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Three new species of *Saccharomyces sensu lato* van der Walt from Yaku Island in Japan: *Saccharomyces naganishii* sp. nov., *Saccharomyces humaticus* sp. nov. and *Saccharomyces yakushimaensis* sp. nov.

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Three new yeast species were isolated from soil and partially decayed leaves in Yaku Island and Iriomote Island in the Nansei Islands of Japan. Based on DNA hybridization and physiological characters, these represent novel taxa. These are designated *Saccharomyces naganishii* sp. nov. (type strain IFO 10181^T = CBS 8797^T), *Saccharomyces humaticus* sp. nov. (type strain IFO 10673^T = CBS 8893^T) and *Saccharomyces yakushimaensis* sp. nov. (type strain IFO 1889^T = CBS 8894^T). The phylogenetic relationships of the three new species with members of other ascomycetous genera (e.g. *Kluyveromyces*, *Saccharomyces*, *Torulaspora* and *Zygosaccharomyces*) were estimated by 18S rDNA gene sequence analysis.

Keywords: *Saccharomyces naganishii* sp. nov., *Saccharomyces humaticus* sp. nov., *Saccharomyces yakushimaensis* sp. nov., DNA–DNA hybridization, electrophoretic karyotype

INTRODUCTION

The generic name *Saccharomyces* was introduced by Meyen (1838) and defined by Reess (1870). In the 4th edition of *The Yeasts, a Taxonomic Study*, 14 species (*Saccharomyces barnettii*, *Saccharomyces bayanus*, *Saccharomyces castellii*, *Saccharomyces cerevisiae*, *Saccharomyces dairenensis*, *Saccharomyces exiguus*, *Saccharomyces kluyveri*, *Saccharomyces paradoxus*, *Saccharomyces pastorianus*, *Saccharomyces rosinii*, *Saccharomyces servazzii*, *Saccharomyces spencerorum*, *Saccharomyces transvaalensis* and *Saccharomyces unisporus*) were described for the genus (Vaughan-Martini & Martini, 1998). James *et al.* (1997) analysed relationships among *Saccharomyces* species based on 18S rDNA sequences and described two new species, *Saccharomyces kunashirensis* and *Saccharomyces martiniae*; while Wyder *et al.* (1999) recently described the new species *Saccharomyces turicensis* isolated from kefyr.

In this study, it is shown that nine isolates from Japan represent three new species of *Saccharomyces sensu lato*. These are proposed as *Saccharomyces naganishii*, *Saccharomyces humaticus* and *Saccharomyces yakushimaensis*.

METHODS

Yeast strains and identification. The procedure used for yeast isolation and purification has been described previously (Banno & Mikata, 1981). The strains isolated are listed in Table 1. *Saccharomyces* type strains used for reference were obtained from the culture collection of the Institute for Fermentation, Osaka (IFO), Japan. Physiological, morphological and cultural characteristics were investigated according to the methods of Yarrow (1998).

Major ubiquinone analysis. The yeast strains were grown at 28 °C with shaking for 2 d in 1 l Erlenmeyer flasks containing 400 ml YPD broth (1% Bacto-yeast extract, 2% Bacto-peptone and 2% glucose). The extraction, purification and analysis of ubiquinone were performed as described by Mikata & Yamada (1999).

DNA preparation. The yeast strains were grown at 28 °C with shaking for 10–24 h in 200 ml Erlenmeyer flasks containing 50 ml of YPD broth. Protoplast preparation and DNA extraction followed the protocol of Holm *et al.* (1986) as modified by Kaneko & Banno (1991).

The GenBank/EMBL/DDBJ accession numbers sequences are AB016512 (IFO 10181^T), AB016513 (IFO 10673^T) and AB016514 (IFO 1889^T) for the 18S rDNA sequences and AB040999 (IFO 10673^T) and AB041000 (IFO 1889^T) for the 26S rDNA domain D1/D2 sequences.

Table 1. Isolated yeast strains

Strain		Sample material	Locality (Japan)
Original no.	IFO no.		
Yp74L-3^T	10181^T	Decayed leaf	Yaku Island
Yp20d	10922	Decayed leaf	Yaku Island
Ys63a	10923	Soil	Yaku Island
U97-433-1	10924	Leaf of *Kandelia candel*	Iriomote Island
Ys79a^T	10673^T	Soil	Yaku Island
Yp53c-2^T	1889^T	Decayed leaf	Yaku Island
Yp3a1	10674	Decayed leaf	Yaku Island
Yp31b	10675	Decayed leaf	Yaku Island
Ys21b	10676	Soil	Yaku Island

DNA G+C determination. The nuclear DNA base compositions were determined by HPLC as described by Tamaoka & Komagata (1984).

DNA–DNA hybridization. DNA–DNA base sequence similarity between strains was studied by the photobiotin microplate-hybridization method of Ezaki *et al.* (1988, 1989) as modified by Kaneko & Banno (1991).

PFGE. Yeast cells were harvested from 5 ml of a culture grown overnight in YPD broth. Agarose blocks containing chromosomal DNA were prepared according to the method of Carle & Olson (1984). Electrophoresis was carried out following the method of Ueda-Nishimura & Mikata (1999). DNA bands stained in ethidium bromide were observed on a transilluminator (TL-33; Ultra-Violet Products) and photographed.

18S rRNA gene sequencing and phylogenetic analysis. 18S rRNA gene (rDNA) sequences were determined using a Thermo Sequenase fluorescent labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia Biotech) following the supplier's protocol. PCR for amplification of 18S rDNA and cycle sequencing using PCR products were performed as described by Ueda-Nishimura & Mikata (1999).

Sequence data were aligned manually with various 18S rDNA sequences for representatives of related genera obtained from the GenBank. A phylogenetic tree was constructed by Kimura's two-parameter method (Kimura, 1980) and the neighbour-joining method (Saitou & Nei, 1987) using CLUSTAL W. Bootstrap values (Felsenstein, 1985) were calculated from 1000 replicates.

