ISSN 0026-2617, Microbiology, 2012, Vol. 81, No. 2, pp. 216–223. © Pleiades Publishng, Ltd., 2012.
Original Russian Text © E.S. Naumova, G.I. Naumov, T.N. Nikitina, A.Zh. Sadykova, V.I. Kondratieva, 2012, published in Mikrobiologiya, 2012, Vol. 81, No. 2, pp. 236–243.

EXPERIMENTAL ARTICLES

Molecular Genetic and Physiological Differentiation of *Kluyveromyces lactis* and *Kluyveromyces marxianus*: Analysis of Strains from the All-Russian Collection of Microorganisms (VKM)

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Received May 15, 2011

Abstract—Molecular genetic identification of 52 *Kluyveromyces* strains from VKM, mainly of dairy origin, was carried out. Restriction analysis of 5.8S-ITS rDNA fragments was used to differentiate between *Kl. lactis* var. *lactis*, *Kl. lactis* var. *drosophilarum* (European population of "krassilnikovii"), and *Kl. marxianus*. *Kl. lactis* was shown to differ from *Kl. marxianus* in its ability to assimilate α-glucosides: maltose, melezitose, and α-methyl-glucoside.

Keywords: dairy yeasts, *Kluyveromyces marxianus, Kluyveromyces lactis*, 5.8S-ITS fragment, IGS2 rDNA, maltose and lactose utilization.

DOI: 10.1134/S0026261712020087

Dairy strains of *Kluyveromyces marxianus* and *Kl. lactis* are among the few microorganisms able to ferment lactose (Lac^+). This feature is rare in yeasts: only 1% of over 700 known yeast species possess it [1]. These yeasts are important in fundamental and applied research.

Mammals are the main sources of lactose. The origin of the dairy yeasts is obviously evolutionary linked with the origin of mammals. Comparative molecular genetics of lactose fermentation in *Kl. marxianus* and *Kl. lactis* may reveal the intra- and interspecies evolution of these yeasts [2–10].

Kluyveromyces (syn. *Zygofabospora, Fabospora*) utilize milk whey lactose and are used for forage, enzyme production, ethanol and low-alcohol drinks production [11–14]. Their role as probiotics able to form antibacterial [15, 16] and antiyeast [17–19] toxins can not be excluded. In addition to their food importance, these yeasts have sanitary significance for humans and animals being, the component of dairy products and their waste, milk whey.

Morphological and physiological tests for yeast taxonomy have been recently supplemented by various molecular methods including sequencing and restriction analysis of ribosomal DNA fragments. Analysis of the D1/D2 26S rDNA domain is most frequently applied in molecular taxonomy of yeasts [20, 21]. The scale is accepted, according to which the differences in 6 and more nucleotides (>1%) in D1/D2 region indicate that the strains belong to different species, while identical sequences in this region or differences in 1 to 3 nucleotides usually indicate conspecificity of the analyzed strains. In closely related species *Kl. lactis* and *Kl. marxianus*, the D1/D2 regions differ only in one nucleotide, while other four species of the genus *Kluyveromyces* (*Kl. dobzhanskii, Kl. wickerhamii, Kl. aestuarii*, and *Kl. nonfermentas*) exhibit up to 7–19 nucleotide substitutions. To analyze the closely related yeast taxa, restriction analysis of non-coding rDNA regions is used more frequently: the 5.8S-ITS fragment including the 5.8S rRNA gene and internal transcribed spacers ITS1/ITS2, as well as the intergenic spacer 2 (IGS2) [21, 22].

To widen the scientific and practical application of the natural gene pool of *Kl. marxianus* and *Kl. lactis*, we reidentified the original strains from the All-Russian Collection of Microorganisms (VKM) using modern molecular techniques. Most of these strains are presented in the catalogue [23], and their initial description is given in the Yeast manual [24]. Previously the yeasts which efficiently fermented lactose were selected from these strains [11].

