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ORIGINAL PAPER

Phylogenetic study of *Lactobacillus acidophilus* group, *L. casei* group and *L. plantarum* group based on partial *hsp*60, *phe*S and *tuf* gene sequences

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Abstract The partial nucleotide sequences encoding elongation factor Tu (*tuf* gene), 60-kDa heat shock protein (*hsp*60 gene) and phenylalanyl-tRNA synthase (*phe*S gene) were determined to assess the suitability as phylogenetic markers for discriminating the closely related species in *Lactobacillus acidophilus* group, *L. casei* group and *L. plantarum* group. A total of 234 lactobacilli were chosen from traditional fermented dairy products that were not exactly assigned to species based on biochemical tests and 16S rRNA gene sequences. The sequencing of partial *tuf*, *hsp*60 and *phe*S gene of all strains was performed, and then, the phylogenetic trees were constructed by neighbor-joining method. Phylogenetic tree revealed three genes provided better resolution of each *Lactobacillus* species than 16S rDNA, and all of strains were clearly identified as *L. casei* (63 strains), *L. plantarum* (58 strains) and *L. helveticus* (113 strains) by comparison of sequences with the type strains. From our results, the partial sequences of three genes had a higher discriminatory power than 16S rRNA gene sequences and were an alternative molecular tool for the taxonomical analysis of *L. casei* group, *L. plantarum* group and *L. acidophilus* group.

Keywords *Lactobacillus* · *hsp*60 gene · *phe*S gene · *tuf* gene

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Introduction

Lactobacilli is an important taxa involved in food microbiology and human nutrition owing to their role in food and feed production and preservation [1]. Therefore, precise identification and classification of lactobacilli to the species level are required. Established methodologies, routinely used for assessing the evolution of lactic acid bacteria (LAB), rely on morphological descriptors and phenotypic methods based on biochemical systems such as API 50CH carbohydrate tests (API Products, Bio-Merieux, France) and phenotypic arrays (Biolog, Hayward, CA, USA) [2, 3]. Although phenotypic tests provide evidence of the metabolic capabilities of different strains, these methods have problems such as non-reproducibility and lack of discriminatory power [4].

Identification of LAB is most commonly based on the variability in their ribosomal RNA genes. Comparison of the gene sequences of bacterial species showed that the 16S rRNA gene is highly conserved within a species and among species of the same genus, so 16S rRNA gene can be used as the "gold standard" for the determination of the bacteria [5]. However, genotypic identification based on 16S rRNA sequences has limited discriminating power for closely related *Lactobacillus* species [6, 7]. Recently, more protein encoding genes have been applied for classification of closely related species: the *tuf* gene (encoding elongation factor Tu) [8], the *hsp*60 gene (encoding 60-kDa heat shock protein) [9], the *htrA* gene (encoding stress-inducible trypsin-like serine protease) [10], the *recA* gene (encoding recombinase A) [11], and the *rpoA* gene (encoding RNA polymerase beta subunit) [12].

The *tuf* gene encodes the elongation factor Tu, involved in protein biosynthesis, which facilitates the elongation of polypeptides from the ribosome and aminoacyl-tRNA during translation. It is universally distributed, and in most gram-positive bacteria, only one *tuf* gene per genome has been

found [13]; thus, it is ideally suited for phylogenetic studies. The *tuf* gene has been used as a target gene for phylogenetic studies [14]. The *hsp*60 gene, which encodes a 60-kDa subunit (known as GroEL, 60-kDa chaperonin, and heat shock protein 60) of a complex that assists with the three-dimensional folding of bacterial proteins, has the potential to serve as a general phylogenetic marker because of its ubiquity and conservation in nature. Recent studies [9, 15, 16] have shown that *hsp*60 gene may be an alternate DNA target for species-specific identification of microbial species. Phenylalanyl-tRNA synthase gene (*phe*S) also been proven to be a valuable tool for the identification of *Lactobacillus* species and the delineation of novel taxa [17, 18].

The objective of this study was to design an accurate and rapid method to identify closely related lactobacilli isolated from traditional fermented dairy samples in six different regions and to assess the usefulness of the partial sequence of *tuf*, *hsp*60 and *phe*S for the differentiation of closely related species. The combination of *tuf*, *hsp*60 and *phe*S sequences alignments and phylogenetic analysis proved that the three genes were able to identify the species of *L. casei* group, *L. plantarum* group and *L. acidophilus* group at the species level.

