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EXPERIMENTAL ARTICLES

The Yeast *Komagataella*: A Genetic Genus in Accordance with Interspecies Hybridization

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Abstract—Using induced complementary auxotrophic mutants and selective growth of prototrophic hybrids on minimal medium, hybridization of the type strain of *Komagataella kurtzmanii* VKPM Y-727 with the type strains of *K. pastoris* VKPM Y-3262, *K. phaffii* NRRL Y-7556, *K. populi* NRRL YB-455, *K. pseudopastoris* NRRL Y-27603, and *K. ulmi* NRRL YB-407 was demonstrated. The data obtained suggest that the genus *Komagataella*, established previously by phylogenetic analysis, corresponds well to the concept of genetic genus in ascomycetous fungi. According to this concept, a genetic genus is a group of hybridized species having a common mating type system. Application of the concept of genetic genus for different yeast genera is discussed.

Keywords: Komagataella yeasts, *Pichia pastoris*, interspecies hybridization, auxotrophic mutants, sibling species

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The yeasts of the genus *Komagataella* Yamada et al. 1995 [1–5], which is better known under the names *Pichia pastoris* (Guilliermond) Phaff 1956 [6] and *Zygowillia pastori* (Guilliermond) Kudriavzev 1960 [7, 8], are of interest to molecular biologists and biotechnologists. High applied significance of this methanol-assimilating yeast naturally resulted in its thorough classification and identification [9–11]. It was established that the previous taxonomic species *Komagataella pastoris* (Guilliermond) Yamada et al. 1995 [5] was a generic complex of at least six phenotypically similar species: *K. pastoris, K. pseudopastoris* Dlauchi et al. 2003, *K. phaffii* Kurtzman 2005, *K. populi* Kurtzman 2012 [14], and the recently described species *K. kurtzmanii* Naumov et al. 2013 [11]. The phylogenetic relatedness of these sibling species was established on the basis of comparative analysis of the nucleotide sequences of the D1/D2 domain and ITS1/ITS2 rDNA, the EF-1α elongation factor, RNA polymerase II, and mitochondrial rRNA SSU [10, 11, 14]. The first two above-mentioned species are of European origin; the remaining ones are of North American origin. Most *Komagataella* strains were isolated from sap exudates of trees and decaying wood.

In this work, we continued to study the interspecies hybridization of the yeast *Komagataella* [11] and revealed the predicted community of the mating type system of different species of this genus, which allows interspecies hybridization.

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MATERIALS AND METHODS

The studied species of the genus *Komagataella* are listed in the table. The strains were obtained from the All-Russian Collection of Industrial Microorganisms (VKPM), Moscow and the ARS Culture Collection (NRRL), National Center for Agricultural Utilization Research, Peoria, Illinois, United States. The table also shows the strain numbers in the Collection of Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands. The yeasts were grown on complete YPD medium containing the following (g/L): bacto-agar (Difco, United States), 20; glucose (REAKHIM, USSR), 20; yeast extract (Difco), 10; and peptone (Difco), 10. Zygote formation was induced on acetate medium [15] containing the following (g/L): bacto-agar, 20; CH_3COONa, 5; KCl, 10; and glucose, 10. Prototrophic hybrids were selected on minimal medium containing the following (g/L): bacto-agar, 20; glucose, 20; and Difco nitrogen base (without amino acids), 6.7. The yeasts were cultivated in all the media at 25°C. Auxotrophic mutants were induced by UV irradiation in all the type cultures with 10% cell viability. For each strain, about ten different auxotrophic mutants were obtained.

RESULTS AND DISCUSSION

The haplont life cycle of the *Komagataella* yeasts significantly simplifies the performance of their hybridization. The hybrids were obtained by mass

Origin and characteristics of the studied strains of the genus *Komagataella* and other reference yeasts (*Phaffomyces*, *Ogataea*, and *Pichia*)

Species identity	Strain number in the collections			Source and site of isolation	GenBank accession no. of the sequence		
	VKPM	NRRL	CBS		D1/D2	Mt SSU	EF-1a
K. kurtzmanii	Y-727	Y-63667 ^{T}	12817	Sap exudation of the fir *Abies* sp., South Arizona, United States	KC715720	KC715723	KC715721
K. pastoris	Y-3262	Y-1603^{T}	704	Sap exudation of the chestnut *Castanea*, France	U75963	EF547704	EF552478
K. phaffii	–	Y-7556^{T}	2612	Sap exudation of the oak *Quercus kelloggii*, California, United States	AF017407	EF547706	EF552480
K. populi	–	YB-455^{T}	12362	Sap exudation of the poplar *Populus deltoides*, Illinois, United States	JN234404	JN234406	JN234408
K. pseudopastoris	–	Y-27603^{T}	9187	Decaying trunk of the willow *Salix alba*, Hungary	AF403149	EF547705	EF552479
K. ulmi	–	YB-407^{T}	12361	Sap exudation of the elm *Ulmus americana*, Illinois, United States	JN234403	JN234405	JN234407
Ph. antillensis	–	Y-12881^{T}	7111	Damaged cactus *Cephalocereus royenii*, Virgin Islands	EU011660	EU014771	EU018559
Ph. opuntiae	–	Y-11707^{T}	7010	Decaying cactus *Opuntia inermis*, Australia	EU011661	EU014772	EU018560
Ph. thermotolerans	–	Y-11709^{T}	7012	Cactus *Lophocereus schottii*, Mexico	EU011662	EU014773	EU018561
O. glucozyma	–	YB-2185^{T}	5766	Insect excrements from the spruce *Picea engelmannii*, Wyoming, United States	U75520	EU018527	EU014736
P. membranifaciens	–	Y-2026^{T}	107	Unknown	U75725	EF547677	EF552451

