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EXPERIMENTAL ARTICLES

Finding of Dairy Yeasts *Kluyveromyces lactis* var. *lactis* in Natural Habitats

G. I. Naumov[a, b, 1], E. S. Naumova[a, b], A. M. Glushakova[c], A. V. Kachalkin[c], and I. Yu. Chernov[c]

[a] *Research and Educational Center for Biomedical Technologies, All-Russian Research Institute for Medicinal and Aromatic Plants, Russian Academy of Sciences, Moscow, Russia*

[b] *Research Institute for Genetics and Selection of Industrial Microorganisms, Moscow, Russia*

[c] *Faculty of Soil Sciences, Lomonosov Moscow State University, Moscow, Russia*

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Abstract—Well-known yeasts *Kluyveromyces lactis* var. *lactis*, which are usually associated with dairy products, were discovered in nature: in woodland park soil under *Impatiens glandulifera* Royle plants. Reliable identification of the yeasts was carried out using physiological criteria (lactose and maltose utilization) and molecular markers (nucleotide sequence of the 5.8S-ITS rDNA fragment, pulsed-field gel electrophoresis, and Southern hybridization of chromosomal DNA with the *LAC4* probe). Ecology of *Kl. lactis* var. *lactis* is discussed.

Keywords: dairy yeasts, *Kluyveromyces lactis*, ITS1/ITS2 rDNA, molecular karyotyping, lactose and maltose utilization, the *LAC4* gene

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The dairy yeast *Kluyveromyces lactis* (Dombrowski) van der Walt attracts attention of molecular biologists, geneticists, and biotechnologists due to its ability to ferment lactose, a mammalian β-galactoside disaccharide [1–6]. Virtually only domesticated *K. lactis* var. *lactis* strains isolated from milk foods possess this fermentation property. Their closest wild relatives *K. lactis* var. *drosophilarum* (Shehata et al.) Sidenberg et Lachance [7–9] are not only unable to assimilate lactose, but lack the silent sequences of the lactose genes *LAC4* and *LAC12* encoding β-galactosidase and lactose permease, respectively [10]. Nevertheless, the related species *Kl. marxianus* (Hansen) van der Walt is represented by both environmental and dairy lactose-utilizing strains [11]. In the latter species, only the strains of nondairy origin from the South African population do not assimilate lactose due to the absence of the active *LAC12* gene [12]. Finally, another species, *Kl. wickerhamii*, represented exclusively by the wild strains, is capable only of lactose assimilation due to respiration-dependent low-affinity lactose transport [13]. All these evidences together indicate the possibility that the lactose-fermenting strains of *K. lactis* var. *lactis* may occur in nature. Otherwise, the origin of dairy strains of this variety is not clear.

The goal of the present work was to carry out comparative molecular genetic analysis of environmental and cultured populations of the yeasts of the genus *Kluyveromyces*. This work is the first report of a population of *Kl. lactis* var. *lactis* found in natural habitats.

MATERIALS AND METHODS

Media. Yeasts were cultivated at 28°C on complete YPD medium of the following composition (g/L): glucose, 20; peptone, 10; yeast extract, 10; agar, 20. The following main sugars were used for diagnosing the yeast: maltose (Sigma, United States) and lactose (Merk, Germany).

Yeast isolation was carried out from humus gley soil in the thickets of the Indian balsam *Impatients glandulifera* Royle in Izmailovskii Forest Park, Moscow. The isolation procedure was described in detail earlier [14]. The reference strain no. 297 (=KBP 4003 = VKPM Y-3737) was isolated on October 21, 2009. Here and hereinafter, the following abbreviations of the collection names are used: KBP, the Department of Soil Biology, Moscow State University; VKPM, All-Russian Collection of Industrial Microorganisms, Moscow; NRRL, Agricultural Research Service Culture Collection, National Center for Agricultural Utilization Research, Peoria, Illinois, United States.

