Wickerhamomyces siamensis sp. nov., a novel yeast species isolated from the phylloplane in Thailand

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Strain DMKU-RK359⁷, representing a novel yeast species, was isolated from the external surface of a sugar-cane leaf collected in Thailand. On the basis of morphological, biochemical, physiological and chemotaxonomic characteristics, sequence analysis of the D1/D2 region of the large-subunit (LSU) rRNA gene and the internal transcribed spacer (ITS) region, strain DMKU-RK359⁷ was assigned to a novel Wickerhamomyces species. The novel species was closest to Wickerhamomyces ciferrii, but differed from it by 0.7 % nucleotide substitutions in the D1/D2 region of the LSU rRNA gene and 6 % nucleotide substitutions in the ITS region. The name Wickerhamomyces siamensis sp. nov. is proposed (type strain DMKU-RK359⁷ = BCC 50732⁷ = NBRC 108900⁷ = CBS 12570⁷).

The genus Wickerhamomyces was proposed to accommodate 17 species that were transferred from the genera Pichia, Williopsis and Hansenula as a result of phylogenetic analysis of nucleotide divergence in the genes coding for the large-subunit (LSU) and small-subunit rRNAs and for elongation factor 1β (Kurtzman et al., 2008; Kurtzman, 2011). The number of Wickerhamomyces species was subsequently increased by the discovery of Wickerhamomyces edaphicus (Limtong et al., 2009), W. querolae (Rosa et al., 2009), W. patagonicus (de García et al., 2010), W. chaumieriensis (Groenewald et al., 2011), W. ochangensis (Shin et al., 2011), W. tratensis (Nakase et al., 2012) and W. xylosica (Limtong et al., 2012).

The phylloplane, the external surface of the plant leaf, has been recognized as an important habitat for epiphytic micro-organisms (Phaff & Starmer, 1987; Fonseca & Inácio, 2006). Both basidiomycete and ascomycete yeasts have been found to be phylloplane colonizers (Nakase et al., 2001; Fonseca & Inácio, 2006; Glushakova et al., 2007; Slavikova et al., 2009; Landell et al., 2010). Although most common phylloplane yeasts are members of basidiomycete genera such as Cryptococcus, Rhodotorula, Sporobolomyces and Trichosporon (de Azeredo et al., 1998; Nakase et al., 2001; Fonseca & Inácio, 2006; Slavikova et al., 2009; Glushakova & Chernov, 2010), various ascomycete yeast species have also been reported, e.g. Debaryomyces hansenii, Hanseniaspora uvarum, Kazachstanbaurna barnetti, Metschnikowia lopurienis, M. pulcherrima, M. saccharicola, Pichia membranifaciens, Saccharomyces cerevisiae and various Candida species including Candida aethiopae, C. olephiila, C. chumphonensis, C. matrarenensis and C. vriesae (Glushakova et al., 2007; Slavikova et al., 2009; Glushakova & Chernov, 2010; Landell et al., 2010; Kaewwichian et al., 2012; Koowadjanakul et al., 2011).

During an investigation of yeasts on the external leaf surfaces of sugar cane in Thailand, strain DMKU-RK359⁷, representing a novel species of Wickerhamomyces, was obtained. In this paper, it is described as the type strain of Wickerhamomyces siamensis sp. nov.

One hundred and sixty-six yeast strains were isolated from the external surfaces of 95 samples of sugar cane (Saccharum officinarum L.) leaf collected in Thailand. Strain DMKU-RK359⁷ was isolated from a sample collected from Nong Muang district, Lop Buri Province, on 5 May 2010, by an enrichment technique using acidified yeast extract-malt extract (YM) broth (0.3 % yeast extract, 0.3 % malt extract, 0.5 % peptone and 1 % glucose) supplemented with 0.025 % sodium propionate and 0.02 % chloramphenicol (Limtong et al., 2007). Cut leaves (3 g) were placed aseptically in a 250-ml Erlenmeyer flask containing 50 ml acidified YM broth and incubated on a rotary shaker at room temperature (27±3 °C) for 3 days. The enrichment culture was then spread on YM agar supplemented with 0.025 % sodium propionate and 0.02 % chloramphenicol and incubated at room temperature (27±3 °C) until yeast colonies appeared. Yeast colonies of different morphologies were picked and...
purified by cross-streaking on YM agar. Purified yeast strains were suspended in YM broth supplemented with 10 % glycerol and maintained at −80 °C.

