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EXPERIMENTAL ARTICLES

Molecular Polymorphism of α-Galactosidase *MEL* Genes of *Saccharomyces* Yeasts

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Abstract—Molecular genetic analysis of melibiose-fermenting *Saccharomyces* strains isolated from fermentative processes and natural sources in different world regions was conducted to deduce the evolutionary diversity of *Saccharomyces* yeasts and find new α-galactosidase *MEL* genes. The species *S. bayanus, S. mikatae*, and *S. paradoxus* were shown to have a single copy of *MEL* and not accumulate polymeric genes, unlike some *S. cerevisiae* populations. The polymeric genes *MELp1* and *MELp2* were identified in *S. paradoxus* for the first time. Genes identical by 98.7% are located on the chromosomes X and VI, respectively. Phylogenetic analysis indicates that *MEL* genes of the *Saccharomyces* yeasts are species-specific.

Keywords: Saccharomyces, α-galactosidase *MEL* genes, 5.8S-ITS, molecular karyotyping, phylogenetic analysis.

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Yeasts are widespread in many ecosystems. The most thoroughly studied genus, *Saccharomyces*, includes the species *S. cerevisiae, S. arboricola, S. bayanus, S. cariocanus, S. kudriavzezii, S. mikatae*, and *S. paradoxus* [1–4]. The complete genomic nucleotide sequence of the yeast *S. cerevisiae* was the first one to be determined among eukaryotic organisms. It was found to contain 55 duplicated blocks comprising several hundred homologous gene pairs [5]. The similar order and orientation of the paralogous genes in the duplicated blocks indicate that the *S. cerevisiae* genome was formed as an ancestral duplication followed by massive chromosome rearrangements and deletion loss of individual genes. During the last decade, full-size genomes of over 20 other species of ascomycetous yeasts have been sequenced. Comparative analysis of genomic sequences in *S. cerevisiae, Lachancea waltii*, and *Eremothecium gossypii* have revealed that complete duplication of eight ancestral chromosomes in *S. cerevisiae* occurred after their divergence from *L. waltii* [6]. In addition to the full genome duplication, multiple duplications of individual chromosomes, segments thereof, and individual genes occurred in the course of the evolution of *S. cerevisiae*. Extremely high concentrations of repeating elements at every level of molecular genetic organization are characteristic of all eukaryotic species, since duplication is the major mechanism of acquisition of new genes in eukaryotes [6].

In the genome of *Saccharomyces* yeasts, genes represented by multiple copies are grouped into multigene families. Genes of ribosomal RNA are located on chromosome XII in tandem repeats forming the sequence gene–spacer–gene–spacer. The haploid genome of *S. cerevisiae* contains approximately 120–200 sets of rRNA genes [7]. Analysis of rRNA sequences is presently widely used to establish the phylogenetic relationships between yeasts at the species and higher taxonomical levels. While the biological species of the genus *Saccharomyces* are practically identical in their 18S and 26S rRNA gene sequences [1–4], they may be easily differentiated by the sequences of noncoding rRNA fragments, namely, of the internal transcribed spacers ITS1/ITS2 and the intergenic spacer IGS2. The ITS region is characterized by significant interspecies divergence and a low level of intraspecific polymorphism. The length of the ITS fragment is constant in the strains of the same species, while the sequence may vary [8].

Nontandem repeats spread over the whole genome have a different type of organization. Polymeric genes of fermentation of various sugars belong to these multigenic families [9]. As a rule, the genes are located in the subtelomeric regions of the chromosomes, which are the most dynamic parts of the yeast genome [7]. The α-galactosidase genes of melibiose fermentation are of particular interest. α-Galactosidases (EC 3.2.1.22) are the enzymes widespread in mycelial fungi, plants, and animals, but are uncommon in yeasts. There are both melibiose-fermenting and nonfermenting strains among the *Saccharomyces* yeasts. α-Galactosidase genes of *S. cerevisiae* are the best-studied. Most strains of the species are not able to fer-

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ment melibiose and lack even the silent *MEL* sequence. Meanwhile, polymeric *MEL* genes are known to accumulate in the *S. cerevisiae* strains from the gastrointestinal tract of mammals and the wastes of the olive oil production [10–12]. Eleven structural α-galactosidase genes *MEL1–MEL11* in various combinations were identified in *S. cerevisiae* strains and mapped in the telomeric regions of 11 different chromosomes [13–15]. Sequencing of the individual genes of this family revealed high identity (96–100%) between their nucleotide sequences [16].

Study of the α-galactosidase genes of other *Saccharomyces* species was performed only on individual strains. A single *MEL* gene was sequenced in *S. paradoxus* (*MELp*), *S. bayanus* var. *bayanus* (*MELb*), *S. bayanus* var. *uvarum* (*MELu*), and *S. mikatae* (*MELj*), as well as in a hybrid taxon *S. pastorianus* (*MELpt*) with a *S. carlsbergensis* synonim (*MELx*) [12, 17–20].

In the present work, the α-galactosidase genes of yeast strains *S. bayanus, S. mikatae,* and *S. paradoxus* of various origins were investigated. The presence of polymeric *MEL* genes in *S. paradoxus* strains was discovered. All known α-galactosidase genes of the *Saccharomyces* genus yeasts were compared.

MATERIALS AND METHODS

Strains and cultivation media. The strains used in the work are listed in Table 1. The yeasts were grown at 28°C on a complete YPD medium containing the following (g/l): bactoagar (Difco, United States), 20; glucose (Merck, Germany), 20; yeast extract (Difco), 10; and bacto peptone (Difco), 20. Sporulation in *S. parasdoxus* was induced on a standard starvation medium containing the following (g/l): bactoagar, 20; CH_3COONa, 10; and KCl, 5. Spore-to-spore hybridization was carried out with a micromanipulator. The spores' isolation was also performed with a manipulator upon the enzymatic digestion of the ascus walls with the stomach juice of the garden snail *Helix pomatia*. *S. paradoxus* strains UCD 61-359, UCD 61-248, and 95-1 marked with auxotrophic mutations *lys* and *ade* were selected on a medium containing D,L-aminoadipiic acid and induced by UV irradiation.

