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Studies on Molecular Identification of Anabaena Cyanobionts of Azolla Species

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Abstract*: Azolla is a genus of free-floating water fern which is widely distributed in aquatic habitats of the tropics and temperate regions. It is one of few vascular plants known to exhibit a symbiotic association with N2-fixing blue - green algae, Anabaena sp. In view of this, a study was carried out on molecular identification of Anabaena strains isolated from three Azolla species. The selected Azolla species were grown in culture medium prepared with garden soil, goat manure, super phosphate and its combinations. The three strains of Anabaena cyanobiants present in the leaf cavity of three different Azolla species were isolated and grown in BG11 medium. Out of three Anabaena isolates, only two isolates (i.e., strains -1 & 2 isolated from Azolla microphylla and A.filiculoides respectively), were able to grow an BG11 medium while other strain isolated from A.pinnata have not grown on the culture medium. These two Anabaena cyanobiants were further mass cultured in BG11 medium and were identified through molecular studies using 23S r RNA sequencing. Thus, the two selected strains were identified as Anabaena sphaerica (**KX055938**) and A.variabilis (**KX055939**).*

Keywords: *Azolla, Anabaena, N_2 fixers, 23S rRNA*

I. Introduction

Azolla, a free floating aquatic fern fixes atmospheric nitrogen in its symbiotic association with blue green algae the *Anabaena*. The importance of *Azolla* as an organic input to soil was first introduced in North Vietnam during 1957. Subsequently its potentially has been recognized in USA, Japan and China as a green manure for rice cultivation [1]. The practical use of *Azolla* as a source of organic nitrogen fertilizer for rice culture has been recognized in Asia, particularly China and Vietnam [2]. In India, International Rice Research Institute made extensive studies on *Azolla* and its application in tropical rice culture. *Azolla* is potential to be used as natural nitrogen source and observed from the study that it was able to increase the growth of the plant. Nitrogen fixation in the cultivated land by *Azolla* cultures reaches 1.1 kg N_2/day and hence it has been used as green manure and soil fertilizer [3]. The mass cultivation of *Azolla* sps., were already achieved in freshwater under strictly controlled condition [4]. The optimum water temperature of 25^0C and pH optimum 5 to 7 were best support the growth of *Azolla* species [5].The *Azolla* inside dorsal leaf lobe exists a specialized cavity that contains a permanent endosymbiotic prokaryotic community (cyanobacteria). This cavity can be considered as the basic physiological unit of the symbiotic association. [6]. The morphological characteristics of different *Anabaena* cyanobionts inhabited in different *Azolla* fern were well documented[6].The potential of sequencing 16S rDNA of bacteria for establishing phylogenetic relationships and for use in molecular identification was well-documented. In a recent report on direct sequencing of the PCR-amplified 23S rDNA, the method involves three amplification steps to obtain overlapping fragments and the subsequent use of 33 sequence primers to achieve the complete sequencing of the 23S rRNA gene [8]. Therefore, the present study was focused on isolation and molecular identification of potential cyanobacterial strains isolated from different species of *Azolla* cultures.

II. Materials And Methods

Azolla culture collection and Mass multiplication

Pure cultures of *Azolla* fern such as *A.filliculoides, A.pinnata* and *A.microphylla* were Collected from TNAU, Coimbatore, Tamil Nadu, India. All the cultures three cultures were mass multiplied using standard procedures [7 and 9]. The 5 ltr capacity of plastic container was filled with 500 gram of garden soil, the substrates were mixed together and made it slurry using water and adjusted pH 5.0 to 5.7.Three different species of *Azolla* were inoculated in separate containers and allowed to grow for three weeks. The optimum temperature of about 25^0C is maintained in green house for Mass multiplication [9].

Isolation and mass culture of *Anabaena* cyanobiants

Ten grams of fresh samples each from three species of *Azolla* were ground in crucible to squeeze the cells forming suspension. 5 ml of cell-free culture suspension of each *Azolla* species was transfer to a 100 ml conical flask containing BG11 medium [8] and incubated in a growth chamber under continuous illumination

(2000 lux) with a temperature of 25°C± 2°C for 30 days. A well developed cyanobacterial colony from each petridishes inoculated with cell-free extract of three different *Azolla* cultures taken with a sterilized inoculation needle and reinoculated to three conicalflask containing liquid BG11 medium. The culture flasks were incubated in the illuminated growth chamber (2000 lux) for 30 days for mass culture.

