Starmerella meliponinorum sp. nov., a novel ascomycetous yeast species associated with stingless bees

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Thirty-two strains of the novel species *Starmerella meliponinorum* sp. nov. were isolated from various substrates associated with three stingless bee species (tribe Meliponini) in Brazil and one in Costa Rica. The strains were found in garbage pellets (faecal material, discarded pollen or food, etc.), pollen provisions, adult bees, honey and propolis of *Tetragonisca angustula*, in honey from *Melipona quadrifasciata* and in adults of *Melipona rufiventris* and *Trigona fulviventris*. The sequence of the D1/D2 domains of the large-subunit rDNA showed that the novel species belongs to the *Starmerella* clade and is most closely related to *Candida etchellsii*, although the two differ in their sequences by 7 % base substitutions. *S. meliponinorum* is homothallic and assimilates few carbon sources. Nitrate is utilized as a sole nitrogen source. The type strain of *S. meliponinorum* is strain UFMG-01-J26.1^T (= CBS 9117^T).

INTRODUCTION

The stingless honeybees (tribe Meliponini), often simply called stingless bees, are highly eusocial forms living in permanent colonies often composed of many thousands of individuals. The tribe Meliponini is a much more diverse group than the tribe Apini (*Apis*), and its many species are restricted to the tropical regions of the world. In the neotropics, the Meliponini are extremely numerous in species and individuals; in fact, they are the most conspicuous bees (Michener, 1974). Nests of stingless bees are usually in pre-existing cavities (subterranean or arboreal) and are occasionally associated with ant or termite nests. The entrance is either a small opening, just large enough to allow one bee at a time to pass, or a more elongated, tube-like entrance that may have a flared opening. Foraging bees visit a wide variety of resources and large amounts of food are stored in the nests (Wille, 1983; Nogueira-Neto, 1997).

Nests of eusocial bees usually harbour a diverse microbiota. The microbial community associated with bees, their food and their environment has been studied with the ultimate aim of determining the role of micro-organisms in the biochemistry, nutrition and physiology of these insects (Gilliam *et al.*, 1988). Various yeast species are associated

with honey bees (*Apis mellifera*) and solitary bees (Inglis *et al.*, 1993; Gilliam, 1997; Rosa *et al.*, 1999; Lachance *et al.*, 2001a, b). Gilliam *et al.* (1984, 1985) isolated bacteria of the genus *Bacillus* in tropical species of stingless bees, but they did not observe yeasts. A few individuals of *Trigona* spp. collected on various flowers in Costa Rica were found to harbour several novel yeast species in the *Starmerella* clade, only two of which have been described (Lachance *et al.*, 2001a). Another yeast, represented by a single strain, has been reported as '*Candida etchellsii* sister 7·0 %' (Lachance *et al.*, 2001b). During a study of yeasts associated with stingless bees in Brazil, an ascospore-forming species was frequently isolated from food resources and adults of these insects. The sequences of the D1/D2 domains of the large subunit rDNA showed that the Brazilian isolates and the Costa Rica strain represent a second species in the recently described genus *Starmerella* (Rosa & Lachance, 1998). The novel species *Starmerella meliponinorum* sp. nov., named for its association with the four meliponine (stingless) bee species *Melipona quadrifasciata*, *Melipona rufiventris*, *Tetragonisca angustula* and *Trigona fulviventris* (Hymenoptera: Apidae: Meliponini), is described herein.

METHODS

Isolation and characterization of yeasts. The strains considered in this study are listed in Table 1. The samples were collected

The GenBank/EMBL/DDBJ accession number for the large-subunit rRNA gene sequence of strain UWO(PS)00-227.1 is AF313354.

Table 1. Origin of strains of *Starmerella meliponinorum* sp. nov.

