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RESEARCH ARTICLE

Diversity Analysis of Lactic Acid Bacteria in Korean Rice Wines by Culture-independent Method Using PCR-denaturing Gradient Gel Electrophoresis

So-Young Kim, Ki-Seon Yoo, Ji Eun Kim, Ji-Sun Kim, Jee Yun Jung, Qing Jin, Hyun-Ju Eom, and Nam Soo Han

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Abstract A polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) method was used to determine the presence and diversity of lactic acid bacteria (LAB) in *takju,* a traditional Korean rice wine. Bacterial DNAs were extracted from 15 commercial rice wines and amplicons of partial 16S rRNA genes were separated by DGGE and intense bands were sequenced. *Lactobacillus* (*Lb.*) *paracasei*, *Lb. plantarum*, and *Leuconostoc pseudomesenteroides* were detected in all samples and *Lb. harbinensis* and *Lb. parabuchneri* were found with above 80% frequency of occurrence. Unknown species of *Lactobacillus* were also widely detected. This result revealed that, regardless of products and raw materials, the distribution profiles of LAB in *takju* products have a common pattern comprising of above predominant species and, furthermore, *takju* can be regarded as a LAB-rich fermented food providing various probiotics.

Keywords: 16S rRNA gene, lactic acid bacteria, polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE), *takju*

Ki-Seon Yoo, Ji Eun Kim, Ji-Sun Kim, Jee Yun Jung, Hyun-Ju Eom, Nam Soo Han (✉)
Department of Food Science and Technology, Research Center for Bioresource and Health, Chungbuk National University, Cheongju, Chungbuk 361-763, Korea
Tel: +82-43-261-2567 Fax: +82-43-271-4412
E-mail: namsoo@chungbuk.ac.kr

So-Young Kim
Food Nutrition and Functionality Division, Department of Agrofood Resources, NAAS, RDA, Suwon, Gyeonggi 441-863, Korea

Qing Jin
Department of Food Science, Yanbian University, Longjing, Jilin 133-400, PR China

Introduction

Takju is a Korean traditional rice wine brewed by using rice and *nuruk* as ingredients, which has milky and turbid color due to the absence of clarification process after the fermentation for 4-7 days (in case of filtered one, it is named as *yakju*). In addition to the alcoholic fermentation of yeast, the brewing process of *takju* involves the use of *nuruk*, a starter culture made from wheat flour and grits (1, 2). Traditionally, the *nuruk* is made by spontaneous solid fermentation, allowing the growth of fungi, yeasts, and bacteria inoculated in nature. In *nuruk*, the fungi such as *Rhizopus* sp., *Mucor* sp., and *Aspergillus* sp., *Saccharomyces cerevisiae*, *Bacillus subtilis*, and various lactic acid bacteria (LAB) were previously reported (3). During *takju* fermentation, fungi, *B. subtilis*, and LAB secret various amylolytic enzymes for saccharification process converting starch into fermentable sugars, and subsequently yeast conducts alcoholic fermentation using sugar to produce ethanol and carbon dioxide (4). At the end of alcohol fermentation, the ethanol concentration usually reaches above 15%(v/v) and the *takju* products are distributed after dilution to about 6%(v/v). Throughout the above periods, LAB conducts acid fermentation to accumulate various organic acids so that *takju* gives a carbonated sourish-sweet taste like a milk punch. However, contrary to this key role of LAB in *takju* fermentation, they have not led attentions of researchers. For investigation of their roles and functional mechanisms on the quality of *takju*, ecological study of the bacterial population should be performed by using reliable and efficient methods.

Recent researches have focused on the application of culture-independent methods such as polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) for more rapid and reliable identification of the

microbial species in foods (4-7). This method is more frequently applied to detect almost unknown bacterial species involving in strict anaerobes and non-cultivable microbes by specific amplification of bacterial 16S rRNA gene sequences (8-10) or monitoring of the dynamic changes of bacterial community in foods, soil, sea, river, and lake waters, and gastrointestinal tract (11,12). The V3 region of 16S rRNA gene has been the most popular target to start investigating an unknown and complicated bacterial population (13).

