International Journal of Systematic and Evolutionary Microbiology (2008), 58, 510–514 DOI 10.1099/ijs.0.65331-0

Saccharomyces arboricolus sp. nov., a yeast species from tree bark

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Three ascomycetous yeast strains, H-6^T, ZX-15 and ZX-20, isolated from the bark of two tree species of the family Fagaceae collected from different regions of China, formed unconjugated and persistent asci containing two to four globose ascospores. 26S rDNA D1/D2 domain and internal transcribed spacer (ITS) region (including 5.8S rDNA) sequence analysis showed that they were closely related to the currently accepted *Saccharomyces* species with strong support. Comparisons of the rDNA sequences, electrophoretic karyotypes and physiological characters indicated that the three strains represent a novel species in the genus *Saccharomyces*. The name *Saccharomyces arboricolus* sp. nov. was proposed for the novel species, with H-6^T (=AS 2.3317^T=CBS 10644^T) isolated from the bark of *Quercus fabri* as the type strain.

Seven species are currently included in the genus *Saccharomyces* Meyen ex Reess as redefined recently by Kurtzman (2003), based on multigene sequence analysis (Kurtzman & Robnett, 2003). During the investigation of the diversity of ascomycetous yeasts associated with plant materials from China, three strains, H-6^T, ZX-15 and ZX-20, isolated from the bark of broadleaf trees, were found to represent a novel species of the genus *Saccharomyces* by physiological characterization, rRNA gene sequencing and electrophoretic karyotyping.

Yeast strains living in bark were isolated by using the enrichment method. Pieces of bark with or without exudates from independent trees were cut with a sterile scalpel and placed into sterile plastic tubes containing 3 ml liquid enrichment medium. The medium was made according to Sniegowski *et al.* (2002) with minor modifications, containing (w/v) 0.3 % yeast extract, 0.3 % malt extract, 0.5 % peptone, 0.5 % sucrose, 0.5 % galactose, 7.6 % (v/v) ethanol, 200 μg chloramphenicol ml^{-1} and 1 ml 1 M HCl per litre. The cultures were incubated at room temperature without shaking for 7 to 14 days. Aliquots (100 μl) of the 10^{-2} to 10^{-4} diluted enrichment culture was spread on plate medium made by adding 2 % agar to the liquid enrichment medium. After 2 days incubation at room temperature or 25 °C, colonies with different morphological characters were transferred into malt extract agar slants for further purification and examination.

Strain H-6^T was isolated from the bark of *Quercus fabri* Hance collected in the Qinling Mountains, Shaanxi Province in western China. Strains ZX-15 and ZX-20 were isolated from the bark of *Castanopsis orthacantha* Franch collected from Yunnan Province in south-west China. Morphological, physiological and biochemical characteristics were examined according to standard methods commonly used in yeast taxonomy (Yarrow, 1998). Assimilation of nitrogen compounds was investigated on solid media with starved inocula (Nakase & Suzuki, 1986).

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 as described previously (Lu *et al.*, 2004). 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). Chromosomal DNA bands were separated in 1 % (w/v) agarose gel 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). Electrophoresis was carried out at 6 V cm^{-1} for 16 h with a switch time of 60 s and then for 11 h with a switch time of 90 s. The temperature of the running buffer was maintained at 12–14 °C. After electrophoresis, the gel was stained in ethidium bromide solution (0.5 μg ml^{-1}) for 30 min,

Abbreviation: ITS, internal transcribed spacer.

The GenBank/EMBL/DDBJ accession numbers for the ITS region and 26S rRNA gene D1/D2 domain sequences of strain H-6^T are EF580917 and EF580918, respectively.

A supplementary table showing nucleotide mismatches in the D1/D2 domain and ITS region sequences between the type strains of *Saccharomyces* species is available with the online version of this paper.

destained in distilled water and viewed under UV light (302 nm) with the AlphaImager 2200 gel documentation system (Alpha Innotech). *Saccharomyces cerevisiae* (YNN 295) chromosomal DNA (Bio-Rad) was used as the molecular size marker.

