

Phylogenetic analysis of symbiotic and free-living cyanobacterial cultures using DNA amplification fingerprinting

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DNA amplification fingerprinting (DAF) of 17 cyanobacterial isolates belonging to symbiotic and free-living forms of 6 different genera was done. The dendrogram analysis of 17 cyanobacterial cultures revealed three major clusters. All *Westiellopsis* cultures formed the first major cluster and their nucleotide relatedness ranged between 71-93%. In the second major cluster, the symbiotic cyanobacterium *Anabaena* sp dominated and all *Anabaena* cultures showed 60-75% similarity with each other. *Nostoc muscorum* is related to *Anabaena variabilis* by 85% and formed the third major cluster. The dendrogram analysis of cyanobacterial isolates clearly revealed that free-living cyanobacterial cultures were closely related with each other and were diverse from the symbiotic forms.

Keywords: Cyanobacteria, DNA amplification finger printing, Free living cyanobacteria, Phylogenetic analysis, Symbiotic cyanobacteria

Development of modern techniques, especially the polymerase chain reaction (PCR), has made it possible to compare more carefully, the differences between free-living and symbiotic cyanobacteria¹. DNA fingerprinting strategy is based on amplification of characteristic DNA fragments by a thermostable DNA polymerase directed by a single oligonucleotide primer of arbitrary sequence in a thermocycling reaction². DNA amplification fingerprinting (DAF) has been useful for distinguishing closely related and diverse organisms. Nishihara *et al.*³ have used RAPD analysis to discriminate genotypes of *Microcystis*. PCR amplification and sequencing of *rpoC1* (DNA dependent RNA polymerase) gene from 19 isolates of *Cylindrospermum raciborskii* from Australia revealed 99 to 100% nucleotide sequence identity indicating that the isolates belong to one species, despite being morphologically distinct⁴. In the present investigation, an attempt was made to find the phylogenic relation among 17 isolates of symbiotic and free-living cyanobacteria belonging to 6 genera, viz. *Anabaena*, *Aulosira*, *Fischerella*, *Nostoc*, *Tolypothrix* and *Westiellopsis* by DNA amplification fingerprinting with random primers.

Materials and Methods

Cyanobacterial cultures and culture conditions—Cyanobacterial cultures used in this study were

collected from different biofertilizer production centres of India. The cultures of *Anabaena azollae*-SK-SL-TNAU1, *A. azollae*-MPK-SK-AM-24, *A. azollae*-MPK-SK-AM-25, *A. azollae*-MPK-SK-AF-38, *Anabaena*-TR-52-ST1, *Anabaena* sp., *Nostoc muscorum* DOH, *Westiellopsis*-4A₂, *Westiellopsis*-C₁₀₀-TR₅-ST₃-PA-SK, *Anabaena variabilis*-SA₀, *Nostoc* sp., *Westiellopsis* sp. were selected from the germplasm collection at the *Azolla* Laboratory, Tamil Nadu Agricultural University (TNAU), Coimbatore, while *Westiellopsis*-ARM48, was obtained from Department of Microbiology, PSG College of Arts and Science, Coimbatore and *Aulosira pseudoramosa*, *Tolypothrix tenuis*, *Westiellopsis prolifica*, and *Fischerella* sp were collected from National Centre for Conservation and Utilization of BGA (NCCUBGA), IARI, New Delhi. The cultures were maintained on BG-11 medium as liquid culture in flasks (100 ml) for DAF analysis. The inoculated flasks were incubated under light intensity of 3000 lux (16/8 hr day and night cycle) at 25° ± 1°C for 30 days. Among the cultures used, *Anabaena azollae*-SK-SL-TNAU1, *A. azollae*-MPK-SK-AM-24, *A. azollae*-MPK-SK-AM-25 and *A. azollae*-MPK-SK-AF-38 are symbiotic forms and the remaining cultures are free-living cultures.

