Yeasts isolated from a fungus-growing ant nest, including the description of *Trichosporon chiarellii* sp. nov., an anamorphic basidiomycetous yeast

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Thirty-nine yeast strains were recovered from a field nest of a primitive and putative novel attine ant species in the genus *Myrmicocrypta* (Hymenoptera: Formicidae: tribe Attini). Yeasts isolated from the fungus garden and waste deposit included *Candida dubliniensis*, *Candida oleophila*, *Cryptococcus haglerorum* and *Hanseniaspora uvarum*. In addition, one morphological type was isolated overwhelmingly. Sequencing data of partial large-subunit (LSU) rDNA and the internal transcribed spacer (ITS) region coupled with morphological and physiological characterization accommodated this morphotype in a separate taxonomic position in relation to the known species of *Trichosporon* (Basidiomycota: Trichosporonales). Here, we propose a novel yeast species named *Trichosporon chiarellii* sp. nov. based on the description of 34 isolates; the type strain is strain FCP 540806^T (=CBS 11177^T).

Fungus-growing ants (Hymenoptera: Formicidae, tribe Attini) are social insects that maintain an obligate mutualism with basidiomycetous fungi (mostly in the Lepiotaceae), which they cultivate as food (Mueller *et al.*, 1998). After 50 million years of co-evolution, the two partners have developed a successful integration, leading to 13 genera and more than 230 described ant species, all exclusive to the New World (Schultz & Brady, 2008). The fungus provides its host with suitable nutrients such as amino acids, simple sugars and enzymes (Silva *et al.*, 2006). In turn, the fungus is manured by the ants, and the ants promote its dispersion when reproductives found a new colony (Weber, 1972).

Based on molecular and morphological evidence, attine ants are usually classified in two phylogenetic groups: the lower and higher attines (Schultz & Meier, 1995; Schultz & Brady, 2008). Nests of lower attine genera are frequently cryptic and small (usually fewer than 100 workers) and

workers do not attack plants (Mueller *et al.*, 2001). These genera include *Cyphomyrmex*, *Mycetophylax*, *Mycocepurus*, *Myrmicocrypta*, *Apterostigma*, *Mycetagroicus*, *Mycetosoritis* and *Mycetarotes*. The genera *Sericomyrmex*, *Trachymyrmex*, *Acromyrmex* and *Atta* make up the higher attines, which usually form larger nests; the number of workers may reach millions in *Atta* species. The latter two genera may be serious pests of agricultural crops as a result of the large numbers of leaves that these insects cut and use as substrate for their fungus (Hölldobler & Wilson, 1990).

The fungus garden of attine ants is considered a microbial consortium because several microbes can be found in this particular environment in addition to the symbiotic fungus (Currie *et al.*, 1999; Mueller *et al.*, 2005; Rodrigues *et al.*, 2008). For example, several authors have reported the presence of yeasts associated with field and laboratory nests of higher attines (Craven *et al.*, 1970; Carreiro *et al.*, 1997; Pagnocca *et al.*, 1996, 2008). In the course of these studies, two novel yeast species, *Cryptococcus haglerorum* (Middelhoven *et al.*, 2003) and *Symphodiomyces attinorum* (Carreiro *et al.*, 2004), were described in association with *Atta sexdens rubropilosa* nests. Recently, black yeasts closely related to the genus *Phialophora* were reported from the bodies of attine ants as a new symbiont (Little & Currie, 2007). Considering that no studies on yeast diversity have been conducted on the lower attines to date, the aim of the present work was to profile yeasts associated with a

Abbreviation: MSP-PCR, microsatellite-primed PCR.

The GenBank/EMBL/DDBJ accession numbers for the 26S rDNA D1/D2 domain and ITS1–5.8S rDNA–ITS2 sequences of strain FCP 540806^T are EU030272 and GQ338074, respectively.

Mycobank accession no. MB514465.

Neighbour-joining trees based on ITS1–5.8S rDNA–ITS2 region sequences and this sequence concatenated with that of the LSU rDNA D1/D2 domain are available as supplementary material with the online version of this paper.

putative novel attine species in the genus *Myrmicocrypta* (J. Sosa-Calvo and T. R. Schultz, personal communication). Here, we introduce a novel anamorphic basidiomycetous yeast species that represented the vast majority of isolates obtained from the ant nest.

