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EXPERIMENTAL ARTICLES

Heterogeneity of the Yeasts *Zygowilliopsis californica*: *Z. californica* var. *dimennae* comb. nov., stat. nov. and *Z. californica* var. *fukushimae* comb. nov., stat. nov.

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Abstract—A significant heterogeneity of the species *Zygowilliopsis californica* was revealed using RFLP-analysis of the PCR-amplified rDNA fragment spanning the 5.8S rRNA gene and the internal transcribed spacers ITS1 and ITS2. Phylogenetic analysis of the nucleotide sequences of ITS1 and ITS2 rDNA differentiated three varieties: *Z. californica* var. *californica, Z. californica* var. *dimennae*, and *Z. californica* var. *fukushimae.* The most variable was the ITS2 region, where 7–26 nucleotide substitutions were revealed. The varieties formed semisterile hybrids with meiotic segregation of control markers. The limits of the phylogenetic species concept are discussed.

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Key words: Zygowilliopsis californica, ITS1 and ITS2 rDNA, phylogenetic analysis.

The genus *Zygowilliopsis* Kudriavzev is monotypic: it includes the species *Z. californica* and three synonymous taxa: *Hansenula dimennae, Endomycopsis fukushimae*, and *Pichia saturnospora* [1, 2]. *Zygowilliopsis* yeasts have been isolated from soils of various regions of the world [2, 3]. These yeasts are known to be associated with various invertebrates and cultivated plants [3–6].

Genetic and molecular data indicate the possible heterogeneity of the genus *Zygowilliopsis* [1, 7–9]. It has been shown that, within one biological species *Zygowilliopsis californica*, the synonymous taxa *Z. californica* and *H. dimennae* are partially isolated genetically: their hybrids are semisterile, with a low ascospore survival rate (17–21%), whereas the viability of the ascospores of parent strains and intrastrain hybrids is 57–83% [7].

To determine the phylogenetic relationships of yeasts, the analysis of ribosomal RNA gene sequences is widely used. Kurtzman and Robnett [10] carried out a comparative analysis of nucleotide sequences of the D1/D2 domain of 26S rDNA in the type cultures of all currently known ascomycetous yeasts and showed that the strains of one species usually have identical sequences of this rDNA region or, in rare cases, differ in 1–3 nucleotide positions. It should be noted that identical 26S rDNA D1/D2 sequences do not always mean strain conspecificity. The analysis of the rDNA fragment spanning the 5.8S rDNA gene and the ITS1 and ITS2 internal transcribed spacers (the 5.8S–ITS fragment) is more suitable for determining the degree of relatedness of close species or varieties. This variable rDNA region is characterized by considerable interspecies divergence and a low level of intraspecies polymorphism [11, 12].

The aim of this work was to conduct a molecular study of 26 strains of *Z. californica* yeasts of different geographical origin. RFLP analysis and sequencing of the 5.8S–ITS rDNA fragment revealed a significant genetic polymorphism of *Z. californica* yeasts, which corresponded to the variety level.

MATERIALS AND METHODS

The strains used in this work and their origins are shown in the table. The yeasts were cultivated at 28°C on complete YPD medium of the following composition (g/l): glucose, 20; peptone, 10; yeast extract, 10; agar, 20. Sporulation was induced in a starvation medium containing 30 g/l of maltose and 20 g/l agar at 28°C.

PCR analysis. The DNA was isolated as described earlier [13]. The polymerase chain reaction was performed using a Tertsik DNA amplifier (DNK-Tekhnologiya, Russia). D1/D2 26S rDNA was amplified with the primers NL-1 (5'-GCATATCAATAAGCGGAGGAAAG-3') and NL-4 (5'-GGTCCGTGTTTCAAGACGG-3') [14]. The PCR regime included initial denaturation at 94°C for 1 min, followed by 36 cycles each including DNA denaturation at 94°C for 1 min,

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Origin of *Z. Zygowilliopsis californica* strains used in this study