Domain D1/D2 26S rRNA gene sequencing. The 600 nucleotide 26S rDNA domain D1/D2 sequences were determined by using a BigDye Terminator Cycle Sequencing FS Ready Reaction Kit (PE Biosystems), CentriSep spin column (PE Biosystems) and the ABI PRISM 310 Genetic Analyzer (PE Biosystems) following the manufacturer's protocol. For amplification of domain D1/D2 26S rDNA sequences, PCR (Saiki *et al.*, 1988) was performed for 30 cycles of denaturation at 94 °C for 30 s, annealing at 57 °C for 30 s, and extension at 72 °C for 45 s with TaKaRa *Taq* DNA polymerase (Takara) using the primer pair NL-1 (5′-GCA TAT CAA TAA GCG GAG GAA AAG-3′) and NL-4 (5′-GGT CCG TGT TTC AAG ACG G-3′) (Kurtzman & Robnett, 1997). The PCR products were purified with GFX PCR DNA and Gel Band Purification Kit (Amersham Pharmacia Biotech) following the supplier's protocol. For sequencing, purified PCR products were used as a template and NL-1 and NL-4 were used as primers.

RESULTS AND DISCUSSION

Morphology and major ubiquinone of isolated strains

In all isolates, persistent asci arose directly from diploid cells, forming 1–4 globose to subglobose ascospores (Fig. 1, lower). As monosporic cultures from four-spored asci could sporulate, they were judged to be homothallic. The major ubiquinone of all nine isolates was Q-6. These results suggested that all isolates might be put in the genus *Saccharomyces*.

Electrophoretic karyotypes

Electrophoretic karyotyping has been used as a tool for the distinction of species in the genus *Saccharomyces* (Naumov *et al.*, 1995; Naumova *et al.*, 1996; Vaughan-Martini, 1995; Vaughan-Martini & Barcaccia, 1996; Vaughan-Martini *et al.*, 1993, 1996). The nine isolates were categorized into three groups based on their electrophoretic karyotypes, which differed from those of the type strains for all currently described *Saccharomyces* species (data not shown). The karyotype patterns of IFO 10181^T, IFO 10922, IFO 10923 and IFO 10924 were similar to each other and displayed 12 bands in a wide range of sizes (0·5–2·2 Mb) (Fig. 2, lane 2). That of IFO 10673^T was unique having 10 bands ranging from 0·5 to 2·2 Mb (Fig. 2, lane 3). The patterns of IFO 1889^T, IFO 10674, IFO 10675 and IFO 10676 were similar and displayed 10 bands in a wide range of sizes (0·4–2·2 Mb) (Fig. 2, lane 4). As a consequence, when strain IFO 10181^T, initially classified as *S. exiguus* based on its physiological characteristics, showed a chromosomal pattern significantly different from that species, it was listed as

