Multilocus sequence typing of *Lactococcus lactis* from naturally fermented milk foods in ethnic minority areas of China

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ABSTRACT

To determine the genetic diversity and phylogenetic relationships among *Lactococcus lactis* isolates, 197 strains isolated from naturally homemade yogurt in 9 ethnic minority areas of 6 provinces of China were subjected to multilocus sequence typing (MLST). The MLST analysis was performed using internal fragment sequences of 12 housekeeping genes (*carB*, *clpX*, *dnaA*, *groEL*, *murC*, *murE*, *pepN*, *pepX*, *pyrG*, *recA*, *rpoB*, and *pheS*). Six (*dnaA*) to 8 (*murC*) different alleles were detected for these genes, which ranged from 33.62 (*clpX*) to 41.95% (*recA*) GC (guanine-cytosine) content. The nucleotide diversity (π) ranged from 0.00362 (*murE*) to 0.08439 (*carB*). Despite this limited allelic diversity, the allele combinations of each strain revealed 72 different sequence types, which denoted significant genotypic diversity. The d_N/d_S ratios (where d_S is the number of synonymous substitutions per synonymous site, and d_N is the number of nonsynonymous substitutions per nonsynonymous site) were lower than 1, suggesting potential negative selection for these genes. The standardized index of association of the alleles ($I_A^S = 0.3038$) supported the clonality of *Lc. lactis*, but the presence of network structure revealed by the split decomposition analysis of the concatenated sequence was strong evidence for intraspecies recombination. Therefore, this suggests that recombination contributed to the evolution of *Lc. lactis*. A minimum spanning tree analysis of the 197 isolates identified 14 clonal complexes and 23 singletons. Phylogenetic trees were constructed based on the sequence types, using the minimum evolution algorithm, and on the concatenated sequence (6,192 bp), using the unweighted pair-group method with arithmetic mean, and these trees indicated that the evolution of our *Lc. lactis* population was cor-

related with geographic origin. Taken together, our results demonstrated that MLST could provide a better understanding of *Lc. lactis* genome evolution, as well as useful information for future studies on global *Lc. lactis* structure and genetic evolution, which will lay the foundation for screening *Lc. lactis* as starter cultures in fermented dairy products.

Key words: traditional fermented milk food, *Lactococcus lactis*, multilocus sequence typing, housekeeping gene

INTRODUCTION

Lactococcus lactis is a species of lactic acid bacteria (LAB) used in dairy starter cultures, especially hard and semihard cheeses. This species is composed of 4 known subspecies: *Lc. lactis* ssp. *cremoris*, *Lc. lactis* ssp. *lactis*, *Lc. lactis* ssp. *hordniae*, and *Lc. lactis* ssp. *tructae* (Schleifer, 1987; Pérez et al., 2011). *Lactococcus lactis* ssp. *hordniae* isolated from the leafhopper *Hordnia circellata* has not been detected in dairy products (Schleifer et al., 1985), and *Lc. lactis* ssp. *tructae* was isolated from the intestinal mucus of brown trout (*Salmo trutta*) and rainbow trout (*Oncorhynchus mykiss*) species (Pérez et al., 2011). *Lactococcus lactis* ssp. *lactis* is found in a variety of environments, including animal sources, dairy products, and plant surfaces (Klijn et al., 1995; Nomura et al., 2006), whereas *Lc. lactis* ssp. *cremoris* is isolated primarily from raw milk and other dairy products (Urbach et al., 1997; Nomura et al., 2006). *Lactococcus lactis* ssp. *lactis* and ssp. *cremoris* are widely used for industry and research and play a key role in the determination of shelf life, preservation, and organoleptic quality, thereby influencing the quality and safety of these fermented products (Smit et al., 2005). In addition, many strains of *Lc. lactis* ssp. *lactis* and ssp. *cremoris* carry plasmids encoding important traits, such as lactose catabolism, citrate utilization, proteinase production, bacteriocin production and immunity, bacteriophage resistance, exopolysaccharide production, as well as heavy metal resistance (McKay, 1983; Davidson et al., 1996). These broad applications

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might be attributed to their remarkable ecological adaptability to diverse habitats.

Lactococcus lactis ssp. *lactis* and ssp. *cremoris* differ by no more than 0.7% in their 16S rDNA sequences (Salama et al., 1991) but display an average of only 85% DNA identity at the genome level (Wegmann et al., 2007). Some strains of *Lc. lactis* showing an *Lc. lactis* ssp. *lactis* phenotype according to classical distinction criteria, show an *Lc. lactis* ssp. *cremoris* genotype (*cremoris* genotype; Jarvis and Jarvis, 1981), meanwhile, phenotypic *Lc. lactis* ssp. *cremoris* showing an *Lc. lactis* ssp. *lactis* genotype (*lactis* genotype) have also been reported (Kelly et al., 2010; Tanigawa et al., 2010). Therefore, *Lc. lactis* has an unusual structure with 2 phenotypically distinct groups, *Lc. lactis* ssp. *lactis* and *Lc. lactis* ssp. *cremoris*, which may differ in genotype. Consequently, it has been difficult to accurately distinguish the 2 subspecies. Numerous methods, including DNA-DNA hybridization, small subunit rRNA gene sequencing, and PCR fingerprint analyses (Salama et al., 1991; Erlandson and Batt, 1997; Mangin et al., 1999; Pu et al., 2002), have been used for identification of *Lc. lactis* subspecies. However, their resolutions were low and the resulting data proved difficult for public database curation.

