Four ascomycetous yeast strains (D4W13, D9W2, D9W4 and D9W17T) were isolated from Botrytis-affected fermenting grape juice originating from Attica Province, Greece. Phylogenetic analysis of rRNA gene sequences (18S, 26S and 5.8S–ITS) showed that the four strains represent a distinct species within the genus Kazachstania, closely related to Kazachstania zonata NBRC 100504T and Kazachstania gamospora NBRC 110565. Electrophoretic karyotyping and physiological analysis support the affiliation of the four strains in a novel species for which the name Kazachstania hellenica sp. nov. is proposed, with D9W17T (=CBS 10706T=NBRC 103637T) as the type strain.

The genus Kazachstania within the family Saccharomycetaeae was first described in 1971 on the basis of a single species, namely Kazachstania viticola, isolated from fermenting grapes in Kazakhstan (Zubkova, 1971). Recently, multigene sequence analysis has redefined phylogenetic relationships among yeasts of the Saccharomyces complex and several former species of the genera Saccharomyces, Klyveromyces, Arxiozyma and Pachytrichospora have been transferred to the genus Kazachstania (Kurtzman, 2003; Kurtzman & Robnett, 2003). Since then, several novel species of the genus Kazachstania have been described, representing either non-pathogenic (Lu et al., 2004; Wu & Bai, 2005; Imanishi et al., 2007; Limtong et al., 2007) or pathogenic species (Kurtzman et al., 2005).

Strains D4W13 (=CBS 10704=NBRC 103634), D9W2 (=CBS10738=NBRC 103635), D9W4 (=CBS 10705=NBRC 103636) and D9W17T (=CBS 10706T=NBRC 103637T) were isolated during an investigation of yeast species diversity in must fermentations from Botrytis-infected grapes (Nisiotou et al., 2007). Grape berries (Vitis vinifera L.) were collected from the experimental vineyard of the Agricultural University of Athens, Attica, Greece, and transferred aseptically to the nearby laboratory where juice was extracted and left to ferment spontaneously (without any starter cultures or chemical additives). Botrytis infection of grapes was verified as shown elsewhere (Nisiotou & Nychas, 2007). Samples of fermenting juice were decimally diluted in Ringer’s solution and 100 μl portions were spread onto Wallerstein laboratory nutrient agar (Oxoid) supplemented with chloramphenicol (Sigma) at 100 mg l⁻¹. Colonies were restreaked onto YPD agar (1 % yeast extract, 2 % peptone, 2 % glucose, 2 % agar) and stored at −80 °C until further analysis. Morphological, physiological and biochemical characteristics were examined according to standard methods (Yarrow, 1998). Morphology of cells was observed under an Olympus BX50 microscope and images were acquired using an Olympus DP71 digital camera.

Genomic DNA was isolated as described previously (Burke et al., 2000). The 18S rRNA gene was amplified according to Ueda-Nishimura & Mikata (1999), the D1/D2 domain of the 26S rRNA gene was amplified according to Kurtzman & Robnett (1998) and the 5.8S–internal transcribed spacer (ITS) rRNA region was amplified as described previously (Nisiotou & Gibson, 2005). PCR products were checked by agarose gel electrophoresis and purified using the QIAquick PCR purification kit (Qiagen). Both DNA strands were directly sequenced with an ABI 3730 XL automatic DNA sequencer by Macrogen (http://www.macrogen.com). BLAST searches of sequences from GenBank were carried out and CLUSTAL_X (1.83) software (http://www-igbmc.u-strasbg.fr/BioInfo) was used to perform sequence alignments. Phylogenetic analysis was conducted using the MEGA version 4 software package (Tamura et al., 2007) after multiple alignment of data by CLUSTAL_X (Thompson et al., 1997). Distances (distance options according to the Kimura two-parameter model) and clustering with the neighbour-joining and
maximum-parsimony methods were determined using bootstrap values based on 1000 replications.

Intact yeast chromosomal DNA was prepared for pulsed-field gel electrophoresis using the method described by Bai et al. (2000). Chromosomal DNA bands were separated as described by Imanishi et al. (2007) in a contour-clamped homogeneous electric field electrophoresis apparatus (CHEF II; Bio-Rad). After electrophoresis, the gel was stained in ethidium bromide solution (0.5 mg ml⁻¹) for 1 h, destained in distilled water and viewed under UV light (302 nm). Saccharomyces cerevisiae YNN 295 chromosomal DNA (Bio-Rad) was used as a molecular size marker.

rRNA sequence comparisons of the 18S, 26S and 5.8S–ITS regions of strains D4W13, D9W2, D9W4 and D9W17T and other strains available in GenBank revealed that the novel isolates were identical to each other and closely related to Kazachstania zonata and Kazachstania gamospora. In the D1/D2 domain, the isolates differed from K. zonata and K. gamospora by 5 noncontiguous substitutions (0.9 %) and by 10 noncontiguous substitutions plus 1 indel (2 %) in 554 nt, respectively. In general, strains of a species show no more than 0–3 nt differences (0–0.5 %) and strains showing 6 or more noncontiguous substitutions (1 %) are separate species, whereas strains with an intermediate number of nucleotide substitutions are generally also separate species (Kurtzman, 2006a).

