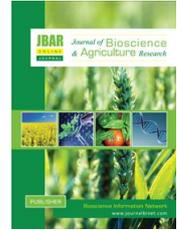


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Molecular analysis of the isolated probiotic microorganisms from yoghurt samples, distributed in Khulna and Chittagong City of Bangladesh, using RAPD marker

Mithila Farjana, Khondoker Moazzem Hossain and Sana Mohammad Mahbubur Rahman

Biotechnology and Genetic Engineering Discipline, Life Science School, Khulna University, Khulna-9208, Bangladesh

✉ For any information: xyz007.bio@gmail.com (Farjana, M)

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ABSTRACT

Yogurt is among the most common dairy products being consumed around the world. During the last decade, probiotics have become an important trend and being used as a useful component regarding the development of functional foods and as substitutes of antibiotics. Probiotics are living, non-pathogenic microorganisms which has profuse beneficial health benefits. The study was aimed at isolation, DNA extraction and finally, molecular characterization using RAPD based PCR from 10 yogurt samples from Khulna and Chittagong Divisions of Bangladesh. Four random 10-mer RAPD primers viz. OPA 2, OPA 5, OPA 9, and OPA 18 were used for amplification. Among them, OPA 18 showed the highest number of amplified bands. The RAPD profiles were demonstrated by Neighbor-joining algorithms. According to the phylogenetic tree, the pair having lower genetic distance was closely related and made cluster. The analysis manifested that, the genetic distance value ranged from 0.14286 to 0.85714. Both genetic distances and dendrograms were determined by using PAUP software. Although most RAPD fragments were common to all ten isolates, where a lower number of polymorphic fragments (lower genetic diversity) were observed that allowed the clear distinction of the isolates. The results indicate that RAPD analysis could be a useful tool to study the molecular landscape on regional native probiotics of Bangladesh.

Key Words: Probiotics microorganisms, RAPD marker, PCR, Primer and Molecular landscape

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I. Introduction

Living microorganisms which render beneficial health effects through maintaining a healthy gut environment, when consumed as food supplementary, are considered as probiotics (Hotel and Cordoba, 2001). Probiotics are considered 'Generally Regarded as Safe' (Liong, 2008). Proper restoration of gut micro-flora and nutritional metabolism, probiotics act as fundamental precursors (Hill et al., 2014). The

beneficial effects of probiotics after consumption directly linked with the doses they are ingested for (Ouweland, 2017). The beneficial facets of probiotics were unraveled by Eli Metchnikoff in the early 20th century, who is known as the forefather of modern probiotics. He first elucidated the function of probiotics in modulating the immune system attributed by Lactic acid bacteria LAB (Anukam and Reid, 2007). There is a myriad of probiotics among which two particular species such as *Lactobacillus* and *Bifidobacterium* tend to exert fundamental treatments against intestinal pandemonium (Marco et al., 2006). Therefore, probiotics supposed to be a central subject of study nowadays and in the last century, a plethora of studies are presenting the crucial role of this type of bacteria in regulating respiratory, gastrointestinal, and immunological functions (Floch et al., 2011). Furthermore, antibacterial substances, for instance, bacteriocins (Cotter et al., 2005) and basic metabolites like, lactic acid and acetic acid are also released by probiotics as a shield of defense by both competing with internal and external pathogens (Servin, 2004). Similarly, selective probiotic strains have reported manipulating the activation of complement system (Jeon et al., 2016), as the complement system act as opsonins which are responsible for inducing secondary immune response inside the body (Al Azad et al., 2016) as part of immune activation after particular pathogenic attack.

Yogurt, cheese, and fermented milk products are the main food sources of probiotics (Mazza, 1998; Balakrishnan and Floch, 2012). Fermented milk products and raw milk possess an enormous number of microbiota encompassing genera *Lactobacillus*, *Enterococcus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Oenococcus*, *Carnobacterium*, *Streptococcus*, and *Weissella* (Federici et al., 2014; Henríquez-Aedo et al., 2016 and Nero et al., 2008). Nowadays, the prominent and relentless health benefits are provoking mass population to take probiotics as a top concern which is leading global demand for it correspondingly. According to the report of the “probiotics market by application”, the market for probiotics was estimated at USD 49.4 billion in 2018, and it is projected to a high rate grow to reach USD 69.3 billion by 2023 (<https://www.marketsandmarkets.com/PressReleases/probiotics.asp>).

