Microbiology, Vol. 74, No. 3, 2005, pp. 329–335. Translated from Mikrobiologiya, Vol. 74, No. 3, 2005, pp. 387–393.

EXPERIMENTAL ARTICLES

Molecular Markers for Differentiation between the Closely Related Dairy Yeast *Kluyveromyces lactis* var. *lactis* and Wild *Kluyveromyces lactis* Strains from the European "Krassilnikovii" Population

E. S. Naumova, N. N. Sukhotina, and G. I. Naumov[1]

State Research Institute of Genetics and Selection of Industrial Microorganisms (GosNIIGenetika), Pervyi Dorozhnyi proezd 1, Moscow, 117545 Russia

Received November 18, 2004

Abstract—A comparative molecular genetic study of 37 *Kluyveromyces* strains of different origin has made it possible to find molecular markers that can differentiate between the dairy yeast *Kluyveromyces lactis* var. *lactis* and the genetically close wild *Kl. lactis* strains from the European "krassilnikovii" population, which are unable to ferment lactose. A restriction fragment length polymorphism analysis of the IGS2 rDNA region reveals two different *Alu*I profiles, one of which corresponds to *Kl. lactis* var. *lactis* while the other corresponds to yeasts from the "krassilnikovii" population. The *Alu*I restriction profile of the IGS2 region of the rDNA also makes it possible to differentiate between the physiologically similar species *Kl. marxianus* and *Kl. lactis.* The origin of clinical *Kl. lactis* var. *lactis* isolates is discussed.

Key words: dairy yeasts, *Kl. lactis* var. *lactis, Kl. marxianus*, "krassilnikovii" population, RFLP analysis, IGS2 rDNA.

The taxonomic position of yeasts of the genus *Kluyveromyces* has been repeatedly revised. The yeast species *Kl. lactis* and *Kl. marxianus*, as well as their wild relatives *Kl. dobzhanskii* and *Kl. wickerhamii*, were originally assigned to the genus *Saccharomyces* [1]. However, in 1965, van der Walt revised the original description of the genus *Kluyveromyces.* In particular, he enriched this initially polysporous genus with the oligosporous species *Saccharomyces marxianus* and other related yeasts [2]. A recent genetic analysis and sequencing of ribosomal genes have revealed a polyphily in the genus *Kluyveromyces* van der Walt emend. van der Walt [3–7]. These studies showed that a group of four allied species (*Kl. lactis, Kl. marxianus, Kl. dobzhanskii*, and *Kl. wickerhamii*), together with the taxonomically close species *Kl. aestuarii* and *Kl. nonfermentas*, forms a separate cluster with a bootstrap value of 96% within the *"Saccharomyces"* clade [6]. These six species were combined into a separate genus, *Zygofabospora* Kudriavzev emend. G. Naumov [3, 4, 7]. Recently, Kurtzman *et al.* [8, 9] have proposed conserving the genus *Kluyveromyces.* It should be noted that the species composition and the type species of the genera *Zygofabospora* and *Kluyveromyces* Kurtzman *et al.* nom. cons. are the same.

Genetic hybridization analysis and molecular karyotyping have shown that the species *Kl. lactis* includes not only lactose-fermenting (Lac^+) cultured yeasts, but also wild Lac^- strains [7], the latter being represented by the genetically isolated (in part) populations "krassilnikovii," "drosophilarum," "phaseolosporus," and "vanudenii." Hybridization analysis has also shown that wild yeasts from the European "krassilnikovii" population are ancestors of the cultured dairy yeast *Kl. lactis* var. *lactis* [7, 10]. "Krassilnikovii" × var. *lactis* hybrids are highly fertile and exhibit a regular segregation of control markers. The yeasts of the "krassilnikovii" population differ from *Kl. lactis* var. *lactis* only in their inability to ferment lactose.

Along with morphological and physiological criteria, the recent systematics of yeasts has widely employed various molecular genetic methods, including sequencing and restriction fragment length polymorphism (RFLP) analysis of various regions of ribosomal DNA. Closely related yeast taxa can be differentiated by an RFLP analysis of the noncoding regions of their rDNA: internal transcribed spacers (ITS1 and ITS2) and intergenic spacer 2 (IGS2) [11–13].

The aim of this work was a comparative study of the collection strains of *Kl. lactis* by means of RFLP analysis of the IGS2 region of their rDNA, molecular karyotyping, and PCR analysis with the microsatellite primer $(GTG)_5$. The molecular markers that we revealed make it possible to differentiate between wild *Kl. lactis* strains

[1] Corresponding author; e-mail: gnaumov@yahoo.com

0026-2617/05/7403-0329

from the "krassilnikovii" population and the dairy yeasts *Kl. marxianus* and *Kl. lactis* var. *lactis.*

MATERIALS AND METHODS

Strains and cultivation media. The experiments were carried out with 37 strains of *Kl. lactis* and *Kl. marxianus*, whose origins are shown in the table. The strains were cultivated at 28°C on a complete yeast extract–peptone–dextrose (YPD) medium containing (g/l) yeast extract, 10; peptone, 10; glucose, 20; and agar, 20.

