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EXPERIMENTAL ARTICLES

Identification of Phenotypically and Genotypically Related *Lactobacillus* Strains Based on Nucleotide Sequence Analysis of the *groEL, rpoB, rplB*, and *16S rRNA* Genes

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Abstract—Sixty-eight cultures of lactic acid bacteria were isolated and identified from national fermented milk drinks (airan, koumiss, kurunga, shubat) home-made in different regions of the Republic of Kazakhstan and the Buryat Republic of Russia. The cultures of lactic acid bacteria of the genus *Lactobacillus* were identified as *L. paracasei* and *L. rhamnosus* related to the *L. casei* group and as *L. brevis, L. buchneri, L. diolivorans*, and *L. parabuchneri* (the *L. buchneri* group) using the classical microbiological methods and on the basis of the 16S rRNA gene sequence analysis. The polymorphism of the nucleotide sequences of the genes *groEL, rpoB*, and *rplB* encoding specific proteins was studied for intraspecific differentiation of the lactobacilli. The analysis of these genes allowed a more accurate identification of the lactobacilli that are genetically and phenotypically related to the *L. casei* group as *L. paracasei* subsp. *paracasei* and *L. paracasei* subsp. *tolerans*. The gene nucleotide sequences of all the genotyped strains were deposited in the GenBank database.

Keywords: national fermented milk drinks, identification, genotyping, *Lactobacillus*, 16S rRNA genes, *groEL, rplB, rpoB* genes.

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Traditional fermented milk products of mixed lactic acid and alcoholic fermentation made according to ancient traditional recipes on the basis of camel (shubat), mare (koumiss), and cow (kurunga) milk have long been used by many nations not only as foodstuffs, but also as therapeutic–prophylactic remedies and medications for treating cardiovascular and respiratory diseases and in gastrointestinal infections [1, 2]. At present, researchers are strongly interested in these drinks and their naturally formed microbiota species composition. These unique natural consortia serve as one of the main substrates for obtaining bacterial cultures with valuable properties for development of new foodstuffs, probiotics, and preservatives. The identification of lactic acid bacteria only on the basis of their morphological, cultural, physiological, and biochemical characteristics is presently insufficient, due to a high level of phenotypic variability exhibited by many species affected by various factors [3]. The molecular genetic methods of identification proved to be reliable and independent of external factors. The high stability of the nucleotide sequence of the 16S rRNA gene prevents its application for unambiguous identification of closely related species. Many species and subspecies of lactobacilli belong to the phylogenetically closely related groups of *L. casei, L. plantarum, L. buchneri,* and *L. acidophilus*, which are not amenable to accurate identification, which requires the search for new genetic markers. For the nucleotide sequence analysis to be used for the purpose of species identification, application of the marker genes the nucleotide sequence of which analysis makes it possible to assess the genetic relatedness of the whole genome within this group of microorganisms was recommended [4, 5]. The importance of lactobacilli as starter cultures and probiotic strains in food and medical industries calls for special attention to their proper taxonomic identification, as this is the main proof of safety of the foodstuffs that include living microorganisms or the products of their vital activity.

The goal of the present work was to identify genetically and phenotypically closely related *Lactobacillus* species isolated from national fermented milk drinks using the nucleotide sequence analysis of the genes *groEL, rpoB*, and *rplB* in comparison with their identification carried out on the basis of the 16S rRNA nucleotide sequence alone.

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MATERIALS AND METHODS

Sixty-eight strains of microorganisms were used, of which 66 were lactic acid bacterial strains from the working collection of the Branch State Enterprise The Republican Collection of Microorganisms (National Center of Biotechnology, Committee of Science of the Ministry of Education and Science of the Republic of Kazakhstan) and were isolated from traditional Kazakh drinks (koumiss, shubat, airan), and two strains were isolated from the Buryat national fermented milk product kurunga. The lactococci were cultivated under static conditions in sterile fat-free milk (skimmed milk) at 30°C [6], and the lactobacilli were cultivated under anaerobic conditions in test tubes with a liquid MRS medium under a layer of starvation agar at 37°C for 36–120 h. The solid MRS medium with 1.5% agar was then inoculated with a series of dilutions of the MRS-grown bacterial cultures. Prior to identification the isolates were transferred twice and incubated for 18–24 h under the same conditions. The strain morphology was studied under an MBI-15 light microscope. For this purpose a fixed stained specimen was prepared. Guided by a list of culture characteristics for the identification of bacteria, they were assessed by the isolate growth in liquid media with a different sodium chloride content—4.0 and 6.5%, at pH 5.5 and 9.6, and at 10 and 45°C, as well as by the shape of colonies on agar media [7].

All the cultures were identified with classical microbiological methods: by their cultural characteristics, morphology, Gram staining, cell motility, the presence of catalase, and the spectrum of fermented carbohydrates [8]. The molecular genetic identification was carried out based on the nucleotide sequence analysis of the 16S rRNA gene using the MegAlign 6.00 DNASTAR Inc. software package.