Strain(s) (UFMG no.)	Locality	Bee species*	Substrate
00-J1, 00-J2.1, 00-J3.1, 00-J4, 00-J6.1, 00-J8, 00-J9.1, 00-J17.1, 00-J18	UFMG, Brazil	<i>Tetragonisca angustula</i> (Jataí)	Adult bees
00-J11.1	UFMG, Brazil	<i>Tetragonisca angustula</i> (Jataí)	Honey
00-J12.1	UFMG, Brazil	<i>Tetragonisca angustula</i> (Jataí)	Pollen provisions
00-J13.2	UFMG, Brazil	<i>Tetragonisca angustula</i> (Jataí)	Garbage
01-J23.1, 01-J25.1, 01-J75.1	Betim, Brazil	<i>Tetragonisca angustula</i> (Jataí)	Adult bees
01-J26.1 ^T , 01-J56.1, 01-J56.2, 01-J67.1	Betim, Brazil	<i>Tetragonisca angustula</i> (Jataí)	Pollen provisions
01-J51.3, 01-J53.1	Betim, Brazil	<i>Tetragonisca angustula</i> (Jataí)	Propolis
01-J52.1, 01-J54.1, 01-J57.2, 01-J60.2, 01-J65.2	Betim, Brazil	<i>Tetragonisca angustula</i> (Jataí)	Honey
01-J55.2, 01-J58.1, 01-J68.2	Betim, Brazil	<i>Tetragonisca angustula</i> (Jataí)	Garbage
01-J74.1	Betim, Brazil	<i>Melipona quadrifasciata</i> (Mandaçaia)	Honey
01-J83.2	Manesmann Reserve, Brazil	<i>Melipona rufiventris</i> (Uruçu)	Adult bee
UWO(PS)00-227.1	Guanacaste, Costa Rica	<i>Trigona fulviventris</i> (Arapuá)	Adult bee on <i>Merremia quinquefolia</i>

*Vernacular names of bees are given in parentheses.

in 2000 and 2001 in three locations in the state of Minas Gerais, Brazil, and in the Area de Conservación Guanacaste, Sector Santa Rosa, Costa Rica. Honey, propolis, pollen provisions and garbage pellets (faecal material, discarded pollen or food, etc.) were usually cultured a few hours after sampling. The samples were diluted in approximately 10 vols sterile water and vortexed for 1 min. One loopful was streaked out or 0.1 ml of successive decimal dilutions was spread on YM agar (1 % glucose, 0.5 % peptone, 0.3 % malt extract, 0.3 % yeast extract, 2 % agar) supplemented with 100 mg chloramphenicol l⁻¹. Adult bees were allowed to walk for 15–30 min on YM plates before being removed and identified. The plates were examined periodically and representative yeast colonies were purified and maintained in YM slants or liquid nitrogen. The yeasts were characterized by standard methods (Yarrow, 1998). Identities were verified using the keys of Kurtzman & Fell (1998) and also with the computer program YEASTCOMPARE (Ciriello & Lachance, 2001), which compares the nutritional characteristics of any yeast with those of known species.

DNA sequence analysis. The D1 and D2 variable domains of the large-subunit rDNA were amplified by PCR from whole cells as described previously (Lachance *et al.*, 1999). Amplified DNA was concentrated and cleaned on QIAquick PCR columns (Qiagen) and sequenced in an ABI sequencer at the John P. Robarts Research Institute, London, Ontario, Canada. The sequences were edited with the program DNAMAN version 4.0 (Lynnon BioSoft). Existing sequences for other yeasts were retrieved from GenBank. The CLUSTAL W (Thompson *et al.*, 1994) algorithm provided in the DNAMAN package was used to align the sequences and to construct a neighbour-joining tree with 1000 bootstrap iterations.

RESULTS AND DISCUSSION

Classification and ecology

D1/D2 sequence analysis showed that the novel species belongs to a clade of several species related to the ascospore-germinating genus *Starmerella*. Although these yeasts form two subclades (Lachance *et al.*, 2001a), the species shown in

Fig. 1 were chosen to represent only one subclade, the other being represented only by *Candida vaccinii*, which also serves as the outgroup. Strain numbers identify species that have not yet been described but whose sequences have been deposited in GenBank (Lachance *et al.*, 2001b) along with that of *S. meliponinorum* strain UWO(PS)00-227.1. The sequences of strains UFMG-01-J12.1 and UFMG-01-J26.1^T were identical to that of the former strain. The novel species is most closely related to *Candida etchellsii* and two as yet undescribed species, but differs from them by 5.7–9.1 %

Fig. 1. Neighbour-joining phylogram based on the D1/D2 divergent domains of the large subunit rDNA of *S. meliponinorum* sp. nov. and its closest relatives. Percentage bootstrap values were obtained from 1000 iterations. Bar, 5 % sequence divergence. All named strains shown are type strains.

base substitutions. *S. meliponinorum* fits sufficiently well into the generic description (Rosa & Lachance, 1998) to make an emendation unnecessary. Its discovery further reinforces the belief that intensified sampling of yeasts in this clade will eventually cause the genus *Starmerella* to reach significant proportions.

