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Morphology and genetic diversity studies of potential cyanobacteria isolated from Loktak Lake using RFLP markers

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ABSTRACT

In the present study, the morphology and genetic diversity of ten (10) potential cyanobacterial strains isolated from fresh water habitats of Loktak Lake was investigated by Restriction Fragment Length Polymorphism of 16S rRNA genes using four different enzymes viz., HinfI, AluI, EcoRI and TaqI. These enzymes digested the 16S rRNA PCR products and yielded different profiles. These strains showed high phycobili proteins content, extracellular ammonium excretion and nitrogenase activity when preliminary screening was conducted. The strains Nostoc spp. (BTA-60, 61), Nostoc commune (BTA-67) and Nostocmuscorum(BTA-950) were similar and delineated from the rest by the enzymes EcoRI and AluI. Other digests which characterized Calothrix sp. BTA-73 as a distinct taxonomic group from the rest was catalyzed by the enzymes EcoRI and AluI. On the basis of the genetic polymorphism band, HinfI, TaqI and AluI were also able to discriminate Anabaena sp. (BTA-964)from the other cyanobacterial strains. Phormidium spp. (BTA-52, 75, 1048) was similar and different from the rest of other strains as indicated by the enzymes EcoRI, TaqI and AluI. Within Nostocspp. group, there was no definite clustering for the morphological speciation of N. commune (BTA-67), N. muscorum (BTA-950) and other Nostoc spp. (BTA-60, 61, 80). Two Nostocstrains (BTA-61 and 67) with exactly the same profiles by digested banding pattern in EcoRIandHinfIwere confirmed as belonging to the same species. Non-heterocystous, filamentous Phormidiumout grouped from the heterocystous cluster but were still closely related to them and to each other. The clusters for four different enzymes yielded heterogenous groupings of the morphotypes and resulted in unclear delineation of the studied cyanobacterial strains.

Keywords: Cyanobacteria, Loktak Lake, Morphology, North-East India, Phylogeny, RFLP

INTRODUCTION

Cyanobacteria are large group of phototrophic microorganisms with variable morphological characters. For a long time, morphological characteristics were taken into account for a taxonomical classification of cyanobacteria [1, 2].The application of genetic methods to the taxonomy, phylogeny, and biotechnology of cyanobacteria has increased dramatically in the past decade, particularly with the advent of polymerase chain reaction methods [3].

The development of new molecular techniques have been introduced to the phylogeny and taxonomy of cyanobacteria. The use of DNA-based genetic markers [4] has changed the practice of genetics. Over the past 20 years since that discovery, many different types of DNA-based genetic markers have been used for the analysis of genetic diversity and applied diagnostic purposes [5]. The use of modern molecular techniques to determine the degree of sequence conservation between bacterial genomes has led to the development of methods based solely on the detection of naturally occurring DNA polymorphisms. These polymorphisms are a result of point mutations or re-arrangements in the DNA and it can be detected by scoring presence or absence of bands in banding patterns that are generated by restriction enzyme digestion and DNA amplification procedures.

Restriction Fragment Length Polymorphism is a technique in which organisms may be differentiated by analysis of patterns derived from cleavage of their DNA. The similarity of the banding patterns generated can be used to differentiate species (and even strains) from one another. RFLP is generated by the presence and absence of a recognition site for the same restriction endonuclease in the same region of a chromosome from different individuals of a species. As a result, the concerned restriction enzyme produces fragments of different length representing the same chromosome region of different individuals. The lanes of the different strains/selected species are compared and RFLPs are detected due to differential movement of a band on their gel lanes and each of such band is regarded as a single RFLP locus. Amplification and restriction enzyme digestion or sequencing of PCR products has provided a specific method for the delineation of cyanobacterial genera [6,7,8,9].

In the present study, the objective was to analyze the morphological and genetic variations using RFLP analysis of 16S rRNA genes. Preliminary screening of these strains was done based on their biochemical components such as pigment composition, extracellular ammonium excretion and nitrogenase activity.

