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Genomic Fingerprinting Patterns of *Bifidobacteria* Isolated from Healthy Koreans Using ERIC-, TAP-, and BOX-PCR

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건강한 한국인으로부터 분리된 비피도박테리아의 ERIC-, TAP-, BOX- 중합효소연쇄반응을 이용한 유전자 지문 분석

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요 약

유산균인 비피도박테리아는 사람과 동물에서 유익한 프로바이오틱 미생물로 알려져 있다. 본 연구에서는 이러한 비피도박테리아 균주의 분류를 위한 repetitive DNA element PCR fingerprinting (ERIC- 또는 TAP-PCR)의 사용을 평가하였다. 사람분변으로부터 분리한 알려지지 않은 비피도박테리움 균주와 한국생명공학연구원 생물자원센터로부터 분양받은 표준균주를 가지고 분류 및 동정에 ERIC-PCR과 TAP-PCR을 이용한 RAPD-fingerprinting을 수행하였다. 그 결과 비피도박테리움 균주에 대한 속과 종단위의 분류가 가능하였으며, 실험에 사용된 모든 비피도박테리움 균주는 RAPD-fingerprinting 분석을 통해 유전적 다양성을 확인하였다. 또한 ERIC2와 TAP1 프라이머를 이용한 실험에서는 *Bifidobacterium adolescentis* 특이 유전자 단편을 확인하였으며 이는 *B. adolescentis* 균주의 동정에 유용할 것으로 사료된다.

Key words : antibacterial resistance, *Bifidobacterium adolescentis*, RAPD-fingerprinting

INTRODUCTION

Lactic acid bacteria (LAB) are beneficial probiotic organisms that inhibit harmful intestinal bacteria, improve of lactose tolerance, synthesize vitamins, and

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reduce serum cholesterol levels (Mitsuoka *et al.*, 1990; Modler *et al.*, 1990; Gopal *et al.*, 1996; Homma, 1998; Rhee *et al.*, 2002; Choi *et al.*, 2005). LAB can also improve immune function and help prevent cancer in humans (Salminen *et al.*, 1974; Sekine *et al.*, 1985; Park *et al.*, 1999; Rafter, 1999). LAB are major components in food fermentation, especially dairy foods, and are also natural components of the gastrointestinal microflora (Fuller, 1992; Drake *et al.*, 1996; Klein *et al.*, 1998; Yoon *et al.*, 2004).

Bifidobacterium spp. is a common probiotic LAB in humans, with various biological activities (Buchanon and Gibbons, 1976; Kohwi *et al.*, 1978; Kato *et al.*, 1981; Perdigon *et al.*, 1986; Modler *et al.*, 1994; Gibson *et al.*, 2003; Masco *et al.*, 2003; You *et al.*, 2004). The development of further uses for this species requires knowledge of antibacterial resistance patterns and genetic diversity in *Bifidobacterium* spp.

Traditionally, LAB were identified by phenotypic properties, such as sugar-fermentation patterns (Pot *et al.*, 1994; Vandamme *et al.*, 1996; Gevers *et al.*, 2001). However, these tests can be difficult to interpret and are time-consuming without the support of genotyping techniques (Gancheva *et al.*, 1999; Tynkkynen *et al.*, 1999). Other profiling techniques, such as protein profiling, 16S rRNA sequencing, and pulsed-field gel electrophoresis (PFGE) (Collins *et al.*, 1991; Pot *et al.*, 1994; Tenover *et al.*, 1995), are too laborious, limited in their resolving power, or require a species-specific methodology. Alternatively, PCR amplification of repetitive bacterial DNA elements such as ERIC-, TAP-, BOX- and REP-PCR (De Urraza *et al.*, 2000) is: (a) rapid, and easy to perform, (b) low cost, (c) suitable for a high-throughput testing of strains, and (d) able to classify and type a wide range of LAB (Versalovic *et al.*, 1994; Olive and Bean, 1999; Englund, 2003; Masco *et al.*, 2003).

The aim of the present study was to assess the applicability of ERIC- and TAP-PCR fingerprinting for the genotypic differentiation of *Bifidobacteria* species. We also determined antibacterial resistance patterns to several antibiotics, including anti-tuberculosis agents, and measured genetic diversity among several *Bifidobacterium* species.

