Microbiology, Vol. 73, No. 6, 2004, pp. 658–665. Translated from Mikrobiologiya, Vol. 73, No. 6, 2004, pp. 768–776.

EXPERIMENTAL ARTICLES

Molecular Divergence of the Soil Yeasts *Williopsis* Sensu Stricto

E. S. Naumova*, D. O. Gazdiev*,,***, and G. I. Naumov*,[1]**

**State Research Institute of Genetics and Selection of Industrial Microorganisms, Pervyi Dorozhnyi proezd 1, Moscow, 113545 Russia*

***Botanical Garden, Amur Scientific Center, Far East Division, Russian Academy of Sciences, Blagoveshchensk, 675000 Russia*

****Amur Integrated Research Institute, Amur Scientific Center, Far Eastern Division, Russian Academy of Sciences, Relochnyi per. 1, Blagoveshchensk, 675000 Russia*

Received March 16, 2004

Abstract—Fifty-three strains of Saturn-spored yeasts were analyzed by means of restriction analysis of the amplified fragment of rDNA comprising the 5.8S rRNA gene and the internal transcribed spacers ITS1 and ITS2. The use of endonucleases *Hae*III and *Msp*I enabled clear differentiation of yeast species *Williopsis mucosa, W. salicorniae, Zygowilliopsis californica,* and *Komagataea pratensis* and the *Williopsis* sensu stricto complex. The minisatellite primer M13 was proposed for differentiation between sibling species of *Williopsis* sensu stricto, which have identical restriction profiles. PCR with primer M13 enabled reidentification of a number of collection strains, species identification of Saturn-spored isolates from the Far East, and detection of three strains affiliated to novel taxa. The latter have unique PCR profiles and differ in the nucleotide sequences of ITS1 and ITS2 fragments of rDNA. Possible variations in the results obtained with different molecular methods are discussed.

Key words: Williopsis sensu stricto, *Zygowilliopsis, Komagataea*, RFLP analysis, ITS1 and ITS2 rDNA.

Progress in the field of molecular biology has generated a range of new methods that are now being widely used in yeast taxonomy along with traditional morphological and physiological methods. Differentiation between yeast taxa and phylogenetic analysis are carried out by means of rRNA gene sequencing, which enables determination of phylogenetic relations at both species and genus levels. Various rDNA fragments have different phylogenetic importance. Traditionally, 18S and 26S rRNA genes and internal transcribed spacers ITS1 and ITS2 are sequenced [1]. Kurtzman and Robnett [2] sequenced the variable fragment D1/D2 of 26S rDNA (600 nucleotides) in most of the known ascomycete species. That study allowed creation of a computer database for taxonomic differentiation of yeasts. rRNA gene sequencing is mainly used for the characterization of type cultures or of a limited number of strains, as it is expensive and time-consuming. In case of a large number of strains, it is preferable to use restriction analysis of amplified rDNA fragments, including the 18S rRNA gene [3] and internal transcribed spacers ITS1 and ITS2 [4].

Saturn-spored yeasts can be found in different genera: *Williopsis, Saccharomycopsis, Pichia*, and *Saturnispora* [5]. The species structure of these genera is often revised as a result of the constant changes in the criteria for evaluation of phylogenetic relatedness. The genus *Williopsis* Zender, included in the latest yeast taxonomic manual [5], comprises five species: *Williopsis californica, W. mucosa, W. pratensis, W. salicorniae*, and *W. saturnus,* with five varieties: var. *saturnus*, var. *mrakii*, var. *sargentensis*, var. *suaveolens*, and var. *subsufficiens.* However, molecular and genetic data indicate that the genus *Williopsis* sensu Kurtzman 1998 is highly heterogeneous. Comparison of the sequences of 18S and 26S rRNA genes showed significant divergence between the species *W. californica, W. mucosa, W. pratensis, W. salicorniae*, and *W. saturnus* [6–9], each of which probably represents an individual genus. On the basis of the significant difference in rDNA sequences and morphological and physiological peculiarities, Yamada *et al.* [7] created a new genus, *Komagataea*, for the species *K. pratensis* and proposed to restore the genus *Zygowilliopsis* Kudriavzev, which includes the species *Z. californica.* The necessity to restore the latter genus was demonstrated earlier by hybridological analysis [10, 11]. The classification of species of the *W. saturnus* complex (*Williopsis* sensu stricto) is also equivocal. The latest yeast taxonomic manual presents these species as varieties, and *W. beijerinckii* is presented as a synonym of *W. saturnus* var. *saturnus* [5]. Genetic analysis suggests species status of the yeasts *W. saturnus, W. beijerinckii, W. mrakii, W. suaveolens*, and *W. subsufficiens* [11–15]. According to DNA–DNA reassociation data, the yeast *W. sargentensis* is also a part of this complex [11, 16, 17].

[1] Corresponding author; e-mail: gnaumov@yahoo.com

0026-2617/04/7306-0658 © 2004 MAIK "Nauka/Interperiodica"

These six sibling species are characterized by 36–78% DNA–DNA reassociation and by identical 18S and 26S sequences and can be differentiated only through sequencing of the internal transcribed spacers ITS1 and ITS2 [7, 9, 16, 17].

In this work, we used restriction analysis of an amplified fragment of nuclear ribosomal DNA comprising the 5.8S rRNA gene and internal transcribed spacers ITS1 and ITS2 (5.8S–ITS fragments) for a comparative study of collection strains of the genera *Williopsis, Zygowilliopsis*, and *Komagataea* and newly isolated Saturn-spored yeasts.

