Cryptococcus surugaensis sp. nov., a novel yeast species from sediment collected on the deep-sea floor of Suruga Bay

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A novel species of the genus *Cryptococcus* was isolated from sediment collected on the deep-sea floor of Suruga Bay, Japan. Nucleotide sequences of 18S rDNA, internal transcribed spacers, 5·8S rDNA and the D1/D2 region of 26S rDNA of strain SY-260^T suggested affinities to a phylogenetic lineage that includes *Cryptococcus luteolus*. Comparisons of the rDNA sequences of each region clarified that strain SY-260^T is related distantly to *Bullera coprosmaensis* and *Bullera oryzae*, but is distinct at the species level. As ballistoconidia and sexual reproduction were not observed in strain SY-260^T, this strain is described as *Cryptococcus surugaensis* sp. nov. (type strain, SY-260^T = JCM 11903^T = CBS 9426^T).

Members of the genus *Cryptococcus* are reported commonly among yeasts isolated from sea water (e.g. Yamasato *et al.*, 1974; Fell, 1976; Hagler & Ahearn, 1987). This anamorphic genus in the Hymenomycetes is characterized mainly by the assimilation of inositol and D-glucuronate and production of starch (Fell & Statzell-Tallman, 1998), but is known to be polyphyletic (Takashima & Nakase, 1999; Fell *et al.*, 2000). We isolated *Cryptococcus* strains in a survey of yeasts from various deep-sea floors in the north-west Pacific Ocean (Nagahama *et al.*, 2001a). A strain from sediment collected at a depth of 2406 m in Suruga Bay (34° 36′ 55 N, 138° 34′ 77 E; temperature, 3 °C) is described here as a novel yeast species of the genus *Cryptococcus*.

Sample collection and isolation

Samples were collected from Suruga Bay by using an unmanned submersible vessel without contamination by open water, as described previously (Takami *et al.*, 1997; Nagahama *et al.*, 2001a). Yeasts were isolated from fresh deep-sea sediments and cultured on YM agar, 1/5 YM agar, potato dextrose agar, cornmeal agar or marine agar (all from Difco), dissolved in artificial sea water supplemented with 0·01 % chloramphenicol. Agar plates were incubated at low temperature (5–10 °C) for the first 2 weeks and then at 20 °C for 1 month.

Physiological and biochemical characteristics

Strains were characterized morphologically and physiologically by using standard methods with some modifications (Yarrow, 1998). Assimilation of nitrogen compounds was examined on solid media by using a starved inoculum (Nakase & Suzuki, 1986). Vitamin requirements were investigated according to the method of Komagata & Nakase (1967). Ubiquinones were extracted by the method of Yamada & Kondo (1973) with slight modifications and determined by HPLC as described previously (Hamamoto & Nakase, 1995). DNA was extracted and purified following the procedure described by Hamamoto & Nakase (1995) and DNA base composition was determined by using the HPLC method of Tamaoka & Komagata (1984).

Phylogenetic analysis

DNA extraction for PCR was performed by using a QIAamp DNeasy Tissue kit (Qiagen) with some modifications (Nagahama *et al.*, 2001b). Primers used for amplification and sequencing of the 18S rDNA, 5·8S rDNA and internal transcribed spacer (ITS) regions were those described by White *et al.* (1990); primers for the D1/D2 region of 26S rDNA were those described by Fell *et al.* (2000). PCR products were purified by using ExoSAP-IT (USB) and sequenced by using a model 4000L (LI-COR) or MegaBACE 1000 (Pharmacia) DNA sequencer.

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Abbreviations: ITS, internal transcribed spacer; ML, maximum-likelihood.

The GenBank/EMBL/DDBJ accession number for the 18S rDNA, D1/D2 region of 26S rDNA and internal transcribed spacer sequences of SY-260^T is AB100440.

