


# Analysis of *Leuconostoc citreum* strains using multilocus sequence typing

Anshul Sharma<sup>1</sup> · Jasmine Kaur<sup>1</sup> · Sulhee Lee<sup>1</sup> · Young-Seo Park<sup>1</sup> 

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**Abstract** The objective of this study was to perform genetic diversity analysis of 13 strains isolated from South Korean foods by multilocus sequence typing (MLST). For typing, seven housekeeping loci (*atpA*, *dnaA*, *dnaK*, *gyrB*, *pheS*, *pyrG*, and *rpoA*) were selected, amplified and analyzed. Fifty-one polymorphic sites varying from 1 to 22 in each species were identified. Thirteen sequence types were generated with allele numbers ranged from 2 to 10. The overall relationship between strains was assessed by unweighted pair group method with arithmetic mean dendrogram and minimum spanning tree. In addition, combined spits tree analysis revealed intragenic recombination. No clear relationship was observed between the isolation sources and strains. The developed MLST scheme enhanced our knowledge of the population diversity of *Leu. citreum* strains and will be used further for the selection of industrially important strain.

**Keywords** Lactic acid bacteria · *Leuconostoc citreum* · MLST · Sequence type · Alleles

## Introduction

The genus *Leuconostoc* consisted of 24 different species characterized as Gram-positive, non-motile, catalase-negative and facultative anaerobes (Kot et al., 2014). This genus has been found to be associated with dairy products, meat, fish and poultry and various other plant-originated

materials (Björkroth and Holzapfel, 2006). It has also been used as starter cultures in fermented milk and vegetables. The important species of this genus is *Leuconostoc citreum* (formerly *Leu. amelibiosum*) considered as one of the most predominant lactic acid bacteria (LAB) which helps in the production of kimchi (Korean traditional fermented cabbages), the top-known Korean traditional dish (Chang and Chang, 2010). It is a heterofermentative lactic acid bacterium which is an inhabitant of various fermented foods of plant and dairy origins, such as Pozol (a fermented corn Beverage-Mexican), cassava starch, sourdough, and recently used for the direct fermentation of semolina (Björkroth and Holzapfel, 2006).

Commonly, typing methods of bacteria include phenotyping and genotyping. Phenotypic methods are traditional (biotypes, serotypes, phage-types, and antibiograms), which often lead to an uncertain identification of the LAB (Dan et al., 2014). Therefore, genotypic methods are playing a significant role in phylogenetic classification and identification of the bacterial species. Till date, many molecular methods have been used for the typing of *Leuconostoc* genus like restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD)-PCR, amplified fragment length polymorphism (AFLP), pulsed-field gel electrophoresis (PFGE), repetitive element palindromic PCR (rep-PCR) using different primers, and multilocus sequence typing (MLST) (Sharma et al., 2018). MLST is based on the principles of multilocus enzyme electrophoresis (MLEE) (Maiden et al., 1998). For characterization of bacteria on the basis of alleles, this technique utilizes the polymorphisms in the sequences of the candidate housekeeping genes. Compared to other methods, this typing tool is showing clear results and the sequence data can be transferred worldwide. It gives valuable information about the species regarding

✉ Young-Seo Park  
ypark@gachon.ac.kr

<sup>1</sup> Department of Food Science and Biotechnology, Gachon University, Seongnam 13120, South Korea

their population organization and evolution. Initially, the technique was described in 1998 for the identification of virulent strains of *Neisseria meningitidis* (Maiden et al., 1998). Continuously, several MLST schemes have been created for bacterial species including the LAB (Sharma et al., 2018). Identification of industrially important bacterial strain is essential in both basic and applied research. MLST may help to differentiate strains at the genetic level and also to identify an industrially important strain. By considering the importance of the technique, the present study was carried out to develop an MLST scheme to determine the genetic diversity of *Leu. citreum* strains at species level from South Korea.

