Kazachstania aquatica sp. nov. and *Kazachstania solicola* sp. nov., novel ascomycetous yeast species

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The unidentified strains AS 2.0706^T, preserved in the China General Microbiological Culture Collection Center (CGMCC), Academia Sinica, Beijing, China, and CBS 6904^T, preserved in the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands, were shown to represent two novel ascomycetous yeast species of the genus *Kazachstania* by 18S rDNA, internal transcribed spacer (ITS) region (including 5.8S rDNA) and 26S rDNA D1/D2 domain sequence analysis and electrophoretic karyotype comparison. The names *Kazachstania aquatica* sp. nov. and *Kazachstania solicola* sp. nov. are proposed for strains AS 2.0706^T and CBS 6904^T, respectively. Phylogenetically, the two novel species are closely related to *Kazachstania aerobia*, *Kazachstania servazzii* and *Kazachstania unispora*.

Recent studies on the molecular taxonomy of yeasts have shown that identification of yeast species based on morphological and physiological criteria is often problematic. In order to authenticate the identification of catalogued yeast strains preserved in the China General Microbiological Culture Collection Center (CGMCC), strains that lacked molecular data were selected for rRNA gene sequencing. One strain, AS 2.0706^T, which was originally labelled as *Torulopsis* sp., was found to represent a novel ascomycetous yeast species based on rRNA gene sequence comparisons. This novel species is closely related to three previously described *Kazachstania* species and to strain CBS 6904^T as revealed by sequence analysis of the small-subunit (18S) rRNA gene, the internal transcribed spacer (ITS) region (including the 5.8S rRNA gene) and the large-subunit (26S) rRNA gene D1/D2 domain. Strain CBS 6904^T, which was assigned to *Saccharomyces dairenensis* (*Naumovia dairenensis*) by Vaughan-Martini & Martini (1998), has recently been shown to represent a separate species by Lu *et al.* (2004). The novel species represented by strains AS 2.0706^T and CBS 6904^T are described in the present study.

Strain AS 2.0706^T was isolated from the wastewater of a paper mill in north-east China. The type strain of *Kazachstania aerobia* (AS 2.2384^T) was obtained from the CGMCC. Strain CBS 6904^T and the type strains of *Kazachstania unispora* (CBS 398^T) and *Kazachstania servazzii* (CBS 4311^T) were obtained from the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands.

Most of the morphological, physiological and biochemical characteristics were examined according to standard methods (Yarrow, 1998). Assimilation of nitrogen compounds was investigated on solid media with starved inocula (Nakase & Suzuki, 1986). Extraction, purification and identification of ubiquinones were carried out according to Yamada & Kondo (1973).

Nuclear DNA was extracted by the method of Makimura *et al.* (1994). The DNA fragment covering the ITS region (including 5.8S rDNA) and the large-subunit rDNA D1/D2 domain was amplified and sequenced according to Lu *et al.*, 2004. The small-subunit rRNA gene was amplified and sequenced according to Sugita & Nakase (1999). Molecular phylogenetic analysis was performed by the methods described by Bai *et al.* (2002). Reference sequences were retrieved from GenBank under the accession numbers indicated in the trees.

Intact yeast chromosomal DNA was prepared by the method of Bai *et al.* (2000). Pulsed-field gel electrophoresis (PFGE) was performed according to Lu *et al.* (2004).

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Abbreviation: ITS, internal transcribed spacer.

The GenBank/EMBL/DDBJ accession numbers for the 18S rRNA, ITS and 26S rRNA gene sequences of *Kazachstania aquatica* sp. nov. and *K. solicola* sp. nov. are AY881650–AY881652.

A more detailed phylogenetic tree based on 18S rRNA gene sequence analysis is available as supplementary material in IJSEM Online.

Fig. 1. Phylogenetic tree drawn from neighbour-joining analysis of the combined sequences of ITS (including 5.8S rDNA) region and 26S rDNA D1/D2 domain sequences, depicting the relationships of *Kazachstania aquatica* sp. nov. and *Kazachstania solicola* sp. nov. with closely related taxa. Bootstrap percentages over 50% from 1000 bootstrap replicates are shown.

Sequence analyses

The close phylogenetic relationship of strain CBS 6904^T with *K. aerobia*, *K. servazzii* and *K. unispora*, based on D1/D2 and ITS sequence analysis, has been shown by Lu *et al.* (2004). The position of strain AS 2.0706^T was not clearly resolved in the tree drawn from the sequences of D1/D2 (data not shown). In trees drawn from the combined ITS (including 5.8S rDNA) and D1/D2 regions (Fig. 1) and from the small-subunit rDNA (see Supplementary Fig. S1 in IJSEM Online), the strain was located in a basal position relative to a small clade containing strain CBS 6904^T and three previously described *Kazachstania* species.

