Cryptococcus bromeliarum sp. nov., an orange-coloured basidiomycetous yeast isolated from bromeliads in Brazil

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During a survey of yeasts associated with the phylloplane of several bromeliad species in Itapuã Park in southern Brazil, we isolated four orange-coloured strains which were found to represent a novel anamorphic tremellaceous (Tremellales, Agaricomycotina, Basidiomycota) yeast species, *Cryptococcus bromeliarum* sp. nov. (type strain BI20^T = CBS 10424^T = NRRL Y-48112^T). PCR-fingerprinting profiles of the four strains with primers M13 and (GTG)₅ were almost identical, which suggested conspecificity among the isolates. On the basis of D1/D2 26S rDNA sequence analysis, *C. bromeliarum* is phylogenetically closely related to other orange-coloured *Cryptococcus* species, namely *Cryptococcus armeniacus*, *C. amylolyticus*, *C. tibetensis* and *C. cistialbidi*, but differed from these species by at least six nucleotide substitutions and was thus considered a separate species. Physiological differences from *C. armeniacus*, *C. amylolyticus* and *C. cistialbidi* included the inability of *C. bromeliarum* to assimilate citrate and to form starch-like compounds. Differentiation from *C. tibetensis* can be achieved by the ability of the latter to assimilate ethylamine.

In recent years, Inácio *et al.* (2005) and Wang *et al.* (2007) have described novel tremellaceous yeast species with orange-coloured colonies, a characteristic that was previously considered rare among these yeasts. Four of these species were classified in the genus *Cryptococcus*, mainly due to their phenotypic characteristics and their placement on phylogenetic trees constructed on the basis of D1/D2 26S rDNA sequence analysis. *Cryptococcus cistialbidi* and *Cryptococcus amylolyticus* were isolated from leaves of *Cistus albidus*, while *Cryptococcus armeniacus* was a culture contaminant from air, all of them isolated in Portugal (Inácio *et al.*, 2005). Phylogenetic analysis of the D1/D2 sequences of these species demonstrated that *C. cistialbidi* appears to be loosely connected to the Aurantia clade of *Tremella*, whereas the other two species seem to be sister species that are not linked to any particular clade of the Tremellales. *Cryptococcus tibetensis*, isolated from leaves of different plants in Tibet, formed a well-supported clade with *C. armeniacus* and *C. amylolyticus* (Wang *et al.*, 2007).

Abbreviation: ITS, internal transcribed spacer.

The GenBank/EMBL/DDBJ accession numbers for the sequences of the D1/D2 domain of the 26S rDNA and the ITS region of strain BI20^T are DQ784566 and EU386359, respectively.

During a survey of yeasts associated with the phylloplane of bromeliads in the south of Brazil, four orange-coloured isolates were obtained that belong to this clade (Landell *et al.*, 2006). As they could not be assigned to any known species, we propose to accommodate these strains in a novel species, *Cryptococcus bromeliarum* sp. nov.

Samples of the bromeliads *Vriesea procera*, *Vriesea friburgensis* and *Tillandsia gardneri* were collected in April and May 2004 in Pedreira Beach in Itapuã Park in southern Brazil (approx. coordinates 30° 22' S 51° 04' W). The leaves were processed as described previously (Landell *et al.*, 2006). Representative colonies of the different morphological types were purified and maintained on agar slants at 4 °C, covered with sterile mineral oil. Strain BI20^T was obtained from leaves of *V. procera*, strain BI68 from *V. friburgensis* and strains BI79 and BI133 from *T. gardneri*. Phenotypic characterization of the isolates was performed according to Yarrow (1998) and Barnett *et al.* (2000).

The D1/D2 domains of the 26S rDNA and the internal transcribed spacer (ITS) region of strain BI20^T were amplified by PCR as described previously (Lachance *et al.*, 1999). The resulting amplicons were concentrated and purified with either Qiaquick PCR columns (Qiagen) or

the GFX Band Purification kit (Amersham Biosciences). Cycle sequencing employed standard protocols with primers NL1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL4 (5'-GGTCCGTGTTTCAAGACGG-3') for the D1/D2 domains and ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') for the ITS region. Sequences were obtained with either an ALFexpress II DNA Analyser (Amersham Pharmacia Biotech) or an ABI DNA Sequencer (Applied Biosystems), according to the manufacturers' instructions. Sequences were aligned with the CLUSTAL algorithm of MEGALIGN (DNASTAR). Phylogenetic trees were computed with MEGA 4.0 using the neighbour-joining method (Tamura *et al.*, 2007). Gaps were treated as missing data.

