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Three new species in the *Saccharomyces sensu stricto* complex: *Saccharomyces cariocanus*, *Saccharomyces kudriavzevii* and *Saccharomyces mikatae*

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On the basis of genetic analysis, molecular karyotyping and sequence analyses of the 18S rRNA and internal transcribed spacer (ITS) region, three new *Saccharomyces* species are described, *Saccharomyces cariocanus* (with type strain NCYC 2890^T), *Saccharomyces kudriavzevii* (with type strain NCYC 2889^T) and *Saccharomyces mikatae* (with type strain NCYC 2888^T). Genetic and molecular analyses did not confirm the previously observed conspecificity of *Saccharomyces paradoxus* and *S. cariocanus*. The latter species exhibits post-zygotic isolation from representative strains from all known geographical populations of *S. paradoxus*: European, Far-East Asian, North American and Hawaiian.

Keywords: *Saccharomyces cariocanus*, *Saccharomyces kudriavzevii*, *Saccharomyces mikatae*, 18S rRNA gene sequence, ITS region

INTRODUCTION

On the basis of genetic hybridization analysis, six reproductively isolated populations representing different biological species have been confirmed in the *Saccharomyces sensu stricto* complex (Naumov, 1987, 1996; Naumov *et al*., 1995a, b). Three of them have been described as *Saccharomyces bayanus*, *Saccharomyces cerevisiae* and *Saccharomyces paradoxus*. Their species status was demonstrated by DNA–DNA reassociation (Vaughan Martini, 1989; Vaughan Martini & Kurtzman, 1985). The taxon *Saccharomyces pastorianus* (syn. *Saccharomyces carlsbergensis*) is considered to be a natural hybrid of *S. bayanus* and *S. cerevisiae* (Kielland-Brandt *et al*., 1995; Masneuf *et al*., 1998; Vaughan Martini & Kurtzman, 1985; Vaughan Martini & Martini, 1987). Recently, one such strain has been shown to be an allotetraploid (Naumov *et al*., 2000). To date, several molecular approaches have been developed to distinguish among *S. cerevisiae*, *S. bayanus*, *S. paradoxus* and *S. pastorianus*: electrophoretic karyotyping, PCR fingerprinting, RFLP analysis of mitochondrial DNA and rRNA sequence analysis (de Barros Lopes *et al*., 1998; Guillamón *et al*., 1994; James *et al*., 1997; McCullough *et al*., 1998; Masneuf *et al*., 1996; Molnar *et al*., 1995; Montrocher *et al*., 1998; Naumov *et al*., 1992b; Nguyen & Gaillardin, 1997; Oda *et al*., 1997; Ryu *et al*., 1998; Valente *et al*., 1996).

The biological species concept (Mayr, 1942) worked out on higher eukaryotes has been applied successfully to *Saccharomyces* yeasts. The gene pool of yeasts is discrete, as it is represented by reproductively isolated, biological species (Naumov, 1987, 1996). All six member species comprising the *Saccharomyces sensu stricto* complex can easily be crossed in any combination. The interspecific hybrids formed are sterile, having non-viable ascospores, whereas intraspecific hybrids produce highly viable ascospores and show regular segregation of control auxotrophic markers. Recently, three genetically isolated *Saccharomyces* populations, two in Japan and one in Brazil, were identified as representing three new unknown species (Naumov *et al*., 1995a, b). The genomic divergence of the two Japanese *Saccharomyces* species has been confirmed by DNA–DNA reassociation data (Yamada *et al*., 1993).

Abbreviation: ITS, internal transcribed spacer.

The EMBL accession numbers for the 18S rRNA and ITS sequences of strains IFO 1802^T, IFO 1815^T and UFRJ 50816^T are AJ271805–AJ271813.

In the present work, we conducted further genetic and molecular studies on these Brazilian and Japanese *Saccharomyces sensu stricto* yeasts. On the basis of the results of the present and previous studies, three new species are formally described as *Saccharomyces cariocanus*, *Saccharomyces kudriavzevii* and *Saccharomyces mikatae*.

METHODS

Yeast strains. The strains studied and their origins are presented in Table 1. All were maintained as monosporic homothallic cultures. The yeast cells were grown and mated on a complete YPD medium at 28 °C, while sporulation was induced on an acetate medium (Naumov *et al*., 1986). A micromanipulator equipped with a glass needle was used to isolate spores from asci. The ascus walls were removed using a crude stomach enzyme complex prepared from *Helix pomatia* snails. Hybrids were obtained by the spore-to-spore method using a micromanipulator or by mass-mating spores marked with complementary auxotrophic mutations on minimal medium. Strains UFRJ 50791 and UFRJ 50816^T were marked with auxotrophic *lys* mutations selected on a medium containing DL-aminoadipic acid. Hawaiian strain 72-142 was a natural auxotroph of unknown identity. Strains IFO 1802^T, IFO 1815^T and UFRJ 50816^T were characterized phenotypically using the standard yeast identification methods as described by Yarrow (1998).

Southern blot analysis of chromosomal DNA. The preparation of chromosomal DNA has been described elsewhere (Naumov *et al*., 1992a). A CHEF-DR II apparatus (Bio-Rad) was used to separate chromosomal DNA. The electrophoresis buffer (0·5 × TBE) was circulated around the gel and cooled to 14 °C. Electrophoresis was conducted at 200 V for 15 h with a switching time of 60 s followed by 9 h with a switching time of 90 s. *S. cerevisiae* strain YNN 295 was used as a karyotype standard (Bio-Rad). The chromosomal DNA separated by CHEF was blotted onto nitrocellulose filters (Maniatis *et al*., 1982). pEL50 was a 1·1 kb *Bam*HI–*Hin*dIII fragment isolated from pGEM3Zf(−) (Louis *et al*., 1994). The Y probe, pEL16, was described by Naumov *et al*. (1992b). The probes were labelled with digoxigenin-11-dUTP using the non-radioactive DNA labelling kit (Boehringer Mannheim). Hybridization and chemiluminescent detection were performed according to the manufacturer's protocol.

PCR amplification of 18S rDNA and the internal transcribed spacer (ITS) region. The 18S rRNA gene was amplified as two overlapping fragments using the primer combinations P108/M3490 and P1190/M3989 as described by James *et al*. (1994). The entire ITS region was amplified as described previously (James *et al*., 1996), with the exception that primer P3490 (5′-CCGCACGCGCGCTACACTGA; positions 1454–1473 of the *S. cerevisiae* numbering of Mankin *et al*., 1986) was used in place of primer pITS1 (White *et al*., 1990). The amplified products were purified using a QIAGEN QIAquick PCR purification kit according to the manufacturer's protocol.

Sequence determination and analysis. Direct sequencing of both the 18S rRNA gene and ITS PCR products was performed using a *Taq* DyeDeoxy terminator cycle sequencing kit (Applied Biosystems) and an Omnigene thermocycler (Hybaid) according to the manufacturers' recommendations. Nearly complete 18S rDNA and ITS sequences were determined by using the primers described previously (James *et al*., 1994, 1996). Purified sequence reaction mixtures were electrophoresed with an Applied Biosystems model 373A automatic DNA sequencer.

Analysis of sequence data. The 18S rRNA gene sequences were aligned using the multiple-sequence alignment program PILEUP (Feng & Doolittle, 1987) contained within the GCG software (Genetics Computer Group, 1991) version 8.1. The alignments were adjusted manually. 18S rRNA gene sequence similarity values were calculated using the program GAP. Phylogenetic analyses were performed by using the PHYLIP phylogeny inference package (Felsenstein, 1993) version 3.572. A distance matrix was obtained by using the DNADIST program and an unrooted phylogenetic tree was constructed by using the neighbour-joining method (Saitou & Nei, 1987) and the NEIGHBOR program. The stability of the individual branches was assessed by using the bootstrap method (Felsenstein, 1985) with the programs SEQBOOT, DNADIST, NEIGHBOR and CONSENSE. The ITS1 and ITS2 sequences of strains IFO 1802^T, IFO 1815^T and UFRJ 50816^T and other *Saccharomyces sensu stricto* strains were aligned using PILEUP and phylogenetic analyses were performed as described above.

The nucleotide sequences determined in this study have been deposited with EMBL and assigned the following accession numbers: strain IFO 1802^T, AJ271811 (18S rRNA gene), AJ271805 (ITS1) and AJ271806 (ITS2); strain IFO 1815^T, AJ271812 (18S rRNA gene), AJ271807 (ITS1) and AJ271808 (ITS2); and strain UFRJ 50816^T, AJ271813 (18S rRNA gene), AJ271809 (ITS1) and AJ271810 (ITS2).

