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Characterization of Isolated *Lactobacillus* spp. and Classification by RAPD-PCR Analysis

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The genetic relationships of six *Lactobacillus* strains and five laboratory isolates from fermented milk were determined by a random amplified polymorphic DNA (RAPD)-Polymease chan reaction (PCR) method. With 42 random primers, the results were analyzed by using the NTSYS-PC software for phenetic analysis. It revealed that all tested bacteria were divided into three distinct clusters. The clusters implied three subgenuses existed for the genus *Lactobacillus*, which were previously proposed by Rogosa and Sharpe. From the results, it was also possible to determine that the isolated *Lactobacillus* strains from fermented milk were grouped into *L. acidophilus* or *L. bulgaricus*. Interestingly, the three tested *L. casei* strains were divided into different clusters implying different subgenuses, i.e., *Thermobacterium* (*L. casei* YIT 9018) and *Streptobacterium* (*L. casei* CHR. Hansen and *L. casei* ATCC 4646). According to the distance matrix generated by an UPGMA program, the isolated bacteria LT01 and LT02 were determined as a subspecies of *L. bulgaricus*. The HK01, HK02 and HK03 were very closely related to either *L. acidophilus* or *L. casei* YIT 9018. Hence, RAPD-PCR appears to be a very practical method to determine the genetic relationships of the *Lactobacillus* species and to characterize the unknown *Lactobacillus* strains at the subspecies level.

Key words: Classification, Lactic acid bacteria, *Lactobacillus,* RAPD-PCR

Lactic acid bacteria (LAB) are Gram positive bacteria showing a DNA G+C content of less than 50 mol% (15, 23) and organisms of interest in the food processing industries because of their typical roles in inhibiting the growth of food spoilage bacteria (20). Among the organisms, the genus *Lactobacillus* contains many commercially useful species for food industries (1, 6) but it consists of more than 64 species (15). According to a proposal by Rogosa and Sharpe (16), the genus could be divided into three subgenuses such as *Thermobacterium, Streptobacterium* and *Betabacterium,* that was determined by their physiological and biochemical characteristics.

Since the classification of the genus *Lactobacillus* remains uncertain (19) and it consists of genetically heterogeneous strains which are difficult to classify by physiological and biochemical tests (5), classification of the genus *Lactobacillus* is a complex task. Thus, many LAB taxonomists recently attempted to determine its phylogenetic relationship at molecular levels including DNA-DNA hybridization with mol% G+C (4), restriction fragment length polymorphism (RFLP) (21) and mostly 16S rRNA sequence analysis (2, 3, 20). From their results, it was concluded that the classical taxonomy for LAB was not close to its molecular taxonomy.

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Among the molecular methods, RAPD-PCR is not a well understood method in the direct use of classifying the *Lactobacillus* species although it is known to be a PCR based DNA fingerprinting method (12, 25, 26). However, the method is now successfully used in classifying and determining genetic relationships of many organisms such as fungi (13, 27), viruses (9) and some *Lactobacillus* species (5, 11, 22, 24).

In this study, we attempted to determine the use of RAPD-PCR as an effective tool for classifying the *Lactobacillus* species (including three *L. casei* strains) using five laboratory isolates from fermented milk of two milk processing companies. The results of this attempt were compared with data from a traditional method (i.e., fermentative characteristics) for *Lactobacillus* classification. Simultaneously, we attempted to establish a systemic order of the tested *Lactobacillus* species and the isolated *Lactobacillus* strains by computer analysis using an UPGMA program from the NTSYS-PC software package (Exter software, USA).

Materials and Methods

Bacterial strains

The strains of *Lactobacillus* species used in this study are listed in Table 1. Among them, *L. acidophilus, L. brevis* (full name, *L. brevis* subsp. *brevis*) and *L. bulgaricus* (new

Table 1. List of *Lactobacillus* strains and isolates used in this study

Strains	Source	Subgenus[2)]
L. casei CHR. Hansen	HYI[1)]	*Streptobacterium*
L. casei ATCC 4646	HYI[1)]	*Streptobacterium*
L. casei YIT 9018	HYI[1)]	*Streptobacterium*
L. brevis	KCTC 3102	*Betabacterium*
L. acidophilus	KCTC 3173	*Thermobacterium*
L. bulgaricus	KCTC 3188	*Thermobacterium*
HK01	commercial	N.D.[3)]
HK02	commercial	N.D.[3)]
HK03	commercial	N.D.[3)]
LT01	commercial	N.D.[3)]
LT02	commercial	N.D.[3)]