Sequence analyses

Strains H-6^T, ZX-15 and ZX-20 have identical sequences in both the D1/D2 domain and ITS region, indicating their conspecificity. They were clustered in the *S. cerevisiae* clade (Kurtzman & Robnett, 2003) with 100 % bootstrap support in the tree constructed from the concatenated sequences of the ITS region and the D1/D2 domain (Fig. 1). However, their phylogenetic position among accepted *Saccharomyces* species was not resolved. Satisfactory resolution of the phylogenetic relationships among *Saccharomyces* species is problematic. The combined 18S–5.8S–26S rDNA sequence analysis did not resolve the species relationships of the genus (Kurtzman & Robnett, 2003). The phylogeny of *Saccharomyces* species determined from a dataset consisting of nucleotide sequences from 18S, 5.8S/alignable ITS and 26S (three regions) rDNAs, EF-1α, as well as mitochondrial small-subunit rDNA and COX II (Kurtzman & Robnett, 2003) was not congruent with that determined from a concatenated dataset composed of 106 genes (Rokas *et al.*, 2003). Nevertheless, the inclusion of the species represented by these three strains in the genus *Saccharomyces* is clear because, even in the tree drawn from either the D1/D2 or the ITS data, they were located in the *S. cerevisiae* clade of the '*Saccharomyces* complex' with 100 % bootstrap support (data not shown).

The result of a BLAST search in GenBank with the D1/D2 sequence of strain H-6^T as the query showed that the closest match was the sequence of *Saccharomyces kudriavzevii* NRRL Y-27340 (Kurtzman & Robnett, 2003) (incorrectly named *Saccharomyces kunashirensis* in the GenBank record). The sequences differed by 3 substitutions. In the ITS region, strain H-6^T differed from strain NRRL Y-27340 by 18 substitutions and two indels. In the phylogenetic tree based on the combined sequences of the rDNA ITS region and large subunit D1/D2 domain, strain NRRL Y-27340 formed a subclade with the type strain of *S. kudriavzevii*, IFO 1802^T, and was clearly separated from strain H-6^T (Fig. 1).

Large subunit D1/D2 domain sequence comparison between strain H-6^T and the type strains of accepted *Saccharomyces* species showed that strain H-6^T was most similar to *Saccharomyces paradoxus*, *Saccharomyces cariocanus* and *S. kudriavzevii*; it differed from the three described species by four, five and six substitutions, respectively. However, in the ITS region, strain H-6^T differed from these three species more significantly (19–20 substitutions) than from the other species of the genus (14–18 substitutions) (see Supplementary Table S1).

Electrophoretic karyotyping

The electrophoretic karyotypes of strains H-6^T, ZX-15 and ZX-20 were compared with those of the type strains of the species currently accepted in the genus *Saccharomyces* (Fig. 2). The three strains showed similar chromosomal banding patterns which differed clearly from those of the other species compared, especially in the molecular sizes of the largest chromosomes. Twelve to thirteen chromosome bands were resolved for the three strains, with molecular sizes ranging approximately from 250 to 2000 kb. The molecular sizes of the largest bands of the other species compared were usually about 2200 kb. Strains ZX-15 and ZX-20 have almost identical patterns, which differed slightly from that of strain H-6^T. This is in agreement with their sources. As wider bands or bands with stronger relative intensity may correspond to doublets or triplets, it is reasonable to infer that each of the three strains contains at least 16 chromosomes (Fig. 2).