Focusing on the high demand for probiotics, now it has been one of the topmost research topics to study about probiotic bacteria (Lactic Acid Bacteria) at the molecular level. As part of the evaluation, it is not nonredundant to explore the properties of probiotic strains and establish their probiotic character thereby, an accurate method of strain detection and identification should be considered. Biochemical tests of probiotics such as- catalase test, sugar fermentation test, arginine hydrolysis, aesculin hydrolysis, nitrate reduction, citrate utilization (Kavitha et al., 2016); pH tolerance, bile tolerance, and phenol tolerance (Abdullah-Al-Mamun et al., 2016) are conventionally used for phenotypically characterization purposes. Despite having some limitations in the staggering of many isolates having similar physiological characteristics, these tests are considered inadequate. To overcome this obstacle, several DNA-based approaches have been developed (Mohania et al., 2008).

It is worthy to mention that, Random Amplification of Polymorphic DNA (RAPD) is one of the most widely used molecular methods among existing techniques, which has already proved its efficacy for the assessment of genetic diversity (Williams et al., 1990) bypassing 16S rRNA gene sequencing. The RAPD technique is a PCR-based discrimination method in which short arbitrary primers anneal to multiple random target sequences (Welsh et al., 1990) resulting in patterns of diagnostic value. Even, interspecific and intraspecific differentiation might be obtained following RAPD-PCR typing (Du Plessis and Dicks, 1995). Using poultry sources in Mymensingh, Bangladesh, the detection, molecular characterization, and screening of probiotic functions of *Lactobacillus* species have been conducted partially (Lutful Kabir et al., 2016). Literature reveals that research regarding the molecular characterization of probiotic isolates from yogurt sources has not been conducted so far in Bangladesh. Observing all the aforementioned facts, the current research work was designed to the objectives of DNA extraction of probiotic bacteria isolated from different regional yogurt samples and molecular analysis of the probiotic isolates based on the RAPD-PCR method.

II. Materials and Methods

Sample collection

In the experiment, yogurt samples were collected within a couple of hours of their preparation from several sweetmeat shops of Khulna and Chittagong City of Bangladesh to isolate as the main intended source of probiotics which were stored at 4°C in the refrigerator. The investigation length was from

October, 2018 to March, 2019 and was conducted at the Animal Cell Culture Laboratory of Biotechnology and Genetic Engineering Discipline at Khulna University, Bangladesh.

Isolation of presumptive probiotics

The collected ten samples were thawed at room temperature and 200mg of each sample were taken for serial dilution using peptone water to ensure the primary culture in De Man, Rogosa, and Sharpe (MRS) agar medium. The Primary cultures of the samples were taken for primary subculture preparation after incubating for 24hr at 37°C in the incubator (INCUCCELL V 55 - ECO line, Spain). Gradually, 8-10 times of subculture were commenced from the primary subculture of each of the isolates to generate pure cultures. A total of 10 different pure isolates of the presumptive probiotics were prepared and their morphological and biochemical properties were characterized following (Abdullah-Al-Mamun et al., 2016).

Extraction of bacterial DNA

The purity of the colonies was maintained through repeated streak plate method for continuous cell passaging. After purity confirmation, 10 µl of active broth culture was re-inoculated into 10 ml sterile MRS broth and incubated at 37°C for 24 hours. Then 2ml aliquots of active log-phase cultures from final broth were used to isolate genomic DNA. DNA isolation was performed following the Ampicillin-Lysozyme tandem lysis method (De et al., 2010). Afterward, the supernatant was discarded and the DNA pellet was washed once with freshly prepared 70% ethanol and air-dried. The final pellet thus obtained was dissolved in 50 µl Tris-EDTA (10:1, pH 8.00) and stored frozen at -20°C till further analysis.

