Wickerhamiella siamensis f.a., sp. nov., an endophytic and epiphytic yeast species isolated from sugar cane leaf

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Six strains representing a novel yeast species were isolated from tissue (DMKU-SE106T, DMKU-SE110, DMKU-SE112 and DMKU-SE132) and the external surface (DMKU-SP335 and DMKU-SP406) 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, the six strains were found to represent a single novel species of the genus Wickerhamiella although the formation of ascospores was not observed. The sequences of the D1/D2 region of the LSU rRNA gene and ITS region of the six strains differed from each other by 0–2 and 2–3 nt substitutions, respectively. The novel species was related most closely to Candida infantilis but with 4.5–4.6% nucleotide substitutions in the D1/D2 region of the LSU rRNA gene and 6.6–7.1% nucleotide substitutions in the ITS region. The name Wickerhamiella siamensis f.a., sp. nov. is proposed. The type strain is DMKU-SE106T (=BCC 61185T=NBRC 109697T=CBS 13331T).

The Wickerhamiella clade appears to be monophyletic from the phylogenetic analysis of sequences from a nearly complete LSU rRNA gene, the mitochondrial small subunit (MtSM) rRNA gene and the cytochrome oxidase II (COXII) gene (Kurtzman & Robnett, 2007). At the time of writing, the Wickerhamiella clade consisted of 30 species and included six species with a sexual state viz. Wickerhamiella australiensis, Wickerhamiella cacticola, Wickerhamiella domercqiae, Wickerhamiella lipophila, Wickerhamiella occidentalis and Wickerhamiella pagnoccae and 24 anamorphic species. (Kurtzman et al., 2011; Barbosa et al., 2012; Badotti et al., 2013; Hagler et al., 2013; Dayo-Owoyemi et al., 2014). Based on the phylogenetic analysis of the D1/D2 region of the LSU rRNA gene and the combined sequences of the nearly complete LSU rRNA gene, MtSM rRNA gene and COXII gene, the Wickerhamiella species with a sexual state are separated into two subclades.

Wickerhamiella domercqiae, the type species of the genus Wickerhamiella, is placed in one subclade while the other five species are in another subclade (Kurtzman & Robnett, 2007; Kurtzman et al., 2011; Hagler et al., 2013).

Endophytic micro-organisms are micro-organisms including bacteria, yeast and filamentous fungi that can colonize healthy plant tissues during at least part of their life cycle without causing any damage to the host plant or developing external structures (Petrini, 1991; Azevedo et al., 2000; Linnakoski et al., 2012). On the other hand, epiphytic micro-organisms are bacteria, filamentous fungi, yeasts and algae that inhabit the external surface of various parts of plants (Andrews & Harris, 2000; Inácio et al., 2002; Lindow & Brandl, 2003). Both ascomycete and basidiomycete yeasts have been found to be endophytes and epiphytes, and one yeast species can be both an endophyte and an epiphyte (Isaeva et al., 2010). Endophytic yeasts which have been found in plant leaves include both basidiomycetous yeast viz. Cryptococcus albidus, Cryptococcus flavesens, Cryptococcus laurentii, Cryptococcus magnus, Rhodotorula graminis, Rhodotorula mucilaginosa, Rhodotorula pinicola and Rhodotorula rubra (Gai et al., 2009; Abdel-Motaal et al., 2009; Isaeva et al., 2010; Khan et al., 2012; Akhtyamova & Sattarov, 2013), and ascomycetous yeasts viz. Candida guilliermondii, Candida oleophila, Candida

Abbreviations: COXII, cytochrome oxidase II; ITS, internal transcribed spacer; MtSM, mitochondrial small subunit.

