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RESEARCH ARTICLE OPEN ACCESS

Applicability of *rpoB* Gene for PCR-RFLP based Discrimination of Bifidobacterial Species Isolated from Human and Animal Sources

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Abstract

Bifidobacteria are widely used as probiotics for their application in the development of functional food and prophylactic therapy. This has necessitated the development of a molecular approach for the genera to be widely identified up to species and subspecies level. In the current study, PCR-RFLP of the partial RNA polymerase β-subunit (*rpoB*) gene fragment was evaluated for differential identification of *Bifidobacterium* species. The *rpoB* gene partial sequences of 575 bp were amplified from 93 previously identified isolates collected from various sources of human and animal origin along with 12 standard reference strains. The PCR amplified products were digested with three restriction endonucleases HhaI, HinfI and BanI separately. Dendrograms constructed from the patterns of HhaI, were found to be more discriminatory and successfully differentiated all the twelve species and also at sub-species level in between *B. longum subsp. longum* and *B. longum* subsp. *infantis*. However, *B. adolescentis* and *B. pseudocatenulatum* group clusters were not separated and represented by one group. The groups were further discriminated by HinfI restriction digestion. A separate combination thereof may be used for inferring the classification of bifidobacterial species targeted on *rpoB* PCR-RFLP analysis. To our knowledge, this work is the first report based on use of *rpoB* PCR-RFLP for discrimination of the isolates of genus *Bifidobacterium* and also provides insights into specific advantages of this method over *hsp60* PCR-RFLP in differentiating *B. longum* subsp. *longum* and *B. longum* subsp. *infantis.*

Keywords: *Bifidobacterium*, PCR-RFLP, HhaI, *rpoB* gene, probiotics

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INTRODUCTION

Gastrointestinal tract comprises of a complex ecosystem where intestinal microbiota has been revealed to exert numerous metabolic, nutritional, physiological and immunological effects to human and animal health.[1] The microbiota is further modulated by the effect of probiotics (live supplemented microorganisms) for maintaining and improving the health integrity.[2-5] Amongst these microorganisms, genus *Bifidobacterium* which are generically defined as Gram-positive, anaerobic and bifid shaped bacteria are found in the gastrointestinal tract (GIT) of humans and animals.[4-7] Many species were recognized and classified under this genus after it was designated as an independent taxon by Orla-Jensen.[8,9] A few of the species among this genus are important contributors to the gut microbiome which have focused interest in characterization up to species and subspecies level due to their potential use as probiotic organisms.[10-12] The classification is important in the food and pharmaceutical industries as some of developed products require specific strains. Thus, the immense contributions to human health[13] imposed for precise taxonomic identification and molecular based discrimination of genus *Bifidobacterium*.

Traditional techniques for identification of bifidobacterial species have limited application due to low reproducible and indecisive results.[14] The current understanding on the complexity and diversity within the genus *Bifidobacterium* has targeted the use of sophisticated molecular techniques for the differentiation of species and subspecies. Stackebrandt and Ludwig[15] reported that DNA hybridization >70% between two species possessed more than 97% 16S rRNA sequence similarity. However, 16S-based classification of genus *Bifidobacterium* is sometimes not discriminative enough to allow differentiation between certain species.[16] The rate of evolutionary substitution in protein-encoding genes has higher importance and provides improved resolution than those in 16S rRNA genes.[17,18] So, modern taxonomic approaches classified bifidobacterial phylogeny based on these highly conserved protein coding genes like *tuf, atpD, recA, dnaK.*[19] In microbial ecological studies, the use of a single copy gene with increased resolution has highlighted enhanced phylogenetic relationships and diversity measurements at subspecies level in comparison to 16S rRNA gene.[20] Among these conserved macromolecules, *rpoB* gene encoding the RNA polymerase β-subunit has come up into view as one of the major genes which is correlated with distinct phylogenetic analysis and identification of firmly related bacterial species.[21] Adekambi et al.[22] proposed entire *rpoB* gene sequencing as a suitable approach to DNA-DNA hybridization technique for differentiation of bacterial genus and species. In recent decades, PCR-RFLP technique of *rpoB* gene has been implemented for species identification in genera viz. *Legionella*,[23] *Mycobacterium*,[24] *Cronobacter*,[25] *Leptospira*,[26] Lactic acid bacteria[27] etc. Kim et al.[28] reported that *rpoB* gene sequences are appropriate molecular markers for differentiation and determining the relationships of bifidobacterial species. Mainly this gene is responsible for rifaximin resistance in *Bifidobacterium* species.[29] So far, no information is available with reference to the context of PCR-RFLP of *rpoB* gene in genus *Bifidobacterium*. Therefore, in the current study we have tried to build up a PCR-RFLP based identification strategy targeting partial *rpoB* gene sequence of bifidobacterial isolates, isolated from different sources of human and animal origin.

