the parental nests for the nuptial flight. As expected in this special moment of the colony's life cycle, the following observations were made: (i) the

nest openings were enlarged, indicating preparation to release a great number of alates; (ii) it had rained significantly on the previous day; (iii) as soon as the rain stopped there was a increased amount of soil deposition outside the nest due to the cleaning of the large openings (Fig. 1a); (iv) a huge amount of workers appeared, including soldiers, walking on the nest mound; (v) winged males first appeared in the openings followed by gynes (Fig. 1b). On October 26th, 2007 the nuptial flight events were similar for the same nests, except for nest #1 of *A. capiguara,* which was not releasing gynes.

Overall, the number of yeasts recovered in our survey was relatively low (Table 1). From 577 isolation trials made from the material collected in 2006 and 2007, only 22 yeast strains were isolated. Considering the 2006 material, a total of 19 yeasts were recovered from 297 isolation attempts (6.4% success, Table 1). Specifically, 191 isolation trials were made with *A. laevigata* gynes from which 12 yeasts were recovered by the P (84 trials; 3 strains), E1 (84 trials; 4 strains) and E2 (23 trials; 5 strains) methods. Additionally, 88 isolation trials were conducted from gynes from *A. capiguara* nest #1. Seven yeasts were isolated by the E1 method (45 trials) and no yeast was isolated by the P method (43 trials; infrabuccal pellets were not found in two out of 45 gynes). No isolation through the E2 method was carried out from gynes of this nest. Moreover, yeast isolation by the E1 method from 18 gynes of *A. capiguara* nest #2 was also unsuccessful (Table 1).

The isolated yeasts strains were identified using both phenotypic and molecular markers (Table 2). The species found in the 2006 collection were: *Rhodotorula glutinis*, *Candida parapsilosis*, *Cr. laurentii* and the black yeast *Aureobasidium pullulans* (Table 2). Five out of seven strains of the latter species were isolated from both ant species by the E1 isolation method, whereas the two remaining strains were isolated by the E2 and by the P methods from the nest of *A. laevigata* (Table 2). *C. parapsilosis* was not isolated from the fungal pellet.

Six *Cr. laurentii* strains were isolated from 2006 offspring. Four out of six strains were isolated from *A. capiguara* exoskeleton and one strain from *A. laevigata* exoeskeleton both using the E1 method. The remaining strain of this group was isolated from the infrabuccal pellet of *A. laevigata*. This was the most prevalent species among yeasts in this survey.

In 2007, a total of 280 isolation trials were performed, of which 140 (60, 50 and 30 through the E1, E2 and P methods, respectively) were from the same *A. laevigata* nest and 140 (60, 50 and 30 through the E1, E2 and P methods, respectively) were from nest 2 of *A. capiguara*. Only three yeasts were recovered from these procedures, all of them identified as *Cr. laurentii* (strains R20, R21 and R22 in Table 2).

From the 2006 collection, we observed the presence of several filamentous microfungi in gynes of all nests sampled. These filamentous fungi were mostly observed in plates using the E1 method (Fig. 1d) but no further subcultures were carried out with these microbes; however, based on morphological characteristics the genera *Cladosporium* and *Paecilomyces* were prevalent. Accordingly with the 2006 observations, we concentrated our efforts in the isolation and identification of filamentous fungi during the 2007 mating flight. A total of 149 strains were isolated by the E1 method ($n = 142$ strains) and by the P method ($n = 7$ strains). Eleven genera and three unidentified fungi resulted from these isolations. Interestingly, the genus *Cladosporium* was prevalent (78%, Table 3) but no further identification at the species level was conducted for most isolates. However, other more common species of this genus such as *Cladosporium cladosporioides* were isolated from the fungal pellet. Interestingly, the fungal parasite *Escovopsis* was not isolated from any source.