[1)] Hankuk Yakult Institute
[2)] Based on a proposal of Rogosa and Sharpe (16)
[3)] Not determined and isolated from commercial yoghurt (liquid-type)

name, *L. delbrueckii* subsp. *bulgaricus*) were obtained from the Korean Collection for Type Cultures (KCTC), Genetic Resources Center, Daegeon, Korea. *L. casei* ATCC 4646, *L. casei* CHR. Hansen and *L. casei* YIT 9018 were kindly provided by Dr. Min Yoo (Department of Biology, Keimyung University, Daegu, Korea). Five bacterial strains were isolated from commercial fermented milk and tentatively named as HK01, HK02, HK03, LT01 and LT02.

Random primers

Random primers were purchased from the Biotechnology Laboratory, University of British Colombia (Vancouver, Canada). Among them, oligonucleotides (10 mer) having GC contents of 50%~80% were applicable for the purpose of *Lactobacillus* genomic DNA amplification. Nucleotide sequences of the primers are listed in Table 2.

Table 2. List of primers and their nucleotide sequences used in this study

Primers	Nucleotide Sequences[a]	Primers	Nucleotide Sequences[a]
LAB01	GCGGCTGGAG	LAB49	AGCAGCGTGG
LAB02	GGTGGGGACT	LAB50	GAAGGCTCTG
LAB08	GTATTGCCCT	LAB51	GCTGTAGTGT
LAB09	TGTACGTGAC	LAB52	CGCACCGCAC
LAB11	AGTAGACGGG	LAB53	GAGTCACGAG
LAB12	GCTTGTGAAC	LAB55	CTGGCGGCTG
LAB13	ATCCCAAGAG	LAB56	GCCTGGTTGC
LAB14	TGACCGAGAC	LAB57	CGTGGGCAGG
LAB15	TTCCGCGGGC	LAB58	TAGCCGTGGC
LAB16	TACGATGACG	LAB59	GAGCCCGTAG
LAB17	TTAGCGGTCT	LAB65	GAAGGCACTG
LAB25	GCGGTTGAGG	LAB71	TGACCCCTCC
LAB26	CTTTCGTGCT	LAB73	CAGGCGGCGT
LAB27	ATCTGGCAGC	LAB74	AACGGGCAGC
LAB28	GCATATTCCG	LAB80	GGGCCACGCT
LAB29	GCGGTATAGT	LAB86	GTGCGTCGCT
LAB30	GGTTATCCTC	LAB94	AGGACGTGCC
LAB44	AGAGGGTTCT	LAB96	CTCCTCCCCC
LAB45	TGTCGGTTGC	LAB97	TCCCCGTTCC
LAB46	ATGTGTTGCG	LAB98	GCAGGACTGC
LAB47	GTGCGTCCTC	LAB99	GCTCCCCCAC

[a]Oligonucleotide sequences are from 5' to 3'.

Enzymatic genomic DNA extraction

Sufficient quantities of genomic DNA of tested *Lactobacillus* strains were extracted by a method described by Kwon *et al.* (10). With this method, pure and intact genomic DNA was easily obtained for the purpose of RAPD-PCR.

RAPD-PCR

RAPD-PCR reactions were carried out in the following conditions. Each 10 μl of purified genomicDNA was added to 40 μl of the PCR reaction mixture, which contained 10X *Taq* buffer, 40 mM dNTPs, oligonucleotide primers (200 pmol each), and 1 unit of *Taq* DNA polymerase (Perkin-Elmer, USA). The 10X *Taq* buffer contained 500 mM Tris-HCl (pH 8.3), 25 mM $MgCl_2$ and 0.1% gelatin. Two drops of mineral oil were added to each reaction tube. In an automatic thermocycler (Perkin-Elmer Model 480), the reaction tubes were subjected to the following temperature cycles: 95°C for one minute, 40~44°C for one minute and 72°C for two minutes. After 40 cycles of amplification, the samples were incubated for 10 minutes at 72°C.

Agarose gel electrophoresis and UV photography

One fifth of the PCR reaction (10 μl) was loaded onto a gel containing 2% NuSieve GTG agarose in Tris-borate buffer (8.9 mM Tris-HCl, 8.9 mM boric acid, pH 8.3) for electrophoresis. For convenience, 0.5 μl of ethidium bromide solution (10 mg/ml) was directly added to each 10 ml agarose solution for DNA staining. The resulting gel was subjected to UV photography as described in Sambrook *et al.* (18) in order to verify the PCR results.