MATERIALS AND METHODS

Bifidobacterial isolates and standards used in the study

Previously identified 93 bifidobacterial isolates comprised of 59 isolates from human sources (milk samples and faeces) and 34 isolates from animal origin (rumen samples, chicken faeces) as reported in our earlier study of *hsp60* PCR-RFLP analysis 30 were included in this work. Standard strains representing 12 different *Bifidobacterium* species were procured from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Germany) were included for validation, as listed in Table 1. Standard strains and our isolated 93 isolates were cultured and revived in M-58 medium whenever required, and stored supplemented with 10% glycerol (v/v) at -80 °C.

Genomic DNA extraction

Genomic DNA extraction was carried out from the cultures following the protocols of Brookman and Nicholson,[31] with some modifications. Bacterial strains were grown in

Bifidobacterium medium (DSMZ- specification) and conditions as described in our previous study.[30] After incubation, the bacterial pellets were collected by centrifugation at 10,000 rpm for15 min, and to it 800 µL of CTAB buffer (Cetyl trimethylammonium bromide) was added and mixed thoroughly. The suspension was kept for 1 h at 70°C and then mixed thoroughly. Then around 500 µL of chloroform: isoamyl alcohol (24:1) was added to the suspended cells and blended to form a white emulsion. In the subsequent stage centrifugation was done at 10,000 rpm for 20 min. The aqueous layer was moved and DNA was precipitated with addition of 300 µL of isopropanol and centrifuged at 10,000 rpm for 10 min). At that point 500 µL of 70% ethanol was added to the white pellet, mixed and afterward kept at 60°C for 10 min in an incubator.

Air dried pellet was dissolved in 60 µL TE buffer [10 mmol Tris-HCl and 1mM EDTA (pH 8.0)]. The quality of the isolated DNA was checked by 0.8% agarose gel electrophoresis and quantified by Nanodrop spectrophotometer (ND-1000; V3.5.2, Nanodrop Technology, Cambridge, UK). For working conditions, measurement was trailed by weakening DNA in Milli Q water. For working conditions, quantification was followed by diluting DNA in Milli Q water.

Amplification of *rpoB* gene

Amplification of *rpoB* gene fragment with primers Bilon-*rpoB*-F-5'-AGACCGACAGCTTCGATTGG-3'; Bilon-*rpoB*-R 5'-AACACGATGGCGGACTGCTT-3' (Sigma, Aldrich) were carried out as mentioned by Makino et al.[32] The composition of PCR mixture (50 µL) was 10X buffer (10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl2 (Genei, Bangalore), 50 mM KCl, 200 µM each dNTP (Genei, Bangalore), template DNA (50 ng), primer (20 pmol each), Taq DNA polymerase (1 unit Sigma, Aldrich) and the volume was made up with sterile PCR grade water. The reaction was subjected to 35 cycles of amplification: initial denaturation 95°C for 8 min, denaturation 95°C for 45 sec, annealing 57°C for 30 sec, extension 72°C for 1 min and this was completed by final extension of 72°C for 10 min. Amplified PCR products were separated on 1.2% agarose gel (Sigma, Aldrich) containing 10 µg/mL ethidium bromide in 1X TBE electrophoresis buffer (10.8 g/L Trizma base (Sigma, Aldrich), 5.5 g/L Boric acid (SRL Ltd., Mumbai), 2.0 ml/L 0.5 M EDTA at 100 V. 100 bp DNA ladder (Promega, #G2101) was used as a molecular base size marker. Photographs of the amplified products were captured using Syngene G-Box, UK gel documentation system.

