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ORIGINAL PAPER

Diversity and evolution of *Lactobacillus casei* group isolated from fermented dairy products in Tibet

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Abstract

Bacteria in *Lactobacillus casei* group, including *Lactobacillus casei* (*L. casei*), *Lactobacillus paracasei* (*L. paracasei*), and *Lactobacillus rhamnosus* (*L. rhamnosus*) are important lactic acid bacteria in the production of fermented dairy products and are faced with the controversial nomenclatural status due to their close phylogenetic similarity. To probe the evolution and phylogeny of *L. casei* group, 100 isolates of lactic acid bacteria originated from naturally fermented dairy products in Tibet of China were subjected to multilocus sequence typing (MLST). The MLST scheme, based on analysis of the housekeeping genes *fusA, ileS, lepA, leuS, pyrG, recA* and *recG*, revealed that all the isolates belonged to a group containing the *L. paracasei* reference strains and were clearly different from the strains of *L. casei* and *L. rhamnosus*. Although nucleotide diversity (π) was low for the seven genes (ranging from 0.00341 for *fusA* to 0.01307 for *recG*), high genetic diversity represented by 83 sequence types (STs) with a discriminatory index of 0.98 was detected. A network-like structure based on split decomposition analysis, and the high values of the relative effect of recombination and mutation in the diversification of the lineages ($r/m = 4.76$) and the relative frequency of occurrence of recombination and mutation ($\rho/\theta = 2.62$) indicated that intra-species recombination occurred frequently and homologous recombination played a key role in generating genotypic diversity amongst *L. paracasei* strains in Tibet. The discovery of 51 new STs and the results of STRUCTURE analysis suggested that the *L. casei* group in Tibet had an individual and particular population structure in comparison to European isolates. Overall, this research might be the first report about genetic diversity and population structure of *Lactobacillus* populations isolated from naturally fermented dairy products in Tibet based on MLST scheme.

Keywords Multilocus sequence typing · *Lactobacillus casei* group · Genetic diversity · Homologous recombination

Introduction

Lactobacillus species are facultative heterofermentative bacteria naturally found in a variety of environmental habitats (Hammes and Vogel 1995), and are widely used for the production of fermented dairy food (Corsetti et al. 2008; Steele et al. 2007; Tan et al. 2012). As probiotics, some

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Lactobacillus casei strains have been implemented in alleviation of diarrhea (Nagata et al. 2011; Wong et al. 2014), modulation of immune response (Lim et al. 2009; Aragón et al. 2014) and reduction of infection risk (Guillemard et al. 2010; Gleeson et al. 2011). Based on the sequence analysis of 16S rRNA gene, *L. casei, Lactobacillus paracasei* and *Lactobacillus rhamnosus* are generally regarded as the *L. casei* group (Salvetti et al. 2012). Although the comparative analysis of 16S rRNA gene sequences is a conventional molecular method for bacterial identification and phylogenetic analysis (Petti et al. 2005), it is not suitable to distinguish between the species of the *L. casei* group. For example, the strains of BD-II, BL23, LC2W, and Zhang, previously defined as *L. casei* by 16S rRNA gene sequence analysis, have been reclassified as members of *L. paracasei* based on phylogeny of concatenated amino acid sequences of different proteins (Toh et al. 2013).

To improve the discrimination of closely related lactobacilli species, many molecular typing methods have been developed, such as pulsed-field gel electrophoresis (PFGE) of plasmid and genomic macrorestriction (Herrerofresno et al. 2012), randomly amplified polymorphic DNA (RAPD) (Capra et al. 2011), amplified fragment length polymorphisms (AFLP) and ribotyping (Coudeyras et al. 2008). However, these methods are difficult to facilitate data-sharing between laboratories and to investigate microbial population biology. To overcome the inherent limitations of the typing methods above, multilocus sequence typing (MLST) based on a set of 6–10 housekeeping genes has been developed to characterize the genetic diversity and phylogenetic structure of lactic acid bacteria (Aanensen and Spratt 2005; Bao et al. 2016; Cai et al. 2007; Diancourt et al. 2007; Ramachandran et al. 2013; Tanigawa and Watanabe 2011; Sun et al. 2015).

High diversity and specificity have been discovered in different niches in *L. casei* group. Previously, 36 sequence types (STs) and frequent intra-species recombination were found based on MLST method among 40 *L. casei* strains isolated from cheese, human GI tract and fermented plant products in different geographical origins (Cai et al. 2007). Similarly, 31 STs were detected among 52 strains of *L. casei* group from different sources (such as human, dairy food and fermented plant products), which indicated homologous recombination was not frequent enough to break the population structure of *L. casei* (Diancourt et al. 2007). Recently, 224 *L. casei* isolated from naturally fermented foods from different regions in China and Mongolia were divided into 171 STs. The results confirmed that recombination occurred more frequently than mutation during the evolution of *L. casei*, and clearly showed most of the strains isolated from acidic gruel, pickles, and traditional fermented dairy products formed different subgroups, respectively (Bao et al. 2016). These previous studies generated controversial conclusions about the frequency of homologous recombination and indicated that the *L. casei* population was specific to different ecological niches (Kimura 1979).