Identification of *Anabaena* isolates using 23S rRNA Sequencing Studies

Identification of *Anabaena* isolates were performed using morphological and molecular studies using standard procedures [8,10]. Fresh isolates of *Anabaena* spp. From two *Azolla* sp. strains were pelleted and resuspended in 1 mL TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The suspension was heated to 80°C for 30 min and centrifuged at 10 000´ g for 15 min, and 1 mL of the supernatant was added to a polymerase chain reaction (PCR). Master mixture (50 ml) containing 1´ PCR buffer [20 mM Tris-HCl, 10 mM KCl, 10 mM (NH4)2SO4 , 1% TritonÒ X-100, 1.5 mM MgSO4 and 1 mg/mL of bovine serum albumin], 200 mM of each dNTP and 10 pmol of each primer. The mixture was overlaid with 50 mL of mineral oil and heated to 80°C before 1 U each of Taq DNA polymerase and Taq extender (Stratagene, La Jolla, CA, USA) was added. Immediately after adding the enzymes, the tubes were subjected to 30 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 60°C and extension for 2.5 min at 72°C on a Model 480 Thermal Cycler (PerkinElmer, Norwalk, CT, USA). The amplification process was terminated by a 10-min extension at 72°C, and tubes were rapidly cooled to 4°C. The primers used were chosen so that the 16S–23S spacer region and virtually the complete 23S rRNA gene would be amplified, thereby enabling determination of the 5' end of the gene. The resulting fragment is approximately 3 kb because of a rather short spacer of 400 bp. The primers are complementary to positions 2669–2654 of the 23S rRNA genes as follows:

Designation	Primer Sequence	dNTP Mixture
1091R	5′(AG)GT GAG CT(AG) TTA CGC3′	dCTP, dTTP, [α-^{33}P]dATP
1055F	5′GGA TGT TGG CTT AGA AGC AG3′	dCTP, dTTP, [α-^{33}P]dATP

Fragments were separated by electrophoresis on a 1% agarose gel. Amplification products were excised and purified using the GENECLEANÒ Kit (BIO 101, Vista, CA, USA) as directed by the manufacturer. The fragment was sequenced directly using the Thermo Sequence Cycle Sequencing Kit (Amersham International plc), which contains all necessary reagents for sequencing except the radiolabel, in a two-step sequence strategy as recommended by the manufacturer. The following reagents were combined in a 0.5-mL PCR tube: 1´ Thermo Sequenase buffer, 1 mL of each of three dNTPs (3 mM) including either [a- 33P] dATP or [a-33P]dCTP, 0.4 pmol sequencing primer, 100 ng purified template and 2 mL of Thermo Sequenase (4 U/mL) in a total volume of 17.5 mL). The tubes were immediately transferred to a thermal cycler programmed for 60 cycles at 94°C for 30 s and 55°C for 30 s, followed by rapid cooling to 4°C. During cycling, tubes containing termination mixtures were prepared and kept on ice. After completion of the labeling step, the reagents were divided (3.5 mL) into four tubes containing the termination mixtures and subjected to 50 cycles at 94°C for 30 s and 72°C for 90s, followed by rapid cooling to 4°C. Sequence reactions were terminated by adding 4 mL stop buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol) to each tube. Before electrophoresis, the reaction mixtures were denatured at 80°C for 4 min and kept on ice while 3 mL were loaded on a 6% polyacrylamide sequencing gel. The gel was fixed, dried and autoradiographed on BioMax MR Film (Eastman Kodak, Rochester, NY, USA). The adapted sequence protocol we describe relies on two separate steps. The first is a labeling step, where the primer is extended using three of the four dNTPs and one a- 33P-labeled dNTP (dCTP or dATP). This step involves thermal cycling and results in the production of labeled, extended primer, the length of which is dependent on the template sequence. In the second step, the concentration of all the dNTPs is increased, a ddNTP is added, and the reaction undergoes thermal cycling as described above. DNA synthesis during this step continues until all chains are terminated by a dideoxynucleotide.The 23S rRNA of two *Anabaena* strains were sequenced individually using sanger dedioxy method and the sequences were submitted in National Center for Biotechnology Information (NCBI), USA and the phylogenitic tree was constructed.