RAPD-PCR data analysis

After RAPD-PCR, the polymorphic DNA band patterns were photographed by a Polaroid camera (film #667). Using a two-digit number system, 358 polymorphic DNA bands were transformed into a binomial matrix code consisting of '1 (band present)' and '0 (band absent)'. In order to examine genetic relationships among the *Lactobacillus* strains and isolated bacteria, NTSYS-PC (numerical taxonomy analysis program package, Exeter software, USA) was used. A dendrogram and a distance matrix were calculated by an UPGMA (Unweighted Pair-Group Method by Arithmetic Average) program in order to determine genetic relationships among *Lactobacillus* species and the isolated strains.

Determination of fermentative characteristics

In order to verify the results of RAPD-PCR, fermentative

characteristics of the tested *Lactobacillus* species were checked with various fermentable carbon sources (arabinose, fructose, galactose, glucose, lactose, maltose, mannose, raffinose, sucrose, and xylose). Each carbon source (10 g/L) was added to the MRS media (10) which did not contain glucose. Each 2×10^7 Colony forming unit (CFU) of the *Lactobacillus* strains and isolated strains were inoculated into 40 ml medium and incubated at 37°C. The results were analyzed after 72 hours.

Results

RAPD-PCR

Among 100 random primers, only 42 primers (Table 2) gave the results which showed a total of 358 polymorphic DNA bands. A typical polymorphic DNA band pattern was presented in Fig. 1 made from primer LAB49. All eleven samples revealed their characteristic band patterns. In general, the isolated HK01, HK02 and HK03 showed exactly the same polymorphic DNA bands (lanes 7, 8, 9). The LT01 and LT02 also showed band patterns which were indistinguishable from each other (lanes 10, 11). Interestingly, the band patterns of LT01 and LT02 were the same as lanes 3, 5 and 6 representing *L. casei* YIT 9018, *L. acidophilus* and *L. bulgaricus*, respectively. This result shows that all five bacteria have very similar genetic constitutions. The most distinct polymorphic DNA band pattern was noted in *L. brevis* (lane 4). Three strains of *L. casei* (lanes 1, 2 and 3) did not show distinct differences but they had specific polymorphic DNA bands as marked by arrows (→) which can differentiate each other at the gene level.

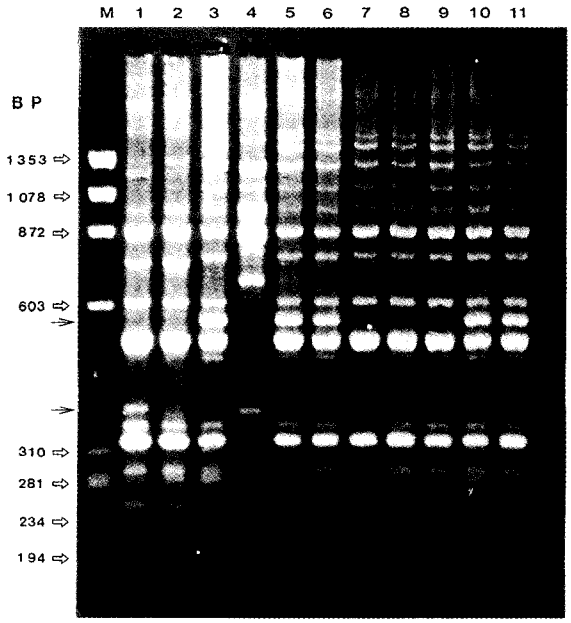

Fig. 1. Typical RAPD-PCR results of various *Lactobacillus* strains with primer LAB49. Lanes: *L. casei* CHR. Hansen, lane 1; *L. casei* ATCC 4646, lane 2; *L. casei* YIT 9018, lane 3; *L. brevis*, lane 4; *L. acidophilus*, lane 5; *L. bulgaricus*, lane 6; HK01, lane 7; HK02, lane 8; HK03, lane 9; LT01, lane 10; LT02, lane 11. M, ΦX174 *Hae*III digested DNA (0.5 μg). Arrows (→) indicate different polymorphic DNA appeared among *L. casei* strains. The full name of *L. brevis* is *L. brevis* subsp. *brevis*. The new name of *L. bulgaricus* is *L. delbrueckii* subsp. *bulgaricus*.