MATERIALS AND METHODS

Fifty collection strains were used in this study, including the type cultures *Williopsis saturnus* CBS 254, *W. suaveolens* CBS 255, *W. beijerinckii* CBS 2564, *W. mrakii* CBS 1707, *W. subsufficiens* CBS 5763, *W. sargentensis* CBS 6342, *Zygowilliopsis californica* CBS 252, *W. mucosa* CBS 6341, *W. salicorniae* CBS 8071, and *Komagataea pratensis* CBS 7079. Most of the strains were isolated from soils of different geographic regions (table). Three strains (01.02, 09.02, and 48.02) were isolated from floodplain alluvial soils in the south of the Far East of Russia in 2002. Samples were taken from a depth of 0–15 cm and plated onto complete YPD agar of the following composition (g/l): glucose, 20; peptone, 10; yeast extract, 5; agar, 20. All types of colonies were subjected to microscopy. Yeast identification was carried out using standard methods and the taxonomic manual [5]. Yeasts were cultivated at 28°C on complete YPD medium. For the induction of spore formation, the strains were cultivated at 28°C on minimal medium of the following composition (g/l): maltose, 30; agar, 20.

PCR analysis. DNA was isolated as described previously [18]. The polymerase chain reaction was carried out on a Tertsik DNA amplifier (DNK-Tekhnologiya, Russia). For the amplification of the 5.8S–ITS fragment of rDNA, the primers pITS1 (5'-TCCGTAGGTGAACCTGGGG-3') and pITS4 (5'-CCTCCGCTTATTGATATGC-3') were used [19]. The reaction mixture (30 μl) contained PCR buffer, 20 mM $(NH_4)_2SO_4$, 3 mM $MgCl_2$, 0.25 mM dNTP, 0.30 μM of each primer, 1.25 units of *Taq* DNA polymerase (Sintol, Russia), and from 20 to 200 ng of the analyzed genomic DNA. Initial DNA denaturation was performed at 94°C for 3 min and was followed by 30 cycles: 2-min DNA denaturation at 94°C, 1-min primer annealing at 60°C, and 2.5-min DNA synthesis at 72°C (10 min for the last cycle). PCR with primer M13 (5'-GAGGGTGGCGGTTCT-3') was carried out in 30 μl of a reaction mixture containing PCR buffer, 20 mM $(NH_4)_2SO_4$, 3 mM $MgCl_2$, 0.25 mM dNTP, 0.30 μM of each primer, 1.25 units of *Taq* DNA polymerase (Sintol, Russia), and from 20 to 200 ng of the analyzed genomic DNA. PCR was performed as 35 cycles of 45-s DNA denaturation at 94°C, 30-s primer annealing at 52°C, and 2-min DNA synthesis at 72°C. Amplification products were separated by electrophoresis through 1% agarose gel at 60–65 V in 0.5× TBE buffer (45 mM Tris, 10 mM EDTA, and 45 mM boric acid) for 2 h and stained with ethidium bromide. For restriction fragment length polymorphism analysis (RFLP analysis), endonucleases *Hae*III, *Hin*fI, *Mbo*I, and *Msp*I (Fermentas, Lithuania) were used. Restriction fragments were separated in 1.6% agarose gel at 60–65 V in 0.5× TBE buffer for 3 h. The gel was stained with ethidium bromide and photographed in UV light on a Vilber Lourmat transilluminator (France).

Cloning. PCR products were eluted from the gel using a GeneClean kit (Bio 101 Inc., United States), following the manufacturer's recommendations, and blunt-end inserted into the pTZ57R/T vector linearized at the *Eco*321 site (*Eco*RV isoschizomer). Competent cells of *E. coli* TG1 were transformed using an InsT/Aclone™ PCR Product Cloning Kit (MBI Fermentas, Lithuania) according to the manufacturer's recommendations. Transformants with the insertion were selected on LB medium containing 100 μg/ml ampicillin, X-gal substrate, and IPTG inducer.

Sequencing. Nucleotide sequences were determined by the Sanger method on an ABI 373 A automatic sequencer (Applied Biosystems) and deposited with the Genbank database under the following accession numbers: AY563508 (strain CBS 254), AY 563509 (CBS 2564), AY563510 (CBS 2876), AY563511 (CBS 1669), AY563512 (CBS 6291), and AY563513 (IFO 1776).

Phylogenetic analysis. For determination of the homology with known nucleotide sequences, the BLAST program was used. Multiple sequence alignment was performed using the CLUSTAL W program.

RESULTS

Restriction analysis of amplified ITS fragments. First of all, we performed amplification of the 5.8S–ITS fragments on the template DNA of ten type cultures of the yeasts *W. saturnus, W. suaveolens, W. beijerinckii, W. mrakii, W. subsufficiens, W. sargentensis, Z. californica, W. mucosa, W. salicorniae*, and *K. pratensis.* The size of the PCR product varied in different species from 550 to 800 bp (Fig. 1). In the six species of the *Williopsis* sensu stricto complex (lanes *1–6*), the sizes of the PCR products were the same (about 650 bp). The type culture of *Z. californica* had a somewhat smaller 5.8S–ITS fragment (about 600 bp, lane *7*). The type culture of *K. pratensis* yielded the smallest PCR product (about 550 bp, lane *8*), and *W. salicorniae* yielded the largest product (about 800 bp, lane *10*). *W. mucosa* had a 5.8S–ITS fragment of about 700 bp (lane *9*).

