Antonie van Leeuwenhoek (2018) 111:525–531
https://doi.org/10.1007/s10482-017-0974-8

ORIGINAL PAPER

Spathaspora piracicabensis f. a., sp. nov., a D-xylose-fermenting yeast species isolated from rotting wood in Brazil

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Received: 29 August 2017 / Accepted: 31 October 2017 / Published online: 9 November 2017

Abstract Two strains of a novel yeast species were isolated from rotting wood of an ornamental tree (purple quaresmeira, *Tibouchina granulosa*, Melastomataceae) in an Atlantic Rainforest area in Brazil. Analysis of the sequences of the internal transcribed spacer (ITS-5.8S) region and the D1/D2 domains of the large subunit rRNA gene showed that this species belongs to the *Spathaspora* clade, and is phylogenetically related to *Spathaspora brasiliensis, Candida materiae* and *Sp. girioi*. The novel species ferments D-xylose, producing ethanol, with amounts between 3.37 and 3.48 g L^{-1} ethanol from 2% D-xylose. Ascospores were not observed from this new species. The name *Spathaspora piracicabensis* f. a., sp. nov. is proposed to accommodate these isolates. The type strain is UFMG-CM-Y5867^T (= CBS 15054^T - = ESALQ-I54^T). The MycoBank number is MB 822,320.

Keywords Atlantic Rainforest · Novel yeast species · Rotting wood · *Spathaspora* · D-xylose-fermenting yeast

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10482-017-0974-8) contains supplementary material, which is available to authorized users.

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Introduction

The *Spathaspora* clade contains several D-xylose-fermenting yeast species isolated from rotting wood or wood-boring insects (Lopes et al. 2016; Cadete and Rosa 2017). *Spathaspora* was proposed by Nguyen et al. (2006) to accommodate the teleomorphic species *Spathaspora passalidarum*, isolated from a passalid beetle in Louisiana, USA, and the anamorphic species *Candida jeffriesii*, isolated from a beetle in Chiriqui, Panama. The genus is composed of a core group that is well supported by phylogenetic analyses of D1/D2 LSU sequences and includes *Sp. passalidarum,* the type species of the genus, *C. jeffriesii*, *Candida materiae*, *Sp. arborariae*, *Sp. brasiliensis*, *Sp. girioi,* and *Sp. suhii* (Barbosa et al. 2009; Cadete et al. 2009, 2013; Daniel et al. 2014; Lopes et al. 2016; Cadete and Rosa 2017). The species *Sp. roraimanensis* shares the unique ascospore morphology of the genus and is closely related to *Sp. gorwiae*, *Sp. hagerdaliae*

and *Sp. xylofermentans*, in which ascospore production have not been observed. Lopes et al. (2016), using phylogenomic analyses, showed that *Sp. gorwiae* and *Sp. hagerdaliae* form a subclade within the *Spathaspora* clade. *Spathaspora allomyrinae* was described by Wang et al. (2016) from three strains isolated from the gut of *Allomyrina dichotoma* beetles in China. This species forms two elongated ascospores similar to those of the *Spathaspora* clade. However, its phylogenetic position within this clade is less clear (Wang et al. 2016; Lopes et al. 2016). Morais et al. (2017) described the species *Sp. boniae* based on two strains producing asci containing elongate ascospores with curved ends typical of the genus *Spathaspora.* That species was isolated from rotting wood in Brazil. A phylogenomic analysis placed *Sp. boniae* in an early-emerging position relative to the larger *Candida albicans*/*Lodderomyces* clade. Based on these results, the authors suggested that the genus *Spathaspora* is, in fact, paraphyletic. Cadete and Rosa (2017) suggested that whole genome sequencing of all *Spathaspora* species and those of related genera, combined with the discovery of new species of the clade will help to elucidate the phylogeny of this genus.