Band pattern characteristics of eleven samples were repeatedly produced by RAPD-PCR as showed in Fig. 2. However, another incredible result was come from the primer LAB98 that presented in Fig. 3. It showed a very thick polymorphic DNA band (→) in lanes 3, 5, 6, 7, 8, 9, 10 and 11 that represented *L. casei* YIT 9018, *L. acidophilus*, *L. bulgaricus*, HK01, HK02, HK03, LT01 and LT02. In this figure, *L. casei* CHR. Hansen (lane 1) and *L. casei* ATCC 4646 (lane 2) revealed indistinguishable band patterns but they were very different from *L. casei* YIT 9018 (lane 3) although they belonged to a group of *L. casei*.

Analysis for genetic relationships among tested Lactobacilli

With the results of RAPD-PCR, we constructed a binomial matrix code depending on the presence or absence of a non-specific polymorphic DNA band that appeared at a specific location. From this data, genetic relationships among the tested *Lactobacillus* species including isolated strains were determined by computer analysis using a NTSYS-PC software package and a dendrogram was generated by an UPGMA program. The result is shown in Fig. 4.

In brief, the tested strains of *Lactobacillus* species diverged into three distinct branches. *L. casei* CHR. Hansen and *L. casei* ATCC 4646 constituted one cluster. Another big cluster consisted of *L. casei* YIT 9018, *L. acidophilus*, *L. bulgaricus*, HK01, HK02, HK03, LT01 and LT02 strains. Only *L. brevis* was very distant from the other strains. In the big cluster, strains of HK01, HK02 and HK03 made one subcluster. *L. casei* YIT 9018, *L. acidophilus*, *L. bulgaricus*, LT01 and LT02 made another subcluster. Among them, *L. casei* YIT 9018 and *L. acidophilus* were grouped together, and the *L. bulgaricus*, LT01 and LT02 were grouped together. That is, the isolated strains LT01 and LT02 were subspecies of *L. bulgaricus*, and the HK01, HK02 and HK03 were very closely related to a group of *L. casei* YIT 9018 and *L. acidophilus*.

When the results were converted into a distance matrix, the genetic similarity among the bacteria was easily determined (Table 3). In particular, the subgroup LT01 and LT02 gave a distance value of 0.037 that implied an insignificant difference existed between them although they showed slightly different colony characteristics (data not shown). Like the distance values of LT01 and LT02, the isolated HK01, HK02 and HK03 gave values of 0.029 ~0.050. Thus, they can be regarded as the same bacteria (or variants) and they were very closely related to