Primary identification of the yeast was performed by the morphological and physiological characteristics [15], as well as by using the rDNA sequence analysis. The conditions for amplification of the 26S rDNA D1/D2 domain and the 5.8S-ITS region comprising the 5.8S rRNA gene and the internal transcribed ITS1

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

and ITS2 spacers were described earlier [14]. The search for homology with the known nucleotide sequences was carried out using the BLAST software. Multiple alignments of the obtained and the already known nucleotide sequences were carried out manually using BioEdit. The phylogenetic tree was constructed using the neighbor-joining algorithm implemented in the MEGA 5 software package [16]. The bootstrap indices determining the statistical significance of group identification were determined for 1000 pseudoreplicates.

Molecular karyotyping and Southern hybridization. Preparation of the chromosomal DNA was described earlier [17]. The apparatus CHEF-DRTM III (Bio-Rad, United States) was used for chromosomal DNA separation. Pulsed-field gel electrophoresis was performed at 200 V in the following mode: (1) at 175 V for 8 h with a field switching time of 40–120 s; (2) at 130 V for 24 h with a field switching time of 120–360 s; and (3) at 100 V for 8 h with a field switching time of 360–1200 s. After electrophoresis, the gel was stained with ethidium bromide and photographed in the ultraviolet light. The buffer used was 0.5× TBE (45 mM Tris, 45 mM boric acid, 10 mM EDTA, pH 8.2) cooled to 14°C. The chromosomal DNA of the strains *Saccharomyces cerevisiae* YNN 295 (Bio-Rad, United States) and *Kl. lactis* var. *lactis* NRRL Y-1118 (*LAC1*) and NRRL Y-1140 (*LAC2*) served as the karyotypic standards. After electrophoresis, the gel was stained with ethidium bromide, washed with distilled water, and photographed.

The chromosomal DNA was transferred onto the nitrocellulose membrane with a vacuum technique using the Vacuum blotter apparatus (Bio-Rad, United States). The DNA was fixed on the membrane by annealing at 80°C for 2 h. The *LAC4* PCR fragment was used as a probe. The label was introduced with a nonradioactive method using dUTP labeled with digoxigenin (dig-II-dUTP) from the DIG High Prime DNA Labeling and Detection Starter Kit I (Roche Applied Science, Switzerland) according to the manufacturer's instruction. Hybridization and development of the hybridization signals were carried out according to the instructions of the same company.

Polymerase chain reaction was performed using a Tercyc DNA amplifier. The primers MR66 (5'-ATGCTTTTGCAAGCTTTC-3') and MR67 (5'-GGTCATGTTCACAGATCC-3') were used for amplification of the *LAC4* gene [18]. PCR was performed in 30 μL of the $(NH_4)_2SO_4$ buffer containing 2.5 mM $MgCl_2$, 0.1 mM of each dNTP, 50 pmol of each primer, 2.5 units of *Taq* polymerase (Syntol, Russia), and 20–200 ng of DNA. Initial denaturation was performed at 94°C for 5 min followed by 36 cycles in the following mode: denaturation at 94°C for 60 s; annealing of the primers at 52°C for 60 s; DNA synthesis at 72°C for 120 s; final elongation, 72°C for 10 min [10].

RESULTS AND DISCUSSION

Four similar strains, no. 297, H1, H2, and H3, were isolated from different soil samples collected in Izmailovskii Park. According to the physiological differentiation tests [19, 20], i.e., by the ability to assimilate maltose and lactose, all the yeast strains indicated belonged to *Kl. lactis* var. *lactis*. This yeast species occurred extremely rarely in the samples analyzed (frequency of occurrence was less than 0.1%). Analysis of the 5.8S-ITS nucleotide sequence (Fig. 1) of the reference strain no. 297 confirmed its species identification as *Kl. lactis* var. *lactis*. It should be noted that the species *Kl. lactis* and *Kl. marxianus* possess virtually identical 26S rDNA D1/D2 sequences [19], but significantly differ in the 5.8S-ITS sequences.