Extraction of total genomic DNA for RAPD analysis—Total genomic DNA from cyanobacterial cultures was isolated by cetyl trimethyl ammonium bromide (CTAB; 2%) procedure with some modification⁵. Blot

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dried cyanobacterial culture (2 g) was ground in an autoclaved and pre-chilled mortar using liquid nitrogen. Macerated samples along with 2 ml of CTAB buffer were transferred to eppendorf tubes and incubated at 65°C for 30 min. Protein was removed by treating with equal volumes of chloroform and isoamyl alcohol (1:1). DNA was precipitated with addition of ice cold isopropanol. The precipitated DNA was hooked out with a bent Pasteur pipette and dissolved in 500 µl of 10 mM Tris-1 mM EDTA buffer. DNA was then concentrated by allowing it to precipitate with absolute alcohol and sodium acetate. The pellet was dissolved in 50 µl of Tris-EDTA buffer and stored at -20°C for further use.

Oligonucleotide primers and PCR amplification—Oligonucleotide primers used were obtained from OPERON-Techniques Inc., USA with known sequence (Table 1). DNA amplification was performed in a PTC-100 TM Programmable Thermocycler [PTC: MJ Research Inc., Water Town, Mass]. The reaction mixture consisted of 3 µl of 25 ng µl⁻¹ DNA; 1 µl of 10 mM dNTPs; 1.2 µl of primer; 2 µl of 10 X assay buffer; 0.2 µl of Taq polymerase (Bangalore Genei Inc.); and 12.6 µl of sterile distilled water. The incubation cycle was followed as—initial denaturation at 95°C for 2 min; denaturation at 94°C for 1 min; annealing at 37°C for 1 min; extension at 72°C for 2 min; and final extension at 72°C for 5 min. Thirty five incubation cycles were followed for denaturation, annealing and extension steps. After the reaction, 15 µl of PCR product was separated using 1.5% agarose gels, stained with ethidium bromide, viewed and photographed using alpha imager TM 1200 documentation and analysis system.

Dendrogram analysis—Each lane of RAPD profiles was scored for presence (1) and absence (0) of specific bands. The cluster analysis was carried out using NTSYS pc version 1.7 (ref. 6).

Results

In this study, RAPD profile of symbiotic and free-living cyanobacterial cultures showed DNA fingerprinting characteristic of each culture. Typical RAPD profile for different cultures using primer OPB 09 and OPF 05 are depicted in Fig. 1A,B. Number of products generated in RAPD analysis for different primers and the number of DNA fragments generated by different primers in different cultures are given in Tables 2 and 3 respectively. Similarity indices for all

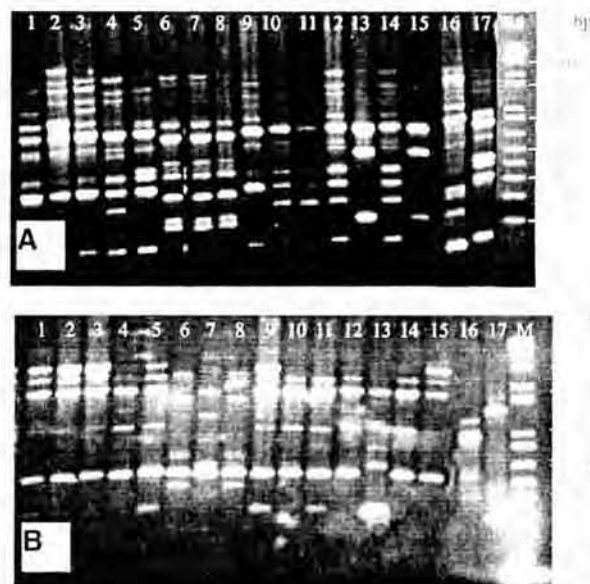


Fig. 1—RAPD profile of cyanobacterial cultures for primer, (A) OPB 09 and (B) OPF 05 [1—*Westiellopsis*—ARM 48; 2—*Westiellopsis* sp.; 3—*Westiellopsis*—C100 TR₅ ST₃ PA-SK; 4—*Westiellopsis prolifica*; 5—*Westiellopsis*—4A₂; 6—*Nostoc muscorum*; 7—*Nostoc* sp.; 8—*Anabaena variabilis*; 9—*A. azollae*—SK—SL—TNAU 1; 10—*A. azollae*—MPK—SK—AM-24; 11—*A. azollae*—MPK—SK—AM-25; 12—*A. azollae*—MPK—SK—AF-38; 13—*Anabaena* sp.; 14—*Anabaena*—TR52-ST1; 15—*Tolypothrix tenuis*; 16—*Aulosira pseudoramosa*; 17—*Fischerella* sp.; and M. Marker]