DNA was isolated from the 24-h cultures incubated at 37°C on the MRS 1 medium (HiMedia) using Wilson's method [9]. The *16S rRNA* gene fragment was amplified according to the method proposed by Vegas [10]. The *groEL* gene was amplified using the primers and conditions proposed by Dellaglio et al. [11]; the *rplB* gene was amplified according to Diancourt et al. [12]. Amplification of the *rpoB* gene was performed with the primers For rpoB (5'-TAACCGTGGTGCTTGGCTDGAATWYGAAAC-3') and Rev- rpoB (5'-ATCAAACCAATGTTAGGNCCTTCWGGDGTTTC-3"). The reaction was carried out in a total volume of 30 μl. The PCR mixture contained 150 ng of DNA, 1 U of Maxima Hot Start *Taq* DNA polymerase (Fermentas), 0.2 mM of each dNTP, 1 PCR buffer (Fermentas), 1.5 mM of $MgCl_2$, and 15 pmol of each primer. The PCR amplification protocol included long-term denaturation at 95°C for 7 min; 30 cycles: at 95°C, 50 s; at 59°C, 60 s; at 72°C, 90 s; and final elongation, 7 min at 72°C. The PCR protocol was performed using the DNA Engine Tetrad 2 Cycler PTC-0240 amplifier (Bio-Rad).

The PCR products were purified from the primers and dNTP using the enzymatic method [13]. The sequencing was carried out using the BigDye(r)Terminator v3.1.Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's instruction with subsequent fragment separation on an automatic 3730x1 genetic analyzer (DNA Analyzer, Applied Biosystems).

The nucleotide sequences were analyzed and combined into a general sequence using the SeqScape 2.6.0 software package (Applied BioSystems). All the sequences obtained in this study were deposited in GenBank (JF520433-JF520627).

The nucleotide sequences of *16S rRNA* genes were identified using the MicroSeqID (Applied BioSystems) and GenBank software. Phylogenetic analysis was carried out using the Mega 3.1 software package [14]. The sequence alignment was carried out using the ClustalW algorithm [15]. The Neighbor-Joining (NJ) algorithm was used for constructing phylogenetic trees [16]. The nucleotide sequences of the closely related species of the *L. casei* phylogenetic group *L. paracasei* and *L. rhamnosus,* as well as *L. sakei, L. plantarum, L. reuteri,* and *L. buchneri*, deposited in the international databases were additionally included in the analysis.

RESULTS AND DISCUSSION

As a result of classical microbiological identification, 61 isolates were differentiated and assigned to 14 *Lactobacillus* spp. species, and 1 isolate was identified as *Lactococcus lactis* subsp. *lactis.* The results of the phenotypic identification are shown in Tables 1 and 2. Thirty-one strains were isolated from koumiss, and 35 strains were isolated and identified from shubat and airan; mesophilic lactococci *L. lactis* subsp. *lactis*, strain K-205 (GenBank, no. EF114305) [2] and lactobacilli were isolated from kurunga, a Buryat national dairy drink.

All the isolates were represented by gram-positive nonmotile cells, which grew well in the presence of 4.0% NaCl and did not grow at 6.5% NaCl. The lactobacilli, unlike *L. lactis* subsp. *lactis*, did not grow at 10°C, grew poorly at 15°C, and decreased the medium pH from 5.5 to 4.0. During glucose fermentation, the lactobacilli formed CO_2 bubbles (Table 1).

Based on analysis of the 16S rRNA gene sequence, 67 strains were assigned to the genus *Lactobacillus*. Forty-three strains of them were assigned to the phylogenetic group of *L. casei* (*L. paracasei,* 35 strains and *L. rhamnosus*, 8 strains); to *L. buchneri*, 14 strains (*L. brevis*, 9; *L. buchneri*, 2; *L. parabuchneri*, 2; and *L. diolivorans*, 1); to *L. plantarum,* 9 strains (*L. plantarum/L. pentosus*). The *L. reuteri* phylogenetic group was represented by a single culture, P-59, isolated form shubat and phenotypically and genetically identified as *L. fermentum.*

Table 1. Differentiating characteristics of *Lactococcus lactis* subsp. *lactis* and *Lactobacillus* ssp. isolated from kurunga

Characteristics	*L. lactis* subsp. *lactis*	*Lactobacillus* spp.
Predominant position of the cells	Chains of up to eight cocci	Long thin rods, single or forming short chains
Motility	–	–
Growth at 10°C	+	–
Growth at 45°C	–	+
pH 9.6	–	–
pH 5.5	+	+
Growth in the presence of 4% NaCl	+	+
Growth in the presence of 4% NaCl	–	–
Gas formation during glucose fermentation	–	+

Note: The sign "+" denotes the presence of a characteristic; the sign "–" denotes the absence of a characteristic.