## Materials and methods

### Bacterial strains and DNA isolation

Bacterial strains were isolated from six food sources of Korea i.e., kimchi (6), young radish kimchi (1), salted oyster (1), salted fish (1), and pear (1) from Seongnam and salted small octopus (3) from Sunchang and Seongnam. The strains were grown in MRS broth (BD, Franklin Lakes, NJ, USA) at 37 °C overnight. Total DNA was extracted from 3.0 mL of fresh cultures using the DNA isolation kit (Bioneer, AccuPrep Genomic DNA Extraction Kit, Daejeon, Korea), as described in the previous study (Kaur et al., 2017a). The good quality DNA was obtained; estimated in ethidium bromide stained 0.8% (w/v) agarose (Seakem, Lonza Ltd., Basel, Switzerland) gel.

### PCR amplification and sequencing

The strains were already identified using 16S rRNA gene sequencing as described in the previous publication (Kaur et al., 2017b). For MLST gene amplification, the primers were designed using the primer blast tool from the NCBI website ([www.ncbi.nlm.nih.gov/](http://www.ncbi.nlm.nih.gov/)) (Table 2). The PCR amplifications of the MLST loci, i.e., ATP synthase subunit alpha (*atpA*), chromosomal replication initiation protein (*dnaA*), chaperone hsp70 (*dnaK*), gyrase subunit B (*gyrB*), phenylalanyl-tRNA synthetase subunit  $\alpha$  (*pheS*), CTP synthase (*pyrG*), and RNA polymerase (*rpoA*) were performed in 20  $\mu$ L PCR reaction mixture premix kit (Bioneer, Daejeon, Korea) containing 1  $\mu$ L of genomic DNA, 1  $\mu$ L of each (forward and reverse) primer (10 pmol/ $\mu$ L) and 17  $\mu$ L of distilled water. Amplification was performed with the automatic thermal cycler (MyCycler<sup>TM</sup>, BioRad, Hercules, CA, USA) following conditions: 94 °C for 2 min, 35 cycles of denaturation at 95 °C for 20 s, annealing temperature different for each locus, i.e., *atpA*, 51.1 °C; *dnaA*, 54.2 °C; *dnaK*, 56.8 °C; *gyrB*, 54.6 °C;

*pheS*, 46.6 °C; *pyrG*, 58.0 °C; *rpoA*, 56.0 °C for 30 s, extension at 72 °C for 30 s, followed by a final elongation at 72 °C for 7 min. The amplified products were purified using a Wizard SV Gel and PCR Cleanup system kit (Promega, Madison, WI, USA) and sequenced by Macro-gen-Humanizing Genomics (Seoul, Korea) and Bioneer (Bioneer, Daejeon, Korea).

### Data analysis

For MLST analysis, forward and reverse sequences were cropped and examined with Bioedit Sequence Alignment Editor ver. 7.2.5 for every gene (Hall, 1999) and aligned with the ClustalX ver. 1.83 software. The GC content was calculated with DnaSP ver. 5.10 (Librado and Rozas, 2009) and the polymorphic sites, Tajima's D value and nucleotide diversity per site ( $\pi$ ) by MEGA software ver. 7 (Kumar et al., 2016). The ratios of the synonymous substitutions ( $d_S$ ) to non-synonymous substitutions ( $d_N$ ) were analyzed online by the SNAP tool ([www.hiv.lanl.gov](http://www.hiv.lanl.gov)) (Korber, 2000). Generation of allelic profiles, sequence types (STs), dendrogram and minimum spanning tree (MST) were done using Bionumerics ver. 7.6 (Applied-Maths, Sint Maartens-Latem, Belgium) software. Split decomposition analysis was completed with SplitsTree ver. 4.14 software (Huson and Bryant, 2006).

