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CHARACTERIZATION OF CYANOBACTERIA WITHIN THE GENUS *Anabaena* BASED ON SDS-PAGE OF WHOLE CELL PROTEIN AND RFLP OF THE 16S rRNA GENE

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ABSTRACT

In this study, ten isolates of freshwater filamentous heterocystous *Anabaena* spp. has been studied by amplified 16S rRNA gene restriction analysis and compared with morphological characters. They 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. The amplicons were digested with three restriction enzymes (*Alu*I, *Hhe*III, *Taq* I) and the banding patterns obtained were analyzed. Cluster analysis showed the separation of all the strains into two main clusters. The clusters for three different enzymes yielded heterogenous groupings of the morphotypes and resulted in unclear delineation of tested organisms.

Keywords: cyanobacteria, a*nabaena*, protein, 16S rRNA gene, RFLP, phylogeny, taxonomy.

INTRODUCTION

Cyanobacteria (Blue-green algae) are large group of phototrophic microorganisms with highly variable morphological futures. For long time, mainly morphological characteristics were taken into account for a taxonomical classification of cyanobacteria (Rippka *et al*., 1979, Schopf, 2000). Cyanobacteria comprise one of the major eubacterial lineages. The diversity with in a lineage, including both that of morphology (single cells, branching filaments, akinets, etc.) and physiology (nitrogen fixation, heterotrophy, motility, etc.) has fascinated microbiologists (Bryant, 1994; Palenik *et al*., 1999). 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. These relationships between morphology and physiological characteristics and the evolution of various group has been investigated using a number of phylogenetic tools, some based on protein sequences, some on 16S rRNA gene sequences.

The uses of DNA sequences for the taxonomic and phylogenetic analysis of cyanobacterial isolates have been carried out by several workers. A genotypic characteristic-DNA base composition was examined for 50 planktonic strains of *Anabaena* assigned to 22 different morphological species (Li *et al.,* 2000). 16S rRNA and ITS-1 sequence analysis were determined in *Synechococcu*s and sequences for the 16S-23S rDNA internal transcribed spacer (ITS) region were determined in 32 *Prochlorococcus* isolates and 25 *Synechococcus* isolates (Rocap *et al.,* 2002; Ernst *et al.,* 2003). rRNA gene sequences have now become the most widely used methods for identification, classification and phylogeny of cyanobacteria. Studies based on restriction fragment length polymorphism (RFLP) and PCR techniques have been used to examine the *Anabaena-Azolla* symbiosis species (Coppenolle *et al.*, 1995; Eskew *et al.*, 1993) and isolates from cycads and *Gunnera* have been studied with respect to genetic diversity by using protein profiles and the RFLP technique (Lupski *et al*., 1992). Fresh water isolates of *Synechococcus* spp. have previously been characterized as distinct genotypes by a genomic fingerprinting technique, RFLP of *psbA* genes (Ernst *et al.*, 1995; Postius *et al*., 1996). The amplified 16S - 23S rDNA spacer (ITS-1) of cyanobacteria has been used in several studies to genetically characterize strains by PCR-RFLP (West and Adams, 1997) or by sequence analyses (Boyer *et al*., 2001; Rocap *et al*., 2002). 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 1978, 1979). 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.

MATERIALS AND METHODS

Organisms and culture conditions

Ten 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 11_o medium (Rippka *et al*., 1979).