Multilocus sequence typing (MLST) is considered the gold standard for characterization of bacterial isolates and is based on the partial nucleotide sequences of multiple housekeeping genes (Maiden, et al., 1998; Urwin and Maiden, 2003; Aanensen and Spratt, 2005). It was recently shown to be a powerful technique for bacterial typing (Enright and Spratt, 1999), providing critical information for evolutionary history, population structure, and long-term epidemiology of bacterial species (Maiden, 2006; Turner and Feil, 2007). Although MLST has been used principally to study major bacterial pathogens, several recent MLST schemes were developed for LAB species, including *Oenococcus oeni* (de las Rivas et al., 2004), *Lactobacillus plantarum* (de las Rivas et al., 2006), *Lactobacillus casei* (Cai et al., 2007), *Streptococcus thermophilus* (Delorme et al., 2010), and *Lactobacillus sanfranciscensis* (Picozzi et al., 2010).

We examined the diversity and relationships of 197 *Lc. lactis* strains isolated from traditional fermented dairy products in different regions of China by characterizing population structure using an MLST scheme. The main aims of the present study were to apply MLST to assess phylogenetic relationship and evolutionary characteristics of these isolates and further reexamine the subspecies composition of *Lc. lactis*, which will contribute to screen starter cultures in the future.

MATERIALS AND METHODS

Bacterial Strains and Genomic DNA Extraction

A total of 197 *Lc. lactis* strains were selected from Collection Centre of Lactic Acid Bacteria of Inner Mongolia Agriculture University in China. Those included strains isolated from naturally fermented yogurt made by the Mongolian peoples in Chifeng, Xilin Gol, Hulunbeir, Bayan Nur of Inner Mongolia, by the Bai peoples in Dali of YunNan, and by the Tibetan peoples in Qinghai, Szechwan, Gansu, and Tibet of China from 2005 to 2009. Other than that, all strains had been achieved by 16S rDNA sequences. Details information of strains are listed in Table 1.

Lactococcus lactis strains were maintained in M17-broth (Oxoid, CM0817B, Wesel, Germany) supplemented with 5.0 g/L (wt/vol) of lactose at 30°C for 18 to 24 h, and then strains were harvested by centrifugation and cell pellets were used for DNA extraction. Total genomic DNA was extracted from cultures by using a previously reported method (Yu et al., 2012). Purified DNA was diluted to a final concentration of 100 ng/μL for application.

DNA Amplification and Sequencing for MLST

The MLST analyses traditionally focus on allelic diversity of housekeeping genes, which match admitted criteria, including their presence as a single copy in all strains, their conserved sequence, and their mutually unlinked location. The final selection included 12 housekeeping genes: *dnaA*, *pyrG*, *rpoB*, *groEL*, *recA*, *clpX*, *carB*, *murC*, *pepN*, *pepX*, *murE*, and *pheS*. In addition, *pyrG*, *groEL*, and *recA* were selected based on the results of a previous study on *Lactobacillus delbrueckii* (Kana and Watanabe, 2011), and *pepN*, *pepX*, and *pheS* were selected based on a study with *Lc. lactis* (Rademaker et al., 2007). Selection of the remaining loci (*dnaA*, *rpoB*, *clpX*, *carB*, *murC*, and *murE*) was based on the presence of SNP between *Lc. lactis* ssp. *lactis* IL-1403 and *Lc. lactis* ssp. *cremoris* SK11. These SNP were identified using comparative genome microarrays. These primers of 12 housekeeping genes were designed by Premier 5.0 (Premier Biosoft International, Palo Alto, CA) based on the known genome of *Lc. lactis* ssp. *lactis* IL1403, and the information of primers is listed in Table 2.

For each strain, the genomic DNA was used as a template for PCR amplification of MLST loci on the automatic thermal cycler (PTC-200, MJ Research,

Table 1. Bacterial strains used in this study (16S rDNA sequences results)