In this study, diversity analysis of LAB flora were conducted in 15 *takju* products by employing PCR-DGGE method with a universal 338f-GC and 518r primer pair for the V3 region of 16S rRNA gene for better understanding of bacterial diversity in *takju*.

Materials and Methods

***Takju* products** The commercial *takju* products that were made of rice and/or flour, after purchasing from local markets, were used to investigate LAB population. The pHs and total acidity of *takju* were determined. The microbial cells in the *takju* samples were harvested by centrifugation at 10,000×g for 10 min. After discarding the supernatants, the solid fractions were resuspended in DNA extraction TE buffer containing 100 mM Tris-HCl and 40 mM ethylenediamine tetraacetic acid (EDTA) at room temperature and stored at −80°C until use.

Bacterial strains and growth conditions The reference strains used in this study were *Lactobacillus* (*Lb.*) *plantarum* KCTC 3104, *Oenococcus oeni* KCTC 3091, *Lb. fermentum* KCTC 3112, *Leuconostoc* (*Leuc.*) *citreum* KACC 91035, *Leuc. pseudomesenteroides* KCTC 3652, *Lb. harbinensis* KCTC 13106, *Lb. casei* KCTC 3109, and *Lb. paracasei* KCTC 13076. All strains were grown on MRS agar (Difco, Sparks, MD, USA). *O. oeni* KCTC 3091 was cultivated on modified MRS agar supplemented with 20%(v/v) tomato juice (pH 4.8) under the anaerobic conditions by using AnaeroPouch® System (Mitsubishi Gas Chemical Company, Inc., Tokyo, Japan) at 37°C for 3 days.

Genomic DNA extraction and PCR amplification The genomic DNA extraction from microbial cells and the reference strains was performed using Genomic DNA Prep kit for bacteria (SolGent, Daejeon, Korea) according to the manufacturer's instruction. Total DNAs extracted from cells were used as template for PCR and the 16S rRNA gene V3 regions of them were amplified using the bacterial universal primers 338f and 518r (14). The sequences of the primers were as follows: 338f, 5'-ACTCCTACGGGAGG CAGCAG-3' (*Escherichia coli* positions 338 to 357) and 518r, 5'-ATTACCGCGGCTGCTGG-3' (*E. coli* positions 518 to 534) (1,13). The forward primer, 338f had a GC clamp sequence at the 5' end: CGCCCGCCGCGCGCGG CGGGCGGGGCGGGGGCACGGGGGG. PCR mixtures were prepared with a 2× master mix (Uni Tap PLUS version, Bioprogen, Daejeon, Korea), 2.5 pmol of each primer and 1 μL of genomic DNA template, in a final volume of 25 μL. After initial denaturing at 94°C for 5 min, 30 cycles of PCRs were performed at 94°C for 1 min, at 52°C for 1 min, and at 72°C for 1 min. The cycling was concluded with a final extension step of 10 min at 72°C. The PCR products were confirmed by electrophoresis in 1%(v/v) agarose gel before they were applied to DGGE (5).

DGGE analysis PCR-amplified 16S rRNA V3 regions were analyzed by DGGE with a DCode system (Bio-Rad, Hercules, CA, USA) (8). The PCR products were loaded onto 8%(v/v) polyacrylamide (37.5:1 acrylamide-bisacrylamide) gels in 1× TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, Bio-Rad). Parallel electrophoresis experiments were performed at 60°C by using gels containing 30 to 60%(v/v) urea: formamide denaturing gradient [100%(v/v) corresponding to 7 M urea and 40%(v/v) formamide] increasing in the direction of electrophoresis. The gels were electrophoresed for 30 min at 20 V and for 15 hr at 60 V, stained with ethidium bromide for 30 min and photographed under ultraviolet (UV) illumination (14,15).

Sequencing of DNA bands and phylogenic analysis The gel bands were excised in 20 μL of TE buffer, overnight and the DNA was recovered (6). For DNA sequence analysis, PCR re-amplification was performed with the same primers (without GC-clamp). The re-amplified PCR products were purified with DNA purification kit (SolGent) and sequenced using the BigDye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA). The 16S rRNA sequences were compared with those from the DDBJ nucleotide sequence database (www.ddbj.nig.jp) using the program FASTA.

