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Debaryomyces renaii sp. nov., an ascomycetous yeast species isolated from soil in Taiwan

Ching-Fu LEE[1,*], Yi-Ru LIU[1], Shuh-Sen YOUNG[1], and Ku-Shang CHANG[2]

[1]*Department of Applied Science, National Hsinchu University of Education, 521 Nanda Road, Hsinchu, 300 Taiwan*
[2]*Graduate Institute of Food Science, Yuanpei University, 306 Yuanpei Street, Hsinchu, 300 Taiwan*

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ABSTRACT. Two strains (SU11S03 and SD6S03) of a proposed new species of the genus *Debaryomyces* were isolated from soil in Renai, Nantou and Dasi, Touyuan, Taiwan. Their identical physiology, morphology and the sequence of the D1/D2 domain of the large subunit (LSU) rRNA gene indicated the two strains were conspecific. The yeast species produced a single warty, spheroidal ascospore per ascus, followed by conjugation between a cell and its bud. Phylogenetic analysis using the D1/D2 domain of the LSU rRNA gene demonstrated that the close relatives of the novel species were the species of the *Debaryomyces hansenii* cluster (10-12 nucleotide difference) and *Debaryomyces mycophilus* (18 nucleotide difference). The species differed from the other *Debaryomyces* species by carbon and nitrogen assimilation patterns and growth on vitamin-free medium. The physiological, morphological, and molecular data described above suggest that these strains belong to a new species, and the name *Debaryomyces renaii* sp. nov. is proposed. The type strain of the new species is SU11S03 (=CBS 10891 =BCRC 23137), which was isolated from soil in Renai, Nantou, Taiwan.

Keywords: *Debaryomyces renaii*; 26S rDNA; New yeast species.

INTRODUCTION

The genus *Debaryomyces* Lodder & Kreger-van Rij Nom. Cons. was accepted against *Debaryomyces* Klöcker in the International Code of Botanical Nomenclature (Greuter et al., 1988). In a comprehensive revision, 15 species previously assigned in the genus *Debaryomyces* Klöcker, *Schwanniomyces*, and *Debaryozyma*, were accepted in the genus *Debaryomyces* Lodder & Kreger-van Rij Nom. Cons (Nakase et al., 1998). Later, an additional three new species were reported, including *Debaryomyces prosopidis* (Phaff et al., 1998), *Debaryomyces mycophilus* (Thanh et al., 2002), and *Debaryomyces singareniensis* (Saluja and Prasad, 2007). The genus *Debaryomyces* is characterized by multilateral budding, an inability to assimilate nitrate, having coenzyme Q-9 as the major ubiquinone, and conjugation between its cell and its bud (Nakase et al., 1998). *Debaryomyces* species can be found in diverse substrates, type strains of over eight species were isolated from soil and include *D. castellii, D. nepalensis, D. occidentalis, D. polymorphus, D. singareniensis, D. udenii, D. vanrijae* and *D. yamadae* (Nakase et al., 1998; Barnett et al., 2000).

During an investigation of yeast diversity in Taiwan's soil, two strains, SU11S03 and SD6S03, showed identical morphological and molecular characteristics, indicating possible conspecificity. The species were determined to be members of the genus *Debaryomyces* and distinct from recognized species, based on the morphological and physiological characteristics and the sequences of the D1/D2 domain of the LSU rRNA gene. The species was closely related to species of the *D. hansenii* cluster and *D. mycophilus*. The present paper proposes a new species, *Debaryomyces renaii*, to accommodate the two strains.

*Corresponding author: E-mail: leecf@mail.nhcue.edu.tw; Tel: 886-3-5213132 ex 2713; Fax: 886-3-5257178.

MATERIALS AND METHODS

Yeast strains and growth conditions

The two strains, SU11S03 (=CBS 10891 =BCRC 23137) and SD6S03, examined in this study, were isolated from Taiwanese forest soil samples collected in Nantou and Touyuan, respectively, in 2006. The yeasts were isolated with the spread plate method on acidified YMA (1% glucose, 0.5% peptone, 0.3% yeast extract, 0.3% malt extract, 1.5% agar, pH 3.5) or DRBC (Dichloran rose Bengal chloramphenicol agar, Merck, Darmstadt, Germany) as described by Liu et al. (2008). The pH of acidified YMA was adjusted by 10% tartaric acid. The yeast colonies were picked and purified by streaking them onto YM agar, followed by preservation on YMA at 4°C or in the freezer at -70°C. The type strain of the species ($SU11S03^T$) was deposited in Bioresources Collection and Research Center, Food Industry Research and

Development Institute (BCRC), Hsinchu, Taiwan, and Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands.

