Microbiology, Vol. 70, No. 5, 2001, pp. 576–582. Translated from Mikrobiologiya, Vol. 70, No. 5, 2001, pp. 668–674.

EXPERIMENTAL ARTICLES

Identification of *Zygowilliopsis californica* Strains of Different Origin by Means of Polymerase Chain Reaction with Universal Primers

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Received December 18, 2000

Abstract—After reevaluation of the taxonomic position of 27 yeast collection strains of different origin by UP-PCR followed by dot-hybridization, only 22 strains were assigned to the biological species *Zygowilliopsis californica* (Lodder) Kudriavzev. Four strains were identified as *Williopsis suaveolens* (Klöcker) Naumov *et al*. Universal primers L45 and N21 are recommended for identification of the *Z. californica* yeasts.

Key words: *Zygowilliopsis californica*, *Williopsis suaveolens*, UP-PCR analysis.

The taxonomic position of the *Zygowilliopsis californica* (Lodder) Kudriavzev, haploid yeast characterized by Saturn-shaped ascospores, has often been reevaluated. This yeast was first described in 1932 by J. Lodder as *Zygohansenula californica*, and recognized later as a special genus *Zygowilliopsis* by V.I. Kudrjawzev [1]. Von Arx *et al.* [2] assigned this haploid yeast to the genus *Williopsis* Zender, whereas Kurtzman [3] assigned it to the genus *Hansenula* H. et P. Sydow. However, in the latest yeast guide, the latter author [4] included the species *Zygowilliopsis californica* into the heterogeneous genus *Williopsis* comprising mostly diploid species. In the 1980s, genetic analysis provided the basis for expelling Saturn-spored yeasts (both haploid and diploid) from the genus *Hansenula* and for assigning them to the genera *Zygowilliopsis* and *Williopsis*, respectively [5–7]. As determined by genetic methods, the species *Hansenula dimennae* Wickerham, *Pichia saturnospora* Soneda, and *Endomycopsis fukushimae* Soneda are synonyms of the species *Z. californica*. The genetic classification well agrees with evidence obtained later by molecular methods. Nuclear DNA homology between *Z. californica* and species of the genus *Williopsis* is very low (from 6 to 16%) [8]; the mitochondrial DNAs also proved to differ significantly [9]. Sequencing of the rRNA genes showed that the species *Z. californica* forms a phylogenetic line remote from other species of the genus *Williopsis* [10–12]. In the present paper, we adhere to the generic name *Zygowilliopsis*.

Z. californica representatives are often isolated from soils (and sometimes from other sources) in various world areas: Australia, Belgium, India, Canada, New Zealand, Russia, United States, Ukraine [4, 13, 14]. In this work, various collection strains of *Z. californica* isolated from soil, rhizosphere of cultivated plants, and other sources were studied by the PCR method using nonspecific primers with random nucleotide sequences. This approach has been widely used for yeast identification [15, 16]; however, *Z. californica* has never been studied by this method.

MATERIALS AND METHODS

This study used 27 yeast strains stored as *Z. californica* in the following collections: KBP (Yeast Collection of the Department of Soil Biology, Moscow State University), CBS (Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands), and VKM (All-Russia Collection of Microorganisms, Moscow) (table). These strains were compared with the type cultures of several yeast species: *Williopsis beijerinckii* (van der Walt) Naumov et Vustin CBS 2564; *W. mrakii* (Wickerham) Naumov et Vustin CBS 1707; *W. sargentensis* (Wickerham et Kurtzman) Naumov CBS 6342; *W. saturnus* (Klöcker) Zender CBS 254; *W. suaveolens* (Klöcker) Naumov *et al*. CBS 255; *W. subsufficiens* (Wikherham) Vustin *et al*. CBS 5763; *W. mucosa* (Wickerham et Kurtzman) Kurtzman CBS 6341; *W. salicorniae* Hinzelin *et al.* CBS 8071; *Komagataea pratensis* (Bab'eva et Reshetova) Yamada *et al*. CBS 7079, and *W. suaveolens* KBP 1118.

Yeasts were cultivated at 28°C on complete YPD medium (g/l): glucose, 20; peptone, 10; yeast extract, 10; agar, 20. Physiological testing followed the conventional procedure.