MATERIALS AND METHODS

1. Isolation and identification of *Bifidobacterium* spp.

Fecal samples of 20 healthy Koreans (20 ~ 30 years old) were collected by BBL's anaerobic sample collection, transported under anaerobic conditions, and used within 24 hrs. Fecal samples were serially diluted 10-fold from 10^{-1} to 10^{-8}, and 100 μL was spread onto selective BL agar (Nissui, Japan) containing 5% sheep blood. After 48 hrs of incubation in anaerobic conditions (Bactron Anaerobic Chamber, Sheldon Manufacturing Inc., USA), brown or reddish-brown colonies 2 ~ 3 mm in diameter were selected for further study (Scardovi, 1986).

A fructose-6-phosphate phosphoketolase (F6PPK) test was performed (Lee *et al.*, 2001) to ensure that the colonies selected were Bifidobacteria. To identify the isolated *Bifidobacterium* spp. at the species level, 16S rRNA sequencing was performed by Bioleaders (Daejeon, Korea)

Table 1. List of *Bifidobacterium* spp. and isolates used in this study

Strain	Source
B. adolescentis SPM0212	Isolated[1]
B. adolescentis SPM1005	Isolated
B. adolescentis SPM1207	Isolated
B. adolescentis SPM1601	Isolated
B. adolescentis KCTC3325	Commercial[2]
B. infantis KCTC3127	Commercial
B. catenulatum KCTC3221	Commercial
B. thermophilum KCTC3225	Commercial
B. ruminantium KCTC3425	Commercial
B. bifidum (BF)	Isolated
B. lactis (BL)	Isolated
B. pseudocatenulatum KCTC3223	Commercial
B. pseudocatenulatum SPM1204	Isolated
B. longum KCTC3128	Commercial
B. longum SPM1205	Isolated

Abbreviations: [1]isolated from human feces and [2]purchased from Korean Collection for Type Culture

Table 2. Primers for PCR-RAPD

Primers	Sequence (5′→3′)
ERIC1R	5′-ATG TAA GCT CCT GGG GAT TCA C-3′
ERIC2	5′-AAG TAA GTG ACT GGG GTG AGC G-3′
TAP1	5′-CAG CAG CCG CGG TAA TAC-3′
TAP2	5′-CAG CAG CCG CGG TAA TTC-3′
BOXA1R	5′-CTA CGG CAA GGC GAC GCT GAC G-3′
BOXA2R	5′-ACG TGG TTT GAA GAG ATT TTC G-3′
REP1R	5′-III ICG ICG ICA TCI GGC-3′
REP2I	5′-ICG ICT TAT CIG GCC TAC-3′

2. Bacterial strains and extraction of genomic DNA

Fifteen strains of *Bifidobacterium* spp. were analyzed by RAPD-PCR (Table 1). All *Bifidobacterium* strains were grown overnight at 37°C on general anaerobic medium (GAM, Nissui Pharm. Co. Ltd., Japan) under anaerobic conditions (90% N_2, 5% H_2, 5% CO_2). The complete genomic DNA of all *Bifidobacterium* strains was isolated with the Wizard genomic DNA purification kit (Promega, Co. Ltd., Madison, WI, USA).

3. PCR-RAPD analysis

The primers used for ERIC-, TAP-, BOX-, and REP-PCR are listed in Table 2. PCR reactions were performed in 30 μL-reaction mixtures containing the DNA template, 10 mM Tris-HCl, 50 mM KCL, 1.5 mM $MgCl_2$, 0.01% gelatin, 200 μM of each dNTP, primers, and 2.5 units of *Taq* DNA polymerase (Promega, Co. Ltd., Madison, U.S.A). The reaction mixture was overlaid with a thin layer of sterile mineral oil to prevent evaporation.

