

NOTE

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Cryptococcus nyarrowii sp. nov., a basidiomycetous yeast from Antarctica

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In December 1997, 196 soil and snow samples were collected from Vestfold Hills, Davis Base, Antarctica. Two isolates, CBS 8804^T (pink colonies) and CBS 8805 (yellow colonies), were shown by proteome analysis and DNA sequencing to represent the same species. Results from the sequencing of the D1/D2 region of the large rDNA subunit placed this species in the hymenomycetous tree in a unique sister clade to the Trichosporonales and the Tremellales. The clade consists of *Holtermannia corniformis* CBS 6979 and CBS strains 8804^T, 8805, 8016, 7712, 7713 and 7743. Morphological and physiological characteristics placed this species in the genus *Cryptococcus*, with characteristics including the assimilation of D-glucuronate and myo-inositol, no fermentation, positive Diazonium blue B and urease reactions, absence of sexual reproduction and production of starch-like compounds. Fatty acid analysis identified large proportions of polyunsaturated lipids, mainly linoleic (C_{18:2}) and, to a lesser extent, linolenic (C_{18:3}) acids. On the basis of the physiological and phylogenetic data, isolates CBS 8804^T and CBS 8805 are described as *Cryptococcus nyarrowii* sp. nov.

Keywords: *Cryptococcus nyarrowii*, rDNA sequencing, fatty acids, proteome analysis, scanning electron microscopy

The genus *Cryptococcus* consists of a heterogeneous group of yeasts, colonies of which may be white, cream, red, yellow or brown in colour. Cells are globose or subglobose, ovoidal to elongate, with polar or multilateral budding, hyphae and pseudohyphae may be present and most species have a capsule. Members of the genus *Cryptococcus* do not produce a sexual state and are defined by the following physiological characteristics: lack of fermentation, assimilation of D-glucuronate and usually myo-inositol, positive reactions for Diazonium blue B and urease tests and presence of xylose in whole-cell hydrolysates (Barnett *et al.*, 1990; Boekhout, 1998; Fell & Statzell-Tallman, 1998; Kurtzman & Fell, 1998; Montes *et al.*, 1999). All members of the genus *Cryptococcus* represent anamorphs of the hymenomycetous yeasts and are represented throughout the Tremellales, Filo-

basidiales, Trichosporonales and Cystofilobasidiales clades.

In our study, 500 yeasts were isolated from 196 soil and snow samples from Antarctica; 120 morphologically similar isolates were selected for further analysis on the basis of a maximum growth temperature less than 25 °C and colony and cell morphology. One-dimensional (1D) SDS-PAGE of whole-cell proteins was then used to select and group isolates. SDS-PAGE allowed the rapid identification of the degree of similarity among unknown isolates by comparing protein fingerprints. Multiple isolates of a species were identified and similar species were grouped for subsequent analysis, reducing the test group of 120 isolates to 36. Morphologically similar species have been shown by this procedure to be unrelated and this method has been useful in distinguishing between closely related species (Pennington *et al.*, 1997; Tsakalidou *et al.*, 1997; Vancanneyt *et al.*, 1994). Sequencing of the internal transcribed spacer (ITS) 1–5.8S rRNA gene–ITS2 (ITS1–5.8S–ITS2) region and the D1/D2 region of the 26S rDNA was performed on the 36 isolates that were identified by proteome analysis as possible different

Published online ahead of print on 21 December 2001 as DOI 10.1099/ijs.0.01940-0.

Abbreviation: ITS, internal transcribed spacer.

The GenBank/EMBL/DDBJ accession numbers for the rDNA D1/D2 and ITS sequences of strains CBS 8804^T and CBS 8805 are respectively AY006480 and AY400696 (D1/D2) and AY006481 and AY400697 (ITS).

