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Full Length Research Paper

Identification and phylogenetic analysis of filamentous Cyanobacteria using random amplified polymorphic DNA (RAPD) fingerprinting

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RAPD techniques were used for the detection of genetic heterogeneticity among the axenic culture of fresh water cyanobacterial isolates. The electrophoretic patterns for 12 cyanobacterial strains belonging to 2 genera (8 *Oscillatoria* strains and 4 *Lyngbya* strains) were used for molecular analysis using the RAPD technique. A total of 12 cyanobacterial isolates were selected and mass cultured in BG11 medium. Genomic DNA was extracted from fresh water cyanobacterial isolates and was amplified using primers D7, M13, OPC11, OPC12, OPC13, OPC14 and OPC15 and distinct PCR fingerprint were generated. Unique banding patterns were observed from all tested cyanobacterial species and their molecular weights of each band were used to calculate their genetic distance among them. Random amplification of polymorphic DNA (RAPD) was carried out for the phylogenetic characterization of these strains. RAPD fingerprinting results clearly showed the genetic variation among the cyanobacterial isolates.

Key words*:* Cyanobacteria, phylogenetic analysis, RAPD, PCR, primers.

INTRODUCTION

Cyanobacteria are unique among the prokaryotes due to their capacity for oxygenic photosynthesis. An important feature of many cyanobacteria is their ability to fix atmospheric nitrogen both under free-living and symbiotic conditions. The species of Cyanobacteria which are known to fix atmospheric nitrogen are classified into three groups; heterocystous aerobic forms, aerobic unicellular forms and non-heterocystous filamentous microaerophilic forms. Nitrogen-fixing cyanobacteria can use sunlight as the energy source for the fixation of carbon and nitrogen, therefore are potential biofertilizers (Kannaiyan, 1985).

The morphology of cyanobacteria in laboratory cultures is known to be modified and the variation between same species become reduced due to the controlled culture conditions (Dores and Parker, 1988).

For example many species of the genera *Oscillatoria*, *Lyngbya*, *Phormidium*, *Schizothrix, Plectonema* were included in *Schizothrix calcicola* (Drouet, 1968), which was originally classified on the basis of sheath characterization and the presence or absence of false branching (Gomont 1892). Rippka et al. (1979) considered strains within the Oscillatoriales under a new group designated as LPP-group B (*Lyngbya*, *Phormidium* and *Plectonema*). There has been increased interest in applying molecular techniques to resolve many of the issues and problems in cyanobacterial taxonomy (Giovannoni et al., 1988; Wilmotte and Golubic, 1991).

Molecular polymorphisms can be identified at random and used to discriminate the phylogenetic relatedness of microorganisms. Using this clue a renowned molecular technique was developed. The technique is Randomly Amplified Polymorphic DNA assay. Actually this is a PCR reaction but it amplify segment of DNA, which are essentially unknown to the researcher (random). Often, PCR is used to amplify a known sequence of DNA that want to amplify, then design and makes primers which

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Abbreviations: RAPD, Random amplification of polymorphic DNA; **ERIC**, enterobacterial repetitive intergenic consensus; **REP**, repetitive extragenic palindromic; **TBE**, tris borate-EDTA; **UPGMA**, unweighted pair–group method using arithmetic averages.

Table 1. Algal strains used as sources of DNA.

S/N	Name of the strain	Nature of the sample	Place of collection
1	*Oscillatoria acuminata* Gomont	Paddy field soil	Aduthurai
2	*Oscillatoria foreaui* Fremy	Water canal	Tenkasi
3	*Oscillatoria animalis* Ag.	Lake	Chegalpattu
4	*Oscillatoria obscura* Bruhl et Biswas	Paddy field	Poondi
5	*Oscillatoria acutissima* Kuff	Canal	Mettur dam
6	*Oscillatoria formosa* Bory	Paddy field	Salem
7	*Oscillatoria boryana* Bory ex Gomont	Lake	Tanjore
8	*Oscillatoria earlei* Gardner	Paddy field	Vedanthangal
9	*Lyngbya lagerheimii* (Möbius) Gomont	Paddy field	Mysore
10	*Lyngbya major* Meneghini ex Gomont	Paddy field	Maduravoyal
11	*Lyngbya martensiana* Meneghini	Paddy field	Mysore
12	*Lyngbya spiralis* Geitler	Pool	Vanduloor

will anneal to sequences flanking the sequence of interest. Thus, PCR leads to the amplification of a particular segment of DNA. This makes it a tool of great power and general applicability.