III. Result And Discussion

Azolla is an aquatic pteridophyte that forms a permanent, hereditary symbiosis with a nitrogen fixing, heterocyst-forming cyanobacterium, *Anabaena azollae*. The *Azolla – A. azollae* symbiosis is the only known mutualistic symbiosis between a pteridophyte and a diazotrophic prokaryote [11,12]. The various morphometric characteristic of symbiotic cyanobacteria, *A.azollae* present in two species of *Azolla* cultures were analysed through observing average number of *Anabaena* filament in each leaf cavity of *Azolla*, length of each cyanobaterial filament, number of vegetative and heterocyst cells present in each cyanobiant filament. In the

present study[8]. Out of three *Anabaena* isolates, only two strains isolated from *A.microphylla* and *A.filiculoides* were selected and mass multiplied in BG11 medium.In order to identify bacterial strains, several researchers undertaken molecular sequencing of 16s and 23s rRNA and also established phylogenetic relationship [7].A simple protocol that allows amplification of almost the complete 23S rRNA gene and the subsequent direct sequencing of the double stranded fragment, thereby eliminating the need for cloning or preparation of single-stranded template [13]. In this study The two *Anabaena* strains were selected and identified through 23S rRNA sequencing. By exploring database available at NCBI, USA, the two selected strains were authenticated as *A.sphaerica* (KX055938) and *A.variabilis* (KX055939) and their phylogenitic tree presented in Plates 2 and 3.

IV. Conclusion

The present investigation clearly evident that *Anabaena* strains present in leaf cavities of *Azolla* fern are sole responsible for fixing atmospheric N2 and support better growth of *Azolla.* Hence these cultures could be used as effective biofertilizer towards sustainable agriculture.

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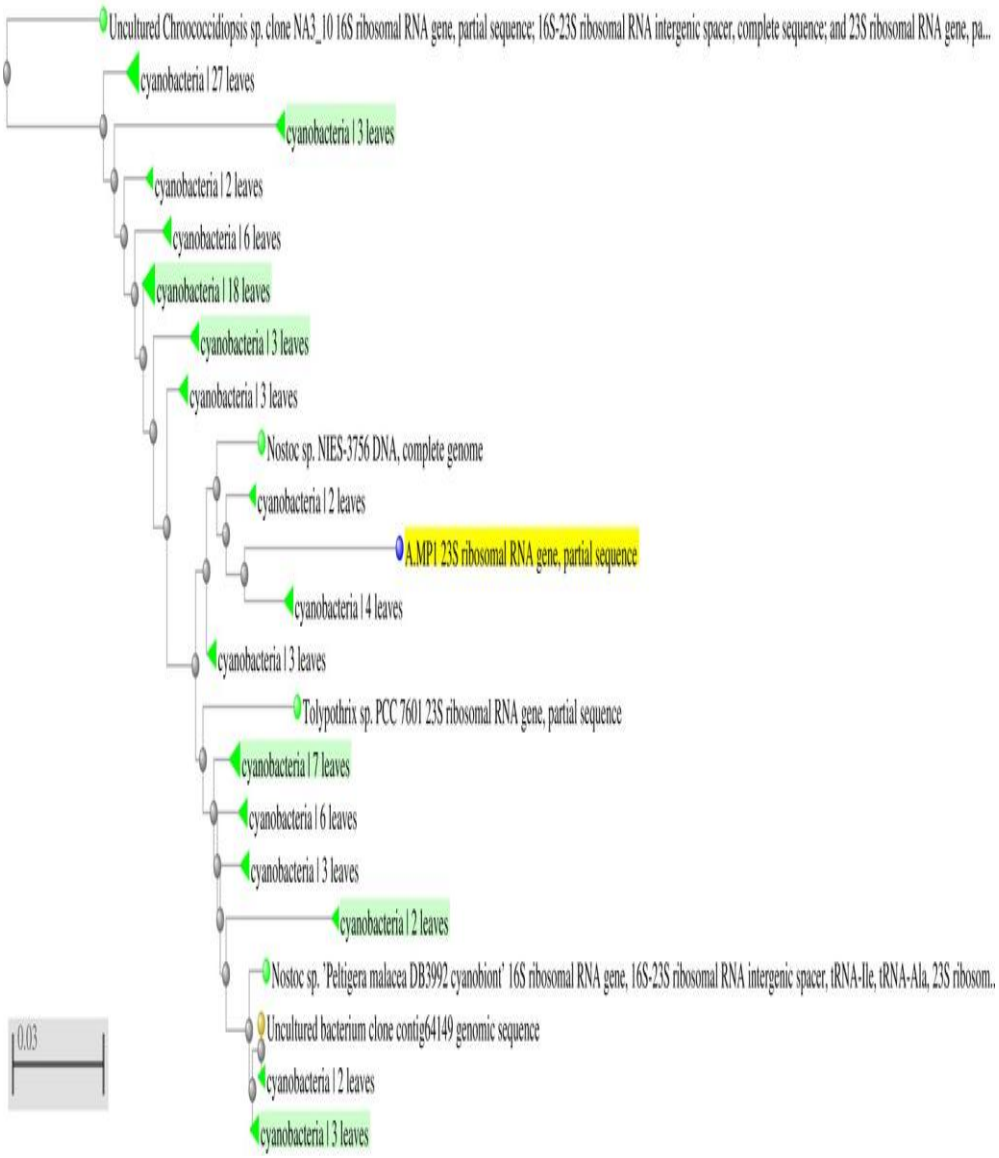

