

Biodiversity of *Anabaena azollae* isolates from different *Azolla* cultures

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The random amplified polymorphic DNA (RAPD) profile of *A. azollae* strains isolated from four different *Azolla* cultures was studied by using different primers. The objective of this study was to determine whether polymerase chain reaction (PCR) with different primers could differentiate the isolated *A. azollae* strains from one another. The primers amplified specific sequences of the isolates and generated fingerprinting pattern characteristic of each isolate. Clear polymorphism was noticed among all the strains which depends on the primer sequence.

The *Azolla-Anabaena* association has a tremendous potential for providing photosynthetic production of N fertilizer to rice crop¹. *Anabaena azollae* is a symbiont residing in the upper leaf cavity of nitrogen fixing aquatic water fern which plays a key role in contributing nitrogen for rice². Despite the long standing and wide-spread use of this symbiotic association to supply nitrogen in rice soils, very little is known on the symbiont. A major constraint to *Anabaena - Azolla* utilization as a rice biofertilizer is the inability to distinguish strains of the symbiont which is very critical for nitrogen fixation in *Azolla*.

Molecular approach and genetic engineering have provided new tools to identify genetic information in cyanobacteria. The use of molecular methods to study the genotypic relationships is underway and initial results are promising. Use of DNA-DNA hybridization, DNA amplification fingerprinting and monoclonal antibodies revealed that the cyanobacterial partner is not uniform throughout the genus *Azolla* and seems promising for strain identification³.

The cyanobacterial partner in different *Azolla* sp. was not uniform throughout and substantial diversification had occurred. Using DNA-DNA hybridization, it will be possible to characterize genotypes of the cyanobacterium. DNA amplification fingerprinting of the *Azolla -Anabaena* symbiosis showed that the contribution of *Anabaena* sequences to the fingerprint of the intact symbiosis ranged from 0.77% depending on the primer sequence and that the fingerprints of *Anabaena* strains were used to confirm the maternal pattern of transmission of *Anabaena* in a sexual hybrid³. The present study was initiated to identify the variations in the *Anabaena azollae* strains of different *Azolla* cultures using four different

primers. The assay was conducted on genomic DNA isolated from the *Anabaena azollae* strains.

Materials and Methods

Algal symbiont of *Azolla*—Four different *Azolla* cultures viz. *A. filiculoides* Lamarck (Italy), *A. microphylla* Kaulfuss (Galapagos Islands), *Azolla* hybrids, RS-SK-TNAU (*A. microphylla* X *A. pinnata*) and Rongping (*A. microphylla* X *A. filiculoides*) were used, and the algal symbiont *Anabaena azollae* isolated from them were designated as *A. azollae*-RS-KK-SK-AF, *A. azollae*-RS-KK-SK-AM, *A. azollae*-RS-KK-SK-TH and *A. azollae*-RS-KK-SK-RP respectively.

Isolation of genomic DNA—The genomic DNA from the isolated *A. azollae* strains was extracted as per the standard procedure⁴. The extracted DNA samples were quantified using Hoefer DyNA Quant 200 Fluorometer by the standard DNA Quantification method⁵.

Oligonucleotide Primers—Four different oligonucleotide primers were screened, and the primer sequences are presented as below:

Primer	Sequence
1.	5' CGAGCTG 3'
2.	5' GTAACCCC 3'
3.	5' CCTGGAGG 3'
4.	5' AAAGCTGCGG 3'