Yeasts capable of metabolizing D-xylose, the second most abundant sugar in lignocellulosic based-feedstocks, may produce ethanol or xylitol as main value-added products (Cadete et al. 2017; Cadete and Rosa 2017). This biotechnological trait has encouraged efforts to search for novel D-xylose-fermenting yeasts, leading to the description of new species endowed with this ability. Some of the most promising yeasts showing these properties are members of the *Spathaspora* clade, including *Sp. passalidarum* and *Sp. arborariae*, which has stimulated studies on yeasts of this genus (Cadete and Rosa 2017).

During an exploration of yeasts associated with rotting wood in an Atlantic Rainforest area in the municipality of Piracicaba, São Paulo state, Brazil, many D-xylose-fermenting strains were isolated. Analyses of ITS-5.8S and D1/D2 sequences of the large subunit rRNA gene showed that two of these strains represent a new species belonging to the *Spathaspora* clade. The strains did not produce ascospores when examined individually or mixed in pair. We propose the name *Spathaspora piracicabensis* sp. nov. for this species. We also characterized ethanol production from D-xylose by the two strains.

Materials and methods

Yeast isolation and identification

The yeasts were isolated from samples of rotting wood collected from an ornamental tree (purple quaresmeira, *Tibouchina granulosa*) in an area (22°42′33″S and 47°37′58″W) of Atlantic Rainforest in the state of São Paulo, Brazil. The area is located on the campus of the Escola Superior de Agricultura Luiz de Queiroz (ESALQ, Universidade de São Paulo), in the municipality of Piracicaba. The predominant vegetation is characterized as an Atlantic Rainforest biome. Twelve rotting wood samples were collected from decayed branches (15–20 cm length) of the tree *T. granulosa* in March 2013. The samples were stored in sterile plastic bags and transported under refrigeration to the laboratory over a period of no more than 24 h. Approximately 3 g of each sample were placed separately in 250 mL Erlenmeyer flasks with 50 mL sterile YNB-xylose medium (yeast nitrogen base 0.67%, D-xylose 0.5%, chloramphenicol 0.02% and tetracycline 0.02%, pH 5.0), as described previously (Cadete et al. 2017). The flasks were incubated at 25 °C in an orbital shaker (New Brunswick™ Innova® 40/40R, USA) at 150 rpm for 5 days. When growth was detected, 0.5 mL of each culture was transferred to flat-bottomed tubes containing 5 mL sterile YEPX broth (yeast extract 1%, peptone 1%, D-xylose 2%, chloramphenicol 0.02% and tetracycline 0.02%, pH 5.0). The tubes were incubated in an orbital shaker at 150 rpm, 25 °C during 48 h. After growth detection in YEPX broth, serial decimal dilutions were performed in sterile distilled water to obtain cell concentrations of 2–4 $\times$ 10^2 cells mL^{-1}. An aliquot of 100 µL of the appropriated dilution was seeded on YEPX agar and the plates were incubated at 28 °C for 5 days, until yeast colonies developed.

The different yeast morphotypes were purified by repeated plating on YEPX agar, and preserved at − 80 °C. Yeasts showing potential for ethanol production from D-xylose in the screening tests described below were morphologically and physiologically characterized using the methods described in Kurtzman et al. (2011). Sporulation was investigated using 1% glucose, 0.01% yeast extract (GY), yeast carbon base plus 0.01% ammonium sulfate (YCBAS), dilute (1:9 and 1:19) V8, Fowell's acetate and YM agars at 15 and 25 °C for up to 4 weeks.

Species identification was performed by the analysis of the D1/D2 variable domains of the 26S ribosomal RNA gene and the ITS-5.8S region of the large subunit of the rRNA gene, as described previously (White et al. 1990; O'Donnell 1993; Lachance et al. 1999). The amplified DNA was concentrated, cleaned and sequenced in an ABI 3130 Genetic Analyzer automated sequencing system (Life Technologies, USA) using BigDye v3.1 and POP7 polymer. The sequences were assembled, edited and aligned with the program MEGA6 (Tamura et al. 2013). The phylogenetic trees were constructed based on LSU rRNA gene D1/D2 domains and ITS-5.8S sequences by Maximum Likelihood analysis of 510 and 379 aligned positions, respectively, using the Tamura-Nei substitution model with a gamma rate distribution and invariant sites. Bootstrap values were determined from 1000 pseudoreplicates.