Phylogenetic analysis of partial 16S rRNA gene sequences was performed using the Clustal W program in the DDBJ database. The phylogenetic tree was made by using a neighbor-joining method in the Phylip 3.6 program (16). The sequences obtained in FASTA format were compared with those deposited in the DDBJ database using the basic BLAST search tools (17) and were assigned the accession number.

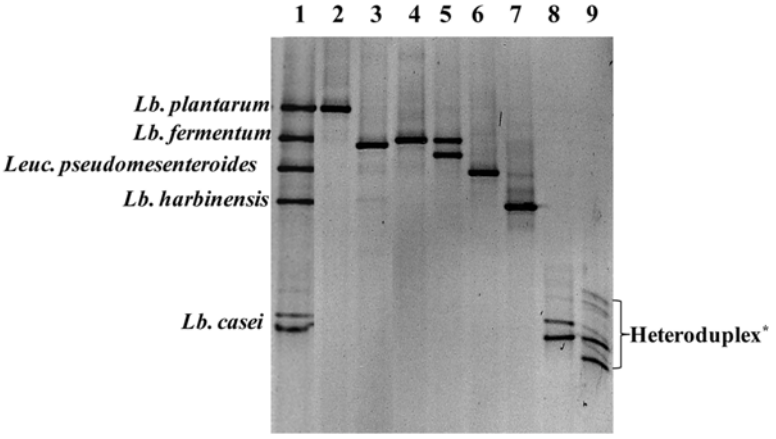

Fig. 1. PCR-DGGE profiles of 16S V3 rDNA sequences among the reference strains using a 338f-GC clamp and 518r primer pair. Lanes: 1, a ladder consisting of reference strains, 2, *Lactobacillus plantarum* KCTC 3104; 3, *Oenococcus oeni* KCTC 3091; 4, *Lb. fermentum* KCTC 3112, 5, *Leuconostoc citreum* KACC 91035; 6, *Leuc. pseudomesenteroides* KCTC 3652; 7, *Lb. harbinensis* KCTC 13106; 8, *Lb. casei* KCTC 3109; 9, *Lb. paracasei* KCTC 13076. *Heteroduplex bands formed in the 16S rRNA gene amplification

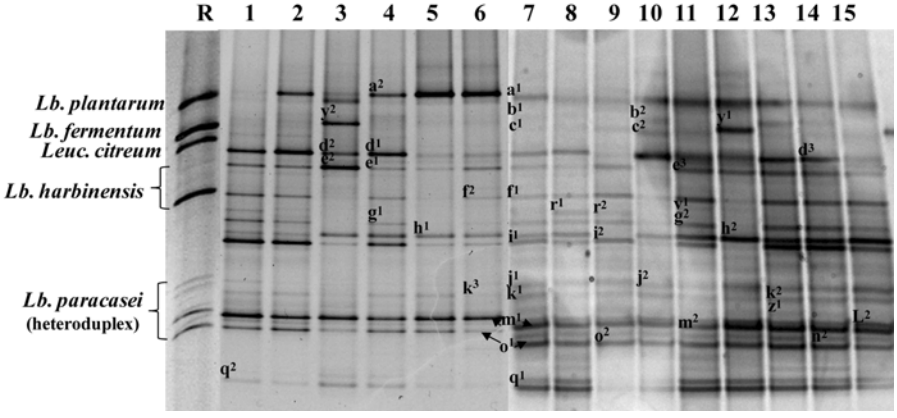

Fig. 2. PCR-DGGE profiles of 16S V3 rDNA sequences among *takju* samples (*n*=15) using a 338f-GC clamp and 518r primer pair. Sequences of the excised fragments indicated as a lowercase letter are listed in Table 1. Numbers of the same band refer to repeated band sequencing at 3 times.

Results and Discussion

Identification of LAB in *takju* by PCR-DGGE The pHs of all *takju* samples used in this study were in between 3.4 and 3.9. The total acidities were also shown in the values of 0.89 to 1.0 and all samples had the alcohol content of 6.0 to 7.5%(v/v).

