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Phylogenetic analysis of the genus *Anabaena* based on PCR fingerprinting

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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. For morphological classification, strains were categorized into two different groups based on the whether or not the akinetes were directly adjacent to heterocysts in the trichome. Genetic diversity was assessed using the banding patterns of repetitive DNA sequences including the short tandemly repeated repetitive (STRR) sequences and long tandemly repeated repetitive (LTRR) sequences that are present in the cyanobacterial genome. The phylogenetic relationships inferred from comparison of the STRR sequences generally supported the traditional classification of cyanobacteria based on morphological criteria. The dendrograms based on the LTRR sequences did not show a clear correlation with the dendrogram based on morphology.

Key words: *Anabaena*, cyanobacteria, short tandemly repeated repetitive (STRR) sequences, long tandemly repeated repetitive (LTRR) sequences, PCR (Polymerase chain reactions), phylogenetic analysis.

INTRODUCTION

Cyanobacteria (blue-green algae) are photosynthetic organisms of cosmopolitan distribution in terrestrial and aquatic habitats and are known to have existed since the Precambrian era, some 3.5 billion years ago (Schopf, 2000; Holt et al., 1994). Cyanobacteria are also involved in symbiotic associations with an exceptionally broad range of representatives within the plant kingdom (Fogg et al., 1973). Cyanobacteria are among the most wide-spread, morphologically distinct and abundant prokaryotes known (Whitton, 1992).

Molecular systematics has been used to understand the phylogenetic divergence within the cyanobacteria (Rajaniemi et al., 2005). Different kinds of information can now be derived from phenotypic and genotypic data (Vandamme et al., 1996). The traditional classification scheme given by Geitler (1932) served as the basis for the later treatments of Elenkin (1938), Desikachary (1959) and Starmach (1966). In more recent years, DNA sequences have been used for the taxonomic and phylo-

genetic analysis of cyanobacterial isolates by several laboratories. DNA base composition is also a very important genetic characteristic used in taxonomic studies of cyanobacteria (Wilmotte, 1994). 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. STRR sequences have also been detected in the genomes of other filamentous, heterocystous genera such as C*alothrix* (Mazel et al., 1991), *Anabaena, Nostoc* (Rouhiainen et al., 1995), *Calothrix* and *Fischerella* (Vioque, 1997) and *Cylindro-spermopsis* (Wilson et al., 2000).

Rouhiainen et al. (1995) detected toxin-producing strains of the genera *Anabaena* and *Nostoc* in a Finnish lake using STRR sequences. STRR IA sequences showed higher diversity in free-living cyanobacteria (Rasmussen and Svenning, 1998) and STRR markers showed that the *Nostoc* symbionts of G*unnera magellanica* were different from those that are symbiotic with G*unnera tinctoria* (Guevara et al., 2002). Zheng et al. (2002)

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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, France
A. variabilis	heterocysts away from the akinetes	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.

synthesized a modified STRR primer, STRR*mod*, based 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; Nayak et al., 2009*)*. The LTRR sequences were used for the specific fingerprinting of individual cyanobacterial isolates of *Anabaena* strains by Masepohl et al. (1996). DNA amplification finger-printing (DAF), a PCR-based method using short oligonucleotides for production of characteristic banding patterns, was used by Eskew et al. (1993) for taxonomic studies of *Anabaena azollae* strains. DAF has been very useful for distinguishing closely related genotypes (Welsh and McClelland 1990). 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).

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., STRR, LTRR sequences).

MATERIALS AND METHODS

Anabaena cultures

Ten strains of *Anabaena* sp. 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). 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., 1989). 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.

STRR sequence profiles

For the PCR-STRR assay, the primers used by Mazel et al. (1990) (Table 2) were used. Each 50-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 II and 2.5 U of Taq DNA polymerase (Genei, Bangalore, India). Thermal cycling conditions were 94°C for 10 min, 1 cycle; 94°C for 30 s, 40°C for 1 min and 65°C for 4 min, 35 cycles; 65°C for 7 min, 1 cycle; hold at 4°C. Each PCR reaction was then electrophoresed directly on a 1% agarose gel containing 0.5 X TBE (Tris Borate-EDTA) and 0.5 µg/ml ethidium bromide.