Table 2—Number of PCR products generated in RAPD analysis of cyanobacterial cultures with different primers

Primers	No. of monomorphic bands	No. of polymorphic bands	Total No. of bands	% polymorphism
OPG 01	0	11	11	100
OPAH 02	0	13	13	100
OPAG 03	0	16	16	100
OPB 09	1	23	24	95.8
OPC 09	0	12	12	100
OPF 15	0	10	10	100
OPG 04	0	18	18	100
OPF 05	0	13	13	100
OPAG 07	0	12	12	100
OPF 04	0	18	18	100
OPF 03	0	12	12	100

Table 1—Primers used for RAPD—PCR analysis of cyanobacterial cultures

Primer	Sequence
OPG01	CTACGGAGGA
OPAH02	CACTTCCGCT
OPAG03	TGCGGGAGTG
OPB09	TGGGGGACTC
OPC09	CTCACCGTCC
OPF15	CCAGTACTCC
OPG04	AGCGTGTCTG
OPF05	CCGAATTCCC
OPAG07	CACAGACCTG
OPF04	GGTGATCAGG
OPF03	CCTGATCACC

pair wise combination among the seventeen cultures were computed and presented in Table 4.

Cyanobacterial cultures, *Westiellopsis* sp. and *Westiellopsis*-C₁₀₀-TR₅-ST₃-PA-SK shared the maximum

similarity which accounted for 93.1%, followed by *Westiellopsis* -ARM48 and *Westiellopsis* sp. (89.3%).

Among the *Westiellopsis* cultures, *W. prolifica* and *Westiellopsis* sp. had the least similarity index of

Table 3—Primer-wise and culture-wise PCR products generated in RAPD analysis with different primers for cyanobacterial cultures

Cyanobacterial cultures	Primers										
	OPG01	OPAH02	OPAG03	OPB09	OPC09	OPF15	OPG04	OPF05	OPAG07	OPF04	OPF03
<i>Westiellopsis</i> – ARM48	2	2	6	5	4	1	1	4	4	8	2
<i>Westiellopsis</i> sp.	2	2	7	8	4	2	4	4	5	10	2
<i>Westiellopsis</i> -C ₁₀₀ TR ₅ ST ₃ PA-SK	2	2	5	9	3	1	3	4	5	13	2
<i>Westiellopsis prolifica</i>	2	3	5	7	3	1	4	3	5	5	3
<i>Westiellopsis</i> 4A ₂	12	4	3	7	6	1	1	6	8	6	3
<i>Nostoc muscorum</i>	14	4	4	7	1	1	1	5	4	5	1
<i>Nostoc</i> sp.	11	6	2	8	2	2	3	4	4	4	2
<i>Anabaena variabilis</i>	9	4	2	6	1	2	5	5	5	5	1
<i>A. azollae</i> –SK-SLTNAU 1	4	5	2	4	1	2	2	5	5	5	2
<i>A. azollae</i> –MPK-SK-AM-24	3	3	5	5	1	6	1	4	5	4	2
<i>A. azollae</i> –MPK-SK-AM-25	3	2	8	2	1	1	3	4	8	4	2
<i>A. azollae</i> –MPK SK-AF –38	3	2	6	11	1	3	4	4	7	4	2
<i>Anabaena</i> sp.	3	5	8	3	3	2	3	4	6	4	1
<i>Anabaena</i> –TR-52 ST 1	3	2	8	5	6	2	1	4	1	4	2
<i>Tolypothrix tenuis</i>	5	0	1	3	1	2	5	4	3	4	1
<i>Aulosira pseudoramosa</i>	5	3	6	7	1	36	4	5	3	5	4
<i>Fischerella</i>	1	6	4	6	3	6	2	2	2	2	5
Total	33	68	99	103	42	38	47	71	90	82	37