Qualitative detection of DNA

Qualification of extracted DNA was done following 1.2% Agarose gel electrophoresis. The mixture of prepared sample and dye was loaded in holes of gel carefully. The electrophoresis chamber was prepared by taking a 1-time TAE buffer for making the electric field on the gel. The loaded gel was placed in an electrophoresis chamber containing a buffer solution. The power supply started to run the gel with electric fields ensuring the gel was submerged into buffer inside the electrophoresis chamber. The machine was set at 120 volts; 20 mA for 90 minutes. After staining with ethidium-bromide-stock-solution (10 mg/ml), the gel was placed in the dark chamber of UV trans-illuminator to observe the DNA bands at 260 nm against the applied UV light.

Measuring the concentration of DNA (quantification)

At 260 nm, the absorbance readings were collected by viewing the monitor of the spectrophotometer. In each time, the cuvette was rinsed out twice with molecular biology grade distilled water and air-dried. The DNA concentration was calculated by multiplying the absorbance at 260 nm by a constant. Obtaining the absorbance reading at 260 nm, the original concentrations were determined according to the following formula (Barbas et al., 2007):

$$\begin{aligned} \text{DNA Concentration} &= \frac{\text{Abs. at 260 nm}}{\text{Path length (1cm)}} \times \text{Dilution factor} \times \text{Conversion factor} \\ &= A_{260} \times 200 \times 50 \mu\text{g/ml} \\ &= \text{ng}/\mu\text{l} \end{aligned}$$

Here, A_{260} = Spectrophotometric absorbance reading at 260 nm of DNA sample
Dilution factor = $1/0.005=200$.

In the present experiment, 10µl of the DNA solution was taken and diluted to a final volume of 2 ng/µl.

PCR reaction

Before running the samples in the PCR machine, the DNA concentrations were adjusted to 2ng/µl by using the following formula:

$$S1.V1 = S2.V2$$

Where, S1= Initial concentration of DNA (ng/µl) (varied for ten samples)

V1= Required volume of DNA (µl)

S2= Final concentration of DNA (ng/µl) = 2 ng/µl

V2= Final volume of DNA solution (µl) = 1500µl

Four 10 bp random primers OPA 2 (5/-TGC CGA GCT G-3/, Tm 340C); OPA 5 (5/-AGG GGT CTT G -3/, Tm 320C); OPA 18 (5/-AGG TGA CCG T-3/, Tm 320C) and OPA 9 (5/-GGG TAA CGC C -3/, Tm 340C) were used. PCR reactions were performed on each DNA sample in a total of 15 µl reaction mix (PCR master mixture 13 µl + template DNA 2 µl). Then the 0.2 ml PCR tube was briefly centrifuged and placed into the Veriti® 99 well Thermal Cycler (Model No. 9902) (Al Azad et al., 2020a). PCR optimization was necessary to adjust to the different concentrations of DNA.

Electrophoresis of the amplified products

The PCR products were mixed with 4µl gel loading dye to run in Agarose gel (1.2%) (Al Azad et al., 2020b). 24µl of the mixture was loaded gently per well on the gel. The molecular weight marker (100bp DNA Ladder) was loaded at the first well on the gel hole. The gel was sunk in the electrophoresis tank with the 1XTAE buffer. The electrophoresis machine was run for 90 min (at 120 volts and 20 mA). The separation process was monitored by the migration of dyes. When the bromophenol blue dye had reached about three-fourths of the gel width & electrophoresis was stopped. After completion of electrophoresis, the gel was stained in ethidium bromide solution with gentle agitation for 45 min.