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MATERIALS AND METHODS

Strains and culture media. The strains of the genus *Kluyveromyces* used in this study and their origin are shown in the table. The type cultures of *Kl. lactis* VKM Y-868 and *Kl. marxianus* CBS 712 (=VKM Y-876) were used as the controls. Yeasts were cultivated at 28°C on a complete YPD culture medium containing the following (g/L): glucose, 20; yeast extract, 10; agar, 20. Physiological and biochemical characterization of the yeasts was carried out under the standard conditions [25]. For identification of yeasts, maltose (Sigma, United States), lactose (Merk, Germany), α-methyl-glucoside (Calbiochem, United States), and melezitose (Serva, The Netherlands) were used.

Molecular methods. PCR was carried out directly on yeast cells using a Bio-Rad DNA cycler (United States). A small amount of yeast biomass (on the tip of a microbiological loop) was resuspended in 30 μL of the PCR buffer containing 3 mM of $MgCl_2$, 0.3 mM of dNTP, and 50 pmol of each primer. The mixture was incubated at 95°C for 15 min for the cell lysis and then supplemented with 2.5 U of *Taq* DNA polymerase (Syntol, Russia). For amplification of the 5.8S-ITS-fragment, primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') were used. For amplification of the intergenic spacer 2 (IGS2), primers NTS2 (5'-AACGGTGCTTTCTGGTAG-3') and ETS1 (5'-TGTCTTCAACTGCTTT-3') were used. Amplification conditions (30 cycles) were as follows: initial denaturing at 95°C for 30 s, annealing at 56°C for 30 s, and the DNA elongation at 72°C for 60 s. The PCR products were separated in a 1% agarose gel at 60–65 V in 0.5× TBE buffer (45 mM of Tris, 10 mM of EDTA, 45 mM of boric acid) for 1.5 h and stained with ethidium bromide.

The analysis of polymorphism of restriction fragments (RFLP analysis) of 5.8S-ITS and IGS2 rDNA regions was carried out using *Hin*dIII and *Alu*I (Fermentas, Lithuania) endonucleases, respectively. Restriction fragments were separated in a 2.5% agarose gel at 50–55 V in 0.5× TBE buffer for 4 h. The gel was stained with ethidium bromide for 2–3 h, washed with distilled water, and photographed using a Vilber Lourmat UV transilluminator (France).

RESULTS

Molecular identification of the strains. *Kl. lactis* and *Kl. marxianus* species with almost identical sequences of the 26S rDNA D1/D2 domains showed significant differences in the sequences of the ITS1/ITS2 region: 23 nucleotide substitutions were identified (Fig. 1). These strains may be differentiated by RFLP analysis using *Hin*dIII endonuclease. *Kl. marxianus* have a *Hin*dIII restriction site (a/agctt) in the ITS fragment, while in *Kl. lactis* this site is absent due to the T–G transversion in position 548 (the numeration is given according to the ITS sequence of *Kl. marxianus* CBS 712 type culture) (Fig. 1).

Amplification of the 5.8S-ITS rDNA region in 52 yeast strains obtained from the All-Russian Collection of Microorganisms was carried out (table). The size of the resulting PCR products (~720 bp) was equal for the studied and control strains. This suggests the assignment of the yeasts under study to the genus *Kluyveromyces* [26]. The subsequent restriction analysis was carried out using the *Hin*dIII endonuclease. According to the similarity of restriction profiles, the yeasts under study formed two groups. The RFLP profiles of some strains are shown in Figure 2. According to their restriction profiles, most of the strains did not differ from the type culture of *Kl. marxianus* CBS 712: two *Hin*dIII fragments of approximately 570 and 150 bp (Fig. 2, lanes *1–6*). The type culture *Kl. lactis* VKM Y-868 and 12 investigated strains lacking the *Hin*dIII restriction sites (VKM Y-869, VKM Y-870, VKM Y-1186, VKM Y-1333, VKM Y-1334, VKM Y-1339, VKM Y-1343, VKM Y-1868, VKM Y-830, VKM Y-831, VKM Y-834, and VKM Y-1890) belonged to the second group (Fig. 2, lanes *7–9*). According to the RFLP analysis, eight strains which have been deposited in the VKM as *Zygofabospora marxiana* and *Fabospora fragilis* were identified as *Kl. marxianus* (table). Among 37 of the investigated *Kl. lactis* strains, 29 were reidentified by molecular analysis as *Kl. marxianus*. Among seven *Zygofabospora krassilnikovii* strains, three were identified as *Kl. marxianus* (VKM Y-835, VKM Y-836, VKM Y-837), and others (VKM Y-830, VKM Y-831, VKM Y-834, and VKM Y-1890) were identified as *Kl. lactis*. Notably, the latter four strains were isolated from natural sources and lack the ability to ferment lactose (table).

