Wickerhamiella dulcicola sp. nov. and Wickerhamiella cachassae sp. nov., yeasts isolated from cachaça fermentation in Brazil

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Six strains of two novel yeast species were isolated from sugar-cane juice and fermentation vats of cachaça production in Brazil. The sequences of the D1/D2 domains of the large-subunit rRNA gene showed that these species belong to the Wickerhamiella clade, and their closest described relative in terms of sequence similarity is Candida (iter. nom. Wickerhamiella) drosophilae. The type strain of Wickerhamiella cachassae sp. nov. is UFMG-D5L7T (CBS 12587T = CBMAI 1469T) and the type strain of Wickerhamiella dulcicola sp. nov. is UFMG-TOL15T (CBS 12588T = CBMAI 1468T).

Cachaça is the most traditional distilled beverage produced in Brazil. In most of the traditional distilleries, the beverage is made by the spontaneous fermentation of fresh sugar-cane juice diluted to 16° Brix (Rosa et al., 2009). Fermentation cycles normally last 20–30 h, after which four-fifths of the fermented must is distilled in copper alembics, and fresh sugar-cane juice is added to the remainder to start a new fermentation cycle (Gomes et al., 2007). Saccharomyces cerevisiae is the prevalent yeast during the fermentation process, but other species can occur at lower frequencies (Gomes et al., 2007; Silva et al., 2009; Vila Nova et al., 2009). Yeasts of the genera Candida, Debaryomyces, Hanseniaspora, Kluyveromyces, Pichia, Torulaspora and Wickerhamomyces are commonly isolated during cachaça fermentation (Morais et al., 1997; Guerra et al., 2001; Gomes et al., 2007; Marini et al., 2009; Silva et al., 2009; Vila Nova et al., 2009). Wild yeasts can be introduced daily with the addition of sugar-cane juice, but they do not normally prevail in the process. During our studies of the cachaça fermentation process in Brazil, we have isolated six strains of two novel species belonging to the Wickerhamiella clade. The sequences of the D1/D2 domain of the large-subunit rRNA gene showed that these yeasts represent novel species. Their closest described relative as determined by sequence similarity is Candida (iter. nom. Wickerhamiella) drosophilae. The first novel species differs by 47 substitutions and 13 indels and the second novel species by 41 substitutions and 7 indels from C. drosophilae in the D1/D2 domains of the large-subunit rRNA gene. In this study, we describe these species as Wickerhamiella dulcicola sp. nov. and Wickerhamiella cachassae sp. nov., respectively.

Six yeast isolates representing possible novel species were isolated from must and sugar-cane juice. The samples were collected from traditional cachaça distilleries in the states of Minas Gerais and Tocantins, Brazil, in October 2005 and August 2007. Isolates UFMG-MGL63, UFMG-D6L7, UFMG-D5L7T and UFMG-D3L2 were recovered from fermented must in two distilleries located in the cities of Salinas and Jequitibá, Minas Gerais. Isolate UFMG-TOL15T was isolated from fermented must in a vat of a distillery in the city of Palmas, Tocantins. Strain UFMG-C5BS was found in sugar-cane juice from a distillery in Jequitibá, Minas Gerais. The yeasts were isolated using yeast carbon base (YCB; Difco) supplemented with 0.056% lysine, 2% agar and 100 mg chloramphenicol l⁻¹. Appropriate decimal

Abbreviation: ITS, internal transcribed spacer.

The GenBank/EMBL/DDBJ accession numbers for the 26S rRNA D1/D2 domain and ITS region sequences determined in this study are JQ180255 and JQ780059 (strain UFMG-TOL15T) and JQ180256 and JQ780060 (strain UFMG-D5L7T), respectively.

The Mycobank numbers of Wickerhamiella cachassae sp. nov. and Wickerhamiella dulcicola are MB 801229 and MB 801228, respectively.
dilutions \(10^{-2}\) and \(10^{-4}\) of the fermented must and sugarcane juice were inoculated onto plates that were then incubated at room temperature \((25 \pm 3 ^\circ C)\) for 3–8 days. A representative of each different yeast morphotype was purified and maintained on YM agar \((0.3 %\) yeast extract, \(0.3 %\) malt extract, \(0.5 %\) peptone, \(1 %\) glucose and \(2 %\) agar) slants or liquid nitrogen for later identification. The yeasts were characterized by standard methods (Kurtzman et al., 2011). Ethanol tolerance was determined as described by Pataro et al. (2002).