Restriction fragment length polymorphism (RFLP) analysis of the IGS2 region of rDNA. The IGS2 region was amplified by using NTS2 (5'-AACGGTGSTTTSTGGTAG-3') and ETS1 (5'-TGTCTTCAACTGCTTT-3') primers [12]. PCR amplifications were conducted in a reaction mixture (30 μl) containing 3 mM $MgCl_2$, 0.3 mM each dNTP, 50 pmol each primer, 1.25 U *Taq* polymerase (Sintol, Russia), and 20–200 ng of genomic DNA. The IGS2 fragment was amplified with the aid of a Tertsik thermocycler (DNK-tekhnologiya, Russia) with 25 cycles of DNA denaturation at 94°C for 1 min, primer annealing at 48°C for 30 s, and DNA synthesis at 72°C for 1 min. The amplification products were subjected to electrophoresis in 1% agarose gel at 60 V in 0.5× TBE buffer for 1.5 h. The buffer contained 45 mM Tris–HCl, 10 mM EDTA, and 45 mM boric acid (pH 8.0). The developed gel was stained with ethidium bromide. The RFLP analysis was performed with *Alu*I restriction endonuclease (Fermentas, Lithuania). The restriction fragments were separated by electrophoresis in 1.6% agarose gel, which was carried out at 50–55 V in the 0.5× TBE buffer for 4 h. The developed gel was stained with ethidium bromide for 2–3 h, destained with distilled water, and photographed under UV light using a Vilber Lourmat transilluminator (France).

PCR with the microsatellite primer $(GTG)_5$ was carried out in a reaction mixture (30 μl) containing 3 mM $MgCl_2$, 0.3 mM each dNTP, 30 pmol each primer, 1.25 U *Taq* polymerase (Sintol, Russia), and 20–200 ng of genomic DNA. The PCR amplifications were performed in the Tertsik thermocycler with 30 cycles of DNA denaturation at 94°C for 30 s, primer annealing at 52°C for 30 s, and DNA synthesis at 72°C for 1 min. The amplification products were separated by electrophoresis in 1.6% agarose gel, which was carried out at 55–60 V in the 0.5× TBE buffer for 3.5–4 h. The developed gel was stained with ethidium bromide for 2–3 h, destained with distilled water, and photographed under UV light.

The isolation of chromosomal DNA. To isolate chromosomal DNA, yeast cells were grown overnight in 10 ml of the liquid YPD medium at 28°C. The cells were precipitated by centrifugation, washed in 1 ml of 10 mM Tris–HCl buffer (pH 7.5) containing 50 mM EDTA, and resuspended in 0.2 ml of the same buffer containing 4 μg of Novozyme 234 preparation (Novo Industri A/S, Denmark). The cell suspension was incubated in a water bath at 42°C for 10–15 min and then mixed with 0.8 ml of 1% molten low-melt agarose (Bio-Rad, United States) cooled to 38–42°C. The mixture was kept on ice for 40–60 min. The agarose gel blocks were incubated overnight in 1–2 ml of LET buffer (0.5 M EDTA and 10 mM Tris–HCl (pH 7.5)) at 37°C; then, they were incubated overnight in an NDS lysis buffer (0.5 M EDTA, 10 mM Tris–HCl (pH 7.5), 1% sarcosine, and 1 mg/ml proteinase K (pH 9.5)) at 48°C. Finally the agarose blocks were washed in an EDTA–Tris buffer and stored at 4°C until further use.

Pulsed-field electrophoresis. The chromosomal DNA was separated using a CHEF-DRIII apparatus (Bio-Rad, United States). Pulsed-field electrophoresis was carried out at 14°C in 0.8% agarose in the 0.5× TBE buffer (45 mM Tris, 45 mM boric acid, and 10 mM EDTA (pH 8.2)). The voltage across the gel was kept at 65 V for 50 h (with a switching time of 1600–2000 s), at 70 V for 48 h (switching time of 800–1600 s), and at 75 V for 22 h (switching time of 120–600 s). The gel was then stained with ethidium bromide and photographed under UV light. The standard karyotypes were