Table 4—Similarity indices of cyanobacterial cultures assessed by RAPD analysis

Cyanobacterial cultures	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1	1.000																
2	0.893	1.000															
3	0.874	0.931	1.000														
4	0.761	0.755	0.774	1.000													
5	0.717	0.686	0.692	0.755	1.000												
6	0.654	0.597	0.604	0.604	0.673	1.000											
7	0.673	0.629	0.623	0.648	0.704	0.855	1.000										
8	0.723	0.692	0.648	0.648	0.654	0.792	0.799	1.000									
9	0.717	0.686	0.667	0.667	0.711	0.673	0.730	0.755	1.000								
10	0.717	0.698	0.679	0.717	0.711	0.623	0.667	0.704	0.811	1.000							
11	0.679	0.648	0.642	0.704	0.698	0.635	0.717	0.692	0.799	0.874	1.000						
12	0.654	0.648	0.629	0.704	0.673	0.597	0.654	0.654	0.723	0.811	0.824	1.000					
13	0.673	0.629	0.623	0.648	0.642	0.604	0.673	0.648	0.704	0.755	0.767	0.717	1.000				
14	0.730	0.686	0.679	0.730	0.723	0.623	0.667	0.679	0.711	0.799	0.761	0.774	0.742	1.000			
15	0.711	0.692	0.698	0.660	0.654	0.654	0.686	0.723	0.717	0.780	0.730	0.692	0.761	0.818	1.000		
16	0.648	0.642	0.660	0.673	0.654	0.604	0.648	0.686	0.704	0.717	0.667	0.667	0.673	0.767	0.786	1.000	
17	0.642	0.635	0.616	0.629	0.610	0.572	0.604	0.642	0.698	0.761	0.686	0.635	0.692	0.711	0.717	0.730	1.000

1—*Westiellopsis* — ARM48

2—*Westiellopsis* sp.

3—*Westiellopsis* — C₁₀₀ TR₅ ST₃ PA-SK

4—*Westiellopsis prolifica*

5—*Westiellopsis* — 4A₂

6—*Nostoc muscorum*

7—*Nostoc* sp.

8—*Anabaena variabilis*

9—*A. azollae* — SK-SL TNAU 1

10—*A. azollae* — MPK-SK-AM — 24

11—*A. azollae* — MPK-SK-AM — 25

12—*A. azollae* — MPK SK-AF — 38

13—*Anabaena* sp.

14—*Anabaena* — TR-52-ST1

15—*Tolypothrix tenuis*

16—*Aulosira pseudoramosa*

17—*Fischerella* sp.

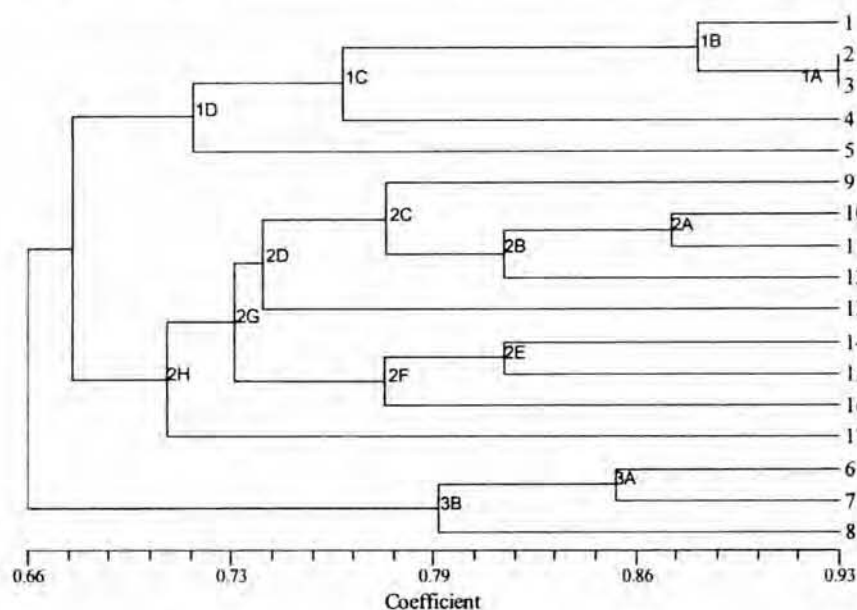
68.6%. *Nostoc muscorum* and *Nostoc* sp. shared a similarity of 85.5%. Considering *Nostoc* and *Westiellopsis* cultures, *Nostoc* sp. and *Westiellopsis*-4A₂ had higher similarity of 70.4%.

