Geotrichum silvicola sp. nov., a novel asexual arthroconidial yeast species related to the genus *Galactomyces*

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Four strains of an asexual arthroconidial yeast species were isolated from *Drosophila* flies in two Atlantic rain forest sites in Brazil and two strains from oak tasar silkworm larvae (*Antheraea proylei*) in India. Analysis of the sequences of the D1/D2 large subunit rRNA gene showed that this yeast represented a novel species of the genus *Geotrichum*, described as *Geotrichum silvicola* sp. nov. The novel species was related to the ascogenous genus *Galactomyces*. The closest relatives of *Geotrichum silvicola* were *Galactomyces* sp. strain NRRL Y-6418 and *Galactomyces geotrichum*. The type culture of *Geotrichum silvicola* is UFMG-354-2^T (= CBS 9194^T = NRRL Y-27641^T).

Yeasts from the arthroconidial ascomycetous genus *Geotrichum* Link:Fries represent the anamorphic state of species of the genera *Dipodascus* Lagerheim and *Galactomyces* Redhead & Malloch (Kurtzman & Robnett, 1998; Ueda-Nishimura & Mikata, 2000). The genus *Geotrichum* has 11 species of which five have their teleomorph in the genus *Dipodascus* and two in the genus *Galactomyces*. Four *Geotrichum* species have no known sexual state (de Hoog *et al.*, 1998).

During a survey of yeasts associated with flies of the genus *Drosophila* in the Atlantic rain forest of Brazil, we isolated four strains of a yeast identified as *Geotrichum* sp. Three strains were isolated from the gut and one from external surfaces of the flies. In another study of yeast communities associated with insects, two strains also identified as

Geotrichum sp. were isolated from oak tasar silkworm larvae (*Antheraea proylei*) in India. The sequence of the D1/D2 domains of the large subunit (LSU) rRNA gene of a Brazilian strain and an Indian strain showed that they represented the same species, related to the genus *Galactomyces*. The closest relative of this novel species was a strain designated by Kurtzman & Robnett (1998) as *Galactomyces* sp. NRRL Y-6418. The D1/D2 domain of the LSU rRNA gene of this novel species differed by 15 substitutions from that of *Galactomyces* sp. NRRL Y-6418. In this report, we describe the novel species *Geotrichum silvicola* sp. nov.

Isolation and characterization of yeasts

The strains considered in this study are listed in Table 1. The Brazilian strains were collected in 2000 and 2001 in two Atlantic rain forest sites in the state of Minas Gerais, Brazil. One site was localized in the Parque Estadual do Rio Doce, while the other was the Ecological Station of the Universidade Federal de Minas Gerais. *Drosophila* species specimens were captured directly from the surface of decaying fruits using sterile plastic bags. Adult flies were allowed to walk on YM agar (1 % glucose, 0.5 % peptone,

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Abbreviation: LSU, large subunit.

The GenBank/EMBL/DDBJ accession number for the large subunit rRNA gene sequence of strain UFMG-354-2^T is AY158042 and of strain MTCC 3974 is AY225313.

Table 1. Sources of isolation of *Geotrichum silvicola*

Locality	Strain no.*	Origin
UFMG Ecological Station	UFMG-DC13-2	Surface of <i>Drosophila</i>
	UFMG-DC49-5	Gut of <i>Drosophila</i>
	UFMG-DC71.1	Gut of <i>Drosophila</i>
Parque Estadual do Rio Doce	UFMG-354-2 ^T	Gut of <i>Drosophila</i>
India	MTCC 3974	Silkworm larva
	MTCC 6224	Silkworm larva

*UFMG, Universidade Federal de Minas Gerais, Brazil; MTCC, Microbial Type Culture Collection and Gene Bank, India.