DNA amplification was performed in a programmable PTC-200 thermal cycler (MJ Research, USA) under the following cycling conditions: (a) for ERIC-PCR: initial denaturation at 94°C for 3 min; followed by 35 cycles consisting of 30 s at 94°C, 60 s at 48°C, and 5 min at 72°C; and a final cycle of 72°C for 7 min; an additional step chilled the PCR products to 4°C. (b) For TAP-PCR: initial denaturation at 92°C for 2 min; followed by 40 cycles consisting of 30 s at 92°C, 1 min at 38°C, and 1.5 min at 68°C; and a final cycle of 68°C for 10 min; an additional step chilled the PCR products to 4°C. (c) For BOXA1R: initial denaturation at 92°C for 2 min; followed by 35 cycles consisting of 30 s at 92°C, 1 min at 52°C, and 2 min at 72°C; and a final cycle of 72° C for 5 min; an additional step chilled the PCR products to 4°C. (d) For BOXA2R: initial denaturation at 95°C for 3 min; followed by 30 cycles consisting of 45 s at 95°C, 1 min at 35°C, and 2 min at 65°C; and a final cycle of 65°C for 5 min; an additional step chilled the PCR products to 4°C. (e) For REP-PCR: initial denaturation at 94°C for 5 min; followed by 30 cycles consisting of 1 min at 94°C, 1 min at 58°C, 8 min at 65°C; and a final cycle of 65°C for 15 min. An additional step chilled the PCR products to 4°C.

All amplified PCR products were resolved by electrophoresis on a 1.5% agarose gel in TAE buffer. PCR products were stained with ethidium bromide and visualized under UV light at 254 nm. The amplification reaction was performed twice to establish reproducibility.

4. Measurement of Minimum Inhibitory Concentrations (MICs)

The following fourteen antimicrobial agents were provided by their manufacturers for use in this study: amoxicillin/clavulanic acid (Ilsung, Korea), cefotaxime (Whan-In, Korea), clindamycin (Yuhan, Korea), ciprofloxacin (Ildong, Korea), ethambutol (Ildong, Korea), rifampicin (Chongkundang, Korea), streptomycin (Chongkundang, Korea), cycloserine (Donga, Korea), gentamicin (Kuk-Je, Korea), meropenem (Yuhan, Korea), mupirocin (Hanol, Korea), quinupristin/dalfopristin (Rhone-Poulenc Rorer, West Malling, Kent ME, UK), vancomycin (Lilly, USA), teicoplanin (Gruppo Lepetit S.p.A., Italy).

MICs were determined by the agar dilution method according to the guidelines established by the National Committee for Clinical Laboratory Standards (NCCLS, 2003), and defined as the lowest concentration of antimicrobial agent producing no visible grow-

Table 3. MIC values for *Bifidobacterium* spp.

Strain	MIC (μg/mL)						
	AMOXI	CEF	CIF	CLIN	CYCS	SM	GEN
B. adolescentis SPM0212	1.6	6.25	6.25	50	>100	50	>100
B. adolescentis SPM1005	0.2	0.2	0.8	50	100	50	100
B. adolescentis SPM1207	0.4	6.25	0.4	6.25	>100	12.5	>100
B. adolescentis SPM1601	0.2	0.1	0.4	6.25	>100	>100	0.2
B. infantis KCTC3127	0.4	<0.05	0.1	1.6	>100	50	<0.05
B. catenulatum KCTC3221	1.6	3.1	0.8	1.6	>100	50	100
B. thermophilum KCTC3225	0.4	3.1	1.6	12.5	>100	100	6.25
B. ruminantium KCTC3425	0.4	<0.05	0.8	6.25	>100	50	1.6
B. bifidum (BF)	1.6	0.4	3.1	6.25	>100	100	100
B. lactis (BL)	1.6	0.4	3.1	6.25	>100	100	100
B. pseudocatenulatum KCTC3223	0.8	0.1	<0.05	3.1	>100	>100	<0.05
B. pseudocatenulatum SPM1204	1.6	0.8	0.8	6.26	>100	50	50
B. longum KCTC3128	0.8	0.2	6.25	3.1	>100	>100	<0.05
B. longum SPM1205	1.6	3.1	3.1	1.6	>100	6.25	6.25

