Candida aechmeae sp. nov. and *Candida vrieseae* sp. nov., novel yeast species isolated from the phylloplane of bromeliads in Southern Brazil

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Two novel yeast species, *Candida aechmeae* sp. nov. and *Candida vrieseae* sp. nov., were isolated from bromeliads in Itapuã Park, Rio Grande do Sul, Brazil. These species are genetically isolated from all other currently recognized ascomycetous yeasts based on their sequence divergence in the D1/D2 domain of the LSU rRNA gene. *C. aechmeae* sp. nov. is phylogenetically close to *Candida ubatubensis*, a species also isolated from bromeliads in Brazil, but the novel species can be differentiated on the basis of differences in the D1/D2 domain and positive results for the assimilation of L-arabinose, raffinose, inulin and citrate. *Candida vrieseae* sp. nov. is phylogenetically placed in a clade near *Candida membranifaciens* that is composed of several species associated with insects, but the novel species can be differentiated from them by the D1/D2 and ITS gene sequences, positive results for the assimilation of nitrite and a negative result for the assimilation of ethylamine. The type strain for *Candida aechmeae* sp. nov. is BI153^T (=CBS 10831^T=NRRL Y-48456^T) and the type strain for *C. vrieseae* sp. nov. is BI146^T (=CBS 10829^T=NRRL Y-48461^T).

The Atlantic forest is one of the richest in terms of biodiversity and is also one of the most threatened. Bromeliads are typically abundant plants in the Atlantic Forest and sustain a great diversity of organisms, including yeasts and animals that can act as yeast vectors (Hagler *et al.*, 1993; Araújo *et al.*, 1998; Landell *et al.*, 2006). Novel yeast species have been isolated from the water tanks and leaves of bromeliads in Brazil, showing the high microbial diversity associated with this substrate (Ruivo *et al.*, 2005; Inácio *et al.*, 2008; Landell *et al.*, 2009).

During a survey of yeasts associated with bromeliads in South Brazil, two novel species of ascomycetous yeasts were isolated from the phylloplane and tank water of different bromeliads. The sequences of the D1/D2 domain of the large subunit (LSU) rRNA gene showed that these species are genetically distinct from all currently accepted ascomycetous yeasts.

Abbreviation: LSU, large subunit.

The GenBank/EMBL/DDBJ accession numbers for the sequences of the D1/D2 domain of the LSU rRNA gene of strains BI153^T (=CBS 10831^T=NRRL Y-48456^T) and BI146^T (=CBS 10829^T=NRRL Y-48461^T) are EU678950 and EU200785, respectively.

Methods

Leaves of the bromeliads *Aechmea recurvata* and *Billbergia nutans* and samples of the tank water of the bromeliad *Vriesea gigantea* were collected aseptically in April and May 2004 in Itapuã Park, South of Brazil (approx. coordinates: 30° 22′ S 51° 04′ W). Leaves were collected in polyethylene plastic bags, transported to the laboratory and washed with sterile distilled water. Bromeliad fragments totalling 10 cm^2 were placed in Erlenmeyer flasks with 50 ml sterile distilled water and maintained on a shaker for 10 min. The water was discarded and replaced by 30 ml 0.5 % Tween 20, followed by vigorous shaking for 30 min. This last step was repeated and decimal dilutions of the last washing were spread on acidified YM agar (1 % glucose, 0.3 % malt extract, 0.3 % yeast extract, 0.5 % peptone, 2 % agar, acidified to pH 4.0 and supplemented with 0.04 % chloramphenicol) plates. After incubation at 20–25 °C for up to 7 days, representative colonies of the different morphological types were purified and maintained on agar slants at 4 °C, covered with sterile mineral oil. Phenotypic characterization of the isolates was performed according to Yarrow (1998) and Barnett *et al.* (2000). Ascospore formation was determined for all isolates with similar or identical D1/D2 sequences in all combina-

tions on acetate agar and diluted (1 : 19) V8 agar at 25 °C (Barnett *et al.*, 2000) and cultures were observed periodically for up to one month.

Yeast DNA was extracted and purified according to Ramos *et al.* (2001). The divergent D1/D2 domain of the LSU rRNA gene was amplified with NL1 and NL4 primers (O'Donnell, 1993). Amplification conditions were as follows: one initial cycle at 94 °C for 3 min; 33 cycles at 95 °C for 1 min, 56 °C for 30 s, 72 °C for 1 min and a final extension cycle at 72 °C for 6 min. The PCR products were examined by electrophoresis on a 1 % agarose gel at 100 V for 45 min and stained with ethidium bromide for visualization under UV light.