LTRR sequence profiles

For PCR amplification of LTRR sequences, the LTRR1 and LTRR2 primers (Table 2) were used (Rasmussen and Svenning, 1998). Each 50 µl reaction mixture contained the similar reaction agents mentioned above in the STRR assay method. Thermal cycles were as follows: 1 cycle at 95°C for 6 min; 30 cycles of 94°C for 1 min, 45°C for 1 min and 65°C for 5 min; 1 cycle at 65°C for 16 min and a final step at 4°C. Each PCR reaction was electrophoresed directly

Table 2. Constructed Primers.

Primers	Sequence	Reference
STRR 1F	5'- CCC CAR TCC CCA RT -3'	
STRR 2F	5'- TTG GTC ATT GGT CA -3'	
STRR 3F	5'- CAA CAG TCA ACA GT -3'	
STRR 1R	5'-GGG GAY TGG GGA YT -3'	Mazel et al.1990
STRR 2R	5'-TGA CCA ATG ACC AA -3'	
STRR 3R	5'-ACT GTT GAC TGT TG -3'	
LTRR1	3'-CAA AAT TGA TTG TTT TTA GG-5'	Rasmussen and
LTRR2	5'- CTA TCA GGG ATT GAA AG-3'	Svenning, 1998

on a 1% agarose gel containing 0.5 X TBE (Tris Borate-EDTA) and 0.5 µg/ml ethidium bromide.

Data analysis

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

STRR sequence profiles were generated using various combinations of the primers for each isolate. This analysis revealed unique band profiles among each of the different *Anabaena* sp. examined, with the extent of the variation dependent on the primer combination used. The combinations were STRR 1F and STRR 3F, STRR 1F and STRR 3R and STRR 1R and STRR 3R. *Anabaena ambigua*, *Anabaena torulosa*, *Anabaena cylindrica*, *Anabaena augstumalis* and *Anabaena sphaerica* only yielded few bands. *Anabaena inaequalis*, *Anabaena variabilis*, *Anabaena fertilissima*, *Anabaena subtropica* and *Anabaena verrucosa* had multiple distinct DNA products (Figures 1 and 2). When combination of primers, STRR 1F and STRR 3F, STRR 1F and STRR 3R and STRR 1R and STRR 3R, was used, an obvious clustering of all *Anabaena* isolates was observed (Figure 4). The dendrogram (Figure 4) constructed for all of the STRR primer sets consisted of a major cluster with eight species (*A. torulosa*, *A. cylindrica*, *A. augstumalis*, *A. sphaerica*, *A. inaequalis*, *A. variabilis*, *A. subtropica* and *A. verrucosa*) and a minor cluster with two species (*A. ambigua* and *A. fertilissima*). The major cluster was clearly separated into two subclusters of four species each. The relationships among the isolates based on the STRR fingerprinting supported the relationships determined using the traditional morphological classification scheme. Only *A. fertilissima* and *A. ambigua* were outgrouped and these were the only two species whose

phylogenetic relationships were not consistent with those from the morphological scheme of classification.

The use of primers LTRR1 and LTRR2 in the PCR analysis of the ten *Anabaena* sp. yielded multiple distinct DNA products (Figure 3). When this combination of primers was used, an obvious clustering among all *Anabaena* isolates was observed (Figure 5). Dendrograms constructed from the LTRR fingerprint patterns produced two major clusters. The pattern of clustering did not show any clear correlation with that based on the morphological type.

