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EFFECTIVE IDENTIFICATION OF *LACTOBACILLUS PARACASEI* SSP. *PARACASEI-1* BY 16S-23S rRNA INTERGENIC SPACER REGION SEQUENCING

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ABSTRACT

Fermentation profiling is a common tool to identify *Lactobacillus paracasei* from other related members of *Lactobacillus* genus. Because of the proximate biochemical characteristics, identification based on the fermentation pattern within *L. casei* group (*L. casei*, *L. paracasei* and *L. rahmnosus*) has considerable limitation. On the other hand, members of *L. casei* group are genetically very closely related and sequencing of signature region is the most reliable and rapid method of differentiation. In this study, instead of sequencing entire 16S or 23S ribosomal RNA gene, we have sequenced 16s-23s rRNA intergenic spacer region and compared them across the members of *L. casei* group. We also compiled thirteen signature sequences within 16s-23s ISR rRNA gene of *L. casei* group members. Our isolated strain (*L. paracasei* ssp. *paracasei-1*) resembled 100% and 99% similarity when compared with 16s-23s rRNA intergenic spacer region of *L. paracasei* and *L. casei* respectively. Our study summarizes that sequencing of short 16s-23s rRNA intergenic spacer region carries great significance in identification of closely related probiotic bacteria such as the members of *L. casei* group. The findings of this research could be very much helpful for food and pharmaceutical industries who are dealing with probiotic bacteria.

Keywords: Identification, *L. paracasei* ssp. *paracasei-1*, 16s-23s rRNA, signature sequence, probiotic

INTRODUCTION

The genus *Lactobacillus* is subject to continual taxonomic changes. It is often thorny to use classical microbiological methods to identify lactic acid bacteria even to genus level because of their similar nutritional and growth requirements (**Dubernet *et al.*, 2002**). Moreover, identifying a *Lactobacillus* isolate accurately to the species level requires about 17 phenotypic tests (**Tannock *et al.*, 1999**). Even bigger challenge is to accurately identify the species of *L. casei* group based on phenotypic tests (**Ward and Timmins, 1999**). However, confirming the identification of diverse species of lactic acid bacteria is very important for quality control and quality assurance perspective when it is intended for human use (**Tilsala *et al.*, 1997**). Very often identification of *Lactobacillus* spp. appears as a dilemma to the researchers. *L. casei* group is an ideal example which must overcome some unusual and difficult obstacles in order to pacify confusion in their identification, especially within the *L. casei* group members. The taxonomy of *L. casei* group has been in dispute over the transfer of some species and strains from *L. casei* to *L. paracasei* (**Kisworo *et al.*, 2008**). *L. casei* group gone through several rearrangement notably from five member group to four members enlisted in *Bergy's Manual* of *Systematic Bacteriologv* (*L. casei* ssp. *alactosus* was combined with *L. casei* ssp. *casei*) (**Mori *et al.*, 1997**). In 1989 **Collins *et al.* (1989)** reclassified the subspecies of *L. casei* into *L. casei*, *Lactobacillus paracasei*, and *Lactobacillus rhamnosus* on the basis of DNA-DNA relatedness data. Although **Coudeyras *et al.* (2008)** classified *L. casei* into four group members, however, we have considered *L. casei* with three group members including *L. casei*, *L. paracasei* and *L. rhamnosus* based on the latest phylogenic structure revealed by **Salvetti *et al.* (2012).**

Considering these identification enigma, use of a diagnostic marker specific to the probiotic strain of interest carries significant importance. Moreover, the beneficial characteristics associated with the probiotic are strain specific (**Flint *et al.*, 2005).** Therefore nucleotide base sequences of *Lactobacillus* 16S ribosomal DNA (rDNA) is an important reliable analysis which provides an accurate basis for identification (**Tannock *et al.*, 1999**). **Barry *et al.* (1991)** have shown that the ribosomal intergenic regions are more variable between bacterial species compared to 16S and 23S rRNA genes. **Tannock *et al.* (1999)** documented several studies which have demonstrated that the DNA sequence between the 16S and 23S genes of lactobacilli is hypervariable. This intergenic spacer region (ISR) is about 200 bases in length. The 16s-23s spacer sequences of lactobacilli are adequately species specific which can be used as a marker to identify *Lactobacillus* species. In fact, using primer that will amplify 16s-23s ISR region is a relatively simple and quick method through which lactobacilli can be identified without resorting to the use of species-specific PCR primers (**Tannock *et al.*, 1999**). While drawing significant relationship within closely related bacterial species, sequencing comparisons are often applied. **Mori *et al.* (1997)** compared the 16S rRNA sequences of all strains of the *L. casei* subspecies. **Chen *et al.* (2000)** reported intergroup comparisons of the 23S-5S rRNA spacer genes from *Lactobacillus casei* related strains. In this study we have compared the sequences of 16s-23s ISR region of *L. casei* group along with the identification of *L. paracasei* ssp. *paracasei-1*.