PCR fingerprinting followed the protocols described in Sampaio *et al.* (2001), using the primers M13 (5'-GAGGGTGCGGTTCT) and (GTG)₅. Gel electrophoresis images were acquired with the GelDoc XR System software (Bio-Rad).

The four strains isolated from bromeliad species presented a unique combination of characteristics: production of orange-coloured colonies, assimilation of inositol and inability to produce ballistoconidia. PCR-fingerprinting profiles with primers M13 and (GTG)₅ were almost identical, suggesting conspecificity (Fig. 1). Sequencing of isolate BI20^T confirmed that they belong to a previously undescribed species, with 6 substitutions in the D1/D2 26S rDNA and 8 substitutions in the ITS region compared with the closest sequence match, *C. armeniacus* PYCC 5855^T (Fig. 2). The novel species *C. bromeliarum* is phylogeneti-

cally close to *C. armeniacus*, *C. amylolyticus* and *C. tibetensis*, three orange-coloured *Cryptococcus* species that form an isolated cluster in the Tremellales (Fig. 2). Trees constructed using maximum-parsimony showed a similar topology to those constructed using neighbour-joining (not shown). The relatedness to the Aurantia/*C. cistialbidi* clade has low bootstrap support. *C. bromeliarum* differs from *C. amylolyticus*, *C. cistialbidi* and *C. tibetensis* by 22, 42 and 16 nucleotide substitutions in the D1/D2 region, respectively, and also differs from *C. tibetensis* by 46 substitutions plus several deletions in the ITS region. ITS sequences from the other species are not available in GenBank. Physiological differentiation from *C. armeniacus*, *C. amylolyticus* and *C. cistialbidi* is possible by means of negative citrate assimilation and no formation of starch-like compounds, while differentiation from *C. tibetensis* can be achieved by negative ethylamine assimilation (Table 1).

Inácio *et al.* (2005) chose to include *C. armeniacus* and *C. amylolyticus*, as well as *C. cistialbidi*, in the genus *Cryptococcus* because the species fit its current delineation closely, in spite of the loose definition of the genus. Wang *et al.* (2007) included *C. tibetensis* in the same genus because it clustered with *C. armeniacus* and *C. amylolyticus* in the D1/D2 phylogenetic tree, but both groups of authors suggest that this cluster could represent a new yeast genus. *C. bromeliarum* is the fourth described species in the *C. armeniacus* clade. A better circumscription of this clade would clearly benefit from the description of additional species in this group of orange-coloured tremellaceous yeasts, as well as the determination of sequences from additional genes that could provide the necessary phylogenetic support for the creation of a new genus.

C. bromeliarum was isolated from different bromeliads (*V. procera*, *V. friburgensis* and *T. gardneri*) on a trail located at Pedreira Beach in Itapuã Park. Although sampling was performed from the same bromeliads at another site in the park (Fora Beach), we were unable to find any representatives of *C. bromeliarum*. More sampling should be performed in order to confirm whether *C. bromeliarum* really has a limited distribution in the park, information that is crucial for the study of its ecology.

Description of *Cryptococcus bromeliarum* M. F. Landell & P. Valente sp. nov.

Cryptococcus bromeliarum (bro.me'li.ar.um. N.L. masc. adj. *bromeliarum* associated with bromelias, the substrate from which the first strains were isolated).

In GYP broth after 7 days at 25 °C, vegetative cells are ellipsoidal and ovoid, 3 × 6 µm, and occur singly, in parent-bud pairs. Sexual reproduction has not been observed. Sediment is formed. On GYP agar, after 1 week at 25 °C, the streak culture is light orange in colour, the surface is glossy and smooth, the texture is mucous and margins are entire. In Dalmau plate culture, true hyphae are not formed. Ballistoconidia are not produced. Fermentation ability is negative. Assimilation of carbon

Fig. 1. PCR fingerprinting of strains of *Cryptococcus bromeliarum* sp. nov. (a) DNA banding patterns obtained with primer (GTG)₅. Lanes: M, markers (1 kb Plus DNA ladder); 1, strain BI20^T; 2, strain BI68; 3, strain BI79; 4, strain BI133. (b) DNA banding patterns obtained with primer M13. Lanes: 1, strain BI20^T; 2, strain BI68; 3, strain BI79; 4, strain BI133.

