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Full Length Research Paper

Fingerprinting of repetitive DNA sequences in the genus *Anabaena* using PCR- based techniques

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In this study, ten species of *Anabaena* were used to test the congruence between the traditional morphological classification system and the present molecular classification system. The electrophoretic patterns for 10 cyanobacterial strains belonging to the genera (*Anabaena*) were used for molecular analysis using the repeated PCR technique. Genetic diversity was assessed using the banding patterns of repetitive DNA sequences including the HIP1 sequences, enterobacterial repetitive intergenic consensus [ERIC] sequences and REP sequences that are present in the cyanobacterial genome. PCR with different sets of repetitive oligonucleotides as primers were used to evaluate the various repetitive DNA sequences. The dendrograms based on the HIP1, ERIC and REP sequences did not show a clear correlation with morphological dendrogram based on traditional classification.

Key words: *Anabaena*, cyanobacteria, highly iterated palindromic sequences, enterobacterial repetitive intergenic consensus sequences, repetitive extragenic palindromic sequences, polymerase chain reaction.

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 atomspheric nitrogen both under free-living and symbiotic conditions. The species of cyanobacteria which are known to fix atmospheric nitrogen are classified into three groups viz., heterocystous-aerobic forms, aerobic unicellular forms and non-heterocystous filamentous microaerophilic forms. Nitrogen-fixing cyanobacteria can use sunlight as the sole energy source for the fixation of carbon and nitrogen and therefore have potential as biofertilizers (Kannaiyan, 1985). Cyanobacteria are also involved in symbiotic associations with an exceptionally broad range of representatives within the plant kingdom (Fogg et al., 1993) Cyanobacteria are among the most widespread, morphologically distinct and abundant prokaryotes known (Whitton, 1992). Molecular systematics has 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 (Vandamme et al., 1996). In more recent years, DNA sequences have been used for the taxonomic and phylogenetic analysis of cyanobacterial isolates by several labs. DNA base composition is also a very important genetic characteristic used in taxonomic studies of cyanobacteria. Large differences in the DNA base composition indicate that the strains are not closely related, whereas similar G+C percentages provide no evidence concerning genotypic relationships (Wilmotte, 1994).

Repetitive DNA sequences have been found in filamentous cyanobacteria (Holland and Wolk, 1990). It was suggested that the banding patterns could be used to distinguish strains at the species and genus levels. Repetitive sequences constitute an important part of the prokaryotic genome. The REP (Stern et al., 1984) and ERIC (Hulton et al., 1991) sequences were originally described for the family *Enterobacteriaceae* but later found in several gram-negative bacteria and close relatives in the same phyla (Versalovic et al., 1991; Zheng et al., 2002) synthesized a modified STRR primer, STRR*mod*, based

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Abbreviations: HIP1, Highly iterated palindromic; **ERIC,** enterobacterial repetitive intergenic consensus; **REP,** repetitive extragenic palindromic; **PCR,** polymerase chain reactions.

Table 1. *Anabaena* strains selected for the study.

Taxonomic designation of *Anabaena* Bory (No. strains)	Position of heterocysts and akinetes	Origin/Source
A. ambigua	heterocysts adjacent to akinetes	1403/7 CCAP, UK
A. torulosa	heterocysts adjacent to akinetes	M2/2 aS2T2 Gif Sur Yvette from, France
A. cylindrica	heterocysts adjacent to akinetes	Isolate175,Kantz(1403/2a),ICC, U.S.A
A. augstumalis	heterocysts adjacent to akinetes	Czech.Jahnke 4a
A. sphaerica	heterocysts adjacent to akinetes	1616 ICC, U.S.A
A. inaequalis	heterocysts away from the akinetes	1403/9 CCAP, UK
A. fertilissima	heterocysts away from the akinetes	M2/3b Gif Sur Yvette PCC,
A. variabilis	heterocysts away from the akinetes	France 1403/12 CCAP, UK
A. subtropica	heterocysts away from the akinetes	Isolate 45 Kantz Feb.71 ICC, U.S.A
A. verrucosa	heterocysts away from the akinetes	ICC, U.S.A