MATERIALS AND METHODS

Strains and growth conditions

Cyanobacterial strains used in this study were obtained from Freshwater Cyanobacterial and Microalgal Repository (National facility created by the Department of Biotechnology, Government of India with reference No. BT/PR 11323/PBD/26/171/2008 dated 31-03-2009), Institute of Bioresources and Sustainable Development (IBSD), Imphal, Manipur, India. These strains were previously isolated from Loktak Lake, the only largest freshwater lake in the North-Eastern region of India. The morphological study of the strains was carried out using trinocular research microscope (NIKON Eclipse 80*i*) and Carl Zeiss fluorescence microscope, Axio Scope A1 coupled with Carl Zeiss Imaging Systems 32 software AxioVision 4.7.2 followed by taxonomical characterization referring to key [10].The strains were allowed to grow in BG-11 medium [11]with light intensity of 54-67 µmol photons $m^{-2}s^{-1}$ provided by cool white fluorescent tubes following light:dark cycles of 14:10h condition maintained at 28±2ºC. The cyanobacterial flasks were shaken manually for two to four times daily to prevent cell clumping.

DNA extraction and PCR amplification

Exponentially growing cells was subjected for isolation of genomic DNA according to the Xanthogenate-SDS (XS) extraction protocol [12] with slight modifications. Amplification of the 16S rRNA gene was carried out by PCR using primers (IDT-Integrated DNA Technologies) forward primer 536F (5'-GTGCCAGCAGCCGCGGTRATA-3') and reverse primer 1488R (5'-CGGTTACCTTGTTACGACTTCACC-3') [13]. The PCR mixture contained 5 µl of 1X reaction buffer, 5 µl of 200 µM of each dNTPs, 1.5 µl of 0.3 µM of each primer, 0.25 µl of 5U *Taq*polymerase, 2 µl (50 ng) of DNA with 34.75 µl of sterile double distilled water. Total reaction volume was 50 µl. The PCR reaction was started as initial denaturation step for 5 min at 95°C followed amplification with by 27 cycles of cyclic denaturation for 1 min at 95°C, 1 min at 55°C for annealing and 1 min at 72°C for extension. The final extension of 10 min at 72°C.Subsequently, the PCR amplicons were migrated at 80V for 1 h on 2 % (w/v) agarose gel contained 1X TAE buffer and ethidium bromide (10 mg ml^{-1}).

RFLP of PCR product

Four restriction enzymes: *Hin*fI, *Alu*I, *Eco*RI and *Taq*I were used for the digestion of the amplified product and to generate RFLP patterns specific to the cyanobacterial strains. Restriction digestion was performed using 1 µl of enzyme, 5 µl of DNA (PCR product), 1 µl of BSA, 1 µl of buffer-H, 2 µl of sterile double distilled water so that total volume of 10 µl was incubated overnight at 37°C in water bath to achieve complete fragmentation. The restriction fragments were separated by electrophoresis in 2.0 % (w/v) agarose gel in 80V contained 1X TAE buffer and 2 µl of ethidium bromide (10 mg ml^{-1}) with 100 bp DNA ladder as the size marker. The patterns of the restriction fragments were visualized and documented using a Vilber Lourmat gel documentation system with Quantum-Capt software.

Phylogenetic tree construction

RFLP profiles were converted to binary data by scoring the presence or absence of bands for each isolate as one or zero. Each lane of the PCR product for different cyanobacterial strains with different primers was scored and cluster analysis was carried out using NTSYSpc version 2.21 software. The combined *Hin*fI, *Alu*I, *Eco*RI and *Taq*I restriction patterns were used for cluster analysis. Keeping all the conditions identical, a phylogenetic tree for calculating the character [14] differences was constructed using unweighted pair group arithmetic mean clustering (UPGMA).

RESULTS AND DISCUSSION

The details of growth, habitats and taxonomic enumeration of the strains were presented (Fig. 1 and Table 1). In the present study, genetic distances between the ten (10)cyanobacterial strains tested by RFLP analysis of 16S rRNA genes using four different enzymes viz., *Hin*fI, *Alu*I, *Eco*RI and *Taq*Iwere executed. The enzymes *Eco*RI, *Hin*fI, *Taq*I and *Alu*I produced 14, 13, 9 and 17 bands(Fig. 2-5).RFLP profiles were converted to binary data by scoring the presence or absence of bands for each strain as 1 or 0 and a phylogenetic tree was constructed(Fig. 6).The strains *Nostoc* spp. (BTA-60, 61), *Nostoc commune* (BTA-67) and *Nostocmuscorum* (BTA-950) were similar and delineated from the rest by the enzymes *Eco*RI and *Alu*I. Other digests which characterized *Calothrix* sp. BTA-73 as a distinct taxonomic group from the rest wascatalyzed by the enzymes *Eco*RI and *Alu*I. On the basis of the genetic polymorphism band, *Hin*fI, *Taq*I and *Alu*I were also able to discriminate *Anabaena* sp. (BTA-964)from the other cyanobacterial strains. *Phormidium* spp. (BTA-52, 75, 1048) was similar and different from the rest of other strains as indicated by the enzymes *Eco*RI, *Taq*I and *Alu*I.