Screening of D-xylose-fermenting yeasts

To assess the ability to ferment D-xylose, the yeasts obtained were subjected to fermentation assays carried out on complex medium containing D-xylose as sole carbon source. *Spathaspora arborariae* (UFMG-CM-Y352^T = CBS 11463^T), *Sp. passalidarum* (UFMG-CM-Y470), *Scheffersomyces parashehatae* (UFMG-CM-Y507) and *Sc. stipitis* NRRL Y-7124^T were used as positive controls for D-xylose fermentation (Cadete et al. 2012, 2017).

Yeast inocula were prepared by adding one loopfull of a recent yeast growth culture (24–48 h) in test tubes containing 3 mL of YEPD broth (yeast extract 1%, bacteriological peptone 1%, dextrose 2%) incubated at 28 °C for 48 h without shaking. After growth, 100 µL of each culture were re-inoculated in test tubes containing 3 mL of YEPD medium and incubated at 28 °C for 36 h. Screening of D-xylose-fermenting yeasts were performed by adding 100 µL of the inoculum (OD_{600nm} = 0.1) in 35 mL flat bottomed tubes containing 6 mL of YEPX medium (D-xylose 2%). The tubes were incubated at 28 °C on an orbital shaker at 150 rpm. Samples (1 mL) were taken regularly at ranges of 12 h to quantify the cell biomass, xylose and metabolite concentration. After this first screening step, a second D-xylose fermentation assay with selected strains was performed by adding 200 µL of the inoculum with (OD_{600nm} = 0.1) in 35 mL flat bottomed tubes containing 6 mL of YEPX medium (D-xylose 2%). The tubes were incubated and sampled as described above.

Cell concentration was determined in spectrophotometer (Micronal B-380, Brazil) by optical density at 600 nm. Afterwards, samples were centrifuged and the resulting supernatants were diluted and filtered using a 0.2 µm cellulose acetate filter (Sartorius, Germany). Levels of D-xylose, xylitol, and ethanol were determined by a CG480C high performance liquid chromatography (HPLC) system with a Bio-Rad Aminex HPX-87H column (Hercules, USA) and a 410 GC refractive index detector (Scientific Instruments CG LTDA, Brazil). The mobile phase was 5 mM H_2SO_4 at a flow rate of 0.6 mL min^{-1}, 65 °C, and a sample injection of 5 µL (Basso et al. 2008).

Results and discussion

Yeast isolation and screening

A total of 83 yeast strains were obtained from the 12 rotting wood samples collected.

After the screening test and performing a second D-xylose fermentation assay (D-xylose 2%), the strains UFMG-CM-Y5867^T (= ESALQ-I54^T) and UFMG-CM-Y6112 (= ESALQ-I38) showed the most substantial ethanol production from D-xylose, with titers of 3.37 and 3.48 g L^{-1} ethanol, respectively, in 24 h. These results were higher to those observed at 24 h of fermentation for *Sc. stipitis* NRRL Y-7124 (2.47 g L^{-1}) and *Sp. arborariae* UFMG-CM-Y352 (3.03 gL^{-1}) but lower than the ethanol titers obtained by *Sp. passalidarum* UFMG-CM-Y470 (4.63 g L^{-1}) and *Sc. parashehatae* UFMG-CM-Y507 (4.58 g L^{-1}). Xylitol production in 24 h for UFMG-CM-Y5867^T and UFMG-CM-Y6112 was equivalent to 1.32 and 1.08 g L^{-1}, respectively, whereas for the remaining yeasts it varied from zero to 0.43 g L^{-1} xylitol (data not showed). In this preliminary D-xylose culture assay, it was possible to observe that UFMG-CM-Y5867^T and UFMG-CM-Y6112 were able to produce higher ethanol than xylitol titers. However, other D-xylose fermentation assays are necessary to verify the real and potential biotechnological application of these strains with regard to the production of bioethanol from lignocellulosic sugars.