The GenBank/EMBL/DDBJ accession numbers for the D1/D2 region sequences of the LSU rRNA gene are AB915236–AB915239 and AB924020–AB924021, and for the ITS region are AB915240–AB915243 and AB924024–AB924025. The MycoBank number for Wickerhamiella siamensis is MB 808650.
railenensis, Cyberlindnera saturnus, Metschnikowia pulcherrima and Wickerhamomyces anomalus (Nassar et al., 2005; Isave et al., 2010; Rodríguez et al., 2011; Oliveira et al., 2012). Epiphytic yeasts which have been found associated with plant leaves include Cryptococcus aurius, Pseudozyma vetuvera, Pseudozyma graminicola, Rhodotorula glutinis, Rhodotorula colostri, Rhodosporidium fluviale and Sporobolomyces fischeri, which belong to Basidimycota (Golubev et al., 2007; Sląwiková et al., 2009; Pozo et al., 2011; Chamnanpa et al., 2013; Limtong et al., 2014) and Candida tropicalis, Metschnikowia saccharicola, Ogataea phyllophila and Yamazadya sianensis, which belong to Ascomycota (de Azeredo et al., 1998; Limtong et al., 2012; Kaewwichian et al., 2012, 2013). Reports of endophytic yeasts, especially those associated with plant leaves, are fewer compared with reports of epiphytic yeasts.

During the investigation of endophytic and epiphytic yeasts of sugar cane leaves in Thailand, four endophytic yeast strains (DMKU-SE106, DMKU-SE110, DMKU-SE112 and DMKU-SE132) and two epiphytic yeast strains (DMKU-SP335 and DMKU-SP406) representing a novel Wickerhamiella species were obtained. In this article, these six strains are described as representatives of Wickerhamiella siamensis f.a., sp. nov.

Endophytic yeasts were isolated from surface sterilized sugar cane (Saccharum officinarum) leaves by the method described by Abdel-Motaal et al. (2009) with slight modification. Three grams of leaf were cut into fragments (3 x 3 cm) and surface sterilization was conducted by immersion in 70 % ethanol for 3 min, then in 5 % sodium hypochlorite solution for 3 min and then rinsing five times for 5 min with sterile deionized water. The effectiveness of the surface sterilization procedure was verified by spreading the final rinse water onto yeast extract-malt extract (YM) agar (0.3 %, w/v, yeast extract; 0.3 %, w/v, malt extract; 0.5 %, w/v, peptone; 2.0 %, w/v, agar) and placing a few leaf fragments directly onto YM agar; no growth of micro-organisms indicated effective sterilization. After surface sterilization, leaves were cut into smaller fragments (0.5 x 0.5 cm), slightly ground in a sterile mortar to expose the inner tissue and then five fragments were placed onto YM agar supplemented with 0.02 % chloramphenicol in a Petri dish. A total of 15 leaf fragments per sample were used for yeast isolation. The Petri dishes were incubated at 25 °C until yeast colonies appeared. Yeast colonies of different morphologies were selected and purified by cross streaking on YM agar. Purified yeast strains were suspended in YM broth supplemented with 10 % (v/v) glycerol and maintained at −80 °C. Yeast cells were enumerated by mixing 1 g of slightly ground leaf fragments with 9 ml of 0.85 % normal saline solution in a test tube and an aliquot (100 µl) was spread on YM agar supplemented with 0.02 % chloramphenicol in a Petri dish and incubated at 25 °C for 3–7 days. Yeast colonies were counted and reported as c.f.u. per g of fresh leaf weight. Epiphytic yeasts were isolated by plating the leaf washings as described by Inácio et al. (2002). Three grams of leaf were suspended in 50 ml of 0.85 % saline solution in a 250 ml Erlenmeyer flask and shaken on a rotary shaker at 150 r.p.m. and 25 °C for 1 h to detach yeast cells from the surface. An aliquot of 100 µl of the washing solution was then spread on YM agar supplemented with 0.025 % sodium propionate and 0.02 % chloramphenicol and incubated at 25 °C until yeast colonies appeared. Yeast colonies were counted and colonies of different morphologies were purified and maintained as described above.

In total, 127 endophytic yeast strains and 267 epiphytic yeast strains were isolated from tissue and external surfaces, respectively, of 102 samples of sugar cane leaf collected in the central and north-eastern parts of Thailand between October 2011 and March 2012.

Endophytic yeast enumeration revealed low yeast numbers in sugar cane leaf tissue, 6.0 x 10^2 to 9.3 x 10^3 c.f.u. (g fresh leaf)^−1 was found. The number of endophytic yeast in plant leaf was not different from the other parts of plant. One study reported the endophytic yeast number of 2.7 x 10^3 c.f.u. (g fresh maize root)^−1 (Nassar et al., 2005), a second study reported that the number of yeast cells in storage tissues was 10^2−10^4 (Isave et al., 2010); however, in some cases they reached up to 10^6–10^7 c.f.u. (g storage tissue)^−1. On the other hand, the number of epiphytic yeast of sugar cane leaf found in this study was in the range of 4.5 x 10^2–3.7 x 10^6 c.f.u. (g leaf)^−1.