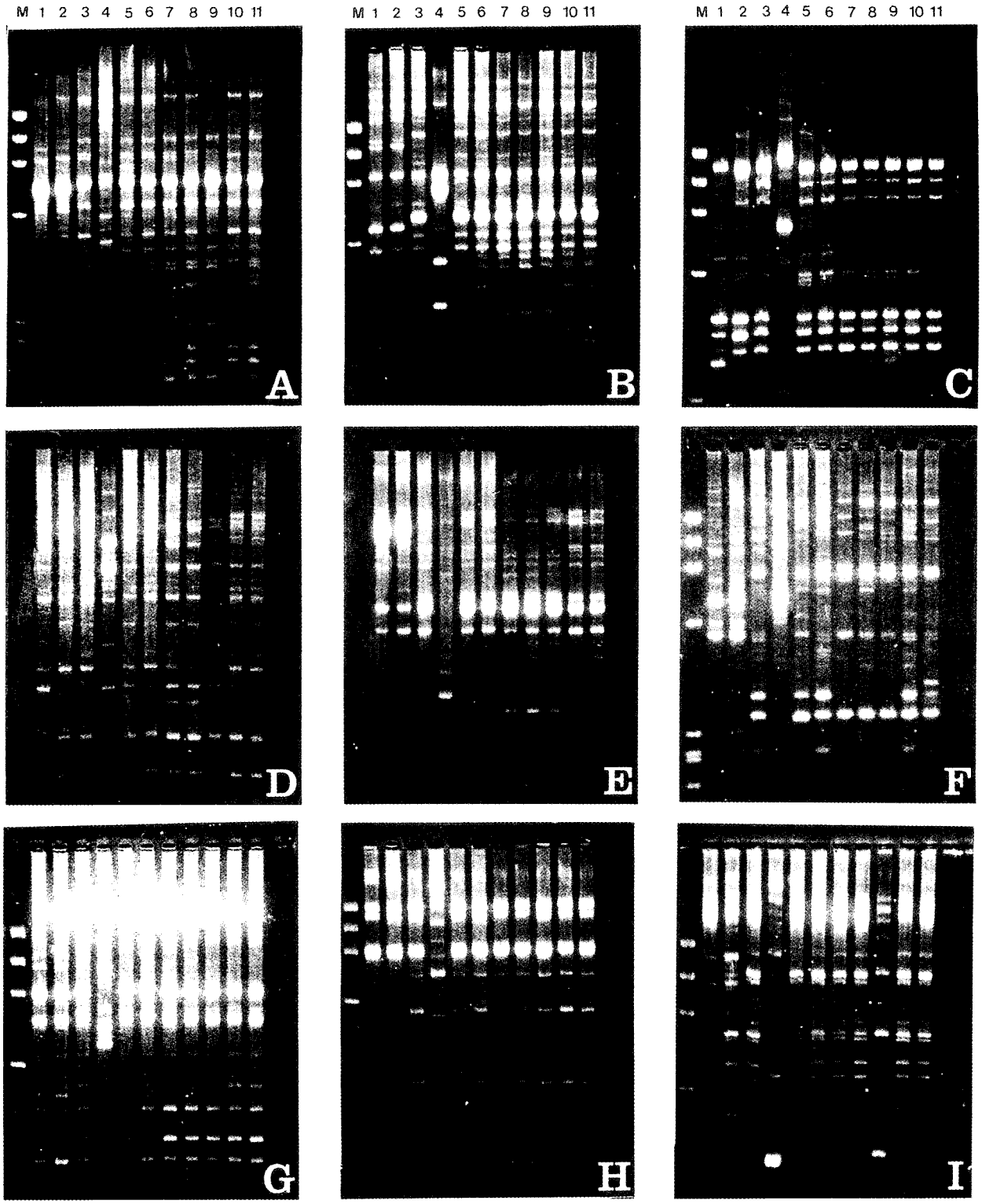

Fig. 2. Pictorial presentation of RAPD band patterns with random primers used in this study. **A,** LAB01; **B,** LAB50; **C,** LAB53; **D,** LAB55; **E,** LAB57; **F,** LAB59; **G,** LAB73; **H,** LAB74; **I,** LAB80; **J,** LAB94; **K,** LAB97; **L,** LAB99 (large gels). **M,** LAB25; **N,** LAB52; **O,** LAB58; **P,** LAB65; **Q,** LAB86; **R,** LAB96 (small gels). Lanes: *L. casei* CHR. Hansen, lane 1; *L. casei* ATCC 4646, lane 2; *L. casei* YIT 9018, lane 3; *L. brevis,* lane 4; *L. acidophilus,* lane 5; *L. bulgaricus,* lane 6; HK01, lane 7; HK02, lane 8; HK03, lane 9; LT01, lane 10; LT02, lane 11. M, ΦX174 *Hae*III digested DNA (sizes from top to bottom: 1,353 bp, 1,078 bp, 872 bp, 603 bp, 310 bp, 281 bp, 271 bp and 194 bp).

