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Molecular Identification of Marine Cyanobacterium-Phormidium ARKK2 from Mangrove Environment

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Abstract

Cyanobacteria are the largest group of photosynthetic prokaryotes, capable of fixing both atmospheric carbon and nitrogen. They are unique in their widespread occurrence abundance and morphological diversity, similar to gram negative bacteria in their cell structure and biochemistry. Only few studies are available on marine cyanobacteria from mangrove habitats. The present study was therefore, made to analyze the marine cyanobacterial population in mangrove rhizosphere soil. The marine cyanobacterium *Phormidium* species *ARKK2* was isolated from sediments of Vellar mangrove forest situated in south east coast of India. Five spices belonging to family Oscillatoriaceae and Chroococcaceae were identified based on the size and shape and they were *Phormidium* sp., *Oscillatoria willei*, *Phormidium fragile*, *Synechococcus elongatus* and *Gloeocapsa* sp., Of the five species, *Phormidium* sp., were analyzed for 16S rDNA, and their phylogeny.

Key wards: Mangroves, Cyanobacteria, Phormidium sp., 16SrDNA.

1. Introduction

Cyanobacteria are the largest group of photosynthetic prokaryotes, capable of fixing both atmospheric carbon and nitrogen [1]. Marine cyanobacteria are abundantly present in both coastal and oligotrophic envzironments [2]. The cyanobacteria constitute one of the important resources of mangrove ecosystem along the tropical coasts [3]. The current classification of cyanobacteria relies heavily upon morphological observations such as cell size, shape and arrangement (filamentous, colonial or single cells), colouration and the presence of characters such as gas vacuoles and a sheath [4]. Although morphologically similar, it has been shown that this order contains organisms originating from several evolutionarily distinct and deeply branching groups within the cyanobacterial phylum [5].

DNA base composition is a very important genetic character to study the taxonomy not only for cyanobacteria but also for all other living organisms [6]. Based on the DNA base composition (Mol % G+C) almost 200 cyanobacterial strains have been determined through molecular character analysis [7]. Large differences in DNA base composition revealed that the strains cannot be closely related, whereas similar G+C percentages give no clue concerning genotyping relationships [8]. Grouping sequence identity is supported by morphological features (size and morphology of vegetative cells, heterocyst and akinetes, and diameter and morphology of trichomes) [9]. The present study attempted to isolate, the non-heterocyst filamentous marine cyanobacteria derived from mangrove biotope, and to identify the predominant species among the total species from the mangrove environment based on morphology and molecular phylogenic characters [10].

2. Materials and methods

2.1. Sample collection

Samples were collected from Parangipettai mangrove forest situated in south east coast of India (Lat. 11° 29’25.1 N; Long. 79º 45’ 51.9 E) by using a corer (1.5m long stainless steel corer with 50cm dia.) during low tide. The samples were transferred to 4°C and analyzed for microbial groups within 4-6 hours of sampling.

2.2. Soil microbiological analysis

A known weight of sediment (1g) was aseptically weighed and transferred to a stopper (150ml) sterile conical flask containing 99ml of sterile diluents. The sediment- diluents mixture was agitated by means of mechanical shaking for about 5-10 minutes and later it was subjected to cyanobacterial examination [11].

Samples were serially diluted up to 10^{-5} with sterilized 50% seawater and plated with BG 11 (Blue-Green) medium for cyanobacteria [12].

2.3. Isolation and maintenance of cyanobacteria

The Cyanobacteria cultures were grown in BG11 medium under laboratory conditions at a light intensity of 3000 lux and room temperature of 24±2˚C with a 14 h light/10 h dark cycle [13]. After a week, the cultures were picked up from the water surface and from the sides of the flasks and were examined under a microscope. The cyanobacteria were identified by using the standard references [14]. The cyanobacterial isolates were obtained by pour plating [15] and isolation of single colonies on Petri dishes. The cultures were sub-cultured once in 7 days for 5 times and pure cultures were obtained. The pure stocks of cyanobacterial cultures were maintained on agar slants. They were incubated in low light at a temperature of 24±2°C. The successive transfers of stock cultures were made for every month [16] and microscopic examination**.**

2.4. Identification of Oscillatoriaceae and Chroococcaceae group members

Based on morphological characteristics under light microscopic examination, a total of five cyanobacterial species belonging to family Oscillatoriaceae and Chroococcaceae were identified in the present study.