Yeast isolation and taxonomy

On 14 August 2006, a *Myrmicocrypta* sp. nest was found at Botucatu, São Paulo State, Brazil (22° 50.600' S 48° 26.091' W; altitude 800 m), at a depth of about 1 m while digging a mature nest of the leafcutter ant *Atta capiguara*. The fungus garden, workers and queen were collected in a sterile container and brought to the laboratory.

No food was provided for 48 h while the ants reconstructed their nest. Samples of approximately 500 mg of the fungal garden and waste material were suspended in peptone water (0.1 %, w/v), gently disrupted with a sterile glass rod and vortexed for 30 s. Three culture media were used for yeast isolation: YMA (1 % glucose, 0.5 % peptone, 0.3 % yeast extract, 0.3 % malt extract, 2 % agar; Yarrow, 1998), MYP (0.7 % malt extract, 0.05 % yeast extract, 0.25 % soytone, 1.5 % agar; Sampaio *et al.*, 2004) and yeast nitrogen base (YNB; Difco) with 0.5 % *myo*-inositol as carbon source. The media were supplemented with 150 µg chloramphenicol ml⁻¹ (US Biological) and isolation was achieved by spreading 100 µl aliquots on the surface of solid medium followed by incubation at 20 °C for 7 days.

Yeast strains were examined under light microscopy and physiological characterization followed the replica plate method (Yarrow, 1998). The tests were carried out in duplicate, and dubious results were resolved using liquid media. The temperature used in the assays was 25 °C and readings were taken at 7, 14 and 21 days of incubation. The following media used for analysis of sporulation were prepared according to Yarrow (1998): Gorodkova agar (0.1 % glucose, 1 % peptone, 0.5 % sodium chloride and 1.5 % agar), McClary acetate agar (0.1 % glucose, 0.18 % potassium chloride, 0.25 % yeast extract and 1.5 % agar), MYP and an additional medium consisting of 1 % glucose, 0.01 % yeast extract and 1.5 % agar.

For molecular characterization, we followed the protocol described by Pagnocca *et al.* (2008). Briefly, genomic DNA from 2- to 3-day-old cultures was extracted and aliquots were used to amplify the D1/D2 domain of the large-subunit (LSU) rDNA with primers NL1 and NL4 and the ITS1–5.8S rDNA–ITS2 region using primers ITS1 and ITS4. DNA aliquots were also used in microsatellite-primed PCR (MSP-PCR) using the primer (GTG)₅ following the protocol of Sampaio *et al.* (2001). Sequences were generated in an ABI 377 automated sequencer (Applied Biosystems).

Both DNA strands were assembled in BioEdit version 7.0.5.3 (Hall, 1999) and aligned with additional sequences of *Trichosporon* species and their relatives retrieved from GenBank. Phylogenetic reconstructions were performed

separately using the D1/D2 domain and the ITS1–5.8S rDNA–ITS2 region. In addition, the two datasets were merged and the concatenated sequences were used for a joint analysis. Phylogenetic inference was conducted in PAUP version 4.0b10 (Swofford, 2002) under the neighbour-joining criterion using Kimura's two-parameter model as the evolutionary model for calculating distances. Gaps were treated as missing data and bootstrap support was calculated under 1000 pseudoreplicates by the neighbour-joining criterion.

Yeasts isolated from a *Myrmicocrypta* sp. nest

In our survey of yeasts associated with *Myrmicocrypta* sp., 39 yeast colonies were selected from inoculated plates. Thirty-six of 39 colonies were identified as basidiomycetes yeasts, and three were ascomycetes. The latter yeasts included one isolate each of *Candida dubliniensis*, *Candida oleophila* (both species were isolated from the waste deposit on MYP) and *Hanseniaspora uvarum* (isolated from the waste deposit on YMA). Of the basidiomycetes, two of the 36 strains were identified as *Cryptococcus haglerorum* (isolated from the fungus garden on MYP and YMA). The remaining 34 strains were identified as a single morphotype, which we name *Trichosporon chiarellii* sp. nov. This species was recovered from all media used in the isolation procedures and from both kinds of substrate (fungus garden and waste deposit). The selected type strain FCP 540806^T was isolated from the waste deposit on MYP.

