A novel psychrotolerant member of the hymenomycetous yeasts from Antarctica: *Cryptococcus waticus* sp. nov.

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Soil, snow and organic material, collected in November 1997 from the Vestvold Hills, Davis Base, Antarctica, were screened for yeasts. Two isolates, which were shown to be indistinguishable by rDNA sequencing and protein analysis by SDS-PAGE, are described in this communication as a novel species, *Cryptococcus waticus* sp. nov. (type culture, CBS 9496^T = NRRL Y-27556^T). Sequence analyses of the 26S rDNA D1/D2 region placed *C. waticus* in the hymenomycetous yeasts in a cluster with *Holtermannia corniformis* and *Cryptococcus nyarrowii*. This species has been allocated to the genus *Cryptococcus* on the basis of physiological and morphological characteristics.

The genus *Cryptococcus* is polyphyletic and is distributed throughout the Hymenomycetous yeasts, with representatives in all four clades (Tremellales, Trichosporonales, Filobasidiales and Cystofilobasidiales). Sequencing of the D1/D2 domain of the large rDNA subunit has been accomplished for all known ascomycete (Kurtzman & Robnett, 1998) and basidiomycete (Fell *et al.*, 2000) species. Strains that differ in this region by at least 2 nt can be considered as potentially novel taxa (Fell *et al.*, 2000). In a previous study by Thomas-Hall & Watson (2002), over 500 yeasts were isolated from Antarctic soil and snow samples, from which 60 isolates were selected (on the basis of morphological characteristics) for further analysis in the current study. One-dimensional SDS-PAGE of whole-cell protein extracts was employed to identify yeast isolates with similar or visually identical protein banding patterns. This condensed the study group to 27. Phylogenetic analysis of the D1/D2 region of the large rDNA subunit sequence of two strains, 22c and 41b, showed that both strains had identical sequences and occurred in a cluster that consisted of *Holtermannia corniformis*, *Cryptococcus nyarrowii* and *Cryptococcus* sp. CBS 7712, CBS 7713, CBS 7743, CBS 8016, KCTC 17061, KCTC 17062 and KCTC 17063, within the Tremellales clade. These two Antarctic isolates are described in this communication as *Cryptococcus waticus* sp. nov.

Isolation and characterization

Samples of soil and snow were collected in November 1997 and stored at -10°C . Yeasts were isolated as described previously (Thomas-Hall & Watson, 2002). Strain 41b was isolated from soil and strain 22c was isolated from stromatolite and shell, from samples that were taken from Watts Lake, Vestvold Hills ($68^{\circ}29'S$ $78^{\circ}25'E$), Davis Base, Antarctica. Cultures were maintained on yeast extract/peptone (YEP) plates [2% (w/v) glucose, 0.5% bacteriological peptone, 0.5% yeast extract, 0.3% KH_2PO_4 , 0.3% $(\text{NH}_4)_2\text{SO}_4$, 1.5% agar] and in aqueous stocks at 6°C , with long-term storage in 15% glycerol at -80°C . Isolates were characterized by the standard methods described by Yarrow (1998).

DNA sequence analysis

An AquaPure Genomic DNA Isolation kit from Bio-Rad was employed to extract DNA from the yeast isolates. PCR products were obtained by utilizing the Qiagen HotStart PCR kit [2 μl purified DNA, 0.5 μl forward primer (5'-TC-CGTGTTTCATGAACCTGCGG-3'), 0.5 μl reverse primer (5'-TCCTCCGCTTATTGATATGC-3'), 21.5 μl MilliQ water, 2.5 μl Q-solution, 25 μl HotStart Mastermix], followed by thermal cycling using the following conditions: 95°C for 15 min, followed by 30 cycles of 94°C for 1 min, 52°C for 1 min and 72°C for 1 min, with a final 10 min at 72°C . PCR products were purified with the Prep-a-gene-clean system (Bio-Rad). A Beckman CEQ2000 Dye Terminator Quick Start kit was used for the sequencing reaction of the D1/D2 domain in a total volume of 20 μl [purified PCR product, 2 μl primer (forward primer MLF