## Discussion

Many insect species are associated with fungi which they feed on and occasionally disperse to new substrates (Phaff and Starmer 1987, Ganter 2006). Insects can harbour several species of filamentous fungi and yeasts on their exoskeleton (Rosa et al. 2003), in their gut (Suh et al. 2005) or specialized structures adapted to transport fungi (e.g. mycangia), as is the case of the bark beetle-fungal association (Six and Klepzig 2004). In all of these insect-microbial associations hosts have an impact in the dispersal or survival of the associated microbes. In leaf-cutting ants, microbes have been found in the fungus gardens (Currie et al. 1999a, Santos et al. 2004, Rodrigues et al. 2008), on the ant's exoskeleton (Currie et al. 1999b, Little and Currie 2007) and in

**Table 3** Filamentous fungi isolated from gynes exoskeleton (by E1 method) and infrabuccal pellets of leaf-cutting ants prior to the mating flight in October, 2007

| Filamentous fungus species | Number of isolates (exoskeleton/pellet)[a] | |
|---|---|---|
| | *A. laevigata* | *A. capiguara* |
| *Absidia corymbifera* | 0/0 | 1/0 |
| *Aspergillus* sp. | 1/0 | 0/0 |
| *Chaetomium funicola* | 0/1 | 0/0 |
| *Cladosporium* sp. | 50/0 | 56/0 |
| *Cladosporium cladosporioides* | 4/3 | 0/3 |
| *Cladosporium subtilissimum* | 0/0 | 1/0 |
| *Cladophialophora* sp. | 1/0 | 0/0 |
| *Cunninghamella echinulata* | 0/0 | 1/0 |
| *Fusarium* sp. | 1/0 | 0/0 |
| *Monilia* sp. | 5/0 | 0/0 |
| *Mucor* sp. | 4/0 | 0/0 |
| *Penicillium* sp. | 3/0 | 3/0 |
| *Trichoderma* sp. | 7/0 | 1/0 |
| Not identified | 1/0 | 2/0 |
| Total | 77/4 | 65/3 |

[a] 60 Gynes and 30 infrabuccal pellets were sampled from both *A. laevigata* and *A. capiguara* ant species

fungal pellets carried by nest-founding gynes (Quinlan and Cherrett 1978).

Of the yeasts isolated in this study (Table 2), two species, *Cr. laurentii* and *A. pullulans,* are common inhabitants of soil and the surfaces of leaves (Slaviková and Vadkertiová 2000, Fonseca and Inácio 2006) and are probably carried to the nest in the plant material collected by foraging workers. It is interesting to note that *Cr. laurentii* has also been found in the infrabuccal pocket of the carpenter ant *Camponotus vicinus* (Mankowski and Morrell 2004) together with several other yeast species. It is likely that attine gynes are in contact with these yeast species since the beginning of their life cycle and it may explain how they have been acquired by these ants. *Cr. laurentii*, *Candida* spp., *Rhodotorula glutinis* and black yeasts have been also found in *A. sexdens rubropilosa* nests (Carreiro et al. 1997).

*Candida parapsilosis* is considered an opportunistic human pathogen associated with several kinds of illnesses. In humans, it has been isolated from nosocomial infections (Kocsube et al. 2007) and vulvovaginites (Cooper and Ginnis 1996), but is also obtained from insects (Suh et al. 2005, Ganter 2006). In addition, Ba and Phillips (1996) and Ba et al. (2000) reported the occurrence of *C. parapsilosis* in association with the red imported fire ant *Solenopsis invicta*.

The low occurrence of yeasts associated with the body surface and the fungal pellets of leaf-cutting ants suggests the absence of species-specific vertical transmission of yeasts by young queens. Thus, the most probable scenario is that yeasts enter the nests after worker emergence when ants start adding new substrates to the young fungus garden and/or from the environment surrounding the nest. This view is supported by the fact that young field nests (i.e. five to six months old with just one fungus chamber) of *A. sexdens rubropilosa* ($n = 14$ nests sampled) and *A. laevigata* ($n = 27$ nests sampled) contain approximately 10–100 yeast CFU/g of fungus garden (as determined by the most probable number method—Fernando C. Pagnocca, unpublished data). On the other hand, Craven et al. (1970) found a minimum of $4.0 \times 10^4$ yeast/g in adult nests of *A. cephalotes.*

Further research is required to determine the roles performed by the various microbes found in attine fungus gardens. According to Carreiro (2000), yeasts in nests of *A. sexdens rubropilosa* are potential producers of hydrolytic enzymes that break down plant polysaccharides into simple sugars making them available for the cultivated fungus and ants. Additionally, Silva et al. (2006) observed that some of the amylolytic enzymes found in the fungal garden could be produced by microorganisms other than the symbiotic fungus.

