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BIODIVERSITY OF *Azolla* AND ITS ALGAL SYMBIONT *Anabaena Azollae*

S. KANNAIYAN and **K. KUMAR**

2006

National Biodiversity Authority

CHENNAI, TAMILNADU, INDIA

NBA Scientific Bulletin Number - 2

Citation : **KANNAIYAN, S.** and **K. KUMAR. 2006.** Biodiversity of *Azolla* and its algal symbiont, *Anabaena azollae*
NBA Scientific Bulletin Number - 2,
National Biodiversity Authority, Chennai, TamilNadu,
Page 1 - 31.

For further information, please contact

The Chairperson
National Biodiversity Authority
475, 9th south cross street,
Kapaleeswarar Nagar,
Neelangarai,
Chennai – 600 041.

Printed by :

Frontline Offset Printers
26, New Street, Llyods Road,
Triplicane, Chennai - 600 005.
Ph : 28470052

Nierwicki-Bauer, A. and Haselkorn, R. 1986. Differences in rRNA levels in *Anabaena* living freely or in symbiotic association with *Azolla*. **Embo J.** 5(1): 29-35.

Neirzwicki-Baue,S.A. and Kannaiyan, S.1990. Molecular and ultrastructural studies of the *Azolla–Anabaena* association. In: **Biotechnology of Biofertilizers** (ed.) S. Kannaiyan, TamilNadu Agrl.Univ. Coimbatore, Tamil Nadu, India.pp.44-58.

Plazinski, J, Franche, C, Liu, C. C, Lin, T, Shaw, W, Gunning B.E.S. and Rolfe, B. G. 1988. Taxonomic status of *Anabaena azollae*: An overview**. Plant and Soil** 108: 185-190.

Plazinski, J, Zheng, Q, Taylor, R, Croft, C, Rolfe B.G. and Gunning. B.C.S. 1990. DNA probes show genetic variation in cyanobacterial symbionts of the *Azolla* fern and a closer relationship to free living *Nostoc* strains than to free-living *Anabaena* strains. **Appl. Environ. Microbiol.** 56(5): 1263-1270.

Rasmussen, U. and. Svenning, M. M. 1998. Fingerprinting of cyanobacteria based on PCR with primers derived from short and long tandemly repeated repetitive sequences. **Appl. Environ. Microbiol.** 64 (1): 265-272.

Sterigianou,K.K. and Fowler, K.1990. Chromosome numbers and taxonomic implications in the fern genus *Azolla* (Azollaceae). **Pl. Syst. Evol 1.173:** 223-229.

Subhashini, R, Kumar, K. and Kannaiyan. S. 2003. Intrinsic antibiotic resistance and biochemical characteristics of *Anabaena azolla* isolated from *Azolla*–cultures. **Ind.J.Microbial.**43 (3):

Zimmerman, W.J, Rosen, B. H. and Lumpkin. T. A. 1989. Enzymatic, Lectin and morphological characterization and classification of presumptive cyanobionts from *Azolla* Lam**. New Phytol.** 113 (4): 497-503.

Zimmerman, W. J, Watanabe, I. and Lumpkin. T. A.1991. The *Anabaena Azolla* symbiosis: Diversity and relatedness of neotropical host taxa. **Plant and Soil** 137: 161-170.

Gates, J.E, Fisher, R. W, Goggin T. W. and Azrolan. N. I. 1980. Antigenic differences between *Anabaena azollae* fresh from the *Azolla* fern leaf cavity and free-living cyanobacteria. **Arch. Microbiology** 128: 126-129.

Glaszmann, J.E. 1987. Isozymes and classification of Asian rice varieties. **Theor. Appl. Genet.** 74: 21-30.

Gebhardt, J.S. and Nierzwicki-Bauer. S.A. 1991. Identification of a common cyanobacterial symbiont associated with *Azolla* spp. through molecular and morphological characterization of free living and symbiotic cyanobacteria. **Appl. Environ. Microbiol.** 57(8): 2141 - 2146.

Kannaiyan, S. and K. Kumar. 2005. Azolla Biofertilizer for sustainable rice production. Daya Publishing House, New Delhi, p.450

Kim, J. J., Krawczyk, N. Lorenty, K and Zimmerman, W. P.1997. Fingerprinting cyanobionts and hosts of the *Azolla* symbiosis by DNA amplification. **World J. Microbiol. Biotechnol.** 13 (1): 97-101

Konde, V.B. 2000. Studies on the biochemical and Molecular characterization of the nitrogen fixing water fern *Azolla* the algal symbiont *Anabaena azollae* and free-living cyanobacteria. Ph.D. Thesis, Tamil Nadu Agrl. Univ. Coimbatore, Tamil Nadu, India.

Kumar, K. and Kannaiyan, S. 1999. Characterization of chemical mutants of *Azolla microphylla* and *Azolla filiculoides* obtained through chemical mutagenesis. **Indian J. Plant Physiol.** 4: 242-245.

Ladha, J.K and Watanabe, I.1982. Antigenic similarity among *Anabaena azollae* separated from different species of *Azolla*. **Biochem. Biophys. Res. Commun.** 109: 675-682.

Liu, C.C, Chen, Y, Tang, L, Zheng, Q, Song, T, Clhem, M, Li, Y. and Lin. T. 1989. Study on preparation and application of monoclonal antibodies to *Anabaena azollae*. **Sci. China (Ser. B)** 32:562-569.

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BIODIVERSITY OF *Azolla* AND ITS ALGAL SYMBIONT *Anabaena azollae*

Introduction

Azolla is a free-floating water fern that floats on the water and fixes atmospheric nitrogen in association with nitrogen fixing cyanobacterium, *Anabaena azollae. Azolla-Anabaena* symbiosis is the only plant-cyanobacterial symbiosis used as biofertilizer in agriculture. The *Azolla-Anabaena* association has a long history as a green manure for rice and as a fodder for poultry and livestock in China and other Far East Countries. The natural occurrence of *Azolla* in rice fields and ponds probably contributed to its eventual recognition leading to the cultivation of *Azolla* as a green manure in Asia. The exact period when *Azolla* cultivation began is not found in history but it is reported to date back to the 11th century in Vietnam. The genus *Azolla* was established by Lamarck in 1893. The name *Azolla* is derived from two Greek words azo means to dry and allyo means to kill. It is a genus of heterosporous and leptosporangiate ferns from aquatic and semi aquatic habitats. These habitats include ponds ditaches, canals and paddy fields from temperate to tropical regions, countless places, where agricultural run off water or urban effluent accumulate, are seasonally covered by a thick mat of *Azolla*, usually in conjunction or succession with other free-floating aquatic plants such as *Lemna, Pistia, Salvinia, Spirodela, Ricciocarpus* and *Riccia.*

Azolla is the most important symbiotic system of the aquatic environment, which is believed to consist of seven species. Accurate taxonomic identification has been a constraint in the genetic improvement of the strains in this agronomically important aquatic fern. Recent applications of molecular technology suggest some arrangements in the *Azolla* taxonomy, particularly in the section Euazolla and also elucidate the genetic diversity of the host, *Azolla* and its algal symbiont, *Anabaena azollae* (Kannaiyan and Kumar 2005).

Distribution of *Azolla*

Azolla species are found in fresh water ecosystems of temperate and tropical regions throughout the world (Fig.1). An analysis of the distribution of *Azolla* shows that four species of the section Euazolla are originally found in North and South America i.e., (i) *A. filiculoides*, Southern South America to Alaska (ii) *A. caroliniana*, Eastern North America, the Caribbean, Mexico and West Indies (iii) *A. mexicana*, Northern South America to British Columbia, Western North

REFERENCES

Braun-Howland,E and Nierzwicki - Bauer. S. A. 1990. Biochemistry, Physiology, Ultrastructure and Molecular Biology of the *Azolla Anabaena* Symbiosis. In : Handbook of symbiotic cyanobacteria (ed.) A. N. Rai, CRC Press Inc., **Boca Raton.** 65-118.