The species *Kl. lactis* has a complex composition and includes two varieties: *Kl. lactis* var. *lactis* and *Kl. lactis* var. *drosophilarum* [26–28]. The latter taxon consists of eight genetic populations: European "krassilnikovii", African "vanudenii", Asian "oriental", and of five North American populations—"drosophilarum", "phaseolosporus", "pseudovanudenii", "aquatic", and "new" [4]. At least some of the populations show partial genetic isolation. Using restriction analysis of the intergenic IGS2 spacer, it is possible to differentiate between the dairy yeast *Kl. lactis* var. *lactis* and the wild yeast *Kl. lactis* var. *drosophilarum* which are not able to ferment lactose [4, 7]. Amplification of the IGS2 rDNA fragment in 12 strains identified previously as *Kl. lactis* was carried out with the NTS2 and ETS1 primers. The size of amplified IGS2 fragments was equal for the strains under study and *Kl. lactis* VKM Y-868 type culture: ~1200 bp. The PCR products were analyzed by enzymatic cleavage with the *Alu*I endonuclease (Fig. 3). According to the *Alu*I profiles, the strains VKM Y-869, VKM Y-870, VKM Y-1186, VKM Y-1333, VKM Y-1334, VKM Y-1339, VKM Y-1343, VKM Y-1868 belonged to *Kl. lactis* var. *lactis*. They had the same

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CBS 712      AAGATTATGAATGAATAGATTACTGGGGGAATCGTCTGAACAAGGCCTGCGCTTAATTGC  60
BKM Y-868    .............G.................-.....T.T.................

CBS 712      GCGGCCAGTTCTTGATTCTCTGCTATCAGTTTTCTATTTCTCATCCTAAACACAATGGAG 120
BKM Y-868    .....T.A.........T.................T.C.....................

CBS 712      TTTTTTCTCTATGAACTACTTCCCTGGAGAGCTCGTCTCTCCAGTGGACATAAACACAAA 180
BKM Y-868    ............................................................

CBS 712      CAATATTTTGTATTATGAAAAACTATTATACTATAAAATTTAATATTCAAAACTTTCAAC 240
BKM Y-868    ...C......C.................-..TC.AG.......................

CBS 712      AACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCGAATTGCGATATGTATTGTGA 300
BKM Y-868    ............................................................

CBS 712      ATTGCAGATTTTCGTGAATCATCAAATCTTTGAACGCACATTGCGCCCTCTGGTATTCCA 360
BKM Y-868    ............................................................

CBS 712      GGGGGCATGCCTGTTTGAGCGTCATTTCTCTCTCAAACCTTTGGGTTTGGTAGTGAGTGA 420
BKM Y-868    ............................................................

CBS 712      TACTCGTCTC–GGGTTAACTTGAAAAGTGGCTAGCCGTTGCCATCTGCGTGAGCAGGGCTGC480
BKM Y-868    .......T.TTC.............................T...................

