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Diversity and phylogenetic analysis of heterocystous cyanobacteria using morphological, nitrogen fixation and molecular attributes

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Diversity and molecular phylogeny of 22 heterocystous cyanobacteria (Nostocales and Stigonematales), isolated from different paddy fields of Chhattisgarh, India, have been assessed on the basis of microscopic analysis, nitrogen fixing abilities, 16S rRNA and *nif*H gene sequences in conjunction with phylogenetic tools. Variations in morphological attributes along with nitrogen fixing capabilities suggested that these attributes are strains specific. Further, it was supported by the principal component analysis (PCA) plot which showed scattered placement of these heterocystous cyanobacterial strains. The monophyletic origin of the heterocystous clade and the polyphyly of the true branching Stigonematales were observed in the constructed dendrograms based on 16S rRNA and the *nif*H gene sequences. Thus, the use of morphological, physiological (nitrogen fixation) and molecular (16S rRNA and *nif*H gene sequences) parameters uncovered the better resolution of taxonomic and phylogenetic issues of heterocystous cyanobacteria.

Keywords: Cyanobacterial diversity, 16S rRNA, *nif*H gene, PCA, phylogeny

Introduction

With an evolutionary history tracing back to the archaean period, cyanobacteria are no doubt, amongst the most ancient organisms inhabiting the earth's environment[1,2]. The evolutionary history of cyanobacteria is thus, very old and they have played a major role in the evolution of life on Earth. The traditional scheme of cyanobacterial classification based on phenotypic characters has lately been observed to have some anomalies where morphological and molecular data have been found to be incoherent[3-7]. Hence, after deep exploration in cyanobacterial taxonomy, most of the studies have led to the application of an assortment/combination of various morphological, ecological and molecular data in which phylogenetic studies based on the 16S rRNA gene have been centrally used for generic definitions[8-14]. The cyanobacterial *nif*H gene has been well studied and well characterized particularly in the members of the Nostocales and Stigonematales orders[6,15,16]. As a consequence of the above mentioned works, a fair amount of debate has been done in assessing cyanobacterial systematics and phylogeny, particularly the heterocystous clades. Considerable high resolution taxonomic depths have been explored in the form of some monumental revisionary works that have been undertaken in the recent years[3,17-20]. According to the most recent classification of cyanobacteria[21], broadly four subclasses of cyanobacteria have been described: Gloeobacteriophycidae, Synechococcophycidae, Oscillatoriophycidae and Nostochopycidae. The order Nostocales a member of the subclass Nostochophycidae, is an assemblage of a large and monophyletic cluster of filamentous cyanobacteria characterized by the presence of heterocytes and akinetes along with a much diversified thallus structure.

Despite the importance of the heterocystous cyanobacteria in various ecological niches, cyanobacteria from Chhattisgarh, India have been poorly studied[22]. Therefore, the present communication deals with the assessment of diversity and molecular phylogeny of twenty two heterocystous cyanobacteria isolated from the different paddy fields of Chhattisgarh, India, using morphological attributes, and nitrogenase activity 16S rRNA, *nif*H gene sequences, and phylogenetic tools. The microscopic analysis, nitrogen fixation abilities along with the use of 16S rRNA gene and *nif*H gene sequences could be an important criterion for the resolution of taxonomic and phylogenetic issues of heterocystous cyanobacteria of these unexplored paddy fields of Chhattisgarh, India.

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Materials and Methods

Growth and Maintenance of Cyanobacteria

Twenty two heterocystous cyanobacterial strains from different paddy fields of Chhattisgarh, India were collected (Table 1). Pure colonies of cyanobacterial strains were raised on sterilized petri plates by the streak plate technique. After 14 days of growth one or two colonies were picked up, washed thrice with deionized water and transferred to fresh liquid medium. For maintenance of bacteria-free cultures, the colonies, which appeared free of bacteria, were isolated and tested for bacterial contamination in dextrose-peptone broth and caseinate-glucose agar media. Thereafter, the pure cultures were grown axenically in 150 ml basal medium (BG-11 medium) in Erlenmeyer flasks (capacity 250 ml) and were maintained in culture room at standard growth condition[5,22]. The identification of the heterocystous cyanobacteria was done using the keys of Desikachary[23-24].