CCAP: Culture collection of algae and protozoa, Cambridge, U.K; ICC: Indiana culture collection, U.S.A.

on the consensus sequence and observed that it could produce distinct and reproducible PCR patterns. Analyses of molecular polymorphisms in a set of *Anabaena* strains were done using STRR, LTRR and Hip1 primers (Prasanna et al., 2006). REP and ERIC sequences in the genomes of a number of gram- negative soil bacteria, like *Rhizobium meliloti,* and in the genomes of a variety of eubacteria were examined using conserved primers (Mullis and Faloona, 1987; Bruijn, 1992; Versalovic et al., 1991; Rasmussen and Svenning, 1998). Fingerprinting of the REP elements and ERIC sequences has been used for identification of symbiotic (Rasmussen and Svenning, 1998) and free-living cyanobacteria (Rasmussen and Svenning, 1998; Lehtimaki et al., 2000).

HIP1 was first identified in association with a gene deletion in mutants of *Synechococcus* PCC 6301 (Gupta et al., 1993). Two novel sets of highly repetitive sequences were found in unicellular cyanobacterial strains (Asayama et al., 1996).

Highly repetitive DNA sequences have been used for the identification of toxic planktonic *Cylindrospermopsis* (Wilson et al., 2000; Chonudomkul et al., 2004) and of *Anabaena* and *Nostoc* strains (Rouhiainen et al., 1995; Lyra et al., 2001). A PCR-based DNA fingerprinting method using base pair extended short oligonucleotide primers for HIP1 was used to distinguish between *Anabaena* species by Prasanna et al. (2006). HIP1 sequences, found in many, but not all, cyanobacteria, were used in DNA fingerprinting assays to evaluate the genetic diversity of cyanobacterial species and strains (Smith et al., 1998; Robinson et al., 1995; Orcutt et al., 2002; Wilson, 2005).

The morphological criteria traditionally used for identification of *Anabaena* species include the biometric characteristics of vegetative cells, heterocysts and akinetes. An important feature for species identification is the proximity of the akinetes (spores) to heterocysts (Anand, 1978, 1979). In the present study, we determined the differences between classification of cyanobacteria based on morphological and molecular traits (e.g., HIP1, ERIC and REP sequences).

MATERIALS AND METHODS

Anabaena cultures

Ten strains of *Anabaena* spp. were selected from the Culture Collection of Algae maintained at the Centre for Advanced Studies in Botany of the University of Madras, India. The original habitats, taxonomic details and akinete positions of the strains are listed in Table 1 (Anand, 1979; Ezhilarasi and Anand, 2009a, b). Axenic cultures of *Anabaena* species were grown in BG 11_0 medium (Rippka et al., 1979).

Preparation of DNA samples

DNA extraction was carried out according to standard procedures (Sambrook et al., 19899). Exponentially growing cells (50 ml) 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 proteinase 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. PCR assays were performed using an ERICOMP Delta cycler I™ Easy cycler ™ PCR system.

Repeat sequences in the cyanobacterial genome

The PCR conditions for these primers (Table 2) were as specified by de Brujin (1992). Each 25 µl reaction mixture contained 50 pmol of the two opposing primers, 50 ng of template (genomic) DNA, 1.25 mM each deoxynucleotide triphosphate (dATP, dCTP, dGTP and dTTP), 250 µM magnesium chloride, 1X PCR buffer and 2 U of Taq DNA polymerase (Genei, Bangalore, India). Thermal cycling began with an initial denaturation at 95°C for 7 min, followed by 30 cycles of denaturation at 90°C for 30 s, annealing 40°C (REP) or 52°C (ERIC) for 1 min and extension at 65°C for 8 min with a

Table 2. Constructed primers.

