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Molecular Phylogeny of Morphologically Diverse Cyanobacteria Based on Ribosomal Conserved Sequence

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ABSTRACT

Morphology and cytological studies are the basis for the conventional identification and taxonomy of cyanobacteria. But it is found that most of the cyanobacterial species change their morphological features in accordance with critical conditions of the ecosystem. Therefore, conventional methods may not be sufficient for the identification of the organism at the species level. In this study, molecular characterization is recommended as a much more reliable technique for better identification of cyanobacteria. Marine and fresh water samples were collected from Kurumadai and Tiruchirappalli respectively. From the purified samples, three strains, *Leptolynbya valderiana* NTDM10, *Oscillatoria boryana* NTDM11and *Planktothrix pseudoagardhii* NTRD01 were selected for the further molecular characterization based on the 16S rDNA sequences. The sequences were deposited to GenBank with accession numbers JQ867392, JQ867393 and JQ867394. Evolutionary relationships and secondary structure prediction was carried out with the sequences. Biomolecular characteristics of the three cyanobacterial strains were also studied by FTIR spectroscopy.

Key words: Cyanobacteria, phylogeny, ribosomal conserved sequence, 16S rDNA, FTIR

INTRODUCTION

Cyanobacteria are morphologically distinct group of oxygenic photosynthetic organisms which inhabit terrestrial and aquatic ecosystem; they are unique among the prokaryotes in possessing the capacity of oxygenic photosynthesis (Thajuddin *et al*., 2010). Various strains of cyanobacteria are known to produce intracellular and extracellular metabolites with diverse biological activities such as antialgal, antibacterial, antifungal, antiviral and immunomodulatory compounds (Baldev *et al*., 2015; Thajuddin and Subramanian, 2005). It has been reported that the cyanobacteria and diatoms are widely used in nanobiotechnological application (MubarakAli, 2012). As, cyanobacteria possess unique bioactive properties, cyanobacterial taxonomy is considered to be important.

The classification of cyanobacteria is based on morphological characters such as trichome width, cell size, division planes, shape and arrangement, pigmentation and the presence of characters such as gas vacuoles and a sheath (Baker, 1991). In order to overcome some problems and to permit identification of cyanobacteria at the genetic level, different molecular tools have been applied for their distinction at different taxonomic levels (Turner, 1997). The use of modern molecular biological 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 (Pandiaraj *et al*., 2012).

It seems to be difficult to define a taxonomic or phylogenetic relationship within the cyanobacteria due to the unavailability of a distinct, consistent characters that leads to a good taxonomic scheme. To overcome these problems, the molecular techniques, including DNA base composition, DNA and RNA hybridization, gene sequences and PCR fingerprinting were developed for cyanobacterial taxonomy. Nucleic acid technology, especially the Polymerase Chain Reaction (PCR), has advanced to the point that it is feasible to amplify and sequence genes and other conserved region from a single cell. However, the molecular results obtained have integrated with other characteristics as the base for polyphasic taxonomy (Vandamme *et al*., 1996).

A molecular approach to the systematics of cyanobacteria may be most appropriate for analysing the phylogenetic relationships (Wilmotte, 1994). Macromolecules, such as nucleic and proteins, are copies or translations of genetic information. The methods applied involve direct studies of the relevant macromolecules by sequencing or indirectly by electrophoresis, hybridization or immunological procedures (Wilmotte, 1994). Cyanobacteria with the same genetic makeup may appear quite different under various physiological conditions as a result of differential gene expression (Castenholz and Norris, 2005). Molecular assessment of cyanobacteria biodiversity were studied in using the specific marker like 16S rDNA, phycocyanin locus, nif gene, rpo gene, ITS region, phosphoenolpyruvate carboxylase gene etc (Smith *et al*., 2008). It is expected that the modern molecular phylogenetic techniques in accordance with the classical taxonomical approaches will improve the classification of these organisms to a great extent. The present study was focused on the molecular approaches of taxonomy and the methodologies of phylogenetic analysis.