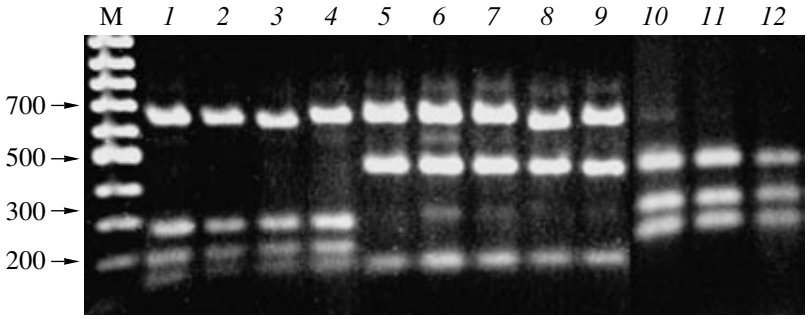

Fig. 1. *Alu*I restriction enzyme analysis of amplified fragments of the intergenic spacer IGS2 of the rDNA taken from the *Kl. lactis* var. *lactis* strains, wild yeasts from the "krassilnikovii" population, and *Kl. marxianus* strains. *Kl. lactis* var. *lactis* lanes: (*1*) VKM Y-868, (*2*) VKM Y-1527, (*3*) NRRL Y-1118, and (*4*) NRRL Y-1140. "Krassilnikovii" lanes: (*5*) VKM Y-831, (*6*) VKM Y-834, (*7*) Vor86, (*8*) CECT 1122, and (*9*) CBS 2877. *Kl. marxianus* lanes: (*10*) CBS 712, (*11*) VKM Y-2454, and (*12*) VKM Y-454. M is the molecular weight marker "100 bp DNA Ladder" (Fermentas, Lithuania).

The origin of the yeast strains under study

Strain	Location and source	Species, variety, or population
VKM Y-831 (T)	Oak exudate, Kaluga	"krassilnikovii"
VKM Y-834	Oak exudate, Kaluga	"krassilnikovii"
CECT 1122	Frass on *Quercus ruber*, Spain	"krassilnikovii"
CBS 2877	Cow intestines, Portugal	"krassilnikovii"
Est86	Oak exudate, Estonia	"krassilnikovii"
Vor86	Oak exudate, Voronezh	"krassilnikovii"
UCM Y-329	Soil, Kiev	"krassilnikovii"
VKM Y-868 (T)	Soft cheese, United Kingdom	*Kl. lactis* var. *lactis*
VKM Y-869	Sour milk, Kola Peninsula	*Kl. lactis* var. *lactis*
VKM Y-870	Sour camel milk	*Kl. lactis* var. *lactis*
VKM Y-896	Soft cheese, Italy	*Kl. lactis* var. *lactis*
VKM Y-1186	Unknown	*Kl. lactis* var. *lactis*
VKM Y-1333	Sour milk, Stavropol kray	*Kl. lactis* var. *lactis*
VKM Y-1339	Sour cream, St. Petersburg	*Kl. lactis* var. *lactis*
VKM Y-1527	Phlegm	*Kl. lactis* var. *lactis*
VKM Y-1868	Unknown	*Kl. lactis* var. *lactis*
NRRL Y-1118	Cream, United States	*Kl. lactis* var. *lactis*
NRRL Y-1140	Cream, United States	*Kl. lactis* var. *lactis*
CBS 762	Cream, United States	*Kl. lactis* var. *lactis*
CBS 1065	Milk	*Kl. lactis* var. *lactis*
CBS 1067	Milk, Netherlands	*Kl. lactis* var. *lactis*
CBS 1797	Phlegm, Norway	*Kl. lactis* var. *lactis*
CBS 2360	Unknown	*Kl. lactis* var. *lactis*
CBS 2619	Cream, United States	*Kl. lactis* var. *lactis*
CBS 2620	Cream, United States	*Kl. lactis* var. *lactis*
CBS 2621	Sour milk, Manchuria	*Kl. lactis* var. *lactis*
CBS 5618	Phlegm, Norway	*Kl. lactis* var. *lactis*
CBS 8043	Child intestines, New Zealand	*Kl. lactis* var. *lactis*
SM 3.8	Camembert cheese, France	*Kl. lactis* var. *lactis*
SM 5.8	Camembert cheese, France	*Kl. lactis* var. *lactis*
SM 6.7	Camembert cheese, France	*Kl. lactis* var. *lactis*
SM 16.9	Camembert cheese, France	*Kl. lactis* var. *lactis*
SM 48.7	Camembert cheese, France	*Kl. lactis* var. *lactis*
UCM Y-328	Kefir, Kiev	*Kl. lactis* var. *lactis*
CBS 712	Unknown	*Kl. marxianus*
Y-454	Matsun cheese, Armenia	*Kl. marxianus*
VKM Y-2454	Unknown	*Kl. marxianus*