Primers	Sequence	Reference
REPIR-I	3'-CGG ICT ACI GCI GCI III-5'	(de Brujin, 1992)
REP2-I	5'-ICG ICT TAT CIG GCC TAC-3'	
ERICIR	3'-CAC TTA GGG GTC CTC GAA TGT A-5'	
ERIC2	5'- AAG TAA GTG ACT GGG GTG AGC G-3'	
HIPCA	5'- GCGATCGCCA - 3'	(Smith et al., 1998)
HIPAT	5'- GCGATCGCAT -3'	
HIPTG	5'- GCGATCGCTG -3'	
HIPGC	5'- GCGATCGCGC -3'	

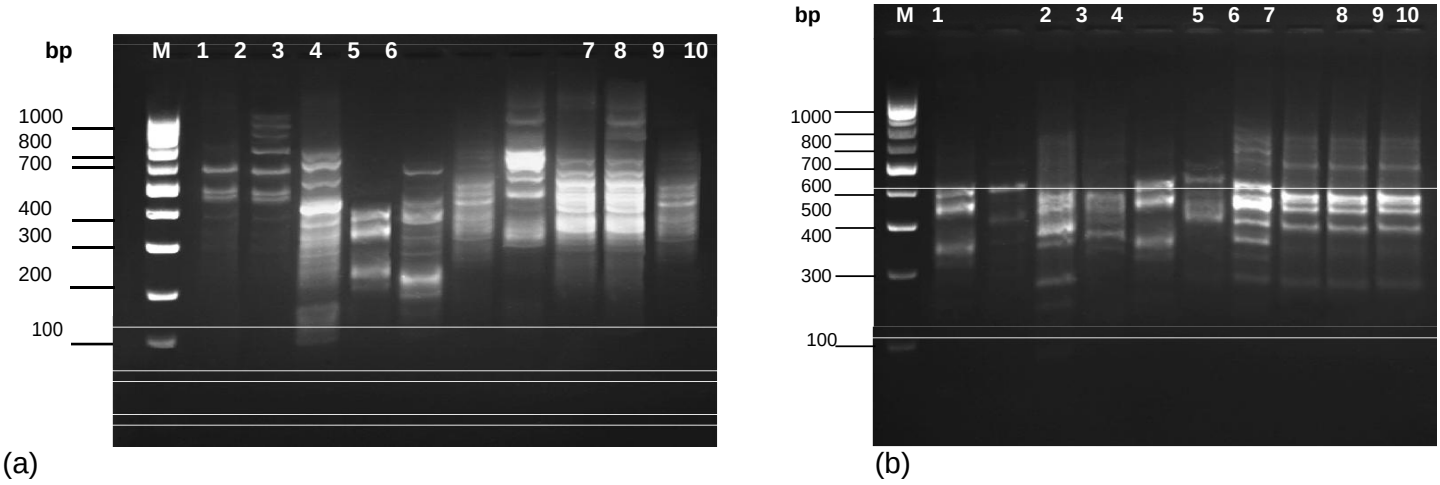

Figure 1. Hip1 gene sequence profile analysis of ten *Anabaena* spp. PCR amplification with primers HIPCA a) and HIPAT (b). M: DNA Ladder 100 bp; Lane 1. *A. ambigua*; Lane 2. *A. torulosa*; Lane 3. *A. cylindrica*; Lane 4. *A. augstumalis*; Lane 5. *A. sphaerica*; Lane 6. *A. inaequalis*; Lane 7. *A. variabilis*; Lane 8. *A. fertilissima*; Lane 9. *A. subtropica*; Lane 10. *A. verrucosa*.

single final extension at 65°C for 16 min and 4°C hold. Each PCR reaction was then electrophoresed directly on a 1% agarose gel containing 0.5 X TBE and 0.5 µg/ml ethidium bromide.

HIP1 sequence profiles

Primers based on the HIP1 sequence GCGATCGC that contained two additional nucleotides at the 3' end were used for this study (Table 2). Each 25 µl reaction mixture contained 20 pmol of the two opposing primers, 50 ng of template (genomic) DNA, 200 µM each deoxynucleotide triphosphate (dATP, dCTP, dGTP and dTTP), 250 µM magnesium chloride, 1X PCR buffer and 1U of Taq DNA polymerase (Genei, Bangalore, India). Thermal cycling conditions began with an initial denaturation at 95°C for 5 min; 30 cycles of 95°C for 30 s, 30°C for 30 s, 72°C for 60 s; one cycle of 72°C for 5 min. PCR products were electrophoresed in TBE buffer. All assays were performed at least in triplicate.

