Cryptococcus keelungensis sp. nov., an anamorphic basidiomycetous yeast isolated from the sea-surface microlayer of the north-east coast of Taiwan

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Strain SN-82^T was isolated from the sea-surface microlayer at Keelung on the north-east coast of Taiwan. Sequence analysis of the D1/D2 domain of the 26S rDNA of strain SN-82^T suggested that this strain is related to the aerius clade in the Filobasidiales. Phenotypic characteristics such as the absence of sexual structures and ballistoconidia, the assimilation of *myo*-inositol and D-glucuronate, the inability to ferment glucose, the possession of coenzyme Q-10 and positive Diazonium blue B and urease reactions also indicated that this strain belongs to the genus *Cryptococcus*. However, divergences of more than 3.6% were observed in the D1/D2 domain when compared with other described *Cryptococcus* species, which indicated that the isolated yeast represents a previously unrecognized member of this genus. Therefore, the novel yeast species *Cryptococcus keelungensis* sp. nov. is proposed, with strain SN-82^T (=CBS 10876^T =BCRC 23107^T =JCM 14893^T) as the type strain.

Members of the genus *Cryptococcus* are commonly isolated from seawater (Yamasato *et al.*, 1974; Fell, 1976; Hagler & Ahearn, 1987; Nagahama *et al.*, 2003). This basidiomycetous yeast genus is characterized mainly by the lack of sexual spores and ballistoconidia, utilization of D-glucuronate, the absence of fermentative ability, the presence of xylose in cell hydrolysates, positive Diazonium blue B and urease tests and the possession of coenzyme Q-9 or Q-10 (Fell & Statzell-Tallman, 1998). The genus *Cryptococcus* is also known to be polyphyletic (Fell *et al.*, 2000; Scorzetti *et al.*, 2002) and its members are heterogeneous in terms of their nutritional abilities.

The sea-surface microlayer (SSML) is a thin film of liquid at the interface of the sea and the atmosphere and is of considerable importance in exchanges that occur between them. Conditions at the SSML are harsh, with high levels of visible and UV radiation and a high concentration of pollutants (Varnam & Evans, 2000). Therefore, the SSML has often been considered an extreme environment for micro-organisms and may contain unusual species and taxa (Maki, 2002).

Abbreviations: ITS, internal transcribed spacer; SSML, sea-surface microlayer.

The GenBank/EMBL/DDBJ accession numbers for the 26S rDNA D1/D2 and ITS region sequences of strain SN-82^T are EF621562 and EF621565, respectively.

During a survey of the yeast community associated with the SSML in Taiwan, strain SN-82^T was isolated together with more than 200 other yeast strains at Keelung on the north-east coast (25° 09' 27" N 120° 09' 22" E). Based on the sequences of the internal transcribed spacer (ITS) region and the 26S rDNA D1/D2 domain, these 200 isolates were identified as belonging to 36 yeast species of the genera *Aureobasidium* (1), *Candida* (10), *Cryptococcus* (5), *Hanseniaspora* (1), *Lodderomyces* (1), *Pichia* (3), *Pseudozyma* (1), *Rhodospiridium* (4), *Rhodotorula* (5), *Sporobolomyces* (1), *Sporidiobolus* (1), *Trichosporon* (1) and *Yarrowia* (2). Four strains isolated from the SSML might represent undescribed taxa. For the novel anamorphic basidiomycetous isolate SN-82^T, the name *Cryptococcus keelungensis* sp. nov. is proposed on the basis of its morphological, physiological and molecular characteristics.

