Phylogenetic Analysis of *Anabaena* spp. (Cyanobacteria) Using Sequences of 16S rRNA Gene.

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**Abstract:** Molecular taxonomic studies were conducted in 8 isolates of freshwater filamentous heterocystous *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 the sequences were compared with those of representative heterocystous cyanobacteria available in GenBank. Phylogenetic tree was inferred by NJ distance method. The clusters are well supported by bootstrap analysis and partly reflect the morphological similarity of the organisms.

**Key words:** *Anabaena*, Cyanobacteria, Sequencing of the 16S rRNA gene, Phylogeny, Taxonomy

**INTRODUCTION**

Cyanobacteria are among the most widespread, morphologically distinct and abundant prokaryotes known. They are oxygenic photosynthetic autotrophs with the unique ability to fix atmospheric nitrogen, (Rippka, R., J. Deruelles, 1979; Whitton, B.A., 1992; Holt, J.G., N.R. Krieg., 1994) originally considered as a class of algae, the blue-green algae. They have an extraordinary biosynthetic potential and a repertoire of diverse metabolic activities. They are one of the dominant genera in various ecological habitats, especially in rice fields, where they are found as both free-living and symbiotic with the water fern, *Azolla*. The genus *Anabaena* was established by Bory in 1922. Geitler (1932) described 57 European species of *Anabaena*, while Desikachary (1959) designated 25 species in Cyanophyta. Morphology, developmental and biochemical parameters may vary with environmental or culture conditions. They can be classified on the basis of morphology, cellular differentiation, biochemical, physiological and genetic criteria.

DNA base composition is a very important genetic character 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 (Herdman, M., M. Janvier, 1979). Large differences in DNA base composition indicate that the strains cannot be closely related, whereas similar G+C percentages give no clue concerning genotyping relationships (Wilmotte, A., 1994). Grouping based on the sequence identity was supported by morphological features (size and morphology of vegetative cells, heterocyst and akinetes, and diameter and morphology of trichomes) (Bolch, C.J.S., S.L. Blackburn, 1996; Bolch, C.J.S., P.T. Orr, 1999).

Phylogenetic characterizations of natural microbial communities of unknown diversity were done through analysis of 1 6S rRNA gene sequence (Stackebrundt, E. and C.R.Woese, 1981). Oxygenic photosynthetic prokaryotes, cyanobacteria and prochlorophytes are genetically related on the basis of 1 6S rRNA sequences (Woese, C.R., 1987). It has now been revealed that *Prochlorococcus* is closely related to the marine clusterA *Synechococcus*, based on analyses using gene sequences from 16S rRNA (16S rDNA) and *rpoC1*, a subunit of DNA-dependent RNA polymerase (Palenik, B. and R. Haselkorn, 1992; Urbach, E., D.L. Robertson, 1992; Urbach, E., D.J. Scanlan, 1998; Toledo, G. and B. Palanik, 1997; Toledo, G., B. Palanik, 1997). Such studies have been performed using 16S rDNA (SSU) sequences. A phylogenetic tree was constructed by Wilmotte (1994). 16S rRNA has been frequently used to establish a gene based phylogeny of cyanobacteria (Giovannoni, S.J., S. Turner, 1988; Wilmotte, A., J.M. Neefs, 1994; Nelissen, B., R. De Baere, 1996; Turner, S., 1997; Lehtimaki, J., C. Lyra., 2000; Lyra, C., S. Suomalainen, 2001; Robertson, B.R., N. Tezuka, 2001). Cyanobacterial genera namely *Anabaena*, *Nostoc*, *Phormidium*, *Microcystis*, *Synechococcus* and *Synechocystis* have been analyzed using molecular techniques such as DNA sequencing (Neilan, B.A., D. Jacobs., 1997).
The amplified 16S - 23S rDNA spacer (ITS-1) of cyanobacteria has been used in several studies to genetically characterize strains by PCR-RFLP (West, N.J. and D.G. Adams, 1997) or by sequence analyses (Wilmotte, A., J.M. Neefs, 1994; Otsuka, S., S. Suda, 1999; Boyer, S.L., V.R. Flechtner, 2001; Rocap, G., D.L. Listel, 2002). A genotypic characteristic-DNA base composition was examined for 50 planktonic strains of *Anabaena* assigned to 22 different morphological species (Li, R., M. Wantanabe, 2000). The genetic variability of the cosmopolitan, ubiquitous fresh water cyanobacterium *Phormedium retzii* was assessed using random amplified polymorphic DNA (RAPD) marker and 16S rDNA sequences (Casamatta, D.A., M.L. Vis, 2003). 16S rRNA and ITS-1 sequence analysis were determined in *Synechococcus* (Rocap, G., D.L. Listel, 2002; Ernst, A., S. Becker, 2003) and sequences for the 16S-23S rDNA internal transcribed spacer (ITS) region were determined in 32 *Prochlorococcus* isolates and 25 *Synechococcus* isolates (Rocap, G., D.L. Listel, 2002; Ernst, A., S. Becker, 2003). rRNA gene sequences have now become the most widely used methods for identification, classification and phylogeny of cyanobacteria.