Strain	MIC (μg/mL)						
	LIN	MEN	MUP	RIF	SYN	TEI	VAN
B. adolescentis SPM0212	0.8	6.25	0.8	0.8	1.6	0.8	3.1
B. adolescentis SPM1005	<0.05	0.2	>100	0.2	0.4	0.4	1.6
B. adolescentis SPM1207	6.25	12.5	12.5	0.2	6.25	0.8	3.1
B. adolescentis SPM1601	0.1	<0.05	0.8	0.8	0.2	<0.05	0.2
B. infantis KCTC3127	<0.05	<0.05	<0.05	1.6	<0.05	<0.05	1.6
B. catenulatum KCTC3221	<0.05	0.2	3.1	<0.05	0.4	100	1.6
B. thermophilum KCTC3225	<0.05	<0.05	0.8	6.25	0.4	0.1	0.8
B. ruminantium KCTC3425	<0.05	0.2	0.2	1.6	0.2	0.4	0.1
B. bifidum (BF)	0.1	0.4	>100	6.25	0.4	0.4	1.6
B. lactis (BL)	0.1	0.4	>100	6.25	0.4	0.4	1.6
B. pseudocatenulatum KCTC3223	<0.05	<0.05	<0.05	6.25	0.4	<0.05	<0.05
B. pseudocatenulatum SPM1204	0.1	3.1	>100	1.6	0.4	0.4	1.6
B. longum KCTC3128	<0.05	0.2	0.1	6.25	0.4	<0.05	<0.05
B. longum SPM1205	0.1	3.1	>100	0.2	0.4	0.2	0.8

Abbreviations: AMOXI, amoxicillin/clavulanic acid; CEF, cefotaxime; CIF, ciprofloxacin; CLIN, clindamycin; CYCS, cycloserine; SM, streptomycin; GEN, gentamycin; LIN, lincomycin; MEN, meropenem; MUP, mupirocin, RIF, rifampicin; SYN, synercid; TEI, teicoplanin; VAN, vancomycin

th of the microorganism.

RESULTS AND DISCUSSION

According to the 16S rRNA sequence analysis, the isolates contained 4 strains of *B. adolecentis* and 2 strains each of *B. pseudocatenulatum* and *B. longum* (Table 1). Other strains were purchased from the Korean Collection for Type Culture (KCTC). To investigate the phenotype of *Bifidobacterium* spp. antimicrobial susceptibilities of these bacteria were tested. All *Bifidobacterium* spp. were sensitive to quinupristin/dalfopristin, vancomycin, teicoplanin and ciprofloxacin, while *B. difidum* (BF) and *B. lactis* (BL) were resistant to mupirocin (Table 3).

The RAPD technique can identify bacterial species or strains within species (Welsh and McClelland, 1990; MacGowan *et al.*, 1993; Sandery *et al.*, 1994). We analyzed 15 *Bifidobacterium* spp. with eight