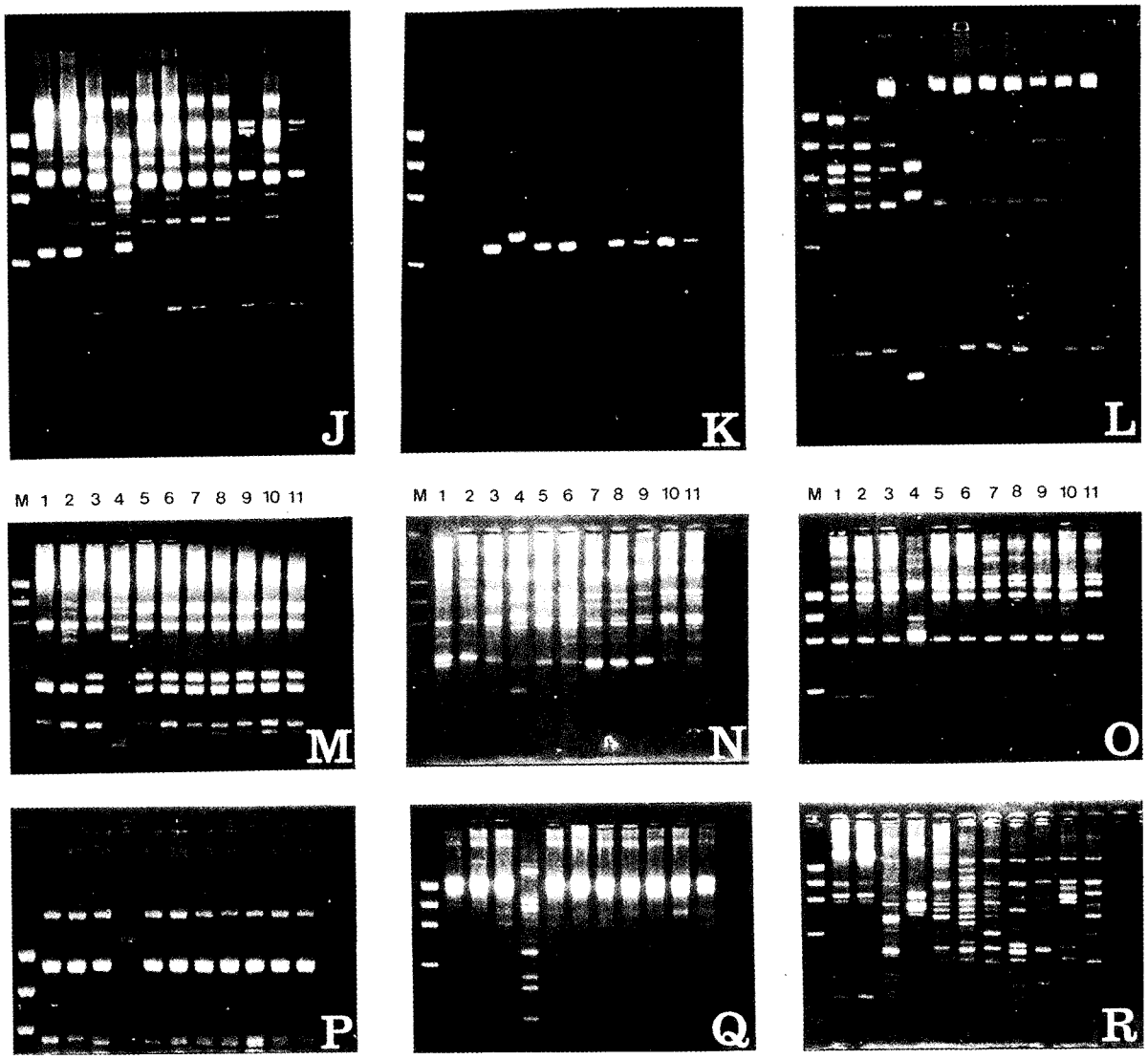

Fig. 2. Continued

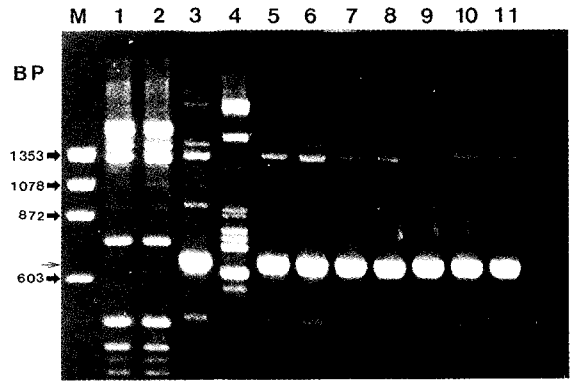

Fig. 3. RAPD-PCR of various *Lactobacillus* strains with primer LAB98. It showed a common RAPD marker (→) that can distinguish the isolated bacteria. Lanes: *L. casei* CHR. Hansen, lane 1; *L. casei* ATCC 4646, lane 2; *L. casei* YIT 9018, lane 3; *L. brevis*, lane 4; *L. acidophilus*, lane 5; *L. bulgaricus*, lane 6; HK01, lane 7; HK02, lane 8; HK03, lane 9; LT01, lane 10; LT02, lane 11. M, ΦX174 *Hae*III digested DNA (0.5 μg).

