Cryptococcus yokohamensis sp. nov., a basidiomycetous yeast isolated from trees and a Queensland koala kept in a Japanese zoological park

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Three strains were isolated from the nostrils of a koala and the surrounding environment in a Japanese zoological park. Sequence analysis of the nuclear rDNA internal transcribed spacer (ITS) region and the large subunit rDNA D1/D2 domains in addition to physiological and morphological studies indicated that the isolates represent a single novel species belonging to the basidiomycetous genus *Cryptococcus* (Tremellales, Tremellomycetes, Agaricomycotina). Phylogenetic analysis based on D1/D2 and ITS regions revealed that the novel species belongs to the Fuciformis clade. The name *Cryptococcus yokohamensis* sp. nov. is proposed to accommodate these isolates with strain JCM 16989^T (=TIMM 10001^T=CBS 11776^T=DSM 23671^T) as the type strain.

The genus *Cryptococcus* Vuillemin includes anamorphic basidiomycetous yeasts that are characterized by the presence of coenzymes Q-9 and Q-10, positivity for urease and diazonium blue B reactions, the absence of fermentation, the presence of xylose in cell-wall hydrolysates and the ability to assimilate D-glucuronate (Fell & Statzell-Tallman, 1998). Furthermore, most species of the genus produce extracellular starch-like compounds. Species forming the polyphyletic genus *Cryptococcus* are dispersed in the orders Tremellales, Trichosporonales, Filobasidiales and Cystofilobasidiales (Fell *et al.*, 2000; Golubev *et al.*, 2006).

In the present study, isolation of three novel basidiomycetous yeast strains during a yeast survey of the Kanazawa Zoological Park in Yokohama city, Japan, is reported. Sequence analysis of the large subunit rDNA D1/D2 domains showed that the strains represent a single species (99% similarity), which was genetically distinct from other species of the Tremellales. The isolates were considered to belong to the genus *Cryptococcus* based on physiological, morphological

and molecular features. In this paper, the novel species *Cryptococcus yokohamensis* sp. nov. is described.

Strains JCM 16990 and JCM 16989^T were isolated from the trunk of trees (*Eucalyptus* spp.), whereas strain JCM 16991 was isolated from the nostril of a Queensland koala (*Phascolarctos cinereus*) kept in a Kanazawa zoo. The nasal smear was sampled with sterilized swabs and then inoculated onto CHROMagar Candida. Morphological, biochemical and physiological characterizations of the isolates were performed using the methods and protocols described by Yarrow (1998). The presence of a capsule was demonstrated by microscopic examination of cells mounted in dilute Indian ink. Scanning electron microscopy of the isolate was performed as described previously (Nishiyama *et al.*, 2005). Two media were used to test mating compatibility among the three isolates as described previously (Bandoni, 1963; Kwon-Chung, 1976).

For molecular biological identification, direct colony PCR (Alshahni *et al.*, 2009) was used to amplify the internal transcribed spacer (ITS) region and the 26S rDNA D1/D2 domains using two pairs of primers, ITS1/ITS4 and 28SF1/635, respectively (Mirhendi *et al.*, 2005; Sugita *et al.*, 2003; White *et al.*, 1990). Amplified fragments were sequenced with an Applied Biosystems model 3130 sequencer. Phylogenetic analysis according to the neighbour-joining

Abbreviation: ITS, internal transcribed spacer.

The GenBank/EMBL/DDBJ accession numbers for the 26S rDNA D1/D2 domain and ITS sequences of JCM 16989^T are HM222927 and HM222926, respectively.

A supplementary figure is available with the online version of this paper.

method (Saitou & Nei, 1987) was performed with MEGA 4.0.2 software (Tamura *et al.*, 2007). A set of 38 taxa which included the major clades in the Tremellales (Scorzetti *et al.*, 2002) was used to construct phylogenetic trees. Data consistency was tested by bootstrapping the alignments with 1000 replicates with correction for multiple substitutions. The quinone profile was determined by TechnoSuruga Laboratory (Shizuoka, Japan). Extraction and purification of the coenzyme Q system was performed according to Yamada & Kondo (1973). HPLC (Waters 600 series) was used to identify coenzyme Q.