Caudales, D, Wells, J.M, Antonie, A.D. and Butterfield. J.E. 1995. Fatty acid composition of symbiotic cyanobacteria from different host plant (*Azolla*) species; Evidence for coevolution of host and symbiont. **Int. J. Syst. Bacteriol** 45(1): 364-370.

Coppenolle, B.V, Watanabe, I, Van Hove, C, Second, G, Huang N. and McCouch. S.R. 1993. Genetic diversity and phylogeny analysis of *Azolla* based on DNA amplification by arbitrary primers. **Genome** 36(4): 686-693.

Coppenolle, B.V, McCouch, S. R, Watanabe, I, Huang, N. and Van Hove. C. 1995. Genetic diversity and phylogeny analysis of *Anabaena azollae* based on RFLPs detected in *Azolla – Anabaena azollae* DNA complexes using *nif* gene probes. **Theor. Appl. Genet.** 91 (4): 589-597.

Dunham, D.G. and Fowler, K.1987. Taxonomy and species recognition in *Azolla* Lam. In: ***Azolla*** utilization. Inte. Rice. Res. Inst. Philippiness, pp. 7-16.

Eskew, D.L, Caetano-Anolles, G, Bassam, B. J. and Gresshoff. P.M. 1993. DNA amplification fingerprinting of the *Azolla Anabaena* symbiosis. **Plant Molecular Biology** 21:363-373.

Franche, C. and Cohen-Bazire. G. 1985. The structural *nif* genes of four symbiotic *Anabaena azollae* show a highly conserved physical arrangement. **Plant Science** 39: 125 - 131.

Franche, C. and Cohen-Bazire. G. 1987. Evolutionary divergence in the *nif* H, D, K gene region among nine symbiotic *Anabaena azollae* and between *Anabaena azollae* and some free-living heterocystous cyanobacteria. **Symbiosis** 3: 159-178.

shared identical and highly specific antigens. Antibodies made against free-living *A. azollae* did not cross–react with any of the symbiotic *A. azollae* indicating either these isolates are not true isolates or their antigenic properties were altered during isolation and culturing (Ladha and Watanabe, 1982).

In China, 13 hybridoma cell lines secreting monoclonal antibodies of *A. azollae* isolates have been established (Liu *et al.,* 1989). They have concluded that there are at least four subgroups of *A. azollae* in *Azolla* species. Fluorescent antibody staining indicated differences in surface antigenicity in *A. azollae* cells fresh from the leaf cavities of the fern *Azolla caroliniana* and algae which were isolated and subcultured from this fern (Gates *et al*., 1980). These results suggested that either changes in antigenicity occur in this symbiont during culturing or that isolation selects for an antigenically different mutant strains capable of *in vitro* growth. The presumptive symbionts from *Azolla* species based on the morphology, lectin binding and enzyme electrophoresis indicated that culturable cyanobacteria from *Azolla* might be either *Anabaena* or *Nostoc*.

Conclusion

In nitrogen-fixing symbiotic system, host has got recognition factor and therefore, specific compatible microbe docks to the host in a way to build up symbiotic phenomenon. Gene(s) and corresponding protein products get shared by both macrosymbiont and microsymbiont for establishing symbiotic association and carrying out nitrogen fixation. *Azolla – Anabaena* symbiosis is although well known, we still do not have a clear understanding on whether the system works in the same manner with similar biochemical and genetic activities as in higher plants, and why only specific *Anabaena* imparts, or shares opportunities for symbiosis.

The major drawback with the utilization of *Anabaena- Azolla* symbiotic system as biofertilizer for rice is the difficulty in distinguishing strains. Biochemical and molecular diversity allowed the differentiation of strains of cyanobacteria and served as useful taxonomic markers to infer phylogenetic relationships and to synthesize new combination of *Azolla* and *Anabaena azollae* strains. More information on the diversity of the symbionts should further our understanding of the intricate interactions of the association. The potential benefits of the system can be further improved by manipulating the nitrogen fixing ability of the algal symbiont in this agronomically useful association.

Fig. 1. World map showing the occurrence and distribution of *Azolla* species

America and Eastward to Illinois (iv) *A. microphylla*, Western and Northern South America to North America and the West Indies. The section Rhizosperma covers only two species and is distributed as follows: (i) A. *pinnata*, Tropical Africa and Southern Africa, South East Asia, Japan and Australia and (ii) *A. nilotica*, Central Africa, upper Nile Sudan, Uganda, Tanzania, Congo and Namibia.

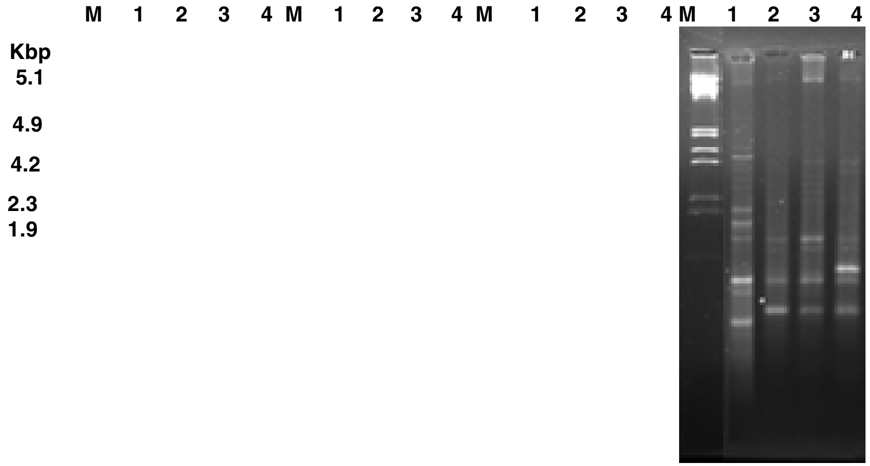
A. filiculoides was formerly native to Europe but disappeared subsequently. In the 19th century, it was reintroduced into Western Europe along with *A. caroliniana* and *A. pinnata*. The species *A. pinnata* has two morphologically distinct forms *viz., A. pinnata* var. *pinnata* and *A. pinnata* var. *imbricata*. The two forms have a basically different distribution pattern, which are disturbed by human interference. The highly imbricate forms show a clear disjointed distribution by their occurrence on the West Coast of Africa and Madagascar and in Australia, whereas the *A. pinnata* forms occur in a conjoined area in Southeast Asia from India to Japan and from Indonesia to Papua, New Guinea.

Survey of geographical distribution of the extant *Azolla* species, along with a map of their world distribution shows that human activity has significantly altered the original species distribution. *A. filiculoides* is now found in Europe (where it may have been indigenous prior to the last Ice Age) Asia and Australia and *A. caroliniana* occurs in Asia, South America and Europe. Human activity appears to have had less effect on the distribution of *A. mexicana, A. microphylla* and *A. pinnata* and no effect on the distribution of *A. nilotica. A.microphylla,* a strain resistant to temperature and salinity with profuse sporulation character, was introduced from Davis, California to Tamil Nadu Province of India which later spread across India.