Samples were collected in the morning during four field campaigns (November 2005, February 2006, May 2006 and August 2006). Seawater from the SSML was collected with a metal screen (Garrett, 1967) and a glass plate (Harvey & Burzell, 1972). One litre of each seawater sample was concentrated to 1 ml by centrifugation at 2000 *g* for 20 min. After serial dilution of the concentrated seawater samples, subsamples (100 µl each) were spread onto acidic yeast extract-malt extract (YM) agar plates (pH 4.5–5.0) (Difco) supplemented with 0.01% (w/v) chloramphenicol and then incubated at 25 °C in the dark. All of the colonies

that appeared on the plates over a period of 3–5 days were transferred to YM broth for growth. The strains were then purified by repeated streaking of an isolated colony onto YM agar followed by incubation at 25 °C. Thereafter, the strains were maintained at 4 °C. For long-term preservation, cell suspensions were stored at –80 °C in broth cultures that were supplemented with 30 % (w/v) glycerol. Isolated strains were characterized morphologically, physiologically and biochemically using standard methods for current yeast taxonomy (Yarrow, 1998). Ubiquinones were extracted and purified following the method of Yamada & Kondo (1973) and then determined by HPLC as described previously (Nakase & Suzuki, 1988). The DNA base composition was determined by HPLC after enzymic digestion of the DNA to deoxyribonucleosides as described by Tamaoka & Komagata (1984).

The 26S rDNA D1/D2 domain and ITS regions were amplified and sequenced by PCR using the primer pairs F63 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and LR3 (5'-GGTCCGTGTTTCAAGACGG-3') and ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'), respectively (Scorzetti *et al.*, 2002). The resulting sequences were compared with those of reference organisms that were retrieved from GenBank. Sequences for phylogenetic analysis were aligned automatically with the program CLUSTAL_X 1.83 (Thompson *et al.*, 1997). Phylogenetic and molecular evolutionary analyses were conducted with MEGA version 3.1 (Kumar *et al.*, 2004) and evolutionary distances were calculated using the neighbour-joining method with Kimura's two-parameter distance measure. Confidence values were estimated from bootstrap analyses of 1000 replicates. *Cryptococcus aquaticus* CBS 5443^T and *Mrakia gelida* CBS 5272^T were the designated outgroup in the analyses.

Phylogenetic analysis based on sequences of the 26S rDNA D1/D2 domain showed that the novel strain belongs to the aerius clade in the Filobasidiales (Fig. 1) as described by Fonseca *et al.* (2000) and Scorzetti *et al.* (2002). Within the aerius clade, the novel strain occupied a relatively isolated position with *Cryptococcus* sp. SDY 170 (3.6 % divergence; 6 substitutions, 16 gaps), *Cryptococcus* sp. SDY 235 and SDY 169 (3.8 % divergence; 7 substitutions, 16 gaps), *Filobasidium* sp. CBS 10190 (3.7 % divergence; 6 substitutions, 16 gaps), *Filobasidium* sp. CBS 10189 (3.7 % divergence; 6 substitutions, 17 gaps) and *Filobasidium* sp. CBS 10188 (4 % divergence; 7 substitutions, 17 gaps) and formed a subclade of its nearest relatives. Strain SN-82^T and an unnamed taxon, represented by strains isolated in Portugal from acid mine drainage (*Cryptococcus* sp. strains SDY 169, 170, 235; Gadanho *et al.*, 2006) and plant leaves (*Filobasidium* sp. strains CBS 10188, 10189, 10190), formed an independent cluster with other aerius strains in the Filobasidiales; strain SN-82^T has sufficiently different sequences to be considered a member of a separate species from these yeast strains. These different isolation sources also illustrate that members of the genus *Cryptococcus* are heterogeneous in terms of their nutritional abilities and have been isolated from diverse habitats. Physiologically, strain SN-82^T could be distinguished from these related taxa as indicated in Table 1. Based on these differences, it is concluded that strain SN-82^T represents a novel species, *Cryptococcus keelungensis* sp. nov.

Latin diagnosis of *Cryptococcus keelungensis* Chang et Liu sp. nov.