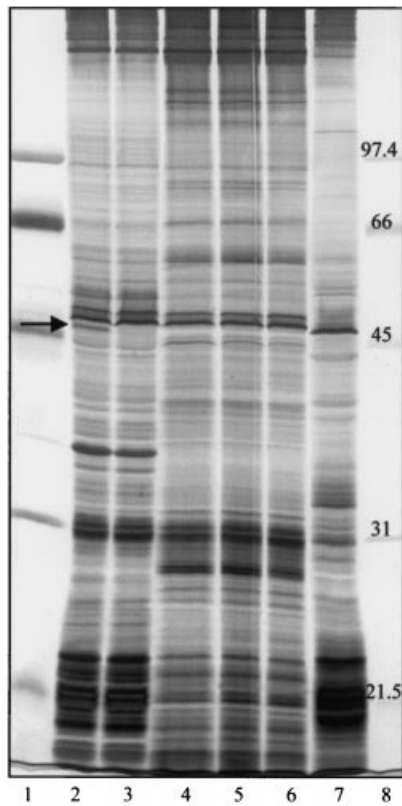


Fig. 1. Proteome analysis of protein extracts by 1D SDS-PAGE (12.5% acrylamide). Lanes: 1 and 8, low-molecular-mass standards (sizes indicated in kDa); 2–7, protein extracts of CBS strains 8804^T, 8805, 7712, 7713 and 7743 and *H. corniformis*. Arrow indicates extra band at 45 kDa.

species. Twelve of these isolates showed at least 2 nt difference from the sequence of any other known species in the D1/D2 phylogenetic trees of the Basidiomycetes and Ascomycetes (Fell *et al.*, 2000; Kurtzman & Robnett, 1998). Two isolates, CBS 8804^T and CBS 8805, are described in this communication as *Cryptococcus nyarrowii* sp. nov., in honour of Nicholas Yarrow, schoolteacher at Murwillumbah High, NSW, Australia.

Strain CBS 8804^T was isolated from snow petrel carnage and strain CBS 8805 was isolated from soil and lichen. Both samples were taken from Lichen Valley (68° 29' S 78° 25' E), Vestvold Hills, Davis Base, Antarctica, in December 1997, stored at –10 °C and screened in April 1998. Soil samples (0.1 g) were added to YEP broth (0.5% yeast extract, 0.5% bacteriological peptone, 0.3% KH₂PO₄, 0.3% (NH₄)₂SO₄ and 2% glucose, all w/v) containing 250 µg streptomycin (strep) ml⁻¹ and 500 µg ampicillin (amp) ml⁻¹ to prevent bacterial growth and samples were incubated at 10 °C on an orbital shaker (150 r.p.m.). Aliquots were taken on days 0, 5 and 10 and serial dilutions were plated on YEP + strep + amp agar plates and incubated at 10 °C until colony growth was visible. Yeast isolates were streaked onto YEP agar plates,

incubated at 10 °C for 1 week and then restreaked if necessary. Cultures were maintained on YEP plates and as aqueous stocks at 6 °C, with long-term storage in glycerol stocks at –80 °C. Cell size and morphology were determined by scanning electron microscopy (JEOL model JSM 5800LV, accelerating voltage 15 kV) using an improved fixation procedure for yeast (Hanschke & Schauer, 1996). Physiological characterizations were performed in duplicate, according to the methods described by Yarrow (1998).

Cells in the exponential growth phase (YEP broth at 15 °C for 3–5 days) were pelleted by centrifugation at 2000 *g* for 5 min, washed once with distilled water and then centrifuged. To the pellet was added 150 µl ice-cold lysis buffer (0.1% Triton X-100, 100 mM KCl, 8 mM MgCl₂, 150 mM NaCl, 20 mM Tris/HCl, pH 7.4, and 1 mM PMSF) with an equal volume of 0.5 mm glass beads. Samples were vortexed vigorously for 30 s followed by 30 s on ice and the procedure was repeated eight times. The lysed cells were centrifuged (1500 *g* for 5 min) and the supernatant was taken for protein analysis. Protein concentrations were determined by a modified Bradford Coomassie method (Pierce). Equal volumes of 3 × gel-loading buffer (150 mM Tris/HCl, pH 6.8, 300 mM DTT, 6% SDS, 0.3% bromophenol blue, 30% glycerol) were added and samples were boiled for 3 min to denature proteins. 1D SDS-PAGE was performed using standard procedures as outlined by Laemmli (1970). Protein (10 µg) was loaded into each well of a 12 or 7% SDS resolving acrylamide gel with a 4% stacking gel. Gels were run at 30 mA, 400 V at 10 °C for about 4 h until the dye migrated to the bottom of the gel. Gels were silver-stained (Bio-Rad silver stain kit) and dried and images were scanned for recording purposes.

