Kazachstania aerobia sp. nov., an ascomycetous yeast species from aerobically deteriorating corn silage

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In an investigation of the yeast biota involved in silage deterioration, a considerable number of strains belonging to Saccharomyces and related genera were isolated from aerobically deteriorating corn silage in Tochigi, Japan. Analysis of sequences of the internal transcribed spacer and the large-subunit rRNA gene D1/D2 domain and electrophoretic karyotyping indicated that two of the strains, NS 14T and NS 26, represent a novel species with close phylogenetic relationships to Kazachstania servazzii and Kazachstania unispora. It is proposed that the novel species be named Kazachstania aerobia sp. nov., with NS 14T (＝AS 2.2384T＝CBS 9918T) as the type strain.

Yeasts are common members of the microbiota of forage crops. Various facultatively anaerobic and acid-tolerant yeasts are involved in the process of silage fermentation. Their activity is considered undesirable. Under anaerobic conditions, yeasts ferment sugars to ethanol and CO2, resulting in a decrease in the sugar available for acid production and an increase in dry-matter loss during ensilage. Under aerobic conditions, yeasts utilize lactic acid, causing an increase in silage pH, which becomes favourable for the growth of many other spoilage organisms (Driehuis & Oude Elferink, 2000). Yeasts, therefore, are recognized as the most important group of microorganisms responsible for the initiation of aerobic spoilage processes in silage (Ohmomo et al., 2002; Woolford, 1990).

In an investigation of the yeast biota involved in the deterioration of corn silage, a considerable number of the strains isolated were identified as members of Saccharomyces and related genera in the ‘Saccharomyces complex’ (Kurtzman & Robnett, 2003) by means of 26S rRNA gene D1/D2 domain sequencing. Two strains, NS 14T and NS 26, selected from one group of these yeasts, were shown to represent a novel species closely related to Saccharomyces servazzii and Saccharomyces unisporus by rRNA gene sequencing and electrophoretic karyotyping. The latter two species have been transferred to the phylogenetically redefined genus Kazachstania Zubkova as Kazachstania servazzii and Kazachstania unispora (Kurtzman, 2003). The novel species is therefore assigned to the same genus.

Strains NS 14T and NS 26 were isolated from aerobically deteriorating corn silage samples by the dilution-plate technique, using potato/glucose agar (Difco). Reference strains CBS 398T (K. unispora), CBS 4311T (K. servazzii) and CBS 6904 (Kazachstania sp.) were obtained from the Centraalbureau voor Schimmelcultures, 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 internal transcribed spacer (ITS) region (including the 5-8S rRNA gene) and the 26S rRNA gene D1/D2 domain was amplified with the primer pair ITS1 (5’-GTC GTA ACA AGG TTT CCG TAG GTG-3’) and NL4 (5’-GTT CCG TGT TTC AAG...
ACG G-3'). The PCR was performed for 36 cycles, with denaturation at 94 °C for 1 min, anealing at 55 °C for 1 min and extension at 72 °C for 2 min. PCR products were purified by using SUPREC-02 centrifugal filter tubes (Takara Shuzo) according to the instructions of the manufacturer. After purification, the PCR products were directly sequenced with the forward primers ITS1 and NL1 (5'-GCA TAT CAA TAA GCG GAG GAA AAG-3') and the reverse primers ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') and NL4, using the ABI BigDye Terminator cycle sequencing kit (Applied Biosystems). Electrophoresis was done on an ABI Prism 377 or 310 DNA sequencer (Applied Biosystems). Phylogenetic analysis was performed using methods described previously (Bai et al., 2002). Reference sequences were retrieved from GenBank under the accession numbers indicated in Fig. 1.

Intact yeast chromosomal DNA was prepared for PFGE using the method of Bai et al. (2000). Chromosomal DNA bands were separated on 1% (w/v) agarose gels in 0.5× TBE buffer (45 mM Tris/borate, 1 mM EDTA, pH 8.0) in a contour-clamped homogeneous electric field electrophoresis apparatus (CHEF Mapper XA; Bio-Rad). The electrophoresis programme was divided into four steps with a total run time of 54 h: step 1 had a pulse time of 240 s for 12 h, step 2 had a pulse time of 160 s for 16 h, step 3 had a pulse time of 90 s for 16 h, and step 4 had a pulse time of 60 s for 10 h. The temperature of the running buffer was maintained at 14 °C and the power supply was set to 4.5 V cm⁻¹. After electrophoresis, the gel was stained in ethidium bromide solution (0.5 μg ml⁻¹) for 1 h, de-stained in distilled water and viewed under UV light (302 nm). Saccharomyces cerevisiae (YNN 295) chromosomal DNA (Bio-Rad) was used as the molecular size marker.

