Cryptococcus taeenensis sp. nov., a new anamorphic basidiomycetous yeast isolated from a salt farm

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Cryptococcus taeenensis, a new anamorphic yeast species originating from a salt farm on the Taeon peninsula in Korea, is described. Strain 3-12^T grew by budding, contained ubiquinone Q-10 and xylose in cell hydrolysates, utilized D-glucuronate and did not ferment D-glucose. A molecular phylogenetic analysis based on the large-subunit rRNA D1/D2 domain and ITS region sequences placed *C. taeenensis* near *Auriculibuller fuscus* and *Bullera japonica*, recently proposed taxa of the Tremellales. However, these species were distinguishable based on standard physiological tests used for yeast identification, with characteristics including the assimilation of L-sorbose, absence of ballistoconidia, no arbutin hydrolysis and no growth in the presence of 0.01 % cycloheximide. The isolate exhibited the typical physiology of the genus *Cryptococcus* Vuillemin, but its large-subunit rRNA D1/D2 domain sequence was clearly distinct from previously described species in the genus. Therefore, on the basis of these results, *Cryptococcus taeenensis* sp. nov. is proposed; the type strain is 3-12^T (=KCTC 17149^T=CBS 9742^T).

The genus *Cryptococcus* Vuillemin is characterized by the lack of sexual spores and ballistoconidia, utilization of D-glucuronate, absence of fermentative ability, presence of xylose in cell hydrolysates, positive Diazonium blue B and urease reactions and the possession of coenzyme Q-9 or Q-10 (Fell & Statzell-Tallman, 1998). Members of the genus are found in a wide variety of habitats, and the genus has been recognized as markedly heterogeneous on the basis of a number of biochemical and molecular studies (Fell *et al.*, 1995; Fell & Statzell-Tallman, 1998; Vancanneyt *et al.*, 1994). More recently, several novel *Cryptococcus* strains isolated from plants and soil appear to be phylogenetically close to the *Cryptococcus laurentii* complex (Golubev *et al.*, 2003; Sato *et al.*, 1999; Scorzetti *et al.*, 2002; Vishniac, 2002).

During a survey of the yeast community associated with salt farms in Korea, many basidiomycetous yeast strains were isolated from soil collected from the Taeon peninsula and characterized by partial 26S rRNA gene sequencing. Comparative partial 26S rRNA gene sequence analysis showed that one strain, designated 3-12^T, exhibited a

specific phylogenetic association with taxa related to the Tremellales, while also displaying over 2 % sequence divergence from established species. A further comparison of the phenotypic properties of the new isolate and its levels of sequence similarity with *Cryptococcus* species revealed that the isolate should be assigned to a novel species, for which the name *Cryptococcus taeenensis* sp. nov. is proposed.

Strain 3-12^T was isolated from a soil sample collected from a salt farm on the Taeon peninsula in Korea. One gram of each soil sample was suspended in 30 ml sterile water and stirred for 10 min, then 100 µl of the suspension was spread onto YM agar (Difco) supplemented with 5 µg gentamicin ml⁻¹ and 16 µg chloramphenicol ml⁻¹. Next, the strain was purified by streaking an isolated colony onto the YM agar without antibiotics and incubating at 25 °C. Thereafter, the strain was maintained at 4 °C; the cell suspensions were stored at -80 °C in broth cultures supplemented with 10 % (w/v) glycerol, for long-term maintenance. The methods used to determine the morphological, physiological and biochemical properties were as described by Yarrow (1998). All assimilation tests were performed twice, in separate experiments. The results were read after 1 and 3 weeks incubation. Coenzyme Q was prepared and analysed as described by Nakase & Suzuki (1988). The presence of xylose in the cell wall was investigated by a TLC analysis, as described by Komagata & Suzuki (1987). Isolation of

The GenBank/EMBL/DDBJ accession numbers for the 26S rRNA D1/D2 domain and ITS region sequences of *Cryptococcus taeenensis* 3-12^T are AY422719 and AY686645, respectively.