Analysis of protein profiles by SDS-PAGE

Sodium dodecyl sulphate - polyacrylamide gel electrophoresis [SDS-PAGE] was carried out by the modified method of Laemmli (1970). Cells were harvested by centrifugation at 2,856 x g for 10 minutes, washed twice in extraction buffer [50 mM Tris-HCl pH 7.8, 0.3 mM $MgSO_4$ and 0.1 mM EDTA] and suspended in 5 ml of the same buffer. Cells were disrupted in a Labsonic 2000 sonicator until the cells were completely broken. The culture was kept on ice while sonication. The homogenate was centrifuged at 2,856 x g for 5 minutes at 4°C in a Beckman J2-21 centrifuge, to remove cell debris. The supernatant was collected in a fresh tube and an excess of ice-cold acetone [100%] was added to precipitate proteins. The precipitated proteins were pelletted by centrifugation at 16,450 x g for 30 min. The tubes were inverted to drain acetone and air dried to remove traces of acetone. The protein pellet was then dissolved in minimum volume of 50 mM Tris buffer and the protein content estimated. Approximately 50 µg protein was loaded in each well. Molecular weight marker was run along with the sample to determine the molecular weight of the polypeptides. Protein molecular weight marker included proteins ranging from 97.4 to 14.3 kDa [Bangalore Genei Pvt. Ltd). An equal volume of 2 x sample buffer was added to the protein sample, mixed well by vortex and heated in a boiling water bath for 5 min. This was allowed to cool before loading on to the gel. Electrophoresis was carried out at room temperature at 50 volts until the bromophenol dye reached the bottom of the resolving gel. Once the separation was over, power supply was switched off, clamps removed and the glass plates were separated by removing the space bars. At the end of electrophoresis, gel was removed and stained with silver staining method of Blum *et al*. (1987). After staining, the gel was stored in 7% (v/v) acetic acid. The gels were scanned by Vilber Lourmat Gel documentation system with Bioimage software and the molecular weights of the protein bands were determined using gel documentation with Bio-1D. The patterns were compared by using the information on apparent molecular masses of the bands, band spacing and band intensity. The results were used to construct a UPGMA (unweighted pair-group method using arithmetic averages) dendrogram program in phylogeny inference package (PHYLIP) (Felsenstein, 1993).

Preparation of DNA sample for electrophoresis

DNA extraction was carried out according to standard procedures (Sambrook *et al.,* 1989). 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 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 I™ system, Easy cycler ™ PCR system. Oligonucleotides were purchased from DDT, UK.

RFLP of PCR products corresponding to the 16S rRNA gene

PCR amplification was performed on purified DNA of *Anabaena* spp. (Itman *et al*., 2000). Amplification of the 16S rRNA gene was carried out by PCR using primers A2 (AGAGTTTGATCCTGGCTCAG) and S8 (TCTACGCATTTCACCGCTAC). 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 95ºC, 2 min at 55ºC and 30 s at 72ºC, 30 cycles of amplification were started (1.5 min at 95ºC, 2.5 min at 55ºC and 3 min at 72ºC). The termination cycle was 7 min at 72ºC. The PCR products were analyzed in horizontal 1.5% (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 (Nei and Li, 1979) differences, which were subsequently used to construct a neighbor-joining tree analysis.

Amplified PCR products (5-10 µl) were digested with following enzymes; *Alu*I, *Hae*III, *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 (Banglore Genei). The gel was documented by phographing under a UV - illuminator using a Vilber Loumart gel documentation system.

RESULTS AND DISCUSSIONS

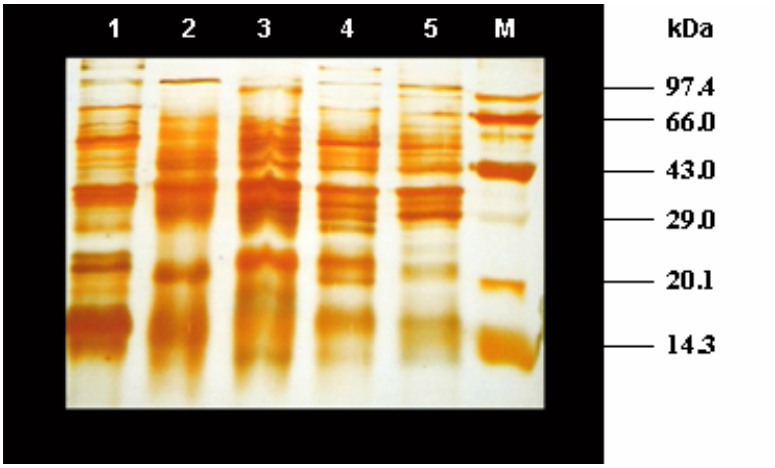
The protein profiles were studied by SDS-PAGE. Exponentially grown cultures were taken for extraction of protein. The molecular weights of the protein were determined with medium range marker protein. The approximate molecular weight of the polypeptide ranged from 97.4 kDa to 14.3 kDa. Maximum of 27 protein bands in *Anabaena ambigua,* 23 bands in *Anabaena augstumalis*, 21 bands in *Anabaena subtropica,* 20 bands in *Anabaena cylindrica* and *Anabaena inaequalis*, 19 bands in *Anabaena fertilissima* and 18 bands in *Anabaena torulosa, Anabaena sphaerica, Anabaena variabilis* and *Anabaena verrucosa* were analyzed (Figure-1). The analysis of the protein profiles by SDS-PAGE showed several proteins to be common among most of the organisms selected for the study. The peptide band of approx. 21 KDa was present in almost all the ten organisms studied. When combination of protein profiles for all *Anabaena* species, an obvious