PCR-RFLP and dendrogram construction

Appropriate restriction enzymes were identified using different accessible nucleotide sequences from NCBI database through Cleaver software (http://cleaver.sourceforge.net/) so as to evaluate different restriction polymorphisms. HhaI (GCG↓C), HinfI (G↓ANTC) and BanI (G↓GYRCC) were selected as appropriate enzymes which were expected to give up distinct and sufficient fragment sizes to produce species specific restriction profiles. Restriction digestion was performed in a reaction volume of 20 µL having HhaI (1unit, Gene Mark; #E143) 0.05 µL; 10X Y buffer 2 µL; BSA 2 µL (0.1%); *rpoB* gene amplified PCR product (1 µg

Table 1. Standard strains of the *Bifidobacterium* species included in this study

No.	Species	Origin	Strain No.
1.	*B. longum* subsp. *longum*	Intestine of adult	DSM-20219
2.	*B. boum*	Bovine rumen	DSM-20432
3.	*B. pseudocatenulatum*	Sewage	DSM-20439
4.	*B. pullorum*	Chicken faeces	DSM-20433
5.	*B. longum* subsp. *infantis*	Intestine of infant	DSM-20088
6.	*B. breve*	Intestine of infant	DSM-20213
7.	*B. animalis*	Chicken faeces	DSM-20105
8.	*B. adolescentis*	Intestine of adult	DSM-20083
9.	*B. bifidum*	Breast-fed infant faeces	DSM-20456
10.	*B. catenulatum*	Human faeces	DSM-16992
11.	*B. thermophilum*	Pig faeces	DSM-20210
12.	*B. merycicum*	Rumen of cattle	DSM-6492

approx). For HinfI and BanI the restriction digestion mixture was prepared having 0.1 µL HinfI (Takara, Shiga, Japan; #1238A) and BanI (1unit, Thermo Scientific; #ER1001), 2 µL 10X H buffer and the *rpoB* gene amplified PCR product (approximately 1 µg.). A total volume of 20 µL was adjusted with Milli Q water (sterile) for the above reactions and incubated for 2 h at 50°C in HhaI digestion and 37°C for HinfI and BanI digestion respectively. After the incubation period completion, the digested PCR products were separated using 3% agarose gel (1X TBE) for 4 h supplemented with ethidium bromide at a final concentration of 10 µg/mL and photographs of the patterns were visualized under gel documentation system. 50 bp DNA ladder (Thermo Fisher Scientific; #SM0371) was used as a molecular marker for estimation of migration distance of individual bands of DNA fragments. RFLP restriction patterns generated from enzyme digestion of all the isolated bifidobacterial isolates and reference standard strains of DSMZ were analysed.

To estimate the group specific positions of the isolates, and their similarity with standard reference strains, fingerprinting profiles generated with each enzyme (HhaI, HinfI and BanI) were evaluated with the restriction profiles of 12 reference cultures. Based on the RFLP fingerprints, binary matrixes were generated using Gel Quest software for each enzyme separately. The restriction fragments which are shorter than 40 bp were not included in this analysis. The data matrices were transferred to MEGA6 software[33] including the standards to estimate distances and the Unweighted Pair Group Method with Arithmetic averages (UPGMA) was implemented for cluster analysis.

RESULTS

Amplification and PCR-RFLP of *rpoB* gene

The DNA extracted from the standard cultures amplified with *rpoB* gene primers (Bilon-*rpoB*-F and Bilon-*rpoB*-R) produced amplicons of 575 bp size in all the isolates. The *rpoB* amplification of bifidobacterial isolates has been depicted in Fig. 1 and PCR-RFLP fingerprinting patterns of standard cultures with HhaI, HinfI and BanI are shown in Fig. 2, 3, 4 respectively.

Dendrogram Analysis

Three dendrograms in respect to each enzyme (HhaI, HinfI and BanI) were constructed on the basis of digested amplified *rpoB* PCR products. HhaI restriction enzyme based fingerprinting patterns of the isolates with standard cultures