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 (Anand, N., 1978; Anand, N., 1979). In the investigation, our objective was to develop an easy and reliable method to analyze the morphological and genetic variations using sequencing of the 16S rRNA gene fingerprinting, all of which are PCR based methods, have been employed for the species level variations of *Anabaena*. CLUSTAL W analysis (Thompson, J.D., D.G. Higgins, 1994) of the sequence results were carried out for the phenograms for the eight isolates used in the study.

**MATERIALS AND METHODS**

**Organisms and Culture Conditions:**

Eight cultures of *Anabaena* were selected from the Culture Collection of Algae, Centre for Advanced Studies in Botany, University of Madras, India. The original habitats, taxonomic details and akinete positions of the strains are listed in Table 1. Axenic cultures of *Anabaena* species were grown in BG 11o medium (Rippka, R., J. Deruelles, 1979).

**Preparation of DNA Sample for Electrophoresis:**

DNA extraction was carried out according to standard procedures (Sambrook, J., E.F. Fritsch, 1989). Exponentially growing (50 ml) cells were pelleted 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 protease K were added to final concentrations of 1% and 100 μg 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™ system, Easy cycler™ PCR system. Oligonucleotides were purchased from DDT, UK.

**Amplification of the 16S rRNA Gene:**

PCR amplification was performed on purified DNA of *Anabaena* spp. (Itelman, I., R. Rippka, 2000). Amplification of the 16S rRNA gene was carried out by PCR using primers A2 (AGAGTTGTATCCAGCTCAG) and S8 (TCTACGACTTCACCAGCTAC). The PCR mixture contained 10 μl *Taq* (10 X) commercial buffer, 10 μl purified DNA (50-100 ng), 150 μM of each dNTP, 500 ng of each...
primer and 2.5 U Taq polymerase. Total reaction volume was 100 μl after an initial cycle consisting of 3 min at 95°C, 2 min at 55°C and 30 s at 72°C, 30 cycles of amplification were started (1.5 min at 95°C, 2.5 min at 55°C and 3 min at 72°C). The termination cycle was 7 min at 72°C. The PCR products were migrated either on 1.5 % (w/v) agarose gel containing 0.5 X TBE and visualized by staining with 0.5 μg/ml ethidium bromide.

**Sequencing of the 16S rRNA Gene:**

After purification [PCR purification kit (Genei, Bangalore)] PCR products of the 16S rRNA (1,500 bp PCR products were amplified) were directly sequenced at a commercial facility (Avestha Gengraine Technologies, Bangalore). The sequences (16S ribosomal RNA gene, partial sequence) determined have been deposited in Gene Bank with the following accession numbers: *Anabaena torulosa* (EF375610), *Anabaena cylindrica* (EF375611), *Anabaena augustumalis* (EF488830), *Anabaena sphaerica* (EF375612), *Anabaena inaequalis* (EF583842), *Anabaena variabilis* (EF488831), *Anabaena subtropica* (EF375613) and *Anabaena verrucosa* (EF375614).

**Phylogenetic Analyses:**

Sequences were aligned using the CLUSTAL W Multiple Sequence Alignment Program (Thompson, J.D., D.G. Higgins, 1994). Phylogenetic tree was constructed including the available cyanobacterial gene sequences along with the sequences determined in this study using the neighbor-joining method. In this program, bootstrap analysis was used to evaluate the tree topologies by performing 1000 resamplings.

**RESULTS AND DISCUSSION**

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.