Table 1 — List of cyanobacterial strains along with 16S rRNA and *nif*H NCBI Accession numbers

S. No	Cyanobacterial Strain	16S rRNA NCBI Accession Numbers	*nif*H NCBI Accession Numbers
1	*Nostoc punctiforme* CG1	KJ774509	KT074971
2	*Nostoc* sp. CG2	KJ774510	KT074972
3	*Nostoc commune* CG3	KJ774511	KT074973
4	*Anabaena variabilis* CG4	KJ774512	KT074974
5	*Nostoc linckia* CG5	KJ774513	KT074975
6	*Nostoc* sp. CG6	KJ774514	KT074976
7	*Nostoc carneum*	KJ774515	KT074977
8	*Nostoc*sp. CG8	KJ774516	KT074978
9	*Nostoc calcicola* CG9	KJ774517	KT074979
10	*Nostoc*sp. CG10	KJ774518	KT074980
11	*Nostoc khannae* CG11	KJ774519	KT074981
12	*Anabaena doliolum* CG12	KJ774520	KT074982
13	*Anabaena sphaerica* CG13	KJ774521	KT074983
14	*Anabaena oryzae* CG14	KJ774522	KT074984
15	*Scytonema bohnerii* CG15	KJ774523	KT074985
16	*Cylindrospermum muscicola*	KJ774524	KT074986
17	*Cylindrospermum* sp. CG17	KJ774525	KT074987
18	*Cylindrospermum stagnale* CG18	KJ774526	KT074988
19	*Westiellopsis* sp. CG19	KJ774527	KT074989
20	*Fischerella muscicola* CG20	KJ774528	KT074990
21	*Tolypothrix tenuis* CG21	KJ774529	KT074991
22	*Mastigocladus laminosus* CG22	KJ774530	KT074992

Microscopic Analysis of Cyanobacteria

Morphological analysis of the cyanobacterial strains was done on the basis of shape and dimension (length x breadth) of the vegetative cells, shape, dimension and position of the heterocysts and akinetes by using Leica DM 2000 microscope fitted with digital camera (Table 1). Cyanobacterial images were captured at 40 X magnification.

Nitrogenase Activity

Nitrogenase (EC 1.18.6.1) activity of the cyanobacterial cultures was estimated by the acetylene reduction assay (ARA)[25]. Cyanobacterial culture (2 ml) was taken in a calibrated stoppered vessel with 10% v/v acetylene gas and was incubated at 29°C for 6 h. Then, ethylene (C_2H_4) produced in the vessel was analyzed by a gas chromatograph. Subsequently, ethylene (100 µl) gas was withdrawn and injected directly onto a gas chromatograph and nitrogenase activity of cyanobacteria was expressed in nmole C_2H_4 mg^{-1} protein h^{-1}.

Principal Component Analysis

To describe the variability among all the morphological and physiological data and also to evaluate the importance of these data, principle component analysis (PCA) was performed using Biodiversity Professional version 2 program. The graphical representation of the values generated by PCA was done by Sigmaplot version 12.1 (Fig. 2).

Genomic DNA Isolation and PCR Conditions

The genomic DNA was isolated from only 12-14 days old log phase cultures using Hi-media Ultrasensitive Spin Purification Kit (MB505). The eluted DNA was stored at -20°C. Oligonucleotide primers fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rD1 (5'-AAGGAGGTGATCCAGCC-3') were used for the amplification of nearly 1600 bp fragments[26]. PCR amplification of the 16S rRNA gene was done (BioRad, DNA Engine, Peltier Thermal cycler) according to the following program: initial denaturation at 95°C for 3 minutes, 30 cycles of 30 seconds denaturation at 94°C, 40 seconds annealing at 55°C, and 1.5 minute extension at 72°C. A final extension at 72°C for 3 minutes was done. Degenerate primers flanking a conserved region of the *nif*H gene were selected and cross-checked using BLASTp algorithm. The primers used were *nif*Hf (5′-CGTAGGTTGCGACCCTAAGGC-

