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EXPERIMENTAL ARTICLES

Chromosomal Differentiation of the Sibling Species *Pichia membranifaciens* and *Pichia manshurica*

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Abstract—The molecular karyotyping analysis of 21 strains within the taxonomic complex *Pichia membranifaciens* allowed the sibling species *P. membranifaciens* and *P. manshurica*, as well as *P. deserticola* and *P. punctispora,* to be differentiated. Heterogeneity of the species *P. membranifaciens* at the variety level is discussed.

Key words: yeast, sibling species, *Pichia membranifaciens, Pichia manshurica*, molecular karyotyping.

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The type species of the genus *Pichia*, *P. membranifaciens*, has attracted close attention of taxonomists, geneticists, molecular biologists, and even biotechnologists over the last two decades [1–9]. Scientific classification and identification of this yeast was initiated by the discovery in them of heterothallism and isolation of monospore cultures with two mating types [10]. Testers for mating types were used to characterize 62 strains from the collection of the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands [11, 12]. Among them, 46 sporulated by themselves or after being mixed with a culture of a suitable mating type; 22 strains had one mating type, and ten had the other mating type. Some strains that poorly sporulated by themselves (after copulation of the maternal cell with the bud) exhibited a pronounced mating type. Standard sex testers were successfully used for the reidentification of the Ukrainian collection of the yeasts *Candida valida* (anamorph *P. membranifaciens*) and *C. vini* [1, 2].

The application of molecular methods revealed the heterogeneity of the taxonomic species *P. membranifaciens*. First, we will refer to the incompletely published CBS DNA–DNA reassociation data [3, 13, 14] which showed that, of 48 strains, only 27, including the type strain *P. membranifaciens* CBS 107, exhibit a high DNA–DNA reassociation (77–100%) with the type strain *C. valida* CBS 638. The use of karyotyping analysis also convincingly showed heterogeneity of the species *P. membranifaciens* and the necessity for the reinstatement of the species *P. manshurica* and *P. punctispora* [4]. Recently, a large collection of *P. membranifaciens* yeasts from the Institute for Fermentation, Osaka, Japan, has been studied by the DNA–DNA reassociation method [7]. According to the high (72–98%) reassociation level with the type strains, the taxonomic species *P. membranifaciens* was divided into two large groups: *P. membranifaciens* proper (25 strains) and the reinstated species *P. manshurica* (18 strains). Based on a low DNA–DNA reassociation level, six strains were not included in either of these groups; one of them, CBS 637=VKM Y-246, was assigned to the known species *P. deserticola*; and it was shown that *P. galeiformis* (IFO10718) and *P. scaptomyzae* (IFO10731) are synonyms of *P. manshurica* and *P. membranifaciens*, respectively [8].

Due to the fact that our preliminary publication [4] on the chromosomal polymorphism of *P. membranifaciens* did not lose its significance [14] and the DNA–DNA reassociation data [7, 14] only support it, we thought it necessary, considering all the evidence available, to publish a special article on the karyotypic identification of the species *P. membranifaciens* and *P. manshurica*.

MATERIALS AND METHODS

The strains of the taxonomic complex *P. membranifaciens* under study and their origin are shown in the table. The yeasts were cultivated for 24 h at 28°C on a complete medium of the following composition (g/l): glucose, 20; peptone, 10; yeast extract, 10; agar, 20.

The CHEF-DR III apparatus (Bio-Rad, United States) was used for separating chromosomal DNA. The isolation of chromosomal DNA was carried out according to the technique described earlier [15]. Pulsed-field electrophoresis in 0.8% agarose was carried out at 100 V for 46 h with the field switching time of 500 s. The chamber was cooled to 12°C. The commercial DNA preparations of strains YNN 295 and

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Origin of the strains of the taxonomic complex *Pichia membranifaciens* studied