Among various *Anabaena* cultures, *A. azollae*-MPK-SK-AM-24 and *A. azollae*-MPK-SK-AM-25, showed 87.4% similarity followed by *A. azollae*-MPK-SK-AM-25 and *A. azollae*-MPK-SK-AF-38 (82.4%). All *Anabaena* cultures showed 60-75% similarity with each other. Among *Anabaena* cultures, *Anabaena variabilis* and *Anabaena* sp. recorded the least similarity value of 64.8%. Considering *Anabaena* and *Westiellopsis* cultures, the saline tolerant culture *Anabaena*-TR-52-ST1 and *Westiellopsis prolifica* had 73% similarity. The acid tolerant *Westiellopsis*-4A₂ shared 65.4% similarity with *Tolypothrix tenuis* and *Aulosira pseudoramosa*.

Fischerella was similar to *Aulosira pseudoramosa* by 73 and 57.2% with *Nostoc muscorum*. *Aulosira pseudoramosa* had higher similarity with *Tolypothrix tenuis*.

In general there was a similarity of 60-90% within *Westiellopsis* cultures. *Nostoc* cultures shared 50-80% similarity with *Westiellopsis* cultures. *Anabaena* cultures were similar to *Westiellopsis* cultures by 60-70%. The least similarity was exhibited between *Fischerella* and *Nostoc muscorum* indicating that they were distantly related.

Dendrogram analysis of seventeen cyanobacterial cultures revealed three major clusters (Fig. 2). The first major cluster was divided into subclusters in which the two cultures *Westiellopsis* sp. and *Westiellopsis*-C₁₀₀-TR₅-ST₃-PA-SK were closely related by 93.1% and forms the 1st subcluster (1A),



- | | |
|---|--------------------------------------|
| 1 <i>Westiellopsis</i> - ARM48 | 9 <i>A. azollae</i> -SK-SLTNAU 1 |
| 2 <i>Westiellopsis</i> sp | 10 <i>A. azollae</i> -MPK-SK-AM 24 |
| 3 <i>Westiellopsis</i> - C ₁₀₀ TR ₅ ST ₃ PA-SK | 11 <i>A. azollae</i> - MPK-SK-AM25 |
| 4 <i>Westiellopsis prolifica</i> | 12 <i>A. azollae</i> - MPK SK-AF -38 |
| 5 <i>Westiellopsis</i> 4A ₂ | 13 <i>Anabaena</i> sp |
| 6 <i>Nostoc muscorum</i> | 14 <i>Anabaena</i> -TR 52 ST 1 |
| 7 <i>Nostoc</i> sp | 15 <i>Tolypothrix tenuis</i> |
| 8 <i>Anabaena variabilis</i> | 16 <i>Aulosira pseudoramosa</i> |
| | 17 <i>Fischerella</i> sp |

Fig. 2—Phylogenetic relationship between the cyanobacterial cultures by RAPD analysis. [1—*Westiellopsis*—ARM48; 2—*Westiellopsis* sp.; 3—*Westiellopsis*—C₁₀₀ TR₅ ST₃ PA-SK; 4—*Westiellopsis prolifica*; 5—*Westiellopsis* 4A₂; 6—*Nostoc muscorum*; 7—*Nostoc* sp.; 8—*Anabaena variabilis*; 9—*A. azollae*—SK-SL TNAU 1; 10—*A. azollae*—MPK-SK-AM 24; 11—*A. azollae*—MPK-SK-AM 25; 12—*A. azollae*—MPK-SK-AF-38; 13—*Anabaena* sp.; 14—*Anabaena*—TR 52 ST 1; 15—*Tolypothrix tenuis*; 16—*Aulosira pseudoramosa*; and 17—*Fischerella* sp.]

followed by their relation with *Westiellopsis*-ARM-48 (88%) and formed the 2nd subcluster (1B). The culture of *W. prolifica* along with 1B formed the 3rd subcluster (1C) showing relation to 77%. The acid tolerant culture *Westiellopsis* 4A₂ along with 1C formed the 4th subcluster (1D) and were related by 71%.