RAPD technique in conjunction with PCR has been employed to identify many organisms to the strain level (Welsh and McClelland, 1990). Based on RAPD markers seventeen different cyanobacterial cultures derived from 6 different decamer primers were analysed to provide diagnostic fingerprints for each culture and their genetic distances. The study revealed that these RAPD markers could be further used to identify and establish the genetic purity of the strains in the cyanobacterial inoculum. A similarity of 60 – 90% was observed within *Westiellopsis* cultures. *Nostoc* cultures shared 50 – 80% similarity with *Westiellopsis* cultures. *Anabaena* cultures were 60 - 70% similar to *Westiellopsis* cultures. The markers produced for each culture were also applied to phylogenetic analysis to infer genetic relatedness in this group of prokaryotes. (Jeberlin Prabina et al., 2005).

The genetic diversity of symbiotic cyanobacteria in coralloid roots of cycads was examined using PCR fingerprinting with primers derived from repetitive sequences. The highest genetic resolution was achieved using the primer corresponding to the short tandemly repeated repetitive sequences (Weiwen et al., 2002). The Repetitive Extragenic Palindromic (REP) and Enterobacterial Repetitive Intergenic Consensus (ERIC) sequences were originally described for the family Enterobacteriaceae but later found in several gram-negative bacteria and close relatives in the same phyla. ERIC sequences were first reported in *E. coli* and other members of *Enterobacteriaceae* as well as *Vibrio cholerae* (Sharples and Lloyd, 1990; Hulton et al., 1991; Wilson and Sharp, 2006). Lyra et al. (2001) reported the use of ERIC PCR for the genotyping of cyanobacterial strains. Widespread distribution of these repetitive DNA elements in the genomes of various microorganisms should enable rapid identification of bacterial species and strains and be useful for the analysis of prokaryotic genomes (James et al., 1991). Among the cyanobacteria, the family Oscillatoriae consists of uniseriate and differentiated trichomes (with or without mucilage sheath). In these cultures there is always a difficulty in identification and it was considered to analyze the molecular features with available tools to understand their relatedness.

MATERIALS AND METHODS

Cyanobacterial cultures and culture conditions

The cultures used in the study were obtained from cyanobacterial culture collection of CAS in Botany, University of Madras. Eight strains of non heterocystous, filamentous *Oscillatoria* spp. and four strains of *Lyngbya* spp. were used. The details of the cultures used as sources of DNA are presented in Table 1.

These cultures were axenized by Imipenem (Merck), a broad spectrum β-lactam antibiotic (Ferris and Hirsch, 1991). All the isolates were maintained in BG 11 medium under a light intensity of 40 µE m^{-2} s^{-1} and 25 ± 1ºC. The culture rack was fitted with Sangmo Weston S650 313F automatic model timer to provide alternative light and dark phases of 12 h each. Batch cultures of the isolates were maintained in BG 11 medium in 250 ml Erlenmeyer flasks. Sixteen days old cultures were used for the study.