The PCR products were analyzed by enzymatic digestion with four restriction endonucleases: *Hae*III, *Hin*fI, *Mbo*I, and *Msp*I (Fig. 2). The six type cultures of *Williopsis* sensu stricto exhibited identical restriction profiles irrespective of the endonuclease used. Thus,

Strains of the genera *Williopsis*, *Zygowilliopsis*, and *Komagataea* used in this work

Original species name	Strain	Isolation substrate and geographical place	Species affiliation established in this work
W. saturnus	CBS 254 (T)	Soil, Himalayas	*W. saturnus*
	CBS 5761	United States	"
	CBS 112	Unknown	"
	CBS 6291	Soil, Nigeria	*Williopsis* sp. N1
	IFO 0811	Soil, Japan	*W. saturnus*
	IFO 0993	"	"
	IFO 1772	"	"
	IFO 1773	Semidecomposed leaf litter, Japan	"
	IFO 1774	"	"
	IFO 1775	Soil, Japan	"
	IFO 1776	"	*Williopsis* sp. N2
	CCY 81-2-1	Soil under mosses, Man Pocton, North Korea	*W. saturnus*
	CCY 81-2-2	Soil under mosses, Pionsan, North Korea	"
W. beijerinckii	CBS 2564 (T)	Soil, South Africa	*W. beijerinckii*
	CBS 2876	"	"
	CBS 4549	Elephant dung, Japan	"
	CBS 4304	Garden soil, Japan	"
	IFO 1191	Soil, Japan	"
	IFO 1762	"	"
	IFO 1763	"	"
W. suaveolens	CBS 255 (T)	Soil, Denmark	*W. suaveolens*
	CBS 1669	Soil, Minnesota, United States	*Williopsis* sp. N3
	IFO 0809	Soil, Japan	*W. saturnus*
	KBP 1097	Peat soil, Tver oblast	*W. suaveolens*
	KBP 1579	Peat soil, Moscow oblast	"
	KBP 1580	"	"
	KBP 1581	"	"
W. mrakii	CBS 1707 (T)	Soil, Papua New Guinea	*W. mrakii*
	CBS 7192	Fruit juice, Italy	"
	IFO 0895	Soil, Japan	*W. beijerinckii*
	IFO 0896	"	"
Williopsis sp.	01.02	Soil, Amur oblast	*W. suaveolens*
	09.02	"	"
	48.02	Soil, Jewish Autonomous oblast	"
W. subsufficiens	CBS 5763 (T)	Soil, Liberia	*W. subsufficiens*
W. sargentensis	CBS 6342	Lake water, New Hampshire, United States	*W. sargentensis*
W. mucosa	CBS 6341 (T)	Soil, United States	*W. mucosa*
W. salicorniae	CBS 8071 (T)	Salt spring water, France	*W. salicorniae*
Z. californica	CBS 252 (T)	Leaves, United States	*Z. californica*
	VKM Y-167	Soil, Japan	"
	VKM Y-168	Soil, Portugal	"
	VKM Y-1918	Berries, Kola Peninsula	"
	KBP 398	Beet rhizosphere, Moscow oblast	"
	KBP 440	Cabbage rhizosphere, Moscow oblast	"
	KBP 3708	*Pachyiulus flavipes* intestine, Gurzuf, Crimea	"
	KBP 3712	Southern chernozems	"
K. pratensis	CBS 7079 (T)	Soil, Dagestan, Caucasian Preserve	*K. pratensis*
	KBP 2691	"	"
	KBP 2690	"	"
	KBP 2680	Soil, Zailiiskii Alatau, northern Tien Shan	"
	KBP 2681	"	"
	KBP 2682	"	"
	KBP 2683	"	"

Note: CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; IFO, Institute for Fermentation, Osaka, Japan; CCY, Culture Collection of Yeasts, Institute of Chemistry, Slovak Academy of Sciences, Bratislava, Slovakia; VKM, All-Russia Collection of Microorganisms; KBP, Collection of the Department of Soil Biology, Moscow State University. CBS 255 = NCYC 2586; CBS 1707 = NCYC 500; CBS 6342 = VKM Y-2140; CBS 7079 = KBP 2692. (T) means "type culture."

*Hae*III restriction of the six type cultures yielded three fragments with sizes of about 350, 180, and 120 bp (Fig. 2a, lanes *1–6*). The restriction profiles of the type cultures of *W. mucosa, W. salicorniae, K. pratensis*, and *Z. californica* were species-specific. *W. mucosa* CBS 6341 was characterized by the absence of the *Msp*I site (Fig. 2d, lane *9*). The PCR product of *Z. californica* was not cut by either *Msp*I or *Hae*III (Figs. 2a, 2d, lane *7*). All of the four endonucleases used cut the 5.8S–ITS fragments of *K. pratensis* and *W. salicorniae* (Fig. 2, lanes *8* and *10*, respectively). To evaluate the intraspecies polymorphism of the restriction profiles, we conducted RFLP analysis of 23 yeast strains belonging to *Williopsis* sensu stricto: *W. saturnus* (two strains), *W. beijerinckii* (three), *W. suaveolens* (four), *W. mrakii* (one), *Z. californica* (seven), and *K. pratensis* (six). The species affiliation of these strains had been established earlier by genetic analysis and/or PCR with nonspecific primers [10, 12–15, 18, 20]. The sizes of the amplified 5.8S–ITS fragments and the restriction profiles for these strains were in full accord with their species affiliation (data not shown).

Thus, RFLP analysis allows the yeasts *W. saturnus, W. mucosa, W. salicorniae, Z. californica*, and *K. pratensis* to be differentiated. The most clear-cut distinctions in the restriction profiles were recorded with the use of endonucleases *Hae*III and *Msp*I (Figs. 2a, 2d). In further experiments, these endonucleases were used for differentiation of Saturn-spored collection strains and new isolates. It should be noted that the six sibling species of *Williopsis* sensu stricto cannot be distinguished by their *Hin*fI, *Hae*III, *Mbo*I, or *Msp*I restriction profiles (Fig. 2, lanes *1–6*). According to our data, these yeasts can be differentiated by using PCR analysis with nonspecific primers [18].