Examination of morphological, physiological, and biochemical characteristics

Morphological, physiological, and biochemical characteristics of the species were determined by the methods described by Yarrow (1998). Sporulation tests were performed on different media, including Fowell acetate agar, corn meal agar (CMA), 2% malt extract agar, and YM agar. The morphological characteristics of vegetative cells and ascospores were examined using an Nikon 80i light microscope.

Phylogenetic analysis

Molecular sequencing of the D1/D2 domain of the LSU rRNA gene was carried out from PCR products of genomic DNA that was extracted from yeast cells with a Biokit Genome DNA Extraction Kit (Biokit Co., Taiwan). The D1/D2 rRNA gene, SSU rRNA gene, and ITS fragments were amplified using primers NL1 and NL4 (Kurtzman and Robnett, 1998), and ITS1 and ITS4 (White et al., 1990), respectively, with a Peltier thermal cycler (PTC-200, MJ Research). The amplification products were confirmed with agarose gel electrophoresis. Sequencing of the fragments was performed with an automatic sequencer (Applied Biosystems 3730 DNA Analysis System, Lincoln Centre Drive, Foster City, CA, USA). Both DNA strands were sequenced, and the reactions were carried out with a Dye Terminator cycle sequencing kit (Applied Biosystems, Lincoln Centre Drive, Foster City, CA, USA). The sequences were initially aligned with the multiple alignment program CLUSTAL X 1.83 (Thompson et al., 1997). A phylogenetic tree was constructed by the neighbor-joining method with the MEGA (version 4.0) software package (Kumar et al., 2004). *Schizosaccharomyces pombe* NRRL Y- 12796^T was used as an outgroup. Bootstrap analysis was performed from 1,000 bootstrap replications (Felsenstein, 1995). The sequence data for *Debaryomyces renaii* were deposited in GenBank: D1/D2, EF653953 for SU11S03^T and EU547811 for SD6S03; ITS, FJ527037 for SU11S03^T and FJ527036 for SD6S03. The reference sequences used in this paper were retrieved from GenBank under the accession numbers indicated in Figure 1.

RESULTS AND DISCUSSION

Sequence comparison and species delineation

The two strains, SU11S03 and SD6S03, isolated from forest soil in Nantou and Touyuan in 2006, showed identical physiology, morphology, and the sequence of the D1/D2 domain of the large subunit (LSU) rRNA gene, indicating that the two strains were conspecific. The species is characterized by the inability to ferment carbon sources, by the formation of asci containing a single spheroidal ascospore with a warty surface followed by conjugation between the cell and its bud, and by growth on a vitamin-free medium. Based on morphological, physiological, biochemical, and molecular characteristics, the species was classified as a member of the genus *Debaryomyces* because it demonstrated morphological characteristics typical of the genus (Nakase et al., 1998). For comparison of the sequences of the D1/D2 domain of the LSU rRNA gene, the species is closely related to those of the *D. hansenii* cluster (*D. coudertii, D. hansenii, D. maramus, D. nepalensis, D. prosopidis, D. robertsiae,* and *D. udenii*) and to *D. etchellsii*, *D. mycophilus*, and *D. singareniensis* (Figure 1). However, the species differed from the other previously described species of the *D. hansenii* cluster and from *D. mycophilus* in regard to fermentation, assimilation of carbon sources, and physiology (Table 1). The species can be differentiated from those of the *D. hansenii* cluster, except for *D. robertsiae*, in its ability to grow on vitamin-free medium, and from *D. robertsiae* in its inability to ferment glucose and to assimilate gluconate and succinate. Also the species can be easily differentiated from *D. mycophilus*, *D. etchellsii* and *D. singareniensis* in assimilation and fermentation of some carbon sources (Table 1).

The neighbour-joining phylogenetic tree based on the D1/D2 domain of the large subunit LSU rRNA gene is illustrated as Figure 1. The *Debaryomyces* species were clustered into three clades. The proposed species, *Debaryomyces renaii*, was clustered with *D. etchellsii*, *D. mycophilus*, and *D. singareniensis* and found to be related to the other seven published species of the *D. hansenii* cluster as described above, confirming that the new species is a member of this genus. In addition to the morphological and physiological evidence described above, the proposed species also proved to be novel based on the neighbour-joining phylogenetic tree (Figure 1). In the analysis, the species differs from its phylogenetic relatives, *D. etchellsii*, *D. mycophilus* and species of the *D. hansenii* cluster by a 3.54% (19 substitutions), 3.36% (18 substitutions) and 1.87-2.24% divergence (10-12 substitutions), respectively, in the D1/D2 domain of the LSU rRNA gene. Meanwhile, the species can be differentiated from the species of the *D. hansenii* cluster by a 1.47-3.85% divergence (8-21 substitutions) in the ITS sequences. Based on the evidence described above, a new species, *Debaryomyces renaii* is proposed to accommodate the two strains.