Previous studies have shown that the species of *Saccharomyces sensu stricto* exhibit relatively homogeneous karyotypes compared with those of species of

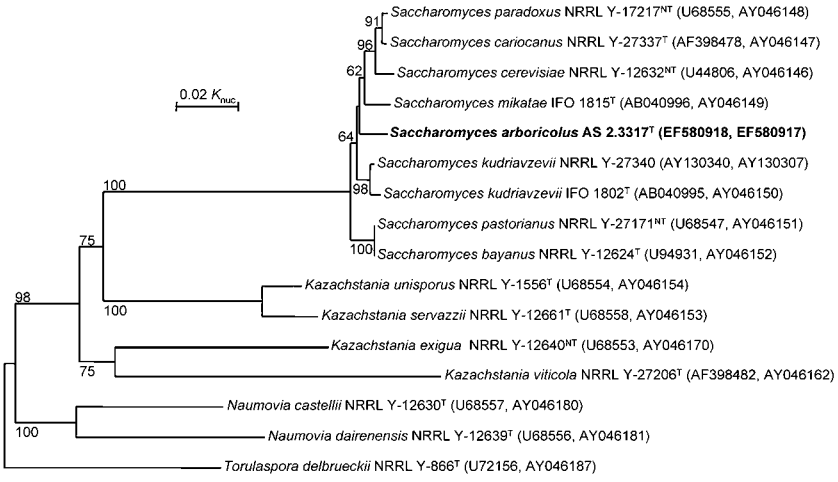

Fig. 1. Phylogenetic tree drawn from neighbour-joining analysis of the combined sequences of the ITS (including 5.8S rDNA) region and 26S rDNA D1/D2 domain, depicting the relationships of *Saccharomyces arboricolus* sp. nov. with closely related taxa. Bootstrap percentages over 50 % from 1000 bootstrap replicates are shown. Reference sequences were retrieved from GenBank under the accession numbers indicated.

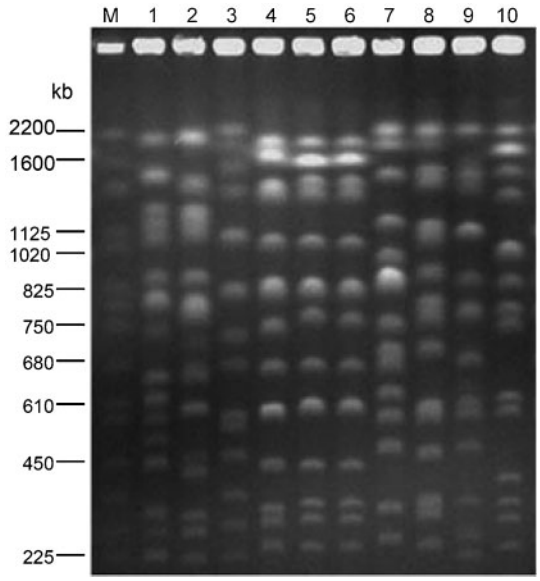

Fig. 2. Electrophoretic karyotypes. Lanes: M, *Saccharomyces cerevisiae* YNN 295; 1, *S. bayanus* AS 2.1885^T; 2, *S. pastorianus* AS 2.2402^T; 3, *S. kudriavzevii* AS 2.2408^T; 4, 5 and 6, *S. arboricolus* sp. nov. H-6^T, ZX-15 and ZX-20, respectively; 7, *S. mikatae* AS 2.2407^T; 8, *S. paradoxus* AS 2.2401^T; 9, *S. cerevisiae* AS 2.1882^T; 10, *S. cariocanus* AS 2.2374^T.

Saccharomyces sensu lato (Vaughan-Martini *et al.*, 1993; Fischer *et al.*, 2000; Naumov *et al.*, 1992, 1995; Petersen *et al.*, 1999; Lu *et al.*, 2004; Wu & Bai, 2005). The electrophoretic karyotypes of strains H-6^T, ZX-15 and ZX-20 bore a superficial resemblance to those of the accepted *Saccharomyces* species, being consistent with their placement in this genus based on rDNA sequence analysis (Fig. 1).