Hoque *et al.* (2010) reported the genus of a bacterial isolates as *Lactobacillus* spp. in their study. Based on classical microbiology and biochemical reaction, **Islam *et al.* (2012)** identified the same isolated bacteria reported by **Hoque *et al.* (2010)** as *L. paracasei* ssp. *paracasei-1*. Following that, **Honi *et al.* (2013)** also bolstered the identification, reported by **Islam *et al.* (2012),** as *L. paracasei* ssp. *paracasei-1* on the basis of PCR amplification of 16s-23s ISR region**.** However, none of these reports include sequencing of the ISR region of *L. paracasei* ssp. *paracasei-1*. *L. paracasei* and other members of *L. casei* group share extremely high similarities within the 16s-23s ISR region**.** So there is every possibility of getting a false positive result from PCR amplification based identification while identifying *L. paracasei* without the sequencing of 16s-23s ISR region. Sequencing of the 16s-23s ISR region of previously identified *L. paracasei* ssp. *paracasei-1* (**Islam *et al.*, 2012; Honi *et al.*, 2013**) and comparing the sequenced results across the already reported 16s-23s ISR region sequences of *L. casei* group members, in order to determine signature sequences, are the main focus of this research.

MATERIAL AND METHODS

Collection of test organism

The test organism (*L. paracasei* ssp. *paracasei-1*, isolated from regional yogurt) (**Islam *et al*., 2012; Honi *et al*. 2013**) was collected from the stock culture maintained by the Biochemistry and Molecular biology Laboratory of Biotechnology & Genetic Engineering Discipline, Khulna University.

Subculture of the bacteria

The test organism was grown in MRS broth, added with 0.05% cysteine, (HiMedia/India) and MRS agar (HiMedia/India) plated in an incubator (Shellab/USA) at 37^0C for 16 hrs. pH (Mettler Toledo/Switzerland) of the media was adjusted to 6.5 (**Islam *et al*., 2012; Honi *et al*., 2013**).

Morphological, physiological and biochemical identification

Identification of *Lactobacillus* spp. was verified via gram staining, cell morphology, endospore assay, catalase test, motility test, colony morphology (**Hoque *et al*., 2010; Islam *et al*., 2012; Honi *et al*., 2013**).

Extraction of chromosomal DNA

Chromosomal DNA was extracted from overnight MRS culture, stored at 37^0C, using bacterial DNA preparation kit (jena bioscience/Germany) according to the manufacturer's instructions. Quantity of DNA extracts was checked with a spectrophotometer (Shimadzu: UV-1800 /Japan) (**Skelin *et al*., 2012**).

Quantification of genomic DNA of *Lactobacillus* spp.

Isolation of genomic DNA was confirmed by using horizontal gel electrophoresis system (BioRad: MINI SUB CELL /USA) and gel documentation system (CB Scientfic: ALPHAIMAGER HP /USA). In this case 2% agarose was used. Then quantification of DNA was done by using spectrophotometer. Absorption reading was taken at 260, 280 and 320 nm. Purity, concentration and total yield of DNA were calculated from these readings which are detailed in Table 1.

Table 1 Concentration and purity of extracted DNA

Sample	A260	A280	A320	Concentration[1], µg/ml	Purity[2]	Purity[3]	DNA Yield[4], µg
A	0.412	0.24	0.008	505	1.71	1.74	1010
B	0.424	0.24	0.012	515	1.76	1.80	1030
C	0.448	0.28	0.02	535	1.60	1.64	1070

1 = [(A260-A320) × Dilution Factor (= 25) × 50 µg/ml pure ds DNA]
2 = (A260÷A280)
3 = [(A260-A320)÷(A280-A320)]
4 = [(DNA Concentration in µg/ml × Total Sample volume (=2 ml)]
A260 = Absorption at 260 nm, A280 = Absorption at 280 nm, A320 = Absorption at 320 nm

PCR amplification

To determine the identification up to species level of *L. paracasei* isolates, species-specific PCR assay was carried out with *Lactobacillus paracasei* species-specific primer set, supplied by Bioneer Corp., USA. Primer set Lp1F- (5'-GGGGATCACCCTCAAGCACCCT -3') and Lp1R-(5'-GCGTCAGCGGTTATGCGATGC -3') along with Applied Biosystem's thermal cycler (VERITY/USA) were used **(Honi *et al*., 2013)**. Amplification of DNA from the 16s-23s ISR rRNA gene was carried out using promega PCR kit (Promega Corporation/USA) and PCR operating protocol described by **Honi *et al.* (2013). Table 2** gives information on primer details and **Table 3** explains the PCR sample loading detail. Amplification products were separated on a 2% agarose gel using horizontal gel Electrophoresis System (BioRad: MINI SUB CELL /USA), stained with 2.5% ethidium bromide, visualized and documented using a Gel Documentation System (CB Scientfic: ALPHAIMAGER HP /USA) **(Honi *et al*., 2013).** The amplified PCR products were purified using conventional ethanol based purification method and manufacturer's instruction of BigDye XTerminator Purification kit (AB Science, USA).