As shown in Fig. 1 and 2, when PCR-DGGE was performed, amplicons of each sample (T1 to T15) and 5 reference strains were migrated with different distances and, by matching the band distances, species-identification of DNA fragments in samples was accomplished. A number of visible DNA bands were generated from the *takju* samples and their numbers were between 11 to 17. The general DGGE profiles obtained from 15 commercial *takju* were similar, with bands *a, d, e, f, g, m,* and *o* being the most intense. In comparison of the bands with those of reference strains, *a, d, f, m,* and *y* were matched to *Lb. plantarum, Leuc. pseudomesenteroides, Lb. harbinensis, Lb. paracasei,* and *Lb. fermentum*, respectively. For band *m*, *Lb. casei* and *Lb. paracasei* shared a common band at the position but the occurrence of *o* band proved the existence of *Lb. paracasei*. When the occurrence of each band was compared, bands *a, d, k, m,* and *o* were present in all samples revealing their ubiquitous existence in *takju* products. Meanwhile, bands *e, f, g, i, j, l, n,* and *p* were present in most samples except 2 or 3 cases, and bands *b, c, r, y,* and *z* only existed in 2 to 5 samples, revealing their product-dependent existence.

The results indicated that 3 species of *Lb. plantarum*, *Leuc. pseudomesenteroides*, and *Lb. paracasei* were present in all *takju* products. They were also found in *moto* and *moromi* prepared by inoculation of rice with *koji* in the production of *sake* (Japanese rice wine) and *takju* as previously described (18-22). In case of *Lb. plantarum*, it is a widespread LAB commonly found in many fermented

Table 1. Species-identification of the fragments excised from PCR-DGGE by 16S rDNA sequencing[1)]

Bands	Samples no.	Species-identification	Identity (%)	Accession no.
a	7	*Lactobacillus plantarum*	100	AB360552
b	10	*Lactobacillus* sp. (unknowm)	97.9	AB360555
c	7	*Lactobacillus* sp. (unknowm)	97.5	AB360556
d	14	*Leuconostoc pseudomesenteroides*	100	AB360559
e, f, v	3	*Lactobacillus harbinensis*	100-98.5	AB360561
g	11	*Lactobacillus* sp. (unknowm)	97.9	AB361046
h*	12	Zea mays chloroplast	98.3	AB360582
i	9	*Lactobacillus parabuchneri*	100	AB360564
j, k, l, m, n, o, z	10	*Lactobacillus paracasei*	100	AB360566
q*	7	Zea mays subsp. mays mitochondrion	98.9	AB360581
r	8	*Lactobacillus* sp. (unknowm)	97.9	AB360577
y	12	*Lactobacillus fermentum*	100	AB360578

[1)]Identities are obtained from the closest sequences to compare with the databases in DDBJ or GenBank; *Commonly called to sweet corn (or maize)

Table 2. LAB species found in *takju* products by both culture-dependent (23) and culture-independent methods (this study)

Species-identification	Culture-dependent			Culture-independent		
		No. of detected samples	Distribution[1)] (%)		No. of detected samples	Distribution (%)
Leuconostoc pseudomesenteroides	-	nt	ND	+	15	100
Lactobacillus paracasei	+	14	93	+	15	100
Lactobacillus arizonensis	+	13	87	-	ND	ND
Lactobacillus platarum	+	3	27	+	15	100
Lactobacillus harbinensis	+	3	20	+	13	87
Lactobacillus parabuchneri	+	2	13	+	13	87
Lactobacillus brevis	+	1	7	-	ND	ND
Lactobacillus hilgardii	+	1	7	-	ND	ND
Lactobacillus fermentum	-	ND	ND	+	3	20