Morphological characters of *Azolla*

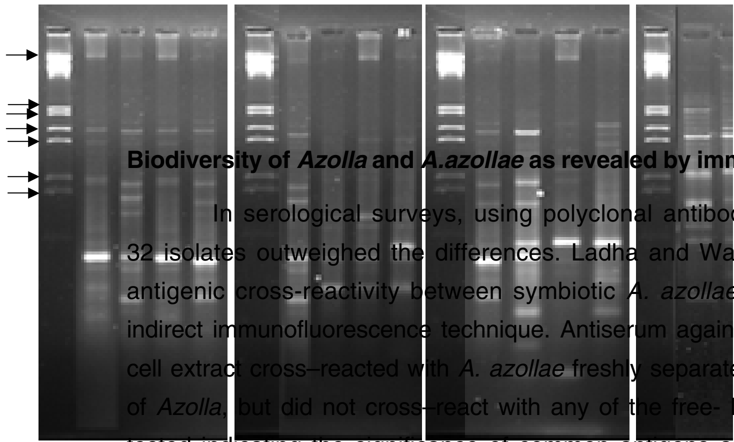
The diploid, spore – producing generation of *Azolla* is called sporophyte, which consists of a horizontal to vertical main rhizome, multibranched, prostrate, floating stems that bear deeply bilobed leaves and determinant, adventitious roots (Plates1 to 5). The extensive branching pattern results in numerous stem apices and a growth habit that ranges from flabellate to polygonal, depending upon the degree and pattern of fragmentation. The presence of abscission layer at the point of root and branch attachment facilitates vegetative propagation through fragmentation.

Plate 8. RAPD profile of *Anabaena azollae* strains using random primers



M-Marker 1 - *A. azollae*-RS-KK-AF 3 - *A. azollae*-RS-KK-TH
2 - *A. azollae*-RS-KK-AM 4 - *A. azollae*-RS-KK-RP

Primer 1 5' CGAGCTG 3' Primer 2 5' GTAACCC 3' Primer 3 5' CCTGGAGG 3' Primer 4 (OPC 11) 5' AAAGCTGCGG 3'



Biodiversity of *Azolla* and *A.azollae* as revealed by immunological studies

In serological surveys, using polyclonal antibodies, similarities among 32 isolates outweighed the differences. Ladha and Watanabe (1982) detected antigenic cross-reactivity between symbiotic *A. azollae* and its *Azolla* host by indirect immunofluorescence technique. Antiserum against *Anabaena*-free *Azolla* cell extract cross–reacted with *A. azollae* freshly separated from different species of *Azolla*, but did not cross–react with any of the free- living *Anabaena* species tested indicating the significance of common antigens and lectin in the *Azolla* – *Anabaena* symbiosis. *Azolla* fern lectins specifically recognize the endosymbiotic cyanobacteria. *A. pinnata* and *A. filiculoides* lectins agglutinated their symbionts and exhibited specificity. Neither lectin reacted with cultured symbionts from either fern species. Direct fluorescent antibody studies against symbiotic *Anabaena azollae* indicated that all the *A. azollae* isolates from 32 specimens of *Azolla*

Nierwicki-Bauer and Haselkorn (1986) studied the differences in mRNA level in *Anabaena* living freely or in symbiotic association with *Azolla* by northern hybridization. The genetic diversity and specificity of symbiotic cyanobacteria in some lichens have also been studied using tRNA *Leu* as a marker to demonstrate the sequence variation in symbiotic cyanobacterial cultures. In the phylogenetic analysis, the cyanobacterial-lichen sequences grouped together with the sequences from free- living *Nostoc* strains. DNA amplification fingerprinting could be a useful tool to study the phylogenetic relationship among the *A*. a*zolla*e isolates and confirmed the inheritance pattern of algal symbionts in a sexual hybrid of *Azolla*.

The RAPD profile of *Anabaena azollae* strains isolated from four different *Azolla* cultures was studied using four different primers. The results revealed that the isolates of *A*. *azollae* generated fingerprinting pattern characteristic of each isolate. Clear polymorphism as well as maternal inheritance of genes was observed among all the strains and this largely depends on the primer sequence. The results distinguished the different strains of *Anabaena azollae* among symbiotic cyanobacteria associated with *Azolla* and indicated the genetic diversity among the isolated strains (Plate 8).

Twenty-two isolates of *Anabaena azollae* derived from 7 *Azolla* species from various geographic and ecological sources were characterized by DNA-DNA hybridization. Each of the symbiont was identified as a unique genotype and it was found that the dominant symbiotic organism in association with *Azolla* sp. was more closely related to *Nostoc* spp. than to free–living *Anabaena* spp. (Plazinski *et al*., 1990).

The fingerprinting pattern is only an indication that the sequence used as primer is present in the genome. DAF offers several advantages over isozyme and RFLP analysis for identification of accessions of the *Azolla-Anabaena* symbiosis. Isozyme analysis requires live healthy cultures and that the identity of these cultures is not beyond doubt. RFLP work requires 1000 fold larger amounts of DNA, much more preliminary work to identify specific probes and the use of radioisotopes for optimum sensitivity. In contrast, DNA preparations for DAF can be stored indefinitely and with little preliminary work it is possible to differentiate *Anabaena* strains.

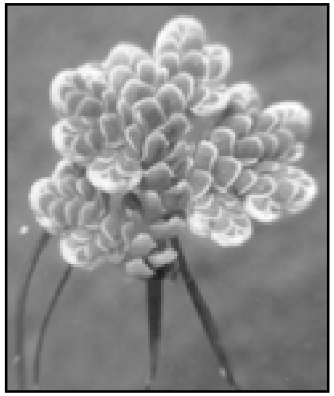


Plate 1. Azolla plant with roots

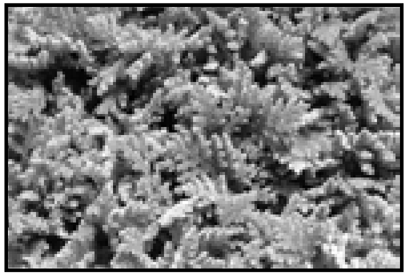


Plate 2. *Azolla filiculoides*

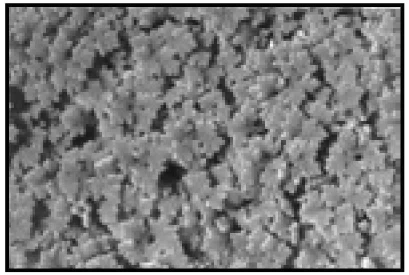


Plate 3. *Azolla microphylla*

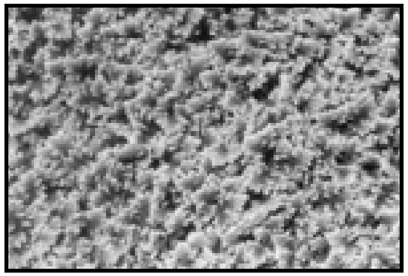


Plate 4. Azolla hybrid – Rong Ping

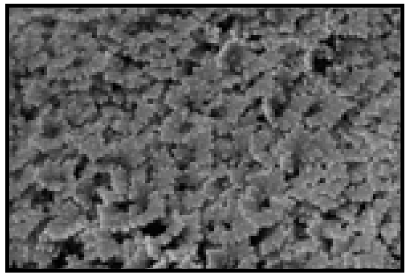


Plate 5. Azolla hybrid – TNAU 1

Leaves on the rhizome are bilobed with alternate pinnation. Rhizome also bears individual roots or root bundles at branch points. The main rhizome when mature may range in size from 0.5 to 7 cm in diameter with individual roots 1 to 5 cm long except *A. nilotica* which produces a trailing rhizome upto 40 cm or more long with root bundles upto 15 cm or more.

The main rhizome is usually achlorophyllous and has alternating branches with several orders of lateral branches. The species that can mature into a nearly vertical morphology when crowded, such as *A. filiculoides* and *A. nilotica,* have a more highly developed vascular system. Well developed plants of *A. nilotica* have branches, which often lack their own roots and are thus dependent on the root system of the main rhizome. The rhizome lacks stomata but occasionally has chloroplasts near the shoot tips. In section, Rhizosperma, the rhizome has trichomes either on the ventral surface or on both the surfaces while plants in section Euazolla lack trichomes on their rhizomes. The roots are initiated at branch points along the rhizome and bear root hairs upto 1 cm long, which emerge from under a root cap. Root initials eventually give rise to a single layered sheath over a two layer cap, followed by an epidermal layer and two layers of cortical cells containing chloroplasts. The occurrence of nitrogen fixing *Azotobacter*, *Azospirillum* and phosphobacteria on the roots of *A. filiculoides* has been reported.

