**Kluyveromyces siamensis** sp. nov., an ascomycetous yeast isolated from water in a mangrove forest in Ranong Province, Thailand

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**Keywords**
*Kluyveromyces siamensis* sp. nov.; novel species; ascomycetous yeast; water; mangrove forest and Thailand.

**Abstract**
Seven strains of a novel *Kluyveromyces* species were isolated from seven water samples collected from a mangrove forest. Analysis of the D1/D2 domain of the large subunit (LSU) rRNA gene sequences revealed that the sequences of five strains (RS8T, RS20, RS54, RV42 and RV89) were identical and differed from the other two strains (RS65 and RV153) by only one nucleotide substitution in 544 nucleotides (nt). The closest species in terms of pairwise sequences similarity was *Kluyveromyces aestuarii*, but the level of nucleotide substitution (six to seven nucleotide substitutions in 544 nt) was sufficient to justify the description of a separate species. The phylogenetic tree based on the sequence of the D1/D2 domain of the LSU rRNA gene rather suggested that the new species is a sister species of *K. aestuarii* and forms a clade with the other six recognized species of *Kluyveromyces*. Sequence analysis of the internal transcribed spacer (ITS1–5.8S rRNA gene–ITS2) region supported their distinct status as a species. The phenotypic characteristics of the seven strains were typical of the genus *Kluyveromyces*. On this basis, the seven strains were assigned to a single novel species of the genus *Kluyveromyces*, for which the name *Kluyveromyces siamensis* sp. nov. is proposed. The type strain is RS8T (BCC 25962T = NBRC 103859T = CBS 10860T).

**Introduction**
The ascomycetous yeast genus *Kluyveromyces* was first proposed by van der Walt (1956) to accommodate *Kluyveromyces polysporus*. Van der Walt (1965) transferred a number of *Saccharomyces* species to the genus *Kluyveromyces*. In the description of the genus *Kluyveromyces* in *The Yeasts: A Taxonomic Study*, 4th edition, *Kluyveromyces* consisted of 15 species in which *K. polysporus*, the type species, was included (Lachance, 1998). Nagahama et al. (1999) described a novel species of the genus *Kluyveromyces* as *Kluyveromyces nonfermentans*. On the basis of multigene analyses of Kurtzman (2003), only six species, *Kluyveromyces aestuarii*, *Kluyveromyces dozhanskii*, *Kluyveromyces lactis*, *Kluyveromyces maxianus*, *Kluyveromyces wickerhamii* and *K. nonfermentans*, continue to be the recognized species of the genus *Kluyveromyces*. Although the original type species *K. polysporus* was transferred to the genus *Vanderwaltozyma*, Kurtzman et al. (2001) proposed to conserve the genus *Kluyveromyces*. The proposal has been officially accepted by the International Botanical Congress and *K. maxianus* was chosen as the new type species (Lachance, 2007).

In mangrove ecosystems, yeasts play an important role in the detrital food web in that they may be a food source for some marine invertebrates and zooplanktons (Nagahama, 2006). Yeasts are abundant in the water adjacent to and within mangrove swamps (Fell et al., 2004). Recently, *Lachancea meyersii* was described for 18 strains isolated from waters in mangrove habitats in the northern Bahamas (Fell et al., 2004). In Thailand, we reported that yeasts could be isolated from all 32 water samples collected from the surface and below the surface in two mangrove forests in Khao Lumpee-Haad Thaimueang National Park (8°N, 98°E) and Mu Ko Ra-Ko Prathong National Park (9°N, 98°E), Phang-Nga Province, and the two novel species, *Candida thaimueangensis* and *Candida phangngensis*, were described (Limtong et al., 2007b, 2008).

During the course of investigation of yeasts in waters in a mangrove forest in Laem Son National Park (9°N, 98°E), Ranong Province, Thailand, seven strains (RS8T, RS20,
RS54, RS65, RV42, RV89 and RV153) were found to represent a novel species of the genus Kluyveromyces; therefore, in this study we describe and propose *Kluyveromyces siamensis* sp. nov.

**Materials and methods**

**Yeast strains**

Seven yeast strains, RS8<sup>T</sup>, RS20, RS54, RS65, RV42, RV89 and RV153, were isolated from seven water samples collected from a mangrove forest in Laem Son National Park (9°N, 98°E), Ranong Province, Thailand, by membrane filtration following the method of Limtong et al. (2007b). Purified yeast strains were suspended in yeast extract malt extract (YM) broth (0.3% yeast extract, 0.3% malt extract, 0.5% peptone and 1% glucose) supplemented with 10% glycerol and maintained at −80°C.

