Nakazawaea siamensis f.a., sp. nov., a yeast species isolated from phylloplane

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Strain DMKU-RK467\textsuperscript{T}, representing a novel yeast species, was isolated from the external surface of sugar cane leaves collected in Thailand. On the basis of morphological, biochemical, physiological and chemotaxonomic characteristics, and sequence analysis of the D1/D2 region of the LSU rRNA gene and the internal transcribed spacer (ITS) region, strain DMKU-RK467\textsuperscript{T} was assigned to a novel species of the genus Nakazawaea. The novel species was related most closely to the type strain of Candida wickerhamii but they differed by 1.9\% nucleotide substitutions in the D1/D2 region of the LSU rRNA gene and by 5.2\% nucleotide substitutions in the ITS region. The name Nakazawaea siamensis f.a., sp. nov. is proposed (type strain DMKU-RK467\textsuperscript{T}=BCC 50734\textsuperscript{T}=NBRC 108903\textsuperscript{T}=CBS 12569\textsuperscript{T}).

The phylloplane, an external surface of plant leaves (Phaff & Starmer, 1987; Fonseca & Inácio, 2006), has been recognized as an important habitat for epiphytic microorganisms. Both basidiomycete and ascomycete yeasts have been found to be phylloplane colonizers (Nakase \textit{et al.}, 2001; Fonseca & Inácio, 2006; Glushakova \textit{et al.}, 2007; Slavikova \textit{et al.}, 2009; Landell \textit{et al.} 2010). Although most common phylloplane yeasts are members of the basidiomycete genera \textit{viz.} Cryptococcus, \textit{Rhodotorula}, \textit{Sporobolomyces} and \textit{Trichosporon} (de Azeredo \textit{et al.}, 1998; Nakase \textit{et al.}, 2001; Fonseca & Inácio, 2006; Slavikova \textit{et al.}, 2009; Glushakova \& Chernov, 2010), various ascomycete yeast species have also been reported, for example \textit{Debaryomyces hansenii}, \textit{Hanseniaspora uvarum}, \textit{Kazachstania barnettii}, \textit{Metschnikowia lopburiensis}, \textit{Metschnikowia pulcherrima}, \textit{Metschnikowia saccharicola}, \textit{Pichia membranifaciens}, \textit{Saccharomyces cerevisiae}, \textit{Wickerhamomyces siamensis}, \textit{Yamadazyma phyllophilae}, \textit{Yamadazyma phyllophila} and some species of the genus \textit{Candida} including \textit{Candida achtemae}, \textit{Candida oleophila}, \textit{Candida chumphonensis}, \textit{Candida mattranensis} and \textit{Candida vrieseae} (Glushakova \textit{et al.}, 2007; Slavikova \textit{et al.}, 2009; Glushakova \& Chernov, 2010; Landell \textit{et al.}, 2010; Koowadjanakul \textit{et al.}, 2011; Kaewwichian \textit{et al.}, 2012; Kaewwichian \textit{et al.}, 2013a, \textit{b}).

The genus Nakazawaea was first proposed to accommodate \textit{Pichia holstii} by Yamada \textit{et al.} (1994) based on data obtained from partial sequences of the LSU and SSU rRNA genes that separate \textit{Pichia holstii} from other hat-shaped ascospore-forming and nitrate-accumulating species of the genus \textit{Pichia}, and Nakazawaea holstii was proposed as the type species. This reclassification was not initially accepted because only a few species were included in the rRNA gene sequence analysis (Kurtzman, 1998). Moreover, sequence analysis of the D1/D2 region of the LSU rRNA gene of all recognized species of the genus \textit{Pichia} by Kurtzman \& Robnett (1998) and of the SSU rRNA gene by Suzuki \& Nakase (1999) did not support the reclassification. However, later analysis of multigene sequences including nuclear sequences of the largest subunit and second largest subunit of the RNA polymerase II gene, actin, the second subunit of the mitochondrial cytochrome oxidase gene, and D1/D2 LSU rRNA gene provided much stronger support for the proposal of the genus Nakazawaea (Tsui \textit{et al.}, 2008).

