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ORIGINAL PAPER

Komagataella kurtzmanii sp. nov., a new sibling species of *Komagataella* (*Pichia*) *pastoris* based on multigene sequence analysis

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Abstract A novel methanol assimilating yeast species *Komagataella kurtzmanii* is described using the type strain VKPM Y-727 (=KBP Y-2878 = UCD-FST 76-20 = Starmer #75-208.2 = CBS 12817 = NRRL Y-63667) isolated by W.T. Starmer from a fir flux in the Catalina Mountains, Southern AZ, USA. The new species is registered in MycoBank under MB 803919. The species was differentiated by divergence in gene sequences for D1/D2 LSU rRNA, ITS1-5.8S-ITS2, RNA polymerase subunit I, translation elongation factor-1α and mitochondrial small subunit rRNA. *K. kurtzmanii* differs from its phenotypically similar sibling species *Komagataella pastoris*, *Komagataella pseudopastoris*, *Komagataella phaffii*, *Komagataella populi* and *Komagataella ulmi* by absence of growth at 35 °C and inability to assimilate trehalose.

Keywords New ascosporic yeast · Sibling species of *Komagataella pastoris* · Methanol yeast

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Introduction

The heterogeneous genus *Pichia* Hansen has been frequently revised (Kurtzman 1984, 1998, 2011; Kurtzman et al. 2008). In particular, Yamada et al. (1995) described a new monotypic genus *Komagataella*, into which the methanol assimilating yeast *Pichia pastoris* has been transferred. The latter species is of great industrial importance, viz. it is widely used in applied genetic engineering and biotechnology (Wegner 1983; Cregg and Madden 1988; Cregg et al. 1993, 2012; Macauley-Patrick et al. 2005; Chen et al. 2012). After discovery of the European species *Komagataella pseudopastoris* (Dlauchy et al. 2003), the genus *Komagataella* has been generally accepted (Kurtzman 2005, 2011). Moreover, a new sibling species of *K. pastoris* was described in North America: *Komagataella phaffii* (Kurtzman 2005). It was shown that biotechnologically important strains *P. pastoris* represent both *K. pastoris* and *K. phaffii*, and the latter species was used in the commercialized Invitrogen Expression Kit (Kurtzman 2009). Quite recently, two more *Komagataella* species have been described using single strains: *Komagataella populi* and *Komagataella ulmi* (Kurtzman 2012). Both are from North America. In the course of molecular genetic study of methanol assimilating yeasts identified phenotypically as *K. pastoris*, previously undescribed *Komagataella* species was found. Based on multigene sequence comparisons, a novel species *Komagataella kurtzmanii* is proposed.

Materials and methods

Yeast strains, culture conditions and phenotypic characterization

The strains used and their origins are listed in Table 1. Phenotypic characterization of strain VKPM Y-727, representing a novel species of the genus *Komagataella*, was carried out according to Yarrow (1998) and Kurtzman et al. (2011). Strains of *K. pastoris* VKPM Y-3262 and *K. phaffii* VKPM Y-3489 were used as reference testers. Yeast cells were grown at 25 °C on the YPD complete medium (2 % glucose, 1 % peptone, 1 % yeast extract and 2 % agar). Sporulation and zygote formation were induced at 25 °C on different acetate agar media used in genetics and taxonomy of yeasts: (1) 1 % CH3COONa, 0.5 % KCl and 2 % agar; (2) 0.82 % CH3COONa, 0.18 % KCl, 0.25 % yeast extract, 0.1 % glucose and 1.5 % agar (McClary et al. 1959); (3) 0.5 % CH3COONa, 1 % KCl, 1 % glucose and 2 % agar (Chen et al. 2012). Also, ME medium was used (5 % malt extract and 2 % agar).

