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EXPERIMENTAL ARTICLES

Discrimination between the Soil Yeast Species *Williopsis saturnus* and *Williopsis suaveolens* by the Polymerase Chain Reaction with the Universal Primer N21

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Abstract—Thirty-five yeast strains of the genus *Williopsis*, analyzed by the polymerase chain reaction with the universal primer N21, were found to belong to two sibling species, *W. saturnus* and *W. suaveolens*. Such affiliation of the strains studied agrees well with the results of genetic and physiological investigations.

Key words: *Williopsis saturnus*, *Williopsis suaveolens*, biological species, UP-PCR

The generic and species taxonomy of saturn-spored soil yeasts is often revised. The genus *Williopsis* Zender with the type species *W. saturnus* was first described in 1925 based on the ability to form saturn-shaped ascospores and assimilate nitrates. Later, this genus was divided into two genera, *Hansenula* and *Pichia*, with the affiliation of *W. saturnus* to the genus *Hansenula*. This genus also comprised some new saturn-spored species, including *H. suaveolens* [1]. In 1977, the genus *Williopsis* was reestablished [2]. Genomic hybridization studies performed in our laboratory proved the validity of the reestablishment of the genus *Williopsis* [3] and allowed the discrimination of five sibling species, *W. beijerinckii*, *W. mrakii*, *W. saturnus*, *W. suaveolens*, and *W. subsufficiens* [4–7]. Later, the sixth sibling species *W. sargentensis* was described [8].

However, the classification of saturn-spored yeasts was revised again, in our opinion, without reasonable grounds [9, 10]. For instance, the species *W. mrakii*, *W. sargentensis*, and *W. subsufficiens*, showing merely 43–56% DNA homology with the species *W. saturnus*, were reclassified as *W. saturnus* varieties. Only the pair *W. mrakii* and *W. sargentensis* and the pair *W. suaveolens* and *W. saturnus* showed DNA homology levels (68 and 72%, respectively) that were sufficient to consider them varieties. DNA homology between *W. beijerinckii* and *W. saturnus* was from 79% [11] to 88–100% [9].

It should be noted that such reclassification of species of the genus *Williopsis* does not agree well with the results of genomic hybridization studies [8] and the results of the hybridization of mitochondrial DNA [12]. Indeed, the dot hybridization of the mitochondrial ORF1 probe of *W. mrakii* CBS 1707 with the mitochondrial DNA of *W. beijerinckii* CBS 2564, *W. suaveolens* CBS 255 and CBS 1670, and *W. sargentensis* CBS 6342 was very high, while the level of hybridization with *W. saturnus* strains was either weak (strains NRRL YB-3985, NRRL YB-4818, and NRRL YB-838) or even zero (strains CBS 5761 and NRRL YB-3285). Furthermore, hybridization with the mitochondrial DNA of *W. subsufficiens* CBS 5763 was also absent.

Traditionally, yeasts are classified based on their morphological and physiological characteristics, including the pattern of utilizable carbon and nitrogen sources. Because of the considerable variability of nutritional requirements, physiological tests do not always lead to reliable taxonomic affiliation. Moreover, these tests are time-consuming. On the other hand, progress in the field of molecular biology gave rise to new methods of yeast identification based on the comparison of nucleic acids and proteins. First of all, these are various types of the polymerase chain reaction method, including RAPD-PCR (random amplified polymorphic DNA analysis), AP-PCR (arbitrarily primed PCR), and UP-PCR (PCR with universal primers) [13, 14], as well as the methods of DNA reassociation and ribosomal gene sequencing [15–17]. To the best of our knowledge, PCR has not yet been applied to the classification of yeasts of the genus *Williopsis*.

In the present work, we used UP-PCR to investigate a large collection of *Williopsis* strains isolated from soils in various regions of Russia and other countries.

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Some properties of *W. saturnus* and *W. suaveolens* strains used in this study

Strain	Source location	Growth		
		on L-rhamnose	on citric acid	on mannitol
CBS 254 (type strain)	The Himalayas	+	+	+
CBS 112	Unknown	+	+	+
CBS 258	The Netherlands	+	+	+
CBS 1994	United States	+	+	+
CBS 5761	The Slovenian Alps	+	+	+
KBP 3655	Russia, Krasnodar krai	+	+	+
VKM Y: 1635, 1636 1637, 1638	Denmark	+	+	+
CBS 255 (type strain)	Russia, Smolensk oblast	–	–	–
CBS 1670	Russia, Tver oblast	–	–	–
KBP:1103, 1104	Russia, Novgorod oblast	–	–	–
1116, 1117, 1118, 1119, 1121, 1134	Russia, Tula oblast	–	–	–
1951, 1952, 1955, 1957, 1958, 1959, 1961, 1962, 1964, 1966, 1967, 1970	Russia, Rostov oblast	–	–	–
2706	Russia, Tula oblast	–	–	–
2709	Russia, Rostov oblast	–	–	–
CCY 38-4-1	unknown	–	–	–