Table 2 Primers used for PCR amplification of 16s-23s intergenic spacer region

Primer	Sequences (5' > 3')	Length	Start	Stop	Tm	GC%
Lp1F	GGGGATCACCCTCAAGCACCCT	22	63	84	64.0	63.64
Lp1R	GCGTCAGCGGTTATGCGATGC	21	208	188	64.3	61.90

Table 3 PCR reaction mixture

Sl No.	Reagents	PCR Master Mix (µl)	Forward Primer[1] (µl)	Reverse Primer[1] (µl)	DNA Template[2] (µl)	DNA Template[3] (µl)	Nuclease Free Water (µl)	Total Vol. (µl)
	Sample							
1	Sample 1	12.5	0.5	0.5	3	0	8.5	25
2	Sample 2	12.5	0.5	0.5	3	0	8.5	25
3	Sample 3	12.5	0.5	0.5	3	0	8.5	25
4	Sample 4	12.5	0.5	0.5	0	3	8.5	25
5	Sample 5	12.5	0.5	0.5	0	3	8.5	25
6	Sample 6	12.5	0.5	0.5	3	0	8.5	25
7	Sample 7	12.5	0.5	0.5	3	0	8.5	25
8	Sample 8	12.5	0.5	0.5	3	0	8.5	25
9	Sample 9	12.5	0.5	0.5	0	3	8.5	25
10	Sample10	12.5	0.5	0.5	0	3	8.5	25

1= Stock Concentration (100 nano mole)
2= Sample-B of Table 1
3= Sample-C of Table 1

16s-23s ISR DNA sequence

The PCR products of the amplification were used for re-identification at the species level and to recheck the primer specificity for *Lactobacillus paracasei* ssp. *paracasei-1* as reported by **Honi *et al.* (2013)**. For DNA sequencing we have used sequencing kit (AB science, USA). An in-house developed protocol was used in determining the sequencing. Briefly, in each cycle, during initial denaturation the temperature was increased to 96°C for 1 minute, followed by 96°C and 10 seconds denaturation cycle. Then temperature decreased to 50°C for 5 seconds to anneal the primer to the template, and finally increased to 60°C for 4 minutes during elongation step involving enzymatic synthesis. A total of 25 cycles were completed. Finally the reaction was kept at 4°C. The PCR product was purified using ethanol precipitation method and purification kit. **Table 4** contains the cycle sequencing sample loading detail which was performed in a DNA analyzer unit of Applied Biosystem science (Applied Biosystem: 3500 GENETIC ANALYZER /USA). The obtained sequences were aligned and compared with the *L. casei* group member's 16s-23s rRNA ISR sequences (accession number U32964.1, U32966.1 and AF121200.1) stored at GenBank database (National Center for Biotechnology Information, Bethesda, MD, USA). BLAST and ClustalW2 algorithm were used in this comparison. To test the reliability of the cycle sequencing method, we have sequenced pGEM vector (AB Science, USA) using the same sequencing protocol described above.

Table 4 Cycle sequencing sample preparation:

Sl No.	Reagents	Ready Reaction Mix (µl)	Dilution Buffer (µl)	DNA Template[1], µl	Forward Primer[2], µl	Reverse Primer[2], µl	Nuclease Free Water (µl)	Total Vol. (µl)
	Sample							
1	Sample 1	1.0	1.5	1.5[a]	2.0[b]	0	4.0	10
2	Sample 2	1.0	1.5	1.5	2.0	0	4.0	10
3	Sample 3	1.0	1.5	1.5	0	2.0	4.0	10

1= Sample B of Table 1, 2= Concentration is 100 nano mole, a= pGEM control, b= Forward primer for pGEM

Comparison of 16s-23s ISR of *L. casei* group

Tilsala *et al.* (1997) determined the 16s-23s rRNA sequence of *L. paracasei* (accession number U32964.1) and *L. Rhamnosus* (accession number U32966.1). **Tannock *et al.* (1999)** determined the 16s-23s rRNA sequence of *L. casei* ATCC 334 (accession number AF121200.1). We tried to identify the signature sequences of ISR region within the *L. casei* group. The homology between *L. paracasei* ssp. *paracasei-1* and *L. casei* group members were determined in order to differentiate *L. paracasei* ssp. *paracasei-1* from *L. casei* group members based on 16s-23s ISR rRNA sequencing. Finally we compared 16s-23s ISR rRNA gene sequence of *L. paracasei* ssp. *paracasei-1* with the 16s-23s ISR rRNA regions of *L. casei* and *L. paracasei* to confirm the identity based on sequencing similarity pattern. All the FASTA sequences were obtained from National Center for Biotechnology Information (NCBI).

Three types of alignment were performed using Clustal W2:

1. Alignment of 16s-23s ISR sequence of *L. casei* group (*L. paracasei*, GenBank U32964.1, *L. rhamnosus* (U 32966.1) and *L. casei*, (AF121200.1))
2. Alignment of 16s-23s *L. paracasei* (U32964.1) and *L. casei* (AF121200.1)
3. Alignment of 16s-23s *L. paracasei* (U32964.1), *L. casei* (AF121200.1) and *L. paracasei* ssp. *paracasei-1*

RESULTS

Morphological, physiological and biochemical characterization

The isolate showed their colonies morphology small, circular, white-creamy in colors. Microscopically they were gram-positive rod shaped, non-motile, catalase negative and non spore forming. Their colony and microbiological characteristics have been summarized in **Table 5**. According to their characteristics, the isolates were identified as *Lactobacillus* spp.

Table 5 Different characteristics of isolated *Lactobacillus* spp.

Morphological, physiological and biochemical test	Findings
Gram staining	Positive
Cell morphology	Rod shaped
Endospore staining	Negative
Catalase test	Negative
Motility test	Negative
Colony morphology	Small round, creamy white

Extraction and quantification of genomic DNA from *Lactobacillus* spp.