Fig. 2. Phylogenetic tree based on sequences of the D1/D2 region of the 26S rDNA showing the phylogenetic placement of *C. bromeliarum* sp. nov. BI20^T. The tree was obtained by neighbour-joining analysis using MEGA 4.0. Bar, 0.01 substitutions per nucleotide position.

compounds: *N*-acetylglucosamine, D-arabinose, L-arabinose, cellobiose (variable), galactose, D-glucitol, D-gluconic acid, D-glucosamine (variable), D-glucose, glycerol (variable), inositol, lactose, maltose, mannitol, melezitose, melibiose, raffinose, L-rhamnose, ribitol (variable), ribose,

Table 1. Differential physiological characteristics of *C. bromeliarum* sp. nov. compared with related orange-coloured species

Species: 1, *C. bromeliarum* sp. nov. (data for strains BI20^T, BI68, BI79 and BI133); 2, *C. armeniacus*; 3, *C. amylolyticus*; 4, *C. tibetensis*; 5, *C. cistialbidi*. Data for reference species were taken from Wang *et al.* (2007) (*C. tibetensis*) and Inácio *et al.* (2005) (remaining species). +, Positive; D, delayed; w, weak; –, negative; v, variable; ND, no data available.

Characteristic	1	2	3	4	5
Assimilation of:					
Cellobiose	v	+	+	+	+
Citrate	–	D	+	–	+
Galactitol	– (w, D)	w	D	v	+
D-Gluconate	+	D	D	ND	– (D)
D-Glucosamine	v	+	+	+	+
Glycerol	v	w	D	–	D
Ribitol	+	– (D)	D	+	+
Soluble starch	v	w	+	+	–
Ethylamine	–	–	+	+	–
Lysine	v	D	+	– (w)	+
Nitrite	v	–	+	–	–
Production of starch-like compounds	–	+	+	–	+

salicin (variable), soluble starch (variable), succinic acid, sucrose, trehalose, xylitol and xylose are assimilated; no growth occurs on acetone, citrate, *meso*-erythritol, ethanol, ethyl acetate, galactitol, hexadecane, inulin, lactate, methanol, 2-propanol or L-sorbose. Assimilation of nitrogen compounds: lysine (variable) and sodium nitrite (variable) are assimilated; no growth on creatine, creatinine, ethylamine or sodium nitrate. No growth at 37 °C or on 1 % acetic acid. Acid formation is negative. Gelatin liquefaction is negative and casein hydrolysis is positive. No growth on GYP broth with 10 or 16 % NaCl or on 50 % glucose/yeast extract/peptone agar. Production of starch-like compounds is negative. Urease activity is positive. Diazonium blue B reaction is positive.

The type strain, BI20^T, and three other strains (BI68, BI79 and BI133) were isolated from leaves of bromeliads in Itapuã Park, Rio Grande do Sul, Brazil. Strains BI20 and BI79 have been deposited in the Yeast Collection of the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands, and the ARS Culture Collection, Peoria, IL, USA, as CBS 10424^T = NRRL Y-48112^T and CBS 10427 = NRRL Y-48113, respectively.

Latin diagnosis of *Cryptococcus bromeliarum*
M. F. Landell et P. Valente sp. nov.

In medium liquido dextrosum et peptonum et extractum fermenti continente post 7 dies ad 25 °C cellulae vegetativae ellipsoideae et ovoideae, 3 × 6 µm, singulae vel binae. Sedimentum formatur. Cultura in GYP agaro post dies 7 ad 25 °C mucosescens, aurantiaca, glabra, nitida et margo

integer. In Dalmau plate nec mycelium nec pseudomycelium formatur. Ballistosporae nullae. Fermentatio nulla. N-Acetylglucosaminum, acidum gluconicum, acidum succinicum, amylum solubile (variabile), D-arabinosum, L-arabinosum, cellobiosum (variabile), galactosum, D-glucitolium, glucosaminum (variabile), glucosum, glycerolum (variabile), inositolium, lactosum, maltosum, D-mannitolium, melezitium, melibiosum, raffinolum, L-rhamnosum, ribitolium (variabile), D-ribosum, salicinum (variabile), sucrosum, trehalosum, xylitolium, D-xylosum assimilantur. Acetinum, citratum, erythritolum, ethanolum, galactitolium, hexadecanum, inulinum, DL-lactatum, methanolium, 2-propanolum, L-sorbosum non assimilantur. Lysinum (variabile) et natrii nitritum (variabile) assimilantur, sed non creatinum, creatininum, ethylaminum, et natrii nitratum. Non crescit in 37 °C. In medio cum 50 % glucoso non crescit. In medio cum 10 % et 16 % NaCl non crescit. Materia amyloidea iodophila non formatur. Ureum finditur. Reactio Diazonii coerulei B positivum.

Typus: BI20^T (=NRRL Y-48112^T =CBS 10424^T), designat stirpem typicam. Isolata ex folio bromeliaceae, Itapuã Park, Brazil. Preservatus in Centraalbureau voor Schimmecultures, Utrecht, The Netherlands et in ARS Culture Collection, Peoria, IL, USA.

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