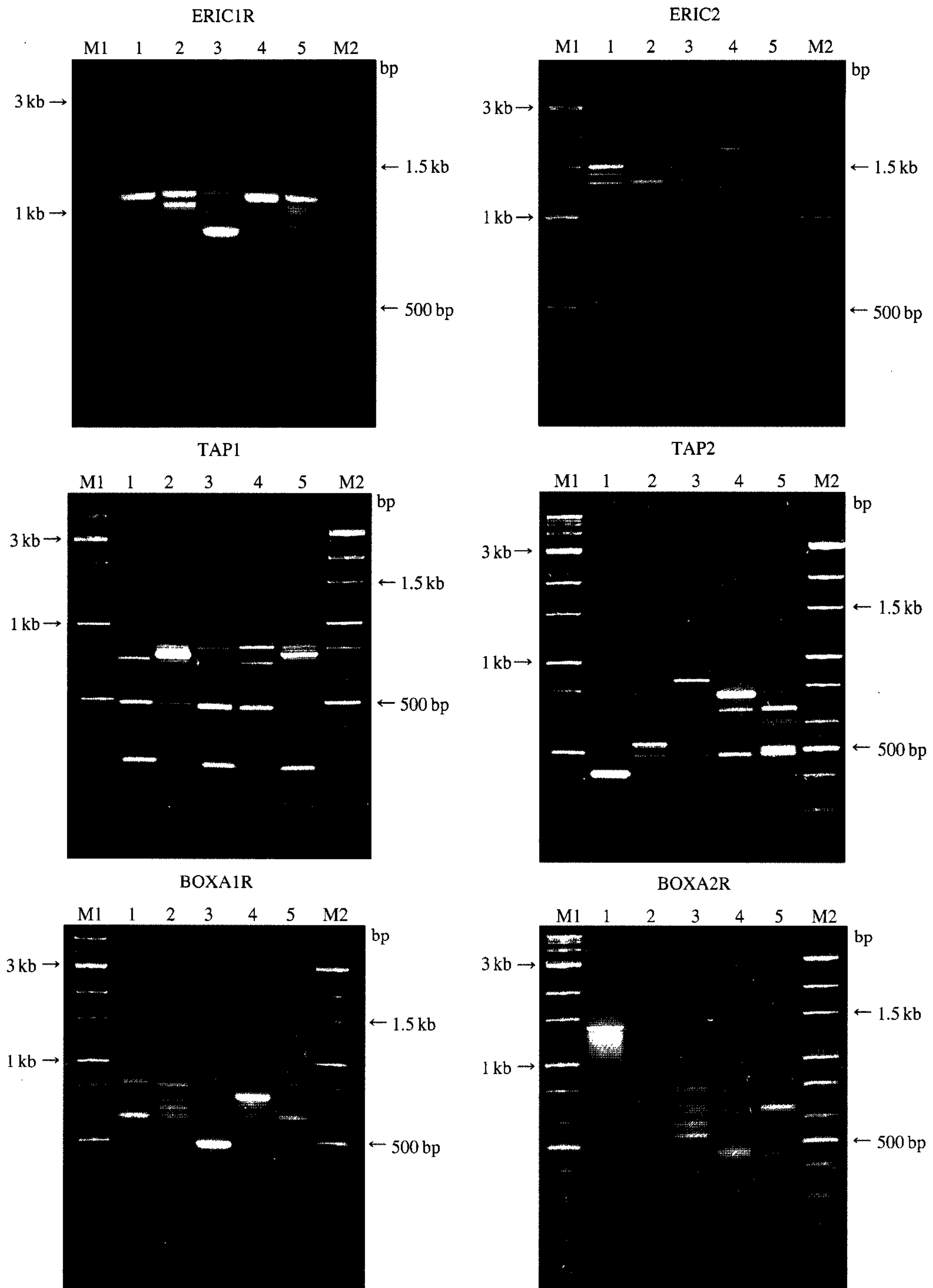

Fig. 1. Genomic fingerprinting patterns of *B. adolescentis* with the microbial uniprimer kit. Lane M1, 1 kb ladder size marker; lane 1, *B. adolescentis* SPM 0212; lane 2, *B. adolescentis* SPM 1005; lane 3, *B. adolescentis* SPM 1207; lane 4, *B. adolescentis* SPM 1601; lane 5, *B. adolescentis* KCTC 3325; lane M2, 100 bp ladder size marker.

