Candida bracarensis sp. nov., a novel anamorphic yeast species phenotypically similar to *Candida glabrata*

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Two yeast strains, 153M^T and NCYC 3133, isolated from clinical sources in separate hospitals were found to be almost identical in the sequences of the D1/D2 domain of large-subunit rDNA, the PCR fingerprinting profiles and physiological characteristics. The isolates are phenotypically similar, although not identical, to *Candida glabrata* and *Kluyveromyces delphensis* (recently renamed *Nakaseomyces delphensis*). Sequence analysis of the 26S rDNA D1/D2 gene variable region revealed that the two clinical isolates were closely related phylogenetically to *C. glabrata* and *K. delphensis*, but differed sufficiently to justify their assignment as representatives of a separate species. The name *Candida bracarensis* sp. nov. is proposed for the novel species with the type strain 153M^T (= CBS 10154^T = NCYC D3853^T = CECT 12000^T).

The genus *Candida* belongs to the ascomycetous yeasts and includes some of the most common human pathogens [e.g. *Candida albicans* and *Candida tropicalis* (Ahearn, 1998)]. About 20 species of *Candida* have been shown to cause disease in humans, but the list of medically important yeasts continues to grow, mostly due to developments in medical interventions and to the increasing number of immunodeficient patients. Improved molecular methods for detecting and differentiating yeasts are also able to distinguish closely related species, providing evidence for the existence of new *Candida* species (e.g. *Candida dubliniensis*, *Candida orthopsilosis*) previously misidentified by conventional chemotaxonomic criteria and which may represent new emerging pathogens (Sullivan *et al.*, 1995; Tavanti *et al.*, 2005).

Following an epidemiological study of candidiasis in the north of Portugal (Correia *et al.*, 2004), one of the clinical isolates (strain 153M^T), identified originally as *Candida glabrata*, was found to have a different and quite unique

T3B fingerprinting pattern. In order to clarify its taxonomic position, conventional physiological and biochemical characterization (Yarrow, 1998) as well as rDNA sequencing was performed. A search for similar sequences in the GenBank sequence database revealed the existence of a second strain [NCYC 3133 (formerly NCYC D3411)], isolated from a patient in a UK hospital, with high sequence similarity in the 26S rDNA D1/D2 region. The two clinical isolates shared identical physiological and molecular characteristics and were related to *C. glabrata*, a species of growing medical concern (Fidel *et al.*, 1999), and to *Kluyveromyces delphensis* [recently renamed *Nakaseomyces delphensis* (Kurtzman, 2003)]. However, the extent of sequence divergence was large enough to propose the assignment of these isolates to a novel species, described in this paper as *Candida bracarensis* sp. nov.

Yeast isolates and their characterization

The strains under study were isolated from patients suffering from candidiasis. Strain 153M^T was collected from a vaginal exudate in a Portuguese hospital and strain NCYC 3133 was isolated from a blood culture from a UK hospital.

The isolates were characterized by using standard chemotaxonomic methods (Yarrow, 1998) and by PCR fingerprinting with primer T3B (Correia *et al.*, 2004).

rDNA sequencing and sequence analysis

The D1/D2 domain of the 26S rDNA was amplified and sequenced according to the procedures described by

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Abbreviation: ITS, internal transcribed spacer.

The GenBank/EMBL/DDBJ accession number for the 26S rDNA D1/D2 domain and complete ITS and 5-8S rDNA gene sequences of *Candida bracarensis* 153M^T are AY589572 and AY589573, respectively.

A figure showing the pairwise alignment of the 26S rDNA D1/D2 sequences of *C. glabrata*, *K. delphensis* and strain 153M^T is available as supplementary material in IJSEM Online.

Sampaio *et al.* (2001). The entire ribosomal internal transcribed spacer (ITS) region, including the 5·8S rDNA, was amplified using the forward primer ITS1 (5'-TCCGTAG-GTGAACCTGCGG-3') and reverse primer ITS4 (5'-TCCT-CCGCTTATTGATATGC-3') and sequenced using the amplification primers (ITS1 and ITS4) as well as two additional internal primers, ITS2 (5'-GCTGCGTTCTTCATCGA-TGC-3') and ITS3 (5'-GCATCGATGAAGAACGCAGC-3') (White *et al.*, 1990). In the case of NCYC 3133, PCR amplification of both rDNA regions was carried out directly from whole yeast cells using the protocol described by James *et al.* (2005). Sequencing was performed with an ABI 310 Genetic Analyzer (Applied Biosystems) using standard protocols. Forward and reverse sequence alignments were made with MegAlign (DNASTAR) and corrected visually.

