Kazachstania ichnusensis sp. nov., a diploid homothallic ascomycetous yeast from Sardinian lentisk rhizosphere

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During an investigation of yeast biota in the rhizosphere of lentisk in Sardinian semi-arid areas, a strain was isolated that could not be assigned to any known species. The sequence of the D1/D2 domain of the large subunit rDNA gene revealed that the strain belonged to the genus *Kazachstania* and was phylogenetically related to a clade including *Kazachstania aerobia*, *Kazachstania servazzii*, *Kazachstania solicola* and *Kazachstania unispora*. The novel isolate differed from members of this clade in its ability to assimilate D-glucono-1,5-lactone and its very weak fermentation of glucose and sucrose; its assimilation profile was unique within the genus *Kazachstania*. Monosporal colonies were able to sporulate, indicating that the species is homothallic. It is proposed that the isolate represents a novel species, *Kazachstania ichnusensis* sp. nov., with LCF 1675^T (=CBS 11859^T) as type strain.

Lentisk (*Pistacia lentiscus* L.) or mastic is a shrub commonly present in semi-arid areas in Sardinia that has the ability to withstand drought and varying salinity conditions (Barazani & Golan-Goldhirsh, 2009). Yeasts have not previously been observed during studies on the phylloplane of lentisk, although yeasts have been found on the leaves of Mediterranean plants, with a clear prevalence of basidiomycetous species (Inácio *et al.*, 2002, 2010). Both ascomycetous and basidiomycetous yeast have been isolated from the rhizosphere of some plants, including cassava and ginseng (Ferreira *et al.*, 2010; Hong *et al.*, 2006), but no information is currently available on the presence of any yeast in the rhizosphere of lentisk. During an isolation campaign from the rhizosphere of different plants, a yeast strain related to members of the genus *Kazachstania* was isolated.

The genus *Kazachstania* was proposed by Zubkova in 1971 with the description of the novel species *Kazachstania viticola* (Zubkova, 1971), which was then considered to be a synonym of *Saccharomyces dairenensis* (Von Arx *et al.*, 1977). Naumov *et al.* (1995) found that the electrophoretic karyotype of *K. viticola* was dissimilar from that of the other *S. dairenensis* strains. Kurtzman & Robnett (2003) carried out a phylogenetic analysis of the D1/D2 domain of the large subunit rDNA gene, revealing that *K. viticola* belonged to a composite clade including species of the genera *Kluyveromyces*, *Saccharomyces* (*sensu lato*) and *Pachytiospora*. Due to taxonomic priority,

the members of the clade were associated with the genus *Kazachstania*. Since then, novel species of this genus have been found in silage (Lu *et al.*, 2004), wastewater (Wu & Bai, 2005), natural substrates such as humus and mushrooms (Imanishi *et al.*, 2007), soil of a deciduous forest (Limtong *et al.*, 2007), soil (Chen *et al.*, 2010; Lee *et al.*, 2008, 2009) and *Botrytis*-affected grape must (Nisiotou & Nychas, 2008). In general, species of the genus have been isolated from soil and natural or anthropized environments. One species is apparently involved in pathogenicity (Flahou *et al.*, 2010). Classification on the basis of morphological and physiological traits is difficult because there are no distinctive characters (Kurtzman & Robnett, 2003). Some species are heterothallic (Imanishi *et al.*, 2007), whereas others are homothallic (Nisiotou & Nychas, 2008).

The novel yeast strain was isolated from soil surrounding lentisk roots. Soil (5 g) adhering to the roots was scraped off gently with sterile gloves and resuspended in 10 ml physiological solution [aqueous isotonic 0.9 % (w/v) NaCl solution]. Different dilutions of this suspension were spread onto YEPDA (2 % glucose, 1 % yeast extract, 1 % peptone and 1.7 % agar) containing 50 mg Rose Bengal l⁻¹ and 500 mg chloramphenicol l⁻¹. Forty-seven isolates were selected after visual inspection of the plates and stored prior to study; for storage, isolates were cultured overnight in YEPD (YEPDA without agar) and 1 ml overnight YEPD culture was mixed with 0.5 ml 50 % sterile glycerol in a 1.7 ml sterile polypropylene tube immediately placed at –80 °C. All isolates were subsequently grown in YEPD and D1/D2 domain sequencing was carried out as described below.

The GenBank/EMBL/DBJ accession number for the 26S rDNA sequence of LCF 1675^T is HQ616669.

Physiological and morphological tests were carried out on the novel isolate in accordance with previously described methods (Kurtzman & Fell, 1998; Yarrow, 1998).