RESULTS AND DISCUSSION

Genetic hybridization analysis

Three taxa of the *Saccharomyces sensu stricto* complex that have not yet been formally described, one isolated in Brazil and two isolated in Japan, represent divergent species, since they exhibit post-zygotic isolation from one another and from the other members of the complex. The strains studied yielded sterile hybrids with the reference test strains of *S. cerevisiae*, *S. bayanus* and *S. paradoxus* and with one another, while intraspecific hybrids were fertile (Table 2). The details of these crosses are given elsewhere (Naumov *et al*., 1995a, b). It should be noted that only monosporic, highly fertile cultures of parental strains were used in the crosses. The hybrid taxon *S. pastorianus* (syn. *S. carlsbergensis*) was not included in the genetic crosses due to its self-sterility. The genetic data on the two Japanese *Saccharomyces* taxa (one comprising strains IFO 1802^T and IFO 1803 and the other represented by strains IFO 1815^T and IFO 1816) are in good agreement with the results from two recent nuclear (n) DNA–nDNA reassociation studies by Kaneko & Banno (1991) and Yamada *et al*. (1993), who compared these yeasts with other *Saccharomyces sensu stricto* strains. In the case of IFO 1802^T and IFO 1803, these two strains were found to exhibit high nDNA/nDNA homology with one another (Kaneko & Banno, 1991; exact value not provided by authors), but displayed only low to moderate homology with strains of *S. bayanus*, *S. cerevisiae* and *S. paradoxus* [values ranged from 26% (IFO 1803/*S. bayanus* CBS 380^T) to 51%

Table 1. *Saccharomyces* strains from which monosporic cultures were used

Culture collections are abbreviated as follows: UFRJ, Instituto de Microbiologia, Universidade Federal do Rio de Janeiro, Brazil; ATCC, American Type Culture Collection, Manassas, VA, USA; IFO, Institute for Fermentation, Osaka, Japan; CBS, Centraalbureau voor Schimmelcultures, Delft, The Netherlands; UCD-FST, Department of Food Science and Technology Collection, University of California, Davis, USA; JCM, Japan Collection of Microorganisms, RIKEN, Saitama, Japan; UWO-PS, Culture Collection of the Department of Plant Sciences, University of Western Ontario, London, Ontario, Canada; NCYC, National Collection of Yeast Cultures, Norwich, UK; VKM, All-Russian Collection of Microorganisms, Moscow, Russia; M, Magarach Scientific Research Institute of Viticulture and Wine Making, Yalta, Ukraine.

Species and strain designation	Source	Reference
S. cariocanus		
UFRJ 50791 (ATCC 201562)	*Drosophila* sp., Catalao point, Rio de Janeiro, Brazil	Morais *et al*. (1992)
UFRJ 50816^T (ATCC 201563^T)	*Drosophila* sp., Tijuca forest, Rio de Janeiro, Brazil	Morais *et al*. (1992)
S. kudriavzevii		
IFO 1802^T	Decayed leaf	Kaneko & Banno (1991)
IFO 1803	Decayed leaf	Kaneko & Banno (1991)
S. mikatae		
IFO 1815^T	Soil	Yamada *et al*. (1993)
IFO 1816	Decayed leaf	Yamada *et al*. (1993)
S. paradoxus		
N7 (ATCC 96885)	Exudate of *Quercus robur*, St Petersburg, Russia	Naumov (1986)
N9 (ATCC 96886)	Exudate of *Quercus* sp., Tashkent, Uzbekistan	Naumov (1986)
N12 (ATCC 96978)	Exudate of *Quercus* sp., Lenkoran, Azerbaijan	Naumov (1986)
N50 (CBS 8444)	Exudate of *Quercus mongolica*, Chajka, Vladivostok, Russia	Naumov *et al*. (1993)
UCD-FST 61-248 (JCM 10617)	*Drosophila pseudoobscura*, Cedar Pass, CA, USA	Naumov *et al*. (1996)
UCD-FST 72-145	Exudate of *Myoporum* sp., Ahumoa, HI, USA	Naumov (1999)
UWO-PS 79-65	*Prunus virginiana*, Ontario, Canada	Naumov *et al*. (1996)
CBS 432^T	Unknown	Naumov (1987)
CBS 5829	Moor soil, Denmark	Naumov (1987)
S. cerevisiae		
NCYC 505^T	Beer, Netherlands	Vaughan Martini & Kurtzman (1985)
YNN 295 (ATCC 200358)	Genetic line	Mortimer & Contopoulou (1991)
VKM Y-502 (CBS 5287)	Grape berries, Russia	Naumov (1987)
S. bayanus		
CBS 380^T	Beer	Vaughan Martini & Kurtzman (1985)
VKM Y-1146 (CBS 8687)	Grape berries, Russia	Naumov (1987)
M300	Sparkling wine, Russia	Naumov *et al*. (1995b)
NCYC 509	Blackcurrant juice, Netherlands	Vaughan Martini & Kurtzman (1985)
NCYC 686	Coca-Cola	–
S. pastorianus		
NCYC 392^T	Beer, Denmark	Vaughan Martini & Martini (1987)
NCYC 396	Beer, Denmark	Vaughan Martini & Martini (1987)
CBS 1503	Beer, Denmark	Vaughan Martini & Martini (1987)

(IFO 1802^T/*S. paradoxus* CBS 432^T)] (Kaneko & Banno, 1991). In the case of IFO 1815^T and IFO 1816, these strains also exhibited high nDNA/nDNA homology with one another (Yamada *et al*., 1993; exact value not provided by authors), while again displaying only low to moderate nDNA/nDNA homology with other *Saccharomyces sensu stricto* strains [values ranged from 26% (IFO 1815^T/*S. bayanus* CBS 380^T) to 46% (IFO 1816/*S. cerevisiae* CBS 1171^T)] (Yamada *et al*., 1993).

Table 2. Genetic analysis of *Saccharomyces* hybrids

Hybrids of Brazilian *Saccharomyces* strains (UFRJ 50971, UFRJ 50816^T) and Japanese *Saccharomyces* sp. N1 (IFO 1802^T, IFO 1803) and N2 (IFO 1815^T, IFO 1816) with reference strains of biological species *S. cerevisiae* (VKM Y-502), *S. bayanus* (M300) and *S. paradoxus* (CBS 5829) were analysed according to Naumov *et al.* (1995a, b).

Hybrid origin	No. of spore pairs crossed	No. of zygotes obtained	No. of tetrads isolated	Proportion of viable ascospores of hybrids (%)
50816^T × 502	59	2	40	0
50816^T × 300	41	4	28	0
50816^T × 5829	38	5	67	0*
50816^T × 50791	46	3	41	95
1802^T × 502	40	2	34	0
1802^T × 300	51	3	40	0
1802^T × 5829	40	6	35	0
1802^T × 1803	42	1	52	50
1815^T × 502	44	2	29	0
1815^T × 300	39	6	31	0
1815^T × 5829	45	5	40	0
1815^T × 1816	34	6	38	44

* One spore formed a microcolony.

For the two Brazilian strains, UFRJ 50791 and UFRJ 50816^T, data published as an abstract by Lemos *et al.* (1995) indicated that these strains were closely related to *S. paradoxus*. Until now, only one European strain of *S. paradoxus*, CBS 5829, has been used in interspecific crosses with the Brazilian strains to demonstrate species isolation (Naumov *et al.*, 1995a). In the *S. paradoxus* species, four geographical populations with different levels of divergence have been detected: European, Far-East Asian, North American and Hawaiian (Naumov, 1999; Naumov *et al.*, 1993, 1996, 1997, 1998). The interpopulation fertility varied from 3 to 55%, while fertility was higher in the intrapopulation combinations. For example, hybrids of European isolates showed mean fertility of 67% (Naumov *et al.*, 1997) and hybrids of Hawaiian isolates showed mean fertility of 91% (Naumov, 1999). All four geographical populations of *S. paradoxus* are genetically isolated from the species *S. cerevisiae*; the data are incomplete for hybrids with the other species. The Hawaiian strains of *S. paradoxus* yielded sterile hybrids with all species of the *Saccharomyces sensu stricto* complex (Naumov, 1999; unpublished data), while the North American population of *S. paradoxus* was found to be genetically isolated from *S. bayanus* (Naumov *et al.*, 1996).