The ability of the yeasts to ferment melibiose was inferred (1) from changes in the color of colonies growing on pH indicator agar medium with eosin and methylene blue and (2) from CO_2 production in the liquid YP medium in test tubes with floats. The pH indicator medium contained the following (g/l): bactoagar, 20; peptone, 3.15; yeast extract, 2.7; KH_2PO_4, 1.8; $MgSO_4 \cdot 7H_2O$, 0.9; $(NH_4)_2SO_4$, 0.9; α-D-melibiose monohydrate (Serva, Germany), 20; potassium eosin (Veb Laborchimie Apolda, Germany), 0.4; and methylene blue (Reakhim, Russia), 0.025. Eosin as 2% solution in 50% ethanol, methylene blue as 0.5% solution in water, and 10% melibiose solution were added before pouring the medium. YP medium had the same composition as YPD, but without agar and with glucose substituted by melibiose. Commonly, Mel^+ strains ferment melibiose in 1 day and, rarely, in 2–3 days, whereas Mel^- strains do not ferment melibiose in 10 days. Initially, the segregants were tested for their ability to ferment melibiose on pH indicator medium for 1 day. The strains that failed to ferment melibiose under these conditions were further examined in test tubes with floats over 10 days.

PCR analysis. Yeast DNA was isolated as described previously [20]. Polymerase chain reaction (PCR) was performed using a Tertsic™ thermocycler. The primers pITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and pITS4 (5'-CCTCCGCTTATTGATATGC-3') were used for amplification of the 5.8S rRNA gene and of the internal transcribed spacers ITS1 and ITS2 (5.8S-ITS fragment). The following pairs of primers were used for amplification of *MEL, ACT1*, and *TRP5* genes: DM1/DM2 (TTCGCAGATGGGTTGGGACAA and TAAGCTTGCTGGAACAGTTGTGTT), ACT15/ACT13, (TATCGTCGGTAGACCAAGACA and AGATCCACATTTGTTGGAAGG), and TRP51/TRP52, (TGGTCACATTTATGACCGCA and CAAGGACTCTTTTTGAAAGGC). PCR was carried out in 30 μl buffer containing 2.5 mM $MgCl_2$, 0.1 mM each dNTP, 50 pmol each primer, 2.5 U *Taq* polymerase (Syntol, Russia), and 20–200 ng DNA. Initial denaturing at 94°C for 3 min was followed by 30 cycles of denaturing at 94°C, 30 s; primer annealing at 56°C, 30 s; DNA synthesis at 72°C, 60 s; and another 10 min at 72°C for completion. Restriction fragment length polymorphism (RFLP) analysis of the 5.8S–ITS fragments was performed using *Hae*III and *Hpa*II endonucleases (Fermentas, Lithuania). Restriction fragments were separated in a 2.5% agarose gel at 60–65 V in 0.5× TBE (45 mM Tris, 10 mM EDTA, and 45 mM boric acid) during 4 h. The gels were stained with ethidium bromide and photographed under UV light in a Vilber Lourmat (France) transilluminator.

Molecular karyotyping and Southern hybridization. Chromosomal DNA preparations were obtained and separated by electrophoresis in a CHEF-DR II apparatus (Bio-Rad, United States) as previously described in [21]. Chromosomal DNA was transferred to a nitrocellulose membrane by the vacuum method in a Vacuum blotter apparatus (Bio-Rad). DNA was fixed on the membrane by annealing for 2 h at 80°C. PCR-amplified fragments of α-galactosidase genes *MEL1* (*S. cerevisiae* C.B.11), *MELj* (*S mikatae* NBRC 1815), and *MELx* (*S. pastorianus* CBS 1513), together with those of *S. cereivisiae* genes *ACT1* (chromosome VI) and *TRP5* (chromosome VII), were used as probes. A nonradioactive digoxigenin label dig-11-dUTP was introduced according to the manufacturer's manual (Roche, Switzerland). Hybridization and visualization of the hybridized bands were also performed according to Roche recommendations.

Table 1. *Saccharomyces* strains under study

Strain	Origin	Mel phenotype
	S. cerevisiae	
CBS 1171 (T)	Top brewing, Europe	–
S288c	Genetic line	–
CBS 7961	Fermenting sugarcane syrup, Sao Paolo, Brazil	+
CBS 7962	Fermenting sugarcane syrup, Sao Paolo, Brazil	+
CBS 382	Beer, Brazil	+
CBS 459	Grape pulp, Italy	–
CBS 5378	Alpechin, Spain	+
YNN 295	Genetic line	–
C. B. 11	Genetic line	+
VKRM Y-61	Pig feces	+
UWO 03-429.1	Nectar of the *Bertam* palm tree, Malaysia	+
UWO 03-433.3	Nectar of the *Bertam* palm tree, Malaysia	+
UWO 03-459.1	Nectar of the *Bertam* palm tree, Malaysia	+
UWO 03-461.4	Nectar of the *Bertam* palm tree, Malaysia	+
	S. arboricola	
CBS 10644 (T)	*Quercus fabric* oak bark, China	–
	S. bayanus* var. *bayanus	
CBS 380 (T)	Beer, Europe	+
CBS 424	Pear juice, Switzerland	+
CBS 425	Apple juice, France	+
CBS 1505	Grape juice, Switzerland	+
	S. bayanus* var. *uvarum	
CBS 395	Blackcurrant juice, Netherlands	+
MCYC 623	*Mesophylax adopersus* trichopteran, Spain	+
CCY 21-31-12	*Amanita citrina*, Slovakia	+
UCD 51-206,	*Drosophila persimiles*, United States	+
UCD 61-137	*D. pseudoobscura*, United States	+
NCAIM Y.00789	Exudate of the hornbeam *Carpinus betulus*, Hungary	+
NCYC 686	Spoilt Coca-Cola	+
UWO 99-807.1.1	Exudate of the beechwood *Nothofagus* sp., Patagonia, Argentina	+
UWO 99-808.3	Exudate of the beechwood *Nothofagus* sp., Patagonia, Argentina	+
136.01	Exudate of the elm *Ulmus pumila*, Blagoveshchensk, Russia	+
148.01	Exudate of the elm *Ulmus pumila*, Blagoveshchensk, Russia	+
VKM Y-1146	Cultivated grape, Michurinsk, Russia	+
	S. cariocanus	
UFRJ 50816 (T)	*Drosophila* sp., Rio de Janeiro, Brazil	–
UFRJ 50791	*Drosophila* sp., Rio de Janeiro, Brazil	–
	S. kudriavzevii	
NBRC 1802 (T)	Decayed leaves, Japan	–
NBRC 1803	Decayed leaves, Japan	–

Table 1. (Contd.)