All sequences were aligned by using CLUSTAL W 1.81 (Thompson *et al.*, 1994) and adjusted manually. Positions where one or more species contained a length mutation and a region that was aligned ambiguously were not included in subsequent phylogenetic analysis. Phylogenetic trees were constructed by utilizing the maximum-likelihood (ML) method (Felsenstein, 1981) with the optimal Ts/Tv ratio of the HKY85 model (Hasegawa *et al.*, 1985), estimated from the neighbour-joining tree (Saitou & Nei, 1987) in PAUP 4.0b8 (Swofford, 1998). This tree was derived by a heuristic search with random stepwise addition of 100 replicates. Robustness of branches in the tree was evaluated by bootstrap analysis (Felsenstein, 1985) with 100 resamplings.

Sequences of the 18S rDNA, 26S rDNA, 5·8S rDNA and ITS regions of SY-260^T were deposited in GenBank/DDBJ under accession number AB100440.

Phylogenetic position of strain SY-260^T isolated from deep-sea sediment

We sequenced a DNA fragment of strain SY-260^T that included 18S rDNA, ITS1, 5·8S rDNA, ITS2 and the D1/D2 region of 26S rDNA. This sequence had some affinity to that of species in the *Cryptococcus luteolus* lineage (Takashima & Nakase, 1999) or *Luteolus* clade (Scorzetti *et al.*, 2002).

The relationship between members of this group and strain SY-260^T was estimated, based on 2077 bp in the 18S rDNA, 5·8S rDNA and ITS regions (Fig. 1). Overall, this branching order was statistically robust and consistent with those based on 18S rDNA in the phylogenetic tree drawn by Bai *et al.* (2001a, b). Species used in this study were divided into the subclades *Luteolus*, *Mrakii* and *Dioszegia* (Fig. 1), which were supported strongly by bootstrap values. Strain SY-260^T was obviously placed into the subclade *Luteolus*. Strain SY-260^T and two *Bullera* species, *Bullera coprosmaensis* and *Bullera oryzae*, formed a cluster that was separate from the other members of the subclade *Luteolus*. The subclade *Mrakii*, which consists of eight *Bullera* species, is relatively coherent with the subclade *Luteolus*. These two subclades appeared to be related to each other and were separate from the subclade *Dioszegia*.

The phylogenetic tree of species in the *Luteolus* subclade with nine undescribed species of *Cryptococcus* based on 603 bp in the D1/D2 region of the 26S rDNA sequences (Fig. 2) had few branches that were supported strongly by bootstrap values and appeared to be relatively ambiguous for the 18S–ITS tree drawn above. Two species with identical sequences, *Bullera derxii* and *Bullera sinensis* var. *sinensis*, differed from *B. sinensis* var. *lactis* at only one base, although these three species were well-differentiated based on 18S rDNA and ITS sequences. The relationship between strain SY-260^T, *B. coprosmaensis* and *B. oryzae* was uncertain in this tree, in contrast to that in the 18S–ITS tree. This disagreement seemed to be caused by the remarkably long lineage of *Cryptococcus* sp. CBS 8369, as a tree that excluded CBS 8369 supported the cluster of strain SY-260^T with the two *Bullera* species. In the nine strains of undescribed *Cryptococcus* species, CBS 8356 and CBS 8367 were identical to *Bullera kunmingensis*. The seven remaining strains were divided into three groups and each appeared to qualify as a novel species.

The closest species to strain SY-260^T in each rDNA sequence region were not identical. They were *B. oryzae* in the 18S, 5·8S rDNA and ITS2 regions, *B. coprosmaensis* in the ITS1