Strain	Substrate and isolation place	Variety	Registration no. in GenBank	
			D1/D2	ITS
CBS 252 (T)	Leaves, USA	*californica*	U75957	DQ137886
CBS 5760	Soil, South Australia	*californica*		
IFO 1764	Soil, Japan	*californica*		
IFO 1766	Soil, Japan	*californica*		
NRRL Y-1681	Soil, Sweden	*californica*		
NRRL YB-2998	Beech frass, Sweden	*californica*		
NRRL Y-5861	Soil, New Zealand	*californica*		
NRRL Y-1999	Soil, California, USA	*californica*		
NRRL YB-3178	Elm frass, Washington USA	*californica*		
VKM Y-167	Soil	*californica*		
VKM Y-168	Soil, Portugal	*californica*		
VKM Y-1918	Kola Peninsula, Russia	*californica*		
VKM Y-839	Self-fermented blueberry juice, Kola Peninsula, Russia	*californica*	DQ137884	DQ137887
KBP 398	Beet rhizosphere, Moscow oblast, Russia	*californica*		
KBP 440	Cabbage rhizosphere, Moscow oblast, Russia	*californica*		
KBP 3708	*Pachyiulus flavipes* intestine, Gurzuf, Crimea	*californica*		
KBP 3712	Southern chernozems, Russia	*californica*		
CBS 5762	Soil, India	*dimennae*	DQ137882	DQ137889
IFO 1765	Soil, Japan	*dimennae*	DQ137885	DQ137890
NRRL YB-2757	Fir frass, Japan	*dimennae*		
NRRL YB-4713	Soil under oats, Canada	*dimennae*		
NRRL YB-1863	Soil, Michigan, USA	*dimennae*		
NRRL YB-1873	Soil, Michigan, USA	*dimennae*		
NRRL Y-1709	Soil, Minnesota, USA	*dimennae*		
NRRL YB-4269	Wet soil, New Zealand	*dimennae*		
CBS 5782	Brown bear dung, Japan	*fukushimae*	DQ137883	DQ137888

Note: Abbreviations: CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; IFO, Institute for Fermentation, Osaka, Japan; NRRL, Agricultural Research Service Culture Collection, National Center for Agricultural Utilization Research, Peoria, Illinois, USA; T, type culture.

annealing of the primers at 52°C for 1 min, and DNA synthesis at 72°C for 1 min; final elongation was allowed to proceed at 72°C for 1 min. The 5.8S–ITS rDNA fragments were amplified using the primers pITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and pITS4 (5'-CCTCCGCTTATTGATATGC-3') [15]. The PCR was performed as described earlier [16]. The amplification products were subjected to electrophoresis in 1% agarose gel at 60–65 V in 0.5× TBE (45 mM Tris–HCl buffer, 10 mM EDTA, 45 mM boric acid) for 2 h and stained with ethidium bromide. Restriction fragment length polymorphism (RFLP) analysis of PCR-amplified 5.8S–ITS rDNA fragments was carried out using the *Hae*III, *Hinf*I, *Mbo*I, *Msp*I, and *Alu*I restriction endonucleases (Fermentas, Lithuania). The restriction fragments were separated in 1.6% agarose gel at 60–65 V in 0.5× TBE for 3 h. The gel was stained with ethidium bromide and photographed in ultraviolet light using a Vilber Lourmat transilluminator (France).

Sequencing. The PCR products were eluted from the gel using a GeneClean Kit according to the instructions of the manufacturer (Bio 101 Inc., USA) and subjected to direct sequencing. The 26S rDNA D1/D2 domains and 5.8S–ITS fragments were sequenced in both directions by Sanger's method on an ABI 373 A automatic sequencer (Applied Biosystems). The determined nucleotide sequences were deposited in the GenBank (table).