PCR amplification of genomic DNA—The genomic DNA extracted was tested for intactness by electrophoresis. The quantified DNA was diluted to a concentration of 25 ng μ l⁻¹ and used for the RAPD-PCR studies by following the method described by Saiki *et al*⁶. The reactions were carried out in a total

volume of 15 μ l using 25 ng μ l⁻¹ genomic DNA as template. The PCR cocktail contained 1 μ l template DNA, 1.2 μ l of 2.5 mM dNTPs, 0.6 μ l primer, 1.5 μ l 10 X assay buffer, 0.18 μ l Taq DNA polymerase, 0.18 μ l of 20 mM magnesium chloride and 10.34 μ l sterile distilled water. The reaction mixture was given a momentary spin for thorough mixing and the amplifications were carried out in thermocycler (Perkin-Elmer, GENE AMP 2400). The thermocycler was programmed to run for 35 cycles and the programme consists of initial denaturing of the samples at 94°C for 5 min, denaturing at 94°C for 1 min, annealing at 37°C for 1 min, extension at 72°C for 2 min, final extension at 72°C for 7 min and the samples were held at 4°C.

Analysis of PCR products—PCR amplified product (10 μ l) was electrophoresed on 1.5 % agarose gel stained with 0.5 μ g ml⁻¹ ethidium bromide as described by Sambrook *et al.*⁷. λ Eco RI + Hind III double digest DNA was run as a marker along with the samples. After the run is complete, the gel was viewed in a UV transilluminator and the bands were observed.

Construction of dendrogram—All the electromorphs for different *Anabaena azollae* strains were scored for their presence or absence of bands using binary codes and the similarity coefficients were calculated by following the formula proposed by Nei and Lei⁸. Based on these coefficients, a dendrogram was constructed using a computer software, "NTSYS pc 2.02i".

Results and Discussion

Four different primers were used on the *A. azollae* DNA samples, and in general, the molecular weight of the amplified products ranged from 0.56 kb to 21.2 kb. The results of the bands generated for individual primers are presented in Table 1. High molecular weight polymorphism was noticed between the strains. Different polymorphic fragments of varying molecular weight were formed at different loci for the different primers tested. Among the four primers

tried, number of RAPD markers generated was found to be more for primer No. 4, and the primer No. 1 registered the least number of fragments. More number of polymorphic bands was generated by primer No.3 followed by primer No. 2. Minimum polymorphic bands was observed in RAPD fragments generated with primer No.1

With the primer numbered 3, there was 100 % polymorphism among all the isolates which proved that the algal symbionts are genetically different although there were some common bands among few isolates. Analysing twenty two isolates of *Anabaena azollae* derived from seven *Azolla* species, each of the symbiont was identified as a unique genotype by DNA-DNA hybridization, and in another study, three groups of *A. azollae* strains belonging to different sections of *Azolla* has been identified by RFLP studies⁹. Using the primers numbered 1 and 4, the fingerprints of *A. azollae* strains were almost identical but the same isolates amplified with primers 2 and 3 were unequivocally distinguishable from one another. The variation in banding pattern among isolates reflects possible divergence in DNA sequence.

In case of primer 3, more intense band was observed in *A. azollae*-RS-KK-SK-AM, *A. azollae* - RS-KK-SK-TH and *A. azollae* - RS-KK-SK-RP and this band was not generated in *A. azollae*-RS-KK-SK-AF (Fig.1). This might probably be due to the fact that *A. microphylla*, being the maternal parent of the 2 hybrids, must have shared the common band. This was a critical example of maternal inheritance of DNA fragments to progenies. The same result was demonstrated showing the maternal inheritance of *Azolla microphylla* to its progeny, Rongping hybrid which is also applicable for the algal symbionts, showing their genetic relatedness¹⁰.

In RAPD profile generated with primer 1, common band sharing was observed between *A. azollae*- RS-KK-SK-AM and *A. azollae*- RS-KK-SK-RP fragments of size 1904bp and 564bp. (Fig.1). The presence of these fingerprints in *A. azollae*-RS-KK-SK-RP similar to that in *A. azollae*-RS-KK-SK-AM

Table 1 — Number of RAPD products generated in different *A. azollae* strains for different primers