MATERIAL AND METHODS

Sample collection: Marine water samples were collected from Kurumadai and fresh water sample collected from Railway workshop at Tiruchirappalli Zone. The collected samples were isolated and purified in laboratory conditions with specific media. Marine and fresh water cyanobacterial samples were grown in MN+ media and BG11 medium respectively. Purified samples were maintained in the germplasm of Department of Microbiology Bharathidasan University, India at 3000 Lux light intensity and a temperature condition of 24±2°C.

Morphological characterization: Microphotographs of isolated cyanobacteria were taken using light microscope (Optika Photomicroscope unit) and the identification of cyanobacteria in marine water samples was achieved using standard monographs.

DNA extraction and amplification: The genomic DNA was isolated from the cyanobacteria (Smoker and Barnum, 1988). The PCR amplification was performed for the respective samples of purified DNA, using the primers CYA 106 F (5'-CGG ACG GGT GAG TAA CGC GTGT-3') and CYA 781 R (5'-GAC TAC TGG GGT ATC TAA TCC CA T-3') (Pandiaraj *et al*., 2012). The polymerase chain reaction condition includes an initial denaturation at 94°C for 1 min. Further denaturation was carried out at 94°C for 15 sec, annealing at 47°C for 15 sec, elongation at 72°C for 45 sec for 35 cycles and a final elongation at 72°C for 5 min. Amplified products were isolated by electrophoresis on 1.2% agarose gel using 1X TBE buffer at a constant supply of 100 V for 30 min. The resolved bands were documented under Slite 140 UV gel documentation system. 16S rDNA gene sequencing was done with amplified samples with respective forward and reverse primers. These sequences were further deposited in GenBank.

In silico analysis of gene sequences

Construction of phylogeny: Based on the obtained nucleotide sequences data, the phylogenetic tree was constructed. The evolutionary distances were calculated using the Neighbor Joining method (Tamura *et al*., 2004).

Secondary structure prediction: The translated protein sequence was analyzed for secondary structure prediction. The query sequence was uploaded in alignment box and the query was submitted to GOR secondary structure prediction method version IV for structural analysis. The structure was predicted and compared with their models (Chou and Fasman, 1974).

Restriction site analysis: The restriction sites in 16s rRNA of selected cyanobacteria species were analyzed using NEBCUTTER program version tools in online http://tools.neb.com/NEBcutter2/ index.pnp.

Biomolecule characterization using FTIR: All cultivated cyanobacterial strains were prepared by an identical procedure for FTIR spectroscopy. One gram of the cell suspension was harvested by centrifugation for 5 min at 14,000 rpm. The pellets were washed twice with distilled water in order to remove the ions and other particles from the cell surface. Then, the pellet was collected and ground into fine particles using mortar and pestle. Then, the finely ground biomass was dried in hot air oven for 1 h at 50°C. These finely dried cells were then subjected to FTIR analysis. Sacksteder and Barry (2001) describe FTIR as a form of vibrational spectroscopy whose spectrum reflects both molecular structure and molecular environment.

RESULTS AND DISCUSSION

Isolation and purification of cyanobacteria: Fresh water sample was collected from Railway workshop station at Tiruchirappalli and marine sample was collected from Kurumadai, Rameshwaram. The purified cultures were inoculated in BG-11 and MN medium for fresh water and marine water sample respectively for biomass production.