[1)]Distribution (%)=sample number with the corresponding species/total number of subjects (n=15)×100; ND, not detected

food products as well as plant materials. Recently this species was divided into *Lb. plantarum* and *Lb. arizonensis.* In our previous report, we detected these 2 species by culture-dependent approach, such as sorting by whole-cell protein patterns using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis (23). On the other hand, PCR-DGGE fingerprinting technique was unable to distinguish the PCR amplicons corresponding to *Lb. plantarum* and *Lb. arizonensis*, because they have very close relationship in 16S rRNA gene sequences. Our data support the suggestion of Swezey *et al.* (24) that *Lb. arizonensis* represents a new species, based on biochemical characteristics. Further biochemical analyses are needed to distinguish these species from *Lb. plantarum* group. As seen in Table 2, it is interesting to find the occurrence of *Leuc. pseudomesenteroides,* which was not detected in the culture-dependent method. This result indicates that this strain may be active in the early stage of fermentation, but cannot survive for long periods in *takju* because of the inhibitory activities of the alcohols or acids produced during the fermentation. The genus *Leuconostoc* is a widespread bacterium in LAB, commonly found in plant-based fermented foods such as *kimchi* (4,16) and sauerkraut (7). *Lb. fermentum* was also detected with low possibility of 20%, which was not found in the culture-dependent method. *O. oeni* is frequently detected as an alcohol-tolerant LAB responsible for malolactic fermentation and only found in grape wine, must, and winemaking (25, 26). During *takju* fermentation, the amylolytic LAB may be involved because they can directly produce lactic acid from starch (27,28). According to Sanoja *et al.* (29), amylase activity was exhibited by various *Lactobacillus* spp. such as *Lb. plantarum*, *Lb. cellobiosus, Lb. amylovorus, Lb. amylophilus,* and *Lb. amylolyticus*.

Species-identification by 16S rDNA sequencing As shown in Fig. 1, 2 and Table 1, we confirmed that 10 bacterial species contained both matched and unmatched bands compared with those of the reference strains using partial 16S rDNA sequencing. The sequences exhibited greater than 97.5% identity with sequences in database. Noticeably, as seen in the previous analysis using references,