Species and their strains in the collections			Source of isolation	Region of isolation	Reference
BKM Y-	CBS	IFO			
P. membranifaciens					
166	184	–	Belgian beer	Belgium	[4, 14]
242	635	–	Wine	–	[4, 14]
243	636	–	Brewing	–	[4]
248	598	–	Lambic beer	Belgium	[4, 14]
288	207	10561	–	Manchuria	[4, 7]
292	214	–	Grape must	Italy	[4, 14]
299 (T)	107	10215	–	Denmark	[4, 7, 14]
303	191	–	Wine	Italy	[4, 14]
1105	1329	10725	Soil	Denmark	[4, 7]
1107	244	–	Banana	–	[4, 14]
1258	5567	–	Lemonade	The Netherlands	[4, 14]
1259	5568	–	Lemonade	The Netherlands	[4, 14]
1493	638	10318	Wine	Germany	[4, 7, 14]
P. manshurica					
298 (T)	209	10726	Alcoholic beverage	Manchuria	[4, 7, 14]
316	2284 (?)	1284 (?)	Exudation sap	United States	[4, 7, 14]
840	240	10562	Bili wine	Western Africa	[4, 7, 14]
844	241	–	Brewing	–	[4, 14]
1115	1367 (?)	–	Excrement	United States	[4]
P. deserticola					
246	637	0162	Tan liquid	Japan	[4, 7, 14]
P. punctispora					
312 (T)	190	–	–	United States	[3, 4]
Pichia sp.					
1108	1331	–	Pineapple	–	[4]

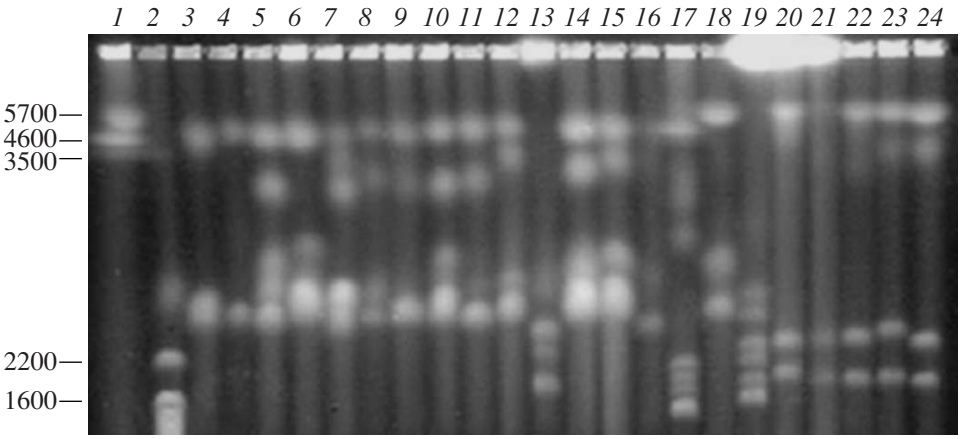
Notes: The following abbreviations are used: T, type culture; VKM Y-, the All-Russian Collection of Microorganisms, Moscow, Russia; CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; IFO, Institute for Fermentation, Osaka, Japan. At present, all the IFO strains are kept under the same numbers at the National Institute of Technology and Evaluation--Biological Center (NBRC), Chiba, Japan. Strain *P. silvestris* VKM Y-316 originates from H.J. Phaff's collection.

Schizosaccharomyces pombe 972 h$^-$ (Bio-Rad) were used as the chromosomal standards.

RESULTS AND DISCUSSION

In the available publications [7, 16] on karyotyping of the yeast *P. membranifaciens*, imperfect regimes of chromosome separation were used, more suitable for *Saccharomyces* yeasts with their relatively small chromosomes. As a result, it was possible to reveal only one to three chromosomes in *P. membranifaciens*. Using a fundamentally different model of an electrophoretic apparatus (CHEF-DR III, Bio-Rad) and special conditions for separating large chromosomes, we managed to reveal from two to eight chromosomes in *P. membranifaciens* yeasts (see figure).

According to data from pulsed-field electrophoresis of chromosomal DNA, the taxon *P. membranifaciens* under study could be separated into at least three groups. The first group, the most voluminous and rather heterogeneous, was represented by the type culture of *P. membranifaciens* VKM Y-299 and by the following strains VKM Y: 166, 288, 292, 303, 898, 1105, 1107, 1258, 1259, 1493, 242, 243, and 248. Upon electrophoresis, all chromosomes of the yeasts of this group were located within the separation of the two VKM Y-299 electrophoretic bands (figure). In this group, the number of bands varies between two to five. As judged from the intensity of band coloration, a number of strains may have complex bands, double or triple (see, e.g., the lower band of strain VKM Y-229 in the figure). The second largest group (VKM Y: 298, 316, 840, 844,