clustering among the isolates were observed (Figure-6). Dendrogram constructs from protein profiles pattern produced 2 major clusters and was heterogeneous in distribution for morphological type. DNAs from the 10 strains studied were amplified with the conserved A2 and S8 primers. At the annealing temperature of 55ºC, all the strains produced a single band of similar size (approximately 1,500bp) (Figure-2). *Alu*I (37 ºC for 2h), *Hae III* (65ºC for 2h) and *Taq I* (37 ºC for 2h) restriction enzymes used digested the 16S rRNA PCR products and yielded different profiles (Figures 3 to 5). Genetic distances between the ten cyanobacteria tested by RFLP analysis of 16S rRNA genes using three different enzymes namely *Alu*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 (Figure-7).

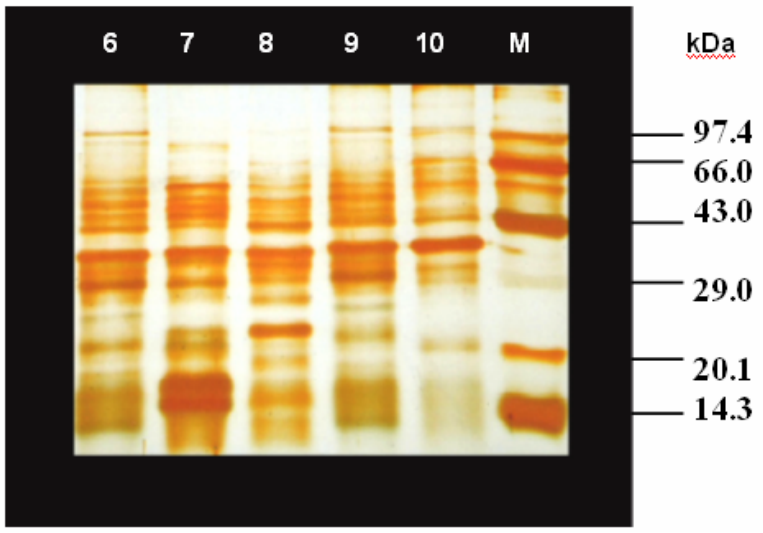
Molecular studies for the determination of the relationships of the 10 *Anabaena* species were carried out. In several studies the amplified 16S/23S ITS of cyanobacteria has been used to genetically characterize strains by PCR- RFLP (Lu *et al*., 1997; West and Adams 1997) and symbiotic *Nostoc* strains (Rasmussen and Svenning 2001). Iteman *et al*. (2002) 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. The restriction enzymes digested the 16S rRNA PCR products and yielded different profiles. Genetic distances found in the RFLP study were short, confirming that close genotypic relationship exists among the cyanobacterial genera (Lyra *et al*., 1997). In the present study genetic distances between the ten *Anabaena* spp tested by RFLP analysis of 16S rRNA genes using three different enzymes *viz.*, *Alu*I, *Taq* I and *Hae* III were done. The clusters for three different enzymes yielded heterogeneous groupings of the morphotypes and did not result in clear delineation of the species. Similar findings have been reported for *Prochlorococcus* strains (Urbach *et al*., 1998). 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 (Lyra *et al*., 2001). Lyra *et al*. (1997, 2001) and Lehtimaki *et al*. (2000) reported close relationship between strains of *Anabaena* and *Aphanizomenon* in a 16S rDNA after the RFLP study.