Data analyses

Fingerprints generated from different *Anabaena* species were compared and all bands were scored. The presence or absence of particular DNA fragments was converted in to binary data and the Jaccard distance index was used to obtain the similarity matrix. The tree was constructed using the unweighted pair-group method using arithmetic averages (UPGMA) program in phylogeny inference package (PHYLIP) (Felsenstein, 1993).

RESULTS

The single HIPCA, HIPAT, HIPTG and HIPGC extended primers gave distinct fingerprints for each of the strains. Very few products were of similar size for all of the species. The DNA fingerprint products ranged in size from approximately 100 to 1200 bp (Figures 1a, b, 2a, and b). The phenogram constructed from the distance matrix of all HIP1 primer sets resulted in two clusters: a major cluster with seven species (*Anabaena ambigua*, *Anabaena torulosa*, *Anabaena inaequalis, Anabaena variabilis*, *Anabaena fertilissima*, *Anabaena subtropica* and *Anabaena verrucosa*) and a minor cluster with three species (*Anabaena cylindrica*, *Anabaena sphaerica* and *Anabaena augstumalis*). The minor cluster contained species of similar morphotypes (Figure 3), but the major cluster showed a mixture of the two morphological categories.

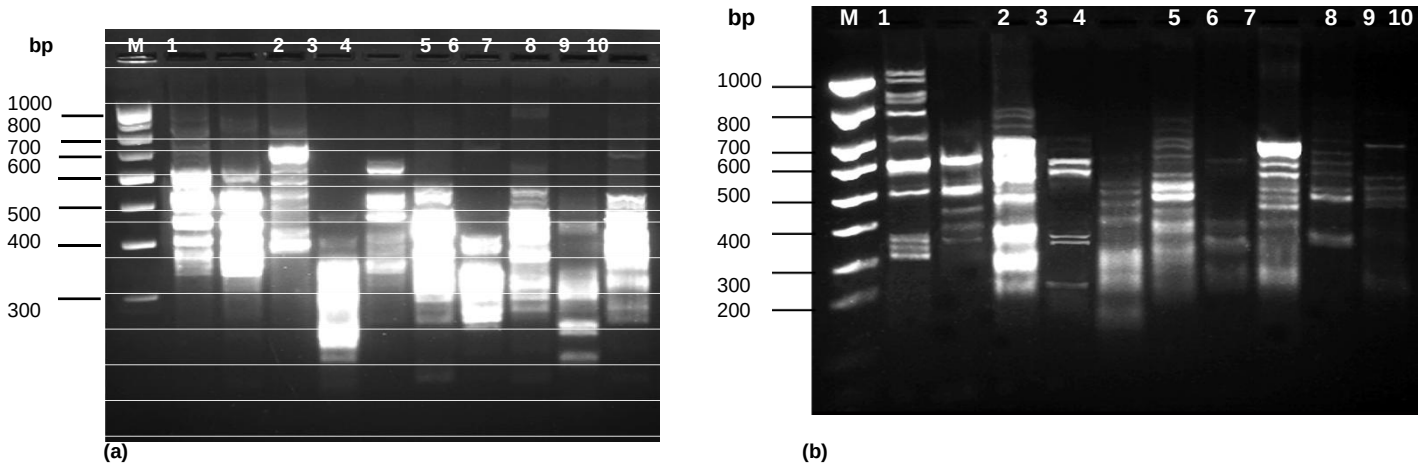

Figure 2. HIP1 gene sequence profile analysis of ten *Anabaena* spp. PCR amplification with primers HIPTG (a) and HIPGC (b). M: DNA Ladder 100 bp; Lane 1. *A. ambigua*; Lane 2. *A. torulosa*; Lane 3. *A. cylindrica*; Lane 4. *A. augstumalis*; Lane 5. *A. sphaerica*; Lane 6. *A. inaequalis*; Lane 7. *A. variabilis*; Lane 8. *A. fertilissima*; Lane 9. *A. subtropica*; Lane 10. *A. verrucosa*.