Molecular analysis of collection strains and new isolates. With the use of RFLP analysis, we studied 20 collection strains of the genus *Williopsis*, obtained from three collections—IFO, CBS, and CCY—under different species names, and three Saturn-spored yeast strains that we had isolated in the Far East (table). All of the strains studied exhibited identical *Hae*III and *Msp*I restriction profiles, typical of the *Williopsis* sensu stricto yeasts (data not shown). To determine the species affiliation of the strains, we used PCR analysis with the minisatellite primer M13 (Fig. 3). The six type cultures of *Williopsis* sensu stricto (*W. saturnus* CBS 254, *W. beijerinckii* CBS 2564, *W. mrakii* CBS 1707, *W. subsufficiens* CBS 5763, *W. sargentensis* CBS 6342, and *W. suaveolens* CBS 255) differed in the number and size of amplified bands (Fig. 3a, lanes *1, 8, 13–15*; Fig. 3b, lane *5*).

By their PCR profiles, the 20 strains studied can be divided into four groups. The first group includes the type culture *W. saturnus* CBS 254, seven Japanese strains ((all IFO) 0811, 0993, 1772, 1773, 1774, 1775, and 0809), and two North Korean strains (CCY 81-2-1 and CCY 81-2-2) (Fig. 3a, lanes *1–7*; Fig. 3b, lanes *1–4*).

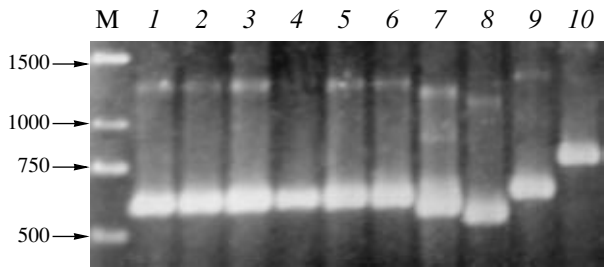

Fig. 1. Size of amplified 5.8S–ITS fragments of rDNA in strains of yeasts of the genera *Williopsis, Zygowilliopsis*, and *Komagataea.* Lanes: (*1*) *W. saturnus* CBS 254; (*2*) *W. beijerinckii* CBS 2564; (*3*) *W. suaveolens* CBS 255; (*4*) *W. mrakii* CBS 1707; (*5*) *W. subsufficiens* CBS 5763; (*6*) *W. sargentensis* CBS 6342; (*7*) *Z. californica* CBS 252; (*8*) *K. pratensis* CBS 7079; (*9*) *W. mucosa* CBS 6341; (*10*) *W. salicorniae* CBS 8071. M, 100-bp DNA Ladder molecular weight marker (Fermentas, Lithuania).

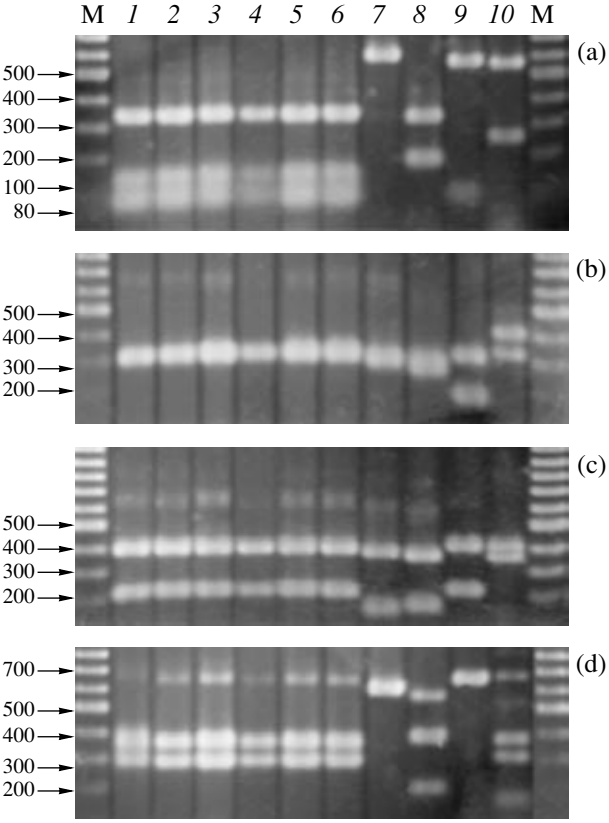

Fig. 2. Restriction analysis of amplified 5.8S–ITS fragments of rDNA in strains of yeasts of the genera *Williopsis, Zygowilliopsis*, and *Komagataea* performed with the use of endonucleases (a) *Hae*III, (b) *Hin*fI, (c) *Mbo*I, and (d) *Msp*I. Lanes: (*1*) *W. saturnus* CBS 254; (*2*) *W. beijerinckii* CBS 2564; (*3*) *W. suaveolens* CBS 255; (*4*) *W. mrakii* CBS 1707; (*5*) *W. subsufficiens* CBS 5763; (*6*) *W. sargentensis* CBS 6342; (*7*) *Z. californica* CBS 252; (*8*) *K. pratensis* CBS 7079; (*9*) *W. mucosa* CBS 6341; (*10*) *W. salicorniae* CBS 8071. M, 100-bp DNA Ladder molecular weight marker (Fermentas, Lithuania).