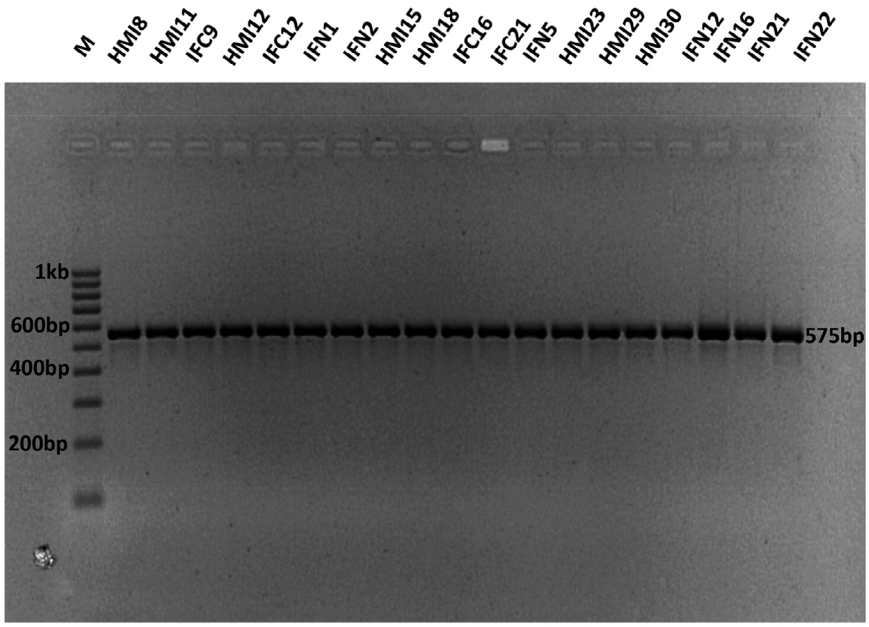

Fig. 1. Agarose gel photographs showing *rpoB* gene amplification of bifidobacterial isolates (M-100 bp DNA size ladder; Lane 1-19 respective isolates)

produced 10 different clusters (Fig. 5). The study conducted on the constructed dendrogram showed 13 isolates clustering with *B. bifidum* in group-I. Nine isolates showed similarity pattern with *B. animalis* in group-II. Three isolates grouped together with *B. catenulatum* in group-III whereas a total of 15 isolates were clustered under *B. longum* subsp. *infantis* in group-IV. Group-V contained 4 isolates which showed similarity outline with *B. longum* subsp. *longum.* The digestion performed with standard strains depicted the clear differentiation between *B. longum* subsp. *longum* and *B. longum* subsp. *infantis*. Eleven isolates with similar RFLP patterns clustered with *B. breve* in group-VI. Five isolates grouped to *B. pullorum* in group-VII. In group-IX both *B. adolescentis* and *B. pseudocatenulatum* were grouped together. Eight isolates clustered with *B. merycicum* in Group-X. Group-VIII contained same 12 isolates as obtained in a separate group in *hsp60* which were identified as *B. ruminantium* in our previous study.[30] None of our isolates grouped with the restriction patterns of *B. boum* and *B. thermophilum* included from the standard isolates list.

Cluster analysis of HinfI based fingerprinting patterns yielded 5 different clusters (Fig. 6). Dendrogram showed a total of 29 isolates clustering with *B. bifidum, B. catenulatum, B. pullorum, B. merycicum, B. boum* and *B. pseudocatenulatum* in Group-I. Thirty isolates grouped mutually with *B. breve*, *B. longum* subsp. *longum* and *B. longum* subsp. *infantis* having same restriction patterns in Group-II. Nine isolates showed a same similarity pattern with *B. animalis* species in Group-III and 13 isolates were clustered under *B. adolescentis* in Group-V. Group-IV contained 12 isolates which did not cluster with any of the standard strain. It was observed that none of our isolated species grouped with *B. thermophilum*. Grouping of two to three species in a cluster was found in HinfI restriction digestion. To some extent HinfI restriction endonuclease does not provide sufficient discriminatory power for differentiation of species and subspecies. Most of the species can be identified by using a single enzyme, HhaI except for a few. But genetically it may be possible that close subspecies and species can be distinguished by *rpoB* PCR-RFLP with the combination of these two enzymes. BanI based fingerprinting patterns of our isolates with standard cultures yielded 5 different clusters (Fig. 7) in the dendrogram. The study conducted on the constructed dendrogram showed 36 isolates clustered with *B. catenulatum*, *B. adolescentis*, *B.*