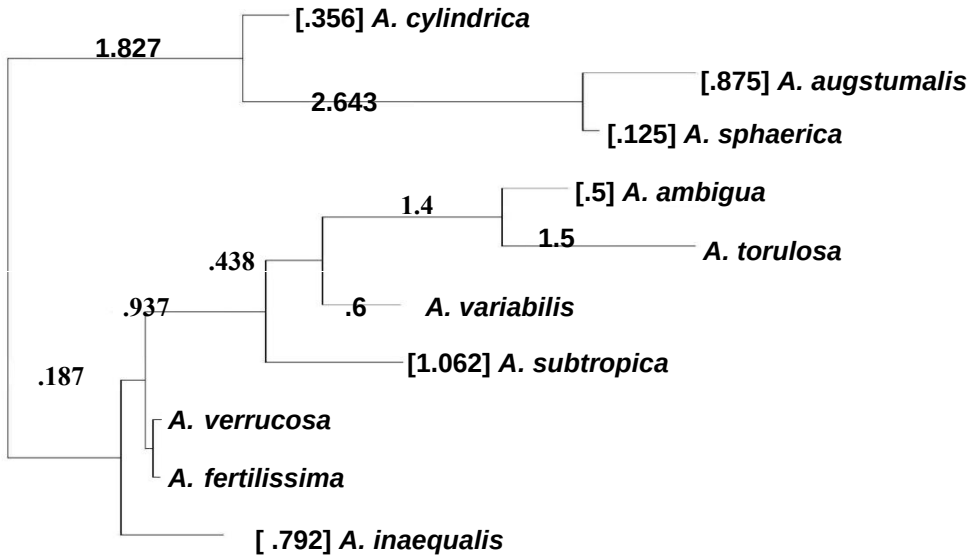

Figure 3. HIP1-PCR primers set values at nodes indicate genetic distance measurements as calculated by the algorithm of Nei and Li (1979).

Distinct REP and ERIC sequence profiles were obtained with the combination of the REP1R-I and REP2-I (Figure 4) and the ERIC1R and ERIC2 (Figure 5) primer sets. Products ranged in size from approximately 126 to 2645 bp. For the REP-PCR fingerprints, the dendrogram showed two clusters (Figure 6).

The major cluster consisted of three species (*A. torulosa* , *A. inaequalis* and *A. verrucosa*). Not all ten species of *Anabaena* were clearly separated, and there was no clear correlation between the clusturing based on REP sequence profiles and the clustering based on morphology. The dendrogram for the ERIC fingerprints showed no correlation with respect to morphological characterization (Figure 7).

DISCUSSION

Species of the genus *Anabaena* have traditionally been distinguished based on morphological characteristics. One of the many characteristics used to differentiate species is the position of the akinete relative to the heterocyst. This characteristic is useful when identifying samples collected from nature, but in cultured strains, it is likely that either sporulation is delayed or that heterocyst formation occurs in response to the nutrients available in

