Kazachstania siamensis sp. nov., an ascomycetous yeast species from forest soil in Thailand

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Two strains (S-34^T and S-35) of a novel ascomycetous yeast species belonging to the genus *Kazachstania* were isolated from soil from a mixed deciduous forest in Amphoe Wang Nam Khiao, Nakhon Ratchasima Province, Thailand. The D1/D2 domains of the large-subunit rDNA sequences of the two strains were identical and also indicated a close relationship with respect to *Kazachstania aquatica*, *Kazachstania unispora*, *Kazachstania aerobia*, *Kazachstania servazzii* and *Kazachstania solicola*. The most closely related species, *K. aquatica*, has 14 nucleotide substitutions and three gaps in 566 nt. The phenotypic characteristics of the two strains were typical of those of members of the genus *Kazachstania*. These characteristics include the formation of a single globose ascospore in an unconjugated and persistent ascus, multilateral budding, the absence of arthrospores and ballistospores, the fermentation of glucose, the inability to assimilate nitrate, negative diazonium blue B and urease reactions, and the presence of ubiquinone Q-6. The novel strains can be distinguished from *K. aquatica* on the basis of a number of phenotypic characteristics and represent a novel species in the genus *Kazachstania*, for which the name *Kazachstania siamensis* sp. nov. is proposed. The type strain is S-34^T (=CBS 10361^T=NBRC 101968^T=BCC 21230^T).

The genus *Kazachstania*, which is a member of the family Saccharomycetaceae, was first described on the basis of the single species, *Kazachstania viticola* (Zubkova, 1971). In 2003, several species previously assigned to *Saccharomyces* and *Kluyveromyces* were transferred to *Kazachstania* on the basis of a multigene sequence analysis that included genes of the rDNA repeat (18S, 26S, internal transcribed spacer), single-copy nuclear genes (translation elongation factor 1α, actin-1, RNA polymerase II) and mitochondrially encoded genes [small-subunit (SSU) rDNA, cytochrome oxidase II] (Kurtzman, 2003; Kurtzman & Robnett, 2003). Recently, three non-pathogenic species (Lu *et al.*, 2004; Wu & Bai, 2005) and four pathogenic species (Kurtzman *et al.*, 2005) of the genus *Kazachstania* were described. During an investigation of yeasts in soil, two novel strains, S-34^T and S-35, were isolated and found to represent a novel species of the genus *Kazachstania*. The novel species is closely related to *Kazachstania aquatica*, *Kazachstania aerobia*, *Kazachstania solicola*, *Kazachstania servazzii* and *Kazachstania unispora*; the first three of these were described only recently (Lu *et al.*, 2004; Wu & Bai, 2005) and the last two were transferred from the genus *Saccharomyces* (Kurtzman, 2003; Kurtzman & Robnett, 2003). *K. aquatica* and *K. solicola* were identified as novel species by sequence analysis of the SSU rDNA, the internal transcribed spacer region (including 5.8S rDNA) and the large-subunit (LSU) D1/D2 domain (Wu & Bai, 2005), whereas *K. aerobia* was circumscribed on the basis of the internal transcribed spacer and D1/D2 sequences and electrophoretic karyotypes (Lu *et al.*, 2004). *K. servazzii* and *K. unispora* were transferred from the genus *Saccharomyces* on the basis of a multigene sequence analysis (Kurtzman, 2003; Kurtzman & Robnett, 2003).

Strains S-34^T and S-35 were isolated from two soil samples collected from a mixed deciduous forest in Amphoe Wang Nam Khiao, Nakhon Ratchasima Province, Thailand, by using an enrichment technique. Each soil sample (5 g) was added to 50 ml acidified yeast extract/malt extract (YM) broth (0.3 % yeast extract, 0.3 % malt extract, 0.5 % peptone, 1 % glucose; adjusted to pH 3.7–3.8 with 1 M

Abbreviations: LSU, large subunit; SSU, small subunit.

The GenBank/EMBL/DDBJ accession numbers for the D1/D2 domains of the LSU rDNA sequences of strains S-34^T and S-35 are AB258462 and AB258463, respectively.

A neighbour-joining phylogenetic tree based on SSU rDNA sequences is available as a supplementary figure in IJSEM Online.

HCl) supplemented with 0.025 % sodium propionate and 200 mg chloramphenicol l^{-1} in a 250 ml Erlenmeyer flask and incubated at room temperature for 3–4 days on a rotary shaker. The enrichment culture was then spread out on YM agar supplemented with 0.025 % sodium propionate and 200 mg chloramphenicol l^{-1}. When necessary, colonies were restreaked onto YM agar.