Table-1. Strains used in this study.

Strain	Taxonomic assignment	Position of heterocysts and akinetes	Source	Reference
A485	*A.ambigua*	heterocysts adjacent to akinetes	1403/7 CCAP, UK	Anand, 1978, 1979.
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	
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	



(a)



(b)

Figure-1. a and b. Protein profiles by SDS-PAGE analysis; M-Markers,
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*.

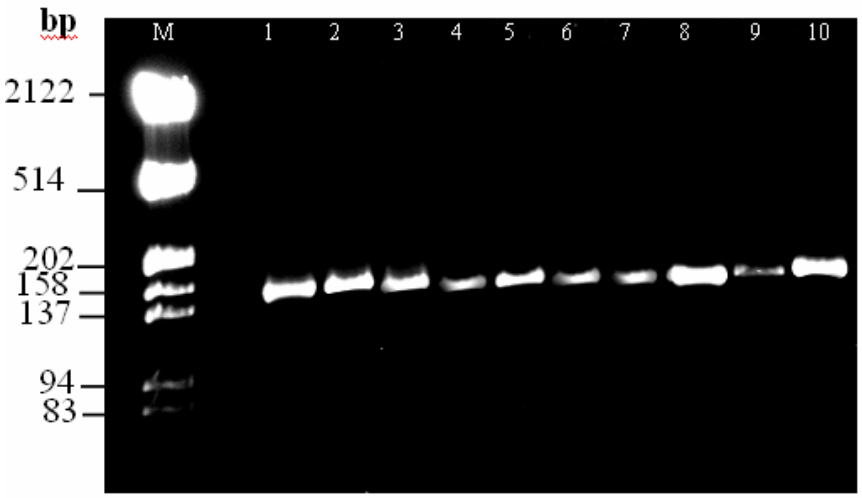


Figure-2. Amplification of the 16S rRNA gene in 10 *Anabaena* spp. and PCR amplification with primers A2 and S8. M-λ DNA/Hind III Digest; Lane 1. *A. ambigua*; Lane 2. *A. torulosa*; Lane 3. *A. cylindrica*; Lane 4. *A. augstumalis*; Lane 5. *A. sphaerica*; Lane 6. *A. inaequalis*; Lane 7. *A. variabilis*; Lane 8. *A. fertilissima*; Lane 9. *A. subtropica*; Lane 10. *A. verrucosa*.

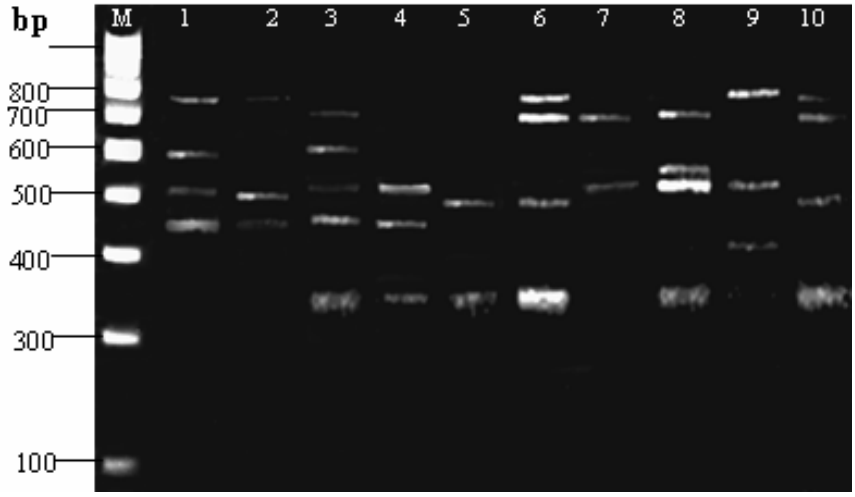


Figure-3. RFLP-16S rRNA genes digested with *Alu*I at 37°C for 2h. M-DNA Ladder 100 bp; Lane 1. *A. ambigua*; Lane 2. *A. torulosa;* Lane 3. *A. cylindrica;* Lane 4. *A. augstumalis*; Lane 5. *A. sphaerica;* Lane 6. *A. inaequalis;* Lane 7. *A. variabilis;* Lane 8. *A. fertilissima;* Lane 9. *A. subtropica;* Lane 10. *A. verrucosa.*

