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# *Zygosaccharomyces machadoi* sp. n., a yeast species isolated from a nest of the stingless bee *Tetragonisca angustula*

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**Abstract**

A new yeast species, *Zygosaccharomyces machadoi*, was discovered in garbage pellets of the stingless bee *Tetragosnica angustula* in Brazil. Analysis of the sequence of the D1/D2 domains of the large-subunit rDNA showed that the new species is related to *Zygosaccharomyces rouxii*. *Z. machadoi* probably causes food spoilage in hives of stingless bees. It assimilates only a few carbon sources such that it is difficult to distinguish it from other *Zygosaccharomyces* species based on conventional physiological tests. The type strain of *Z. machadoi* is UFMG-J01-63.2$^T$ (CBS10264$^T$).

**Keywords**: *Zygosaccharomyces machadoi*, stingless bees, honey, garbage pellet.

## Introduction

The ascomycetous yeast genus *Zygosaccharomyces* is well supported by multi-gene phylogenetic analysis (Kurtzman, 2003). Six species are currently recognized, namely *Z. bailii, Z. bisporus, Z. kombuchaensis, Z. lentus, Z. mellis*, and *Z. rouxii*, the type species. *Zygosaccharomyces* species are involved in the spoilage of many different foods (Snowdon & Cliver, 1996; Loureiro & Malfeito-Ferreira, 2003), in particular some that are rich in sugars, such as honey. *Z. rouxii* is the commonest causative agent of fermentative spoilage of the honey of *Apis mellifera*, due mostly to its tolerance to high osmotic pressures (Snowdon & Cliver, 1996; White, 1978).

During a survey of yeasts associated with stingless bees in Brazil (Rosa et al., 2003), we isolated a yeast that forms ascospores typical of the genus *Zygosaccharomyces*. The strain was obtained from a garbage pellet of the stingless bee *Tetragonisca angustula* (Hymenoptera, Apidae, Miliponini). The sequences of the D1/D2 domains of the large subunit ribosomal DNA showed that the strain represents a new genetically distinct species of the genus *Zygosaccharomyces*, related to *Z. rouxii*. In this paper, we describe the new species *Zygosaccharomyces machadoi*.

## Material and methods

*Yeast isolation and characterization*

The strain considered in this study was collected from a hive of the stingless bee *Tetragonisca angustula* (Hymenoptera, Apidae, Meliponinae) in the state of Minas Gerais, Brazil. The sample was collected during the year 2000 in the Ecological Station of the Universidade Federal de Minas Gerais and consisted of garbage pellets of *T. angustula*. The pellet was aseptically collected from the hive and transported to the laboratory. A small amount of garbage pellet was diluted in sterile distilled water and the suspension was spread on YM agar (1.0% glucose, 0.5% peptone, 0.3% malt extract, 0.3% yeast extract, 2.0% agar) supplemented with 100 mg/L chloramphenicol. The plates were incubated for 5 days at room temperature (25 $\pm$3ºC). The yeast colonies were counted and representatives of each morphological type were purified and maintained on YM slants or frozen in liquid nitrogen. The yeast was characterized by standard methods (Yarrow, 1998). Phenetic similarity to other described yeasts was examined by using 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/D2 variable domains of the large-subunit rDNA were amplified by whole cell PCR 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. Robarts Research Institute, London, Ontario, Canada. The sequence was edited with the program DNAMAN, version 4.1 (Lynnon BioSoft). Existing sequences for other yeasts were retrieved from GenBank. The CLUSTAL W (Thompson et al., 1994) and neighbour-joining (Saitou & Nei, 1987) algorithms provided in DNAMAN were used to align the sequences and construct a phylogram with 1000 bootstrap iterations. The sequence of the type strain has been deposited in GenBank under the accession number AF432228

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**Results and discussion**

*Phylogenetic placement and ecology*

The relationship between the new *Zygosaccharomyces* species and their closest relatives is shown in Fig. 1. The new species *Z. machadoi* is closely related to *Z. rouxii*. The two species differ by 21 substitutions and three gaps in the D1/D2 large-subunit rDNA sequence. The closest relatives are *Z. rouxii* and *Z. mellis*, both of which have been reported as spoilage agents of the honey of *Apis mellifera* (Snowdon & Cliver, 1996; Kurtzman, 1998). *Z. machadoi* was isolated from garbage pellets of *T. angustula*. The cell count of *Z. machadoi* was 1.4 x $10^5$ colony forming unit (cfu)/g in the garbage pellets. This result suggests that the yeast is metabolically active in this substrate, and further that the yeast may be an agent of spoilage in nests of stingless bees.