A loopful of yeast cells from the appropriate plate was washed with distilled water and then resuspended in 1 ml distilled water. Amplicons were obtained using 5 µl of this cell suspension and the Finnzyme system (Geneworks) [5 µl 10 × buffer 511, 1 µl dNTP mix, 1 µl Dynazyme II (polymerase), 25 pmol forward primer NS7 (5'-GAGGCAATAACAGGTCTGTGATGC-3') and 25 pmol reverse primer LR6 (5'-CGCCAGTTCTGCTTACC-3') in a final volume of 50 µl made up with distilled water]. The PCR program was as follows: initial denaturation at 94 °C for 1 min, 30 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min, with a final 8 min step at 70 °C. Amplicons of approximately 2.2 kb were confirmed by agarose gel electrophoresis and purified using the QIAquick purification kit (Qiagen). The D1/D2 region of the 26S rDNA was cycle-sequenced using forward primer F63 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and reverse primer LR3 (5'-GGTCCGTGTTTCAAGACGG-3'). The ITS1–5.8S–ITS2 region was analysed using forward primer ITS1 (5'-TCCGTAGG-TGAACCTGCGG-3') and reverse primer ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). The sequences were obtained with a LI-COR automated sequencer using a standard protocol, analysed and corrected

Table 1. Yeasts examined in the D1/D2 region of the large-subunit rDNA and ITS rDNA regions

Strain	GenBank accession no.	
	D1/D2	ITS
<i>Bullera dendrophila</i> CBS 6074 ^T	AF189870	
<i>Cryptococcus albidus</i> CBS 142 ^T	AF075474	AF145321
<i>Cryptococcus aquaticus</i> CBS 5443 ^T	AF075470	AF410469
<i>Cryptococcus curvatus</i> CBS 570 ^T	AF189834	AF410467
<i>Cryptococcus heveanensis</i> CBS 569 ^T	AF075467	
<i>Cryptococcus himalayensis</i> CBS 6293 ^T	AF181502	
<i>Cryptococcus humicola</i> CBS 571 ^T	AF189836	AF410470
<i>Cryptococcus laurentii</i> CBS 139 ^T	AF075469	AF410468
<i>Cryptococcus nyarrowii</i> CBS 8804 ^T	AY006480	AY006481
<i>Cryptococcus nyarrowii</i> CBS 8805	AY400696	AY400697
<i>Cryptococcus</i> sp. CBS 7712	AJ311450	AF408417
<i>Cryptococcus</i> sp. CBS 7713	AJ311451	AF408418
<i>Cryptococcus</i> sp. CBS 7743	AJ311452	AF408419
<i>Cryptococcus</i> sp. CBS 8016	AF360844	AF410905
<i>Cryptococcus vishniacii</i> CBS 7110 ^T	AF075473	AF145320
<i>Filobasidium capsuligenum</i> CBS 4736	AF075501	
<i>Holtermannia corniformis</i> CBS 6979	AF189843	AF410472
<i>Mrakia gelida</i> CBS 5272 ^T	AF189831	AF144485
<i>Tremella encephala</i> CBS 6968	AF189867	AF410474
<i>Trichosporon aquatile</i> CBS 5973 ^T	AF075520	AF410475
<i>Trichosporon guehoae</i> CBS 8521 ^T	AF1054014	AF410476

using LI-COR AlignIR and aligned to the closest phylogenetic relatives using MegAlign (DNASTar). Phylogenetic analyses were computed with PAUP 4.0 using parsimony analysis (heuristic search, random stepwise additions).