<i>Lactococcus lactis</i> ssp. <i>lactis</i>	<i>Lactococcus lactis</i> ssp. <i>cremoris</i> ¹	Source of isolate		
		Region	Number	Year
IMAU80707, IMAU80786, IMAU80782, IMAU80785, IMAU80754, IMAU80737, IMAU80680, IMAU80789, IMAU10765, IMAU10951, IMAU10962, IMAU10987, IMAU10994, IMAU10940, IMAU10941, IMAU10943, IMAU10946, IMAU10950, IMAU10955, IMAU10978, IMAU10979, IMAU10982, IMAU10984, IMAU10937, IMAU10939, IMAU10944, IMAU10945, IMAU10947, IMAU10936, IMAU11008, IMAU11039, IMAU11040, IMAU11043, IMAU11049, IMAU11009, IMAU11055, IMAU10876, IMAU10856, IMAU10857, IMAU10859, IMAU10864, IMAU10869, IMAU10872, IMAU10878, IMAU10881, IMAU10860, IMAU10865, IMAU10842, IMAU10844, IMAU10845, IMAU10848, IMAU10850, IMAU10852, IMAU10855, IMAU10874, IMAU10877, IMAU10897, IMAU10862, IMAU10880, IMAU10884, IMAU10887, IMAU10888, IMAU10889, IMAU10892, IMAU10893, IMAU10894, IMAU10895, IMAU10896, IMAU10899, IMAU10900, IMAU10906, IMAU10854, IMAU10093, IMAU10056, IMAU10065, IMAU10066, IMAU10059, IMAU10061, IMAU10067, IMAU10071, IMAU10086, IMAU70097	IMAU80703	Gannan of Gansu	9	2009
		Chifeng of Inner Mongolia	64	2009
	—			
		Bayan Nur of Inner Mongolia	10	2002
	—			
		Hulunbeir of Inner Mongolia	14	2009
	—			
IMAU11105, IMAU11119, IMAU10347, IMAU11058, IMAU11060, IMAU11062, IMAU11069, IMAU11070, IMAU11064, IMAU11067, IMAU11072, IMAU11076, IMAU11077, IMAU11081, IMAU11083, IMAU11085, IMAU11087, IMAU11089, IMAU11100, IMAU11110, IMAU11115, IMAU11118, IMAU11116, IMAU11079, IMAU11082	IMAU11120, IMAU11075	Xilin Gol of Inner Mongolia	27	2009
IMAU40134, IMAU40059, IMAU40053, IMAU40054, IMAU40055, IMAU40049, IMAU40064, IMAU40029, IMAU40121, IMAU40131, IMAU40066	IMAU40058, IMAU40061	Haibei of Qinghai	13	2005
IMAU80309, IMAU80260, IMAU80272	IMAU80338	Xigaze of Szechwan	4	2009
IMAU60156, IMAU60050, IMAU60009, IMAU60010, IMAU60011, IMAU60195, IMAU60034, IMAU60035, IMAU60036, IMAU60037	IMAU60067, IMAU60024	Xigaze of Tibet	12	2007
IMAU50049, IMAU50037, IMAU50152, IMAU50126, IMAU50130, IMAU50131, IMAU50115, IMAU50118, IMAU50124, IMAU50133, IMAU50140, IMAU50142, IMAU50144, IMAU50107, IMAU50108, IMAU50110, IMAU50114, IMAU50111, IMAU50112, IMAU50113, IMAU50117, IMAU50119, IMAU50120, IMAU50121, IMAU50122, IMAU50129, IMAU50134, IMAU50136, IMAU50141, IMAU50153, IMAU50156, IMAU50159, IMAU50161, IMAU50163, IMAU50095, IMAU50103, IMAU50105, IMAU50101, IMAU50104, IMAU50106, IMAU50166, IMAU50170	IMAU50149, IMAU50150	Dali of YunNan	44	2006

¹Where no data is entered, no strains were used.

Waltham, MA). For each target, a PCR mixture (50 µL) containing 150 ng of genomic DNA, 10 mM deoxy-ribonucleotide, 10 pmol of each primer, 2.5 U of Taq polymerase (TransGen Biotech, Beijing City, China) in 1× PCR buffer (with Mg²⁺). The PCR amplification program consisted of an initial denaturation step at 94°C for 4 min; 30 cycles of 94°C for 1 min, 50 to 60°C (optimal annealing temperatures for each locus are listed in Table 2) for 45 s, and 72°C for 1 min; and a final extension step at 72°C for 7 min. The PCR

products were electrophoresed in a 1.2% agarose gel. Sequencing of the PCR products was performed by Shanghai Majorbio Bio-pharm Technology Corporation (Shanghai, China). The same primers were used for PCR and sequencing on both DNA strands.

Data Analysis for MLST

For MLST analysis, forward and reverse sequences were trimmed, aligned, and analyzed using MEGA 5.0

Table 2. Genes and primers used for multilocus sequence typing¹

Gene	Gene product	Primer ²	Sequence (5'→3')	Length (bp)	Annealing temperature (°C)
<i>dnaA</i>	Chromosomal replication initiator protein dnaA	<i>dnaA</i> /F <i>dnaA</i> /R	CTATTTATTTATGGTGGAGC ACATCGCAATCTGTCTTG	745	52
<i>pyrG</i>	CTP synthase (UTP-ammonia lyase)	<i>pyrG</i> /F <i>pyrG</i> /R	GTGACGTAGCACCAGAAGC CGCCTGAGAATACGAAACC	765	58
<i>rpoB</i>	RNA polymerase β subunit	<i>rpoB</i> /F <i>rpoB</i> /R	GACTCCAACCGTGCCCTCA AACAATACCGCCACCTCC	741	60
<i>groEL</i>	60 kDa heat-shock protein	<i>groEL</i> /F <i>groEL</i> /R	AGGATTTGGTGATCGTCGTA GGGTCCATTGGAGGCATA	771	55
<i>recA</i>	Recombinase protein A	<i>recA</i> /F <i>recA</i> /R	TTCACTTATGCGACTTGG TTGGGTCTTGTTATCTCC	652	50
<i>clpX</i>	ATP-dependent Clp protease subunit X	<i>clpX</i> /F <i>clpX</i> /R	TAGCGAAATCACTCAACG AGGGCTTCATCTTCAAAT	658	50
<i>carB</i>	Carbamoyl phosphate synthetase	<i>carB</i> /F <i>carB</i> /R	CAGAAGCGATTCTGTCGTG GCTCCTGGTGGTTGTGGA	748	55
<i>murC</i>	UDP-N-acetyl muramate-alanine ligase	<i>murC</i> /F <i>murC</i> /R	AATATCGTGGACACTTCTT CTAAATCTTGTCGCGTAA	790	55
<i>pepN</i>	Lysyl-aminopeptidase, aminopeptidase N	<i>pepN</i> /F <i>pepN</i> /R	ACCCAGCAATCGTCTATG CAGCAACTTTATCCCAAC	765	53
<i>pepX</i>	X-prolyl dipeptidyl aminopeptidase	<i>pepX</i> /F <i>pepX</i> /R	GTGAGGTCGTTTGGGTTG CGGTCTAAAGCAGTGGTCAT	802	55
<i>murE</i>	UDP-N-acetylmuramyl tripeptide synthase	<i>murE</i> /F <i>murE</i> /R	TTTTCTTTGCCAAGGGTT GTCCTAATAAGCGAATGT	741	55
<i>pheS</i>	Phenylalanyl-tRNA synthase α subunit	<i>pheS</i> /F <i>pheS</i> /R	AAAGGGTGAATTGACTGA ATGGGAAATAACTTGGAC	659	52

¹*Lactococcus lactis* ssp. *lactis* IL1403 was used as the template for primer design.