Extraction of total genomic DNA from cyanobacterial strains

Total DNA was isolated using the protocol by Wu and Sammy (2000) with few modifications. 5 ml of exponentially growing cyanobacterial cells were harvested by centrifugation, washed twice in wash buffer (Tris buffer (pH 8.0) – 100 mM, EDTA (pH 8.0) - 50 mM, NaCl, - 100 mM) and resuspended in 200 µl of solution I (Tris buffer (pH 8.0) – 25 mM, Glucose-50 mM, EDTA (pH 8.0) - 10 mM) followed by the addition of 2 mg of lysozyme and incubated at 37°C for 1 h. SDS was added to a final concentration of 2% and mixed vigorously and 100 µl of 5M NaCl was added and mixed, left at -20°C for 10 min. The mixture was centrifuged at 12000 rpm for 5 min and to the supernatant, equal volume of phenol: chloroform (1:1) was added and the contents mixed well and centrifuged at 12000 rpm for 5 mins followed by one chloroform wash and DNA was precipitated with 2.5 volumes of 95% ethanol. The pellet was

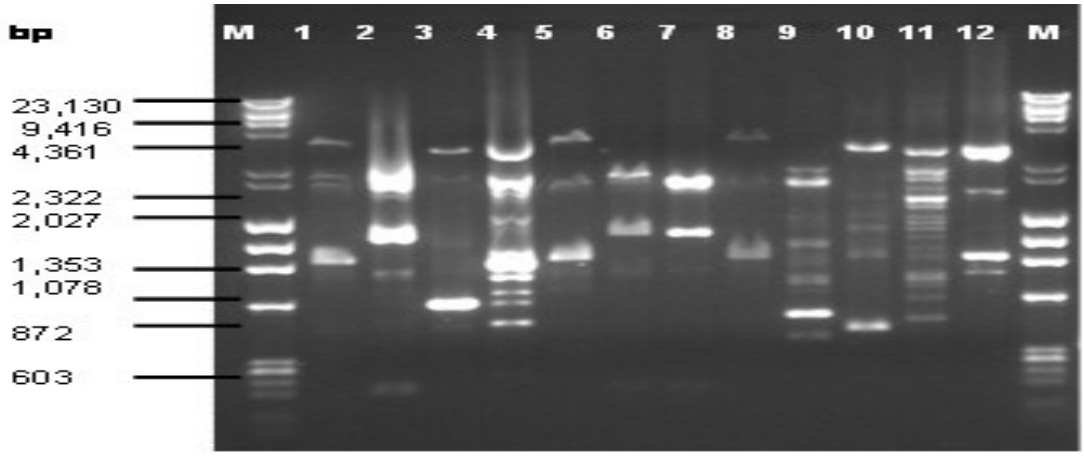

Figure 1. RAPD profile analysis of PCR amplification with primer D7. Lane M, λ DNA–*Hind* III digest and øX174 DNA *Hae* III digest Mix (Finnzymes). Lane 1, *Oscillatoria acuminata* Gomont Lane 2, *Oscillatoria foreaui* Fremy; Lane 3*, Oscillatoria animalis* Ag; Lane 4, *Oscillatoria obscura* Bruhl et Biswas; Lane 5, *Oscillatoria acutissima* Kuff; Lane 6, *Oscillatoria formosa* Bory; Lane 7, *Oscillatoria boryana* Bory ex Gomont; Lane 8, *Oscillatoria earlei* Gardner; Lane 9, *Lyngbya lagerheimii* (Möbius) Gomont; Lane 10, *Lyngbya major* Meneghini ex Gomont; Lane 11, *Lyngbya martensiana* Meneghini; Lane 12, *Lyngbya spiralis* Geitler,

dissolved in 25 µl of STE buffer, followed by RNase (5 µg) treatment and then reprecipitated with 95% ethanol after phenol treatment. The DNA was resuspended in 0.1X TE and used for further analysis.