Cultures of strain LCF 1675^T (=CBS 11859^T) were grown both on McClary's acetate agar (5 days at 25 °C) and on YEPDA (for 1–2 days at 25 °C) and then daily examined microscopically for 1 week for the presence of ascospores.

To induce sporulation of the culture maintained in YEPDA, cells were grown for 2 days in yeast nitrogen base (Sacco) medium containing 2 % glycerol, with shaking at 150 r.p.m. at 25 °C. For short-term storage, the strain was maintained in YEPDA, but for long-term storage, 1 ml fresh overnight culture was frozen with 0.5 ml sterile 50 % glycerol at –80 °C.

Genomic DNA was extracted and sequenced from all 47 isolates (Bolano *et al.*, 2001; Cardinali *et al.*, 2001). Amplification of the D1/D2 domain of the 26S rDNA was performed using primers NL-1 (5'-GCATATCAATAAGCGGAGGAAAAG) and NL-4 (5'-GGTCCGTGTTTCAA-GACGG) (O'Donnell, 1993) with EuroTaq enzyme (EuroClone) and a PTC-100 Peltier Thermal Cycler (MJ Research) according to the following procedure [adapted from the original of Kurtzman & Robnett (1998)]: initial denaturation at 95 °C for 4 min, 35 amplification cycles

(94 °C for 1 min, 53 °C for 1 min and 72 °C for 1 min) and final extension at 72 °C for 10 min. Amplicons were purified with a GFX PCR DNA purification kit (GE Healthcare) and electrophoresis was performed on agarose gels at 1.5 % (Gellyphor; EuroClone). Sequence data were processed with Geneious (Drummond *et al.*, 2009). Alignment of the D1/D2 domain of the rDNA sequence was carried out using Muscle (Edgar, 2004). Phylogenetic analysis was conducted in MEGA4 (Tamura *et al.*, 2007).

Strain CBS 11859^T exhibited vigorous sporulation on YEPDA medium (2 % glucose) after 4 days at 25 °C. After three passages on glucose-containing medium, this ability was strongly reduced and eventually lost. Growth for 3 days on glycerol-containing medium partially restored the ability to sporulate on glucose, although only 20 % of the cells sporulated, rather than 90 % as observed with fresh cultures. Yeast cultures were frozen at –80 °C in 17 % glycerol immediately upon isolation; cultures revitalized from frozen samples exhibited high sporulation ability, which was comparable to that of fresh cultures.

In order to test the possibility that this strain is homothallic, eight spores from two different asci were dissected and grown on YEPDA medium until visible colonies were obtained. All cultures transferred onto McClary's medium

Table 1. Comparison of the assimilation and fermentation profiles of selected substrates by *K. ichnusensis* and related *Kazachstania* species

Strains: 1, *K. ichnusensis* LCF 1675^T (=CBS 11859^T); 2, *K. aerobia* CBS 9918^T; 3, *K. solicola* CBS 6904^T; 4, *K. servazzii* CBS 4311^T; 5, *K. unispora* CBS 398^T; 6, *K. siamensis* CBS 10361^T; 7, *K. aquatica* CBS 10102^T; 8, *K. africana* CBS 2517^T; 9, *K. barnettii* CBS 5648^T; 10, *K. exigua* CBS 379^T; 11, *K. yakushimaensis* CBS 8388^T; 12, *K. naganishii* CBS 8797^T; 13, *K. gamospora* CBS 10328^T; 14, *K. zonata* CBS 10326^T; 15, *K. pintolopesii* CBS 2985^T; 16, *K. hellenica* CBS 10706^T. +, Growth; –, no growth; w, weak growth; d, delayed growth; ND, not determined. Data for the novel isolate were from this study; all other data were from the CBS database except where marked.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Assimilation of:																
D-Galactose	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+
D-Ribose	–	w	–	d, w*	–	–	–	–	–	–	–	–	–	–	–	–
Sucrose	w	–	–	–	–	–	–	–	d	+	–	+	+	+	–	+
α,α-Trehalose	–	+	+	d	–	+	w	–	+	+	–	–	–	–	–	w
Raffinose	–	–	–	–	–	ND	–	–	+	+	–	+	+	+	–	+
Inulin	–	–	–	–	–	–	–	–	–	–	–	–†	+	+	–	+
Glycerol	+	+	–	d	–	–	–	–	–	–	–	–	+	+	–	–
D-Glucono-1,5-lactone	+	ND	ND	–	–	–	ND	–	+	+	–	+	+	–	–	ND
Succinate	–	w	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Ethylamine	–	ND	–	–	–	–	–	–	–	–	+	+	+	+	–	+
Glucosamine	–	ND	ND	–	+	–	–	–	–	–	–	–	ND	ND	–	–
Fermentation of:																
Glucose	w, d	+	+	+	+	w	+	+	+	+	+	+	+	+	+	+
Sucrose	w	–	–	–	–	–	–	–	+	+	–	+	+	+	–	+
α,α-Trehalose	–	ND	ND	–	–	–	ND	–	+	+	ND	ND	–	–	ND	–
Maltose	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

*Data from Wu & Bai (2005).