The Tibet region of China lies in the southwest of Qinghai-Tibet plateau, where the traditional fermented dairy products from yaks and goats are popular local food (Wu 2001). Due to the peculiar and individual state of climate (temperature, moisture, pressure, oxygen concentration, etc.) and geographic conditions, these dairy products exhibit unique flavour, probiotic properties and diverse microbial resource (Luo et al. 2011). However, no study on the population structure and diversity of single *Lactobacillus* group has been reported in this particular region. In this study, 100 lactic acid bacteria were isolated from traditional fermented dairy products in Tibet and the MLST scheme was used to understand the genetic characteristics of them.

Materials and methods

Sampling of fermented dairy products and bacterial isolation

For isolation of bacteria, traditional fermented dairy products, including cow yogurt, cow milk residue, dairy foods, ghee, goat yogurt, goat-yak yogurt, kurut, starter, urum, and yak milk residue were sampled from rural Tibetan households, at 13 towns and cities of Tibet in August of 2014 (Supplementary Table S1). The solid samples were sealed in sterilized plastic bags and liquid samples were introduced into sterilized test tubes with crew cap, unless the products have been packed by the producer. All the samples stored on ice and transported to our laboratory by plane. In the laboratory, those were stored at 4 °C until isolation of the bacterial strains.

Each of the samples was used to prepare a decimal dilution up to 10^{-6} and 0.1 mL of the last three dilutions were individually spread on MRS agar (Difco Laboratories, Detroit, MI, USA) plate and incubated at 28 °C for 48 h under an anaerobic environment created using Anaeropack system. After incubation *L. casei* colonies characterized with round, smooth and creamy surfaces (Vinderola and Reinheimer 2000) were picked up and purified by streaking repeatedly on the same medium. All the pure isolates were initially confirmed as members of the *L. casei* group using carbohydrate fermentation tests (API 50CHL), using as inoculants of cultures grown overnight at 37 °C in MRS Broth (Difco Laboratories, Detroit, Mich).

DNA extraction

A total of 100 isolates belonging to *L. casei* group were obtained from traditional fermented dairy products in Tibet

(Supplementary Table S1). To further identify the isolates by phylogenetic analyses, the genomic DNA was extracted from each isolate using a modified Chelex method (Phillips et al. 2012). Briefly, the bacteria collected from 5 mL of overnight culture at 37 °C in MRS Broth by centrifugation were resuspended in 200 µL of digestion buffer (50 mM Tris–HCl, 1 mM EDTA, 0.5% Nonidet P-40) containing 25% w/v Chelex®100 and incubated in a water bath at 56 °C for 30 min followed by boiling for 10 min. The lysates were stored at − 20 °C as DNA extracts until further analysis.

Amplification and phylogeny of 16S rRNA genes

The 16S rRNA gene fragments of all strains were amplified with primers 27F and 1492R (Kim et al. 2007) using the DNA extracts mentioned above as templates. The obtained PCR products were analyzed by electrophoresis in 1% (w/v) agarose gels (Schneider et al. 1993). Both DNA strands of the extracted gene fragments were directly sequenced in Genewiz (Beijing, China) by following the procedures as described by Cui et al. (2014). The acquired sequences were aligned using ClustalW (Chenna et al. 2003) with 16S rRNA gene sequences of the type or reference strains in *L. casei* group obtained from the GenBank database and Danone Research, Centre de Recherche Daniel Carasso (Diancourt et al. 2007). The phylogenetic analysis of these sequences was processed by MEGA 6.0 software (Tamura et al. 2013) to construct phylogenetic trees with the neighbor-joining method and the Kimura 2-parameter model (Kimura 1979) with 1000 bootstrap replications.

Multilocus sequence typing (MLST)

The MLST scheme proposed by Diancourt et al. was used to characterize the *L. casei* strains. The following seven housekeeping genes were: *fusA* (elongation factor EF-2), *ileS* (isoleucyl-tRNA synthetase), *lepA* (GTP-binding protein LepA), *leuS* (leucyl-tRNA synthetase), *pyrG* (CTP synthetase), *recA* (recombinase A) and *recG* (ATP-dependent DNA helicase). The PCR conditions used for the amplification reactions were as follows: 94 °C for 5 min, followed by 30 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, and a final extension at 72 °C for 5 min. The PCR products were examined by agarose gel electrophoresis and purified with the QIAquick PCR purification kit (Qiagen, Hilden, Germany). Both strands of the purified PCR products were sequenced by Genewiz (Beijing, China). Similar to the 16S rRNA gene analysis, sequences of the seven genes obtained in this study, together with those extracted from database (Diancourt et al. 2007), were processed by MEGA 6.0 software (Tamura et al. 2013) to construct phylogenetic trees with the neighbor-joining method and the Kimura 2-parameter model (Kimura 1979) with 1000 bootstrap replications for all the single gene sequences, as well as for the combined sequences.