DNA extraction and sequencing

Genomic DNA was extracted from yeast cells by using Genomic DNA Purification Kit (Fermentas, Lithuania). The genes for D1/D2 26S rRNA, translation elongation factor-1α (EF-1α), RNA polymerase II (subunit RPB1), mitochondrial small subunit (Mt SSU) rRNA and ITS1-5.8S-ITS2 were amplified and sequenced using the oligonucleotide primers (Kurtzman and Robnett 1998, 2003; Kurtzman 2009). Amplification reactions were performed in a volume of 30 µl containing 100 ng of genomic DNA, *Taq* polymerase (0.05 U, Syntol, Moscow), and the primers (50 pmol each). A thermal cycler Tertsik (Russia) was programmed for 30 cycles of 45 s at 94 °C, 30 s at 52 °C and 2 min at 72 °C. Amplification products were separated by electrophoresis in 1 % agarose gels and detected by staining with ethidium bromide. For sequencing, the amplified products were purified using the GeneClean Purification Kit (Bio101, USA) according to the manufacturer's instructions. Direct sequencing of both strands of the genes analyzed was performed using Beckman-Coulter automated DNA sequencer (USA). Sequences obtained were analysed with the SeqMan package (DNAStar Inc., Madison, WI, USA). An alignment was done visually using the program BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). The MEGA 5 package (Tamura et al. 2011) was applied to generate a distance tree using the neighbour-joining algorithm with Kimura 2-parameter correction. Alignment gaps were deleted pairwise. Type cultures of *Ogataea glucozyma* NRRL YB-2185 and *P. membranifaciens* NRRL Y-2026 were used as outgroup species. A total of 1,000 bootstrap replicates were used for analysis. The nucleotide sequences determined in this study have been deposited with GenBank (Table 1). The reference sequences used in the phylogenetic analysis were retrieved from GenBank under the accession numbers indicated in Table 1.

Results

The D1/D2 nucleotide sequence obtained for strain VKPM Y-727 differs by 4–6 nucleotide substitutions from the corresponding sequences of the five known *Komagataella* species (Table 2). To elucidate a taxonomic status of VKPM Y-727 we conducted multigene sequence analysis. ITS1-5.8S-ITS2, EF-1α and RPB1 sequences of strain VKPM Y-727 differ markedly from those of *K. pastoris*, *K. phaffii*, *K. ulmi*, *K. populi* and *K. pseudopastoris* (Table 2). On the other hand, VKPM Y-727 and *K. ulmi* NRRL YB-407 showed identical Mt SSU sequences, which differed by a single nucleotide substitution from *K. phaffii* NRRL Y-7576. In terms of pairwise multigene sequence similarity the closest relative to the proposed new *Komagataella* species is *K. phaffii*. Based on the multigene sequence comparisons a phylogenetic tree was depicted (Fig. 1). Three pairs of the most closely related species can be distinguished with high bootstrap support: *K. pastoris*/*K. ulmi*, *K. kurtzmanii*/*K. phaffii* and *K. pseudopastoris*/*K.populi.* The data suggest that strain VKPM Y-727 represents a novel species of the genus *Komagataella*.

Description of *Komagataella kurtzmanii* G.I. Naumov, E.S. Naumova, O.V. Tyurin and D.G. Kozlov sp. nov.

Growth on 5 % malt extract (ME) agar

After 3 days at 25 °C, cells divide by multilateral budding and are spherical (2–7 µm) to ovoid (2–7 × 3–6 µm), occur singly and in pairs (Fig. 2a). Colony growth is white, butyrous and with a smooth semi-glistening surface.