III. Results and Discussion

Isolation and identification

The developed pure culture single colonies of each sample showed the same morphological characteristics with round shaped and white (Figure 01), prepared from all the collected samples from Khulna and Chittagong Cities. The isolates were also found gram-positive, motile, rod-shaped, catalase-negative, 0.3% bile salt-tolerant, phenol tolerant, and pH 3.0 tolerant, in the MRS medium, which has initially proved their probiotic properties and potentialities. The subculture process was repeated 4-5 times, while the bacterial culture was allowed to grow in incubation for 24-36 hours each time. The final pure colony of probiotic bacteria was obtained after a week. The late log phase re-inoculated pure single colony into MRS broth for each sample showed very opaque appearance in the test tubes after incubating 24hr at 360C, from which the DNA was extracted.

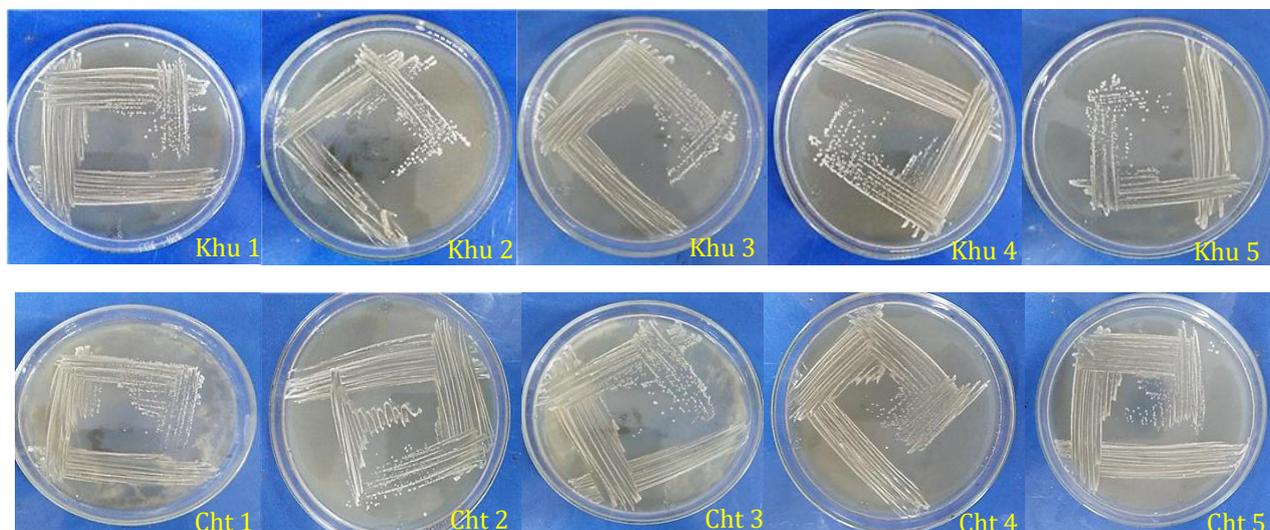


Figure 01. Pure culture plates with phenomenal number of same size-shaped and colored fresh single colonies prepared from the yoghurt samples of Khulna (Khu) and Chittagong (Cht) City.

Isolates from samples of Dahi were assembled for analysis using MRS agar media for the screening of *Lactobacillus* species and then the same morphological shaped colonies were obtained and a series of biochemical tests proved the presence of *Lactobacillus* in Dahi (Puniya et al., 2012). The same procedure was followed LAB from traditional Persian pickled vegetables (Dallal et al., 2017). A study for probiotic potential from raw & fermented milk, MRS solid medium, and M17 agar was used to determine the colony morphology visually thereby hanging drop technic recorded the motility (Bin Masalam et al., 2018). In our study, only MRS media was used. A similar experiment was demonstrated by several studies (Kwon et al., 2000; Abdullah-Al-Mamun et al., 2016). The result of biochemical tests ensured the presence of *Lactobacillus* spp in similar studies on probiotics species (Mannan et al., 2017; Du Plessis

and Dicks, 1995; Puniya et al., 2012). The molecular characterization of another species was described and several biochemical methods were conducted by (Al Azad et al., 2020a).