**DNA sequencing and phylogenetic analysis**

The sequences of the D1/D2 domain of the large subunit (LSU) rRNA gene and the internal transcribed spacer (ITS) (ITS1–5.8S rRNA gene–ITS2) region were determined from PCR products from genomic DNA extracted from yeast cells using a slightly modified version of the method described by Lachance et al. (1999). The D1/D2 domain of the LSU rRNA gene was amplified by a PCR with the forward primer NL1 and the reverse primer NL4 (O’Donnell, 1993); amplification of the ITS region including the 5.8S rRNA gene was performed with the forward primer pITS-F and the reverse primer pITS-R (Sugita et al., 1999). The PCR product was checked by agarose gel electrophoresis, purified using the QIAquick purification kit (Qiagen) and cycle-sequenced using an ABI BigDye terminator cycle sequencing kit, checked by agarose gel electrophoresis, purified using the primer pITS-R (Sugita et al., 1999). The LSU rRNA gene was amplified by a PCR with the forward primer NL1 and the reverse primer NL4 (O’Donnell, 1993); amplification of the ITS region including the 5.8S rRNA gene was performed with the forward primer pITS-F and the reverse primer pITS-R (Sugita et al., 1999). The sequences were determined using an ABI PRISM 3100 automated DNA sequencer (Applied Biosystems), with the external primers, NL1 and NL4, for the D1/D2 domain (Kurtzman & Robnett, 1998) and the external primers, pITS-F and pITS-R, for the ITS region (Sugita et al., 1999). The sequences were determined using an ABI PRISM 3100 automated DNA sequencer (Applied Biosystems) according to the manufacturer’s instructions. The sequences were compared pairwise using BLASTN homology 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). A phylogenetic tree was constructed from the evolutionary distance data with Kimura’s two-parameter correction (Kimura, 1980), using the neighbour-joining method (Saitou & Nei, 1987). Confidence levels of the clades were estimated from bootstrap analysis (1000 replicates) (Felsenstein, 1985).

**Examination of taxonomic characteristics**

The strains were characterized morphologically, biochemically and physiologically according to the standard methods described by Yarrow (1998). Assimilation of nitrogen compounds was investigated on solid media with starved inocula following the method of Nakase & Suzuki (1986). Vitamin requirement was determined according to the method of Komaga & Nakase (1967). Growth at various temperatures was determined by cultivation in YM broth.

**Ubiquinone system**

Ubiquinones were extracted from intact cells cultivated in yeast extract peptone dextrose (YPD) broth (1% yeast extract, 2% peptone and 2% dextrose) 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., 2007a).

**Results and discussion**

**Phylogenetic analysis**

The sequences of the D1/D2 domain of the LSU rRNA gene of the five strains (RS8<sup>T</sup>, RS20, RS54, RV42 and RV89) were identical and differed from the other two strains (RS65 and RV153) by only one nucleotide substitution in 544 nucleotides (nt). The closest species in terms of pairwise sequence similarity was *K. aestuarii* but with 1.1–1.3% nucleotide substitutions (six to seven nucleotide substitutions in 544 nt). According to Kurtzman & Robnett (1998), yeast strains showing nucleotide substitutions >1% in the D1/D2 domain of the LSU rRNA gene are usually different species. The phylogenetic tree based on the sequence of the D1/D2 domain of the LSU rRNA gene rather suggested that the new species is a sister species of *K. aestuarii* and forms a clade with the other six recognized species of the genus *Kluyveromyces*, *K. aestuarii*, *K. dobzhanskii*, *K. lactis*, *K. maxianus*, *K. wickerhamii* and *K. nonfermentans* that constitute a monophyletic clade (Fig. 1). Therefore, the seven novel strains are considered to represent a single novel phylogenetically distinct species. As the difference in the D1/D2 domain of the LSU rRNA gene was relatively small, therefore, the ITS region was determined to confirm the distinct status of the novel species. The sequences of the ITS region of the five strains (RS8<sup>T</sup>, RS54, RV42, RV89 and RV153) were identical and differed from the other two strains (RS20 and RS65) by only one nucleotide substitution in 636 nucleotides. The closest species to the seven strains in terms of pairwise ITS region sequence similarity was *K. aestuarii* but with 1.0–1.1% nucleotide substitutions (six to seven nucleotide substitutions in 636 nt). Sugita et al. (1999)
indicated that conspecific yeast strains usually have a nucleotide difference of less than 1% in the ITS (ITS1–5.8S rRNA gene–ITS2) region. These results lend further support to the conclusion that the seven strains represent a single novel species of the genus *Kluyveromyces* closely related to *K. aestuarii*.