The sequences of two strains, BI146^T and BI153^T, were obtained with Amersham MegaBACE 1000 automated sequencers using standard protocols at the facilities of the Brazilian Genome Network at the Center of Biotechnology, Cbiot-UFRGS-RS, Brazil, and Instituto do Cancer, RJ, Brazil, respectively. Alignments and phylogenetic trees were constructed with MEGA4 (Tamura *et al.*, 2007) using the neighbour-joining and maximum-parsimony methods, with bootstrap values based on 1000 random samplings.

PCR-fingerprinting followed the protocols described by Sampaio *et al.* (2001) using the primer $(GTG)_5$. Gel electrophoresis images were acquired with GelDoc XR System Software (Bio-Rad).

Results and discussion

Three anamorphic ascomycetous yeast strains isolated from bromeliads, strains BI153^T, BI259 and BI146^T, were characterized by PCR fingerprinting with primer $(GTG)_5$. The profiles of strains BI153^T and BI259, isolated from the phylloplane of *A. recurvata* and *B. nutans*, respectively, were identical, suggesting conspecificity. The profile of strain BI146^T, isolated from the water tank of *V. gigantea* differed from those of strains BI153^T and BI259 (data not shown). D1/D2 sequencing of isolate BI153^T confirmed that it belonged to a novel yeast species. When compared with the closest match for the D1/D2 domain of the LSU rRNA gene sequence, *Candida ubatubensis*, 18 base substitutions were found, indicating that these species are phylogenetically distinct. Phylogenetic placement of strain BI153^T differed in neighbour-joining (Fig. 1) and maximum-parsimony (data not shown) trees, but it always clustered with *C. ubatubensis*. Strain BI259 was considered to belong to the same taxon as strain BI153^T based on their GTG_5 fingerprinting patterns and their identical phenotypic characteristics. This novel species was named *Candida aechmeae* sp. nov. and it could be differentiated from *C. ubatubensis* by differing results in tests for the assimilation of L-arabinose, raffinose, inulin and citrate. *C. ubatubensis* was isolated from the water tank of the bromeliad *Canistropsis sidelii* in an Atlantic rainforest in South-east Brazil (Ruivo *et al.*, 2005), suggesting that *C. ubatubensis* and *Candida aechmeae* sp. nov. may represent a cluster of novel ascomycetic yeast species associated with bromeliads.

D1/D2 sequencing of isolate BI146^T confirmed that it also belonged to a novel yeast species, named *Candida vrieseae* sp. nov. Phylogenetic trees constructed using neighbour-joining (Fig. 2) and maximum-parsimony (data not shown) methods placed the strain within a group of species isolated from the guts of a variety of insects and

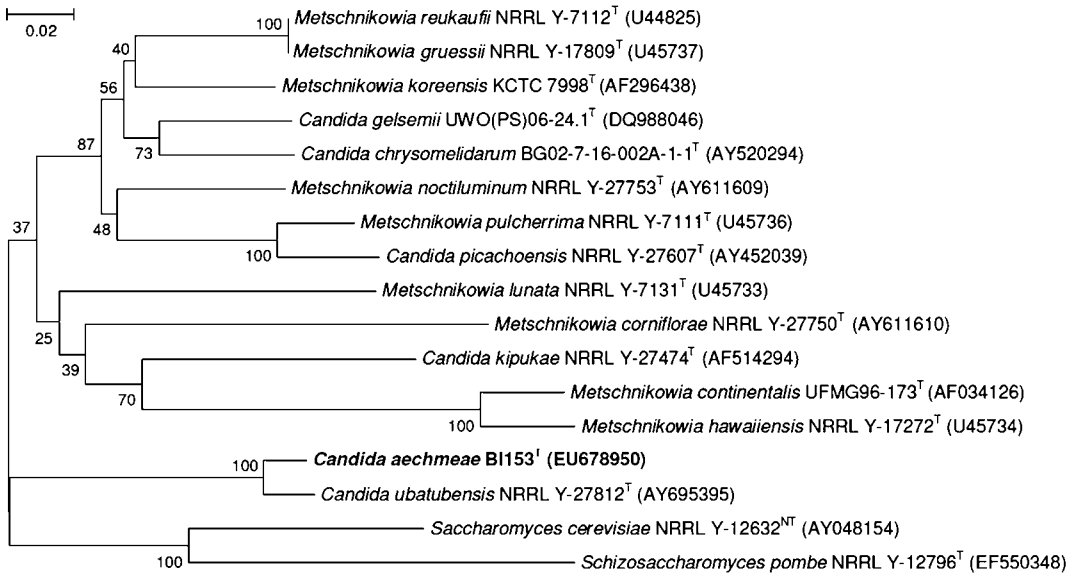

Fig. 1. D1/D2 tree showing the phylogenetic placement of *Candida aechmeae* sp. nov., obtained by neighbour-joining analysis using MEGA 4.0. The numbers given on the branches are the frequencies with which a given branch appeared in 1000 bootstrap replications. Bar, 0.02 substitutions per nucleotide position.