The sequences of the D1/D2 domain of the LSU rDNA and the SSU rDNA were determined from PCR products from genomic DNA extracted from yeast cells by using a slightly modified version of the method described by Lachance *et al.* (1999). The D1/D2 domain of the LSU rDNA was amplified by a PCR with the forward primer NL-1 and the reverse primer NL-4 (O'Donnell, 1993); amplification of the SSU rDNA was done with the forward primer P1 and the reverse primer P2 (Sjamsuridzal *et al.*, 1997). The PCR product was checked by agarose gel electrophoresis, purified using the QIAquick purification kit (Qiagen) and cycle-sequenced using the ABI BigDye terminator cycle sequencing kit, version 3.1 (Applied Biosystems), with the external primers NL-1 and NL-4 for the D1/D2 domain (Kurtzman & Robnett, 1998) and eight primers, P1–P8, for the SSU rDNA (Yamada *et al.*, 1999). The sequences were determined with an ABI PRISM 3100 automated DNA sequencer (Applied Biosystems) according to the instructions of the manufacturer. The sequences were compared pairwise by the BLASTN homology search program (Altschul *et al.*, 1990) 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 according to the two-parameter method of Kimura (1980) and the neighbour-joining method (Saitou & Nei, 1987). The robustness of the phylogenetic trees was estimated using bootstrap analysis (1000 replicates) (Felsenstein, 1985).

The strains were characterized morphologically, biochemically and physiologically according to the standard methods described by Yarrow (1998). Assimilation of nitrogen compounds was examined on solid media with starved inocula, using the method of Nakase & Suzuki (1986). Growth at various temperatures was determined by cultivation on yeast extract/peptone/dextrose (YPD) agar (1 % yeast extract, 2 % peptone, 2 % glucose and 2 % agar). Ubiquinones were extracted from intact packed cells cultivated in YPD broth on a rotary shaker at 28 °C for 24–48 h and then purified according to the method described by Yamada & Kondo (1973). The isoprenologues were identified by HPLC using a Cosmosil (5C18; Waters) 4.6 × 250 mm column and methanol/2-propanol (2 : 1) at 1 ml min^{-1} as the elution system, with spectrophotometric detection (275 nm wavelength), according to the method of Kuraishi *et al.* (1985).

The sequences of the D1/D2 domains of the LSU rDNAs of strain S-34^T and S-35 were identical. The two strains clustered with *K. aquatica*, *K. unispora*, *K. aerobia*, *K. servazzii* and *K. solicola* with high bootstrap values and were separate from *Saccharomyces* species (Fig. 1) and as well as from the pathogenic species of the *Kazachstania telluris* complex (*Kazachstania bovina*, *Kazachstania heterogenica*, *Kazachstania pintolopesii* and *Kazachstania slooffiae*) recently described by Kurtzman *et al.* (2005) (data not shown). Strain S-34^T and S-35 showed 3 % divergence (14 nucleotide substitutions and three gaps out of 566 nt) from *K. aquatica*, the closest species in terms of pairwise sequence similarity, indicating that the two strains could represent a novel species. According to Kurtzman & Robnett (1998), yeast strains that show nucleotide substitution greater than 1 % in the D1/D2 domain of the LSU rDNA usually represent different species. However, to confirm the novelty of the two strains, their SSU rDNA sequences were determined: they were found to be identical. The closest relative of the two strains in terms of pairwise sequence similarity was *K. aquatica*, which differed by nine nucleotide substitutions and three gaps out of 1781 nt. In the phylogenetic analysis based on the SSU rDNA sequences, the two novel strains clustered with *K. aquatica*, *K. unispora*, *K. aerobia*, *K. servazzii* and *K. solicola* (see Supplementary Fig. S1 available in IJSEM Online), which is similar to what is seen in the tree based on the D1/D2 LSU rDNA sequences. These results lend further support to the conclusion that the two strains represent a novel species closely related to *K. aquatica*.