Strain	Origin	Mel phenotype
	S. mikatae	
NBRC 1815 (T)	Soil, Japan	+
NBRC 1816	Decayed leaves, Japan	+
NBRC 10992	Decayed leaves, Japan	+
NBRC 10993	Decayed leaves, Japan	+
NBRC 10994	Decayed leaves, Japan	+
NBRC 10995	Decayed leaves, Japan	+
NBRC 10996	Soil, Japan	+
NBRC 10997	Decayed leaves, Japan	+
NBRC 10998	Decayed leaves, Japan	+
NBRC 10999	Soil, Japan	+
NBRC 11000	Exudate of *Quercus crispula*, Japan	+
NBRC 11001	Exudate of *Camellia japonica*, Japan	+
NBRC 11002	Exudate of *Fagus crenata*, Japan	+
NBRC 11003	Exudate of *Quercus myrsinaefolia*, Japan	+
	S. paradoxus	
CBS 432 (T)	Unknown, Europe	–
CBS 5829	Soil, Denmark	–
CBS 8436	Exudate of the oak *Quercus mongolica*, Vladivostok, Russia	–
CBS 8437	Exudate of the oak *Quercus mongolica*, Vladivostok, Russia	–
CBS 8438	Exudate of the oak *Quercus mongolica*, Vladivostok, Russia	–
UWO 80-13	*Prunus virginiana*, Ontario, Canada	+
UCD 52-153	*Drosophila* sp., United States	–
UCD 61-359	*Ulmus* sp., United States	+
UCD 61-248	*D. pseudoobscura*, California	+
95-1	*Quercus* sp., Michigan, United States	–
	S. pastorianus	
CBS 1513	Beer, Denmark	+
CBS 1538 (T)	Beer, Denmark	+

Notes: Acronyms of culture collections: VKPM, All-Russian Collection of Industrial Microorganisms, Moscow; CBS, Centraalbureau voor Schimmelcultures, Utrecht, Netherlands; NBRC (ex-IFO), NITE Biological Resource Center, Chiba, Japan; MCYC, Departamento de Microbiologia, Escuela Tecnica Superior de Ingenieros Agronomos, Universidad Politecnica de Madrid, Spain; UWO, University of Western Ontario, Department of Biology culture collection, Canada; UCD, Herman J. Phaff Yeast Culture Collection, Department of Food Science and Technology, University of California at Davis, United States. Matching strains: VKPM Y-61 = CBS 4411, CBS 8436 = N42, CBS 8437 = N 43, CBS 8438 = N44, S288c = CBS 8803, MCYC 623 = CBS 7001. C.B.11 is an inbred line of the strain NCYC 74. Strain 95-1 was isolated by G.I. Naumov. T, type strain.

Sequencing and phylogenetic analysis. Nucleotide sequences of α-galactosidase *MEL* genes and 5.8S-ITS fragments over two strands were defined by the Sanger method of direct sequencing on an automated Beckman Coulter sequencer (United States). The homology rate with the known sequences was evaluated using the BLAST software package. Multiple alignment of the obtained and previously known nucleotide and amino acid sequences (for the ITS and *MEL* regions, respectively) was performed manually using the BioEdit software package. The phylogenetic tree was constructed using a neighbor-joining algorithm implemented in the MEGA 3 software package.

RESULTS AND DISCUSSION

The species affiliation of the yeasts was carried out using RFLP analysis of the amplified 5.8S-ITS fragments and by molecular karyotyping. Restriction analysis with endonucleases *Hae*III and *Hpa*II made it possible to differentiate *S. cerevisiae* and *S. mikatae* from the species groups *S. paradoxus*/*S. cariocanus* and *S. bayanus*/*S. kudryavzevii*. In turn, *S. bayanus*