Data analysis

For MLST analysis, novel allelic sequences were assigned consecutive numbers above those in the existing allele sequences. On the basis of their allelic profile, isolates were assigned to an ST that had already been described or to a new ST if the allelic profile was novel. Clonal complexes (CCs) were defined as groups of related STs differing by no more than one of the seven loci, which were considered as descendants from a common ancestor (Feil et al. 2004). The Simpson's index of diversity was calculated to evaluate the discriminatory power of the MLST scheme (Hunter 1990). The equation is $D = 1 - 1/[N(N-1)]\sum_{j=1}^{S} n_j(n_j - 1)$, where N is the number of bacterial strains, S is the number of sequence types, and n_j is the number of strains belonging to the type j, $j = 1$ to S. Nucleotide diversity (π) and nonsynonymous substitutions to synonymous substitutions (dN/dS) were calculated using DnaSP 5.0 with Jukes–Cantor correction (Rozas et al. 2003). The Tajima's D value and GC value were estimated with DnaSP 5.0 (Jukes and Cantor 1969; Rozas et al. 2003). To explore the relationship among the detected STs on the basis of allelic profiles, clonal complexes were analysed using the eBURST v3.0 program (http://eburst.mlst.net) (Feil et al. 2004). Neighbor-joining tree analysis was constructed by MEGA 6.0 with 1000 bootstrap replications (Tamura et al. 2013). Split decompositions and phi test for recombination were performed with SplitsTree4 (Huson and Bryant 2006). The r/m and ρ/θ were accounted by ClonalFrame with three independent runs of 100,000 Markov Chain Monte Carlo (MCMC) iterations plus 10,000 burn-in iterations (Didelot and Falush 2006). Structure v2.3 with linkage model (Falush et al. 2007) was performed to identify the number of ancestral subpopulations (K) with three independent runs consisted of 100,000 (MCMC) iterations.

Nucleotide sequence accession numbers

The GenBank accession numbers for the *16S rRNA, fusA, ileS, lepA, leuS, pyrG, recA* and *recG* gene sequences determined in this study are KU955744–KU955843, KU511387–KU511486, KU511387–KU511486, KU511487–KU511586, KU511587–KU511686, KU511687–KU511786, KU511787–KU511886, and KU511887–KU511986, respectively.

Results

16S rRNA analysis of the isolates

The 16S rRNA sequence similarities varied from 98.7 to 99.9% among the isolates. In the 16S rRNA phylogenetic tree (Supplementary Fig. S2), all the isolates formed a cluster together with reference of *L. rhamnosus* JCM 1136^T, *L. zeae* ATCC 15820^T, *L. casei* ATCC 393^T, *L. paracasei* JCM 8130^T, that were distinct from *L. sakei* DSM 20017^T and *L. plantarum* JCM 1149^T. These results confirmed that all the isolates were members of the *L. casei* group.

MLST loci and allelic diversity of the isolates and reference strains

The seven genes were successfully amplified for all 100 isolated strains. Supplementary Fig. S1 presented polymorphic nucleotide sites for each locus including the original and the newly discovered, and only the variable sites were shown. A total of 132 polymorphisms sites in the 132 strains were detected based upon the 7 sequenced gene fragments. Across the seven genes, the frequency of polymorphic sites ranged from 4.51% (*pyrG*) to 21.8% (*lepA*) and the number of alleles varied from 7 (*pyrG*) to 24 (*lepA*). The length of the sequences for the MLST scheme ranged from 315 bp for *recA* to 663 bp for *fusA* (Table 1). The average nucleotide diversity (π) was 0.00786, ranging from 0.00341 (*fusA*) to 0.01307 (*recG*). The ratio of nonsynonymous to synonymous substitutions (d*N*/d*S*) was operated to estimate the selection pressure on each locus. The results remained less than 1 for each locus and ranged between 0.073 (*lepA*) and 0.426 (*recA*). Accordingly, the values from Tajima's *D* test were between − 2.0075 (*recA*) to − 1.4005(*leuS*). The GC value of all alleles ranged from 46.8% (*pyrG*) to 50.4% (*lepA*).

Allelic profile of the isolates and reference strains

In this study, a total of 54 STs were assigned, based on the allele combination for the seven MLST gene fragments, in the 100 isolates from naturally fermented dairy products in Tibet (Supplementary Table S1). Four strains belonging to three STs (ST9, ST16 and ST19) found earlier in the findings of Diancourt et al. (2007), and 51 STs (ST33–83) were not previously identified. ST33, ST36, ST39, ST40, ST65 and ST71 represented new combinations of existing alleles, while ST34, ST35, ST37, ST38, ST41–64, ST66–69, ST70 and ST72–83 contained a variable number of new alleles not previously documented (Supplementary Table S1).

The Simpson's index of diversity with 98% confidence intervals manifested that the *L. casei* group could be typed by the MLST scheme. As shown in Fig. 1, the resolution power and the number of STs also decreased with the number reduction of locus. When one locus was removed in the MLST scheme, the discriminatory power was although not less than the 0.95 threshold (Belkum et al. 2007), the number of STs was decreased based on concatenated sequence of remaining six loci (Fig. 1). Among 35 traditional fermented milk samples studied, each included more than one STs. Approximately 86% STs of isolates comprised only one isolate, 6% STs were represented by at least three sample sources. ST33 was remarkably prevalent type in this study and contained 13 strains, followed by ST69 (8 strains), and ST45 (7 strains).

