ISSN NO. 2320-5407

Journal homepage: http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

Genetic diversity analysis of cyanobacteria from acidic rice fields of Manipur, India falling under Indo-Burma biodiversity hotspots

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Manuscript Info

Manuscript History:

Received: 18 March 2015
Final Accepted: 29 April 2015
Published Online: May 2015

Key words:

Heterocystous cyanobacteria, Diversity, PCR-fingerprinting, STRR

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Abstract

The genetical diversity of morphologically distinct thirty two strains of four cyanobacterial genera (*Nostoc*, *Anabaena*, *Calothrix* and *Microchaete*) isolated from rice fields of seven different districts of Manipur, India was studied. The identity of strains was confirmed through 16S rRNA gene sequencing and the sequences obtained were deposited in NCBI data bank. The genetic diversity of different genera obtained by STRR PCR fingerprinting technique was correlated to the results of certain morphological features and molecular data (16S rRNA partial sequencing) of the present cyanobacterial species. Cyanobacterial genomic DNA isolated from the different species were used as templates and polymerase chain reaction (PCR) fingerprinting with 16S rRNA was performed. The genetic diversity was analysed using short tandemly repeated repetitive (STRR) sequences as primer and PCR fingerprinting patterns were used to evaluate the genetic closeness. Based upon the fingerprinting patterns, the divergence amongst distant strains and closeness in similar strains was clearly understood. Unweighted pair-group method of arithmetic mean (UPGMA) analysis showed an accurate estimate of genetic diversity, and relationships within and amongst all species which correlated with the morphological parameters and 16S sequencing.

INTRODUCTION

Cyanobacteria (blue-green algae) are capable of both carbon assimilation and N_2 fixation, thereby enhancing productivity in almost all conceivable environments. They are morphologically diverse group of phototrophic prokaryotes, which occur in almost every habitat on earth and are useful in different ways (Thajuddin and Subramanian, 2005). Cyanobacteria are one of the main components of the microbiota in rice fields (Ladha and Reddy, 2003) and play an important role in the maintenance and build-up of soil fertility, consequently increasing rice growth and yield (Roger and Ladha, 1992; Kaushik and Prasanna, 2002; Peoples et al., 1995). Indian rice fields have been studied for cyanobacteria by different workers during the past half century (Aiyer et al., 1972; Shukla et al., 1971; Tiwari and Pandey, 1976; Devi et al., 1999; Nayak et al., 2001). Diazotrophic cyanobacteria are the main contributors to photodependent N_2 fixation in rice fields and among them; heterocystous cyanobacteria are quite ubiquitous (Sinha and Hader, 1996). The dominating heterocystous nitrogen fixing cyanobacterial species of *Aulosira*, *Cylindrospermum*, *Nostoc*, *Anabaena*, *Tolypothrix* and *Calothrix* were reported from soils of Cuttack and Orissa (Singh, 1961).

Our knowledge of the native cyanobacterial populations in rice fields has so far exclusively been achieved by means of cultivation-based analysis, followed by morphological identification of individual isolates (Khan et al., 1994; Quesada and Valiente, 1996). Such indirect approaches have certain limitations for studying diversity in

complex natural systems, as only a limited number of bacteria can be recovered from the soil by traditional cultivation techniques (Garcia Pichel et al., 2001; Smit et al., 2001). Moreover, some diagnostic features, such as gas vacuoles or akinetes can show variations with different environmental or growth conditions and even be lost during cultivation (Rudi et al., 1997; Lyra et al., 2001). Such limitations of phenotypic characters have highlighted the requirement for more reliable methods and promoted molecular approaches in cyanobacterial taxonomy, including DNA base composition (Kaneko et al., 1996, 2001), DNA hybridizations (Kondo et al., 2000), gene sequencing (Nubel et al., 1997) and PCR fingerprinting (Rasmussen and Svenning, 1998; Versalovic et al., 1991).

Repetitive sequences constitute an important part of the prokaryotic genome. For cyanobacteria, distinct families of repetitive sequences, the short tandemly repeated repetitive (STRR) sequences, have been described (Jackman and Mulligan, 1995; Mazel et al., 1990). The STRR sequences have been identified in a number of cyanobacterial genera and species, all belonging to the heterocystous cyanobacteria. Initially the sequences were described for *Calothrix* species, where the copy number was estimated to about 100 per genome (Mazel et al., 1990). A large number of paddy fields from various locations of Manipur belonging to Indo-burma Biodiversity spots have not been explored for the presence of cyanobacteria. The present endeavour was aimed to study the heterocystous cyanobacterial diversity and their relatedness utilizing morphological and STRR fingerprinting approaches.

Materials and methods

Cyanobacterial culture conditions

Thirty two morphologically distinct heterocystous cyanobacterial strains belonging to four different genera originally isolated from rice fields of Manipur during 2010-2012 were obtained from the National freshwater cyanobacterial and microalgal repository of IBSD, Manipur. The details of the selected strains along with the other details are presented in Table1. All the strains were grown at a temperature of 28±2˚C, light intensity 54-67 µmol photon m^{-2} s^{-1} and 14h/10h light and dark in nitrate free BG-11 liquid medium (Stanier et al., 1971).

Morphological evaluation

Isolated cyanobacterial strains were identified using the diacritical morphological features for genera devised by Desikachary (1959). Microscopic inspections were conducted on a CARL ZEISS FLUORESCENCE MICROSCOPE, AXIO SCOPE A1 coupled with Carl Zeiss Imaging Systems 32 software AxioVision 4.7.2 (CARL ZEISS, INDIA PVT. LTD.) (Figs 8, 9 and 10).