TGA-3′) and *nif*Hr (5′-GCATACATCGCCATCATTT CACC-3′), which targeted a fragment of approximately 297 bp[27]. The PCR amplification of *nif*H was performed in 25-µl aliquots containing 10–25 ng DNA template, 0.5 µM of each primer, 1.5 mM $MgCl_2$, 200 µM dNTPs, and 1 U/µl *Taq* polymerase. The template was initially denatured at 95°C for 5 min. This was followed by 30 cycles of denaturation for 15 s at 95°C, 30 s of annealing at 48°C and 1 min of extension at 72°C, followed at last by the final extension step of 5 min at 72°C. The amplified products were visualized on Bio-Rad Gel Documentation System after running in 1.2 % agarose gel.

Sequence Analysis and Submission of Sequences to NCBI

The DNA sequences obtained were cross checked through the nucleotide blast (*blastn*) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) program for assessing the relatedness of the heterocystous cyanobacterial strains with each other. The sequences were submitted to the NCBI database using the online program Bank. It with proper annotations in the standard format. The list of cyanobacterial strains used in the present investigation along with their accession numbers have been given in Table 2.

Table 2—Morphological characteristics of cyanobacterial strains used in the present study.

Cyanobacterial Strains	Vegetative Cells			Heterocysts			Akinetes			N_2 Fixation			
	Shape	Dimension (µm) (Length)	(Width)	Shape	Dimension (µm) (Length)	(Width)	Position	Shape	Dimension (µm) (Length)	(Width)	Position	µmole C_2H_4 produced mg^{-1} Chl*a* min^{-1}
Nostoc punctiforme CG1	Ellipsoidal	3.5±0.11	3.00±0.09	Oblong	5.0±0.12	4.5±0.19	Intercalary	Subspherical	5.0± 0.10	7.0±0.15	Intercalary	6.92 ±0.42
Nostoc sp.CG2	Spherical	3.5±0.12	3.5±0.11	Spherical	5.7±0.21	6.0±0.20	Intercalary	Oval	7.5±0.24	8.0±0.22	Intercalary	7.62 ±0.65
Nostoc commune CG3	Subspherical	5.0±0.12	4.75±0.08	Spherical	8.0±0.30	8.2±0.19	Intercalary	Oval	9.2±0.12	7.0±0.15	Intercalary	6.84±0.34
Nostoc linckia CG5	Oval	4.5±0.15	4.0±0.13	Subspherical	7.0±0.23	6.7±0.20	Intercalary	Spherical	8.0±0.20	7.5±0.22	Intercalary	7.04±0.25
Nostoc sp.CG6	Spherical	4.0±0.05	3.75±0.02	Spherical	6.0±0.02	6.2±0.03	Intercalary	Subspherical	8.0±0.29	8.5±0.30	Intercalary	8.92±0.64
Nostoc carneum CG7	Cylindrical	4.0±0.15	3.5±0.14	Oblong	7.0±0.20	6.5±0.30	Intercalary	Ellipsoidal	8.0±0.32	6.0±0.2	Intercalary	6.34±0.48
Nostoc sp.CG8	Cylindrical	4.0±0.11	3.0±0.08	Oblong	8.0±0.29	6.0±0.22	Intercalary	Oval	9.0±0.25	6.5±0.13	Intercalary	6.72±0.28
Nostoc calcicola CG9	Barrel	2.0±0.09	2.5±0.01	Subspherical	4.0±0.14	4.5±0.15	Intercalary	Oval	7.0±0.21	5.0±0.12	Intercalary	7.54±0.52
Nostoc sp.CG10	Cylindrical	4.5±0.19	4.0±0.12	Oblong	6.5±0.20	5.0±0.19	Intercalary	Oval	7.2±0.14	6.0±0.15	Intercalary	6.81±0.65