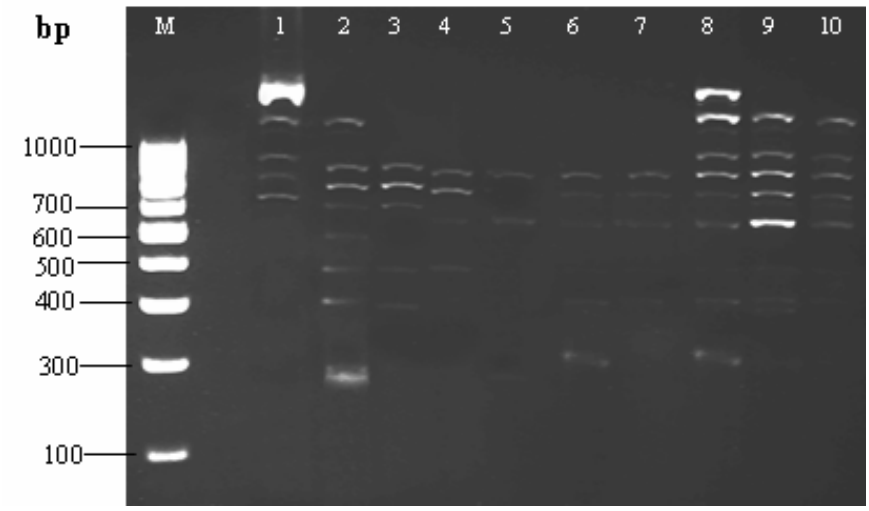


Figure-4. RFLP - 16S rRNA genes digested with *Taq* I at 65°C for 2h. M-DNA Ladder 100 bp; Lane 1. *A. ambigua*; Lane. *A. torulosa*; Lane 3. *A. cylindrica*; Lane 4. *A. augstumalis*; Lane 5. *A. sphaerica*; Lane 6. *A. inaequalis*; Lane 7. *A. variabilis*; Lane 8. *A. fertilissima*; Lane 9. *A. subtropica*; Lane 10. *A. verrucosa*.

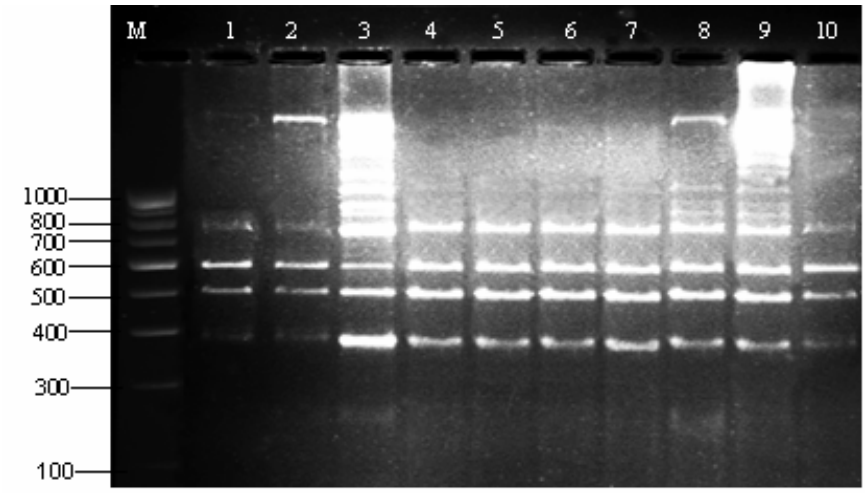


Figure-5. RFLP - 16S rRNA genes digested with *Hae* III at 65°C for 2h. M-DNA Ladder 100 bp; Lane 1. *A. ambigua*; Lane. *A. torulosa*; Lane 3. *A. cylindrica*; Lane 4. *A. augstumalis*; Lane 5. *A. sphaerica*; Lane 6. *A. inaequalis*; Lane 7. *A. variabilis*; Lane 8. *A. fertilissima*; Lane 9. *A. subtropica*; Lane 10. *A. verrucosa*.