Table 2. Phenotypic identification of the lactic acid bacteria isolated from the fermented milk drinks

Names of species	Codes of the cultures studied
L. paracasei	P-003, P-010, P-018, P-019, P-020, P-021, P-025, P-027, P-159, P-034, P-96, P-41, P-51, K-0201, K-0202, K-0208
L. casei	P-001, P-006, P-012, P-043, P-66
L. rhamnosus	P-67, P-73, P-78, P-79, P-85, P-98, P-039
L. plantarum	P-040, K-0015, K-0026
L. buchneri	P-024, P-025-2
L. parabuchneri	P-005, P-231
L. fermentum	P-59, K-0025, K-0103, K-0155, K-0203
L. brevis	P-032, P-042, K-0004, K-0005, K-0006, K-0007, K-0010, K-0105, K-0154
L. hilgardia	P-014
L. acidophilus	P-028
L. cellobiosus	K-001, K-0012
L. delbrueckii	K-0021, K-0023, K-0044
L. diolivorans	KP-2
Lactococcus lactis subsp. *lactis*	K-0024, K-0041, K-0043, K-0149, K-0153, K-0048, K-205
L. leichmani	K-0104, K-0106, K-0108, K-0110

Assessment of the nucleotide identity of the sequences of the *16S rRNA* gene fragments with the reference sequences of the phylogenetically closely related species is shown in Table 3. In order to rule out a possible methodical error of gene analysis of this gene [17], phylogenetic trees were additionally constructed (Fig. 1a, 1b).

Taking into account the mode of the *16S rRNA* gene-based assessment of the species identity of bacteria in which the boundary species identity value is 97.0% of the nucleotide identity, in our study it was possible to unambiguously identify only strain P-59 as *L. fermentum*. Reliable identification of the other species included in the groups was not possible due to the high interspecific identity of the *16S rRNA* gene sequences [18].

The strains classified as *L. casei* according to their *16S rRNA* gene sequences were used for further identification by analysis of the sequences of the fragments of the *groEL, rplB*, and *rpoB* genes.

The strains exhibiting the highest identity to *L. paracasei* were additionally phenotypically identified down to the subspecies level: *L. paracasei* subsp. *paracasei*/*L. paracasei* subsp. *tolerans*. The identifiable strains isolated from shubat and airan that fermented cellobiose, esculin, maltose, mannitol, melezitose, salicyl, and trehalose were assigned to *L. paracasei* subsp. *paracasei* (K-0015, K-0024, K=h0041, K-0043, K-0103, K-0105, K-0106, K-0108, K-0153, K-0154, K-0201, K-0208, P-001, P-003, P-006, P-010, P-012, P-019, P-020, P-021, P-025, P-027, P-028, P-159, P-034, P-96, P-039, P-41, P-043, P-51). The identifiable strains K-0023, K-0149, K-0155, and K-0104 isolated from koumiss and not fermenting cellobiose, esculin, maltose, man-

Table 3. Comparative analysis of the similarity of the *16S rRNA* gene nucleotide sequences between the lactobacilli isolated from fermened milk drinks and reference strains

Names of cultures	Identification by *16S rRNA* gene analysis, (%)	
K-0007, K-0010, K-0006, K-0005, K-0004, K-001, P-014, P-032, P-042	*L. brevis*	99.6 ± 0.4
	L. parabrevis	97.5 ± 0.3
	L. hammesii	97.4 ± 0.3
P-040, K-0202, K-0021, K-0012, K-0026, K-0044, K-0110, K-0048, K-0203	*L. plantarum*	99.8 ± 0.2
	L. pentosus	99.8 ± 0.2
	L. paraplantarum	99.5 ± 0.2
K-0015, K-0023, K-0024, K-0025, K-0041, K-0043, K-0103, K-0104, K-0105, K-0106, K-0108, K-0149, K-0153, K-0154, K-0155, K-0201, K-0208, P-001, P-003, P-006, P-010, P-012, P-019, P-020, P-021, P-025, P-027, P-028, P-159, P-034, P-96, P-039, P-41, P-043, P-51	*L. paracasei*	99.8 ± 0.2
	L. rhamnosus	98.1 ± 0.2
	L. casei	98.7 ± 0.2
P-005, P-231	*L. parabuchneri*	99.8 ± 0.2
	L. kefiri	98.4 ± 0.1
	L. buchneri	97.5 ± 0.4
P-024, P-025-2	*L. buchneri*	99.3 ± 0.5
	L. kefiri	97.6 ± 0.1
	L. parabuchneri	97.5 ± 0.4
P-86, P-67, P-73, P-85, P-78, P-79, P-98, P-66	*L. rhamnosus*	99.8 ± 0.2
	L. paracasei	98.1 ± 0.2
	L. casei	98.1 ± 0.2
KP-2	*L. diolivorans*	100
	L. genomosp.	98.0
P-59	*L. fermentum*	100
K-205	*L. lactis* subsp. *lactis*	99.7

nitol, melezitose, salicyl, and trehalose were assigned to *L. paracasei* subsp. *tolerans*.

The *groEL* gene fragment about 1000 bp in size was amplified by PCR. After removing the primer sequence, 802-bp nucleotide sequences were obtained in the not overlapping regions. Their alignment revealed that the sizes of the *groEL* fragments of the reference strains *L. zeae* ATCC 393 (GenBank, no. AF429674) and *L. casei* subsp. *casei* ATCC 393 versus strain DSM 20011 (GenBank, no. AY424336) were 552 and 506 bp, respectively. To obtain a homogeneous 506 bp sample, part of the nucleotide sequences were therefore removed from the strains studied. The gene *groEL* encodes the 60-kDa heat shock protein, which is involved in the protein life cycle, from the polypeptide chain synthesis to assemblage into the multimer complexes, and is very important for maintaining the cell activity. Earlier, this gene was used in the works of Dellaglio et al. [11] and Blaiotta et al. [18] for identification of lactic acid bacteria.