²F = forward; R = reverse.

software package (version 5.0, www.megasoftware.net). Definition of alleles using a nonredundant data set and minimum spanning tree analysis were performed using the BioNumerics Software (version 6.0, Applied-Maths, Sint Maartens-Latem, Belgium). For each MLST locus, each different allele was assigned a different arbitrary number, and a unique combination of 12 allele numbers (allelic profile) unambiguously defined the sequence type (ST) of a strain. The same ST was used for several strains when they shared the same allelic profiles. Allelic profiles were used for subsequent analysis. Groups of isolates with closely related allelic profiles have been called clonal complexes (CC). The ST were grouped into CC with the BURST program developed by E. Feil (Feil et al., 2004) and located in the BioNumerics program.

The mean GC (guanine-cytosine) content of the DNA and the d_N/d_S ratios (where d_S is the number of synonymous substitutions per synonymous site, and d_N is the number of nonsynonymous substitutions per nonsynonymous site) were calculated by using START 2.0 (Nei and Gojobori, 1986). This software was also used to calculate the index of association (I_A) by the LIAN linkage analysis (Smith et al., 1993); nucleotide diversity (π) per site was calculated using DnaSP version 5.0 (Rozas et al., 2003). The split decomposition method was used to assess the degree of tree-like structure for alleles of each locus and all ST using SplitsTree 4.0 (Huson, 1998; Huson and Bryant, 2006). The unweight-

ed-pair group method using average linkages tree of ST was drawn with the START version 2.0 program (Jolley et al., 2001)

Nucleotide Sequence Accession Numbers

The sequences of the 12 MLST loci have been deposited in the GenBank database under accession numbers KF837137 to KF839500.

RESULTS

Genetic Diversity

We developed a new MLST scheme for 197 *Lc. lactis* strains based on the fragments of 12 housekeeping genes. By combining the 12 gene loci using the Bionumerics 6.0 software, MLST identified 72 different ST (Table 3), indicating high genotypic diversity. Of these ST, 51 corresponded to single isolates and 12 corresponded to 2 to 4 isolates. The best represented was ST5, identified in 38 isolates (19.28%) of strains from as many as 6 regions across 4 provinces (Chifeng, Bayannur, and Xilin Gol of Inner Mongolia, Haibei of Qinghai, Gannan of Gansu, and Xigaze of Tibet), followed by ST13 (7.6%), ST17 (5.58%) and ST34 (5.58%); the remaining ST accounted for less than 5% of isolates. Seven ST were associated with strains isolated from more than one region. In contrast, we found 65 ST assigned to

strains isolated from one specific region, as was the case for ST12, ST13, ST17, and ST19, which were identified only in strains from Chifeng, Inner Mongolia, and for ST31, ST34, and ST42, which were identified in strains collected from Dali, Yunnan.

The number of alleles per locus ranged from 6 (*dnaA*) to 18 (*murC*). The GC content of all alleles ranged from 33.62% (*clpX*) to 41.95% (*recA*), consistent with the 38.42% GC content that has been observed across the entire *Lc. lactis* genome. The housekeeping genes examined by MLST had GC contents similar to those of *Lc. lactis* ssp. *lactis* (strain IL1403), suggesting that these genes have been present in *Lc. lactis* for some time (Bolotin et al., 2001). The nucleotide diversity (π , the average number of nucleotide differences per site between 2 randomly-selected isolates) ranged from 0.00362 (*murE*) to 0.08439 (*carB*; Table 4), which demonstrated the different evolution rates of 12 genes used in the new MLST scheme. The d_N (number of non-synonymous changes per nonsynonymous site) and d_S (number of synonymous changes per synonymous site) values were calculated for each gene. The d_N/d_S ratios were determined to be lower than 1 for all loci (Table 4), indicating the predominance of purifying selection preferentially associated with reducing AA variation.

Recombination and Linkage Disequilibrium

We measured intergenic recombination by estimating the linkage disequilibrium between the 12 loci using START2.0 (Nei and Gojobori, 1986). The I_A (Smith et al., 1993) and standardized index of association (I_A^S ; Haubold and Hudson, 2000) are measures of the extent of linkage equilibrium within a population determined by quantifying the amount of recombination in a set of sequences and detecting association among alleles at different loci. If linkage equilibrium is evident due to frequent recombination events, the expected value of I_A is zero. Clonal populations are identified by an I_A value that differs significantly from zero. We calculated the I_A and I_A^S values to be 3.3423 and 0.3038, respectively, for the 12 loci, indicating linkage disequilibrium or association among the alleles (Haubold and Hudson, 2000).

The method of split decomposition (Huson, 1998; Rozas et al., 2003) was used to assess the degree of tree-like structures present in the alleles at each locus in all 197 bacterial isolates. The algorithm used in this software can display conflicting results in the phylogenetic descent of sequences. A tree-like structure is created if the descent is clonal, whereas an interconnecting network resembling a parallelogram will appear whenever recombination has influenced the evolution of that gene (de las Rivas et al., 2006). The split graphs for all 12 loci showed different network structures, except for

the *murE* gene (Figure 1a), suggesting that intragenic recombination occurred during the evolution of the other 11 loci. Yet, the parallelogram network of the 11 genes was not very apparent, indicating that intragenic recombination was not remarkable. A combined split graph based on a distance matrix comprising pairwise distances of all alleles of the 12 loci also revealed a network-like structure; the multiple parallel paths were indicative of incompatibilities resulting from recombination or recurrent mutation (Figure 1b).