Latin diagnosis of *Debaryomyces renaii* Lee & Liu sp. nov.

In medio liquido cum glucoso et peptono et extracto levidinis post dies 3 ad 25°C, cellulae ovoideae vel globosae (3.2-5.0 × 3.6-5.2 μm), singulae aut binae, per gemmationem reproducentes. Post 1 mensem sedimentum formatur. Cultura in agaro cum glucoso et peptono et extracto levidinis post dies 7 ad 25°C, cremea, butyrosa, glabra et nitida. In agaro farinae Zea mays post dies 10 ad 25°C, mycelium et pseudomycelium nulla. Asci

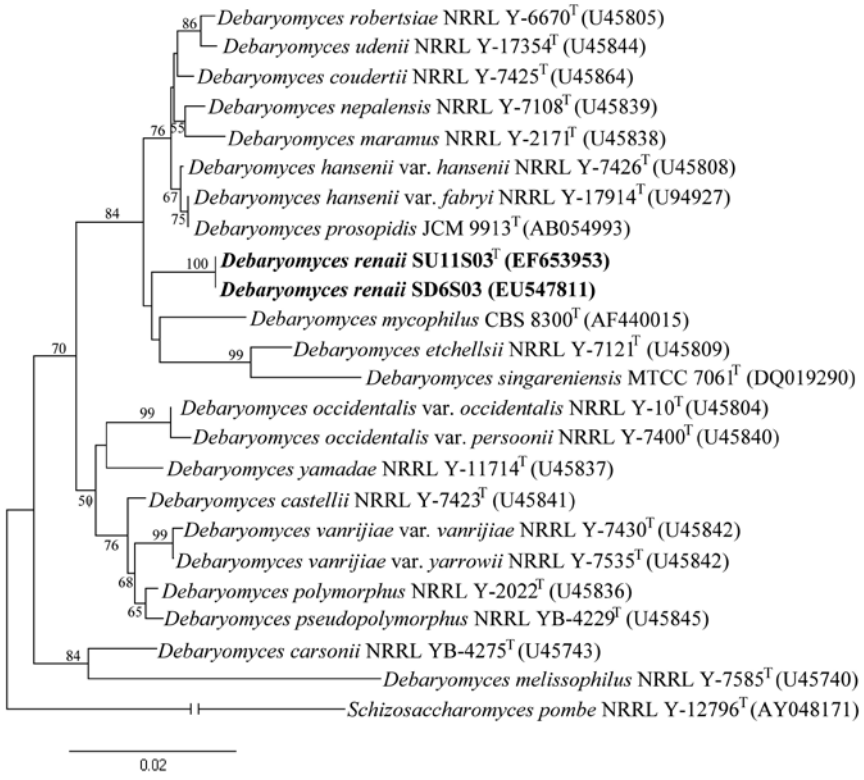

Figure 1. Neighbor-joining phylogenetic tree based on the domains D1/D2 of the large subunit (26S) rDNA showing the relationship of *D. renaii* sp. nov. and related *Debaryomyces* species. *Schizosaccharomyces pombe* is used as an outgroup in the phylogenetic tree. Bootstrap values based on 1,000 replicates are given near the branches. All taxa are represented by type strains. Bar: 0.02 substitutions per nucleotide position.