The sequences of the D1/D2 region of the LSU rRNA gene and the ITS (ITS1-5.8S rRNA-ITS2) region were determined from PCR products amplified from genomic DNA, using the primers NL1 and NL4 (Kurtzman & Robnett, 1998), and ITS1 and ITS4 (White et al., 1990), respectively. DNA extraction and amplification of the D1/D2 region of the LSU rRNA gene and the ITS region were performed as described previously (Limtong et al., 2007).

The PCR product was checked by agarose gel electrophoresis and purified using the HiYield Gel/PCR DNA Fragments Extraction kit (RBC Bioscience), according to the manufacturer’s protocol. The purified products were sequenced (Macrogen, Korea) with primers NL1 and NL4 for the D1/D2 region of the LSU rRNA gene, and with ITS1 and ITS4 for the ITS region. The sequences were compared pairwise using the BLAST search program (Altschul et al., 1997) and were aligned with the sequences of related species retrieved from GenBank using the multiple alignment program CLUSTAL_X version 1.81 (Thompson et al., 1997). The 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 the MEGA software version 5.03 (Tamura et al., 2011). Confidence levels of the clades were estimated from bootstrap analysis (1000 replicates) (Felsenstein, 1985).

The strains of the novel yeast species were characterized morphologically, biochemically and physiologically according to the standard method described by Kurtzman et al. (2011). Mycelium formation was investigated by cultivation on potato dextrose agar (PDA; 20 %, w/v, potato
Analysis of the D1/D2 region of the LSU rRNA gene revealed that the six strains represented a single novel species. Four strains (DMKU-SE106T, DMKU-SE110, DMKU-SE112 and DMKU-SE132) were isolated from tissue and two strains (DMKU-SP406 and DMKU-SP335) were isolated from the external surface of sugar cane leaves (Table 1). The sequence of the D1/D2 region of the LSU rRNA gene of the endophytic yeast strain DMKU-SE106T differed by only one nucleotide substitution from the sequences of the other three strains (DMKU-SE110, DMKU-SE112 and DMKU-SE132) and by two nucleotide substitutions from the sequences of the two epiphytic strains (DMKU-SP406 and DMKU-SP335). The sequences of the D1/D2 region of the LSU rRNA gene of these six strains differed by 4.5–4.6 % nucleotide substitutions (25–26 nt substitutions and five gaps out of 562 nt) from Candida infanticola NRRL Y-17858\textsuperscript{T} (DQ438230), the closest species in terms of pairwise sequence similarity. The nucleotide sequence of the ITS region of strain DMKU-SE106T differed by only two nucleotide substitutions from that of two strains (DMKU-SE110 and DMKU-SE132) and by three nucleotide substitutions from the sequences of the other three strains (DMKU-SE112, DMKU-SP335 and DMKU-SP406). The ITS sequences of the six strains differed by 6.6–7.1 % nucleotide substitutions (27–29 nt substitutions and 64–69 gaps out of 409 nt) from the sequences of Candida infanticola NRRL Y-17858\textsuperscript{T} (DQ911458).

A phylogenetic analysis based on sequences of the D1/D2 region of the LSU rRNA gene placed the six strains (DMKU-SE106\textsuperscript{T}, DMKU-SE110, DMKU-SE112, DMKU-SE132, DMKU-SP335 and DMKU-SP406) as a sister clade to Candida infanticola NRRL Y-17858\textsuperscript{T} (DQ911458) and Candida sorbophila NRRLY-1792\textsuperscript{1}\textsuperscript{T} (U45852) with strong bootstrap support (Fig. 1). Although the sequence is polymorphic for the six strains, strong bootstrap values also support their reciprocal monophyly with respect to the sister species. A phylogenetic species concept therefore applies.