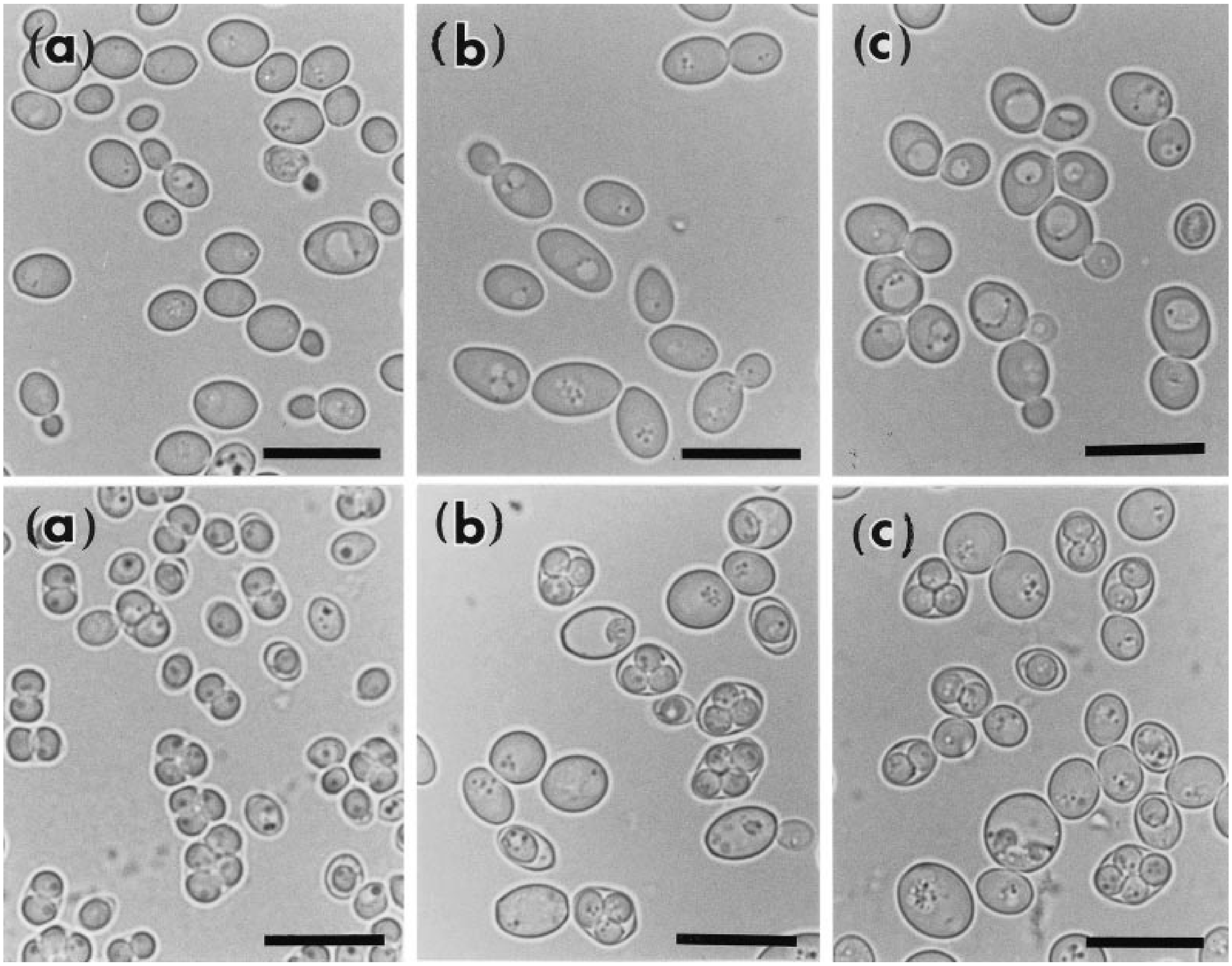

Fig. 1. Photomicrographs of vegetative cells grown on YM broth for 3 d at 24 °C (upper) and sporulated cells on potassium acetate agar for 7 d at 24 °C (lower). (a) *S. naganishii* IFO 10181^T; (b) *S. humaticus* IFO 10673^T; (c) *S. yakushimaensis* IFO 1889^T. Bars, 10 μm.

Saccharomyces sp. in the 10th edition of the IFO list of cultures (1996). In addition, all of the new species had karyotypes sufficiently different from those of *S. cerevisiae* and related species to indicate that the isolates did not belong to the *Saccharomyces sensu stricto* complex (Vaughan-Martini *et al.*, 1993).

DNA base compositions and DNA–DNA hybridization

The DNA G+C contents of the isolates were 31·0–31·5, 33·8 and 44·0–44·7 mol %, respectively (Table 2). DNA base sequence similarity tested against type strains of *Saccharomyces sensu lato* species by the microplate-hybridization method showed that IFO 10673^T had low similarity to all tested strains (Table 2), while IFO 10181^T, IFO 10922, IFO 10923 and IFO 10924 are conspecific, with similarity levels ranging from 82·7 to 97·2 %. Strains IFO 1889^T, IFO 10674, IFO 10675 and IFO 10676 also showed high similarity with one another (88·0–97·0 %). These were the same groupings revealed by PFGE patterns. A representative strain of each group showed mostly low base sequence similarity (2·4–49·7 %) to all other *Saccharomyces sensu lato* species.

Based on the results of these determinations, three new species are proposed as *Saccharomyces naganishii* sp. nov., *Saccharomyces humaticus* sp. nov. and *Saccharomyces yakushimaensis* sp. nov.

Latin diagnosis of *Saccharomyces naganishii* Mikata, Ueda-Nishimura et Hisatomi sp. nov.

In medio liquido YM post dies 3 *ad* 24 °C, *cellulae singulae aut binae*, *cellulae globosae vel ellipsoideae* (2·7–6·0) × (3·5–8·0) μm. *Post* 1 *mensem sedimentum formatur*. *In agaro YM post* 1 *memsum ad* 17 °C, *cremea aut suffuscus*, *glabra*, *butyrosa*, *nitida*, *margine glabra*. *In agaro farinae Zea mays post dies* 7, *pseudohyphae nullae*. *Asci inconjugatio fiunt*. *Ascosporae*

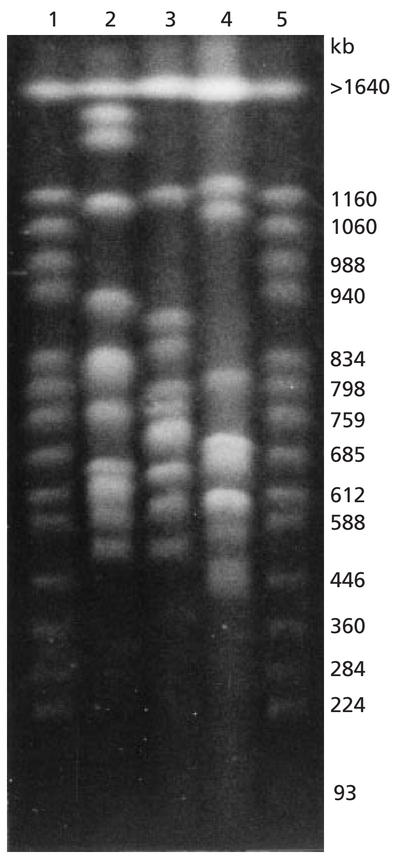

Fig. 2. Electrophoretic karyotypes of *Saccharomyces sensu lato*. Lanes: 1 & 5, *S. cerevisiae* IFO 10613 (size marker); 2, *S. naganishii* IFO 10181^T; 3, *S. humaticus* IFO 10673^T; 4, *S. yakushimaensis* IFO 1889^T.

rotundae aut ovoidae (2·3–3·8) × (2·3–4·2) µm, 1–4 *in quoque asco*.

Glucosum, *galactosum*, *sucrosum*, *trehalosum et raffinosum fermentatur*, *at non maltosum*, *lactosum*, *inulinum nec amylum solubile*. *Glucosum*, *galactosum*, *sucrosum*, *trehalosum*, *raffinosum*, D-*gluconatum*, 5-*ketogluconatum et* D-*glucono*-1,5-*lactonum assimilantur*, *at non* L-*sorbosum*, *maltosum*, *cellobiosum*, *lactosum*, *melibiosum*, *melezitosum*, *inulinum*, *amylum solubile*, D-*xylosum*, L-*arabinosum*, D-*arabinosum*, D-*ribosum*, L-*rhamnosum*, *ethanolum*, *methanolum*, *glycerolum*, *erythritolum*, *ribitolum*, *galactitolum*, D-*mannitolum*, D-*glucitolum*, *methyl* α-D-*glucosidum*, *salicinum*, *acidum* DL-*lacticum*, *acidum succinicum*, *acidum citricum*, *arbutinum*, *inositolum*, 2-*ketogluconatum*, *glucosaminum*, *N*-*acetyl*-D-*glucosaminum nec hexadecanum*. *Ethylaminum*, L-*lysinum et cadaverinum assimilantur*, *at non kalium nitricum*. *Ad crescentiam vitaminae externae necessariae sunt*. *Augmentum in* 34 °C, *raro in* 37 °C. *In* 10 % *natrium chloridum*/5 % *glucoso crescentiae at non* 0·1 % *cycloheximido*. *Ureum non hydrolysature*. *Diazonium caeruleum B non respondens*. *Amylum non formatur*. *Guaninum et cytosinum acidi deoxyribonucleati* 44–45 mol %. *Systema coenzymatis* Q-6 *adest*.