CBS 712      GTGTCAAGTCTATGGACTCGACTCTTGCACATCTACGTCTTAGGTTTGCGCCAATTCGTG 540
BKM Y-868    ............................................................
                ↓HindIII
CBS 712      GTAAGCTT-GGGTCATAGAGACTCATAGGTGTTATAAAGACTCGCTGGTGTTTGTCTCCTT 600
BKM Y-868    ......GA......AT...-..T.....................................

CBS 712      GAGGCATACGGCTTTAACCAAAACTCTCAAAGT 633
BKM Y-868    ....-.........-..T..............
```

Fig. 1. Nucleotide sequences of the 5.8S-ITS rDNA region of *Kluyveromyces marxianus* CBS 712 and *Kl. lactis* VKM Y-868 type cultures. Identical nucleotide sequences are indicated with points. Numbering of the sequences is given according to the strain CBS 712. The *Hin*dIII restriction site is shown in grey.

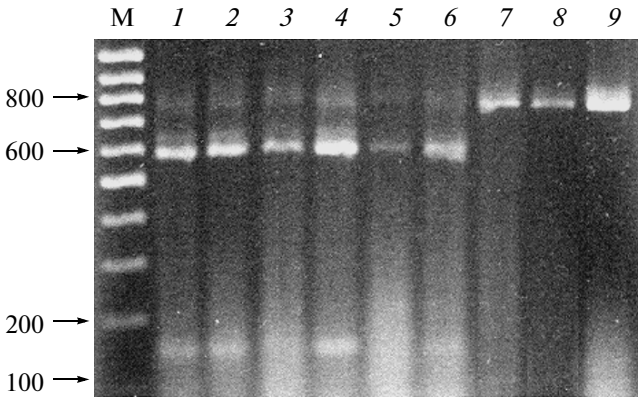

Fig. 2. RFLP analysis of the amplified 5.8S-ITS rDNA fragments of *Kl. marxianus* and *Kl. lactis* using *Hin*dIII restriction endonuclease: CBS 712 (*1*); VKM Y-453 (*2*); VKM Y-470 (*3*); VKM Y-883 (*4*); VKM Y-1332 (*5*); VKM Y-2013 *Kl. lactis* (*6*); VKM Y-868 (*7*); VKM Y-1333 (*8*); VKM Y-1890 (*9*). M is the molecular weight marker (bp) 100 bp DNA Ladder (Fermentas, Lithuania).

restriction profiles as the VKM Y-868 type culture: four restriction fragments of ca. 650, 250, 200, and 100 bp (Fig. 3, lanes *1*–*4*). The strains VKM Y-830, VKM Y-831, VKM Y-834, and VKM Y-1890, which are unable to utilize lactose, formed the second group, characterized by three fragments of ~650, 450, and 100 bp (Fig. 3, lanes *5*–*8*). These strains belonged to the European "krassilnikovii" population of the *Kl. lactis* var. *drosophilarum*.

Physiological features of the investigated strains. The possibility of using physiological characteristics for identification of the dairy yeasts species was reinvestigated [29]. Earlier, the ability of the strains to assimilate some α-glucosides (maltose, melezitose, α-methyl-glucoside) and the absence of growth at elevated temperatures (37–42°C) have been suggested for the differentiation of *Kl. lactis* and *Kl. marxianus* [30–32]. According to the assimilation of α-glucosides, the strains were precisely divided into two groups (table). All the strains identified by molecular

analysis as *Kl. marxianus* were unable to assimilate maltose, melezitose and α-methyl-glucoside. The strains of *Kl. lactis* var. *lactis* and *Kl. lactis* var. *drosophilarum* (European population "krassilnikovii"), which were able to assimilate these α-glucosides, formed the second group.

It is important to note that phenotypic determination of *Kluyveromyces* species deposited in the VKM by assimilation of α-glucosides which has been carried out previously [29] mainly gives identical results with their molecular identification (except the strains whose ability to assimilate some α-glucosides was probably incorrectly determined). It is well known that determination of yeast growth on the minimal agar media frequently gives uncertain results.