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 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* Bory were collected: five species that have akinetes adjacent to heterocysts and five species whose akinetes are not adjacent to heterocysts. The distribution of STRR sequences among cyanobacteria has been investigated by southern blot analysis in the genomes of many filamentous, heterocystous genera. These include *Calothrix* sp. (Mazel et al., 1990); *Anabaena* sp. and *Nostoc* sp. (Rouhianinen et al., 1995); unicellular *Microcystis* strains (Asayama et al., 1996); *Anabaena* sp., *Nostoc* sp., *Calothrix* sp. and *Fischerella* sp. (Vioque, 1997);
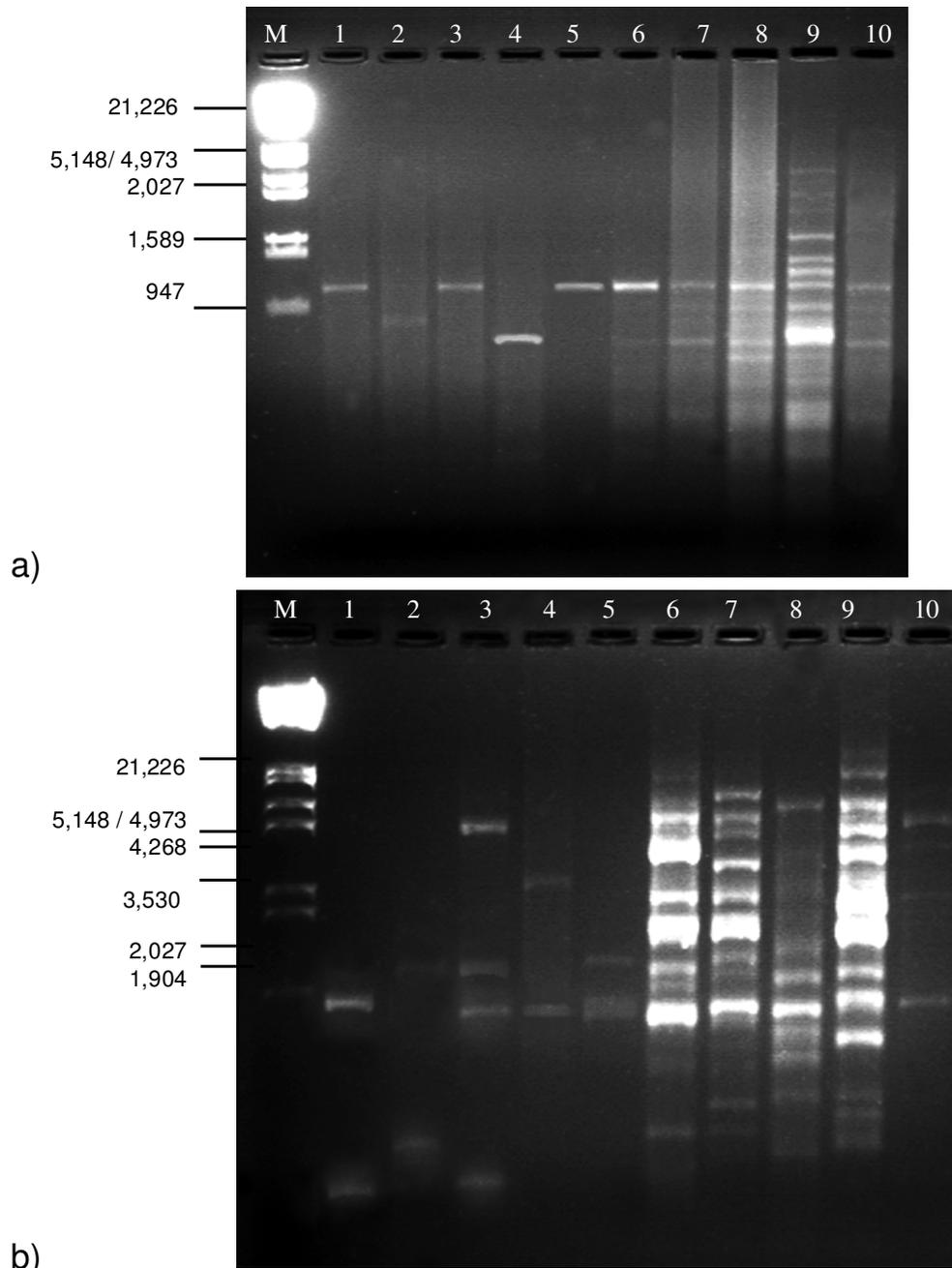
Figure 1. STRR sequence profile analysis of ten *Anabaena* spp. PCR amplification with primers STRR 1F and STRR3F (a) and STRR1R and STRR3R (b). M: λ DNA/EcoRI/Hind III Digest; Lane 1: *A. ambigua*; Lane 2: *A. torulosa;* Lane 3: *A. cylindrica*; Lane 4: *A. augstumalis*; Lane 5: *A. sphaerica*; Lane 6: *A. inaequalis*; Lane 7: *A. variabilis*; Lane 8: *A. fertilissima*; Lane 9: *A. subtropica*; and Lane 10: *A. verrucosa*.