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0026-2617/01/7005-0576$25.00

Yeast strains studied in this work

Strain designation	Source and area of strain isolation	Year of isolation	Author or reference
CBS 252 (T)	Leaves, United States	1931	Muller F.M.
CBS 5760	Soil, Southern Australia	Unknown	Unknown
CBS 5762	Soil, India	1952	Mathur R.N.
CBS 5769, CBS 5782	Brown bear excrement	1959	Soneda M.
VKM Y-167	Soil	Unknown	Kodama K.
VKM Y-839	Self-fermented blueberry juice, Kola Peninsula, Russia	1936	Rautenstein Ya.I.
VKM Y-1917, VKM Y-1918	Berries, Kola Peninsula, Russia	1936	Rautenstein Ya.I.
VKM Y-168	Soil, Portugal	Unknown	Kodama K.
KBP 398	Beet rhizosphere, Moscow oblast	1961	[19]
KBP 399	Carrot rhizosphere, Moscow oblast	1961	[19]
KBP 440, KBP 441	Cabbage rhizosphere, Moscow oblast	1961	[19]
KBP 1426	Oat rhizosphere, Moscow oblast	1961	[20]
KBP 3707, KBP 3708,	Millipede intestine	1992	Vu Nguen Tkhan
KBP 3809, KBP 3710	*Pachyiulus flavipes*, Gurzuf, the Crimea		
KBP 3547, KBP 3548	Millipede excrement	1992	Vu Nguen Tkhan
	Pachyiulus flavipes, Gurzuf, the Crimea		
KBP 3711, KBP 3712	Southern chernozems, Russia	Unknown	Unknown
KBP 1097	Peat bogs, Tver oblast	1961	[21]
KBP 1579, KBP 1580, KBP 1581	Peat bogs, Moscow oblast	1966	Kartintsev A.V.

Note: CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; VKM, All-Russian Collection of Microorganisms; KBP, collection of the Department of Soil Biology, Moscow State University; T, type culture; KBP 1581 = VKM Y-2036.

PCR analysis. DNA was isolated as described previously [16]. Polymerase chain reaction was conducted on a Techne PHC-2 amplifier using universal primers L45 (5'-GTAAAACGACGGCCAGT) and N21 (5'-CGAGAACGACGGTTCT). The reaction mixture (20 μl) contained 2.5 mM $MgCl_2$, 0.4 mM (each) dNTP; 0.2 mM primer; 0.05 units/μl of DNA polymerase (Fermentans, Vilnius); 20 to 200 ng of DNA. The temperature cycling (30 cycles) was as follows: DNA denaturation for 50 s at 94°C, primer annealing for 80 s at 55°C; DNA synthesis for 60 s at 70°C. With the microsatellite primer $(CAC)_5$, the PCR conditions were as described previously [16]. With the M13 primer (5'-GAGGGTGGCGGTTCT), the reaction was carried out in 20 μl of a buffer containing 1.5 mM $MgCl_2$, 0.4 mM (each) dNTP; 0.1 units/μl of DNA polymerase (Super Tag); 0.25 mM primer; 20 to 200 ng of DNA. PCR cycling (40 cycles) was as follows: DNA denaturation for 1 min at 94°C; primer annealing for 2 min at 52°C; DNA synthesis for 3 min at 74°C. Amplification products were analyzed by electrophoresis in 1.2% agarose for 4–5 h (1 TBE buffer, 60 V). After electrophoresis, DNA was stained with ethidium bromide to be visualized and photographed in UV light.

After amplification with the universal primers L45 and N21, the PCR products were analyzed by dot-hybridization to reveal DNA homology. Amplified DNA of the type culture *Z. californica* CBS 252 served as the probe, as well as the DNAs of strains KBP 1581 and VKM Y-839. Amplification products were transferred onto a nitrocellulose membrane (Sigma) and analyzed by dot-hybridization as described previously [16].