D1/D2 is the 26S rDNA D1/D2 domain; Mt SSU is a mitochondrial rRNA small subunit gene; EF-1α, the translational elongation factor. T indicates a type strain.

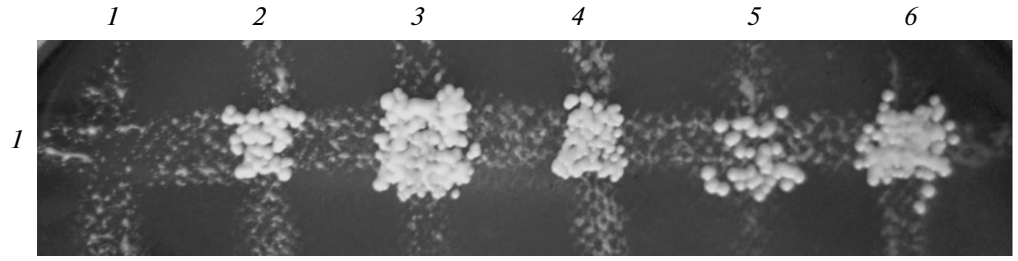

Fig. 1. Hybridization of auxotrophic mutants of *Komagataella* species in accordance with prototrophic growth on minimal medium at the sites of intersection of parental strains. Yeast replica: *K. kurtzmanii* VKPM Y-727 (arg16) (*1*); *K. pastoris* VKPM Y-3262 (his2) (*2*); *K. phaffii* NRRL Y-7556 (lys3) (*3*); *K. populi* NRRL YB-455 (ade6) (*4*); *K. pseudopastoris* NRRL Y-27603 (met2) (*5*); and *K. ulmi* NRRL YB-407 (arg8) (*6*). The auxotrophic mutants used are listed in parentheses.

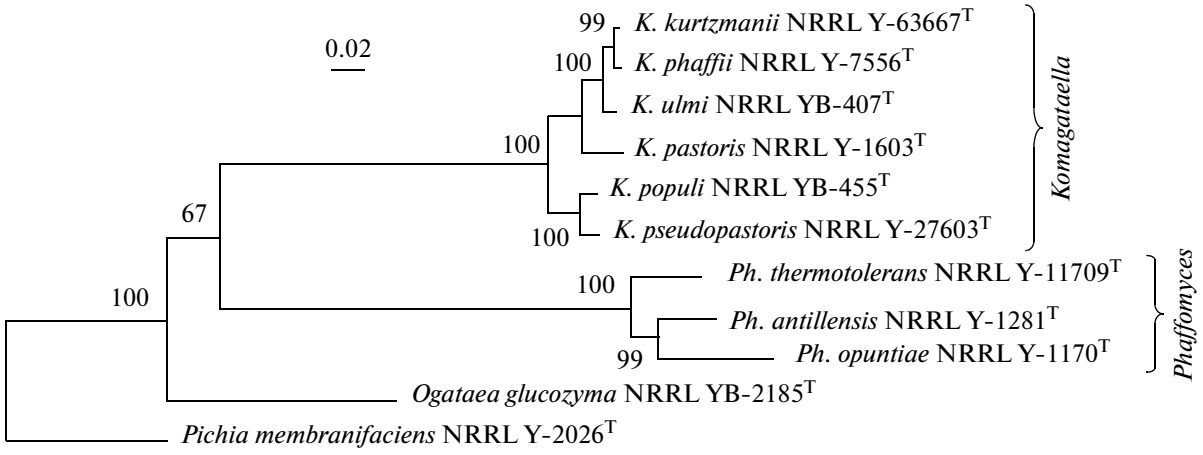

Fig. 2. Molecular differentiation of the yeasts of the genus *Komagataella* according to phylogenetic analysis of the 26S rDNA D1/D2 domain, the translational elongation factor EF-1α, and the mitochondrial rRNA small subunit gene. The scale corresponds to 20 nucleotide substitutions per 1000 nucleotide positions. The tree was constructed using the Neighbor-Joining method in MEGA 5 software package [16]. T is the type culture.

hybridization of haploid cells possessing complementary auxotrophic mutations on starvation acetate medium. For this purpose, 24-h yeast cultures were transferred criss-cross from complete YPD medium to starvation medium using a velvet replicator. After 24-h exposure, the yeasts were repeatedly transferred to the minimal medium with the velvet replicator. The growth of prototrophic hybrids was observed after 48–72 h at the places where the replica crossing took place. First, we tested the capacity for hybridization in the auxotrophic mutants of each of the strains analyzed. The complementary auxotrophic mutants of the homothallic species *K. kurtzmanii, K. pastoris, K. phaffii, K. populi*, and *K. ulmi* formed intrastrain hybrids with varying frequency. Yeast development (from several prototrophic colonies to massive growth) was observed at the intersection of the streaks. Auxotrophic mutants of the strain *K. pseudopastoris* NRRL Y-27603, which did not hybridize with each other, were exceptional in this respect, presumably due to the presence of the cells of only one mating type.