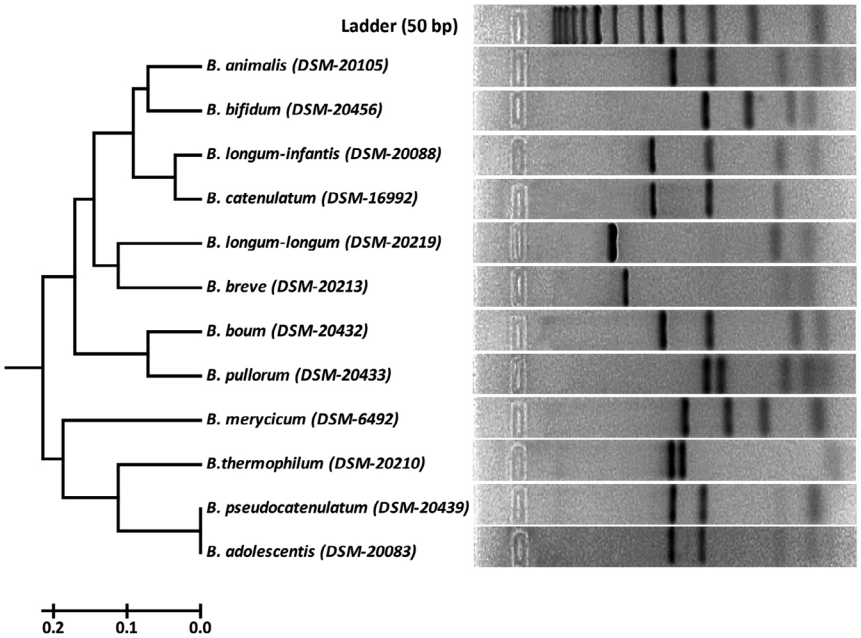

Fig. 2. RFLP fingerprints of *rpoB* PCR amplified products with HhaI (GCG/C) of standard reference isolates.

pseudocatenulatum, *B. boum*, *B. merycicum* and *B. thermophilum* in Group-I. Thirteen isolates grouped with *B. bifidum* in Group-II. Five isolates displayed a same resemblance pattern with *B. pullorum* in Group-III and 30 isolates were clustered under *B. breve*, *B. longum* subsp. *infantis* and *B. longum* subsp. *longum* in Group-IV. Group-V contained 9 isolates which exhibited similarity pattern with *B. animalis*. Group specific band patterns estimated through Gel Quest software for each enzyme are represented in Table 2.

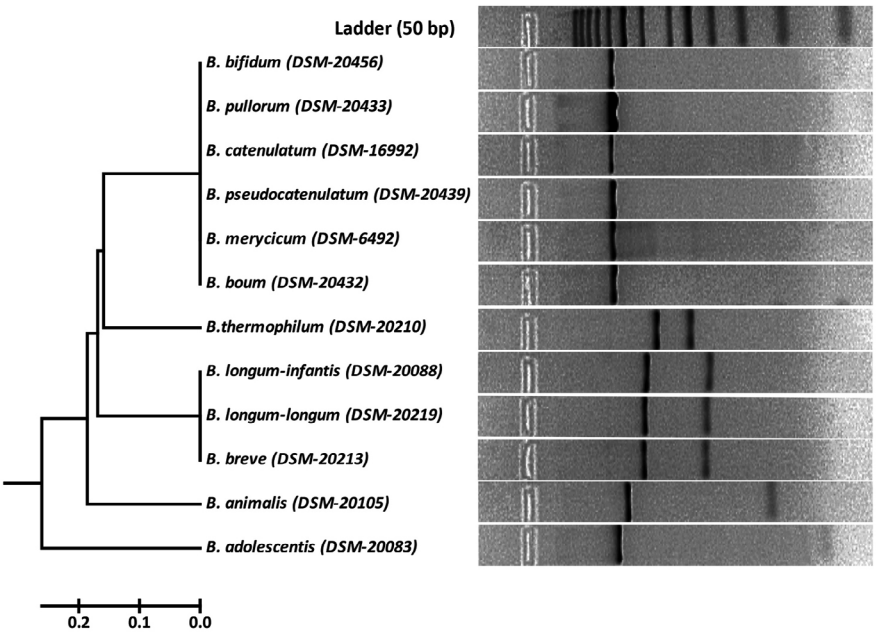

Fig. 3. RFLP fingerprints of *rpoB* PCR amplified products with HinfI (G/ANTC) of standard reference isolates.