Materials and methods

Bacterial strains

All lactobacilli were isolated from traditional fermented dairy samples collected from Tibet, Qinghai, Inner Mongolia, Yunnan and Xinjiang province of China and Mongolia. A total of 234 lactobacilli were chosen from isolates (Table 1), which were preliminary classified and identified as *L. casei* group, *L. plantarum* group and *L. acidophilus* group by biochemical tests and 16S rRNA gene sequences in our laboratory [19–22].

DNA extraction

Total genomic DNA was extracted from overnight cultures by the previous method [23]. Briefly, 1 ml liquid culture of each strain incubated overnight in MRS broth was pelleted by centrifugation at 8,000*g* for 5 min. The pellets were washed with 500 µl TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0) in a clean 1.5-ml microcentrifuge tube and repelleted by centrifugation. Washed cell pellets were resuspended in 500 µl TE buffer; tubes were frozen for 5 min by liquid nitrogen and then incubated at 65 °C for 5 min. This freezing–thawing step was repeated 4 times; 10 µl proteinase K solution (20 mg proteinase K/ml in TE; Amresco Inc.) and 60 µl SDS solution (10 %) were added. After incubated at 37 °C for 1 h, 100 µl NaCl (5 M) and 80 µl CTAB/NaCl (10 % cetyltrimethylammonium bromide, 0.7 M NaCl) were added, and tubes were incubated at 65 °C for 30 min. The mixture was extracted with an equal volume of phenol/chloroform/isoamylalcohol. After centrifugation, DNA was obtained by the addition of isopropanol (one equal volume) and then washed in 500 µl ethanol (70 %). DNA was pelleted, dried and dissolved in 100 µl RNase solution (100 µg/ml RNase in TE; Sigma-Aldrich). After incubation for 1 h at 37 °C, the sample volumes were adjusted to 400 µl with TE. Then, phenol/chloroform extraction was performed, and DNA was precipitated with 0.5 M NaAc (final concentration) and two volumes of ethanol (99 %). Pellets were washed in 500 µl ethanol (70 %). Finally, DNA was solubilized as above in 50–250 µl TE, and stock solution were stored at −20 °C.

PCR amplification

For each strain, the genomic DNA was used as a template for PCR amplification of a segment of *tuf*, *hsp*60 and *phe*S genes on the automatic thermal cycler (PTC-200, MJ Research, Waltham, MA). The sequences of the primers

Table 1 Strains and numbers of lactobacilli used in this study

Sample	Source	Number of isolates		
		Lactobacillus helveticus	*Lactobacillus plantarum*	*Lactobacillus casei*
Koumiss	Inner Mongolia	8	6	13
Koumiss	Xinjiang	48	8	1
Koumiss	Mongolia	21	7	–
Fermented yak milk	Inner Mongolia	5	12	5
Koumiss	Qinghai	1	7	–
Fermented yak milk	Qinghai	3	10	–
Dairy fan	Yunnan	19	1	–
Kurut	Tibet	3	2	21
Fermented Mongolian cattle milk	Tibet	5	5	23
Total	–	113	58	63

Table 2 Oligonucleotide primers used in this study

Primer	Oligonucleotide sequence (5′–3′)	References
TUF-1	GATGCTGCTCCAGAAGA	[8]
TUF-2	ACCTTCTGGCAATTCAATC	
PheS-21FA	cgccagggttttcccagtcacgacCAYCCNGCHCGYGAYATGC	[24]
PheS-22RA	agcggataacaatttcacacaggaCCWARVCCRAARGCAAARCC	
H729	cgccagggttttcccagtcacgacGAIIIIGCIGGIGA(T/C)GGIACIACIAC	[33]
H730	agcggataacaatttcacacagga(T/C)(T/G)I(T/C)(T/G)ITCICC(A/G)A AICCIGGIGC(T/C)TT	

were listed in Table 2. For each target, PCR mixture (50 μl) contained 200 ng template DNA, 5 μl 10 × buffer with 3.0 mM $MgCl_2$, 6.0 unit *Taq* DNA polymerase, 0.4 mM of dNTP and 20 pmol of each primer.