formantur ex conjugatione inter cellulam maternam et gemmam, 1 ascosporusin asco continentes. Ascosporae sphaericae. Fermentatio nulla. Glucosum, galactosum, L-sorbosum (exigue), D-xylosum, sucrosum, maltosum, α, α-trehalosum, methyl α-D-glucosidum, cellobiosum, salicinum, arbutinum, raffinosum, melezitosum, glycerolum, erythritolum, ribitolum, xylitolum, L-arabinitolum, D-glucitolum, D-mannitolum, D-glucono-1,5-lactonum, acidum 2-keto-D-gluconicum, acidum D-glucuronicum, acidum DL-lacticum (exigue), ethanolum, et N-acetylglucosaminum assimilantur, at non D-glucosaminum, L-arabinosum, D-ribosum, D-arabinosum, L-rhamnosum, melibiosum, lactosum, inulinum, amylum, galactitolum, myo-inositolum, 5-keto-D-gluconicum, acidum D-gluconicum, acidum D-galacturonicum, acidum succinicum, acidum citricum, methanolum, propanum 1,2 diolum, nec butanum 2,3 diolum. L-lysinum, ethylaminum, et cadaverinum assimilantur, at non natrium nitrosum, kalium nitricum, nec creatinum. Vitaminae externae ad crescentiam necessaria non sunt. Non crescit in substrato 10% sal / 5% glucosum continente. Non crescit in 50% glucosum addito. Non crescere potest in 0.01% cycloheximido. In 25°C crescere potest at non in 30°C. Materia amyloidae iodophila non formatur. Ureum nonhydrolysatur. Diazonium caeruleum B est negativum.

Typus stirpis SU11S03^T (=CBS 10891^T =BCRC 23137^T) *isolatus ex terea,* Renaii, Nantou, Taiwan, Bioresources Collection and Research Center, Food Industry Research and Development Institute (BCRC), Hsinchu, Taiwan *et* Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands *deposita est*.

Description of *Debaryomyces renaii* Lee & Liu sp. nov.

Debaryomyces renaii (re. nai'i. L. adj. renaii, Renaii referring to Renai, Nantou, Taiwan, where the yeast was originally isolated). After growth in YM broth at 25°C for 3 days, the vegetative cells are ovoidal to spheroidal (3.2-5.0 × 3.6-5.2 μm), single or in pairs (Figure 2A). Vegetative reproduction is accomplished by multilateral budding. Sediment is present. After growth on YM agar for 7 days at 25°C, streak cultures are creamy, butyrous, smooth, and glistening. On Dalmau plate cultures on corn meal agar after 10 days at 25°C, pseudomycelium and mycelium are absent. Asci resulting from cell-bud conjugation containing a single warty, spheroidal ascospore were observed on YM agar, Fowell's acetate agar, and malt extract agar after incubation at 18°C and 25°C for 14 days (Figure 2B). Fermentation is absent. The following carbon compounds are assimilated: glucose, D-galactose, L-sorbose (weak), D-xylose, sucrose, maltose, α, α-trehalose, methyl α-D-glucoside, cellobiose, salicin, arbutin, raffinose, melezitose, glycerol, erythritol, ribitol, xylitol, L-arabinitol, D-glucitol, D-mannitol, D-glucono-1,5-lactone, 2-keto-D-gluconate, D-glucuronate, DL-lactate (weak), ethanol, and N-acetylglucosamine. No growth occurs on D-glucosamine, L-arabinose, D-ribose, D-arabinose, L-rhamnose, melibiose, lactose, inulin, starch, galactitol, *myo*-inositol, 5-keto-D-gluconate, D-gluconate, D-galacturonic acid, succinate, citrate, methanol, propane 1,2 diol, or butane 2,3 diol. L-Lysine, cadaverine, and ethylamine are assimilated, but nitrate, nitrite, and creatine

Table 1. Comparison of phenotypic characteristics of *Debaryomyces renaii* with other genetically related *Debaryomyces* species.

Species	Fermentation			Assimilation of											Growth		
	Gu	Su	Tr	Su	Me	La	Ra	Mz	St	Gy	Er	Gl	Sa	Lt	NG	Vit	35°C
D. renaii	-	-	-	+	-	-	+	+	-	+	+	-	-	w	-	+	-
D. robertsiae[a]	+	+	+	+	-	-	+	+	-	+	+	+	+	+	+	+/s	-
D. udenii[a]	ws	w/-	w/-	+	+	-	+	+	+	+	+	s	s	-	+	-	-
D. coudertii[a]	-	-	-	-	-	-	-	-	+	+	+	s	+	-	+	-	-
D. nepalensis[a]	w/-	w/-	w/-	+	+	v	+	+	+	+	+	s	+	v	+	-	+
D. maramus[a]	w/-	-	-	+	+	+	s	+	+	+	+	+	+	-	+	-	-
D. hansenii var. *hansenii*[a]	w/-	w/-	w/-	+	v	v	+	v	v	+	v	+	+	v	+	-	-
D. hansenii var. *fabryi*[a]	w/-	w/-	w/-	+	v	v	+	v	v	+	v	+	+	v	+	-	+
D. prosopidis[b]	-	-	-	+	-	-	+	+	+	+	+	+	+	-	-	-	+
D. mycophilus[c]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
D. etchellsii[a]	+/w	w/-	-	+	-	-	-	+	-	+	-	-	+	v	+	-	+
D. singareniensis[d]	+	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-