Nostoc khannae CG11	Spherical	4.0±0.11	3.7±0.09	Spherical	5.0±0.20	5.0±0.19	Intercalary	Spherical	6.2±0.25	6.0±0.21	Intercalary	8.42±0.46
Anabaena doliolum CG12	Barrel	3.5±0.12	4.0±0.13	Barrel	7.5±0.22	5.5±0.15	Intercalary	Barrel	9.0±0.25	6.3±0.21	Intercalary	8.82±0.36
*Anabaena variabilis*CG4	Cylindrical	5.0±0.20	4.5±0.19	Oval	8.0±0.30	6.0±0.12	Intercalary	Spherical	11.0±0.35	5.0±0.24	Intercalary	7.19±0.28
Anabaena sphaerica CG13	Spherical	6.5±0.23	5.0±0.18	Oval	8.0±0.21	6.5±0.20	Intercalary	Spherical	12.0±0.21	10.5±0.32	Intercalary	8.26±0.58
Anabaena oryzae CG14	Barrel	3.0±0.08	3.5±0.11	Ellipsoidal	5.0±0.12	5.5±0.10	Intercalary	Spherical	6.5±0.25	7.0±0.20	Intercalary	10.12±0.54
Scytonema bohnerii CG15	Cylindrical	5.0±0.15	6.0±0.10	Cylindrical	10.0±0.33	8.0±0.20	Intercalary	-	-	-	-	4.68±0.22
Cylindrospermum muscicola CG16	Cylindrical	5.0±0.12	4.0±0.14	Barrel	7.0±0.11	5.0±0.18	Terminal	-	-	-	-	5.12±0.16
Cylindrospermum sp.CG17	Hemispherical	5.0±0.10	5.5±0.15	Barrel	9.0±0.35	10.5±0.22	Terminal	-	-	-	-	5.62±0.37
Cylindrospermum stagnale CG18	Elliptical	4.0±0.08	4.50±0.13	Barrel	8.5±0.19	7.0±0.25	Terminal	Oval	35.0±0.35	12.0±0.32	Basal	5.96±0.28
Fischerella muscicola CG20	Subspherical	8.0±0.25	7.5±0.24	Spherical	10.0±0.75	7.0±0.25	Intercalary	-	-	-	-	4.12±0.16
Tolypothrix tenuis CG21	Quadrate	6.0±0.11	5.0±0.16	Cylindrical	7.0±0.15	10.0±0.35	Intercalary	-	-	-	-	4.52±0.32
Mastigocladus laminosus CG22	Oblong	5.0±0.14	3.0±0.03	Cylindrical	9.0±0.20	6.5±0.05	Intercalary	-	-	-	-	5.18±0.38
Westiellopsis sp.CG19	Barrel	8.0±0.27	9.5±0.15	Oblong	11.0±0.22	5.9±0.17	Intercalary	-	-	-	-	5.17±0.27

Phylogenomics of Heterocystous Cyanobacteria Using the 16S rRNA Gene and *nif*H Gene Sequences

16S rRNA and *nif*H gene sequences were used to construct the phylogenetic tree using MEGA 5.1 Beta2 version in accordance with the interior test of phylogeny using the maximum parsimony[28]. Thus, for the present analyses, we adopted an approach that was robust along with presenting a balanced scheme of phylogeny of heterocystous cyanobacteria.

Results

Morphological Analysis and N_2 Fixation Abilities

All the twenty two strains which were studied on the basis of their morphology exhibited varying degrees of dimensions in their length and width. The vegetative cells, heterocysts, akinetes along with their shape, size and positions in conjunction with nitrogen fixing abilities were assessed and the trends obtained clearly reflected the amount of diversity present amongst the twenty two strains. The different families represented in this study *viz*., Nostocaceae, Scytonemataceae, Aphanizomenonaceae, Hapalosiphonacaeae, and Tolypothricaceae were demarkable by the characteristic features of the representatives of these families (Fig. 1a-d; Table 2).