Genomic DNA isolation and PCR amplification using 16S rRNA and STRR primers

Genomic DNA extracted by a modified Xanthogenate method (Avijeet et al., 2013) was used as a template for PCR amplification of 16S rRNA gene and STRR in the selected cyanobacterial strains. For 16S rRNA gene amplification, universal primers (IDT- Integrated DNA technologies) namely forward primer (536f-5´-GTGCCAGCAGCCGCGGTRATA-3´) and reverse primer (1488R-5´-CGGTTACCTTGTTACGACTTCACC-3´) (Nubel et al., 1997) were used along with 1X PCR buffer, dNTPs mix, Taq DNA polymerase, template DNA and sterile double distilled water. All the PCRs were carried out in a 25µl volume in a Thermal cycler (VERITI 96 WELL THERMAL CYCLER, APPLIED BIOSYSTEMS, CANADA). After the reaction was completed, 5 µl of amplified DNA was separated on 2% agarose gel (SIGMA ALDRICH CHEMICALS PVT. LTD., BANGALORE, INDIA) with 1X TAE stained with ethidium bromide for 1-h at 60volt. The 16S rRNA gene sequences were aligned with reference sequences retrieved from GenBank and analysed by using the BLASTn (Altschul et al., 1990) through NCBI. The sequences obtained were submitted in NCBI GenBank database through GenBank submission tools sequin software V12.30.

The STRR oligonucleotide primer (SIGMA ALDRICH CHEMICALS PVT. LTD., BANGALORE, INDIA) having the sequence: 5´-CCTRACCCTRACC-3´was used and PCR reaction was carried out in a 25µl volume containing 50 pmole primer, 1.25 mM deoxynucleotide triphosphate, 1µl cyanobacterial genomic DNA and 1U DNA polymerase (SIGMA ALDRICH CHEMICALS PVT. LTD., BANGALORE, INDIA). The buffer supplied with the enzyme was used according to the manufacturer's protocol. Reaction was carried out with the conditions of 1 cycle at 95˚C for 6 min; 35 cycles at 94˚C for 1 min, 56˚C for 1 min, and 65˚C for 5 min and1 cycle at 65˚C for 16 min with a final step at 4˚C using as indicated earlier. After the reaction was completed, 10 µl of amplified DNA was separated on 1.5% agarose (SIGMA ALDRICH CHEMICALS PVT. LTD., BANGALORE, INDIA) with 1X TAE stained with ethidium bromide for 2-h at 80 volt. Separated amplified fragments were recorded and analysed using Quantum Capt version 15.12 software.

Phylogenetic analysis

The genetic diversity and the relatedness within and amongst thirty two heterocystous cyanobacterial strains was estimated by means of dendrogram constructed on the basis of STRR fingerprint patterns. The dendrogram was constructed using the unweighted pair-group method of arithmetic mean (UPGMA) (Sneath and Sokal, 1973)

employing sequential, agglomerative, hierarchical and nested clustering (SAHN) of NTSYSpc version 2.21m software (Rohlt, 1994).

Results

The morphologically characterized cyanobacterial strains were further confirmed by 16S rRNA sequence analysis and the accession numbers obtained for the selected thirty two cyanobacterial strains are provided in Table 2.

The PCR amplification with STRR primers yielded multiple distinct DNA products and banding patterns with size ranging from 160bp to 790bp (Fig 5 and Fig 6) exhibiting inter-generic diversity. The similar fingerprint patterns were obtained from the strains within each species from different geographical origin, while different patterns were obtained amongst strains of a species isolated from same geographical area.

Genetic variability amongst *Anabaena* species

The results of PCR fingerprint patterns obtained for selected *Anabaena* strains from rice fields depicted distinct differences. The fragments showed molecular size of 740, 600, 534, 400, 348, 272, 222 and 164bp and all the studied strains showed a common fragment of 740bp (Fig 5: Lane no. 2 to Lane no. 17). *Anabaena* strain BTA19 showed only single band of 740bp. Other strains examined namely BTA03 (*Anabaena variabilis*), BTA990 (*Anabaena variabilis*), BTA04 (*Anabaena dolionum*), BTA31 (*Anabaena flos-aquae*), BTA919 (*Anabaena flos-aquae*), BTA14 (*Anabaena oryzae*), BTA50 (*Anabaena oryzae*), BTA35 (*Anabaena fertilissima*), BTA1050 (*Anabaena* sp.), BTA880 (*Anabaena* sp.), BTA903 (*Anabaena* sp.), BTA84 (*Anabaena* sp.), BTA653 (*Anabaena* sp.) and BTA1131 (*Anabaena* sp.) showed almost similar banding patterns with STRR primers. The fragment of 164bp was absent in BTA653 while the other fragments were found to be present in the above mentioned species. BTA06 (*Anabaena variabilis*) was characterized by the presence of three fragments with the size of 740, 534 and 400bp.

Genetic variability amongst *Nostoc* species

Electrophoretic patterns exhibited the molecular fragments with the size of 763, 609, 553, 400, 353, 271, 221 and 161bp amongst *Nostoc* species (Fig 6: Lane no. 2 to Lane no. 10). These eight fragments were present in the selected strains of *Nostoc* namely BTA12 (*Nostoc hatei*), BTA27 (*Nostoc muscorum*), BTA29 (*Nostoc parmelioides*), BTA37 (*Nostoc hatei*), BTA38 (*Nostoc carneum*), BTA947 (*Nostoc* sp.), and BTA1016 (*Nostoc* sp.). On the other hand, BTA87 (*Nostoc muscorum*) showed only three bands having size of 763, 609 and 400bp only. Further, strain BTA923 (*Nostoc* sp.) exhibited fragments size of 763, 609, 553, 400, 271 and 161bp respectively.