After performing gel electrophoresis (2% agarose, voltage: 85V) there was a distinctive band of genomic DNA (Figure 1). Quantification of DNA concentration carried out by spectrophotometer. The purity range of isolated DNA of the test organism was within 1.60 to 1.80 **(Table 1)**

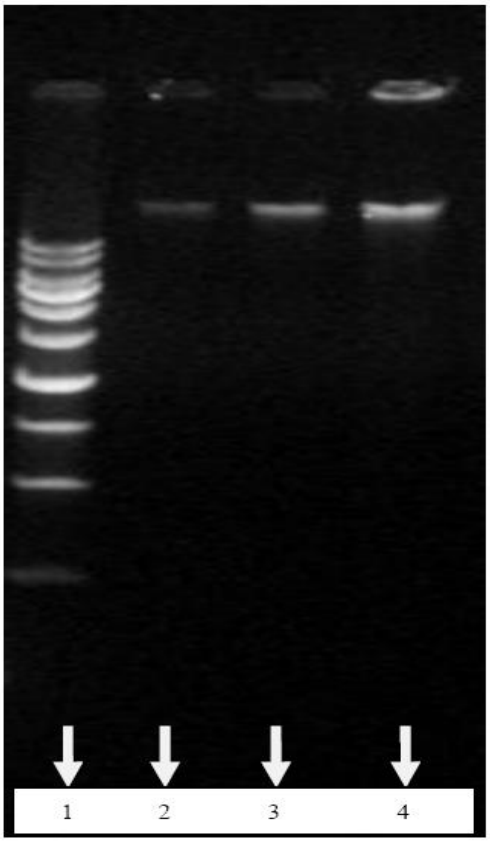

Figure 1 Isolated genomic DNA of *Lactobacillus* spp. (1= 1Kb ladder, 2= sample A, 3= sample B and 4= sample C of Table 1

PCR amplification of the 16s-23s rRNA ISR from *Lactobacillus paracasei* DNA

One primer pair (Lp1F & Lp1R) was used for PCR amplification of spacer region of our *Lactobacillus* spp. PCR product was obtained with the primer pair (Figure 2). The amplified product with the same primer pair has been reported to be 146 bp long (**Honi *et al.*, 2013**).

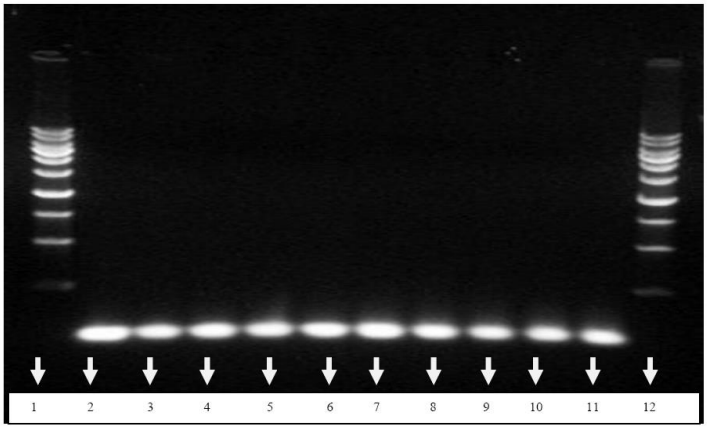

Figure 2 Gel run of PCR amplified sample

Lane 1: 1Kb Ladder	Lane 2: Sample-1	Lane 3: Sample-2	Lane 4: Sample-3	Lane 5: Sample-4	Lane 6: Sample-5
Lane 7: Sample-6	Lane 8: Sample-7	Lane 9: Sample-8	Lane 10: Sample-9	Lane 11: Sample-10	Lane 12: 1Kb Ladder

16s-23s ISR rRNA gene sequencing result

Obtained forward sequence using forward primer Lp1F

>

TTGAAAACTGGATATCATTGTATTAATTGTTTTAAATTGCCGAGAACACAGCGTATTTGTATGAGTTTCTGAA
AAAGAAATTCGCATCGCATAACCGCTGACGCAA

Query sequence (obtained forward sequence) **:**

1 TTGAAAACTG GATATCATTG TATTAATTGT TTTAAATTGC CGAGAACACA GCGTATTTGT
61 ATGAGTTTCT GAAAAAGAAA TTCGCATCGC ATAACCGCTG ACGCAA

Subject sequence (*Lactobacillus paracasei paracasei*, 16s-23s rRNA spacer region, U32964.1)

1 CTAAGGAAAC AGACTGAAAG TCTGACGGAA ACCTGCACAC ACGAAACTTT GTTTAGTTTT
61 GAGGGGATCA CCCTCAAGCA CCCTARCGGG TGCGACTTTG TTCTTTGAAA ACTGGATATC
121 ATTGTATTAA TTGTTTTAAA TTGCCGAGAA CACAGCGTAT TTGTATGAGT TTCTGAAAAA
181 GAAATTCGCA TCGCATAACC GCTGACGCAA GTCAGTACAG G

Sequence ID: lcl|49579Length: 221Number of Matches: 1

Score	Expect	Identities	Gaps
196 bits(106)	1e-55	106/106(100%)	0/106(0%)

Query 1 TTGAAAACTGGATATCATTGTATTAATTGTTTTAAATTGCCGAGAACACAGCGTATTTGT 60
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 105 TTGAAAACTGGATATCATTGTATTAATTGTTTTAAATTGCCGAGAACACAGCGTATTTGT 164

Query 61 ATGAGTTTCTGAAAAAGAAATTCGCATCGCATAACCGCTGACGCAA 106
||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 165 ATGAGTTTCTGAAAAAGAAATTCGCATCGCATAACCGCTGACGCAA 210

Among 221 bases of reference sequence 106 bases perfectly matched with the results of sample's forward sequence.