An eBURST population snapshot was performed to investigate the clonal relatedness of 132 strains on the basis of allelic profiles (Fig. 2). The sequences of 83 STs were divided into 16 CCs and 23 singletons, the latter were all from fermented dairy products in Tibet, the only two exceptions correspond to the strains D699 (ST30) and SB3888 (ST31) isolated from traditional food and

Table 1 Nucleotide and allelic diversity of seven housekeeping genes for *L. casei* group

Locus	Sequence length (bp)	No. of alleles	Number of polymorphic sites	Tajima's *D* value	GC content (%)[a]	d*N*/d*S*[b]	Nucleotide diversity (π)
fusA	663	16	14	− 1.80726	49.3	0.127	0.00341
ileS	360	17	17	− 1.80726	46.9	0.208	0.00907
lepA	549	24	29	− 1.42391	50.4	0.073	0.00879
leuS	642	21	26	− 1.40048	48.4	0.114	0.00715
pyrG	345	7	6	− 1.52412	46.8	0.147	0.00497
recA	315	13	16	− 2.00753	48.2	0.426	0.00855
recG	342	18	25	− 1.52761	48.4	0.136	0.01307

[a]Guanine-cytosine content

[b]The ratio of nonsynonymous to synonymous substitutions

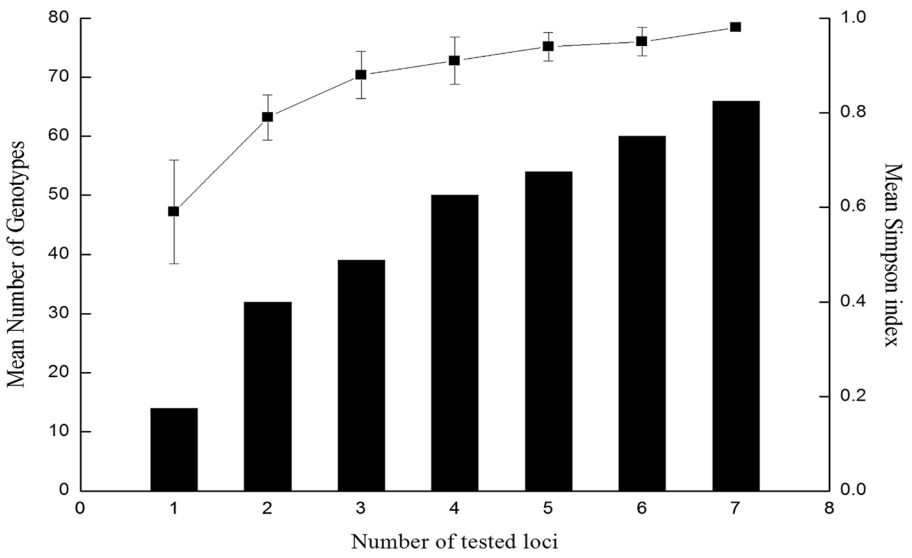


Fig. 1 Discriminatory power calculated with Simpson's index of diversity, and the number of sequence types (STs) based on the various combinations of housekeeping genes of MLST scheme. When the seven MLST genes were concatenated, the Simpson's index of diversity achieved to the highest confidence intervals