Fig. 1. Phylogenetic tree showing placement of *C. watticus* among related basidiomycetous yeasts, derived from maximum-parsimony analysis of the 26S rDNA D1/D2 domain. Bootstrap replications from 100 full heuristic replications using the neighbour-joining method are displayed. *Cryptococcus aquaticus* and *Mrakia gelida*, which represent the Cystofilobasidiales, are the designated outgroup species in this analysis. Labelled clades have representative species only.

5'-GCATATCAAGCGGAGGAAAAG-3', reverse primer MLR 5'-GGTCCGTGTTTCAAGACGG-3'), 4 µl Beckman CEQ DTCS Quickstart mix, 14 µl MilliQ water] and was placed in the thermal cycler for 30 cycles of 96 °C for 20 s, 50 °C for 20 s and 60 °C for 4 min. Sequences were obtained with a Beckman CEQ2000 automatic sequencer and aligned visually with BioEdit. Phylogenetic relationships were assessed with MegAlign (DNASTar). Phylogenetic analyses employed the maximum-parsimony program of PAUP 4.0 (Sinauer Associates) with heuristic searches and neighbour-joining analysis. Bootstrap values were determined by using PAUP; values of < 50 % were not included in tree figures.

Fatty acid and protein pattern analyses

Cells were grown to stationary phase in 100 ml YEP broth and fatty acid composition and whole-cell protein patterns were determined as described previously (Thomas-Hall & Watson, 2002).

Sixty isolates from the original 500 yeasts that were isolated in a previous study (Thomas-Hall & Watson, 2002) were chosen for further analysis on the basis of morphological characteristics. One-dimensional SDS-PAGE of whole-cell proteins was employed initially to characterize relatedness among unknown isolates by comparing protein banding patterns. Isolates with visually identical or similar protein banding patterns were grouped together. Two isolates, 22c and 41b, were shown to have protein banding patterns that were indistinguishable from each other, but differed significantly from those of *C. nyarrowii* and *H. corniformis* (results not shown). Phylogenetic analyses revealed that the two isolates have identical sequences and have 2 nt difference from the Korean strains KCTC 17061, KCTC 17062 and KCTC 17063, 15 nt difference from *H. corniformis* and 16 nt difference from *C. nyarrowii*, resulting in their placement in the Tremellales clade with other isolates from Antarctica (*C. nyarrowii* and *Cryptococcus* strains CBS

7712, CBS 7713 and CBS 7743) (Fig. 1). Fatty acid analysis of strain 41b revealed oleic acid (C_{18:1}) to be the predominant fatty acid present (61 %), together with the polyunsaturated fatty acids C_{18:2} (5 %) and C_{18:3} (3 %), whereas strain 22c showed significant amounts of C_{18:1} (28 %) and the polyunsaturated fatty acids C_{18:2} (31 %) and C_{18:3} (11 %). These fatty acid compositions confirmed previous studies that have shown that psychrophilic Antarctic yeasts, such as species of the genera *Candida*, *Leucosporidium* and *Mrakia*, have a high unsaturated fatty acid content (Watson, 1987). The new yeast isolates were placed in the genus *Cryptococcus*, as no sexual state was observed under a variety of conditions. Vegetative reproduction is by multilateral budding (Fig. 2), D-glucuronate and inositol are assimilated and urease and Diazonium blue B reactions are positive (Fell & Statzell-Tallman, 1998; Kurtzman & Fell, 1998).

Fig. 2. *Cryptococcus watticus* after 3 days at 15 °C in YEP broth, showing budding cells. Bar, 10 µm.

Description of *Cryptococcus watticus* Guffogg, Thomas-Hall, Holloway & Watson sp. nov.