Obtained reverse sequence using reverse primer Lp1 R:

>

ATACAAATACGCTGTGTTCTCGGCAATTTAAAACAATTAATACAATGATATCCAGTTTTCAAAGAACAAAGT
CGCACCCGYTAGG

Blasting was performed in following way

Query sequence (obtained reverse sequence)**:**

1 ATACAAATAC GCTGTGTTCT CGGCAATTTA AAACAATTAA TACAATGATA TCCAGTTTTC
61 AAAGAACAAA GTCGCACCCG YTAGG

Subject sequence (*Lactobacillus paracasei paracasei*, 16s-23s rRNA Spacer Region, U32964.1):

1 CTAAGGAAAC AGACTGAAAG TCTGACGGAA ACCTGCACAC ACGAAACTTT GTTTAGTTTT
61 GAGGGGATCA CCCTCAAGCA CCCTARCGGG TGCGACTTTG TTCTTTGAAA ACTGGATATC
121 ATTGTATTAA TTGTTTTAAA TTGCCGAGAA CACAGCGTAT TTGTATGAGT TTCTGAAAAA
181 GAAATTCGCA TCGCATAACC GCTGACGCAA GTCAGTACAG G

Sequence ID: lcl|19777Length: 221Number of Matches: 1

Score	Expect	Identities	Gaps
154 bits(83)	6e-43	85/85(100%)	0/85(0%)

Query 1 ATACAAATACGCTGTGTTCTCGGCAATTTAAAACAATTAATACAATGATATCCAGTTTTC 60
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 166 ATACAAATACGCTGTGTTCTCGGCAATTTAAAACAATTAATACAATGATATCCAGTTTTC 107

Query 61 AAAGAACAAAGTCGCACCCGYTAGG 85
|||||||||||||||||||||||||
Sbjct 106 AAAGAACAAAGTCGCACCCGYTAGG 82

Among 221 bases of reference sequence 85 bases perfectly matched, shown in bold, with the results of sample's reverse sequence. Sequence given below, shown within box, shows the forward primer position within the reference sequence. Good signal sequence was obtained after 20 initial bases which continued up to 210 no. base.

Forward

1 CTAAGGAAAC AGACTGAAAG TCTGACGGAA ACCTGCACAC ACGAAACTTT GTTTAGTTTT
61 GAGGGGATCA CCCTCAAGCA CCCTARCGGG TGCGACTTTG TTCTTTGAAA ACTGGATATC
121 ATTGTATTAA TTGTTTTAAA TTGCCGAGAA CACAGCGTAT TTGTATGAGT TTCTGAAAAA
181 GAAATTCGCA TCGCATAACC GCTGACGCAA GTCAGTACAG G

The following sequence, shown within box, shows the reverse primer position within the reference sequence. Good signal sequence was obtained after 21 bases which continued up to 82 no. base.

Reverse

1 CTAAGGAAAC AGACTGAAAG TCTGACGGAA ACCTGCACAC ACGAAACTTT GTTTAGTTTT
61 GAGGGGATCA CCCTCAAGCA CCCTARCGGG TGCGACTTTG TTCTTTGAAA ACTGGATATC
121 ATTGTATTAA TTGTTTTAAA TTGCCGAGAA CACAGCGTAT TTGTATGAGT TTCTGAAAAA
181 GAAATTCGCA TCGCATAACC GCTGACGCAA GTCAGTACAG G

After combining both the results it was found that almost 148 bases, shown within box, were completely matched with the reference sequence.

Combined result:

1 CTAAGGAAAC AGACTGAAAG TCTGACGGAAACCTGCACAC ACGAAACTTT GTTTAGTTTT
61 GAGGGGATCA CCCTCAAGCA CCCTARCGGG TGCGACTTTG TTCTTTGAAA ACTGGATATC
121 ATTGTATTAA TTGTTTTAAA TTGCCGAGAA CACAGCGTAT TTGTATGAGT TTCTGAAAAA
181 GAAATTCGCA TCGCATAACC GCTGACGCAA GTCAGTACAG

Sequences of the 16s-23s ISR rRNA gene of isolated *L. paracasei* ssp. *paracasei-1*

From the combined cycle sequencing result we did determine the intended region designed for our primer. Sequence of *L. paracasei* ssp. *paracasei-1* showed 100% similarity (comparison - 3) with the 16s-23s ISR sequence of *L. paracasei* (accession number U32964.1).