## Results and discussion

The present study describes the MLST scheme of 13 bacterial strains isolated from South Korea. Isolated strains were examined for their genetic diversity using comparative sequence analysis of selected seven housekeeping genes. Gene sequences of the seven loci have been deposited in GenBank under the accession numbers KX286339-KX286348, KX286350-KX286352 for 16S rRNA gene and KY763993-KY764057 and KY767960-KY767985 for MLST loci.

### MLST typing and locus variation

Seven target genes were amplified, and the length of genes varied from 286 bp for *pheS* to 1647 bp for *dnaK*. The MLST scheme defined alleles between 264 (*pheS*) and 1100 bp (*dnaA*). The number of alleles per gene varied between 2 for *gyrB* and 10 for *dnaK*. From the gene sequence data of the seven loci, 13 different sequence types (STs) were obtained. Each ST was represented by a single strain of *Leu. citreum*, indicating about the genetic diversity in selected strains. The genetic variations of the *Leu. citreum* strains for the different loci have been shown in Table 1. All STs were differed at various loci, whereas ST-

**Table 1** Source and typing data of *Leu. citreum* strains analyzed in this study

Strain	KCCP number	Source	MLST (sequence type)							
			ST	<i>atpA</i>	<i>dnaA</i>	<i>dnaK</i>	<i>gyrB</i>	<i>pheS</i>	<i>pyrG</i>	<i>rpoA</i>
SC53	11037	Salted small octopus	1	1	1	1	1	1	1	1
YKC002	11076	Kimchi	2	2	2	2	1	1	2	2
YKC003	11077	Kimchi	3	2	1	3	1	2	2	3
YKC019	11082	Kimchi	4	2	1	2	1	1	3	2
PEAR008	11093	Pear	5	1	3	4	1	3	3	2
NJ1	11382	Salted small octopus	6	1	1	4	2	4	4	2
CMK2	11388	Young reddish kimchi	7	3	1	4	2	4	2	2
NJG3-1	11391	Salted small octopus	8	1	4	5	2	5	5	2
KCF001	11413	Kimchi	9	1	1	6	2	5	6	2
KCF002	11414	Kimchi	10	1	1	7	2	2	7	2
KCG001	11422	Kimchi	11	1	1	8	1	6	7	2
OJ1	11432	Salted oyster	12	4	4	9	1	7	8	2
SF4	11447	Salted fish	13	5	2	10	2	8	9	2

KCCP Korean Culture Collection of Probiotics

2, ST-4 and ST-6, ST-7 were having 5 alleles identical out of 7, depicting around 71% similarity. In addition, it can be observed that the allelic frequency was dominated for alleles 1 and 2 compared to other alleles among the selected few gene loci for MLST analysis. For example, dominance in frequencies was observed in allele 1 for *atpA*, *dnaA*, and *gyrB* loci and in allele 2 for *gyrB* and *rpoA* loci (Table 1). The number of polymorphic sites varied

from 1 for *gyrB* to 22 for the *dnaK*, the most polymorphic locus, therefore, a total of 51 SNPs has been detected (Table 2). Out of 7, three loci namely *gyrB*, *rpoA*, and *dnaA* had low polymorphism showing 1, 2, and 3 polymorphic sites respectively. The apparent low levels of biodiversity in *gyrB*, *rpoA*, and *dnaA* suggested that the partial gene sequences of the loci were somewhat conserved amongst 13 strains, and have the lower