Table 1 Strains of *Komagataella* species compared

Species	Strain designation[a]			Source of strain	GenBank accession numbers[b,c]				
	VKPM	NRRL	CBS		D1/D2 LSU	ITS	Mt SSU	EF-1α	RPB1
Komagataella kurtzmanii	Y-727	63667	12817	Fir (*Abies* sp.) flux, Catalina mountains, Southern AZ, USA	KC715720	KC771256	KC715723	KC715721	KC715722
Komagataella pastoris	Y-3262	Y-1603^T	704	Exudate, chestnut tree (*Castanea* sp.), France	U75963	JQ398742	EF547704	EF552478	GQ327955
Komagataella phaffii	–	Y-7556^T	2612	Exudate, black oak (*Quercus kelloggii*), California, USA	AF017407	JQ398743	EF547706	EF552480	GQ327957
	Y-3489	Y-11430	–	U.S. Patent 4.414.329					
Komagataella populi	–	YB-455^T	12362	Exudate, cottonwood tree (*Populus deltoides*), Peoria, Illinois, USA	JN234404	JQ398744	JN234406	JN234408	JN234410
Komagataella pseudopastoris	–	Y-27603^T	9187	Rotted willow tree (*Salix alba*), Hungary	AF403149	JQ398745	EF547705	EF552479	GQ327956
Komagataella ulmi	–	YB-407^T	12361	Exudate, elm tree (*Ulmus americana*), Peoria, Illinois, USA	JN234403	JQ398746	JN234405	JN234407	JN234409
Ogataea glucozyma	–	YB-2185^T	5766	Insect frass, Engelmann spruce (*Picea engelmannii*), Wyoming, USA	U75520	–	EU018527	EU014736	GQ327959
Pichia membranifaciens	–	Y-2026^T	107	Substrate unknown	U75725	–	EF547677	EF552451	GQ327958

[a] VKPM, All-Russian Collection of Industrial Microorganisms, Moscow; NRRL, ARS Culture Collection, National Center for Agricultural Utilization Research, Peoria, Illinois, USA; CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands, *T* type strain

[b] Gene sequence accession numbers (GenBank) for D1/D2 LSU, domains 1 and 2, LSU rRNA; ITS, internal transcribed spacer ITS1-5,8S-ITS2; Mt SSU, mitochondrial SSU rRNA; EF-1α, translation elongation factor-1α; RPB1, subunit RPB1 of RNA polymerase II

[c] Both strains of *K. phaffii* had identical sequences for all five genes

Table 2 Genetic divergence of six *Komagataella* species based on pairwise comparison of nucleotide sequences for D1/D2 domain (341 bp), ITS1-5.8S-ITS2 region (277 bp), translation elongation factor-1α (876 bp), subunit RPB1 of RNA polymerase II (828 bp) and mitochondrial SSU rRNA (630 bp), including substitutions (s) and indels (i)

Species pair		*K. phaffii*	*K. populi*	*K. pseudopastoris*	*K. ulmi*	*K. kurtzmanii*
K. pastoris	D1/D2	6s + 4i	4s + 6i	7s + 4i	7s + 3i	5s + 3i
	ITS1-5.8S-ITS2	21s + 11i	18s + 9i	33s + 14i	23s + 21i	21s + 8i
	EF-1α	10s	28s	23s	3s	10s
	RPB1	55s	83s	82s	21s	52s
	Mt SSU	23s + 19i	21s + 19i	33s + 10i	24s + 19i	24s + 19i
K. phaffii	D1/D2		6s + 6i	7s + 6i	4s + 3i	4s + 1i
	ITS1-5.8S-ITS2		24s + 8i	32s + 5i	21s + 14i	13s + 9i
	EF-1α		33s	27s	13s	4s
	RPB1		97s	94s	52s	9s
	Mt SSU		23s + 24i	23s + 19i	1s	1s
K. populi	D1/D2			3s	5s + 7i	5s + 5i
	ITS1-5.8S-ITS2			23s + 12i	35s + 10i	21s + 13i
	EF-1α			13s	29s	32s
	RPB1			11s	73s	93s
	Mt SSU			7s + 17i	22s + 24i	22s + 24i
K. pseudopastoris	D1/D2				6s + 7i	6s + 5i
	ITS1-5.8S-ITS2				20s + 15i	29s + 4i
	EF-1α				24s	27s
	RPB1				74s	90s
	Mt SSU				22s + 19i	22s + 19i
K. ulmi	D1/D2					4s + 4i
	ITS1-5.8S-ITS2					16s + 16i
	EF-1α					13s
	RPB1					49s
	Mt SSU					0