Found in many heterogeneous sources (Meyer *et al.*, 1998), *C. etchellsii* has also been recovered from a stingless bee (M.-A. Lachance, unpublished results), as have the other two sister species shown in Fig. 1 (Lachance *et al.*, 2001b). In fact, most yeasts in the *Starmerella* clade were isolated from bees or sugar-rich microhabitats visited by bees. The occurrence of *S. meliponinorum* in association with meliponine bees and their food resources indicates that the yeast may play an important role in the nutrition of the bees. *S. meliponinorum* was found not only in adult workers of *Tetragonisca angustula*, but also in their pollen provisions, honey, propolis and garbage pellets, suggesting a particularly close association with this bee. The novel species was found less frequently in *Melipona quadrifasciata* and *Melipona rufiventris*, which appear to be dominated by other yeasts (C. A. Rosa, unpublished results).

The population sizes of *S. meliponinorum* in hives of *Tetragonisca angustula* were large: $\sim 2.6 \times 10^4$ c.f.u. ml⁻¹ were found in honey, 7.9×10^5 c.f.u. g⁻¹ in garbage pellets and 1.3×10^6 c.f.u. g⁻¹ in pollen provisions. This indicates that the yeast is metabolically active and can grow at the expense of the sugars present in these resources, improving their nutritional quality. Yeasts are known to be a rich source of proteins and vitamins for other insects such as *Drosophila* and beetles (Starmer, 1981; Morais *et al.*, 1994; Lachance *et al.*, 2001b). The high counts further suggest that *S. meliponinorum* is not an agent of food spoilage for these bees, but rather that a close mutualistic association exists between the yeast and the insects. Gilliam (1979) suggested that *Candida magnoliae*, a member of the *Starmerella* clade, is actively added to trapped pollen by *Apis mellifera*. The yeast was absent in the pollen of almond flowers before they were visited by the honey bees, but occurred in the guts of adult workers, trapped pollen and bee bread samples. Similar results were obtained by Rosa *et al.* (1999) with two solitary digger nesting bees in Brazil. *Candida batistae*, also in the *Starmerella* clade, was frequently isolated from nests at high levels, suggesting that it plays an essential role in improving the nutritional quality of the provisioned pollen. Clearly, such symbiotic interactions should be studied more intensively across the geographical range of tropical bees.

Identification

Most yeasts in the *Starmerella* clade are physiologically similar and are fermentative and utilize few carbon compounds. The ascosporeogenous species *Starmerella bombicola* and *S. meliponinorum* can be separated on the basis of utilization of nitrate and nitrite as nitrogen sources and the assimilation of maltose as sole carbon source, both of which are exclusive to the latter. *S. bombicola* is heterothallic and

S. meliponinorum is homothallic. All strains of *S. meliponinorum* listed in Table 1 produced asci and ascospores on yeast carbon base with 0.01 % ammonium sulfate. Among the closest relatives of *S. meliponinorum* (Fig. 1), strain UWO(PS)00-102.2 is nearly identical physiologically. Strain UWO(PS)00-226.2 failed to grow on maltose, D-ribose, nitrate or nitrite, grew at 37 °C and was resistant to 1000 µg cycloheximide ml⁻¹. *C. etchellsii* did not assimilate sucrose or raffinose (Meyer *et al.*, 1998). Unfortunately, these growth responses were variable (the Costa Rican isolate was negative).

Latin diagnosis of *Starmerella meliponinorum* Teixeira, Marini, Lachance et Rosa sp. nov.