Candida oleophila and *H. uvarum* are ascomycetous yeasts that are commonly found on the surfaces of leaves and fruits; thus, their presence in the fungus garden is not surprising, as ants may carry such yeasts during foraging for substrate. The remaining ascomycetous strain showed chlamydospore production on cornmeal agar plates incubated at 25 °C for 72 h, and it was identified as *Candida dubliniensis*. The majority of *Candida dubliniensis* strains have been isolated from clinical cases, including the type strain (Sullivan *et al.*, 1997). The interesting report of Nunn *et al.* (2007) revealed the presence of this species in marine bird excrement.

Previous studies have revealed that nests of *Atta sexdens rubropilosa* reared in the laboratory have a high prevalence of ascomycetous yeasts (Carreiro *et al.*, 1997); however, in field nests of *Acromyrmex* and *Atta* species, most of the yeasts isolated belong to the basidiomycetes, with *Cryptococcus* and *Trichosporon* species found irrespective of foraging behaviour or geographical location (unpublished results). Both genera are common inhabitants of soils and plant surfaces, probably explaining their presence inside the nests. The isolation of *Cryptococcus haglerorum* in this study was noteworthy. This particular yeast was originally isolated from a laboratory nest of *Atta sexdens rubropilosa* (Middelhoven *et al.*, 2003) and, as far as we know, it has not been found anywhere else. This species has now been isolated from a field nest of a lower attine ant

almost 200 km from the original site of isolation. The association of this yeast species with attine ants is unclear, but additional studies may reveal whether its occurrence is occasional or specific to attine nests.

The high proportion of *T. chiarellii* sp. nov. isolates found in this survey (20 strains isolated from the fungus garden and 14 from the waste deposit) was surprising. Yeasts in the genus *Trichosporon* are anamorphic basidiomycete fungi (Hymenomycetes, Tremelloidae, Trichosporonales) with variable cell morphology and are characterized by the production of arthroconidia (Middelhoven *et al.*, 2004). Species of this genus are widely distributed in the environment, mostly occurring in soil and/or water, but some species are considered opportunistic pathogens (Sugita *et al.*, 2002). In addition, *Trichosporon* species may be found in association with insects; *Trichosporon insectorum*, a recently described species, was isolated from the guts of beetles collected in Panama (Fuentefria *et al.*, 2008) and *Trichosporon scarabaeorum* was also isolated from insects (Middelhoven *et al.*, 2004).

Species delineation and classification

The 34 strains of *T. chiarellii* sp. nov. presented identical morphological and physiological characteristics. Colonies were butyrous, light-cream and round with a radiating margin (Fig. 1a, b). This anamorphic yeast produces true mycelium that rarely breaks into rectangular arthroconidia (Fig. 1d), and the vegetative cell takes on a variety of sizes and shapes (Fig. 1c). The genetic similarity among strains was confirmed by MSP-PCR fingerprints, which were identical for nine randomly selected strains (not shown). Microsatellite fingerprints have been used successfully for

the differentiation of yeast species in other studies (Inácio *et al.*, 2004). All the strains analysed by MSP-PCR were tested on sporulation media, alone or mixed in pairs, and no signs of sexual spores were observed after 10 days of incubation at 25 °C.

NCBI BLASTN results using data from the D1/D2 domains from five of the nine strains subjected to MSP-PCR profiling indicated that *T. scarabaeorum* CBS 5601^T (GenBank accession no. AB164372) is the closest relative of *T. chiarellii*, differing by 30 substitutions in 635 bp (95 % identity), indicating that they clearly represent distinct species. This discrepancy was confirmed using data from the ITS region from three of the nine strains. NCBI BLASTN results indicated *Trichosporon gamsii* CBS 8245^T (GenBank accession no. AF444424; Middelhoven *et al.*, 2004) as the closest relative, differing by 38 substitutions in 527 bp (92 % identity).

The genus *Trichosporon* is monophyletic except for *Trichosporon pullulans* (= *Guehomyces pullulans*) according to SSU and LSU rDNA and ITS sequences (see Middelhoven *et al.*, 2004 and references therein). However, in recent years, some *Bullera* and *Cryptococcus* species as well as the recently erected genus *Cryptotrichosporon* (Okoli *et al.*, 2007) have been placed within the Trichosporonales clade based on several molecular markers. The presence of such yeasts in the Trichosporonales reflects the plasticity of species within this order and suggests the need for a future revision of the clade, including *Trichosporon* species.