Species delineation and classification

Sequence comparison of the ITS and D1/D2 domains of the three isolates showed high levels of similarity and confirmed their conspecificity. A similarity search of these regions using BLAST indicated that the isolated strains represent a novel yeast species.

Tremella species are found in four main clades in the phylogenetic trees constructed based on ITS and D1/D2 regions (Scorzetti *et al.*, 2002). The three strains of *Cryptococcus yokohamensis* sp. nov. clustered in the Fuciformis clade as shown in trees generated for the D1/D2 (Fig. 1) and ITS (Supplementary Fig. S1 available in IJSEM Online) regions. The strains appear to be related to *Tremella* species, similar to *Cryptococcus skinneri*, which is in the Foliacea clade (Scorzetti *et al.*, 2002). Strains of the novel yeast species were isolated from the trunk of trees and the nostril of a koala bear. The latter isolation might be fortuitous and the result of contamination of the animal from its feeding site. Many other *Tremella* species have been recovered from trees.

Phenotypic characteristics of this yeast are consistent with the current phenotypic characteristics of the genus *Cryptococcus* (Fell & Statzell-Tallman, 1998), i.e. utilization of D-glucuronate, production of extracellular starch, the absence of fermentation, and Q-9 and Q-10 as major coenzymes. Moreover, the novel yeast is distinct from other basidiomycetous genera in the absence of ballistoconidia (*Udeniomyces*, *Bensingtonia*, *Sporobolomyces* and *Bullera*) or by the absence of hyphae (*Trichosporon*, *Moniliella* and *Hyalodendron*). In addition, buds of the novel yeast are not presented on elongated stalks unlike some basidiomycetes (*Kockovaella*, *Sterigmatosporidium*, *Fellomyces*, *Tsuchiyaea*, *Kurtzmanomyces* and *Sterigmatomyces*). Moreover, the isolates differ from *Phaffia* species by the absence of fermentation and from *Rhodotorula* species by production of starch-like compounds and assimilation of myo-inositol. Therefore, despite their relatedness to *Tremella* species, the anamorphic isolates were assigned to the genus *Cryptococcus* based on the key identification features of basidiomycetes, morphological characterization of the type strain and the absence of any sexual structures of Tremellales formed during mating tests for 30 days of incubation at 28 °C (data not shown).

It is noteworthy that even though *C. yokohamensis* sp. nov. shares most of the phenotypic properties of the genus *Cryptococcus*, i.e. possesses a capsule, utilizes D-glucuronate

and produces extracellular starch, it has two major coenzymes, Q-9 (38.1 %) and Q-10 (61.9 %). This has not been reported previously in any tremellaceous *Cryptococcus* species, whereas *Tremella mesenterica* and *Tremella coalescens* are known to have both Q-9 and Q-10 (Vancanneyt *et al.*, 1994). Furthermore, the ability of our novel strains to grow with 0.1 % cycloheximide is also found in some *Tremella* species but is rarely observed in *Cryptococcus* species (Barnett *et al.*, 2000). Although the novel isolates are phenotypically best accommodated in the genus *Cryptococcus*, other unique properties may warrant further taxonomic consideration and classification in a new genus. Therefore, the discovery of new related strains would be desirable to complete the sexual cycle and the description of this novel species.

Latin diagnosis of *Cryptococcus yokohamensis* Alshahni, Satoh & Makimura sp. nov.