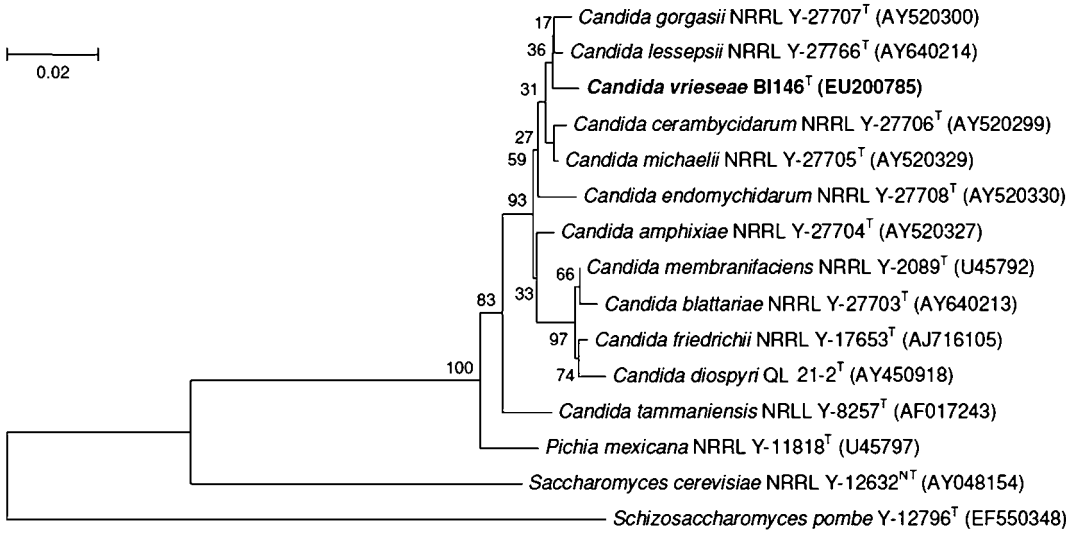

Fig. 2. D1/D2 tree showing the phylogenetic placement of *Candida vrieseae* sp. nov. obtained by neighbour-joining analysis using MEGA 4.0. The numbers given on the branches are the frequencies with which a given branch appeared in 1000 bootstrap replications. Bar, 0.02 substitutions per nucleotide position.

closely related to *Candida membranifaciens* (Suh *et al.*, 2005), although the bootstrap values were low. This phylogenetic placement was confirmed by the ITS tree (data not shown). Strain BI146^T had 5 base substitutions in the D1/D2 region and 11 and 13 substitutions in the ITS region compared with the closest sequence matches, *Candida gorgasii* and *Candida lessepsii*, respectively. The physiological characteristics that differentiate the novel isolate from the recognized species can be seen in Table 1. It has been suggested by Suh *et al.* (2005) that this group of yeasts diversified in association with insects. Therefore it cannot be ruled out that strain BI146^T may have reached the water tank of *V. giganteae* by means of an insect vector.

Although it is desirable that the description of novel species is based on a number of isolates that could represent the variability within the species, rare species are not uncommon in nature. *C. aechmeae* sp. nov. and *C. vrieseae* sp. nov. are two novel rare ascomycetous yeast species isolated from bromeliads during a three-year survey in Itapuã Park, Brazil. Therefore, their descriptions are based on only a few isolates as a direct consequence of the rarity of both novel species.

Latin diagnosis of *Candida aechmeae* Landell *et* Valente sp. nov.