The leaf consists of two lobes, (i) a thick aerial dorsal lobe and (ii) a thin ventral lobe occasionally of a slightly larger size. The dorsal lobe is chlorophyllous, except in the transparent margin and contains a colony of *Anabaena* within a basal cavity connected to the atmosphere by a pore on the adaxial side. The surface of the dorsal lobe has an epidermis covered with vertical rows of single celled stomata and trichomes of one or more cells. The dorsal lobe tissue consists of a single layer of elongated palisade parenchyma with prominent intercellular spaces, one or more layers of sparsely branched spongy mesophyll and a single concentric vascular bundle.

The thin ventral lobe is nearly achlorophyllous with few stomata and trichomes and several chambers. A single vascular bundle consists of several tracheary and sieve elements, but lacks parenchyma cells. The ventral lobe provides buoyancy as a result of its convex surface touching the water. It may also function in absorption, since *Azolla* plants are known to survive without roots. The dorsal leaf lobes of *Azolla* possess a cavity for housing the algal symbiont *Anabaena azollae* (Plates 6&7). As the leaf primordium differentiates at the growing point, a slight depression is formed near the base on the adaxial side of the

clearly indicated that DAF should help to accelerate progress in improving this agronomically important symbiosis.

During the past 10 years, several research groups working on the process of nitrogen fixation and its regulation have isolated nitrogenase of *nif* genes from nitrogen – fixation organisms, including *Anabaena*. Reports indicated that these genes were organized in clustered array and that similar physical arrangement of the genes existed in free-living *Anabaena* and other N_2- fixing cyanobacteria like *Nostoc*. Evolutionary studies were found to be consistent with those based on 16S rRNA sequences. It is now recognized that *nif* genes are also useful in studying bacterial taxonomy. In one experiment, cloned *nif* genes from free-living *Anabaena* PCC 7120 were used as RFLP probes on cyanobacterial DNA extracted from isolates of different *Azolla* species. The resulting grouping of symbionts showed a certain degree of consistency with the actual *Azolla* taxonomic framework (Franche and Cohen – Bazire, 1987, Plazinski *et al.*, 1988).

In a study conducted by Konde (2000), a total of 282 RAPD markers were scored at an average of 47 markers per primer. The phylogenetic relationship was evident from the dendrogram developed by using Nei's genetic distances among the cyanobacterial cultures. The results indicated three groups of cyanobacterial cultures. First group consists of symbiotic *A. azollae* isolates, second group comprised of free – living *Nostoc muscorum* (DOH), *A. variabilis* (SA0) and *Oscillatoria* sp. (Kew-SK), whereas *Westiellopsis* sp. (GG-SK) was placed in third group of dendrogram generated (Fig 7). Interestingly, the results also demonstrated the usefulness of RAPD studies in cyanobacteria to differentiate the symbiotic *Anabaena* isolates from free–living cyanobacterial cultures.

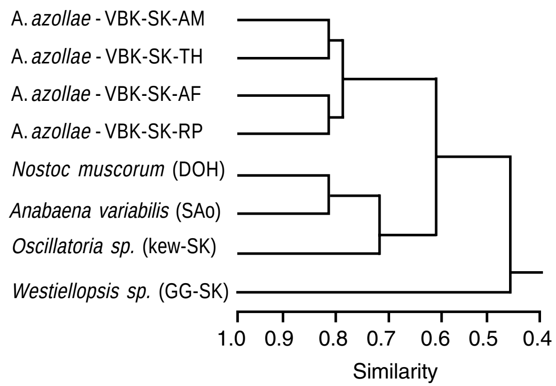


Fig 7. Phylogenetic relationship between *A. azollae* and free- living cyanobacterial cultures

DNA sequences of a fragment of *nif* H from diverse cyanobacteria were amplified, cloned and sequenced. The analysis showed that the *nif* H sequences of filamentous non-heterocystous cyanobacteria were not closely related and that the phylogeny of *nif* H is largely consistent with the phylogeny of 16S rRNA and is used to identify uncultivated N_2 fixing cyanobacteria. Plazinski *et al.* (1990) characterized 22 isolates of *A.* a*zolla*e derived from seven *Azolla* species from various geographical and ecological sources by DNA – DNA hybridization. Cloned DNA fragments derived from the genomic sequences of three different *A. azollae* isolates were used to detect RFLP among all symbiont *Anabaenas*. These cloned genomic DNA probes identified 11 different genotypes of *A. azollae* isolates. The data also indicated that the dominant symbiotic organism in association with *Azolla* spp. is more closely related to *Nostoc* spp. than to free – living *Anabaena* spp.

Gebhardt and Nierzwicki- Bauer (1991) have isolated and cultured symbiotically associated cyanobacteria from *A. mexicana* and *A. pinnata* in a free-living state. Morphological analysis revealed differences between the free-living isolates and their symbiotic counterparts, as did RFLP analysis with both single copy *gln* A and *rbe* S probes and a multicopy *psb* A gene probe. The results suggested the ubiquitous presence of a culturable minor cyanobacterial symbiont in at least three species of *Azolla*. The nitrogen fixation (*nif*) gene cluster from *Anabaena azollae* showed that the closely related cyanobacteria have highly variable but structured intergenic regions.

The genetic diversity of *Anabaena* strains symbiotically associated with the aquatic fern *Azolla* and evolution of relationships among these symbionts were evaluated by means of RFLP using *nif* gene probes cloned from the free-living cyanobacterium *Anabaena* PCC 7120. The results revealed three groups of cyanobacteria associated with *Azolla* species from different sections (Coppenolle *et al*., 1995). They have also suggested that total DNA extracted from *Azolla-Anabaena* complexes can be used equally for RFLP analysis of host plant or symbiotic cyanobacteria.

The findings indicated that the various primer sets have different degrees of resolution, and in order to draw a more specific conclusion about diversity or similarity among closely related isolates, different primers have to be included in the PCR analysis (Rasmussen and Svenning, 1998). A greater number of *Azolla* – *Anabaena* accessions will have to be examined and more primers screened, before the full potential of the DAF technique can be assessed. The current results

dorsal lobe. A portion of the *Anabaena* colony generating at the shoot apex above the dorsal lobe primordia is scooped into the enlarging depression by a glove shaped transfer hair. The colony becomes entrapped in the cavity by a ring of meristematic epidermal cells originating around the circumference of the depression. The cells grow inward to cover the depression, leaving a pore over the center where they meet. The interior of the cavity is lined by a porous envelope, which develops only if the symbiont is present

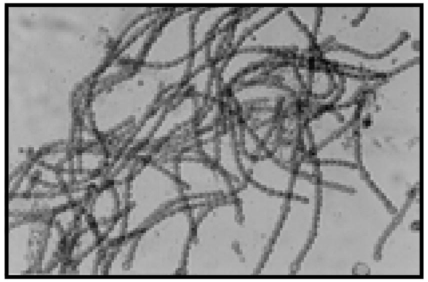


Plate 6. Microscopic view of ***Anabaena azollae***

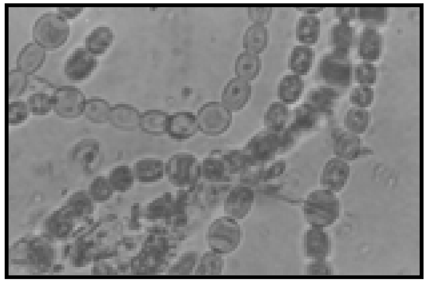


Plate 7. Close view of the vegetative and heterocyst cells of ***Anabaena azollae***

During sporogenesis, the endosymbiont is packaged in sporocarps such that continuity of the symbiosis is maintained during sexual reproduction also. The ultra structural changes in the vegetative cells and heterocysts during the germination of megaspore of *A. microphylla* and during heterocyst formation of endosymbiont have been studied in detail (Neirzwicki- Bauer and Kannaiyan, 1990)

Varieties of certain species such as *A. filiculoides* and *A. nilotica* become fertile only after attaining a mature morphology; however, mature morphology is only a precondition but not necessarily concurrent with fertility in these species. Like fertility, initiation of the mature morphology is also environmentally dependent. The failure to initiate mature morphology has a positive agronomic value, because multilayered immature fronds have a higher nitrogen content and lower lignin content and thus decompose more easily than do matured fronds.