The *Saccharomyces* species can easily be crossed in any combination, though interspecific hybrids formed are sterile (Naumov *et al.*, 2000). This has probably resulted in the common introgression of genetic material in these species (Fischer *et al.*, 2000) and in the ambiguous sequence differences between some of the species. For example, the two mating types of *S. kudriavzevii* have remarkable 26S rDNA D1/D2 domain, protein gene EF-1α and mitochondrial COX II sequence divergences, while *S. bayanus* and *S. pastorianus* possess identical D1/D2 and ITS sequences (Fischer *et al.*, 2000; Kurtzman & Robnett, 2003). Nevertheless, the genetic isolation of the population represented by the three Chinese strains from other *Saccharomyces* species seems clear, for the new strains differed from all the accepted *Saccharomyces* species significantly in ITS sequences (supplementary data) and retained a specific electrophoretic karyotyping profile (Fig. 2).

Phenotypic characterization

Strains H-6^T, ZX-15 and ZX-20 exhibited similar morphological and physiological characters that are typical for the genus *Saccharomyces*. Each of them readily formed unconjugated asci containing two to four globose ascospores on YM (Yarrow medium) or acetate agar. The asci were persistent (Fig. 3). The strains vigorously fermented glucose, galactose, sucrose and raffinose. However, they can be differentiated from the type strains of the other species of the genus *Saccharomyces* by several key phenotypical characters (Naumov *et al.*, 2000), as listed in Table 1.

The molecular and phenotypic comparison showed that strains H-6^T, ZX-15 and ZX-20 represent a novel species of the genus *Saccharomyces*, for which the name *Saccharomyces arboricolus* sp. nov. is proposed.

Latin diagnosis of *Saccharomyces arboricolus* F.-Y. Bai & S.-A. Wang sp. nov.

In media liquido YM *post dies* 3 *ad* 25 °C, *cellulae ellipsoideae*, 2.5–7.5 × 2.5–10 μm, *singulae*, *binae et adhaerentes. Per gemmationem multipolarem reproducentes. Post* 1 *mensem sedimentum formatur. In agaro farinae Zea maydis confecto pseudomycelium observatae. Ascosporae ovoideae*, 2–4 *in asco*, *ex ascis non liberantur.*

Glucosum, *galactosum*, *sucrosum et raffinosum fermentantur at non maltosum nec lactosum. Glucosum*, *galactosum*, *sucrosum*, *trehalosum* (*lente*), *melibiosum*, *raffinosum*, *melezitosum*, D-*mannitolum* (*varium*) *et* α-*methyl*-D-*glucosidum assimilantur at non* L-*sorbosum*, *maltosum* (*varium*),

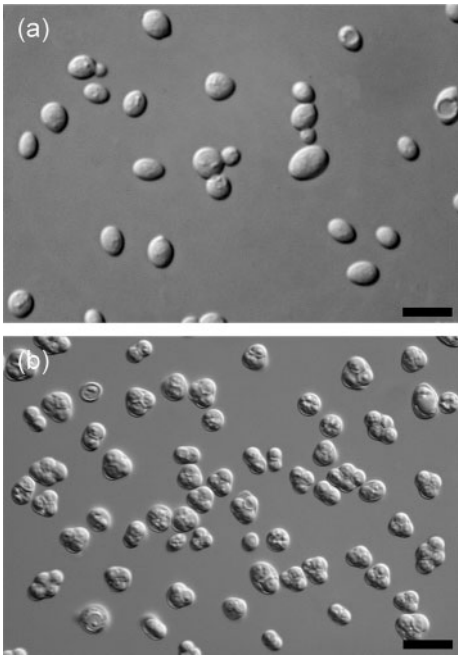

Fig. 3. *Saccharomyces arboricolus* sp. nov. H-6^T. (a) Vegetative cells grown in YM broth for 3 days at 25 °C. (b) Asci formed on acetate agar after 5 days at 25 °C. Bars, 10 μm.

Table 1. Physiological characteristics that distinguish the type strains of *Saccharomyces* species

Strains: 1, *S. arboricolus* sp. nov. AS 2.3317^T; 2, *S. bayanus* CBS 380^T; 3, *S. cerevisiae* NCYC 505^T; 4, *S. paradoxus* CBS 432^T; 5, *S. pastorianus* NCYC 392^T; 6, *S. cariocanus* UFRJ 50816^T; 7, *S. kudriavzevii* IFO 1802^T; 8, *S. mikatae* IFO 1815^T. +, Positive; −, negative; D, delayed positive; S, slow.