The sequence of the 16S rRNA gene was determined for eight *Anabaena* spp. (*Anabaena torulosa* A525, *A. cylindrica* A621, *A. augustumalis* A802, *A. sphaerica* A904, *A. inaequalis* A487, *A. variabilis* A514, *A. subtropica* A618, and *A. verrucosa* A622). The sequences were compared with those of representative heterocystous cyanobacteria (*Anabaena*) available in GenBank. Phylogenetic tree was inferred by NJ distance method. The clusters are well supported by bootstrap analysis and partly reflect the morphological similarity of the organisms (Fig. 1 and Fig. 2). The cyanobacteria studied here were divided into two branches (Fig. 1). The first branch constituted the six of the eight sequences and included *Anabaena* forming heterocysts away from the akinetes. The second minor branch contained *A. sphaerica* and *A. torulosa* which form heterocysts adjacent to akinetes. The NJ tree resulted by the combination of the eight sequences determined in this study with other *Anabaena* spp. sequences also showed two major branches. Within the first major cluster the two morphoforms of *Anabaena* with respect to heterocysts position adjacent or away from akinetes were seen (Fig. 2). The results indicated that morphological differences within the *Anabaena* genus with respect to the positioning of heterocysts are not reflected at the 16S rRNA gene level.

In the present study, Neighbor-Joining method is used for analysis of the 16S rRNA and for the phylogenetic tree constructions. Two branches of the *Anabaena* tested were found in 16S rRNA gene sequence analysis. Though morphologically similar *A. inaequalis*, *A. subtropica*, *A. variabilis* and *A. verrucosa* formed the major first cluster, the other morphoform *A. cylindrica* and *A. augustumalis* were intermixed with the first major cluster. Previously, molecular taxonomic studies have indicated the grouping of *Anabaena* together with *Aphanizomenon* strains (Neilan, B.A., 1995; Rudi, K., O.M. Skulberg, 1997; Lu, W., H.E. Evans, 1997; Lyra, C. J. Hantula, 1997; Rudi, K. and K.S. Jakobsen, 1999). The 16S rRNA sequences were combined with other selected *Anabaena* species available in the database and the CLUSTAL W (Thompson, J.D., D.G. Higgins, 1994) analysis showed the presence of distinct groups. A BLAST search performed on the 16S rRNA primary sequences resulted in the highest similarity scores for cyanobacteria. 16S rRNA gene sequence similarities of 98% within *Anabaena cylindrica*, *Anabaena sphaerica*, *Anabaena variabilis* and *Anabaena verrucosa*, 97% in *Anabaena augustumalis*, 96% in *Anabaena torulosa*, *Anabaena subtropica* and 93% in *Anabaena inaequalis* were observed.
Fig. 1: Neighbor joining phylogenetic tree for the selected Anabaena spp. 16S rRNA gene sequences. Bootstrap values are indicated in the point at nodes.

Fig. 2: Neighbor joining phylogenetic tree for the tested Anabaena spp. 16S rRNA gene sequences with other sequences available in public databases. Bootstrap values are indicated in the point at nodes.

It needs mention that morphological characterization of unicellular *Merismopedia* and *Microcystis* strains was also unsuccessful in distinguishing genetic subcultures created by 16S rDNA sequencing (Palinska, K.A., W. Liesack, 1996; Otsuka, S., S. Suda, 1998). Giovannoni *et al.* (1988) and Wilmotte (1994) have found grouping of *Nodularia* PCC 73104 with *Anabaena cylindrica* PCC 7122 and *Prochlorococcus* is phylogenetically related to cyanobacteria and closest to *Synechococcus* (Urbach, 1998). Hepatotoxic *Anabaena* strains were phylogenetically different from neurotoxin producing strains using 16S rDNA sequence (Lyra, 2001). The 16S rDNA sequence analysis data in this report confirm that heterocyst-forming cyanobacteria consist of a monophyletic group. Phylogenetic data do not correlate with the bacteriological and traditional classifications, which distinguish filamentous heterocystous cyanobacteria with or without true branching (Nostocales/Stigonematales) (Gugger, M.F. and L. Hoffmann, 2004). Sequence analysis of 16S rRNA gene revealed that *Nodularia* is closely related to *Nostoc*, *Aphanizomenon* and *Anabaena* (Lehtimaki, 2000). It needs mention here that a new genus *Spirirestis rafaelensis* shared morphological characters with both
Scytonemaceae and Microchaetaceae but was found to be closely related to Microchaetacea at the molecular level (Flechtner, V.R., S.L. Boyer, 2002).

REFERENCES