To study the genetic relatedness of strains UFRJ 50791 and UFRJ 50816^T to *S. paradoxus* in more detail, we here used reference strains from all four populations: three European strains (N7, N9 and N12), two North American strains (61-248 and 79-65), one Far-East Asian strain, N50, and one Hawaiian strain, 72-145 (Table 3). All matings yielded sterile hybrids. In some crossing combinations, one to four microcolonies with poor growth were found. Formation of a few microcolonies has been observed previously in interspecific crosses between *S. cerevisiae* and *S. paradoxus*, but was not considered to be significant (Naumov, 1987; Naumov *et al.*, 1992a, 1993, 1996, 1998). The data obtained indicated that strains UFRJ 50816^T and UFRJ 50791 are genetically divergent from all four known geographical populations of *S. paradoxus* and therefore represent a separate species. Further nDNA–nDNA reassociation analysis needs to be conducted on the two Brazilian strains to establish the extent of nDNA/nDNA homology between them and representative strains of the four *S. paradoxus* populations.

Molecular karyotyping

The karyotype patterns of the Japanese (IFO 1802^T, IFO 1803, IFO 1815^T and IFO 1816) and Brazilian (UFRJ 50816^T, UFRJ 50791) strains have been compared previously with those of *S. cerevisiae*, *S. bayanus* and *S. paradoxus* (Naumov *et al.*, 1995a, b). Despite their highly divergent genomes and reproductive isolation, the six biological species have similar basic karyotypic characteristics, i.e. the same haploid number of chromosomes ($n = 16$) and the same range of chromosomal bands (from 250 to 2200 kb). However, the order and sizes of the homologous chromosomes can vary in the six sibling species. Earlier Southern hybridization experiments using different cloned genes of *S. cerevisiae* clearly demonstrated the identity of the karyotypes of *S. cerevisiae* and *S. paradoxus* and the species specificity of the *S. bayanus* karyotype: at least four homologous chromosomes, II, IV, VIII and XV, are of different sizes (Naumov *et al.*, 1992b, 1994; Ryu *et al.*, 1996). The sizes of some homologous chromo-

Table 3. Genetic analysis of interspecific hybrids between *S. paradoxus* strains and the new Brazilian *Saccharomyces* species

Hybrids of the Brazilian *Saccharomyces* taxon (UFRJ 50971, UFRJ 50816^T) with *S. paradoxus* strains from different geographical populations, European (N7, N9, N12), Far-East Asian (N50), North American (61-248, 79-65) and Hawaiian (72-145), were analysed.

Hybrid origin	No. of spore pairs crossed	No. of zygotes obtained	No. of tetrads isolated	Proportion of viable ascospores of hybrids (%)
N7 × 50791	38	2	29	0
N9 × 50791	36	3	29	0
N12 × 50791	26	4	29	0*
N50 × 50791	30	5	27	0*
61–248 × 50816^T	40	1	28	0*
79–65 × 50816^T	43	2	28	0*
72–145 × 50816^T	–†	–	23	0*
72–145 × 50791	–†	–	23	0

* One to four spores formed microcolonies.

† Hybrids between auxotrophic strains were obtained on minimal selection medium.

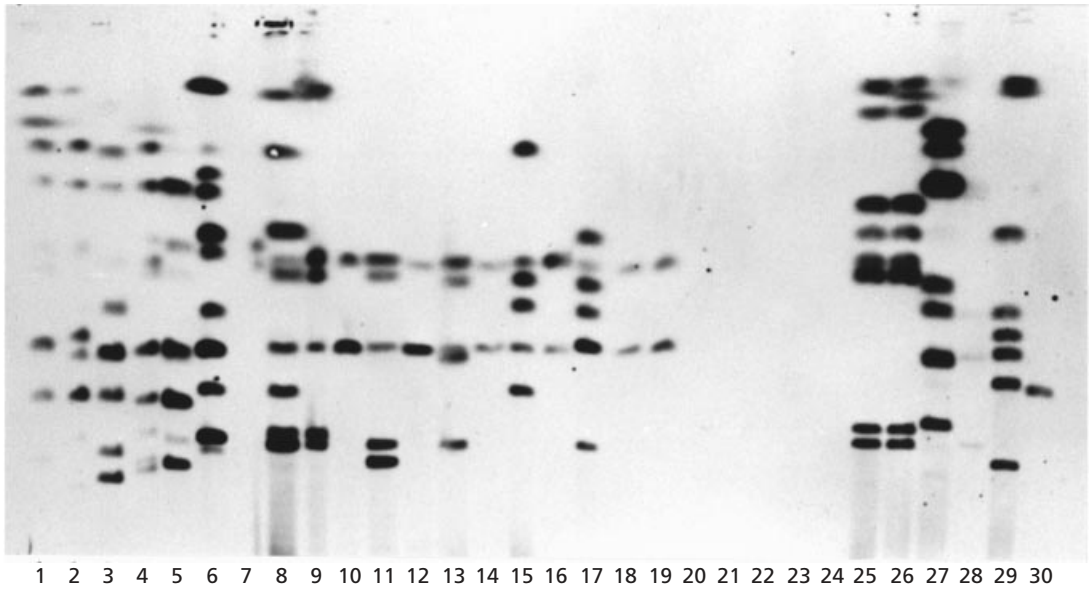

Fig. 1. Southern blot of a CHEF gel showing Y hybridization using pEL16 (Naumov *et al.*, 1992b) of the following *Saccharomyces sensu stricto* strains. Lanes: 1–6, *S. cerevisiae* S288C, A364a, Y55, SK1, MCYC 2756 and VKM Y-502; 7–19, *S. paradoxus* CBS 5829, N7, N8, N9, N11, N12, N15, N17, N18, N25, N34 and N36; 20–24, *S. bayanus* NRRLY 969, MCYC 623, VKM Y-1146, VKMY 361 and VKM Y-508; 25–26, *S. cariocanus* UFRJ 50816^T and UFRJ 50791; 27–28, *S. kudriavzevii* IFO 1802^T and IFO 1803; 29–30, *S. mikatae* IFO 1815^T and IFO 1816. All strains not listed in Table 1 have been used in karyotypic analysis in the past (Naumov *et al.*, 1992b; Louis *et al.*, 1994). All strains except *S. bayanus* have homology to the Y probe at one or more chromosomes. The weak hybridization seen for IFO 1803 (lane 28) is due to underloading of the lane.

somes in *S. cerevisiae* and in the Brazilian *Saccharomyces* isolates are also different (Naumov *et al.*, 1995a). The karyotype patterns of the Japanese strains IFO 1802^T, IFO 1803, IFO 1815^T and IFO 1816 are similar to those of *S. cerevisiae* and *S. paradoxus* (Naumov *et al.*, 1995b).

A number of telomere-associated repeat sequences have been documented in *S. cerevisiae*; some of these are unique to *S. cerevisiae*, while others are found in both *S. cerevisiae* and *S. paradoxus*, but none are found in *S. bayanus* (Naumov *et al.*, 1992b; Louis *et al.*, 1994; Pryde *et al.*, 1995). Chromosomal DNAs of

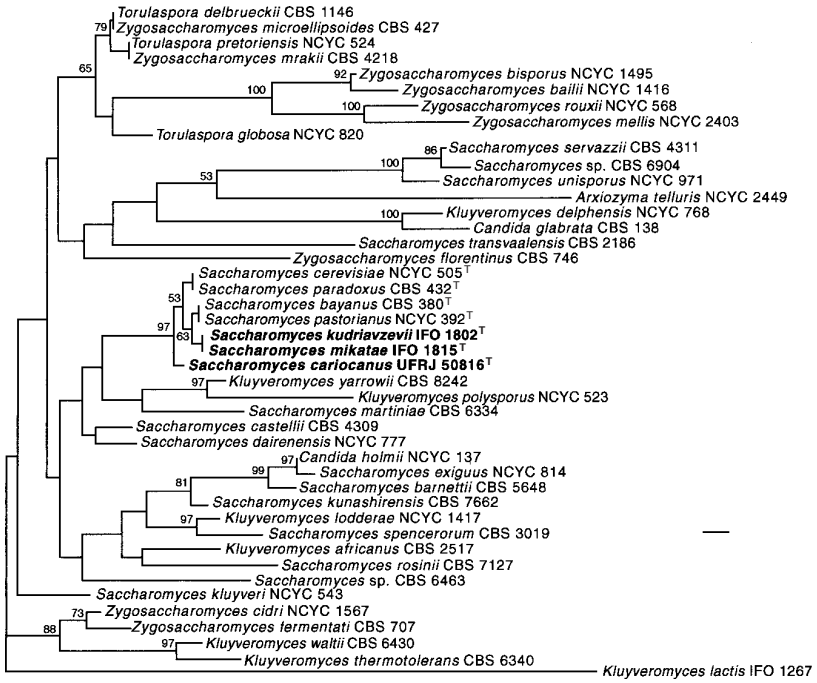

Fig. 2. Dendrogram showing the phylogenetic relationship of strains IFO 1802^T, IFO 1815^T and UFRJ 50816^T to other *Saccharomyces* and non-*Saccharomyces* species on the basis of 18S rRNA gene sequences. The tree was constructed by the neighbour-joining method (Saitou & Nei, 1987). Bootstrap values, expressed as percentages of 200 replications, are given at branch points (only values greater than 50 % are shown). Bar, one estimated base substitution per 1000 nucleotide positions. All strains shown are type strains with the exception of *Kluyveromyces lactis* IFO 1267.