On the basis of analysis of multigenes, including the D1/D2 region of the LSU rRNA gene, translation elongation factor-1\textalpha{} (EF-1\textalpha{}) gene and mitochondrial SSU rRNA gene, the Nakazawaea clade was found to be basal to the \textit{Petrozyla} clade that accommodates two species transferred from the genus \textit{Pichia}, namely \textit{Petrozyla toletana} and \textit{Petrozyla xylosa}, and close to \textit{Pachysolen tannophilus} (Kurtzman \& Robnett, 2010). The Nakazawaea clade as defined by Kurtzman (2011) comprises \textit{N. holstii}, which is the only known ascospore-producing species in the genus Nakazawaea, and nine species of the genus \textit{Candida}, namely \textit{Candida anastomae}, \textit{Candida chilenis}, \textit{Candida helicenia}, \textit{Candida ishiwadae}, \textit{Candida peltata}, \textit{Candida pomicola}, \textit{Candida populi}, \textit{Candida wickerhamii} and \textit{Candida wyomingensis}. \textit{N. holstii} is heterothallic and only asporogenous haploid strains have been isolated from nature.
During an investigation of yeasts in the external leaf surfaces of sugar cane in Thailand, strain DMKU-RK467T, representing a novel species of the genus Nakazawaea, was obtained. After isolation of yeasts using a technique including leaf washing followed by plating, we obtained 372 strains from 102 leaf samples but we were unable to derive a second strain of this novel species. Moreover, we have identified more than 900 yeast strains isolated from the phylloplane of various plants species both by an enrichment technique and by leaf washing followed by plating but we did not obtain another strain of the novel species. According to Kurtzman (2010), the description of species based on a single strain will add to an understanding of yeast phylogeny and species diversity, and we thus decided to describe the novel species based on this single strain. In this paper, strain DMKU-RK467T is described as the type strain of Nakazawaea siamensis f.a., sp. nov.

In total, 166 yeast strains were isolated from the external surfaces of 95 samples of sugar cane (Saccharum officinarum L.) leaves collected in Thailand. Strain DMKU-RK467T was isolated from a sample collected from Chokchaisri district, Nakhon Ratchasima province (14° 43’ 56” N 102° 9’ 47” E), on 6 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). Three grams of leaves, cut to a size appropriate for placement in a 250 ml Erlenmeyer flask, was inoculated to 50 ml of acidified YM broth in the flask 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, 1998) 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 (Seoul, Republic of Korea) for sequencing with primers NL1 and NL4 for the D1/D2 region and primers ITS1 and ITS4 for the ITS region. 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-RK467T was characterized morphologically, biochemically and physiologically according to standard methods described by Yarrow (1998). Hyphae formation was investigated in slide culture on potato dextrose agar (PDA; 20% potato infusion, 2% glucose and 1.5% agar) 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), 5% malt extract agar (5% malt extract and 1.5% agar), McClary’s acetate agar (0.1% glucose, 0.18% potassium chloride, 0.82% sodium acetate trihydrate, 0.25% yeast extract and 1.5% agar), Gorodkowa agar (0.1% glucose, 0.5% sodium chloride, 1% peptone and 2% agar) and corn meal agar (1.7% corn meal agar) at 15 and 25°C for up to 4 weeks. 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 ml Erlenmeyer flask containing 250 ml YPD broth on a rotary shaker at 28°C for 24–48 h and purified according to the methods described by Yamada & Kondo (1973) and Kuraishi et al. (1985). Isoprenologues were identified by HPLC as described previously (Limtong et al., 2007).