Note: Abbreviated culture collection names: VKM, All-Russia Collection of Microorganisms, Moscow, Russia; CBS, Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands; CECT, Spanish Type Culture Collection, University of Valencia, Spain; SM, J.P. Schmidt, Institut National Agronomique, Paris-Grignon, France; NRRL, Northern Region Research Center, Peoria Ill., USA; and UCM, Ukrainian Collection of Microorganisms, Zabolotny Institute of Microbiology and Virology, Kiev, Ukraine. Strains NRRL Y-1118 = CBS 6315 and NRRL Y-1140 = CBS 2359 were represented in the work by, respectively, the auxotrophic mutants wm27 (α *lys*) and wm37 (a *his*) derived by I.A. Herman. Est86 = CBS 9057 and Vor 86 = CBS 9058. Strains VKM Y-1527, VKM Y-896, and CBS 2621 were earlier assigned to the species *Saccharomyces sociasi*, *Zygosaccharomyces versicolor*, and *Torulopsis manchurica*, respectively. According to their passports, strains UCM Y-328 and UCM Y-329 were isolated from soil and kefir, respectively. This, however, contradicts our observations that UCM Y-328 ferments lactose whereas UCM Y-329 does not. We assume that the passports of these strains were confused and, based on this assumption, exchanged their origins.

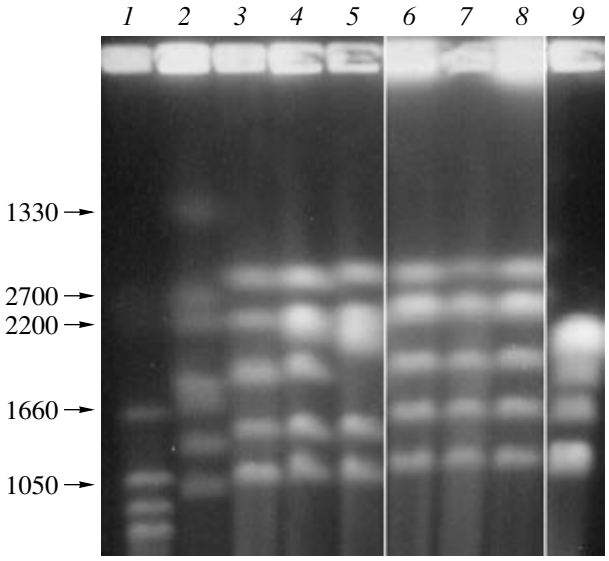

Fig. 2. Molecular karyotypes of the *Kl. lactis* var. *lactis* strains, wild yeasts from the "krassilnikovii" population, and *Kl. marxianus* strains. Lanes with standard karyotypes: (*1*) *S. cerevisiae* YNN 295 and (*2*) *P. canadensis* YB-4662-VIA. *Kl. lactis* var. *lactis* lanes: (*3*) VKM Y-868, (*4*) NRRL Y-1118, and (*5*) NRRL Y-1140. "Krassilnikovii" lanes: (*6*) VKM Y-831, (*7*) VKM Y-834, and (*8*) Vor86. *Kl. marxianus* lane: (*9*) CBS 712.

those of strains *Saccharomyces cerevisiae* YNN 295 and *Pichia canadensis* (syn. *Hansenula wingei*) YB-4662-VIA (Bio-Rad, United States).

Phylogenetic analysis. The strain relatedness was evaluated by comparing the PCR products amplified with the microsatellite primer $(GTG)_5$. A dendrogram was constructed by applying the neighbor-joining method with the aid of the TREECON software package [14] and using type strain *Kl. marxianus* CBS 712 as the outgroup. The bootstrap values, which indicate the statistical significance of a strain grouping on the tree, are expressed as a percentage of 100 replications.

RESULTS AND DISCUSSION

The IGS2 regions of the rDNA of 37 *Kluyveromyces* strains of different origin were studied using three methods: RFLP analysis, molecular karyotyping, and PCR analysis with the microsatellite primer $(GTG)_5$.