16S rRNA Gene based Phylogenetic Analysis

The 16S rRNA based maximum parsimony (MP) tree (Fig. 3) consisted of many small clusters and sub-

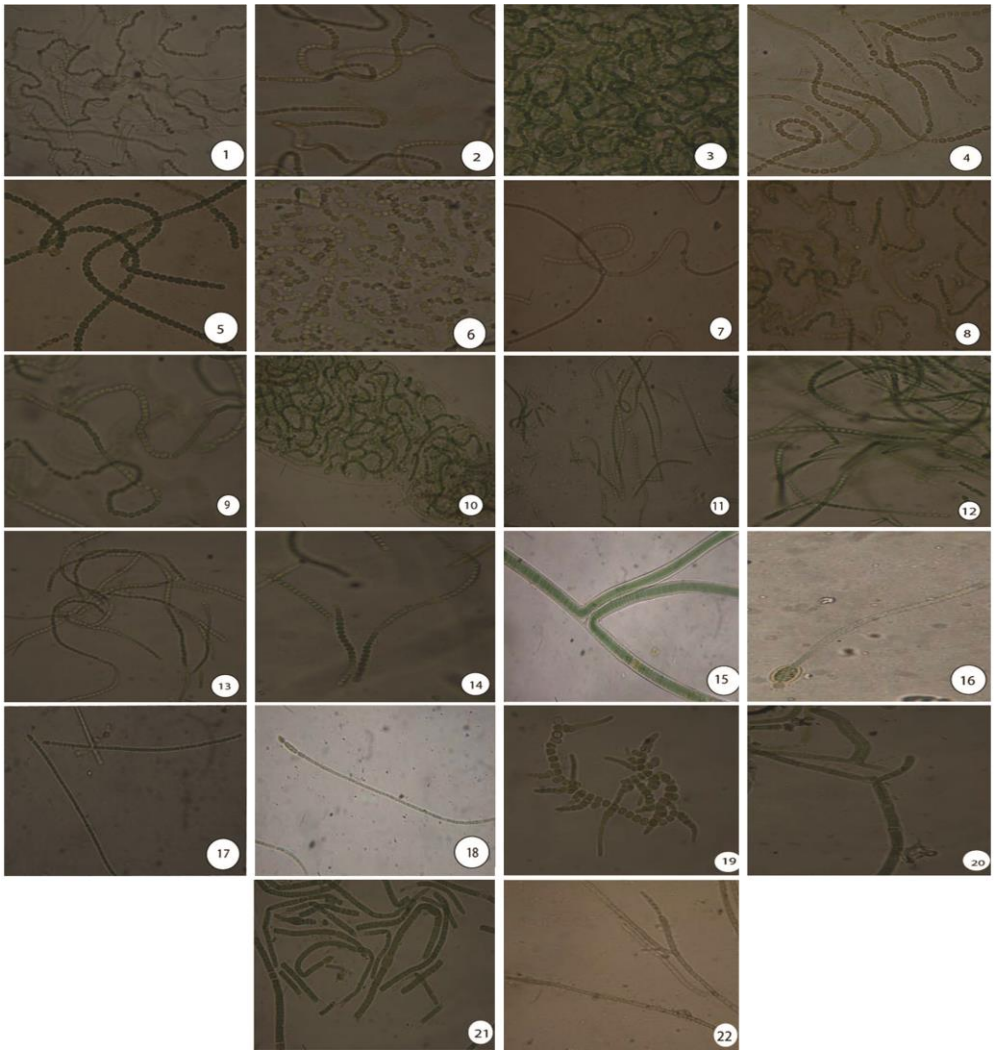

Fig. 1a — (1) *Nostoc punctiforme* CG 1 (2) *Nostoc* sp. CG 2 (3) *Nostoc commune* CG 3 (4) *Anabaena variabilis* CG 4 (5) *Nostoc linckia* CG 5 (6) *Nostoc* sp. CG 6; 1b — (7) *Nostoc carneum* CG 7 (8) *Nostoc* sp. CG 8 (9) *Nostoc calcicola* CG 9 (10) *Nostoc* sp. CG 10 (11) *Nostoc khannae* CG 11 (12) *Anabaena doliolum* CG 12; 1c — (13) *Anabaena sphaerica* CG 13 (14) *Anabaena oryzae* CG 14 (15) *Scytonema bohnerii* CG 15 (16) *Cylindrospermum muscicola* CG 16 (17) *Cylindrospermum* sp. CG 17 (18) *Cylindrospermum stagnale* CG 18; 1d — (19) *Westiellopsis* sp. CG 19 (20) *Fischerella muscicola* CG 20 (21) *Tolypothrix tenuis* CG 21 (22) *Mastigocladus laminosus* CG 22.

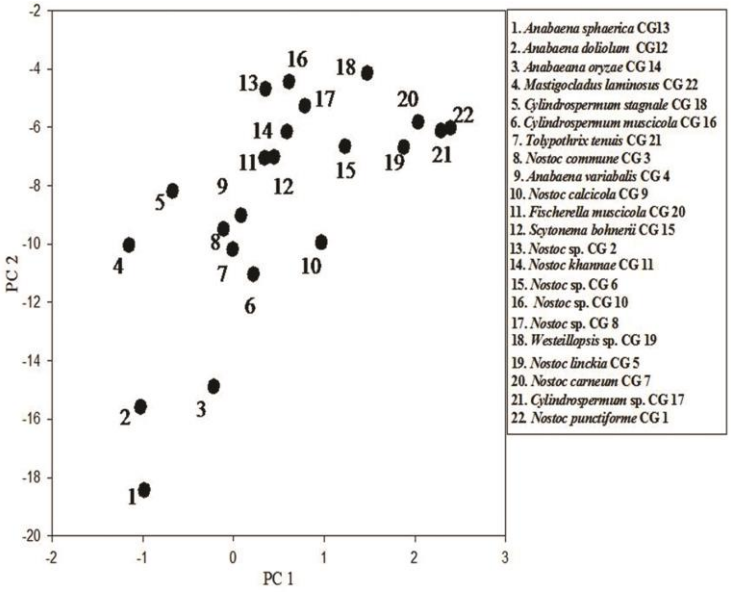

Fig. 2 — Representation of principle component analysis on the basis of morphological features among the cyanobacterial strains.