Cells for fatty acid analysis were grown in 100 ml YEP broth and washed twice with distilled water. In a 10 ml glass test tube, the pellet was resuspended in 2 ml 14% BF₃ in methanol plus 2 ml methanol, gassed with nitrogen, capped and heated at 80 °C for 2 h, with vortexing every 20 min. Tubes were then cooled and 3 ml distilled water and 3 ml hexane were added and samples were shaken. The upper, hexane phase was removed and concentrated for fatty acid analysis (Rule, 1996). Samples were analysed using a Hewlett Packard 5890 series II GC equipped with a flame-ionization detector. The fatty acids were separated on a 30 m × 0.53 mm FFAP (Polyethylene glycol ester; Alltech) column with a carrier gas (helium) flow rate of 10 ml min⁻¹. The oven temperature was set at 200 °C for detection and injection. Fatty acids were identified from retention times relative to appropriate standards (Sigma) and the percentage fatty acid composition was determined by integration (Swan & Watson, 1997).

Proteome analysis

Proteome fingerprinting by 1D SDS-PAGE was used as a rapid initial screening step for the preliminary characterization of the Antarctic yeasts isolated in the

present studies (data not shown). Protein profiles of CBS strains 8804^T, 8805, 7712, 7713 and 7743 and *Holtermannia corniformis* CBS 6979 were obtained by 1D SDS-PAGE for comparative proteome fingerprinting. A protein band at 45 kDa, not seen in CBS 8805, was noted in strain CBS 8804^T (Fig. 1). Although some similarities were observed between the two strains CBS 8804^T and CBS 8805 (lanes 2–3) and the related CBS strains 7712, 7713 and 7743 (lanes 4–6) in the 31 and 45 kDa regions, the overall protein-banding patterns revealed little similarity between these groups of strains and even less similarity to *H. corniformis* (lane 7). Vancanneyt *et al.* (1994) stated in their study that yeast strains including *Cryptococcus* were delineated on the basis of whole-cell protein patterns. The distinctly different protein-banding pattern of CBS strains 8804^T and 8805 compared with those of CBS strains 7712, 7713 and 7743 supports their classification as different species.

Fatty acid analysis

Cells were grown to exponential phase and fatty acid profiles were analysed. In the case of strain CBS 8804^T, there were no marked changes in fatty acid profile in cells grown to exponential phase at 2, 6, 15 or 22 °C. Cells grown to stationary phase at 6 °C (14 days) or 15 °C (5 days) showed a higher percentage (50–52%) of C_{18:2} than cells grown to exponential phase, with corresponding decreases in C_{18:1} (18–22%) and C_{16:0}

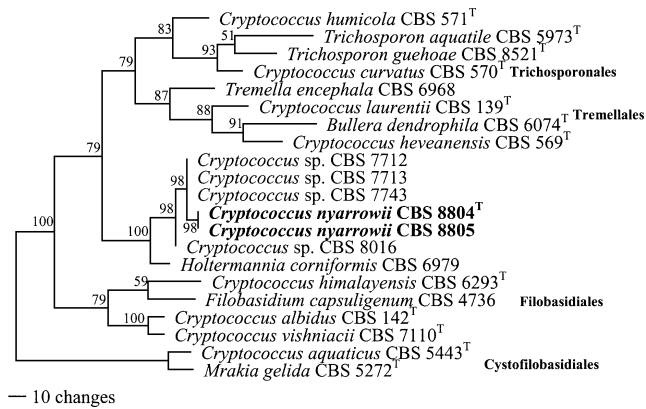


Fig. 2. Phylogenetic analysis of the D1/D2 region of the large-subunit rDNA. Bootstrap percentages from 100 full heuristic bootstrap replications are shown. The Cystofilobasidiales clade was used as the outgroup. Labeled clades have representative species only.