**Novel species delineation and identification**

Analysis of the D1/D2 region of the LSU rRNA gene revealed that the sequence of strain DMKU-RK467T was closest to that of Candida wickerhamii CBS 2928T, with 1.9% nucleotide substitutions (11 nt substitutions out of 569 nt). The sequence of the ITS region of strain DMKU-RK467T differed by 5.2% nucleotide substitutions (35 nt substitutions and 99 gaps out of 680 nt) from that of Candida wickerhamii CBS 2928T.

The phylogenetic tree based on sequences of the D1/D2 region of the LSU rRNA gene further demonstrated that strain DMKU-RK467T was in the subclade that contained Candida wickerhamii, N. holstii, Candida anatomiae, Candida ishiwadae, Candida pomicola, Candida populi and Candida wyomingensis at a position distinct from the other members of the clade (Fig. 1).

Cells of strain DMKU-RK467T were globose to subglobose and proliferated by multilateral budding (Fig. 2). Pseudohyphae and true hyphae were not formed in slide culture on PDA. Ascospores were not produced on YPD agar, 5% malt extract agar, McClary’s acetate agar, Gorodkowa agar or corn meal agar after 4 weeks at 15 and 25°C.
On the basis of morphological, biochemical, physiological and chemotaxonomic characteristics and sequence analysis of the D1/D2 region of the LSU rRNA gene and ITS region, we conclude that strain DMKU-RK467\textsuperscript{T} represents a novel species. Although formation of ascospores was not observed, according to the International Code of Nomenclatural for algae, fungi and plants concerning the nomenclatural rules for fungi that the most important is the adoption of ‘one fungus, one name’ (Miller et al., 2011), the novel species is assigned to the genus Nakazawaea, and the designation forma asexualis (f.a.) is included following the recommendation of Lachance (2012). The name Nakazawaea siamensis f.a., sp. nov. (MB 803008) is proposed.

Description of Nakazawaea siamensis

Kaewwichian & Limtong f.a., sp. nov.

Nakazawaea siamensis (si.am.en’sis. N.L. fem. adj. siamen-sis of or belonging to Siam, the old name of Thailand, where the type strain was isolated).

After 3 days at 25 °C in YM broth, cells are globose to subglobose (3–5 μm × 3–5 μm) and occur singly, in pairs or in groups (Fig. 2). Budding is multilateral. After 3 days at 25 °C in YM agar, the streak culture is white to cream, smooth, soft and has an entire margin. Pseudohyphae and true hyphae are not formed in slide culture on PDA after 7 days at 25 °C. Ascospores are not produced on YPD agar, 5 % malt extract agar, McClary’s acetate agar, Gorodkowa agar or corn meal agar after 4 weeks at 15 and 25 °C.

Positive for fermentation of D-glucose but negative for D-galactose, sucrose, maltose, raffinose, lactose, trehalose, xylose and cellobiose. D-Glucose, D-galactose (slow), L-sorbose (slow), sucrose, maltose, cellobiose, trehalose, melezitose, soluble starch, inulin, D-xyllose, L-arabinose (latent), D-arabinose (slow), L-rhamnose, D-ribose, ethanol, glycerol, erythritol, ribitol, galactitol (latent), D-mannitol, D-glucitol, methyl α-D-glucoside, salicin, D-gluconic acid (slow), succinic acid, citric acid, arbutin, N-acetyl-D-glucosamine, 2-keto-D-gluconic acid (weak), 1,2-propanediol (weak), 2,3-butanediol (weak), D-glucono-δ-lactone, xylitol, D-galacturonic acid, arabinose, ethylamine, L-lysine (weak) and cadaverine are assimilated but lactose, melibiose, raffinose, DL-lactic acid, methanol, D-gluconic acid, 5-keto-D-gluconic acid, inositol, D-gluconolactone, hexadecane, nitrate and nitrite are not. No growth in vitamin-free medium. Growth on medium containing 50 % glucose, 60 % glucose and 10 % sodium chloride/5 % glucose is positive, but on 16 % sodium chloride/5 % glucose is negative. Growth with 0.01 and 0.1 % cycloheximide is positive (weak). Grows at 25, 30, 35, 37 and

