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TAXONOMIC DESCRIPTION

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Zygotorulaspora cariocana sp. nov., a yeast species isolated from tree bark in Brazil

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Abstract

Six strains of a novel yeast species were isolated from tree bark collected in the Atlantic Forest and the Amazon Rainforest in Brazil. Analyses of the sequences of D1/D2 domains of the large subunit rRNA gene showed that the strains belong to a species in the genus *Zygotorulaspora*. The species differed by 5.54% sequence divergence (25 substitutions and five indels out of 542 bp) in the D1/D2 sequences from *Zygotorulaspora mrakii*, its closest relative. The ITS sequence of the type strain of the novel species differs by 27–69 nucleotide substitutions/indels from the other *Zygotorulaspora* species. The novel species is able to grow on trehalose, maltose, L-sorbose, inulin and at 37 °C, which are negative in *Z. mrakii*. The name *Zygotorulaspora cariocana* sp. nov. is proposed. The holotype of *Z. cariocana* sp. nov. is CBS 16118^T. The MycoBank number is MB 833702.

INTRODUCTION

Kurtzman [1], based on multigene phylogenetic analysis, proposed the genus *Zygotorulaspora* to accommodate the species *Zygotorulaspora florentina* and *Zygotorulaspora mrakii*. In a phylogenomic analysis of 332 species of the subphylum Saccharomycotina, Shen *et al.* [2] showed that *Z. florentina* and *Z. mrakii* form a distinct clade, with affinities with both *Torulaspora* and *Zygosaccharomyces*. Kurtzman [1], based on multigene analyses, also reported a weak association of *Zygotorulaspora* with these two genera. Carvalho *et al.* [3] described two additional species of this genus, *Zygotorulaspora chibaensis* and *Zygotorulaspora danielsina*. *Z. chibaensis* was isolated from bark of *Quercus salicina* and soil underneath *Castanopsis sieboldii* in Japan. *Z. danielsina* was isolated from bark of *Nothofagus menziesii* and soil underneath this tree species in New Zealand. More generally, *Zygotorulaspora* species are isolated as minor components of the yeast communities associated with fruits, insects, silage, soft drinks, tree bark and soil [3, 4]. One species of this genus, *Zygotorulaspora florentina*, was reported as having the potential to reduce volatile acidity in wine [5] and in beer [6].

During studies aimed at isolating yeasts of the genus *Saccharomyces* in Brazil, we collected approximately 700 samples of tree bark in different ecosystems [7, Rosa, C. A., unpublished results]. Six strains of a possible novel species of the genus *Zygotorulaspora* were isolated using an enrichment step with a liquid medium containing raffinose or glucose as sole carbon sources and supplemented with ethanol to inhibit sensitive yeasts [7]. This procedure is similar to that used by Carvalho *et al.* [3] to isolate *Z. chibaensis* and *Z. danielsina*. Analyses of the sequences of the D1/D2 domains of the large subunit rRNA gene showed that the Brazilian strains represent a novel species of the genus *Zygotorulaspora*, and suggest an early emerging position with respect to the four previously known species. In this work, we describe these isolates as a novel species of *Zygotorulaspora*.

METHODS

The samples were collected in two areas of the Atlantic Forest and one area of the Amazon Rainforest in Brazil. The Atlantic Forest areas were the Ilha Grande State Park (12.052 ha; 23° 10′ S, 44° 16′ W) and the National Park of Tijuca (3.953 ha; 22°

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Keywords: *Zygotorulaspora cariocana* sp. nov; yeast species; Atlantic Forest; tree bark.
Abbreviations: CBS, CBS Yeast Collection; ITS, Intergenic spacer; LSU rRNA, Large subunit ribosomal ribonucleic acid; T, Type strain; UFMG-CM, Collection of Microorganisms and Cells of Federal University of Minas Gerais; YM, Yeast extract - Malt extract agar; YNB, yeast–nitrogen base.
The GenBank/EMBL/DDBJ accession number for the sequences of the ITS region and the D1/D2 domains of the large subunit rRNA gene determined in this study is MN721358.
A supplementary figure is available with the online version of this article.