strains belonging to the different sibling species were hybridized with the *S. cerevisiae* telomeric sequence pEL50 (enolase-like gene). This sequence was found only in *S. cerevisiae* strains YNN 295, NCYC 505^T and VKM Y-502 on the same three chromosomes (XV, doublet XVI/XIII) and was absent from the other strains studied (data not shown). Therefore, the pEL50 sequence appears to be specific for *S. cerevisiae* and can be used to differentiate *S. cerevisiae* from the other sibling species. The *S. cerevisiae* telomeric sequence pEL16 (Y probe), on the other hand, hybridized with all strains except *S. bayanus* and therefore can be used to distinguish the rest of the *Saccharomyces sensu stricto* species from *S. bayanus* (Fig. 1). This is consistent with *S. bayanus* being the most distantly related member of the species complex.

18S rRNA sequence analysis

The nearly complete (> 95 % of the primary structure) 18S rRNA gene sequences of Japanese strains IFO 1802^T and IFO 1815^T and Brazilian strain UFRJ 50816^T were determined and aligned with the complete or nearly complete 18S rRNA gene sequences for all *Saccharomyces* species and their close relatives, as determined in earlier studies (James *et al.*, 1994, 1996, 1997; Cai *et al.*, 1996). Levels of sequence similarity were calculated and the derived distances were used to infer phylogenetic relationships. Fig. 2 shows a rooted tree (with *Kluyveromyces lactis* used as the outgroup) constructed by using the neighbour-joining method (Saitou & Nei, 1987) and shows the phylogenetic relationships of strains IFO 1802^T, IFO 1815^T and UFRJ 50816^T to other *Saccharomyces* and non-*Saccharomyces* yeasts.

Detailed sequence analysis revealed that strains IFO 1802^T and IFO 1815^T have identical 18S rRNA gene sequences, which differed from that of strain UFRJ 50816^T by two base substitutions (positions 191 and 645 of the *S. cerevisiae* numbering; Mankin *et al.*, 1986). When these 18S rRNA gene sequences were compared with those for the type strains of the four *Saccharomyces sensu stricto* species (*S. bayanus*, *S. cerevisiae*, *S. paradoxus* and *S. pastorianus*), it was found that all seven strains were highly related to one another. As shown previously by James *et al.* (1997), *S. cerevisiae* and *S. paradoxus*, two species which display intermediate nDNA/nDNA homology with one another (46–59 %: Vaughan Martini, 1989), were phylogenetically inseparable from one another on the basis of 18S rRNA gene sequences, as were *S. bayanus* and *S. pastorianus*, with the two species pairs differing from each other by only two base substitutions (at positions 645 and 713 of the *S. cerevisiae* numbering).

In the case of the two Japanese isolates IFO 1802^T and IFO 1815^T, these strains differed from the *S. cerevisiae*/*S. paradoxus* and *S. bayanus*/*S. pastorianus* species pairs by single base substitutions (at positions 645 and 713, respectively). The Brazilian isolate UFRJ

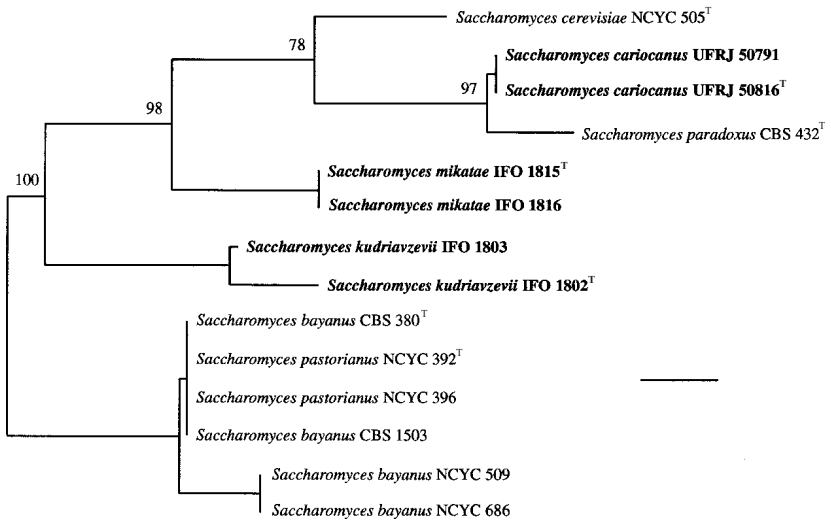

Fig. 3. Unrooted dendrogram constructed by the neighbour-joining method from ITS1 sequences, showing the phylogenetic relationship of strains IFO 1802^T, IFO 1803, IFO 1815^T, IFO 1816, UFRJ 50791 and UFRJ 50816^T to other *Saccharomyces sensu stricto* strains. Bootstrap values, expressed as percentages of 200 replications, are given at branch points (only values greater than 50 % are shown). Bar, two estimated base substitutions per 1000 nucleotide positions.

50816^T differed by a single base substitution (at position 191) from the *S. cerevisiae*/*S. paradoxus* species pair and by three base substitutions (at positions 191, 645 and 713) from the *S. bayanus*/*S. pastorianus* species pair. As shown in Fig. 2, strains IFO 1802^T, IFO 1815^T and UFRJ 50816^T, along with *S. bayanus*, *S. cerevisiae*, *S. paradoxus* and *S. pastorianus*, are phylogenetically separate from all other *Saccharomyces* and non-*Saccharomyces* taxa examined and form a distinct species complex (bootstrap value 97 %). However, the placement of the individual species within the complex as shown in Fig. 2 cannot be deemed accurate or reliable, due to the poor statistical support (i.e. low bootstrap values), and the more variable ITS regions were therefore analysed.

ITS sequence analysis

The nucleotide sequences of the ITS1 and ITS2 regions for all four Japanese isolates (IFO 1802^T, IFO 1803, IFO 1815^T and IFO 1816) and both Brazilian isolates (UFRJ 50791 and UFRJ 50816^T) were determined by direct sequencing of PCR-amplified fragments. For ITS1, strains IFO 1802^T, IFO 1803, UFRJ 50791 and UFRJ 50816^T have a spacer length of 362 bp while strains IFO 1815^T and IFO 1816 have a spacer length of 360 bp. For ITS2, strains IFO 1802^T, IFO 1803, UFRJ 50791 and UFRJ 50816^T have a spacer length of 233 bp while strains IFO 1815^T and IFO 1816 have a spacer length of 232 bp.

The ITS1 and ITS2 sequences of these six strains were aligned with those of other *Saccharomyces sensu stricto* strains, including the type strains of *S. bayanus* (CBS 380^T), *S. cerevisiae* (NCYC 505^T), *S. paradoxus* (CBS 432^T) and *S. pastorianus* (NCYC 392^T). In contrast to the 18S rRNA gene sequence data, comparative ITS sequence analysis revealed that these six strains could readily be subdivided into the three separate taxa as determined by genetic hybridization analyses (i.e. IFO 1802^T and IFO 1803; IFO 1815^T and IFO 1816; UFRJ 50791 and UFRJ 50816^T) on the basis of their ITS sequences (particularly ITS1). In turn, these three taxa could be differentiated from *S. bayanus*, *S. cerevisiae*, *S. paradoxus* and *S. pastorianus*.