A phylogenetic tree based on analysis of ITS region (including 5.8S rRNA gene) sequences showing the position of *C. taeenensis* 3-12^T in relation to taxa of the Tremellales is available in IJSEM Online.

genomic DNA and PCR amplification of the D1/D2 domain of the 26S rRNA gene were performed using the protocols developed by Kurtzman & Robnett (1998). The primers used to amplify and sequence the 5.8S rRNA and ITS regions were those described by White *et al.* (1990). The amplified fragments were purified by using a QIAquick PCR purification kit (Qiagen); then, the purified fragment was sequenced directly by using an ABI PRISM BigDye Terminator Cycle Sequencing FS Ready Reaction kit (PE Biosystems) and analysed with an ABI PRISM 310 Genetic Analyser (PE Biosystems).

The resultant sequences were compared with those of reference organisms retrieved from the GenBank database. The sequences were aligned automatically by using the multiple-sequence alignment program CLUSTAL W (Thompson *et al.*, 1994), then corrected manually. Phylogenetic analyses were performed with the PHYLIP 3.5c package (Felsenstein, 1993), and the evolutionary distances were calculated by using the program DNADIST according to the algorithm of Jukes & Cantor (1969). A phylogenetic tree was constructed by using the neighbour-joining method and Jukes–Cantor correction (Jukes & Cantor, 1969). The tree was visualized with the program TreeView (Page, 1996), and the bootstrap analysis was done based on 1000 resamplings. *Tremella encephala* CBS 6968 and *Fellomyces polyborus* CBS 6072^T were the designated outgroup in the analysis. Other related sequences were obtained from GenBank.

In the present study, a total of 74 strains was isolated from three soil samples collected at the Chunghwa salt farm, Taean peninsula, Korea in November 2002. The samples were all collected during a rest period from salt production. The salinity, temperature and pH values of the soil samples were 10–15 ‰, 10–15 °C and 8.2–8.5, respectively. Sequencing of the D1/D2 domain of the 26S rRNA gene was performed for all the isolates. The majority of the isolates

(*n* = 43) belonged to the genus *Cryptococcus* or *Rhodospodium*, while the rest were identified as *Aureobasidium* (*n* = 8), *Bullera* (*n* = 2), *Bulleromyces* (*n* = 2), *Dioszegia* (*n* = 3), *Pseudogymnoascus* (*n* = 1), *Pseudozyma* (*n* = 5), *Rhodotorula* (*n* = 7), *Sporisorium* (*n* = 2) and *Sterigmatomyces* (*n* = 1) species. In the course of identification, one strain, 3-12^T, belonging to the genus *Cryptococcus* was characterized further due to its unique large-subunit rRNA D1/D2 domain sequence. This isolate grew over a temperature range of 10–30 °C and in the presence of 10 % NaCl + 5 % glucose; it was, also, a mesophile and moderately halotolerant yeast, in agreement with the temperature and salinity of its salt-farm habitat. A solar saltern consists of shallow ponds connected in a sequence of increasingly saline brines, and it is used for the commercial production of salt from sea water. As such, with regard to this ecosystem, the isolate seemingly originated from the coastal marine environment and would appear to have adapted to or survived in a salt-farm environment.

The phylogenetic analysis based on the D1/D2 domain sequence showed that *C. taeanensis* belongs to the *Bulleromyces* clade in the Tremellales (Fig. 1) that includes species able to produce ballistospores together with species that do not present this characteristic (Scorzetti *et al.*, 2002). *C. taeanensis* was also found to form a distinct lineage with *Auriculibuller fuscus* and *Bullera japonica*, with a 77 % bootstrap confidence level, in contrast to other *Cryptococcus* species. The closest relatives of *C. taeanensis* were *A. fuscus* and *B. japonica*, which are ballistoconidia-forming species, as described in a recent study by Sampaio *et al.* (2004). However, when testing the ability of *C. taeanensis* to produce ballistoconidia with various media (malt agar, potato dextrose agar, corn meal agar and morphology agar), no ballistoconidia were observed. Moreover, *C. taeanensis* did not produce a brownish pigment when grown on potato