Contrary to our expectations, considering the highly evolved defense system of these insects against alien microorganisms, the number and diversity of filamentous fungi isolated in our study was high, especially on the plates where gynes had walked. Despite the prevalence of filamentous fungi in comparison with yeasts on the ants' exoskeleton, the presence of most of the filamentous fungi species suggest they may be transient, even though the hypothesis that some become harmful after dispersal should be considered. However, when compared with yeasts, the number of filamentous fungi isolated from infrabuccal pellets was low ($n = 7$ isolates, Table 1) suggesting that the ants provide special care to prevent fungal pellet contamination. The massive occurrence of *Cladosporium* sp. in both collections suggests that these insects may be an important

mechanism of dispersal for these fungi, but we cannot yet determine whether they are intentionally transmitted. Indeed, *Cladosporium* spp. can be found as epiphytes on plant leaves and are also considered a common inhabitant of the soil usually as a saprophytic life-style (Domsch et al. 1980), and our study found no evidence for a species-specific relationship between this fungal species and the leafcutter ant symbiosis.

The filamentous fungi found in our survey can be characterized as members of the soil community (Table 2). Except for the soil-borne zygomycetes *Absidia corymbifera* and *Cunninghamella echinulata*, all of the other fungi recovered are ascomycetes that are commonly isolated from soil samples worldwide (Sun and Liu 2008). Interestingly, some soil-borne species of the genera *Cladosporium*, *Cunninghamella*, *Fusarium* and *Trichoderma* were considered to be potential antagonists in leafcutter ants gardens (Rodrigues et al. 2008).

Our results suggest that leafcutter gynes do not harbour entomopathogenic fungi before the mating flight, including common species such as *Beauveria* sp. and *Metarhizium* sp. This finding contrasts with observations that gynes collected immediately before the mating flight and reared in the lab are commonly threatened by entomopathogenic fungi (A. Silva, personal observation).

Currie (2001b) found that *Escovopsis* is a specific parasite that causes a loss of the fungus garden mass and a decrease in the ants' work force in fungus-growing ant nests. *Escovopsis* prevalence was determined to be variable throughout the fungus-growing ants including the leafcutters (Currie et al. 1999a). Despite the high incidence of filamentous fungi in our samples, no *Escovopsis* strains were isolated, providing further support for the hypothesis that this parasite is horizontally transmitted (Currie et al. 1999a). In addition, the fact that no *Escovopsis* isolates were found in the fungal pellets of both ant species is consistent with the findings by Currie et al. (1999a) who did not find any *Escovopsis* associated with foundress queens of *Atta columbica*, a Central American leaf-cutting ant species. It is still unknown how *Escovopsis* is transmitted between fungus-growing ant nests, though it is generally assumed that invertebrates, such as mites, that occasionally visit attine nests are the possible source of *Escovopsis* dispersion (Currie at al. 1999a, Currie 2001a, b).

The production of winged females and males by the colony demands large amounts of energy and according to observations made in laboratory colonies there is a decline in fungus garden biomass during this time in the colony's lifecycle (Bueno et al. 2002), possibly due to increased cultivar consumption by reproductive ants (males and gynes). Thus, it's possible that some microbes, especially filamentous fungi, can escape from the ant's control during this period thus explaining why they were prevalent in this study.

**Acknowledgments** We are grateful to the "Conselho Nacional de Desenvolvimento Científico e Tecnológico" (CNPq–Proc. 301713/2005-7 and 479990/2006-9) and "Fundação de Amparo a Pesquisa do Estado de São Paulo" (FAPESP–Proc. 50139/4-2007 and 08/00185-7) for financial support. We also like to thank Dr. Scott E. Solomon from The University of Texas at Austin for his valuable review of this manuscript and Dr. Luiz Carlos Forti from the Department of Vegetal Production, UNESP - São Paulo State University for providing the facilities during field collection.