A DNA fragment corresponding to partial of *tuf* gene was amplified; each PCR cycling profile consisted of an initial denaturation step of 3 min at 95 °C, followed by amplification for 35 cycles as follows: denaturation (30 s at 95 °C), annealing (30 s at 52 °C) and extension (2 min at 72 °C). Amplification was completed with an elongation phase (10 min at 72 °C).

Universal *hsp*60 oligonucleotide primers H729 and H730 (Table 2) were used to amplify the *hsp*60 gene. The cycling conditions were 5 min at 95 °C for 1 cycle, followed by 30 cycles of 1 min at 95 °C, 30 s at 37 °C and 1 min at 72 °C. The last cycle was performed at 10 min at 72 °C.

For the convenience of sequencing, 24-bp nucleotide sequences were inserted in front of the primers PheS-21FA and PheS-21RA as described in the study conducted by Naser et al. [24], and they were used to amplify the *phe*S gene. The program consisted of 30 cycles of 30 s at 94 °C, 30 s at 35 °C and 2 min at 72 °C. PCR products were electrophoresed in a 1.0 % agarose gel and visualized by UV transillumination after ethidium bromide staining.

Nucleotide sequencing and phylogeny study

Sequencing of the PCR products was performed in Shanghai Sangni Biosciences Corporation. To confirm the species, the nucleotide sequences of the *tuf*, *hsp*60 and *phe*S genes of all the tested strains were analyzed and determined by BLAST program on NCBI. Consensus sequences were imported into MEGA version 4.0 software (http://www.megasoftware.net) [25], with which a sequence alignment and the representative sequences of each group were selected for phyletic tree construction based on neighbor-joining method, *Bifidobacterium longum* was considered as an outlier.

Nucleotide sequence accession numbers

All sequences determined in this study were deposited in GenBank under the following accession numbers: FJ983574 to FJ984182, FJ825030 to FJ825054, FJ825056 to FJ825086, FJ825088 to FJ825118 and FJ825120 to FJ825125.

Results

The three genomic regions of 234 isolates targeted were successfully amplified. The expected fragment lengths were observed for the PCR products. Removed the biased primer regions and ambiguous single-strand data, about 760 bp for *tuf*, 450 bp for *phe*S and 570 bp for *hsp*60 were subjected to phylogenetic analysis.

*hsp*60 sequence and phylogenetic analysis

Partial *hsp*60 gene was determined from closely related species including *L. casei* group, *L. acidophilus* group and *L. plantarum* group. Furthermore, nucleotide sequence homology searches of databases available through the BLAST yielded the highest matching scores for the corresponding reference strains; 113 strains shared 100 % identity with the *hsp*60 gene sequence of *L. helveticus* ATCC15009^T, *and* 63 isolates showed a similarity of 100 % to *L. paracasei* ATCC 25302^T, and less than 90 % to the other species (*L. rhamnosus* ATCC 7469^T, 88 %; *L. casei* ATCC 393^T, 85 %; *L. zeae* ATCC 15820^T, 86 %); 58 isolates was close to *L. plantarum* ATCC 14917^T, and they shared 99.9 % homology; however, all isolates only shared 95 and 94 % homology with *L. paraplantarum* DSM 10667^T and *L. pentosus* ATCC 8041^T, respectively. The phylogenetic tree constructed from the alignment of the representative *hsp*60 gene sequences is shown in Fig. 1. All strains were divided into two large branches, the first branch contained two groups, the *L. acidophilus* group and the *L. casei* group. In the *L. acidophilus* group, representative IMAU60068 and type strain *L. helveticus* ATCC15009^T were clustered into a group, IMAU10126 of *L. casei* group was closely related to *L. paracasei* ATCC 25302^T in 100 % of bootstrap analyses. The second branch contained *L. plantarum* group, where representative IMAU50045 was placed in the cluster of *L. plantarum*