**Table 2** Primers used and nucleotide diversity observed within the *Leu. citreum* strains

Locus	Primer sequence (5'-3')	Length (bp)	Sequence used (bp)	Polymorphic sites	No. of alleles	G + C content	$\pi$ Value	Tajima's D value	$d_N/d_S$ ratio
<i>atpA</i>	F-GTTTTTCGAGCCATTACAA	590	567	7	5	40.74	0.00529	- 0.746820	0
	R-GTGATTGAGATAACGTTTG								
<i>dnaA</i>	F-CCAATTACAAAAGAGGAACTA	1261	1100	3	4	37.79	0.00151	0.167656	0
	R-TTATCTTGTTGGTTGCGTG								
<i>dnaK</i>	F-GAAGGTGGCGAACCACAAAA	1647	806	22	10	41.28	0.00896	- 0.337078	1.1951
	R-AGCCAATGCTTCTGTCTTAG								
<i>gyrB</i>	F-ATAAAGTCTCTGGTGGATT	1022	800	1	2	41.62	0.00067	1.475424	0
	R-TGGTAGGATAGCTTGTGTTA								
<i>pheS</i>	F-CACAACTTCACCTGT	286	264	5	8	41.71	0.00757	0.168432	0.3874
	R-ACGTCTACTTCAACTG								
<i>pyrG</i>	F-ACCGTGGCTTAAAATTGGC	415	398	11	9	39.06	0.01060	0.203116	0.1152
	R-TCACTGCCAAGATCTGATTCAT								
<i>rpoA</i>	F-CGTTGTTGAAGATGTCACAC	291	271	2	3	42.9	0.00113	- 1.468006	0.2459
	R-GCCAACACGCCGATAG								

$\pi$ , nucleotide diversity per site;  $d_N$ , number of nonsynonymous changes per nonsynonymous site;  $d_S$ , number of synonymous changes per synonymous site

discriminatory ability than the other housekeeping loci used in the study. The remaining four loci namely *atpA*, *dnaK*, *pheS*, and *pyrG* had more polymorphic sites (between 5 and 22), suggesting their good discriminatory ability. Fifty-one polymorphic sites were detected among 4206 nucleotides giving polymorphism rate of 1.21%. This value was higher than that observed for *Leu. lactis*, which showed 47 SNPs (0.88%) amongst 5325 nucleotides (Dan et al., 2014). Comparatively low number of polymorphic sites among seven housekeeping loci suggested that these are conserved amongst the 13 *Leu. citreum* isolates. The Tajima's D value (Table 2) ranged between  $-1.46$  to  $1.47$ , which supported the complete fitting of the population in the model system (Andreani et al., 2014) and since the values are between  $-2$  and  $2$  the evolution of these genes was likely driven by neutral selection. The mean GC content of the MLST gene fragments varied from the 37.79% for *dnaA* to 42.9% for *rpoA* (Table 2), which was compared with already reported GC content (38–44%) for the genus *Leuconostoc* (De Bruyne et al., 2007). The nucleotide diversity index ( $\pi$ ) ranging from the 0.00067 for *gyrB* to 0.01060 for *pyrG* (Table 2). It has been reported that the higher value of  $\pi$  is related to high intragenic nucleotide polymorphism (Cai et al., 2007). Similarly, in this study, the loci with high  $\pi$  value were represented with more polymorphic sites compared to the others. Analysis of synonymous substitutions to non-synonymous substitutions in the allele sequence of a locus can be used to determine whether the genes are under positive selection, therefore a ratio greater than 1 implies selection for amino acid changes. In this genetic analysis, six housekeeping loci had  $d_N/d_S$  ratio lower than 1. Even for three loci, i.e., *atpA*, *dnaA*, and *gyrB* the ratio was found equal to zero, where substitution was synonymous indicating that the amino acid composition of these genes did not change (Table 2). However, one exception was observed for locus *dnaK*, which had a  $d_N/d_S$  ratio,  $1.1951 > 1$ , this is not typically observed for housekeeping genes. In addition, a similar exception was also reported earlier in a study of *Leu. mesenteroides* using 9 housekeeping loci,  $d_N/d_S$  ratio varied from 0.0219 to 2.4379 and was found to be  $> 1$  only for one locus, i.e., *murC* (2.4379) (Zhang et al., 2015).