DISCUSSION

The molecular markers have been presently found that make it possible to differentiate the dairy yeast *Kl. marxianus* and *Kl. lactis* var. *lactis* [22], as well as the latter and *Kl. lactis* var. *drosophilarum* (European population "krassilnikovii") [4, 7]. This allows us to carry out reliable molecular identification of *Kl. lactis* and *Kl. marxianus*, especially in the case of strains of dairy origin.

Using RFLP analysis of the non-coding rDNA regions and some physiological features, 52 yeast strains of *Kluyveromyces* were studied (table). The molecular approach made it possible to conduct total reidentification of the Russian dairy yeast strains of the genus *Kluyveromyces* deposited in the All-Russian Collection of Microorganisms. Molecular analyses demonstrated that most dairy strains previously assigned to the species *Kl. lactis* belonged to the species *Kl. marxianus* (table). In addition, the yeasts *Zygofabospora krassilnikovii* were shown to be heterogeneous. Of seven strains, only four were assigned to *Kl. lactis* var. *drosophilarum* (population "krassilnikovii"), and three strains, to *Kl. marxianus*. Notably, similar mistakes in identification of *Kl. marxianus* and *Kl. lactis* have been made in other collections, such as Centraalbureau voor Schimmelcultures (CBS). Van der Walt [29] previously has identified a set of strains as *Kl. vanudenii* (syn. *Kl. lactis* var. *drosophilarum*—an African population of "vanudenii" [4]). Only the type culture CBS 4372 was identified correctly. Molecular identification demonstrated [3, 33] the strains CBS 5669 and CBS 5670 to belong to *Kl. marxianus* [34, 35] but not to *Kl. lactis*, as has been suggested earlier [36].

A correlation between the results of molecular analyses and physiological tests for α-glucoside assimilation was revealed. According to RFLP analysis, all the strains able to assimilate α-glucosides were related to the varieties of *Kl. lactis* var. *lactis* and *Kl. lactis* var. *drosophilarum* (European population "krassilnikovii"). Taking into account the availability and low price of maltose, we suggest using the test for maltose assimilation for the differentiation of *Kl. marxianus* and *Kl. lactis*.

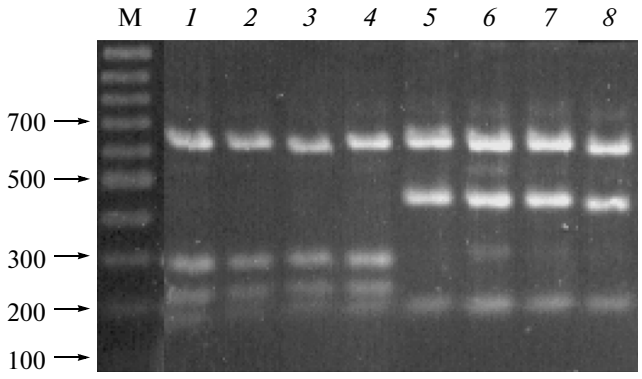

Fig. 3. RFLP analysis of the amplified fragments of the rDNA intergenic spacer IGS2 of *Kl. lactis* var. *lactis* and *Kl. lactis* var. *drosophilarum* (European population "krassilnikovii") with *Alu*I restriction endonuclease. *Kl. lactis* var. *lactis*: VKM Y-868 (*1*); VKM Y-870 (*2*); VKM Y-1333 (*3*); VKM Y-1343 (*4*); *Kl. lactis* var. *drosophilarum* (population "krasilnikovii"): VKM Y-830 (*5*); VKM Y-831 (*6*); VKM Y-834 (*7*); VKM Y-1890 (*8*). M is the molecular weight marker (bp) 100 bp DNA Ladder (Fermentas, Lithuania).