(12–15%). Similar changes were noted in the fatty acid composition of strain CBS 8805, except that growth temperature appeared to have a significant effect on cell fatty-acid composition. For example, cells grown to stationary phase at 6 °C were relatively enriched (12%) in $C_{18:3}$ compared with cells grown at 15 °C (6%). However, the overall fatty acid composition did not vary significantly between the two strains. For comparative purposes, all strains were grown to stationary phase with *Saccharomyces cerevisiae* strain K7 used as a control. The Antarctic strains (CBS strains 8804^T, 8805, 7712, 7713 and 7743) were shown to have significant amounts of linolenic acid ($C_{18:3}$) (3–10%) and no $C_{16:1}$ in comparison with *H. corniformis* CBS 6979 and *S. cerevisiae* K7, both of which had 30–40% $C_{16:1}$ but no $C_{18:3}$. Overall, the relatively high percentages of polyunsaturated fatty acids ($C_{18:2}$ and $C_{18:3}$) in strains CBS 8804^T and CBS 8805 were in agreement with the reported high polyunsaturated fatty acid contents of yeasts isolated from the Antarctic, which include *Mrakia*, *Candida*, *Torulopsis* and *Leucosporidium* species (Watson, 1987).

Sequence analysis

Strains used in the analysis of rDNA sequences are listed in Table 1. Phylogenetic analyses based on the D1/D2 region of the large rDNA subunit sequence showed that strains CBS 8804^T and CBS 8805 have identical sequences and occur with *H. corniformis* in a cluster of the hymenomycetous yeasts as a sister clade to the Trichosporonales and the Tremellales (Fell *et al.*, 2000). This cluster (Fig. 2) also contains three strains of *Cryptococcus* isolated by Golubev (1977), CBS 7712 and 7713 (from soil, East Falkland, Antarctica) and 7743 (from a herbaceous plant, South Georgia Island, Antarctica). These strains differ in sequence by 4 nt from strains CBS 8804^T and CBS 8805. The next closest phylogenetic relative is *Cryptococcus* strain CBS 8016 (isolated from seawater off

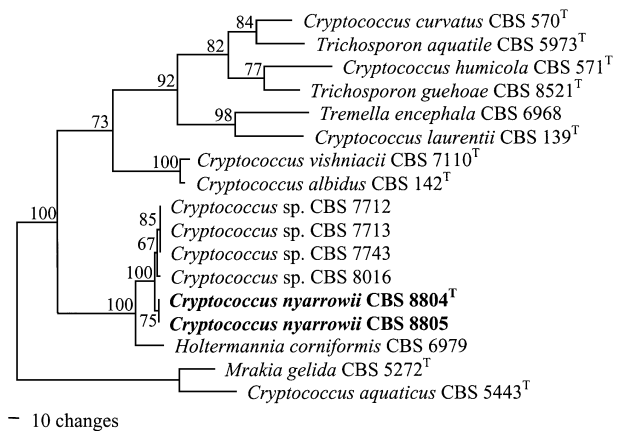


Fig. 3. Phylogenetic analysis of the ITS1–5.8S–ITS2 region. Bootstrap percentages from 100 full heuristic bootstrap replications are shown. The Cystofilobasidiales clade was used as the outgroup.

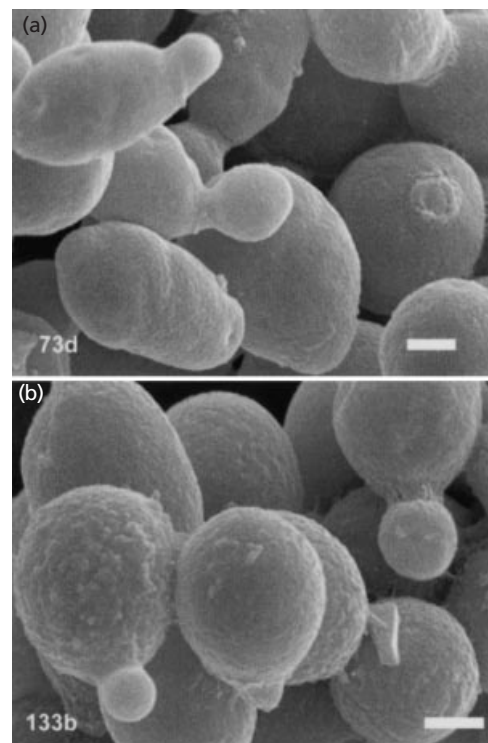


Fig. 4. Scanning electron micrographs of strains CBS 8804^T (a) and CBS 8805 (b), showing budding cells and bud scars. Bars, 1 µm.