### Splits tree analysis

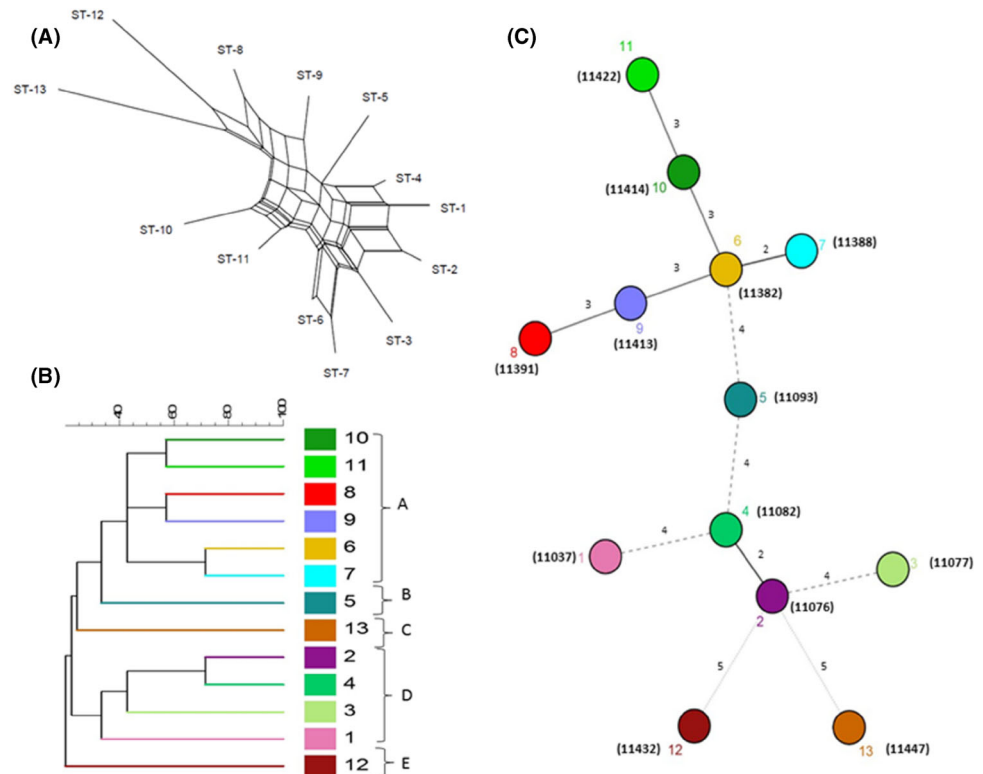
In order to further analyze the population structure of *Leu. citreum* we used split decomposition analysis. A tree-like structure is created when the descent is clonal, but an interconnecting network will appear when recombination plays a role in the evolutionary history of *Leu. citreum* genes. A combined split graph based on all alleles in the seven MLST loci displayed a network-like structure with different rays of length [Fig. 1(A)]. Parallelogram-shaped

groupings were observed suggesting their descents were originated from interspecies recombination events. It has been reported in the previous literature that usually in the genus *Leuconostoc* recombination could occur due to various mobile elements (transposable elements, genomic islands, and bacteriophages) in their genome sequences (Meslier et al., 2012). In addition, plasmid has also been identified from *Leu. citreum* (Chang and Chang, 2009). Furthermore, it can be observed from the figure that all the STs were showing some degree of relationship, except ST-12 and ST-13, which were distantly related to remaining 11 isolates.