A comparison of the ITS1 and ITS2 sequence alignments of these *Saccharomyces sensu stricto* strains revealed that the ITS1 region displays far greater sequence variation than the ITS2 region, thus permitting a more detailed resolution of this species complex than could otherwise be achieved on the basis of the more conserved 18S rRNA gene (Fig. 2). Consequently, only the ITS1 sequences were used to investigate the genealogical relationships of strains IFO 1802^T, IFO 1803, IFO 1815^T, IFO 1816, UFRJ 50791 and UFRJ 50816^T to other *Saccharomyces sensu stricto* strains. Fig. 3 shows the resulting unrooted phylogenetic tree constructed by using the neighbour-joining method. In contrast to the 18S rRNA tree (Fig. 2), both Japanese taxa (as represented by strains IFO 1802^T and IFO 1803 and by IFO 1815^T and IFO 1816) can clearly be distinguished, as they form separate lineages from the other *Saccharomyces sensu stricto* species. Despite possessing identical ITS2 sequences (data not shown), strains IFO 1802^T and IFO 1803 can be further differentiated from one another, as their ITS1 sequences differ by two base substitutions (Fig. 3). The Brazilian taxon (as represented by strains UFRJ 50791 and UFRJ 50816^T) is most closely related to *S. paradoxus*, with its ITS1 and ITS2 sequences differing by two and one base substitutions, respect-

Table 4. Physiological characteristics that distinguish between the type strains of members of the *Saccharomyces sensu stricto* complex

Type strains are abbreviated as Sb (*S. bayanus* CBS 380^T), Sca (*S. cariocanus* UFRJ 50816^T), Sc (*S. cerevisiae* NCYC 505^T), Sk (*S. kudriavzevii* IFO 1802^T), Sm (*S. mikatae* IFO 1815^T), Sp (*S. pastorianus* NCYC 392^T) and Spd (*S. paradoxus* CBS 432^T). Growth is scored as: +, positive; L, delayed positive (latent); s, slow; −, negative.

Characteristic	Sb	Sca	Sc	Sk	Sm	Sp	Spd
Fermentation of:							
D-Galactose	−	+	−	−	+	−	−
Maltose	−	−	−	s	−	−	−
Melibiose	−	−	−	−	+	−	−
α-Methyl D-glucoside	−	−	−	+	+	−	−
Assimilation of:							
D-Galactose	+	+	+	−	+	+	+
Maltose	+	−	+	−	+	+	+
Trehalose	+	−	+	−	L	+	+
Melibiose	−	−	−	−	L	−	−
Inulin	−	L	−	+	−	−	−
Ribitol	+	−	+	L	+	+	+
Galactitol	−	−	−	+	+	−	−
α-Methyl D-glucoside	+	−	+	+	+	+	+
Growth on 10% NaCl/5% glucose	−	L	−	−	−	−	+

ively, from those of the *S. paradoxus* type strain, CBS 432^T. Like strains IFO 1815^T and IFO 1816, both Brazilian strains have identical ITS1 and ITS2 sequences. Together, strains CBS 432^T, UFRJ 50791 and UFRJ 50816^T form a distinct group (bootstrap value 97%) that is closely related to, but nevertheless separate from, *S. cerevisiae*.

Collectively, strains IFO 1802^T, IFO 1803, IFO 1815^T, IFO 1816, UFRJ 50791 and UFRJ 50816^T, along with the type strains of *S. cerevisiae* (NCYC 505^T) and *S. paradoxus* (CBS 432^T), form a distinct species group (bootstrap value 100%) that is separate from the species pair of *S. bayanus* and *S. pastorianus*. In turn, the strains of these latter two species can be subdivided into those strains that have ITS1 sequences identical to that of the *S. bayanus* type strain, CBS 380^T [*S. bayanus* CBS 1503 and *S. pastorianus* strains NCYC 392^T and NCYC 396 (*S. carlsbergensis* type strain)] and those strains originally described as belonging to *Saccharomyces uvarum* (NCYC 509^T and NCYC 686). However, further *S. bayanus* and *S. uvarum* strains will need to be analysed in order to investigate this possible subdivision of *S. bayanus* in more detail.

Phenotypic characterization

The morphological and physiological characteristics of strains IFO 1802^T, IFO 1815^T and UFRJ 50816^T were determined by using standard methods, as described by Yarrow (1998). The key physiological characteristics that permit the discrimination of the Brazilian and Japanese strains from both one another and from the other *Saccharomyces sensu stricto* type strains are shown in Table 4. Despite having identical 18S rRNA gene sequences, the two Japanese strains IFO 1802^T and IFO 1815^T can readily be distinguished from one another on the basis of their differing physiological profiles, as well as mating analysis. For example, strain IFO 1815^T ferments D-galactose and melibiose and grows on maltose, trehalose (delayed) and melibiose (delayed), whereas strain IFO 1802^T does not (Table 4). Likewise, although UFRJ 50816^T was shown to be closely related to *S. paradoxus* on the basis of ITS1 sequences (Fig. 2), this strain can nevertheless be distinguished from the *S. paradoxus* type strain (CBS 432^T) by its inability to grow on maltose, trehalose, ribitol or α-methyl D-glucoside (Table 4).

On the basis of the data reported here and earlier (Kaneko & Banno, 1991; Naumov *et al.*, 1995a, b; Yamada *et al.*, 1993), we formally describe three new members of the *Saccharomyces sensu stricto* complex.

Latin diagnosis of *Saccharomyces cariocanus* sp. nov.

Cultura in agaro morphologico (Difco) *post* 48 *horas ad* 24 °C: *cellulae rotundae vel ovoideae* (4·0–9·0 × 5·0–7·0 μm), *singulae vel binae*, *per gemmationem multipolari reproducentes*. *Pseudohyphae nullae*. *Ascosporae ovoideae*, 2–4 *in asco*, *ex ascis non liberantur*.

Glucosum, *galactosum*, *sucrosum et raffinosum fermentantur at non maltosum*, *cellobiosum*, *trehalosum*, *lactosum*, *melibiosum*, *inulinum*, *amylum nec α-methylum* D-*glucosidum*. *Glucosum*, *galactosum*, *sucrosum*, *raffinosum*, *melezitosum*, *alcohol aethylicum*, D-*mannitolum et acidum lacticum assimilantur at non* L-*sorbosum*, *maltosum*, *cellobiosum*, *trehalosum*, *lactosum*, *melibiosum*, *inulinum*, *amylum*, *xylosum*, L-*arabinosum*, D-

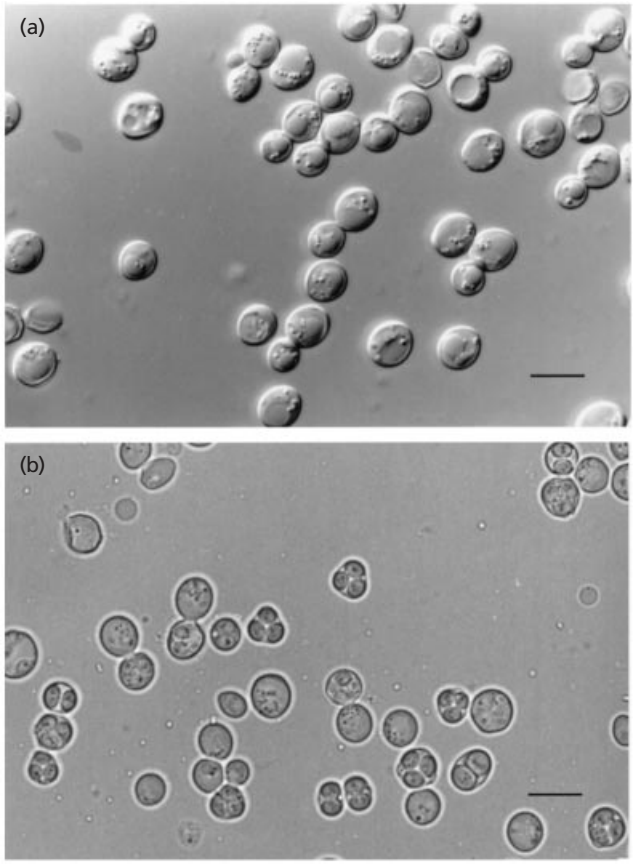

Fig. 4. *Saccharomyces cariocanus* UFRJ 50816^T. (a) Budding yeast cells, 1 d, YNBG medium (25 mM glucose), 25 °C; (b) asci with ascospores, 2 weeks, YM broth (unshaken), 20 °C. Bars, 10 μm.

arabinosum, L-*rhamnosum*, *glycerinum*, *erythritolum*, *ribitolum*, *galactitolum*, D-*glucitolum*, α-*methylum* D-*glucosidum*, *salicinum*, *acidum succinicum*, *acidum citricum*, *inositolum*, *glucono*-D-*lactonum*, D-*glucosaminum*, *methanolum*, *xylitolum*, *nitras kalicus*, *ethylaminum*, *cadaverinum nec lysinum*. *Crescit in medio cum* 50 % *glucoso et in* 10 % NaCl/5 % *glucoso*. *Non crescit in medio* 0·01 % *cycloheximido addito neque in medio* 1 % *acido acetico addito*.