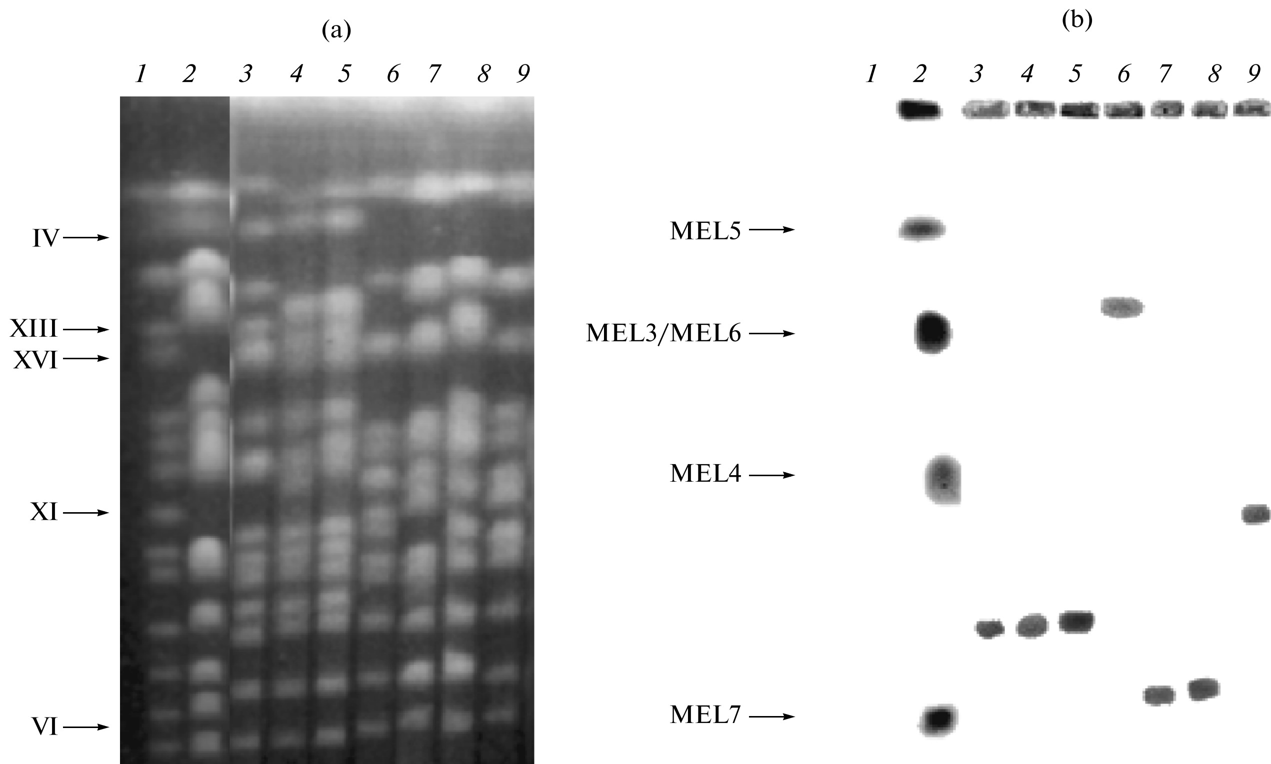

Fig. 1. Pulsed-feld gel electrophoresis of chromosomal DNA of Mel$^+$ strains of the *Saccharomyces* yeasts (a) and Southern hybridization with the *MEL1* probe (b). Lanes: *S. cerevisiae:* YNN 295 (chromosomal standard) (*1*), VKPM Y-61 (control strain) (*2*); *S. bayanus* var. *uvarum*: MCYC 623 (*3*), UWO 99-807.1.1 (*4*), UWO 99-808.3 (*5*); *S. mikatae*: NBRC 1816 (*6*); *S. paradoxus:* UWO 80-13 (*7*), UCD 61-359 (*8*), and UCD 61-248 (*9*). The order of chromosomes follows that of the strain *S. cerevisiae* YNN 295 (lane *1*).

and *S. cariocanus* species may be differentiated by their species-specific molecular karyotypes [1]. Type cultures *S. cerevisiae* CBS 1171, *S. arboricola* CBS 10644, *S. bayanus* CBS 380, *S. cariocanus* UFRJ 50816, *S. kudriavzevii* NBRC 1802, *S. mikatae* NBRC 1815, and *S. paradoxus* CBS 432 were used as species testers. Pulsed-field gel electrophoresis of native chromosomal DNA followed by Southern hybridization with *MEL1, MELx*, and *MELj* probes was used for mapping of *MEL* genes.

***MEL* genes of *S. bayanus* and *S. pastorianus*.** Melibiose fermentation is characteristic of two *Saccharomyces* species, *S. bayanus*, and *S. mikatae*, together with a hybrid bottom-fermentation brewing yeast *S. pastorianus* (syn. *S. carlsbergensis*). The biological species *S. bayanus* is heterogeneous and is represented by two varieties which are partially genetically isolated, namely, *S. bayanus* var. *bayanus* and *S. bayanus* var. *uvarum* [22, 23]. The strains isolated under beer fermentation conditions, including the type strain CBS 380, belong mainly to *S. bayanus* var. *bayanus*. Beer strains of bottom fermentation are hybrids between *S. cerevisiae* and *S. bayanus* var. *bayanus* [23]. The specific ecological niche for *S. bayanus* var. *uvarum* is low-temperature wine production. Melibiose-fermenting *Saccharomyces* strains typically belong to *S. bayanus* var. *uvarum* and rarely, to *S. cerevisiae*. For example, among the 45 Mel$^+$ strains isolated in Russia, Moldova, Slovakia, Switzerland, France, Italy, and Spain, only 3 were identified as *S. cerevisiae*, while the rest were classified as *S. bayanus* [24, 25].

In the present work, we performed molecular karyotyping of over 60 *S. bayanus* var. *uvarum* strains of different origin. To establish the chromosome localization of the *MEL* genes, chromosomal DNA of the strains under study were transferred to nitrocellulose membranes by Southern blot and hybridized with the *MELx* probes. Strain VKPM Y-61 (see Table 1) possessing five *MEL* genes of different chromosome localization, namely, *MEL3* (chromosome XVI), *MEL4* (XI), *MEL5* (IV), *MEL6* (XIII), and *MEL7* (VI) (Figs. 1a and 1b, lane *2*) was used as the control. It should be noted that the genes *MEL3* and *MEL6* are located in the doublet containing the chromosomes XIII and XVI. Southern blot analysis revealed that all strains under study have a single *MEL* gene of the same chromosome localization. The results of Southern hybridization of some strains are presented in Figs. 1 and 2. In *S. bayanus* var. *uvarum* strains, the *MELx* probe hybridized with the chromosomal band that corresponded to the chromosome VI of the control strain

S. cerevisiae YNN 295 (Fig. 1, lanes *3–5*, Fig. 2, lanes *4–10*) while, in *S. pastorianus* CBS 1513 and *S. bayanus* var. *bayanus*, CBS 380 the hybridization signal was located at the level of chromosome X of the control strain YNN 295 (Fig. 2, lanes *2* and *3*, respectively). Earlier, in a hybridization analysis employing the strain *S. cerevisiae* S7 with the chromosome X substituted via cytoduction by the homologous chromosome of *S. pastorianus* containing the *MELx* gene [26], the latter was found to be localized in the right arm of chromosome X [19]. Taking into account that the reciprocal translocation characteristic of *S. bayanus* var. *uvarum* involves the right arm of chromosome X and the left one of chromosome VI [23, 27], it is most likely that the *MEL* genes are also localized in the right arm of chromosome X.

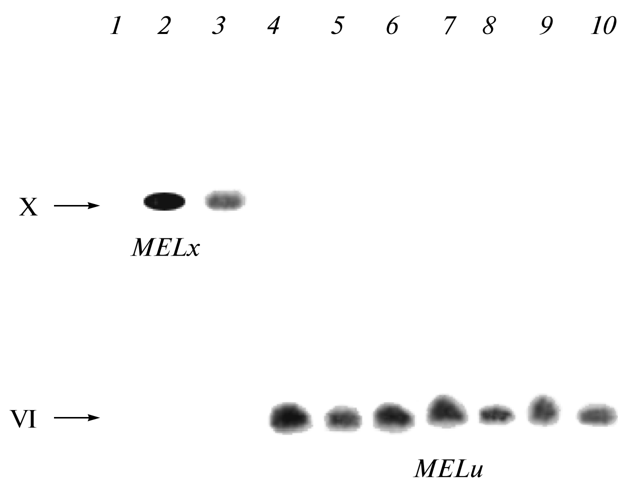

Fig. 2. Southern hybridization of chromosomal DNA of the strain *S. bayanus* var. *uvarum* with the *MELx* probe. Lanes: *S. cerevisiae*: YNN 295 (*1*); *S. pastorianus*: CBS 1513 (*MELx*) (*2*); *S. bayanus* var. *bayanus*: CBS 380 (*3*); *S. bayanus* var. *uvarum*: UCD 51-206 (*4*), UCD 61-137 (*5*), NCAIM Y.00789 (*6*), 136.01 (*7*), 148.01 (*8*), VKM Y-1146 (*9*), and CBS 395 (*10*).