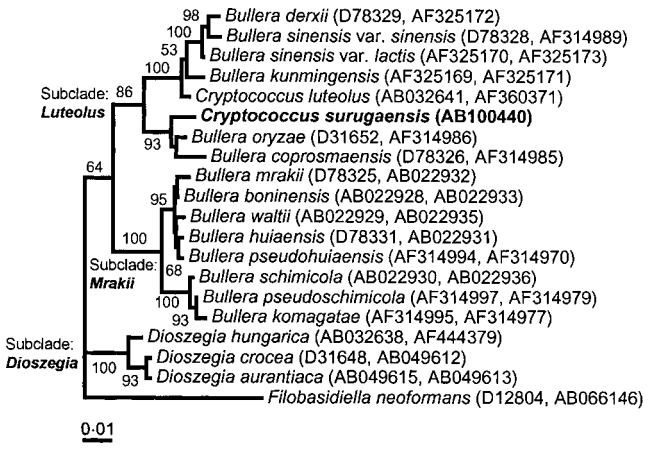

Fig. 1. Phylogenetic tree within the *C. luteolus* lineage on the basis of 2077 aligned nucleotide sites in 18S rDNA, ITS and 5·8S rDNA. The ML tree with the HKY85 model (Ts/Tv, 1·175931) was constructed as described in the text. Numbers are bootstrap values for nodes supported by >50% (100 replicates). Bar, 0·01 substitutions per site.

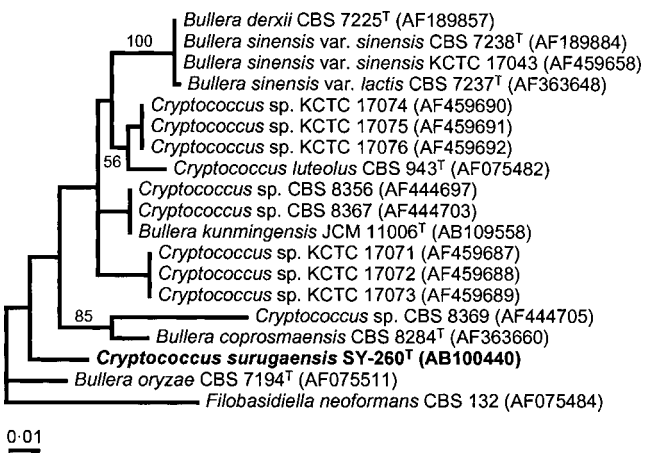

Fig. 2. Phylogenetic tree of *C. surugaensis* and related species on the basis of 603 aligned nucleotide sites in the D1/D2 region of 26S rDNA. The ML tree with the HKY85 model (Ts/Tv, 3·136150) was constructed as described in the text. Numbers are bootstrap values for nodes supported by >50% (100 replicates). Bar, 0·01 substitutions per site.

region and *B. sinensis* var. *lactis* in the D1/D2 region. Nucleotide substitutions of 0·6 % in the 18S and 5·8S rDNA, 13·4 % in the ITS1, 10·5 % in the ITS2 and 4·5 % in the D1/D2 regions between strain SY-260^T and its most closely related species indicated that this strain is sufficiently separated from known species. Differences of <1 % in the D1/D2 region or of 1–2 % in ITS sequences were generally recognized to correspond to the borderline between conspecific and different species (Fonseca *et al.*, 2000; Fell *et al.*, 2000; Bai *et al.*, 2001a, 2001b; Hamamoto *et al.*, 2002; Nagahama *et al.*, 2003).

Comparison of physiological and biochemical characteristics between strain SY-260^T and related species

Phylogenetically, the species most closely related to strain SY-260^T was considered to be *B. oryzae* (Fig. 1) but their DNA G+C contents had differences of >10 %, whereas *B. coprosmaensis* had a DNA G+C content similar to that of strain SY-260^T. The evolutionary distances among these three species appear to be too great to estimate relationships based on G+C contents. Strain SY-260^T can be discriminated physiologically from *B. coprosmaensis* and *B. oryzae*, based on the assimilation of L-sorbose and soluble starch. As ballistoconidia and sexual reproduction were not observed in strain SY-260^T, we describe this strain as a novel species of the genus *Cryptococcus*.