57′ S, 43° 14′ W), both in Rio de Janeiro state. The Ilha Grande State Park is a pristine Atlantic Forest ecosystem, whereas the National Park of Tijuca is claimed to be one of the largest urban forests in the world. The Tijuca Park is a reforested area, mainly with species of the Atlantic Forest, located in a place affected in the nineteenth century by deforestation caused by sugarcane and coffee cultivation. The samples were collected in these areas in July 2017. The Amazon Rainforest area is located in the Protected Ecological Reserve of Serra do Lajeado (10° 19′ S, 42° 9′ W), in the municipality of Taquaruçu, state of Tocantins, North Brazil, and represents transition zones of Amazon Rainforest and the Cerrado. The samples were collected in this area in October 2011. Forty samples of tree bark were collected in each area. Approximately 1 g samples of tree bark were collected and stored in sterile plastic bags. The samples were transported refrigerated to the laboratory and processed within 24 h. For yeast isolation, the bark samples were inoculated, in duplicate, in tubes containing 15 ml yeast–nitrogen base (YNB; Difco) supplemented with 1% raffinose, 8% ethanol and 0.02% chloramphenicol, as described by Sampaio and Gonçalves [8] and Carvalho *et al.* [3]. Another set of the samples was inoculated, in duplicate, in tubes containing 15 ml YNB supplemented with 1% glucose, 6% ethanol and 0.02% chloramphenicol with the intent of isolating strains of *Schizosaccharomyces*. One tube of each medium was incubated at 10 °C and another at 30 °C. Upon observation of yeast growth, the tubes were shaken and decimal dilutions were prepared for each sample. Aliquots of 100 µl of appropriate decimal dilutions were spread on YM agar (1% glucose, 0.3% yeast extract,0.3% malt extract, 0.5% peptone, 2% agar and 0.01% chloramphenicol) plates. The plates were incubated at 25 °C for 10 days. The different yeast morphotypes were purified by repeated streak-inoculation on YM agar plates and preserved at −80 °C. The yeasts were characterized morphologically and physiologically as described by Kurtzman *et al.* [9].

Species identification was performed by analysis of the sequences of the DNA region spanning the ITS-5.8S and D1/D2 variable domains of the large subunit rRNA gene, as described previously [10–13]. The amplified DNA was concentrated, cleaned and sequenced in an ABI 3130 Genetic Analyzer automated sequencing system (Life Technologies) by using BigDye version 3.1 and POP7 polymer. The sequences were edited and aligned using the program MUSCLE provides in the MEGA6 package [14]. They were compared with those in the GenBank database using the Basic Local Alignment Search Tool (BLAST; www.ncbi.nlm.nih.gov/pubmed/2231712) [15]. A phylogenetic tree based on the D1/D2 domains of the large subunit rRNA gene sequences was reconstructed by using the neighbour-joining analysis (in the MEGA6 software package) of 540 aligned positions and Kimura's two-parameter distance correction. In addition, a phylogenetic tree showing the placement of the novel species was reconstructed based on maximum-likelihood analysis of D1/D2 sequences totalling 523 aligned positions, with the Tamura–Nei distance and Gamma distributed rates, with invariant sites. Bootstrap values are shown for 1000 replicates.

RESULTS AND DISCUSSION

Species delineation and phylogenetic placement

Five isolates of the novel species were obtained from tree bark collected in two different Atlantic Rainforest areas in Rio de Janeiro. Strains UFMG-CM-Y6404 and Y6621 were isolated in Ilha Grande State Park and strains UFMG-CM-Y6340, Y6619 and Y6620 in the Tijuca Forest (Table 1). Another strain, UFMG-CM-Y6047 (=C103), was isolated from a bark of the tree *Tapirira guianensis* in an area of Amazon Rainforest in North Brazil. Strains UFMG-CM-Y6404, Y 6621and Y6047 have identical D1/D2 and ITS sequences, whereas strains UFMG-CM-Y6340, Y6619 and Y6620 differ of the strains above by three nucleotide substitutions and one indel in D1/D2 domains and seven differences in the ITS region. Analyses of the sequences of D1/D2 domains of the large subunit rRNA gene showed that the strains belong to a species in the genus *Zygotorulaspora* (Fig. 1 and Fig. S1), available in the online version of this article). Analyses of the sequence of the D1/D2 domains of the type strain of this novel species with the type strains of its closest relatives showed that this novel *Zygotorulaspora* species differs by 25 substitutions and five indels (5.54% sequence divergence) from *Z. mrakii*, 27 substitutions and six indels (6.11% sequence divergence) from *Z. danielsina*, 25 substitutions and 10 indels (6.45% sequence divergence) from *Z. chibaensis*, and 31 substitutions and six indels

Table 1. Origin and isolation media of the strains of *Zygotorulaspora cariocana* sp. nov.