## References

Almeida JMGCF (2005) Yeast community survey in the Tagus estuary. FEMS Microbiol Lett 53:295–303

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W et al (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389–3402. doi:10.1093/nar/25.17.3389

Autuori M (1941) Contribuição para o conhecimento da saúva (*Atta* spp.–Hymenoptera: Formicidae). I–Evolução do sauveiro (*Atta sexdens rubropilosa* Forel, 1908). Arq Inst Biol (São Paulo) 12:197–228

Ba AS, Phillips SA Jr (1996) Yeast biota of the red imported fire ant. Mycol Res 100:740–746

Ba AS, Phillips SA, Anderson JT (2000) Yeasts in the mound soil of the red imported fire ant. Mycol Res 104:969–973. doi:10.1017/S0953756299002385

Barnett JA, Payne RW, Yarrow D (2000) Yeasts: characteristics and identification, 2nd edn. Cambridge University Press, Cambrigde

Berkov A, Feinstein J, Small J, Nkmany M, Centeno P (2007) Yeasts isolated from neotropical wood-boring beetles in SE Peru. Biotropica 39:530–538. doi:10.1111/j.1744-7429.2007.00280.x

Bueno OC, Hebling MJA, Schneider MO, Pagnocca FC, Bacci M Jr (2002) Occurrence of winged forms of *Atta sexdens rubropilosa* Forel (Hymenoptera: Formicidae) in laboratory colonies. Neotrop Entomol 31:469–473. doi:10.1590/S1519-566X2002000300019

Cafaro MJ, Currie CR (2005) Phylogenetic analysis of mutualistic filamentous bacteria associated with fungus-growing ants. Can J Microbiol 51:441–446. doi:10.1139/w05-023

Carreiro SC (2000) Pesquisa do fator killer e análise de degradação de polissacarídeos vegetais por leveduras associadas aos ninhos de *Atta sexdens*. Ph.D. dissertation, Universidade Estadual Paulista

Carreiro SC, Pagnocca FC, Bueno OC, Bacci M Jr, Hebling MJA, Silva OA (1997) Yeasts associated with nests of the leaf-cutting ant *Atta sexdens rubropilosa* Forel, 1908. Antonie Van Leeuwenhoek 71:243–248. doi:10.1023/A:1000182108648

Carreiro SC, Pagnocca FC, Bacci M Jr, Lachance MA, Bueno OC, Hebling MJA et al (2004) *Sympodiomyces attinorum* sp. nov., a yeast species associated with nests of the leaf-cutting ant *Atta sexdens*. Int J Syst Evol Microbiol 54:1891–1894. doi:10.1099/ijs.0.63200-0

Chapela IH, Rehner SA, Schultz TR, Mueller UG (1994) Evolutionary history of the symbiosis between fungus-growing ants and their fungi. Science 266:1691–1694. doi:10.1126/science.266.5191.1691

Cooper CR, Ginnis Mc (1996) In vitro susceptibility of clinical yeast isolates to fluconazole and terconazole. Am J Obstet Gynecol 175:1626–1631. doi:10.1016/S0002-9378(96)70116-3

Craven SE, Dix MW, Michaels GE (1970) Attine fungus gardens contains yeasts. Science 169:184–186

Currie CR (2001a) A community of ants, fungi and bacteria: A multilateral approach to studying symbiosis. Annu Rev Microbiol 55:357–380. doi:10.1146/annurev.micro.55.1.357

Currie CR (2001b) Prevalence and impact of a virulent parasite on a tripartite mutualism. Oecologia 128:99–106. doi:10.1007/s004420100630

Currie CR, Mueller UG, Malloch D (1999a) The agricultural pathology of ant fungus gardens. Proc Natl Acad Sci USA 96:7998–8002. doi:10.1073/pnas.96.14.7998

Currie CR, Scott JA, Summerbell RC, Malloch D (1999b) Fungus-growing ants use antibiotic-producing bacteria to control garden parasites. Nature 398:701–704. doi:10.1038/19519

Currie CR, Bot ANM, Boomsma JJ (2003a) Experimental evidence of a tripartite mutualism: bacteria protect ant fungus gardens from specialized parasites. Oikos 101:91–102. doi:10.1034/j.1600-0706.2003.12036.x

Currie CR, Scott JA, Summerbell RC, Malloch D (2003b) Corrigendum: Fungus-growing ants use antibiotic-producing bacteria to control garden parasites. Nature 423:461. doi:10.1038/nature01563

Currie CR, Wong B, Stuart AE, Schultz TR, Rehner SA, Mueller UG et al (2003c) Ancient tripartite co-evolution in the attine ant–microbe symbiosis. Science 299:386–388. doi:10.1126/science.1078155