L. acidophilus and *L. bulagricus* at distance values of 0.11 ± 0.01. Since all bacteria in the big cluster showed distance values within 0.10 ± 0.05, they could be classified in the same subgenus *Thermobacterium*. That is, the isolated strains were grouped into the genus *Lactobacillus* and classified into either *L. acidophilus* or *L. bulgaricus*. Also, it was confirmed that *L. brevis* was most distant from other *Lactobacillus* bacteria tested (values 0.75 ± 0.02), genetically. On the other hand, *L. casei* YIT 9018 was more related to *L. acidophilus* or *L. bulgaricus* (values 0.082, 0.089) rather than *L. casei* CHR. Hansen and *L. casei* ATCC 4646 (values 0.405, 0.363).

Classical method

In order to prove the results of RAPD-PCR, a classical test such as fermentative characteristics for each *Lactobacillus* species and isolated strains was carried out

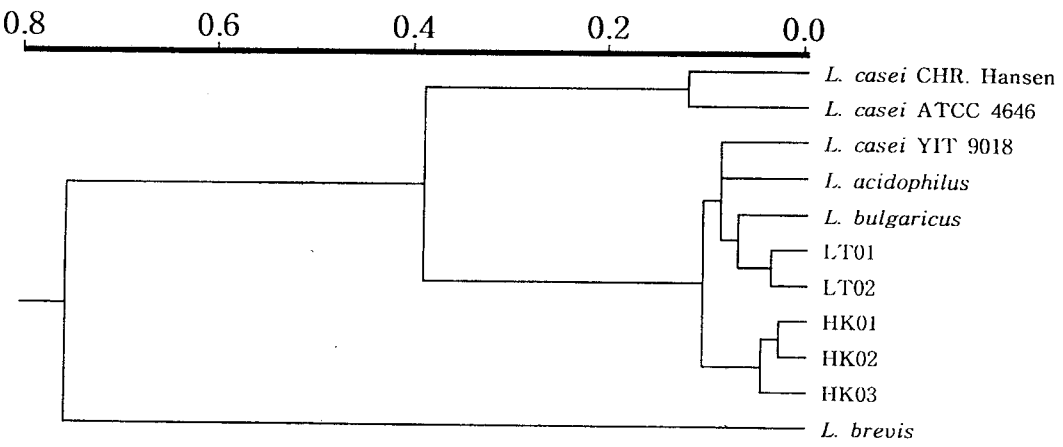

Fig. 4. Dendrogram of *Lactobacillus* species and isolated *Lactobacillus* strains based on RAPD-PCR. The dendrogram was constructed from a distance matrix using an UPGMA program.

Table 3. Distance matrix among the 11 strains of *Lactobacillus* species

Strains	Hansen	4646	9018	brevis	acidophilus	bulgaricus	HK01	HK02	HK03	LT01	LT02
Hansen	0.000										
4646	0.123	0.000									
9018	0.405	0.363	0.000								
brevis	0.730	0.730	0.767	0.000							
acidophilus	0.420	0.393	0.089	0.767	0.000						
bulgaricus	0.428	0.380	0.082	0.765	0.075	0.000					
HK01	0.406	0.368	0.130	0.760	0.102	0.095	0.000				
HK02	0.388	0.347	0.118	0.763	0.112	0.094	0.029	0.000			
HK03	0.411	0.391	0.154	0.761	0.119	0.123	0.045	0.050	0.000		
LT01	0.411	0.363	0.088	0.761	0.093	0.065	0.085	0.079	0.107	0.000	
LT02	0.405	0.356	0.099	0.749	0.098	0.075	0.090	0.083	0.107	0.037	0.000

*Hansen, *L. casei* CHR. Hansen; 4646, *L. casei* ATCC 4646; 9018, *L. casei* YIT 9018; brevis, *L. brevis*; acidophilus, *L. acidophilus*; bulgaricus, *L. bulgaricus*; LT01, LT02, HK01, HK02 and HK03 are laboratory isolates.

Table 4. Fermentative characteristics of tested *Lactobacillus* strains with various carbon sources

Lactobacillus strains	Ara	Fru	Gal	Glu	Lac	Mal	Man	Raf	Suc	Xyl
L. casei CHR. Hansen	-	+	+	+	+	+	+	-	+	-
L. casei ATCC 4646	-	-	+	+	+	+	+	-	+	-
L. casei YIT 9018	-	+	+	+	+	+	+	-	+/-	-
L. brevis	+	+	-	+	-	+	-	-	+	+
L. acidophilus	-	+	+	+	+	+	+	+	+	-
L. bulgaricus	-	+	+	+	+	+/-	+	-	+/-	-
HK01	-	+	+	+	+	+	+	+	+	-
HK02	-	+	+	+	+	+	+	+	+	-
HK03	-	+	+	+	+	+	+	+	+	-
LT01	-	+	+	+	+	+	+	-	+	-
LT02	-	+	+	+	+	+	+	-	+	-

*Ara, arabinose; Fru, fructose; Gal, galactose; Glu, glucose; Lac, lactose; Mal, maltose; Man, mannose; Raf, raffinose; Suc, sucrose; Xyl, xylose. +/-: variable reaction.