Computer treatment of the results. To reveal relatedness between strains, the electrophoretic patterns of amplification products obtained with the L45 primer were compared. A matrix of distinctions was calculated by the formula $D = 1 - (a + b)/n$, where D is the coefficient of distinction, a is the total of fragments present in both strains, b is the total of fragments absent from both strains; and n is the total number of fragments. Fragment intensity was ignored. Dendrograms were constructed using the UPGMA program (version 3.572) from the PHYLIP 3.5 software package [17]. Soil yeasts *Lipomyces starkeyi* Lodder et Kreger-van Rij (strain KBP 3713), which are evolutionarily remote from *Z. californica*, were used for molecular comparison.

RESULTS AND DISCUSSION

Species affiliation for 27 strains was determined by UP-PCR using the L45 primer (Figs. 1a, 1b). All strains were divided into two groups based on the electro-

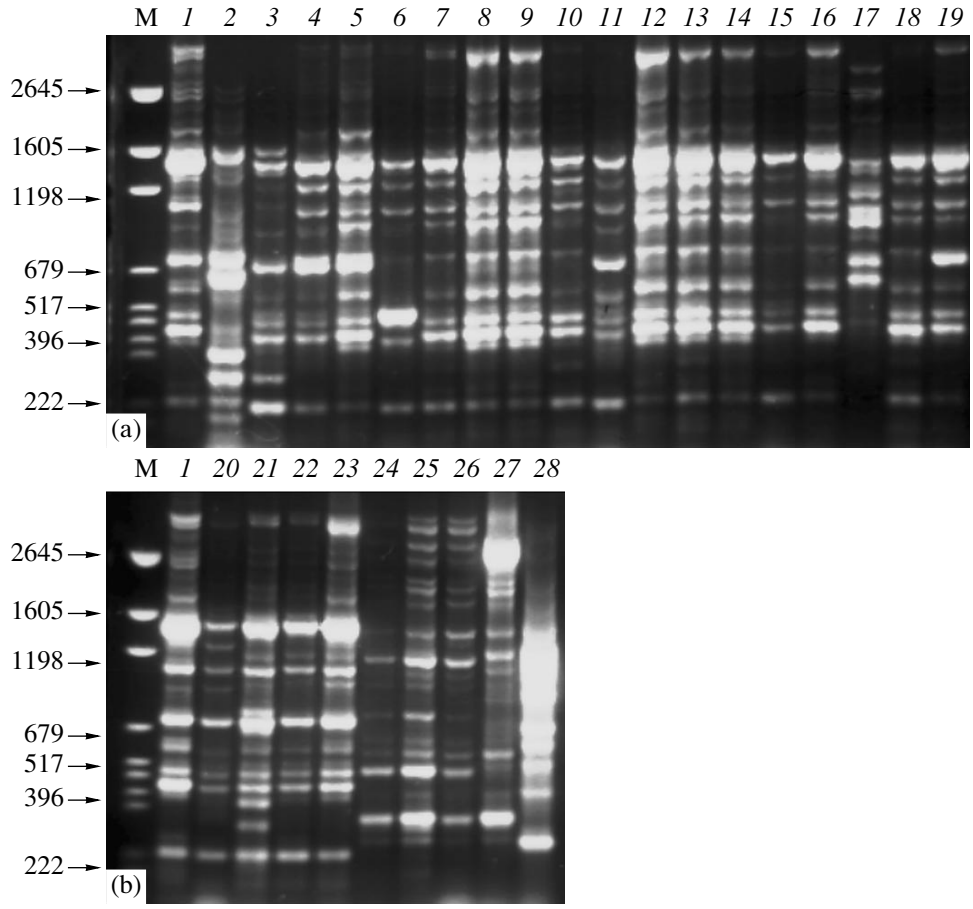

Fig. 1. UP-PCR with the universal primer L45 of the DNAs from *Z. californica* strains. (a) *1*, CBS 252; *2*, CBS 5760; *3*, CBS 5762; *4*, CBS 5769; *5*, CBS 5782; *6*, VKM Y-167; *7*, KBP 3708; *8*, KBP 3707; *9*, KBP 3710; *10*, KBP 3709; *11*, KBP 399; *12*, KBP 440; *13*, KBP 441; *14*, KBP 398; *15*, KBP 1426; *16*, KBP 3548; *18*, KBP 3711; *19*, KBP 3712. Lane *17*, *Lipomyces starkeyi* KBP 3713. M, molecular weight markers (bp). (b) *1*, CBS 252; *20*, KBP 3547; *21*, VKM Y-1918; *22*, VKM Y-1917; *23*, VKM Y-168; *24*, KBP 1579; *25*, KBP 1581; *26*, KBP 1580; *27*, KBP 1097; *28*, VKM Y-839; M, molecular weight markers (bp).