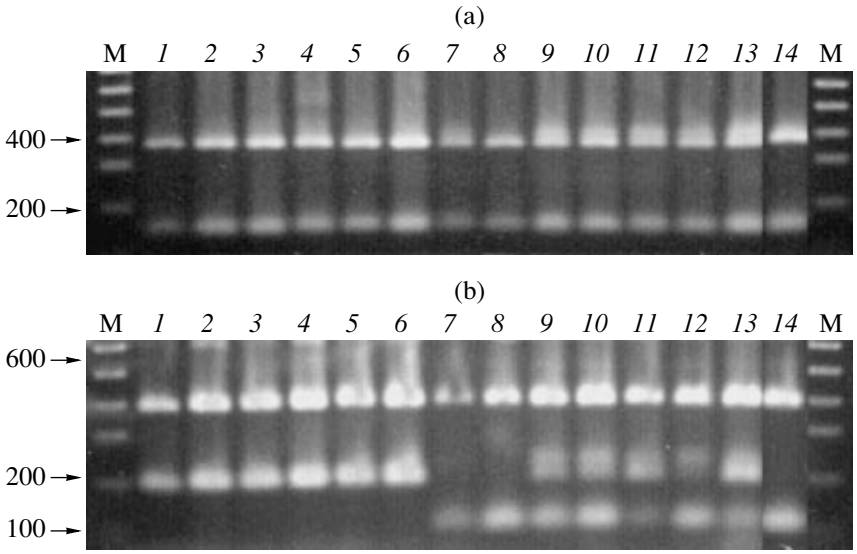

Fig. 1. Restriction analysis of amplified 5.8S–ITS rDNA fragments of the yeast *Z. californica* performed with the use of endonucleases (a) *Mbo*I and (b) *Alu*I. Lanes: *1*, CBS 252; *2*, NRRL Y-1681; *3*, NRRL YB-2998; *4*, NRRL Y-5861; *5*, NRRL Y-1999; *6*, NRRL YB-3178; *7*, IFO 1765; *8*, NRRL YB-2757; *9*, NRRL YB-4713; *10*, NRRL YB-1873; *11*, NRRL YB-1863; *12*, NRRL Y-1709; *13*, NRRL YB-4269; *14*, CBS 5762. M, the 100 bp DNA Ladder (Fermentas, Lithuania) molecular weight marker (bp).

Phylogenetic analysis. The search for homology with known nucleotide sequences was carried out using the BLAST program. Multiple alignment of the newly determined and already known nucleotide sequences was performed manually using the BioEdit software package. The phylogenetic tree was constructed using the neighbor-joining method implemented in the TREECON software package [17]. The bootstrap values, showing the statistical significance of group outlining, were determined for 100 replicates.

RESULTS AND DISCUSSION

Preliminary generic-level identification of the strains studied was performed based on the morphology of colonies, vegetative cells, zygotes, and ascospores. All the strains had Saturn-like spores and were characterized by the haploid developmental cycle (ascospore formation from zygotes), which was indicative of their affiliation with the genus *Zygowilliopsis*.

Restriction analysis of amplified 5.8S–ITS fragments. Further strain identification was performed by means of RFLP-analysis of PCR-amplified 5.8S–ITS fragments. The size of the PCR products was the same (about 600 bp) in the 26 strains studied, including the type cultures *Z. californica* CBS 252, *E. fukushimae* CBS 5782, and *H. dimennae* CBS 5762. The PCR products were analyzed using five enzymes. In all of the strains, the endonucleases *Hae*III, *Hpa*II, and *Msp*I failed to cut the amplified rDNA fragment, which is characteristic of *Zygowilliopsis* yeasts [16]. After *Mbo*I restriction, all the strains yielded two fragments measuring about 400 and 200 bp (Fig. 1a). After *Alu*I restriction, two restriction patterns were observed (Fig. 1b). The first group included the type culture *Z. californica* CBS 252 and 17 more strains (CBS 5760, CBS 5782, IFO 1764, IFO 1766, NRRL Y-1681, NRRL YB-2998, NRRL Y-5861, NRRL Y-1999, NRRL YB-3178, VKM Y-167, VKM Y-168, VKM Y-1918, VKM Y-839, KBP 398, KBP 440, KBP 3708, and KBP 3712); their restriction patterns exhibited two fragments measuring approximately 400 and 200 bp (Fig. 1b, lanes *1–6*). The type culture *H. dimennae* CBS 5762 and seven more strains were characterized by profiles with three fragments measuring approximately 400, 120, and 80 bp (Fig. 1b, lanes *7–14*). In some of the strains of this group, the 120-bp fragment was not always clearly seen in the gel.