In the case of, RAPD based pattern analysis; the maximum number of polymorphic bands was recorded by OPA 18 (Figure 02), while OPA 2 & OPA 9 didn't generate satisfactory polymorphic bands. OPA 5 generated the least banding pattern and the average band per primer was above 2.0. The frequencies of polymorphic bands varied from primer to primer (Figure 02). DNA extraction was done following the Ampicillin Lysozyme Tandem Lysis method. The protocol was developed by Sachinandan et al, 2010. The extracted DNA was evaluated by using Spectrophotometer. The highest absorbance was 0.334 & the lowest was 0.017. Based on their absorbance value, the concentration was determined. Sample Chi 10 had lower concentrated DNA. The DNA yield from 10 probiotic bacteria was average 1225 ng/μl with the range of 170-3340 ng/μl. According to a study in 2010, a total of 269 *Lactobacillus* isolates were tested and the mean amount of DNA isolated from these isolates was 2187.654 ng/μl with a range of 293.58–5353.9 ng/μl (De et al., 2010). The variation in the DNA yield was due to handling errors, pipetting, and differences in the number of bacteria in a fixed volume of inoculum.

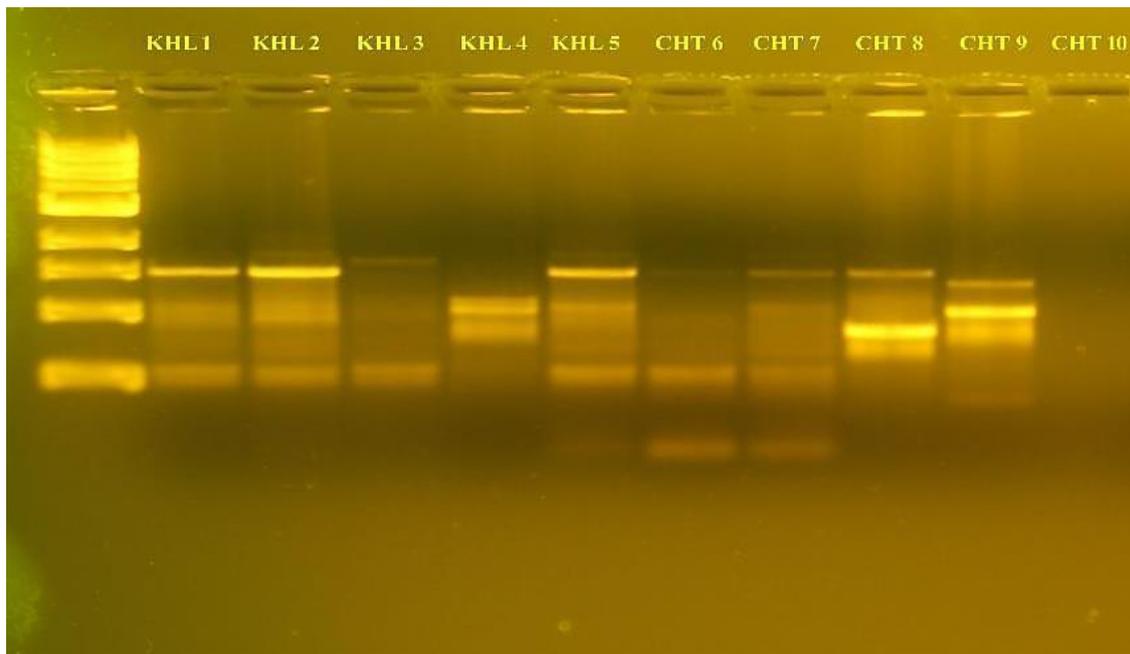


Figure 02. RAPD profiles of ten samples of probiotic bacteria from yogurt using random primer (OPA-18). KHL and CHT indicate Khulna and Chittagong Region, respectively.

RAPD-PCR method was used in different investigations (Kwon et al., 2000; Saxami et al., 2016). In known's study, the genetic relationship of 6 *Lactobacillus* strains was determined where five isolates were extracted from fermented milk and a total number of 42 primers were utilized in the study and the results were analyzed using NTSYS software. All *Lactobacillus* isolates were categorized into 3 separate groups (Kwon et al., 2000) and all primers except for the OPA 02 presented the maximum percentage of polymorphism (100%) indicating the high efficiency of such primers in discriminating *Lactobacillus* isolates.