The percentage of similarity between the nucleotide sequences of the *groEL* gene fragments for *L. casei* and *L. paracasei* was 87.0%; for *L. paracasei* and *L. rhamnosus*, 88.5%; for *L. casei* and *L. rhamnosus*, 90.0%; and for *L. paracasei* and *L. sakei*, 74.0%. In the process, the intraspecific polymorphism of the *groEL* nucleotide sequence of *L. paracasei* did not exceed 1.2%; that of *L. rhamnosus* was below 1.8%. At the same time, the identity of the nucleotide sequences of the 16S rRNA gene between the genetically and phenotypically closely related *L. casei* strains (*L. paracasei* and *L. rhamnosus*) exceeded 98.0% (Fig. 2).

In the same strains belonging to the *L. casei* group, a 480-bp *rplB* nucleotide sequence fragment was amplified. Similarly to the *groEL* gene, the 366-bp *rplB* nucleotide sequences were obtained using the direct and the reverse primers. Earlier, Diancourt et al. [12] used this *rplB* gene fragment for identifying the phenotypically and genetically closely related species of the *L. casei* group, as well as *L. plantarum, L. brevis, L. animalis*, and *L. sakei* subsp. *sakei*. One-hundred-eighty-four polymorphisms (50.3%) were found between these species on the 366-bp nucleotide seg-

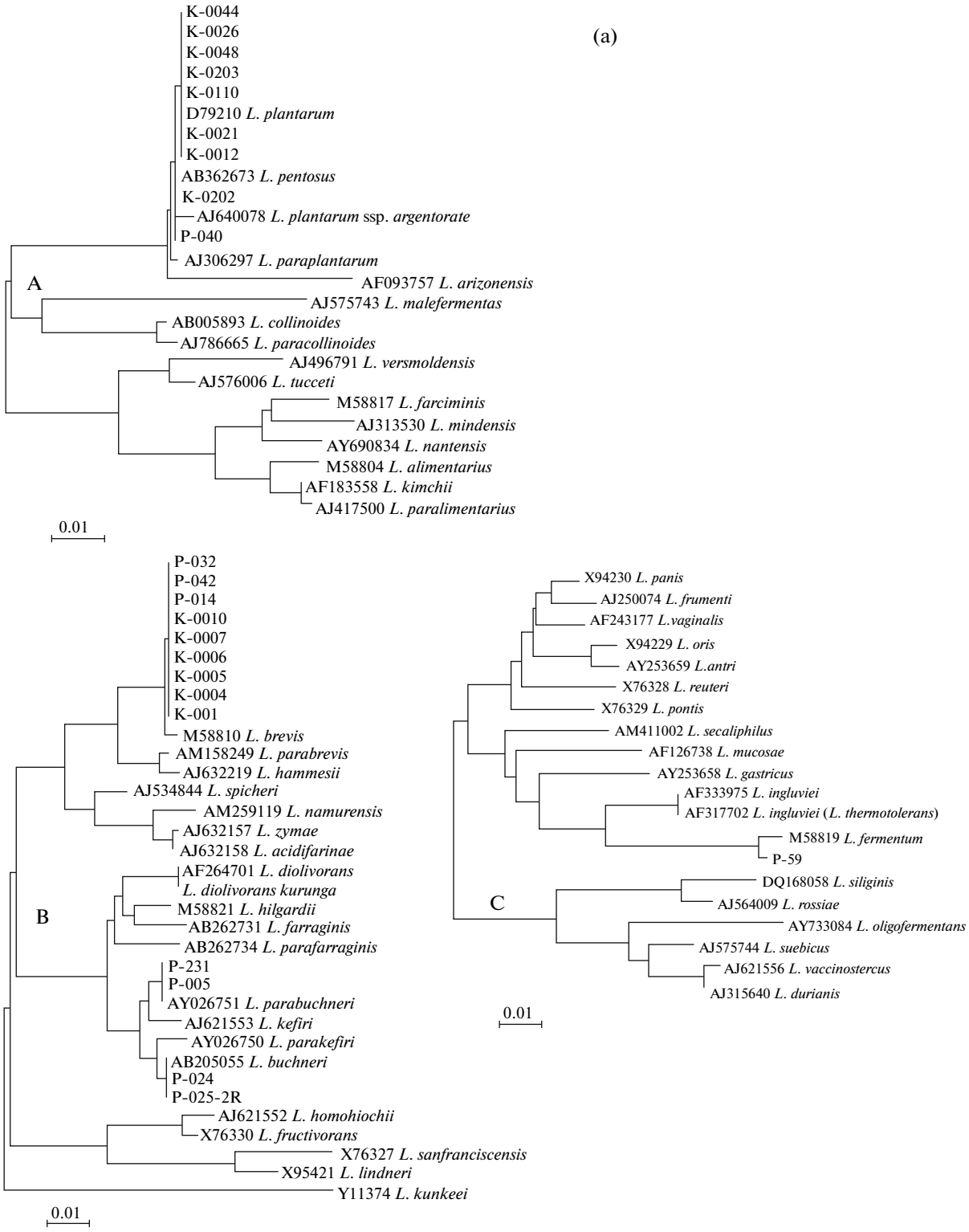


Fig. 1a. Phylogenetic position of the isolated strains of lactobacilli constructed on the basis of the *16S rRNA* nucleotide sequences: *L. plantarum* group (A), *L. buchneri* group (B), and *L. reuteri* group (C).