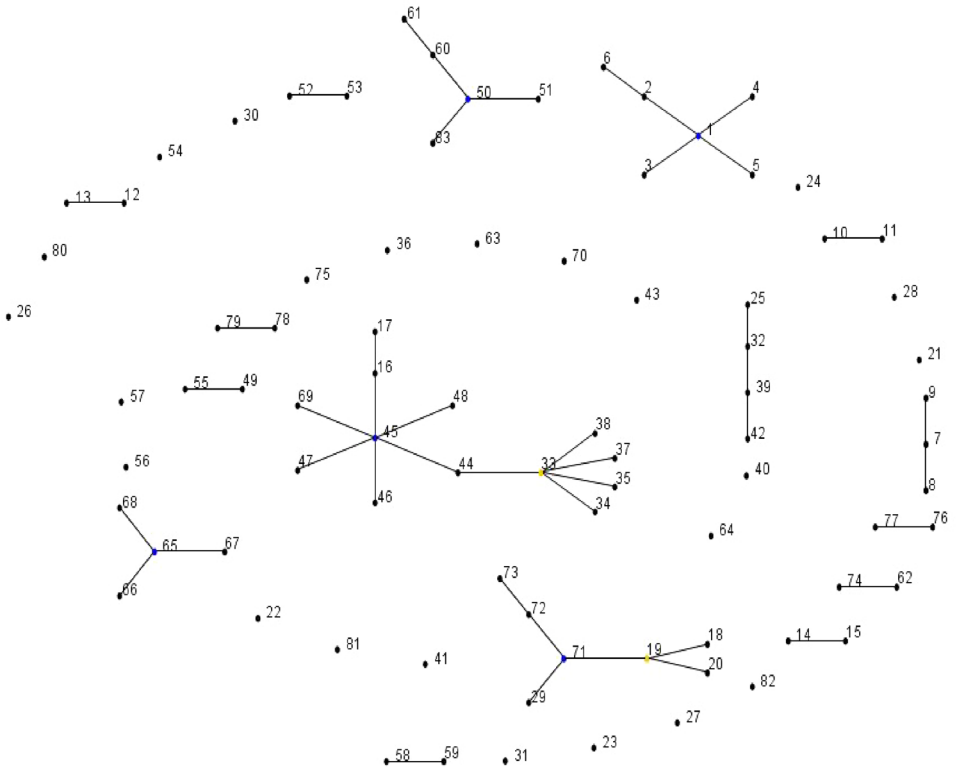
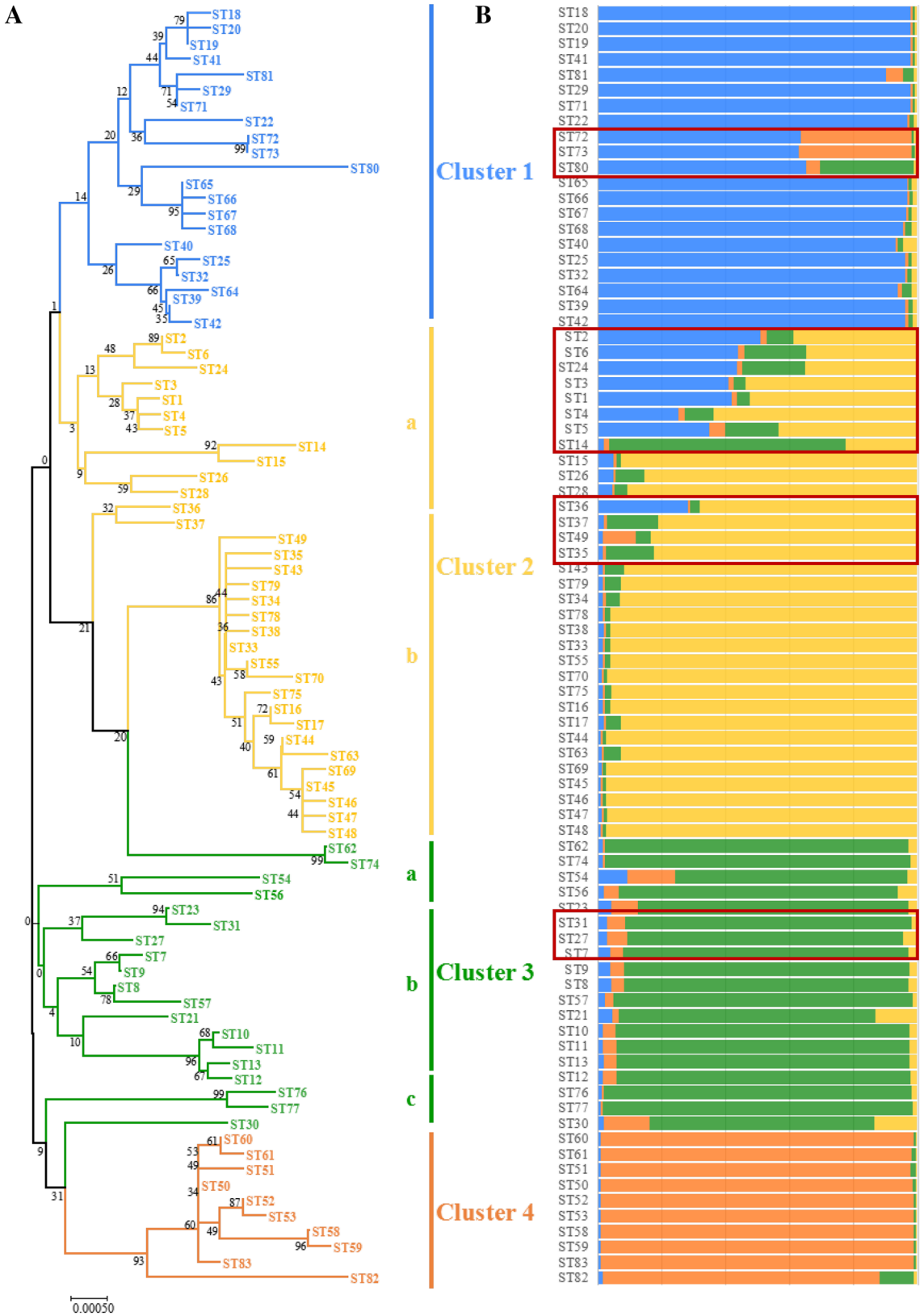


Fig. 2 Population structure of 132 *L. casei–paracasei* strains based on allelic profiles of seven housekeeping genes by eBURST analysis. Each circle corresponds to a sequence type (ST), and the sizes of the circle are associated with the number of isolates. Blue circles are indicated of group founder, and yellow circles represent subgroup founder of the clone complex. (Color figure online)

human in Europe, respectively. The 16 CCs comprised 72% STs, which were differed by at least four out of the seven loci from all other profiles. Eight CCs (CC4, CC6 and CC11–CC16) were all from Tibet, and stains of five CCs (CC3 and CC7–CC10) were all from Europe. The predominant clonal complex of *L. casei* group was CC1, consisting of 13 (16%) STs with 40 (30%) strains, 95% of which were isolate strains in addition to the two strains D661 (ST16) and D645 (ST17) from European dairy products. ST45 was assigned as the common ancestry of CC1 on the basis of the highest number of single-locus variants (SLVs), which comprised six SLVs and two double-locus variants (DLVs). Moreover, ST33 was identified as predicted subgroup founder associated with five SLVs and three DLVs. The CC2 was the second largest and comprised seven STs representing 8 *L. paracasei* strains, half of which were isolated from Tibet. Moreover, *L. paracasei* ATCC 334 (ST32) was assigned to CC5 which were all