Typus depositus in collectionis National Collection of Yeast Cultures, Norwich, *Britannia* (NCYC 2890^T).

Description of *Saccharomyces cariocanus* sp. nov.

Saccharomyces cariocanus (car.i.o.can′us. M.L. adj. *cariocanus* referring to the inhabitants of Rio de Janeiro, known as 'carioca').

After 48 h growth on morphology agar at 24 °C, the cells are spherical to ovoid (4·0–9·0 × 5·0–7·0 μm) and occur singly or in pairs (Fig. 4a). Budding is multipolar. No pseudohyphae are observed in cultures grown on corn-meal agar and potato agar. Oval asci containing two to four round ascospores are formed after incubation for 1–3 weeks at 24 °C on corn-meal agar (Fig. 4b). Asci are persistent.

Ferments glucose, galactose, sucrose and raffinose. Does not ferment maltose, cellobiose, trehalose, lactose, melibiose, inulin, α-methyl D-glucoside or starch. Assimilates glucose, galactose, sucrose, raffinose, melezitose, ethanol, mannitol and DL-lactate. Does not assimilate L-sorbose, maltose, cellobiose, trehalose, lactose, melibiose, inulin, starch, xylose, L-arabinose, D-arabinose, L-rhamnose, glycerol, erythritol, ribitol, galactitol, D-glucitol, α-methyl D-glucoside, salicin, succinate, citrate, inositol, D-glucono-1,5-lactone, D-glucosamine, methanol or xylitol. Does not assimilate cadaverine, ethylamine hydrogen chloride, lysine or potassium nitrate. Starch-like compounds are not produced. Growth occurs in the presence of 50 % glucose and on 10 % (w/w) NaCl/5 % glucose agar. No growth occurs in the presence of 0·01 % cycloheximide, 1 % acetic acid or at 37 °C. Cultures of the type strain, NCYC 2890^T (a monosporic culture of UFRJ 50816^T), have been deposited in the National Collection of Yeast Cultures, Norwich, UK.

Latin diagnosis of *Saccharomyces kudriavzevii* sp. nov.

Cultura in agaro morphologico (Difco) *post* 48 *horas ad* 24 °C: *cellulae ovoideae* (5·0–8·0 × 6·0–10·0 μm), *singulae*, *binae et paucae aggregatae*, *per gemmationem multipolarem reproducentes*. *In agaro farina Zeae maydis confecto pseudomycelium primitivum adest*. *Ascosporae rotundae*, 4 *in asco*, *ex ascis non liberantur*.

Glucosum, *sucrosum*, *maltosum*, *raffinosum*, *melezitosum et* α-*methylum* D-*glucosidum fermentantur at non galactosum*, *cellobiosum*, *trehalosum*, *lactosum*, *melibiosum*, *inulinum nec amylum*. *Glucosum*, *sucrosum*, *raffinosum*, *melezitosum*, *inulinum*, *alcohol aethylicum*, *galactitolum*, D-*mannitolum*, α-*methylum* D-*glucosidum et acidum lacticum assimilantur at non galactosum*, L-*sorbosum*, *maltosum*, *cellobiosum*, *trehalosum*, *lactosum*, *melibiosum*, *amylum*, *xylosum*, L-*arabinosum*, D-*arabinosum*, L-*rhamnosum*, *glycerinum*, *erythritolum*, *ribitolum*, D-*glucitolum*, *salicinum*, *acidum succinicum*, *acidum citricum*, *inositolum*, *glucono*-D-*lactonum*, D-*glucosaminum*, *methanolum*, *xylitolum*, *nitras kalicum*, *ethylaminum*, *cadaverinum nec lysinum*. *Crescit in medio cum* 50 % *glucoso*. *Non crescit in medio* 0·01 % *cycloheximido addito*, *neque in medio* 1 % *acido acetico addito*, *neque in* 10 % NaCl/*glucoso*.

Typus depositus in collectionis National Collection of Yeast Cultures, Norwich, *Britannia* (NCYC 2889^T).

Description *of Saccharomyces kudriavzevii* sp. nov.

Saccharomyces kudriavzevii (kud.ri.av′ze.vi.i. L. gen. m. n. *kudriavzevii* of Kudriavzev, in honour of the Russian yeast taxonomist and ecologist V. I. Kudriavzev, who did a great deal to introduce the wild *S. paradoxus* species into science and for his con-

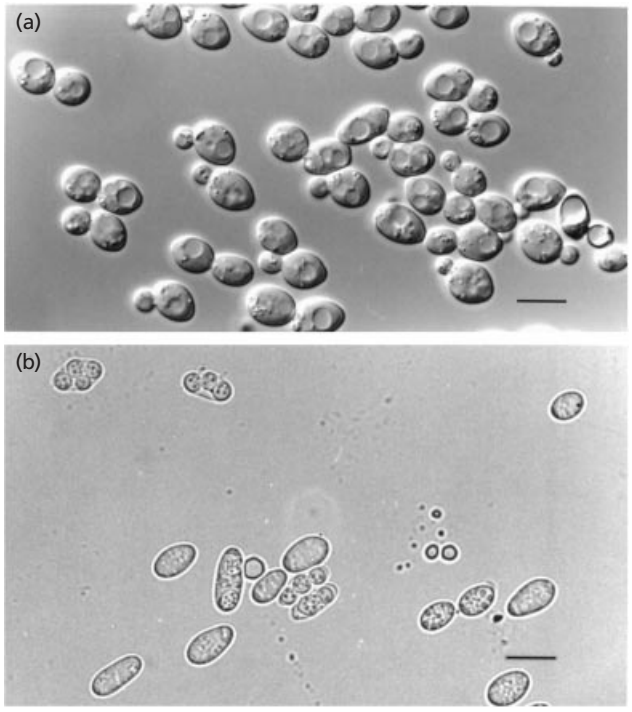

Fig. 5. *Saccharomyces kudriavzevii* IFO 1802^T. (a) Budding yeast cells, 1 d, YNBG, 25 °C; (b) asci with ascospores, 1 month, corn-meal agar, 25 °C. Bars, 10 μm.

tributions to the yeast studies of Russian Far-East Asia).

After 48 h growth at 24 °C on morphology agar, the cells are ovoid (5·0–8·0 × 6·0–10·0 μm) and occur singly, in pairs and in small groups (Fig. 5a). Budding is multipolar. Ill-formed pseudohyphae are observed in cultures grown on corn-meal agar. Oval to elongated oval asci containing four round ascospores are formed after incubation for 3 weeks at 24 °C on corn-meal agar and Gorodkowa agar (Fig. 5b). Asci are persistent.

Ferments glucose, sucrose, maltose (slowly), raffinose, melezitose and α-methyl D-glucoside. Does not ferment galactose, cellobiose, trehalose, lactose, melibiose, inulin or starch. Assimilates glucose, sucrose, raffinose, melezitose, inulin, ethanol, galactitol, D-mannitol (slowly), α-methyl D-glucoside and DL-lactate. Does not assimilate galactose, L-sorbose, maltose, cellobiose, trehalose, lactose, melibiose, starch, xylose, L-arabinose, D-arabinose, L-rhamnose, glycerol, erythritol, ribitol, D-glucitol, salicin, succinate, citrate, inositol, D-glucono-1,5-lactone, D-glucosamine, methanol, xylitol, cadaverine, ethylamine hydrogen chloride, lysine or potassium nitrate. Starch-like compounds are produced. Growth occurs in the presence of 50 % glucose. No growth occurs in the presence of 0·01 % cycloheximide or 1 % acetic acid. Cultures of the type strain, NCYC 2889^T (a monosporic culture of IFO 1802^T), have been deposited in the National Collection of Yeast Cultures, Norwich, UK.

Latin diagnosis of *Saccharomyces mikatae* sp. nov.

Cultura in agaro morphologico (Difco) *post* 48 *horas ad* 24 °C: *cellulae rotundae vel ovoideae* (4·0–6·0 × 5·0–9·0 μm), *singulae vel binae*, *per gemmationem multipolarem reproducentes*. *In agaro farina Zeae maydis confecto pseudomycelium primitivum adest*. *Ascosporae ovoideae*, 4 *in asco*, *ex ascis non liberantur*.