The primary criterion of proposing the six strains as representatives of a single novel species was the sequence analysis of the D1/D2 region of the LSU rRNA gene and ITS region. Although formation of ascospores was not observed, according to the nomenclatural rules for fungi described within the International Code of Nomenclature for Algae, Fungi and Plants, the most important requirement is the adoption of 'one fungus, one name' (Miller \textit{et al.}, 2011). Consequently, the novel species was assigned to the genus \textit{Wickerhamiella}, and the designation \textit{forma sexualis} (f.a.) was included following the recommendation as described by Lachance (2012). The name \textit{Wickerhamiella siamensis} f.a., sp. nov. (MB 808650) is proposed.

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### Table 1. Strains isolated from tissue and the external surface of sugar cane leaves collected on 19 April 2012 used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Endophyte/epiphyte</th>
<th>Location of sample collection</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMKU-SE106\textsuperscript{T}=BCC 6118\textsuperscript{T}=NBRC 109697\textsuperscript{T}=CBS 13331\textsuperscript{T}</td>
<td>Endophyte</td>
<td>Chaloem Phra Kiat district, Saraburi province, Thailand (14° 36’ 30 N 100° 58’ 6 E)</td>
</tr>
<tr>
<td>DMKU-SE110</td>
<td>Endophyte</td>
<td>Chaloem Phra Kiat district, Saraburi province, Thailand (14° 36’ 30 N 100° 58’ 6 E)</td>
</tr>
<tr>
<td>DMKU-SE112</td>
<td>Endophyte</td>
<td>Chaloem Phra Kiat district, Saraburi province, Thailand (14° 36’ 30 N 100° 58’ 6 E)</td>
</tr>
<tr>
<td>DMKU-SP335</td>
<td>Epiphyte</td>
<td>Kaeng Khoi district, Saraburi province, Thailand (14° 36’ 30 N 100° 58’ 6 E)</td>
</tr>
<tr>
<td>DMKU-SE132</td>
<td>Endophyte</td>
<td>Nong Muang district, Lopburi province, Thailand (15° 7’ 9 N 100° 53’ 10 E)</td>
</tr>
<tr>
<td>DMKU-SP406</td>
<td>Epiphyte</td>
<td>Nong Muang district, Lopburi province, Thailand (15° 7’ 19 N 100° 53’ 10 E)</td>
</tr>
</tbody>
</table>

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References:
- Miller \textit{et al.}, 2011.
- Lachance (2012).
- Lachance (2012).
Fig. 1. Phylogenetic tree based on the sequence of the D1/D2 region of the LSU rRNA gene, showing positions of strains DMKU-SE110T, DMKU-SE112, DMKU-SE132, DMKU-SP335 and DMKU-SP406 with respect to closely related species. The phylogenetic tree was reconstructed using the neighbour-joining method by MEGA software version 5.03, and the nucleotide substitution rate ($K_{\text{nuc}}$ value) was computed from evolutionary distance data by Kimura’s two-parameter model. Bar, evolutionary distance of 0.02 $K_{\text{nuc}}$. Numbers at nodes indicate percentages of bootstrap sampling, derived from 1000 samples. Numbers in parentheses are GenBank accession numbers. Starmerella bombicola NRRL Y-17069T was the outgroup in the analysis.
Description of *Wickerhamiella siamensis*
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*Wickerhamiella siamensis* (si.am.en’sis. N.L. fem. adj. *siamensis* of or belonging to Siam, the old name of Thailand, where the type strain was isolated).