Strain AS 2.0706^T differed from strain CBS 6904^T, *K. aerobia*, *K. servazzii* and *K. unispora* by 3% in the D1/D2 regions and by more than 20 nucleotides in the ITS region. Strain CBS 6904^T and *K. aerobia* had identical D1/D2 sequences, but they differed by 18 base substitutions and 10 indels (6.7% mismatch) in the ITS region. Strain CBS 6904^T differed from *K. servazzii* and *K. unispora* by two and four base substitutions, respectively, in the D1/D2 region and by 21 substitutions and 13 indels (8.1%) and by 34 substitutions and 24 indels (13.8%), respectively, in the ITS region (Lu *et al.*, 2004).

Electrophoretic karyotyping

The electrophoretic karyotypes of strains AS 2.0706^T and CBS 6904^T bore a superficial resemblance to those of the phylogenetically closely related species *K. aerobia*, *K. servazzii* and *K. unispora* (Fig. 2). Approximately 9 (600 to 2200 kb) and 11 (585 to 2200 kb) bands were resolved for AS 2.0706^T and CBS 6904^T, respectively. As wider bands or bands with higher intensities may correspond to doublets or triplets, the actual number of chromosomes may be higher. The patterns were nonetheless sufficiently distinct to differentiate the species from one another. Although strain CBS 6904^T was more closely related to *K. aerobia* than to *K. servazzii* and *K. unispora* based on the sequence data, its chromosomal banding profile had more pronounced differences from that of *K. aerobia* compared with *K. servazzii* and *K. unispora* (Fig. 2).

Taxonomy

On the basis of rRNA sequences and chromosomal DNA banding profiles, we regard strains AS 2.0706^T and CBS 6904^T as representing two novel species of the genus *Kazachstania* and propose the names *Kazachstania aquatica* sp. nov. and *Kazachstania solicola* sp. nov., respectively. Phenotypically, *K. aquatica* sp. nov. is similar to *K. unispora*, the two differing slightly in the assimilation reactions of trehalose and ethanol. *K. solicola* is similar to *K. servazzii*, but the two differ in their responses in the ethanol assimilation test (Table 1).

Fig. 2. Electrophoretic karyotypes. Lanes: 1, *Kazachstania aquatica* sp. nov. AS 2.0706^T; 2, *Kazachstania unispora* CBS 398^T; 3, *Kazachstania servazzii* CBS 4311^T; 4, *Kazachstania aerobia* AS 2.2384^T; 5, *Kazachstania solicola* sp. nov. CBS 6904^T; 6, *Saccharomyces cerevisiae* YNN 295.

Table 1. Physiological characteristics that differentiate *Kazachstania aquatica* sp. nov. and *Kazachstania solicola* sp. nov. from closely related species

+, Positive; W, weak; D, delayed positive; –, negative.

Assimilation of:	<i>K. aquatica</i>	<i>K. solicola</i>	<i>K. aerobia</i>	<i>K. unispora</i>	<i>K. servazzii</i>
Trehalose	W, D	D	+	–	D
D-Ribose	–	D	D	–	W, D
D-Glucosamine	–	D	–	–	–
Ethanol	W, D	–	–	+	+
Glycerol	–	D	+	–	D
D-Mannitol	–	–	D	–	–
Succinic acid	–	–	W	–	–
Ethylamine	+	–	–	+	–
L-Lysine	+	–	+	+	–
Cadaverine	+	–	–	+	–

ITS sequencing can be used to differentiate the novel species from their close relatives, although the amount of intra-specific variation will remain unknown until other strains are available. However the following data suggest that ITS sequences are generally conserved within *Kazachstania* species. Two strains of *K. aquatica* sp. nov. with slightly different karyotypes have been shown to have identical ITS sequences (Lu *et al.*, 2004). Kurtzman & Robnett (2003) examined two strains of *K. unispora* and found them to have identical ITS sequences. The ITS sequences of two additional strains of *K. unispora* (GenBank accession nos AF321542 and AF455430) differ from that of the type strain by only one indel. Nevertheless, since the D1/D2 sequences of *K. solicola* sp. nov. and *K. aerobia* are identical and only one strain is available for the former at present, DNA–DNA hybridization would certainly be helpful to justify the establishment of this novel species.