Earlier, we revealed considerable similarity (93.6–99.3%) between the genes *MELb* (*S. bayanus* var. *bayanus*), *MELu* (*S. bayanus* var. *uvarum*), *MELpt* (*S. pastorianus*), and *MELx* (*S. carlsbergensis*) [20]. In the present work, *MEL* genes in seven strains of *S. bayanus* var. *uvarum* (CBS 395, MCYC 623, UWO 99-807.1.1, UWO 99-808.2, 136.01, 148.01, and VKM Y-1146) and two strains of *S. bayanus* var. *bayanus* (CBS 425 and CBS 1505) were sequenced. The obtained nucleotide sequences were compared with each other and with other *MEL* gene sequences of the *Saccharomyces* yeasts deposited in GenBank. The nucleotide sequences of the *MEL* genes in strains CBS 425 and CBS 1505 did not differ from the *MELb* gene of the type strain CBS 380, whereas the similarity level of the analyzed α-galactosidase sequences of seven *S. bayanus* var. *uvarum* strains was 98–100%. Strains UWO 99-807.1.1 and UWO 99-808.3 had identical *MEL* gene sequences, with 99% similarity to the *MELb* gene. The α-galactosidase genes of the Far Eastern isolates 136.01 and 148.01, differing from the *MELb* gene by 23 and 24 nucleotide substitutions, respectively, were the most divergent. In other *S. bayanus* var. *uvarum* strains, the level of similarity with the *MEL* genes was 98%.

Amino acid sequences of the relevant proteins were determined from the nucleotide sequences of *MEL* genes in *S. bayanus* var. *uvarum* strains. They were found to be identical by 98–100%. Their similarity to the MELb protein of *S. bayanus* var. *bayanus*, and MELpt and MELx of *S. pastorianus*/*S. carlsbergensis* was 99, 94, and 93%, respectively. Altogether, five types of α-galactosidase amino acid sequences were revealed in *S. bayanus* var. *uvarum* and designated MELu1 (strain NCYC 686), MELu2 (VKM Y-1146, MCYC 623, and CBS 395), MELu3 (UWO 99-807.1.1 and UWO 99-808.3), MELu4 (136.01), and MELu5 (148.01).

The *MEL* genes in *S. mikatae*. Fourteen strains of *S. mitakae* isolated from various natural sources in Japan are presently known (Table 1). All these strains are able to ferment melibiose. Mapping of the *MEL* genes in strains *S. mitakae* was performed using molecular karyotyping and Southern hybridization with the *MELj* probe (Fig. 3). In 13 strains a single hybridization band was detected around 1125 kb (Fig. 3b, lanes *2–10* and *12–15*) corresponding to the doublet chromosome VII/XV of the control strain *S. cerevisiae* YNN 295 (Fig. 3a, lane *1*). In the strain NBRC 10999, an additional hybridization band matching by size the chromosome XVI of the control strain *S. cerevisiae* YNN 295 was detected (Fig. 3b, lane *11*). *S. mitakae* strains NBRC 1815 and NBRC 1816 are both known to possess a reciprocal translocation between chromosomes VI and VII, and an additional translocation involving chromosome XVI is characteristic of NBRC 1815 [27]. The results of Southern hybridization of chromosomal DNA of the *S. mitakae* strains are presented in Figs. 3c and 3d. Species-specific molecular markers of *S. cerevisiae, TRP5* of chromosome VII and *ACT1* of chromosome VI, were used as probes. Reciprocal translocation between chromosomes V and VII was detected in all *S. mitakae* strains, while an additional translocation involving chromosome XVI occurred only in the type strain NBRC 1815 (Fig. 3, lane *2*). Comparison of the results of Southern hybridization with the probes *MELj, TRP5*, and *ACT1* suggests that the *MEL* gene in *S. mitakae* is located in chromosome XV. Apparently, the second *MEL* gene of strain NBRC 10999 is localized in chromosome XVI.

Sequencing of the *MEL* gene was performed in strains NBRC 10993, NBRC 11002, and NBRC 10999. The sequences were identical and differed from the nucleotide sequence of *MELj* of the type strain NBRC 1815 by two nucleotide substitutions that did

