Kazachstania bromeliacearum sp. nov., a yeast species from water tanks of bromeliads

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Cultures of a novel nutritionally specialized, fermentative yeast species were isolated from 34 water tanks of five bromeliad species, two mangrove sediment samples and one swamp water sample in Rio de Janeiro, Brazil. Sequence analysis of the D1/D2 domains of the large subunit of the rRNA gene showed that the novel species belongs to the genus Kazachstania. The novel species differs from Kazachstania martiniae by 11 substitutions and 2 gaps in the sequence of the domains D1/D2 of the LSU rRNA gene. The name Kazachstania bromeliacearum sp. nov. is proposed for the novel species. The type strain is IMUFRJ 51496T (=CBS 7996T =DBVPG 6864T =UFMG BR-174).

Water tanks of bromeliads are dynamic and complex environments inhabited by communities of different organisms including endemic species (Benzing, 1990; Lopez et al., 2009; Whittman, 2000). The presence of trapped water and organic detritus (phytotelmata) in tanks formed in bromeliad leaf rosettes is a major source of nutrients for these organisms and communities associated with the phytotelmata (Richardson et al., 2000). Hagler et al. (1993) isolated, from phytotelmata of the bromeliad Quesnelia quesneliana in the Coroa Grande mangrove, Rio de Janeiro, two cultures that were identified by physiological and morphological tests as Saccharomyces unisporus-like. Araujo et al. (1998) isolated additional strains of the same yeast from water tanks of five bromeliad species in mangroves, rain forest and coastal sand dune ecosystems of Rio de Janeiro. Sequence analysis of the D1/D2 regions of the large subunit of the rRNA gene showed that these strains belong to the genus Kazachstania. The novel species, Kazachstania bromeliacearum sp. nov., is proposed to accommodate these isolates.

Abbreviation: ITS, internal transcribed spacer.

The GenBank/EMBL/DDBJ accession number for the D1/D2 domain sequence of the large subunit of the rRNA gene of strain IMUFRJ 51496T is HO412595.

Water samples from tanks of five bromeliad species, Q. quesneliana, Nidularium procerum, Neoregelia cruenta, Aechmea nudicaulis and Vriesia proceria, were collected from sites in the mangrove of Coroa Grande, a swamp located in the Atlantic Forest Ecological Reserve of Poça das Antas and in the sand dune area of Maricá. All collection sites were located in the state of Rio de Janeiro, Brazil. Collections were done between January 1990 and September 1997. Water samples were collected aseptically with a sterile pipette and transferred to sterile flasks that were transported to the laboratory on wet ice for processing within 8 h. Aliquots of 0.1 ml of appropriate decimal dilutions were spread on YM agar supplemented with 0.04 % chloramphenicol (Yarrow, 1998). The plates were incubated at 25 °C for 3 to 8 days and selected colonies were purified and maintained on 1 % glucose, 0.5 % yeast extract, 0.5 % malt extract, 0.2 % mono sodium phosphate and 2 % agar (GYMP) agar slants or in liquid nitrogen for later identification. The yeasts were characterized by standard methods (Yarrow, 1998), and identification followed the keys of Kurtzman & Fell (1998). The rDNA internal transcribed spacer (ITS) region was amplified by PCR using ITS1 and ITS4 primers and the number of base pairs was estimated on an agarose gel (Valente et al., 1996). The D1/D2 domains of the large subunit rRNA gene...
were amplified by PCR directly from whole cells as described previously (Lachance et al., 1999). The sequence was edited with the program MEGA5 (Tamura et al., 2007), which was also used to reconstruct a phylogenetic tree by the maximum-parsimony method, based on 522 aligned positions.

High molecular mass nuclear DNA for optical reassociation experiments was obtained from 24–48 h cultures grown in YEPG (1.0 % yeast extract, 1.0 % peptone, 2.0 % glucose) at 25 °C. Cells were suspended in a sucrose buffer (0.02 M Tris, 0.02 M EDTA, 15 % sucrose) together with an equal volume of 0.45–0.50 mm Ø glass beads and were disrupted mechanically using a Bead-Beater Cell Disrupter (Biospec Products). DNA was purified according to Bernardi et al. (1970) with modifications by Price et al. (1978). DNA–DNA reassociation experiments were performed according to the optical method of Kurtzman et al. (1980) using a Gilford 250 spectrophotometer equipped with a model 2527 Gilford thermoprogrammer (Gilford Instruments).