**Phenotypic characteristics**

Cells of *K. siamensis* were spherical to ellipsoidal, proliferated by multilateral budding, and formed one to four spherical ascospores in a conjugated and evanescent ascus. Spherical ascospores in a conjugated and evanescent ascus are produced parthenogenetically or by conjugation between a cell and its bud or between independent cells. Pseudo hyphae were formed but true hyphae were not formed. *Kluyveromyces siamensis* fermented glucose, did not assimilate nitrate, gave negative results for the diazonium blue B and urease reactions and had Q-6 as the major ubiquinone as do other members of the genus *Kluyveromyces*.

The novel species, *K. siamensis*, differed from *K. aestuarii*, its closest phylogenetic relative by some phenotypic characteristics as shown in Table 1.

On the basis of the data reported above, it is therefore concluded that the seven strains represent a single novel species of the genus *Kluyveromyces*. The name *K. siamensis* sp. nov. is proposed for these strains.

**Latin diagnosis of *K. siamensis* Am-In, Yongmanitchai et Limtong sp. nov.**

In medio liquido: ‘YM’, post dies 3 ad 28°C culturae globosae aut ellipsoidae (2.3–5.4 μm × 2.3–6.9 μm), singulae aut binae, per germinacionem multipolarem reproductae. In agar ‘YM’, post dies 3 ad 28°C, cultura butyrosa, creema, sublatum, glabra et margine glabra. Pseudo hyphae formantur nec hyphae non formantur. Ascus formatur per parthenogenesis vel conjunctio. Ascosporae globosae, 1–4 in asco.

D-Glucosum, sucrosum et raffinosum (exique) fermentantur at non D-galactosum, maltosum, lactosum, trehalose nec melibiosum. D-Glucosum, D-galactosum, L-sorbosem, succrosum, cellobiosum, lactosum, raffinosum, D-xyllosum, ethanolum, glycerolum, ribitolum (lente), D-mannitolum, D-glucitolum, salicinum, D-glucono-δ-lactonum, acidum DL-lacticum, acidum succinicum assimilantur at non maltosum, trehalosum, melibiosum, melizitosum, inulinum, amylo specule, L-arabinosum, D-arabinosum, D-ribosum, L-rhamnosum, N-acetyl-D-glucosaminum, methanolum, erytritolum, galactitolum, α-methyl-D-glucosidum, acidum D-glucanicum, acidum D-glucononicum, acidum D-galactoronicum, acidum 2-keto-D-gluconicum, acidum 5-keto-D-gluconicum, acidum citricum nec inositolum. Ethylaminum, L-lysinum et cadaverinum assimilantur at non nitrosum nec nitricum. Vitamina externa ad crescentiam necessaria non sunt. Crescit in 10% NaCl/5% glucosum et non crescit in 15% NaCl/5% glucosum. Non crescit in 0.01% cycloheximido et 0.1% cycloheximido. Crescit in 50% glucosum et 60% glucosum. Crescere potest in temperatura 37 (infirme) et 42°C (infirme). Diazonium caeruleum B non respondens. Ureum non hydrolysatur. Ubiquinonum majus: Q-6.

**Table 1.** Characteristics that distinguish *Kluyveromyces siamensis* sp. nov. from *Kluyveromyces aestuarii*.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th><em>K. siamensis</em></th>
<th><em>K. aestuarii</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Assimilation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-Xylose</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Ribitol</td>
<td>1</td>
<td>–</td>
</tr>
<tr>
<td>Growth</td>
<td></td>
<td></td>
</tr>
<tr>
<td>At 37°C</td>
<td>w</td>
<td>–</td>
</tr>
<tr>
<td>At 42°C</td>
<td>w</td>
<td>–</td>
</tr>
<tr>
<td>With 50% glucose</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>With 60% glucose</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Vitamin-free medium</td>
<td>+</td>
<td>–</td>
</tr>
</tbody>
</table>

+, positive; –, negative; l, latent; w, weak; v, variable.

*Data from Lachance (1998).*
Holotypus: Stirps RS8 isolatus aqua, Ranong Provincia, Thailandia. Cultura et conservatus in Collectionie Culturarum in BIOTEC Culture Collection (BCC), National Center for Genetic Engineering and Biotechnology (BIOTEC), Pathumthani, Thailandia ut BCC 25962T; NITE Biological Resources Center (NBRC), Department of Biotechnology, National Institute of Technology and Evaluation, Chiba, Japonia conservatus ut NBRC 103859T et Centraalbureau voor Schimmelcultures (CBS), Utrecht, the Netherlands, ut CBS 10860T.