Sweden), which differs by 9 nt. *H. corniformis* differs by 24 nt. The sequence of the ITS1–5.8S–ITS2 regions is identical for strains CBS 8804^T and CBS 8805 and differs by 5 nt from the sequence of CBS strains 7712, 7713 and 7743, by 5 nt from the sequence of CBS 8016 and by 30 nt from *H. corniformis*. The data for the ITS regions consolidated the phylogenetic relationship of the D1/D2 tree (Fig. 3).

Table 2. Biochemical and physiological characteristics that vary between CBS strains 8804^T, 8805, 7712, 7713, 7743 and 8016

+, Positive; -, negative; W, weak; ND, no data available; D, delayed; D/W, delayed and weak.

Characteristic	CBS 8804 ^T	CBS 8805	CBS 7712	CBS 7713	CBS 7743	CBS 8016
Assimilation of:						
Galactose	+	+	-	D	D	+
L-Sorbose	D/W	D/W	-	-	-	-
Cellobiose	+	W	+	+	+	+
Lactose	-	-	D	D	+	+
Raffinose	-	-	+	+	+	+
Soluble starch	W	W	+	+	+	+
D-Arabinose	W	W	-	-	D	+
L-Rhamnose	+	W	D	D	D	+
Ethanol	W	W	-	D	D	-
Ribitol	W	D/W	-	-	D	+
Galactitol	W	W	-	-	D	+
D-Glucitol	W	+	+	+	+	+
Methyl α -D-glucoside	+	W	+	D	+	+
D-Gluconate	D	D/W	+	+	+	+
Citrate	W	-	-	D	D	+
Hexadecane	-	W	ND	ND	ND	ND
Growth on:						
Vitamin-free medium	W	W	-	-	-	-
10% NaCl, 5% glucose	+	W	-	-	-	ND
Biotin-free medium	+	+	-	-	+	+
Thiamin-free medium	+	W	-	-	-	-
Growth at 25 °C	-	-	+	+	+	ND

Latin diagnosis of *Cryptococcus nyarrowii* Thomas-Hall et Watson sp. nov.

Cryptococcus nyarrowii, (olim in principio CBS 8804^T et CBS 8805 nominatus) e Lichen Valley (68° 29' S 78° 25' E), Vestfold Hills, Davis Base, Antarctica, inventus est. In YEP liquido post dies 3 (15 °C), cellulae globosae vel ovoidae sunt singulae vel binae (2–4 × 3–5 µm). Flosculi polares, quos iungit breve tuber, cicatrices linquunt. In YEP agar CBS 8804^T fert convexas, rotundas, luteas colonias, textu glutineo. CBS 8805 fert convexas, rotundas, luteas colonias, textu glutineo quae dimidio incremento sunt. Ex agar farinatio parvum incrementum. In agar farinae *Zea mays* incrementum paulo minus sed coloniae similes crescunt. Hyphae vel pseudohyphae numquam crescunt neque status sexualis apparet. Assimilat: glucosum, galactosum, sucrosum, maltosum, trehalosum, melezitium, D-xylosum, L-arabiosum, D-ribosum, N-acetyl-D-glucosaminum, D-mannitolium, salicinum, succinatum, inositolium, D-glucoronatum, sine biotino. Omnia respondent exigue: amyllum solubile, D-arabiosum, ethanolium, glycerolum, ribitolium, galactolum, invalide respondent. L-Sorbosum, D-gluconatum sine vitamini invalide tardeque respondent. Cellobiosum, L-rhamnosum, methyl α -D-glucosidum, sine thiamin, 10% NaCl, 5% glucosum, D-glucitolium positive vel invalide respondent. Hexadecanum et citratum invalide vel non

respondent. Lactosum, melibiosum, raffinatum, inulinum, D-glucosaminum, methanolium, erythritolum, DL-lactatum, nitratum non respondent. Amyli instar composita creantur. Fermentatio nulla. Liquefactio gelatinis non respondet. Incrementum in 25 °C non respondet, maxima incrementi temperatio est 22 °C, optime crescit in 15–18 °C. In collectione zymotica Centraalbureau voor Schimmelcultures, Uppsalalaan 8, 3584 CT Utrecht, Neerlandia, CBS 8804^T est *Cryptococcus nyarrowii*, et CBS 8805 est *Cryptococcus nyarrowii*.