The sequences of the D1/D2 region of the LSU rRNA gene and the internal transcribed spacer (ITS) region were determined from PCR products amplified from genomic DNA, using primers NL1 and NL4 (Kurtzman & Robnett, 1997) and ITS1 and ITS4 (White et al., 1990), respectively, for sequencing and amplification. Methods of DNA extraction and amplification were as described previously (Limtong et al., 1998) and ITS1 and ITS4 (White et al., 1990), respectively, for sequencing. The sequences were determined from PCR products amplified from genomic DNA, using primers NL1 and NL4 (Kurtzman & Robnett, 1997) and ITS1 and ITS4 (White et al., 1990), respectively, for sequencing and amplification. Methods of DNA extraction and amplification were as described previously (Limtong et al., 2007). The PCR products were checked by agarose gel electrophoresis and purified by using the QIAquick purification kit (Qiagen) and the purified products were submitted to Macrogen Inc. (Seoul, Republic of Korea) for sequencing. The sequences were compared pairwise using a BLAST search (Altschul et al., 1997) and were aligned with the sequences of related strains retrieved from GenBank using the multiple alignment program CLUSTAL_X version 1.81 (Thompson et al., 1997). A phylogenetic tree was reconstructed from the evolutionary distance data with Kimura’s two-parameter correction (Kimura, 1980) using the neighbour-joining method (Saitou & Nei, 1987) and MEGA software version 5.0 (Tamura et al., 2011). Confidence levels of the clades were estimated from bootstrap analysis (1000 replicates) (Felsenstein, 1985).

Strain DMKU-RK359T was characterized morphologically, biochemically and physiologically according to standard methods described by Yarrow (1998). Mycelium formation was investigated on potato dextrose agar (PDA; 20 % potato infusion, 2 % glucose and 1.5 % agar) in slide culture at 25 °C for up to 7 days. Ascospore formation was investigated on YPD agar (1 % yeast extract, 2 % peptone, 2 % glucose and 1.5 % agar) and 5 % malt extract agar (5 % malt extract and 1.5 % agar) at 25 °C. Carbon assimilation tests were conducted in liquid medium according to the method described by Yarrow (1998). Assimilation of nitrogen compounds was examined on solid medium with starved inocula following the method of Nakase & Suzuki (1986). Growth at various temperatures was determined by cultivation in YM broth. Ubiquinones were extracted from cells cultivated in a 500-mI Erlenmeyer flask containing 250 ml YPD broth on a rotary shaker at 28 °C for 24–48 h and purified according to the method described by Yamada & Kondo (1973) and Kuraishi et al. (1985). Isoprenologues were identified by HPLC as described previously (Limtong et al., 2007). The DNA base composition was determined by HPLC as described by Tamaoka & Komagata (1984) using the DNA-GC kit (Yamasu Shoyu).

Morphological examination of ascospores under the scanning electron microscope was performed as follows. Strain DMKU-RK359T was grown on YPD agar at 25 °C for 24–48 h and purified according to the method described by Yamada & Kondo (1973). Isoprenologues were identified by HPLC as described by Tamaoka & Komagata (1984) using the DNA-GC kit (Yamasu Shoyu).
for 3 days or until ascospores were produced. An ascospore suspension was prepared in 1 ml normal saline and then centrifuged. An aliquot (0.5 ml) of 0.1 M phosphate buffer, pH 7.0, was added to the cell suspension and the cells were harvested by centrifugation. Ascospores were fixed with 0.5 ml of 3.5 % glutaraldehyde in 0.1 M phosphate buffer for 3 days or until ascospores were produced. An ascospore suspension was prepared in 1 ml normal saline and then centrifuged. Ascospores were moved to a critical-point dryer and then coated with platinum. Finally, ascospores were examined under a scanning electron microscope (Hitachi S-5200).