phoretic patterns of the amplification products (PCR patterns). Twenty two strains constituted the first group. They had similar PCR patterns with major fragments of 200, 400, 500, 700, 1000, 1600, and 3000 bp in size (Figs. 1a, 1b, lanes *1–16* and *18–23*). Strains of the first group differed only in the number of minor bands. Strains CBS 5762, CBS 5769, and CBS 5782 (Fig. 1a, lanes *3*, *4*, and *5*) are the *Hansenula dimennae*, *Pichia saturnospora*, and *Endomycopsis fukushimae* type cultures, respectively. Their PCR patterns were quite similar to that of the *Z. californica* type strain. The PCR pattern of strain CBS 5760 (Fig. 1a, lane *2*) was somewhat different, with additional major fragments of about 100, 300, 350, and 676 bp. Note that this strain also differs from *Z. californica* CBS 5762 and NRRL Y-5863 in the electrophoretic patterns of two out of seven enzymes studied, esterase (Est) and catalase (Cat) [18].

Four strains, KBP 1579, KBP 1581, KBP 1580, and KBP 1097, constituted the second group (Fig. 1b, lanes *24–27*). Their PCR patterns were characterized by fragments of about 250, 500, 700, and 1200 bp and six to seven minor fragments from 1300 to 3000 bp. An additional major 2700-bp band was characteristic of strain KBP 1097. All four strains were isolated from peat bogs. The PCR pattern of strain VKM Y-839 differed from those characteristic of either of the aforementioned groups (Fig. 1b, lane *28*). The control strain *L. starkeyi* KBP 3713 displayed a unique PCR pattern quite different from the rest 27 strains studied (Fig. 1, lane *17*).

To determine the degree of genetic relationships of the 27 strains, their PCR products were transferred onto a membrane and hybridized with the amplified DNA of the type culture *Z. californica* CBS 252 (Fig. 2). DNA of all 22 strains assigned to the first group based on their PCR patterns displayed strong hybridization. DNA of five strains, KBP 1579, KBP 1581, KBP 1580, KBP 1097 and VKM Y-839, produced slight hybridization signals (Fig. 2, *24–28*). No hybridization was observed with DNA of strain *L. starkeyi* KBP 3713 (Fig. 2, *17*). Species specificity of the hybridization of UP-PCR products was previously demonstrated for

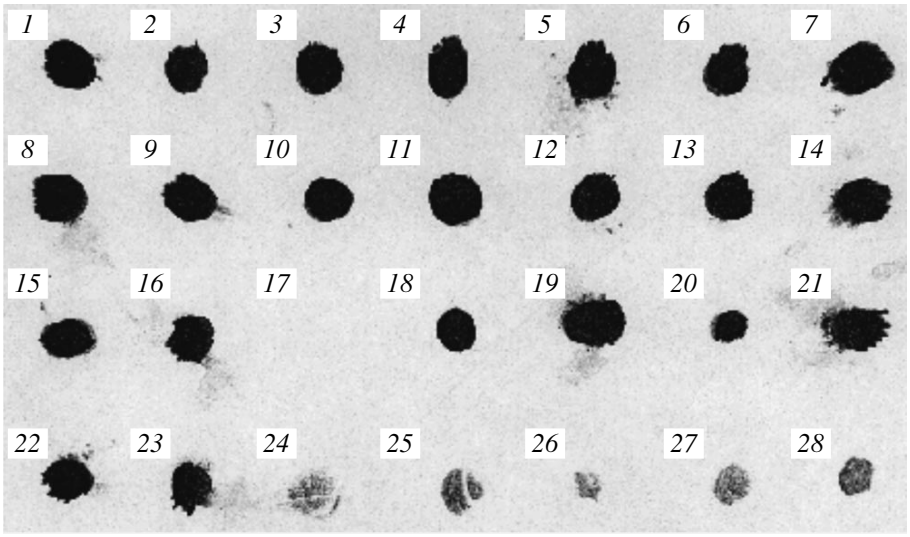

Fig. 2. Dot-hybridization of the DNAs from 27 strains amplified with the universal primer L45. Total amplified DNA of the type strain CBS 252 served as the probe. Dot numbers corresponds to the lane numbers in Fig. 1.