Generally, RFLP analysis supports strain similarity as shown by the 16S rRNA gene sequence (data not shown). The disparity could be due to errors in the RFLP method caused by undetectable restriction fragments (small fragments) or fragment length differences. At the species level, the current, morphology based taxonomy was not supported by the RFLP data. *Aphanizomenonflos-aquae*, *Anabaena flos-aquae*, *Anabaenopsis*, *Cyanospira* and *Nodularia* are distinct and consistent with their position in trees obtained from the 16S rRNA sequences by RFLP as reported by [15]. The morphological characteristics (e.g. *Anabaena* and *Aphanizomenon*), the physiological characteristics or the geographical origins did not reflect the level of 16S rRNA gene relatedness of the closely related strains studied [16].

In the present study, the clusters yielded different groupings of the morphotypes and did not result in clear delineation of the species. Similar findings have been reported for *Prochlorococcus*strains [17]. [18] reported close relationship between strains of *Anabaena* and *Aphanizomenon*in a 16S rRNA after the RFLP study. In this study, RFLP data are typically used to infer nucleotide substitution rates, which manifest the presence or absence of defined restriction fragments instead of restriction digest profiles.

The phenetic relationships inferred an essentially bifurcating phylogeny with one cluster dominated by filamentous heterocystous strains and the other consisted mainly of both heterocystous and non-heterocystous strains. Within *Nostoc*spp. group, there was no definite clustering for the morphological speciation of *N. commune* (BTA-67), *N. muscorum* (BTA-950) and other *Nostoc* spp. (BTA-60, 61, 80). Two *Nostoc*strains (BTA-61 and BTA-67) with exactly the same profiles by digested banding pattern in EcoRIand*Hin*fI were confirmed as belonging to the same species. *Nostoc*strains were dispersed throughout this cluster, once again indicated the inadequacies of the current taxonomy which relies on the subjective observation of microscopic morphology. In the present study, non-heterocystous, filamentous *Phormidium*outgrouped from the heterocystous cluster but were still closely related to them and to each other. These results are in agreement with the previous cyanobacterial partial 16S rRNA gene sequencing studies of [19], which have revealed that relatively close evolutionary relationships underlie the extensive diversity of cyanobacterial morphological features.

The uses of DNA sequences for the taxonomic and phylogenetic analysis of cyanobacterial isolates have been carried out by several workers. Studies based on restriction fragment length polymorphism (RFLP) and PCR techniques have been used to examine the *Anabaena-Azolla* symbiosis species [20, 21] and isolates from cycads and *Gunnera* have been studied with respect to genetic diversity by using protein profiles and the RFLP technique [22]. The amplified 16S-23S rRNA spacer (ITS-1) of cyanobacteria has been used in several studies to genetically characterize strains by sequence analyses [23] or by PCR-RFLP [24].The investigations of [25] showed that morphological differences do not necessarily appear at the 16S rRNA gene level. However, the use of more restriction enzymes, the analysis of the sequence of the whole 16S rRNA gene, or the analysis of more variable intergenic spacers between 16S rRNA and 23S rRNA genes may reveal differences between our closely related genera. More than 50% of the strains in the culture collections have taxonomic names which do not agree with the morphological description of the taxon [26]. Numerical analysis of RFLP of the 16S rRNA gene provide broader

taxonomic applications. The closeness amongst the strains on the basis of RFLP data indicate that the strains may belong to single species or two species and the use of additional restriction enzymes may depict better results to make firm taxonomic conclusions.

