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EXPERIMENTAL ARTICLES

Williopsis saturnus and *Williopsis beijerinckii* Are Recognized as Distinct Taxa by Means of the Polymerase Chain Reaction with Nonspecific Primers

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Abstract—Fifteen strains of the yeast *Williopsis* sensu stricto were analyzed by means of UP-PCR. With the N21 universal primer, this approach showed that the strains could be clearly divided into two groups corresponding to the species *W. saturnus* (Klöcker) Zender and *W. beijerinckii* (van der Walt) Naumov et Vustin. The results obtained are in good agreement with data of genetic and isoenzyme analyses and provide no support for the conspecificity of *W. saturnus* and *W. beijerinckii* commonly accepted in modern yeast taxonomic manuals.

Key words: *Williopsis saturnus*, *Williopsis beijerinckii*, UP-PCR, dot-hybridization.

The genus *Williopsis* Zender is genetically extremely heterogeneous. The four species, *W. mucosa* (Wickerhan et Kurtzman) Kurtzman, *W. salicorniae* Hinzelin et al., *W. pratensis* Babjeva et Reshetova, and *W. californica* (Lodder) von Arx, differ significantly from one another, as well as from the type species of the genus *Williopsis*, *W. saturnus* (Klöcker) Zender [1–3], by nuclotide sequences of their 26S and 18S rRNA. These species probably belong to different genera, and, therefore, Yamada *et al.* [2] proposed a new monotypic genus (*Komagataea*) for the species *K. pratensis* (Babjeva et Reshetova) Yamada *et al*. These authors have also supported Naumov's *et al.* proposal [4] (based on genetic hybridization analysis) to restore another monotypic genus, *Zygowilliopsis* Kudriavzev, which includes the species *Z. californica* (Lodder) Kudriavzev.

As confirmed by genetic analysis [5], the *Williopsis* sensu stricto complex comprises the type species *W. saturnus* and five sibling species, *W. beijerinckii* (van der Walt) Naumov et Vustin, *W. mrakii* (Wickerham) Krassilnikov, *W. sargentensis* (Wickerham et Kurtzman) Naumov, *W. suaveolens* (Klöcker) Naumov *et al.*, and *W. subsufficiens* (Wickerham) Vustin *et al.* Interspecific hybrids of *W. saturnus*, *W. subsufficiens*, *W. suaveolens*, *W. beijerinckii*, and *W. mrakii* are sterile: the viability of their ascospores ranges from 0 to 4% [6–9]. The genomes of these sibling species share 36 to 72% homology [10], and their ribosomal RNAs have identical nucleotide sequences [2, 3]. Contrary to the universally adopted species definition based on DNA–DNA reassociation values, the latest yeast taxonomic manual [11] recognizes the species of *Williopsis* sensu stricto as the following varieties: *W. saturnus* var. *saturnus*, *W. saturnus* var. *mrakii* (Wickerham) Kurtzman, *W. saturnus* var. *sargentensis* (Wickerham et Kurtzman) Kurtzman, *W. saturnus* var. *suaveolens* (Klöcker) Kurtzman, and *W. saturnus* var. *subsufficiens* (Wickerham) Kurtzman. The species *W. saturnus* and *W. beijerinckii*, with their genome homology of 79% [12] or 88 to 100% [10] were hitherto treated as synonyms [11]. Note that the type strain of *W. saturnus* was not used for the DNA–DNA reassociation experiments.

To differentiate among various yeast species, the polymerase chain reaction with universal primers (UP-PCR) can be applied [13–15]. In this study, we used this approach to evaluate the extent of divergence between the genomes of the yeasts *W. saturnus* and *W. beijerinckii*.

MATERIALS AND METHODS

Yeast strains from the following collections were used in this study: CBS (Centraalbureau voor Schimmelcultures, Delft, The Netherlands), VKM (All-Russia Collection of Microorganisms, Moscow), and KBP (Yeast Collection of the Department of Soil Biology, Moscow State University). The strains studied are listed in the table. Strain KBP 3655 was kindly provided by I.P. Bab'eva.