Note: CBS, Centraalbureau voor Schimmelcultures (Delft, the Netherlands); KBP, the Collection of Yeasts of the Department of Soil Biology, Faculty of Biology, Moscow State University; VKM, All-Russia Collection of Microorganisms; CCY, Culture Collection of Yeasts, Institute of Chemistry, Slovak Academy of Sciences, Bratislava, Slovakia.

MATERIALS AND METHODS

The strains of the genus *Williopsis* used in this study are listed in the table.

Isolation of DNA. Strains were grown at 28°C for 24 h on complete YPD medium of the following composition (g/l): glucose, 20; peptone, 10; yeast extract, 10; agar, 20. A loopful of yeast cells was suspended in 200 µl of 50 mM Tris–HCl buffer (pH 7.8) containing 50 mM NaCl, 500 mM β-mercaptoethanol, 2% *N*-lauroylsarcosine, and 500 µg/ml proteinase K. The suspension was incubated at 65°C for 1 h to induce cell lysis, and the concentration of NaCl in the lysate was increased to 1 M. The lysate was mixed with an equal volume of a chloroform–isoamyl alcohol (24 : 1) mixture and shaken for 15 min. After centrifugation at 12000 *g* for 5 min, the upper phase was transferred to another tube, and DNA was precipitated by adding 0.6 volume of isopropanol and centrifugating for 15 min. The DNA precipitate was washed with 70% ethanol and dissolved in 50 µl of TE buffer (1 mM Tris–HCl with 0.1 mM EDTA, pH 7.8). The final concentration of DNA in TE buffer was about 200 ng/µl.

Polymerase chain reaction analysis. PCR amplification with the universal primer N21 (5'-GGATCCGAGGGTGGCGGTTCT) was carried out in a mixture (20 µl) containing 2.5 mM $MgCl_2$, 0.4 mM of each dNTP, 0.2 mM primer, 0.05 U/µl DNA polymerase (Dynazyme II), and 20–200 ng of DNA. PCR amplification (30 cycles) was performed in a PHC-3 thermal cycler (Techne Inc.) with the following steps: 50-s DNA denaturation at 94°C, 80-s primer annealing at 55°C, and 60-s DNA synthesis at 70°C. It should be noted that primer N21 was already successfully used for the discrimination of three sibling species in the *Hansenula polymorpha* complex [18].

PCR amplification with the microsatellite primer $(CAC)_5$ was carried out in a mixture (20 µl) containing 1.5 mM $MgCl_2$, 0.4 mM of each dNTP, 0.25 mM primer, 0.1 U/µl SuperTaq DNA polymerase, and 20–200 ng of DNA. PCR amplification (30 cycles) was performed in the same thermal cycler with the following steps: 30-s DNA denaturation at 94°C, 30-s primer annealing at 50°C, and 60-s DNA synthesis at 72°C.

PCR products were separated by electrophoresis through 1.2% agarose gel run at 60 V in 1 × TBE buffer (90 mM Tris, 20 mM EDTA, and 90 mM boric acid) for 4–5 h. After electrophoresis, the gel was stained with ethidium bromide and photographed under UV light.

Dot hybridization. The homology of DNA samples amplified with primer N21 was determined by hybridizing them with the PCR products of strain CBS 255. The PCR products were transferred to a Hybond N^+ membrane (Amersham, United Kingdom) according to the recommendation of the manufacturer. DNA was fixed on the membrane by annealing at 80°C for 2 h and labeled with digoxigenin-11-dUTP (Boehringer Mannheim, Germany). The amplified DNA of strain CBS 255 purified on a QIAGEN column (Germany) was

used as a DNA probe. DNA hybridization and the detection of hybridization products were carried out according to the recommendations of Boehringer Mannheim.

RESULTS AND DISCUSSION

The 35 strains used in this study were isolated from soils in European Russia, Denmark, the United States, Slovenia, the Netherlands, and the Himalayas (see table). In contrast to the earlier studies, where the nontype strains *W. saturnus* CBS 5761 and *W. suaveolens* CBS 1670 were used, in the present work, we investigated the type strains *W. saturnus* CBS 254 and *W. suaveolens* CBS 255.