Middelhoven *et al.* (2004) accommodated the *Trichosporon* species in four groups based on sequences of the LSU rDNA. Phylogenetic analysis based on the same sequence resolved *T. chiarellii* on a separate branch as the sister group of the ovoides clade, but with low (<50 %)

Fig. 1. Morphology of *Trichosporon chiarellii* sp. nov. FCP 540806^T. (a) Light-cream-coloured colonies with fimbriate edges grown for 5 days at 25 °C on YMA. (b) Detail of true mycelium after 5 days at 25 °C on YMA. Bar, 0.5 mm. (c) Vegetative cells incubated on 2 % glucose YNB for 2 days at 25 °C. Bar, 7 µm. (d) Arthroconidia formed on 2 % glucose YNB for 2 days at 25 °C. Bar, 11.5 µm.

bootstrap support (Fig. 2). On the other hand, *T. chiarellii* is clearly different from species of the ovoides clade in its ability to assimilate D-galacturonic acid and its inability to assimilate lactose, methyl α -D-glucoside and D-gluconate. In addition, *T. chiarellii* displayed weak growth on creatine and creatinine and no growth at temperatures above 32 °C and no vitamins were required. Additional analysis using the sequence of the ITS region also accommodated *T. chiarellii* on a separate branch as a sister group of the cutaneum and humicola clades with low (<50 %) bootstrap support (Supplementary Fig. S1, available in IJSEM Online). Analysis using the concatenated dataset accommodated *T. chiarellii* as the sister group of the gracile/brassicae and porosum clades, again with low (<50 %) bootstrap support (Supplementary Fig. S2). Despite the apparently conflicting results of the three analyses regard-

ing the closest clade as the sister group of *T. chiarellii*, the molecular markers suggest that this yeast species belongs to the Trichosporonales, clustering within the *Trichosporon* species but in a position separate from its close relatives. Thus, multiple lines of evidence (i.e. molecular analyses and morphological/physiological traits) support the designation of a novel species to accommodate these strains.

Latin diagnosis of *Trichosporon chiarellii* Pagnocca, Legaspe, Rodrigues et Ruivo sp. nov.

In medio liquido post dies tres ad 25 °C, cellulae ovoidae, ellipsoideae aut elongatae, singulae aut in catenis brevis (27.0 × 5.6 µm). Cultura in agar extracta malti et levidinis continente post dies 3 ad 25 °C, candida aut cremata, aliquando cum lineis radiabus, butyrosa, hyphae fragmen-

Fig. 2. Phylogenetic tree of *T. chiarellii* sp. nov. FCP 540806^T and close relatives in the Trichosporonales. The tree was inferred from a 589 bp fragment of the LSU rDNA D1/D2 domain under the neighbour-joining criterion. Numbers on branches represent percentage bootstrap support (values of less than 50 % not shown). Species names are followed by the voucher accession number and the GenBank sequence accession number. Clade names are shown on the right according to Middelhoven *et al.* (2004) and Taj-Aldeen *et al.* (2009). Bar, 0.1 substitutions per nucleotide position.

tantes formantur. Sedimentum et pellicula repens formantur. In agaro farinae Zea mays post dies 3 arthroconidia formatur. Fermentatio nulla. Glucosum, galactosum (exigüe), L-sorbose, D-glucosaminum, D-xylosum, L-rhamnosum, sucrosum, maltosum, trehalosum, cellobiosum, salicinum, melezitotum, ribitolum, D-ribosum, L-arabinitolum, xylitolum, mannitolum, glucitolum, inositolum, N-acetylglucosaminum, acidum glucuronicum, acidum lacticum, acidum succinicum, acidum citricum, glucono- δ -lactonum, glycerolum, acidum galacturonicum et ethanolum assimilantur. Non assimilantur L-arabinosum, D-arabinosum, melibiosum, lactosum, raffinotum, inulinum, amyllum solubile, galactitolum, acidum gluconicum, 10 partes natrium chloratum per centum plus 5 partes glucosi per centum, n-hexadecanum, imidazolium, 2-ketogluconatum, methanolum, 5-ketogluconatum, propano-1,2-diolum, methyl α -D-glucosidum et erythritolum. Creatinum et creatininum (exigüe), butano-2,3-diolum (variabile), D-glucosaminum, L-lysinum et cada-verinum assimilantur at non ethylaminum, natrium nitrosum nec natrium nitricum. Ad crescentiam vitaminæ externæ non necessariae sunt. Augmentum in 32 °C, at non 35 °C. Materia amyloidea non formantur. Non crescit in agaro extrato fermenti confecto 50 partes glucosi per centum et medio 100 mg cycloheximido addito. Ureum finditur.