PCR analysis. Yeasts were cultivated at 28°C on a complete medium of the following composition (g/l): glucose, 20; peptone, 10; yeast extract, 10; agar, 20. DNA was isolated as described previously [15]. The

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Origin of the *Williopsis* strains used in this study

Species	Strains	Isolation source
W. saturnus	CBS254 (T)	Soil, the Himalayas
	CBS 5761	Soil
	CBS 112	Kral collection, Austria
	CBS 1994	Soil, The Netherlands
	CBS 258	Unknown
	KBP 3655	Soil, Alps, Slovenia
	VKM Y-1635	Soil, Krasnodar krai, Russia
	VKM Y-1636	Soil, Krasnodar krai, Russia
	VKM Y-1637	Soil, Krasnodar krai, Russia
	VKM Y-1638	Soil, Krasnodar krai, Russia
W. beijerinckii	CBS 2564 (T)	Soil, South Africa
	CBS 2876	Soil, South Africa
	CBS 4549	Elephant dung, zoological garden, Japan
	CBS 4304	Garden soil, Japan
	CBS 5260	Dead leaves, Japan

Note: VKM, All-Russian Collection of Microorganisms, Moscow; CBS, Centraalbureau voor Schimmelcultures, Delft, The Netherlands; KBP, Yeast Collection of the Department of Soil Biology, Moscow State University; T, type culture; CBS 112 = VKM Y-167; CBS 4304 = VKM Y-1097; VKM Y-1635 = CBS 8881; VKM Y-1638 = CBS 8882.

polymerase chain reaction was carried out on a Techne PHC-3 DNA amplifier in 20 μl of a buffer containing 2.5 mM $MgCl_2$, 0.4 mM of each dNTP, 0.2 mM of a primer, 0.05 units/μl of DNA polymerase (Dynazyme II, Finland), and from 20 to 200 ng of DNA. The universal primers N21 (5'-GGATCCGAGGGTGGCGGTTCT) and L45 (5'-GTAAAACGACGGCCAGT) were used. Temperature cycling comprised 30 amplification cycles (DNA denaturation for 50 s at 94°C, primer annealing for 80 s at 55°C, and DNA synthesis for 60 s at 70°C). PCR products were analyzed by electrophoresis in 1.2% agarose and 1 × TBE buffer at 60 V for 4–5 h. Amplified DNA was visualized by staining with ethidium bromide and photographed in UV light. When using a microsatellite primer $(CAC)_5$, PCR conditions were the same as described previously [15].

To determine DNA homology, the PCR products that were amplified using primer N21 were hybridized with labeled amplified DNA from the strains CBS 254 and CBS 2564. The transfer of PCR products onto nitrocellulose membrane (Sigma) and hybridization were performed as previously described [15].

RESULTS AND DISCUSSION

Fifteen collection yeast strains assigned to the species *W. saturnus* were examined by the UP-PCR method using the universal primer N21. Analysis of the PCR products showed that the strains could be divided into two clearly distinct groups with regard to the corresponding electrophoretic patterns (Fig. 1). The first group comprised the type culture *W. saturnus* CBS 254 and nine more strains with identical electrophoretic patterns (CBS 5761, CBS 112, CBS 1994, CBS 258, KBP 3655, VKM Y-1635, VKM Y-1636, VKM Y-1637, and VKM Y-1638) (Fig. 1, lanes *1–10*, respectively). Five strains constituted the second group (CBS 2876, CBS 2564, CBS 4549, CBS 4304, and CBS 5260) (Fig. 1, lanes *11–15*, respectively). Note that until the latest taxonomic revision of the genus *Williopsis* [11], all strains of the second group belonged to the species *W. beijerinckii*. According to our data, a major 450-bp fragment was among the PCR products obtained with the DNA of these strains. Within the second group, the patterns of the PCR products were somewhat polymorphic. Those of the type strain *W. beijerinckii* CBS 2564 and strains CBS 2876 were identical and contained major 1800- and 450-bp bands (Fig. 1, lanes *11* and *12*, respectively). The patterns of strains CBS 4549 and CBS 4304 contained 2500- and 1500-bp DNA fragments (Fig. 1, lanes *13* and *14*, respectively), and the pattern of strain CBS 4549 had an additional 1250-bp major band. Note that the latter is the type strain of *Hansenula coprophila* Soneda (synonym of *W. beijerinckii*). The pattern corresponding to another strain, *W. beijerinckii* CBS 5260, contained 5000- and 1400-bp fragments (Fig. 1, lane *15*).

The results obtained with the universal primer L45 and the microsatellite primer $(CAC)_5$ were analogous: based on the patterns of the amplification products, the strains could be divided into two clearly distinct groups corresponding to the species *W. saturnus* and *W. beijerinckii* (data not shown).