Unlike *W. suaveolens* CBS 255, *W. saturnus* CBS 254 can assimilate L-rhamnose, citric acid, and mannitol [7, 10, 19]. Based on the ability to assimilate these carbon sources, we divided all the strains studied into two groups (table). The first group included nine strains (CBS 112, CBS 258, CBS 1994, CBS 5761, VKM Y-1635, VKM Y-1636, VKM Y-1637, VKM Y-1638, and KBP 3655), which, similarly to *W. saturnus* CBS 254, were able to utilize L-rhamnose, citric acid, and mannitol. The second group included the remaining 25 strains, which, similarly to *W. suaveolens* CBS 255, were unable to assimilate these carbon sources.

The UP-PCR analysis of strains with the use of primer N21 (Figs. 1 and 2) showed that the molecular weight of PCR products varied from 0.4 to 3.0 kb, depending on the particular strain. In the similarity of their PCR product patterns, strains again fell into two groups. All ten strains of group I had identical PCR product patterns characterized by the presence of three major DNA fragments 0.6, 0.8, and 1.1 kb in size (Fig. 1) and exhibited the range of utilizable carbon sources typical of *W. saturnus* CBS 254.

On the other hand, the remaining 25 strains comprised group II with the PCR product patterns characterized by the presence of a major DNA fragment 0.4 kb in size (Fig. 2) and the range of utilizable carbon sources typical of *W. suaveolens* CBS 255. Inside group II, two subgroups could be distinguished, one subgroup characterized by the presence of a second major 1.0-kb DNA fragment in the PCR product pattern (Fig. 2, lanes *2–18*) and the other subgroup, by a 1.8-kb DNA fragment (Fig. 2, lanes *19–24*). The PCR product patterns inside the subgroups differed only in the presence or absence of some minor bands. At the same time, strain KBP 1117 (Fig. 2, lane *25*) differed from the strains of both subgroups by exhibiting two additional major DNA fragments about 0.8 and 2.5 kb in size. The dot hybridization analysis of the PCR products of the strains of both subgroups and the type strain CBS 255 confirmed that they all belong to the species *W. suaveolens* (data are not presented).

In our opinion, the minor differences in the PCR product patterns of strains comprising the subgroups of

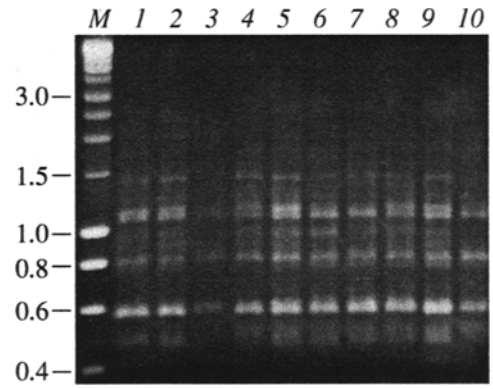

Fig. 1. Electrophoresis of the UP-PCR products of *W. saturnus* strains. Lanes: *1*, CBS 254; *2*, CBS 5761; *3*, CBS 112; *4*, CBS 1994; *5*, CBS 258; *6*, KBP 3655; *7*, VKM Y-1635; *8*, VKM Y-1636; *9*, VKM Y-1637; *10*, VKM Y-1638. *M* denotes molecular weight markers (kb).

group II are due to intraspecies polymorphism. The hybridization species specificity of the UP-PCR products, which manifests itself in the absence of cross-hybridization of DNA fragments from different PCR product patterns, has been shown for many species of yeasts and mycelial fungi [13, 14]. By comparison, we performed the PCR-based analysis of strains comprising group II with the use of the microsatellite primer $(CAC)_5$. Again, these strains fell into two subgroups, although the differences in the PCR product patterns of these subgroups were not so pronounced as in the case of analysis with primer N21. In addition to the DNA fragments typical of the strains of the second subgroup, the strains of the first subgroup exhibited a 0.65-kb DNA fragment (data are not presented).

We were not able to reveal a correlation between the intraspecies polymorphism of *W. suaveolens* strains and the sites from which they were isolated, since the strains of subgroup I were isolated from soils in the Netherlands, the United States, and the Smolensk, Tver, and Novgorod oblasts of Russia, and the strains of subgroup II, from soils in the Tula, Rostov, and Tver oblasts of Russia.

Based on the results of UP-PCR analysis and physiological tests (see table), we transferred strain CCY 38-4-1 from the species *W. saturnus* to the species *W. suaveolens*.