Growth occurs in YM broth. After 3 days at 25 °C, cells are subglobose to ovoid (1.6–3 × 2–4 μm) and occur singly or in pairs (Fig. 2a). Budding is multilateral. After 3 days of growth on YM agar at 25 °C, the streak culture is white to cream in colour, soft with a smooth surface and has an entire margin. Rudimentary pseudohyphae are formed in slide culture on PDA after 14 days at 25 °C (Fig. 2b), but true hyphae are not formed in slide culture on PDA or corn meal agar after 28 days at 25 °C. Ascospores are not produced for individual strains or strain pairs on PDA, corn meal agar, 5 % malt extract agar, YPD agar, Fowell’s acetate agar at 15 °C or 25 °C after 4 weeks. Fermentation is absent. D-Glucose, D-galactose, L-sorbose, D-xylitol (weak), soluble starch, glycerol, D-glucitol, D-mannitol, D-glucono-1,5-lactone, 5-ketogluconic acid, succinate, ethanol (weak), xylitol (latent) are assimilated, but N-acetylg glucosamine, D-ribose, L-arabinose, D-arabino-bose, L-rhamnose, sucrose, maltose, α-α-trehalose, methyl α-D-glucoside, cellobiose, salicin, melibiose, lactose, raffinose, melazitose, inulin, ribitol, erytritol, galactitol, myo-inositol, 2-ketogluconic acid, D-gluconate, D-gluconurate, D-galacturonic acid, D-lactate, citrate and methanol are not assimilated. Ammonium sulfate, ethylamine hydrochloride, L-lysine and cadaverine are assimilated, but potassium nitrate is not assimilated. No growth occurs in vitamin-free medium or with 16 % (w/v) sodium chloride/5 % (w/v) glucose, 0.01 % cycloheximide or 0.1 % cycloheximide. Growth on medium containing 50 % (w/v) glucose, 60 % (w/v) glucose and 10 % (w/v) sodium chloride/5 % (w/v) glucose is present. Growth is present at 15, 25, 30, 37 °C, but absent at 40, 42 and 45 °C. Acid formation is absent. Starch-like compounds are not produced. Diazonium blue B colour and urease reaction are negative. The major ubiquinone is Q-9.

The type strain, DMKU-SE106ᵀ (=BCC 61185ᵀ=NBRC 109697ᵀ= CBS 13331ᵀ), was isolated from the tissue of a sugar cane (*Saccharum officinarum*) leaf collected from Chaloem Phra Kiat district, Saraburi province, Thailand. The MycoBank accession number is MB 808650.

In practice, *Wickerhamiella siamensis* can be distinguished from the closest related species, *Candida infanticola*, not only by the analysis of the sequence of the D1/D2 region of the LSU rRNA gene and the ITS region but also by some phenotypic characteristics. Rudimentary pseudohyphae are formed by *Wickerhamiella siamensis* but are not formed by *Candida infanticola*. *Wickerhamiella siamensis* assimilates D-xylitol (weakly), soluble starch, 5-ketogluconic acid, ethanol (weakly) and xylitol (latent) while *Candida infanticola* does not. Growth with 0.01 % cycloheximide is absent for *Wickerhamiella siamensis* but is present for *Candida infanticola*.

The members of the *Wickerhamiella* clade have been obtained from various parts of plants such as flowers (*Candida aloacastica*, *Candida musiphila*, *Wickerhamiella cacticola*, *Wickerhamiella kiyanii* and *Wickerhamiella pagnoccae*) and fruits (*Candida sergipensis* and *Wickerhamiella fructicola*) (Lachance et al., 1998; Trindade et al., 2004; Wang et al., 2008; Barbosa et al., 2012; Dayo-Owoyemi et al., 2014). Interestingly, among the plant-associated species, *Wickerhamiella slavikovae* and *Wickerhamiella goesii* were isolated from the surface of sugar cane leaves (Hagler et al., 2013), the same source as the novel species in this study. Some of the members have been found associated with insects, e.g. *Wickerhamiella occidentalis* was reported to have been isolated from morning glories and their associated insects in Hawaii, and *Candida kazou* and *Candida hasegawai* were isolated from insect frass in Thailand (Nakase et al., 2007; Kurtzman et al., 2011). Therefore, plants and associated insects may be important habitats for yeasts in this clade. However, no member of the *Wickerhamiella* clade isolated from plant leaf tissue has ever been reported. In this study, among the 127 endophytic
yeast strains obtained from the tissue of 102 samples of sugar cane leaves, four strains (DMKU-SE106, DMKU-SE110, DMKU-SE112 and DMKU-SE132) represented a novel species, Wickerhamiella siamensis. The most frequently isolated yeast species from sugar cane leaf tissue was Meyerozyma caribbica (18 strains, equivalent to 14.2 % frequency of isolation). The novel species was found among 42 endophytic yeast species including 29 known species, eight undescribed species and five novel species, which was calculated to be 2.4 % of total species. At the same time, two yeast strains (DMKU-SP335 and DMKU-SP406) were isolated from the external surface of sugar cane leaves. Therefore, it is clear that the novel species was present not only in tissue but also on the external surface of plant leaves. In fact, in one sample, strain DMKU-SE132 was detected in the tissue and strain DMKU-SP406 was found on the external surface of sugar cane leaves.

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