Genetic variability amongst *Calothrix* species

The amplification of primer STRR in the PCR amongst four strains of *Calothrix* species yielded multiple distinct bands (Fig 6: Lane no. 12 to Lane no. 15). The strains BTA24 (*Calothrix javanica*) and BTA206 (*Calothrix marchica*) showed identical banding patterns with the size of 790, 600, 553, 400, 340, 259, 200 and 160bp. BTA1059 (*Calothrix* sp.) showed only three specific bands at 790, 600 and 553bp. However, BTA15 (*Calothrix wembaerensis*) was distinct from BTA24 and BTA206 by missing the two bands of size 430bp and 160bp.

Genetic variability amongst *Microchaete* species

The three *Microchaete* species generated individual and unique banding patterns (Fig 6: Lane no. 17 to Lane no. 19). BTA01 (*Microchaete uberrima*), BTA07 (*Microchaete grisea*) and BTA44 (*Microchaete loktakensis*) showed four specific bands of 418, 550, 598 and 750bp size while BTA01 was distinct and did not show these four fragments in its profile. BTA07 and BTA44 showed additional molecular bands of the size of 370, 273, 206 and 160bp.

Phylogenic tree construction

A dendrogram (Fig 7) was created based on the generated fingerprints of the individual cyanobacterial strains of four different genera. Two clusters were obtained, cluster I and cluster II. Cluster I divided into two (Ia and Ib) subclusters and subcluster Ib was further divided into Ib1 and Ib2. Strains of *Anabaena* species belonged to Group Ia and *Nostoc* strains exhibited in subgroup Ib1. On the other hand, subgroup Ib2 depicted four strains of *Calothrix* sp. and the strains of *Microchaete* species were present in cluster II.

Table 1 Selected heterocystous cyanobacterial strains and the habitat details

Name of strains	Repository Reference no*.	Habitat details
Anabaena variabilis	BTA03	Ricefields, Imphal East, Manipur, Altitude: 215m, Latitude/longitude: N 24°15′11.1″ and E093°17′50.5″
Anabaena dolionum	BTA04	Ricefields, Imphal East, Manipur, Altitude: 775m,

		Latitude/longitude: N 24°49′26.4″ and E093°57′52.0
Anabaena variabilis	BTA06	Ricefields, Imphal East, Manipur, Altitude: 782m, Latitude/longitude: N 24°52′09.0″ and E094°01′12.9″
Anabaena oryzae	BTA14	Ricefields, Imphal East, Manipur, Altitude: 775m, Latitude/longitude: N 24°49′26.4″ and E093°57′52.0″
Anabaena oryzae	BTA19	Ricefields, Imphal West, Manipur, Altitude: 769m Latitude/longitude: N 24°46′06.9″ and E093°54′14.3″
Anabaena flos-aquae	BTA31	Ricefields, Imphal West, Manipur, Altitude: 782m Latitude/longitude: N 24°48′14.3″ and E093°54′18.3″
Anabaena fertilissima	BTA35	Ricefields, Bishnupur, Manipur, Altitude: 776m Latitude/longitude: N 24°43′15.2″ and E093°50′27.0″
Anabaena oryzae	BTA50	Ricefields, Bishnupur, Manipur, Altitude: 767m Latitude/longitude: N 24°33′39.0″ and E093^{0}45′42.3″
Anabaena sp.	BTA84	Ricefields, Thoubal, Manipur, Altitude: 769m Latitude/longitude: N 24°29′25.1″ and E094°00′43.7″
Anabaena sp.	BTA653	Ricefields, Senapati, Manipur, Altitude: 933m Latitude/longitude: N 25°02′19.1″ and E093°55′34.9″
Anabaena sp.	BTA880	Ricefields, Imphal East, Manipur, Altitude : 765m Latitude/longitude: N 24°50′49.7″ and E093°56′22.7″
Anabaena sp.	BTA903	Ricefields, Imphal East, Manipur, Altitude : 775m Latitude/longitude: N 24°49′26.4″ and E093°57′52.0″
Anabaena flos-aquae	BTA919	Ricefields, Thoubal, Manipur, Altitude:805m Latitude/longitude: N 24°29′28.7″ and E094°00′24.1″
Anabaena variabilis	BTA990	Ricefields, Bishnupur, Manipur, Altitude: 805m Latitude/longitude: N 24°42′21.2″ and E093°49′03.7″
Anabaena sp.	BTA1050	Ricefields, Bishnupur, Manipur, Altitude: 773m Latitude/longitude: N 24°42′09.6″ and E093°48′22.3″
Anabaena sp.	BTA1131	Ricefields, Bishnupur, Manipur, Altitude: 773m Latitude/longitude: N 24°42′09.6″ and E093°48′22.3″
Nostoc hatei	BTA12	Ricefields, Imphal East, Manipur, Altitude: 775m Latitude/longitude: N 24°49′26.4″ and E093°57′52.0″
Nostoc muscorum	BTA27	Ricefields, Imphal West, Manipur, Altitude: 792m Latitude/longitude: N 24°50′33.6″ and E093°56′23.4″
Nostoc parmelioides	BTA29	Ricefields, Imphal West, Manipur, Altitude: 792m Latitude/longitude: N 24°50′33.6″ and E093°56′23.4″
Nostoc hatei	BTA37	Ricefields, Nambol, Bishnupur, Manipur, Altitude :776m Latitude/longitude: N 24°43′15.5″ and E093°50′27.8″
Nostoc carneum	BTA38	Ricefields, Bishnupur, Manipur, Altitude: 776m Latitude/longitude: N 24°43′15.5″ and E093°50′27.8″
Nostoc muscorum	BTA87	Ricefields, Thoubal, Manipur, Altitude : 769m Latitude/longitude: N 24°29′25.1″ and E094°00′43.7″
Nostoc sp.	BTA923	Ricefields, Kakching, Thoubal, Manipur, Altitude: 782m Latitude/longitude: N 24°39′18.5″ and E093°59′18.6″
Nostoc sp.	BTA947	Ricefields, Thoubal, Manipur, Altitude: 782m Latitude/longitude: N 24°39′18.5″ and E093°59′18.6″
Nostoc sp.	BTA1016	Ricefields, Bishnupur, Manipur, Altitude: 773m Latitude/longitude: N 24°42′09.6″ and E093°48′22.3″
Calothrix wembaerensis	BTA15	Ricefields, Imphal East, Manipur, Altitude: 775m Latitude/longitude: N 24°49′26.4″ and E093°57′52.0″
Calothrix javanica	BTA24	Ricefields, Imphal west, Manipur, Altitude: 780m Latitude/longitude: N 24°47′36.0″ and E093°53′25.5″
Calothrix marchica	BTA206	Ricefields, Churachandpur, Manipur, Altitude: 835m Latitude/longitude: N 24°20′36.7″ and E093°41′50.3″
Calothrix sp.	BTA1059	Ricefields, Bishnupur, Manipur, Altitude: 773m Latitude/longitude: N 24°42′09.6″ and E093°48′22.3″