Morphological identification of cyanobacteria: Isolated cultures was morphologically identified as *Leptolynbya valderiana* NTDM10, *Oscillatoria boryana* NTDM11 and *Planktothrix pseudoagardhii* NTRD01 (Fig. 1). The identified strains were maintained with alternative illumination (i.e., 16 h: 8 h light: dark cycles) in germ plasm at 24°C. The morphological features were determined with their cell shape and cell size (Table 1). Cyanobacteria are known to be a group of organisms having enormous potentials. So their isolation and identification always seems to be a challenging topic among the researchers. Diversity and molecular systematics of cyanobacteria and diatoms have been reported (MubarakAli, 2012). Molecular phylogenetics is the most appropriate approach for the identification of cyanobacteria. Within the last decade a number

Table 1: Cyanobacterial species and their morphological characteristics

Taxons	Morphological characteristics
Leptolynbya valderiana	Trichome; 0.5-3.2 μm wide, filamentous, colorless facultative sheaths, the apical end, pale blue-green, grayish, olive-green, not constricted or constricted at the cross walls, nonmotile, end cells without thickened cell walls or calyptras
Oscillatoria boryana	Trichome; 4-6 μm broad, unbranched filaments, spices end, lightly granulated at the cross- walls calyptra absent, blue-green, brownish, without sheaths, end cells widely rounded, sometimes capitates or with narrow calyptra
Planktothrix pseudoagardhii	Trichome: 3-6.4 μm broad, colour: pale blue-green, blue-green or yellowish constrictions: trichome ends: slightly attenuated, apical cells: tapered-rounded, bluntly conical; sometimes small calyptras, straight and solitary filaments, cells are slightly constricted at the cross walls

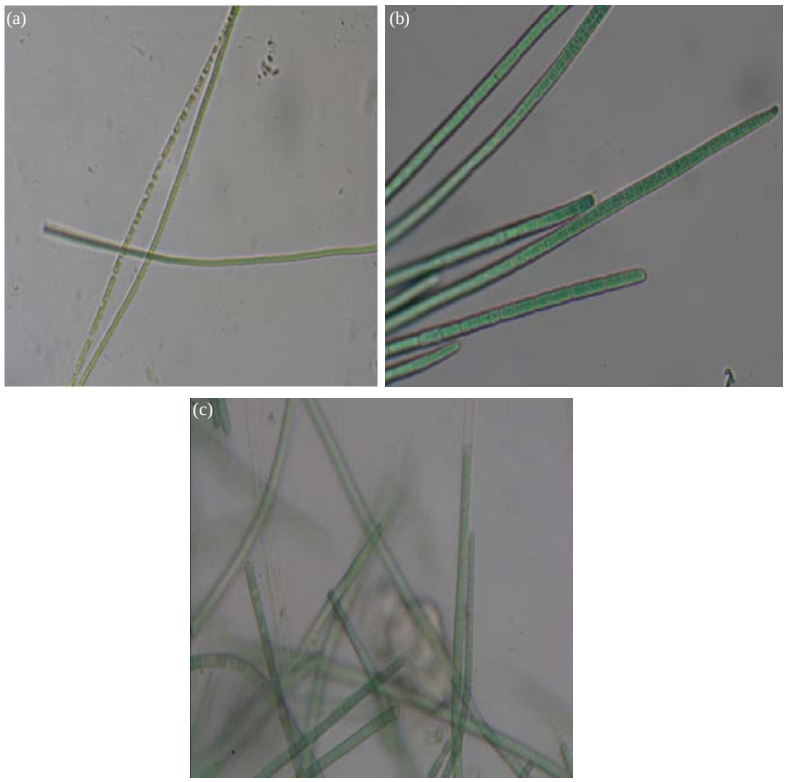

Fig. 1(a-c): (a) *Leptolynbya valderiana* NTDM10, (b) *Oscillatoria boryana* NTDM11 and (c) *Planktothrix pseudoagardhii* NTRD01

of psychologists began using molecular techniques to answer questions dealing with the taxonomy, population dynamics and the evolution of cyanobacteria. Among the most popular molecular techniques employed by bacteriologists are DNA-DNA hybridization, sequence determination of small ribosomal subunit ribonucleic acids (16S rRNAs) (Pandiaraj *et al*., 2012).