In medio liquido dextroso, peptono et extracto fermenti continente post 7 *dies ad* 25 °C *cellulae vegetativae ellipsoideae et ovoideae,* 3–6.5 × 2–4.5 μm, *singulae vel binae. Cultura in GYP agaro post dies* 7 *ad* 25 °C *albida et butyrosa. In agaro farinae Zea mays post dies* 21 *mycelium et pseudomycelium formantur. Asci non formantur. Glucosum et galactosum fermentantur. Glucosum, galactosum,* D-*ribosum,* D-*xylosum,* L-*arabinosum,* L-*rhamnosum, sucrosum, maltosum, cellobiosum, salicinum, raffinosum, inulinum, glycerolum, erythritolum, ribitolum, glucitolum, mannitolum, acidum succinicum, citratum, N-acetylglucosaminum assimilantur.* D-*Arabinosum, trehalosum, melibiosum, lactosum, amylum solubile, meso-inositolum, acidum gluconicum, acidum lacticum non assimilantur. Natrii nitritum et lysinum assimilantur, sed non*

Table 1. Differential physiological characteristics of *Candida vrieseae* sp. nov. and related species

Taxa; 1, *Candida vrieseae* sp. nov.; 2, *Candida cerambycidarum*; 3, *Candida gorgasii*; 4, *Candida michaelii*; 5, *Candida endomychidarum*; 6, *Candida lessepsii*. Data for taxa 2–5 are taken from Suh *et al.* (2005). +, Positive; −, negative; D, delayed reaction; W, weak reaction.

Characteristic	1	2	3	4	5	6
Assimilation of carbon compounds						
D-Ribose	−	+	D	−	D	+
L-Arabinose	+	+	+	+	+	D
D-Arabinose	−	W	D	−	+	+
L-Rhamnose	+	+	+	+	+	−
Lactose	−	−	+	−	−	−
DL-Lactate	−	D	W	+	D	−
Assimilation of nitrogen compounds						
Nitrite	+	−	−	−	−	−
Ethylamine	−	+	+	+	+	+
Growth with						
50 % D-Glucose	−	+	−	+	+	+
10 % NaCl	+	+	+	+	+	+
16 % NaCl	−	W	−	−	+	W

natrii nitratum, ethylaminum, creatinum et creatininum. Non crescit in 37 °C. *In medio cum* 50 % *glucosum non crescit. Materia amyloidea iodophila non formatur. Ureum non hydrolysatur. Reactio Diazonii coerulei B non respondens.*

Typus: BI153^T (=CBS 10831^T=NRRL Y-48456^T) *designat stirpem typicam. Isolata ex folio bromeliaceae*, Itapuã Park, Brazil. *Preservatus in collectione* Centraalbureau voor Schimmelcultures, *Trajectum ad Rhenum.*

Description of *Candida aechmeae* Landell & Valente sp. nov.

Candida aechmeae (aech.me′ae. N.L. n. *Aechmea* a botanical genus name; N.L. gen. n. *aechmeae* of *Aechmea*, isolated from the bromeliad *Aechmea recurvata*).

In GYP broth after 7 days at 25 °C, the asexual cells are ellipsoidal and ovoid, 3–6.5 × 2–4.5 μm and occur singly, in parent-bud pairs (Fig. 3a). On GYP agar, after 1 week at 25 °C, the streak culture is white, smooth and butyrous. After 3 weeks in Dalmau plate culture on cornmeal agar, pseudomycelium and true mycelium are formed (Fig. 3b). Asci are not formed on common sporulation media. Glucose and galactose fermentation are positive. Assimilation of carbon compounds: D-glucose, galactose, ribose, xylose, L-arabinose, L-rhamnose, sucrose, maltose, cellobiose, salicin, raffinose, inulin, glycerol, *meso*-erythritol, ribitol, D-glucitol, mannitol, succinic acid, citrate and *N*-acetylglucosamine are assimilated; no growth occurs on D-arabinose, trehalose, melibiose, lactose, soluble starch, inositol, D-gluconic acid or lactate. Assimilation of nitrogen compounds: sodium nitrite (variable) and lysine are assimilated; no growth on sodium nitrate, ethylamine, creatine or creatinine. No growth at 37 °C. Gelatin liquefaction and casein hydrolysis are negative. Growth on GYP broth with 10 % (w/v) NaCl is positive, but no growth with 16 % NaCl. Growth on 50 % glucose/yeast extract/peptone agar is negative. Production of starch-like compounds is negative. Urease activity and Diazonium Blue B reaction are negative.

The type strain was isolated from leaves of bromeliads in Itapuã Park, Rio Grande do Sul, Brazil. The type strain, BI153^T (=CBS 10831^T=NRRL Y-48456^T), has been deposited in the Yeast Collection of the Centraalbureau voor Schimmecultures, Utrecht, The Netherlands, and the ARS Culture Collection, USA.

Latin diagnosis of *Candida vrieseae* Landell *et* Valente sp. nov.