Phylogenetic Analysis

To explore the relationships among the 197 strains, the population structure was analyzed using minimum spanning trees with an algorithm modified to take into account the number of mutations among allelic profiles (Bionumerics v. 6.0; Schouls et al., 2004). In this representation, strains with the same ST are grouped in one circle, the size of which is proportional to the number of strains. Assignment of ST to CC using BURST analysis (Feil et al., 2004) revealed that the 72 ST were divided among 14 CC (CC1 to CC14) and 23 singletons (Table 3), and CC can be defined as groups of profiles differing by no more than one gene from at least one other profile of the group. Strains within CC represented 88.3% of the total 197. In Figure 2, the shaded zones between certain groups of circles indicate that these profiles belong to the same CC. The most common CC was CC1 (pink zone), represented by 13 ST, and ST5 was most prevalent among the 197 strains, seen in 38 (19.28%) total. Sequence type 5 had 7 single locus variants, ST18 had 6 single locus variants and 2 double-locus variants (Katherine et al., 2007), whereas ST6, ST23, and ST66 appeared to be closely related to CC1, as they differed from ST5 in only 2 loci. Sequence type 8, ST13, ST14, ST16, and ST19 were all isolated from strains in Chifeng of Inner Mongolia; ST22, ST23, ST25, ST26, ST27, and ST28 from Xilin Gol of Inner Mongolia; ST71 and ST72 from Gannan of Gansu; ST3 and ST6 from Bayannur of Inner Mongolia; and ST39 and ST66 were isolated from strains in Dali of Yunnan and in Aba of Szechwan, respectively (Figure 2).

All other CC comprised only 2 to 4 ST and a limited number of strains; in particular, those of the 4 branches of ST5 (Figure 2). From left to right, the first branch consisted of CC3 and CC4, which were connected by discontinuous links. The second branch consisted of CC6, CC7, and single ST; most strains with these ST were *Lc. lactis* ssp. *cremoris*, according to 16S rRNA sequences, with the exception of ST56 and ST70, which identified as *Lc. lactis* ssp. *lactis*. Six CC (CC8, CC9, CC10, CC11, CC12, and CC14) and a few single ST were identified in the third branch, of

Figure 1. Split decomposition analysis based on the allelic profiles of the *Lactococcus lactis* strains. (a) Split decomposition of alleles for individual multilocus sequence typing loci and (b) combined split decomposition of alleles for all 12 multilocus sequence typing loci.

which all but CC9 were isolated from strains in Dali of Yunnan. The fourth branch comprised CC5 and CC13 with strains from Dali of Yunnan and Hulun Buir of Inner Mongolia.

We concatenated the 12 loci sequences, obtaining a much longer sequence alignment of 6,192 bp. This procedure is advantageous in that it can buffer local recombination consequences that potentially affect the recovery of true phylogenetic relationships among single or small numbers of genes (Hanage et al., 2005). An unweighted-pair group method using average linkages dendrogram of the concatenated sequence demonstrated the genetic relatedness among the *Lc. lactis*

strains investigated in this study (Figure 3). In this tree, the 197 strains were separated into 2 major clusters, designated A and B. Most strains of cluster A belonged to *Lc. lactis* ssp. *cremoris*, except those of ST56 and ST70; whereas the strains of cluster B included *Lc. lactis* ssp. *lactis*, with the exception of ST28. Cluster B was further divided into 2 subclusters. Cluster B1 consisted of one group representing ST of strains from Dali of Yunnan and Hulun Buir of Inner Mongolia and a second group representing the majority of strains from Xilin Gol and Chifeng of Inner Mongolia, Xigaze of Tibet, Haibei of Qinghai, and Gannan of Gansu; 4 strains from Bayannur of Inner Mongolia; and 2 strains from

b

Figure 1 (Continued). Split decomposition analysis based on the allelic profiles of the *Lactococcus lactis* strains. (a) Split decomposition of alleles for individual multilocus sequence typing loci and (b) combined split decomposition of alleles for all 12 multilocus sequence typing loci.

Aba of Szechwan. Cluster B2 predominantly comprised strains from Dali of Yunnan, as well as 9 strains from Chifeng of Inner Mongolia, 6 from Bayannur of Inner Mongolia, and 1 from Aba of Szechwan.

DISCUSSION

A previous analysis of a collection of strains sampled from traditional fermented dairy products of a wide range of geographic locations, including 5 regions of Inner Mongolia, Tibet, Qinghai, Gansu, Szechwan, and Yunnan, revealed high genotypic diversity among *Lc. lactis*. Multilocus sequence typing, which allows high-

resolution analysis of molecular diversity, was used to evaluate the diversity and relationships of this species. However, few studies have characterized the genetic population diversity and evolutionary patterns of *Lc. lactis* strains from different ecological origins. To contribute to the characterization of the *Lc. lactis* population structure, we designed an MLST typing scheme to examine 197 strains isolated from different geographical areas.