The genus *Kazachstania* was redefined recently based on multigene sequencing. A relatively large number of species in an only moderately supported clade resolved from the combined sequences of six regions or genes were assigned into the genus (Kurtzman, 2003; Kurtzman & Robnett, 2003). This was apparently a provisional treatment. The phylogenies of these yeasts resolved from the sequences of the small-subunit rRNA gene and other single genes were not congruent with that resolved from the combined gene analysis (James *et al.*, 1997; Kurtzman & Robnett, 2003; Mikata *et al.*, 2001; Špirek *et al.*, 2003). The addition of novel species may help to reclassify the species in this genus into more homogeneous groups. The two novel species described in the present study and the three already described species, *K. aerobia*, *K. servazzii* and *K. unispora*, formed a strongly supported clade in the trees based on the small-subunit rRNA and combined ITS and D1/D2 sequences (see Supplementary Fig. S1 in IJSEM Online and Fig. 1). Interestingly, the species in this group have similar karyotypes (Fig. 2), as do those of *Saccharomyces sensu stricto* (Fischer *et al.*, 2000; Vaughan-Martini *et al.*, 1993).

Latin diagnosis of *Kazachstania aquatica* Bai & Wu sp. nov.

In medio liquido YM post dies 3 ad 25 °C, cellulae globosae vel subglobosae, 3·7–6·2 × 5·0–7·5 µm, singulae, binae et adhaerentes. Per gemmationem multipolarem reproducentes. Post 1 mensem sedimentum formatur. In agarō YM post 1 mensem ad 25 °C, butyrosa, crenea, initida, verrucosa, glabra cum radiatis, margo undulato. In agarō farinae Zea mays post dies 7, pseudohyphae nullae. Asci inconjugatio fiunt. Ascosporae globosae, 1 in quoque asco.

Glucosum et galactosum fermentantur at non sucrosum, maltosum, lactosum nec raffinsum. Glucosum, galactosum, trehalosum (infirme, lente), ethanolum (infirme, lente) assimilantur at non L-sorbosum, sucrosum, maltosum, cellobiosum, lactosum, melibiosum, raffinsum, melezitum, inulinum, amyllum solubile, D-xylosum, L-arabiosum, D-arabiosum, D-ribosum, L-rhamnosum, D-glucosaminum, methanolum, glycerolum, erythritolum, ribitolum, galactitolum, D-mannitolum, D-glucitolum, methyl α-D-glucosidum, salicinum, acidum DL-lacticum, acidum succinicum, acidum citricum, inositolum nec hexadecanum. Ammonium sulfatum, ethylaminum, L-lysinum et cadaverinum assimilantur at non natrum nitrosum nec kalium nitricum. Ad crescentiam vitaminarum externarum necessariae sunt. Maxima temperatura crescentiae: 37 °C. Materia amyloidea iodophila non formantur. Diazonium caeruleum B non respondens. Ureum non hydrolysat. Systema coenzymatis Q-6 adest. Typus depositus in collectione China General Microbiological Culture Collection Center, Academia Sinica (AS 2.0706^T).

Description of *Kazachstania aquatica* Bai & Wu sp. nov.

Kazachstania aquatica (L. fem. adj. *aquatica* aquatic, referring to the source of the type strain).

In YM broth (Yarrow, 1998), after 3 days at 25 °C, the cells are globose to subglobose, 3·7–6·2 × 5·0–7·5 µm and occur singly, in pairs or in groups (Fig. 3). Budding is multilateral.

Fig. 3. *Kazachstania aquatica* sp. nov. AS 2.0706^T. (a) Vegetative cells grown in YM broth for 3 days at 25 °C. (b) Asci formed on acetate agar after 5 days at 25 °C. Bars, 10 µm.

After 1 month at 25 °C, sediment is present. On YM agar (Yarrow, 1998), after 1 month at 25 °C, the streak culture is butyrous, cream-coloured, raised, glossy, verruciform and smooth with faint striations; the margin is undulating. In Dalmau plate culture on cornmeal agar, pseudohyphae are not formed. Sporulation was observed on acetate agar (Fowell, 1952) after 5 days at 25 °C; vegetative cells transform directly into persistent asci each containing one globose ascospore (Fig. 3).

Glucose and galactose are fermented; sucrose, maltose, lactose and raffinose are not fermented. Glucose, galactose, trehalose (weak, delayed), ethanol (weak, delayed) are assimilated; L-sorbose, sucrose, maltose, cellobiose, lactose, melibiose, raffinose, melezitose, inulin, soluble starch, D-xylose, L-arabinose, D-arabinose, D-ribose, L-rhamnose, D-glucosamine, methanol, glycerol, erythritol, ribitol, galactitol, D-mannitol, D-glucitol, methyl α -D-glucoside, salicin, DL-lactic acid, succinic acid, citric acid, inositol and hexadecane are not assimilated. Ammonium sulfate, ethylamine hydrochloride, L-lysine and cadaverine dihydrochloride are assimilated; sodium nitrite and potassium nitrate are not assimilated. Growth in vitamin-free medium is negative. Maximum growth temperature is 37 °C. Starch-like compounds are not produced. Diazonium blue B reaction is negative. Urease activity is negative. Ubiquinone type is Q-6.