Microchaete uberrima	BTA01	Ricefields, Imphal East, Manipur, Altitude: 775m Latitude/longitude: N 24°49′26.4″ and E093°57′52.0″
Microchaete grisea	BTA07	Ricefields, Imphal East, Manipur, Altitude: 782m Latitude/longitude: N 24°52′09.0″ and E094°01′12.9″
Microchaete loktakensis	BTA44	Ricefields, Bishnupur, Manipur, Altitude: 764m Latitude/longitude: N 24°32′21.9″ and E093°45′27.6″

* BTA – Biotechnological algae

Table 2 Details of the accession numbers of cyanobacterial strains submitted in NCBI GenBank

BTA No.	**GenBank Accession No.**	**BTA No.**	**GenBank Accession No.**
BTA03	KM010233	BTA27	KM435246
BTA04	KM010234	BTA29	KM435247
BTA06	KM435244	BTA37	KF953531
BTA14	KM010231	BTA38	KM435249
BTA19	KM435245	BTA87	KF953527
BTA31	KM435248	BTA923	KM435252
BTA35	KJF62184	BTA947	KF953524
BTA50	KM435250	BTA1016	KJ511804
BTA84	KF953534	BTA15	KM010232
BTA653	KM435255	BTA24	KM953528
BTA880	KM435256	BTA206	KF953525
BTA903	KJ652540	BTA1059	KF953526
BTA990	KJ830948	BTA01	KM010230
BTA1050	KM435253	BTA07	KM010235
BTA1131	KJ830949	BTA44	KF953529
BTA12	KM010236	BTA919	KM435251

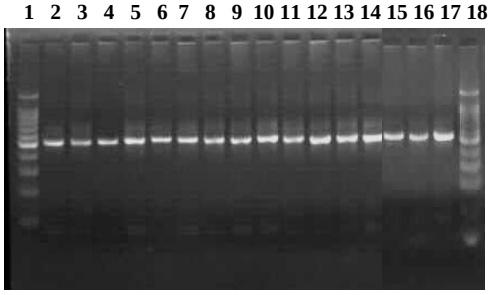

Fig 1

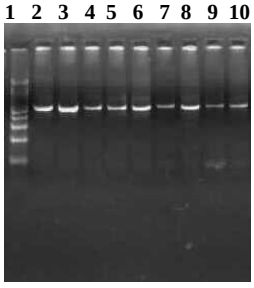

Fig 2

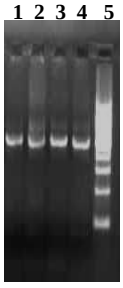

Fig 3

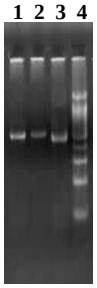

Fig 4

Fig 1 16S rRNA PCR fingerprint patterns generated from strains of *Anabaena* sp. of rice fields of Manipur (Lane1-DNA molecular weight standard in bp, Lane2-BTA03, Lane3-BTA06, Lane4-BTA990, Lane5-BTA04, Lane6-BTA31, Lane7-BTA919, Lane8-BTA14, Lane9-BTA50, Lane10-BTA19, Lane11-BTA35, Lane12 BTA1050, Lane13-BTA880, Lane14-BTA903, Lane15-BTA84, Lane16-BTA653, Lane17-BTA1131 and Lane18-DNA molecular weight standard in bp).

Fig 2 16S rRNA PCR fingerprint patterns generated from strains of *Nostoc* sp. of rice fields of Manipur (Lane1-DNA molecular weight standard in bp, Lane2-BTA923, Lane3-BTA947, Lane4-BTA12, Lane5-BTA37, Lane6-BTA1016, Lane7-BTA38, Lane8-BTA27, Lane9-BTA29, Lane10-BTA87).