0.3 % malt extract, 0.3 % yeast extract, 2 % agar, supplemented with 100 mg chloramphenicol l⁻¹) for 1–2 h before being removed and identified. Yeasts from these plates were predominantly carried on the external surfaces and should include as minor components cells regurgitated by the flies, cells present in faecal pellets and cells carried on the ovipositor of females. Another set of adult flies was surface-sterilized in the field by immersion in 70 % ethanol for 1 min and transported to the laboratory within 2 h in tubes containing sterile water on ice. These flies were directly streaked on to YM agar (Morais *et al.*, 1995). Plates were incubated at room temperature (25 ± 3 °C). The plates were examined periodically and representative yeast colonies were purified and maintained on YM slants or in liquid nitrogen. Two strains from India were isolated from cocoons of dead larvae of *Antheraea proylei*. These larvae feed on leaves of the kharsu oak (*Quercus semecarpifolia*) and abound in the rainy season (June–July). Strain MTCC 3974 was isolated in February 2002 and strain MTCC 6224 was recovered in July 2002, from Joginder Nagar, Himachal Pradesh, India, at an altitude of 2500 m. The relative humidity was 90–100 % at the time of collection. The larvae are normally infected in the 4th or 5th stage. Although cocoon formation may occur, extensive infection causes pupae to dissolve completely. The dissolved contents of larvae were streaked on YM agar. Yeasts were characterized by standard methods (Yarrow, 1998). Identities were verified using the keys of Kurtzman & Fell (1998) and also using the program YEASTCOMPARE (Ciriello & Lachance, 2001), which compares the nutritional characteristics of any yeast with those of known species.

DNA sequence and PCR analysis

The D1/D2 divergent domains of the LSU rRNA gene were amplified by PCR from whole cells as described previously (Lachance *et al.*, 1999). The amplified DNA was concentrated and cleaned on QIAquick PCR columns (Qiagen) and sequenced in an ABI sequencer at the John P. Roberts Research Institute (London, Ontario, Canada). Sequences were edited with the program DNAMAN, version

4.15 (Lynnon BioSoft). Existing sequences for other species were retrieved from GenBank. The CLUSTAL W (Thompson *et al.*, 1994) algorithm provided in the DNAMAN package was used to align the sequences and construct a neighbour-joining tree with 1000 bootstrap iterations.

Geotrichum silvicola strains UFMG-354-2^T, UFMG-DC13-2, UFMG-DC49-5, UFMG-DC71.1 and MTCC 3974 were used for PCR fingerprinting. Yeast DNA was purified as described by Pataro *et al.* (2000). The primer EI1 (5'-CTGGCTTGGTGTATGT-3'; de Barros Lopes *et al.*, 1998) targets intron-splicing sites in mutable regions of the *Saccharomyces* genome. Each PCR assay was performed in a 10 µl reaction mixture containing 1 µl DNA, 1 µl 10 µM EI1 primer, 1 µl 10 × PCR buffer, 0.5 µl dNTPs (2.5 mM each) and 0.3 µl *Taq* DNA polymerase (5 U µl⁻¹). Samples were overlaid with 10 µl mineral oil prior to PCR amplification. PCR conditions were 5 min at 95 °C and two cycles of 30 °C for 2 min, extension at 72 °C for 30 s and denaturation at 95 °C for 30 s. This was followed by 32 cycles with an annealing temperature of 40 °C for 2 min, extension at 72 °C for 30 s and denaturation at 95 °C for 30 s. A last cycle of annealing for 2 min at 40 °C and extension for 5 min at 72 °C was added. The PCR products were analysed by gel electrophoresis on 6 % acrylamide. The gel was stained with silver nitrate and scanned.

Classification and ecology

Strains UFMG-354-2^T and MTCC 3974 differed by only three substitutions in the D1/D2 region of the LSU rRNA gene, indicating that they were closely related. PCR amplification with the intron-splicing-site primer gave similar, but not identical, profiles in the five strains of *Geotrichum silvicola* examined (Fig. 1). Strain MTCC 3974, in particular, lacked one, or possibly two, bands in the 298–344 base range. Isolates from the same species generally give similar PCR profiles (de Barros Lopes *et al.*, 1998). The small amount of polymorphism observed here was consistent with the variation observed between conspecific strains of different origins, as for example in strains of *Saccharomyces cerevisiae*, *Metschnikowia pulcherrima*, *Dekkera bruxellensis*, *Dekkera anomala* and *Hanseniaspora uvarum* (de Barros Lopes *et al.*, 1998; Guerra *et al.*, 2001). The novel species belonged to a clade of several *Galactomyces* species (Fig. 2). Some controversy exists as to the delimitation of *Galactomyces* and *Dipodascus*. Kurtzman & Robnett (1998) suggested that there is no basis for maintaining two separate genera, as the two are phylogenetically intertwined. Naumova *et al.* (2001) argued differently. As ascospore formation has not been observed in *Geotrichum silvicola*, the species was assigned to the anamorph genus *Geotrichum*, independently of the eventual resolution of conflicting views on the nomenclature of its closest relatives. These include an undescribed species named *Galactomyces* sp. NRRL Y-6418 by Kurtzman & Robnett (1998). The latter differs from *Geotrichum silvicola* by 15 substitutions in the D1/D2 region of the LSU rRNA gene.