Sequence comparison

Strains NS 14ᵀ and NS 26 have identical sequences in both the D1/D2 and ITS regions, indicating their conspecificity. The rRNA gene sequence comparison showed that their closest relatives were strain CBS 6904, K. servazzii and K. unispora. In the D1/D2 region, strains NS 14ᵀ and NS 26 were identical to strain CBS 6904, K. servazzii and K. unispora. The electrophoretic karyotypes of strains NS 14ᵀ and NS 26 differed from strain CBS 6904, K. servazzii and K. unispora by 28 (18 base substitutions and 10 indels), 25 (20 substitutions and 5 indels) and 51 (37 substitutions and 14 indels) bases, respectively. Strain CBS 6904 differed from K. servazzii and K. unispora in the same region by 34 (21 substitutions and 13 indels) and 58 (34 substitutions and 24 indels) bases, respectively.

The close phylogenetic relationships of strains NS 14ᵀ and NS 26 with respect to strain CBS 6904, K. servazzii and K. unispora analysed from ITS sequences are illustrated in Fig. 1. In a tree constructed from the D1/D2 sequences of all accepted species in the genus Kazachstania and the type species of other related genera in the family Saccharomycetaceae (Kurtzman, 2003), strains NS 14ᵀ and NS 26 also clustered together with K. servazzii, K. unispora and strain CBS 6904 with strong bootstrap support (see supplementary material in IJSEM Online).

Electrophoretic karyotyping

Previous studies have shown that the Saccharomyces sensu stricto species exhibit relatively homogeneous karyotypes that are clearly different from those of the Saccharomyces sensu lato species (Vaughan-Martini et al., 1993; Fischer et al., 2000; Naumov et al., 1992, 1995; Petersen et al., 1999). Among the Saccharomyces sensu lato species, S. servazzii (K. servazzii) and S. unisporus (K. unispora) exhibit similar karyotypes (Naumov et al., 1995; Petersen et al., 1999). However, when multiple strains were compared, the karyotypes of K. unispora strains were more similar to each other than to those of K. servazzii strains (Naumov et al., 1995).

The electrophoretic karyotypes of strains NS 14ᵀ and NS 26 are similar, but not identical (Fig. 2). In agreement with the phylogenetic relationships established by sequence analysis, these karyotypes resemble those of strain CBS 6904 and the types of K. servazzii and K. unispora, although strains NS 14ᵀ and NS 26 are easily differentiated from the others. Eleven bands were resolved for each of NS 14ᵀ and NS 26, with molecular sizes ranging approximately from 580 to 2000 kb. As bands with stronger relative intensity may correspond to doublets or triplets, it is not easy to

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**Fig. 1.** Phylogenetic tree drawn from neighbour-joining analysis of the ITS (including the 5.8S rRNA gene) sequences, depicting the relationship between K. aerobia sp. nov. and closely related taxa. Bootstrap percentages over 50% from 1000 bootstrap replicates are shown. Reference sequences were retrieved from GenBank under the accession numbers indicated.

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Strain CBS 6904 appears to be more similar to judge the exact chromosomal numbers of these two strains. Electrophoretic karyotypes. Lanes: 1, K. unispora 295; 2, K. unispora NS 26; 5, CBS 6904; 6, K. aerobia.

**Taxonomy**

The D1/D2 and ITS sequence and karyotype comparisons indicated that strains NS 14T and NS 26 represent a novel species closely related to K. servazzii, K. unispora and strain CBS 6904. The two strains reproduce asexually by multi-lateral budding on a narrow base and sexually by forming un conjugated and persistent ascii containing single ascospores. The name _Kazachstania aerobia_ sp. nov. is proposed for the novel species. The novel species differs from _K. servazzii_ by the ability to assimilate D-ribose, D-mannitol and L-lysine, and from _K. unispora_ in the assimilation of ethanol, glycerol, D-ribose, D-mannitol, ethylamine and cadaverine.

Strain CBS 6904 was assigned to _Naumovia dairrenensis_ (formerly _Saccharomyces dairrenensis_) by Vaughan-Martini & Martini (1998) on the basis of its physiological characteristics. However, the molecular karyotype of this strain is strikingly different from that of the type strain of _N. dairrenensis_ (Naumov et al., 1995). Analyses of the 18S rRNA gene (James et al., 1997), the 26S rRNA gene D1/D2 domain and the ATP9 mitochondrial gene (Spírek et al., 2003) sequences indicated that strain CBS 6904 was most closely related to _K. servazzii_. Although the D1/D2 sequence of strain CBS 6904 was identical to that of _K. aerobia_ sp. nov., comparisons of ITS sequences and electrophoretic karyotypes made in the present study suggest that this strain represents a distinct species in the genus _Kazachstania_. Physiologically, _K. aerobia_ sp. nov. differs from strain CBS 6904 by its ability to assimilate D-mannitol and l-lysine.