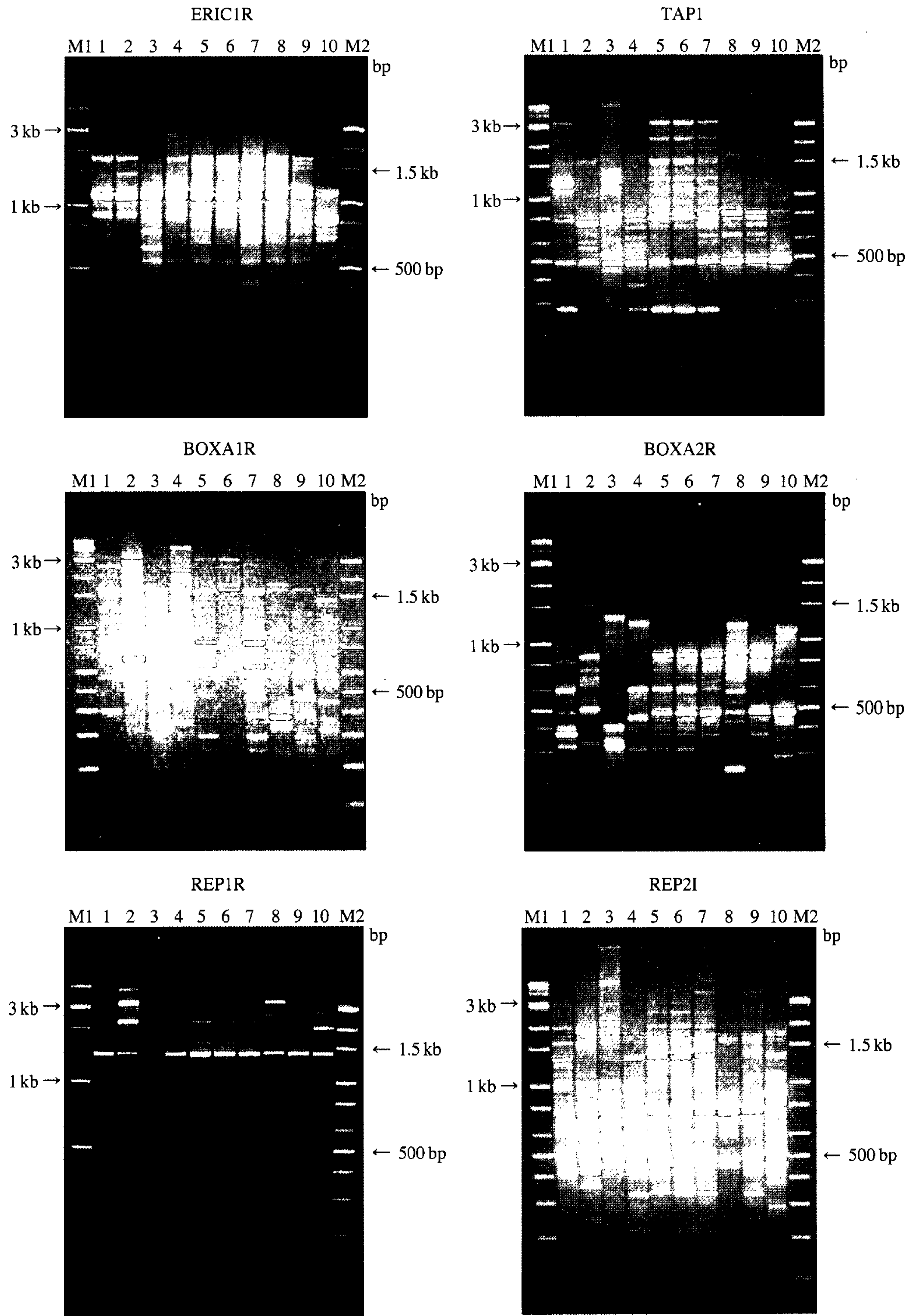

Fig. 2. Genomic fingerprinting patterns of 10 *Bifidobacterium* spp. with RAPD-PCR. Lane M1, 1 kb ladder size marker; lane 1, *B. infantis* KCTC3127; lane 2, *B. catenulatum* KCTC3221; lane 3, *B. thermophilum* KCTC3225; lane 4, *B. ruminantum* KCTC3425; lane 5, *B. bifidum*; lane 6, *B. lactis*; lane 7, *B. pseudocatenulatum* KCTC3223; lane 8, *B. pseudocatenulatum* SPM1204; lane 9, *B. longum* KCTC3128; lane 10, *B. longum* SPM1205; lane M2, 100 bp ladder size marker.

primers using RAPD (Figs. 1 and 2). The size of bands generated in RAPD-PCR varied from 3.0 kb to 200 bp, but all *B. adolescentis* samples produced a common band of approximately 1.2 kb and 1.3 kb in ERIC1R- and ERIC2-PCR, respectively. Other *Bifidobacterium* strains also had a 1.2 kb-band in ERIC1R-PCR.

TAP-PCR uses a specific primer targeting a highly conserved sequence within the 16S rRNA gene to produce PCR amplicons suitable for molecular fingerprinting (Cusick and O'Sullivan, 2000). PCR amplification using the TAP1 primer produced three common bands of 800 bp, 500 bp, and 290 bp in *B. adolescentis* and most *Bifidobacterium* spp., suggesting that their genetic makeup may be related at the genus level. However, PCR amplification using the ERIC1R primer could differentiate *B. adolescentis* from the other *Bifidobacterium* spp. by the presence of a 600 bp band. This specific band could provide a basis for further studies to develop a sequence-characterized amplified region (SCAR) marker through DNA sequencing and database searching or for analyzing the phylogenetic relationships of *B. adolescentis.*

In conclusion, our results show that this RAPD method could discriminate between *B. adolescentis* and other *Bifidobacterium* spp. ERIC-PCR fingerprinting using the ERIC1R primer is a rapid, easy to perform, and reproducible method that is suitable for high-throughput testing of *Bifidobacterium* strains. These genotyping tools also permit differentiation of *Bifidobacteria* at the species, subspecies, and potentially up to the strain level.

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