Intact chromosomal DNA for pulsed field gel electrophoresis was prepared as previously reported (Vaughan-Martini et al., 1993). All analyses were performed on a Chef Mapper (Bio-Rad) using gels composed of 2 % agarose (Type II-A, medium EEO; Sigma) in 0.5 × Tris/Borate/EDTA (TBE) buffer. Temperatures of 12–15 °C were maintained throughout the runs. The run time was 68 h with a ramp of 1–5 min at 4.5 V with an angle of 120 degrees.

### Species delineation and ecology

Although it is clear that *K. bromeliacearum* sp. nov. belongs to the genus *Kazachstania* based on the analysis of the D1/D2 domains of the large subunit rRNA gene, a reliable placement within the genus was not achieved (Fig. 1). Sisterhood with *Kazachstania viticola* was suggested from

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**Fig. 1.** Phylogenetic placement of *K. bromeliacearum* sp. nov. by maximum-parsimony analysis of the D1/D2 regions of the large subunit rRNA gene. A bootstrap consensus tree for 100 replicates is shown with bootstrap values greater than 50 % only shown. A total of 522 aligned positions were analysed. Bar, 5 changes.
analyses using other methods such as maximum-likelihood or neighbour-joining, but always with low bootstrap values and a very short internode subtending the pair of species. Distance-based searches also suggested an affinity of the species with K. martiniae. The most closely related sequence in GenBank was that of an unassigned isolate labelled BG02-7-14-003-2-4, which is closely related to K. martiniae. The novel species differs from K. martiniae by 11 substitutions and 2 gaps and from strain BG02-7-14-003-2-4 by 10 substitutions and 2 gaps in the sequence of the domains D1/D2 of the LSU rRNA gene. CBS culture 7997, which was isolated from the same site but five months later than the type culture, is identical for this sequence (http://www.cbs.knaw.nl/collections/BioloMICS.aspx consulted on 28 October 2002). The current definition of the genus Kazachstania (Kurtzman & Robnett, 2003) is based on a phylogeny reconstructed from a concatenation of the small subunit rRNA gene, the ITS rDNA region, parts of the large subunit rRNA gene, the elongation factor 1-alpha, the mitochondrial small subunit rRNA gene, and cytochrome oxidase II. Such an analysis isolates all species often known as Saccharomyces sensu stricto as distinct from species assigned to the genus Kazachstania, but such a distinction is not possible based on D1/D2 sequences only. Our intent here is purely to demonstrate that strain IMUFRJ 51496T and similar strains are truly representative of a novel species, and not to provide a definitive phylogeny.

Divergence at the level of the ITS-5.8S rDNA region is considerable (in excess of 140 nt differences, depending on the alignment), which attests to the distinct nature of the novel species. However, a credible alignment of the spacers with most other species was not possible as the extent of divergence and the number of indels among congeners is considerable. Some segments found to be unique to Kazachstania bromeliacearum had no detectable similarity to any sequences in GenBank, whereas other segments had clear similarities with those of Candida humilis (AY493349) and Kazachstania piceae (FR716598). Any attempt to infer a phylogeny from other regions than the 5.8S rRNA gene itself would be futile at best because of the poor quality of the spacer alignments.

DNA reassociation values between the type strain of the novel species and other yeasts of the same clade were as follows: Kazachstania martiniae DBVPG 6752T, 63%; Kazachstania transvaalensis DBVPG 6757T, 55%; Naumovozyma dairienensis DBVPG 6366T, 44%; Kazachstania kunashirensis DBVPG 6756T, 39%; Kazachstania spencerorum DBVPG 6746T, 37%; Kazachstania unispora DBVPG 6368T, 35%; Kazachstania barnetti DBVPG 6365T, 30%; Kazachstania exigua DBVPG 6252T, 23%; Kazachstania servazzii DBVPG 6355T, 18%; N. dairienensis DBVPG 6357, 31%; Naumovozyma castellii DBVPG 6298T, 26%; Lachancea cidri DBVPG 6385T, 28%; and Lachancea fermentati DBVPG 6297T, 18%. These results support the view that the novel species is closely related to K. martiniae although distinct from that species, as seen by an intermediate (63%) DNA–DNA reassociation value. The name Kazachstania bromeliacearum sp. nov. is proposed for the novel species.