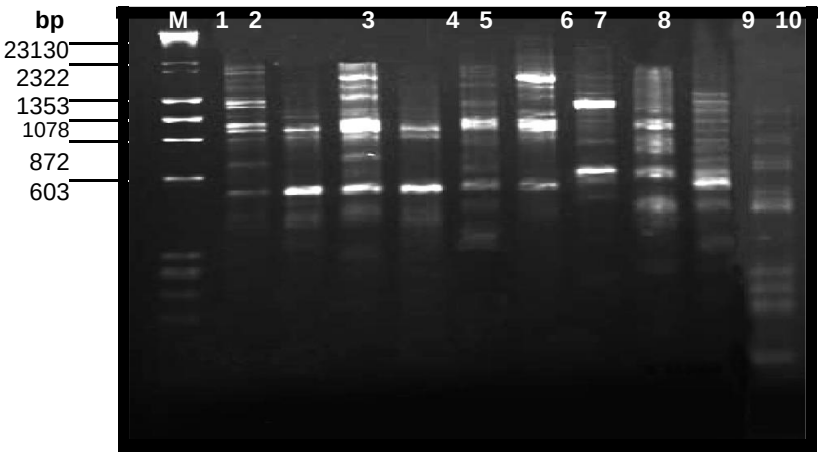

Figure 4. ERIC sequence profile analysis of ten *Anabaena* spp. and PCR amplification with primers ERIC IR and ERIC 2. M: DNA/Hind III Digest; Lane 1. *A. ambigua*; Lane 2. *A. torulosa*; Lane 3. *A. cylindrica*; Lane 4. *A. augstumalis*; Lane 5. *A. sphaerica*; Lane 6. *A. inaequalis*; Lane 7. *A. variabilis*; Lane 8. *A. fertilissima*; Lane 9. *A. subtropica*; Lane 10. *A. verrucosa*.

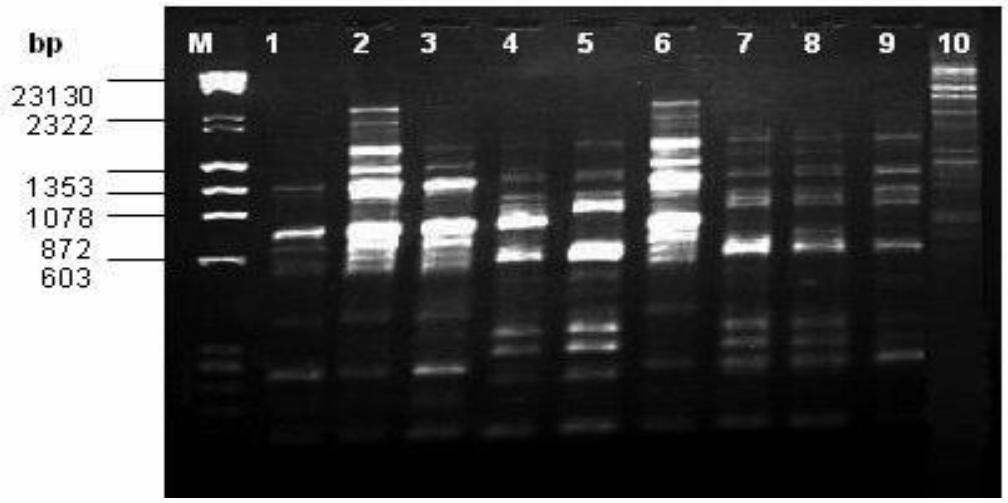

Figure 5. REP sequence profile analysis of ten *Anabaena* spp. and PCR amplification with primers REPIR-I and REP2-I. M: DNA/Hind III Digest; Lane 1. *A. ambigua*; Lane 2. *A. torulosa*; Lane 3. *A. cylindrica*; Lane 4. *A. augstumalis*; Lane 5. *A. sphaerica*; Lane 6. *A. inaequalis*; Lane 7. *A. variabilis*; Lane 8. *A. fertilissima*; Lane 9. *A. subtropica*; Lane 10. *A. verrucosa*.

the medium. Moreover, in culture, the biometric characteristics of vegetative cells, heterocysts and akinetes can vary from those of natural specimens.

Therefore, using morphological characteristics to classify cultured strains may give inaccurate results. The purpose of the present study was to determine whether the morphological characteristics on which the taxonomic identity is based are genetically strong and stable. Several molecular tools are commonly used for the analysis of the taxonomic status of the microbes. In the present study, ten isolates of *Anabaena* were collected: five species that have akinetes adjacent to heterocysts and five species whose akinetes are not adjacent to heterocysts.