For unambiguous molecular identification of the strains no. 297, H1, H2, and H3, karyotypic analysis using Southern hybridization of their chromosomal DNA with the *LAC4* probe was carried out. The results of molecular karyotyping of the strains analyzed and the subsequent Southern hybridization of their chromosomal DNA with the *LAC4* probe are shown in Figs. 2a and 2b. The molecular karyotypes of no. 297, H1, H2, and H3 were similar to the karyotype of the dairy yeast *Kl. lactis* var. *lactis* NRRL Y-1140 (Fig. 2a, lanes 2–6). In all four strains, the *LAC4* gene was located in chromosome II as in the other dairy control strain NRRL Y-1118 (Fig. 2b, lanes 1, 3–6). It should be taken into account that some dairy strains of *Kl. lactis* var. *lactis*, unlike *Kl. lactis* var. *drosophilarum* of the "krassilnikovii" population, may differ in the size of individual chromosomes [7, 21, 22]. Moreover, the closely linked *LAC4* and *LAC12* genes in *Kl. lactis* var. *lactis* dairy strains may be located in different chromosomes: II, III, and IV [22, 23].

The isolation of *Kl. lactis* var. *lactis* from soil under Indian balsam *Imp. glandulifera* is hardly accidental. This invasive ruderal species is used as an ornamental plant but quickly becomes wild and forms, as indicated in [14], massive thickets in the open spaces of rich moist soils. However, after the first light frosts Indian balsam dies off; its aerial parts are lysed very quickly enriching the upper layers of soil with the carbon sources readily accessible to yeasts.

It can not be ruled out that the spread of dairy yeasts in nature may occur with the participation of wild mammals. We link the appearance of *Kl. lactis* var. *lactis*, in soil in particular, to different rodents during milk-feeding the offspring. A high dairy yeast concentration on the breeding-grounds of marine mammals can be predicted. Puddles of milk are known to be formed on them during the season of feeding young

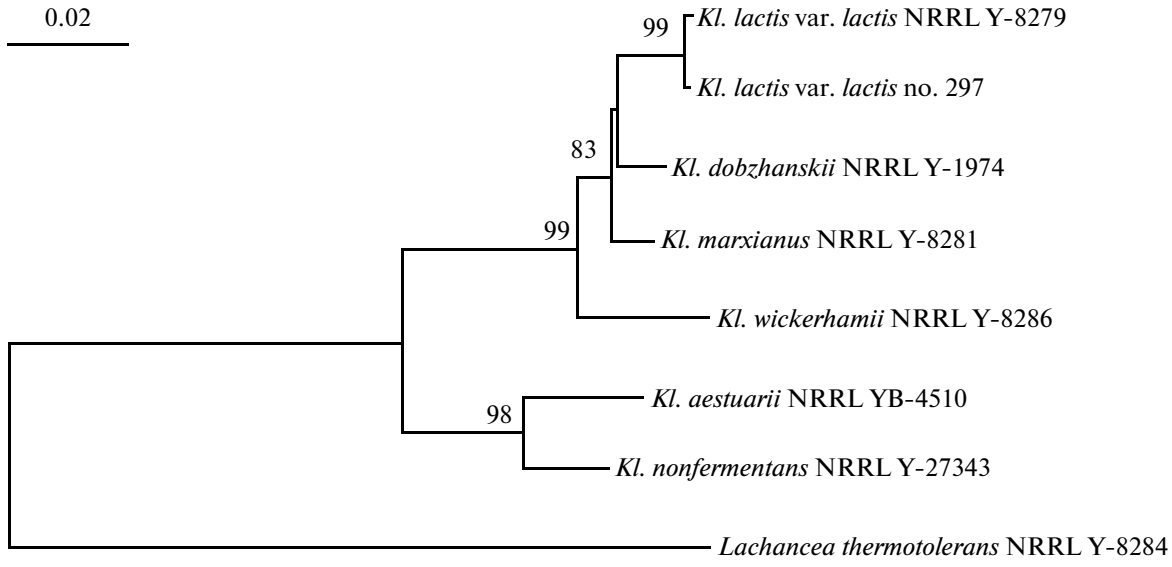

Fig. 1. Phylogenetic analysis of the rDNA 5.8S-ITS nucleotide sequences of the *Kluyveromyces* species and identification of the strain *Kl. lactis* var. *lactis* no. 297. The 5.8S-ITS sequence of the type culture of the yeast *Lachancea thermotolerans* was used as an outgroup. The bootstrap values >50% are given. The scale bar corresponds to 20 nucleotide substitutions per 1000 nucleotide positions.