A
B
ST18
ST20
ST19
ST41
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ST22
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ST73
ST80
ST65
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ST67
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ST76
ST77
ST30
ST60
ST61
ST51
ST50
ST52
ST53
ST58
ST59
ST83
ST82
Cluster 1
Cluster 2
Cluster 3
Cluster 4
a
b
a
b
c
0.00050

◂**Fig. 3** NJ tree and Ancestry of 83 STs of *Lactobacillus casei* group. **a** Neighbor-joining tree based on the concatenated partial sequences of 83 STs. The bootstrap values are shown for all branches. The bootstrap values are shown through the numbers at nodes based on analysis of 1000 replicates. ST are colored in terms of their affiliation to one of the four ancestral lineages. **b** The sources of ancestry of each unique ST from five ancestral populations by the linkage model of STRUCTURE. Each ST is represented by a single line with the ST designation at the top consisting of colour stacked bars that indicate the proportion of ancestry from each of four populations (blue, yellow, green, and orange). STs in the red box represented admixture of ancestral sources. (Color figure online)

isolated from dairy products with three isolates in Tibet and one in Europe.

Phylogenetic and population structure analysis of strains in *L. casei* group

The individual genes *fusA, ileS, lepA, leuS, pyrG, recA*, and *recG* were successfully amplified for the isolates and strains of *L. casei* group, but they were not amplified in species *L. fermentum* and *L. sakei*. The phylogenetic trees were constructed from seven housekeeping genes using the neighbor-joining method, respectively. In all these seven phylogenetic trees, the 132 studied strains were clearly clustered together with *L. paracasei* strains ATCC 334 and JCM 8130^T with 99 or 100% similarity. This group was obviously distinct from *L. rhamnosus* JSM 1136^T and *L. casei* ATCC 393^T (Supplementary Fig. S3).

In the neighbor-joining tree constructed based on the concatenated sequences of the seven protein-coding genes (Fig. 3a), strains representing the 83 STs were divided into four clusters, which were consistent with four major ancestral populations revealed by the STRUCTURE. Cluster 1 (blue) contained three CCs (CC2, CC5 and CC6) and six singletons, 78.5% of these isolates were from Tibet and included reference strain *L. paracasei* ATCC 334. Cluster 2 (yellow) contained two subclusters. Cluster 2a contained 2CCs (CC3 and CC8) and three singletons, and all of these isolates were derived from Europe. Cluster 2b consisted of 4CCs (CC1, CC12, CC14 and CC16) and five singletons were all isolated from dairy products derived from Tibet except ST16 and ST17. Cluster 3 (green) was composed of 5CCs (CC7, CC9, CC10, CC15 and CC16) and eight singletons. Cluster 3a and Cluster 3c contained seven STs with isolates from Tibet except D699 (ST30). Cluster 3b included twelve STs, all of which were from Europe except TD059 (ST57). Cluster 4 (orange) contained 3CCs (CC4, CC11 and CC13) and one singleton, all of which were from traditional fermented dairy products in Tibet.

In the analysis of average proportion of genetic material of each ST according to STRUCTURE with the linkage model, a maximum posterior probability of $K=4$ was found within the genetic population of 83 STs (132 strains), revealing that the primary source of Cluster 1 to Cluster 4 were blue, yellow, green and orange colours ancestral population, respectively (Fig. 3b). The percentage of assignment to a cluster lower than 85% was considered as threshold for admixture. On the whole, 22.9% of all STs (Marked by red box in Fig. 3b) contained a mixture of ancestral sources, representing a high degree of heterogeneity. Little admixture of ancestral sources was observed among the 100 isolates from Tibet, which suggested that high degree of genetic homogeneity of these STs in each lineage.

Gene recombination in the tested populations

Split-decomposition analysis was used to examine the influence of recombination on the evolution of each locus. As shown in the Supplementary Fig. S4, the split graphs for all seven loci showed diverse parallelogram structures, except for the gene of *pyrG* and *recA*, indicating that intragenic recombination occurred during the evolution of the other five loci, especially *lepA* loci. In addition, the combined split graph of alleles for all seven MLST loci displayed a network-like structure (Fig. 4). On the whole, the phylogeny obtained using the neighbor-joining method was very similar to that of split network.

Furthermore, the *P* value for the concatenated sequences of strains was considered to be significant ($P=1.812E{-}7$) by the *phi* test. In addition, the relative contribution of recombination and mutation for population structure were assessed by ClonalFrame. The value of ρ/θ was 2.62 and the r/m rate for nucleotides was 4.76, indicating that nucleotides are approximately fourfold more likely to change by recombination than by mutation during the evolution.