Domsch KH, Gams W, Anderson T (1980) Compendium of soil fungi. Academic, London

Fonseca A, Inácio J (2006) Phylloplane yeasts. In: Rosa CA, Péter G (eds) The yeast handbook: biodiversity and ecophysiology of yeasts. Springer Verlag, Berlin, pp 263–301

Ganter PF (2006) Yeasts and invertebrates associations. In: Rosa CA, Péter G (eds) The yeast handbook: biodiversity and ecophysiology of yeasts. Springer Verlag, Berlin, pp 263–301

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser 41:95–98

Kocsube S, Toth M, Vagvolgyi C, Doczi I, Pesti M, Pocsi I et al (2007) Occurrence and genetic variability of *Candida parapsilosis* sensu lato in Hungary. J Med Microbiol 56:190–195. doi:10.1099/jmm.0.46838-0

Kost C, Lakatos T, Böttcher I, Arendholz WR, Redenbach M, Wirth R (2007) Non-specific association between filamentous bacteria and fungus-growing ants. Naturwissenschaften 94:821–828. doi:10.1007/s00114-007-0262-y

Kurtzman CP, Fell JW (1998) The yeasts: a taxonomic study, 4th edn. Elsevier, Amsterdam

Kurtzman CP, Robnett CJ (1998) Identification and phylogeny of ascomycetous yeasts from analysis of nuclear large subunit (26S) rDNA partial sequences. Antonie Van Leeuwenhoek 73:331–371. doi:10.1023/A:1001761008817

Little AEF, Currie CR (2007) Symbiont complexity: discovery of a fifth symbiont in the attine ant-microbe symbiosis. Biol Lett 3:501–504. doi:10.1098/rsbl.2007.0253

Liu XY, Huang H, Zheng RY (2001) Relationships within *Cunninghamella* based on sequence analysis of ITR rDNA. Mycotaxon 80:77–95

Mankowski ME, Morrell JJ (2004) Yeasts associated with the infrabuccal pocket and colonies of the carpenter ant *Camponotus vicinus*. Mycologia 96:226–231. doi:10.2307/3762058

Middelhoven WJ, Fonseca A, Carreiro SC, Pagnocca FC, Bueno OC (2003) *Cryptococcus haglerorum*, sp. nov., an anamorphic basidiomycetous yeast isolated from nests of the leaf-cutting ant *Atta sexdens*. Antonie Van Leeuwenhoek 83:167–174. doi:10.1023/A:1023384830802

Moser JC (1967) Mating activities of *Atta texana* (Hymenoptera: Formicidae). Insect Soc 14:295–312. doi:10.1007/BF02252831

Mueller UG (2002) Ant versus fungus versus mutualism: ant-cultivar conflict and the deconstruction of the attine ant-fungus symbiosis. Am Nat 160:S67–S98

Mueller UG, Rehner SA, Schultz TR (1998) The evolution of agriculture in ants. Science 281:2034–2038. doi:10.1126/science.281.5385.2034

O'Donnel K (1993) *Fusarium* and its near relatives. In: Reynolds DR, Taylor JW (eds) The fungal holomorph: mitotic and pleomorphic speciation in fungal systematics. CAB International, Wallingford, pp 225–233

Pagnocca FC, Carreiro SC, Bueno OC, Hebling MJ, da Silva OA (1996) Microbiological changes in the nests of leaf-cutting ants fed on sesame leaves. J Appl Entomol 120:317–320

Pagnocca FC, Bacci M Jr, Fungaro MH, Bueno OC, Hebling MJA, Sant'Anna A et al (2001) RAPD analysis of the sexual state and sterile mycelium of the fungus cultivated by the leaf-cutting ant *Acromyrmex hispidus fallax*. Mycol Res 105:173–176. doi:10.1017/S0953756200003191

Phaff J, Starmer WT (1987) Yeast associated with plants, soil and insects. In: Rose AH, Harrinson JS (eds) The yeast: biology of yeast. Academic Press, London, pp 123–180

Quinlan RJ, Cherrett JM (1978) Studies on the role of the infrabuccal pocket of the leaf-cutting ant *Acromyrmex octospinosus* (Reich) (Hym., Formicidae). Insectes Soc 25:237–245. doi:10.1007/BF02224744