In order to determine the genetic relatedness of the strains with different restriction profiles, we sequenced two variable rDNA regions.

Analysis of the nucleotide sequences of the 26S rDNA D1/D2 domain and of the ITS1 and ITS2 internal transcribed spacers. The *dimennae*-group strains of different geographic origin (CBS 5762, NRRL Y-1709, NRRL YB-2757, NRRL YB-4269, and IFO 1765), the type cultures *Z. californica* CBS 252 and *E. fukushimae* CBS 5782, and strain VKM Y-839 were subjected to sequencing. It was shown earlier that the last strain, assigned by us to the first group according to the restriction profile, significantly differed from the type culture CBS 252 in the PCR profile with the universal primers L45 and no. 21 [9].

The comparison of the 26S rDNA D1/D2 nucleotide sequences revealed their high similarity, which is indicative of the close relatedness of the strains studied. The greatest differences from the type culture *Z. californica* CBS 252 were noted in the type cultures *H. dimennae*

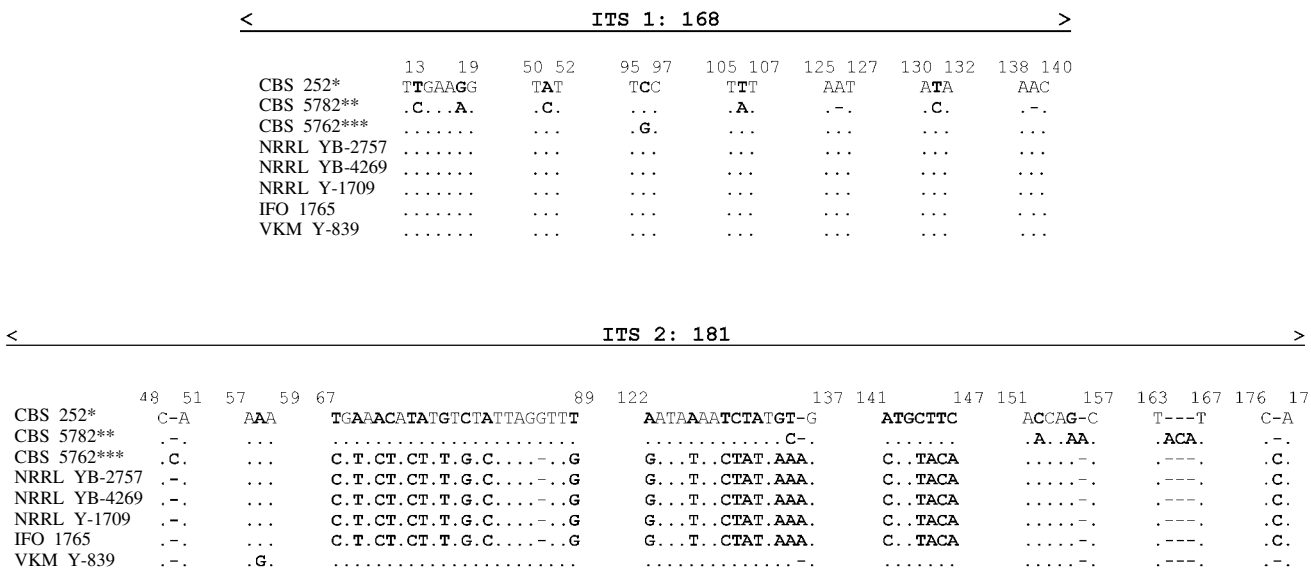

Fig. 2. Multiple alignment of the nucleotide sequences of the ITS1 and ITS2 rDNA of the *Z. californica* yeasts. The asterisks mark the type culture *Z. californica* var. *californica* (*), *Z. californica* var. *fukushimae* (**), and *Z. californica* var. *dimennae* (***).