Description of *Cryptococcus nyarrowii* Thomas-Hall et Watson sp. nov.

Cryptococcus nyarrowii (n.yar'ro.wi.i. N.L. gen. masc. n. *nyarrowii* in honour of Nicholas Yarrow).

Two strains, CBS 8804^T and CBS 8805, were isolated from Lichen Valley (68° 29' S 78° 25' E), Vestfold Hills, Davis Base, Antarctica. In YEP broth after 3 days growth at 15 °C, the cells are spheroidal to ovoid, occur singly or in parent-bud pairs and measure 2–4 × 3–5 µm. Budding is polar and a short protuberance connects budding cells, leaving raised bud scars (Fig. 4). Strain 8804^T gives rise to convex, circular, shiny pink colonies on YEP agar that are pasty in texture with young colonies that are pale yellow, maturing to a deep pink. Under the same growth

conditions, strain 8805 gives rise to convex, circular, shiny yellow colonies, pasty in texture, but half the size of those of strain CBS 8804^T. No sexual state is observed from mixed and pure cultures plated on cornmeal agar, malt agar, YEP agar, carbon base agar or nitrogen base agar. Growth response to corn meal agar is weak. Growth on malt agar is slightly weaker than that on YEP and similar colonies are formed. Under these conditions, no hyphae or pseudohyphae are formed. Carbon compound assimilation: positive for glucose, galactose, sucrose, maltose, trehalose, melezitose, D-xylose, L-arabinose, D-ribose, *N*-acetyl-D-glucosamine, D-mannitol, salicin, succinate, inositol and D-glucuronate; weak for soluble starch, D-arabinose, ethanol, glycerol, ribitol and galactitol; positive or weak for cellobiose, L-rhamnose, methyl α -D-glucoside and D-glucitol; slow and weak for L-sorbose and D-gluconate; weak or negative for citrate and hexadecane; negative for lactose, melibiose, raffinose, inulin, D-glucosamine, methanol, erythritol and DL-lactate. Nitrate assimilation is negative. Growth on biotin-free media is positive, growth on thiamin-free media and with 10% NaCl/5% glucose is positive or weak and growth on vitamin-free media is slow and weak. Starch-like compounds are formed, Diazonium Blue B and urease reactions are positive. Growth on 50% (w/w) glucose/yeast extract agar is negative. Gelatin liquefaction is negative. Fermentation is absent. Growth at 25 °C is negative, the maximum growth temperature is 22 °C and the optimal growth temperature is 15–18 °C. Characteristics that differ between strains CBS 8804^T and CBS 8805 and their closest phylogenetic relatives strains CBS 7712, 7713, 7743 and 8016 are listed in Table 2.

The type strain is strain CBS 8804^T. Strains CBS 8804^T and CBS 8805 have been deposited in the collection of the Yeast Division of the Centraalbureau voor Schimmelfcultures, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands.

Acknowledgements

This work was supported in part by the Australian Research Council (K. W.), a UNE postgraduate scholarship (S. T.-H.) and the National Science Foundation, Division of Ocean Sciences (Jack Fell). We thank John Bowman and Tom McMeekin of the Antarctic CRC, University of Tasmania, Hobart, for supply of soil samples and helpful discussions, Jack Fell for his invaluable comments on the manuscript and the use of his laboratory and database, Gloria Scorzetti for helpful comments and patience, Teun Boekhout for sequencing the D1/D2 and ITS regions of CBS strain 8016 and Allan Treloar for the Latin translation.

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