Strain	Location	Isolation substrate	Isolation media
UFMG-CM-Y6340 (=CBS 16324)	Tijuca Forest, Rio de Janeiro	Unidentified tree	YNB with glucose 1% and ethanol 6%
UFMG-CM-Y6619	Tijuca Forest, Rio de Janeiro	Unidentified tree	YNB with raffinose 1% and ethanol 8%
UFMG-CM-Y6620	Tijuca Forest, Rio de Janeiro	Unidentified tree	YNB with glucose 1% and ethanol 6%
UFMG-CM-Y6047 (former C103;=CBS 16105)	Protected Ecological Reserve of Serra do Lajeado, Tocantins	*Tapirira guianensis*	YNB with raffinose 1% and ethanol 8%
UFMG-CM-Y6404^T (=CBS16118^T)	Ilha Grande Forest, Rio de Janeiro	Unidentified tree	YNB with glucose 1% and ethanol 6%
UFMG-CM-Y6621	Ilha Grande Forest, Rio de Janeiro	Unidentified tree	YNB with raffinose 1% and ethanol 8%

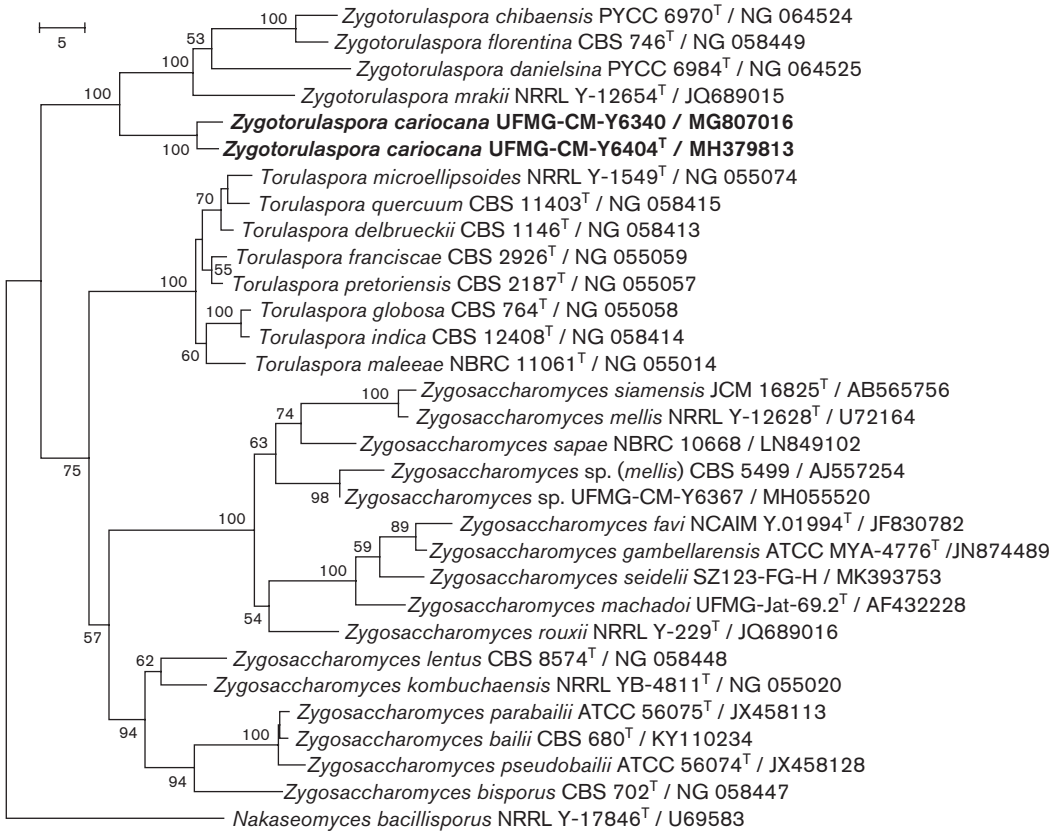

Fig. 1. Phylogram showing the placement of *Zygotorulaspora cariocana* sp. nov. Neighbour-joining tree based on 540 aligned positions for the large subunit rRNA gene D1/D2 region. The branches are scaled to the number of substitutions and bootstrap values are shown for 1000 replicates. Bar, 5 substitutions per site.