Cells of strains S-34^T and S-35 formed single globose ascospores in unconjugated and persistent asci (Fig. 2), proliferated by multilateral budding (Fig. 2), lacked arthrospores and ballistospores, fermented glucose but did not assimilate nitrate, gave negative reactions for the diazonium blue B and urease tests and contained Q-6 as

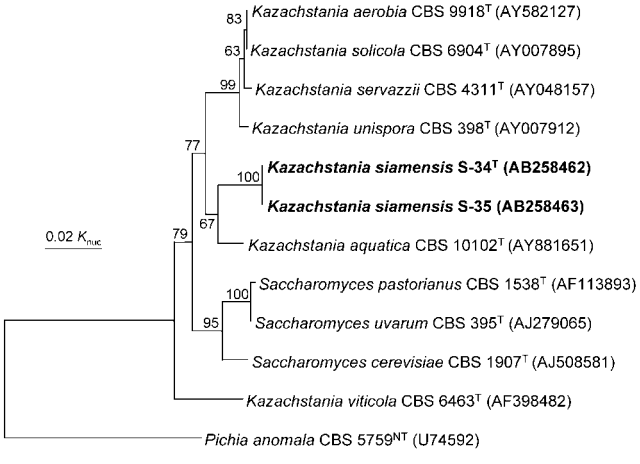

Fig. 1. Phylogenetic tree, based on the D1/D2 LSU rDNA, showing the placement of strains S-34^T and S-35 with respect to closely related species. The tree was constructed with the neighbour-joining method, based on approximately 600 nt, using Kimura's two-parameter distance correction. Numbers at branch points are bootstrap percentages derived from 1000 pseudoreplicates.

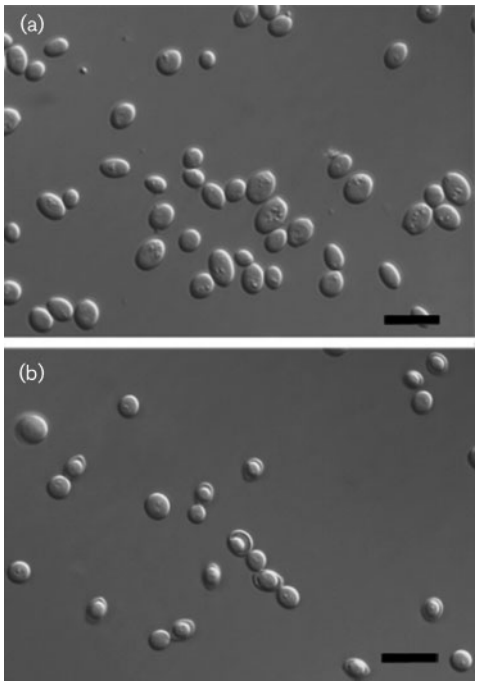

Fig. 2. Micrographs of strain S-34^T showing (a) vegetative cells grown on YM agar for 3 days at 28 °C and (b) ascospores formed on Gorodkowa agar after 7 days at 28 °C. Bars, 10 μm.

the major ubiquinone. These characteristics fit well with those of species of the genus *Kazachstania*. The two strains also shared the same conventional taxonomic characteristics, as shown in Table 1. We conclude, therefore, that the two strains represent a single novel species of the genus *Kazachstania*, for which the name *Kazachstania siamensis* is proposed.

K. siamensis can be distinguished from *K. aquatica*, the species closest to it in the phylogenetic tree, on the basis of a number of phenotypic characteristics, as shown in Table 1.

Latin diagnosis of *Kazachstania siamensis* Limtong, Yongmanitchai, Tun, Kawasaki *et* Seki sp. nov.

In agaro YM *post dies* 3 *ad* 28 °C *cellulae ovoideae aut ellipsoideae*, (2.4–4.8 × 2.9–8.1 μm), *singulae, aut binae, per germinationem multipolarem reproducentes. Cultura cremea, butyrosa, initida, glabra, nitida et margine undulato. In agaro farinae Zea mays post dies* 21 *ad* 28 °C *et agaro* YM *post dies* 21 *ad* 25 °C *pseudomycelium nec mycelium non formantur. In agaro aceti et agaro* Gorodkowa *post dies* 7 *ad* 28 °C *asci formantur. Asci inconjugatio fiunt. Ascosporae globosae*, 1 *in quoque asco. In medio liquido* YM *post unum mensem ad* 28 °C, *sedimentum formantur et pellicula non formantur. Glucosum et galactosum* (*infirme*) *fermentatur at non maltosum, sucrosum, trehalosum, lactosum nec raffinosum. Glucosum, galactosum et trehalosum assimilantur at non* L-*sorbosum*, D-*ribosum*, D-*xylosum*, L-*arabinosum*, D-*arabinosum*, L-*rhamnosum*, *sucrosum*, *maltosum*, *cellobiosum*, *salicinum*, *melezitosum*, *inulinum*, *amylum solubile*, *glycerolum*, *erythritolum*, *ribitolum*, *glucitolum*, D-*mannitolum*, *galactitolum*, *inositolum*, *acidum* 2-*ketogluconicum*, *acidum* D-*gluconicum*, *acidum* D-*glucuronicum*, *acidum* D-*galacturonicum*, *acidum* DL-*lacticum*, *acidum succinicum*, *acidum citricum*, *methanolum nec ethanolum. Ammonium sulfatum assimilatur at non natrum nitrosum*, *kalium nitricum*, *ethylaminum*, L-*lysinum nec cadaverinum. Crescere potest in temperatura* 40 °C *at non in* 42 °C. *Crescit in* 0.01 % *cycloheximido*, 0.1 % *cycloheximido et* 50 % *glucosum* (*infirme*). *Non crescit in* 60 % *glucosum nec* 10 % NaCl/5 % *glucosum* (10 : 5). *Ureum non hydrolysatur. Diazonium caeruleum* B *non respondens. Ubiquinonum majum*: Q-6.

