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Genetic Diversity of Marine and Fresh Water Cyanobacteria from the Gujarat State of India

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Abstract

Cyanobacteria from habitats within Gujarat have been poorly studied with regard to their diversity. In the present investigation eight morphologically distinct cyanobacterial isolates were obtained and characterized from the fresh water and marine habitats. Identification was performed based on morphological features and on 16S rDNA sequences analysis. A phylogenetic tree based on 16S rDNA sequence of cyanobacterial isolates was prepared. Phylogenetic analysis clustered the eight morphologically distinct isolates into two distinct groups thus highlighting the importance of both morphological and genetic methods in studying cyanobacterial diversity.

Keywords

Cyanobacteria, 16S rDNA, Phylogenetic Analysis, Cyanobacterial Diversity

1. Introduction

Cyanobacteria were the first prokaryotic organisms believed to have evolved on earth approximately 3.8 billion years ago. They constitute one of the major gram negative bacterial phyla and occupy the diverse range of habitats inhabiting most of the Earth's environment. They display considerable morphological diversity ranging from unicellular to colonial and filamentous forms. Cyanobacteria are capable of photo-autotrophic growth; perform oxygenic photosynthesis similar to those of eukaryotic algae and plants. They also have the unique ability to fix atmospheric nitrogen [1] [2].

Cyanobacteria can be classified on the basis of morphology, cellular differentiation, biochemical, physiological and genetic criteria. The taxonomy of cyanobacteria until now has been based mainly on their morphology and according to it they are classified into five orders: Chroococcales (I), Pleurocapsales (II), Oscillatoriales (III),

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Nostocales (IV) and Stigonematales (V). However, the morphology and other phenotypic characteristics of cyanobacteria can be dramatically influenced by environmental factors and stage of development [3] [4]. Thus, classifications based on phenotypic characteristics do not represent natural grouping when analyzed based on genetic data.

DNA base composition is important genetic character used to study the taxonomy of cyanobacteria. Analysis of the DNA base composition (Mol % G + C) is one of the few molecular characters that have been determined for almost 200 cyanobacterial strains. Comparative analysis of 16S ribosomal RNA sequence has been used for identification and construction of cyanobacterial phylogeny [5].

Gujarat state of India is rich in both fresh water and marine ecosystems. In the present study we have isolated eight cyanobacterial strains from freshwater and marine ecosystems and tried to explore their diversity using morphology and 16S rRNA sequence analysis.

2. Material and Methods

2.1. Sample Collection

Water samples were collected from five different regions of the Gujarat state namely Rajkot, Bhuj, Positara, Gadhada, Okha, Mithapur, which include both fresh and marine habitats and were stored in a growth chamber at 25˚C with under 8 hours/16 hours dark/light photoperiod. For morphological studies, the strains were cultivated in BG11 medium [6].

Pure cultures of cyanobacteria were obtained by three techniques viz. enrichment, serial dilution and direct streak plate method. In the serial dilution method, the inoculum was prepared by mixing the samples with sterilized BG11 medium and then serial dilutions were made in test tubes containing same medium. The tubes were incubated at 25˚C under 8 hours/16 hours dark/light photoperiod. After growth the culture was streaked on BG11 agar plates to obtain unialgal cultures. In the streak plate method, the crude samples were directly streaked on BG11 agar plates. The plates were incubated at 25˚C under 8 hours/16 hours dark/light photoperiod. Through successive streaking or transferring from one agar plate to another, unialgal cultures were obtained. Unialgal stocks were maintained in 100 ml liquid medium as well as on agar slants and stored at 25˚C under low light intensity.

Axenic culture of cyanobacteria was obtained by treating the cells with standard antibiotic solution (Streptomycin, Nalidixic acid, Ampicillin and Cycloheximide). 400 µL of sterile BG11 medium was added along with 400 µL of sterile antibiotic solution in both solid and liquid media to give a final antibiotic concentration of 50 µg/ml. The culture was then incubated at room temperature under darkness. The morphology of cells and filaments was studied using an Olympus 45× light microscope with a digital camera.

2.2. DNA Extraction, PCRs and Sequencing

Purelink Plant DNA extraction kit (Invitrogen, USA) was used to obtain high quality genomic DNA samples from cyanobacterial isolates, according to the manufacturer's protocol. Electrophoresis was done in 0.8% agarose gel at 5 V/cm for 30 min. Quantity of the genomic DNA was estimated spectrophotometrically by measuring absorbance at 280 nm while the quality was determined by taking ratio of absorbance at 260 and 280 nm in a UV 1800 Shimadzu UV Vis Spectrophometer.