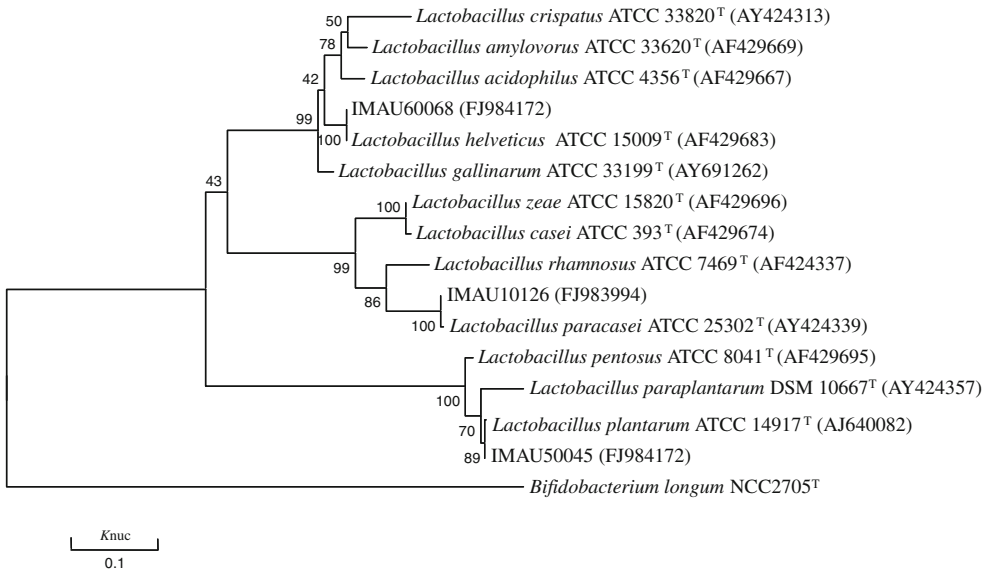

Fig. 1 Neighbor-joining tree showing the phylogenetic relationships between representative strains from each group and the type strains based on *hsp*60 gene sequences. *Bifidobacterium longum* was considered as an outlier. Bootstrap values based on 100 replications are given at the nodes

ATCC 14917^T, which recovered in 100 % of bootstrap analyses. According to the phylogenetic analysis based on the *hsp*60 gene, all isolates could be clearly identified as *L. casei* (63 strains), *L. plantarum* (58 strains) and *L. helveticus* (113 strains).

tuf sequence and phylogenetic analysis

The partial *tuf* gene sequences (760 bp) of all strains were determined. Resulting sequences were compared with related bacteria sequences in the GenBank, and sequence similarities were determined using BLAST program. Phylogenetic trees were constructed with the *tuf* gene sequences (Fig. 2). In this way, 234 lactobacilli belong to three different *tuf* clusters. One *tuf* cluster has showed the highest sequence similarity (100 %) with the type strain of *L. paracasei* ATCC 25302^T and clearly distinct from other species (Similarity: *L. rhamnosus* ATCC 7469^T, 93 %; *L. casei* ATCC 393^T, 91 %; *L. zeae* ATCC 15820^T, 92 %). The other *tuf* cluster has showed the highest sequence similarity of 100 % with the type strain of *L. plantarum* ATCC 14917^T and similarity of 98 % with the other two species. Moreover, the isolates IMAU60068 have high similarity to *L. helveticus* ATCC15009^T (100 %). Phylogenetic analysis showed the same result with *hsp*60 gene and provided higher resolution than the 16S rRNA gene.

*phe*S sequence and phylogenetic analysis

Phylogenetic analysis of the *phe*S gene sequences showed distinct positions of the species (Fig. 3). IMAU60068 shared 100 % homology with *L. helveticus* ATCC15009^T. Strain IMAU50054 and type strain *L. plantarum* ATCC 14917^T were clustered into a group with a similarity of 100 %, and its *phe*S gene sequence showed a similarity of 90 % to *L. paraplantarum* DSM 10667^T and 84 % to *L. pentosus* ATCC 8041^T. IMAU60068 was placed in the cluster of *L. casei* group, which recovered in 100 % of bootstrap analyses, and its *phe*S gene sequence showed a similarity of less than 93 % to the other type strains. The topology of the phylogenetic tree from *phe*S sequences showed a distribution of lactobacilli similar to that based on *hsp*60 gene and *tuf* gene sequence analysis. However, it is more discriminatory than *hsp*60 gene and *tuf* gene.