The octameric palindromic sequence HIP1 is located in the chromosomal DNA of certain cyanobacteria as a highly repetitious interspersed sequence involved in adaptive responses. This sequence was first identified in cells of Cd-tolerant *Synechococcus* strains PCC6301 (Gupta et al., 1993). HIP1 sequences are known to occur frequently in a large number of cyanobacterial strains (Robinson et al., 1995). HIP1 sequences can be used in a way similar to repetitive sequences in PCR-based techniques. In the present study, a PCR-based DNA

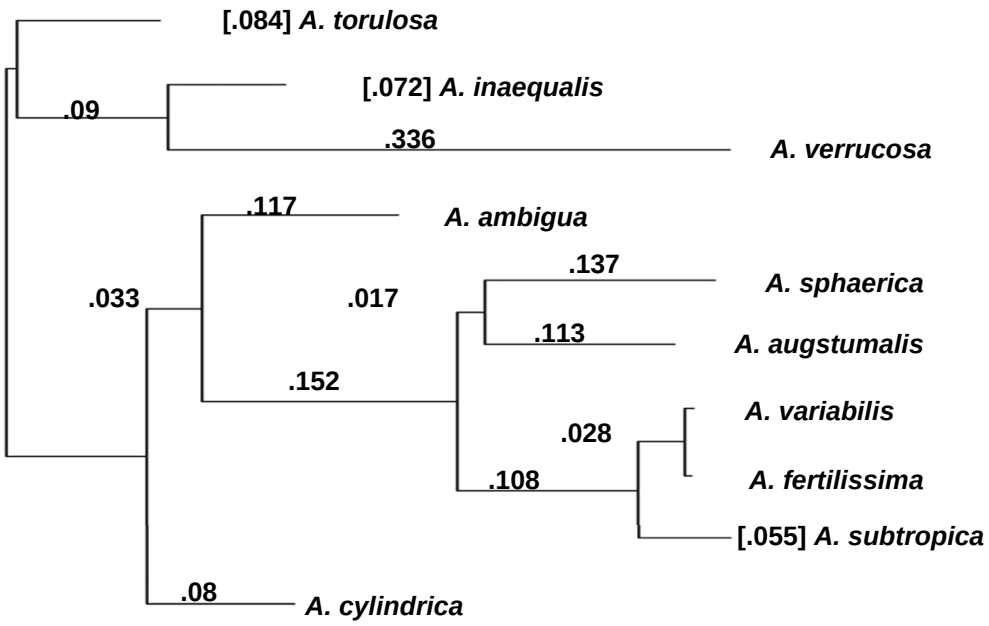

Figure 6. REP-PCR (Primers-REPIR-I and REP2-I) values at nodes indicate genetic distance measurements as calculated by the unweighted pair-group method using arithmetic averages (UPGMA) program in phylogeny inference package (PHYLIP) (Felsenstein, 1993).