Fig. 1. PCR fingerprints of *Geotrichum silvicola* strains obtained using the primer EI1. Lanes: M, 1 kb ladder; 1, UFMG-354-2^T; 2, MTCC 3974; 3, UFMG-DC13-2; 4, UFMG-DC71.1; 5, UFMG-DC49-5.

Three isolates of *Geotrichum silvicola* from the UFMG Ecological Station were obtained from *Drosophila* specimens feeding on fruits of *Acrocomia aculeata* (Arecaceae). The strain isolated in the Ecological Reserve of the Parque Florestal do Rio Doce was obtained from the gut of a fly feeding on an unidentified decaying fruit on the ground. These results suggested that *Geotrichum silvicola* may be part of the yeast community associated with this and other tropical fruits. The yeast may participate in the microbial deterioration of fruits in these forest environments. That three of the four strains of *Geotrichum silvicola* were isolated

Fig. 2. Neighbour-joining phylogram based on the D1/D2 divergent domains of the LSU rRNA gene of *Geotrichum silvicola* and its closest relatives. The percentage bootstrap values were obtained from 1000 iterations. Bar, 5% sequence divergence.

from the guts of the flies suggested that the yeast is consumed as food by the flies. Yeasts are known to be a source of food for *Drosophila* and the flies are important vectors for these micro-organisms (Starmer, 1981; Morais *et al.*, 1994). The Indian strains were isolated from the cocoons of dead larvae of *Antheraea proylei*, which originate from a cross between the two species *Antheraea roylei* (from the Himalayan valley) and *Antheraea pernyi* (from China). These larva feed on leaves of *Q. semecarpifolia*. The isolation of two strains from larvae in different months suggested that the yeast may have a role in the infection of larvae. However, as some other filamentous fungi were also isolated from the infected larvae, the exact role of the yeast species remains to be established.

Identification

Physiologically similar, most species in the *Galactomyces* clade utilize few carbon compounds. Most species of the genus assimilate glucose, galactose, L-sorbose, D-xylose, ethanol, glycerol, D-mannitol, D-glucitol, DL-lactate, succinate and citrate as carbon source. *Geotrichum silvicola* can be separated from *Galactomyces reessii* by the utilization of D-mannitol, which does not occur in the latter. *Galactomyces citri-aurantii* utilizes ribitol, but *Geotrichum silvicola* does not. Physiological separation of *Geotrichum silvicola* and *Galactomyces geotrichum* is more difficult. *Galactomyces geotrichum* gives variable responses for growth on D-ribose, ribitol, D-mannitol, DL-lactate, succinate and citrate, as well as for the fermentation of glucose. Growth on D-ribose and ribitol are negative for *Geotrichum silvicola* and the yeast does not ferment glucose. All six strains of *Geotrichum silvicola* were able to assimilate D-mannitol, DL-lactate, succinate and citrate. The isolates were examined after growth on the most common sporulation media (5% malt extract agar, cornmeal agar, yeast carbon/base agar with 0.1% of ammonium sulfate, dilute V8 agar and potato/dextrose agar), but asci were not found. All isolates were mixed in pairs and no signs of conjugation were observed.

Latin diagnosis of *Geotrichum silvicola* Pimenta, Prasad, Lachance et Rosa sp. nov.