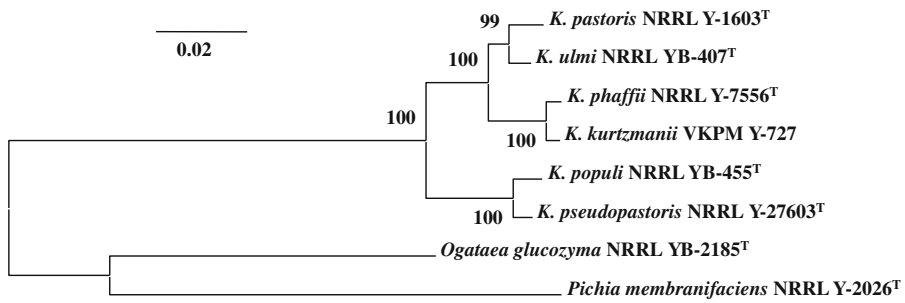

Fig. 1 Neighbour-joining tree showing phylogenetic relationship between the sibling species of the genus *Komagataella* based on combined sequences for D1/D2 and ITS1-5.8S-ITS2 rDNA, translation elongation factor-1α, RNA polymerase II (subunit RPB1), and mitochondrial SSU rRNA. Bootsrap values from 1,000 replications are shown. Bar, 20 estimated base substitutions per 1,000 nucleotide positions. *T* type culture

Dalmau plate culture on morphology agar

After 7 days at 25 °C, growth under the coverglass formed neither hyphae nor pseudohyphae.

Formation of ascospores

Ascosporulation occurs on 5 % ME agar and on different acetate media after 4 days at 25 °C. The best