Latin diagnosis of *Cryptococcus surugaensis* sp. nov. Nagahama, Hamamoto *et* Nakase

In medio liquido YM *post* 3 *dies ad* 25 °C, *cellulae ovoideae vel ellipsoidae* (2–4 × 2–7 μm), *singulae aut binae. Post unum mensem pellicula fragilis et sedimentum formantur. Cultura in agaro* YM *ad* 25 °C, *subflava, nitida, mollis et margine glabra. Hyphae et pseudohyphae non formantur. Fermentatio nulla. Glucosum, galactosum,* L-*sorbosum, saccharosum, maltosum, cellobiosum, trehalosum, melibiosum, raffinosum, melezitosum,* D-*xylosum,* L-*arabinosum,* D-*arabinosum,* D-*ribosum,* L-*rhamnosum, glycerolum, erythritolum, ribitolum* (*exiguum*), *galactitolum,* D-*mannitolum,* D-*glucitolum* (*exiguum*), *methyl-α-*D-*glucosidum, salicinum* (*exiguum*), *glucono-δ-lactonum, acidum* 2-*ketogluconicum, acidum* 5-*ketogluconicum, acidum* DL-*lacticum, acidum succinicum, acidum citricum et inositolum assimilantur, at non lactosum, inulinum, amylum solubile, ethanolum, acidum* D-*glucuronicum nec acidum* D-*galacturonicum. Ethylaminum, lysinum et cadaverinum assimilantur at non kalium nitricum nec natrium nitrosum. Maxima temperatura crescentiae*: 31–34 °C. *Ad crescentiam vitaminum non necessarium est. Materia amyloidea iodophila formantur.* G+C *acidi deoxyribonucleati* 48·3 mol% (*per* HPLC). *Ubiquinonum majus* Q-10.

Typus stirps SY-260^T *ex sedimentum*, Suruga Bay, Japan, *isolata est. In collectionibus culturarum quas* Japan Collection of Microorganisms, Wako, Saitama, Japan, *sustentant*, no. JCM 11903^T (=CBS 9426^T) *deposita est.*

Description of *Cryptococcus surugaensis* sp. nov. Nagahama, Hamamoto & Nakase

Cryptococcus surugaensis (su.ru.ga.en′sis. N.L. masc. adj. *surugaensis* referring to the geographical origin of the species).

In YM broth (Difco) after 3 days culture at 25 °C, cells are ovoidal to ellipsoidal (2–4 × 2–7 μm) and occur singly or in parent-bud pairs. A sediment and fragile pellicle are formed after 1 month. After 1 month on YM agar at 25 °C, streak culture is light yellow, glistening, soft and has an entire margin. In Dalmau plate cultures on cornmeal agar (Difco), no branching hyphae or pseudohyphae are formed. Fermentation ability is negative. The following carbon compounds are assimilated: D-glucose, galactose, L-sorbose, sucrose, maltose, cellobiose, trehalose, melibiose, raffinose, melezitose, D-xylose, L-arabinose, D-arabinose, D-ribose, L-rhamnose, glycerol, erythritol, ribitol (weak), galactitol, D-mannitol, D-glucitol (weak), methyl α-D-glucoside, salicin (weak), glucono-δ-lactone, 2-ketogluconic acid, 5-ketogluconic acid, DL-lactic acid, succinic acid, citric acid and inositol; no growth occurs on lactose, inulin, soluble starch, ethanol, D-glucuronic acid or D-galacturonic acid. The nitrogen compounds ethylamine, lysine and cadaverine are assimilated. No growth occurs on potassium nitrate or sodium nitrite. Maximum temperature for growth is 31–34 °C. Vitamins are not required for growth. No growth occurs on 50 % glucose/yeast extract agar. Growth occurs in the presence of 100 p.p.m. cycloheximide. Growth in the presence of 10 % sodium chloride is negative. Starch-like substances are produced. Diazonium blue B reaction is positive. Urease activity is positive. G+C content of nuclear DNA is 48·3 mol% (by HPLC). Major ubiquinone is Q-10.

The type strain of *C. surugaensis*, SY-260^T, was isolated from sediments collected from the deep-sea floor of Suruga Bay, Japan. This strain has been deposited in the Japan Collection of Microorganisms, Saitama, Japan, as JCM 11903^T (=CBS 9426^T).

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