Fig. 1. Dendrogram showing phylogenetic relationship between *Cryptococcus taeanensis* sp. nov. 3-12^T (=KCTC 17149^T=CBS 9742^T) and type strains of related species of the *Bulleromyces* clade (Tremellales), based on 26S rRNA D1/D2 domain sequences. Bootstrap values were calculated from 1000 trees and values less than 50 % were omitted. GenBank/EMBL/DDBJ accession numbers are given in parentheses. Bar, nucleotide substitutions per site. Outgroup species were *Tremella encephala* CBS 6968 and *Fellomyces polyborus* CBS 6072^T.

dextrose agar, in contrast to *A. fuscus* and *B. japonica*. In a comparison of the 26S rRNA D1/D2 domain sequences, *C. taeanensis* exhibited 12 base substitutions among 607 nucleotide positions with *A. fuscus* and 14 base substitutions with *B. japonica*.

To study the phylogenetic position of *C. taeanensis* more precisely, the ITS region (including 5.8S rRNA gene) sequence was compared with published sequences of strains related to *C. taeanensis*. The resulting phylogenetic relationships based on the ITS sequences confirmed a close phylogenetic relationship among *C. taeanensis*, *A. fuscus* and *B. japonica* (see Supplementary Fig. A available in IJSEM Online). In addition, the close relationship among *Papiliotrema bandonii*, *A. fuscus* and *B. japonica* shown in Supplementary Fig. A agrees well with that described by Sampaio *et al.* (2004). *C. taeanensis* differed from *A. fuscus* by 45 base substitutions and nine indels in the ITS region, while it differed from *B. japonica* by 49 base substitutions and 10 base indels.

Scorzetti *et al.* (2002) recommended a combined sequence analysis of the D1/D2 domain and ITS region for the species identification of basidiomycetous yeasts. As such, the sequence similarities of both the D1/D2 domain and the ITS region between the isolate and related taxa were less than 99 %, thereby indicating distinct species.

Based on the conventional criteria examined, as well as the molecular phylogenetic analysis, the novel isolate was assigned to the anamorphic basidiomycetous yeast genus *Cryptococcus* Vuillemin (Fell & Statzell-Tallman, 1998). However, the novel isolate did not split arbutin and was unable to grow in the presence of 0.01 % cycloheximide, in contrast to *A. fuscus* and *B. japonica*, its nearest phylogenetic neighbours. In addition, *C. taeanensis* was distinct from *A. fuscus* based on its ability to assimilate L-sorbose as a sole carbon source (Sampaio *et al.*, 2004). Therefore, the presented results demonstrate that the isolate was distinguishable from its relatives based on its D1/D2 domain and ITS region sequences and physiological characteristics. Accordingly, it is concluded that the novel isolate should be assigned to a novel species of the genus *Cryptococcus*, *Cryptococcus taeanensis* sp. nov.

Latin diagnosis of *Cryptococcus taeanensis* Shin et Park sp. nov.

In medio agaro YM post dies 3 ad 25 °C cellulae vegetative sphaeroidae vel ovoideae (4.0–6.3 et 2.6–3.6 µm), singulae aut binae. Post unum mensem ad 25 °C, annulus, pelliculum et sedimentum formatur. Cultura in agaro YM post dies 3 (25 °C) parva, glabra, butyrosa, nitida, crenea, et margine glabra. Pseudohyphae nullae. Assimilantur D-glucosum, D-galactosum, L-sorbosum, D-glucosaminum, D-ribosum, D-xylosum, L-arabinosum, D-arabinosum, L-rhamnosum, sucrosum, maltosum, α,α-trehalosum, methyl α-D-glucosidum, cellobiosum, salicinum, melibiosum, lactosum, raffinsum, melezitum, amyllum solubile (exiguum), glycerolum (lente),