Typus stirps IFO 10181^T (= CBS 8797^T) *isolatus ex partim putridus folia*, *in Yuku insula*, *Kagoshima Pref*., *Japonia*, *VIII* 1971, *I. Banno*, *conservatur in collectionibus culturarum quas* 'Institute for Fermentation, Osaka (IFO)', *sub no*. IFO 10181^T *deposita est*.

Description of *Saccharomyces naganishii* Mikata, Ueda-Nishimura et Hisatomi sp. nov.

Saccharomyces naganishii (na.ga.ni.shi.i. L. gen. masc. n. *naganishii* of Naganishi, in honour of Hirosuke Naganishi).

Growth in YM medium: after 3 d at 24 °C, the cells are globose to ellipsoidal (2·7–6·0) × (3·5–8·0) µm, single or in pairs (Fig. 1a, upper). After 1 month at 17 °C, a sediment is present and occasionally a thin film. Growth on YM agar medium: after 1 month at 17 °C the streak culture is butyrous, cream to slightly brownish, smooth, scanty growth, rather flat or raised, dull, with an entire or undulating margin. Dalmau plate culture on corn meal agar: after 7 d at 24 °C, pseudohyphae are not formed. Formation of ascospores: vegetative cells are transformed directly into persistent asci containing 1–4 globose to subglobose (2·3–3·8) × (2·3–4·2) µm, smooth ascospores (Fig. 1a, lower). Sporulation was observed on acetate agar, corn meal agar and YM agar after 7 d incubation at 24 °C.

Glucose, galactose, sucrose, trehalose and raffinose are fermented, but not maltose, lactose, inulin or soluble starch. Glucose, galactose, sucrose, trehalose, raffinose, D-gluconate, 5-ketogluconate and D-glucono-1,5-lactone are assimilated, but not L-sorbose, maltose, cellobiose, lactose, melibiose, melezitose, inulin, soluble starch, D-xylose, L-arabinose, D-arabinose, D-ribose, L-rhamnose, ethanol, methanol, glycerol, erythritol, ribitol, galactitol, D-mannitol, D-glucitol, methyl-α-D-glucoside, salicin, DL-lactic acid, succinic acid, citric acid, arbutin, inositol, 2-ketogluconate, glucosamine, *N*-acetyl-D-glucosamine or hexadecane. Ethylamine, L-lysine and cadaverine are assimilated as a sole nitrogen sources, but not potassium nitrate. Growth in vitamin free-medium is negative. Growth at 34 °C is positive, at 37 °C negative. Growth in the presence of 1000 p.p.m. cycloheximide is negative. Growth on 50 % (w/w) glucose-yeast extract agar is negative. Growth in 10 % sodium chloride plus 5 % glucose in yeast nitrogen base is positive. Diazonium blue B reaction is negative. Production of starch-like substances is negative. Urease activity is negative. Ubiquinone type is Q-6. The G + C content of the nuclear DNA is 44–45 mol %.

Strains IFO 10181^T and IFO 10922 from partially decayed leaves and IFO 10923, from soil were isolated in August 1971 in Yaku Island, Japan. IFO 10924 was isolated in July 1997 from a leaf of *Kandelia candel* in Iriomote Island, Japan. Cultures of the type strain IFO 10181^T (= CBS 8797^T) have been deposited in the

Table 2. G+C content and DNA base sequence similarity between the new species and others of *Saccharomyces*

Bold numbers indicate similarity values for the same species.

Strain	G+C (mol%)	DNA similarity (%)		
		IFO 10181^T	IFO 10673^T	IFO 1889^T
S. yakushimaensis				
IFO 10674	ND	ND	ND	**97·0**
IFO 1889^T	31·0	26·1	30·5	**100**
IFO 10676	31·3	ND	35·0	**90·5**
IFO 10675	31·5	ND	ND	**88·0**
S. unisporus IFO 0316^T	32·3	2·4	19·9	25·0
S. exiguus IFO 1128^T	32·5	9·2	19·8	23·0
S. transvaalensis IFO 1625^T	33·1	26·5	49·7	33·1
S. spencerorum IFO 10851^T	33·5	22·4	21·5	26·6
S. kunashirensis IFO 10915^T	33·5	4·0	5·0	2·4
S. barnettii IFO 10849^T	33·7	21·9	24·0	29·1
S. humaticus IFO 10673^T	33·8	17·9	**100**	38·7
S. dairenensis IFO 0211^T	34·1	27·0	15·6	10·7
S. servazzii IFO 1838^T	34·4	30·4	16·6	15·9
S. martiniae IFO 0752^T	35·2	24·6	23·3	36·5
S. castellii IFO 1992^T	35·3	31·8	20·3	21·5
S. cerevisiae IFO 10217^T	38·0	25·5	29·4	38·4
S. pastorianus IFO 1167	38·3	ND	ND	ND
S. paradoxus IFO 10609^T	38·6	ND	ND	ND
S. bayanus IFO 1127^T	39·0	ND	ND	ND
S. rosinii IFO 10008^T	39·7	18·3	19·9	25·1
S. kluyveri IFO 1685^T	40·0	14·7	15·6	20·6
S. naganishii				
IFO 10924	44·0	**82·7**	ND	ND
IFO 10922	44·5	**97·2**	ND	ND
IFO 10181^T	44·6	**100**	ND	ND
IFO 10923	44·7	**83·9**	ND	ND

ND, Not determined.

culture collection of the Institute for Fermentation, Osaka (IFO).