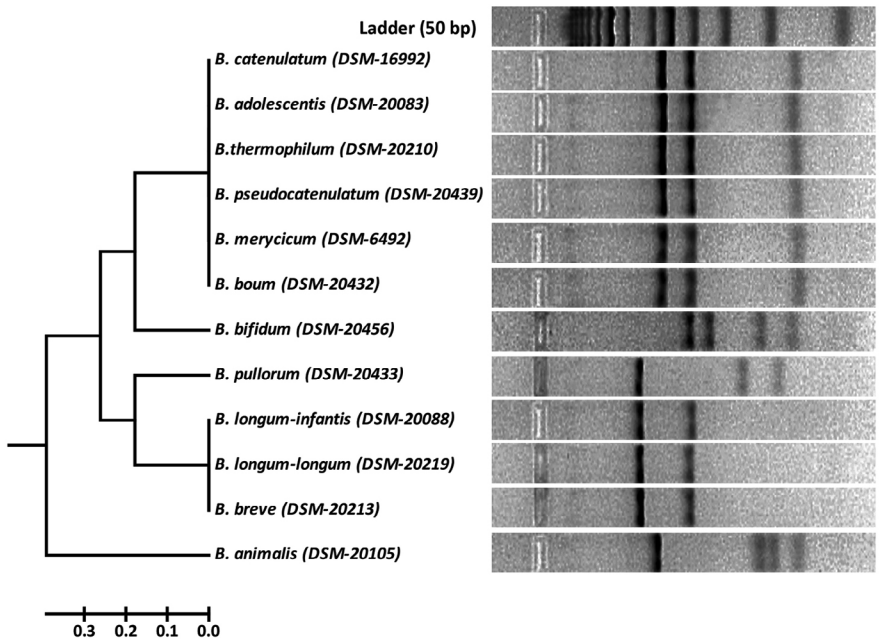

Fig. 4. RFLP fingerprints of *rpoB* PCR amplified products with BanI (G/GYRCC) of standard reference isolates.

DISCUSSION

In the investigation, attempts have been made to demonstrate the efficacy of *rpoB* PCR-RFLP method in discrimination of closely related *Bifidobacterium* up to subspecies level. Baffoni et al.[34] reported that *B. longum* subsp. *infantis* and *B. longum* subsp. *longum* were not identified individually in other genes like *hsp60* PCR-RFLP. But in *rpoB* PCR-RFLP with HhaI enzyme clear differentiation in between *B. longum* subsp. *infantis* and *B. longum* subsp. *longum* was observed (Fig. 2). In another study it was difficult to differentiate in between both the species of *B. longum* using 16S rRNA based

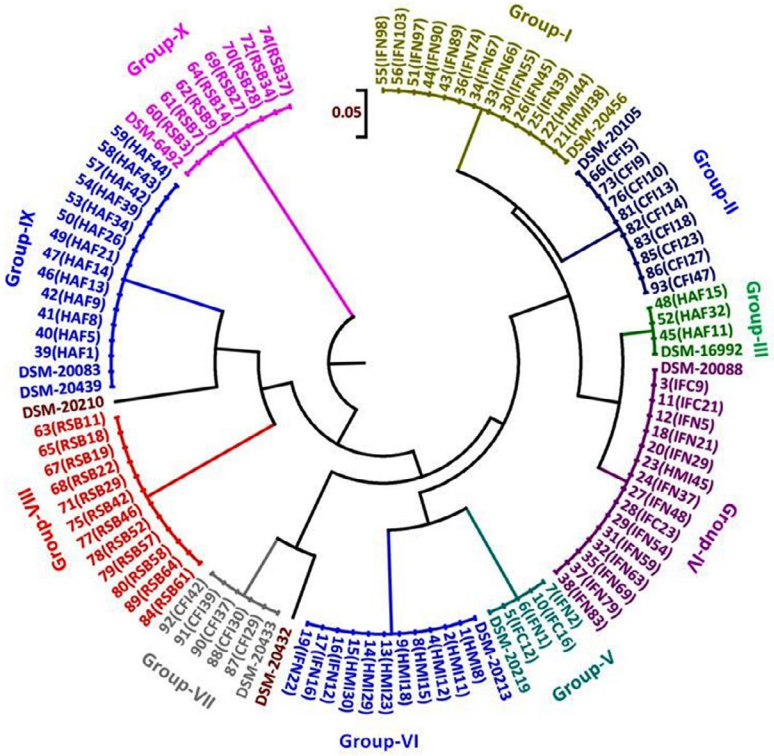

Fig. 5. Dendrogram constructed from RFLP fingerprints of amplified *rpoB* PCR products with HhaI (GCG/C).