The results of this molecular study show that the PCR-RFLP analysis of the rDNA 5.8S-ITS and IGS2 fragments makes it possible to carry out rapid and precise identification of dairy yeasts *Kl. marxianus* and *Kl. lactis*, as well as to differentiate between the varieties of the latter species. The procedure of molecular identification takes no more than 4–5 days, including cultivation of the yeasts on solid culture media (2 days), PCR using the yeast cells, and subsequent RFLP analysis (2–3 days).

Additional study of maltose utilization in 29 strains (as described in [4]) of *Kl. lactis* var. *drosophilarum* from North American populations, demonstrated that all of these strains except the type strain from the "phaseolosporus" population have the phenotype Mal^+. Since any phenotypic feature is subjected to genetic variability, indication of the rare natural mutant Mal^- does not discredit the application of this diagnostic feature for preliminary differentiation of *Kl. lactis* and *Kl. marxianus*.

Thus, according to our reidentification, most *Kluyveromyces* strains from the VKM which are capable of active lactose fermentation [11] were found to belong to *Kl. marxianus*.

ACKNOWLEDGEMENTS

We thank W.I. Golubev for the participation in assimilation and fermentation tests. The work was supported by the Russian Foundation for Basic Research, project no. 09-04-00664. Oligonucleotide primers were synthesized using the equipment of the

Yeasts strains of the genus *Kluyveromyces* from The All-Russian Collection of Microorganisms used in the study

Original species name and VKM Y-number	Author* and number in other collections	Source and site of isolation	Sugar assimilation**				Established species name
			Lac	Mal	Amg	Mez	
		Kluyveromyces lactis					
450	Tsigankov M.F.	Chal, Turkmenistan	+	–	–	–	*Kl. marxianus*
451	Tsigankov M.F., chal 15	The same	+	–	–	–	*Kl. marxianus*
452	Tsigankov M.F.	The same	+	–	–	–	*Kl. marxianus*
453	Unknown	Mazun, Armenia	+	–	–	–	*Kl. marxianus*
454	Unknown	The same	+	–	–	–	*Kl. marxianus*
455	Unknown	The same	+	–	–	–	*Kl. marxianus*
459	Kudriavtsev V.I., no. 701	Curd cheese, Yelets	+	–	–	–	*Kl. marxianus*
460	Kudriavtsev V.I., no. 702	The same	+	–	–	–	*Kl. marxianus*
461	Unknown	Armenia, Yerevan	+	–	–	–	*Kl. marxianus*
462	Kudriavtsev V.I., tvor. 1	Curd cheese	+	–	–	–	*Kl. marxianus*
464	Kudriavtsev V.I., var .6	Fermented boiled milk	+	–	–	–	*Kl. marxianus*
465	Kudriavtsev V.I., no. 1	Chal, Turkmenistan	+	–	–	–	*Kl. marxianus*
466	Tsigankov M.F.	The same	+	–	–	–	*Kl. marxianus*
467	Tsigankov M.F.	The same	+	–	–	–	*Kl. marxianus*
468	Tsigankov M.F.	The same	+	–	–	–	*Kl. marxianus*
469	Tsigankov M.F.	The same	+	–	–	–	*Kl. marxianus*
470	Tsigankov M.F.	The same	+	–	–	–	*Kl. marxianus*
471	Tsigankov M.F.	The same	+	–	–	–	*Kl. marxianus*
472	Tsigankov M.F.	The same	+	–	–	–	*Kl. marxianus*
473	Tsigankov M.F.	The same	+	–	–	–	*Kl. marxianus*
474	Tsigankov M.F.	The same	+	–	–	–	*Kl. marxianus*
476	Tsigankov M.F., no. 37	The same	+	–	–	–	*Kl. marxianus*
869	Kudriavtsev V.I., Z85 sev.	Soured milk, Kola Peninsula	+	+	+	+	*Kl. lactis*