Species delineation and phylogenetic placement

The analyses of the sequences of the ITS-5.8S and the D1/D2 sequences of LSU rRNA of strains UFMG-CM-Y5867^T and UFMG-CM-Y6112 showed that these yeasts represent a novel species of the clade *Spathaspora*, and is phylogeneticly related to *Sp. brasiliensis*, *C. materiae* and *Sp. girioi* (Fig. 1). Sequences of the ITS-5.8S and D1/D2 domains of both isolates were identical. The novel species differs by six substitutions in D1/D2 domains from *Sp. brasiliensis* and ten substitutions from *C. materiae* and *Sp. girioi*. In the ITS-5.8S region, both strains of the novel species differ by 24 substitutions and 13 indels from *Sp. brasiliensis*, six substitutions and three indels from *C. materiae*, and 29 substitutions and 16 indels from *Sp. girioi*.

Phylogenetic analysis of the ITS-5.8S region confirmed that the new species is distinct from closely related species (Supplementary Fig. 1). Despite the description of new species such as *Sp. boniae* improved our knowledge regarding *Spathaspora* phylogeny by suggesting it as a paraphyletic genus (Morais et al. 2017), the new species here described was phylogenetically placed between known *Spathaspora* spp. using both genetic markers (sequences of ITS-5.8S and D1/D2-26S rRNA), not contributing for clarifying the phylogenetic boundaries of the genus *Spathaspora*. Therefore, the description of new species is still needed for a better comprehension of the complex phylogeny of this genus.

Both strains were examined individually, or mixed in pairs on several sporulation media, but asci or signs of conjugation were not seen. The name *Spathaspora piracicabensis* sp. nov. is proposed to accommodate this species. The mention forma asexualis (f.a.) was added in the title as a reminder that a sexual state is not known (Lachance 2012).