(6.84% sequence divergence) from *Z. florentina*. The ITS sequence of the type strain of the novel species differs by 27–69 nucleotide substitutions/indels from its closest relatives. Therefore, we propose the novel species *Zygotorulaspora cariocana* sp. nov. to accommodate these isolates.

A BLAST search of the whole ITS-D1/D2 sequence of the new species ranked some non-members of *Zygotorulaspora* higher in percent identity than for other *Zygotorulaspora* species. For example, *Kazachstania barnettii* (94.1% identity) ranked above *Z. danielsina* (93.7%). This is due in part to variation in query coverage among GenBank entries. GenBank entries vary in coverage, which can affect the magnitude and rank of identity values. For example, if one BLASTS sequence NG_064525 (*Z. danielsina*), one does not get the same confusing results. This also serves as an important reminder that pairwise sequence identity is only a crude estimator of relatedness and can be misleading if interpreted in that manner. Phylogenetic analyses establish relatedness on the basis of shared, derived nucleotides (synapomorphies), and not on the basis of overall pairwise divergence, as does BLAST. Neighbour-joining analysis of the current dataset, with only *K. barnettii* added, listed the latter in a basal position with respect to *Zygotorulaspora*. When, however, all species of *Kazachstania* were added, the latter formed a single clade that was more distant from *Zygotorulaspora* than were *Torulaspora* or *Zygosaccharomyces*. The purpose of the tree presented here is simply to show that *Z. cariocana* is a distinct species. A robust phylogeny would require a much broader sequence sampling and cannot arise from aligned D1/D2 sequences, regardless of the tree reconstruction method.

During our extensive studies of yeasts associated with tree bark in Brazil, approximately 700 samples were collected in different ecosystems. However, only six isolates of *Z. cariocana* were obtained. This result suggests that this species represents a minor component of the yeast communities associated with this substrate. It is likely that tree bark is not the true ecological niche of this species, and these isolates could be considered a transitory species in these communities. However, the isolation of *Z. cariocana, Z. chibaensis* and *Z. danielsina* from samples of tree bark suggests that the vectors of these species visit these substrates.

The six isolates of *Z. cariocana* produced persistent asci formed after conjugation between independent cells, or autogamy between cell and bud. Asci may also be unconjugated. The species appears to be homothallic. Ascospores were observed on YM agar after 5 days at 25 °C (Fig. 2). The novel species can be separated from *Z. mrakii* by the growth