Latin diagnosis of *Saccharomyces humaticus* Mikata et Ueda-Nishimura sp. nov.

In medio liquido YM post dies 3 *ad* 24 °C, *cellulae singulae aut binae*, *cellulae globosae vel ellipsoideae* (4·3–6·4)×(5·9–11·0) µm. *Post* 1 *mensem sedimentum formatur*. *In agaro YM post* 1 *memsum ad* 17 °C, *cremea*, *glabra*, *butyrosa*, *nitida*, *margine glabra*. *In agaro farinae Zea mays post dies* 7, *primitivus pseudomycelium formantur*. *Asci inconjugatio fiunt*. *Ascosporae rotundae aut ovoidae* (2·7–4·3)×(2·7–4·3) µm, 1–4 *in quoque asco*.

Glucosum et galactosum fermentatur, *at non sucrosum*, *maltosum*, *trehalosum*, *lactosum*, *raffinosum*, *inulinum nec amylum solubile*. *Glucosum et galactosum assimilantur*, *at non* L-*sorbosum*, *sucrosum*, *maltosum*, *cellobiosum*, *trehalosum*, *lactosum*, *melibiosum*, *raffinosum*, *melezitosum*, *inulinum*, *amylum solubile*, D-*xylosum*, L-*arabinosum*, D-*arabinosum*, D-*ribosum*, L-*rhamnosum*, *ethanolum*, *methanolum*, *glycerolum*, *erythritolum*, *ribitolum*, *galactitolum*, D-*mannitolum*, D-*glucitolum*, *methyl* α-D-*glucosidum*, *salicinum*, *acidum* DL-*lacticum*, *acidum succinicum*, *acidum citricum*, D-*gluconatum*, *arbutinum*, *inositolum*, 2-*ketogluconatum*, 5-*ketogluconatum*, *glucosaminum*, *glucono*-1,5-*lactonum*, *N*-*acetyl*-D-*glucosaminum nec hexadecanum*. *Ethylaminum et cadaverinum assimilantur*, *at non kalium nitricum nec* L-*lysinum*. *Ad crescentiam vitaminae externae necessariae sunt*. *Augmentum in* 34 °C, *raro in* 37 °C. *Non crescere potest in* 10% *natrium chloridum*/ 5% *glucoso nec* 0·1% *cycloheximido*. *Ureum non hydrolysature*. *Diazonium caeruleum B non respondens*. *Amylum non formatur*. *Guaninum et cytosinum acidi deoxyribonucleati* 34 mol%. *Systema coenzymatis* Q-6 *adest*.

Typus stirps IFO 10673^T (=CBS 8893^T) *isolatus ex terra*, *in Yuku insula*, *Kagoshima Pref*., *Japonia*, *VIII* 1971, *I. Banno*, *conservatur in collectionibus culturarum quas* 'Institute for Fermentation, Osaka (IFO)', *sub no*. IFO 10673^T *deposita est*.

Description of *Saccharomyces humaticus* Mikata et Ueda-Nishimura sp. nov.

Saccharomyces humaticus (hu.ma.ti.cus. L. adj. *humaticus* the soil from isolated).

Growth in YM medium: after 3 d at 24 °C, the cells are globose to ellipsoidal (4·3–6·4) × (5·9–11·0) µm, single or in pairs (Fig. 1b, upper). After 1 month at 17 °C, a sediment is present. Growth on YM agar medium: after 1 month at 17 °C the streak culture is butyrous, cream to slightly brownish, smooth, scanty growth, rather flat or raised, dull, with an entire or undulating margin. Dalmau plate culture on corn meal agar: after 7 d at 24 °C, primitive pseudohyphae are formed. Formation of ascospores: vegetative cells are transformed directly into persistent asci containing 1–4 globose to subglobose (2·7–4·3) × (2·7–4·3) µm, smooth ascospores (Fig. 1b, lower). Sporulation was observed on acetate agar, corn meal agar and YM agar after 7 d incubation at 24 °C.

Glucose and galactose are fermented, but not sucrose, maltose, trehalose, lactose, raffinose, inulin or soluble starch. Glucose and galactose are assimilated, but not L-sorbose, sucrose, maltose, cellobiose, trehalose, lactose, melibiose, raffinose, melezitose, inulin, soluble starch, D-xylose, L-arabinose, D-arabinose, D-ribose, L-rhamnose, ethanol, methanol, glycerol, erythritol, ribitol, galactitol, D-mannitol, D-glucitol, methyl α-D-glucoside, salicin, DL-lactic acid, succinic acid, citric acid, D-gluconate, arbutin, inositol, 2-ketogluconate, 5-ketogluconate, glucosamine, glucono-1,5-lactone, *N*-acetyl-D-glucosamine or hexadecane. Ethylamine and cadaverine are assimilated as a sole nitrogen sources, but not potassium nitrate and L-lysine. Growth in vitamin-free medium is negative. Growth at 34 °C is positive, at 37 °C negative. Growth in the presence of 1000 p.p.m. of cycloheximide is negative. Growth on 50% (w/w) glucose-yeast extract agar is negative. Growth in 10% sodium chloride plus 5% glucose in yeast nitrogen base is negative. Diazonium blue B reaction is negative. Production of starch-like substances is negative. Urease activity is negative. Ubiquinone type is Q-6. The G+C content of the nuclear DNA is 34 mol%.

The type strain IFO 10673^T was isolated in August 1971 from soil in Yaku Island, Japan. Cultures of the type strain IFO 10673^T (= CBS 8893^T) have been deposited in the culture collection of the Institute for Fermentation, Osaka (IFO).

Latin diagnosis of *Saccharomyces yakushimaensis* Mikata et Ueda-Nishimura sp. nov.