*Identification*

*Z. machadoi* grows slowly on YM agar and on yeast nitrogen base agar containing suitable carbon sources such as glucose, galactose, trehalose, D-mannitol, D-glucitol, and xylitol. The physiological separation of *Z. machadoi* from *Z. rouxii* or *Z. mellis* is difficult as the three species have similar physiological profiles. *Z. machadoi* does not grow on YM agar with 10% sodium chloride whereas the other two species give positive response for this physiological test. However, confirmation of identity based on D1/D2 sequencing is recommended.

*Latin diagnosis of* Zygosaccharomyces machadoi *Rosa & Lachance sp. n.*

*In medio liquido post dies tres cellulae singulae aut binae; cellulae ovoidae (2-3 x 2-5* mm*). Post unum mensem sedimentum formatur. 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. Asci stabiles sunt. Species homothallica. Glucosum fermentatur. Glucosum, galactosum (lente), trehalosum (lente), mannitolum (lente), glucitolum(lente) et xylitolum (lente) assimilantur, at non L-sorbosum, maltosum, sucrosum, inulinum, melibiosum, lactosum, trehalosum, melezitosum, cellobiosum, salicinum, amylum solubile, L-rhamnosum, D-xylosum, L-arabinosum, D-arabinosum, D-ribosum, ethanolum, methanolum, L-propanolum, 2- propanolum, erythritolum, ribitolum, galactitolum, meso-inositolum, acidum lacticum, acidum citricum, 2-ketogluconatum, glucosaminum, N-acetylglucosaminum, acetonum, ethyl acetas nec hexadecanum. Ethylaminum, lysinum et cadaverinum assimilantur at non natrium nitricum nec natrium nitrosum. Ad crescentiuam vitaminae externae necessariae sunt. Augmentum in 30°C, at non 37°C. Habitat apes meliponinae in Brazil. Typus UFMG-01-J63-2. In collectione zymotica* Centraalbureau voor Schimmelcultures, *Trajectum ad Rhenum, sub no. CBS 10264 typus stirps deposita est.*

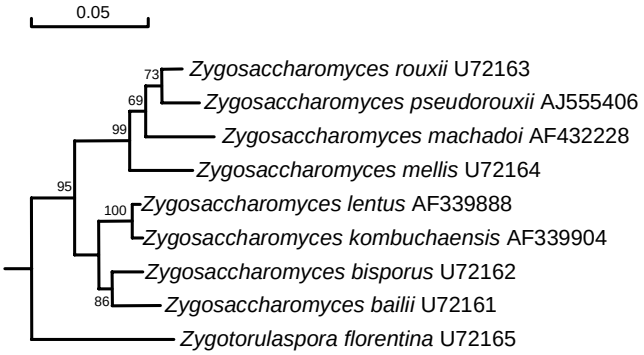


**Figure 1 -** Neighbour joining phylogram based on the D1/D2 divergent domains of the large subunit rDNA of *Zygosaccharomyces machadoi* and its closest relatives. The percentage bootstrap values were obtained from 1000 iterations. The scale bar shows 5% sequence divergence. All strains shown are type strains.