Pulsed-field electrophoresis of the chromosomal DNA of different strains of the taxonomic complex *Pichia membranifaciens.* Lanes: *P. membranifaciens* (*3*) VKM Y-299, (*4*) VKM Y-166, (*5*) VKM Y-288, (*6*) VKM Y-292, (*7*) VKM Y-303, (*8*) VKM Y-898, (*9*) VKM Y-1105, (*10*) VKM Y-1107, (*11*) VKM Y-1258, (*12*) VKM Y-1259, (*14*) VKM Y-1493, (*15*) VKM Y-242, (*16*) VKM Y-243, (*18*) VKM Y-248; *Pichia manshurica* (*20*) VKM Y-298, (*21*) VKM Y-316, (*22*) VKM Y-840, (*23*) VKM Y-844, (*24*) VKM Y-1155; *Pichia* sp. (*13*) VKM Y-1108; *P. deserticola* (*17*) VKM Y-246; *P. punctispora* (*19*) VKM Y-312. The chromosome sizes in megabases are given according to the standards (*1*) *Schizosaccharomyces pombe* 972 h^- and (*2*) *Saccharomyces cerevisiae* YNN 295. The DNA was stained with ethidium bromide.

and 1155) was karyotypically quite homogeneous and may be assigned to the species *P. manshurica* Saito, represented by the authentic strain VKM Y-298 (see the figure). Finally, there were three strains (VKM Y: 1108, 246, and 312) whose karyotypes differed from one another and from those of the above-mentioned two groups. Evidently, these three strains represent distinct species. As already noted, strain VKM Y-246 belongs, according to the DNA-DNA reassociation data [7], to the species *P. deserticola.* According to the karyotypes, only one (the type strain VKM Y-312) of the six reidentified strains [3] belongs to the reinstated species *P. punctispora* (Melard) Dekker; the rest of the strains belong to the species *P. manshurica.* Of our first group, only four strains were studied in [7]. Despite their significant karyotypic distinctions (figure), they have highly homologous genomes. Thus, DNA–DNA reassociation level of the type culture VKM Y-299 with the strains VKM Y: 288, 1105, and 1493 was 78, 72, and 88%, respectively. The representatives of our second group (VKM Y: 298, 316, 840) also exhibit high genome homology with each other. Their DNA–DNA reassociation level with the type culture *P. manshurica* VKM Y-298 was 85 and 87%, respectively [7].

Comparison of the results of the karyotyping (figure) and DNA–DNA reassociation data [7] allows the following conclusions to be made. Strains of *P. mebranifaciens* can be differentiated by the minimal and maximal size of the chromosomes of the type strain VKM Y-299. It should be noted that other strains of this species may differ significantly in intermediate chromosomes. The species *P. manshurica* is sufficiently homogeneous karyotypically and may be well differentiated by at least the two lower chromosomes of the type strain VKM Y-298. Along with karyotyping, DNA homology, the G+C content and the differences in growth temperature may be used for differentiating between *P. membranifaciens* and *P. manshurica* [7].

Taking into account the significant chromosomal polymorphism of *P. membranifaciens* yeasts, the substantial divergence of their rRNA [7], and occurrence of strains with a DNA–DNA reassociation level close to 70% [7], the problem of heterogeneity of this taxon is not dismissed, at least, at the variety level. A diversity of molecular-genetic data that convincingly prove the existence of taxonomic varieties were earlier obtained in our laboratory as exemplified by *Arthroascus*, *Kluyveromyces, Saccharomyces*, and other yeast genera.

Until recently, cell hybridization and spore formation in mixtures with *P. membranifaciens* sex testers were considered sufficient for species identification of strains [1, 2, 11]. According to [11] and the CBS strain catalogue [12, 14], the same system of mating types controls hybridization of not only the *P. membranifaciens* strains VKM Y: 288, 299, 1107, 1258, and 1259, which exhibit chromosomal polymorphism, but also of *P. manshurica* VKM Y-316 and *Pichia* sp. VKM Y-1108. This agrees with the rule earlier established for the species *Saccharomyces* [17], *Kluyveromyces* [18], *Williopsis* [19], *Arthroascus* [20], *Galactomyces* [21], *Metschnikowia* sensu stricto [22], and *Metschnikowia* sensu lato [23] that possession of common mating types is a generic rather than a species feature. The species identity can genetically be determined only with taking into account hybrid fertility (the ascospore survival) and normal recombination of the control markers.

According to the phylogenetic tree constructed based on the D1/D2 domain of 26S ribosomal DNA [24], the closest relatives of the *P. membranifaciens* yeasts are (along with *P. deserticola*), *P. manshurica*

(syn. *P. galeiformis*) and *P. kudriavzevii* (syn. *Isachenkia orientalis, Candida rusei*). It remains to be clarified whether all these species have the same mating type. If they do, then it will be possible to say that, of the hundred known species [24], only those listed by us belong to the genus *Pichia* sensu stricto.

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