In medio extracto fermenti et extracto malti confecto, post dies tres as 28 °C, cellulae incapsulatae, globosae ad cylindricae (1.7–2.4 × 2.4–3.5 µm). In agar malti post dies decem ad 28 °C, striis coloro, glabra, nitida, mucosa, margo integra. Ballistoconidia nulla. Pseudohyphae nullae. Non fermentat. Assimilat sic: glucosum, D-galactosum, L-sorbosum, saccharosum, maltosum, cellobiosum, trehalosum, raffinose, melezitosum, inulin (exiguo), amylo solubile, D-xylosum, L-arabiosum, D-arabiosum, D-ribosum, L-rhamnosum (exiguo), N-acetyl-D-glucosaminum, ethanolum, erythritolum, ribitolum, galactitolum, D-mannitolum, methyl α-D-glucosidum, salicinum, acidum gluconicum, acidum succinicum, acidum citricum, inositolum (exiguo), D-glucuronatum et D-glucitolum; non respondent lactosum, melibiosum, methanolum, glycerolum, acidum DL-lacticum, hexadecanum nec 2-ketogluconatum. L-Lysinum, cadaverinum, ethylaminum et ammonium sulfatum assimilantur at non natrium nitrosum nec kalium nitricum. In medio sine vitaminis externis non crescit. Augmentum in 28 °C; non crescit in 42 °C. Materia amyloidea iodophila formantur. Reactiones diazonium caeruleum B et ureasii positivae. Crescere potest in medio 0.1 % cycloheximido addito et 10 % NaCl + 5 % glucose, non respondet 50 % (w/w) GY medio. Ubiquinonum majus: Q-9 et Q-10. Typus stirps JCM 16989^T ex arbor, Japonia, isolata est. In collectionibus culturarum quas Japan Collection of Microorganisms, Wako, Saitama, sustentant, no. JCM 16989^T, Teikyo Institute of Medical Mycology, Hachioji, Tokyo, sustentant, no. TIMM 10001^T, Centraalbureau voor Schimmelcultures, Utrecht, Neerlandia, sustentant, no. CBS 11776^T, et Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany sustentant, no. DSM 23671^T, deposita est.

Description of *Cryptococcus yokohamensis* Alshahni, Satoh & Makimura sp. nov.

Cryptococcus yokohamensis (yo.ko.ha.men'sis. N.L. masc. adj. *yokohamensis* of or belonging to Yokohama, referring to the name of the place where Kanazawa Zoological Park is located and the isolates were obtained).

Fig. 1. Phylogenetic tree drawn from neighbour-joining analysis of 26S rDNA D1/D2 domain sequences depicting the relationships of *Cryptococcus yokohamensis* sp. nov. with closely related species. Numbers on branches are bootstrap percentages ($\geq 50\%$). *Cryptococcus marinus* CBS 5235^T is the outgroup. Bar, 2 substitutions per 100 nucleotide positions.

After 3 days in 5% malt extract at 28 °C, cells are capsulated, globose to elongate (1.7–2.4 × 2.4–3.5 µm). Polar budding is observed and cells occur as parent bud pairs (Fig. 2). After 10 days on YM agar at 28 °C, colonies are glistening, slimy, mucoid and tan with an entire margin. Pseudohyphae are not formed in Dalmau plate cultures on cornmeal. On

cornmeal agar, ballistoconidia are not formed. Fermentation is absent. The following carbon compounds are assimilated: glucose, galactose, L-sorbose, sucrose, maltose, cellobiose, trehalose, raffinose, melezitose, inulin (weak), soluble starch, xylose, L-arabinose, D-arabinose, ribose, L-rhamnose (weak), N-acetyl-D-glucosamine, ethanol, erythritol, ribitol, galactitol,

Fig. 2. Scanning electron micrograph of cells indicating polar budding of globose to elongated cells. Cells were grown on YM agar for 2 days before being processed for scanning electron microscopy. Bar, 1 µm.

mannitol, methyl α -D-glucoside, salicin, D-gluconate, succinate, citrate, inositol (weak), D-glucuronate and sorbitol. The following are not assimilated: lactose, melibiose, methanol, glycerol, DL-lactate, hexadecane and 2-keto-D-gluconate. L-Lysine, cadaverine, ethylamine and ammonium sulphate are utilized as nitrogen sources, but not sodium nitrite or potassium nitrate. Growth in vitamin-free medium is negative. Optimal growth temperature is 28 °C; delayed growth occurs at 37 °C but no growth is seen at 42 °C. Starch-like compounds are formed. Urease activity is positive. Diazonium blue B reaction is positive. Growth is observed in the presence of 0.1 % (w/v) cycloheximide and in 10 % sodium chloride/5 % glucose media, but not in 50 % glucose. Major ubiquinones are Q-9 and Q-10.

The type strain is JCM 16989^T (=TIMM 10001^T=DSM 23671^T=CBS 11776^T), isolated from the trunk of a *Eucalyptus* tree in Kanazawa Zoological Park (Yokohama, Japan).

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