In medio agaro YM post dies 3 ad 25 °C, cellulae spheroidae vel ovoideae (3.3–5.9 × 2.9–4.4 µm), singulae aut binae. Cultura in agaro YM post dies 3 ad 25 °C, parva, glabra, nitida, cremea

Fig. 1. Neighbour-joining tree based on the D1/D2 divergent domain of large-subunit rDNA sequences of *Cryptococcus keelungensis* sp. nov. SN-82^T and related species. Bootstrap values (%) above 60 % from 1000 samples are shown. Accession numbers are given in parentheses. Bar, 0.01 substitutions per site. *Cryptococcus aquaticus* CBS 5443^T and *Mrakia gelida* CBS 5272^T were used as outgroups.

Table 1. Selected characteristics of strain SN-82^T and type strains of related *Cryptococcus* species

Strains: 1, strain SN-82^T; 2, *C. terreus* CBS 1895^T; 3, *C. phenolicus* CBS 8682^T; 4, *C. fuscescens* CBS 7189^T; 5, *C. aerius* CBS 155^T; 6, *C. terricola* CBS 4517^T. +, Positive; –, negative; w, weak; d, delayed; combinations of symbols indicate that two or more of the responses are observed. Data were obtained in this study.

Characteristic	1	2	3	4	5	6
Assimilation of:						
L-Sorbose	+	+	+	–	w	w/d/+
D-Glucosamine	+	w	+	–	w	w/d/+
D-Arabinose	+	+	+	–	+	w/d/+
L-Rhamnose	+	+	+	–	+	w/d/+
Sucrose	+	–	–	–	+	+
Methyl α -D-glucoside	+	–/d	+	–	w	w/d/+
Melibiose	–	–	–	–	+	w/d/+
Lactose	+	+	+	–	+	+
Raffinose	+	–	–	–	+	+
Melezitose	+	d	+	–	+	+
Starch	w	w/d/+	+	–	+	w/d/+
Glycerol	+	–	–	–	–	w/d/–
Erythritol	–	–	–	–	w	–
Ribitol	+	w/d/+	+	–	w	w/d/–
Xylitol	+	w/d/+	+	–	+	w/d/–
L-Arabitol	+	d	d	–	w	w/d/–
D-Glucitol	+	+	+	–	+	+
D-Mannitol	+	+	+	–	–	+
Galactitol	+	+	+	–	–	+
D-Glucono-1,5-lactone	+	+	–	–	+	+
D-Galacturonic acid	+	d	–	–	–	–
Succinate	+	+	+	d	–	w/d/+
Citrate	–	+	+	+	–	+/-
Ethanol	w	w/d	–	–	w/d	w/d
Nitrite	–	+	+	+	+	–
L-Lysine	–	+	+	–	+	w/d/–
Growth in the absence of vitamins	–	–	+	–	–	+
Growth at/in:						
35 °C	+	–	–	–	–	–
Acetic acid (1 %)	–	–	+	–	–	–
D-Glucose (50 and 60 %)	–	–	+	–	–	–
Starch formation	+	+	+	+	–	w

et margine glabra. Post unum mensem ad 25 °C, annulus, pelliculum et sedimentum formatur. Hyphae et pseudohyphae non formantur. Fermentatio nulla. Assimilantur D-glucosum, D-galactosum (exiguum), L-sorbosum, D-glucosaminum, D-ribosum, D-xylosum, L-arabinosum, D-arabinosum, L-rhamnosum, sucrosum, maltosum, trehalosum, methyl α -D-glucosidum, cellobiosum, salicinum, arbutinum, lactosum, raffinolum, melezitosum, amyllum solubile (exiguum), glycerolum, ribitolum, xylitolum, L-arabitolum, D-glucitolum, D-mannitolum, galactitolum, myo-inositolum, D-glucono-1,5-lactonum, 2-ketogluconatum, 5-ketogluconatum, D-gluconatum, D-glucuronatum, acidum galacturonicum, succinatum, ethanolum, acidum et N-acetyl-D-glucosaminum. Non assimilantur melibiosum, inulinum, erythritolum, DL-lactatum, citratum methanolum,