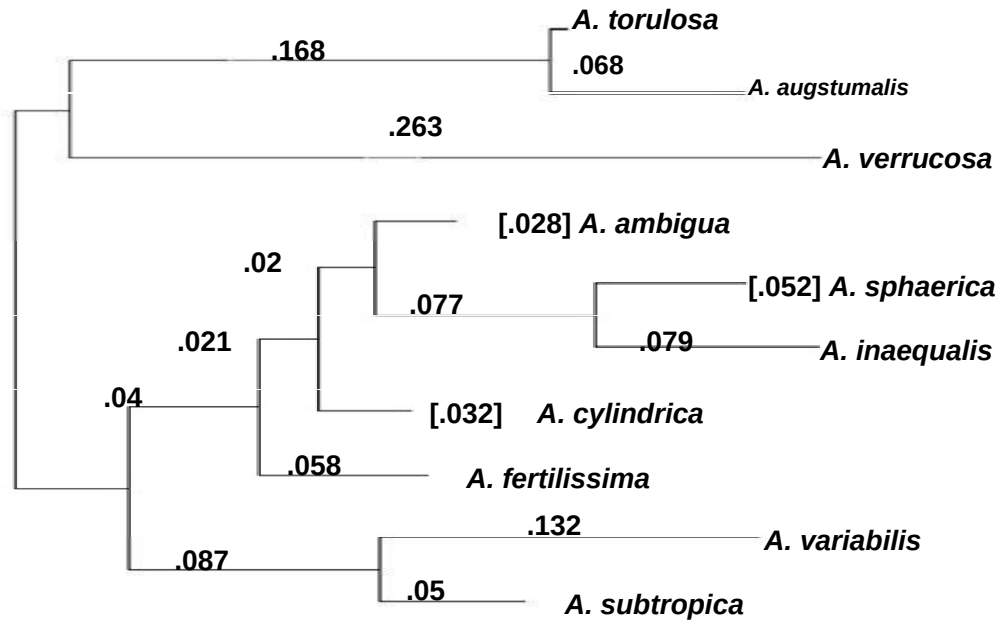

Figure 7. ERIC-PCR (Primers-ERIC IR and ERIC2) values at nodes indicate genetic distance measurements as calculated by the unweighted pair-group method using arithmetic averages (UPGMA) program in phylogeny inference package (PHYLIP) (Felsenstein, 1993).

fingerprinting method using base pair extended short oligonucleotide primers for HIP1 was used to distinguish *Anabaena* spp. and to investigate the genetic diversity within and between species. Each of the four HIP1 extended primers was tested for its ability to distinguish the cultures of the different *Anabaena* sp. These cyanobacteria contain large numbers of HIP1 sequences that could be probed using the four single extended primers that we selected. The single HIPCA, HIPAT, HIPTG and HIPGC extended primers gave distinct fingerprints for

each of the ten *Anabaena* strains tested, but very few products were similar in size for all of the species. The phenogram constructed from the distance matrix of all HIP1 primer sets resulted in two clusters, one major cluster with seven species (*A. ambigua*, *A. torulosa*, *A. inaequalis, A. variabilis*, *A. fertilissima*, *A. subtropica* and *A. verrucosa*) and a minor cluster with three species (*A. cylindrica*, *A. sphaerica* and *A. augstumalis*).

The species in the minor cluster all had similar morphotypes, but the major cluster contained a mixture of species from the two morphological categories. The amplicon sizes and fingerprint patterns observed in the present study were in accordance with the previous observations of Zheng et al. (2002). The frequency of HIP1 repeats in the DNA of some cyanobacteria is high, and analysis of database sequences revealed that the HIP1 sequence occurs on average every 320 bp in the chromosomal DNA of *Synechococcus* PCC 6301 (Robinson et al., 1995).

The present study clearly demonstrated the presence of HIP1 sequences in all of ten *Anabaena* strains, indicating that these sequences can be used to study the diversity of *Anabaena*. The profiles of the REP and ERIC sequences have been investigated in cyanobacterial genomes (Versalovic et al., 1991; Rasmussen and Svenning, 1998). *Anabaena* , *Aphanizomenon*, *Microcystis* and *Planktothrix* strains were differentiated to a high degree by REP and ERIC fingerprinting (Lyra et al., 2001). Genomic DNA from axenic cyanobacterial isolates was assayed to determine these species (*Anabaena* spp.) carry the ERIC and REP sequences. In the current study, PCRs were performed by using these DNAs as template and the combination of either the REP (REP1R-I and REP2-I) or ERIC (ERIC1R and ERIC2) oligonucleotides as primers. The results show that both ERIC and REP sequences generated distinct PCR profiles in the cyano-bacteria. Distinct ERIC and REP profiles were generated for each *Anabaena* strain in the current study. The dendrogram based on the REP fingerprints showed two clusters.

The major cluster consisted of three species (*A. torulosa* , *A. inaequalis* and *A. verrucosa*). Not all ten species of *Anabaena* were clearly separated, and there was no clear correlation between the clustering based on REP sequence profiles and the clustering based on morphology. The dendrogram based on the ERIC fingerprints also showed a heterogeneous distribution of the species with respect to morphological characterization. The use of REP sequences for fingerprinting and diversity studies is a powerful technique for many bacterial taxonomic studies (Laguerre et al., 1992; Bruijn, 1992).

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