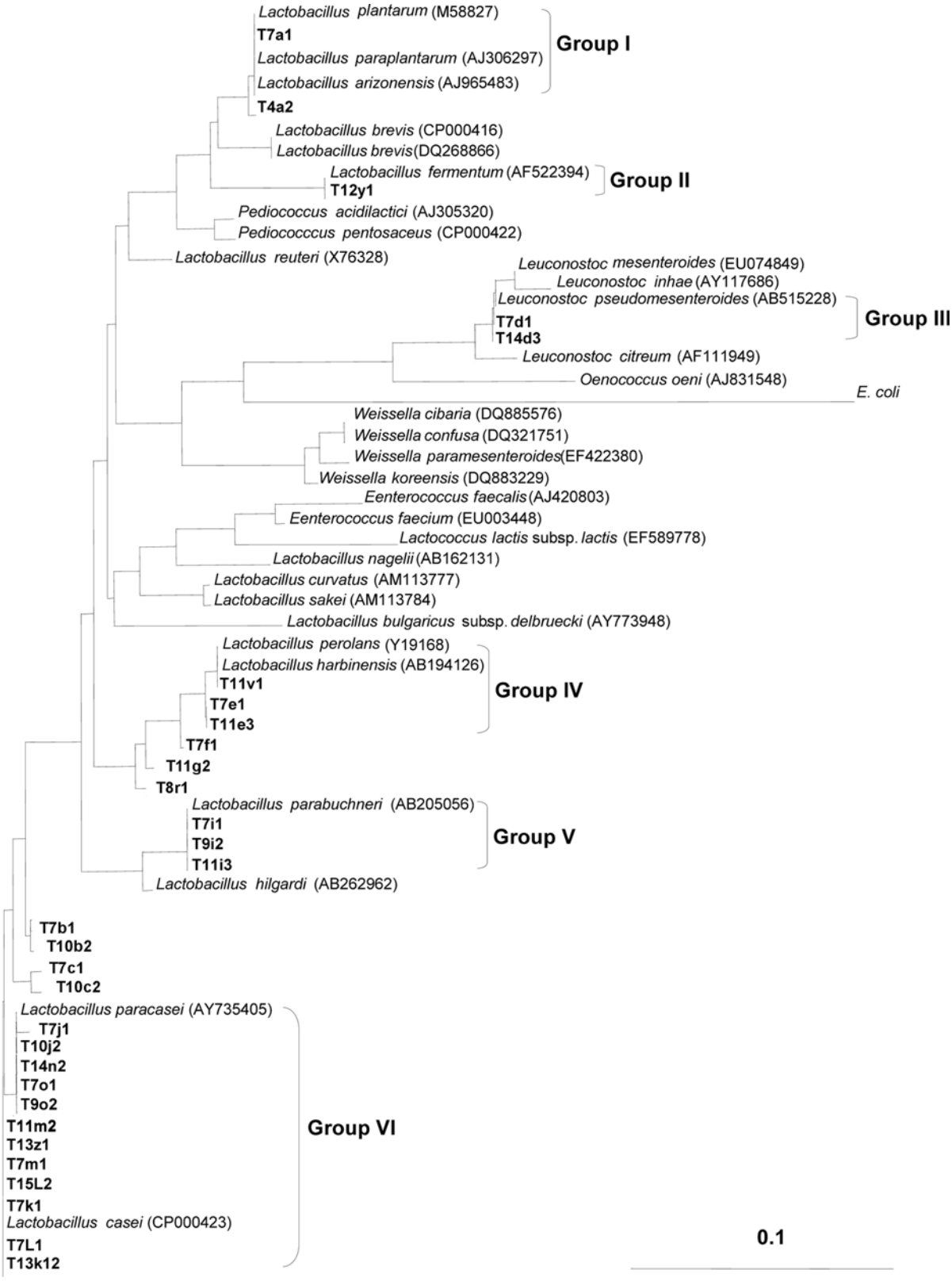

Fig. 3. Phylogenetic tree generated from the neighbor-joining method of 16S V3 rRNA sequences (ca. 197 bp) derived by PCR with a primer set (338f and 518r). Six bacterial groups and unknown bacteria (grey color) are indicated: I, *Lactobacillus* (*Lb.*) *plantarum* group; II, *Lb. fermentum* group; III, *Leuconostoc pseudomesenteroides* group; IV, *Lb. harbinensis*; V, *Lb. parabuchneri* group; VI, *Lb. paracasei* group. *E. coli* was used as an outgroup. Scale bar represents (calculated) distance. Reference strains are closely related to the isolates from the fermented foods. Accession number of each gene is indicated in the parenthesis. Band number isolated from *takju* samples are indicated by bold letters as 'T7a1'.

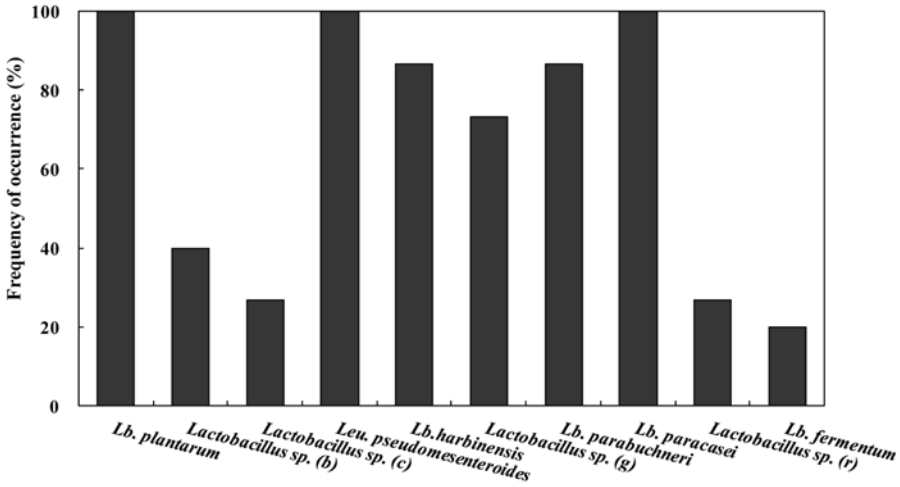

Fig. 4. Frequencies of occurrence of the lactic acid bacteria in rice wines (*n*=15) by the analysis of DNA fragments amplified using 338f and 518r primers.