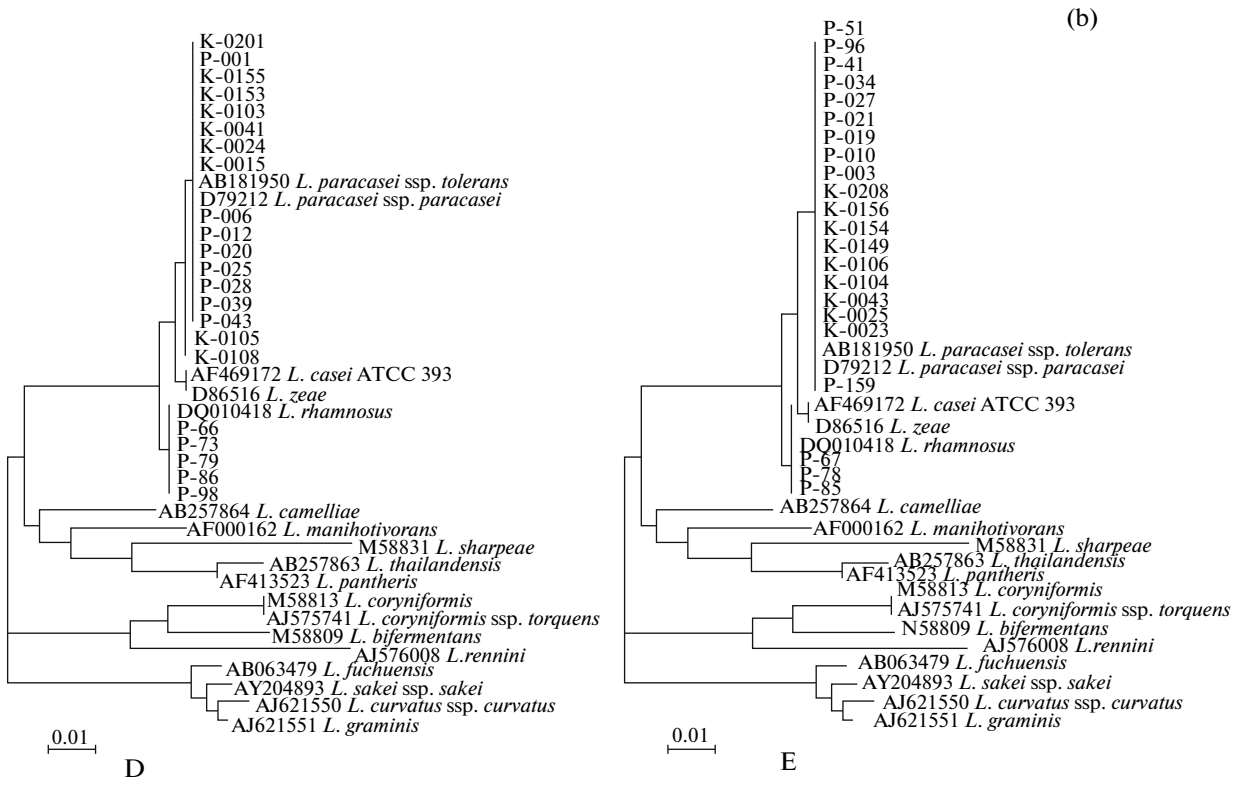


Fig. 1b. Phylogenetic position of the isolated strains of lactobacilli constructed on the basis of the *16S rRNA* gene nucleotide sequences: *L. casei* group (D and E).

ment, and phylogenetic analysis enabled us to differentiate between *L. casei, L. rhamnosus*, and *L. zeae*. In our study, the average similarity of the *rplB* gene fragment nucleotide sequence was 84.2% between *L. casei* and *L. paracasei*, 83.7% between *L. paracasei* and *L. rhamnosus*, and 85.5% between *L. casei* and *L. rhamnosus*. A decrease in the nucleotide similarity of the *rplB* gene fragments was also noted between the other species assigned to the *L. casei* phylogenetic group. Thus, for example, the identity of the *rplB* fragments between *L. paracasei* and *L. sakei* was about 68.9%; between *L. rhamnosus* and *L. sakei*, 66.0%.

The interspecific topology of the phylogenetic trees constructed on the basis of analysis of the nucleotide sequence of the *rplB* gene fragments of *L. sakei* differed from the topology of the tree constructed on the basis of the *16S rRNA* gene sequence (Figs. 1, 3). *L. sakei* formed a cluster together with *B. subtilis*. The *rplB* gene sequence encodes the ribosomal protein L2, which is essential for binding the ribosomal subunits, tRNA binding, and tRNAase transfer [20].