Discussion

As previously reported (Salvetti et al. 2012), the three species in *L. casei* group, *L. casei, L. paracasei*, and *L. rhamnosus*, as well as the reference species *L. zeae*, were undistinguishable by 16S rRNA gene phylogeny in the present study. In contrast, MLST was recognized as the most powerful technique for studying typing and population structure of bacteria. This approach had been widely applied to the studies of diversity and evolution of various lactobacillus, such as *Leuconostoc lactis* (Tong et al. 2014), *Leuconostoc mesenteroides* (Zhang et al. 2014), *Lactococcus lactis* (Xu et al. 2014) and *L. plantarum* (Xu et al. 2015). Up to now, three different MLST schemes had been employed for studying the evolution of *L. casei* group (Bao et al. 2016; Cai et al. 2007; Diancourt et al. 2007). To facilitate comparison and show more clearly the distinction of genetic diversity of Tibetan strains, we used the same system of ST designation, and data from the study

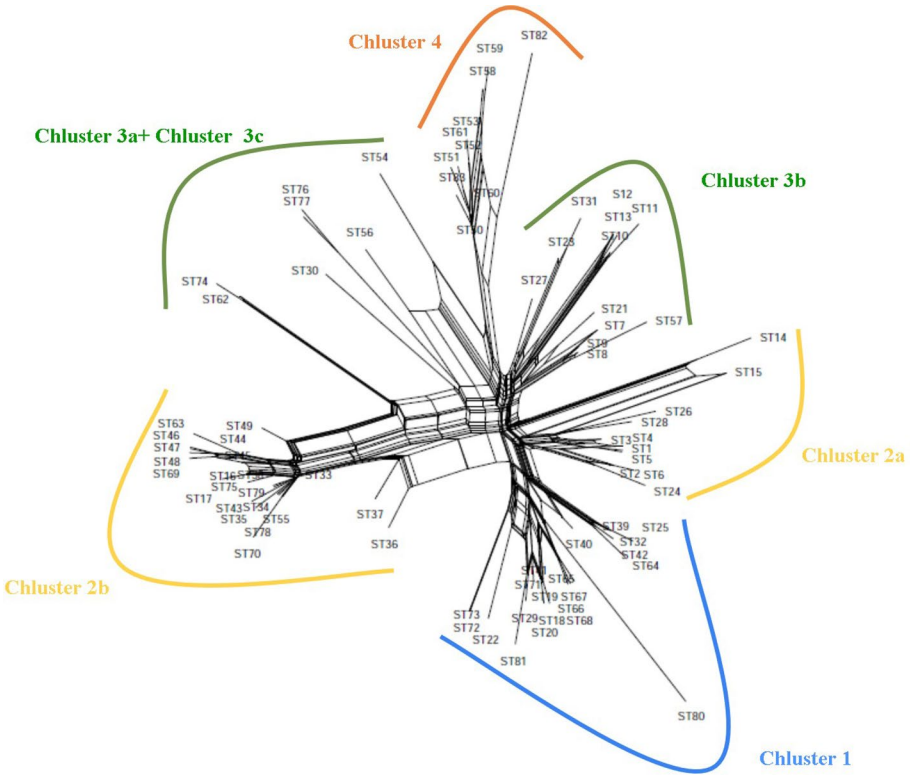

Fig. 4 Split-decomposition analysis of 83 STs for the concatenated sequences of seven loci of *L. casei–paracasei* strains. Parallelogram structures indicate the recombination events

of Diancourt et al. were also included to provide a broader perspective (Diancourt et al. 2007). In this study, all the isolates were identified as members of the *L. casei* group (Supplementary Fig. S2). Of which, the strains belonging to *L. casei, L. paracasei*, and *L. rhamnosus* were clearly differentiated from each other based on the single gene tree. Furthermore, all the isolates were clustered with *L. paracasei* reference strains, which illustrated that the isolates were a single group belonging to *L. paracasei* (Supplementary Fig. S3).

Although only one *L. paracasei* was detected in this study, the dividing of 100 isolates from fermented dairy products in Tibet into 54 different STs based on 7 genetic loci and 44 STs representing by only a single isolate revealed a high intraspecific diversity. The high genetic diversity also was proved by the four ancestral sources of *L. paracasei* populations in the analysis of STRUCTURE (Fig. 3b). The similar results of genetic diversity were found in the MLST analysis of *L. helveticus* (Sun et al. 2015), *L. plantarum* (Zhang et al. 2015) and *L. fermentum* (Tong et al. 2015). Significantly, Tibet isolates of cluster 4 only contained one novel orange ancestral population (Fig. 3b), which was distinguished from the other three clusters. It indicated that *L. casei* group in Tibet had an individual type of population structure in comparison to European isolates. The discovery of many new polymorphic sites and STs also reinforced that point. However, it remained to be determined due to the limitation of the number of isolates.

Combined with the previous studies, our results demonstrated that MLST is a suitable method for studying genetic diversity in populations, with high-resolution power (0.98) displayed by the discriminatory index. The number of STs was decreased when either gene loci removed. This phenomenon indicated that the current MLST scheme was a reliable method for studying genetic diversity and population structure of *L. casei* group from Tibet in this study. Based on the MLST scheme, the phylogenetic tree contained four major branches which were supported by low bootstrap values (Fig. 3a). According to the ancestral source of genetic diversity, the poor bootstrap values of the topology could be explained by the high degree of admixture. Therefore, the results showed that neighbor-joining tree was not suitable for analyzing the phylogenetic relationship of *L. casei* group in our study. The conclusion was consistent with previous reports for *L. casei* (Bao et al. 2016). In other words, this approach was more sensitive to the effects of genetic recombination. Instead, the approach such as eBURST could avoid the problem as much as possible (Diancourt et al. 2007; Bao et al. 2016).