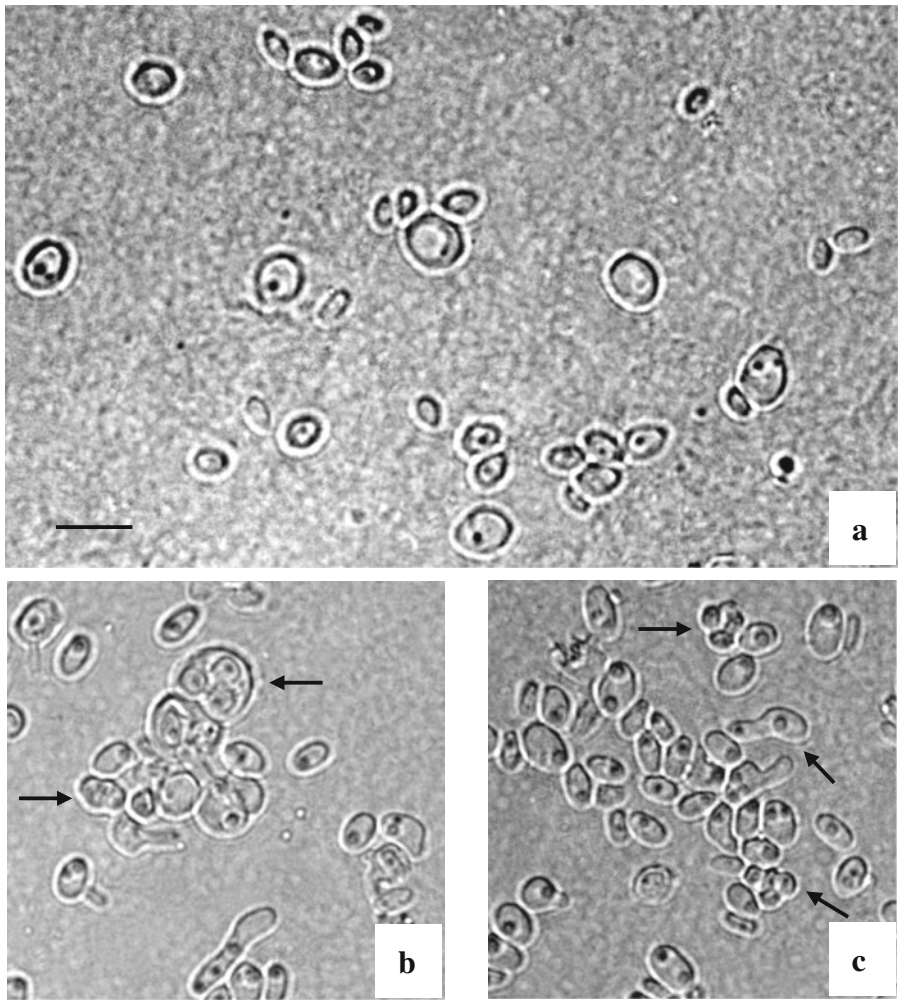

Fig. 2 *Komagataella kurtzmanii* sp. nov. VKPM Y-727. **a** Budding cells at 25 °C on 5 % ME agar at 3 days. **b** Intact asci with four hat-shaped ascospores (the arrows indicate two asci) and **c** zygotes, deliquescent asci (the arrows indicate two asci and a zygote) of 4 days incubation at 25 °C on acetate medium. Bar, 5 μm

sporulation is observed on the medium containing 0.5 % sodium acetate, 1 % potassium chloride, 1 % glucose and 2 % agar. Asci may be unconjugated or show conjugation between a cell and its bud or between independent cells. One to four hat-shaped ascospores are formed in each ascus and they are soon liberated (Fig. 2b, c). In view of conjugation between cells and their buds, the species appears to be homothallic.

Etymology

Komagataella kurtzmanii (kurtz.man'i.i. N.L. gen. masc. n. Kurtzmanii referring to Cletus P. Kurtzman, for his great contributions to yeast taxonomy, in particular studying the genus *Komagatella*.).

Fermentation and growth reactions

Glucose is fermented. Galactose, sucrose, maltose, lactose, raffinose and trehalose are not fermented. Assimilation of carbon compounds: glucose, L-rhamnose, methanol, ethanol, glycerol, DL-lactate and succinate. No growth occurs on galactose, L-sorbose, sucrose, maltose, cellobiose, trehalose, lactose, melibiose, raffinose, melezitose, inulin, soluble starch, D-xylose, L-arabinose, D-arabinose, D-ribose, D-glucosamine, *N*-acetyl-D-glucosamine, erythritol, ribitol, xylitol, L-arabinitol, galactitol, D-mannitol, D-glucitol, methyl α-D-glucoside, salicin, D-gluconate, D-glucoronate, D-galacturonate, 2-keto-D-gluconate, 5-keto-D-gluconate, citrate, inositol and D-glucono-1,5-lacton.

Assimilation of nitrogen compounds: ethylamine and L-lysine. No growth occurs on potassium nitrate, nitrite, D-glucosamine and imidazole.

Growth at 35 °C is negative. Growth on vitamin-free medium is negative. Growth on YM agar with 10 % sodium chloride is negative. Growth in 50 % glucose/yeast extract (0.5 %) is negative. Growth on 1 % acid acetic medium is negative. Growth in the presence of 0.1 % cycloheximide is positive.

Type

VKPM Y-727 (=KBP Y-2878 = UCD-FST 76-20 = Starmer #75-208.2), the type strain, is preserved as a lyophilized preparation in the All-Russian Collection of Industrial Microorganisms, Moscow, Russia, also as CBS 12817, with the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands, and as NRRL Y-63667, with the ARS Culture Collection, National Center for Agricultural Utilization Research, Peoria, Illinois, USA. MycoBank accession number = 803,919. Strain was isolated by W.T. Starmer from a fir flux in the Catalina Mountains, Southern AZ, USA.

The nucleotide sequences of the of 26S rRNA (D1/D2 domain), translation elongation factor-1α (EF-1α), RNA polymerase II (subunit RPB1), Mt SSU rRNA and ITS-region (ITS1-5.8S-ITS2) are deposited in GenBank under the following accession numbers: KC715720, KC715721, KC715722, KC715723 and KC771256, respectively.