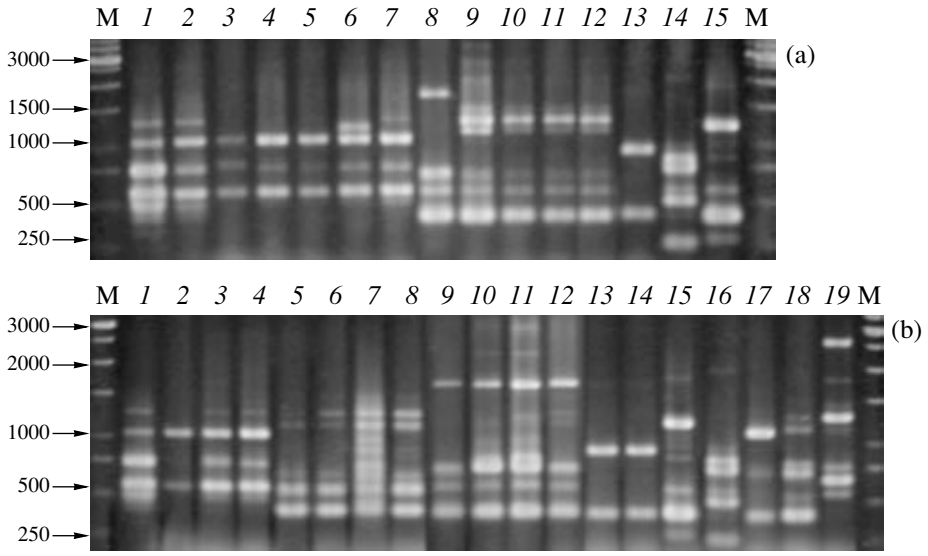

Fig. 3. PCR analysis of strains of the *Williopsis* sensu stricto complex with the use of the nonspecific minisatellite primer M13. (a) *W. saturnus* (*1*) CBS 254, (*2*) IFO 0811, (*3*) IFO 0993, (*4*) IFO 1772, (*5*) IFO 1773, (*6*) IFO 1774, and (*7*) IFO 1775; *W. beijerinckii* (*8*) CBS 2564, (*9*) IFO 1191, (*10*) IFO 1762, (*11*) IFO 1763, and (*12*) CBS 4549; (*13*) *W. mrakii* CBS 1707; (*14*) *W. subsufficiens* CBS 5763; and (*15*) *W. sargentensis* CBS 6342. (b) *W. saturnus* (*1*) CBS 254, (*2*) IFO 0809, (*3*) CCY 81-2-1, and (*4*) CCY 81-2-2; *W. suaveolens* (*5*) CBS 255, (*6*) 01.02, (*7*) 09.02, and (*8*) 48.02; *W. beijerinckii* (*9*) CBS 2564, (*10*) IFO 0895, (*11*) IFO 0896, and (*12*) CBS 2876; *W. mrakii* (*13*) CBS 1707 and (*14*) CBS 7192; (*15*) *W. sargentensis* CBS 6342; (*16*) *W. subsufficiens* CBS 5763; and *Williopsis* sp. (*17*) CBS 6291, (*18*) CBS 1669, and (*19*) IFO 1776. M, 1-kb DNA Ladder molecular weight marker (Fermentas, Lithuania).

These strains exhibited four major bands with approximate product sizes of 500, 600, 750, and 1100 bp. Eight of these strains were earlier identified as *W. saturnus* var. *saturnus*, and strain IFO 0809 was identified as *W. saturnus* var. *suaveolens* (table). The second group is composed of eight strains, including the type culture *W. beijerinckii* CBS 2564 and strains CBS 4549 and CBS 2876, which were assigned to this species based on data of DNA–DNA reassociation and genetic analysis (Fig. 3a, lanes *8, 12*; Fig. 3b, lanes *9, 12*). Earlier, we revealed molecular polymorphism of *W. beijerinckii* strains, which is related to the geographical origin of these strains. Strains isolated in South Africa (CBS 2564 and CBS 2876) and Japan (CBS 4549 and CBS 4304) had somewhat different UP PCR profiles [20]. In the present work, three Japanese strains (IFO 1191, 1762, and 1763) proved to be similar to strain CBS 4549 by their PCR profiles with primer M13 (Fig. 3a, lanes *9–12*). Two other Japanese strains (IFO 0895 and IFO 0896) earlier identified as *W. saturnus* var. *mrakii*, the type culture CBS 2564, and strain CBS 2876 had virtually identical PCR profiles with major fragments of approximate sizes of 350, 600, 700, and 1700 bp (Fig. 3b, lanes *9–12*). The type culture of *W. suaveolens* and the three strains that we isolated in the Far East comprise the third group (Fig. 3b, lanes *5–8*). The fourth group includes the type culture *W. mrakii* CBS 1707 and strain CBS 7192 (Fig. 3b, lanes *13, 14*).

Three strains (CBS 6291, CBS 1669, and IFO 1776) exhibited unique patterns and did not fall into any of the above four groups. Strain CBS 6291 was characterized by a PCR profile with two major fragments sized about 350 and 1250 bp (Fig. 3b, lane *17*). Strain CBS 1669 exhibited four major bands with sizes of about 350, 700, and 750 bp (Fig. 3b, lane *18*). Strain IFO 1776 had a PCR profile with three major bands with sizes of about 700, 1300, and 2600 bp (Fig. 3b, lane *19*). Two of these strains (CBS 6291 and IFO 1776) had earlier been identified as *W. saturnus* var. *saturnus*, and strain CBS 1669 had been identified as *W. saturnus* var. *suaveolens* (table). The type cultures *W. subsufficiens* CBS 5763 and *W. sargentensis* CBS 6342 also exhibited unique PCR profiles (Fig. 3a, lanes *14, 15*; Fig. 3b, lanes *15, 16*).