In medio liquido YM post dies 3 *ad* 24 °C, *cellulae singulae aut binae*, *cellulae globosae vel subglobosae* (4·8–7·0) × (5·9–9·1) µm. *Post* 1 *mensem sedimentum formatur*. *In agaro YM post* 1 *memsum ad* 17 °C, *cremea*, *glabra*, *butyrosa*, *nitida*, *margine glabra*. *In agaro farinae Zea mays post dies* 7, *pseudomycelium nullae*. *Asci inconjugatio fiunt*. *Ascosporae rotundae aut ovoidae* (2·5–4·3) × (3·2–4·8) µm, 1–4 *in quoque asco*. *Glucosum et galactosum fermentatur*, *at non sucrosum*, *maltosum*, *trehalosum*, *lactosum*, *raffinosum*, *inulinum et amylum solubile*. *Glucosum*, *galactosum et trehalosum* (*tardus*) *assimilantur*, *at non* L-*sorbosum*, *sucrosum*, *maltosum*, *cellobiosum*, *lactosum*, *melibiosum*, *raffinosum*, *melezitosum*, *inulinum*, *amylum solubile*, D-*xylosum*, L-*arabinosum*, D-*arabinosum*, D-*ribosum*, L-*rhamnosum*, *ethanolum*, *methanolum*, *glycerolum*, *erythritolum*, *ribitolum*, *galactitolum*, D-*mannitolum*, D-*glucitolum*, *methyl* α-D-*glucosidum*, *salicinum*, *acidum* DL-*lacticum*, *acidum succinicum*, *acidum citricum*, D-*gluconatum*, *arbutinum*, *inositolum*, 2-*ketogluconatum*, 5-*ketogluconatum*, *glucosaminum*, *glucono*-1,5-*lactonum*, *N-acetyl*-D-*glucosaminum nec hexadecanum*. *Ethylaminum et* L-*lysine assimilantur*, *at non kalium nitricum nec cadaverinum*. *Ad crescentiam vitaminae externae necessariae sunt*. *Augmentum in* 34 °C, *raro in* 37 °C. *Non crescere potest in* 10% *natrium chloridum/* 5% *glucoso nec* 0·1% *cycloheximido*. *Ureum non hydrolysature*. *Diazonium caeruleum B non respondens*. *Amylum non formatur*. *Guaninum et cytosinum acidi deoxyribonucleati* 31–32 mol%. *Systema coenzymatis* Q-6 *adest*.

Typus stirps IFO 1889^T (= CBS 8894^T) *isolatus ex partim putridus folia*, *in Yuku insula*, *Kagoshima Pref*., *Japonia*, *VIII* 1971, *I. Banno*, *conservatur in collectionibus culturarum quas* 'Institute for Fermentation, Osaka (IFO)', *sub no*. IFO 1889^T *deposita est*.

Description of *Saccharomyces yakushimaensis* Mikata et Ueda-Nishimura sp. nov.

Saccharomyces yakushimaensis (ya.ku.shi.ma.en′sis L. adj. *yakushimaensis* pertaining to Yaku Island, Japan, where the yeast was originally isolated).

Growth in YM medium: after 3 d at 24 °C, the cells are globose to subglobose (4·8–7·0) × (5·9–9·1) µm, single or in pairs (Fig. 1c, upper). After 1 month at 17 °C, a sediment is present. Growth on YM agar medium: after 1 month at 17 °C the streak culture is butyrous, cream to slightly brownish, smooth, scanty growth, rather flat or raised, dull, with an entire or undulating margin. Dalmau plate culture on corn meal agar: after 7 d at 24 °C, pseudohyphae are not formed. Formation of ascospores: vegetative cells are transformed directly into persistent asci containing 1–4 globose to subglobose (2·5–4·3) × (3·2–4·8) µm, smooth ascospores (Fig. 1c, lower). Sporulation was observed on acetate agar, corn meal agar and YM agar after 7 d incubation at 24 °C.

Glucose and galactose are fermented, but not sucrose, maltose, trehalose, lactose, raffinose, inulin or soluble starch. Glucose, galactose and trehalose (slow) are assimilated, but not L-sorbose, sucrose, maltose, cellobiose, lactose, melibiose, raffinose, melezitose, inulin, soluble starch, D-xylose, L-arabinose, D-arabinose, D-ribose, L-rhamnose, ethanol, methanol, glycerol, erythritol, ribitol, galactitol, D-mannitol, D-glucitol, methyl α-D-glucoside, salicin, DL-lactic acid, succinic acid, citric acid, D-gluconate, arbutin, inositol,

Table 3. Key characters of species of *Saccharomyces sensu lato*

Su, sucrose; Tr, trehalose; Raf, raffinose; Ma, maltose; Eth, ethanol; Gly, glycerol; LA, lactate; SA, succinate; GA, gluconate; Ety, ethylamine; Lys, lysine; Cad, cadaverine; Cyc, resistance to cycloheximide 1000 p.p.m.; 34 and 37, growth at 34 or 37 °C. +, Positive growth; −, negative; s, slow growth; v, variable; NT, not tested.