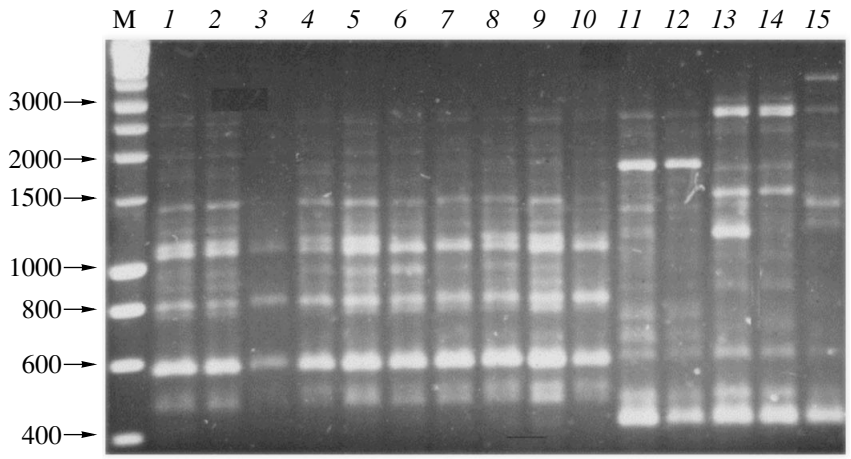

Fig. 1. UP-PCR product polymorphism of the *Williopsis* sensu stricto strains. *W. saturnus*: *1*, CBS 254; *2*, CBS 5761; *3*, CBS 112; *4*, CBS 1994; *5*, CBS 258; *6*, KBP 3655; *7*, VKM Y-1635; *8*, VKM Y-1636; *9*, VKM Y-1637; *10*, VKM Y-1638. *W. beijerinckii*: *11*, CBS 2876; *12*, CBS 2564; *13*, CBS 4549; *14*, CBS 4304; *15*, CBS 5260; M are molecular weight (bp) markers.

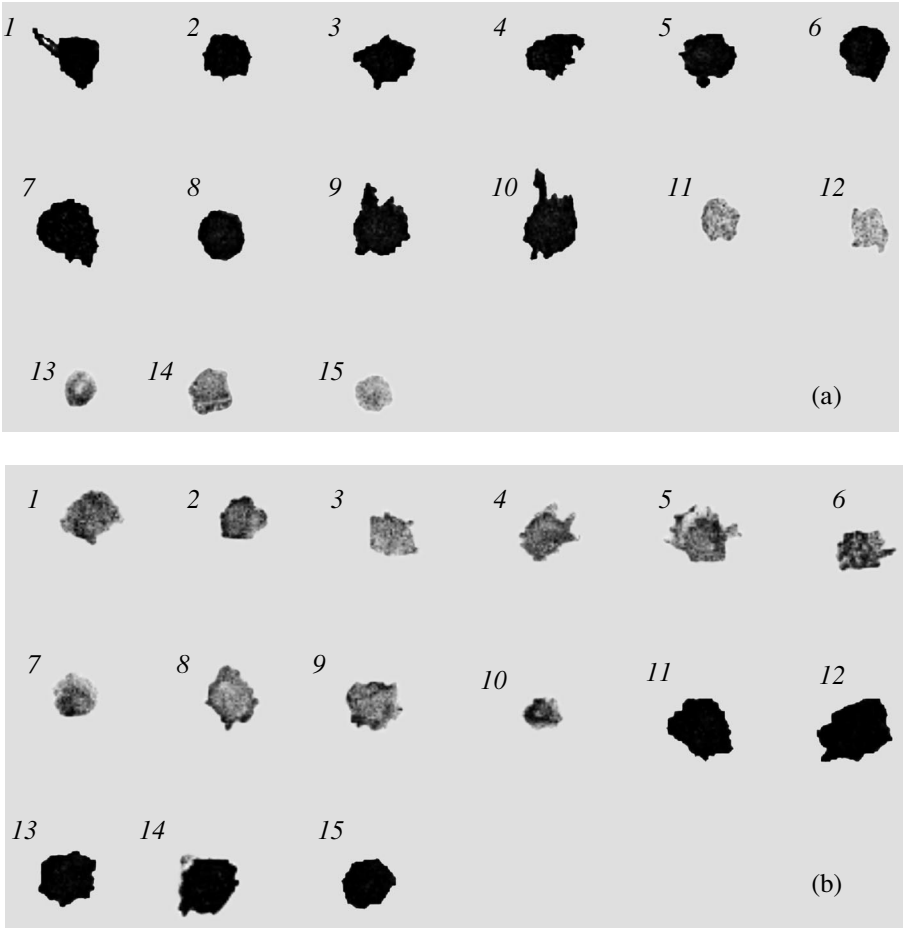

Fig. 2. Cross dot-hybridization between the PCR products of the strains examined and amplified DNA of (a) the type strain *W. saturnus* and (b) the type strain of *W. beijerinckii*. The strains are numbered as in Fig. 1.