2.3. PCR Amplifications

In order to the get the sequence of 16S rDNA from cyanobacterial isolates, the 16s rRNA gene was amplified using two sets of oligonucleotide primers in separate PCR reactions [7]. The sequence of the primer and the approximate length of DNA amplified by them are given in **Table 1**.

Table 1. Primer used for the amplification of 16S rRNA gene.

Set	Primer sequence	Length of PCR amplicon
1	CYA106F-5'CGGACGGGTGAGTAACGCGTGA3' CYA781R-5'GACTACTGGGGTATCTAATCCCATT3'	675
2	CYA106F-5'CGGACGGGTGAGTAACGCGTGA3' CYAN1281R-5'GCAATTACTAGCGATTCCTCC3'	1175

PCR were performed by GeneAmp PCR system 9700. The PCR reaction mixture (20 µL total volume) consisted of 100 ng genomic DNA, 1.5 mM $MgCl_2$, 10 mM of each dNTP, 1xTaq buffer, 10 µM of each primer and 1 unit of Taq polymerase. Samples were subjected to the following thermal profile: 3 min of denaturing at 94˚C and 30 cycles of three steps: 30 sec of denaturing at 94˚C, 30 sec of annealing at 58˚C, and 1 min of elongation at 72˚C followed by final extension of 10 minutes at 72˚C. A digital gel image of the gel was obtained in Gel Doc XR system (Bio-Rad) using QUANTITY ONE® 1-D V 4.6.7 analysis software.

2.4. Sequencing

After purification [PCR purification kit (Genei, Bangalore)] the PCR amplicons were directly sequenced at 1st BASE DNA sequencing Services, Malaysia. The sequence obtained using both set of primers were checked for overlaps and a final sequence of 1175 base pair was obtained. The sequences obtained were submitted to NCBI (KC140132 - KC140134 and KC248207 - KC248211).

2.5. Phylogenetic Analysis

DNA sequence data was analyzed by BLAST. Multiple sequence alignments were generated using the CLUSTALW program [8]. Phylogenetic distance trees were inferred by Neighbour-Joining analyses [9], using MEGA5.10 [10]. Confidence in topologies was assessed using bootstrapping (1000 replicates).

3. Result

3.1. Morphological Characteristics of Isolated Cyanobacteria

On microscopic observation of samples we distinguished eight different cyanobacterial morphotypes. **Figure 1** shows the morphological diversity among the isolates.

3.2. Amplification of 16S rRNA Gene by PCR

The 16S rRNA gene was amplified using hot start PCR with two set of primers, CYA106F and CYA781R; CYA106F and CYA1281R. A PCR product of 675 bp was obtained using the first set and an amplification product 1175 bp was obtained using the second set of primer (**Figure 2**). The sequence obtained using both primers were aligned to get the complete sequence of 1175 bp as sequencing gave a read only up to 700 bp. The complete sequences of 1175 bp were submitted to NCBI.

3.3. Phylogenetic Tree

A phylogenetic tree based on 16S rRNA sequences was constructed using MEGA 5.10 software (**Figure 3**). The