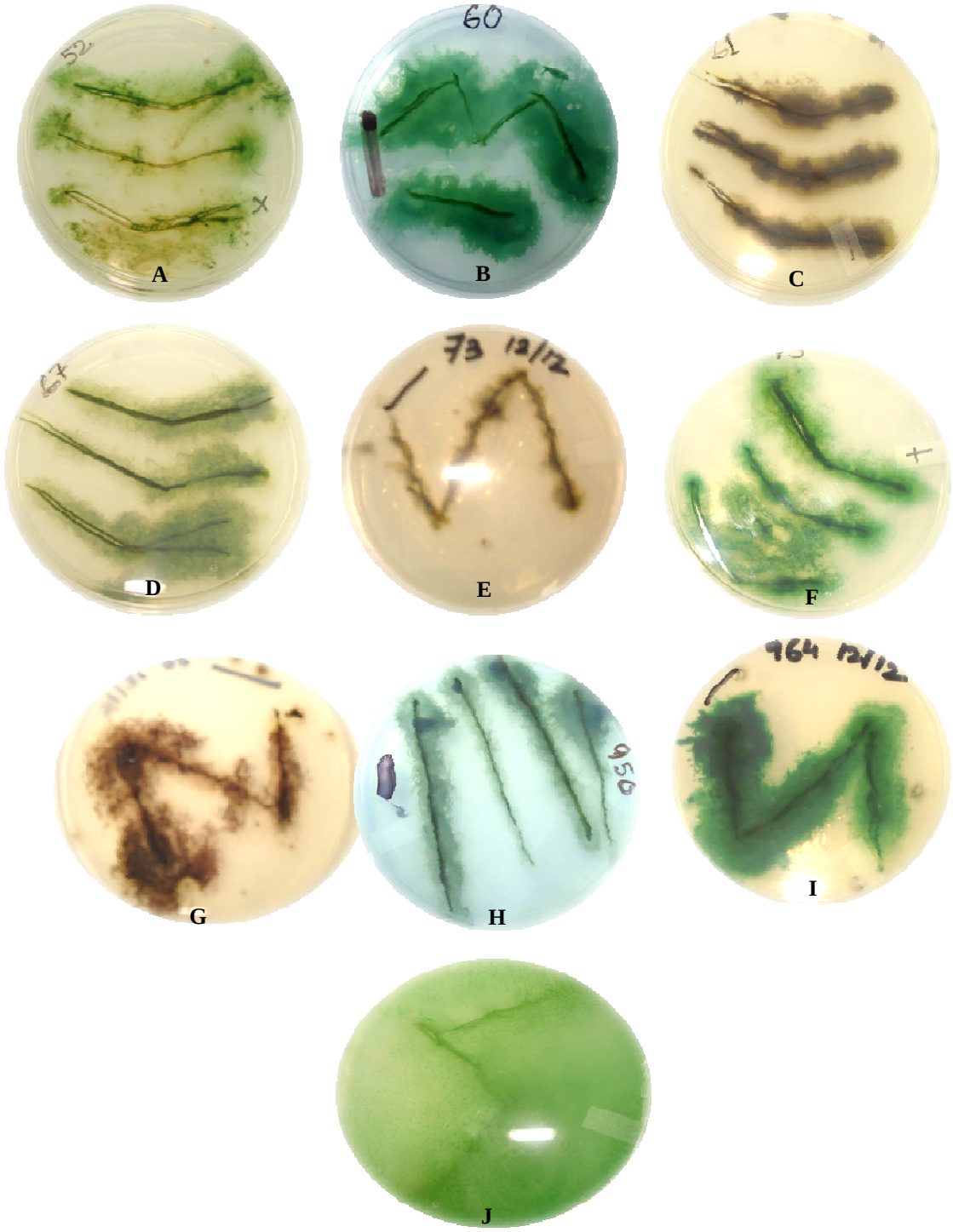

Fig. 1:Growth of cyanobacterial growth on agar plates

A.*Phormidium*sp. BTA-52; **B.** *Nostoc*sp.BTA-60; **C.** *Nostoc* sp.BTA-61; **D.** *Nostoc commune* BTA-67; **E.** *Calothrix* sp. BTA-73; **F.** *Phormidium* sp. BTA-75; **G.** *Nostoc* sp. BTA-80; **H.** *Nostoc muscorum* BTA-950; **I.** *Anabaena* sp. BTA-964; **J.** *Phormidium* sp. BTA-1048