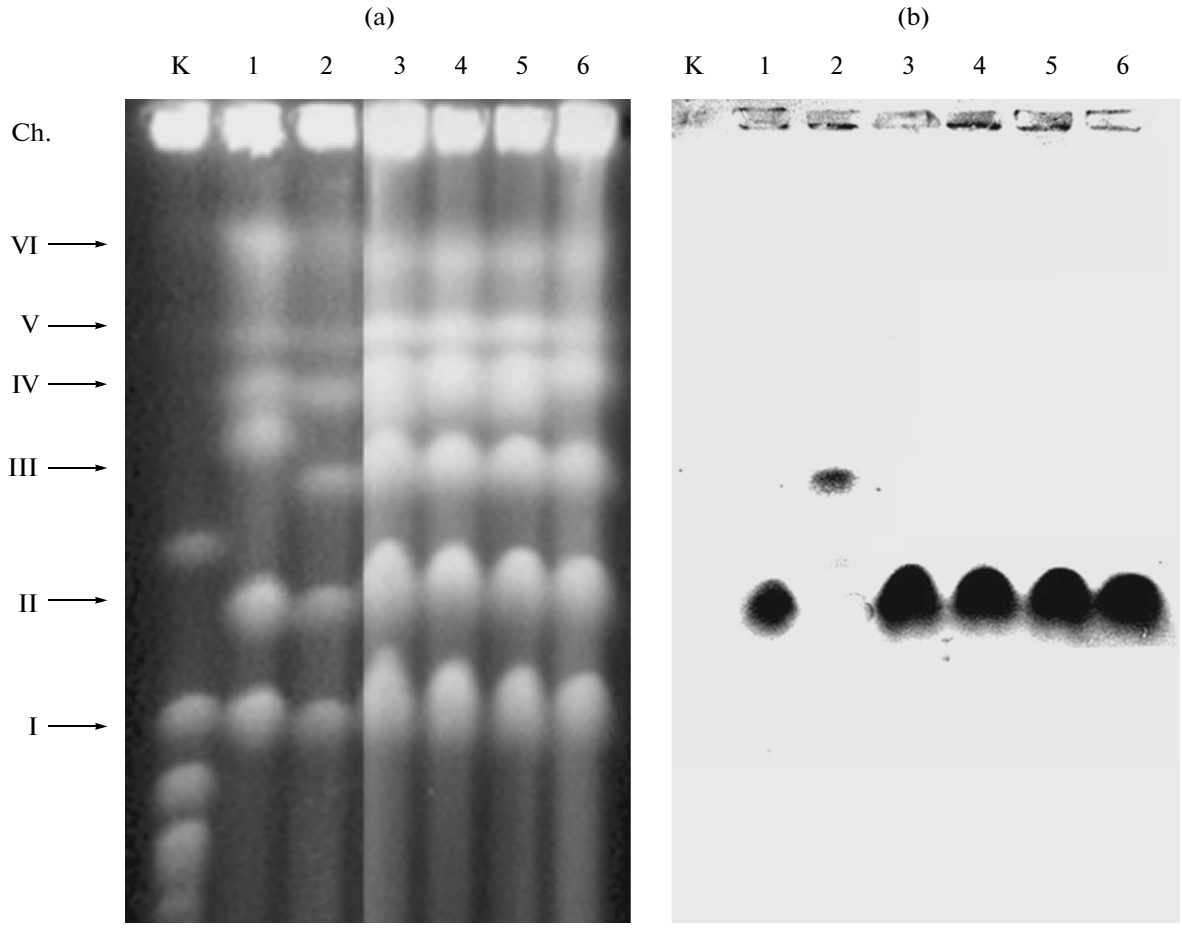

Fig. 2. Pulsed-field electrophoresis of the chromosomal DNA of *Kl. lactis* (a) and Southern-hybridization with the *LAC4* probe (b). Lanes: K, *S. cerevisiae* YNN 295 (chromosomal standard); *Kl. lactis* var. *lactis*: NRRL Y-1140 (1); NRRL Y-1118 (2); H1 (3); H2 (4); H3 (5); and no. 297 (6). In Fig. 2a, the arrow shows *Kl. lactis* var. *lactis* chromosomal numbers.