Primer Number	Sequence	No. polymorphic bands	No. monomorphic bands	Total No. RAPD fragments
1.	5' CGAGCTG 3'	8	12	20
2.	5' GTAACCCC 3'	13	8	21
3.	5'CCTGGAGG 3'	27	—	27
4.	5' AAAGCTGCGG 3'	12	24	36

probably represents a homozygous chromosomal DNA sequence in the maternal parent. The fingerprints obtained with primer 2 showed a new band of 983 nucleotides size in *A. azollae*-RS-KK-SK-RP which was absent in other isolates (Fig.1). This distinguishes it from all other strains and hence can be developed as a marker for this particular

isolate. The fingerprints generated with primer 4, showed a polymorphic fragment of size about 3536bp in *A. azollae*-RS- KK-SK-AM alone which can be developed as a possible marker for this strain (Fig.1).

The similarity coefficient between the four *Anabaena azollae* strains for the random primers are presented in Table 2. The similarity coefficient value

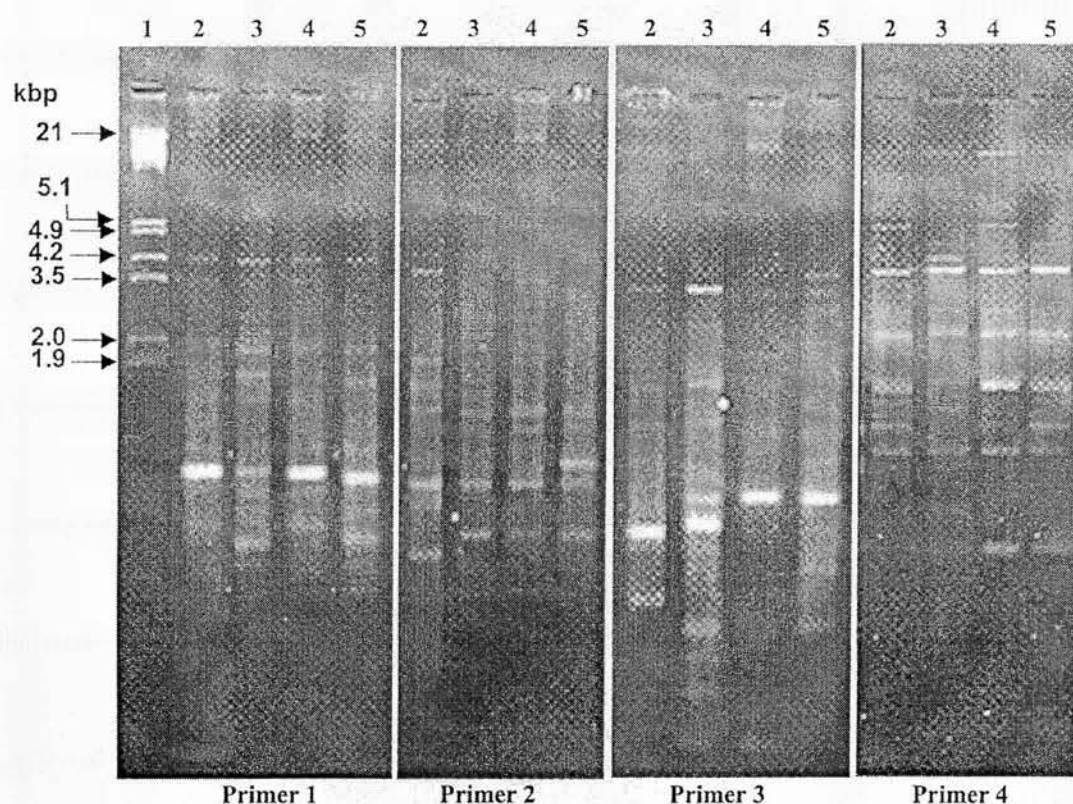


Fig. 1 — RAPD profile of *Anabaena azollae* strains using primer 1-4, Lane 1 — marker; Lane 2 — *A. Azollae*-RS-KK-SK-AF; Lane 3 — *A. Azollae*-RS-KK-SK-AM; Lane 4 — *A. Azollae*-RS-KK-SK-TH and Lane 5 — *A. Azollae*-RS-KK-SK-RP.