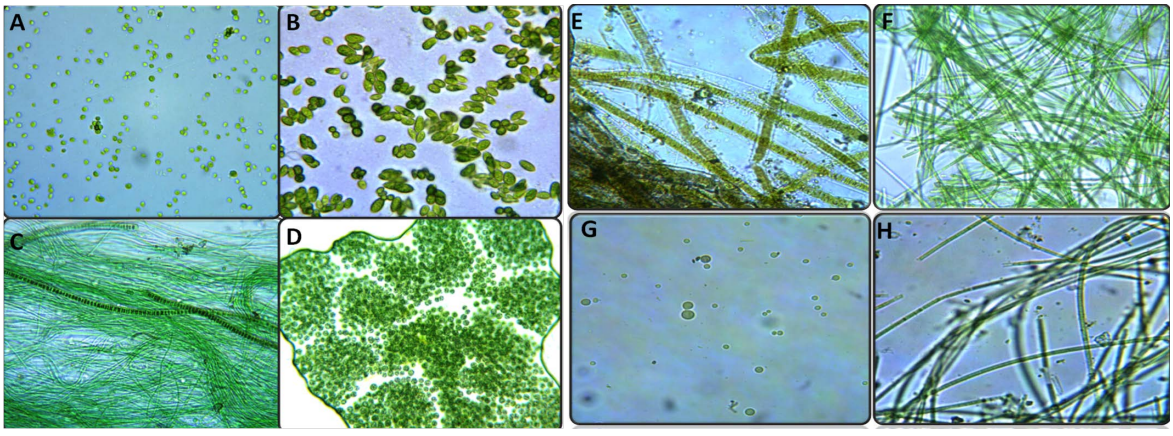
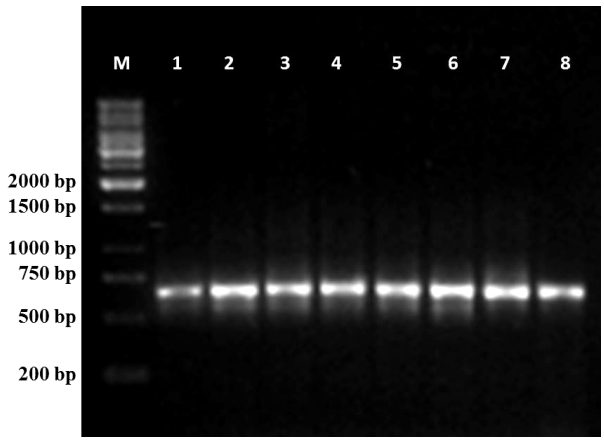
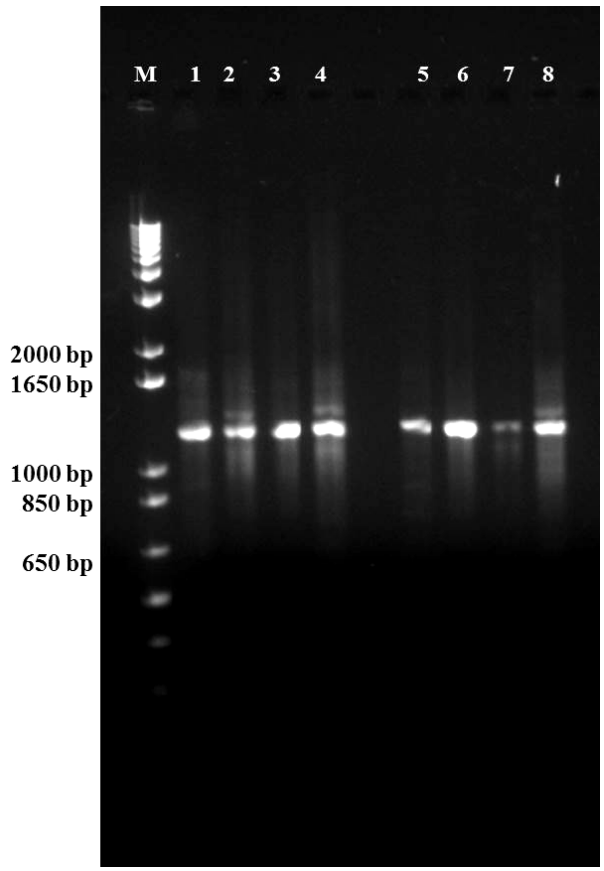


Figure 1. Light micrograph illustrating the diversity of Cyanobacterial morphotypes isolated from the Gujarat state of India. (A) Cyanobacterium sp. PBJ1 KC248207.1; (B) Cyanobacterium sp. PBJ2 KC248208.1; (C) Cyanobacterium sp. PRJT KC140132.1; (D) Cyanobacterium sp. PSGH KC248209.1; (E) Cyanobacterium sp. POKH1 KC140133.1; (F) Cyanobacterium sp. POKH2 KC248210.1; (G) Cyanobacterium sp. PMTP KC248211.1; (H) Cyanobacterium sp. PPST KC140134.1.



(A)



(B)

Figure 2. PCR amplicons of 16S rDNA gene in 1.0% (w/v) agarose gel. (A) PCR amplification using primers CYA106F and CYA781R; (B) PCR amplification using primers CYA106F and CYA1281R (M, Marker; 1, PBJ1; 2, PBJ2; 3, PRJT; 4, PSGH; 5, POKH1; 6, POKH2; 7, PMTP; 8, PPST).

isolates grouped into two clusters, the first cluster, supported by 100% bootstrap value, included isolates Cyanobacterium sp. POKH1, Cyanobacterium sp. PSGH, Cyanobacterium sp. PBJ1, and Cyanobacterium sp. PBJ2 while the other cluster which was not supported by a sufficient bootstrap value included Cyanobacterium sp. PRJT, Cyanobacterium sp. POKH2, Cyanobacterium sp. PPST and Cyanobacterium sp. MTP.