The RFLP analysis of the amplified IGS2 fragments of rDNA. The amplified IGS2 fragments of most of the strains under study were approximately 1200 bp in size. However, two of the strains, VKM Y-2454 and VKM Y-454, had IGS2 fragments of about 1250 bp in size. The PCR products were analyzed with an *Alu*I restriction endonuclease. According to the similarity of their restriction profiles, the strains were

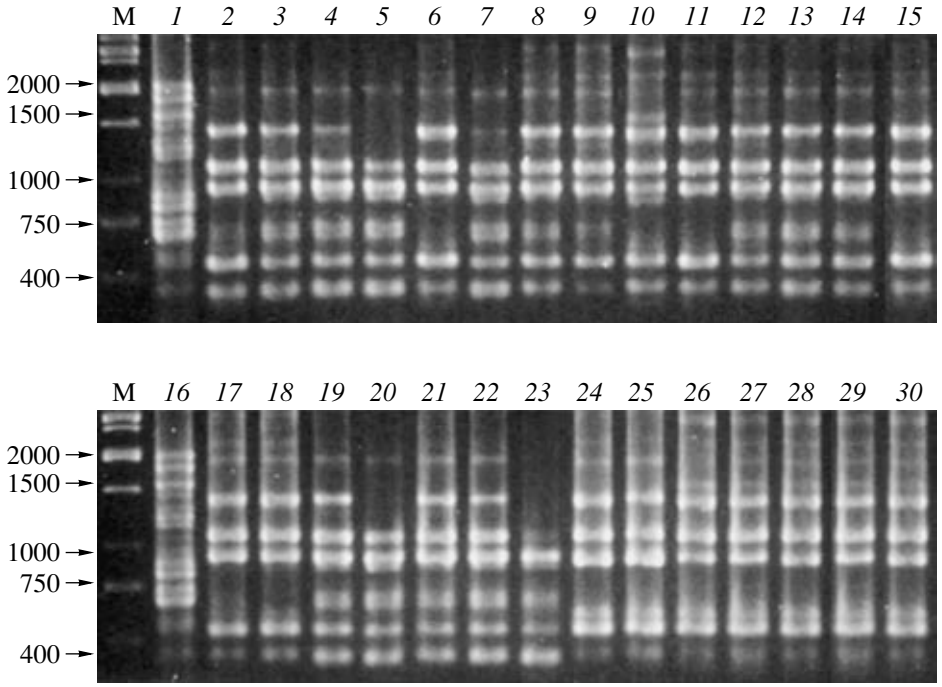

Fig. 3. PCR analysis of the *Kl. lactis* var. *lactis* strains, wild yeasts from the "krassilnikovii" population, and *Kl. marxianus* strains with the microsatellite primer $(GTG)_5$. *Kl. marxianus* lanes: (*1, 16*) CBS 712. *Kl. lactis* var. *lactis* lanes: (*2*) VKM Y-868, (*3*) VKM Y-896, (*4*) SM 3.8, (*5*) SM 6.7, (*6*) VKM Y-1527, (*7*) CBS 1797, (*8*) CBS 5618, (*9*) CBS 8043, (*10*) NRRL Y-1118, (*11*) NRRL Y-1140, (*12*) CBS 762, (*13*) CBS 2619, (*14*) CBS 2620, (*15*) UCM Y-328, (*17*) VKM Y-870, (*18*) CBS 2621, (*19*) CBS 1067, (*20*) CBS 1065, (*21*) VKM Y-1333, (*22*) VKM Y-1339, and (*23*) VKM Y-869. "Krassilnikovii" lanes: (*24*) VKM Y-831, (*25*) VKM Y-834, (*26*) CECT 1122, (*27*) CBS 2877, (*28*) Vor86, (*29*) Est86, and (30) UCM Y-329. M is the molecular weight marker "100 bp DNA Ladder" (Fermentas, Lithuania).