Comparative sequence analyses

Partial DNA sequences of *tuf*, *phe*S and *hsp*60 genes were obtained for 113 *L. helveticus*, 63 *L. casei* and 58 *L. plantarum* isolates. Our data clearly shown that *hsp*60, *tuf* and *phe*S genes sequences are more discriminatory than 16S rRNA, especially in *L. plantarum* group. The average nucleotide sequence similarities of *hsp*60, *tuf* and *phe*S genes among the *L. plantarum* group type strains was significantly less than that of 16S rRNA (96, 98.6, 91.3 and 99.4 %, respectively). On the 16S rRNA gene tree (Fig. 4), *L. paraplantarum*, *L. pentosus* and *L. plantarum* could not be distinguished due to few nucleotide differences in the variable region. However, sequencing of *hsp*60, *tuf* and *phe*S genes from the investigated lactobacilli confirmed the clustering of the *L. plantarum*, which were clearly and easily separated from *L. pentosus* and *L. paraplantarum*. Within *L. casei* group, the average nucleotide sequence similarities of *hsp*60, *tuf* and *phe*S genes were significantly

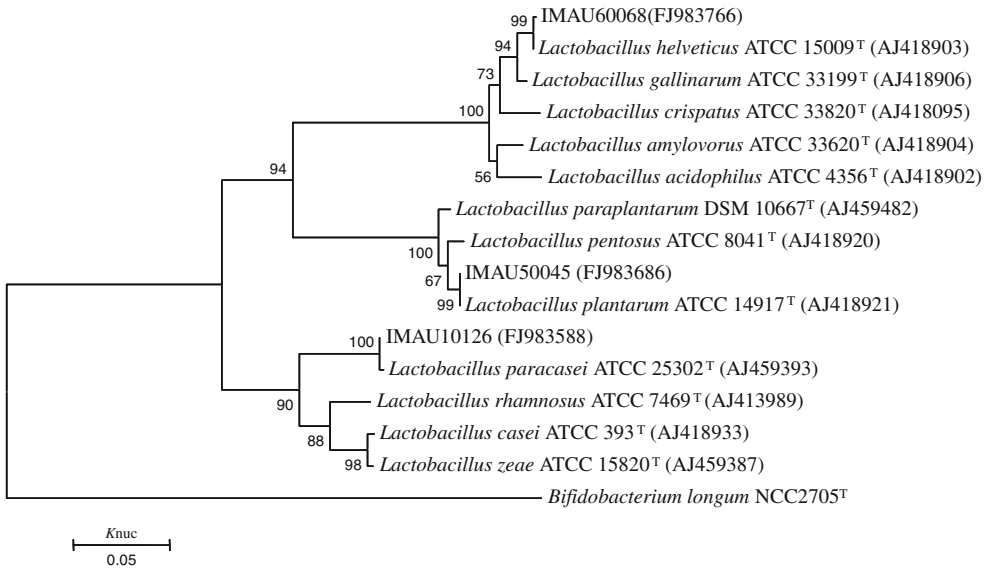

Fig. 2 Neighbor-joining tree showing the phylogenetic relationships between representative strains from each group and the type strains based on *tuf* gene sequences. *Bifidobacterium longum* was considered as an outlier. Bootstrap values based on 100 replications are given at the nodes