CBS 5762 and *E. fukushimae* CBS 5782. The former strain is characterized by the presence of a six-nucleotide deletion; in the latter, a four-nucleotide deletion and two nucleotide substitutions were revealed as compared to the type culture *Z. californica* CBS 252. Strains IFO 1765 and VKM Y-839 had one nucleotide substitution each. Strains NRRL Y-1709, NRRL YB-2757, and NRRL YB-4269 have a 26S rDNA D1/D2 sequence identical to that of the type culture CBS 252.

The length of the ITS region is known to be constant in strains of the same species [12], but its nucleotide sequence may be variable [11]. Comparative analysis of the 5.8S–ITS fragments revealed considerable interstrain differences (Fig. 2). In the ITS1 region, single nucleotide substitutions were noted: one in strain CBS 5762 and seven (including two deletions) in strain CBS 5782. The ITS2 region appeared to be more variable. One nucleotide substitution was revealed in strain VKM Y-839. Strains IFO 1765, NRRL Y-1709, NRRL YB-2757, and NRRL YB-4269 had identical ITS2 sequences that differed from that of the type culture *Z. californica* CBS 252 in 26 nucleotide positions, including one insertion and one deletion. The same ITS2 sequence was revealed in strain *H. dimennae* CBS 5762, except for one additional insertion of a C nucleotide in position 50. Differences in seven nucleotide positions in the ITS2 region were found in the type culture *E. fukushimae* CBS 5782. A total of 14 distinctions were found in the ITS1 and ITS2 regions of this strain. Hybridization experiments performed earlier [18] allowed us to establish only the generic status of this strain. Its ability to hybridize with the *Z. californica* yeasts is indicative of its affiliation with the genus *Zygowilliopsis*. In a number of hybrids, in spite of low fertility, segregation of both parent markers was observed, indicating close relatedness between the strain studied and *Z. californica.* On this basis, a conclusion was reached that this strain is likely to be a synonym of *Z. californica.* DNA–DNA reassociation between strain CBS 5782 and the *Z. californica* type culture was as high as 96% [19].

Thus, the comparative analysis of the 5.8S–ITS rDNA nucleotide sequences gives evidence of the heterogeneity of *Z. californica* yeasts. A separate group is formed by the type culture *H. dimennae* CBS 5762 and seven more strains (IFO 1765, NRRL YB-2757, NRRL YB-4713, NRRL YB-1873, NRRL YB-1863, NRRL Y-1709, and NRRL YB-4269), which exhibit considerable differences from the type culture of *Z. californica* in the nucleotide sequences of the internal transcribed spacers.

Based on the analysis of the 5.8S–ITS rDNA sequences, a phylogenetic tree was constructed (Fig. 3). Three clusters can be identified in the tree at a 100% significance level. The first cluster includes *H. dimennae* strains; the second, the type culture *Z. californica* and strain VKM Y-839. A separate branch is occupied by the type culture *E. fukushimae* CBS 5782. The molecular–biological data and the results of the genetic analysis that we performed [7, 18] give evidence of a complex composition of the species *Z. californica* and allow three varieties to be differentiated: *Zygowilliopsis californica* var. *californica, Zygowilliopsis californica* var. *dimennae*, and *Zygowilliopsis californica* var. *fukushimae.* These varieties form semisterile hybrids with meiotic recombination of control markers [7, 18].

Below are the changes that we propose in the composition of the genus *Zygowilliopsis* Kudriavzev [21].