Species	**Fermentation**			**Assimilation**												**Growth**		
				Carbon									**Nitrogen**					
	Su	**Tr**	**Raf**	**Su**	**Ma**	**Tr**	**Raf**	**Eth**	**Gly**	**LA**	**SA**	**GA**	**Ety**	**Lys**	**Cad**	**Cyc**	**34**	**37**
*S. sensu stricto**	v	−	+	+	+	+s	+	+	v	v	v	v	−	−	−	−	v	v
S. barnettii	+	s	s	+	−	+	+	−	−	+	+	+	−	−	−	−	−	−
S. castellii	−	−	−	−	−	s	−	−	−	−	v	+	−	−	−	−	+	−
S. dairenensis	−	−	−	−	−	+	−	v	v	−	s	v	−	−	−	−	v	−
S. exiguus	+	+	s	+	−	+	+	s	−	−	s	−	−	−	−	v	v	−
S. humaticus	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+	−	+	−
S. kluyveri	+	−	+	+	+	+	+	+	v	+	−	−	+	+	+	−	+	+
S. kunashirensis	−	−	−	−	−	+	−	−	+	−	−	−	−	−	−	−	NT	−
S. martiniae	−	+	−	−	−	+	−	−	v	−	−	+	−	−	−	−	NT	−
S. naganishii	+	+	+	+	−	+	+	−	−	−	−	+	+	+	+	−	+	−
S. rosinii	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
S. servazzii	−	−	−	−	−	+	−	−	+	−	−	−	−	−	−	+	+	+
S. spencerorum	s	+	−	+	−	+	−	−	+	+	s	+	+	+	+	−	+	+
S. transvaalensis	−	−	−	−	−	+	−	v	v	−	−	+	−	v	v	−	+	+
S. turicensis†	+	+	−	+	−	+	−	−	−	−	−	−	−	−	−	−	NT	−
S. unisporus	−	−	−	−	−	v	−	+	−	−	s	−	+	+	+	+	+	v
S. yakushimaensis	−	−	−	−	−	s	−	−	−	−	−	−	+	+	−	−	+	−

* *Saccharomyces sensu stricto*: *S. cerevisiae*, *S. pastorianus*, *S. paradoxus* and *S. bayanus*.

† Data are from Wyder *et al.* (1999).

2-ketogluconate, 5-ketogluconate, glucosamine, glucono-1,5-lactone, *N*-acetyl-D-glucosamine or hexadecane. Ethylamine and L-lysine are assimilated as a sole nitrogen source, but not potassium nitrate and cadaverine. Growth in vitamin-free medium is negative. Growth at 34 °C is positive, at 37 °C negative. Growth in the presence of 1000 p.p.m. of cycloheximide is negative. Growth on 50 % (w/w) glucose-yeast extract agar is negative. Growth in 10 % sodium chloride plus 5 % glucose in yeast nitrogen base is negative. Diazonium blue B reaction is negative. Production of starch like substances is negative. Urease activity is negative. Ubiquinone type is Q-6. The G+C content of the nuclear DNA is 31–32 mol %.

Strains IFO 1889^T, IFO 10674 and IFO 10675 from partially decayed leaves and IFO 10676, from soil were isolated in August 1971 in Yaku Island, Japan. Cultures of the type strain IFO 1889^T (= CBS 8894^T) have been deposited in the culture collection of the Institute for Fermentation, Osaka (IFO).

Physiology

Differential physiological characteristics for the isolates and other *Saccharomyces* species are listed in Table 3. Most physiological analysis corresponded to those of previous studies except for the ability of *S. kunashirensis* CBS 7662^T (= IFO 10915^T) and *S. martiniae* CBS 6334^T (= IFO 0752^T) to utilize and ferment sucrose. Contrary to others who found that these could ferment but not utilize this suger aerobically as a sole carbon source (James *et al.*, 1997), in this study both tests gave negative results. This seems more in keeping with the fact that the 4th edition of *The Yeasts, a Taxonomic Study* (Kurtzman & Fell, 1998) does not list strains able to ferment a compound that it is not able to also use aerobically.

S. naganishii was similar to *S. barnettii* and *S. exiguus* in its ability to ferment glucose, galactose, sucrose, trehalose and raffinose. However, *S. naganishii* could be distinguished from *S. barnettii* and *S. exiguus* by the assimilation of ethylamine, lysine and cadaverine. *S. humaticus* and *S. yakushimaensis* were similar to each other and to *S. castellii*, *S. dairenensis*, *S. kunashirensis*, *S. rosinii*, *S. servazzii*, *S. transvaalensis* and *S. unisporus* in their ability to ferment and assimilate glucose and galactose and their failure to utilize most other compounds. However, *S. humaticus* and *S. yakushimaensis* could be distinguished from the above species by the combination of ethylamine, lysine and cadaverine assimilation. Furthermore, they can be distinguished by trehalose, lysine and cadaverine assimilations and mol % G+C contents, although