Description of *K. siamensis* Am-In, Yongmanitchai & Limtong sp. nov.

Growth in YM broth: After 3 days at 28 °C, cells are spherical to ellipsoidal (2.3–5.4 μm × 2.3–6.9 μm) and occur singly or in pairs (Fig. 2). Budding is multilateral. Growth on YM agar: After 3 days at 28 °C, the streak culture is butyrous, cream-coloured, raised, with a smooth surface and has an entire margin. Formation of hyphae: Slide culture on corn meal agar after 7 days at 28 °C, pseudohyphae are formed but true hyphae are not formed (Fig. 2).

Formation of ascospores: Ascospores are produced on YM agar, 5% malt extract agar, corn meal agar, Gorodkowa agar and Fowell’s acetate agar after 3 days at 28 °C. The ascus is evanescent and formed parthenogenetically or by conjugation between a cell and its bud or between independent cells. Ascospores are spherical and one to four ascospores are formed in one ascus. No pellicle is present on the surface of assimilation medium.

<table>
<thead>
<tr>
<th>Assimilation of carbon compounds</th>
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</table>
| D-Glucose                       | +  
| D-Galactose                     | +  
| L-Sorbose                       | +  
| Sucrose                         | +  
| Maltose                         | -  
| Cellobose                       | +  
| Trehalose                       | -  
| Lactose                         | +  
| Melibiose                       | -  
| Raffinose                       | +  
| Melizitose                      | -  
| Inulin                          | -  
| Soluble starch                  | -  
| D-Xylose                        | +  
| L-Arabinose                     | -  
| D-Arabinose                     | -  
| D-Ribose                        | -  
| L-Rhamnose                      | -  
| N-Acetyl-D-glucosamine          | -  
| Methanol                        | -  
| Ethanol                         | +  
| Glycerol                        | +  
| Erythritol                      | -  
| Adonitol (ribitol)              | Delayed  
| Galactitol                      | -  
| D-Mannitol                      | +  
| D-Glucitol                      | +  
| α-Methyl-D-glucoside            | -  
| Salicin                         | +  
| D-Gluconic acid                 | -  
| D-Glucuronic acid               | -  

Fig. 2. *Kluyveromyces siamensis* sp. nov. RS8T. (a) Vegetative cells and ascospores on YM agar after 4 days at 28 °C. (b) Pseudohyphae formed on corn meal agar after 7 days at 28 °C. Scale bar = 10 μm.
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d-Galacturonic acid –
d-Glucono-δ-lactone +
2-Keto-d-gluconate –
5-Keto-d-gluconate –
dl-Lactic acid +
Sucinic acid +
Citric acid –
Inositol –

Assimilation of nitrogen compounds
Nitrate –
Nitrite –
Ethylamine +
t-Lysine +
Cadaverine +
Growth in vitamin-free medium +
Growth on NaCl 10% and Glucose 5% +
Growth on NaCl 15% and Glucose 5% –
Growth on medium containing 0.01% cycloheximide –
Growth on medium containing 0.1% cycloheximide –
Growth on medium with 50% glucose +
Growth on medium with 60% glucose +
Growth at 20 °C +
Growth at 25 °C +
Growth at 37 °C Weak
Growth at 42 °C Weak
Diazonium blue B color reaction –
Urease –
Acid formation from glucose +
Amyloid production –
Major ubiquinone Q-6

Holotype: RS8 is the holotype of K. siamensis. The strain was isolated from estuarine water collected from a mangrove forest in Laem Son National Park, Ranong Province, Thailand. The living culture from type was deposited at the BIOTEC Culture Collection (BCC), National Center for Genetic Engineering and Biotechnology (BIOTEC), Pathumthani, Thailand, as BCC 103859T, NITE Biological Genetic Engineering and Biotechnology (BIOTEC), BIOTEC Culture Collection (BCC), National Center for Resources Center (NBRC), Department of Biotechnology, National Institute of Technology and Evaluation, Chiba, Japan, as NBRC 103859T and Centraalbureau voor Schimmelcultures (CBS), Utrecht, the Netherlands, as CBS 10860T.

Etymology: The species epithet siamensis (si.a.men.is) N.L. fem. adj. siamensis referring to Siam, the old name of Thailand, where the strains were isolated.

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References


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