Discussion

Based on the results of multigene sequence comparisons and taxonomic phenotypic analysis, a novel member of the genus *Komagataella* is formally described, *K. kurtzmanii* sp. nov. The genus *Komagataella* represents a phylogenetically distinct clade currently containing six sibling species: *K. kurtzmanii*, *K. pastoris*, *P. phaffii*, *K. populi*, *K. pseudopastoris* and *K. ulmi* (Kurtzman 2012; present study). According to our preliminary data, the *Komagataella* species may have the same mating type system. In particular, after mating of auxotrophic mutants of *K. kurtzmanii* and *K. phaffii* on acetate medium, prototrophic hybrids can be selected on minimal medium. The genus *Komagataella* apparently meets the concept of a genetic genus in fungi, which suggests that member species possess a common mating type system allowing them to be crossed in any combination (Naumov 1979, 1988). Due to postzygotic isolation, the resulting interspecies hybrids are sterile, having non-viable ascospores. Note that the genetic genera form well separated clusters in phylogenetic trees based on sequence data of rRNA genes (Kurtzman and Robnett 1998). Initially proposed for some *Saccharomyces* yeasts and mycelium fungus *Neurospora,* the concept of genetic genus was successfully applied to many polytypic ascomycetous genera, viz. *Saccharomyces* (Naumov 1987a, 1996; Naumov et al. 2000, 2010), *Williopsis* (Naumov 1987b), *Arthroascus* (Naumov et al. 2006), *Kluyveromyces* (syn. *Zygofabospora*) (Naumov 1986; Naumov and Naumova 2002), *Zygowilliopsis* (Naumov et al. 2009), *Galactomyces* (Naumova et al. 2001) and *Ogataea* (*Hansenula*) *polymorpha* complex (Naumov et al. 1997).

The six species currently assigned to the genus *Komagataella* are phenotypically very similar (Table 3). The proposed new species *K. kurtzmanii* differs from the others by absence of growth at 35 °C and inability to assimilate trehalose, D-glucitol, D-mannitol. Taking into account a high variability of physiological properties in yeasts (Kudrjawzev 1960; Scheda and Yarrow 1966) and the fact that *K. kurtzmanii*, *K. populi* and *K. ulmi* are described based on single strains, it seems difficult to separate all six *Komagataella* species from one another solely by conventional physiological tests. Thus, multigene sequence comparisons must be used for reliable identification of *Komagataella* species.

Of the five molecular markers used in this study, the D1/D2, ITS1-5.8S-ITS2, EF-1α and RPB1 gave congruent resolutions for species differentiation. In the case of the Mt SSU sequence, it is impossible to distinguish *K. phaffii*, *K. ulmi* and *K. kurtzmanii*. Earlier, it was noticed that species phylogeny based on nuclear and mitochondrial gene sequences can be quite different in fungi (Wu et al. 2008; Schoch et al. 2012). The ITS region is recently recommended as a universal DNA barcode marker for species discrimination in fungi (Schoch et al. 2012). This region of rRNA is characterized by a significant interspecies divergence and a low level of intraspecies polymorphism. Indeed, all six species of *Komagataella* can be clearly differentiated by the ITS-sequences, which differ by 13–35 nucleotides and numerous indels. On the other hand, no intaspecific variation was observed

Table 3 Physiological characteristics of sibling species of the genus *Komagataella*