†Data from Mikata *et al.* (2001).

Fig. 1. Microscopic images of *K. ichnusensis* CBS 11859^T during two different stages of the growth cycle: (a) actively growing culture; (b) spores on McClary's acetate agar after 5 days at 25 °C. Bars, 10 µm.

exhibited the ability to sporulate again, indicating that cells of different mating types were formed within the colonies derived from single spores.

The sequence of the D1/D2 domain of the LSU rDNA showed that strain CBS 11859^T is related, although with moderate bootstrap support (79%), to a clade including *Kazachstania solicola*, *Kazachstania aerobia*, *Kazachstania servazzi* and *Kazachstania unispora*. The moderate level of bootstrap support is shared by other clades within the genus (Fig. 2) and is confirmed by previous phylogenetic reconstructions using the 18S rRNA gene (Imanishi *et al.*, 2007), whereas higher bootstrap values were obtained by combining the D1/D2 and the 18S rDNA sequences (Chen *et al.*, 2010). The use of different algorithms to reconstruct dendrograms resulted in the same topological relations between strain CBS 11859^T and the four species of this clade (data not shown). According to a pairwise comparison, the D1/D2 domain sequences of *K. ichnusensis* displayed nine mismatches (1.67%) from the above species and 10 (1.85%) from *K. servazzi*, which exceeds the difference of 1% suggested as a minimum threshold between species (Kurtzman & Robnett, 1998) and is much larger than many differences observed among members of this genus (Fig. 2).

On the basis of the rDNA distances, we regard strain LCF 1675^T (=CBS 11859^T) as a novel species of the genus

Kazachstania and propose the name *Kazachstania ichnusensis* sp. nov. This novel species status is corroborated by evidence from the assimilation profile of *K. ichnusensis*, which is unique in the genus and differs from those of the four most closely related species, *K. solicola*, *K. aerobia*, *K. servazzi* and *K. unispora*, by the ability to assimilate D-glucono-1,5-lactone vigorously and to assimilate and ferment sucrose weakly. More importantly, the isolate showed weak fermentation of glucose, whereas it was unable to ferment trehalose or maltose. This fermentation behaviour is similar to that of *K. siamensis*, a yeast isolated from soil (Limtong *et al.*, 2007), and contrasts with the general ability displayed by the members of this genus to ferment glucose vigorously (Table 1). The species has been described on the basis of a single strain, as done previously for some members of the genus (Wu & Bai, 2005), but nevertheless, its description should favour the search for other novel strains.

The novel isolate was the only hemiascomycetous yeast isolated from the lentisk rhizosphere in this study, whereas the other isolates belonged to the basidiomycetous species *Cryptococcus festuosus*, *Cryptococcus phenolicus*, *Cryptococcus terreus*, *Cryptococcus terrestris*, *Cryptococcus uzbekistanensis* and *Filobasidium globisporum*. The newly proposed species is one of the few isolated from rhizosphere, e.g. *Kazachstania piceae* (Weber *et al.*, 1992) and *Kazachstania zonata* (Imanishi *et al.*, 2007), whereas most of the other members of the genus were isolated from soil, e.g. *K. servazzii* (Capriotti, 1967) and *K. solicola* (Wu & Bai, 2005).

Latin diagnosis of *Kazachstania ichnusensis* sp. nov.