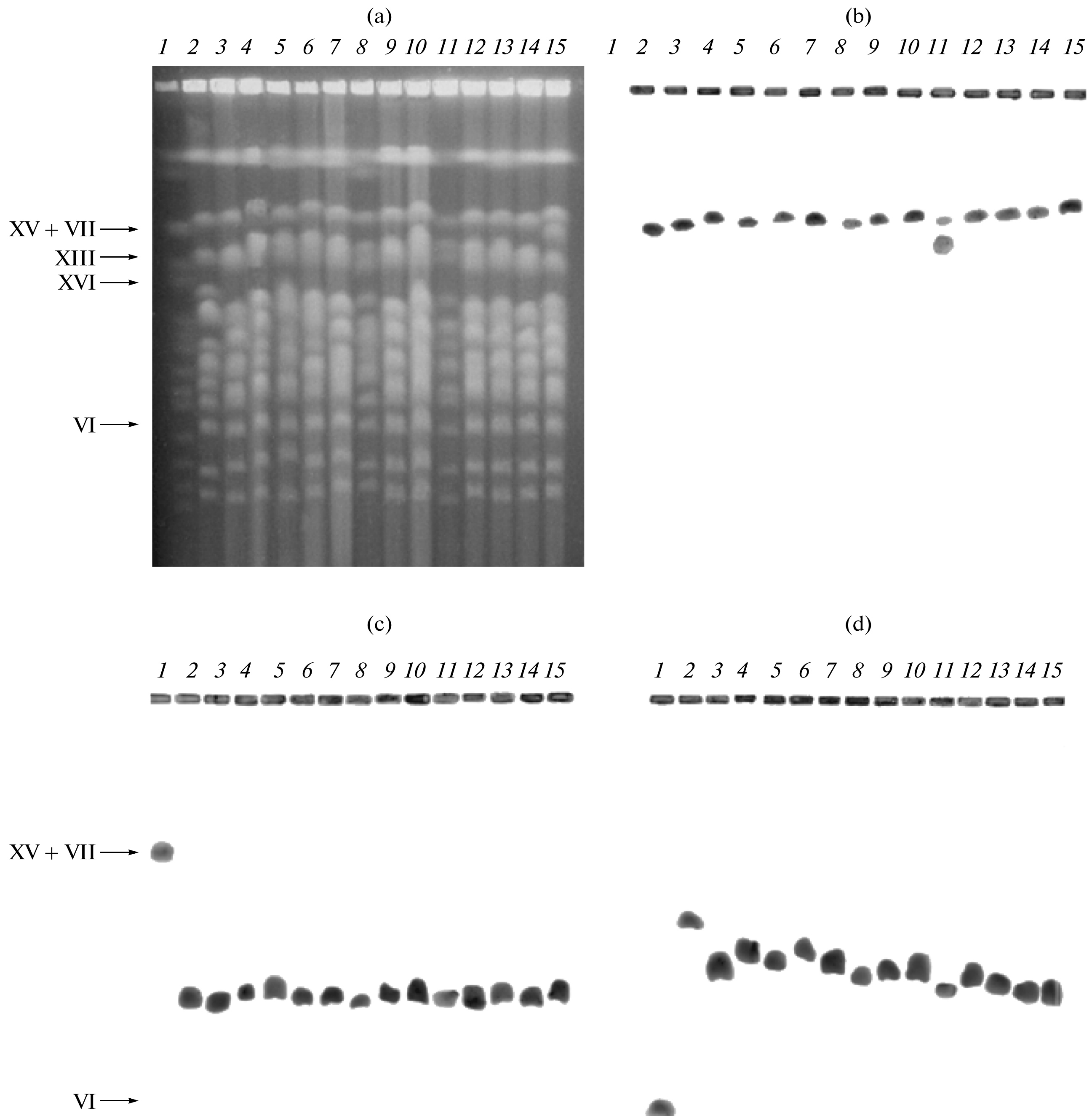

Fig. 3. Pulsed-feld gel electrophoresis of chromosomal DNA of *S. mikatae* strains (a) and Southern hybridization with the probes *MELj* (b) *TRP5* (c), and *ACT1* (d). Lanes: YNN 295 (chromosomal standard) (*1*), NBRC 1815 (T) (*2*), NBRC 1816 (*3*), NBRC 10992 (*4*), NBRC 10993 (*5*), NBRC 10994 (*6*), NBRC 10995 (*7*), NBRC 10996 (*8*), NBRC 10997 (*9*), NBRC 10998 (*10*), NBRC 10999 (*11*), NBRC 11000 (*12*), NBRC 11001 (*13*), NBRC 11002 (*14*), and NBRC 11003 (*15*). The order of chromosomes follows that of the strain *S. cerevisiae* YNN 295 (lane *1*).

not influence the amino acid sequences of the corresponding proteins.

The *MEL* genes in *S. paradoxus*. *S. paradoxus* is a cosmopolitan species represented by several partially genetically isolated populations (European, Far Eastern, and North American) [28–30]. Typically, strains of the species are not able to ferment melibiose and do not contain even a silent copy of the α-galactosidase gene *MEL*. Only three Mel^+ strains were revealed among over 100 isolates of *S. paradoxus* of various origin [17]. All three strains belong to the North American population.

Molecular genetics study of the α-galactosidase genes of the Mel^+ *S. paradoxus* strains was performed.

Table 2. Genetic identification of the *MEL* genes of the strains *S. paradoxus* UCD 61-359 and UCD 61-248

Hybrid origin	Number of tetrades isolated	Ascospore viability, %	Meiotic segregation		Genotype
			$Mel^+ : Mel^-$	of the control markers	
61-248 × 95-1 *ade*	93	42	77 : 81	80 ADE : 78 ade	*MELp1 ADE/mel ade*
61-359 *lys* × 95-1 *ade*	48	87	83 : 84	84 LYS : 83 lys 81 ADE : 86 ade	*MELp2 lys ADE/mel LYS ade*
61-248 × 61-359 *lys*	92	30	80 : 30	54 LYS : 56 lys	*MELp1 LYS/MELp2 lys*

Molecular karyotyping and Southern hybridization with the *MEL1* probe demonstrated that all three strains possess a single copy of the *MEL* gene of different localization (Fig. 1b, lanes *7*–*9*). In strains UWO 80-13 and UCD 61-359, the hybridization signal was detected in the chromosome band corresponding to the chromosome VI of the standard strain *S. cerevisiae* YNN 295 (Fig. 1, lanes *7* and *8*). The earlier sequenced gene *MELp* of strain UCD 61-248 [19] is located in chromosome X (Fig. 1, lane *9*).

Strain UWO 80-13 did not produce spores and therefore was not suitable for genetic analysis. Hybridization analysis of strains UCD 61-359 and UCD 61-248 was performed (Table 2). Strain 95-1 of North American origin not fermenting melibiose was used as the control. Auxotrophic mutants of strains UCD 61-359, UCD 61-248, and 95-1 were characterized by high ascospore viability of 89–91%. Interstrain hybrids UCD 61-359 × 95-1 and UCD 61-248 × 95-1 had normal segregation of the auxotrophic markers; the ascospore viability was 30–87% (Table 2). Monogenic segLregation by melibiose fermentation confirmed that strains UCD 61-359 and UCD 61-248 each have a single copy of the *MEL* gene.

To identify the *MEL* gene in *S. paradoxus* UCD 61-359, its hybridization with strain UCD 61-248 possessing the *MELp* gene was performed. In the hybrid, segregation on a melibiose fermentation feature was observed (Table 2). Therefore, the recombination test for allelism confirmed that strains UCD 61-248 and UCD 610359 possess different α-galactosidase genes, namely, *MELp1* and *MELp2.*

MEL genes in strains UWO 80-13 and UCD 61-359 were amplified using the primers DM-1 and DM-2. The nucleotide sequences of these genes were identical and different from the *MELp1* gene of strain UCD 61-248 by 16 nucleotide substitutions (98.7% similarity).

Comparative analysis of α-galactosidases in the *Saccharomyces* yeasts. On the basis of nucleotide sequences of *MEL* genes in the studied strains of *S. bayanus, S. mikatae*, and *S. paradoxus*, amino acid sequences of the relevant proteins comprising 414 residues were determined. The sequences were then compared with those of *Saccharomyces* α-galactosidases deposited in GenBank. The aligned amino acid sequences of the studied protein fragment different *Saccharomyces* yeasts exhibited 82.0–99.0% identity.

Phylogenetic analysis of the aligned amino acid sequences of the *Saccharomyces* α-galactosidases is presented in Fig. 4. The MELz α-galactosidase of *Lachancea cidri* was used as an outgroup. Three clusters were identified with 100% bootstrap support in the phylogenetic tree upon their analysis relative to the outgroup. The first one comprises the MEL proteins of *S. cerevisiae* with amino acid sequences homologous by 95–100%. The second one contains the MELp1 and MELp2 α-galactosidases of *S. paradoxus* with 99% identity.