Performing the RAPD-PCR method, Saxami's team detected two potential probiotic *lactobacilli* strains from fermented olives (Saxami et al., 2016). Strain-specific primers were designed and applied in a multiplex polymerase chain reaction where a total of 120 arbitrary primers were used in another investigation (Galanis et al., 2015). Characterization of 33 LAB from indigenous fermented products was done using (GTG) 5-based rep-PCR, where the close relationship among species was performed using species-specific PCR techniques after the sequencing of the 16S rRNA gene (Adimpong et al., 2012). In the present investigation, 16S rRNA gene amplification and multiplex PCR were not conducted. Honeybee gut's probiotics were analyzed using PCR-RFLP and phylogenetic analysis, where genomic DNA was extracted from LABs and a distinct band, fragment of 1540 bp in size of 16S rRNA gene was amplified (Sharifpour et al., 2016). In contrast, the present study didn't detect bacteria at the strain level successfully; varieties of primers were not used.

The identification of lactobacillus by RAPD analysis with the OPA primer was described in several papers. Varieties of random primers OPT-14, OPA-11 and OPT-16 (Huang and Lee, 2011), OPA-02 (Björneholm et al., 2002; Coeuret et al., 2003 and Hasslof et al., 2013), OPA-2 and 05 (Weiss et al., 2005) were used for RAPD fingerprints analysis. OPA 02 didn't produce desirable bands in a study which seems similar to our findings (Abdollahniya et al., 2018). In some studies, OPA-02 and 18 (Manan et al., 2009), OPA-18 (Daud Khaled et al., 1997) showed better amplification just like our results. No specific primer was designed for this experiment. Only four random primers were used due to a shortage of time and OPA-02 didn't generate any banding pattern. For Genetic diversity analysis, only the RAPD approach was done & the dendrogram obtained from the RAPD marker was provided lower geographical discrimination. OPA-2 & OPA-9 didn't generate any pattern. The polymorphism showed in the RAPD pattern was quite lower. This was maybe due to time shortage and lacking enough primer trial. A total of 176 isolates of LAB from traditional fermented items demonstrated different degrees of variation across 3 regions of Russia (Yu et al., 2015). The present investigation showed the regional probiotics discrimination more or less. The samples collected from different location demonstrated the meticulous pattern of genetic relationship among the beneficial bacteria. Furthermore, Neighbor-joining based phylogenetic analysis helped to determine the evolutionary relationship of the probiotic isolates (Figure 03) where 'Chi' for Chittagong and 'Khu' for Khulna. Distance from the pair members (Khu 4 and Chi 9) to the new node then the distance of the other taxa from the new node was displayed based on the genetic distance matrix. Genetic distances and Nei's tree were determined by using the software Phylogenetic Analysis Using Parsimony (PAUP, Version 4.0). Neighbor-joining took as input a distance matrix specifying the distance between each pair of taxa. However, the overall experiment carried a landscape of genetic relatedness of probiotic bacteria on regional basis. Following the UPGMA on Nei's genetic distance, Khu 4 and chi 9 were closely related as well as Khu 2 and Khu 5 had the lower genetic distance but in a different node, the genetic distance value ranged from 0.14286 to 0.85714. The polymorphisms showed by amplified bands were lower because enough primers were not available.