In medio liquido cum glucoso et peptono et extracto levidino post dies 3 ad 25°C, cellulae ovoideae (4–5 × 2–3 µm), singulae aut binae, per gemmationem reproducentes. Post dies decem sedimentum formatur. Cultura, in agaro cum glucoso et peptono et extracto levidino post dies 7 ad 25 °C, exhibet rotundas et parvas colonias, cum nitido margine et albo opaco colore. In agaro farinae Zea mays post dies 10 ad 25 °C, mycelium et pseudomycelium non formantur. Asci per transformationem cellularum vegetativarum diploidearum, 1–4 ascosporae continentes stabiles sunt. Ascosporae rotundae (2–3 µm) possunt videri. Glucosum et sucrosus infirme fermentantur. Glucosum, galactosum, glycerolum et D-glucono-1,5-lactonum assimilantur. Sucrosus, acidum DL-lacticum, D-ribosum, maltosum, methyl α-D-glucosidum, salicinum, acidum 2-keto-D-gluconicum, acidum D-glucuronum, acidum succinicum, i-inositolum, acidum citricum et N-acetylglucosaminum infirme assimilantur. α,α-Trehalosum, raffinolum, L-sorbosum, D-glucosaminum, D-xylosum, L-arabinosum, D-arabinosum, L-rhamnosum, cellobiosum, melibiosum, lactosum, melezitolum, inulinum, amyllum, erythritolum, adonitolum, dulcitolum, D-mannitolum, D-sorbitolum, acidum malicum, methanolum, ethanolum, kalium nitrosus, kalium nitricum, ethylaminum et L-lysinum non assimilantur. Crescere potest in 0.1% cycloheximido. Non crescit in substrato

Fig. 2. Phylogenetic tree reconstructed from neighbour-joining analysis (Tamura *et al.*, 2004) of the 26S rDNA D1/D2 domain sequences, computed using the Tamura–Nei method (Tamura & Nei, 1993), depicting the relationship of strain CBS 11859^T with members of closely related taxa. Bootstrap values over 50 % (10 000 bootstrap replicates) are shown (Felsenstein, 1985). Reference sequences, all from type strains of the species included in the tree, were retrieved from GenBank and CBS under the accession numbers indicated. The square bracket indicates the subclade containing the novel isolate. Bar, 0.01 substitutions per nucleotide position.

10 % sal/2 % glucosum continente. Non crescit in 50 % glucosum medio. In 4 °C et 25 °C crescere potest, sed non in 37 °C et 42 °C. Materia amyloidea iodophila non formatur. Ureum non hydrolysat. Diazonium caeruleum B negativum est. Proteinae, sed non lipides, hydrolisantur.

Typus ex tellure radicum Pistaciae lentisci in Sardinia insula isolatus est et in collectione zymotica Centraalbureau voor Schimmelcultures, Utrecht, sub no. CBS 11859^T [in nostro laboratorio LCF 1675^T appellatus] depositus est.

Description of *Kazachstania ichnusensis* sp. nov.

Kazachstania ichnusensis (ich.nu.sen'sis. N.L. fem. adj. *ichnusensis* of or belonging to Ichnusa, the ancient name of the island of Sardinia where the yeast was isolated).

After growth in YM broth at 25 °C for 3 days, cells are ovoid (4–5 × 2–3 µm) (Fig. 1a), and occur singly or in pairs (Fig. 1a). Vegetative reproduction occurs by multilateral budding. Sediment is present. After 7 days at 25 °C

on YM agar, streak cultures present small, round-shaped colonies with sharp edges, and a colour varying from matt to bright white. On Dalmau slide cultures with corn meal agar or rice extract agar after 10 days at 25 °C, pseudo-mycelium or true mycelium is not formed either under the cover glass or without cover glass. Sporulation occurs abundantly on YEPDA and McClary's acetate agar at 25 °C after 4 days. Diploid cells are transformed directly into asci containing four round ascospores (2–3 µm). Ascospores are not liberated from asci after 1 month at 25 °C (Fig. 1b). Glucose and sucrose are fermented weakly. Glucose, galactose, glycerol and D-glucono-1,5-lactone are assimilated. Other carbon compounds tested in this study, including sucrose, DL-lactate, D-ribose, maltose, methyl α -D-glucoside, salicin, 2-keto-D-gluconate, D-glucuronate, succinate, citrate, *i*-inositol and *N*-acetylglucosamine are weakly assimilated. α,α -Trehalose, raffinose, L-sorbose, D-glucosamine, D-xylose, L-arabinose, D-arabinose, L-rhamnose, cellobiose, melibiose, lactose, melezitose, inulin, starch, erythritol, adonitol, dulcitol, D-mannitol, D-sorbitol, malic acid, methanol, ethanol, nitrate, nitrite, ethylamine and L-lysine are not assimilated. Growth on 50 % glucose and 10 % NaCl is negative. Growth occurs on 5 % NaCl and weakly in presence of 0.1 % cycloheximide. Growth occurs at 25 and 4 °C, but not at 37 or 42 °C. No starch-like substance is produced. Urease hydrolysis and Diazonium blue B reaction are negative. Lipase activity is weak, whereas proteinase activity on casein agar is positive.

The type strain is LCF 1675^T (=CBS 11859^T), isolated from lentisk rhizosphere in the island of Sardinia, near the Piscinas dunes (39° 32' 30" N 8° 27' 37" E, 33 m above sea-level).

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