Molecular characterization: The genomic DNA of *Leptolynbya valderiana* NTDM10, *Oscillatoria boryana* NTDM11 and *Planktothrix pseudoagardhii* NTRD01 was extracted. After PCR, approximately 650 bp of amplified locus of 16S rDNA was observed (Fig. 2). The obtained sequences of 16S rDNA of the three isolates were deposited in the GenBank with accession numbers as JQ867392, JQ867393 and JQ867394 for *Leptolynbya valderiana* NTDM10, *Oscillatoria boryana* NTDM11 and *Planktothrix pseudoagardhii* NTRD01, respectively. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 1.81351902 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method (Nei and Kumar, 2000) and are in the units of the number of base differences per site. The analysis involved 9 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated (Tamura *et al*., 2011). There were a total of 46 positions in the

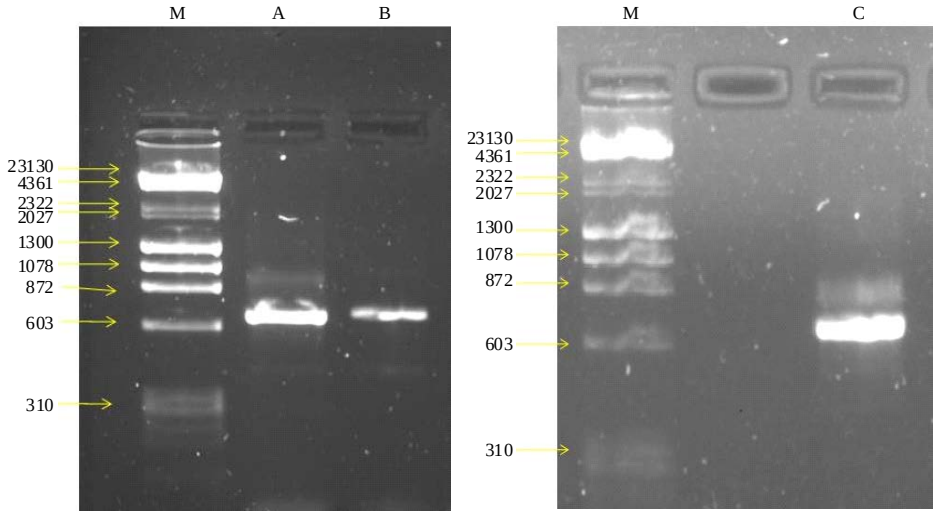

Fig. 2: 16S rDNA gene amplification, (A) *Leptolynbya valderiana* NTDM10,(B) *Oscillatoria boryana* NTDM11, (C) *Planktothrix pseudoagardhii* NTRD01 and (M) Markers

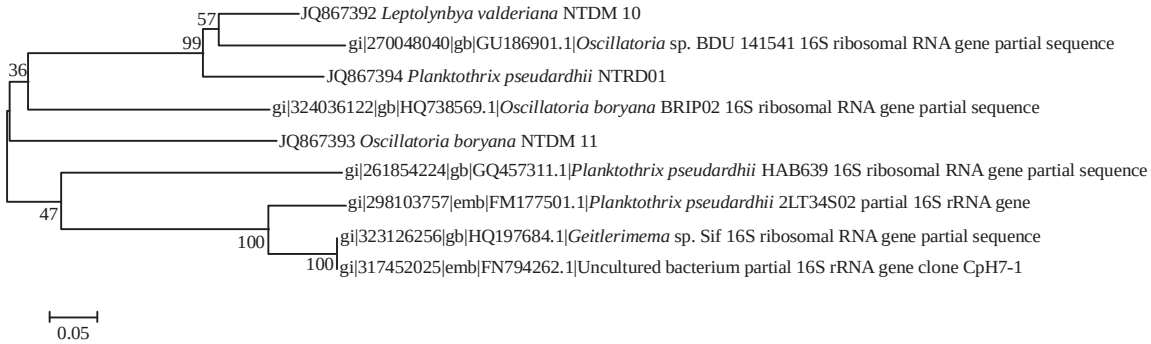

Fig. 3: Evolutionary relationship of cyanobacterial isolates, *Leptolynbya valderiana* NTDM10, *Oscillatoria boryana* NTDM11 and *Planktothrix pseudoagardhii* NTRD01