The type strain, AS 2.0706^T (= CBS 10102^T), was isolated from wastewater collected in a paper mill in north-east China. This strain has been deposited in the CGMCC, Academia Sinica, Beijing, China.

Latin diagnosis of *Kazachstania solicola* Bai & Wu sp. nov.

In medio liquido YM post dies 3 ad 25 °C, cellulae ovoideae vel subglobosae, 2.5–5.0 × 3.7–7.5 µm, singulae, binae et adhaerentes. Per gemmationem multipolarem reproducentes. Post 1 mensem sedimentum formatur. In agarō YM post 1 mensem ad 25 °C, butyrosa, crenea, seminitida, glabra cum radiatis, margo glabro vel undulato. In agarō farinae Zea mays post dies 7, pseudohyphae nullae. Asci inconjugatio et conjugatio fiunt. Ascoaporiae subglobosae, 1 in quoque asco.

Glucosum et galactosum fermentantur at non sucrosus, maltosum, lactosum nec raffinose. Glucosum, galactosum, trehalosum (lente), D-ribosum (lente), D-glucosaminum (lente) et glycerolum (lente) assimilantur at non L-sorbosum, sucrosus, maltosum, cellobiosum, lactosum, melibiosum, raffinose, melezitosum, inulinum, amyllum solubile, D-xylosum, L-arabiosum, D-arabiosum, L-rhamnosum, methanolum, ethanolum, erythritolum, ribitolum, galactitolum, D-mannitolum, D-glucitolum, methyl α -D-glucosidum, salicinum, acidum DL-lacticum, acidum succinicum, acidum citricum, inositolum nec hexadecanum. Ammonium sulfatum assimilantur at non natrum nitrosus, kalium nitricum, ethylaminum, L-lysinum nec cadaverinum. Ad crescentiam vitaminarum externarum necessariae sunt. Maxima temperatura crescentiae: 37 °C. Materia amyloidea iodophila non formatur. Diazonium caeruleum B non respondens. Ureum non hydrolysat. Systema coenzymatis Q-6 adest. Typus depositus in collectione Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands (CBS 6904^T).

Description of *Kazachstania solicola* Bai & Wu sp. nov.

Kazachstania solicola (N.L. n. *solicola* inhabitant of the soil, referring to the source of the type strain).

In YM broth (Yarrow, 1998), after 3 days at 25 °C, the cells are ovoid to subglobose, 2.5–5.0 × 3.7–7.5 µm and occur singly, in pairs or in groups (Fig. 4). Budding is multilateral. After 1 month at 25 °C, sediment is present. On YM agar (Yarrow, 1998), after 1 month at 25 °C, the streak culture is butyrous, cream-coloured, raised, semi-glossy, smooth with faint striations; the margin is entire to slightly undulating. In Dalmau plate culture on corn meal agar, pseudohyphae are not formed. Sporulation is observed on acetate agar (Fowell, 1952) after 7 days at 25 °C, vegetative cells transform directly into persistent asci each containing one subglobose ascospore (Fig. 4).

Glucose and galactose are fermented; sucrose, maltose, lactose and raffinose are not fermented. Glucose, galactose, trehalose (delayed), D-ribose (delayed), D-glucosamine (delayed) and glycerol (delayed) are assimilated; L-sorbose,

Fig. 4. *Kazachstania solicola* sp. nov. CBS 6904^T. (a) Vegetative cells grown in YM broth for 3 days at 25 °C. (b) Asci formed on acetate agar after 7 days at 25 °C. Bars, 10 µm.

sucrose, maltose, cellobiose, lactose, melibiose, raffinose, melezitose, inulin, soluble starch, D-xylose, L-arabinose, D-arabinose, L-rhamnose, methanol, ethanol, erythritol, ribitol, galactitol, D-mannitol, D-glucitol, methyl α -D-glucoside, salicin, DL-lactic acid, succinic acid, citric acid, inositol and hexadecane are not assimilated. Ammonium sulfate is assimilated; sodium nitrite, potassium nitrate, ethylamine hydrochloride, L-lysine and cadaverine dihydrochloride are not assimilated. Growth in vitamin-free medium is negative. Maximum growth temperature is 37 °C. Starch-like compounds are not produced. Diazonium blue B reaction is negative. Urease activity is negative. Ubiquinone type is Q-6.

The type strain, CBS 6904^T (= AS 2.2406^T), was isolated from soil collected from the Elburz Mountains, Iran. This strain has been deposited in the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.

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