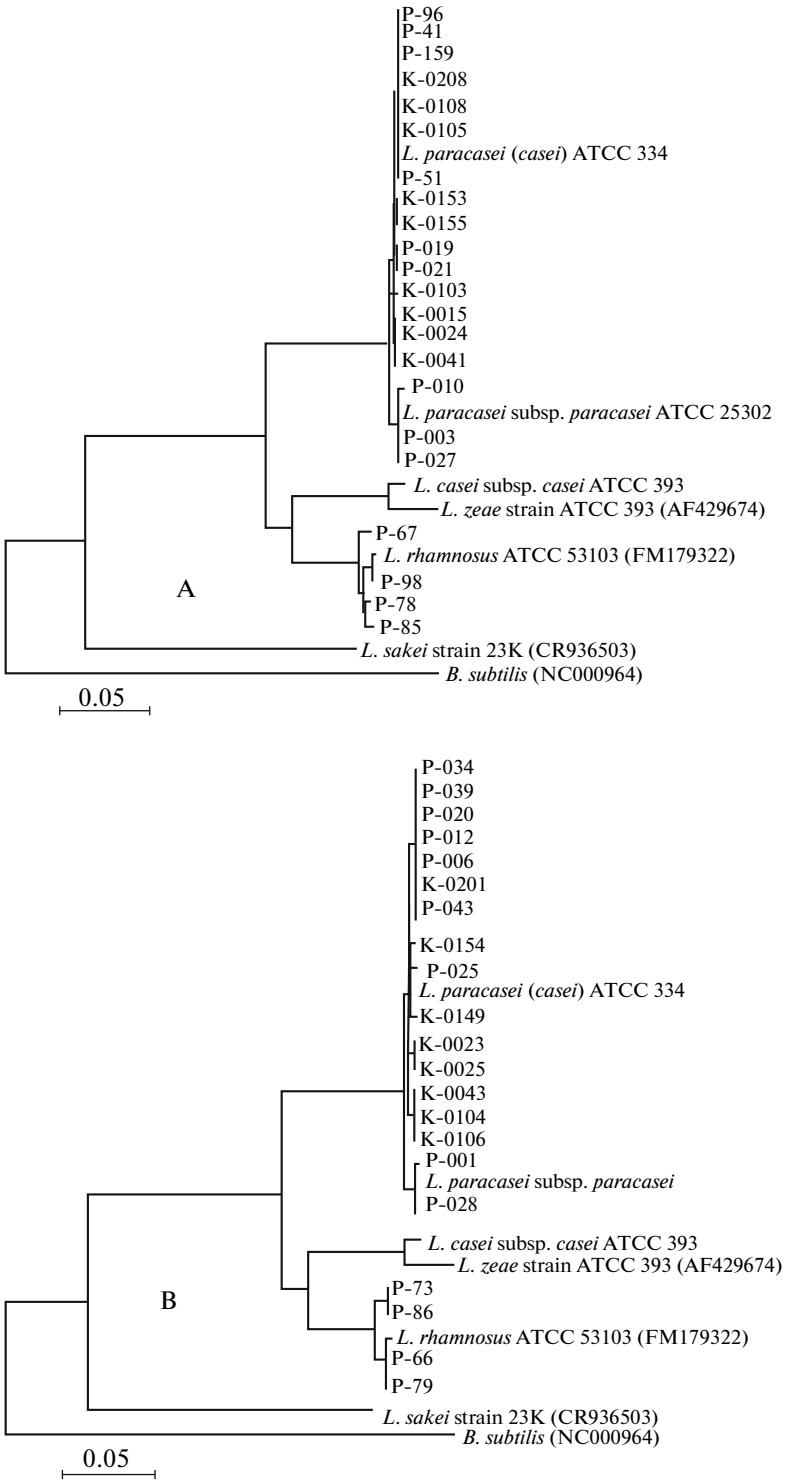
The *rpoB* gene sequence encodes the RNA polymerase β subunit and initiates RNA synthesis. The bacterial *rpoB* gene is universal and occurs in a single copy in the genome. The *rpoB* gene nucleotide sequence makes it possible to assess the relations at the species and intraspecies level, which has been successfully used in the study of the phylogenetic relationship between *Staphylococcus aureus* and *Enterobacteriaceae* [21]. The evolutionarily ancient origin of the gene suggests the possibility of its use as a bacterial chronometer [22].

The 1116-bp fragment of the *rpoB* gene was amplified by PCR in 43 identifiable strains of the *L. casei* group. The processed nucleotide sequences were 771 bp in size. The topology of the phylogenetic trees of the *rpoB* and 16S rRNA genes did not have any differences (Figs. 1, 4).

The average similarity between the *rpoB* gene sequences of *L. paracasei* and *L. rhamnosus* was 84.8%; between *L. paracasei* and *L. sakei* sequences, 74.5%. The intraspecific polymorphism of the *rpoB* gene sequence between *L. paracasei* strains did not exceed 0.8%; within the *L. rhamnosus* species, 0.7% (Fig. 4).