The ribosomal gene region spanning the internal transcribed spacers (ITS), including the 5.8S rRNA gene, and the D1/D2 domains of the large-subunit rRNA gene was amplified by PCR as described previously (Lachance et al., 1999). The amplified DNA was concentrated, purified [Wizard Plus SV Minipreps DNA Purification System (Promega) or Qiagen Qiaquick] and sequenced in an ABI 3130 automated sequencing system (Life Technologies) at the John P. Robarts Research Institute (London, Ontario, Canada) or at the Veterinary Faculty of the Universidade Federal de Minas Gerais (Belo Horizonte, MG, Brazil). The sequences were edited with the program Chromas (version 2.33; Technelysium) and aligned with the procedures contained in the program MEGAS (Tamura et al., 2011), which was also used to generate phylogenetic trees. The tree shown in Fig. 1 was reconstructed by the maximum-likelihood method, using Kimura’s two-parameter substitution model. Evolutionary rate differences were modelled after the gamma distribution with five categories. The final dataset contained 498 aligned positions. Clade consistency was estimated with bootstrap values determined from 100 iterations.

**Species delineation, generic assignment and ecology**

Analysis of the sequences of the ITS region and the D1/D2 domains of the large-subunit rRNA gene showed that the six yeast isolates from cachaça fermentation represented two novel species with affinities to the genus Wickerhamiella. The strains form a subclade within a clade that contains several species associated with floricolous insects including *Wickerhamiella occidentalis* and *Candida azyma* (Fig. 1). The first (strains UFMG-TOL15T, UFMG-MGL63, UFMG-D6L7 and UFMG-C5BS) and second (strains UFMG-D5L7T and UFMG-D3L2) novel species differ by at least 47 substitutions in the D1/D2 sequence from any previously described species. This value was obtained with C. *drosophilae*, although an examination of the tree in Fig. 1 shows that pairwise sequence divergence is not necessarily a perfect reflection of patristic distance. Sequences with the highest identity to the two novel species were those of *Candida* sp. LCF-01, isolated from organic materials in Taiwan (5 substitutions), and strain UWOPS 03-446.4 (8 substitutions), which is representative of a few isolates recovered from bertam palm nectar and associated insects in Malaysia (Wiens et al., 2008). The Taiwan strain differs in the combined ITS and D1/D2 sequences by 32 differences from strain UFMG-TOL15T and by 48 differences from strain UFMG-D5L7T. Strains UFMG-TOL15T and UFMG-D5L7T differ from strain UWOPS 03-466.4 at approximately 70 positions in the ITS and D1/D2 sequences. From this, we conclude that the isolates under consideration are representative of two novel yeast species that we describe here as *Wickerhamiella dulcicola* sp. nov. (four isolates) and *Wickerhamiella cachassae* sp. nov. (two isolates).

*W. dulcicola* sp. nov. and *W. cachassae* sp. nov. were isolated during the fermentative process of cachaça production with counts ranging from \(7.6 \times 10^2\) to \(4.3 \times 10^4\) c.f.u. ml\(^{-1}\). Isolates of both species were able to grow rapidly in the presence of \(4 %\) ethanol and slowly \((144\) h) in \(5 %\) ethanol. The species are probably brought into the fermentation by drosophilids, bees, beetles or other insects that visit the mill used for juice extraction. However, it is likely that their relative abundance declines rapidly as cachaça fermentation proceeds, as a result of the high alcohol concentrations normally encountered by the end of the process \((\text{approx.} \ 8\%)\).

The isolates of *W. dulcicola* and *W. cachassae* were examined after growth on the most common sporulation media (cornmeal agar, dilute V8 agar, \(5 %\) malt extract agar and YCB agar supplemented with \(0.01 %\) ammonium sulphate), alone or mixed in pairs. Asci or signs of conjugation were not seen. In spite of this, the species are assigned to the genus *Wickerhamiella* in conformance with the provisions of the Melbourne Code (Norvell, 2011). Opinions differ as to whether the clade shown in Fig. 1 might be better treated as two separate genera. As the D1/D2 sequences are the only orthologues currently available for all strains included in the analysis, the best inference at this time should take into account the relatively short internodes observed at the base of the clade and the low bootstrap values associated with them, which together do not point to a natural dichotomy. Furthermore, a BLAST search (Altschul et al., 1990) using the *Candida pararugosa* D1/D2 sequence as query yields species of both subclades, indicating that membership of that species in a genus centred around *W. occidentalis* or *C. azyma* as opposed to *Wickerhamiella domercqiae* would be equally tenable. Last, a phylogeny involving 15 species found in Fig. 1 but based on a concatenation of three independent genes (Kurtzman & Robnett, 2007) provided strong evidence that the clade in Fig. 1 is monophyletic and cohesive, which would argue against a split that would result only in nomenclatural confusion.