Multilocus sequence typing is considered the gold standard for characterizing isolates of bacterial species using the DNA sequences of selected housekeeping gene internal fragments (Urwin and Maiden, 2003). Most

Table 3. Allelic profiles of analyzed *Lactococcus lactis* strains

ST	carB	clpX	dnaA	groEL	murC	murE	pepN	pepX	pheS	pyrG	recA	rpoB	Group ¹	Origin (no. of isolates)
1	1	10	5	10	14	8	1	13	8	4	4	7	CC9	Chifeng (5); Bayannur (3)
2	2	8	4	9	15	8	7	9	8	4	6	7	CC8	Bayannur (1)
3	8	7	6	3	2	2	5	6	4	10	7	2	S1	Bayannur (1)
4	1	10	5	10	13	8	1	13	8	4	4	7	CC9	Bayannur (1)
5	3	7	2	3	2	2	5	6	4	10	4	2	CC1	Chifeng (18); Bayannur (1); Haibei (6); Xigaze (9); Xilin Gol (1)
6	3	7	2	3	2	4	5	6	6	10	4	2	S2	Bayannur (1)
7	8	7	6	8	2	2	5	7	4	10	5	2	CC2	Bayannur (1)
8	6	2	3	1	5	2	3	11	4	2	1	2	CC13	Hulun Buir (4)
9	3	7	1	7	2	3	5	8	5	10	4	1	CC4	Hulun Buir (1)
10	7	2	3	10	9	2	6	10	4	8	6	2	CC5	Hulun Buir (8); Dali (1)
11	7	2	3	10	2	2	6	10	4	8	6	2	CC5	Hulun Buir (1)
12	1	9	6	10	8	7	1	10	8	4	4	7	S3	Chifeng (3)
13	8	7	2	3	2	2	5	6	4	9	4	2	CC1	Chifeng (16)
14	3	7	2	3	2	5	5	6	4	10	4	2	CC1	Chifeng (1)
15	9	7	5	10	2	2	11	6	4	10	4	2	S4	Chifeng (1)
16	8	7	2	14	2	2	5	6	4	10	4	2	CC1	Chifeng (1)
17	3	7	2	3	2	3	5	6	5	10	4	1	CC3	Chifeng (11)
18	8	7	2	3	2	2	5	6	4	10	4	2	CC1	Chifeng (1)
19	3	7	2	3	10	2	5	6	4	10	4	2	CC1	Chifeng (5)
20	1	10	5	10	12	8	1	13	8	4	4	7	CC9	Chifeng (1)
21	3	7	5	3	10	2	4	6	2	12	4	2	S5	Chifeng (1)
22	8	7	2	3	2	2	4	6	4	10	4	2	CC1	Xilin Gol (5)
23	3	6	2	3	2	1	5	6	4	10	4	2	S6	Xilin Gol (3)
24	11	7	2	14	17	2	8	2	11	14	11	2	CC6	Xilin Gol (1)
25	3	7	2	3	4	2	5	6	4	10	4	2	CC1	Xilin Gol (9)
26	8	7	2	6	2	2	5	6	4	10	4	2	CC1	Xilin Gol (4)
27	3	7	2	3	2	2	5	6	5	10	4	2	CC1	Xilin Gol (2)
28	8	7	6	3	6	2	5	6	4	10	4	2	S7	Xilin Gol (1)
29	11	7	2	14	17	2	8	2	4	14	11	2	CC6	Xilin Gol (1); Xigaze (1)
30	3	7	2	5	3	2	5	6	3	10	4	2	S8	Haibei (4)
31	1	10	5	10	11	8	1	14	7	4	3	7	CC10	Haibei (1); Dali (2)
32	10	7	2	13	17	2	8	2	4	14	9	2	S9	Haibei (1)
33	11	7	2	13	16	2	8	3	4	14	11	2	S10	Haibei (1)
34	6	2	3	1	5	2	2	11	4	2	1	2	CC13	Dali (11)
35	1	8	5	10	11	8	1	13	2	3	2	5	CC12	Dali (1); Bayannur (1)
36	5	5	4	4	2	2	12	10	9	11	1	6	S11	Dali (1)
37	7	1	2	10	13	8	10	13	8	4	6	7	S12	Dali (1)
38	1	10	5	10	12	8	1	14	7	5	3	7	S13	Dali (1)
39	8	7	2	3	2	2	5	6	4	10	8	2	CC1	Dali (1)
40	6	5	3	11	13	6	10	15	8	6	6	4	S14	Dali (1)
41	1	8	5	2	12	8	2	5	7	1	2	7	CC14	Dali (1)
42	1	8	5	2	11	8	2	5	7	1	2	7	CC14	Dali (1)
43	1	8	5	2	2	8	2	5	7	1	2	7	CC14	Dali (1)
44	1	8	5	2	11	8	2	5	7	10	2	7	CC14	Dali (1)
45	6	2	3	1	2	2	2	11	4	2	1	2	CC13	Dali (1)
46	1	8	4	9	12	8	7	9	8	4	6	7	CC8	Dali (1)
47	1	8	4	9	15	8	7	9	8	4	6	7	CC8	Dali (1)
48	1	8	4	9	16	8	7	9	8	4	6	7	CC8	Dali (1)
49	1	8	4	9	2	8	7	9	8	4	6	7	CC8	Dali (1)
50	8	7	6	8	2	2	5	6	4	10	5	2	CC2	Dali (1)
51	3	7	2	3	7	3	5	6	5	10	4	1	CC3	Dali (1)
52	3	7	2	3	14	3	5	6	5	10	4	1	CC3	Dali (1)
53	1	10	2	10	2	8	1	13	8	4	6	7	S15	Dali (1)
54	12	7	2	13	17	2	8	2	4	13	11	2	CC7	Dali (1)
55	12	7	5	13	17	2	8	2	4	13	11	2	CC7	Dali (1)
56	12	7	5	13	2	8	8	2	1	13	10	7	S16	Dali (1)
57	1	8	5	10	12	8	1	12	2	3	2	5	CC12	Dali (1)
58	1	8	5	10	11	8	1	12	2	3	2	5	CC12	Dali (1)
59	1	3	5	10	11	8	1	13	8	4	6	7	CC11	Dali (1)
60	6	3	5	10	2	8	1	13	8	4	6	7	CC11	Dali (1)
61	6	3	5	10	11	8	1	13	8	4	6	7	CC11	Dali (1)
62	6	4	6	10	9	2	6	10	4	8	4	2	S17	Dali (1)
63	13	7	2	12	18	2	9	4	10	15	12	2	S18	Xigaze (1)