Rodrigues A, Pagnocca FC, Bacci M Jr, Hebling MJA, Bueno OC, Pfenning LH (2005) Variability of non-mutualistic filamentous fungi associated with *Atta sexdens rubropilosa* nests. Folia Microbiol (Praha) 50:421–425

Rodrigues A, Bacci M Jr, Mueller UG, Ortiz A, Pagnocca FC (2008) Microfungal "weeds" in the leafcutter ant symbiosis. Microb Ecol doi:10.1007/s00248-008-9380-0

Rosa CA, Morais PB, Santos SR, Neto PRP, Mendonça-Hagler LC, Hagler AN (1995) Yeast communities associated with different plant resources in sandy coastal plains of south eastern Brazil. Mycol Res 99:1047–1054

Rosa CA, Lachance M-A, Silva JOC, Teixeira ACP, Marini MM, Antonini Y et al (2003) Yeast communities associated with stingless bees. FEMS Yeast Res 4:271–275. doi:10.1016/S1567-1356(03)00173-9

Samson RA, Hoekstra ES, Frisvad JC (2000) Introduction to food-airborne fungi, 6th edn. Centraalbureau voor Schimmelcultures, Baarn

Santos AV, Dillon RJ, Dillon VM, Reynolds SE, Samuels RI (2004) Occurrence of the antibiotic producing bacterium *Burkholderia* sp. in colonies of the leaf-cutting ant *Atta sexdens rubropilosa*. FEMS Microbiol Lett 239:319–323. doi:10.1016/j.femsle.2004.09.005

Schubert K, Groenewald JZ, Braun U, Dijksterhuis J, Starink M, Hill CF et al (2007) Biodiversity in the *Cladosporium herbarum* complex (*Davidiellaceae, Capnodiales*), with standardisation of methods for *Cladosporium* taxonomy and diagnostics. Stud Mycol 58:105–156

Schultz TR, Brady SG (2008) Major evolutionary transitions in ant agriculture. Proc Natl Acad Sci USA doi:10.1073/pnas.0711024105

Schultz TR, Meier R (1995) A phylogenetic analysis of the fungus-growing ants (Formicidae: Attini) based on morphological characters of the larvae. Syst Entomol 20:337–370. doi:10.1111/j.1365-3113.1995.tb00100.x

Silva A, Bacci M Jr, Pagnocca FC, Bueno OC, Hebling MJA (2006) Starch metabolism in *Leucoagaricus gongylophorus*, the symbiotic fungus of leaf-cutting ants. Microbiol Res 161:299–303. doi:10.1016/j.micres.2005.11.001

Six DL, Klepzig KD (2004) *Dendroctonus* bark beetles as model systems for studies on symbiosis. Symbiosis 37:207–232

Slaviková E, Vadkertiová R (2000) The occurrence of yeasts in the forest soils. J Basic Microbiol 40:207–212. doi:10.1002/1521-4028(200007)40:3<207::AID-JOBM207>3.0.CO;2-H

Suh S-O, McHugh JV, Pollock DD, Blackwell M (2005) The beetle gut: a hyperdiverse source of novel yeasts. Mycol Res 109:261–265. doi:10.1017/S0953756205002388

Sun B-D, Liu X-Z (2008) Occurrence and diversity of insect-associated fungi in natural soils in China. Appl Soil Ecol 39:100–108. doi:10.1016/j.apsoil.2007.12.001

Villensen P, Mueller UG, Schultz TR, Adams RMM, Bouck AC (2004) Evolution of ant-cultivar specialization and cultivar switching in *Apterostigma* fungus-growing ants. Evolution Int J Org Evolution 58:2252–2265

Weber NA (1972) Gardening ants: the attines. American Philosophical Society, Philadelphia

Wetterer JK, Schultz TR, Meier R (1998) Phylogeny of fungus-growing ants (Tribe Attini) based on mtDNA sequence and morphology. Mol Phylogenet Evol 9:42–47. doi:10.1006/mpev.1997.0466

Yarrow D (1998) Methods for the isolation, maintenance and identification of yeasts. In: Kurtzman CP, Fell JW (eds) The yeasts: a taxonomic study. Elsevier, Amsterdam, pp 77–100