Taxonomy

Azolla belongs to the order Salviniales and is grouped with the genus *Salvinia*. The genus *Azolla* was originally included in the Salviniaceae, a family of heterosporous free-floating ferns but it is separated into the monotypic family Azollaceae. Part of the reason for grouping *Azolla* with *Salvinia* is that both genera produce two distinct types of spores (heterospory), in contrast to most other ferns producing only one type of spore. In addition, the spores of *Azolla* and *Salvinia* are borne on special stalks and contained in special capsules called sporocarps.

Six extant species of *Azolla* and 25 fossil species have been recorded. The genus is divided into two subgenera *viz*., Euazolla, a new word *Azolla* (3 floats) and Rhizosperma (9 floats), primarily based on the reproductive organs such as megaspore floats and glochidia (Fig.2).

Division	**:**	**Pteridophyta**
Class	**:**	**Filicopsida**
Order	**:**	**Salvinales**
Family	**:**	**Azollaceae**
Genus	**:**	***Azolla***

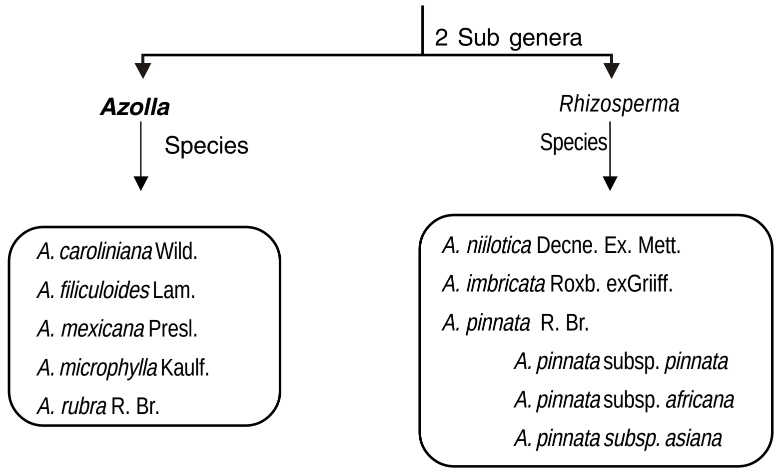


Fig 2. Outline classification of *Azolla*

The genome size of 128 strains of cyanobacteria representing all major taxonomic group lies in the range of 1.6 x 10^9 – 8.6 x 10^9 d. The majority of unicellular cyanobacteria contain genomes of 1.6 x 10^9 – 2.7 x 10^9 d, whereas filamentous strains possessed larger genomes. Revisions were designed to permit generic identification of cyanobacterial cultures on the basis of a comparative study of 178 strains. The genera were placed in different sections, each distinguished by particular patterns of structure and development and reference strains were proposed for each genus. Filamentous organisms possessed large genomes and the heterocyst–forming strains showed a wider variation in genome size (3.2 x 10^9 – 8.6 x 10^9 d) than any other group, accompanied by extensive variation in morphological complexity and physiological properties. It has also been reported that the genome size of *Anabaena* is around 3.2 x 10^9 to 3.9 x 10^9 d and that the DNA base composition is 38 – 44 mol % G + C.

A detailed study of sequence divergence based on such hybridization studies with leaf cavity microsymbionts of 4 species from the sub-section Euazolla and 5 strains of *A. pinnata* from section Rhizosperma, indicated that *Anabaena azollae* associated with different *Azolla* species belong to a common ancester, owing to a great internal similarity, yet these show slightly divergent evolutionary lines (Franche and Cohen-Bazire, 1985).

Plazinski *et al*. (1988) showed that the cyanobacterial partner in different *Azolla* sp. was not uniform throughout and that substantial diversification had occurred. They reported that it would be possible to characterize genotypes of the cyanobacterium using DNA-DNA hybridization techniques and to monitor experiments aimed at synthesizing new combinations of *Azolla* species and *Anabaena azollae* strains.

Franche and Cohen- Bazire (1985) reported that the arrangement of *A. azollae* and the free-living *Anabaena* sp. PCC 7120 *nif* H, D, K genes appeared similar, with *nif* H and D linked to *nif* K some distance away from *nif* D. They also reported that *A. azollae nif* HDK genes appear strongly conserved among the *Azolla* species regardless of the geographical origin of the ferns.

Eskew *et al*. (1993) worked on the DNA amplification fingerprinting of the *Azolla*- *Anabaena* symbiotic system and showed that the contribution of *Anabaena* sequences to the fingerprint of the intact symbiotic system ranged from 0.77% depending on the primer sequence. They also reported that fingerprints of *Anabaena* strains were used to confirm the maternal pattern of transmission of *Anabaena* in a sexual hybrid.

The similarity index values between the *Azolla* cultures indicated that the *A. microphylla* and *A. filiculoides* cultures are distantly related, whereas the *Azolla* hybrids recorded intermediate similarity indices. The dendrogram clearly indicated the grouping of *Azolla* in three clusters *viz*., *A. filiculoides* cultures, *Azolla* hybrids and *A. microphylla* cultures (Fig 6). *A. filiculoides* formed a separate species, which had only 45 per cent genetic similarity with rest of the *Azolla* cultures (Konde, 2000).

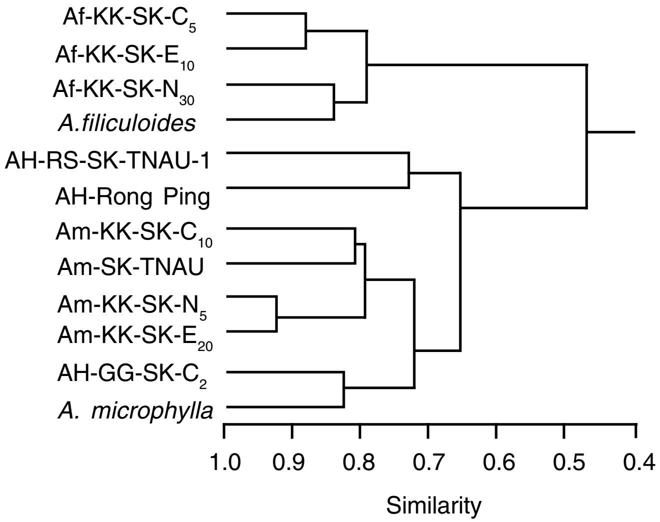


Fig 6. Phylogenetic relationship between the Azolla cultures

Genetic diversity of *Anabaena azollae*

*Anabaena azolla*e is the only species, which has been mentioned to have symbiotic association with *Azolla* sp. There is very little evidence to corroborate that there are different strains of *A. azolla*e, since the isolates obtained from different fern species are morphologically similar.

The *Azolla* – *Anabaena* symbiotic system has been used for centuries as a biofertilizer for enriching the nitrogen nutrition in rice paddies. Genetic improvement of the symbionts has been limited by the difficulty in identifying *Azolla* – *Anabaena* accessions and *Anabaena azollae* strains. Sexual crossing of *Azolla* and cross inoculation of microsymbionts has shown that it is possible to improve the performance of the symbiosis.