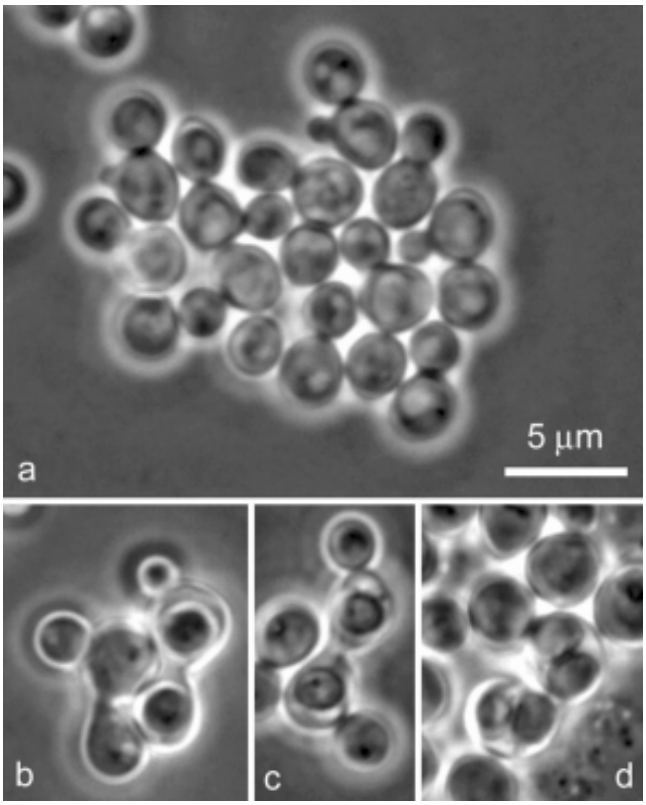


**Figure 2 -** Phase contrast micrographs of *Zygosaccharomyces machadoi*. (a) Vegetative cells after five days on yeast extract-malt extract (YM) agar. Conjugating cells (b) and asci with two (b,c) or four (d) ascospores.

*Description of* Zygosaccharomyces machadoi *Rosa & Lachance sp. n.*

In yeast extract (0.5%), glucose (2%) broth after 3d at 25°C, the cells are ovoid to ellipsoidal (2-3 x 2-5 mm). Budding is

multilateral. A sediment is formed after a month. On YM agar after 2 days at room temperature, colonies are white, convex, smooth and opalescent. In Dalmau plates after two weeks on cornmeal agar, pseudomycelia or true mycelia are not formed. After two days on agar media with a low nitrogen/carbon ratio (*e.g.*, Yeast Carbon Base with 0.01% ammonium sulphate), conjugated pairs of cells give rise to asci containing one or two spheroidal ascospore. Ascospores are not liberated. The species is homothallic, as conjugation takes place between cells of a single haploid population. Glucose is fermented. Glucose, galactose (slow), trehalose (slow), D-mannitol (slow), D-glucitol (slow), and xylitol (weak and slow) are assimilated. No growth occurs on L-sorbose, sucrose, maltose, cellobiose, lactose, melibiose, raffinose, melezitose, inulin, soluble starch, D-xylose, L-arabinose, D-arabinose, D-ribose, L-rhamnose, ethanol, erythritol, ribitol, galactitol, salicin, lactic acid, citric acid, myo-inositol, methanol, hexadecane, glucosamine, acetone, ethyl acetate, isopropanol, gluconic acid and N-acetyl-glucosamine. Assimilation of nitrogen compounds: positive for lysine, ethylamine-HCl and cadaverine, and 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; at 37°C is negative. Growth on YM agar with 10% sodium chloride is negative. Growth in 50% glucose/yeast extract (0.5%) is positive. Starch-like compounds are not produced. In 100 mg cycloheximide $mL^{-1}$ the growth is negative. Urease activity is negative. Diazonium Blue B reaction is negative. The habitat is garbage pellets of the stingless bee *T. angustula*. The type strain of *Zygosaccharomyces machadoi* is strain UFMG-01-J63-$2^T$. It was isolated from garbage pellet of the stingless bee *Tetragonisca angustula* 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 CBS10264. The epithet *machadoi* (ma.cha'do.i) L. gen. masc. sing. n. *machadoi*, is in honour of Professor Angelo B. M. Machado for his contributions to the studies on taxonomy and ecology of insects in Brazil.

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