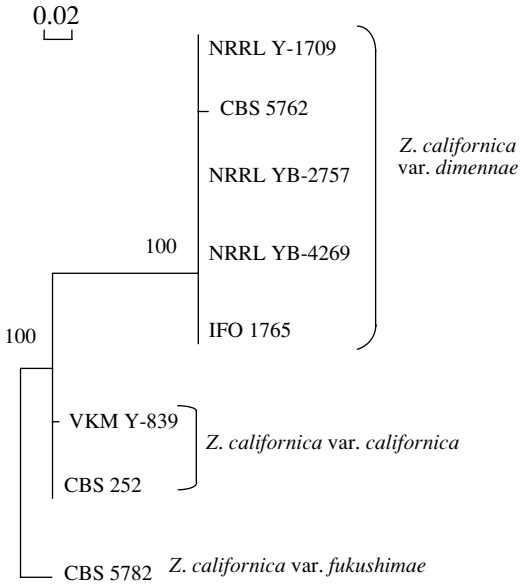

Fig. 3. Phylogenetic tree of the 5.8S–ITS rDNA regions of the *Zygowilliopsis californica* yeasts. The bootstrap values above 70% are shown. The scale bar corresponds to 20 nucleotide substitutions per 1000 nucleotide positions.

Zygowilliopsis californica (Lodder) Kudriavzev var. *dimennae* Naumova, Gazdiev & Naumov G. comb. nov., stat. nov. Basionym: *Hansenula dimennae* Wickerham, Mycopathol. Mycol. Appl. 37, 28, 1969. Type strain: CBS 5762.

Zygowilliopsis californica (Lodder) Kudriavzev var. *fukushimae* Naumova, Gazdiev & Naumov G. comb. nov., stat. nov. Basionym: *Endomycopsis fukushimae* Soneda, Trans. Mycol. Soc. Japan. 3, 36, 1962. Type strain: CBS 5782.

The formal nomenclature announcement of the taxon *Zygowilliopsis californica* (Lodder) Kudriavzev var. *californica* (Lodder, 1932) is as follows. Type strain: CBS 252. Synonyms: *Zygohansenula californica* Lodder (1932), *Hansenula californica* (Lodder) Wickerham (1951), *Hansenula californica* (Lodder) Wickerham var. *maltosa* Capriotti (1959) nom. nud., *Pichia saturnospora* Soneda (1962).

The above-mentioned varieties can be differentiated by their ITS1 and ITS2 rDNA sequences, and *Z. californica* var. *dimennae* can also be differentiated by RFLP-analysis of 5.8S–ITS fragments using the endonuclease *Alu*I. Like *Z. californica* var. *californica, Z. californica* var. *dimennae* occurs in many regions of the world. It has been found in India, Japan, North America, and New Zealand.

Analysis of the data that we obtained and data available in the literature showed the limitations of the phylogenetic species concept. *Z. californica* yeasts exemplify a contradiction between different molecular approaches to the species identification of yeasts. The phylogenetic species concept, based on comparing the nucleotide sequences of ribosomal genes, may contradict the results of determination of species relatedness on the basis of DNA–DNA reassociation. Despite 96–100% DNA–DNA reassociation [19], the three varieties of *Z. californica* significantly differ in the ITS1 and ITS2 rDNA sequences (14–26 nucleotide substitutions), whereas in partially genetically isolated varieties of the yeast *Kluyveromyces lactis*, having a lower DNA–DNA reassociation level (64–78%), the distinctions in the internal transcribed ITS1 and ITS2 sequences constitute only 1 to 3 nucleotides [20]. On the other hand, in the sibling species of *Williopsis* sensu stricto, whose genomes exhibit considerable divergence according to the data of DNA–DNA reassociation (36–78%) and which form sterile interspecies hybrids, the distinctions in the ITS1 and ITS2 sequences do not exceed seven nucleotide positions [16].

From the genetic point of view, even 100% DNA reassociation cannot guarantee strain conspecificity. The yeast *Z. californica* exemplifies the high resolving capacity of genetic analysis, which enables a researcher to identify partially isolated populations, as well as the necessity of simultaneous application of different molecular methods for yeast identification and classification.

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