Randomly amplified polymorphic DNAs (RAPD)

All PCR reactions were carried out in 0.5 ml tubes in either a Perkin Elmer 9600 thermal cycler or in a MJ research PTC 100 cycler. The programs used for amplification are mentioned in detail later along with individual experiments. The list of primers used for the different PCR reaction and their sequences are given in Table 2. All primers were obtained from Sigma Genosys, Bangalore. RAPD was carried out with the primers D7, M13, OPC11, OPC12, OPC13, OPC14 and OPC15.The amplification was carried out with a 20 µL reaction mixture containing: primer (2 µM/µL)- 4.0 µL, 10X buffer-2.0 µL, 2 mM dNTP mix-3.0 µL, *Taq* DNA polymerase (3 U/µL)- 0.6 µL, template DNA (50 ng)- 2.0 µL, Sterile distilled water-8.4 µL. Amplification was carried out with PTC-100 Thermal cycler (MJ Research Inc.).Thermal cycling was performed at 94°C for 2 min, 2 cycles of 94°C for 30 s, 36°C for 30 s and 72°C for 120 s, followed by 30 cycles of 94°C for 20 s, 36°C for 15 s, 45°C for 15 s and 72°C for 90 s and a final extension at 72°C for 10 min. The amplified products were separated on a 2.0% agarose gel in 1XTBE at 75 V for 3 h. The gel was stained with ethidium bromide and the amplified product was visualized and documented using a Vilber Lourmat gel documentation system with Bioimage software.The data obtained for RAPD were arranged to group the strains in a dendrogram separately using UPGMA (Unweighted Pair–Group Method using Arithmetic Averages) programme (using NTSYS software).

RESULTS AND DISCUSSION

Molecular taxonomic analysis of 12 cyanobacterialstrains belonging to the genera *Oscillatoria* and *Lyngbya* were carried out using RAPD. All the analyses were done with total DNA (Wu et al., 2000).

Table 2. Primers used for RAPD-PCR analysis of cyanobacterial strains.

Name	Sequence (5'-3')
D-7	TTGGCACGGG
M-13	GGTGGTCAAG
OPC – 11	AAAGCTGCGG
OPC – 12	TGTCATCCCC
OPC – 13	AAGCCTCGTC
OPC – 14	TGCGTGCTTG
OPC – 15	GACGGATCAG

RAPD was carried out employing seven different primers for the twelve strains and were of high G + C content. Many bands were obtained, ranging from 6.5 kb to 230 bp and were polymorphic. About 21 different molecular bands ranging from 4 kb to 350 bp were observed for the D7 primer (Figure 1). Three unique bands in *Lyngbya martensiana* (1.8,-1.7-and-1.5-kb),-2unique bands *Lyngbya lagerheimii* (350 and 500 bp) and one unique band of 400 bp in *Lyngbya major* were observed. Fourteen different bands were obtained for the M 13 primer (ranging 6.5 kb to 400 bp) in which one unique band of 400 bp was observed in *Lyngbya martensiana*. OPC11 primer produced 21 different bands, ranging from 3 kb to 280 bp. Three unique bands in *L. martensiana* (2.5 kb, 400 and 280 bp) and two unique bands in *Lyngbya lagerheimii* (870 and 700 bp) were ob-