Fig 3 16S rRNA PCR fingerprint patterns generated from strains of *Calothrix* sp. of rice fields of Manipur (Lane1-BTA15, Lane2-BTA24, Lane3-BTA206, Lane4-BTA1059, Lane5-DNA molecular weight standard in bp).

Fig 4 16S rRNA PCR fingerprint patterns generated from strains of *Microchaete* sp. of rice fields of Manipur (Lane1-BTA01, Lane2-BTA07, Lane3-BTA44, Lane4-DNA molecular weight standard in bp).

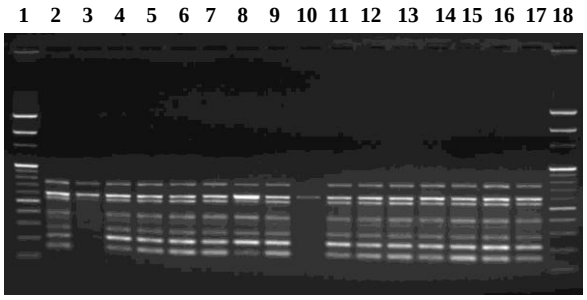

Fig 5

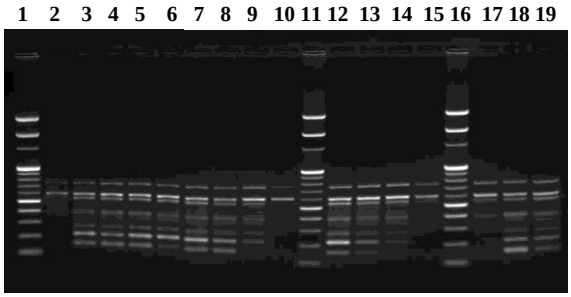

Fig 6

Fig 5 PCR fingerprint patterns generated from strains of *Anabaena* sp. of rice fields of Manipur (Lane1-DNA molecular weight standard in bp, Lane2-BTA03, Lane3-BTA06, Lane4-BTA990, Lane5-BTA04, Lane6-BTA31, Lane7-BTA919, Lane8-BTA14, Lane9-BTA50, Lane10-BTA19, Lane11-BTA35, Lane12-BTA1050, Lane13-BTA880, Lane14-BTA903, Lane15-BTA84, Lane16-BTA653, Lane17-BTA1131, Lane18-DNA molecular weight standard in bp).

Fig 6 PCR fingerprint patterns generated from strains of *Nostoc* sp., *Calothrix* sp. and *Microchaete* sp. of rice fields of Manipur (Lane1-DNA molecular weight standard in bp, Lane2-BTA923, Lane3-BTA947, Lane4-BTA12, Lane5-BTA37, Lane6-BTA1016, Lane7-BTA38, Lane8-BTA27, Lane9-BTA29, Lane10-BTA87, Lane11-DNA molecular weight standard in bp, Lane12-BTA15, Lane13-BTA24, Lane14-BTA206, Lane15-BTA1059, Lane16-DNA molecular weight standard in bp, Lane17-BTA01, Lane18-BTA07, Lane19-BTA44).

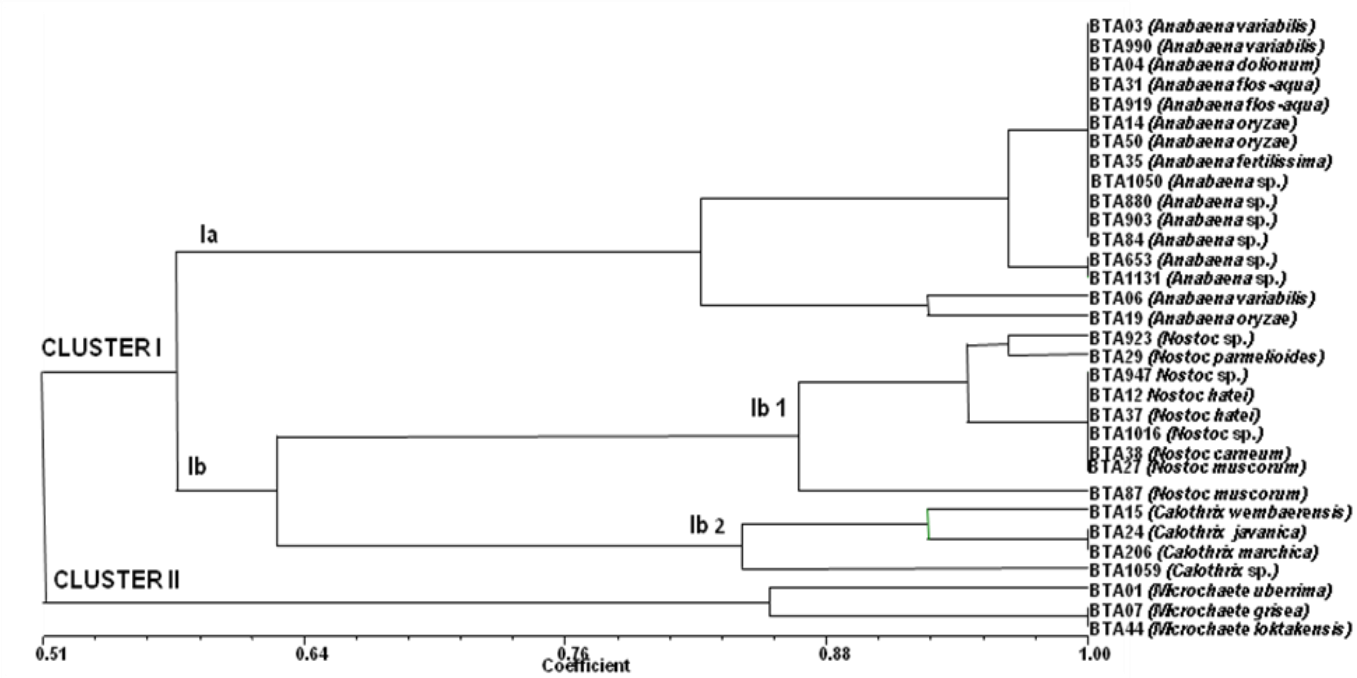

Fig 7 Dendrogram derived from cluster analysis (UPGMA) for thirty two cyanobacterial strains using STRR primer.