Table 2. Group wise estimated band patterns obtained from rpoB PCR-RFLP with respect to each restriction enzyme (approximate DNA fragment size ± 5bp) of the isolated strains

Groups	HhaI		HinfI		BanI	
	Isolates	Band patterns	Isolates	Band patterns	Isolates	Band patterns
Group-I	13	157-106-64-52	29	572	36	265-203
Group-II	9	221-155-76-52	30	363-210	13	203-178-110-76
Group-III	3	272-155-76	9	464-112	5	350-131-85
Group-IV	15	272-154-77-51	12	464-93	30	352-203
Group-V	4	412-77-52	13	518-57	9	282-110-89-76
Group-VI	11	364-76-54				
Group-VII	5	159-138-64-56-43				
Group-VIII	12	220-153-92-74-41				
Group-IX	13	221-166-52				
Group-X	8	196-138-85-43				

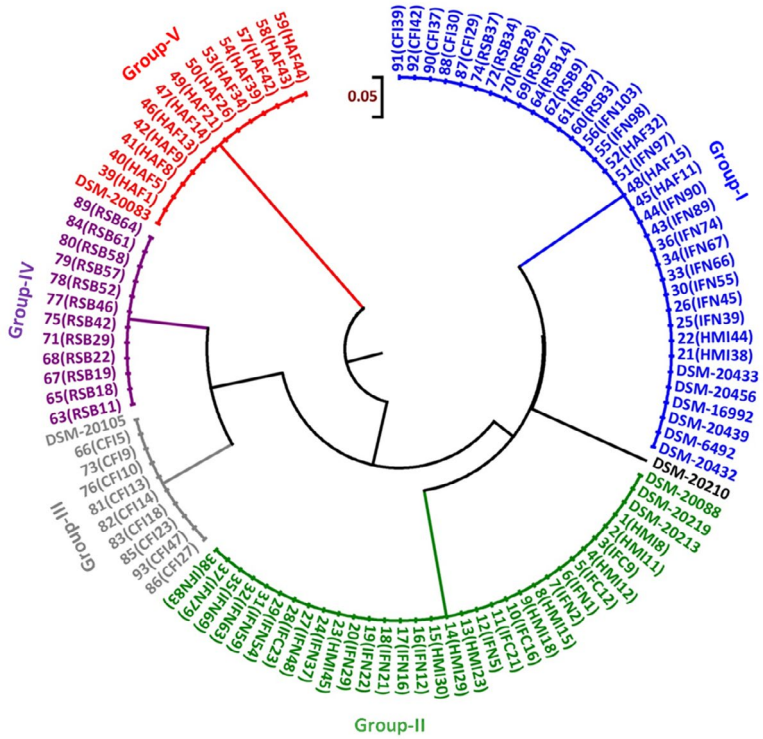

Fig. 6. Dendrogram constructed from RFLP fingerprints of amplified *rpoB* PCR products with HinfI (G/ANTC).

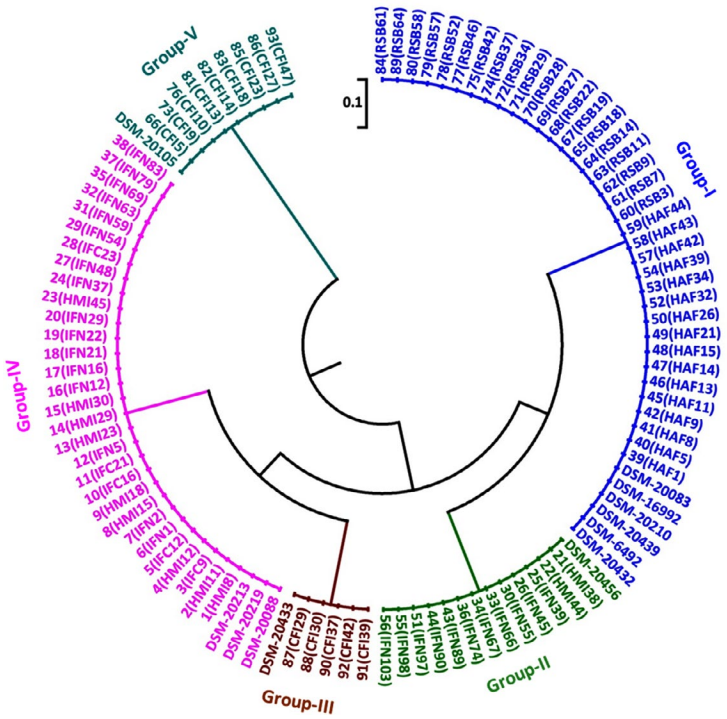

Fig. 7. Dendrogram constructed from RFLP fingerprints of amplified *rpoB* PCR products with BanI (G/GYRCC).