The glochidia of the species belonging to Euazolla (*A. filiculoides* Lamarck, *A. caroliniana* Wild, *A. microphylla* Kaulfuss and *A.mexicana* Presl.) are septate, while those of the *Rhizosperma* are simple (*A. pinnata* R. Brown) and absent in *A. nilotica.* The use of septa in glochidia as a distinguishing characteristic was questioned, because of the presence of extensive morphological variation within a given species

The location of glochidia on the massulae can also be a distinguishing feature and in the subgenus, Rhizosperma, the glochidia are replaced by a root like structure emerging from the massulae in the microsporangium. In *A. nilotica* neither the glochidia nor the root like structure is present in the massulae. The species belonging to the Euazolla (*A. filiculoides, A. caroliniana, A. microphylla* and *A. mexicana*) have glochidia positioned on the entire surface of the massulae, while those of the Rhizosperma are located on the inner surface of *A. pinnata* and are absent in *A. nilotica*.

Although the fossil record of the genus dates back to the late Cretaceous period, the stratigraphic range of individual species is relatively short. The oldest living species are *A. filiculoides* of Euazolla and *A. pinnata* of Rhizosperma, which belong to the Pleistocene era. There has been long confusion about the classification of *Azolla* species, For example some *A. filiculoides* varieties were named as *A. rubra* and *A. japonica* and some *A. pinnata* varieties as *A. africana* and *A. quinensis* .In several cases, a specific epithet was changed to a varietal name such as *A. filiculoides* var. *rubra* and *A. pinnata* var *imbricata*.

The nature of the taxonomic confusion in section *Azolla* is summarized (Table 1) by Dunham and Fowler (1987). Early rationalization of described species recognized only *A. microphylla*. *A. caroliniana*, *A. cristat*, *A. magellanica* and *A. rubra*. Dunham and Fowler (1987) later combined *A. microphylla, A. caroliniana* and *A. cristata* into *A. caroliniana*, at the same time placed *A. magellanica* and *A. rubra* into *A. filiculoides* apparently disregarding reestablished *A. microphylla*, *A. mexicana*, *A. caroliniana* and *A. filiculoides* as distinct species.

Table 1. Taxonomy of *Azolla* and current proposals (Dunham and Fowler, 1987)

Original name and author	Mettenius 1847	Mettenius 1867	Svenson 1944	Present proposal
A. portoricensis	*A. portoricensis*	-	*A. microphylla*	*A.* sp
A. microphylla	*A. microphylla*	-	-	-
A. bonariensis	*A. bonariensis*	-	-	-
A.mexicana	*A.mexicana*	-	*A.mexicana*	*A.mexicana*
A. caroliniana	*A. caroliniana*	*A. microphylla*	-	-
A.densa Desvx,	*A.densa*	*A. caroliniana*	-	-
		A. cristata	A. *caroliniana*	
A. cristata	*A. cristata*	-	-	-
A. arubuscula	*A. arubuscula*	-	-	*A. caroliniana*
A. megellanica	*A. megellanica*	*A. filiculoides*	*A. filiculoides*	*A. filiculoides*
A. filiculoides	*A. filiculoides*	*A. rubra*	-	*A. rubra*
A. rubra	*A. rubra*	-	-	*Azolla* sp

A. caroliniana Sensu is considered equivalent to *A. mexicana*. *A. microphylla* and *A. caroliniana* Sensu, later being redefined from vegetative features and glochidial septation, the megaspore apparatus not even having been found (Dunham and Fowler, 1987).

Tentative taxonomic proposals

Dunham and Fowler (1987) have proposed tentative taxonomic conclusions based on critical assessment of vegetative and reproductive characters using light microscope, thin sectioning, scanning electron microscopy (SEM) and some transmission electron microscopy (TEM). They found that apart from leaf trichomes and possibly root anatomy, vegetative features provide little assistance in taxonomic separation. They conformed that, despite variations

The free-living *A. variabilis* was found to be more resistant than *A. azollae* to most of the different antibiotics but were highly sensitive to erythromycin and streptomycin (Subhashini *et al.,* 2003).

Molecular Taxonomy

Use of DNA-DNA hybridization, DNA amplification fingerprinting (Eskew *et al*., 1993) and monoclonal antibodies show that the cyanobacterial partner is not uniform throughout the genus *Azolla* and seems promising for strain identification (Plazinski *et al*., 1988).

Genetic diversity of *Azolla*

Most accessions of *Azolla* never become fertile under maintenance conditions of the germplasm collections. The identity of any accession may also become suspect after entering a germplasm collection because of cross – contamination with other accessions. A novel tool for DNA fingerprinting of *Azolla* accessions has been proposed .The strategy is based on amplification of characteristic DNA fragments by a thermostable DNA polymerase directed by a single oligonucleotide primer of an arbitrary sequence in a thermocycling reaction. The DNA amplification fingerprinting (DAF) used very short primers (>5 nucleotides in length) to generate complex fingerprint patterns, when amplification products are separated by polyacrylamide gel electrophoresis and detected by staining. The three neotropical species of section *Azolla*, *A. caroliniana, A. mexicana* and *A. microphylla* were very similar in their DNA fingerprints (Zimmerman *et al*., 1991).

Coppennolle *et al.* (1993) demonstrated the use of RAPDs to study phylogenetic relationships among *Azolla* accessions by using random arbitrary primers of 10 nucleotide in length with 70 per cent G + C content. They found a high frequency of polymorphism among accessions and species. This information was used to construct a dendrogram and a principal component analysis (PCA) representation, which indicated that the genus *Azolla* contains three distinct groups: group 1 contains the entire section Euazolla; group 2 contains a cluster of *A. pinnata* species and group 3 contains a cluster of *A. nilotica* species. Kim *et al.* (1997) have generated fingerprints of cyanobionts and its hosts *Azolla* by using specific primers for the chloroplast-encoded intron of the tRNA- leucine (VAA) gene. The restriction fragment length polymorphism (RFLP) of the amplified 16S rRNA gene and random amplified polymorphic DNA (RAPD) demonstrated the capacity of this method for the rapid assessment of similarties among the symbionts and its hosts.

Variation in antibiotic resistance of *Anabaena azollae* isolates

Cyanobacteria generally show a high degree of resistance to a variety of antibiotics. Use of different antibiotics at different concentrations on cyanobacteria serve as efficient markers for strain differentiation and selection of antibiotic resistant strains of same species. The gene symbols, *amp*^{r} (ampicillin or penicillin resistance), *ery*^{r} (erythromycin resistance), *kan*^{r} (kanamycin resistance), *pmr*^{r} (polymyxin resistance), *rif*^{r} (rifampicin resistance) and *str*^{r} (streptomycin resistance) serve as chromosomal genetic markers of cyanobacteria.

Antibiotic resistance genes that are expressed well in cyanobacteria even in low copy have been incorporated into mobilizable vectors. One such gene is *npt* from Tn5, encoding amino glycoside 3'-phosphotransferase and conferring resistance to kanamycin and neomycin. Other antibiotic resistance genes that function well in *Anabaena* sp. strain PCC 7120 are the Ω fragment, that contains *add* A gene conferring resistance to streptomycin and spectinomycin under the control of *psb* A promotor. Erythromycin resistance (*erm*, combined with a *cat* gene encoding chloramphenicol acetyl transferase and conferring resistance to chloramphenicol for selection in *E.coli*) with its native promoter have been used primarily for selection of replicating shuttle vectors in *Anabaena* sp. strains PCC 7120 although it functions well in chromosome.

The study on the response of *Anabaena azollae* to different concentrations of the antibiotic penicillin showed that at higher concentrations (200 mg / ml), the organism synthesized additional proteins, while the inhibitory effect of the antibiotic on total proteins was only marginal. There was a marked reduction in the total nucleic acid at 200 mg/ml of penicillin, which was attributed to the synthesis of additional proteins to withstand the stress created upon exposure of the organisms to higher concentration of the antibiotic.