The 26S rDNA D1/D2 sequences determined in this study were compared with sequences from the GenBank database and aligned with CLUSTAL_X version 1.81 (Thompson *et al.*, 1997). Phylogenetic analysis was carried out using the PAUP* version 4.0b8 software package (Swofford, 2000) and the 26S rDNA D1/D2-derived tree was constructed by using the neighbour-joining method (Saitou & Nei, 1987) with the Kimura two-parameter distance measure. Confidence limits were estimated from bootstrap analysis (1000 replicates) and only values of 50 % or greater were recorded on the tree.

Molecular fingerprinting profiles

When analysed by PCR fingerprinting using primer T3B, strains 153M^T and NCYC 3133 were found to have very similar profiles which were quite distinct from those of *C. glabrata* and *K. delphensis* strains (Fig. 1).

Physiological and biochemical characterization

The physiological and biochemical characteristics of strains 153M^T and NCYC 3133 were found to be similar, but not identical, to those of *C. glabrata* and *K. delphensis*. In fact, the two strains can be separated from *C. glabrata*, their nearest known relative (Fig. 2), on the basis of L-lysine assimilation. Both novel strains are able to utilize L-lysine as a sole nitrogen source, whereas strains of *C. glabrata* assimilate this compound either weakly or not at all (Barnett *et al.*, 2000; Meyer *et al.*, 1998). Similarly, strains 153M^T and NCYC 3133 can be readily separated from *K. delphensis* based on their ability to assimilate both L-lysine and α,α -trehalose, to ferment α,α -trehalose, to grow at temperatures in excess of 40 °C and by an inability to assimilate ethanol (Barnett *et al.*, 2000; Lachance, 1998).

Phylogenetic analysis

Sequence analysis of the D1/D2 domain of 26S rDNA for strain 153M^T showed the highest similarity with strain NCYC 3133 (differing by a single nucleotide substitution). Strain *Candida* cf. *glabrata* UWO(PS)98-110.4 and three clinical strains, designated JAF-2004 isolates 2960 to 2962, also shared a high similarity with strain 153M^T, showing 14

Fig. 1. PCR profiles obtained with primer T3B. Lanes: 1, *C. glabrata* CBS 138^T; 2, *Kluyveromyces delphensis* PYCC 2899; 3, strain 153M^T; 4, strain NCYC 3133. M, Molecular size marker.

nucleotide differences and 14 nucleotide substitutions and two gaps, respectively. Analysis of the phylogenetic tree derived from the alignment of D1/D2 sequences showed that the two closest species to strains 153M^T and NCYC 3133 were *C. glabrata* and *K. delphensis* (Fig. 2). Sequence similarity values were estimated from an alignment of the D1/D2 sequences of the respective type strains with strain 153M^T. The similarity between *C. glabrata* and *K. delphensis* was 92 %, corresponding to 38 nucleotide differences and four gaps. When compared with these species, strain 153M^T displayed 94·8 % sequence similarity with *C. glabrata* (corresponding to 30 nucleotide differences) and 93·8 % similarity with *K. delphensis* (corresponding to 32 nucleotide differences and four gaps). The pairwise alignment of 26S rDNA D1/D2 sequences is presented in Supplementary Fig. S1 available in IJSEM Online).

Sequence analysis was also carried out on the entire ribosomal ITS region (i.e. ITS1/5·8S rDNA/ITS2) of strains 153M^T and NCYC 3133. Both strains were found to have ITS1 sequences of identical length (305 bp), which differed from one another by a total of 13 base substitutions, corresponding to 95·7 % sequence similarity. Likewise, the ITS2 sequences of the two novel strains were found to be 252 bp (153M^T) and 253 bp (NCYC 3133) in length and differed from one another by a single base substitution and a single nucleotide gap (99·2 % sequence similarity). Such levels of ITS1 and ITS2 sequence divergence probably reflect the fact that the strains were isolated from patients in separate hospitals, one in Portugal (153M^T) and the other in the UK (NCYC 3133). Comparison of the complete ITS

Fig. 2. Phylogenetic tree derived from neighbour-joining analysis based on 26S rDNA D1/D2 region sequences showing the placement of *Candida bracarensis* sp. nov. and its closest relatives. Only bootstrap percentages (1000 replicates) of 50% or greater are shown. Bar, 1% nucleotide sequence divergence.

sequences revealed that 153M^T displayed greater sequence similarity to *K. delphensis* (68%) than to *C. glabrata* (65%), while 153M^T and NCYC 3133 displayed 97.9% sequence similarity to each other.