For further assessment of genetic separation, the 5.8S–ITS region was analysed as suggested previously (Kurtzman, 2006b). The respective sequences of strains D4W13, D9W2, D9W4 and D9W17T were identical, but differed significantly from those of K. zonata and K. gamospora by 72 base substitutions and 24 indels (13.6 %) and by 83 substitutions and 31 indels (16.1 %) in 708 nt, respectively. It has been shown that conspecific yeast strains usually have fewer than 1–2 % nucleotide differences in the ITS 1 and 2 regions (Sugita et al., 1999a, b; Nagahama et al., 1999; Bai et al., 2001). Hence, data acquired from D1/D2 and ITS sequence analyses indicate that the four strains represent a genetically separate species. This was further supported by sequence comparisons of the 18S rRNA gene that revealed

---

**Fig. 1.** Bootstrap consensus neighbour-joining tree of the combined D1/D2, ITS (including 5.8S) and 18S rRNA gene sequences depicting the relationships between Kazachstania hellenica sp. nov. and closely related species. The respective GenBank accession numbers are given in parentheses. Numbers at branch points are bootstrap percentages derived from 1000 replicates. Hanseniaspora guilliermondii NRRL Y-1625⁷ was the outgroup species in the analysis. Bar, 0.01 substitutions per nucleotide position.
5 (3 substitutions and 2 indels) and 6 (all substitutions) nucleotide differences in 1755 bp between the novel species and K. zonata and K. gamospora, respectively.

Phylogenetic trees based on D1/D2, ITS (including the 5.8S rRNA gene) or 18S rRNA gene sequences were constructed to visualize interrelationships among the four isolates and other members of the family Kazachstania. In all cases, the novel species, represented by strain D9W17T, was clearly placed within the recently described small clade of K. zonata and K. gamospora. Similar results were obtained when a phylogenetic tree was drawn from the analysis of the combined sequences of D1/D2, ITS and 18S regions. No differences were detected between neighbour-joining and maximum-parsimony methods, particularly regarding the position of strain D9W17T. Therefore, only the neighbour-joining tree is presented (Fig. 1).

Chromosomal banding profiles of the four novel strains clustered into two groups that were quite similar to each other, but profoundly distinct from those of K. zonata and K. gamospora (Fig. 2). Strains D9W2 and D4W13 possessed 12 chromosomes ranging in size from 400 to 2200 kb and strains D9W4 and D9W17 had 11 chromosomes ranging from 400 to 1600 kb. The banding profiles of K. zonata NBRC 101821 and K. gamospora NBRC 11056 consisted of 11 and 14 chromosomes, respectively, ranging from 300 to 2200 kb, as described previously (Imanishi et al., 2007).

The results of the physiological characterization are given in the species description. The four strains exhibited a number of distinct morphological and physiological characters that clearly differentiated them from the closely related species K. zonata and K. gamospora and also from other Kazachstania species (Fig. 3a, b; Table 1). For instance, all four strains produced one to four spheroidal ascospores, whereas both K. zonata and K. gamospora produce two ascospores per ascus. The novel strains were able to assimilate maltose, cellobiose and melezitose, unlike other, but profoundly distinct from those of K. zonata and K. gamospora.

Description of Kazachstania hellenica Nisiotou & Nychas sp. nov.

Kazachstania hellenica [hel.len’i.ca. N.L. fem. adj. hellenica (from Gr. fem. adj. hellenikê) Hellenic, referring to Hellas, where the four strains were isolated].

In YM broth (Yarrow, 1998), after 3 days at 25 °C, cells are ovoid (4.3–7.1 × 6.1–9.0 μm) and occur singly, in pairs or aggregated in large clumps (Fig. 3a). Budding is

Latin diagnosis of Kazachstania hellenica Nisiotou et Nychas sp. nov.


Fig. 2. Electrophoretic karyotypes. Lanes: 1, Saccharomyces cerevisiae YNN 295; 2, K. gamospora NBRC 11056T; 3, K. zonata NBRC 101821; 4, K. hellenica sp. nov. D4W13; 5, Saccharomyces cerevisiae YNN 295; 6, K. hellenica sp. nov. D9W2; 7, K. hellenica sp. nov. D9W4; 8, K. hellenica sp. nov. D9W17T.
multilateral. After 1 month at 25 °C, a flocculent sediment is present. On YM agar (Yarrow, 1998), after 1 month at 25 °C, the streak culture is butyrous, cream-coloured, raised and smooth; the margin is entire to undulating. Sporulation is observed on cornmeal agar after 5 days at 25 °C. Asci may be unconjugated or show bud–parent cell conjugation (Fig. 3b) suggesting that the species is probably homothallic. Asci are persistent, containing 1–4 spheroidal ascospores. Glucose, galactose and sucrose are fermented; maltose, lactose, raffinose and trehalose are not fermented. Glucose, galactose, sucrose, maltose, cellobiose, trehalose (delayed), raffinose, melezitose (weak, delayed), inulin, D-xylose (delayed), L-arabinose, D-glucitol (weak) and arbutin are assimilated; L-sorbitose, lactose, melibiose, D-arabinose, D-ribose, L-rhamnose, D-glucosamine, N-acetyl-D-glucosamine, ethanol, glycerol, D-mannitol, adonitol, salicin, D-glucurate, DL-lactate, succinate, citrate, inositol and 2-ketogluconate are not assimilated. Ethylamine hydrochloride is assimilated; L-lysine, sodium nitrite, potassium nitrate and cadaverine are not assimilated. Does not grow in 10% sodium chloride plus 5% glucose in a nitrogen base. Maximum growth temperature is 34 °C. Starch-like compounds are not produced. Urease activity is negative. Grows in the presence of 50% (w/v) glucose.

The type strain, D9W17^T (=CBS 10706^T=NBRC 103637^T), was isolated from fermenting grape juice in Athens, Greece, in October 2004.

### References


http://ijs.sgmjournals.org


IP: 54.70.40.11

Downloaded from www.microbiologyresearch.org by