The third cluster comprises the α-galactosidases of *S. bayanus* and *S. pastorianus*/*S. carlsbergensis* with a similarity level of 94.9–100%. In this cluster, two groups comprising amino acid sequences of *S. bayanus* var. *bayanus/S. bayanus* var. *uvarum* and *S. pastorianus*, respectively may be distinguished. The level of protein homology within each of the two groups is 99–100%. The MELj α-Galactosidase of *S. mikatae* is close to the latter cluster. Therefore, molecular analysis evidences the species specificity of the *MEL* genes in the species *S. cerevisiae, S. paradoxus, S. bayanus*, and *S. mikatae.*

Comparative analysis of the sequences of internal transcribed spacers ITS1 and ITS2 in *Saccharomyces.* Sequencing of the 5.8S-ITS fragment was performed for eight *S. paradoxus* strains (UWO 80-13, UCD 61-248, UCD 61-359, 95-1, UCD 52-153, CBS 8436, CBS 8437, and CBS 8438) and five *S. bayanus* var. *uvarum* strains (136.01, 148.01, VKM Y-1146, UWO 99-807.1.1, and UWO 99-808.3). The nucleotide sequences were compared with each other and with other ITS sequences of *Saccharomyces* yeasts deposited in GenBank. Importantly, GenBank contains the ITS sequences of *S. cerevisiae* strains of various origin including those of the Mel^+ phenotype. Melibiose-fer-

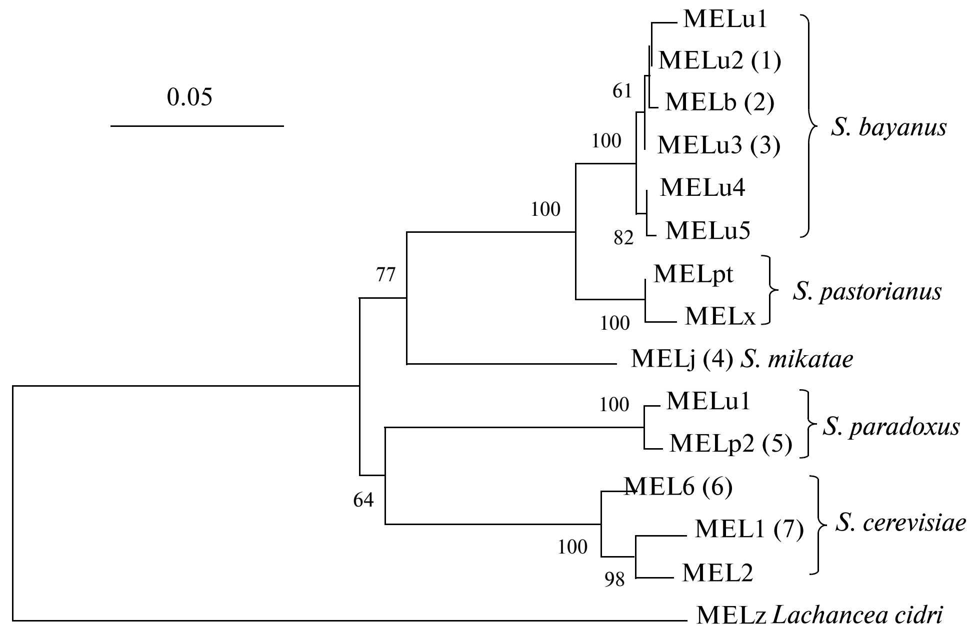

Fig. 4. Phylogenetic analysis of α-galactosidase amino acid sequences of the *Saccharomyces* yeasts. The α-galactosidase of *Lachancea cidri* was used as an outgroup. The bootstrap values >50% are presented. The scale bar corresponds to 50 amino acid substitutions per 1000 residues. The numerals in parentheses indicate groups of strains with identical amino acid sequences: (1) VKM Y-1146, MCYC 623, and CBS 395; (2) CBS 380, CBS 425, and CBS 1505; (3) UWO 99-807.1.1 and UWO 99-808.3; (4) NBRC 10993, NBRC 11002, NBRC 10997, NBRC 1815, and NBRC 1816; (5) UWO(PS) 80-13 and UCD 61-359; (6) MEL4, MEL5, MEL7, and MEL9; and (7) UWO 03-433.3, UWO 03-459.1, and C.B.11.

menting strains discovered earlier among the Malaysian population of *S. cerevisiae* (UWO 03-429.1, UWO 03-433.3, UWO 03-459.1, and UWO 03-461.4) were also included in the analysis [31].

Two major clusters may be identified in the phylogenetic tree constructed on the basis of the nucleotide sequences of the ITS1 region (Fig. 5). The first one comprises *S. cerevisiae, S. paradoxus,* and *S. cariocanus* strains. Three types of ITS1 region sequences were revealed for *S. cerevisiae*. The first type is characteristic of the type strain CBS 1171 and strains S288c and CBS 7961. Strains UWO 03-429.1, UWO 03-433.3, UWO 03-459.1, UWO 03-461.4, CBS 459, CBS 5378, and CBS 382 formed the second group. In these strains, a nucleotide substitution T–C was revealed in position 301 according to the numbering of the ITS1 sequence in the type strain CBS 1171. Nucleotide substitution G–T at position 120 of the ITS1 region is unique for strain CBS 7962. This grouping into three types is not associated with the geographical origin of the strains or their ability to ferment melibiose (Table 1). For example, the Mel$^+$ strains CBS 7961 and CBS 7962, both isolated from sugarcane in Brazil, belong to different groups.

In contrast to *S. cerevisiae*, division of *S. paradoxus* into three groups correlated with their geographical origin (Fig. 5 and Table 1). Independently of the ability to ferment melibiose, North American strains had identical ITS1 region sequences. *S. cariocanus* strains did not differ from *S. paradoxus* in these sequences.

S. mikatae and *S. kudryavzevii*, with *S. arboricola* in between, adhere to the first cluster. Recently, intraspecific polymorphism was revealed in *S. mikatae* in the nucleotide sequences of the ITS1 region [30]. Most strains were demonstrated to be identical to the type strain NBRC 1815. Four strains (NBRC 10992, NBRC 10993, NBRC 10994, and NBRC 10999) are characterized by two T–C transitions at positions 88 and 190 according to the numeration of the ITS1 sequence in the type strain NBRC 1815, and a G–T transversion at position 165 is characteristic of the NBRC 10999 strain.