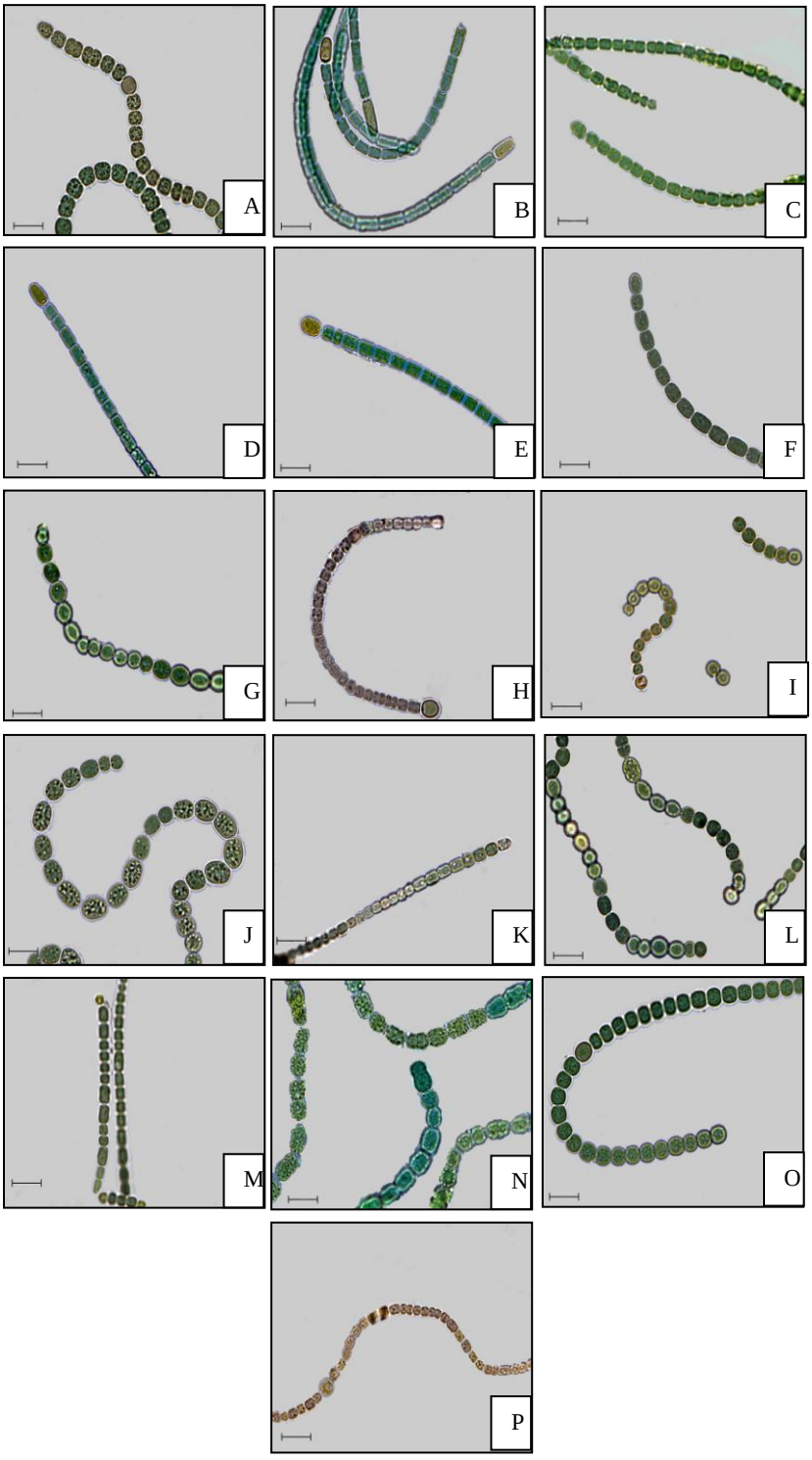

Fig 8 Photomicrograph of *Anabaena* species. A-BTA03 *Anabaena variabilis*, B-BTA04 *Anabaena dolionum*, C-BTA06 *Anabaena variabilis*, D-BTA14 *Anabaena oryzae*, E-BTA19 *Anabaena oryzae*, F-BTA31 *Anabaena flos-aquae*, G-BTA35 *Anabaena fertilissima*, H-BTA50 *Anabaena oryzae*, I-BTA84 *Anabaena* sp., J-BTA653 *Anabaena* sp., K-BTA880 *Anabaena* sp., L-BTA903 *Anabaena* sp., M-BTA919-*Anabaena flos-aquae*, N-BTA990 *Anabaena variabilis*, O-BTA1050 *Anabaena* sp., P-BTA1131 *Anabaena* sp. Scale bar = 10µm.