Characteristic	1	2	3	4	5	6	7	8
Fermentation of:								
D-Galactose	+	−	−	−	−	+	−	+
Maltose	−	−	−	−	−	−	S	−
Assimilation of:								
D-Galactose	+	+	+	+	+	+	−	+
Maltose	−	+	+	+	+	−	−	+
D-Mannitol	D	D	−	+	−	+	D	+
Melibiose	+	−	−	−	−	−	−	D
Trehalose	+	+	+	+	+	−	−	D
Galactitol	−	−	−	+	−	−	+	+
Inulin	−	−	D	−	−	−	+	−
Methyl α-D-glucoside	+	+	+	+	+	−	+	+

cellobiosum, *lactosum*, *inulinum*, *amylum solubile*, D-*xylosum*, L-*arabinosum*, D-*arabinosum*, D-*ribosum*, L-*rhamnosum*, D-*glucosaminum*, *methanolum*, *ethanolum*, *glycerolum*, *erythritolum*, *ribitolum*, *galactitolum*, *salicinum*, DL-*lacticum*, *acidum succinicum*, *acidum citricum*, *inositolum nec hexdecanum. Ammonium sulfatum et ethylaminum assimilantur at non natrum nitrosum*, *kalium nitricum*, L-*lysinum net cadaverinum. Ad crescentiam vitamina externa necessaria sunt. Maxima temperatura crescentiae*: 32 °C. *Materia amyloidea iodophila non formantur. Diazonium caeruleum* B *non respondens. Ureum non hydrolysatur.*

Description of *Saccharomyces arboricolus* F.-Y. Bai & S.-A. Wang sp. nov.

Saccharomyces arboricolus (ar.bo.ri′co.lus. N.L. masc. n. *arboricolus*, living in trees, referring to the source of the type strain).

In YM broth (Yarrow, 1998), after 3 days at 25 °C, the cells are ellipsoid, 2.5–7.5 × 2.5–10 μm and occur singly, in pairs or in groups (Fig. 3a). Budding is multilateral. After 1 month at 25 °C, sediment is present. Pseudohyphae are observed in cultures grown on cornmeal agar. Oval asci containing two to four round ascospores are formed after incubation for 5 days at 25 °C on acetate or YM agar (Fig. 3b). Asci are persistent. Glucose, galactose, sucrose and raffinose are fermented; maltose and lactose are not fermented. Glucose, galactose, sucrose, trehalose (delayed), melibiose, raffinose, melezitose, D-mannitol (variable) and methyl α-D-glucoside are assimilated; L-sorbose, maltose (variable), cellobiose, lactose, inulin, soluble starch, D-xylose, L-arabinose, D-arabinose, D-ribose, L-rhamnose, D-glucosamine, methanol, ethanol, glycerol, erythritol, ribitol, galactitol, salicin, DL-lactic acid, succinic acid, citric acid, inositol and hexadecane are not assimilated. Ammonium sulfate and ethylamine hydrochloride are assimilated; potassium nitrate, sodium nitrite, L-lysine and cadaverine hydrochloride are not assimilated. Growth in vitamin-free medium is positive. Maximum growth temperature is 32 °C. Starch-like compounds are not produced. Diazonium blue B reaction is negative. Urease activity is negative.

The type strain, H-6^T (=AS 2.3317^T=CBS 10644^T), was isolated from the bark of *Quercus fabri* Hance collected in Qinling Mountains, Shaanxi Province in west China in September 2006. This strain has been deposited in the China General Microbiological Culture Collection Center (CGMCC), Academia Sinica, Beijing, China, as AS 2.3317^T (=CBS 10644^T).

Acknowledgements

We thank Professor J.-Y. Zhuang, Institute of Microbiology, Chinese Academy of Sciences, Beijing, China, for identifying the plant samples. This study was supported by grant no. 30470005 from the National Natural Science Foundation of China (NSFC).

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