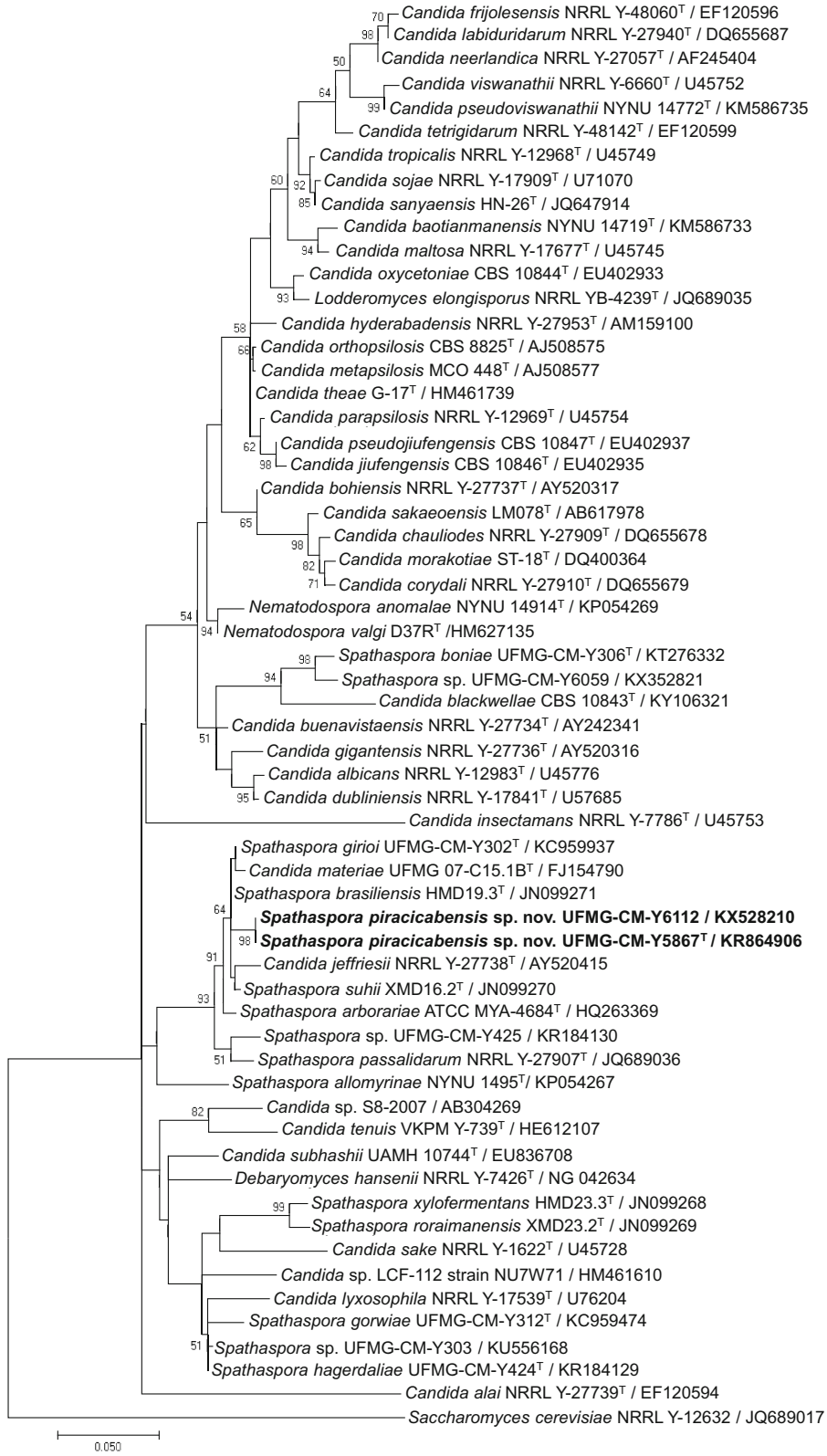
Description of *Spathaspora piracicabensis* sp. nov. C.S. Varize, R.M. Cadete, L.D. Lopes, R.M. Christofoleti-Furlan, M.A. Lachance, L.C. Basso and C.A. Rosa sp. nov. Mycobank number MB 822320

Etymology: The specific epithet *pi.ra.ci.*ca.*ben'sis* N.L. nom. adj., piracicabensis, refers to the Brazilian city of Piracicaba, where this yeast was found.

Fig. 1 Phylogenetic placement of *Spathaspora piracicabensis* ▶ sp. nov. based on LSU rRNA gene D1/D2 domains sequences. The tree was constructed by maximum likelihood analysis of 510 aligned positions using the Tamura-Nei substitution model with a gamma rate distribution and invariant sites. Bootstraps were determined from 1000 pseudoreplicates and values above 50% are indicated in the tree. Bar, 0.05 substitutions per site

On YM agar, after 7 days at 25 °C, the cells are spherical to ovoid (3–4 × 3–4 μm). Budding is multilateral (Fig. 2). Colonies are white, smooth, glistening and convex and pseudohyphae are present. Asci or signs of conjugation were not seen on sporulation media. Fermentation of D-glucose, maltose, trehalose and D-galactose are positive. Xylose fermentation is negative using Durham tubes, but ethanol is produced from xylose when determined by HPLC. D-glucose, D-galactose, L-sorbose (variable), maltose, sucrose, cellobiose, trehalose, melezitose, D-xylose, D-ribose, ethanol, erythritol, ribitol, D-mannitol, D-glucitol, salicin, succinate, hexadecane (variable, slow and weak), xylitol, ethylacetate and *N*-acetyl-D-glucosamine are assimilated. No growth occurs on lactose, melibiose, raffinose, inulin, soluble starch, L-arabinose, D-arabinose, L-rhamnose, glycerol, galactitol, DL-lactate, citrate, *myo*-inositol, methanol, acetone, isopropanol and D-gluconate. Assimilation of nitrogen compounds is positive for lysine and negative for nitrate and nitrite. Growth in amino-acid free medium is positive. Growth at 37 °C is positive, and at 40 °C is negative. No growth is observed on 50% (w/w) glucose, 1% acetic acid or 10% (w/v) sodium chloride/5% (w/v) glucose. Growth on 0.01% cycloheximide is negative. Starch-like compounds are not produced. Acid production is negative.