Plate 1:A Phylogenic tree showing position and evolutionary relationships between the cyanobacteria and *Anabaena sphaerica* (KX055938)

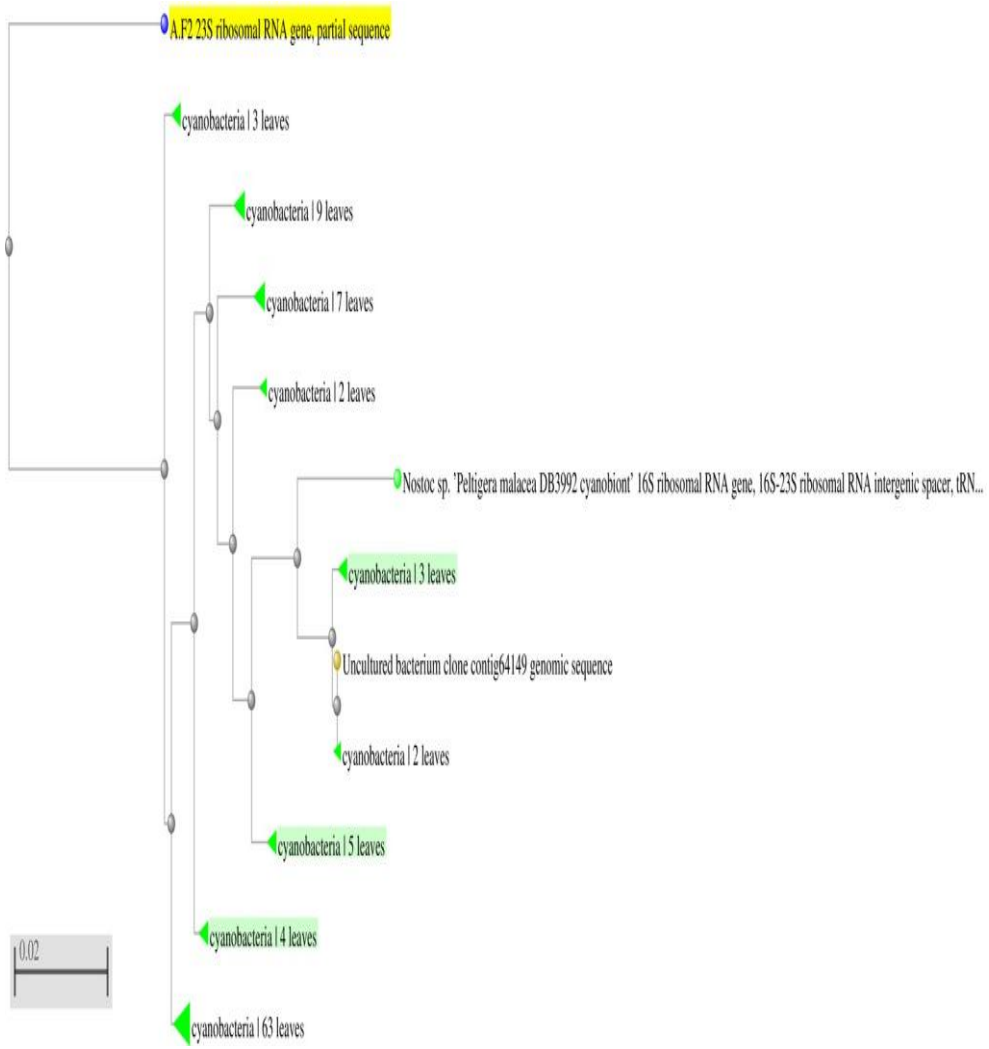

Plate 2:A Phylogenic tree showing position and evolutionary relationships between the cyanobacteria and *Anabaena variabilis* (KX055939)

References

[1]. Lumpkin T.A. and Plucknett, D.L. (1980). *Azolla* Botany, physiology and use as green manure. Eco. Bot. 3, 111 – 153.

[2]. Dao, T.T. and Tran, Q.T. (1979). The use of *Azolla* in rice production in Vietnam, *In* Nitrogen and Rice, International Rice Research Institute, Los Banos, Laguna, Philippines.395-405.

[3]. Arora, P.K. Singh, J. (2003). Comparison of biomass productivity and nitrogen fixing potential of *Azolla* SP. Journal of Biomass and Bioenergy. 24, 175- 178.

[4]. Thangam.P and Mahalingam.P.U.(2010). Whey water as a low cost media for *Azolla* cultivation. J.Bionano Fronitor. 3 (2), 195-195.

[5]. Cary, P.R. and Weerts, P.G.J. (1992). Growth and classification trees to model the Co., (P) Ltd. 3rd edition. distribution pattern of a new exotic species. 43,163-180.

[6]. Desikachary, T. V. (1959). Cyanophyta. ICAR Monograph on Algae. ICAR, New Delhi, India. 1-686.