The species *W. suaveolens* and *W. saturnus* differ in some physiological characteristics, particularly in the ability to utilize L-rhamnose, citric acid, and mannitol [5]. We compared physiological properties of the strains that, according to their PCR profiles, were assigned to the first and third groups. Like the type culture of *W. saturnus*, all of the strains of the first group utilized L-rhamnose, citric acid, and mannitol. Our three isolates (01.02, 09.02, and 48.02), as well as the type culture of *W. suaveolens*, failed to utilize these carbon sources. Thus, the results of physiological tests were in full agreement with the molecular data.

Determination of the nucleotide sequence of internal transcribed spacers ITS1 and ITS2. James and coworkers [9] showed that the sibling species *W. saturnus, W. mrakii, W. sargentensis, W. suaveolens*, and *W. subsufficiens* have identical sequences of 18S rRNA but differ in their ITS1 and ITS2 sequences. It should be noted that these authors used strain CBS

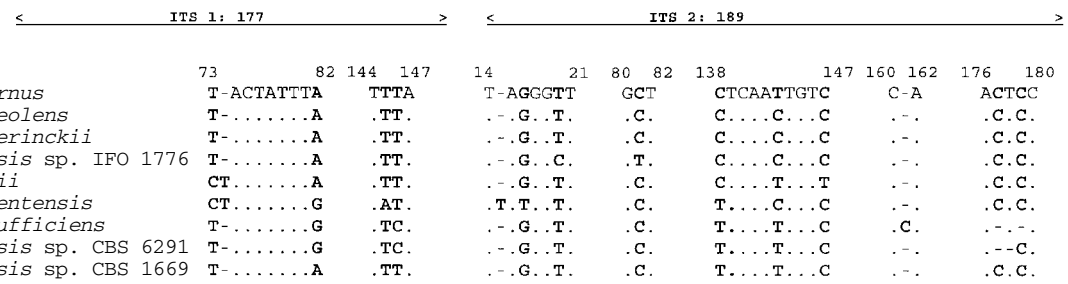

Fig. 4. Multiple alignment of the nucleotide sequences of the ITS1–5.8S–ITS2 region of the rDNA of the yeasts *Williopsis* sensu stricto: *W. saturnus* strains CBS 254(T), CBS 5761, and NCYC 23; *W. suaveolens* CBS 255(T); *W. beijerinckii* CBS 2564(T) and CBS 2876; *W. mrakii* CBS 1707(T) and NCYC 2251; *W. sargentensis* CBS 6342; and *W. subsufficiens* CBS 5763(T). (T) means "type culture."

5761 instead of the type culture of *W. saturnus* and did not use in their work *W. beijerinckii* yeasts.

We performed sequencing of the ITS1 and ITS2 sites in the type culture *W. saturnus* CBS 254 and two strains of *W. beijerinckii* (the type culture CBS 2564 and strain 2876), as well as in three strains that exhibited unique profiles with primer M13: CBS 6291, CBS 1669, and IFO 1776. The nucleotide sequences obtained were compared with the available ITS1 and ITS2 sequences of *Williopsis* sensu stricto yeasts (Figs. 4, 5). The ITS1 and ITS2 sequences of the type culture *W. saturnus* CBS 254 were identical with those of two earlier studied strains of this species, CBS 5761 and NCYC 23. Strains of *W. beijerinckii* were indistinguishable by their ITS1 and ITS2 sequences from the type culture of *W. suaveolens.*

On the whole, comparative analysis of ITS sequences testifies to close relatedness of the strains studied. Strains belonging to the same species exhibited identical ITS1 and ITS2 sequences. Among species, the distinctions in the ITS2 site were greater than in the ITS1 site. The species *W. saturnus, W. suaveolens*, and *W. beijerinckii* and strains IFO 1776 and CBS 1669 have identical ITS1 sequences. The type culture *W. subsufficiens* CBS 5763 and strain CBS 6291 are also indistinguishable by this site. Only *W. mrakii* and *W. sargentensis* exhibited species-specific ITS1 sequences, differing by two and more nucleotide substitutions from other strains (Fig. 5a).

Five out of the six sibling species of *Williopsis* sensu stricto had species-specific ITS2 sequences. In only two species, *W. suaveolens* and *W. beijerinckii*, were the ITS2 sequences identical. Strains IFO 1776 and CBS 1669, possessing identical ITS1 sequences, differed in their ITS2 sequences by four nucleotides (Fig. 5b). Strain CBS 6291 differed from *W. subsufficiens* and other strains studied by no less than three nucleotide substitutions.

DISCUSSION

The results obtained in this work are in good agreement with earlier conducted molecular genetic studies [7, 9, 11] and confirm the heterogeneity of the genus *Williopsis* sensu Kurtzman 1998 [5]. This genus is not monophyletic and should most probably be restricted to the *Williopsis* sensu stricto yeasts, as was suggested by Yamada *et al.* [7] but not accepted in the last manual of yeasts [5]. Like *Zygowilliopsis californica* and *Komagataea pratensis*, the species *W. salicorniae* and *W. mucosa* apparently also represent independent genera. These two taxa differ considerably by their 18S rRNA sequences from each other and from *Z. californica, K. pratensis*, and *Williopsis* sensu stricto [7, 9]. Our comparative study of strains of yeasts with Saturn-shaped spores showed that *Z. californica, K. pratensis, W. salicorniae, W. mucosa*, and *Williopsis* sensu stricto can be clearly distinguished by restriction analysis of the 5.8S–ITS fragment. The distinction was most pronounced when restriction endonucleases *Hae*III and