The differentiation of the novel species from *Sp. brasiliensis* (its near neighbour, Fig. 1) is based on the assimilation of DL-lactate, which is negative for the novel species and positive for *Sp. brasiliensis*, and the assimilation of D-ribose, which is positive for *Sp. piracicabensis* and negative for *Sp. brasiliensis*. *Spathaspora piracicabensis* differs from *C. materiae* based on the assimilation of ethylacetate and ethanol, which are both positive for *Sp. piracicabensis* and negative for *C. materiae*, and the assimilation of glycerol, which is negative for the novel species and positive for *C. materiae*. Lastly, *Sp. piracicabensis*


Candida frijolesensis NRRL Y-48060T / EF120596
Candida labiduridarum NRRL Y-27940T / DQ655687
Candida neerlandica NRRL Y-27057T / AF245404
Candida viswanathii NRRL Y-6660T / U45752
Candida pseudoviswanathii NYNU 14772T / KM586735
Candida tetrigidarum NRRL Y-48142T / EF120599
Candida tropicalis NRRL Y-12968T / U45749
Candida sojae NRRL Y-17909T / U71070
Candida sanyaensis HN-26T / JQ647914
Candida baotianmanensis NYNU 14719T / KM586733
Candida maltosa NRRL Y-17677T / U45745
Candida oxycetoniae CBS 10844T / EU402933
Lodderomyces elongisporus NRRL YB-4239T / JQ689035
Candida hyderabadensis NRRL Y-27953T / AM159100
Candida orthopsilosis CBS 8825T / AJ508575
Candida metapsilosis MCO 448T / AJ508577
Candida theae G-17T / HM461739
Candida parapsilosis NRRL Y-12969T / U45754
Candida pseudojiufengensis CBS 10847T / EU402937
Candida jiufengensis CBS 10846T / EU402935
Candida bohiensis NRRL Y-27737T / AY520317
Candida sakaeoensis LM078T / AB617978
Candida chauliodes NRRL Y-27909T / DQ655678
Candida morakotiae ST-18T / DQ400364
Candida corydali NRRL Y-27910T / DQ655679
Nematodospora anomalae NYNU 14914T / KP054269
Nematodospora valgi D37RT /HM627135
Spathaspora boniae UFMG-CM-Y306T / KT276332
Spathaspora sp. UFMG-CM-Y6059 / KX352821
Candida blackwellae CBS 10843T / KY106321
Candida buenavistaensis NRRL Y-27734T / AY242341
Candida gigantensis NRRL Y-27736T / AY520316
Candida albicans NRRL Y-12983T / U45776
Candida dubliniensis NRRL Y-17841T / U57685
Candida insectamans NRRL Y-7786T / U45753
Spathaspora girioi UFMG-CM-Y302T / KC959937
Candida materiae UFMG 07-C15.1BT / FJ154790
Spathaspora brasiliensis HMD19.3T / JN099271
Spathaspora piracicabensis sp. nov. UFMG-CM-Y6112 / KX528210
Spathaspora piracicabensis sp. nov. UFMG-CM-Y5867T / KR864906
Candida jeffriesii NRRL Y-27738T / AY520415
Spathaspora suhii XMD16.2T / JN099270
Spathaspora arborariae ATCC MYA-4684T / HQ263369
Spathaspora sp. UFMG-CM-Y425 / KR184130
Spathaspora passalidarum NRRL Y-27907T / JQ689036
Spathaspora allomyrinae NYNU 1495T/ KP054267
Candida sp. S8-2007 / AB304269
Candida tenuis VKPM Y-739T / HE612107
Candida subhashii UAMH 10744T / EU836708
Debaryomyces hansenii NRRL Y-7426T / NG 042634
Spathaspora xylofermentans HMD23.3T / JN099268
Spathaspora roraimanensis XMD23.2T / JN099269
Candida sake NRRL Y-1622T / U45728
Candida sp. LCF-112 strain NU7W71 / HM461610
Candida lyxosophila NRRL Y-17539T / U76204
Spathaspora gorwiae UFMG-CM-Y312T / KC959474
Spathaspora sp. UFMG-CM-Y303 / KU556168
Spathaspora hagerdaliae UFMG-CM-Y424T / KR184129
Candida alai NRRL Y-27739T / EF120594
Saccharomyces cerevisiae NRRL Y-12632 / JQ689017
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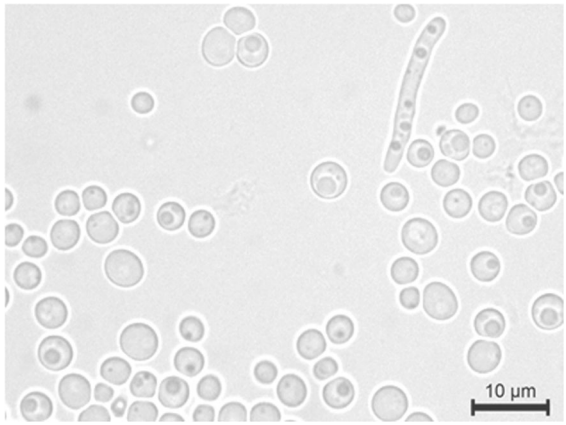