various yeast and fungal species [15]. In this work, yeast strains of the second group, as well as strain VKM Y-839, produced PCR patterns quite different from that characteristic of the species *Z. californica* and showed poor hybridization to the corresponding probe, which indicates that they do not belong to the species *Z. californica*.

From comparison of PCR patterns, a matrix of distinctions was calculated for the strains studied, which served as the basis to construct an unrooted phylogenetic tree (Fig. 3). The evolutionarily distant species *Lipomyces starkeyi* served as an outgroup. The 27 strains studied proved to constitute two clearly distinct clusters. The first one incorporated 22 strains which produced similar PCR patterns and hybridized with the DNA fragment of the type culture *Z. californica* CBS 252. Within this cluster, three subgroups can be differentiated which include strains with similar PCR patterns. Strains CBS 5760, CBS 5762, VKM Y-1918, and KBP 3547, characterized by a PCR-pattern variation from 8.7 to 14.7%, constitute the first subgroup. The second subgroup comprises the type strain CBS 252, as well as strains CBS 5769, KBP 3548, CBS 5782, KBP 3708, VKM Y-168, KBP 398, KBP 441, KBP 3707, KBP 440, KBP 3710, and VKM Y-1917 (PCR-pattern variation from 0 to 5.5%). Strains VKM Y-167, KBP 3709, KBP 399, KBP 1426, KBP 3711, and KBP 3712, characterized by the lowest variation of PCR patterns (from 0 to 3.9%), constitute the third subgroup. The polymorphism of the 22 strains constituting the first cluster was studied using two microsatellite primers $(CAC)_5$ and M13. With both primers, individual strains could be differentiated (data not shown). However, like with the L45 universal primer, no correlation was found between PCR patterns of the strains and their origin (source and area of isolation).

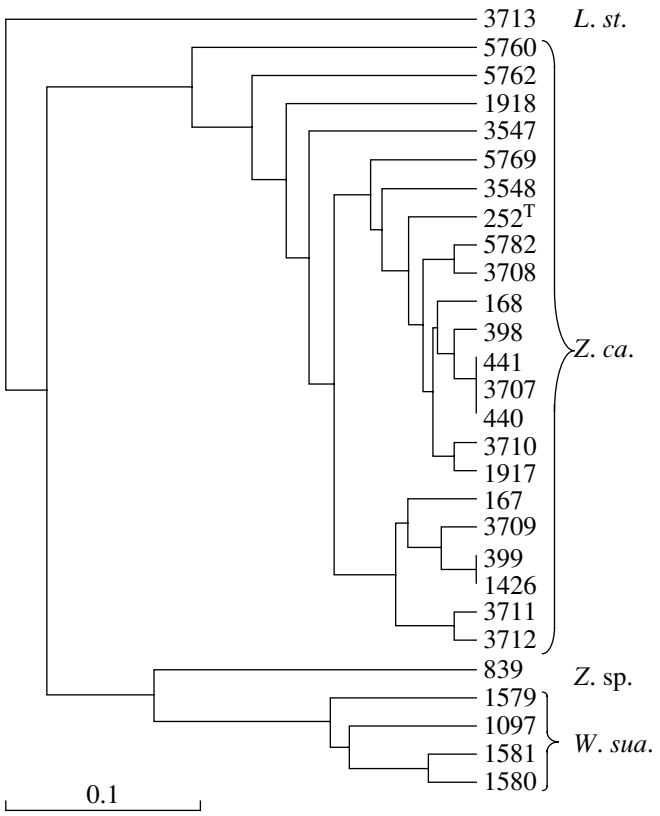

Fig. 3. Dendrogram of *Z. californica* strains based on a matrix of distinctions calculated from the PCR patterns obtained with the universal primer L45. T, type strain; *Z. ca.*, *Zygowilliopsis californica*; *W. sua.*, *Williopsis suaveolens*; *L. st.*, *Lipomyces starkeyi*; *Z.* sp., *Zygowilliopsis* sp. The results were processed using the UPGMA program (version 3.572) from the PHYLIP 3.5 software package.