The second cluster is formed by the most divergent species of the genus *Saccharomyces*, namely *S. bayanus* together with the hybrid taxon *S. pastorianus*. Within the cluster, two groups may be identified. The first one is formed by *S. bayanus* var. *uvarum* strains, which, despite the differences in their geographical origin and *MEL* genes, possess identical ITS1 sequences. The second one is formed by the strains of *S. bayanus* var. *bayanus* and *S. pastorianus*. The ITS1 sequences of *S. bayanus* var. *uvarum* and *S. bayanus* var. *bayanus* differ by two transitions, A–G at position 235 and C–T at position 252.

We compared the results of the phylogenetic analysis of the *Saccharomyces* α-galactosidases and nucle-

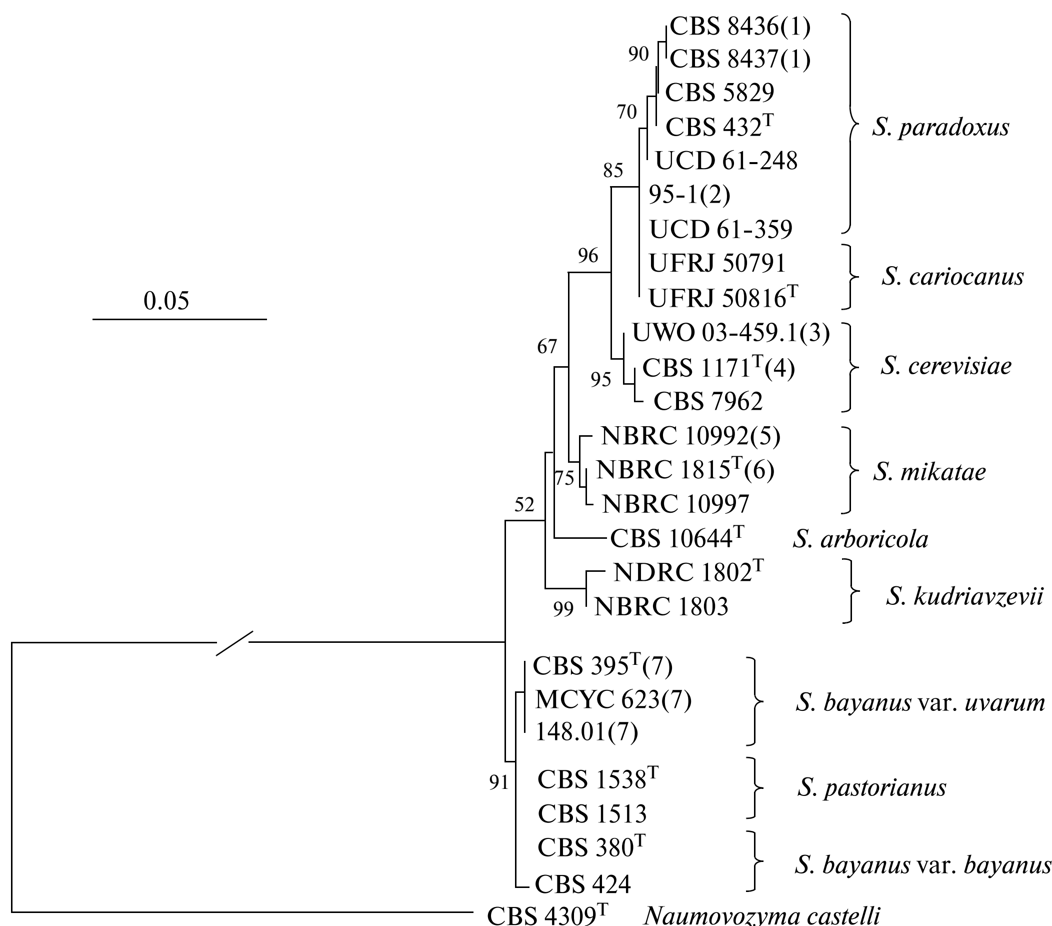

Fig. 5. Phylogenetic analysis of the ITS1 region sequences of the *Saccharomyces* yeasts. The ITS1 sequence of the yeast *Naumovozyma castelii* was used as an outgroup. The bootstrap values >50% are presented. The scale bar corresponds to 50 substitutions per 1000 nucleotides. The numerals in parentheses indicate the groups of strains with identical nucleotide sequences: (1) CBS 8436, CBS 8437, and CBS 8438; (2) 95-1, UCD 52-153, UCD 61-359, and UWO 80-13; (3) UWO 03-429.1, UWO 03-433.3, UWO 03-459.1, UWO 03.461.4, CBS 459, CBS 5378, and CBS 382; (4) CBS 1171, S288c, and CBS 7961; (5) NBRC 10992, NBRC 10993, NBRC 10994, and NBRC 10999; (6) NBRC 1815, NBRC 1816, NBRC 10995, NBRC 10996, NBRC 10998, NBRC 11000, NBRC 11001, NBRC 11002, and NBRC 11003; (7) 148.01, 136.01, VKM Y-1146, UWO 99-807.1.1, UWO 99-808.3, MCYC 686, MCYC 623, and CBS 395.

otide sequences of the internal transcribed spacer ITS1 (Figs. 4 and 5). In general, the topologies of the phylogenetic trees coincide. According to the results of phylogenetic analysis of the ITS1 sequences and α-galactosidase amino acid sequences, the species *S. bayanus* is the most divergent one within the genus *Saccharomyces*. At the same time, the phylogeny of α-galactosidase does not entirely match the phylogeny of the ribosomal sequences. On the phylogenetic tree designed on the basis of the ITS1 region nucleotide sequences, *S. paradoxus* is the species most closely related to *S. cerevisiae* with 96% bootstrap support (Fig. 5). Other molecular data also indicate their close genetic relationship [3, 7, 32]. However, on the phylogenetic tree designed on the basis of α-galactosidase sequences, *S. paradoxus* is a separate entity (Fig. 4). Apparently, this phenomenon is in associated with the increased mutation rate of the α-galactosidase genes due to their localization in the telomeric regions of the chromosomes known as hot points of intra- and interchromosomal recombination events. The fact of the great variety of α-galactosidase sequences in *S. bayanus* var. *uvarum* strains with identical ITS1 sequences supports the idea. The data obtained indicate that the yeasts *S. mikatae, S. bayanus*, and *S. paradoxus* each have a single copy of the *MEL* gene and do not accumulate them, unlike some populations of *S. cerevisiae.* Comparative phylogenetic analysis indicates that *MEL* genes in *Saccharomyces* yeasts are species-specific.

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