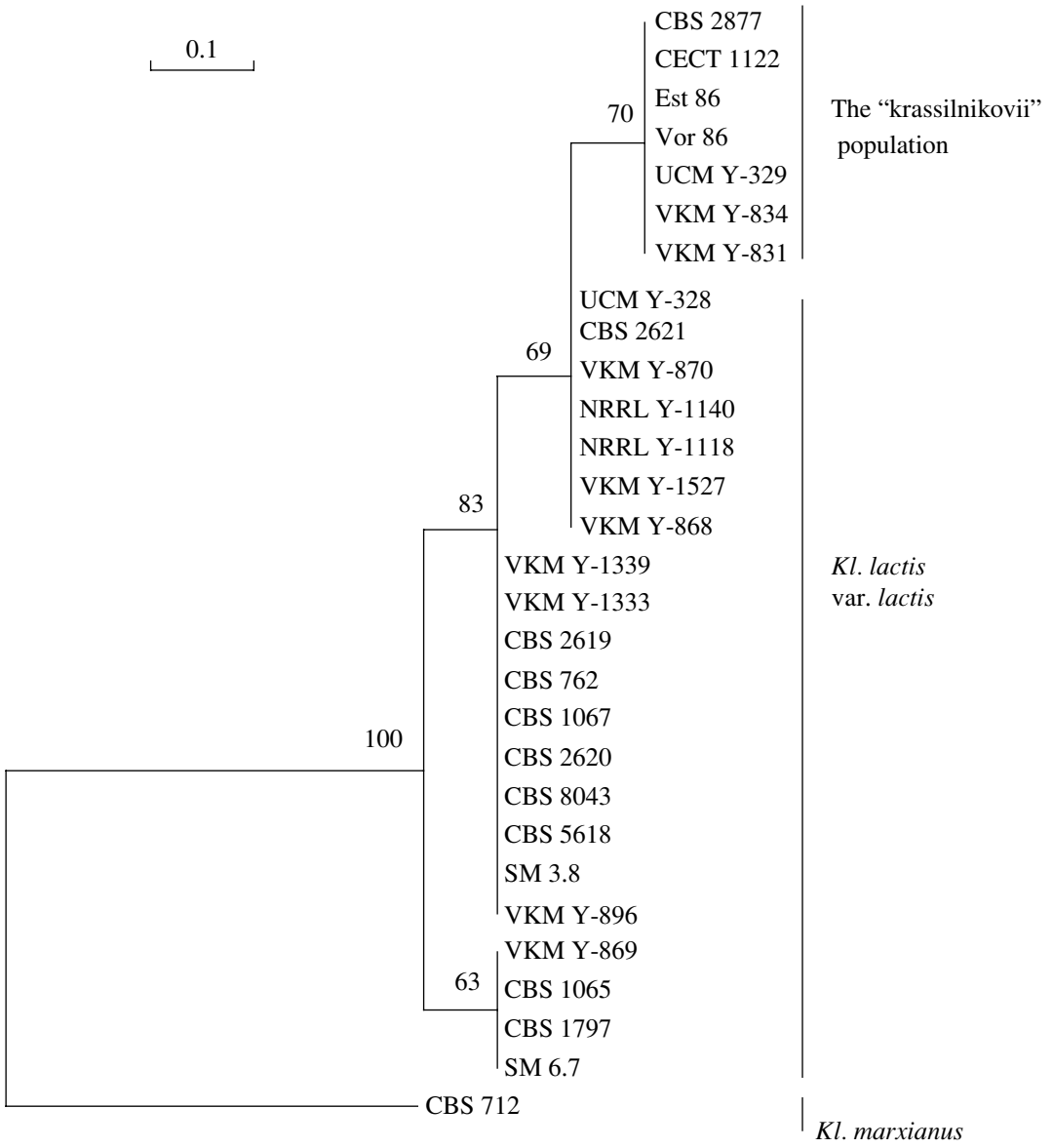

Fig. 4. A dendrogram showing the relatedness of the *Kl. lactis* strains based on their PCR profiles with the microsatellite primer $(GTG)_5$. The dendrogram was constructed with strain *Kl. marxianus* CBS 712 as the outgroup. The data were processed using the neighbor-joining method with the aid of the TREECON software package.

divided into three groups. The first group consisted of 27 *Kl. lactis* var. *lactis* strains with four fragments (approximately 650, 250, 200, and 100 bp in size) in their *Alu*I restriction profiles (Fig. 1, lanes *1–4*). The second group, with three fragments in their restriction profiles (650, 450, and 100 bp), included six wild-type strains from the European "krassilnikovii" population and strain UCM Y-329, which was isolated from soil (Fig. 1, lanes *5–9*). The third group was made up of two strains, VKM Y-2454 and VKM Y-454, with three *Alu*I fragments (approximately 550, 400, and 300 bp in size) in their profiles, which were identical to that of type culture *Kl. marxianus* CBS 712 (Fig. 1, lanes *10–12*).

Molecular karyotyping. The chromosomal DNA of the strains under study, whose size was determined by using standard karyotypes of commercially available strains *S. cerevisiae* YNN 295 and *P. canadensis* YB-4662-VIA, gave rise to 4–5 bands (Fig. 2). The *Kl. lactis* var. *lactis* and "krassilnikovii" strains gave rise to five virtually identical chromosomal bands ranging from 1000 to 2800 bp in size (Fig. 2, lanes *3–5* and *6–8*, respectively). According to the intensity of the ethidium bromide staining, the fourth band from the bottom was a double band (therefore, the actual number of chromosome bands was six). This result agrees with the data of other researchers [15–17]. Strains VKM Y-2454 and VKM Y-454 gave rise to four chromosomal bands

(from 1050 to 2200 bp in size), which were identical to those of type strain *Kl. marxianus* CBS 712 (Fig. 2, lane *9*). Thus, the molecular karyotyping confirmed, as was indicated by the data of the RFLP analysis, that strains VKM Y-2454 and VKM Y-454 belong to the species *Kl. marxianus.*

PCR with the microsatellite primer $(GTG)_5$. Microsatellite primers have multiple locations in yeast genomes, which makes it possible to compare a great number of polymorphic loci. Figure 3 shows the results of the PCR analysis of 21 *Kl. lactis* var. *lactis* strains and 7 "krassilnikovii" strains using the microsatellite primer $(GTG)_5$. The *Kl. lactis* var. *lactis* strains of unknown origin and three of the five yeast strains isolated from Camembert cheese (table) were excluded from this analysis.