*Cultura in agar*o malti post dies 7 (22 °C) plana, sicca, capillata et candida. Hyphae ramosae cum arthroconidiis formantur, at non cellulae gemmant. In agaro farinae *Zea mays* post dies 14 mycelium verum et arthroconidia formantur. Asci nec ascosporae non formantur. Glucosum non fermentatur. Galactosum, L-sorbosum, D-xylosum, ethanolum, glycerolum, mannitolum, glucitolum, ethyl acetat, D-gluconatum (variabile), acidum lacticum (lente), acidum succinicum et acidum citricum assimilantur, at non maltosum, sucrosom, raffinotum, D-ribosom, inulinum, melibiosum, lactosum, trehalosum, melezitotum, cellobiosum, salicinum, amyllum solubile, L-rhamnosum, L-arabinosum, D-arabinosum, methanolum, 2-propanolum, erythritolum, ribitolum, galactitolum, meso-inositolum, 2-keto-gluconatum, glucosaminum, N-acetylglucosaminum, xylitolum, acetonom nec hexadecanum. Lysinum, ethylaminum et cadaverinum

assimilantur et non natrium nitricum et natrium nitrosum. Ad crescentiam vitaminarum externarum non necessariae. Augmentum in 30 °C, at non 37 °C. Habitat Drosophila in Brazil et larvae bomicis Antheraea roylei in India.

Typus UFMG-354-2^T. *In collectione zymotica* Centraal-bureau voor Schimmelcultures, *Trajectum ad Rhenum*, sub no. CBS 9194^T *typus stirps deposita est.*

Description of *Geotrichum silvicola* Pimenta, Prasad, Lachance & Rosa sp. nov.

Geotrichum silvicola (sil.vi'co.la. L. nom. masc. n. *silvicola* inhabiting woods).

After 7 days on malt extract/yeast extract agar at 22 °C, colonies are white, flat, dry and powdery to finely hairy. Budding cells are absent. Hyphae are 6–7 µm wide, with frequent dichotomous branching at the apex, with early disarticulation into cubic arthroconidia. Arthroconidia are hyaline and slightly inflating to 5–6 µm wide and 30–55 µm long. A pellicle is formed after 2 days on fermentation medium. In Dalmau plates after 2 weeks on cornmeal agar, abundant true mycelia and arthroconidia are formed (Fig. 3). Glucose is not fermented. Assimilation of carbon

compounds: galactose, L-sorbose, D-xylose, ethanol, glycerol, D-mannitol, D-glucitol, lactic acid (weak), succinic acid, citric acid, D-gluconate (variable) and ethyl acetate. No growth occurs on maltose, sucrose, raffinose, D-ribose, cellobiose, lactose, melibiose, melezitose, inulin, starch, trehalose, L-arabinose, D-arabinose, L-rhamnose, erythritol, ribitol, galactitol, salicin, *myo*-inositol, methanol, hexadecane, glucosamine, xylitol, acetone, 2-propanol or *N*-acetylglucosamine. Assimilation of nitrogen compounds: positive for lysine, ethylamine/HCl and cadaverine; negative for nitrate and nitrite. Growth in vitamin-free medium is positive. Growth in amino acid-free medium is positive. Growth at 30 °C is positive, but at 37 °C is negative. Growth on YM agar with 10 % sodium chloride is negative. Growth in 50 % glucose/yeast extract (0.5 %) is negative. Starch-like compounds are not produced. Growth on 1 % acetic acid medium is negative. In 100 and 1000 µg cycloheximide ml⁻¹, growth is positive. Urease activity is negative. Diazonium Blue B reaction is negative. The habitat is *Drosophila* flies in the Atlantic rain forest and possibly fruits visited by the flies in Brazil and larva of the oak tasar silkworm, *Antheraea proylei*, in India.

The type strain is UFMG-354-2^T, isolated from the gut of a *Drosophila* fly in Parque Estadual do Rio Doce in State of Minas Gerais, Brazil. It has been deposited in the collection of the Yeast Division of the Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands, as strain CBS 9194^T (=NRRL Y-27641^T).

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Fig. 3. Phase-contrast micrographs of *Geotrichum silvicola* after 2 weeks on cornmeal agar at 25 °C. (a) Vegetative cells, true hyphae and arthrospores; (b) arthrospore formation after 1 day on yeast extract/malt extract agar. Bar, 5 µm.

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