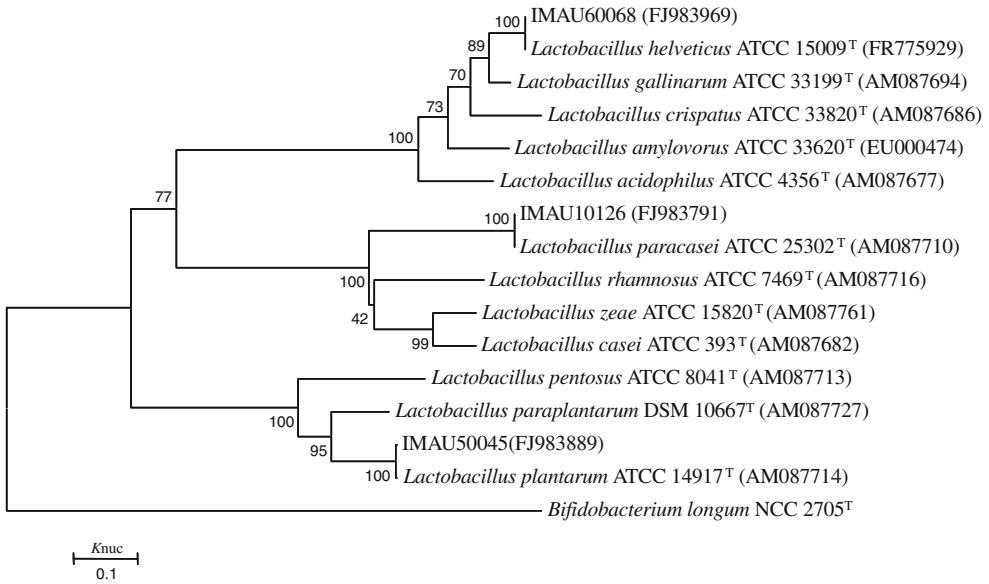

Fig. 3 Neighbor-joining tree showing the phylogenetic relationships between representative strains from each group and the type strains based on *phe*S gene sequences. *Bifidobacterium longum* was considered as an outlier. Bootstrap values based on 100 replications are given at the nodes

less than that of 16S rRNA gene (89.8, 94, 88.3 and 99.1 %, respectively). Although the 16S rRNA gene sequence analysis of *L. acidophilus* group showed the representative strains form a well-defined cluster with their type strains in phylogenetic tree, the average nucleotide sequence similarities of *hsp*60, *tuf* and *phe*S genes among the type strains in *L. acidophilus* group were less than that of 16S rRNA gene (93.8, 97, 92.3 and 98.5 %, respectively). Therefore, the sequence analysis of *hsp*60 gene and *phe*S gene was useful and exact taxonomic criteria for *L. acidophilus* group.

Discussion

The genus *Lactobacillus* is the largest group among the *Lactobacteriaceae* and contains more than 170 species (http://www.bacterio.cict.fr/). Significant changes have occurred in bacterial taxonomy since the introduction of molecular techniques. Currently, several molecular targets have been exploited for the molecular identification of *Lactobacillus* species. The identification of many species can be accomplished by 16S rRNA gene, which is considered an important molecular marker of modern bacterial

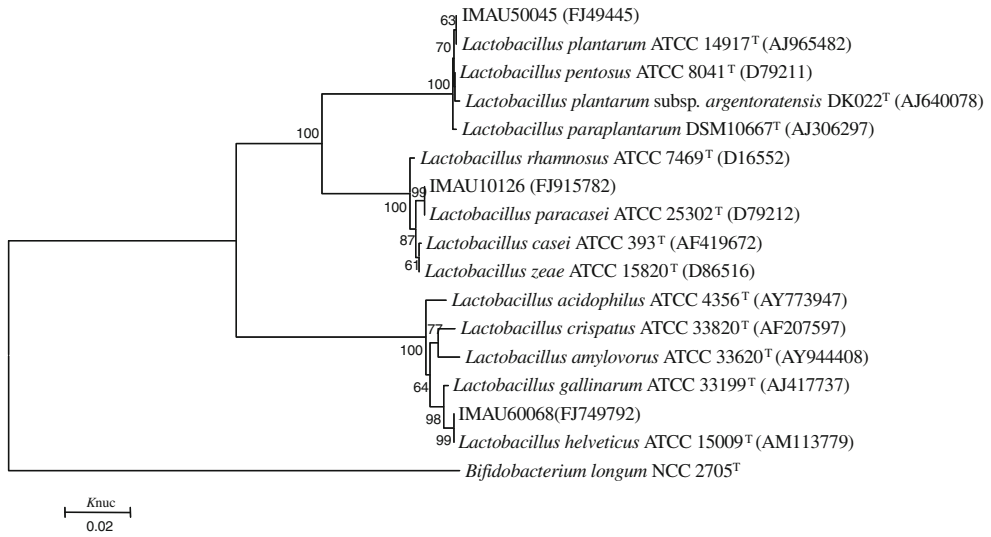

Fig. 4 Neighbor-joining tree showing the phylogenetic relationships between representative strains from each group and the type strains based on 16S rRNA gene sequences. *Bifidobacterium longum* was considered as an outlier. Bootstrap values based on 100 replications are given at the nodes

taxonomy. Although the comparison of the 16S rRNA gene sequences has been useful in phylogenetic studies at the species level, in some cases, closely related species cannot be differentiated from each other by 16S rRNA gene. Therefore, the use of highly conserved protein encoding genes as evolutionary chronometers might have strong applications in the identification and differentiation of species [8]. We studied the partial sequences of *hsp*60, *phe*S and *tuf* genes of 234 isolates with the aim of exploiting a rapid and reliable tool for discrimination very closely related species.