symbiotic *Nostoc* sp. (Rasmussen and Svenning 1998; Guevara et al., 2002); free-living *Nodularia* and *Nostoc* (Rasmussen and Svenning, 1998; Lehtimaki et al., 2000) and *Cylindrospermopsis* (Wilson et al., 2000; Chonudomkul et al. 2004)*.* The trichome morphology (straight or coiled) of *Cylindrospermopsis* strains was shown to be correlated with STRR profiles in the study by

Wilson et al. (2000). STRR sequences might be the target of specific DNA-binding proteins involved in replication during heterocyst differentiation (Lupski and Weinstock, 1992; Masepohl et al., 1996). Rasmussen and Svenning (1998) used STRR1 to fingerprint symbiotic cyanobacterial isolates from the angiosperm *Gunnera* to show the high genetic diversity and distinct clus-
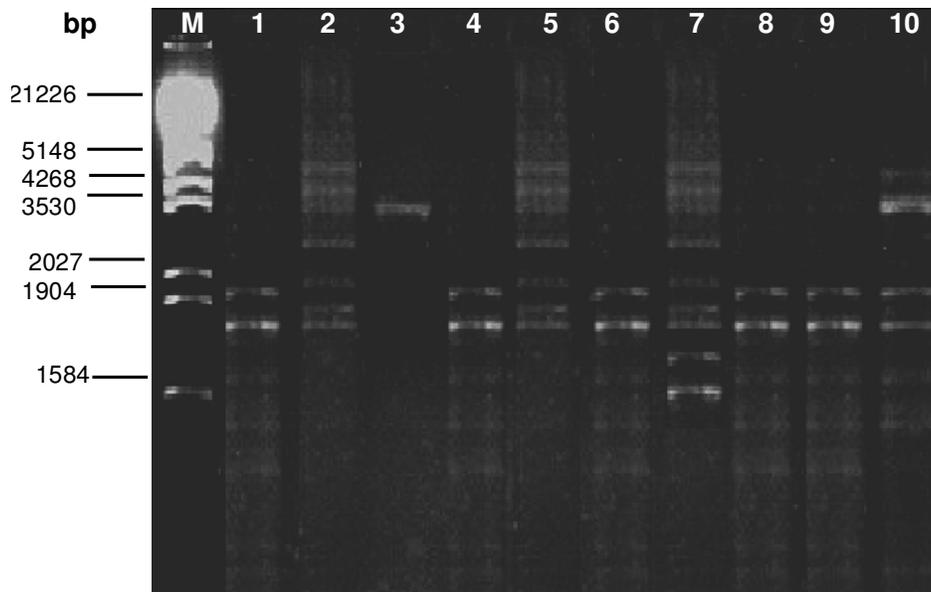
Figure 2. STRR sequence profile analysis of ten *Anabaena* spp. PCR amplification with primers STRR1F and STRR3R. M: λ DNA/EcoRI/Hind III Digest; Lane 1: *A. ambigua*; Lane 2: *A. torulosa;* Lane 3: *A. cylindrica*; Lane 4: *A. augstumalis*; Lane 5: *A. sphaerica*; Lane 6: *A. inaequalis*; Lane 7: *A. variabilis*; Lane 8: *A. fertilissima*; Lane 9: *A. subtropica*; and Lane 10: *A. verrucosa.*
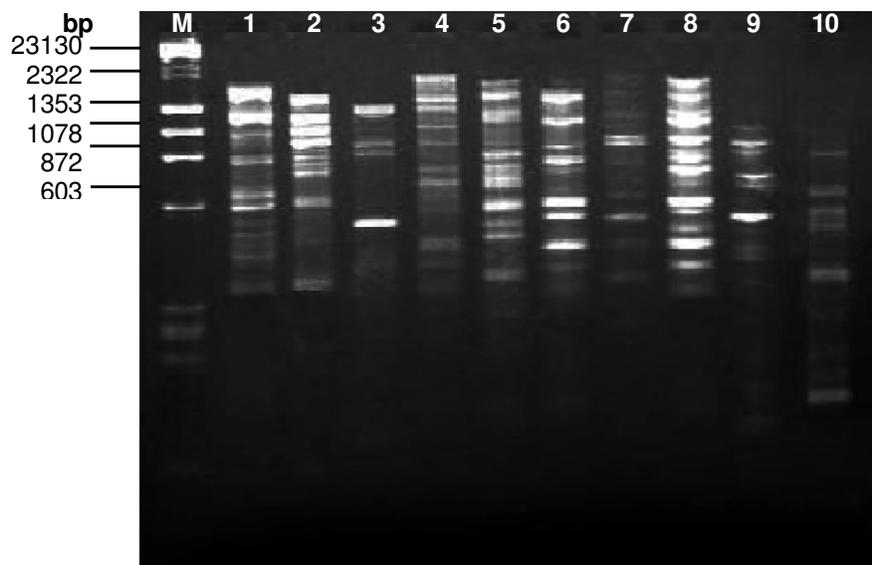