The range of nucleotide diversity (π) from 0.00334 for *fusA* to 0.01337 for *recG* estimated for the 100 noncommercialized dairy isolates of *L. paracasei* (except the reference and type strains) from Tibet revealed relative high nucleotide diversity than those obtained from *L. casei* in 2007 (Diancourt et al. 2007), but little bit lower than those of *L. helveticus* (0.00373 for *murE* to 0.06233 for *pepX*) (Sun

et al. 2015), *L. fermentum* (0.00393 for *murC* to 0.01421 for *dnaA*) (Tong et al. 2015) and *L. plantarum* (0.00401 for *clpX* to 0.03220 *groEL*) (Xu et al. 2015). These differences in distinct studies might be related to the variations in geographic and sample origins of the isolates because our isolates in this study were only isolated from naturally fermented dairy products in Tibet. However, the factors affecting the diversity of bacteria were multiple and complicated, including homologous recombination, gene decay and acquisition, horizontal gene transfers (HGTs) (Cai et al. 2009), and geographic isolation environment (Tong et al. 2014).

The homologous recombination has a significant impact on the accuracy of phylogenetic analysis and evolutionary stability of population structure (Delétoile et al. 2010; Feil and Spratt 2001). However, the rates of homologous recombination varied in different bacterial species (Fraser et al. 2007). In our study, the split graph with parallelograms (Fig. 4) and phi value ($P = 2.452E{-}6$) provided the evidence that homologous recombination occurred in the *L. paracasei* populations. In addition, the parallelogram-shaped structures were found in individual split graphs of *fusA, ileS, lepA, leuS* and *recG* (Supplementary Fig. S4). Thus it can be inferred that the homologous recombination was mainly affected by the five loci. The split-decomposition method for detecting recombination events was also implemented to other lactic acid bacteria, such as *Bifidobacterium* species (Delétoile et al. 2010), *L. sakei* (Chaillou et al. 2013) and *Carnobacterium maltaromaticum* (Rahman et al. 2014).

In our study, 22.9% of STs shared a high degree of admixture, which indicated that recombination has contributed to genetic heterogeneity of *L. paracasei* populations. Furthermore, the high value of r/m and ρ/θ in our study indicated that recombination had a major contribution to shape the population structure of *L. paracasei* populations than point mutation. Moreover, split-decomposition analysis also provided evidences for intra-species recombination among *L. paracasei* populations according to the network-like topology (Fig. 4). These results were consistent with the previous reports for *L. casei* group (Bao et al. 2016; Cai et al. 2007; Diancourt et al. 2007). And, this phenomenon could be interpreted by the research of Broadbent et al. that large shifting of genes was in relation to the evolution of *L. casei* isolates in nutrient-rich dairy niche (Broadbent et al. 2012). In addition, the incongruence of branching of Cluster3a and Cluster3c between the two topology structures could be attributed to HGT. Makarova and Koonin (2007) confirmed that genome diversity of *L. casei* group was mainly driven by horizontal gene transfer from other bacterial species particularly lactobacilli. The similar HGT events were also detected in the MLST analysis of *Listeria monocytogenes* because of the distinct branching between the trees with and without correction for recombination (Ragon et al. 2008). The low d*N*/d*S* values in the MLST analysis indicated that the neutral mutations in the selective pressure dominated the large accumulation of nucleotide changes in the evolution of *L. paracasei* populations. The conclusion was also confirmed by the Tajima's *D* values across seven loci which did not present a significant deviation from neutral expectation (Tajima 1989). Besides, the housekeeping genes from *L. casei* group were driven by neutral selection, which was in accordance with the previous report (Bao et al. 2016; Cai et al. 2007; Diancourt et al. 2007).

The classification and nomenclature of *L. casei* group have always wavered between *L. casei* and *L. paracasei* due to the high genetic similarity of these species (Tindall 2008). The protein-coding genes with a sufficient number of sequence polymorphisms have successfully discriminated the species within *L. casei* group (Felis et al. 2001; Huang and Lee 2011), which are concordant with the phylogenetic results in our study. Thus, the individual genes of the MLST scheme can be proposed as alternative markers for the identification of species in the *L. casei* group. However, the sequence analysis of individual housekeeping genes was hardly performed for intraspecies identification or evolutionary biology compared with MLST. As a suitable alternative molecular marker, the *recA* gene could fully identify the species in *L. casei* group (Felis et al. 2001).

Conclusions

This research might be the first report about genetic diversity and population structure of *Lactobacillus* populations isolated from naturally fermented dairy products in Tibet based on MLST scheme. All the 100 isolates were identified as *L. paracasei* and 54 STs were among the isolates. High degree of homologous recombination was detected within the studied populations. Our results provided a better understanding of the evolution and phylogeny of *L. casei* group of Tibet and contributed for the meaningful information for future studies. Nevertheless, the isolated strains in our study were the only one from the fermented dairy products. It would be necessary to monitor the phylogenetic relationships of *L. casei* group from diverse niches by combined use of MLST and whole-genome resequencing techniques.

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