Determination of whether strain DMKU-RK359<sup>T</sup> was homothallic or heterothallic was performed by the heat-treatment method described by Wickerham & Burton (1954). A loopful of cells from a YM agar plate (24–48 h, 25 °C) was suspended in 2 ml YM broth in a 16-mm test tube and the test tube was placed in a water bath at 62 °C. After 1, 2, 3, 4 and 5 min, loopfuls of the cell suspension were taken and spread on YM agar. The plates were examined after incubation to determine the time taken to kill the vegetative cells. A suspension of a sporulated culture of strain DMKU-RK359<sup>T</sup> (3 days, 25 °C on YM agar) was then prepared in YM broth. The suspension was heated at 62 °C for 7 min and spread on YM agar. Vegetative cells were killed, so that the colonies arising on plates were derived only from surviving ascospores. Twelve to twenty colonies were checked for sporulation. If all or nearly all colonies show good sporulation, the strain is homothallic; otherwise, the culture is heterothallic.

**Novel species delineation and identification**

Analysis of the D1/D2 region of the LSU rRNA gene sequence revealed that the sequence of strain DMKU-RK359<sup>T</sup> was closest to that of *W. ciferrii*, with 0.7 % nucleotide substitutions (four nucleotide substitutions and four gaps out of 571 nt). The sequence of the ITS region of strain DMKU-RK359<sup>T</sup> was also analysed; it differed by 6 % nucleotide substitutions (33 substitutions and eight gaps out of 550 nt) from that of *W. ciferrii*.

The phylogenetic tree based on sequences of the D1/D2 domain of the LSU rRNA gene shows that strain DMKU-RK359<sup>T</sup> is closely related to *W. ciferrii*, *W. edaphicus* and *Candida silvicultrix* with high bootstrap support and belongs to the *Wickerhamomyces* clade at a position distinct from the other members of the clade (Fig. 1).

Ascospores were produced by strain DMKU-RK359<sup>T</sup> on YPD agar and 5 % malt extract agar after 7 days at 25 °C. Ascii were persistent and unconjugated and formed four hat-shaped ascospores. Strain DMKU-RK359<sup>T</sup> was found to be homothallic.

On the basis of morphological, biochemical, physiological and chemotaxonomic characteristics and sequence analysis of the D1/D2 domain of the LSU rRNA gene and ITS region, we conclude that strain DMKU-RK359<sup>T</sup> represents a novel *Wickerhamomyces* species, for which the name *Wickerhamomyces siamensis* sp. nov. is proposed. *W. siamensis* sp. nov. is distinguished from *W. ciferrii*, *W. edaphicus* and *C. silvicultrix*, the most closely related described species, by the phenotypic characteristics shown in Table 1.

Member of *Wickerhamomyces* species are often found associated with plants; however, only a few species have been isolated from the phylloplane, such as *W. edaphicus*, which was isolated from *Terminalia catappa* L. (Limtong & Koowadjanakul, 2012). In this study, of 166 yeast strains obtained from the phylloplane of 95 samples of sugar-cane leaf, strain DMKU-RK359<sup>T</sup> was the only strain of *W. siamensis* sp. nov. that was isolated; therefore, it is clear that the novel species is not common in the phylloplane. *Meyerozyma guilliermondii* was found to be the most

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**Table 1.** Phenotypic characteristics that differentiate *Wickerhamomyces siamensis* sp. nov. DMKU-RK359<sup>T</sup> from closely related species

Species/strains: 1, *W. siamensis* sp. nov. DMKU-RK359<sup>T</sup>; 2, *W. ciferrii* (data from Kurtzman, 2011); 3, *W. edaphicus* (Limtong et al., 2009); 4, *C. silvicultrix* (Lachance et al., 2011). +, Positive; w, weakly positive; –, negative; ND, no data available.
common yeast species in the sugar-cane phylloplane; 25 strains of this species were obtained.