The natural habitats of *W. saturnus* and *W. suaveolens* species are alluvial boggy soils in the river floodplains of the temperate zone [20]. All the strains isolated in the central part of European Russia were found to belong to *W. suaveolens*. On the other hand, only four of the 26 strains isolated from Russian soils could be assigned to *W. saturnus* (see table). All four of these strains were isolated from the chernozem-like soil of a virgin meadow in a warm rice-growing region of Krasnodar krai. The abundance rate of *W. saturnus* in the soils of this region is more than 50% [21]. Therefore, there is a high fidelity of *W. suaveolens* to the soils of

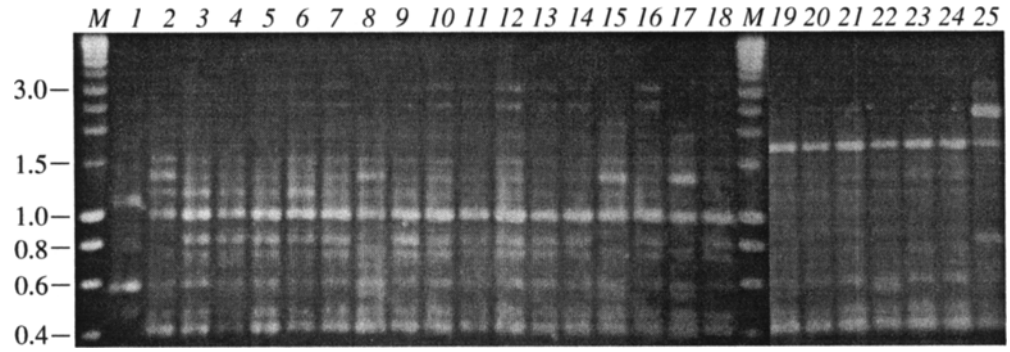

Fig. 2. Electrophoresis of the UP-PCR products of *W. suaveolens* strains. Lanes: *1*, CBS 5761; *2*, CBS 255; *3*, CBS 1670; *4*, KBP 1103; *5*, KBP 1104; *6*, KBP 1116; *7*, KBP 1951; *8*, KBP 1952; *9*, KBP 1955; *10*, KBP 1957; *11*, KBP 1958; *12*, KBP 1959; *13*, KBP 1961; *14*, KBP 1962; *15*, KBP 1964; *16*, KBP 1966; *17*, KBP 1967; *18*, KBP 1970; *19*, KBP 1118; *20*, KBP 1119; *21*, KBP 1121; *22*, KBP 1134; *23*, KBP 2706; *24*, KBP 2709; *25*, KBP 1117. *M* denotes molecular weight markers (kb).

the central part of European Russia, whereas the species *W. saturnus* is predominant in the soils of the southern part of European Russia. Unfortunately, we could not study the divergent strain *W. saturnus* KBP 2708 from the Far Eastern population of soil yeasts [5], since it has been lost.

The species *W. saturnus* and *W. suaveolens* exhibit a high level of DNA homology, are indistinguishable in the 18S and 26S rRNA gene sequences, and differ only slightly in less conservative ITS sequences [15–17]. As already noted, there are two points of view on the taxonomic status of *W. saturnus* and *W. suaveolens:* some authors treat them as distinct species [8], and others consider *W. suaveolens* to be a variety of *W. saturnus* [9, 10]. The results of UP-PCR analysis presented in this paper and the genetic data obtained earlier [5, 7] prove that *W. saturnus* and *W. suaveolens* are distinct species.

Earlier, the four strains of saturn-spored soil yeasts, CBS 1670, KBP 1955, KBP 2706, and KBP 2709, were assigned to the species *W. suaveolens* [7]. Such an affiliation of these species is consistent with the results of their UP-PCR analysis with the use of primer N21 (Fig. 2). It should be emphasized that the PCR-based method has some advantages over the genetic hybridization method. Indeed, the latter method enables the study of only sporulating fertile cultures, whereas the PCR method is applicable to all cultures and allows a great number of strains to be analyzed within a short time period. In particular, the PCR method employing the universal primer N21 is appropriate for discrimination of the sibling species *W. saturnus* and *W. suaveolens*.