(Continued)

Table 3 (Continued). Allelic profiles of analyzed *Lactococcus lactis* strains

ST	<i>carB</i>	<i>clpX</i>	<i>dnaA</i>	<i>groEL</i>	<i>murC</i>	<i>murE</i>	<i>pepN</i>	<i>pepX</i>	<i>pheS</i>	<i>pyrG</i>	<i>recA</i>	<i>rpoB</i>	Group ¹	Origin (no. of isolates)
64	3	7	2	10	2	2	1	13	1	4	6	2	S19	Xigaze (1)
65	3	7	1	7	1	3	5	8	5	10	4	1	CC4	Gannan (2); Aba (1)
66	3	7	2	3	3	2	5	6	4	10	4	3	S20	Aba (1)
67	1	10	5	10	11	8	1	14	7	7	3	7	CC10	Aba (1)
68	3	7	5	3	2	2	1	6	4	14	4	2	S21	Aba (1)
69	11	7	5	3	17	2	8	1	4	14	4	2	ST22	Gannan (1)
70	11	7	2	14	17	2	8	1	4	14	4	2	ST23	Gannan (1)
71	4	7	2	3	2	2	5	6	4	10	4	2	CC1	Gannan (1)
72	3	7	2	7	2	2	5	6	4	10	4	2	CC1	Gannan (1)

¹CC = clonal complex; S = singleton.

MLST schemes are based on 5 to 8 housekeeping genes with 450- to 500-bp fragments of each gene; however, small numbers of gene loci and short fragment lengths may decrease the amount of information obtained and the reliability of the results. In our study, 12 gene targets (*carB*, *clpX*, *dnaA*, *murC*, *murE*, *pepN*, *pepX*, *pyrG*, *recA*, *groEL*, *uvrC*, and *rpoB*) that gave reliable amplification products were used for MLST analysis. By combining the 12 gene loci, 72 ST were identified with an average of 9 distinct alleles per locus, indicating a high level of genetic diversity within slowly evolving loci. A similar finding for *Lc. lactis* was obtained using MLST in previous studies (Delphine et al., 2010). Such differences are partly due to the number of isolates studied and the MLST scheme. The abundance of MLST loci and isolates of a wide geographical range contributed to the high genetic diversity.

The nucleotide diversity per site (π) of the 12 genes varied from 0.00362 in *murE* to 0.08439 in *carB*. These values confirmed the differences in evolutionary rates among the genes used in this MLST scheme. The π values were greater than 0.01 for 9 of the 12 loci, in which cases the variability was strongly correlated with the overall genomic variability. In comparison with other LAB, the allelic diversity values for *Lb. plantarum* (de las Rivas et al., 2006), *Lb. delbrueckii* (Tanigawa and

Watanabe, 2011), and *Oenococcus oeni* (de las Rivas et al., 2004) ranged from 0.0004 to 0.0072, 0.0051 to 0.0096, and 0.0011 to 0.0370, respectively. Higher values were obtained for most of the species with available MLST data. Therefore, our *Lc. lactis* strain population appeared to encompass higher nucleotide diversity relative to these other LAB species, likely attributed to 2 *Lc. lactis* subspecies, which is similar to a report from 2010 (Delphine et al., 2010). The synonymous and nonsynonymous changes in the allelic sequences of a locus can provide information about the degree of selection exerted on a population. The d_N/d_S ratios for 12 loci were <1, indicating a strong purifying selection against changes in AA composition, which is typical of housekeeping genes and desirable in MLST schemes (Sneath and Sokal, 1973).

Multilocus linkage disequilibrium was analyzed using I_A and I_A^S based on the MLST profile. High values are indicative of a strong clonal population. Thus, the analyzed genes in *Lc. lactis* appear far from linkage equilibrium according to our results. The I_A^S (0.3038) value suggested strong clonal population, confirming that recombination played a key role in allelic distribution, but not frequently enough to prevent the emergence of clones. Split decomposition also provided strong evidence for intraspecies recombination according to the

Table 4. Allelic variation in 12 housekeeping genes

Locus	Allele	GC content (%)	π^1	d_N	d_S	d_N/d_S^2
<i>carB</i>	13	36.72	0.08439	0.0199	0.6830	0.0291
<i>clpX</i>	10	33.62	0.01443	0.0006	0.0680	0.0090
<i>dnaA</i>	6	37.35	0.00406	0.0000	0.0181	0.0000
<i>groEL</i>	14	40.13	0.04173	0.0055	0.2092	0.0264
<i>murC</i>	18	37.81	0.03524	0.0038	0.2158	0.0176
<i>murE</i>	8	35.38	0.00362	0.0018	0.0076	0.2436
<i>pepN</i>	12	37.66	0.03757	0.0108	0.1747	0.0618
<i>pepX</i>	15	40.44	0.07644	0.0287	0.3824	0.0751
<i>pheS</i>	11	39.16	0.03009	0.0023	0.1762	0.0132
<i>pyrG</i>	15	41.95	0.03328	0.0052	0.1468	0.0355
<i>recA</i>	12	41.03	0.02580	0.0005	0.1219	0.0041
<i>rpoB</i>	7	39.81	0.00767	0.0008	0.0306	0.0249

¹Mean pairwise nucleotide differences per site.

