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Characterization of *Anabaena* species by RFLP of the 16S rRNA gene

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The genetic diversity studies were conducted in 10 isolates of *Anabaena* spp. Those were morphologically discriminated two groups, each containing five *Anabaena* species based on the proximity of the akinetes to heterocyst, adjacent to or away from the spore in the trichome. Genetic diversity among strains tested was determined with banding patterns from the restriction fragment length polymorphism (RFLP) of the 16S rRNA gene have been used to examine the *Anabaena* spp. Genetic distances between the ten strains tested by RFLP analysis of 16S rRNA genes using three different enzymes *Viz*., *Xba* I, *Taq* I and *Hae* III were used to construct cluster analysis. The clusters for three different enzymes yielded heterogenous groupings of the morphotypes and resulted in unclear delineation of tested organisms.

Key words: Cyanobacteria, *Anabaena*, 16S rRNA gene,
Restriction fragment length polymorphism (RFLP), Phylogeny, Taxonomy.

Cyanobacteria (blue-green algae) are an ancient group of prokaryotic microorganisms exhibiting the general characteristics of gram-negative bacteria. They are unique among the prokaryotes in possessing the capacity of oxygenic photosynthesis. In addition, some cyanobacteria also have the capacity for fixation of atmospheric nitrogen within the same organism. These qualities make cyanobacteria the most successful and widespread group among the prokaryotes found in diverse terrestrial and aquatic environments[1,2,3,4]. Traditionally classified on the basis of their morphology into five orders representing Chroococcales, Pleurocapsales, Oscillatoriales, Nostocales and Stigonematales as given in Bergey's Manual[3].

Molecular systematics has also been used to gain an understanding of phylogenetic divergence within the cyanobacteria. Different kinds of information can now be derived from phenotypic and genotypic data[5]. 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

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the *Anabaena-Azolla* symbiosis species[6,7,8] and isolates from cycads and *Gunnera* have been studied with respect to genetic diversity by using protein profiles and the RFLP technique[9,10,11]. Fresh water isolates of *Synechococcus* spp. have previously been characterized as distinct genotypes by a genomic fingerprinting technique, RFLP of *psbA* genes[12,13]. The amplified ribosomal genes can be digested by restriction enzymes and the analysis of the resulting patterns indicated polymorphisms useful in identification[14]. This technique has been termed restriction fragment length polymorphisms (RFLP). RFLP markers are considered to be a reliable and highly informative tool for characterizing genetic diversity and have been used extensively as an efficient DNA fingerprinting method to identify symbiotic cyanobacteria[15]. RFLP of 16S rRNA is advantageous because the sequencing of 16S rRNA gene is tedious and time consuming especially when one needs to classify a large number of strains[16]. The amplified 16S - 23S rDNA spacer (ITS-1) of cyanobacteria has been used in several studies to genetically characterize strains by PCR-RFLP[17] or by sequence analyses [18, 19, 20, 21]. In the present study the genus *Anabaena* Bory was chosen for taxonomic analysis. The morphological criteria traditionally used for identification of *Anabaena* species are: biometric characters of vegetative cells, heterocysts and spores. An important feature for species identity of the taxa is the proximity of the akinetes to heterocysts[22,23]. In the investigation, our objective was to develop an easy and reliable method to analyze the morphological and genetic variations using RFLP analysis of 16S rRNA genes and finally analyze the genetic diversity in this genus.

MATERIAL AND METHODS

Cultures and Culture conditions

In the study, cultures were chosen from the Culture Collection of Algae, Centre for Advanced Studies in Botany, University of Madras. Axenic cultures of *Anabaena* species were grown in BG 11_o medium[24]. Liquid cultures were grown at 25°C ± 1°C in a growth chamber under fluorescent illumination of 40 $\mu Em^{-2}s^{-1}$, with 12 hrs dark/12 hrs light conditions. The growth chamber was fitted with a Sangmo Weston Ltd., S656 313 model automatic timer. Gentle shaking of the flasks was done to reduce the clumping of cells. The cultures used in the present study are listed and described (Table 1).

Preparation of DNA sample for electrophoresis

DNA extraction was carried out according to standard procedures[25]. Exponentially growing (50 ml) cells were pelletted by centrifugation and resuspended in 0.5 ml of lysis solution (25% sucrose, 50 mM Tris – HCl, 100 mM EDTA). The cells were treated with 5 mg of lysozyme for 30 min at 37°C. Sodium dodecyl sulfate and proteinase K were added to final concentrations of 1% and 100 $\mu g\ ml^{-1}$, respectively and the samples were incubated at 45°C overnight. The DNA was extracted three times with Phenol: Chloroform: Isoamyl alcohol (25:24:1) and twice with Chloroform: Isoamyl alcohol (24:1). The DNA was precipitated, washed with 70% ethanol, resuspended in 100 µL of Tris – EDTA buffer, and stored at -20°C. Polymerase chain reactions (PCRs) were performed on an ERICOMP, Delta cycler I™ system, Easy cycler ™ PCR system.