final dataset. Evolutionary analyses were conducted in MEGA5 (Fig. 3). There are reports stating that grouping of taxa according to morphological characters is not sufficient, where, 16S rRNA gene sequencing was used for better sample recognition (Heidari *et al*., 2013).

Secondary structure prediction: The sequences 16S RNA gene was compared with the secondary structure for better understanding of the gene sequences. The secondary structure will help in determining the exact structure of the gene. The translated protein sequence was uploaded in alignment box and the query was submitted to GOR secondary prediction method version IV for structural analysis. The structure was prediction and compare with their models. Secondary structure for the strains NTDM10, NTDM11 and NTRD01 was prediction and given in the (Fig. 4). The diversity study of different organisms suitably satisfies the above mentioned fact. 16S rDNA, PC and 18S rDNA gene markers are the versatile locus for the identification of cyanobacteria and diatom respectively (MubarakAli, 2012). These molecular techniques have been found to be very useful in the species level differentiation. Unequal evolutionary rate, base frequency bias and even

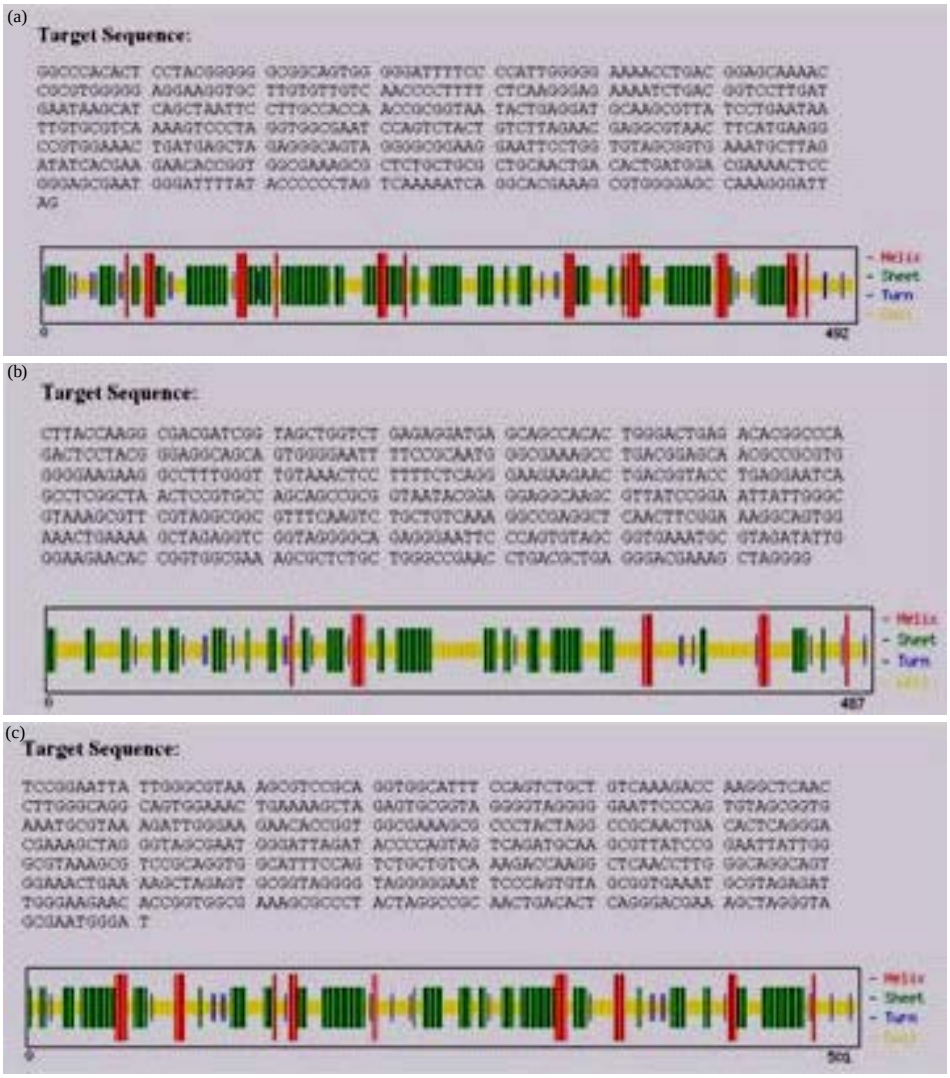

Fig. 4(a-c): Secondary structure of the ribosomal conserved sequence of cyanobacterial isolates, (a) *Leptolynbya valderiana* NTDM10, (b) *Oscillatoria boryana* NTDM11 and (c) *Planktothrix pseudoagardhii* NTRD01

methodological difficulties of tree making algorithms will produce difficulties in phylogenetic analysis. Therefore, molecular techniques can be adopted to resolve many of the issues and problems in cyanobacterial taxonomy.

Restriction site analysis: Restriction enzyme sites and the number of restriction cutting site for the commercially available enzymes were found in NEB Cutter program used. For all cyanobacteria 16S RNA gene sequences selected the GC (%) is higher than AT (%). This is one of the reasons that cyanobacteria are capable of surviving and dominating all kinds of extreme environments. The restriction sites found in select cyanobacteria species taken in this study shows the restriction sites found in namely *Leptolynbya valderiana* NTDM10, *Oscillatoria boryana* NTDM11 and *Planktothrix pseudoagardhii* NTRD01 (Fig. 5).