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REFERENCES

1. Kurtzman, C.P., Genus 11. *Hansenula* H. et P. Sydow. Genus 21. *Pichia* Hansen, *The Yeasts: A Taxonomic Study*, Kreger-van Rij, N.J.W., Ed., Amsterdam: Elsevier, 1984, pp. 165–213, 295-378.
2. Von Arx, J.A., Rodrigues de Miranda, L., Smith, M.Th., and Yarrow, D., The Genera of Yeasts and the Yeast-Like Fungi, *Studies Mycol.*, 1977, vol. 14, pp. 1–42.
3. Naumov, G.I., Vustin, M.M., and Bab'eva, I.P., Sexual Divergence of the Genera *Williopsis* Zender, *Zygowilliopsis* Kudriavzev and *Hansenula* H. et P. Sydow, *Dokl. Akad. Nauk SSSR*, 1980, vol. 255, no. 2, pp. 468–471.
4. Naumov, G.I., Vustin, M.M., and Naumova, T.I., Hybridological 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.
5. Vustin, M.M., Naumov, G.I., Bab'eva, I.P., and Naumova, T.I., Genetic Systematics of *Williopsis saturnus*: New Biological Species, *Dokl. Akad. Nauk SSSR,* 1982, vol. 267, no. 6, pp. 1481–1484.
6. Vustin, M.M. and Naumov, G.I., Anomalous Meiosis in Hybrids of the Yeast Genus *Williopsis, Biol. Nauki*, 1984, no. 7, pp. 88–91.
7. Naumov, G.I., Vustin, M.M., Bab'eva, I.P., and Reshetova, I.S., Supplements to the Genetic Systematics of the Genera *Williopsis* and *Zygowilliopsis, Mikrobiologiya*, 1985, vol. 54, no. 2, pp. 239–244.
8. Naumov, G.I., Results of the Genetic Systematics of the Yeast Genera *Williopsis* Zender and *Zygowilliopsis* Kudriavzev, *Mol. Genet. Mikrobiol. Virusol.*, 1987, no. 2, pp. 3–7.
9. 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.
10. Kurtzman, C.P., *Williopsis* Zender, *The Yeasts: A Taxonomic Study*, 4th Revised and Enlarged Edition, Kurtzman, C.P. and Fell, J.W., Eds., Amsterdam: Elsevier, 1998, pp. 413–419.
11. Kurtzman, C.P., Prediction of Biological Relatedness among Yeasts from Comparisons of Nuclear DNA Complementarity, *Studies Mycol.*, 1987, no. 30, pp. 459–468.

12. Drissi, R., Sor, F., Nosek, J., and Fukujara, H., Genes of the Linear Mitochondrial DNA of *Williopsis mrakii*: Coding Sequences for a Maturase-Like Protein, a Ribosomal Protein VAR1 Homologue, Cytochrome Oxidase Subunit 2, and Methionyl tRNA, *Yeast*, 1994, vol. 10, pp. 391–398.
13. Bulat, S.A. and Mironenko, N.V., Identification of Fungi and Analysis of their Variability by Polymerase Chain Reaction (PCR) with Gene Specific and Universal Primers, *Genetika*, 1996, vol. 32, no. 2, pp. 165–183.
14. Bulat, S.A., Lubeck, M., Mironenko, N., Jensen, D.F., and Lubeck, P.S., UP-PCR Analysis and ITS1 Ribotyping of Strains of *Trichoderma* and *Gliocladium*, *Mycol. Res.*, 1998, vol. 102, no. 8, pp. 933–943.
15. 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.
16. Yamada, Y., Matsuda, M., Maeda, K., Sakakiba, C., and Mikata, K., The Phylogenetic Relationships of the Saturn-Shaped Ascospore-Forming Species of the 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. Biotech. Biochem.*, 1994, vol. 58, no. 7, pp. 1236–1244.
17. 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.
18. Naumov, G.I., Naumova, E.S., Kondratieva, V.I., Bulat, S.A., Mironenko, N.V., Mendonca-Hagler, L.C., and Hagler, A.N., Genetic and Molecular Delineation of Three Sibling Species in the *Hansenula polymorpha* Complex, *Syst. Appl. Microbiol.*, 1997, vol. 20, pp. 50–56.
19. Wickerham, L.J., Taxonomy of Yeast, *U.S. Dept. Agric. Technol. Bull.*, 1951, no. 1029, pp. 1–57.
20. Vustin, M.M. and Bab'eva, I.P., Natural Habitats of the Yeast Genera *Williopsis* Zender and *Zygowilliopsis* Kudriavzev, *Mikrobiologiya*, 1981, vol. 50, no. 6, pp. 1088–1091.
21. Golubev, V.I. and Vdovina, N.V., Yeasts in Herbicide-polluted Soils of Rice Fields, *Materialy I-go Vses. soveshch. "Povedenie, prevrashchenie i analiz pestitsidov i ikh metabolitov v pochve"* (Proc. Ist All-Union conf. "Conversion and Analysis of Pesticides and their Metabolites in Soils"), Pushchino-na-Oke, 1973, pp. 66–73.