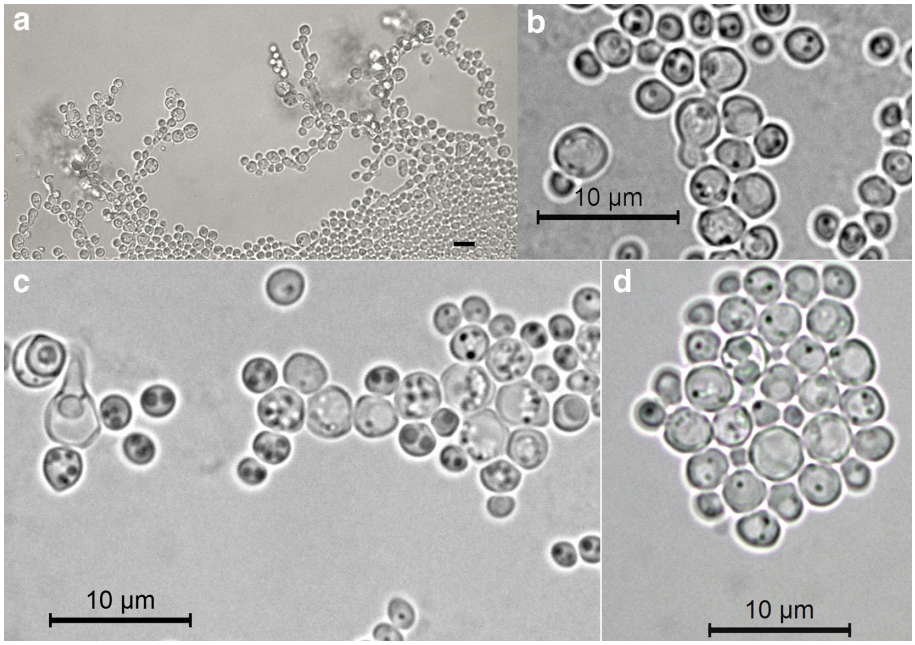

Fig. 2. Micromorphology of *Zygotorulaspora cariocana* UFMG-CM-Y6404^T. (a) Rudimentary pseudophypae on Dalmau plates after 2 weeks at 25 °C; (b) Budding cells and conjugation between independent cells on YM agar incubated at 25 °C after 5 days; (c and d) Budding cells and asci with one (c) and two ascospores (d) on YM agar incubated at 25 °C after 5 days. Bar 10 µm.

on trehalose, maltose, L-sorbose, inulin and at 37 °C, that are positive for *Z. cariocana* and negative for the latter species.

DESCRIPTION OF *ZYGOTORULASPORA CARIOCANA* MOREIRA, SANTOS, MORAIS, LACHANCE AND ROSA SP. NOV.

Zygotorulaspora cariocana (ca.ri.o.ca´na N.L. fem. adj. *cariocana* referring to the inhabitants of Rio de Janeiro, known as 'carioca').

After 5 days on YM agar at 25 °C, cells are spherical to ovoid (1.5–6×2–5 µm) and occur singly, in pairs, or occasionally, in small clusters (Fig. 2). Budding is multilateral. On YM agar after 2 days at 25 °C, the colonies are small, convex, white to tannish-white, and have an entire margin. On Dalmau plates after 2 weeks at 25 °C, rudimentary pseudohyphae are present (Fig. 2a) but true hyphae are not formed. Asci formation was observed on 5% malt and YM agar, after 5 days at 25 °C. Asci are persistent and may be unconjugated or arise following conjugation between independent cells or between a cell and its bud (Fig. 2b). Each ascus forms one to two smooth, spherical ascospores(Fig. 2c, d). The species appears to be homothallic. Fermentation of D-glucose is positive. Glucose, sucrose, raffinose, galactose, trehalose, maltose, melibiose, melezitose (variable), L-sorbose, inulin, ethanol, D-mannitol, D-glucitol, DL-lactate, succinic acid (variable), xylitol and ethyl acetate are assimilated. No growth occurs on D-xylose, D-ribose, lactose, glycerol, ribitol soluble starch, cellobiose, salicin, citrate, D-gluconate, D- glucosamine, *N*-acetyl-D-glucosamine, hexadecane, L-rhamnose, L-arabinose, D-arabinose, methanol, erythritol, galactitol, *myo*-inositol, acetone or isopropanol. Lysine is utilized as the sole nitrogen source, but growth on nitrate and nitrite is negative. Growth on amino acid-free medium is positive. Growth at 37 °C is positive and negative at 40 °C. Growth on 0.01% cycloheximide is positive. No growth is observed on 1% acetic acid, 50% (w/w) glucose or 10% (w/v) sodium chloride/ 5% (w/v) glucose. Starch-like compounds are not produced. Acid production is slow. Urease activity is negative. Diazonium Blue B reaction is negative. The habitat is tree bark collected from the Atlantic Forest and the Amazon Rainforest in Brazil. The designated holotype, CBS 16118^T, is preserved in a metabolically inactive state in the CBS Yeast Collection of the Westerdijk Fungal Diversity Institute, Utrecht, The Netherlands. It was isolated from tree bark collected in the Ilha Grande State Park, state of Rio de Janeiro, Southeast Brazil. An isotype of *Zygotorulaspora cariocana* sp. nov. is deposited in the Collection of Microorganisms and Cells of Federal University of Minas Gerais (Coleção de Microrganismos e Células da Universidade Federal de Minas Gerais, UFMG), Belo Horizonte, Minas Gerais, Brazil, as strain UFMG-CM-Y6404. The Mycobank number is MB 833702. The GenBank/EMBL/DDBJ accession number for the sequences of the ITS region and the D1/D2 domains of the large subunit rRNA gene is MN721358.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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