Cultures of the algal symbiont, *Anabaena azollae*, were isolated from four different *Azolla*- species *viz., A. filiculoides, A. microphylla, Azolla* hybrids *viz*., TNAU and Rongping and designated as *Anabaena azollae* - AF, *Anabaena azollae* - AM, *Anabaena azollae* - TH *Anabaena azollae* – RP respectively. The algal symbionts were characterized for their intrinsic resistance to antibiotics and biochemical constituents. *A.azollae*-AF and *A.azollae*-RP showed almost identical pattern of resistance to most of the antibiotics tested, while *A.azollae*-AM showed different pattern. *A.azollae*-AM was able to tolerate maximum concentration of the antibiotics, while *A.azollae*-TH showed the least resistance.

observed, features of the megaspore apparatus are the most reliable means of separating taxa within *Azolla*. The six main megaspore types are recognized as belonging to *A. filiculoides, A. mexicana, A. microphylla* and *Azolla* sp. within section Euazolla and *A. pinnata* and *A. nilotica* in section Rhizosperma. Within the *A. filiculoides* megaspore type, atleast two subtypes can be distinguished *viz*., *A. filiculoides* subtype *rubra* confined to Western and South Western Australia and New Zealand and *A. filiculoides* subtype *filiculoides* exhibits a clone of variation possible because of its wide geographic distribution (Dunham and Fowler, 1987). They have proposed to divide *A. filiculoides* into the two subspecies *A. filiculoides* subspecies *rubra* and *A. filiculoides* subspecies *filiculoides*.

Evidence from the investigation of Dunham and Fowler (1987) indicates that *A. caroliniana* can no longer be justifiably regarded as a distinct species, but should be considered synonymous with *A. filiculoides*. They also found that Type Specimen should be placed in synonym with *A. filiculoides* thus rendering the name *A. microphylla* invalid.

The precise identification of the members of section *Azolla* is somewhat complex due to similarity in vegetative characters. But in Rhizosperma, it is easy since members are distinct from each other.

With the hope of resolving the complex taxonomical problems, attention has now been focused on the cytology of the genus. The basic chromosomal number in all species of section Euazolla as well as *A. pinnata* of the section Rhizosperma is n=22, whereas in *A. nilotica*, it is n=26. The chromosomes number clearly separated *A. nilotica* from all other species and indicated that *A. pinnata* might have closer phylogenetic affinities with species of the section Euazolla (Sterigianou and Fowler, 1990). This finding was supported further by DNA probe studies, which indicated that the dominant symbiont in the section Euazolla and *A. pinnata* generally belong to divergent evolutionary lines (Plazinski *et al.,* 1990).

Biochemical diversity of *Azolla* and the algal symbiont

Physiological and genotypic characters like pigment composition, fatty acid analysis, nitrogenase activity, heterotrophic growth, DNA base composition and genome length were studied to find out strain differences. The N content of mucilaginous genera of cyanobacteria is lower than that of non-mucilaginous genera and it varies from 3 to12%. In general, *Anabaena* has higher nitrogen content (6 to 7%) compared with other N-fixing algae and laboratory grown strains recorded lower N than those from natural environments.

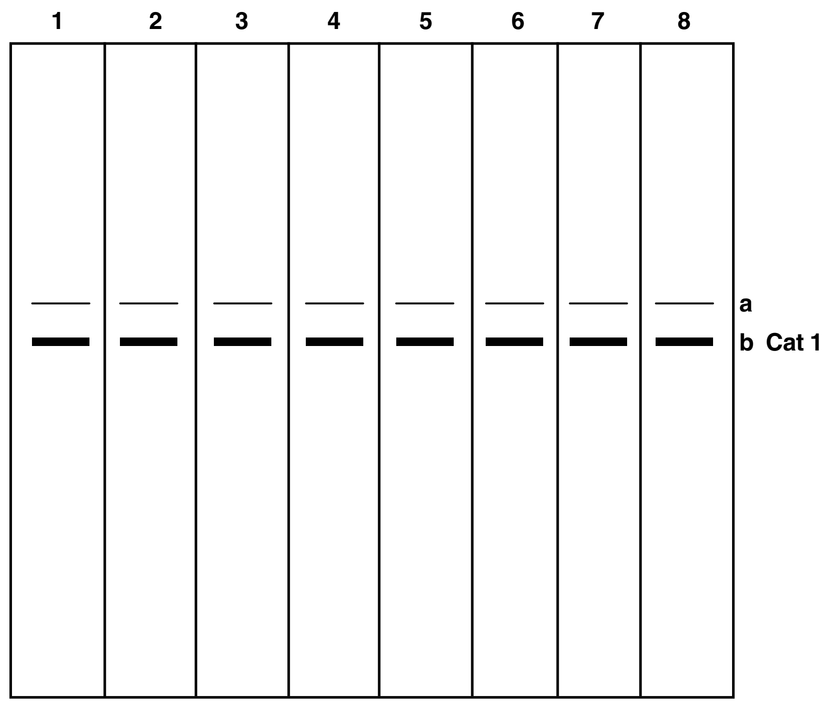
Caudales *et al.* (1995) isolated total cellular fatty acids from 40 cyanobacterial symbionts obtained from seven species of *Azolla* by gas- liquid chromatography- mass spectroscopy. Fatty acid compositions varied among the cyanobacteria depending on the host species. The results suggested that the cyanobacterial symbionts of *Azolla* spp. co-evolved into distinct genetic groups with their hosts. Twenty eight axenic planktonic cyanobacterial strains including *Microcystis, Oscillatoria, Spirulina, Aphanizomenon* and *Anabaena* were analysed for their fatty acid composition by measurement of non-polar and hydroxy fatty acids. The results revealed a better chemical diversity (Chemotaxonomy) among the different cyanobacterial strains.

Environmental stresses like water stress, salinity, frost, high temperature and toxic chemicals elicit cellular and molecular responses in plants and such responses usually lead to alternation of protein constituents. The protein constituents of *A. microphylla,* subjected to stress conditions such as high temperature and mutagenic agents, were analyzed by SDS- PAGE. Prominent heat shock protein bands were detected in *A. microphylla* fronds exposed to 40 ± 1°C. Colchicine mutants of *A. microphylla* recorded higher protein content than wild type cultures of *A. microphylla*.

The mutants of *A. microphylla*- KK-SK-F_{20} and *A. microphylla*- KK-SK-C_{10} showed the presence of a prominent additional protein band, which was absent in wild types. The protein profile of the mutants and wild cultures of *A. filiculoides* and *A. microphylla* by size exclusion chromatography indicated the presence of 3-4 major peaks in the mutants (Kumar and Kannaiyan, 1999). The protein subunits 98 kDa, 180 kDa, 60 kDa and 65 kDa were found to be characteristic of *A. filiculoides, A. microphylla,* and *Azolla* hybrids, *viz*., Rong Ping and AH-RS-SK-TNAU-1, respectively.

The cultures of *Anabaena azollae* exhibited variations in their protein content. A 43kDa protein subunit, found in all the symbiotic *Anabaena* isolates, was absent in free-living cyanobacterial cultures (Konde, 2000). The 32kDa protein, which is encoded by the *psb*A gene, is required for PS II quinone-mediated electron transfer. This protein is made and rapidly turned over in unicellular cyanobacteria and in chloroplasts of higher plants. There is only one copy of the *psb*A gene in plant chloroplast DNA, whereas there are multiple copies in *Anabaena* 7120 and *Anabaena azollae*. It is not known how many of these copies are actually expressed in vegetative cells. Also, although heterocysts reportedly lack PS II reaction centers, it is not known whether they also lack the 32–kDa protein. In the