Glucosum, *galactosum*, *sucrosum*, *melibiosum*, *raffinosum et* α*-methylum* D*-glucosidum fermentantur at non maltosum*, *cellobiosum*, *trehalosum*, *lactosum*, *melezitosum*, *inulinum nec amylum*. *Glucosum*, *galactosum*, *sucrosum*, *maltosum*, *trehalosum*, *melibiosum*, *raffinosum*, *melezitosum*, *alcohol aethylicum*, *ribitolum*, *galactitolum*, D*-mannitolum*, α*-methylum* D*-glucosidum et acidum lacticum assimilantur at non* L*-sorbosum*, *cellobiosum*, *lactosum*, *inulinum*, *amylum*, *xylosum*, L*-arabinosum*, D*-arabinosum*, L*-rhamnosum*, *glycerinum*, *erythritolum*, D*-glucitolum*, *salicinum*, *acidum succinicum*, *acidum citricum*, *inositolum*, *glucono-*D*-lactonum*, D*-glucosaminum*, *methanolum*, *xylitolum*, *nitras kalicus*, *ethylaminum*, *cadaverinum nec lysinum*. *Crescit in medio cum* 50 % *glucoso*. *Non crescit in medio* 0·01 % *cycloheximido addito*, *neque in medio* 1 % *acido acetico addito*, *neque in* 10 % NaCl/*glucoso*.

Typus depositus in collectionis National Collection of Yeast Cultures, Norwich, *Britannia* (NCYC 2888^T).

Description of *Saccharomyces mikatae* sp. nov.

Saccharomyces mikatae (mik.at'ae. L. gen. m. n. *mikatae* of Mikata, in honour of the yeast taxonomist K. Mikata, who isolated the strains of both new Japanese species).

After 48 h growth at 24 °C on morphology agar, the cells are spherical to ovoid (4·0–6·0 × 5·0–9·0 μm) and occur singly or in pairs (Fig. 6a). Budding is multipolar. Ill-formed pseudohyphae are observed in cultures grown on corn-meal agar. Oval asci containing four round ascospores (somewhat granular in appearance) are formed after incubation for 3 weeks at 24 °C on corn-meal agar, Gorodkowa agar and potassium acetate agar (Fig. 6b). Asci are persistent.

Ferments glucose, galactose, sucrose, melibiose, raffinose and α-methyl D-glucoside. Does not ferment maltose, cellobiose, trehalose, lactose, melezitose, inulin or starch. Assimilates glucose, galactose, sucrose, maltose, trehalose (slowly), melibiose, raffinose, melezitose, ethanol, ribitol, galactitol, mannitol, α-methyl D-glucoside and DL-lactate. Does not assimilate L-sorbose, cellobiose, lactose, inulin, starch, xylose, L-arabinose, D-arabinose, L-rhamnose, glycerol, erythritol, D-glucitol, salicin, succinate, citrate, inositol, D-glucono-1,5-lactone, D-glucosamine, methanol or xylitol. Does not assimilate cadaverine, ethylamine hydrogen chloride, lysine or potassium nitrate. Starch-like compounds are not produced. Growth occurs in the presence of 50 % glucose. No growth occurs in the presence of 0·01 % cycloheximide, 1 % acetic acid, on 10 % (w/w) NaCl/5 % glucose agar or at 37 °C.

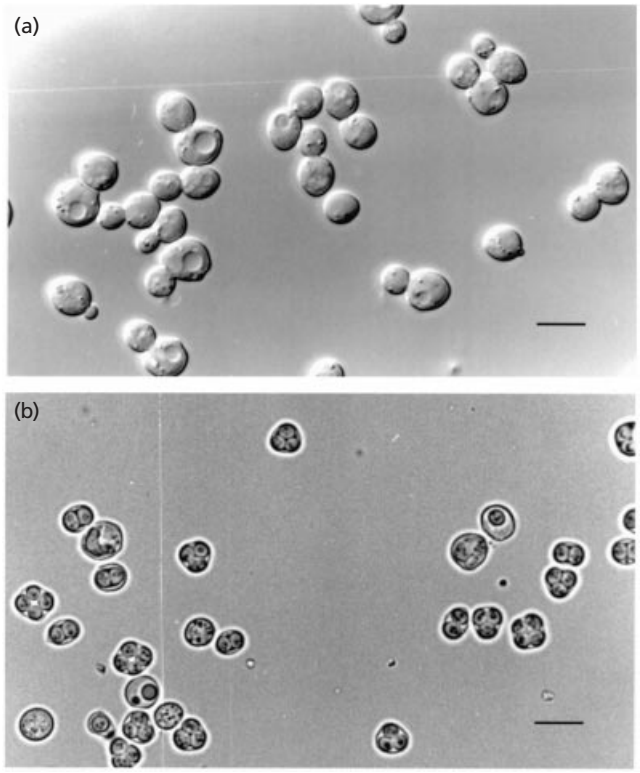

Fig. 6. *Saccharomyces mikatae* IFO 1815^T. (a) Budding yeast cells, 1 d, YNBG, 25 °C; (b) asci with ascospores, 1 month, cornmeal agar, 25 °C. Bars, 10 μm.

Cultures of the type strain, NCYC 2888^T (a monosporic culture of IFO 1815^T), have been deposited in the National Collection of Yeast Cultures, Norwich, UK.

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REFERENCES

de Barros Lopes, M., Soden, A., Martens, A. L., Henschke, P. A. & Langridge, P. (1998). Differentiation and species identification of yeasts using PCR, *Int J Syst Bacteriol* **48**, 279–286.

Cai, J., Roberts, I. N. & Collins, M. D. (1996). Phylogenetic relationships among members of the ascomycetous yeast genera *Brettanomyces*, *Debaryomyces*, *Dekkera*, and *Kluyveromyces* deduced by small-subunit rRNA gene sequences, *Int J Syst Bacteriol* **46**, 542–549.

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap, *Evolution* **39**, 783–791.

Felsenstein, J. (1993). PHYLIP: Phylogenetic Inference Package, version 3.5. Department of Genetics, University of Washington, Seattle, WA, USA.

Feng, D. F. & Doolittle, R. F. (1987). Progressive sequence alignment as a prerequisite to correct phylogenetic trees, *J Mol Evol* **25**, 351–360.

Genetics Computer Group (1991). Program manual for the GCG package, version 7. Genetics Computer Group, Madison, WI, USA.

Guillamón, J. M., Barrio, E., Huerta, T. & Querol, A. (1994). Rapid characterization of four species of the *Saccharomyces* sensu stricto complex according to mitochondrial DNA patterns, *Int J Syst Bacteriol* **44**, 708–714.

James, S. A., Collins, M. D. & Roberts, I. N. (1994). Genetic interrelationship among species of the genus *Zygosaccharomyces* as revealed by small-subunit rRNA gene sequences, *Yeast* **10**, 871–881.

James, S. A., Collins, M. D. & Roberts, I. N. (1996). Use of an rRNA internal transcribed spacer region to distinguish phylogenetically closely related species of the genera *Zygosaccharomyces* and *Torulaspora*, *Int J Syst Bacteriol* **46**, 189–194.

James, S. A., Cai, J., Roberts, I. N. & Collins, M. D. (1997). A phylogenetic analysis of the genus *Saccharomyces* based on 18S rRNA gene sequences: description of *Saccharomyces kunashirensis* sp. nov. and *Saccharomyces martiniae* sp. nov, *Int J Syst Bacteriol* **47**, 453–460.

Kaneko, Y. & Banno, I. (1991). Re-examination of *Saccharomyces bayanus* strains by DNA-DNA hybridization and electrophoretic karyotyping, *IFO Res Comm* **15**, 30–41.

Kielland-Brandt, M. C., Nilsson-Tillgren, T., Gjermansen, C., Holmberg, S. & Pedersen, M. B. (1995). Genetics of brewing yeasts. In *The Yeasts*, 2nd edn, vol. 6, pp. 223–254. Edited by A. Wheals, A. Rose & J. Harrison. New York: Academic Press.

Lemos, G. A., Valente, D., Pimentel, D., Hagler, A. N. & Mendonça-Hagler, L. C. (1995). Characterization of *Saccharomyces paradoxus* and a new species of *Saccharomyces* from Brazilian ecosystem. In *Abstracts of the Seventh International Symposium on Microbial Ecology (ISME-7)*, pp. 3–40.13. Santos-Sa-Paulo, Brazil, 27 August–1 September 1995.

Louis, E. J., Naumova, E. S., Lee, A., Naumov, G. & Haber, J. E. (1994). The chromosome end in yeast: its mosaic nature and influence on recombinational dynamics, *Genetics* **136**, 789–802.

McCullough, M. J., Clemons, K. V., McCusker, J. H. & Stevens, D. A. (1998). Intergenic transcribed spacer PCR ribotyping for differentiation of *Saccharomyces* species and interspecific hybrids, *J Clin Microbiol* **36**, 1035–1038.

Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982). *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Mankin, A. S., Skryabin, K. G. & Rubtsov, P. M. (1986). Identification of ten additional nucleotides in the primary structure of yeast 18S rRNA, *Gene* **44**, 143–145.

Masneuf, I., Aigle, M. & Dubourdieu, D. (1996). Development of a polymerase chain reaction/restriction fragment length polymorphism method for *Saccharomyces cerevisiae* and *Saccharomyces bayanus* identification in enology, *FEMS Microbiol Lett* **138**, 239–244.

Masneuf, I., Hansen, J., Groth, C., Piskur, J. & Dubourdieu, D. (1998). New hybrids between *Saccharomyces* sensu stricto yeast

species found among wine and cider production strains, *Appl Environ Microbiol* **64**, 3887–3892.

Mayr, E. (1942). *Systematics and the Origin of Species*. New York: Columbia University Press.

Molnar, O., Messner, R., Prillinger, H., Stahl, U. & Slavikova, E. (1995). Genotypic identification of *Saccharomyces* species using random amplified polymeric DNA analysis, *Syst Appl Microbiol* **18**, 136–145.

Montrocher, R., Verner, M.-C., Briolay, J., Gautier, C. & Marmeisse, R. (1998). Phylogenetic analysis of the *Saccharomyces cerevisiae* group based on polymorphisms of rDNA spacer sequences, *Int J Syst Bacteriol* **48**, 295–303.

Morais, P. B., Hagler, A. N., Rosa, C. A., Mendonça-Hagler, L. C. & Klaczko, L. B. (1992). Yeasts associated with *Drosophila* in tropical forests of Rio de Janeiro, Brazil, *Can J Microbiol* **38**, 1150–1155.

Mortimer, R. K. & Contopoulou, R. (1991). *Yeast Genetic Stock Catalogue*, 7th edn. Department of Molecular and Cellular Biology, Division of Genetics, University of California at Berkeley, Berkeley, CA, USA.

Naumov, G. I. (1986). Genetic differentiation and ecology of the yeast *Saccharomyces paradoxus* Batschinskaia, *Dokl Biol Sci* **289–291**, 213–216.

Naumov, G. I. (1987). Genetic basis for classification and identification of the ascomycetous yeasts, *Stud Mycol* **30**, 469–475.

Naumov, G. I. (1996). Genetic identification of biological species in the *Saccharomyces* sensu stricto complex, *J Ind Microbiol* **17**, 295–302.

Naumov, G. I. (1999). Divergent population of *Saccharomyces paradoxus* in the Hawaii Islands: an *in statu nascendi* yeast species, *Dokl Biol Sci* **364**, 51–53.

Naumov, G. I., Kondratieva, V. I. & Naumova, E. S. (1986). Methods for hybridization of homothallic yeast diplonts and haplonts, *Soviet Biotechnol* **6**, 29–32.

Naumov, G., Naumova, E. & Korhola, M. (1992a). Genetic identification of natural *Saccharomyces* sensu stricto yeasts from Finland, Holland and Slovakia, *Antonie Leeuwenhoek* **61**, 237–243.

Naumov, G. I., Naumova, E. S., Lantto, R. A., Louis, E. J. & Korhola, M. (1992b). Genetic homology between *Saccharomyces cerevisiae* and its sibling species *S. paradoxus* and *S. bayanus*: electrophoretic karyotypes, *Yeast* **8**, 599–612.

Naumov, G. I., Naumova, E. S., Azbukina, Z. M., Korhola, M. & Gaillardin, C. (1993). Genetic and karyotypic identification of *Saccharomyces* sensu stricto yeasts from Far East Asia, *Cryptogam Mycol* **14**, 85–93.

Naumov, G. I., Naumova, E. S., Gaillardin, C., Turakainen, H. & Korhola, M. (1994). Identification of new chromosomes of *Saccharomyces bayanus* using gene probes from *S. cerevisiae*, *Hereditas* **120**, 121–126.

Naumov, G. I., Naumova, E. S., Hagler, A. N., Mendonça-Hagler, L. C. & Louis, E. J. (1995a). A new genetically isolated population of the *Saccharomyces* sensu stricto complex from Brazil, *Antonie Leeuwenhoek* **67**, 351–355.

Naumov, G. I., Naumova, E. S. & Louis, E. J. (1995b). Two new genetically isolated populations of the *Saccharomyces* sensu stricto complex from Japan, *J Gen Appl Microbiol* **41**, 499–505.

Naumov, G. I., Naumova, E. S. & Sancho, E. D. (1996). Genetic reidentification of *Saccharomyces* strains associated with black knot disease of trees in Ontario and *Drosophila* species in California, *Can J Microbiol* **42**, 335–339.

Naumov, G. I., Naumova, E. S. & Sniegowski, P. D. (1997). Differentiation of European and Far East Asian populations of *Saccharomyces paradoxus* by allozyme analysis, *Int J Syst Bacteriol* **47**, 341–344.

Naumov, G. I., Naumova, E. S. & Sniegowski, P. D. (1998). *Saccharomyces paradoxus* and *Saccharomyces cerevisiae* are associated with exudates of North American oaks, *Can J Microbiol* **44**, 1045–1050.

Naumov, G. I., Naumova, E. S., Masneuf, I., Kondratieva, V. I., Aigle, M. & Dubourdieu, D. (2000). Natural polyploidization of some cultured yeast *Saccharomyces* sensu stricto: auto- and allotetraploidy, *Syst Appl Microbiol* (in press).

Nguyen, H.-V. & Gaillardin, C. (1997). Two subgroups within the *Saccharomyces bayanus* species evidenced by PCR amplification and restriction polymorphism of the non-transcribed spacer 2 in the ribosomal DNA unit, *Syst Appl Microbiol* **20**, 286–294.

Oda, Y., Yabuki, M., Tonomura, K. & Fukunaga, M. (1997). A phylogenetic analysis of *Saccharomyces* species by the sequence of 18S-28S rRNA spacer regions, *Yeast* **13**, 1243–1250.

Pryde, F. E., Huckle, T. C. & Louis, E. J. (1995). Sequence analysis of the right end of chromosome XV in *Saccharomyces cerevisiae*: an insight into the structural and functional significance of sub-telomeric repeat sequences, *Yeast* **11**, 371–382.

Ryu, S.-L., Murooka, Y. & Kaneko, Y. (1996). Genomic reorganization between two sibling yeast species, *Saccharomyces bayanus* and *Saccharomyces cerevisiae*, *Yeast* **12**, 757–764.

Ryu, S.-L., Mikata, K., Murooka, Y. & Kaneko, Y. (1998). A simple PCR method for distinguishing *Saccharomyces cerevisiae* from its sibling species by amplification of the *PRL2* region, *J Ferment Bioeng* **86**, 249–252.

Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees, *Mol Biol Evol* **4**, 406–425.

Valente, P., Gouveia, F. C., de Lemos, G. A., Pimentel, D., van Elsas, J. D., Mendonça-Hagler, L. C. & Hagler, A. N. (1996). PCR amplification of the rDNA internal transcribed spacer region for differentiation of *Saccharomyces* cultures, *FEMS Microbiol Lett* **137**, 253–256.

Vaughan Martini, A. (1989). *Saccharomyces paradoxus* comb nov., a newly separated species of the *Saccharomyces* sensu stricto complex based upon nDNA/nDNA homologies, *Syst Appl Microbiol* **12**, 179–182.

Vaughan Martini, A. & Kurtzman, C. P. (1985). Deoxyribonucleic acid relatedness among species of the genus *Saccharomyces* sensu stricto, *Int J Syst Bacteriol* **35**, 508–511.

Vaughan Martini, A. & Martini, A. (1987). Three newly delimited species of *Saccharomyces* sensu stricto, *Antonie Leeuwenhoek* **53**, 77–84.

White, T. J., Bruns, T., Lee, S. & Taylor, J. (1990). PCR protocols: a guide for methods and applications. In *Amplification and Direct Sequencing of Fungal Ribosomal RNA Genes for Phylogenetics*, pp. 315–322. Edited by M. A. Innis, D. H. Gelfand, J. J. Sninsky & T. J. White. New York: Academic Press.

Yamada, Y., Mikata, K. & Banno, I. (1993). Reidentification of 121 strains of the genus *Saccharomyces*, *Bull JFCC* **9**, 95–119 (in Japanese).

Yarrow, D. (1998). Methods for the isolation, maintenance and identification of yeasts. In *The Yeasts – a Taxonomic Study*, 4th revised edn, pp. 77–100. Edited by C. P. Kurtzman & J. W. Fell. Amsterdam: Elsevier.