(a)

W. saturnus CBS 254*, CBS 5761, NCYC 23
0 *W. suaveolens* CBS 255*
0 0 *W. beijerinckii* CBS 2564*, CBS 2876
0 0 0 *Williopsis* sp. IFO 1776
2 2 2 2 *W. mrakii* CBS 1707*, NCYC 2251
4 4 4 4 2 *W. sargentensis* CBS 6342*
2 2 2 2 4 4 *W. subsufficiens* CBS 5763*
2 2 2 2 4 4 0 *Williopsis* sp. CBS 6291
0 0 0 0 2 4 2 2 *Williopsis* sp. CBS 1669

(b)

W. saturnus CBS 254*, CBS 5761, NCYC 23
1 *W. suaveolens* CBS 255*
1 0 *W. beijerinckii* CBS 2564*, CBS 2876
3 2 2 *Williopsis* sp. IFO 1776
1 2 2 4 *W. mrakii* CBS 1707*, NCYC 2251
4 3 3 5 5 *W. sargentensis* CBS 6342*
4 5 5 7 5 6 *W. subsufficiens* CBS 5763*
3 4 4 6 4 5 3 *Williopsis* sp. CBS 6291
1 2 2 4 2 3 3 2 *Williopsis* sp. CBS 1669

Fig. 5. Number of nucleotide substitutions in the (a) ITS1 and (b) ITS2 sites of rDNA of the yeasts *Williopsis* sensu stricto. Asterisks mark type cultures.

(a)

W. saturnus CBS 5761
72 *W. suaveolens* NRRL Y-838, CBS 1670
78-100 ND *W. beijerinckii* CBS 2564*, CBS 4549
52 54 56 *W. mrakii* CBS 1707*
43 54 50 68 *W. sargentensis* CBS 6342*
56 52 52 44 36 *W. subsufficiens* CBS 5763*, NRRL YB-1718

(b)

W. saturnus CBS 254*, CBS 5761
0 *W. suaveolens* CBS 255*, NRRL Y-838
1 1 *W. beijerinckii* CBS 2564*
1 1 2 *W. mrakii* CBS 1707*, NRRL Y-17814
0 0 1 1 *W. sargentensis* CBS 6342*
5 5 4 4 5 *W. subsufficiens* CBS 5763*, NRRL YB-1718

Fig. 6. DNA–DNA reassociation (data from [16, 17]) (a) and the number of nucleotide substitutions in the D1/D2 region of 26S rDNA (data from [2, 6]) (b) in *Williopsis* sensu stricto yeasts. ND stands for "not determined." Asterisks mark type cultures.

*Msp*I were used. The six sibling species of *Williopsis* sensu stricto, which could not be distinguished by restriction analysis, could be differentiated by PCR with the minisatellite primer M13. The use of PCR analysis allowed us to reidentify three strains from the IFO Japanese collection as *W. saturnus* (IFO 0809) and *W. beijerinckii* (IFO 0895 and IFO 0896). Formerly, the first of these strains had been assigned to *W. suaveolens* and the last two strains had been assigned to *W. mrakii* (table). The three strains of Saturn-spored yeasts that we isolated in the Far East proved to belong to *W. suaveolens.* Strains CBS 1669, CBS 6291, and IFO 1776 possess unique PCR profiles, differ from other strains in the ITS1 and ITS2 sequences, and apparently represent new taxa. The exact determination of the taxonomic status of these strains requires genetic analysis.

We compared the results obtained in the present study with data published on the sequencing of the ITS1 and ITS2 sites and D1/D2 region of 26S rDNA and on DNA–DNA reassociation of sibling species of *Williopsis* sensu stricto (Figs. 5, 6). Comparison of the results obtained by different molecular biological approaches revealed that they were contradictory. Despite the significant divergence of genomes revealed by DNA–DNA reassociation data, the species of *Williopsis* sensu stricto have similar sequences of the ITS1 and ITS2 sites and of the D1/D2 regions of 26S rDNAs. *W. subsufficiens* and *W. mrakii*, exhibiting 44% DNA–DNA reassociation, differ by four or five nucleotide substitutions in the investigated regions of rDNA. Virtually the same level of DNA–DNA reassociation (43%) was recorded in the species pair *W. saturnus*–*W. sargentensis.* However, these species have identical D1/D2 sequences. It is commonly considered that strains of different species differ in more than three nucleotides in the D1/D2 region of 26S rDNA [2]. *W. mrakii*, which exhibits 52 and 68% DNA–DNA reassociation with *W. saturnus* and *W. sargentensis*, respectively, differs from these species by one and five nucleotide substitutions in the ITS2 site. In both cases, only one nucleotide substitution was recorded in the D1/D2 region of 26S rDNA. It should be noted that *W. beijerinckii*, which is presented in the last yeast manual [5] as a synonym of *W. saturnus*, also differs from the latter species by one nucleotide substitution in the D1/D2 region. According to the results of genetic analysis, interspecies hybrids of the *Williopsis* sensu stricto yeasts are sterile in all combinations, which indicates that their species status is the same. The example of the *Williopsis* sensu stricto yeasts demonstrates that, for identification and classification of yeasts, it is necessary to simultaneously use several molecular approaches in combination with genetic analysis.

REFERENCES

1. Valente, P., Ramos, J.P., and Leoncini, O., Sequencing As a Tool in Yeast Molecular Taxonomy, *Can. J. Microbiol.*, 1999, vol. 45, pp. 949–958.