Cryptococcus watticus (wat'ti.cus. N.L. adj. *watticus* referring to Watts Lake, Antarctica, from where the isolates originated).

After 3 days growth in YEP broth at 15 °C, cells are ovoidal and occur singly or in pairs. Budding is polar. Aerobic growth results in pink, convex, circular colonies with an entire margin. Colonies are viscous in consistency. No sexual state is observed from mixed or pure cultures plated for 3–6 months at 6 or 15 °C on YEP agar, cornmeal agar, malt agar or nitrogen base agar. Growth on yeast nitrogen base agar or cornmeal agar is slower than on YEP or malt agar. Assimilation of carbon compounds is as follows: positive reactions are observed for glucose, galactose, sucrose, trehalose, lactose, melibiose, raffinose, inulin, D-xylose, D-ribose, L-rhamnose, N-acetyl-D-glucosamine, D-mannitol, methyl α -D-glucoside, salicin, succinate, citrate, inositol and D-glucuronate; negative or weak reactions are observed for D-arabinose, D-glucosamine, glycerol, erythritol, ribitol, D-glucitol, hexadecane, L-sorbose, maltose, cellobiose, melezitose, L-arabinose, methanol, ethanol, galactitol, D-gluconate and DL-lactate. Assimilation of soluble starch is weak. Assimilation of nitrate is positive. Growth in vitamin-free medium, 50 % (w/w) glucose/yeast extract agar and 10 % NaCl/5 % glucose is negative. Growth is positive in both biotin- and thiamin-free media. Gelatin liquefaction and urease reaction are positive. Diazonium blue B reaction is positive. Starch formation is negative. Growth at 25 °C is weak; no growth occurs at 30 °C.

The type strain is 41b^T (= CBS 9496^T = NRRL Y-27556^T). These strains were isolated from soil (41b) and stromatolite and shell (22c), Watts Lake, Vestvold Hills, Davis Base (68° 29' S 78° 25' E), Antarctica.

Latin diagnosis of *Cryptococcus watticus* Guffogg, Thomas-Hall, Holloway et Watson sp. nov.

In YEP liquido post dies 3 ad 15 °C, cellulae ovoidae sunt, singulae vel binae. Flosculi sunt polares. Incrementum cum aere effecit luteas, convexas, rotundas colonias margine toto. Coloniae sunt consistentiae crassae. Status sexualis non apparet culturis in agaro YEP, farinae Zea mays, malti, vel nitrogeneo 3–6 menses. Ex agaro nitrogeneo vel farinae Zea mays est minus quam ex agaro YEP vel malti. Assimilat sic: glucosum, galactosum, sucrosum, trehalosum, lactosum, melibiosum, raffiniosum, inulinum, D-xylosum, D-ribosum, L-rhamnosum, N-acetyl-D-glucosaminum, D-mannitolum,

methyl- α -D-glucosidum, salicinum, succinatum, citratum, inositolum et D-glucuronatum; non respondent D-arabiosum, D-glucosaminum, glycerolum, erythritolum, ribitolum, D-glucitolum, hexadecanum, L-sorbosum, maltosum, cellobiosum, melezitiosum, L-arabiosum, methanolum, ethanolum, galactitolum, D-gluconatum, DL-lactatum. Exigue respondet amyllum solubile. Respondet nitratum. Incrementum sine vitamina, ex 50 % (w/w) GY agaro, 10 % NaCl, 5 % glucosum non respondet. Incrementum respondet sine biotino et sine thiamin. Liquatio gelatinis et ureum respondent. Amyllum non creatur. Incrementum ad 25 °C exigue est, ad 30 °C non est.

*In collectione zymotica Centraalbureau voor Schimmelcultures, Utrecht, Nederlandia, CBS 9496^T (= NRRL Y-27556^T) est *Cryptococcus watticus*. Haec est isolata ex terra, testa Watts Lake, Vestvold Hills, Davis Base (68° 29' S 78° 25' E), Antarctica.*

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