Table 1. Assimilation of carbon compounds by *T. chiarellii* sp. nov. FCP 540806^T

+, Positive; −, negative; w, weakly positive; v, variable.

Compound	Result	Compound	Result
D-Glucose	+	N-Acetyl-D-glucosamine	+
D-Galactose	w	Starch	−
L-Sorbose	+	Glycerol	+
D-Glucosamine	+	Erythritol	−
D-Ribose	+	Ribitol	+
D-Xylose	+	Xylitol	+
L-Arabinose	−	L-Arabitol	+
D-Arabinose	−	D-Glucitol	+
L-Rhamnose	+	D-Mannitol	+
Sucrose	+	Galactitol	−
Maltose	+	myo-Inositol	+
α,α -Trehalose	+	D-Glucono-1,5-lactone	+
Methyl α -D-glucoside	−	2-Keto-D-gluconate	−
Cellobiose	+	5-Keto-D-gluconate	−
Salicin	+	D-Gluconate	−
Melibiose	−	D-Glucuronate	+
Lactose	−	D-Galacturonate	+
Raffinose	−	DL-Lactate	+
Melezitose	+	Succinate	+
Inulin	−	Citrate	+
Methanol	−	Propane-1,2-diol	−
Ethanol	+	Butane-2,3-diol	v

Diazonium caeruleum B positivum. Typus stirps FCP 540806^T. In collectione zymotica Centraalbureau voor Schimmelcultures, Trajectum ad Rhenum, sub no. CBS 11177^T, typus stirps deposita est.

Description of *Trichosporon chiarellii* Pagnocca, Legaspe, Rodrigues et Ruivo sp. nov.

Trichosporon chiarellii (chi.a.rel/li.i. N.L. gen. sing. adj. *chiarellii* of Chiarelli, in memory of Cid Chiarelli, the father of Mara Fonseca Chiarelli Legaspe, co-author of this work).

On YMA after 3 days at 25 °C, colonies are white to cream, butyrous with a fimbriate margin (Fig. 1a). In 0.5 % yeast extract/2 % glucose/0.75 % peptone broth after 3 days at 25 °C, cells are ovoidal, ellipsoidal or elongated (mean size 27.0 × 5.6 μ m), occurring singly or in budding pairs and in short chains. Sediment and pellicle are observed in the same medium. Details of budding cells in 2 % glucose YNB are shown in Fig. 1(c). After 3 days in Dalmau plate culture on cornmeal agar and in 2 % glucose YNB, septate hyphae and arthroconidia develop (Fig. 1d). No sexual reproduction has been observed. Assimilation tests for the type strain on standard carbon and nitrogen compounds and other characteristics are shown in Tables 1 and 2.

Table 2. Assimilation of nitrogenous compounds and miscellaneous characteristics of *T. chiarellii* sp. nov. FCP 540806^T

+, Positive; −, negative; w, weakly positive response.

Characteristic	Result
Assimilation of:	
Potassium nitrate	−
Potassium nitrite	−
D-Glucosamine	+
Ethylamine	−
L-Lysine	+
Cadaverine	+
Creatine	w, +
Creatinine	w, +
Imidazole	−
Fermentation of D-glucose	−
Growth on n-hexadecane	−*
Growth without vitamins	+
Urea hydrolysis	+
Diazonium blue B colour reaction	+
Production of amyloid compounds	−
Growth at/in:	
25–32 °C	+
35 °C	−
50 % D-Glucose	−
10 % NaCl plus 5 % D-glucose	−
0.01 % Cycloheximide	−
0.1 % Cycloheximide	−

*Slant culture method (Middelhoven *et al.*, 2004).

The type strain, FCP 540806^T (=CBS 11177^T), was isolated from a fungus-growing ant nest of the genus *Myrmicocrypta* located in Botucatu, Brazil.

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