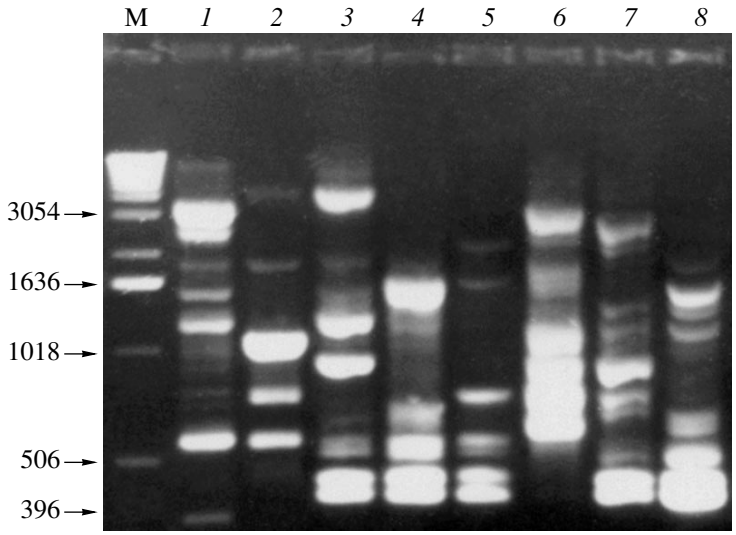

Fig. 4. Comparative UP-PCR with the universal primer N21 of the DNAs from strains VKM Y-839, KBP 1097, KBP 1581, *Z. californica* (CBS 252), *W. saturnus* (CBS 254), and *W. suaveolens* (CBS 255, KBP 1118): *1*, CBS 252; *2*, CBS 254; *3*, CBS 255; *4*, KBP 1118; *5*, KBP 1117; *6*, VKM Y-839; *7*, KBP 1097; *8*, KBP 1581; M, molecular weight markers (bp).