870	Tsigankov M.F., chal 8	Chal, Turkmenistan	+	+	+	+	*Kl. lactis*
1186	Kudriavtsev V.I., CM14	Milk, Kiev, Ukraine	+	+	+	+	*Kl. lactis*
1332	Skorodumova A.M., 153/15	Curd cheese, Kislovodsk	+	–	–	–	*Kl. marxianus*
1333	Skorodumova A.M., 154/21	Soured milk, Budenovsk	+	+	+	+	*Kl. lactis*
1334	Skorodumova A.M., 155/22	The same	+	+	+	+	*Kl. lactis*

Table. (Contd.)

Original species name and VKM Y-number	Author* and number in other collections	Source and site of isolation	Sugar assimilation**				Established species name
			Lac	Mal	Amg	Mez	
1336	Skorodumova A.M., 171/25	Milk, Karachaevo-Cherkessia	+	–	–	–	*Kl. marxianus*
1337	Skorodumova A.M., 159/28	Clabber, Pyatigorsk	+	–	–	–	*Kl. marxianus*
1338	Skorodumova A.M., 160/29	The same	+	–	–	–	*Kl. marxianus*
1339	Skorodumova A.M., 161/33	Sour cream, Leningrad	+	+	–	–	*Kl. lactis*
1341	Skorodumova A.M., 164/36	Milk, Kareliya	+	–	–	–	*Kl. marxianus*
1342	Skorodumova A.M., 165/120	Dairy plant, Tyumen' region	+	–	–	–	*Kl. marxianus*
1343	Romanovich T.G., no. 166	Milk, Gomel region, Belorussia	+	+	+	+	*Kl. lactis*
1868	Tsigankov M.F.	Chal, Turkmenistan	+	+	+	+	*Kl. lactis*
2454	IBPM Y-583=CCY 21-3-1	–	+	–	–	–	*Kl. marxianus*
		Zygofabospora krassilnikovii					
830	Kudriavtsev V.I., No. 1	Air	–	+	+	+	*Kl. lactis****
831	Kudriavtsev V.I., Kaluga 2	Oak exudate, Kaluga	–	+	+	+	*Kl. lactis****
834	Kudriavtsev V.I., Kaluga 1	The same	–	+	+	+	*Kl. lactis****
835	Kudriavtsev V.I., L-1	Hydrolysis plant, Labvinsk	+	–	–	–	*Kl. marxianus*
836	Kudriavtsev V.I., SD-8	Hydrolysis plant, Saratov	+	–	–	–	*Kl. marxianus*
837	Kudriavtsev V.I., T-4	Hydrolysis plant, Tavda	+	–	–	–	*Kl. marxianus*
1890	Kudriavtsev V.I.	Oak exudate, Kaluga, Russia	–	+	+	+	*Kl. lactis****
		Zygofabospora marxiana					
832	Kudriavtsev V.I., no. 734	Soil, Moscow	+	–	–	–	*Kl. marxianus*
833	Kudriavtsev V.I., no. 739	The same	+	–	–	–	*Kl. marxianus*
2013	CCY 50-2-4	Sugar beet, Slovakia	+	–	–	–	*Kl. marxianus*
		Fabospora fragilis					
126	Kudriavtsev V.I., no. 2	Soured milk, Russia	+	–	–	–	*Kl. marxianus*
431	Kudriavtsev V.I., 84 sev.	Milk, Kola Peninsula	+	–	–	–	*Kl. marxianus*
1335	Skorodumova A.M., 156/24	Milk, Karachaevo-Cherkessia	+	–	–	–	*Kl. marxianus*
432	Mrak E.M., no. 104	Spoiled pulled figs, United States	+	–	–	–	*Kl. marxianus*
433	Mrak E.M., no. 106	The same	+	–	–	–	*Kl. marxianus*

Notes: * Description of the strains by Skorodumova A.M. can be found in [16].

** "+" and "–" indicate assimilation of lactose (Lac), maltose (Mal), α-methyl-glucoside (Amg), and melezitose (Mez), and the absence of assimilation, respectively.

*** Complete species name is *Kl. lactis* var. *drosophilarum* (European population "krassilnikovii").

Centre for Collective Use of GosNIIgenetika, having partial financial support from the Ministry of Education and Science of the Russian Federation (state contract no. 16.552.11.7029).

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