DNA Profiling of *Leuconostoc citreum* Strains in Fermented Foods by Repetitive Element Polymerase Chain Reaction ^S

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To identify and discriminate the bacterial species at the subspecific level, rep-PCR is a reliable genomic fingerprinting tool. Fourteen strains of bacteria were isolated from different food sources, identified as *Leuconostoc citreum* using 16S rRNA gene sequencing, and amplified using rep-primers (REP, ERIC, and (GTG)₅). Fingerprinting patterns generated bands in the range of 300–6,000 bp with REP, 150–6,000 bp with ERIC, and 200–1,700 bp with (GTG)₅ primers. In UPGMA dendrogram analysis, 14 strains were clustered into three clades (I, II, and III) with all the primers, thus differentiating them at the molecular level. The present study revealed the differentiation of *L. citreum* strains using rep-PCR.

Keywords: *Leuconostoc citreum*, rep-PCR, ERIC-PCR, (GTG)₅-PCR

The genus *Leuconostoc* is a Gram-positive, non-motile, catalase-negative, facultative anaerobe with a relatively low GC content (37–45 mol%). It consists of 24 different species [1], an important one being *Leuconostoc citreum*, which has recently been established to have four genomic sequences [2]. Although genus *Leuconostoc* is found in human feces, vagina, and breast milk, it is not part of the human microbiota [3, 4]. However, it is extensively used in the production of cheese, butter, kimchi, sourdough, etc. [4, 5]. Along with other *Leuconostoc* spp., *L. citreum* provides the natural flavor and texture to kimchi (a Korean traditional dish) [6–8]. The classification and characterization of lactic acid bacteria (LAB) include phenotyping and genotyping. Compared with phenotypic methods, genotypic ones offer advantages such as general applicability and good discriminatory power [4, 9]. Various methods have been used for typing of bacteria, amongst which repetitive element polymerase chain reaction (rep-PCR) is considered as one of the most robust techniques with high discriminatory power to identify species [10–13] and provide unambiguous results [14]. It amplifies the repetitive intergenic elements of the DNA, which provide information on the genetic

diversity and population structure of the strain.

The objective of this study was to use rep-PCR for the characterization of South Korean *L. citreum* strains isolated from various food items. The 14 bacterial strains (Table 1) were cultured in MRS broth (Difco Laboratories, USA) at 37°C overnight. For DNA isolation and 16S rRNA gene amplification, the protocol used has been described previously [5]. The 16S rRNA gene sequencing results identified the colonies as *L. citreum* (Accession Nos. KX286339–KX286352), and the UPGMA dendrogram covering 16S rRNA gene sequencing analysis for the 14 *L. citreum* strains is shown in the supplementary material. The rep-PCR amplification was carried out as described previously [15] with REP-PCR (primer sets Rep1R-I and Rep2-I), ERIC-PCR (primer sets ERIC1R and ERIC2), and (GTG)₅-PCR (primer (GTG)₅) in a Bio-Rad thermal cycler (Bio-Rad Laboratories, USA) using 35 ng of template DNA and 10 mM concentration of primers (synthesized by Macrogen Inc., Korea). The amplified DNA fragments were separated by electrophoresis on a 1.5% agarose gel [16] with 100 bp and 1 kb DNA ladders (TaKaRa Bio. Inc., Japan) as the size markers. The gel imaging was done with a gel documentation system (Gel Doc XR⁺; Bio-

Rad Laboratories). Finally, the bands were scored 1 for the presence and 0 in the absence, and the dendrogram was

Table 1. Details of *Leu. citreum* strains from Korea.

Strain	Area/locality	Source
11001	Seongnam / Korea	Sweet pumpkin
11037	Seongnam / Korea	Salted small octopus
11076	Seongnam / Korea	Kimchi
11077	Seongnam / Korea	Kimchi
11082	Seongnam / Korea	Kimchi
11093	Seongnam / Korea	Pear
11382	Seongnam / Korea	Salted small octopus
11388	Seongnam / Korea	Young reddish kimchi
11391	Seongnam / Korea	Salted small octopus
11413	Seongnam / Korea	Kimchi
11414	Seongnam / Korea	Kimchi
11422	Seongnam / Korea	Kimchi
11432	Seongnam / Korea	Salted oyster
11447	Seongnam / Korea	Salted fish

constructed with unweighted pair-group method with arithmetic mean method using Numerical Taxonomy System software [17].

The band patterns generated by the three primers were different and contrasting. In addition, phylogenetic trees verified the results obtained from the banding profiles of each strain. The strains were divided into three clades (I, II, and III) with all the primers. Most of the strains showed $\geq 40\%$, $>60\%$, and $>20\%$ similarity with REP-PCR, (GTG)₅-PCR, and ERIC-PCR, respectively.

For REP-PCR, 31 distinct fragments with 191 bands in total were observed with a band range of 300–6,000 bp (Fig. 1A). A maximum of 17 bands was found for strain 11082 (kimchi), and a minimum of 10 were found for strain 11001 (sweet pumpkin). However, REP-PCR showed the presence of 1,050 and 300 bp bands for all 14 strains. A unique fingerprinting pattern was revealed for strain 11001, with the presence of bands at 100, 1,400, 2,200, and 5,000 bp that were not seen in any other sample, along with the absence of bands at 490, 590, and 1,200 bp (Fig. 1A). There

Fig. 1. (A) REP-PCR fingerprint profiles and (B) UPGMA dendrogram showing the different cluster divisions of 14 strains of *Leuconostoc citreum* isolated from food sources.