Fig. 1. Phylogenetic tree based on sequences of the D1/D2 region of the LSU rRNA gene, showing the position of Nakazawaea siamensis sp. nov. DMKU-RK467\textsuperscript{T} with respect to strains of closely related species. The phylogenetic tree was reconstructed from evolutionary distance data with Kimura’s two-parameter correction, using the neighbour-joining method with MEGA version 5.0. Numbers at nodes indicate percentages of bootstrap sampling, derived from 1000 datasets. Pachysolen tannophilus CBS 4044\textsuperscript{T} was used as the outgroup. Bar, 0.005 K\textsubscript{nuc} distance.

Fig. 2. Budding cells of Nakazawaea siamensis sp. nov. DMKU-RK467\textsuperscript{T} in YM broth after 3 days at 25 °C. Bar, 10 μm.
40 °C, but not at 42 or 45 °C. Starch-like compounds are not produced. Diazonium blue B colour and urease reactions are negative. The major ubiquinone is Q-7.

The holotype is DMKU-RK467\(^T\), isolated from the phylloplane of sugar cane (Saccharum officinarum L.) collected from Nakhon Ratchasima province, Thailand. Living cultures from the type have been deposited at the BIOTEC Culture Collection (BCC), National Center for Genetic Engineering and Biotechnology (BIOTEC), Pathumthani, Thailand, as BCC 50734\(^T\), the NITE Biological Resources Center (NBRC), Department of Biotechnology, National Institute of Technology and Evaluation, Chiba, Japan, as NBRC 108903\(^T\) and the Centraalbureau voor Schimmelcultures (CBS), Utrecht, the Netherlands, as CBS 12569\(^T\). The Mycobank registration number is MB 803008.

In practice, \( N. \) siamensis can be distinguished from its closest relative, \( Candida \) wickerhamii, not only on the basis of the sequences of the D1/D2 region of the LSU rRNA gene and ITS region but also on some phenotypic characteristics. \( N. \) siamensis sp. nov. assimilates sucrose, maltose, melezitose, inulin, soluble starch, erythritol (weak), galactitol (latent), methyl \( \alpha\)-D-glucoside and xylitol, in contrast to the type strain of \( Candida \) wickerhamii. The novel species does not assimilate nitrate or nitrite, in contrast to the type strain of \( Candida \) wickerhamii. Growth on medium containing 50 % glucose and 10 % NaCl plus 5 % glucose is positive for \( N. \) siamensis but negative for the type strain of \( Candida \) wickerhamii.

Members of the Nakazawaea clade are often found on plants and substrates associated with plants (Kurtzman, 2011). Many strains of \( N. \) holstii seem to be primarily associated with bark beetles infesting pine, spruce and fir trees (Kurtzman, 2011), but it has also been isolated from other substrates, such as cider and apples, insect frass, streams and lichens. \( Candida \) chilensis was collected from \( Tritoma \) sp., a basidiocarp-feeding beetle, \( Candida \) heliconiaceae was isolated from water accumulated in flower bracts of \( Heliconia \), \( Candida \) populi from sap flux of trembling aspen, \( Candida \) wickerhamii from silage and \( Candida \) wyomingensis from sap fluxes of trees (Kurtzman, 2011; Lachance et al., 2011). However, members of the Nakazawaea clade have not previously been isolated from the leaf surface. In this study of 166 yeast strains obtained from the phylloplane of 95 samples of sugar cane leaf, strain DMKU-RK467\(^T\) was the only strain of Nakazawaea siamensis isolated; therefore, it is clear that the novel species is not common in the phylloplane. \( Meyerozyma \) caribbica was found to be the most common yeast species in the sugar cane phylloplane in this study.

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**References**