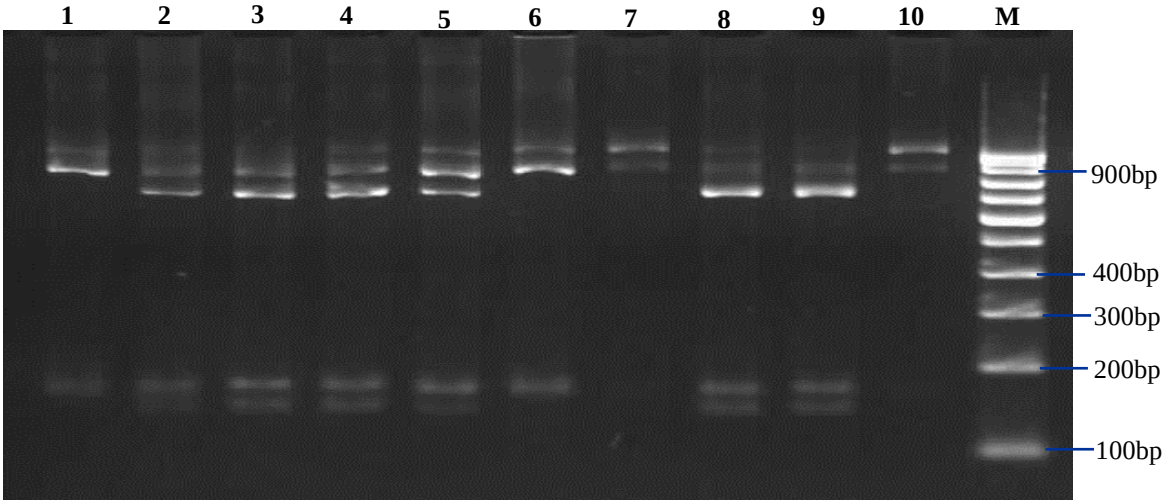

Fig.2: RFLP-16S rRNAproduct digested with *Eco*RI

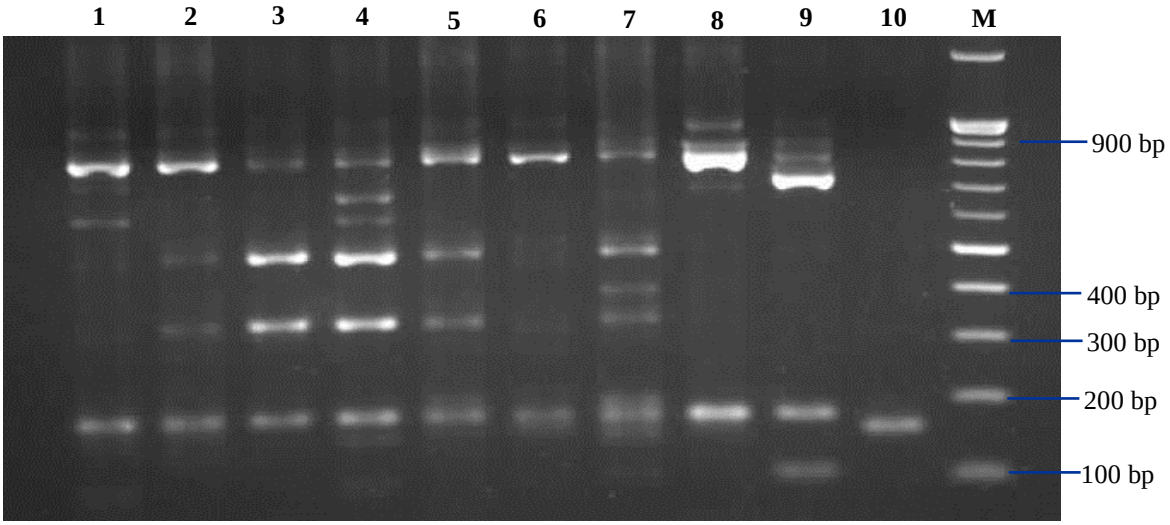

Fig.3: RFLP-16S rRNAproduct digested with *Hin*fI

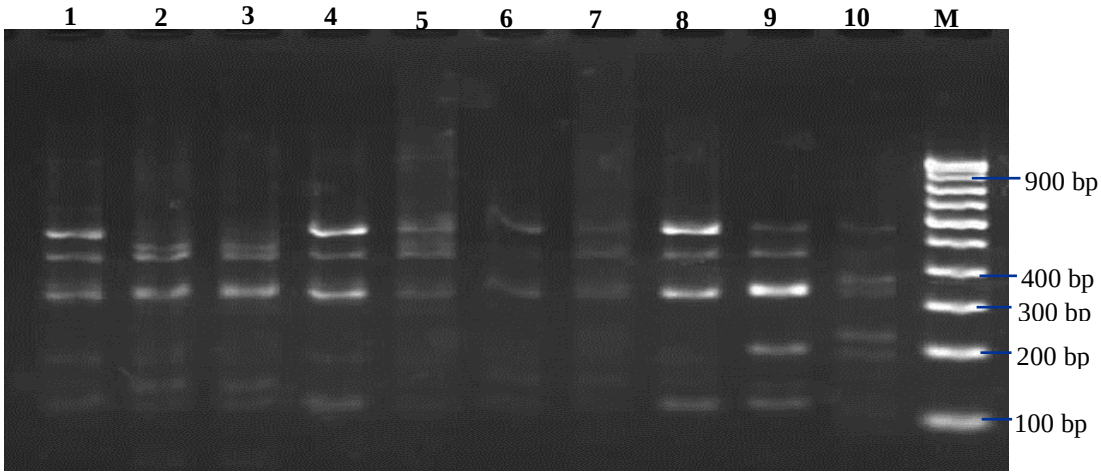

Fig.4: RFLP-16S rRNAproduct digested with *Taq*I

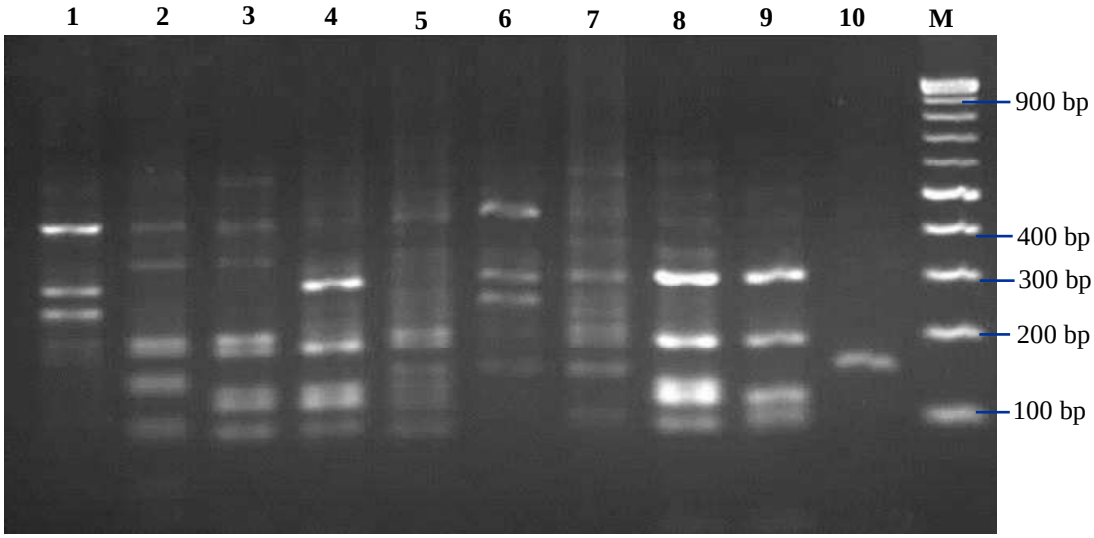

Fig.5: RFLP-16S rRNA product digested with *Alu*I

M-DNA ladder 100 bp; **1**-*Phormidium* sp. BTA-52; **2**-*Nostoc* sp. BTA-60; **3**-*Nostoc* sp. BTA-61; **4**-*Nostoc commune* BTA-67; **5**-*Calothrix* sp. BTA-73; **6**-*Phormidium* sp. BTA-75; **7**-*Nostoc* sp. BTA-80; **8**-*Nostoc muscorum* BTA-950; **9**-*Anabaena* sp. BTA-964; **10**-*Phormidium* sp. BTA-1048