Comparison of 16s-23s ISR rRNA gene of *L. casei* group and *L. paracasei* ssp. *paracasei-1*

The 16s-23s ISR rRNA gene sequences of the *L. casei* group species obtained from the data bank **(**accession number U32964.1, U32966.1 & AF121200.1) and shown in comparison 1. While comparing *L. casei* and *L. paracasei* sequences we saw that *L. paracasei* possess four extra bases at position no 1, 2, 220 & 221 (comparison 2). Apart from that, we observed a single base difference at 86 no. position. Then we blasted ISR sequence of *L. paracasei* ssp. *paracasei-1* with *L. paracasei* and *L. casei* in order to confirm the identification. From these blasts (comparisons 1 – 3, Table 6 & 7) we became 100% ascertain that our isolate was *L. paracsei* ssp. *paracasei-1.*

Table 6 Signature sequences between the members of *L. casei group* and *L. paracasei* ssp. *paracasei-1*

Nucleotide position[1]	**1**	**2**	**69**	**86**	**126**	**127**	**131**	**175**	**177**	**179**	**180**	**221**	**222**
*L. rhamnosus (*U32966.1)	*C*	*T*	*T*	*G*	*T*	*G*	*A*	*A*	*T*	*A*	*T*	*G*	*G*
L. paracasei (U32964.1)	*C*	*T*	*C*	***R***	*A*	*T*	*T*	*G*	*A*	-	*A*	*G*	*G*
L. casei (AF121200.1)	-	-	*C*	***A***	*A*	*T*	*T*	*G*	*A*	-	*A*	-	-
L. paracasei ssp. *paracasei-1*			*C*	***R***	*A*	*T*	*T*	*G*	*A*	-	*A*		

1= From the 5' end of the 16s-23s ISR rRNA sequence of *L. rhamnosus*

Table 7 Comparison of 16s-23s ISR rRNA region of isolated *L. paracasei* ssp. *paracasei-1* with *L. casei* group members

Strain	Homology with the sequence of *L. paracasei*- ssp. *paracasei-1*	Sequencing start-end position[1]	Position no. where difference were found [1]
L. paracasei (U32964.1)	100%	63-210	None
L. casei (AF121200.1)	99%	63-210	86
L. rhamnosus (U32966.1)	93%	63-210	69, 86, 126, 127, 131, 175, 177, 178,179,180,

1= Numbering position with respect to *L. rhamnosus*

Comparison 1 CLUSTAL 2.1 multiple sequence alignment no. 01 (*L. rhamnosus* (U32966.1), *L. paracasei* (U32964.1) and *L. casei* (AF121200.1))

```
gi|1854588|gb|U32966.1|LRU3296      CTAAGGAAACAGACTGAAAGTCTGACGGAAACCTGCACACACGAAACTTT 50
gi|1854587|gb|U32964.1|LPU3296      CTAAGGAAACAGACTGAAAGTCTGACGGAAACCTGCACACACGAAACTTT 50
gi|5902977|gb|AF121200.1|           --AAGGAAACAGACTGAAAGTCTGACGGAAACCTGCACACACGAAACTTT 48
                                      **************************************************

gi|1854588|gb|U32966.1|LRU3296      GTTTAGTTTTGAGGGGATTACCCTCAAGCACCCTAGCGGGTGCGACTTTG 100
gi|1854587|gb|U32964.1|LPU3296      GTTTAGTTTTGAGGGGATCACCCTCAAGCACCCTARCGGGTGCGACTTTG 100
gi|5902977|gb|AF121200.1|           GTTTAGTTTTGAGGGGATCACCCTCAAGCACCCTAACGGGTGCGACTTTG 98
                                    ****************** ***************** **************

gi|1854588|gb|U32966.1|LRU3296      TTCTTTGAAAACTGGATATCATTGTTGTAAATGTTTTAAATTGCCGAGAA 150
gi|1854587|gb|U32964.1|LPU3296      TTCTTTGAAAACTGGATATCATTGTATTAATTGTTTTAAATTGCCGAGAA 150
gi|5902977|gb|AF121200.1|           TTCTTTGAAAACTGGATATCATTGTATTAATTGTTTTAAATTGCCGAGAA 148
                                    **************************: ***:******************

gi|1854588|gb|U32966.1|LRU3296      CACAGCGTATTTGTATGAGTTTCTAATAATAGAAATTCGCATCGCATAAC 200
gi|1854587|gb|U32964.1|LPU3296      CACAGCGTATTTGTATGAGTTTCTGAAA-AAGAAATTCGCATCGCATAAC 199
gi|5902977|gb|AF121200.1|           CACAGCGTATTTGTATGAGTTTCTGAAA-AAGAAATTCGCATCGCATAAC 197
                                    ************************.*:* :********************

gi|1854588|gb|U32966.1|LRU3296      CGCTGACGCAAGTCAGTACAGG 222
gi|1854587|gb|U32964.1|LPU3296      CGCTGACGCAAGTCAGTACAGG 221
gi|5902977|gb|AF121200.1|           CGCTGACGCAAGTCAGTACA-- 217
                                    **********************
```