Lane M1: Marker 1 kb; Lane M2: Marker 100 bp; Lane 1: 11001; Lane 2: 11037; Lane 3: 11076; Lane 4: 11077; Lane 5: 11082; Lane 6: 11093; Lane 7: 11382; Lane 8: 11388; Lane 9: 11391; Lane 10: 11413; Lane 11: 11414; Lane 12: 11422; Lane 13: 11432; Lane 14: 11447.

Fig. 2. (A) ERIC-PCR fingerprint profiles and (B) UPGMA dendrogram showing the different cluster divisions of 14 strains of *Leuconostoc citreum* isolated from food sources.

Lane M1: Marker 1 kb; Lane M2: Marker 100 bp; Lane 1: 11001; Lane 2: 11037; Lane 3: 11076; Lane 4: 11077; Lane 5: 11082; Lane 6: 11093; Lane 7: 11382; Lane 8: 11388; Lane 9: 11391; Lane 10: 11413; Lane 11: 11414; Lane 12: 11422; Lane 13: 11432; Lane 14: 11447.

were other uncommon bands observed in various strains that were not shared with the rest of them. Apart from this, the 1,100 bp band was found absent in strains 11001 and 11388. Interestingly, strain 11001 was seen to be distinguished in the other two cases as well. Phylogenetically, out of the three, clade III of REP-PCR contained strains 11077 and 11414 (Fig. 1B), which were closely related and isolated from a similar food source (*i.e.*, kimchi), whereas strains 11391 and 11413 were found to be similar but were isolated from salted small octopus and kimchi, respectively.

However, ERIC primers generated 25 fragments with 110 bands in a range of 150–6,000 bp (Fig. 2A). In contrast to the REP primer results, a maximum of 13 bands were obtained from strain 11001 with bands of 1,200, 2,900, 5,000, and 6,000 bp, which were absent in the remaining strains. Moreover, the band at 1,000 bp was reported for all strains except for 11001. Phylogenetically, strain 11382 (salted small octopus) was categorized into clade III and showed a strong relationship with strain 11388 (young

radish kimchi) (Fig. 2B). It can be stated that strain 11382 had genetic relatedness with the strains isolated from kimchi.

The (GTG)₅ primers showed 20 distinct fragments with a total of 167 bands in a range of 200–1,700 bp (Fig. 3A). Nevertheless, the highest number of bands (15) was found in strains 11082 (kimchi) and 11388 (young radish kimchi), and the minimum (9 bands) was seen in 11422 (kimchi). Bands of 450, 700, and 1,300 bp were observed for all the strains. Furthermore, bands of 200, 600, and 1,100 bp were reported in all strains except 11001, whereas a 250 bp band was only observed in strain 11001.

Unlike REP-PCR and ERIC-PCR, the phylogenetic analysis with (GTG)₅-PCR revealed that from clade II, strains 11076 and 11077 (both sourced from kimchi) were identical, which were accompanied by strains 11391 and 11447 (isolated from salted small octopus and salted fish, respectively) that exhibited similarity (Fig. 3B). Interestingly, strain 11001 (sweet pumpkin) was represented in a single clade in all three PCR cases, suggesting that it carried a unique and distinctive profile from that of the other strains under study. However, unlike REP-PCR and (GTG)₅-PCR, there was no band common to all strains in ERIC-PCR, but unique bands of 4,500 and 550 bp were observed in strains, 11076 and 11082, respectively.

The different profiles generated can be attributed to the dispersed repetitive sequences in the canonical sequence [12, 18, 19]. Less similarity (more than 50%) was observed among the strains from the same source (*i.e.*, kimchi) and only a few were observed to be similar, which is supported by a study based on *Leuconostoc* strains from sourdough with distinct rep-PCR profiles [20]. Moreover, the differences may arise owing to the effect of the environment (home or industry) provided for kimchi fermentation. In this study, REP-PCR produced complex fingerprint banding patterns, followed by ERIC and then (GTG)₅. This is in contrast to previous studies where the (GTG)₅ primer was found to be the most suitable for identifying *Leuconostoc* species [20, 21] and other LAB [10, 16, 22, 23]. However, several studies have revealed distinctive results with different primers. Comparable profiles were seen with REP, BOX, and (GTG)₅ primers in a study based on the identification of probiotic strains [23], whereas ERIC-PCR was found to generate more complex patterns than REP-PCR in differentiating *Lactobacillus johnsonii* strains [9]. Even the less complex patterns make it easier to analyze the results [24,25], which helped toward understanding the genetic relatedness among the strains [16, 26, 27]. Overall, the ability of rep-PCR to show variations at the strain level proved it to be a reliable technique in the characterization of Gram-positive LAB.

Fig. 3. (A) (GTG)₅-PCR fingerprint profiles and (B) UPGMA dendrogram showing the different cluster divisions of 14 strains of *Leuconostoc citreum* isolated from food sources.

Lane M: Marker 1 kb; Lane 1: 11001; Lane 2: 11037; Lane 3: 11076; Lane 4: 11077; Lane 5: 11082; Lane 6: 11093; Lane 7: 11382; Lane 8: 11388; Lane 9: 11391; Lane 10: 11413; Lane 11: 11414; Lane 12: 11422; Lane 13: 11432; Lane 14: 11447.

Rep-PCR is an easy, rapid, reliable, and reproducible molecular typing method with a practical approach. To our best knowledge, there is no other study that has differentiated *L. citreum* strains only by comparative analysis with rep-PCR.

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