² d_N/d_S = the ratio of nonsynonymous to synonymous substitutions.

Figure 2. Minimum spanning tree analysis of the 197 *Lactococcus lactis* strains based on the allelic profiles of the 12 genes *carB*, *clpX*, *dnaA*, *groEL*, *murC*, *murE*, *pepN*, *pheS*, *pepX*, *pyrG*, *recA*, and *rpoB*. Each circle corresponds to a sequence type (ST), and the circle size corresponds to the number of strains sharing the same ST. The shaded zones between certain groups of circles indicate that these profiles belong to the same clonal complex. Numerals connecting the circles indicate the number of allelic differences between the profiles. The strength of the link (bold, plain, or discontinuous) is correlated to the genetic similarity (number of common alleles) between profiles. CC = clonal complex.

network structure. Split graphs for each locus showed tree-like or slight network-like structures, indicating that most of the genes were not significantly affected by intragenic recombination (Figure 1a). The concatenated sequence of the 12 MLST genes showed a network-like

structure (Figure 1b), suggesting that the 12 gene fragments underwent widespread associative recombination overall. Therefore, our data suggest that recombination and selective pressure likely contributed to the evolution of *L. lactis*.

Figure 3. An unweighted pair-group method with arithmetic mean tree constructed from 72 concatenated nucleotide sequences of 12 multilocus sequence typing loci.

According to minimum spanning tree and unweighted pair-group method with arithmetic mean tree analyses, IMAU50152 (ST56) and IMAU80707 (ST70) were assigned to *Lc. lactis* ssp. *cremoris*, which was classified as *Lc. lactis* ssp. *lactis* based on the 16S rRNA sequence. Furthermore, IMAU80338 appeared to be distantly related to other *cremoris* strains due to the discontinuous link between ST68 and *Lc. lactis* ssp. *cremoris* according to the minimum spanning profile. In addition, the ST28 of the IMAU11116 strain was found in *Lc. lactis* ssp. *cremoris*, which clustered in the *Lc. lactis* ssp. *lactis* group. The 16S rDNA gene sequences of *lactis* and *cremoris* subspecies differ by 9 to 10 bp (depending on strain) within the first 200 bp of the 16S rDNA sequence, the difference is less than 1% for the total 16S rDNA sequence of 1,500 bp. Therefore, 16S rDNA sequence analysis is difficult to distinguish between *lactis* and *cremoris* subspecies (Salama et al., 1991). As early as 1991, Koehler et al. (1991) applied for traditional physiological and biochemical identification method and 16S rDNA sequence analysis technology to identify *Lc. lactis* ssp. *lactis* and ssp. *cremoris*, but their results showed that *Lc. lactis* aren't accurately assigned to the subspecies level. This provides strong evidence for reclassification of *Lc. lactis* subspecies by MLST, consistent with previous reports supporting MLST as a new method for distinguishing subspecies (Rademaker et al., 2007; Kana and Watanabe, 2011).

Meanwhile, we explored the evolutionary relationships among *Lc. lactis* isolates from various regions of China. Based on our findings, CC1 originated from ST5, whose ancestral status suggests it is the most frequent ST in the population, and it was represented in 6 regions across 4 provinces (Feil, 2004). Interestingly, ST18 was only detected in a strain from Chifeng of Inner Mongolia, but it had 6 single locus variants (including ST5) and 2 double-locus variants, which is relatively primitive. In addition, 18 ST5 strains originated from Chifeng; thus, some of these isolates are likely to be ancestors to strains in this region of China. It was also confirmed that CC1 was represented in all regions except for Hulun Buir of Inner Mongolia. Moreover, abundant genetic diversity was observed in the isolates from Dali of Yunnan, which was apparent from the 44 strains that classified into 31 ST, and some isolates were related to the majority of those from Hulun Buir of Inner Mongolia. Our results differed from those obtained for similar species used in food fermentation, such as *Lb. sanfranciscensis* and *Lb. plantarum*, in which no significant clusters were correlated with the geographic origin of the strains (Rozas et al., 2003; Picozzi et al., 2010). Similarly, Delphine et al. (2010) revealed little relationship between core and dispensable genome phylogenies by MLST, indicating

that clonal diversification and phenotypic variability of the domesticated strains essentially arose through substantial genomic flux within the dispensable genome. The use of MLST will be of great help in defining the ecological and phylogenetic status of new lactococcal strains, and may be more informative than other genotyping methods (Delphine et al., 2010). As increasing numbers of isolates are collected from different regions, our findings enable comparisons with *Lc. lactis* isolates collected nationwide as well as providing information regarding the factors involved in the evolution of this important bacterium.

CONCLUSIONS

In conclusion, MLST was used to investigate the genetic polymorphisms and evolutionary relationships among 197 *Lc. lactis* isolates from various regions. First, an MLST scheme was developed for the study of *Lc. lactis*, which contributed data to the MLST database. Second, we demonstrated MLST to be an effective method for distinguishing *Lc. lactis* ssp. *lactis* and ssp. *cremoris*. Last, analysis of geographically diverse populations of bacterial isolates using MLST can provide a better understanding of *Lc. lactis* genomic evolution and useful information for further studies on global *Lc. lactis* structure and genetic evolution.

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