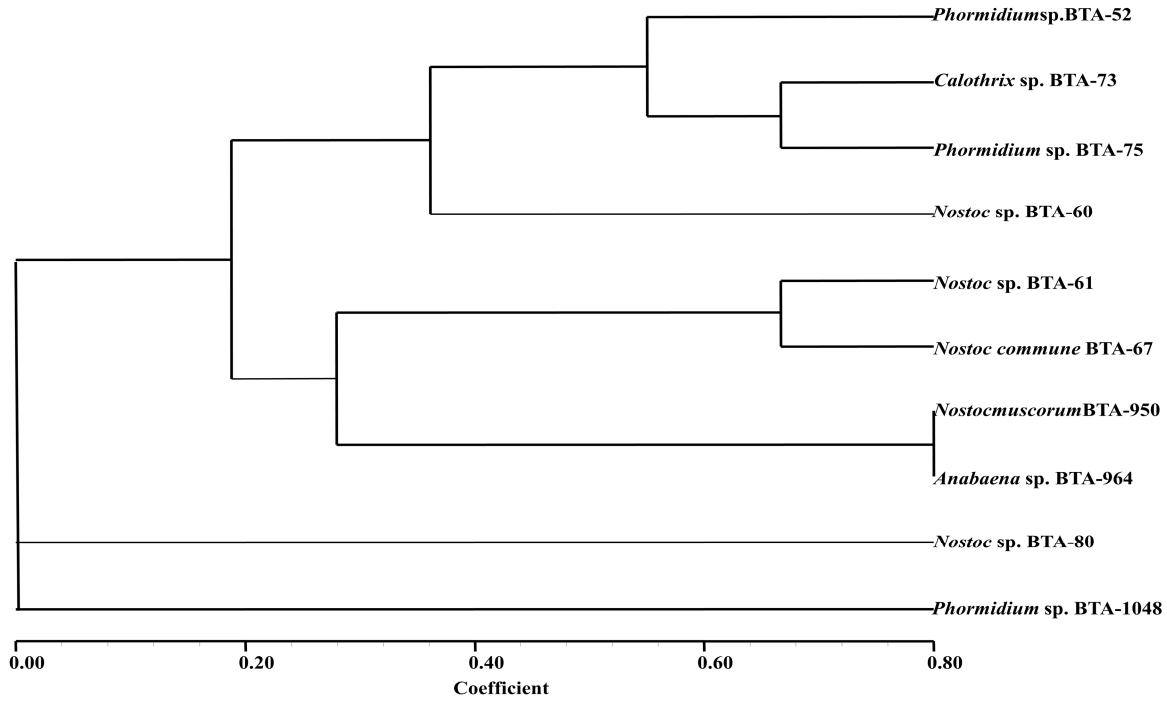

Fig.6: Phylogenetic tree (UPGMA) of RFLP-16S rRNAproduct digested with restriction enzymes indicating genetic distance measurement

Table 1: Origin of cyanobacterial isolates investigated and some of their morphological characters

Taxonomical Assignment	Culture collection and strain number	Cell width (µm)	Cell form	Origin	
				Habitat	Locality
Phormidium sp.	BTA-52	3.55	elongated	freshwater	Loktak Lake
*Nostoc*sp.	BTA-60	4.07	barrel	freshwater	Loktak Lake
*Nostoc*sp.	BTA-61	5.35	quadratic	freshwater	Loktak Lake
Nostoc commune	BTA-67	3.96	barrel	freshwater	Loktak Lake
*Calothrix*sp.	BTA-73	4.52	elongated	freshwater	Loktak Lake
*Phormidium*sp.	BTA-75	3.21	elongated	freshwater	Loktak Lake
*Nostoc*sp.	BTA-80	5.15	quadratic	freshwater	Loktak Lake
Nostocmuscorum	BTA-950	3.22	barrel	freshwater	Loktak Lake
Anabaena sp.	BTA-964	6.91	barrel	freshwater	Loktak Lake
*Phormidium*sp.	BTA-1048	3.30	quadratic	freshwater	Loktak Lake

The study on diversity of cyanobacteria from this lake were contributed earlier by [27, 28,29]. In conclusion, the present analysis with different strains of cyanobacteria using molecular approaches have clearly indicated a high degree of genetic diversity. It may also noted that ITS-RFLP method could be more appropriate to distinguish different strains of cyanobacterial genera and ITS region may show an extremely high genetic diversity which may not co-relate with the diversity of 16S rRNA gene in the further research. This approach should pave the way and prove useful for the further researchers for unraveling the physiological differences and phylogenetic relatedness amongst cyanobacterial population available in nature. It is essential not only important to study the diversity of cyanobacteria in this unexplored habitats but also to exploit them for industrial applications. More research should focus on modifying these cyanobacterial strains for high value-added products by molecular techniques or genetic engineering in the future. Their mass production for biotechnological importance would attract an increasing attention.

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