Cyanobacterial cultures *A. azollae*-MPK-SK-AM-24 and *A. azollae*-MPK-SK-AM-25 were related by 87.4% in the second major cluster and formed the subcluster 2A. The subcluster 2A along with *A. azollae*-MPK-SK-AF-38 formed the subcluster 2B and were related by 80%. *A. azollae*-SK-SL-TNAU1 was in similarity with 2B by 78% and formed the subcluster 2C. The subcluster 2D was formed by *Anabaena* sp. in relation with 2C and were similar by 74%. *Anabaena* -TR-52-ST1 and *Tolypothrix tenuis* were similar by 81% forming the subcluster 2E. The subcluster 2E was similar to *Aulosira pseudoramosa* by 78% and formed the subcluster 2F. The subcluster 2F and 2D were in similarity by 73% and formed the subcluster 2G. *Fischerella* in relation to subcluster 2G formed the subcluster 2H and were related by 70%. The two major clusters, cluster I and II were related by 67.25%. In the third major cluster, *Nostoc muscorum* and *Nostoc* sp. were similar by 85.5% and formed subcluster 3A which in turn was related to *Anabaena variabilis* by 79% and formed the subcluster 3B. The third major cluster was related to first and second cluster by only 66%.

Discussion

Presence of repeated DNA short tandemly repeated repetitive (STRR) and long tandemly repeated repetitive (LTRR) sequence in the genome of cyanobacteria was used to generate a fingerprint method for symbiotic and free-living isolates. Isolates which have been postulated to be closely related or identical revealed similar results by PCR techniques indicating that the technique is useful for clustering of even closely related strains⁷. In our study, dendrogram analysis revealed 3 major clusters. All the *Westiellopsis* cultures form the first major cluster indicating that they are closely related with each other and evolved gradually. The second major cluster is dominated by the isolates of symbiotic cyanobacteria *Anabaena azollae*-SK-SL-TNAU1, *A. azollae*-MPK-SK-AM-24, *A. azollae*-MPK-SK-AM-25, and

A. azollae-MPK-SK-AF-38. The free-living forms *Aulosira pseudoramosa*, *Tolypothrix tenuis* and *Fischerella* sp are similar to *Anabaena*-TR-52-ST1 by 70%. There was a similarity of 60-90% with in *Westiellopsis* cultures. *Nostoc* cultures shared 50-80% similarity with *Westiellopsis* cultures. *Anabaena* cultures were similar to *Westiellopsis* cultures by 60-70%. The dendrogram analysis revealed the genetic diversity between the symbiotic and free-living cyanobacteria. Plazinski *et al.*⁸ have identified 9 different strains of *Anabaena azollae* using DNA/DNA hybridisation. The results of the present study indicated the potential use of RAPD markers as a rapid method to detect genetic variation/relatedness of the cyanobacterial strains. RAPD markers generated may be used as a molecular marker for identification of the cyanobacterial cultures.

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References

- 1 Nierzwicki-Bauer S A & Bushnell T P Jr, Molecular characterization of the *Azolla-Anabaena* symbiosis, in *Cyanobacterial Biotechnology*, edited by G Subramanian, B D Kaushik and G S Venkataraman, (Oxford & IBH Publishing Co. Pvt. Ltd., New Delhi) 1998, 169.
- 2 Caetano-Anolles G, Bassam B J & Gresshoff P M, DNA amplification finger printing using very short arbitrary oligonucleotide primers, *Biotechnology*, 9 (1991) 553.
- 3 Nishihara H, Miwa H, Watanabe M, Nagashima M, Yagi O & Takamura Y, Random amplified polymorphic DNA (RAPD) analysis for discriminating genotypes of *Microcystis* cyanobacteria, *Biosci Biotechnol Biochem*, 61 (1997) 1067.
- 4 Wilson K M, Schenbri M A, Baker P D & Saint C P, Molecular characterization of the toxic cyanobacterium *Cylindrospermopsis raciborskii* and design of a species-specific PCR, *Appl Environ Microbiol*, 66 (2000) 332.
- 5 *Plant molecular biology—A laboratory manual*, edited by S C Melody (New York: Springer-Verlag) 1997, pp.
- 6 Rohlf F J, NTSYS-pc Numerical taxonomic and multivariate analysis system version 1.7, Owner manual, 1992.
- 7 Rasmussen U & Svenning M M, Finger printing of cyanobacteria based on PCR with primers derived from short and long tandemly repeated repetitive sequences, *Appl Environ Microbiol*, 64 (1998) 265.
- 8 Plazinski J, Franche C, Liu C C, Lin T, Sha W, Gunning B E S & Rolfe B G, Taxonomic status of *Anabaena azollae*- An overview, *Plant Soil*, 108 (1988) 185.