2.5. Genomic DNA extraction

DNA was isolated by following xanthogenate-SDS (XS) DNA extraction protocol [17]. Briefly, 1ml volume of the mid to late logarthmic growth phase cyanobacetrial cell culture were harvested by centrifugation and the pellets were resuspended in 50µL of TER (10 Mm tris HCL, pH 7.4: 1 Mm EDTA, Ph 8; 100µg; RNase A) [18]. To each cell suspension in a 1.5 ml microcentrifuge tube was added with 750µL of freshly made XS buffer (1 % potassium ethyl xanthogenate [Hi-media Mumbai, India]; 100 mM Tris-HCL. pH 7.4; 20 mM EDTA. pH 8; 1% sodium dodecylsulfate; 800mM ammonium acetate) and the tubes were inverted several times to mix. The tubes were incubated at 70°C for 10 min in a waterbath. After incubation the tubes were vortexed for 10seconds before being placed on ice for 30 min. Precipitated cell debris was removed by centrifugation at 14,000 rpm for 10 min and the supernatant was carefully transferred to fresh eppendorf tubes containing 750µL of isopropanol sample [19].. Samples were incubated at room temperature for 10 min and centrifuged at 12,000 g. The DNA pellets were washed once with 70 % ethanol, air-dried, and finally resuspended in 100 µL of TE (10Mm Tris-HCL, pH 7.4; 1

Mm EDTA, pH 8). To ensure that the DNA samples were free of exo-and endonucleases, samples were incubated at 37°C for 2h in 1X *HinF I* restriction enzyme buffer before gel electrophoresis [20]..

2.6. Amplification of 16S rDNA gene by PCR

PCR amplifications were performed using the primer pair 29F (5'-GAATTTKCCGYAAKGGGC-3') and 1459R (5'-GGTAAYGACTTCGGGCRT-3) as described by Diaz (1997). For precise annealing of the primers to target DNA, 0.5 U of Taq DNA polymerase (GeNei Biotechnology, Ltd., Bangalore, India) was added to the 24µL reaction mixture after the initial denaturation step at 94°C for 5 min. The PCR was conditioned as, 35 incubation cycles followed by, each step consisting of 1 min at 94°C, 1 min at 60°C, 1 min at 72°C and final extension at 72°C for 8 minutes. The successful amplification was tested in 1.2% china agarose (Bioserve Biotechnologies Pvt. Lt., Hyderabad, India) gel electrophoresis. PCR product purification and DNA sequencing was carried through high throughput MegaBace sequencer (Bioserve Biotechnologies Pvt. Ltd., Hyderabad, India) [21]..

2.7. Nucleotide sequence accession numbers

The nucleotide sequence data derived in the study have been assigned the following GenBank accession number: JN998127.

3. Results

3.1. Morphological characteristics of the isolated cyanobacteria

Based on morphological characteristics under light microscopic examination, a total of five cyanobacterial species belonging to family Oscillatoriaceae and Chroococcaceae were identified in the present study. The species identified were *Phormidium* sp., *Oscillatoria willei, Phormidium fragile, Synechococcus elongatus* and *Gloeocapsa* sp.

1. *Phormidium sp.,*

   (Family – Oscillatoriaceae; order – Nostocales)

Thallus soft blue-green and membranous; filaments 12-14 µ broad; trichome blue-green, interwoven and not attenuating, 8-9.5 µ broad; sheath hyaline.