The 48 cultures of K. bromeliacearum were isolated from 34 separate samples of phytotelmata collected from 158 bromeliad tanks, 2 of mangrove sediments and 1 of swamp water from the same sites where the bromeliads were sampled. The size of the ITS region of the rRNA gene, estimated on agarose gels, was the same (approximately 850 base pairs including the primers) in all 22 samples analysed. ITS rDNA sequences were determined for four available strains in different laboratories, resulting in different degrees of completion, although the sequence for the type covered the entire segment from the end of the small subunit rRNA gene to the end of the D2 domain of the large subunit rRNA gene. The length of the ITS regions including the 5.8S rRNA gene was 786 nt measured from the 3’ end of the binding site for primer IT1 (TAGG-TGAACCTGGCGGAAAGGTACAT) and the 5’ end of the binding site for primer NL1, a result that is compatible with those obtained electrophoretically. The four ITS sequences were identical except for a four-nucleotide deletion starting at position 115 for strain DBVPG 7105. In addition, two of the more recent isolates, including one from a different location and bromeliad host species (DBVPG 7104) from the type culture, had electrophoretic karyotypes, determined by pulsed field gel electrophoresis, that were similar enough to those of the type culture to be considered the same species (Fig. 2). These cultures were mostly from bromeliad tanks that received little sun exposure. The phytotelmata of bromeliads receiving more sunlight are dominated by algal growth and contain few ascomycetous yeasts. Because most isolates of K. bromeliacearum sp. nov. were obtained from phytotelmata of different bromeliad species, it can be assumed that the species is associated with this microhabitat. Phytotelmata have a dynamic influx of diverse arthropod and amphibian visitors, and these organisms could act as vectors of K. bromeliacearum sp. nov. metapopulations among different plants and for other habitats where the yeast was isolated. In addition to the unique habitat, K. bromeliacearum sp.
nov. differs from *K. unispora*, which has the mostly single-spored asc and assimilates ethanol, and from *K. martiniae*, *K. transvaalensis*, *K. kunashirensis* and *K. spencerorum* that are all positive for assimilation of trehalose.

**Latin diagnosis of Kazachstania bromeliacearum**

*Araujo, Rosa, Freitas, Lachance, Vaughan-Martini, Mendonça-Hagler et Hagler* sp. nov.


**Description of Kazachstania bromeliacearum**

*Araujo, Rosa, Freitas, Lachance, Vaughan-Martini, Mendonça-Hagler & Hagler* sp. nov.

*Kazachstania bromeliacearum* (bro.me.lia.ce’a.rum. L. gen. plur. f. n. bromeliacearum of Bromeliaceae, referring to the bromeliads from which most strains of the species were isolated).

In yeast extract (0.5 %), glucose (2 %) broth after 3 days at 25 °C, cells are ovoid to ellipsoid (2–3 × 2–4 μm) and occur singly or in pairs (Fig. 3). Budding is unilateral. A 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. In Dalmau plates after 2 weeks on cornmeal agar, pseudohyphae or true hyphae are not formed. Unconjugated asc containing one to four globose ascospores are formed after 7 days on Fowell’s acetate agar at 20 °C in freshly isolated cultures. After prolonged storage, ascospores are formed after exposure to 55 °C for 4 min followed by incubation for 2 days at 25 °C in GYMP broth prior to inoculation on acetate agar (Fig. 4). Ascospores are not liberated. Glucose, galactose and occasionally sucrose (type culture is negative) are fermented. Glucose, galactose (variable, type culture is positive), sucrose (rare, type culture is negative), glycerol (rare, type culture is negative), glucono-1,5-δ lactone (rare, type culture is delayed weak), lactate (rare, type culture is delayed), succinate (variable, type culture is positive) and ethanol (rare, type culture is negative) are assimilated. Sorbose, glucosamine, ribose, xylose, L-arabinose, D-arabinose, rhamnose, maltose, trehalose, methyl α-D-glucoside, cellobirose, salicin, melibose, lactose, raffinose, melezitose, inulin, soluble starch, erythritol, ribitol, D-glucitol, D-mannitol, galactitol, myo-inositol, 2-ketogluconate, 5-ketogluconate, citrate, methanol and hexadecane are not assimilated. Ethylamine and cadaverine are assimilated, but L-lysine, sodium nitrate and sodium nitrite are not assimilated. Growth at 37 °C is variable. Growth on YM agar
with 10% sodium chloride is negative. Growth in 50% glucose/yeast extract (0.5%) is negative. Starch-like compounds are not produced. With 100 μg cycloheximide ml⁻¹, growth is negative. Urease activity is negative and the Diazonium Blue B reaction is negative. Inhabits phytotelmata in water tanks of shaded bromeliad plants on the south-east coast of Rio de Janeiro, Brazil.

The type strain is CBS 7996T (=IMUFRJ 51496T =UMFG BR-174T =DBVPG 6864T =IFO 10916T), isolated from a water tank of the bromeliad *Quesnelia quesneliana* in Rio de Janeiro, Brazil.

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