As for all the protein-encoding genes described earlier, a change in the percentage of identity toward decreasing was noted when closely related genes in the *L. casei* group were compared.

Thus, the identification of 68 strains made it possible to reveal the unreliable phenotypic identification of 6 strains at the generic level and of 24 strains at the species level, which is associated with the phenotypic variability of lactobacilli. The highest percentage of unreliable strain identification was found in the *L. casei* phylogenetic group, because they had highly



Fig, 2. Phylogenetic position of the isolated strains of lactobacilli constructed on the basis of the *groEL* nucleotide sequences: *L. casei* group (A and B).

identical profiles of carbohydrate fermentation, as well as a high similarity of 16S rRNA genes, which might have been the main cause of the unreliable identification. It is also important to note that, despite the high percentage of 16S rRNA similarity between the subspecies *L. paracasei* subsp. *paracasei* and *L. paracasei* subsp. *tolerans*, they are very different phenotypically, with the phenotype of the latter being dissimilar to any

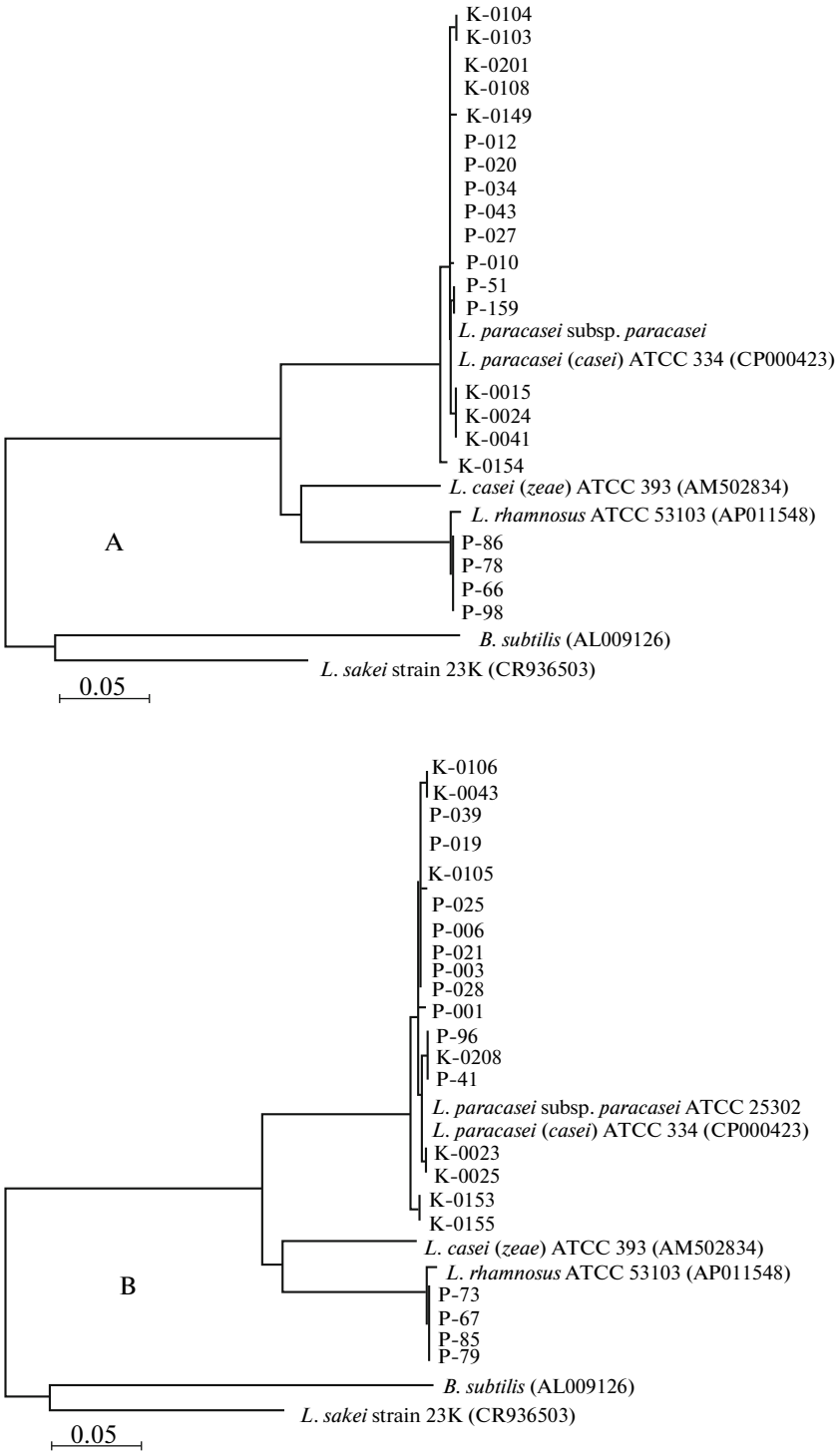


Fig. 3. Phylogenetic position of the isolated strains of lactobacilli constructed on the basis of the *rplB* nucleotide sequences: *L. casei* group (A and B).

phenotype within the *L. casei* group, which was the cause of the unreliable identification of all *L. paracasei* subsp. *tolerans* strains.