2. *Oscillatoria willei* Gardner em.Droute

   (Family – Oscillatoriaceae; order – Nostocales)

Trichome pale blue-green bent at the ends or screw like, 2,4-3.6 µ broad; unconstructed at the cross-wall, ends not attenuated not capitates; cells 1.3 up to twice as long as broad, not granulated at the cross walls, and cells rounded without a thickened membrane.

3. *Phormidium fragile*

(Family – Oscillatoriaceae; order – Nostocales)

Thallus mucilaginous, lamellated, yellowish or brownish blue-green; sheath diffluent, not coloured violet by chlor-zinc-iodide; trichomes more or less flexuous, entangled or nearly parallel, distinctly constructed at the cross walls, septa not granulated at the ends, 1.2-2.3 broad, pale blue green; cells nearly quardrate.

4. *Synechococcus elongatus* Nag.

(Family – Choroococcaceae; order – Choroococales)

Cells cylindrical 1.4-2µ broad, 1.5-3 times as long as broad, single or 2-4 cells together; contents homogenous and light blue green .

5. *Gloeocapsa* sp.,

(Family – Choroococcaceae; order – Choroococales)

Thallus gelatinous olivaceous, when dried blackish, gelatinous, soft; cell in groups of 2-4 rarely more, colonies spherical or oval, 12-24µ diameter, closely arranged in the peripheral regions and loosely arranged in the middle, cells without sheath 4-8 µ in diameter; with a thin yellowish to brownish sheath in the outer regions and colourless or diffluent in the sheath inside, sheath unlamellated; often with nannocyte formation, nannocytes 2.5-3.5µ diameter [22].

Among the filamentous and unicellular forms of marine cyanobacteria, *Phormidium* sp., was found to be abundant and they were *Phormidium* sp., The *Phormidium* sp., was further analysed for molecular phylogency. The predominant *Phormidium* sp., was examined under light microscopic examination by using a fluorescence microscope (Model: DM 2500, Leica, Switzerland) [23]. Figure. 1.

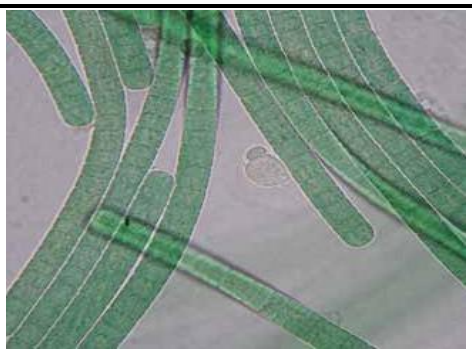
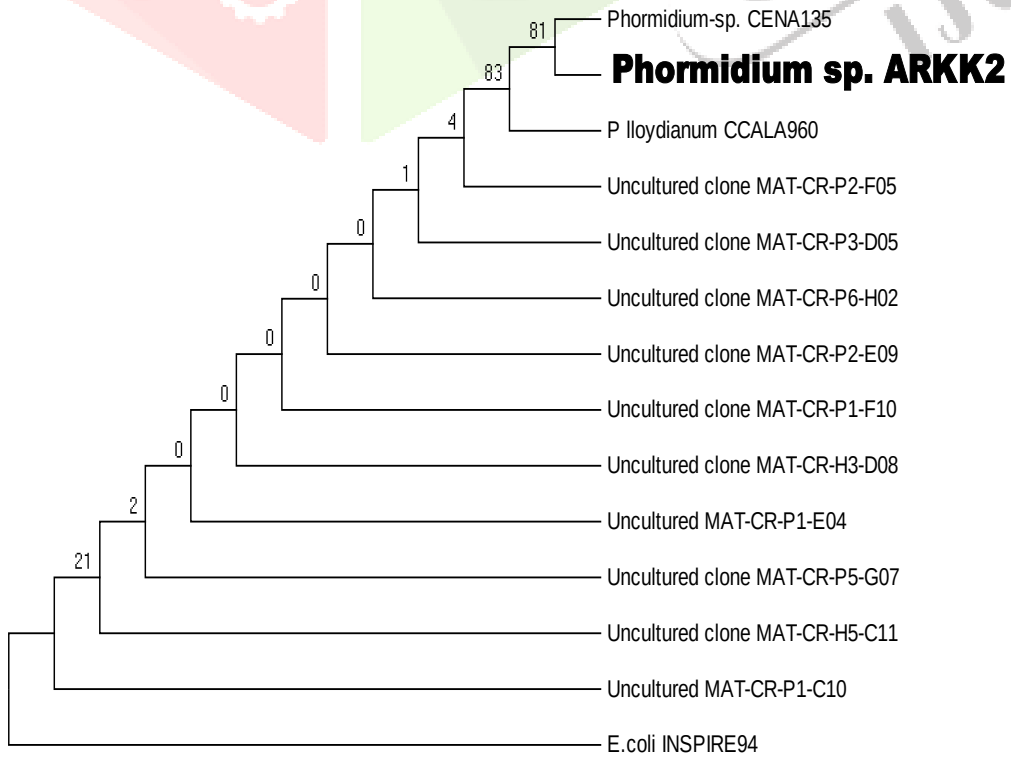