Figure 3. LTRR sequence profile analysis of ten *Anabaena* spp. and PCR amplification with primers LTRR1 and LTRR2. M: λ DNA/Hind III Digest; Lane 1: *A. ambigua*; Lane 2: *A. Torulosa;* Lane 3: *A. cylindrica*; Lane 4*: A. augstumalis*; Lane 5: *A. sphaerica*; Lane 6: *A. inaequalis*; Lane 7: *A. variabilis*; Lane 8: *A. fertilissima*; Lane 9: *A. subtropica*; and Lane 10: *A. verrucosa.*

tering of symbiotic *Nostoc* isolates.

In the present study, unique band profiles for each of the species were observed depending on the primer combination. The phylogenetic relationships inferred from the comparison of STRR sequences generally supported the traditional classification of the genus based on morphological criteria. Multiple distinct DNA products were present and there was distinct clustering of isolates. The dendrogram constructed based on the STRR fingerprints consisted of a minor cluster containing *A. ambigua* and *A.*
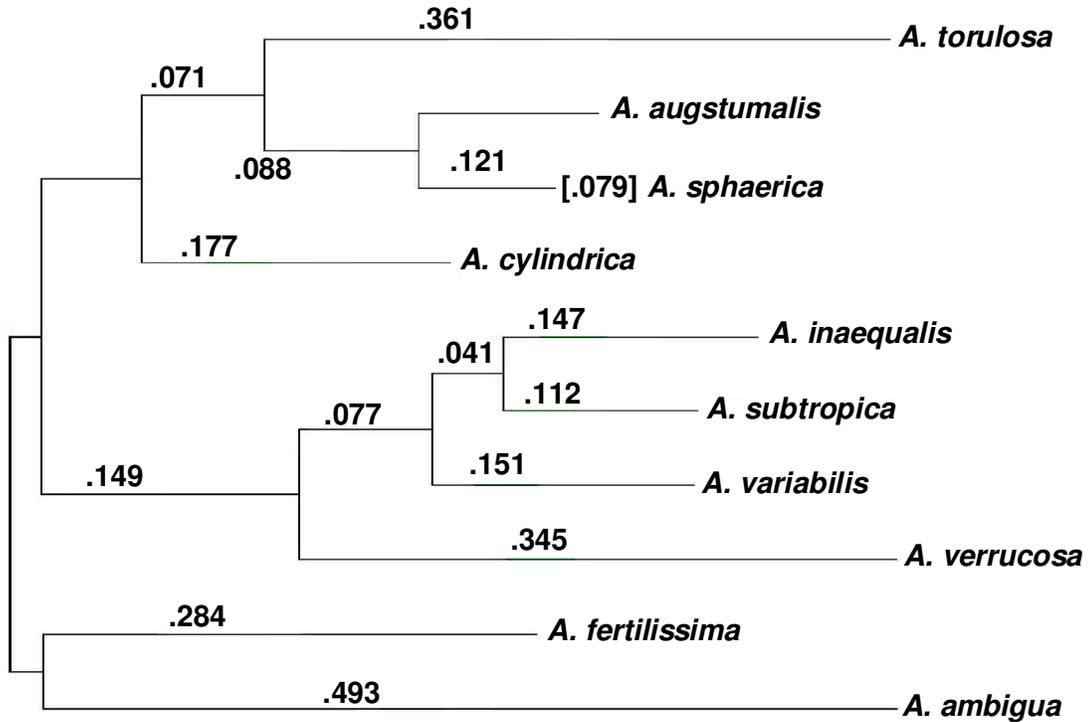
Figure 4. STRR-PCR primers set values at nodes indicate genetic distance measurements as calculated by the algorithm of Nei and Li (1979).
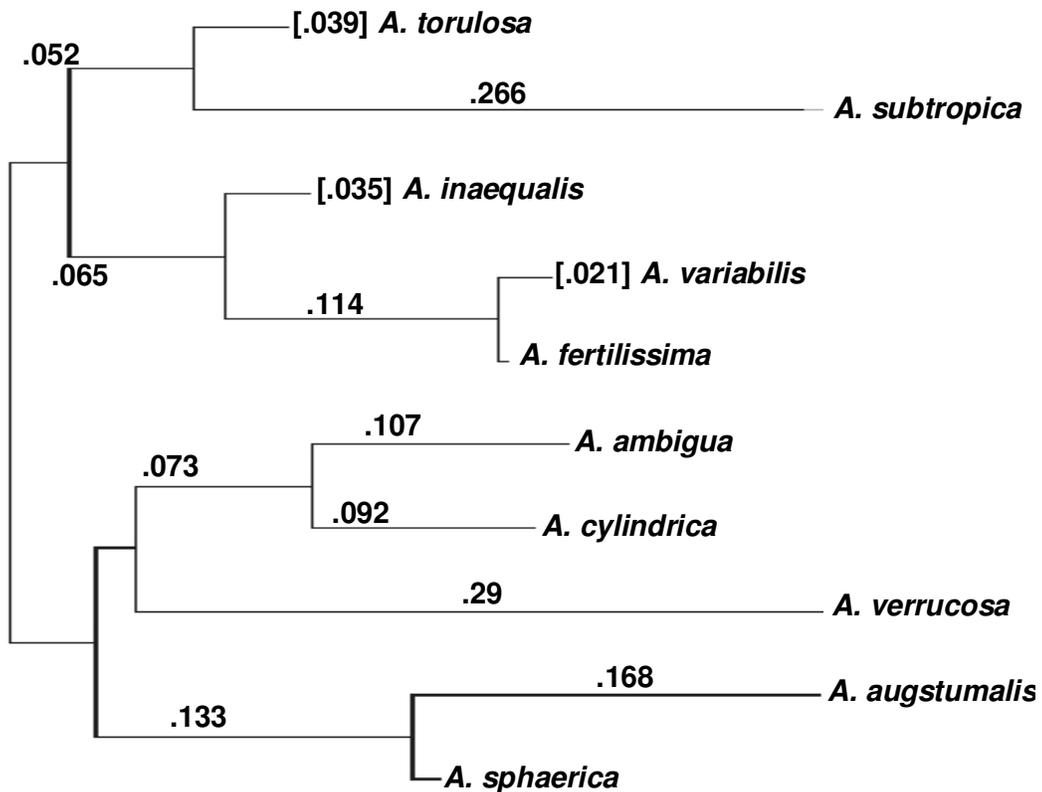