As can be seen from Fig. 3, the size of the amplified DNA fragments from different strains varied from 400 to 2000 bp. The *Kl. lactis* strains considerably differed from type strain *Kl. marxianus* CBS 712 in their PCR profiles (Fig. 3, lane *1*). The *Kl. lactis* var. *lactis* strains gave rise to two major fragments of approximately 400 and 600 bp in size (Fig. 3, lanes *2–15*, *17–23*). The seven strains from the "krassilnikovii" population had identical profiles (Fig. 3, lanes *24–30*), which differed from those of the *Kl. lactis* var. *lactis* strains.

The dendrogram constructed on the basis of the PCR profile similarity (Fig. 4) showed that the *Kl. lactis* strains form a separate cluster relative to test strain *Kl. marxianus* CBS 712. Within this cluster, there are two groups of yeast strains with similar PCR profiles. The first group consists of three subgroups, one of which includes yeast strains from the "krassilnikovii" population while the other two include clinical isolates and strains isolated from dairy products. The second group consists of four *Kl. lactis* var. *lactis* strains of different origin.

The yeasts *Kl. lactis* var. *lactis* and the strains from the "krassilnikovii" population are not genetically isolated, have similar karyotypes, and cannot be differentiated by a restriction enzyme analysis of the ITS regions of their rDNA, as is evident from the data of this research and those available in previous publications [7, 13]. At the same time, these yeasts are isolated ecologically, since *Kl. lactis* var. *lactis* yeasts live in dairy products and "krassilnikovii" yeasts live in habitats containing no lactose. We succeeded in finding the molecular marker that allows the differentiation of the dairy yeasts *Kl. lactis* var. *lactis* and wild yeasts from the European "krassilnikovii" population. Specifically, the *Alu*I restriction analysis of the IGS2 region of rDNA showed that *Kl. lactis* var. *lactis* and the yeast strains from the "krassilnikovii" population have two different *Alu*I restriction profiles. It should be noted that *Alu*I restriction analysis of IGS2 regions also makes it possible to differentiate between the physiologically close species *Kl. marxianus* and *Kl. lactis.* By using this approach, we reclassified two strains of *Kl. lactis* (VKM Y-454 and VKM Y-2454) as *Kl. marxianus* strains.

There is increasing interest among biotechnologists in the dairy yeasts *Kl. lactis* and *Kl. marxianus* due to their role as harmless producers of various useful products [18]. It should be noted, however, that three of the four groups of *Kl. lactis* strains formed on the basis of the similarity of their PCR profiles with the microsatellite primer $(GTG)_5$ contain clinical isolates (see the data of the present work). This molecular genetic approach shows that a close relation exists between the dairy and clinical *Kl. lactis* isolates, as in the case of clinical and nonclinical *S. cerevisiae* isolates [19, 20]. The association of lactose-fermenting yeasts with dairy products and, hence, mammals suggests that they may be the ancestors of clinical strains. Of interest is the fact that some of the characteristics of dairy yeast strains, such as the formation of pseudomycelium, tolerance to elevated temperatures, and high pectolytic activity, are typical of opportunistic pathogens.

ACKNOWLEDGMENTS

We are grateful to S.S. Nagornaya, Kiev, for providing, in exchange, strains UCM Y-328 and UCM Y-329.