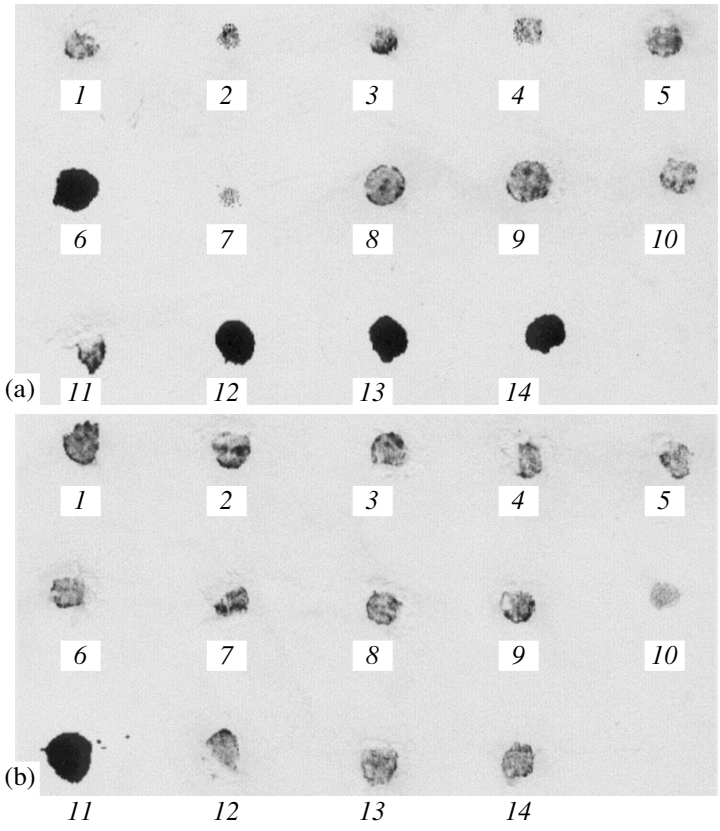

Fig. 5. Dot-hybridization of the DNAs from strains of the second group and from type cultures of ten species of the genera *Williopsis*, *Zygowilliopsis*, and *Komagataea* (the DNAs were amplified using the N21 primer): *1*, *Z. californica* CBS 252; *2*, *K. pratensis* CBS 7079; *3*, *W. mucosa* CBS 6341; *4*, *W. salicorniae* CBS 8071; *5*, *W. saturnus* CBS 254; *6*, *W. suaveolens* CBS 255; *7*, *W. subsufficiens* CBS 5763; *8*, *W.mrakii* CBS 1707; *9*, *W. beijerinckii* CBS 2564; *10*, *W. sargentensis* CBS 6342; *11*, VKM Y-839; *12*, KBP 1097; *13*, KBP 1581; *14*, KBP 1579. Amplified DNAs of strains (a) KBP 1581 and (b) VKM Y-839 served as the probes.

Within the second cluster, four strains were combined: KBP 1097, KBP 1579, KBP 1580, and KBP 1581. Strain VKM Y-839 is located separately (Fig. 3). To determine species affiliation of these strains, an additional analysis was conducted. The four strains isolated from peat bogs were similar to *W. suaveolens* as determined from their physiological characteristics: they were unable to assimilate *L*-rhamnose, citric acid, or mannitol. Strain VKM Y-839 did not differ from the type culture *Z. californica* CBS 252 in its physiological properties. In Fig. 4, PCR patterns of strains VKM Y-839, KBP 1097, and KBP 1581 obtained with the universal primer N21 are compared with those of the type cultures *Z. californica* CBS 252, *W. saturnus* CBS 254, and *W. suaveolens* CBS 255. Since the strains of the latter species display a certain intraspecific polymorphism [16], strains KBP 1118 and KBP 1117 were also used for comparison (Fig. 4, lanes *4*, *5*) in addition to the type culture. Strain KBP 1097 was similar to the type culture *W. suaveolens* CBS 255 in its PCR pattern (Fig. 4, lanes *7* and *3*, respectively). The remaining three strains, isolated from peat bogs and characterized by identical PCR patterns, were similar to strain KBP 1118. The PCR pattern of one of these strains, KBP 1581, is shown in Fig. 4 (lane *8*). Strain VKM Y-839 displayed an individual PCR pattern (Fig. 4, lane *6*).

The DNAs isolated from the strains of the second group and ten type cultures of the species *Z. californica, K. pratensis, W. mucosa, W. salicorniae, W. saturnus, W. suaveolens, W. subsufficiens, W. mrakii, W. beijerinckii,* and *W. sargentensis* was amplified using the N21 primer and hybridized with labeled amplification products of strains KBP 1581 and VKM Y-839 (Figs. 5a, 5b, respectively). The probe derived from strain KBP 1581 produced strong hybridization only with the DNAs of strains isolated from peat bogs and with the DNA of the type culture *W. suaveolens* CBS 255 (Fig. 5a, nos. *12–14* and *6*, respectively). With the DNAs of the rest strains, only slight hybridization signals were obtained. This indicates that the four strains isolated from bog soils of Moscow and Tver oblasts belong to the species *W. suaveolens*. Note that this species dominates the soils of the middle area of Russia [13, 16].

The probe derived from strain VKM Y-839 produced strong hybridization only with its own DNA (Fig. 5b, no. *11*). Cross dot-hybridization of this probe with the DNA of the type culture *Z. californica* CBS 252 produced more intense hybridization signals than with the other species (Figs. 2, 5b). This indicates that strain VKM Y-839 is more related genetically to the species *Z. californica* than to the *Williopsis* and *Komagataea* yeasts.

Thus, our results show that all 22 strains of the first cluster belong to the species *Z. californica*. All four strains of the second cluster were redetermined as *W. suaveolens*. To determine species affiliation of strain VKM Y-839, additional genetic and molecular analysis is required. Primers L45 and N21 can be recommended for the identification of *Z. californica* yeasts.

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