*W. dulcicola* and *W. cachassae* have similar growth profiles, as assessed by standard methods used in yeast taxonomy. The two species differ primarily in the assimilation of nitrate as sole nitrogen source. The growth responses are generally similar to those of *C. azyma*, *C. azymoides* and *C. parazyma*, but distinct from those of *C. drosophilae* and other members of its subclade.

**Description of Wickerhamiella cachassae**

Badotti, Gomes, Lachance & Rosa sp. nov.

*Wickerhamiella cachassae* (ca.chas’sae. N.L. gen. fem. sing. adj. *cachassae* of *cachaça*, referring to the beverage resulting
from the fermentation process in which the yeast was first isolated).

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 (Fig. 2a). Sediment is formed after a month, but no pellicle is observed. On YM agar after 2 days at 17 °C, colonies are white, convex, smooth and opalescent. On Dalmau plates after 2 weeks on cornmeal agar, pseudomycelium or true mycelium is not formed, but chains of budding cells are sometimes present. Fermentation of glucose is negative. Assimilation of glucose, galactose, L-sorbose, maltose, sucrose, trehalose, melezitose, D-xylose,
L- arabino, ribitol (variable), ethanol, glycerol, D-mannitol, D-glucitol, succinic acid, xyitol and D-gluconic acid is positive. No growth is detected with inulin, cellobiose, melibiose, raffinose, soluble starch, D-arabinose, D-ribose, L-rhamnose, erythritol, galactitol, salicin, DL-lactic acid, citric acid, inositol, methanol, hexadecane, D-glucosamine or N-acetyl-D-glucosamine. Assimilation of nitrogen compounds: positive for lysine, nitrite and nitrate. Growth in amino-acid-free medium and at 37 °C is positive. Growth in 50 % glucose is negative. Starch-like compounds are not produced. In 100 μg cycloheximide ml⁻¹, growth is positive. Diazonium blue B reaction is negative.

The type strain is UFMG-D5L7T. It was isolated from sugar-cane must from distilleries in Minas Gerais state, Brazil. It has been deposited in the collection of the Yeast Division of the Centraalbureau voor Schimmelcultures, Utrecht, Netherlands, as CBS 12587T and in the Brazilian Collection of Environmental and Industrial Microorganisms (Colecção Brasileira de Micro-organismos de Ambiente e Indústria, CBMAI), Campinas, São Paulo, Brazil, as CBMAI 1469T. The Mycobank number is MB 801229.

**Description of Wickerhamiella dulcicola Badotti, Gomes, Morais, Lachance & Rosa sp. nov.**

Wickerhamiella dulcicola [dul.ci’ co. la. L. adj. dulcis sweet; L. suff. -cola inhabitant, dweller; N.L. n. dulcicola intended to mean an inhabitant of sugar-cane juice (i.e. sweet juice), the substrate from which this yeast was first isolated].

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 (Fig. 2b). Sediment is formed after a month, but no pellicle is observed. On YM agar after 2 days at 17 °C, colonies are white, convex, smooth and opalescent. On Dalmat plates after 2 weeks on cornmeal agar, pseudomycelium or true mycelium is not formed, but chains of budding cells are sometimes present. Fermentation of glucose is negative. Assimilation of glucose, galactose, L-sorbose, maltose, sucrose, trehalose, melezitose, D-xylose, ethanol, ribitol, D-mannitol, D-glucitol, succinic acid, xyitol and D-gluconic acid is positive. No growth is detected with inulin, cellobiose, melibiose, soluble starch, D-arabinose, D-ribose, L-rhamnose, erythritol, galactitol, salicin, DL-lactic acid, citric acid, inositol, methanol, hexadecane, D-glucosamine or N-acetyl-D-glucosamine. Growth on raffinose, L-arabinose and glycerol is variable. Assimilation of nitrogen compounds: positive for lysine, nitrite and nitrate. Growth in amino-acid-free medium and at 37 °C is positive. Growth on YM agar with 10 % NaCl is variable. Growth in 50 % glucose is negative. Starch-like compounds are not produced. In 100 μg cycloheximide ml⁻¹, growth is positive. Diazonium blue B reaction is negative.

The type strain is UFMG-TOL15T. It was isolated from sugar-cane must from a distillery in the city of Palmas, Tocantins, Brazil. It has been deposited in the collection of the Yeast Division of the Centraalbureau voor Schimmelcultures, Utrecht, Netherlands, as CBS 12588T and in the Brazilian Collection of Environmental and Industrial Microorganisms (Colecção Brasileira de Micro-organismos de Ambiente e Indústria, CBMAI), Campinas, São Paulo, Brazil, as CBMAI 1468T. The Mycobank number is MB 801228.

**References**


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