Lactobacillus plantarum group include *L. plantarum*, *L. pentosus* and *L. paraplantarum*. These three closely related species have very similar fermentation abilities and cannot be distinguished by 16S rRNA gene sequence analysis because they show 99 % sequence similarity [26]. In view of its demonstrated effectiveness, sequence analysis of protein coding genes as alternative phylogenetic markers was applied to difference *L. plantarum* group. Torriani et al. [11] designed species-specific primers based on *recA* gene to distinguish species in *L. plantarum* group. Huang et al. [27] reported that a molecular marker, *dnaK* gene, was used for discriminating phylogenetic relationships among *L. plantarum* group, and the data indicated that phylogenetic relationships between 22 strains are easily resolved using sequencing of the *dnaK* gene. In this study, sequencing of *tuf*, *hsp*60 and *phe*S gene of 58 *L. plantarum* was performed, and reliable phylogenetic trees based on those genes were clearly shown for three species of *L. plantarum* groups (Figs. 1, 2 and 3). The species *L. zeae*, *L. rhamnosus*, *L. paracasei* and *L. casei* are phylogenetically and phenotypically closely related and are regarded as the *L. casei* group. Some strains in this group have been shown to be probiotic and are widely used in the food and feed industries [28]; however, more than 28 % of commercial probiotic products are mislabeled at the genus or species level due to the use of methods that have limited taxonomic resolution or that are unsuitable for reliable identification to the species level [29, 30]. In this study, 63 isolates tentatively identified as belonging to the *L. casei* group by prior sugar fermentation profiles and 16S rRNA gene sequence analysis were examined using *hsp*60, *tuf* and *phe*S genes sequence analysis; three phylogenetic trees based on the partly *hsp*60, *tuf* and *phe*S gene sequences clearly display all isolates belonging to *L. paracasei*.

Lactobacillus helveticus is an important species used as a starter in the dairy industry for many hard cheese productions [31]. However, a clear identification of *L. helveticus* within the genus *Lactobacillus* is sometimes ambiguous and complicated [32]. Therefore, rapid and accurate identification of *L. helveticus* is important. *L. acidophilus*, *L. gallinarum*, *L. crispatus* and *L. helveticus* form a cluster of closely related species based on 16S rRNA gene and those strains that showed more than 98 % homology with each other; we analyzed the *hsp*60, *tuf* and *phe*S genes of 113 *L. helveticus* strains to provide a more accurate method and prove the feasibility of three genes for distinguishing *L. acidophilus* group. The results of phylogenetic analysis confirmed that those genes showed better resolution with a high discrimination level than 16S rRNA gene to differentiate the *L. acidophilus* group species. Similar to our results, Sun et al. [22] used *hsp*60, *tuf* and *phe*S gene to distinct 32 *L. helveticus* strains, and the result shown that the analysis of *tuf*, *hsp*60, especially *phe*S

partial gene sequences, effectively allows *L. helveticus* group to be differentiated at a higher discrimination level. However, based on a large amount of data, we demonstrated *hsp*60, *tuf* and *phe*S genes analysis showed better resolution not only among the *L. acidophilus* group species, but also among the *L. casei* group and *L. plantarum* group species.

In conclusion, in this study, we determined the protein encoding genes sequences of a large number of lactobacilli, increasing the already existent sequence databases of LAB species. We demonstrated a higher distinctness of the *tuf*, *hsp*60 and *phe*S sequences than of the 16S rRNA sequences and offered a valid molecular marker for inferring phylogeny among closely related taxa. Moreover, owing to its specificity, manageability and rapidity, this approach proposed in this study can be considered a valid strategy for typing at the species level lactobacilli isolated from food samples.

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