### UPGMA tree analysis and minimum spanning tree

Genetic relatedness among *Leu. citreum* strains were investigated by constructing a UPGMA (Unweighted Pair Group Method with Arithmetic Mean) dendrogram [Fig. 1(B)]. As it can be observed from the figure, strains were showing the low degree of similarity. As shown in the figure, there were two major groups of the strains on the basis of similarity in their allelic profiles. Larger group A consisted of six strains (ST-6 to ST-11). Groups B and C were represented by ST-5 and ST-13, whereas group D consists of strains (ST-1 to ST-4). ST-6 and ST-7 from group A and ST-2 and ST-4 from group D were showing maximum similarity (71.4%) amongst 13 strains on the basis of 5 common alleles out of seven. Earlier, the use of other genotypic methods namely rep-PCR (Kaur et al., 2017c) and RAPD-PCR (Kaur et al., 2017b) have also been reported for the analysis of *Leu. citreum* using REP, ERIC, (GTG)<sub>5</sub> and 239, Kay3 primers respectively. In addition, minimum spanning tree (MST), an algorithm was also used to perform a phylogenetic analysis of the strains based on the allelic profiles. Usually, in MST, the size of the circle is proportional to the number of isolates with that unique profile. Nevertheless, as represented in the figure, the size of each circle is similar, as there were 13 circles of different color, representing one isolate each. Different circles were connected to each other by different lines representing the degree of relationship between *Leu. citreum* strains [Fig. 1(C)]. The numbers on the lines were depicting the difference in the alleles between two isolates. On the top of the MST, bold lines between strains (11391, 11382, 11388, 11413, 11414, and 11422) indicating a comparatively strong relationship. Among these strains, 2 (11382 and 11388) or 3 (11391, 11413, 11414, and 11422) alleles were found to be different from each other. These strains were isolated from three different sources namely, kimchi, salted small octopus, and young radish kimchi. Similarly, strains 11082 and 11076 were connected with each other with a bold line, both were isolated from kimchi, and showing a difference for two alleles out of seven. Moreover, the

**Fig. 1** (A) Combined split-decomposition analysis based on concatenated sequences of the seven MLST loci. The numbering in the figure refers to ST. (B) UPGMA dendrogram showing the genetic relationship between 13 STs belonging to *Leu. citreum*. The numbering in the figure refers to ST. (C) Minimum-spanning tree analysis of the 13 *Leu. citreum* strains from South Korea. Each circle relates to a sequence type (ST) and a line between isolates indicates the strength of the genetic relationship between these isolates (bold, strong relationship; solid dashed, intermediate relationship; very small dotted line, weak relationship)



strains 11037, 11093, and 11077 were connected with dashed lines to other strains showing they have 4 alleles different and isolated from salted small octopus, pear and kimchi respectively. Lastly, 11432, and 11447 strains isolated from salted oyster and salted fish were connected with dotted lines, showing a very weak relationship on the basis of alleles (maximum allelic difference). The strains were not clustered in the common circle, indicating variability at the genetic level. Comparable results were obtained with MST, UPGMA tree and concatenated splits tree analysis. However, despite the same isolation source for some of the STs, no strong relationship was observed, as can be seen from MST as well as allelic profiles also [Fig. 1(C)]. It can be observed from tree analysis that no appropriate association was found between ST and the source from which the bacteria were isolated (Table 1). Comparable results have been reported in *Leu. lactis*, where no significant associations between STs and the sources of the isolates could be found (Picozzi et al., 2010). The absence of such an association in *Leu. citreum* strains may be because of the genetic diversity of individual *Leu. citreum* strains. The variation in MLST data from one study to another could be because of different bacterial species (habitats), isolation sources and housekeeping genes selected for individual analysis.

In summary, the presented MLST scheme was found to be a useful tool for the typing of *Leu. citreum* strains and showed a sufficient discriminatory power to determine the

genetic variability. The protocol was followed for the structural analysis of 13 bacterial species, which were directed into thirteen sequence types. It was found that the evolution of different *Leuconostoc* strains was not related to respective food sources. For a better understanding of *Leu. citreum* from South Korea, a large number of strains must need to be evaluated which will further contribute details on the evolution and population genetics of the species. As MLST allows precise identification, and easy assessment, the future application of this molecular method could be useful for the identification of valuable *Leu. citreum* strains and nonpathogenic food production bacteria for their use in the food industry with a valuable application.

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#### Compliance with ethical standards

**Conflict of interest** The authors declare no conflict of interest.



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