In medio liquido dextroso, peptono et extracto fermenti continente post 7 *dies ad* 25 °C *cellulae vegetativae ellipsoideae et ovoideae*, 3–4.5 × 2–3.5 μm, *singulae vel binae. Cultura in GYP agaro post dies* 7 *ad* 25 °C *albida et butyrosa. In agaro farinae Zea mays post dies* 21 *mycelium nec pseudomycelium non formantur. Asci non formantur. Glucosum et galactosum fermentantur. Glucosum, galactosum,* D-*xylosum,* L-*arabinosum,* L-*rhamnosum, sucrosum, maltosum, trehalosum, cellobiosum, salicinum, raffinosum, inulinum, glycerolum, erythritolum, ribitolum, glucitolum, mannitolum, acidum gluconicum, acidum succinicum, citratum, N-acetylglucosaminum assimilantur.* D-*Ribosum,* D-*arabinosum, melibiosum, lactosum, amylum solubile, meso-inositolum, acidum lacticum non assimilantur. Natrii nitritum et lysinum assimilantur, sed non natrii nitratum, ethylaminum, creatinum et creatininum. Augmentum in* 37 °C, *non crescit in* 40 °C. *In medio cum* 50 % *glucosum non crescit. Materia amyloidea iodophila non formatur. Ureum non hydrolysatur. Reactio Diazonii coerulei B non respondens.*

Typus: BI146^T (=CBS 10829^T=NRRL Y-48461^T), *designat stirpem typicam. Isolata ex aquam in bromeliaceae*, Itapuã Park, Brazil. *Preservatus in collectione* Centraalbureau voor Schimmelcultures, *Trajectum ad Rhenum.*

Description of *Candida vrieseae* Landell & Valente sp. nov.

Candida vrieseae (vrie.se′ae. N.L. n. *Vriesea* a botanical genus name; N.L. gen. n. *vrieseae* of *Vriesea*, isolated from the tank water of the bromeliad *Vriesea gigantea*).

In GYP broth after 7 days at 25 °C, the vegetative cells are ellipsoidal and ovoid, 3–4.5 × 2–3.5 μm and occur singly, in parent-bud pairs (Fig. 3c). On GYP agar, after 1 week at 25 °C, the streak culture is white, smooth and butyrous. After 3 weeks in Dalmau plate culture on cornmeal agar, pseudomycelium or true mycelium are not formed. Asci are not formed on common sporulation media. Fermentation of glucose and galactose is positive. Assimilation of carbon compounds: D-glucose, galactose, xylose, L-arabinose, L-rhamnose, sucrose, maltose, trehalose, cellobiose, salicin, raffinose, inulin, glycerol, *meso*-erythritol, ribitol, D-glucitol, mannitol, D-gluconic acid, succinic acid, citrate and *N*-acetylglucosamine are assimilated; no growth occurs on ribose, D-arabinose, melibiose,

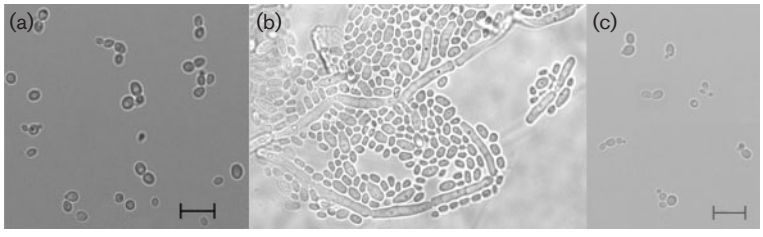

Fig. 3. (a) Budding yeast cells of strain BI153^T on GYP agar at 25 °C after 7 days; (b) pseudomycelium of strain BI153^T on cornmeal agar at 25 °C after three weeks; (c) budding yeast cells of strain BI146^T on GYP agar at 25 °C after 7 days. Bars, 10 μm.

lactose, soluble starch, inositol or lactate. Assimilation of nitrogen compounds: lysine and sodium nitrite are assimilated; no growth on sodium nitrate, ethylamine, creatinine or creatine. Grows at 37 °C. Gelatin liquefaction is negative and casein hydrolysis is positive. Growth on GYP broth with 10 % (w/v) NaCl is positive; no growth with 16 % NaCl. Growth on 50 % glucose/yeast extract/peptone agar is negative. Production of starch-like compounds is negative. Urease activity and Diazonium Blue B reaction are negative.

The type strain, BI146^T (=CBS 10829^T=NRRL Y-48461^T), was isolated from tank water of bromeliads in Itapuã Park, Rio Grande do Sul, Brazil. The type strain has been deposited in the Yeast Collection of the Centraalbureau voor Schimmecultures, Utrecht, The Netherlands, and the ARS Culture Collection, USA.

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