species specific and group specific primers.[35] Sakata et al.[36] unified B. *infantis*, *B. longum* and *B. suis* into a single species but Mattarelli et al.[37] reclassified the 3 biotypes of *B. longum* as 3 subspecies. Deletoile et al.[38] reported MLSA/ MLST method with seven housekeeping genes including *rpoB*, for strain typing in *Bifidobacterium* species and showed clearly demarcated clusters and phylogenetic distinctness of *B. longum* sub-species. Although all the isolates were distinguished into separate groups the two species *B. adolescentis* and *B. pseudocatenulatum* in our study displayed identical restriction patterns which needs successive digestion with other enzyme for both species' differentiation. In our study, *B. pseudocatenulatum* and *B. catenulatum* were distinguished from the restriction profiles in HinfI restriction digestion in spite of having more than 90% similarity in 16S rRNA gene sequence in taken into account. Kim et al.[28] reported that *rpoB* and *hsp60* gene sequences based phylogenetic trees showed more discriminative patterns than the 16S rRNA gene tree. Their finding was especially discernible among related species such as *B. pseudocatenulatum* and *B. catenulatum*, *B. saeculare, B. gallinarum* and *B. pullorum* and *B. pseudolongum* subsp. *pseudolongum* and *B. pseudolongum* subsp. *globosum.* The authors also presented *hsp60* based results which provided clear differentiation between these closely related groups. This substantiated the results of Jian et al.[43] and the present study with respect to discrimination of *B. pseudocatenulatum* and *B. catenulatum* species in different groups.

Matsuki et al.[35] observed that it is very difficult to differentiate *B. adolescentis*, *B. catenulatum* and *B. pseudocatenulatum* based on the conventional sugar fermentation pattern which puzzled the identification between *B. catenulatum* group and *B. adolescentis*. Kim et al.[28] reported 351 bp partial *rpoB* gene sequences to differentiate species of the genus *Bifidobacterium* which proved to be a suitable marker and based on this *B. pseudocatenulatum* and *B. catenulatum* were evidently discriminated. But the authors didn't state their discrimination with regard to *B. longum* subsp. *longum* and *B. longum* subsp. *infantis*. Targeted pyrosequencing method of *rpoB* amplicons (351 bp) revealed the low number of *Bifidobacterium* species with major incidence of *B. longum* and *B. breve* species in the antibiotic-treated infants. [39] In multigenic approach,[40] *rpoB* gene can be a gene of choice for providing detailed molecular analysis due to its easy accessibility and high resolving nucleotide-based molecular typing system.[28] Ventura et al.[41] reported that single gene trees may not provide adequate phylogenetic relationships because of horizontal gene transfer events, inappropriate mutation rates and variable recombination rates. On the other hand, Vos et al.[42] reported that single-copy protein-encoding genes are of benefits in higher sampling efficiency, error-correction and easy survey of homologous recombination rate and mutations effects. But Ventura et al.[17] reported that multigene concatenation approach comprising of conserved orthologous proteins to be more discriminatory for differentiation of bifidobacterial species. However, *rpoB* gene sequences present improved phylogenetic resolution over the 16S rRNA gene.[21] So, *rpoB* gene can be used as a sequence of choice in case of ambiguity when other gene are taken into account for identification.

CONCLUSIONS

PCR-RFLP based on *rpoB* amplified partial gene product can be successfully applied to differentiate in between different species of genus *Bifidobacterium* as well as at sub-species level. The study also indicates that among the restriction enzymes HhaI, was found to be more discriminatory in differentiating different species of the genus as well as at sub species level. In addition to that whenever a single restriction enzyme fails to differentiate in between different species of the genus additional enzymes thereof may be used for inferring the classification of bifidobacterial species targeted on *rpoB* PCR-RFLP analysis.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

None.

DATA AVAILABILITY

All datasets generated or analysed during this study are included in the manuscript.

ETHICS STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors.

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