RFLP of PCR products corresponding to the 16S rRNA gene

16S rRNA genes were amplified by PCR with primers fD1 and rD1[26]. PCR was performed in a total volume of 50 µl containing 10-20 ng of template DNA, primers (0.1 M), dNTPs (200 µM), 1U Taq DNA polymerase (Genei, Bangalore). PCR amplification was performed in a Delta Cycler I ™ system, Easy cycler ™ series PCR system (ERICOMP) according to the following program: an initial denaturation at 94ºC for 3 min, 30 cycles of 30 s denaturation at 94ºC, 40s annealing at 55ºC, and 1.5 min extension at 72ºC. A final extension at 72ºC for 3 min was used. The PCR products were analyzed in horizontal 0.8% (w/v) agarose gel (0.5 mg ethidium bromide/l) in 0.5 X TBE (pH 8.0). RFLP profiles were converted to binary data by scoring the presence or absence of bands for each isolate as one or zero. These data were used to calculate total character[27] differences, which were subsequently used to construct a neighbor–joining tree analysis.

Amplified PCR products (5-10 µl) were digested with following enzymes; Hae I I I, Xba I, Taq I (gene) the restricted fragments were

analyzed by horizontal electrophoresis in 2% agarose gel at 100 V for 2 h and visualized by ethidium bromide (0.5 µg/ml). The molecular weight standard was a 1kb ladder (Genei, Bangalore). The gel was documented by phographing under a UV – illuminator using a Vilber Loumart gel documentation system.

RESULTS AND DISCUSSION

In the present study the genus *Anabaena* Bory was chosen for taxonomic analysis. The morphological criteria traditionally used for identification of *Anabaena* species are: biometric characters of vegetative cells, heterocysts and spores. One important feature for specific identity of the taxa is the proximity of the akinetes to heterocyst, whether adjacent to or away from.

The PCR amplification using the universal primer with the genomic DNA extracted from *Anabaena* spp. resulted in a PCR product of approximately 1450bp (Fig. 1). *Xba I* (37ºC for 2h) , *Taq I* (37ºC for 2h) and *Hae III* (65ºC for 2h) restriction enzymes used digested the 16S rRNA PCR products and yielded different profiles (Fig. 2&3). Genetic distances between the ten cyanobacteria tested by RFLP analysis of 16S rRNA genes using three different enzymes namely *Xba* I, *Taq* I and *Hae* III were used to construct cluster analysis. The clusters for three different enzymes yielded heterogenous groupings of the morphotypes and resulted in unclear delineation of tested organisms (Fig.4).

Species of the genus *Anabaena* Bory are distinguished based on morphological characters and one of the many characters is the position of the spore in relation to the heterocyst. This character is useful when identifying collected samples but in cultured strains it is likely that either sporulation could be delayed or heterocysts formation is in response to the nutrients availability in the medium. Moreover in culture the biometric characters of vegetative cells, heterocysts and spores (akinetes) can vary from that of natural specimens. The present attempt is to look into this aspect to know exactly whether morphological characters on which the taxonomic identity is based are genetically strong and stable. Molecular studies for the determination of the relationships of the 10 *Anabaena* species were carried out. In several studies the amplified 16S/23S ITS of cyanobacteria has been used to genetically characterize strains by PCR- RFLP[14, 17]. Analysis have resulted in distinct banding pattern for *Synechocystis* sp. strain PCC6803, *Nostoc* sp. strain PCC7120, and *Anabaena cylindrica*[17] and symbiotic *Nostoc* strains[28]. The *Nodularia* strains were characterized by using a polyphasic approach, including RFLP of PCR–amplified 16S rRNA genes. The toxic Baltic strains were separated from the non-Baltic strains and from non- toxic Baltic strains[29]. Analyses of the 16S rRNA, 23S rRNA and ITS-1 of 19 picocyanobacteria that was isolated from different sources revealed differences in genotype (RFLP

Table 1. Strains used in this study

Strain No.	Taxonomic designation	Position of heterocysts and akinetes Refference	Origin / Source
A485	*A.ambigua*	heterocysts adjacent to akinetes	1403/7 CCAP, UK
A525	*A. torulosa*	heterocysts adjacent to akinetes	M2/2 aS2T2 Gif Sur Yvette , France
A621	*A. cylindrica*	heterocysts adjacent to akinetes	Isolate175,Kantz(1403/2a),ICC, U.S.A
A802	*A.augstumalis*	heterocysts adjacent to akinetes	Czech.Jahnke 4a
A904	*A. sphaerica*	heterocysts adjacent to akinetes	1616 ICC, U.S.A (23)
A487	*A. inaequalis*	heterocysts away from the akinetes	1403/9 CCAP, UK
A514	*A. variabilis*	heterocysts away from the akinetes	1403/12 CCAP, UK
A549	*A. fertilissima*	heterocysts away from the akinetes	M2/3b Gif Sur Yvette, France
A618	*A. subtropica*	heterocysts away from the akinetes	Isolate 45 Kantz Feb.71 ICC, U.S.A
A622	*A. verrucosa*	heterocysts away from the akinetes	Isolate175,Kantz ,ICC, U.S.A

CCAP: Culture Collection of Algae and Protozoa, Cambridge, U.K.
ICC: Indiana Culture Collection, U.S.A.

Similar findings have been reported for *Prochlorococcus* strains[30]. The morphological characteristics (e.g. *Anabaena* and *Aphanizomenon*), the physiological (toxicity) characteristics or the geographical origins did not reflect the level of 16S rRNA gene relatedness of the closely related strains studied[31]. Lyra *et al.*[16, 31] and Lehtimaki et al. (29) reported close relationship between strains of *Anabaena* and *Aphanizomenon* in a 16S rDNA after the RFLP study. Iteman *et al.*[32] found by RFLP that *Aphanizomenon flos-aquae*, *Anabaena flos-aquae, Anabaenopsis, Cyanospira* and *Nodularia* are distinct and consistent with their position in trees obtained from the 16S rDNA sequences.

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