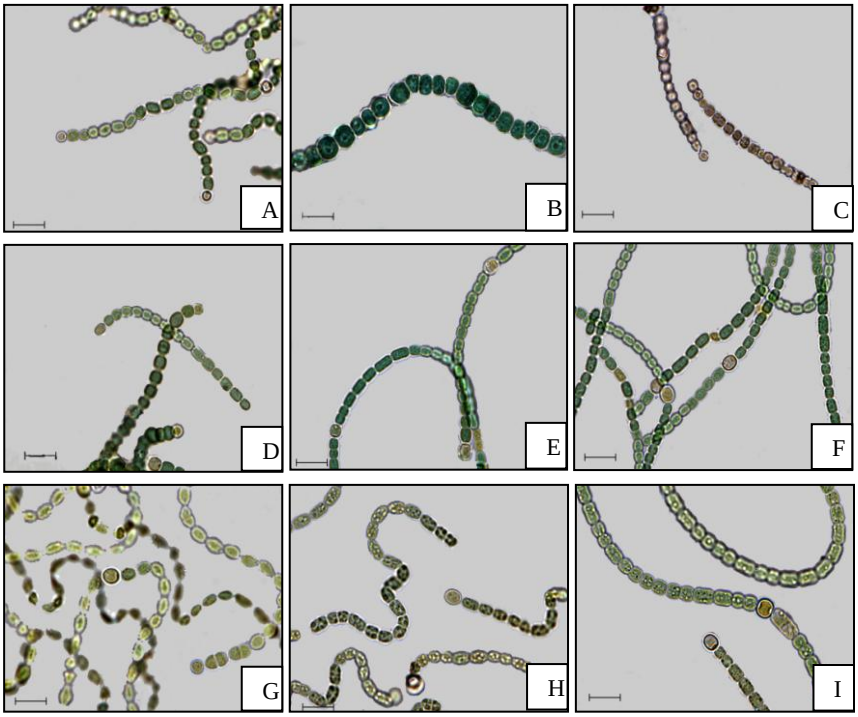

Fig 9 Photomicrograph of *Nostoc* species. A-BTA12 *Nostoc hatei*, B-BTA27 *Nostoc muscorum*, C-BTA29 *Nostoc parmelioides*, D-BTA37 *Nostoc hatei*, E-BTA38 *Nostoc carneum*, F-BTA87 *Nostoc muscorum*, G-BTA923 *Nostoc* sp., H-BTA947 *Nostoc* sp., I-BTA1016 *Nostoc* sp. Scale bar = 10µm.

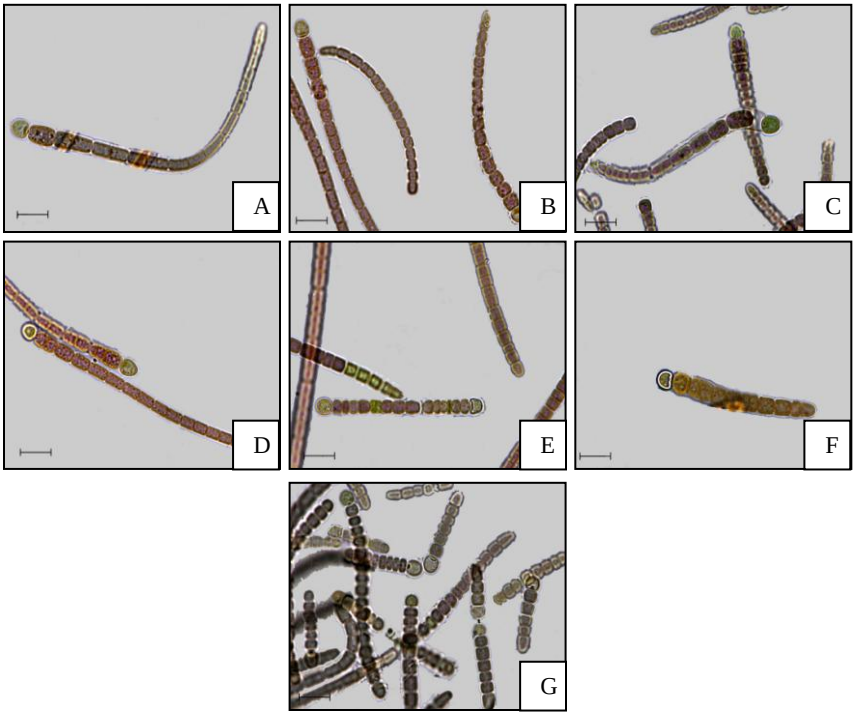

Fig 10 Photomicrograph of *Calothrix* & *Microchaete* species. A-BTA15 *Calothrix wembaerensis*, B-BTA24 *Calothrix javanica*, C-BTA206 *Calothrix marchica*, D-BTA1059 *Calothrix* sp., E-BTA01 *Microchaete uberrima*, F-BTA07 *Microchaete grisea*, G-BTA44 *Microchaete loktakensis*. Scale bar = 10µm.

Discussion

Species of the heterocystous cyanobacteria have traditionally been distinguished based on morphological characteristics. One of the many characteristics used to differentiate species is the position of the akinete relative to the heterocyst. This characteristic is useful when identifying samples collected from nature, but in cultured strains, it is likely that either sporulation is delayed or that heterocyst formation occurs in response to the nutrients available in the medium. Moreover, in culture, the biometric characteristics of vegetative cells, heterocysts and akinetes can vary from those of natural specimens. Therefore, using morphological characteristics to classify cultured strains may give inaccurate results. Several methods have been made to classify the cyanobacteria inhabiting different areas by using serological methods, fatty acid profiling, RFLP analysis or PCR with arbitrary primers (Franche and Cohen-Bazire, 1987; Plazinsky et al., 1998, 1990; Eskew et al., 1993; Caudales et al., 1995; Van Coppenolle et al., 1995). Therefore in the present study, STRR-PCR fingerprinting was used to distinguish heterocystous cyanobacterial genera and to understand intergeneric as well as interspecific variations amongst the strains belonging to four genera.