To confirm the species affiliation of the 15 strains examined, the PCR amplification products obtained with primer N21 were hybridized to the amplified DNA from the type strains *W. saturnus* CBS 254 and *W. beijerinckii* CBS 2564 (Fig. 2a and 2b, respectively). PCR products obtained with the universal primers exhibit species-specific hybridization reactions, as was shown previously by the example of various species of yeasts and mycelial fungi [13, 16, 17].

When hybridized to the amplified DNA of the type culture *W. saturnus* CBS 254, the PCR products of ten strains assigned to the first group showed strong hybridization (Fig. 2a, *1–10*), whereas the PCR products of five strains assigned to the second group (Fig. 1, lanes *11–15*) showed extremely weak hybridization (Fig. 2a, *11–15*). The opposite results were obtained when the amplified DNA of the type strain *W. beijerinckii* served as the hybridization probe. In this case, only the PCR products obtained with the DNA from the five strains contributing to the second group showed strong hybridization (Fig. 2b, *11–15*), whereas those of the ten strains contributing to the first group exhibited weaker hybridization (Fig. 2b, *1–10*). Thus, dot-hybridization of PCR products obtained by the amplification of DNA from the 15 strains examined in this study also confirms that these strains can be divided into two groups corresponding to the species *W. saturnus* and *W. beijerinckii*.

Two strains of the first group (CBS 5761 and CBS 112) and three strains of the second group (CBS 2564, CBS 4549, and CBS 4304) were previously identified by means of genetic hybridization analysis [6]. It should be noted that interstrain hybrids of *W. beijerinckii* showed regular segregation with respect to control auxotrophic markers, but their fertility decreased (20–25%) as compared to intrastrain hybrids (65–93%). This might suggest some genetic distinctions between strains CBS 2564, CBS 4549, and CBS 4304. The UP-PCR analysis also revealed the heterogeneity of the *W. beijerinckii* strains examined. Unlike the ten strains of *W. saturnus*, the five strains of *W. beijerinckii* yielded four patterns of PCR products (Fig. 1). The hybrids *W. saturnus* × *W. beijerinckii* were characterized by ascospore viability from 0 to 1.5% [6] and extremely irregular segregation with respect to the control markers: the bulk of segregants (94.4%) were prototrophic; monoauxotrophic segregants were few (5.5%), and recombinant double auxotrophs were almost absent (0.1%). This indicates that the two species are reproductively isolated. The results of our UP-PCR analysis, as well as our previous genetic data, are in good agreement with data of electrophoretic enzyme comparison [5, 18]. As determined by Japanese researchers, electrophoretic patterns corresponding to the species *W. saturnus*, *W. beijerinckii*, *W. mrakii*, and *W. subsufficiens* differ, as a rule, by several enzymes, whereas strains of the same species are characterized by identical patterns. In particular, the species *W. beijerinckii* and *W. saturnus* differ in the electrophoretic mobility of five enzymes: malate dehydrogenase (MDH), gluconate dehydrogenase (GDH), glucose-6-phosphate dehydrogenase (G6PDH), lactate dehydrogenase (LDH), and esterase (Est) [18]. Different results were also obtained upon dot-hybridization of *W. saturnus* and *W. beijerinckii* with the mitochondrial ORF1 probe derived from the type strain *W. mrakii* CBS 1707 [19]. In these experiments, significant hybridization was observed with the two *W. beijerinckii* strains (CBS 2564 and CBS 45649), whereas no hybridization occurred with *W. saturnus* CBS 5761, and weak hybridization was observed with *W. saturnus* NRRL YB-3985. In addition, *W. beijerinckii* and *W. saturnus* differ in their physiology: the former assimilates maltose and melezitose, whereas the latter does not assimilate these carbon sources [20].

Thus, UP-PCR analysis and data on isoenzyme electrophoresis available in the literature demonstrate that the species *W. beijerinckii* established previously by genetic analysis [6] is not synonymous with the species *W. saturnus.*

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