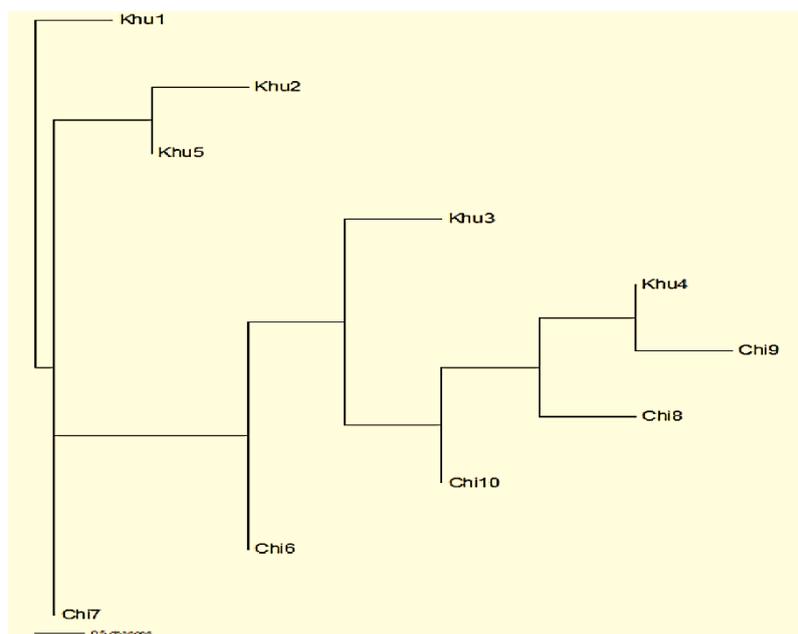


Figure 03. Phylogenetic tree by neighbor-joining method based on genotypes of DNA data obtained from probiotic bacteria of different yogurt samples.

Determination of Genetic Distances

The genetic distances between taxa were determined based on mean character differences between taxa. The analysis pair-wise genetic distance showed that the genetic distance value ranged from 0.14286 to 0.85714. The highest genetic distance was 0.85714 and the lowest genetic distance was 0.14286 (Table 01). The program was accomplished using PAUP software.

Table 01. Summary of pair-wise Genetic Distance values between taxa of probiotic bacteria isolated from yogurt samples of Khulna and Chittagong Divisions

Genotypes	Khu 1	Khu 2	Khu 3	Khu 4	Khu 5	Cht 6	Cht 7	Cht 8	Cht 9	Cht 10
Khu 1	***	0.14286	0.42857	0.42857	0.28571	0.42857	0.14286	0.42857	0.57143	0.42857
Khu 2		***	0.28571	0.57143	0.14286	0.57143	0.28571	0.57143	0.71429	0.57143
Khu 3			***	0.57143	0.42857	0.28571	0.57143	0.57143	0.71429	0.28571
Khu 4				***	0.71429	0.57143	0.57143	0.28571	0.14286	0.28571
Khu 5					***	0.42857	0.14286	0.71429	0.85714	0.71429
Cht 6						***	0.28571	0.57143	0.71429	0.28571
Cht 7							***	0.57143	0.71429	0.57143
Cht 8								***	0.42857	0.28571
Cht 9									***	0.42857
Cht 10										***

Legends: 1-10 counts the total number of samples, where 1-5 from Khulna and 6-10 from Chittagong

Overall, the experiment helped to show the genetic distances as well as genetic diversity among regional probiotic bacteria of two Divisions of Bangladesh and further progress or part of the work, even the relevant trial using these samples is continuing.

IV. Conclusion

In this study, the DNA yield from 10 probiotic bacteria was average 1225 ng/μl with the range of 170-3340 ng/μl after DNA isolation of the probiotic isolates. From the study, it is concluded that RAPD markers can be sensitive, simple, efficient, and powerful tools for genetic diversity analysis, using a larger number of primers would be worthy in future research.

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Competing interest

The authors strongly declare that there is no competing interest.

Abbreviations

RAPD (Rapid Amplified Polymorphic DNA); PCR (Polymerase Chain Reaction); MRS (De Man, Rogosa and Sharpe); Taq (*Thermophilus aquaticus*); TAE (Tris Acid EDTA); SDS (Sodium Dodecyl Sulphate); Rpm (Revolution per minute); TE -Tris EDTA buffer; DNA -Deoxyribo Nucleic Acid; Et-Br -Ethidium Bromide; EDTA -Ethylenediamine Tetraacetic Acid; GS -Genetic Similarity

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