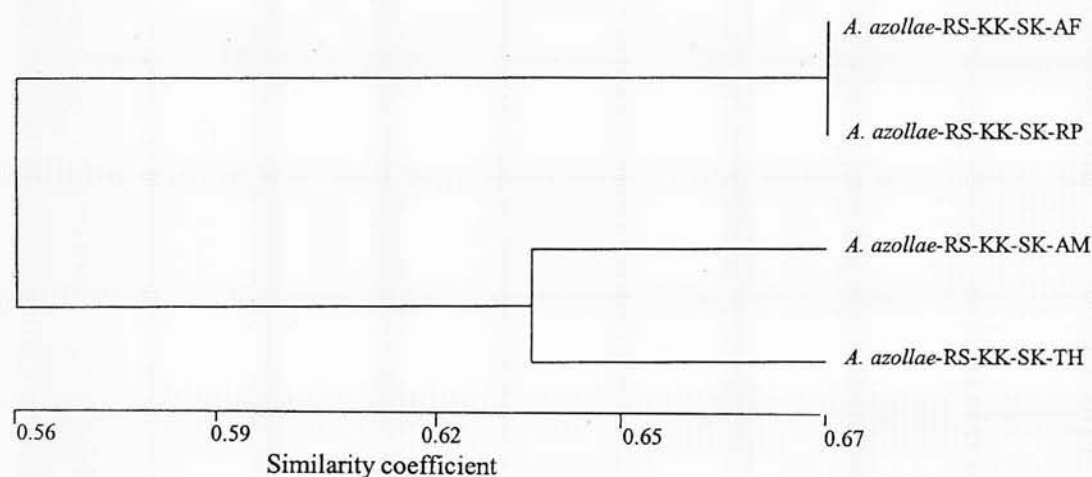


Fig. 2 — Dendrogram of different *A. azollae* strains

Table 2 — Similarity coefficient of different *A. azollae* strains

	C1	C2	C3	C4
C1	1.0000000			
C2	0.5306122	1.0000000		
C3	0.6734694	0.6122449	1.0000000	
C4	0.4897959	0.6326531	0.6122449	1.0000000

C1 - *A. azollae*-RS-KK-SK-AFC2 - *A. azollae*-RS-KK-SK-AMC3 - *A. azollae*-RS-KK-SK-RPC4 - *A. azollae*-RS-KK-SK-TH

between *A. azollae*-RS-KK-SK-AF and *A. azollae*-RS-KK-SK-RP was 0.67 indicating closer relationship between them, whereas *A. azollae*-RS-KK-SK-AM and *A. azollae*-RS-KK-SK-TH shared similarity coefficient of 0.63. The dendrogram (Fig.2) clearly demonstrated the maternal inheritance of *A. azollae*-RS-KK-SK-AF in *A. azollae*-RS-KK-SK-TH and *A. azollae*-RS-KK-SK-AM in *A. azollae*-RS-KK-SK-RP. All the *Anabaena* strains shared similarities at 0.56 similarity coefficient.

The fingerprinting pattern is only an indication that the sequence used as primer is present in the genome. Fingerprinting cyanobionts and hosts of the *Azolla* symbiosis by DNA amplification by RAPD analyses demonstrated the possible role of this method for the rapid assessment of similarities among *A. azollae* and minor *Anabaena* isolates from *Azolla*¹¹. A common cyanobacterial symbiont associated with *A. mexicana*, *A. pinnata* and *A. filiculoides* through molecular and morphological characterization was demonstrated¹².

It has been pointed out 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 analysis¹³. The current results indicated that genetic variation exists among the different *A. azollae* strains of *Azolla*. These studies have clearly indicated that DNA amplification. Fingerprinting (DAF) should help to accelerate progress in improving the potential symbiotic associations of *Azolla* and *Anabaena*.

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