(Table 4). Typically only *L. brevis* fermented arabinose and xylose (i.e., pentoses) but could not ferment some hexoses such as galactose and mannose. Interestingly, the isolated LT01 and LT02 were negative for raffinose similar to *L. bulgaricus,* but HK01, HK02 and HK03 were positive for raffinose similar to *L. acidophilus*. This was the only difference found from the fermentative characteristics between the two groups of isolated strains. These results were identical to the results from RAPD-PCR which revealed that LT01 and LT02 would be same as *L. bulgaricus,* and HK01, HK02 and HK03 would be the same as *L. acidophilus.* These fermentative charac-

teristics results were quite consistent with previous results published (7, 8, 16). In addition, it was notable that some results of fermentative characteristics were ambiguous. For example, the fermentative characteristics of the *L. casei* CHR. Hansen and *L. casei* ATCC 4646 was almost the same as that of *L. casei* YIT 9018. Only *L. casei* YIT 9018 did not grow well on MRS with sucrose. This kind of characteristics was also found in *L. bulgaricus* with maltose and sucrose.

Discussion

The 11 tested strains of *Lactobacillus* were easily grouped into three clusters according to their genetic relationships generated by computer analysis and the results were quite consistent with a proposal of Rogosa and Sharpe (16). They suggested that the genus *Lactobacillus* was grouped into three subgenuses, i.e., *Thermobacterium, Streptobacterium* and *Betabacterium.* Also, the three clusters formed with tested *Lactobacillus* strains were identical to the result of a 16S rRNA sequencing method by Stackebrandt and Teuber (20). However, most LAB taxonomists now disagree with the proposal of Rogosa and Sharpe for many reasons because recent molecular methods gave better definitions on the classification of the genus *Lactobacillus.* On the other hand, their efforts cannot be ignored because most commercially useful *Lactobacillus* species were characterized on the basis of physiological and biochemical analysis (8, 16). Thus more applicable methods for classifying *Lactobacillus* species should be established with comprehensive ways that meet both opinions.

As an alternative choice, we employed RAPD-PCR for classifying the *Lactobacillus*, and it appeared to differentiate some *Lactobacillus* species with ease at the subspecies level (22). For example, our results with RAPD-PCR revealed genetic relatedness among *Lactobacillus casei* strains at a distance value of 0.123 where the values with *L. casei* YIT 9018 were 0.405 and 0.363 (Table 3). These figures were very reliable because the isolated strains gave distance values of only 0.029~0.050 that were regarded as the same bacteria or variants. Also the isolates were related to *L. bulgaricus* or *L. acidophilus* at values of 0.065~0.123 in this study. Thus the isolated strains of LT01 and LT02 appears to be the subspecies of *L. bulgaricus,* and those of HK01, HK02 and HK03 are regarded as the subspecies of *L. acidophilus* with supporting data from the fermentative characteristics test (Table 4). From this study, it was also noted that *L. casei* YIT 9018 was very different from *L. casei* CHR. Hansen and *L. casei* ATCC 4646. This result was consistent with previous studies by Kwon *et al.* (11) and Nam *et al.* (14) who worked on the identification of *L. casei* variants by RAPD and PCR, respectively. However, all three *L. casei* strains showed the same fermentative characteristics and they were only different from *L. acidophilus* with the ability for raffinose fermentation as shown in Table 4.

Conclusively, different strains of *Lactobacillus* were very successfully characterized at the level of subspecies or variants by RAPD-PCR with application of random primers. By the analysis for numerical taxonomy using an UPGMA method, the tested *Lactobacillus* species were grouped into three distinct clusters and the isolated strains, LT01 and LT02 were more closely related to *L. bulgaricus* than the other *Lactobacillus* species tested. The isolates, HK01 and HK02 seemed to be identical organisms but differed slightly from HK03.

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