[7]. Mahalingam.P.U, Muniappan.K Arumugam.N and Senthilmurugan.G.(2014). Use of *Azolla* biofertilizer on the growth studies with paddy crop oryza sativa in the innovative journal of Agricultural sciences. 2(3), 1-6.

[8]. Sallen, B., A. Rajoharison, S. Desvarenne, F. Quinn and C. Mabilat. (1996). Comparative analysis of 16S and 23S rRNA sequences of Listeria monocytogenes. Int. J. Syst. Microbiol. 46,669-674.

[9]. Mahalingam.P.U, Muniappan.K and Therasammal.M (2016). Studies on Morphometric Evaluation of Symbiotic Cyanobiont in two Species of *Azolla* Fern. International Journal of Scientific Research. 5(8):79-81.

[10]. Lawson, V.A., D.A. McPhee and N.J. Deacon. (1996). Elimination of sequence ambiguities by a single-step modification of a solidphase, single-stranded sequencing protocol. BioTechniques. 21,356-358.

[11]. Grilli Caiola.M and Forni.C. (1999). The Hard Life of Prokaryotes in the Leaf Cavities of *Azolla*, Enigmatic Microorganisms and Life in Extreme Environments, J. Seckbach , ed. 629-639.

[12]. Lumpkin, T.A. and Plucknett, D.A. 1982. *Azolla* as green manure: use and management in crop production. *West view Tropical Agricultural Series*, no. 5. Boulder, CO. West view.

[13]. Lawson, V.A., D.A. McPhee and N.J. Deacon. 1996. Elimination of sequence ambiguities by a single-step modification of a solid phase, single-stranded sequencing protocol. Bio Techniques. 21,356-358.