Comparison 2 CLUSTAL 2.1 multiple sequence alignment no. 03 (*L. paracasei* (U32964.1) and *L. casei* (AF121200.1))

```
gi|1854587|gb|U32964.1|LPU3296      CTAAGGAAACAGACTGAAAGTCTGACGGAAACCTGCACACACGAAACTTT 50
gi|5902977|gb|AF121200.1|           --AAGGAAACAGACTGAAAGTCTGACGGAAACCTGCACACACGAAACTTT 48
                                      **************************************************

gi|1854587|gb|U32964.1|LPU3296      GTTTAGTTTTGAGGGGATCACCCTCAAGCACCCTARCGGGTGCGACTTTG 100
gi|5902977|gb|AF121200.1|           GTTTAGTTTTGAGGGGATCACCCTCAAGCACCCTAACGGGTGCGACTTTG 98
                                    ********************************** **************

gi|1854587|gb|U32964.1|LPU3296      TTCTTTGAAAACTGGATATCATTGTATTAATTGTTTTAAATTGCCGAGAA 150
gi|5902977|gb|AF121200.1|           TTCTTTGAAAACTGGATATCATTGTATTAATTGTTTTAAATTGCCGAGAA 148
                                    **************************************************

gi|1854587|gb|U32964.1|LPU3296      CACAGCGTATTTGTATGAGTTTCTGAAAAAGAAATTCGCATCGCATAACC 200
gi|5902977|gb|AF121200.1|           CACAGCGTATTTGTATGAGTTTCTGAAAAAGAAATTCGCATCGCATAACC 198
                                    **************************************************

gi|1854587|gb|U32964.1|LPU3296      GCTGACGCAAGTCAGTACAGG 221
gi|5902977|gb|AF121200.1|           GCTGACGCAAGTCAGTACA-- 217
                                    **********************
```

Comparison 3 CLUSTAL 2.1 multiple sequence alignment (*L. paracasei* (U32964.1), *L. casei* (AF121200.1) and *L. paracasei* ssp. *paracasei-1* (Our isolate))

```
gi|1854587|gb|U32964.1|LPU3296      CTAAGGAAACAGACTGAAAGTCTGACGGAAACCTGCACACACGAAACTTT 50
gi|5902977|gb|AF121200.1|           --AAGGAAACAGACTGAAAGTCTGACGGAAACCTGCACACACGAAACTTT 48
gi|KU|gb|AFKU|                      --------------------------------------------------

gi|1854587|gb|U32964.1|LPU3296      GTTTAGTTTTGAGGGGATCACCCTCAAGCACCCTARCGGGTGCGACTTTG 100
gi|5902977|gb|AF121200.1|           GTTTAGTTTTGAGGGGATCACCCTCAAGCACCCTAACGGGTGCGACTTTG 98
gi|KU|gb|AFKU|                      -----------GGGGATCACCCTCAAGCACCCTARCGGGTGCGACTTTG 38
                                               ********************** **************

gi|1854587|gb|U32964.1|LPU3296      TTCTTTGAAAACTGGATATCATTGTATTAATTGTTTTAAATTGCCGAGAA 150
gi|5902977|gb|AF121200.1|           TTCTTTGAAAACTGGATATCATTGTATTAATTGTTTTAAATTGCCGAGAA 148
gi|KU|gb|AFKU|                      TTCTTTGAAAACTGGATATCATTGTATTAATTGTTTTAAATTGCCGAGAA 88
                                    ************************************************

gi|1854587|gb|U32964.1|LPU3296      CACAGCGTATTTGTATGAGTTTCTGAAAAAGAAATTCGCATCGCATAACC 200
gi|5902977|gb|AF121200.1|           CACAGCGTATTTGTATGAGTTTCTGAAAAAGAAATTCGCATCGCATAACC 198
gi|KU|gb|AFKU|                      CACAGCGTATTTGTATGAGTTTCTGAAAAAGAAATTCGCATCGCATAACC 138
                                    ************************************************

gi|1854587|gb|U32964.1|LPU3296      GCTGACGCAAGTCAGTACAGG 221
gi|5902977|gb|AF121200.1|           GCTGACGCAAGTCAGTACA-- 217
gi|KU|gb|AFKU|                      GCTGACGCAA---------- 148
                                    **********
```

DISCUSSION

Morphological, physiological and biochemical identification of collected test organism

The isolated bacteria were grown on MRS selective media. Addition of 0.05% cysteine in MRS media increased the specificity for isolation of *Lactobacillus* spp. (**Hoque *et al.*, 2010; Hartemink *et al.*, 1997**). Small, rounded, creamy, white colony and rod-shaped cells further support our claim on morphological background. The isolate was found to be gram positive and at the same time, endospore, catalase and motility negative, summing it up to be a *Lactobacillus* sp. These characteristics can be correlated with **Hoque *et al.* (2010)**, **Islam *et al.* (2012)** and **Honi *et al.* (2013)**.

Extraction of chromosomal DNA

In gel electrophoresis, extracted DNA appeared as a distinct band denoting the manufacturer's isolation protocol was mild to generate enough DNA yield. Spectrophotometer revealed good quality DNA ratio was isolated with very good yield (1010-1070 µg/ml). Quantification of DNA concentration was done at 260 nm, 280 nm and 320nm.

PCR amplification of 16s-23s rRNA gene ISR of *Lactobacillus paracasei* ssp. *paracasei*-1

In this study, in order to sequence the 16s-23s ISR rRNA gene, we have used primer pairs Lp1F and Lp1R (**Honi *et al*., 2013**). The primer pairs specifically anneal and amplify 16s-23s ISR rRNA gene when correct genomic DNA was present during the thermal cycling reaction. The PCR condition was same as described by **Honi *et al.* (2013)**. PCR amplification with the primer pairs followed by electrophoresis produced bands. **Honi *et al.* (2013)** reported an outcome of 146 bp band, using the same primer pairs Lp1F and Lp1R, which was in exact concordance to *L. paracasei* ssp.

