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

Gianluigi Cardinali, Livio Antonielli, Laura Corte, Luca Roscini, Ambra Bagnetti, Cristina Pelliccia and Gianfranco Puddu

Department of Applied Biology – Microbiology Division, Borgo 20 Giugno, 74, I – 06121 Perugia, Italy

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 1675T (=CBS 118597) as type strain.

**Correspondence**
Gianluigi Cardinali
gianlu@unipg.it

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 *Klyuyveromyces*, *Saccharomyces* (*sensu lato*) and *Pachytopisospora*. 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 1\(^{-1}\) and 500 mg chloramphenicol 1\(^{-1}\). 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.
Physiological and morphological methods 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’-GGTCCGTGTTTCAA-3’), NL-4 (5’-GGAGGAAAAG-3’) and EuroTaq enzyme (EuroClone) and a PTC-100 Peltier Thermal Cycler (MJ Research) 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 MacClary’s medium

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

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*Data from Wu & Bai (2005).
†Data from Mikata et al. (2001).
showed that strain CBS 11859\textsuperscript{T} is related, although with
The sequence of the D1/D2 domain of the LSU rDNA
of different mating types were formed within the colonies
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\textsuperscript{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\textsuperscript{T} and the four species of this clade
(data not shown). According to a pairwise comparison,
the D1/D2 domain sequences of \textit{K. ichnusensis} displayed
nine mismatches (1.67\%) from the above species and 10
(1.85\%) from \textit{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\textsuperscript{T} (=CBS 11859\textsuperscript{T}) as a novel species of the genus
\textit{Kazachstania} and propose the name \textit{Kazachstania ichnusensis}
sp. nov. This novel species status is corroborated by
evidence from the assimilation profile of \textit{K. ichnusensis},
which is unique in the genus and differs from those of
the four most closely related species, \textit{K. solicola}, \textit{K. aerobia},
\textit{K. servazzi} and \textit{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 beha-
vour is similar to that of \textit{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
\textit{Cryptococcus festucosus}, \textit{Cryptococcus phenolicus}, \textit{Cryptococcus
terreus}, \textit{Cryptococcus terrestris}, \textit{Cryptococcus uzbekistanensis}
and \textit{Filobasidium globisporum}. The newly proposed species is
one of the few isolated from rhizosphere, e.g. \textit{Kazachstania
picae} (Weber et al., 1992) and \textit{Kazachstania zonata}
(Imanishi et al., 2007), whereas most of the other members
of the genus were isolated from soil, e.g. \textit{K. servazzii}
(Capriotti, 1967) and \textit{K. solicola} (Wu & Bai, 2005).

\textbf{Latin diagnosis of \textit{Kazachstania ichnusensis}
sp. nov.}

\textit{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 gennationem 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 formatur. Asci per
transformationem cellularum vegetativarum diploidearum,
1–4 ascospores continentem stabiles sunt. Ascosporae rotundae
(2–3 μm) possunt videri. Glucosum et sucrosum infirme
fermentantur. Glucosum, galactosum, glycerolum et D-
glucono-1,5-lactonum assimilantur. Sucrosum, acidum DL-
lacticicum, D-ribosum, maltosum, methyl α-D-glucosidum,
salicinium, acidum 2-keto-D-gluconicum, acidum D-glucuro-
nicum, acidum succinicum, i-inositolum, acidum citricum et
N-acetylglucosaminum infirme assimilantur. X α-Trehalosum,
raffinosum, L-sorbitosum, D-glucosaminum, D-xylulosum,
L-arabinosum, D-arabinosum, L-rhamnosum, cellobiosum, meli-
biosum, lactosum, melezitosum, inulinum, amyllum, erythri-
tolum, adonitolum, dulcitolum, D-mannitolum, D-sorbitolum,
acidum malicum, methanolum, ethanolum, kalium nitrosum,
kalium nitricum, ethylaminum et L-lysinum non assimilantur.
Crescere potest in 0.1 % cycloheximido. Non crescit in substrato

\textbf{Fig. 1. Microscopic images of \textit{K. ichnusensis} CBS 11859\textsuperscript{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.}
10% sal/2% glucosum continent. Non crescit in 50% glucosum medio. In 4°C et 25°C crescre potest, sed non in 37°C et 42°C. Materia amyloidae iodophila non formatur. Ureum non hydrolysatur. 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 11859T [in nostro laboratorio LCF 1675T appellatus] depositus est.

Description of Kazachstania ichnusensis sp. nov.

Kazachstania ichnusensis (ich.nu.sen’s.isis. 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 z-D-glucoside, salicin, 2-keto-D-gluconate, D-glucuronate, succinate, citrate, i-inositol and N-acetylglucosamine are weakly assimilated. x, z-Trehalose, raffinose, L-sorbos, D-glucosammon, D-xyllose, 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-lysinne 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 1675T (=CBS 11859T), 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).

Acknowledgements

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References