Analysis of the *groEL, rplB*, and *rpoB* genes allowed us to reveal high polymorphism of the nucleotide sequences of these genes in the representatives of the *L. casei* phylogenetic group and reliably identify the phenotypically and genetically closely related species of this group of lactobacilli. The discriminative ability of these genes by several times exceeded that of the

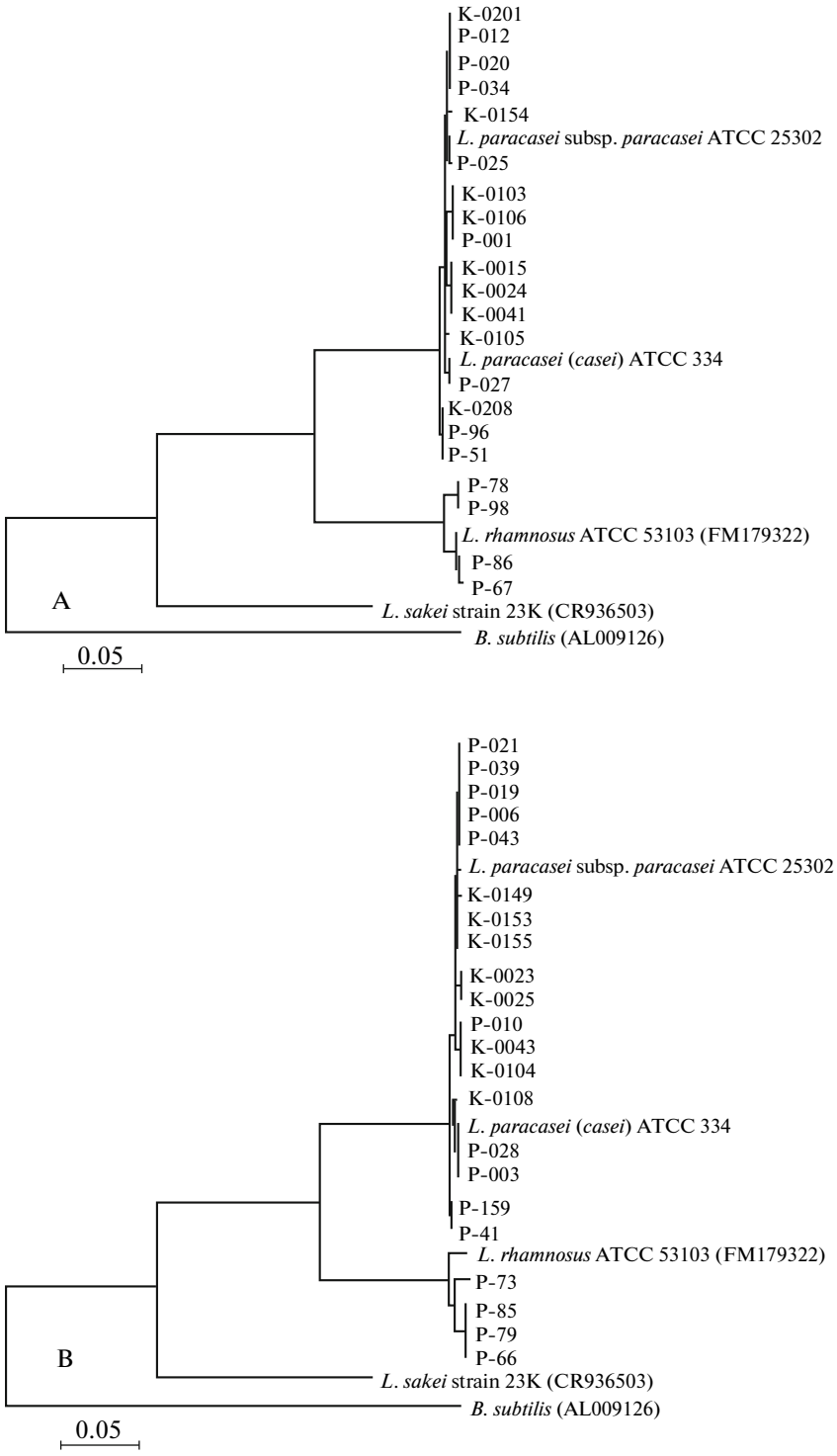


Fig. 4. Phylogenetic position of the isolated strains of lactobacilli constructed on the basis of the *rpoB* nucleotide sequences: *L. casei* group (A and B).

16S rRNA gene. The topology of the phylogenetic trees was identical to the conventional 16S rRNA-based phylogeny, except for the tree constructed on the basis of the *rplB* sequences. Importantly, the addition of the nucleotide sequences of other genes may change the typological structure. The differences between the topological structures of the phylogenetic trees constructed on the basis of analysis of various marked genes do not influence the reliability of species identification [23].

It should be noted that the genetic markers used in our investigations did not allow us to carry out intraspecific identification of *L. paracasei*. Therefore, the subspecies identification of *L. paracasei* subsp. *paracasei* and *L. paracasei* subsp. *tolerans* should be based on phenotypic parameters.

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