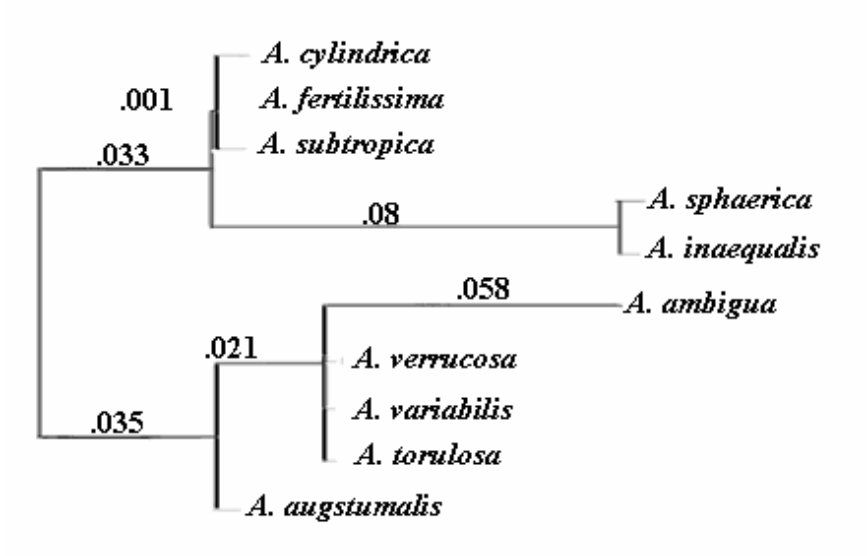


Figure-6. SDS-PAGE values at nodes indicate protein profiles measurements as calculated by algorithm of Nei and Li (1979).

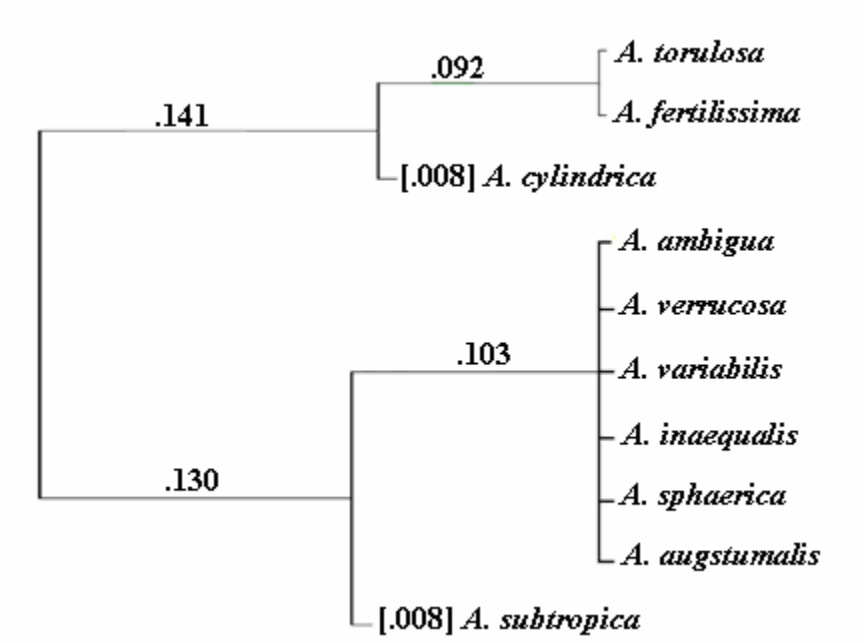


Figure-7. RFLP-16S rRNA genes (digested with *Alu*I, *Hae* III, *Taq* I) values at nodes indicates genetic distance measurements as construct a UPGMA (unweighted pair-group method using arithmetic averages) dendrogram program in phylogeny inference package.

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