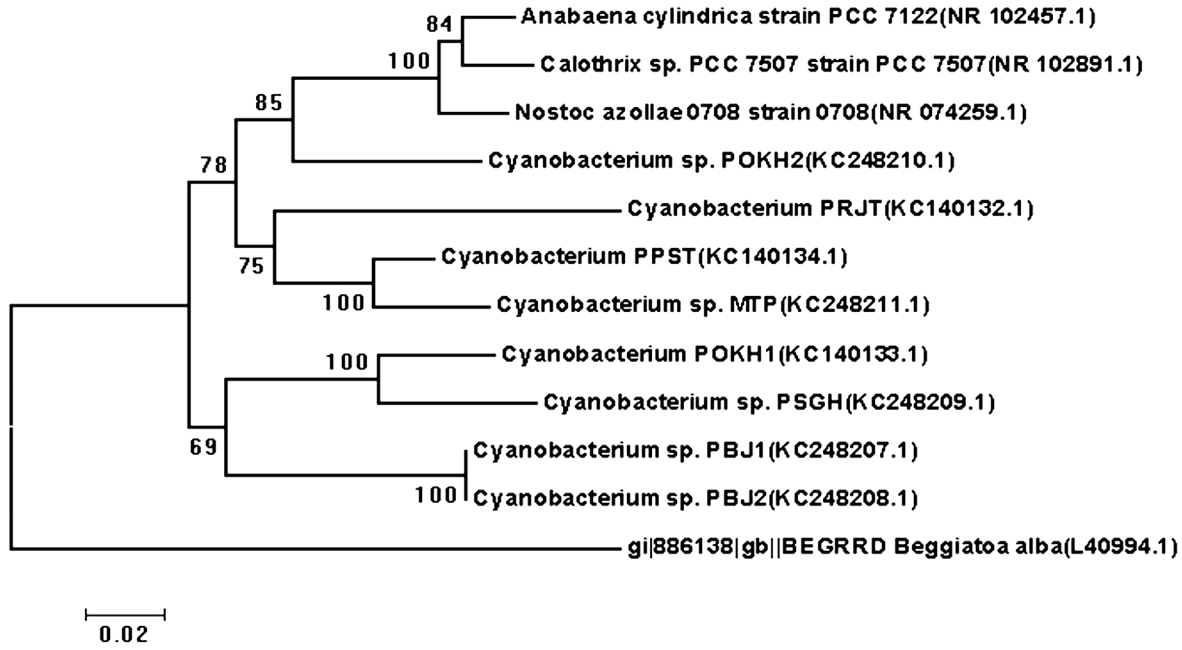


Figure 3. Phylogenetic tree based on an alignment containing the partial 16S rRNA gene sequences from 8 cyanobacteria constructed by neighbour-joining with *Beggiatoa alba* as outgroup.

4. Discussion

Pure cultures of cyanobacteria are essential for studying various aspects of their biology. Gujarat state of India has one of the largest coastlines but still there are very few reports on isolation, characterization and diversity of cyanobacteria from these habitats. Since isolation and culturing of all forms of cyanobacteria are difficult, we are able to isolate only eight morphologically distinct cyanobacterial isolates from water samples collected from different regions of Gujarat. The general inability of cyanobacterial species to grow as unialgal culture has been noted in the literature and has been attributed to the fact that laboratory environment is quite different compared to natural habitats [11]. Also, the variety of biotic interactions that exists among species may be critically important for the survival for cyanobacterial species. The 16S rRNA gene sequence analysis grouped these eight morphologically distinct isolates into two distinct clusters, highlighting the importance of both morphological and genetic methods in studying cyanobacterial diversity. The cyanobacteria has been reported to exhibit pronounced polymorphism with changing environmental conditions, and thus studying diversity just based on morphological characteristics is not sufficient [12]. Garcia-Pichel *et al*. (1998) also demonstrated that morphology is not a phylogenetically reliable character for the taxonomy of cyanobacteria and it has to be substantiated with physiological and genetic characteristics [13]. 16S rRNA gene analyses have been used to study the diversity of cyanobacteria. Analysis of diversity based on comparison 16S rRNA gene sequences is a reliable way of studying cyanobacterial diversity.

Our work is a preliminary report on isolation, characterization and diversity of cyanobacterial isolates from Gujarat state of India.

Conflicts of Interest Statement

The experiments comply with the current laws of the countries in which the experiments were performed. The authors declare no conflict of interest.

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