_Kazachstania_ and other related genera in the family Saccharomycetaceae were redefined on the basis of multigene sequencing (Kurtzman & Robnett, 2003). Analysis of combined gene sequences of the rRNA gene repeat (18S, 26S, ITS), single-copy nuclear genes (translation elongation factor 1α, actin-1, RNA polymerase II) and mitochondrially encoded genes (small-subunit rRNA, cytochrome oxidase II) resolved the Saccharomycetaceae species into 11 clades (Kurtzman & Robnett, 2003). A different genus name was then assigned to each of the clades, resulting in the redefinition of currently accepted genera and the proposal of five novel genera (Kurtzman, 2003). A relatively large number of species in an only moderately supported clade that included _S. servazzii_ and _S. unisporus_ was assigned to the redefined genus _Kazachstania_. This was apparently a provisional treatment, as the species assigned to this genus by Kurtzman (2003) are polyphyletic. The phylogenies of these yeasts resolved from single-gene analyses were not congruent with that resolved from the combined gene analysis, as shown in the present study (see supplementary material in IJSEM Online) and previous studies (James et al., 1997; Kurtzman & Robnett, 2003; Mikata et al., 2001; Spírek et al., 2003). The addition of novel species to existing phylogenies is expected to contribute to a reclassification of species into more homogeneous groups. The present study indicates that the strongly supported clade comprising _K. aerobia_ sp. nov., _K. servazzii_, _K. unispora_ and the species represented by CBS 6904 may represent a separate genus. Multigene sequencing may confirm the distinct status of this clade.

**Ecology**

_K. aerobia_ sp. nov., _K. unispora_ and _S. cerevisiae_ were the dominant species among the yeasts isolated from the aerobically deteriorating corn silage. The silage contained whole-crop corn of cultivar Nasu (Japan) and had been ensiled for about 6 months before being opened to the air. Yeasts were detected from the silage samples on the opening day and after 1, 3, 5, 7 and 10 days exposure to air. On the opening day and after 1 day exposure to air, only _K. unispora_ strains were detected and the counts were $2.5 \times 10^7$ and $3.0 \times 10^4$ cf.u. g$^{-1}$, respectively. After 3, 5 and 7 days exposure, the counts of _K. unispora_ strains were $3.5 \times 10^5$, $2.5 \times 10^5$ and $3.0 \times 10^5$ cf.u. g$^{-1}$, respectively. After 10 days exposure, _K. unispora_ strains became undetectable. _K. aerobia_ and _S. cerevisiae_ strains were
detected after 3 days exposure to air, with counts of $2 \times 10^5$ and $1 \times 10^5$ c.f.u. g$^{-1}$, respectively. After additional exposure to air for 7 and 10 days, the numbers of *K. aerobia* cells increased to $1 \times 10^6$ and $5 \times 10^6$ c.f.u. g$^{-1}$, respectively, and the numbers of *S. cerevisiae* cells increased to $4 \times 10^5$ and $2 \times 10^5$ c.f.u. g$^{-1}$, respectively.

**Latin diagnosis of *Kazachstania aerobia* Bai et Cai sp. nov.**


**Description of *Kazachstania aerobia* Bai & Cai sp. nov.**

*Kazachstania aerobia* (ae.ro'bia.a. Gr. n. aer air; Gr. n. bios life; N.L. fem. adj. aerobia referring to the aerobically deteriorating stage of the corn silage from which the species was isolated).

In YM broth (Yarrow, 1998), after 3 days at 25°C, the cells are ovoid to ellipsoid, 2:5–4:5 x 3:0–6:0 μm and occur singly, in pairs or in groups (Fig. 3a). Budding is multilateral. After 1 month at 25°C, sediment and a thin ring are present. On YM agar (Yarrow, 1998), after 1 month at 25°C, the streak culture is butyrous, cream-coloured, flat, semi-glossy and smooth with faint striations; the margin is entirely to slightly undulating. In Dalmau plate culture on corn-meal agar, pseudohyphae are not formed. Sporulation observed on YM agar after 7 days at 25°C; vegetative cells transform directly into persistent ascii each containing one ellipsoidal ascospore (Fig. 3b). Glucose and galactose are fermented; sucrose, maltose, lactose and raffinose are not fermented. Glucose, galactose, trehalose, D-ribose (delayed), glycerol, D-mannitol (delayed) and succinic acid (weak) are assimilated; L-sorbose, sucrose, maltose, cellobiose, lactose, melibiose, raffinose, melezitose, inulin, soluble starch, D-xyllose, L-arabinose, D-arabinose, L-rhamnose, D-glucosamine, mehtanol, erythritol, ribitol, galactitol, D-glucitol, methyl α-D-glucoside, salicin, DL-lactic acid, inositol, citric acid and hexadecane are not assimilated. Ammonium sulfate and L-lysine are assimilated; sodium nitrate, ethylamine hydrochloride, cadaverine dihydrochloride and potassium nitrate are not assimilated. Growth in vitamin-free medium is negative. Maximum growth temperature is 40°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, NS 14$^T$, was isolated from aerobically deteriorating corn silage in Tochigi, Japan, in April 2002. This strain has been deposited in the China General Microbiological Culture Collection Centre, Academia Sinica, Beijing, China as AS 2.2384$^T$ (=CBS 9918$^T$).
Acknowledgements

This study was supported by grant KSCX2-SW-101C from the Chinese Academy of Sciences and by the Japan Society for the Promotion of Science Postdoctoral Fellowship for Foreign Researchers (FY2001).

References


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