In medio liquido post dies tres cellulae singulae aut binae; cellulae ovoidae (2–3 × 2–5 µm). Post unum mensem sedimentum formantur. Cultura in agaro malti post dies 14 (17 °C) parva, convexa, glabra et candida. In agaro farinae Zea mays post dies 14 mycelium nec pseudomycelium non formantur. Post dies unus in agaro glucosi et extracti levidinis asci formantur. Post dies duo, asci ascosporam una liberant. Species homothallica. Glucosum, sucrosum (variabile), maltosum et raffinotum (variabile) fermentantur. Galactosum, L-sorbotum, maltosum, sucrosum (variabile), raffinotum (variabile), D-ribotum (lente), glycerolum, manitolum, glucitolum, acidum succinicum (variabile) et xylytolum (lente) assimilantur, at non inulinum, melibiosum, lactosum, trehalosum, melezitotum, cellobiosum, salicinum, amyllum solubile, L-rhamnosum, D-xylosum, L-arabinosum, D-arabinosum, ethanolum, methanolum, 2-propanolum, erythritolum, ribitolum, galactitolum, meso-inositolum, acidum lacticum, acidum citricum, 2-ketogluconatum, glucosaminum, N-acetylglucosaminum, acetotum, ethyl acetat nec hexadecanum. Natrium nitricum, natrium nitrosum, ethylaminum (lente), lysinum et cadaverinum et assimilantur. Ad crescentiam vitaminarum externarum necessariae sunt. Augmentum in 30 °C, at non 37 °C. Habitat apes meliponinae in Brazil et Costa Rica. Typus UFMG-01-J26.1^T. In collectione zymotica Centraalbureau voor Schimmelcultures, Trajectum ad Rhenum, sub no. CBS 9117^T typus stirps deposita est.

Description of *Starmerella meliponinorum* Teixeira, Marini, Lachance et Rosa sp. nov.

Starmerella meliponinorum (me.li.po.ni.no'rum. N.L. gen. masc. pl. n. *meliponinorum* of Meliponini, referring to the tribe of bees with which strains of this yeast are associated).

In yeast extract (0.5 %), glucose (2 %) broth after 3 days at 25 °C, the cells are ovoid to ellipsoidal (2–3 × 2–5 µm). Budding is multilateral. A sediment is formed after 1 month. On YM agar after 2 days at room temperature, colonies are white, convex, smooth and opalescent, with an entire edge. In Dalmau plates after 2 weeks on cornmeal agar, pseudomycelia or true mycelia are not formed. After 2 days on agar media with a low nitrogen/carbon ratio (e.g. yeast carbon base with 0.01 % ammonium sulfate),

Fig. 2. Phase-contrast micrographs of *S. meliponinorum* sp. nov. (a) Vegetative cells after 1 day on YM agar. Bar, 5 µm. (b)–(i) Conjugating cells (b, c), zygote (d), mature asci (e–h) and ruptured ascus (i) after 2 days on yeast carbon base with 0.01% ammonium sulfate. Scale as in (a).

conjugated pairs of cells give rise to asci containing one spheroidal to elongate ascospore. Ascospores are released terminally and can agglutinate (Fig. 2). The species is homothallic, as conjugation takes place between cells of a single haploid population. Glucose, sucrose (variable), maltose and raffinose (variable) are fermented; galactose is not fermented. The following carbon compounds are assimilated: galactose, L-sorbose, maltose, sucrose (variable), raffinose (variable), D-ribose (slow and weak), glycerol, D-mannitol, D-glucitol, succinic acid (weak and variable) and xylitol (slow). No growth occurs on cellobiose, lactose, melibiose, melezitose, inulin, starch, trehalose, D-xylose, L-arabinose, D-arabinose, L-rhamnose, ethanol, erythritol, ribitol, galactitol, salicin, lactic acid, citric acid, *meso*-inositol, methanol, hexadecane, glucosamine, acetone, ethyl acetate, 2-propanol, 2-ketogluconic acid or *N*-acetylglucosamine. The following nitrogen compounds are assimilated: nitrate, nitrite, lysine, ethylamine hydrochloride (weak) and cadaverine. Growth in vitamin-free medium and in amino-acid-free medium is positive. Growth at 30 °C is positive and at 37 °C is negative. Growth on YM agar with 10% NaCl is slow. Growth in 50% glucose/yeast extract (0.5%) is positive. Starch-like compounds are not produced. In 100 µg cycloheximide ml⁻¹, growth is negative. Urease activity is negative. Diazonium blue B reaction is negative. Habitat is pollen provisions, honey, propolis, garbage pellets and adult workers of stingless bees in Brazil and Costa Rica. The type strain is strain UFMG-01-J26.1^T. It was isolated from pollen provision of the stingless bee *Tetragonisca angustula* in the 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 9117^T.