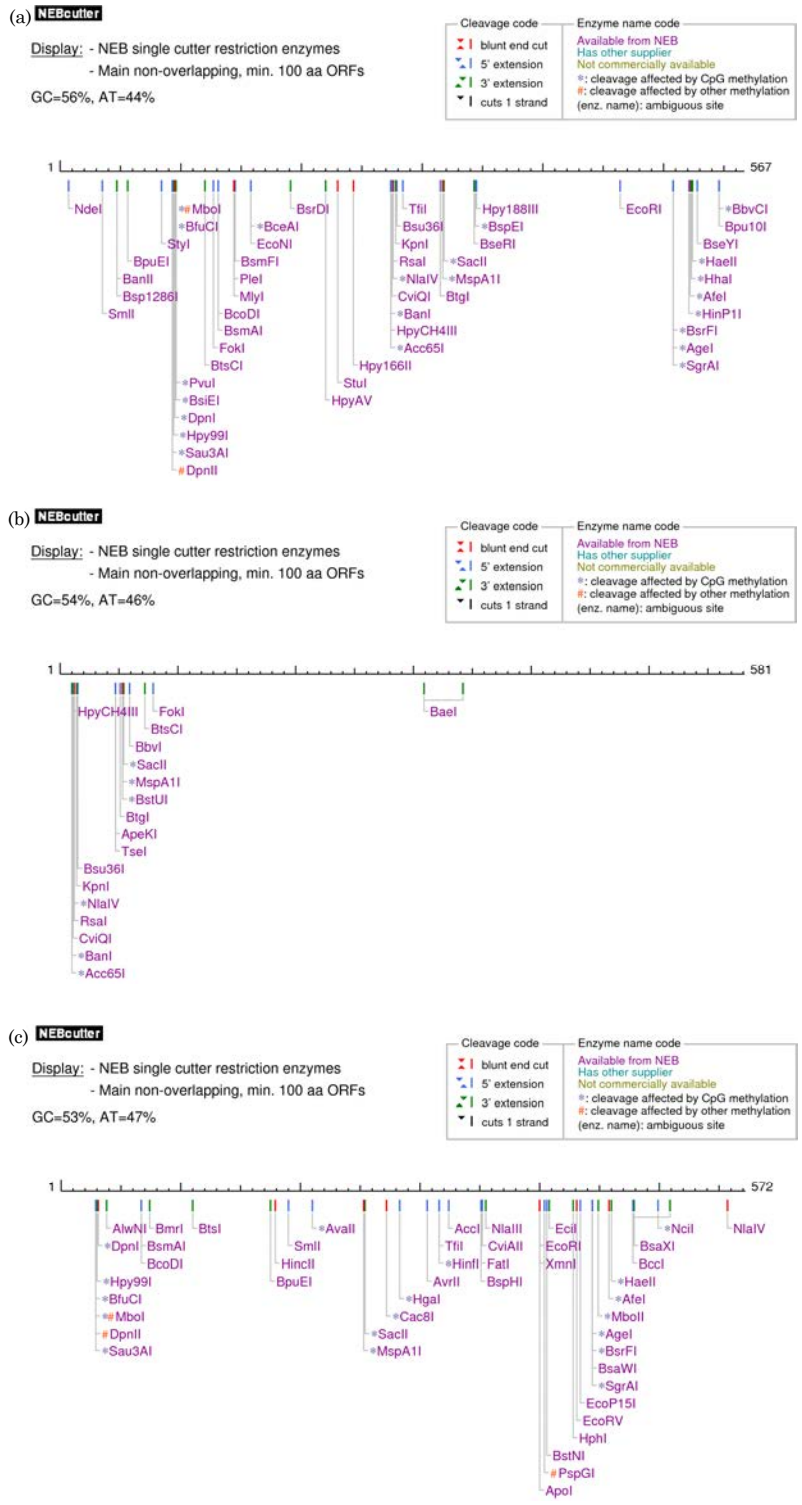

Fig. 5(a-c): Restriction sites of the ribosomal conserved sequence of cyanobacterial isolates, (a) *Leptolynbya valderiana* NTDM10, (b) *Oscillatoria boryana* NTDM11 and (c) *Planktothrix pseudoagardhii* NTRD01