The results of interspecies hybridization are shown on Fig. 1. The tester strain *K. kurtzmanii* VKPM Y-727 was hybridized with all the remaining *Komagataella* species. Thus, it may be stated that the species of this genus have the common mating type system.

In order to demonstrate the high interspecies and intergeneric molecular divergence of *Komagataella* yeasts, Fig. 2 shows the phylogenetic tree including all known species of the genus *Komagataella* and of the closest genus *Phaffomyces* Y. Yamada et al. 1997. The low statistical support of phylogenetic relatedness of these genera does not allow us to consider them as sister genera; this was emphasized by K. Kurtzman himself [10, 17]. The relatedness between the genus *Komagataella* and the heterogeneous genus *Ogataea* Y. Yamada et al. 1994 represented in the phylogenetic tree (Fig. 2) by the type species *O. glucozyma* Y. Yamada et al. 1994 is even lower. Differentiation of *Komagataella* species is well documented with the use of five molecular markers [11], which resulted in the resolution of three clusters, each containing two species: *K. pastoris* and *K. ulmi, K. kurtzmanii* and *K. phaffii, K. pseudopastoris* and *K. populi.*

Our experimental results indicate that the genus *Komagataella* created on the basis of phylogenetic analysis [10, 11, 14, 17] is in agreement with the concept of genetic genus of ascomycetous fungi according to which the species of one genus possess a common mating type system that enables them to hybridize in any combination [18]. Nevertheless, genetic genera incorporate real biological species exhibiting postzygotic isolation—nonviable meiosis products (ascospores) of hybrids. Thirty-five years later we can state that not only the genus *Saccharomyces* [19–22] but also other revised genera correspond to the genetic concept of genus: *Williopsis* Zender 1925, *Arthroascus* von Arx 1972, *Kluyveromyces* Kurtzman et al. 2001 (syn. *Zygofabospora* Kudriavzev 1960 emend. G. Naumov 2002), *Zygowilliopsis* Kudriavzev 1960, *Galactomyces* Readhead, Malloch 1977, and the complex *Ogatae* (*Hansenula*) *polymorpha* (Falcão de Morais, Dália Maia 1959) Y. Yamada et al. 1994 [23–29]. Taking into account the literature data [30–33], the small-spore species of *Metschnikowia* Kamienski 1899 (sensu stricto) and large-spore *Metschnikowia* species (sensu lato) are likely to be considered as different genetic genera. Despite the fact that hybridization was not studied between all small-spore *Metschnikowia* species, the capacity for hybridization of yeasts from different divergent subclusters of their phylogenetic tree is indicative of the common interspecies mating type system [32]. As for the capacity for hybridization of large-spore species [31], we think that in some negative cases visual control of zygote formation should be supplemented by a more sensitive selective method for hybridization of auxotrophic mutants. The taxonomic species *Schizosaccharomyces pombe* Lindler 1893 merits attention. The genetic and molecular-karyotypic analyses showed this yeast to be a genetic genus and numerous species in statu nascendi [34]. Due to chromosomal rearrangements, *Sch. pombe* strains of different geographic origin form almost sterile hybrids, which, however, still have meiotic recombination of the control parental markers. Finally, according to the available literature data, the status of a genetic genus may also be predicted for the yeast *Phaffomyces*. This is evidenced by the possibility of obtaining *Ph. thermotolerans* × *Ph. opuntiae* hybrids [35, 36] with 28% total DNA–DNA parent reassociation. The species *Ph. antillensis* was shown to be related to *Ph. opuntiae* and *Ph. thermotolerans* having 50 and 26% DNA–DNA reassociations, respectively [36, 37]. Since no zygote formation visible under the microscope occurred in *Ph. antillensis* with *Ph. opuntiae* and *Ph. thermotolerans*, it is necessary to study their capacity for hybridization using selective conditions. According to our experience with other yeasts, 50% DNA–DNA reassociation should provide interspecies hybridization of haploid cells and/or spores.

In conclusion, it should be noted that the genetic genus is not only a theoretical but also an operational concept. It allows experimental determination on the basis of sexual hybridization of the species and strain belonging to a certain genus. The phenomenon of genetic genus has a biological role aiding not only in the formation of natural interspecies hybrids but also in the autonomous transfer of plasmids or individual chromosomes known in the *Saccharomyces* yeasts [38, 39]. The discovery of natural hybrid yeast genomes [40–44] proves the evolutionary and applied significance of interspecies hybridization. The mechanisms of conversion of the mating types afford the formation of novel fertile amphidiploid species of yeasts in nature [45, 46]. Further development of the genetic genus concept would be expected.

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