propane-1,2-diolum et butano-2,3-diolum. Assimilantur kalii nitratum. Non assimilantur natrium nitrosum, L-lysinum et creatinum. Vitaminae externae ad crescentiam necessariae sunt. Non crescit in medio 0.1 % cycloheximido addito. Non crescit in medio 1 % acido acetico addito. Non cresceret potest in 10 % NaCl. Non crescit in medio cum 50 % glucoso. Materia amyloidea iodophila formantur. Acidum aceticum haud formatum. Reactiones Diazonium caeruleum B et ureasii positivae. 25 °C et 35 °C crescit, neque 40 °C. Ubiquinonum primus: Q-10. G + C acidica deoxyribonucleati 54.8 mol% (per HPLC). Typus SN-82^T (=CBS 10876^T =BCRC 23107^T =JCM 14893^T) isolatus ex aquamarinus, in Keelung, Taiwan. Conservatur in Bioresource Collection and Research Center, Hsinchu, Taiwan.

Description of *Cryptococcus keelungensis* Chang & Liu sp. nov.

Cryptococcus keelungensis (kee.lung.en'sis. N.L. nom. masc. adj. *keelungensis* referring to Keelung in Taiwan, where the organism was first isolated).

After growth on YM agar for 3 days at 25 °C, cells are spherical to ovoid, 3.3–5.9 μ m long by 2.9–4.4 μ m wide, occurring singly or in pairs (Fig. 2). Streak culture is smooth, glistening and cream coloured, with an entire margin. After 1 month incubation in YM broth at 25 °C, pellicles and sediment are observed. No mycelia or pseudomycelia are formed in Dalmau plate cultures on cornmeal agar after 2 weeks incubation. Fermentation is absent. D-Glucose, D-galactose (weak), L-sorbose, D-glucosamine, D-ribose, D-xylose, L- and D-arabinose, L-rhamnose, sucrose, maltose, trehalose, methyl α -D-glucoside, cellobiose, salicin, arbutin, lactose, raffinose, melezitose, soluble starch (weak), glycerol, ribitol, xylitol, L-arabitol, D-glucitol, D-mannitol, galactitol, myo-inositol, D-glucono-1,5-lactone, 2-keto-D-gluconate, 5-keto-D-gluconate, D-gluconate, D-glucuronate, D-galacturonic acid, succinate, ethanol (weak) and N-acetyl-D-glucosamine are assimilated. Melibiose, inulin,

Fig. 2. Morphology of vegetative cells of *Cryptococcus keelungensis* sp. nov. SN-82^T grown on YM agar for 3 days at 25 °C. Bar, 10 μ m.

erythritol, DL-lactate, citrate, methanol, propane-1,2-diol and butane-2,3-diol are not assimilated. Potassium nitrate is utilized. Sodium nitrite, L-lysine and creatine are not utilized. Does not grow in vitamin-free medium. Growth in the presence of 0.1 % cycloheximide, 1 % acetic acid or 10 % sodium chloride is inhibited and negative. Does not grow on 50 % glucose-yeast extract agar. Produces starch-like compounds. Acetic acid production is negative. Positive for the Diazonium blue B reaction and urease activity. Grows at 25 and 35 °C, but not at 40 °C on YM agar. The major ubiquinone is Q-10.

The type strain is SN-82^T (=CBS 10876^T =BCRC 23107^T =JCM 14893^T), isolated from seawater collected from the SSML in Keelung, Taiwan. The nuclear DNA G + C content of the type strain is 54.8 mol% (by HPLC).