animals. We suggest the following turnover of dairy yeasts in nature: mammals–milk–soil–mammals. The same chain may be maintained by human activities, namely, cattle breeding and preparation of dairy products. Further special study of the natural ecology of *Kl. lactis* var. *lactis* is undoubtedly necessary. As we indicated, the easiest way is to try to establish an association between dairy yeasts and marine mammals on their breeding grounds.

The discovery of *Kl. lactis* var. *lactis* soil strains necessitates their population study in comparison with both the dairy strains of this variety and the strains of *Kl. lactis* var. *drosophilarum*. It is necessary to point out that the name *Kl. lactis* var. *drosophilarum* is somewhat conventional. As was shown earlier, the latter taxon is genetically heterogeneous, combining divergent ecological and geographical populations [7–9]. For example, five divergent populations were revealed in North America. The European "krassilnikovii" population is very close, if not identical, to the dairy yeast *Kl. lactis* var. *lactis* by its genome. This was evidenced by the hybrid fertility test and molecular karyotypes. Nevertheless, they are differentiated by their relation to lactose (the absence of the *LAC* genes in "krassilinikovii" [10, 24]) and restriction analysis of the intergene rDNA IGS2 spacer. The latter analysis revealed two different *Alu*I profiles [17, 20]. It is expedient to go ahead with the study of the population structure of the species *Kl. lactis* using multilocus sequencing (MLST-analysis).