2. Kurtzman, C.P. and Robnett, C.J., Identification and Phylogeny of Ascomycetous Yeasts from Analysis of Nuclear Large Subunit (26S) Ribosomal DNA Partial Sequences, *Antonie van Leeuwenhoek*, 1998, vol. 73, pp. 331–371.

3. Dlauchy, D., Tornai-Lehoczki, J., and Peter, G., Restriction Enzyme Analysis of PCR Amplified rDNA as a Taxonomic Tool in Yeast Identification, *Syst. Appl. Microbiol.*, 1999, vol. 22, pp. 445–453.

4. Esteve-Zarzoso, B., Belloch, C., Uruburu, F., and Querol, A., Identification of Yeasts by RFLP Analysis of the 5.8S rRNA Gene And the Two Ribosomal Internal Transcribed Spacers, *Int. J. Syst. Bacteriol.*, 1999, vol. 49, pp. 329–337.

5. Kurtzman, C.P., *Williopsis* Zender, in *The Yeasts. A Taxonomic Study*, Kurtzman, C.P. and Fell, J.W., Eds., Elsevier, 1998, pp. 413–419.
6. Liu, Z. and Kurtzman, C.P., Phylogenetic Relationships among Species of *Williopsis* and *Saturnospora* gen. nov. as Determined from Partial rRNA Sequences, *Antonie van Leeuwenhoek*, 1991, vol. 60, pp. 21–30.
7. Yamada, Y., Matsuda, M., Maeda, K., Sakakibara, C., and Mikata, K., The Phylogenetic Relationships of the Saturn-Shaped Ascospore–Forming Species of Genus *Williopsis* Zender and Related Genera Based on the Partial Sequences of 18S and 26S Ribosomal RNAs (*Saccharomycetaceae*): the Proposal of *Komagataea* gen. nov., *Biosci. Biotechnol. Biochem.*, 1994, vol. 58, no. 7, pp. 1236–1244.
8. Yamada, Y., Yano, J.-I., Matsuda, M., Higashi, T., and Mikata, K., The Phylogeny of *Williopsis salicorniae* Hinzelin, Kurtzman et Smith Based on the Partial Sequences of 18S and 26S Ribosomal RNAs (*Saccharomycetaceae*), *Biosci. Biotechnol. Biochem.*, 1995, no. 5, pp. 945–948.
9. James, S.A., Roberts, I.N., and Collins, M.D., Phylogenetic Heterogeneity of the Genus *Williopsis* as Revealed by 18S rRNA Gene Sequences, *Int. J. Syst. Bacteriol.*, 1998, vol. 48, pp. 591–596.
10. Naumov, G.I., Vustin, M.M., and Bab'eva, I.P., Sexual Divergence of Yeasts of the Genera *Williopsis* Zender, *Zygowilliopsis* Kudriavzev and *Hansenula* H. et R. Sydow, *Dokl. Akad. Nauk SSSR*, 1980, vol. 255, no. 2, pp. 468–471.
11. Naumov, G.I., Genosystematics of Yeasts of the Genera *Williopsis* Zender and *Zygowilliopsis* Kudriavzev, *Mol. Genet. Mikrobiol. Virusol.*, 1987, no. 2, pp. 3–7.
12. Vustin, M.M. and Naumov, G.I., Anomalous Meiosis in Hybrids of Biological Species of the Genus *Williopsis, Biol. Nauki*, 1974, no. 7, pp. 88–91.
13. Vustin, M.M., Naumov, G.I., Bab'eva, I.P., and Naumova, T.I., Genosystematics of the Yeasts *Williopsis saturnus*: New Biological Species, *Dokl. Akad. Nauk SSSR*, 1982, vol. 267, no. 6, pp. 1481–1484.
14. Naumov, G.I., Vustin, M.M., and Naumova, T.I., A Genetic Study of the Yeast Species *Zygowilliopsis californica, Williopsis saturnus, W. beijerinckii* comb. nov., *W. mrakii* comb. nov., *Dokl. Akad. Nauk SSSR*, 1981, vol. 259, no. 3, pp. 718–722.
15. Naumov, G.I., Vustin, M.M., Bab'eva, I.P., and Reshetova, I.S., Addendum to the Genosystematics of the Yeasts of the genus *Williopsis* and *Zygowilliopsis, Mikrobiologiya*, 1985, vol. 54, no. 2, pp. 239–244.
16. Kurtzman, C.P., Prediction of Biological Relatedness among Yeasts from Comparisons of Nuclear DNA Complementarity, *Studies Mycol.*, 1987, vol. 30, pp. 459–468.
17. Kurtzman, C.P., DNA Relatedness among Saturn-Spored Yeasts Assigned to the Genera *Williopsis* and *Pichia, Antonie van Leeuwenhoek*, 1991, vol. 60, pp. 13–19.
18. Naumov, G.I., Tokareva, N.G., Naumova, E.S., and Bab'eva, I.P., Discrimination between the Soil Yeast Species *Williopsis saturnus* and *Williopsis suaveolens* by the Polymerase Chain Reaction with the Universal Primer N21, *Mikrobiologiya*, 2000, vol. 69, no. 2, pp. 280–285.
19. White, T.J., Bruns, T., Lee, E., and Taylor, J., Amplification and Direct Sequencing of Fungal Ribosomal RNA Genes for Phylogenetics, *PCR Protocols: A Guide to Methods and Applications*, New York: Academic, 1990, pp. 315–322.
20. Naumova, E.S., Tokareva, N.G., and Naumov, G.I., *Williopsis saturnus* and *Williopsis beijerinckii* Are Recognized as Distinct Taxa by Means of the Polymerase Chain Reaction with Nonspecific Primers, *Mikrobiologiya*, 2001, vol. 70, no. 3, pp. 365–369.