REFERENCES

1. Lodder, J. and Kreger-van Rij, N.J.W., *The Yeasts: A Taxonomic Study*, 1st ed., Amsterdam: North-Holland Publ., 1952.
2. van der Walt, J.P., The Emendation of the Genus *Kluyveromyces* v. d. Walt, *Antonie van Leeuwenhoek*, 1965, vol. 31, pp. 341–348.
3. Naumov, G.I., The Genosystematics of Yeasts from the Genus *Zygofabospora* Kudriavzev emend. G. Naumov, *Mol. Genet. Mikrobiol. Virusol.*, 1986, no. 5, pp. 10–13.
4. Naumov, G.I., The Nomenclature of Yeasts from the Genus *Zygofabospora* Kudriavzev emend. G. Naumov, *Mikol. Fitopatol.*, 1987, vol. 21, no. 2, pp. 134–140.
5. Cai, J., Roberts, I.N., and Collins, M.D., Phylogenetic Relationships Among Members of the Ascomycetous Yeast Genera *Brettanomyces, Debaryomyces, Dekkera*, and *Kluyveromyces* Deduced by Small Subunit rRNA Gene Sequences, *Int. J. Syst. Bacteriol.*, 1996, vol. 46, pp. 542–549.
6. Kurtzman, C.P. and Robnett, C.J., Identification and Phylogeny of Ascomycetous Yeasts from Analysis of Nuclear Large Subunit (26S) Ribosomal DNA Partial Sequences, *Antonie van Leeuwenhoek*, 1998, vol. 73, pp. 331–371.
7. Naumov, G.I. and Naumova, E.S., Five New Combinations in the Yeast Genus *Zygofabospora* Kudriavzev emend. G. Naumov (pro parte *Kluyveromyces*) Based on Genetic Data, *FEMS Yeast Research*, 2002, vol. 3, no. 2, pp. 39–46.
8. Kurtzman, C.P., Lachance, M.-A., Nguyen, H.-V., and Prillinger, H., Proposal To Conserve *Kluyveromyces* with a Conserved Type (*Ascomycota: Hemiascomycetes, Saccharomycetaceae*), *Taxon*, 2001, vol. 50, pp. 907–908.
9. Kurtzman, C.P., Phylogenetic Circumscription of *Saccharomyces, Kluyveromyces* and Other Members of the *Saccharomycetaceae*, and the Proposal of the New Gen-

era *Lachancea, Nakaseomyces, Naumovia, Vanderwaltozyma* and *Zygotorulaspora, FEMS Yeast Research*, 2003, vol. 4, pp. 233–245.

10. Naumov, G.I., The Wild European Species *Zygofabospora krassilnikovii* is an Ancestor of the Dairy Yeast *Z. lactis, Dokl. Akad. Nauk*, 2000, vol. 372, no. 6, pp. 846–849.
11. Esteve-Zarzos, B., Belloch, C., Uruburu, F., and Querol, A., Identification of Yeasts by RFLP Analysis of the 5.8S rRNA Gene and the Two Ribosomal Internal Transcribed Spacers, *Int. J. Syst. Bacteriol.*, 1999, vol. 49, pp. 329–337.
12. Nguyen, H.-V., Pulvirenti, A., and Gaillardin, C., Rapid Differentiation of the Closely Related *Kluyveromyces lactis* var. *lactis* and *K. marxianus* Strains Isolated from Dairy Products Using Selective Media and PCR/RFLP of the rDNA Non-Transcribed Spacer 2, *Can. J. Microbiol.*, 2000, vol. 46, pp. 1115–1122.
13. Belloch, C., Fernandes-Espinar, T., Querol, A., Garcia, M.D., and Barrio, E., An Analysis of Inter- and Intraspecific Genetic Variabilities in the *Kluyveromyces marxianus* Group of Yeast Species for the Reconsideration of the *K. lactis* Taxon, *Yeast*, 2002, vol. 19, pp. 257–268.
14. van der Peer, Y. and de Wachter, R., TREECON for Windows: A Software Package for the Construction and Drawing of Evolutionary Trees for the Microsoft Windows Environment, *Comput. Appl. Biosci.*, 1994, vol. 10, pp. 569–570.
15. Sor, F. and Fukuhara, H., Analysis of Chromosomal DNA Patterns of the Genus *Kluyveromyces, Yeast*, 1989, vol. 5, pp. 1–10.
16. Wesolowski-Louvel, M., Breunig, K.D., and Fukuhara, H., *Nonconventional Yeasts in Biotechnology: A Handbook*, Wolf, K., Ed., Berlin: Springer, 1996, pp. 139–201.
17. Belloch, C., Barrio, E., Garcia, M.D., and Querol, A., Inter- and Intraspecific Chromosome Pattern Variation in the Yeast Genus *Kluyveromyces, Yeast*, 1998, vol. 14, pp. 1341–1354.
18. Bonekamp, F.J. and Oosterom, J., On the Safety of *Kluyveromyces lactis*: A Review, *Appl. Microbiol. Biotechnol.*, 1994, vol. 41, pp. 1–3.
19. Hennequin, C., Thierry, A., Richard, G.F., Lecointre, G., Nguyen, H.-V., Gaillardin, C., and Dujon, B., Microsatellite Typing As a New Tool for Identification of *Saccharomyces cerevisiae* Strains, *J. Clin. Microbiol.*, 2001, vol. 3, pp. 551–559.
20. de Llanos, R., Querol, A., Planes, A.M., and Fernandez-Espinar, M.T., Molecular Characterization of Clinical *Saccharomyces cerevisiae* Isolates and Their Association with Non-Clinical Strains, *Syst. Appl. Microbiol.*, 2004, vol. 27, no. 4, pp. 427–435.