the bands of *a, d, f, m,* and *y* were confirmed as those of *Lb. plantarum, Leuc. pseudomesenteroides*, *Lb. harbinensis, Lb. paracasei,* and *Lb. fermentum*, respectively. On the other hand, the bands of *j, k, l, m, n, o,* and *z* indicated *Lb. casei* or *Lb. paracasei* with 100% identity levels. In addition, the bands of *e, f*, and *v* pointed *Lb. harbinensis* and *i* was *Lb. parabuchneri*. In case of bands *b, c, g,* and *r*, they were corresponded to *Lactobacillus* genus but species-identification was not made due to their low identity levels (97.5-97.9%). Although the bands of *h* and *q* were found in DGGE profiles, they were identified as a chloroplast DNA of plant.

Phylogenetic analysis As shown in Fig. 3, the strains identified in PCR-DGGE analysis were classified into 6 bacterial groups. Five groups belonged to the genus *Lactobacillus* and 1 did to *Leuconostoc,* as follows: *Lb. plantarum* group (I), *Lb. fermentum* group (II), *Leuc. pseudomesenteroides* group (III), *Lb. perolans/harbinensis* group (IV), *Lb. parabuchneri* group (V), and *Lb. casei/paracasei/rhamnosus* group (VI). The strains in group I, II, III, V, and VI were corresponded to each of type strain showing close matches between nucleotide sequences. However, the DNA bands *b* (T7b1 and T10b2 in grey), *c* (T7c1 and T10c2 in grey), *g* (T11g2 in grey), and *r* (T8r1 in grey) with lower identity-levels in the previous analysis were not included to any pre-defined group and this result implicated a high possibility of occurrence of novel strains in Korean rice wines.

Bacterial populations in *takju* Based on culture-independent method using PCR-DGGE, distribution of microbial populations in 15 *takju* products was presented in Fig. 4 with their frequency of occurrences. Three species of *Lb. plantarum*, *Leuc. pseudomesenteroides,* and *Lb. paracasei* were present in all *takju* products and another species of *Lb. harbinensis* and *Lb. parabuchneri* were with above 70% frequency of occurrence, revealing their dominance in *takju* fermentation regardless of products. *Lb. fermentum* was also detected with low possibility of 20%. Other frequently detected unknown strains belong to the genus *Lactobacillus* and these strains might be a novel one existing broadly in Korean rice wines. This result implicated that the microbial diversity pattern of *takju* was comparable to that of Japanese rice wine, where *nuruk* and *koji* were used for *takju* and *moromi* (main culture for *sake*), respectively (19). *Nuruk* is usually made by mixed cultures of mold, bacteria, and yeast, while *koji* is cultured with a specific mold (*Aspergillus* sp.). Accordingly, it is regarded that two types of rice wines share a similar LAB composition, despite the difference between two inoculants.

As indicated in Table 2, the results of our culture-independent study support the results of culture-dependent analysis reported from Jin *et al*. (23) and PCR-DGGE method could rapidly identify 3 dominant LAB species in Koreans rice wines. Notably, an interest on the development and use of culture-independent techniques to investigate microbial population has been increasing, because the classical microbiological techniques could not accurately detect total microbial diversity (30,31). Although the heteroduplex formation such as *Lb. paracasei* limits the analytical potential of PCR-DGGE method by leading to an overestimation of the bacterial diversity (32), PCR-DGGE method is to be a preferred tool for rapid analysis of microbial community in a mixed microbial system, at least in the early stage of investigation. Accordingly, in this study, we could confirm the bacterial species regardless of cell survival based on culture-independent method. Besides, this result revealed that, distribution of LAB in *takju* had a common pattern with predominant species

regardless of products, even though starters were not inoculated and raw materials were not homogeneous.

In conclusion, by employing PCR-DGGE method, we elucidated the diversity of lactic acid bacterial population in Korean rice wines and we found the existence of various probiotic LAB in *takju*. For the understanding of their probiotic roles in *takju*, further investigation should be performed.

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