Typus stirpis S-34^T (= CBS 10361^T = NBRC 101968^T = BCC 21230^T) *isolatus e terea*, Nakhon Ratchasima *Provincia*,

Table 1. Growth characteristics of the novel strains and *K. aquatica*

Results for strain S-35 were identical to those given for strain S-34^T. Data for *K. aquatica* were taken from Wu & Bai (2005). Both taxa were positive for the fermentation of D-glucose but negative for the fermentation of D-maltose, sucrose, lactose and raffinose. L-Sorbose, D-ribose, D-xylose, L-arabinose, D-arabinose, L-rhamnose, sucrose, maltose, cellobiose, salicin, melibiose, lactose, raffinose, melezitose, inulin, soluble starch, glycerol, erythritol, ribitol, D-glucitol, D-mannitol, galactitol, *myo*-inositol, DL-lactate, succinic acid, citric acid, methanol, potassium nitrate and sodium nitrite were not assimilated by either taxon. For both taxa, growth did not occur in vitamin-free medium, growth occurred at 25, 30, 35 and 37 °C and growth did not occur at 42 °C. For both taxa, the results for diazonium blue B colour and urease reactions were negative. +, Positive; −, negative; D, delayed positive; ND, no data available; W, weak.

Characteristic	Strain S-34^T	*K. aquatica*
Fermentation of D-galactose	W	+
Assimilation of:		
D-Glucose	+	W, D
D-Galactose	+	W, D
Trehalose	+	W, D
2-Ketogluconic acid	−	ND
D-Gluconic acid	−	ND
D-Glucuronic acid	−	ND
D-Galacturonic acid	−	ND
Ethanol	−	W, D
Ethylamine hydrochloride	−	+
L-Lysine hydrochloride	−	+
Cadaverine	−	+
Growth at/with:		
40 °C	+	−
0.01 % Cycloheximide	+	ND
0.1 % Cycloheximide	+	ND
50 % Glucose	W	ND
60 % Glucose	−	ND
10 % NaCl + 5 % glucose	−	ND

Thailandia, *conservatur in collectionibus culturarum quas* Centraalbureau voor Schimmelcultures (Utrecht, The Netherlands), NITE Biological Resource Center, National Institute of Technology and Evaluation (Chiba, Japan) *et* BIOTEC Culture Collection, National Center for Genetic Engineering and Biotechnology, Thailand (Pathumthani, Thailand) *deposita est.*

Description of *Kazachstania siamensis* Limtong, Yongmanitchai, Tun, Kawasaki & Seki sp. nov.

Kazachstania siamensis (si.am.en′sis. N.L. fem. adj. *siamensis* referring to Siam, the old name of Thailand, where the two strains were isolated).

After 3 days growth on YM agar at 28 °C, cells are ovoid to ellipsoid (2.4–4.8 × 2.9–8.1 μm) and occur singly or in pairs (Fig. 2). Budding is multilateral. Streak culture on YM agar after 3 days at 28 °C is butyrous, cream-coloured, glossy, smooth and raised with undulate margins. In Dalmau plate culture on cornmeal agar at 28 °C and YM agar at 25 °C after 3 weeks, pseudohyphae and true hyphae are not formed. Neither arthrospores nor ballistospores are produced. Ascospores form on Forwell's acetate agar and Gorodkowa agar after 7 days at 28 °C; asci are unconjugated, persistent and each contain a single globose ascospore (Fig. 2). After 1 month in YM broth at 28 °C, sediment is present. A pellicle is not present during growth on the surface of assimilation medium. The major ubiquinone is Q-6. Phenotypic characteristics of the species are shown in Table 1.

The type strain, S-34^T (CBS 10361^T = NBRC 101968^T = BCC 21230^T), was isolated from soil collected in a mixed deciduous forest in Amphoe Wang Nam Khiao, Nakhon Ratchasima Province, Thailand.

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