**Description of Wickerhamomyces siamensis Kaewwichian, Kawasaki & Limtong sp. nov.**

Wickerhamomyces siamensis (si.a.men’sis. N.L. fem. adj. siamensis of or belonging to Siam, the old name of Thailand, where the type strain was isolated).

Growth in YM broth: after 3 days at 25 °C, cells are globose, subglobose to ovoid (3–8 x 4–8 μm) and occur singly, in pairs or in groups (Fig. 2a). Budding is multilateral. Growth on YM agar: after 3 days at 25 °C, the streak culture is greyish white, smooth, soft to butyrous and has an entire margin. Pseudohyphae are formed but true hyphae are not formed in slide culture on PDA after 3 days at 25 °C (Fig. 2b). Ascospores are produced on YPD agar and 5 % malt extract agar after 7 days at 25 °C. Asci are persistent, unconjugated and formed four hat-shaped ascospores (Fig. 2c). D-Glucose, D-galactose, sucrose, maltose (weak) and raffinose (weak) are fermented but lactose, trehalose, xylose and cellobiose are not fermented. Compounds assimilated include D-glucose, D-galactose (slow), sucrose, maltose, cellobiose (weak), trehalose (weak), raffinose (weak), melezitose (slow), soluble starch (weak), D-xylose (slow), ethanol, glycerol, erythritol (weak), D-mannitol (weak), D-glucitol (weak), methyl α-D-glucoside (weak), salicin (weak), D-gluconic acid, D-lactic acid, succinic acid, arbutin (weak), propane-1,2-diol (weak), xyitol (weak), ethylamine, L-lysine (weak) and cadaverine. Compounds that are not assimilated include L-sorbose, lactose, melibiose, inulin, L- and D-arabinose, L-rhamnose, D-ribose, N-acetyl-D-glucosamine, methanol, ribitol, galactitol, D-glucuronic acid, D-galacturonic acid, 2-keto-D-gluconic acid, 5-keto-D-gluconic acid, D-glucono-δ-lactone, citric acid, inositol, D-glucosamine, hexadecane, butane-2,3-diol, arabitol, nitrate and nitrite. No growth in vitamin-free medium. Growth occurs on medium containing 50 and 60 % glucose, but not on 10 % NaCl/5 %
glucose or 16 % NaCl/5 % glucose. No growth with 0.01 or 0.1 % cycloheximide. Growth occurs at 25, 30, 35, 37, 40 and 42 °C, but not at 45 °C. Starch-like compounds are not produced. Diazonium blue B colour and urease reactions are negative. The major ubiquinone is Q-7. The DNA G+C content of the type strain is 46.2 mol%.

The holotype is DMKU-RK359T, isolated from the phylloplane of sugar cane (Saccharum officinarum L.) collected from Lop Buri province, Thailand. Living cultures from the type have been deposited at the BIOTEC Culture Collection, National Center for Genetic Engineering and Biototechnology, Pathumthani, Thailand, as BCC 50732T, the NITE Biological Resources Center, Department of Biototechnology, National Institute of Technology and Evaluation, Chiba, Japan, as NBRC 108900T, and the Centraalbureau voor Schimmelcultures, Utrecht, Netherlands, as CBS 12570T. The Mycobank registration number is MB802886.

Acknowledgements

This work was supported by the Thailand Research Fund through the Royal Golden Jubilee PhD program grant no. PHD/0215/2551, a TRF Research-Team Promotion Grant (RTA5480009) and the NITE Biological Resource Center (NBRC), Japan. The authors would like to thank Dr Ken-ichiro Suzuki, Director of NBRC, for allowing R. K. to do part of this research at NBRC. Special thanks go to Mr Atsushi Yamazaki and Mrs Yumiko Miyazaki for their technical assistance.

References