No[a]	Physiological test[b]	*K. kurtzmanii*	*K. pastoris*	*K. phaffii*	*K. populi*[c]	*K. pseudopastoris*[d]	*K. ulmi*[c]
Fermentation							
F1	Glucose	+	+	+	+	+	+
F2	Galactose	–	–	–	–	–	–
F3	Maltose	–	–	–	–	–	–
F5	Sucrose	–	–	–	–	–	–
F6	Trehalose	–	–	–	w	–	w
F8	Lactose	–	–	–	–	–	–
F11	Raffinose	–	–	–	–	–	–
Growth							
C1	Glucose	+	+	+	+	+	+
C2	Galactose	–	–	–	–	–	–
C3	L-Sorbose	–	–	–	–	–	–
C4	D-Glucosamine	–	–	–	–	–	–
C5	D-Ribose	–	–	–	–	–	–
C6	D-Xylose	–	–	–	–	s	–
C7	L-Arabinose	–	–	–	–	–	–
C8	D-Arabinose	–	–	–	–	s/-	–
C9	L-Rhamnose	+	+	+	+	+	+
C10	Sucrose	–	–	–	–	–	–
C11	Maltose	–	–	–	–	–	–
C12	Trehalose	–	+	+	+	+	+
C13	Methyl-α-D-glucoside	–	–	–	–	–	–
C14	Cellobiose	–	–	–	–	–	–
C15	Salicin	–	–	–	–	–	–
C16	Arbutin	–	–	–	n	–	n
C17	Melibiose	–	–	–	–	–	–
C18	Lactose	–	–	–	–	–	–
C19	Raffinose	–	–	–	–	–	–
C20	Melezitose	–	–	–	–	–	–
C21	Inulin	–	–	–	–	–	–
C22	Soluble starch	–	–	–	–	–	–
C23	Glycerol	+	+	+	+	+	+
C24	Erythritol	–	–	–	–	–	–
C25	Ribitol	–	–	–	–	–	–
C26	Xylitol	–	–	–	n	–	n
C27	L-Arabinitol	–	–	–	n	–	n
C28	D-Glucitol	–	+	+	+	+	+
C29	D-Mannitol	–	+	+	+	+	+
C30	Galactitol	–	–	–	–	–	–
C31	*myo*-Inositol	–	–	–	–	–	–
C32	D-Glucono-1,5-lactone	–	–	–	n	–	n
C33	2-Keto-D-gluconate	–	–	–	–	–	–
C34	5-Keto-D-gluconate	–	–	–	–	n	–
C35	D-Gluconate	–	–	–	–	–	–
C36	D-Glucuronate	–	–	–	n	–	n

Table 3 continued

No[a]	Physiological test[b]	*K. kurtzmanii*	*K. pastoris*	*K. phaffii*	*K. populi*[c]	*K. pseudopastoris*[d]	*K. ulmi*[c]
C37	D-Galacturonate	–	–	–	n	–	n
C38	DL-Lactate	+	+	+	w	+	+
C39	Succinate	+	+	+	+	+	+
C40	Citrate	–	–	–	–	–	–
C41	Methanol	+	+	+	+	+	+
C42	Ethanol	+	+	+	+	+	+
C48	*N*-Acetyl-D-glucosamine	–	–	–	–	–	–
N1	Nitrate	–	–	–	–	–	–
N2	Nitrite	–	–	–	n	–	n
N3	Ethylamine	+	+	+	n	+	n
N4	L-Lysine	+	+	+	n	+	n
N8	Glucosamine	–	–	–	n	–	n
N9	Imidazole	–	–	–	n	–	n
V1	w/o Vitamins	–	–	–	–	+/w	–
02	Cycloheximide 0.1 %	+	+	+	n	+	n
03	Acetic acid 1 %	–	–	–	n	–	n
	10 % NaCl/5 % Glucose	–	–	–	–	–	–
	Growth on 50 % w/w glucose yeast extract agar	–	–	–	–	–	n
	Growth at 35 °C	–	+	+	n	+	n
	Growth at 37 °C	–	+	+	w	n	w

[a] Numbering of physiological tests is given according to the CBS (www.cbs.knaw.nl)

[b] −Negative, + positive, *w* weak, *s* slow, *v* variable, *n* no data

[c] Fermentation and assimilation data are from Kurtzman (2012)

[d] Fermentation and assimilation data are from Dlauchy et al. (2003)

for the ITS-sequences in *K. pastoris* and *K. phaffii* (Kurtzman 2009).

At present only single strains are known for *K. populi*, *K. ulmi* and *K. kurtzmanii*, probably because the complex composition of the genus *Komagataella* has been established recently (Kurtzman 2005, 2011, 2012). ITS sequence comparisons of natural isolates and strains maintained in different yeast collections may result in finding new *Komagataella* species, as well as additional strains of the known species. Taking into account great industrial importance of *K. pastoris* and *K. phaffii*, their sibling species may serve as a new gene pool for basic and applied studies.

Acknowledgments Synthesis of oligonucleotide primers and sequencing of the D1/D2 26S rRNA, translation elongation factor-1α, RNA polymerase II, mitochondrial small subunit rRNA and ITS1-5.8S-ITS2 were performed using the equipment of the Centre for Collective Use of GosNIIgenetika (Moscow).

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