erythritolum, ribitolum, xylitolum, L-arabitolum, D-glucitolum, D-mannitolum, galactitolum, myo-inositolum, glucono-δ-lactonum, acidum 2-keto-D-gluconicum, acidum 5-keto-D-gluconicum (vel exigue), acid gluconicum, acidum glucuronicum, acidum galacturonicum, acidum DL-lacticum, acidum succinicum, acidum citricum (vel exigue), acidum saccharicum, acidum galactonicum (lente), et N-acetyl-D-glucosaminum. Non assimilantur inulinum, methanolum, ethanolum, propane-1,2-diolum, butano-2,3-diolum, et acidum quini. Assimilantur ethylaminum, L-lysinum, cada-verinum, glucosaminum, D-tryptophanum (lente) et prolinum. Non assimilantur kalii nitratum, natrium nitrosum, creatinum, creatininum, et imidazolum.

Vitaminae externae ad crescentiam necessariae sunt. Non crescit in medio 0.01 % cycloheximido addito. Non crescit in medio 1 % acido acetico addito. Cresceret potest in 10 % NaCl + 5 % glucoso. Non cresceret potest in 16 % NaCl + 5 % glucoso. Crescit in medio cum 50 % glucoso. Materia amyloidea iodophila formantur. Diazonium caeruleum B: positivum. Ureum hydrolysatur. 10–30 °C (exiguum) crescit neque 35 °C. Ubiquinonum primum: Q-10. Xylosum in cellulis presens. Typus KCTC 17149^T (= CBS 9742^T) isolatus ex saltern, Taean in Korea, praeservatus in Korean Collection for Type Cultures, Taejon, Korea.

Description of *Cryptococcus taeanensis* Shin & Park sp. nov.

Cryptococcus taeanensis (tae.an.en'sis. N.L. adj. *taeanensis* pertaining to Taean, Korea, where the type strain was isolated).

In YM liquid medium after 3 days at 25 °C, cells are spheroid to ovoid, 4.0–6.3 µm long by 2.6–3.6 µm wide, and occur singly or in pairs. After 1 month at 25 °C, pellicles and sediment are observed. On YM agar after 3 days at 25 °C, the colonies are smooth, butyrous, glistening and cream-coloured with an entire margin. No mycelium or pseudo-mycelium is formed in Dalmau plate cultures on corn meal agar after 2 weeks.

D-Glucose, D-galactose, L-sorbose, D-glucosamine, D-ribose, D-xylose, L-arabinose, D-arabinose, L-rhamnose, sucrose, maltose, α,α-trehalose, methyl α-D-glucoside, cellobiose, salicin, melibiose, lactose, raffinose, melezitose, soluble starch (weak), glycerol (delayed), erythritol, ribitol, xylitol, L-arabitol, D-glucitol, D-mannitol, galactitol, myo-inositol, glucono-δ-lactone, 2-ketogluconic acid, 5-ketogluconic acid (or weak), D-gluconate, D-glucuronate, D-galacturonate, DL-lactate, succinate, citrate (or weak), saccharate, D-galactonate (delayed) and N-acetyl D-glucosamine are assimilated. Inulin, methanol, ethanol, propane-1,2-diol, butane-2,3-diol and quinic acid are not assimilated. Ethylamine hydrochloride, L-lysine, cadaverine, D-glucosamine, D-typtophan (delayed) and D-proline are utilized. Potassium nitrate, sodium nitrite, creatine, creatinine and imidazole are not utilized. Growth does not occur in vitamin-free medium. No growth in the presence of

0.01 % or 0.1 % cycloheximide. No growth in the presence of 1 % acetic acid. Growth occurs in YM broth containing 10 % (w/w) NaCl + 5 % glucose, but not with 16 % (w/w) NaCl + 5 % glucose. Weak growth occurs on 50 % glucose + yeast extract agar. The production of starch-like compounds is positive. Reaction to Diazonium Blue B is positive. Urease-positive. Growth at 10 °C is weak. Growth at 30 °C, but not at 35 °C. The major ubiquinone is Q-10. Xylose is present in the cells.

The type strain, 3-12^T (= KCTC 17149^T = CBS 9742^T), was isolated from a saltern in Taean, Korea and deposited in the Korean Collection for Type Cultures, Taejeon, Korea.

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