Sequences of the 16s-23s ISR rRNA gene of *L. paracasei* ssp. *paracasei-1*

The internal spacer region of 16s-23s rRNA gene sequence of *Lactobacillus* genus is 221 bp long (**Tilsala *et al*., 1997**). Sequence signals of pGEM vector confirmed the reliability of our hardware and the protocol used in the sequencing purpose. We have obtained 148 nucleotides sequences of the 16s-23s ISR rRNA gene homologous to position no. 63 to 210 of the *L. paracasei* ssp. *paracasei-1* using PCR DNA-sequencing method. There is scope, for the future, to improve the primer design so that it can bind more upstream position of the 16s-23s ISR region of *L. paracasei* ssp. *paracasei-1*.

Comparison of 16s-23s ISR rRNA gene of *L. casei* group

There have been lots of controversies over the identification of *L. casei* group *(L. casei*, *L. paracasei and L. rhamnosus*) (**Ward *et al*., 1999**). A few species of *Lactobacillus*, including species of *L. casei* group, may be recovered from a variety of diverse habitats because of their ecological adaptability. It is empirical that bacterial genome adaptation and evolution occur via three major processes: 1) decay of genes that no longer offer a fitness benefit 2) gaining of exogenous genes or gene clusters by bacteria through horizontal gene transfer (HGT) and 3) modification of existing genes by mutation with vertical inheritance (**Broadben *et al*., 2012**). In our previous experiment (**Honi *et al.*, 2013**) we supported that the bacteria reported by **Hoque *et al.* (2010)** and **Islam *et al.* (2012)** was *Lactobacillus paracasei* ssp. *paracasei-1*. We obtained PCR amplification of 16s-23s ISR rRNA gene based on which we reported the isolate as *Lactobacillus paracasei* ssp. *paracasei-1*. Because of the taxonomic dispute and close genetic similarities, however, in this study we extrapolate that the isolate could be one of the members of *L. casei* group rather than *L. paracasei*. We wanted to confirm the identification based on nucleotide sequencing, more specifically by sequencing of 16s-23s ISR rRNA gene, which provides an accurate basis for identification (**Tannock *et al*., 1999**). In doing so, we had to compile the sequence signature of 16s-23s ISR rRNA genes of *L. casei* group obtained from the NCBI data bank (comparison 1). By comparing 16s-23s ISR rRNA gene (217 to 222 bp) across the three members of *L. casei* group we have identified thirteen signature sequence locations. **Mori *et al.* (1997)** also reported thirteen signature sequences in *L. casei* group members by comparing, however, 1.5 Kbp long 16rDNAs of *L. casei* group. The high frequency of variability between 16s-23s ISR rRNA gene could have evolved because of the higher sequence and fragment length polymorphism (**Felis *et al*., 2001**). From the determined signature sequences between these three species of *L. casei* group, we were confirmed that our isolated stain (*L. paracasei* ssp. *paracasei-1*) was *L. paracasei.*

Between *L. paracasei* (U32964.1) and *L. casei* (AF121200.1) we have observed five signature sequence for 16s-23s ISR sequence, based on *L. paracasei* numbering, chronologically which were base position no. 1, 2, 86, 220, and 221 (comparison 2). The base pair location 86, based on *L. paracasei* numbering, appeared to be a critical signature sequence for separating *L. casei* and *L. paracasei*. It suggests that in order to differentiate *L. paracasei* from *L. casei*, based on 16s-23s ISR rRNA gene sequencing, we have to confirm the base pair of location no. 86. When we blasted the sequence of 16s-23s ISR rRNA gene of *L. paracasei* ssp. *paracasei-1* with strain U32964.1 (*L. paracasei*) and AF121200.1 (*L. casei*) we have found only difference at position no 86 (comparison 3). In *L. paracasei* ssp. *paracasei-1* sequence it was R (A or G) which matched with *L. paracasei* strain. The result of comparison-3 (*L. paracasei* ssp. *paracasei-1*, *L. casei* and *L. paracasei*) reveals that our isolate was *L. paracasei* ssp *paracasei-1*.

CONCLUSION

Our study shows that comparison of the percentages of similarity between 16s-23s ISR rRNA gene sequences of lactobacilli provides a practical method of strain identification. The small 16s-23s ISR rRNA gene spacer sequences of lactobacilli are around 200 bp in length. These relatively short sequences can be easily sequenced on both polynucleotide strands and provide reliable information for comparative bacterial identification research. Moreover, sequencing of the spacer region restrains the advantage in distinguishing *Lactobacillus* spp from the members of *L. casei* group which cannot be accomplished by comparison of 16S V2-V3 region sequences. The findings of this research is a very strong bolstering evidence for the research works of **Islam *et al.* (2012)** and **Honi *et al.* (2013)** who also identified the bacteria, in different approaches though, as *L. paracasei*

ssp. *paracasei-1*. We suggest that while differentiating between *L. caesi* and *L. paracasei*, the base position no. 86, based on *L. paracasei* numbering, can solve the identification dilemma. Purine (R) base at position no. 86 confirms the strain as *L. paracasei*. Similarly, it can be suggested that other closely related bacterial group, as like *L. casei* group, can be separated from the group members by sequencing 16s-23s ISR rRNA gene because of the higher sequence and fragment length polymorphism. The series of our research activities (**Hoque *et al.*, 2010; Islam *et al.*, 2012** and **Honi *et al.*, 2013),** including this one, can be utilized in screening potential probiotic bacteria for food and pharmaceuticals application.

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