The identification of cyanobacterial strains procured from designated repository was authenticated on the basis of 16S rRNA gene sequences and the sequences were compared with the GenBank database using BLASTn. These strains were identified initially on the basis of microscopic parameters following Cyanophyta (Desikachary, 1959). The STRR primer was used to distinguish the cyanobacterial strains belonging to four different genera namely *Anabaena*, *Nostoc*, *Calothrix* and *Microchaete*. Specific fingerprint patterns were obtained for thirty two cyanobacterial strains. The dendrogram constructed on the basis of fingerprint patterns showed two clusters. The major cluster, Cluster 1 was divided into two groups, in which group 1 showed sixteen *Anabaena* strains and the group 2 subdivided into two subgroups with subgroup 1 having nine *Nostoc* strains and subgroup 2 with four *Calothrix* strains. The other minor cluster 2 exhibited three strains of *Microchaete* species. The results clearly indicated distinct separation of strains belonging to different cyanobacterial genera. The results obtained based upon STRR fingerprint patterns were consistent with the earlier reports on the family Scytonemataceae along with several other reports from the phylogenetic tree derived from GenBank (Flechtner et al., 2002; Gugger and Hoffmann, 2003; Gugger and Hoffmann, 2004; Lucking et al., 2009; Novis and Visnovski, 2011). Based on the STRR fragments comparisons with each species of four genera, strains belonging to *Microchaete* were genetically distant from the other three genera examined. Interspecific closeness was also depicted amongst four genera based on STRR fragments similarity. The three genera (*Anabaena*, *Nostoc* and *Calothrix*) were found to be genetically close as compared to *Microchaete* indicating that the lineages shared a common and original monophyletic unit. In all the genera surveyed, the level of STRR polymorphism was high amongst the genera whereas high monomorphism was exhibited within the genera. The four genera showed different degrees of genetic variation based on STRR fragments. There was a higher level of intraspecific genetic similarity as compared to interspecific with STRR fingerprints. The genetic variability exhibited based upon STRR fingerprinting patterns clearly depicts the differences amongst the genera. This pattern of genetic variation in the four genera of heterocystous cyanobacteria suggests that genetic diversity and its relatedness in intra-specific strains and inter-specific strains using STRR fingerprinting approaches is consistent with the morphological and molecular (16S rRNA) identification.

The STRR primer gave distinct fingerprints for each of sixteen *Anabaena* species studied and the level of genetic diversity was found to be low among the sixteen *Anabaena* species. Large number of *Anabaena* strains showed closeness in having all of the whole octamer of bands. Fourteen strains of *Anabaena* occupied group Ia of cluster 1 confirming their similarity. Genetic diversity of sixteen *Anabaena* species existed primarily only in two species (*Anabaena oryzae* BTA19 and *Anabaena variabilis* BTA06). This can be attributed to different geographical locations in the rice fields from where these were isolated.

In the present study, the nine *Nostoc* species isolated from the rice fields of Manipur, India were grouped together in one evolutionary line of subgroup Ib of cluster1. BTA12 (*Nostoc hatei*), BTA27 (*Nostoc muscorum*), BTA37 (*Nostoc hatei*), BTA38 (*Nostoc carneum*), BTA947 (*Nostoc* sp.) and BTA1016 (*Nostoc* sp.) shared PCR products of equal mobility, and their overall fingerprint were quite similar. The generally lower level of STRR fragment polymorphism of *Nostoc* species studied may reflect a narrower range of intra-specific variation. In the generated phylogenetic tree, BTA87 (*Nostoc muscorum*) was diverted from the common branch whereas BTA29 (*Nostoc parmelioides*) and BTA923 (*Nostoc* sp.) were grouped in different branch due to low number of bands.

STRR fingerprint analysis exhibited low levels of polymorphism amongst the strains of *Calothrix* which showed eight unique molecular fragments. The major genetic difference in the BTA1059 (*Calothrix* sp.) was largely due to the absence of six unique molecular fragments. The dendrogram generated had a subgroup of Ib2, in which BTA24 and BTA206 were together in a same branch and BTA15 is diverted from the above two species and the

strain BTA1059 was present in separate branch. There was a clear separation of *Microchaete* species from the other three genera. All the three *Microchaete* species (*Microchaete uberrima* BTA01, *Microchaete grisea* BTA07 and *Microchaete loktakensis* BTA44) were in the minor cluster II.

Genetic diversity studies using STRR markers proved to be an effective tool to understand the cyanobacterial genera showing ubiquitous distribution in the rice fields of Manipur. Such an approach has been earlier used in cyanobacterial genera (Mazel et al., 1990). The present study clearly showed the intergeneric differences amongst *Anabaena*, *Nostoc*, *Calothrix* and *Microchaete* through STRR fingerprinting patterns. Distinct banding patterns were obtained for all the cyanobacterial strains and subsequent diversity could be drawn according to the size of fragments recorded.

Conflict of interest

The authors declare that there is no conflict of interest.

Acknowledgements

The authors are thankful to the Director, IBSD, Imphal, Manipur, India for providing laboratory facilities. This work was supported by a grant BT/PR11323/PBD/26/171/2008 from the Department of Biotechnology, Government of India.

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