Fig 5. Zymogram for catalase enzyme of *Azolla* cultures



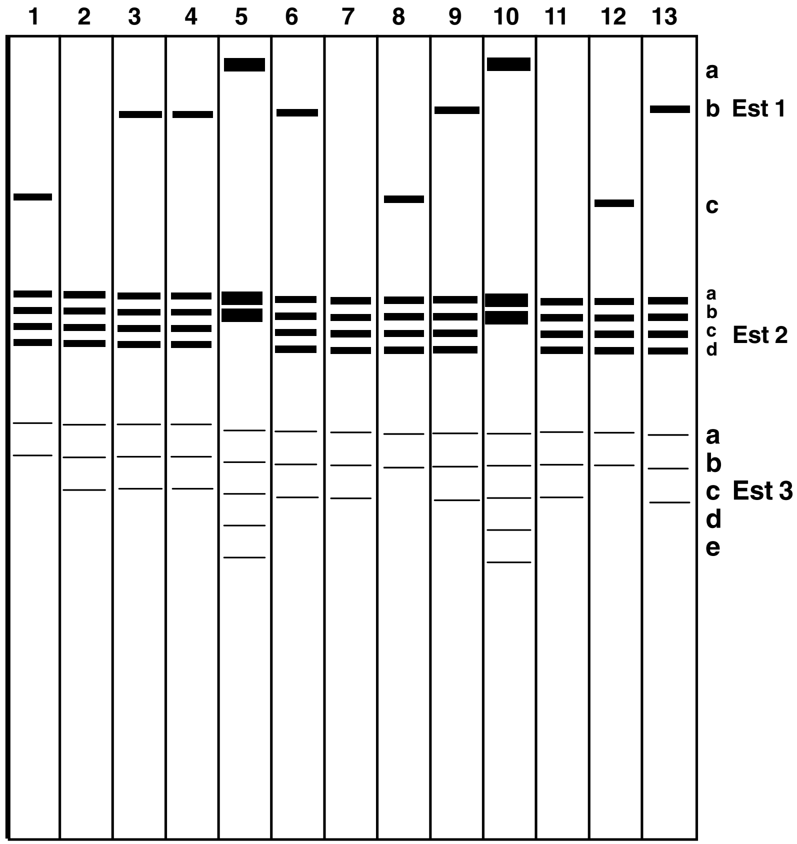
Lanes 1 & 5 - *A. microphylla*

2 & 6 - *A. filiculoides*

3 & 7 - A Hybrid-RS-TNAU-1

4 & 8 - A Hybrid-Rong Ping

Fig 4. Zymogram for esterase enzyme of *Azolla* cultures



Lanes

1,8 and 12 - *A. microphylla*

2,7and 11 - *A. filiculodes*

5 and 10 - A Hybrid-RS-SK-TNAU-1

3,4,6,9 and 13 - A Hybrid-Rong Ping

endosymbiont, the 32-kDa–specific transcripts were approximately seven to ten times more abundant than in free – living*Anabaena*.

The fact that *psb*A expression was so high in the endosymbiont cannot be explained by variations in cell populations (i.e., percentages of heterocysts and vegetative cells). While the only reported function for the 32-kDa proteins is its role in PS II quinone – mediated electron transfer, it seems likely (given the high transcript levels) that this protein has additional functions in the endosymbiont. Consequently, it is now of considerable interest to learn more about the expression and regulation of transcription of the *psb*A genes. It is not known whether different *psb*A copies are selectively expressed under different environmental conditions and whether the 32-KDa protein is present in heterocysts even though they lack an active PS II (Braun- Howland and Nierzwicki- Bauer, 1990).

Isozyme pattern of *Azolla* cultures

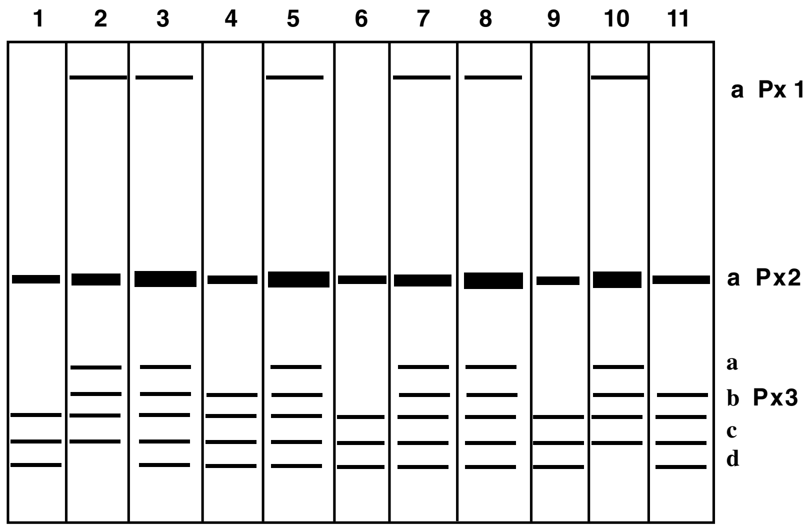
Utilization of isozyme profiles has proved valuable for cultivar identification in many agricultural plants. Correlation between isozyme variation and the geographic origin of plant accessions is also possible (Glaszmann, 1987). Enzyme electrophoresis used by Zimmerman *et al*., (1989) to differentiate sections within the genus *Azolla* Lam demonstrated the value of this method in fingerprinting taxa. Preliminary examination of selected isolates indicated that allozyme diversity was particularly evident in section *Azolla*, where specific classification by morphological means is difficult. Zimmerman *et al*. (1989) also fingerprinted and classified the *Azolla* accessions from the germplasm collections of the International Rice Research Institute and Washington State University by enzyme electrophoresis and leaf trichome morphology. The results of the isozymes and RFLP analysis also indicated that *A. microphylla*, *A. caroliniana* and *A. mexicana* were genetically similar and distinct from other taxa (Zimmerman *et al*., 1991). Classification among neotropical and other species of the aquatic fern *Azolla* Lam was examined using molecular (RFLP) and physiological parameters and demonstrated inbreeding between *A.mexicana*–*A. caroliniana* and *A. microphylla*, which yielded the first fertile *Azolla* hybrids.

Four *Azolla* cultures *viz*., *A. filiculoides*, *A. microphylla*, *Azolla* hybrids *viz*., AH- Rong Ping and AH-RS-SK-TNAU-1 were screened for three isozymes each *viz*., peroxidase, esterase and catalase. The peroxidase and esterase enzymes showed three loci with considerable variation in the mobility and exhibited polymorphic alleles (Fig. 3 & 4). The catalase enzyme showed no variation among the *Azolla* cultures analyzed (Fig.5). The allele Px3/a and Px3/b were common in *A. microphylla* and AH-RS-SK- TNAU-1 and AH- Rong Ping cultures, respectively. In the case of esterase enzyme, the alleles *Est* 3/a and *Est* 3/b were common between *A. filiculoides*, AH- Rong Ping and AH-RS-SK-TNAU-1 cultures. Interestingly, the peroxidase and esterase enzyme banding pattern showed considerable variation among the wild cultures of *A. microphylla* and *A. filiculoides* (Konde, 2000).

Isozyme comparison in different *Azolla* cultures was first attempted using known protocols of gel electrophoresis. Where nine *Azolla* accessions were tested for isozyme pattern, at least 15 loci were observed in 11- enzyme systems. Intraspecifc diversity was depicted using presumptive members of *A. microphylla* for malate dehydrogenase (MDH) enzyme.

In another study, the presence of the enzymes viz., phosphoglucoisomerase (PGI), phosphoglucomutase (PGM) and isocitrate dehydrogenase (IDH) were examined and based upon this study *A. microphylla, A. caroliniana* and *A. mexicana* were closely clustered. *A. filiculoides* was the most easily discernible one of the five species by its zymogram. No appreciable phenetic distances were found between *A.microphylla* and *A.mexicana-A.caroliniana* group, whereas *A. caroliniana* was unrelated to *A. filiculoides*. *A. microphylla* and *A. filiculoides* were identifiable from the others in the banding pattern of certain enzymes.

Fig 3. Zymogram **for peroxidase enzyme of *Azolla* cultures**



Lane 1 and 6 - *A. microphylla*

Lane 2 and 7 - *A. filiculoides*

Lane 4,9 and11 - A Hybrid-Rong Ping

Lane 3,5,8 and 10 - A Hybrid-RS-SK-TNAU-1