Data from reference: [a]Nakase et al. (1998); [b]Phaff et al. (1998); [c]Thanh et al. (2002); [d]Saluja and Prasad (2007).
Gu, glucose; Su, sucrose; Tr, trehalose; Me, melibiose; La, lactose; Ra, raffinose; Mz, melezitose; St, soluble starch; Gy, glycerol; Er, erythritol; Gl, gluconate; Sa, succinate; Lt, DL-lactate; NG, 10% NaCl-5% glucose; Vit, vitamin-free medium; +: positive; -, negative; v, variable; S, slow growth; W, weak growth.

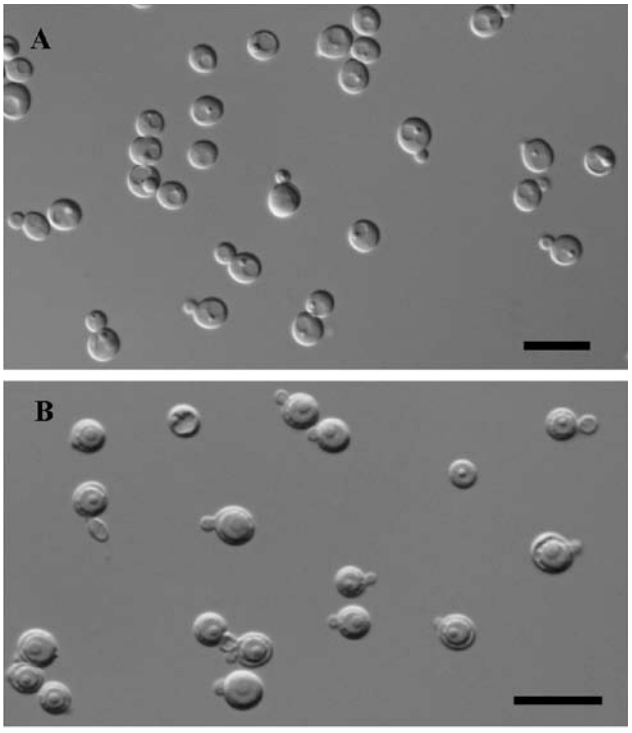

Figure 2. Morphology of *Debaryomyces renaii* SU11S03 (=CBS 10891 =BCRC 23137) as determined by light microscopy. The strain was cultivated in YM broth for 3 days at 25°C (A). Ascospores were found on Fowell's acetate agar after 14 days at 25°C (B). Scale bars: 10 μm.

are not. Growth in vitamin-free medium is positive. Growth does not occur on 50% or 60% glucose or on 10% NaCl plus 5% glucose. No growth occurs in the presence of 0.01% cycloheximide. Growth occurs at 25°C but not at 30°C. No starch-like substance is produced. Acid production on chalk agar is negative. Urease hydrolysis and Diazonium blue B reaction are negative.

The type strain of *Debaryomyces renaii*, SU11S03 (=CBS 10891 =BCRC 23137), was isolated from forest soil in Renai, Nantou, Taiwan in 2006.

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Debaryomyces renaii sp. nov. 台灣土壤分離之子囊酵母菌新種

李清福[1]　柳怡如[1]　楊樹森[1]　張谷昇[2]

[1] 國立新竹教育大學 應用科學系

[2] 元培科技大學 食品科學系

本文描述一從土壤分離的酵母菌新種 *Debaryomyces renaii*。作者共分離出生理、形態及分子特徵皆相同的該種兩菌株 (SU11S03 and SD6S03)，其分別分離自南投縣仁愛鄉及桃園縣大溪鎮山區土壤。該菌種主要特徵為母細胞與子細胞接合生殖後產生子囊，每一個子囊內含單一粗糙表面的球型胞子，且此菌種於不含維生素培養基中生長良好。另經核糖體基因序列分析顯示 ： 該菌種與 *Debaryomyces mycophilus* 和 *Debaryomyces hansenii* 菌群之基因相關性高，但其生理、形態及分子生物特徵與 *Debaryomyces* 屬內現有種皆有明顯差異。因此，本文建議該菌種可視為 *Debaryomyces* 屬之新種。該新種之標準菌株定為 SU11S03^T (=CBS 10891^T =BCRC 23137^T)。

關鍵詞 ：*Debaryomyces renaii*；26S 核糖體基因 ； 酵母菌新種。