Fig. 2 Budding yeast cells and pseudohyphae of *Spathaspora piracicabensis* sp. nov. UFMG-CM-Y5867^T on YM agar after 7 days at 25 °C

differs from *Sp. girioi* based on the assimilation of DL-lactate and citrate, which are both negative for the novel species and positive for *Sp. girioi*, and the assimilation of D-ribose and ethylacetate, which are both positive for *Sp. piracicabensis* and negative for *Sp. girioi*.

The known habitat of the new species is rotting wood collected in Atlantic Rain forest in Brazil. The type strain UFMG-CM-Y5867^T was isolated from rotting wood of an ornamental tree (purple quaresmeira, *Tibouchina granulosa*) in an area of Atlantic Rainforest biome, in Piracicaba, São Paulo state, Brazil. It has been deposited in the Collection of Microorganisms and Cells of Federal University of Minas Gerais (Coleção de Micro-organismos e Células da Universidade Federal de Minas Gerais, UFMG), Belo Horizonte, Minas Gerais, Brazil, as strain UFMG-CM-Y5867^T, and it is permanently preserved in a metabolically inactive state. An ex-type culture has been deposited in the collection of the Westerdijk Fungal Biodiversity Institute, Utrecht, the Netherlands, as strain CBS 15,054. The MycoBank number is MB 822,320. The Genbank accession numbers for the ITS-5.8S and D1/D2-26S rRNA partial gene sequences of strains UFMG-CM-Y5867^T (ESALQ-I54) and UFMG-CM-Y6112 (ESALQ-I38) are KR864907/KR864906 and KX528211/KX528210, respectively.

Funding This work was funded by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq—Brazil, Grant Numbers 457,499/2014-1 and 160143/2014-4), and Fundação do Amparo a Pesquisa do Estado de Minas Gerais (FAPEMIG, Grant Number APQ-01525-14).

Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest.

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