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REFERENCES

- Ciriello, C. J. & Lachance, M. A. (2001). YEASTCOMPARE. University of Western Ontario, London, Canada.
- Gilliam, M. (1979). Microbiology of pollen and bee bread: the yeasts. *Apidologie* 10, 43–53.
- Gilliam, M. (1997). Identification and roles of non-pathogenic microflora associated with honey bees. *FEMS Microbiol Lett* 155, 1–10.
- Gilliam, M., Buchmann, S. L. & Lorenz, B. J. (1984). Microbial flora of the larval provisions of the solitary bees, *Centris pallida* and *Anthophora* sp. *Apidologie* 15, 1–10.
- Gilliam, M., Buchmann, S. L., Lorenz, B. J. & Roubik, D. W. (1985). Microbiology of the larval provisions of the stingless bee, *Trigona hypogaea*, an obligate necrophage. *Biotropica* 71, 28–31.
- Gilliam, M., Lorenz, B. J. & Richardson, G. V. (1988). Digestive enzymes and micro-organisms in honey bees, *Apis mellifera*: influence of streptomycin, age, season and pollen. *Microbios* 55, 95–114.
- Inglis, G. D., Sigler, L. & Goettel, M. S. (1993). Aerobic microorganisms associated with alfalfa leafcutter bees (*Megachile-Rotundata*). *Microb Ecol* 26, 125–143.
- Kurtzman, C. P. & Fell, J. W. (editors) (1998). *The Yeasts, a Taxonomic Study*, 4th edn. Amsterdam: Elsevier.

- Lachance, M. A., Bowles, J. M., Starmer, W. T. & Barker, J. S. F. (1999). *Kodamaea kakaduensis* and *Candida tolerans*, two new ascomycetous yeast species from Australian *Hibiscus* flowers. *Can J Microbiol* **45**, 172–177.
- Lachance, M. A., Bowles, J. M., Chavarria Diaz, M. M. & Janzen, D. H. (2001a). *Candida cleridarum*, *Candida tilneyi* and *Candida powellii*, three new yeast species isolated from insects associated with flowers. *Int J Syst Evol Microbiol* **51**, 1201–1207.
- Lachance, M. A., Starmer, W. T., Rosa, C. A., Bowles, J. M., Barker, J. S. F. & Janzen, D. H. (2001b). Biogeography of the yeasts of ephemeral flowers and their insects. *FEMS Yeast Res* **1**, 1–8.
- Meyer, S. A., Payne, R. W. & Yarrow, D. (1998). *Candida* Berkhout. In *The Yeasts, a Taxonomic Study*, 4th edn, pp. 454–573. Edited by C. P. Kurtzman & J. W. Fell. Amsterdam: Elsevier.
- Michener, C. D. (1974). *The Social Behavior of the Bees*. Cambridge, MA: Belknap Press.
- Morais, P. B., Rosa, C. A., Hagler, A. N. & Mendonça-Hagler, L. C. (1994). Yeast communities of the cactus *Pilosocereus arrabidaei* as resources for larval and adult stages of *Drosophila serido*. *Antonie van Leeuwenhoek* **66**, 313–317.
- Nogueira-Neto, P. (1997). *Vida e Criação de Abelhas Indígenas sem Ferrão*. São Paulo: Nogueirapis.
- Rosa, C. A. & Lachance, M.-A. (1998). The yeast genus *Starmerella* gen. nov. and *Starmerella bombicola* sp. nov., the teleomorph of *Candida bombicola* (Spencer, Gorin & Tullock) Meyer & Yarrow. *Int J Syst Bacteriol* **48**, 1413–1417.
- Rosa, C. A., Viana, E. M., Martins, R. P., Antonini, Y. & Lachance, M. A. (1999). *Candida batistae*, a new yeast species associated with solitary digger nesting bees in Brazil. *Mycologia* **91**, 428–433.
- Starmer, W. T. (1981). A comparison of *Drosophila* habitats according to the physiological attributes of the associated yeast communities. *Evolution* **35**, 38–52.
- Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.
- Wille, A. (1983). Biology of the stingless bees. *Annu Rev Entomol* **28**, 41–64.
- Yarrow, D. (1998). Methods for the isolation and identification of yeasts. In *The Yeasts, a Taxonomic Study*, 4th edn, pp. 77–100. Edited by C. P. Kurtzman & J. W. Fell. Amsterdam: Elsevier.