Figure 5. LTRR-PCR (Primers LTRR 1 and LTRR2) values at nodes indicate genetic distance measurements as calculated by the algorithm of Nei and Li (1979).

fertilissima and a major cluster containing the remaining eight species. The major cluster was clearly separated into two subclusters of four species each. This clustering supported the morphological classification schemes. Only *A. fertilissima* and *A. ambigua* were out-grouped and were not consistent with the morphological classification.

Masepohl et al. (1996) identified a 37-bp LTRR sequence in *Anabaena* strain PCC 7120. Some differences were obtained among symbiotic cyanobacterial isolates from the angiosperm *Gunnera* using LTRR primers (Rasmussen and Svenning, 1998). Prasanna et al. (2006) used two primers each for the LTRR- and HIPTG-based analysis of 13 *Anabaena* strains. In our study, the use of primers LTRR1 and LTRR2 yielded multiple distinct DNA products for all ten strains. Dendrograms of the LTRR fingerprint patterns showed two major clusters that contained a mixture of the morphological groups.

In the present study, the results of the analysis using STRR primers indicate that there is a strong genetic basis for the morphological classification system. Analysis using certain primers for STRR, like STRR 1F, STRR 3F, STRR 1R, STRR 3R, seems to give results similar to those from the conventional methods of morphological characterization. Extensive screening of several strains and many methods would ultimately result in designing protocols for a dependable method that should be reproducible in any laboratory. Molecular taxonomic diagnosis will then become a reality.

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