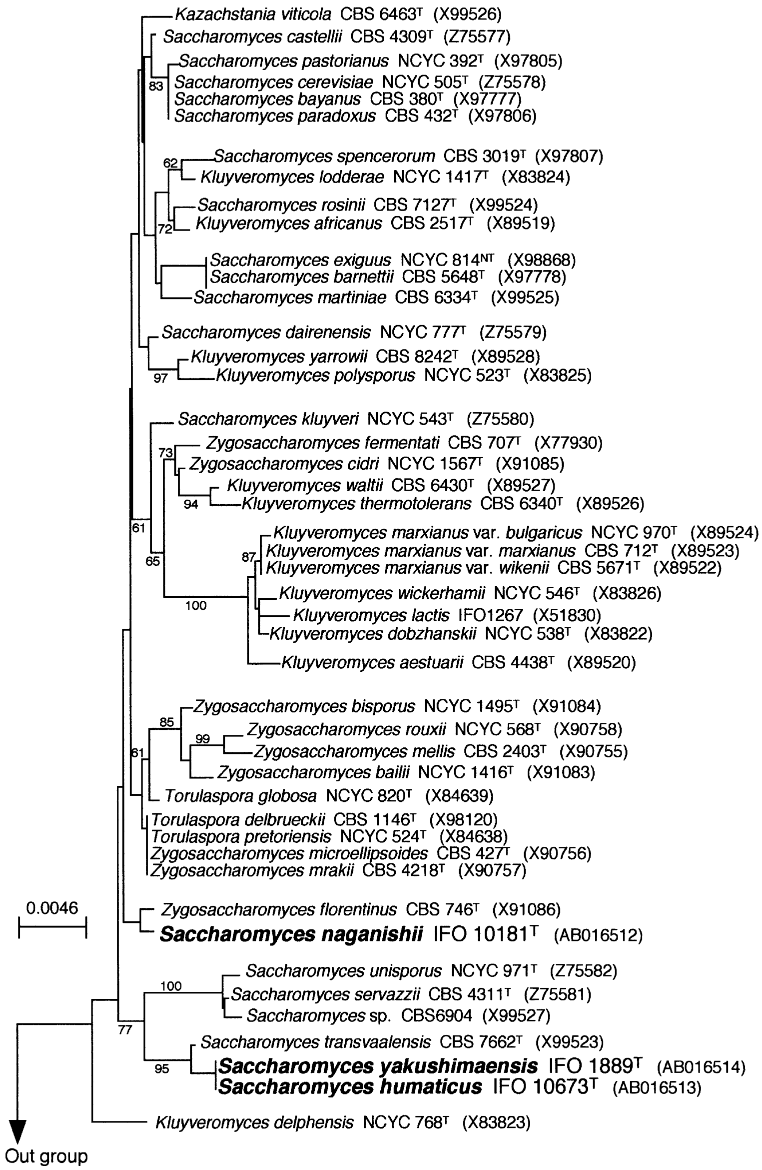

Fig. 3. Phylogenetic tree showing the relationship of the strains isolated to species of the genera *Kluyveromyces*, *Saccharomyces*, *Torulaspora* and *Zygosaccharomyces*. The tree is based on 18S rDNA sequence data and was constructed by using the neighbour-joining method. Bootstrap values were calculated from 1000 trees, and values < 60 % were omitted. GenBank/EMBL/DDBJ accession numbers are shown in parentheses. Bar, sequence dissimilarity value of 0·46 %. The outgroup species were *Tetrapisispora* clade, *Kluyveromyces blattae* and *Candida albicans*.

most other physiological characters were similar (Table 3).

Phylogeny

18S rDNA sequences of *S. naganishii* IFO 10181^T, *S. humaticus* IFO 10673^T and *S. yakushimaensis* IFO 1889^T were determined by the direct method, and a phylogenetic tree was generated by the neighbour-joining method (Fig. 3).

S. naganishii IFO 10181^T displayed no specific affinity with any other species of *Saccharomyces*. However, this strain and *Zygosaccharomyces florentinus* form a cluster at a 45 % bootstrap confidence level (omitted from Fig. 3). Phylogenetic analyses by James *et al.* (1994, 1997) showed that *Z. florentinus* forms a distinct line, showing no specific affinity with any other *Zygosaccharomyces*, *Saccharomyces*, *Kluyveromyces* or *Torulaspora*. In addition, the G+C content of *S. naganishii* (44·0–44·7 mol%), was higher than any other species of *Saccharomyces* (Table 2), while being similar to that of *Z. florentinus* (42·4–43·1 mol%) (Kurtzman, 1998). Nevertheless, since the bootstrap value supporting the joining of *S. naganishii* and *Z. florentinus* was low (45%), suggesting that the topology was variable, together with the fact that *S. naganishii* has a Q-6-type system and forms persistent asci, this new species will be temporarily put in the genus *Saccharomyces*. By the interspecific sexual reactions, it was suggested that *S. naganishii* has some relation to *S. cerevisiae* (Hisatomi *et al.*, 1988). Hisatomi *et al.* (1986) isolated heterothallic strains from the original homothallic strain *S. naganishii* IFO 10181^T by introducing a mutation into a homothallism gene. An **a** mating-type strain THE1-16C (= IFO 10945) and an α mating-type strain THE1-16B (= IFO 10944) derived from *S. naganishii* IFO 10181^T did not sexually agglutinate or mate with either mating-type strain of *S. cerevisiae*, in spite of interspecifically sexual reactions that both α mating pheromones from *S. cerevisiae* and *S. naganishii* induce G1-arrest in both **a** mating-type cells of these two species (Hisatomi *et al*., 1988).

S. humaticus IFO 10673^T and *S. yakushimaensis* IFO 1889^T are found to be closely related to each other (Fig. 3). Although *S. humaticus* IFO 10673^T and *S. yakushimaensis* IFO 1889^T were different species on the basis of low DNA reassociation (30·5–38·7%) (Table 2), it was not possible to separate them on the basis of this analysis, the only difference being one base insertion in *S. yakushimaensis*. As a result, to distinguish these species, their 26S rDNA domain D1/D2 sequences were determined by the direct method and found to differ by three base substitutions. Since both were isolated from the same area, Yaku Island, it could be possible that they descended from a common ancestor. This hypothesis could also be supported by the identity of most physiological characters and chromosome numbers (Table 3, Fig. 2).

S. yakushimaensis and *S. humaticus* are also close to *S. transvaalensis*, forming a cluster supported at a bootstrap level of 95% (Fig. 3). In comparison with the 26S rDNA domain D1/D2 sequence of *S. transvaalensis* (GenBank accession no. U68549), that of *S. humaticus* IFO 10673^T had three base substitutions and one base deletion and that of *S. yakushimaensis* IFO 1889^T had six base substitutions and one base deletion. Similarities among their 26S rDNA domain D1/D2 sequences showed that *S. transvaalensis* is closer to *S. humaticus* IFO 10673^T than to *S. yakushimaensis* IFO 1889^T. This is also supported by DNA–DNA hybridization where *S. humaticus* showed an intermediate relationship to *S. transvaalensis* (49·7%), but only 30·5% to *S. yakushimaensis* (Table 2). This is, however, in contrast with phylogenetic analyses based on rDNA sequences, where *S. humaticus* and *S. yakushimaensis* were closest. One possible explanation for the low DNA–DNA similarities of *S. yakushimaensis* to both *S. humaticus* and *S. transvaalensis* is that *S. yakushimaensis*, which had differentiated from *S. humaticus*, may have evolved more rapidly, resulting in a high frequency of chromosomal rearrangement and decreasing G+C contents in the non-coding regions giving the lowest G+C content for strains of *Saccharomyces* (31·0–31·5 mol%) (Table 2).

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