Figure 1. View under light microscope (40x magnifications).

3.2. Molecular taxonomy

Phormidium ARRK2 showed more similarity with that of *Phormidium* sp. strain CENA135 isolated in Brazil and interestingly the strain CENA135 was also isolated from mangrove environment of Brazil region [24]. The 16S rRNA gene sequence (Sequences were blasted against the complete non-redundant NCBI Genbank database and were aligned using the CLUSTAL W Multiple Sequence Alignment Program by the method of Thompson *et al.,* [1994] of *Phormidium* sp. ARRK2 shared 4% variation with *P. lloydianum* (HQ730084) but could not be consider as same species as the variation is greater than the definition of species boundary which is 3% [25]. The tree topology obtained from phylogenetic analysis of cyanobacterial 16S rDNA sequences was in agreement with that described by Honda *et al*. [1999]. Hence, based on the morphological and phylogenetic analysis (Figure 2) it is being concluded that the species of cyanobacteria were *Phormidium* sp.ARKK2.



Figures 2. Closest similarity (88-99%) in GenBank database with that of query sequence is used for phylogram construction. The optimal tree with the sum of branch length = 7.63913458 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (2000 replicates) is shown next to the branches. The evolutionary distances were in the units of the number of base substitutions per site. There were a total of 1098 positions in the final dataset.

4. Discussion

Cyanobacteria are widespread and abundant in marine habits. They grow in seawater is presumably related to a preference for alkaline condition and an ability to tolerate high salt concentrations. The resistance, which many species show towards osmotic shock, extremes of temperature and reducing conditions, suits their existence in a variety of intertidal habitats. Cyanobacteria are an important microorganisms used to produce number of essential industrial products [26].

This study report has identified the presence of cyanobacteria from the mangrove environment**.** Future studies will be focused on determining the presence and distribution of different species of cyanobacteria from mangrove environment. This is the first report on *Phormidium* sp. ARKK2 have been studied from Parangipettai mangrove forest, In the study area are artificially developed mangrove forest by Prof. K. Kathiresan [32,33].

Thajuddin and Subramanian (1991) have made a detailed survey of marine cyanobacterial biodiversity of a continuous stretch of over 2660 km off the coastline from Tirakol of Goa to Cape Comorin of Tamilnadu and from Cape Comorin to Bhimunipattanam of Andhra Pradesh encompassing the regions such as the Arabian Sea, Indian ocean, Palk Bay, Palk Strait, Gulf of Manner and bay of Bengal including Andaman and nicobar and Lakshadweep [28]. Which revealed that the presences of cyanobacteria [29,30,31,32]. This work will be more used to further fruitful findings.

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