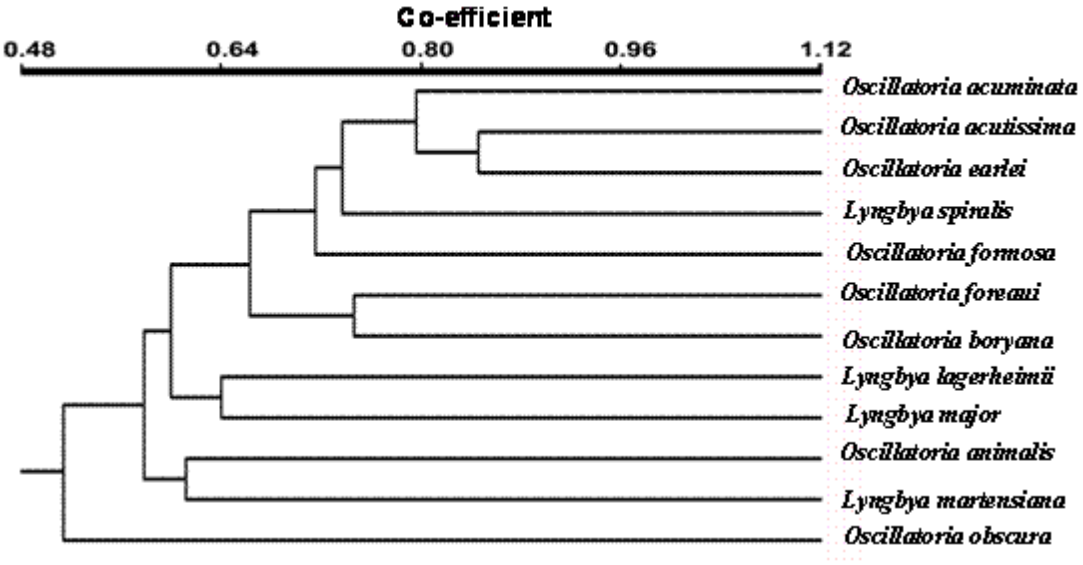

Figure 2. Phylogenetic analysis of cyanobacterial strains' RAPDs.

served. Similarly two unique bands of size 2 and 1.6 kb were observed in *Oscillatoria animalis*. Unique bands of molecular weight of 1.7 kb in were evident. 25 different bands were obtained for OPC 12primer with sizes ranging from 6.5 kb to 230 bp. Twounique bands of sizes, 850 and 230 bp were observed in *O. obscura*. An amplified product of molecular weight 1 kb was found to be unique for *O. acutissima*. OPC 13 primer produced 20 different bands (4.3 kb to 400 bp). Two unique bands were observed for *Oscillatoria obscura* (3 kb and 400 bp) and *L. lagerheimii* (2.3 kb and 550 bp). *Oscillatoria earlei* had a unique band of 450 bp. RAPD primer OPC 14 produced 21 different bands of molecular weight ranging from 3.5 kb to 400 bp. One unique band each was observed for 5 strains namely *Oscillatoria animalis* (2.5 k.b), *Oscillatoria formosa* (1.8 kb), *L. lagerheimii* (600 bp) and *Oscillatoria obscura* (400 bp), *Lyngbya major* had two unique bands of molecular weight 1.3 kb and 750 bp. OPC primer 15 produced 20 different bands of molecular weight 4.3 kb to 270 bp. Only one unique band of molecular weight 3 kb was observed for *Lyngbya spiralis*. Phylogenetic tree constructed had two major clusters, *O. obscura* forming a single major cluster and the rest of the cyanobacterial strains formed the other cluster (Figure 2). RAPD finger printing has been used to differentiate members of the genera *Anabaena* and *Microcystis* by (Neilan et al., 1995). Symbiotic and free living cyanobacterial cultures were identified by Rasmussen and Svenning (1998) and genotypes of *Microcystis* were discriminated by Nishihara et al., (1997). Recently Prabina et al. (2005) has used RAPD finger printing as a tool for checking genetic purity of cyanobacterial strains. In most of these studies, more than ten primers were used to generate RAPD patterns. Otherinteresting features in this study include: (i) D7, M13 and OPC15 primers which produced unique markers only for strains of *Lyngbya* genus; (ii) OPC12 and OPC13 primers produced unique markers only for strains of *Oscillatoria* genus (iii) OPC11 and OPC14 markers produced unique markers for strains belonging to both genera. Since the two genera were closely related as both belong to same family Oscillatoriaceae the strains belonging to *Oscillatoria* and *Lyngbya* were not grouped separately. The number of unique bands produced ranged from one to 8 for all the strains except 3 strains of *Oscillatoria* that did not produce any unique bands. The preliminary results indicate that some
strains of *Oscillatoria* and *Lyngbya* are genetically closer whereas other strains of *Oscillatoria* and *Lyngbya* are genetically separate.

Each band visualized on a gel was considered a RAPD marker and part of the total RAPD fingerprint generated for strains of cyanobacteria. Only two strains of *Lyngbya* got grouped separately. RAPD fingerprinting can be used successfully to differentiate closely related cyanobacterial strains. This method is well suited for fast and accurate strain differentiation and is an alternative and complementary approach to the traditional methods for studying cyanobacterial systematics.

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