References

- Fell, J. W. (1976).** Yeasts in oceanic regions. In *Recent Advances in Aquatic Mycology*, pp. 93–124. Edited by E. B. G. Jones. London: Elek Science.
- Fell, J. W. & Statzell-Tallman, A. (1998).** *Cryptococcus* Vuillemin. In *The Yeasts, a Taxonomic Study*, 4th edn, pp. 742–767. Edited by C. P. Kurtzman & J. W. Fell. Amsterdam: Elsevier.
- Fell, J. W., Boekhout, T., Fonseca, A., Scorzetti, G. & Statzell-Tallman, A. (2000).** Biodiversity and systematics of basidiomycetous yeasts as determined by large-subunit rDNA D1/D2 domain sequence analysis. *Int J Syst Evol Microbiol* **50**, 1351–1371.
- Fonseca, A., Scorzetti, G. & Fell, J. W. (2000).** Diversity in the yeast *Cryptococcus albidus* and related species as revealed by ribosomal DNA sequence analysis. *Can J Microbiol* **46**, 7–27.
- Gadanhó, M., Libkind, D. & Sampaio, J. P. (2006).** Yeast diversity in the extreme acidic environments of the Iberian Pyrite Belt. *Microb Ecol* **52**, 552–563.
- Garrett, W. D. (1967).** The organic chemical composition of the ocean surface. *Deep-Sea Res* **14**, 221–227.
- Hagler, A. N. & Ahearn, D. G. (1987).** Ecology of aquatic yeasts. In *The Yeasts*, 2nd edn, vol. 1, pp. 181–205. Edited by A. H. Rose & J. S. Harrison. London: Academic Press.
- Harvey, G. W. & Burzell, L. A. (1972).** A simple microlayer method for small samples. *Limnol Oceanogr* **17**, 156–157.
- Kumar, S., Tamura, K. & Nei, M. (2004).** MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief Bioinform* **5**, 150–163.
- Maki, J. S. (2002).** Neuston microbiology: life at the air–water interface. In *Encyclopedia of Environmental Microbiology*, pp. 2133–2144. Edited by G. Bitton. New York: Wiley.
- Nagahama, T., Hamamoto, M., Nakase, T., Takaki, Y. & Horikishi, K. (2003).** *Cryptococcus surugaensis* sp. nov., a novel yeast species from sediment collected on the deep-sea floor of Suruga Bay. *Int J Syst Evol Microbiol* **53**, 2095–2098.
- Nakase, T. & Suzuki, M. (1988).** *Sporobolomyces yuccicola*, a new species of ballistosporous yeast equipped with ubiquinone-9. *Antonie van Leeuwenhoek* **54**, 47–55.
- Scorzetti, G., Fell, J. W., Fonseca, A. & Statzell-Tallman, A. (2002).** Systematics of basidiomycetous yeasts: a comparison of large subunit D1/D2 and internal transcribed spacer rDNA regions. *FEMS Yeast Res* **2**, 495–517.
- Tamaoka, J. & Komagata, K. (1984).** Determination of DNA base composition by reversed-phase high-performance liquid chromatography. *FEMS Microbiol Lett* **25**, 125–128.
- Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997).** The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876–4882.
- Varnam, A. H. & Evans, M. G. (2000).** *Environmental Microbiology*. Washington, DC: American Society for Microbiology.
- Yamada, Y. & Kondo, K. (1973).** Coenzyme Q system in the classification of the yeast genera *Rhodotorula* and *Cryptococcus* and the yeast like genera *Sporobolomyces* and *Rhodospiridium*. *J Gen Appl Microbiol* **19**, 59–77.
- Yamasato, K., Goto, S., Ohwada, K., Okuno, D., Araki, H. & Iizuka, H. (1974).** Yeasts from the Pacific Ocean. *J Gen Appl Microbiol* **20**, 289–307.
- Yarrow, D. (1998).** Methods for the isolation, maintenance and identification of yeasts. In *The Yeasts, a Taxonomic Study*, 4th edn, pp. 77–100. Edited by C. P. Kurtzman & J. W. Fell. Amsterdam: Elsevier.