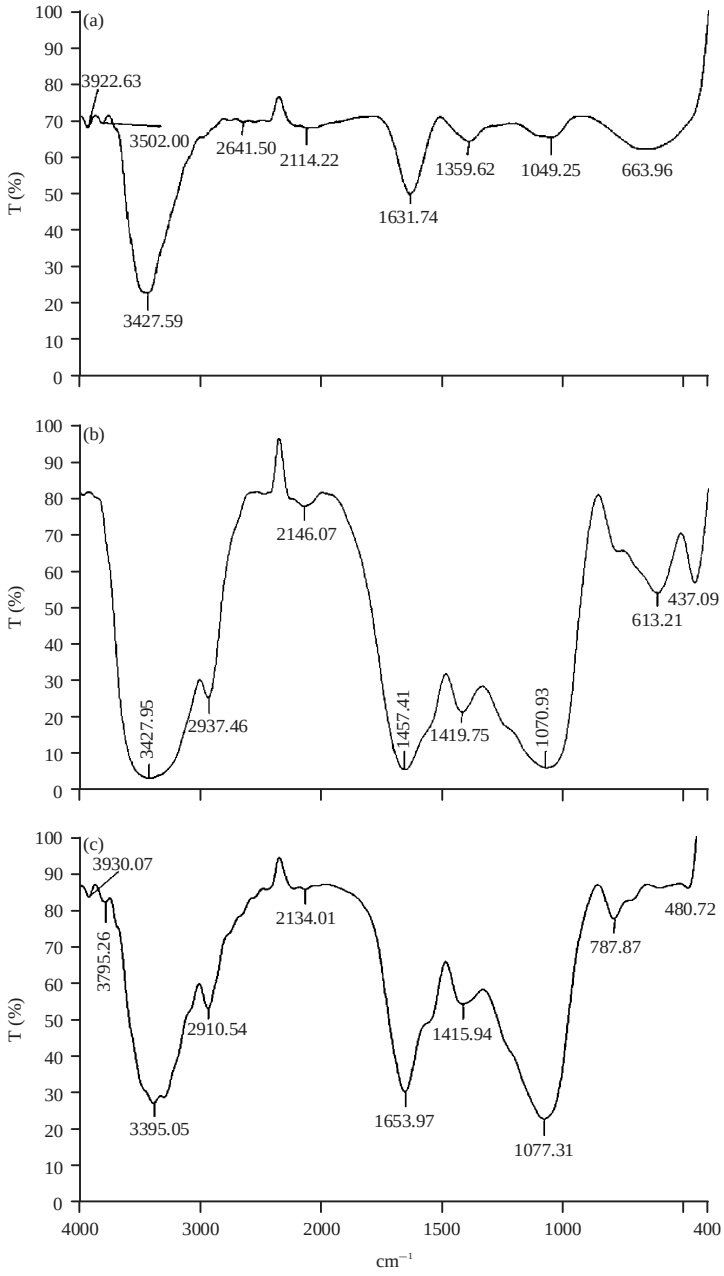

Fig. 6(a-c): FTIR spectrum of cyanobacterial isolates, (a) *Leptolynbya valderiana* NTDM10, (b) *Oscillatoria boryana* NTDM11 and (c) *Planktothrix pseudoagardhii* NTRD01

Biomolecule characterization by FTIR analysis: Cyanobacterial species was further characterized by FTIR spectroscopy and the spectral differences between each species was studied. *Leptolynbya valderiana* NTDM10, *Oscillatoria boryana* NTDM11 and *Planktothrix pseudoagardhii* NTRD01 was spectroscopically analyzed in the spectral range of 4000-400 cm^{-1}. The FTIR spectrums of the three cyanobacterial species were also shown (Fig. 6). Fourier Transform Infrared microspectroscopy (FTIR), in combination with chemometrics, was investigated as a novel method to discriminate between cyanobacterial strains. The FTIR spectroscopy combined with chemometric

classification methods constitute a rapid, reproducible and potentially automated approach to classifying filamentous cyanobacteria (Bounphanmy *et al*., 2010). The total of 810 absorbance spectra were recorded from one eukaryotic and cyanobacterial taxa spanned three genera and include two strains of one species, *Microcystis aeruginosa*. Principal Component Analysis (PCA) based classified techniques such as Soft Independent Modeling of Class Analogy (SIMCA) and K-Nearest Neighbors (KNN) were investigated. Different spectral regions using derivative spectra were investigated to find the best combinations for classification. The highest rate of correct classifications (99-100%) was achieved using first derivative spectra with a spectral region of 1800-950 cm^{-1} for both the SIMCA and KNN. A dendrogram constructed using averaged spectra of the six taxa studied showed that the two strains of *Microcystis aeruginosa* exhibited the highest degree of similarity, while the eukaryotic taxon was the most dissimilar from the prokaryotic (Kansiz *et al*., 1999).

CONCLUSION

The cyanobacteria are widely distributed in all types of ecosystem. This present study emphasized on the genetic variation by sequence variability was found in the cyanobacteria isolated from different ecosystem was assessed based on the ribosomal conserved sequence such as 16S rDNA. Spectrometric study also aid for the identification of cyanobacterial strains. Molecularly identified sequences were deposited in GenBank with accession of JQ867392, JQ867393 and JQ867394. It is suggested that along with ribosomal conserved sequence, IR spectrum would be one of the ideal tool to differentiate the cyanobacterial strains.

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