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REFERENCES

1. Wésolowski-Louvel, M., Breunig, K.D., and Fukuhara, H., *Kluyveromyces lactis*, in *Nonconventional Yeasts in Biotechnology. A Handbook*, Berlin: Springer, 1996, pp. 139–201.
2. Bolotin-Fukuhara, M., Toffano-Nioche, C., Artiguenave, F., Duchateau-Nguyen, G., Lemaire, M., Marmeisse, R., Montrocher, R., Robert, C., Termier, M., Wincker, P., and Wésolowski-Louvel, M., Genomic exploration of the hemiascomycetous yeasts: 11. *Kluyveromyces lactis, FEBS Lett.*, 2000, vol. 487, pp. 66–70.
3. Schaffrath, R. and Breunig, K.D., Genetics and molecular physiology of the yeast *Kluyveromyces lactis, Fungal Genet. Biol.*, 2002, vol. 30, pp. 173–190.
4. Breunig, K.D. and Steensma, H.Y., *Kluyveromyces lactis*: genetics, physiology and application, in *Functional Genetics of Industrial Yeasts,* vol. 2. *Topics in Current Genetics*, Berlin: Springer, 2003, pp. 171–205.
5. Rubio-Texeira, M., Endless versatility in the biotechnological applications of *Kluyveromyces LAC* genes, *Biotechnol. Advanc.*, 2006, vol. 24, pp. 212–225.
6. Fukuhara, H., *Kluyveromyces lactis*—a retrospective, *FEMS Yeast Res.*, 2006, vol. 6, no. 3, pp. 323–324.
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 Res.*, 2002, vol. 2, no. 1, pp. 39–46.
8. Naumova, E.S., Sukhotina, N.N., and Naumov, G.I., Molecular genetic differentiation of the dairy yeast *Kluyveromyces lactis* and its closest wild relatives, *FEMS Yeast Res.*, 2004, vol. 5, no. 3, pp. 263–269.
9. Naumov, G.I., *Zygofabospora krassilnikovii*, a wild European species, is an ancestor of the dairy yeasts *Z. lactis, Dokl. Biol. Sci.*, 2000, vol. 372, no. 6, pp. 421–424.
10. Naumov, G.I., Naumova, E.S., Barrio, E., and Querol, A., Genetic and molecular study of the inability of the yeast *Kluyveromyces lactis* var. *drosophilarum* to ferment lactose, *Microbiology* (Moscow), 2006, vol. 75, no. 3, pp. 248–252.
11. Naumov, G.I., Naumova, E.S., and Choi, E.-S., Natural and industrially important patterns of sugar utilization in the yeast *Kluyveromyces marxianus*, *Biotekhnologiya*, 2010, no. 2, pp. 54–58.
12. Naumov, G.I., Genetics of lactose utilization polymorphism in the yeast *Kluyveromyces marxianus*, *Dokl. Biol. Sci*, 2006, vol. 409, no. 3, pp. 317–319.
13. Naumov, G.I., Why does the yeast *Kluyveromyces wickerhamii* assimilates but not ferments lactose?, *Dokl. Biol. Sci.*, 2005, vol. 403, no. 6, pp. 310–312.
14. Glushakova, A.M., Kachalkin, A.V., and Chernov, I.Yu., Specific features of the dynamics of epiphytic and soil yeast communities in the thickets of Indian balsam on mucky gley soil, *Euras. Soil Sci.*, 2011, vol. 44, no. 8, pp. 886–892.
15. Kurtzman, C.P., Fell, J.W., Boekhout, T., and Robert, V., Methods for isolation, phenotypic characterization and maintenance of yeasts, in *The Yeasts, A Taxonomic Study*, Kurtzman, C.P., Fell, J.W., and Boekhout, T., Eds., Amsterdam: Elsevier, 2011, pp. 87–110.
16. Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S., MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods, *Mol. Biol. Evol.*, 2011, vol. 28, pp. 2731–2739.
17. Naumova, E.S., Sukhotina, N.N., and Naumov, G.I., Molecular markers for differentiation between the closely related dairy yeast *Kluyveromyces lactis* var. *lactis* and wild *Kluyveromyces* lactis strains from the European "krassilnikovii" population, *Microbiology* (Moscow), 2005, vol. 74, no. 3, pp. 329–335.
18. Adam, A.C., Prieto, J.A., Rubio-Texeira, M., and Polaina, J., Construction of a lactose-assimilating strain of baker's yeast, *Yeast*, 1999, vol. 15, pp. 1299–1305.
19. Lachance, M.-A., *Kluyveromyces* van der Walt (1971), in *The Yeasts, A Taxonomic Study*, Kurtzman, C.P.,

Fell, J.W., and Boekhout, T., Eds., Amsterdam: Elsevier, 2011, pp. 471–482.

20. Naumova, E.S., Naumov, G.I., Nikitina, T.N., Sadykova, A.Zh., and Kondratieva, V.I., Molecular genetic and physiological differentiation of *Kluyveromyces lactis* and *Kluyveromyces marxianus*: analysis of strains from the All-Russian collection of microorganisms (VKM), *Microbiology* (Moscow), 2012, vol. 81, no. 2, pp. 216–223.

21. Wésolowski-Louvel, M., Breunig, K.D., and Fukuhara, H., *Kluyveromyces lactis*, in *Nonconventional Yeasts in Biotechnology: A Handbook*, Wolf, K.B., Ed., Berlin: Springer, 1996, pp. 139–201.

22. Naumov, G.I. and Naumova, E.S., Polymeric lactose fermentation genes in the yeast *Kluyveromyces lactis*: a new locus *LAC3, Dokl. Biol. Sci.*, 2014, vol. 435, pp. 106–108.

23. Naumov, G.I., Identification of the lactose *LAC* gene superfamilies in *Kluyveromyces* yeast, *Dokl. Biochem. Biophys*, 2008, vol. 420, no. 6, pp. 158–160.

24. Naumov, G.I., Domestication of dairy yeast *Kluyveromyces lactis*: transfer of the β-galactosidase (*LAC4*) and lactose permease (*LAC12*) gene cluster?, *Dokl. Biol. Sci.*, 2005, vol. 401, no. 2, pp. 120–123.

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