Despite being related to *C. glabrata*, the number of nucleotide substitutions in the D1/D2 region (30 nucleotides over 581) of the novel strains is much higher than the 0–3 nucleotide differences typically observed between either conspecific or sister species (Kurtzman & Robnett, 1997, 1998). Indeed, as the 26S rDNA D1/D2-derived tree in Fig. 2 illustrates, strains 153M^T and NCYC 3133 clearly belong to a phylogenetically distinct species, a fact supported by both ITS sequencing and PCR fingerprint profiling. Consequently, based on the collective results of this study, a novel species, *Candida bracarensis* sp. nov., is proposed. As the novel species exhibited an API 32C profile identical to *C. glabrata*, it seems possible that 153M^T and NCYC 3133 may not be unique isolates of this species and similar clinical isolates may well exist, but may have been misidentified as *C. glabrata*.

This novel species adds to the overall knowledge of yeast biodiversity and represents an additional reference in its phylogenetic clade. This is particularly significant for yeast groups impacting on human health, since modern medical therapy and improved methods for detecting and differentiating yeasts have shown that many novel and unusual species have become clinically important.

Latin diagnosis of *Candida bracarensis* Correia, Sampaio, James et Pais sp. nov.

In medio liquido multi post dies tres ad 30 °C, cellulae sunt globosae (3.0–3.5 µm) ad ellipsoideae (3.0–4.0 × 4.0–4.5 µm) singulae aut binae (Fig. 3). In agar multi post 7 dies ad 30 °C, cultura albida crenea, centrum colonia altum, butyrosa et margine glabro. In agar farinae Zea mays post dies 21 ad 25 °C, pseudohyphae et hyphae verae absentes. Asci non formantur. Glucosum et trehalosum fermentantur. Galactosum, maltosum, sucrosus, lactosum, cellobiosum, melezitium, raffinatum et inulinum non fermentantur. Assimilantur glucosum, trehalosum, glycerolum, D-glucono-1,5-lactonum, D-gluconatum et L-lysinum. Non assimilantur galactosum, L-sorbosum, sucrosus, maltosum, cellobiosum, melezitium, D-xylosum, L-arabinosum, D-arabinosum, D-ribosum, D-glucosaminum, ethanolum, erythritolum, ribitolium, xylitolium, D-mannitolium, D-glucitolium, methyl-α-D-glucosidum, salicinum, acidum lacticum, arbutinum, acidum succinicum, acidum citricum, lactosum, inulinum, raffinatum, melibiosum, amyllum solubile, L-rhamnosum, methanolum, galactitolium, D-glucuronatum, cadaverinum, ethylaminum, D-glucosaminum (nitrogenium), imidazolium, creatinum, creatininum, kalium nitratum, natrium nitritum et D-tryptophanum. Temperatura 42 °C crescit. Materia amyloidea et acidum aceticum non formantur. Diazonium coeruleum B negativum.

Typus CBS 10154^T isolatus ex vaginae. *Depositus et*

Fig. 3. Differential interference contrast micrograph of *Candida bracarensis* 153M^T cells grown in YM broth after 3 days at 30 °C. Bar, 10 µm.

preservatus in *Collectione Zymotica* Centraalbureau voor Schimmelcultures *Culturarum*, Utrecht.

Description of *Candida bracarensis* Correia, Sampaio, James & Pais sp. nov.

Candida bracarensis [brac'ar.en.sis. L. fem. adj. *bracarensis* pertaining to Bracara Augusta, the roman name of the city (Braga, Portugal) from where the type strain was isolated.]

In YM broth, after 3 days at 30 °C, the cells are spherical (3.0–3.5 µm) to ellipsoidal (3.0–4.0 × 4.0–4.5 µm) and occur singly or in pairs (Fig. 3). On YM agar, culture colonies are umbonate, pale cream, glistening and butyrous with an entire margin. Neither pseudohyphae nor true hyphae are formed under the coverglass in Dalmau plate culture on cornmeal agar after 21 days at 25 °C. Ascospores are not detected in the type strain when grown for up to 3 months on YM agar or sodium acetate agar incubated at 25 °C. Glucose and α,α -trehalose are fermented. Carbon compounds sucrose, α,α -trehalose, glycerol, D-gluconolactone and D-gluconate are assimilated. No growth occurs on D-galactose, L-sorbose, D-glucosamine, D-ribose, D-xylose, D-arabinose, L-arabinose, L-rhamnose, maltose, methyl α -D-glucoside, cellobiose, arbutin, salicin, melibiose, lactose, raffinose, melezitose, inulin, starch, erythritol, ribitol, xylitol, D-glucitol, D-mannitol, galactitol, D-glucuronate, DL-lactate, succinate, citrate, methanol or ethanol. The only nitrogen compound assimilated is L-lysine. Tests for the Diazonium blue B reaction, urea hydrolysis and starch and

acetic acid formation are negative. Growth occurs on 0.1% cycloheximide and at 42 °C.

The type strain, 153M^T (CBS 10154^T=CECT 12000^T=NCYC D3853^T), was isolated from a case of vaginal candidiasis in a medical institution, Braga, Portugal.

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