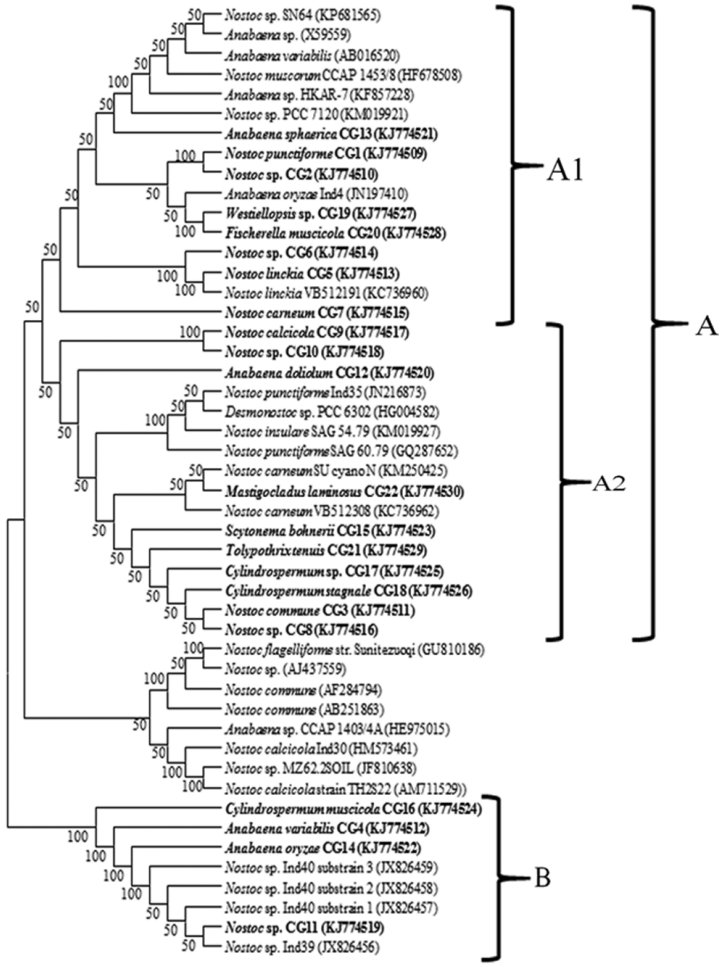

Fig. 3 — Maximum parsimony analysis of heterocystous cyanobacterial strains using 16S rRNA gene sequences.

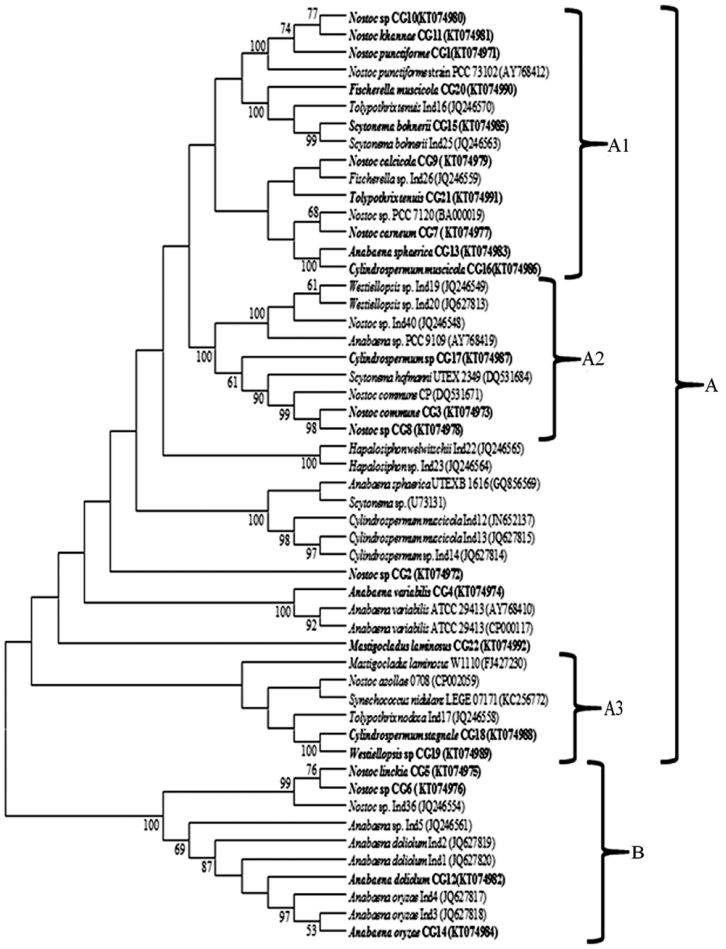

Fig. 4 — Maximum parsimony analysis of heterocystous cyanobacterial strains using *nif*H gene sequences.

clusters with no prominent clustering pattern being visible. Overall, the tree consisted of two major clusters A and B. The cluster A comprised of two sub-clusters A1 and A2. The sub-cluster A1 comprised of the genus *Anabaena*, *Nostoc*, *Westiellopsis*, *Fischerella*. The sub-cluster A2 consisted of the strains represented by the genus *Nostoc*, *Anabaena*, *Mastigocladus*, *Scytonema*, *Tolypothrix* and *Cylindrospermum*. The other major cluster B was represented by the genus *Nostoc*, *Anabaena* and *Cylindrospermum*.

nifH Gene based Phylogenetic Analysis

In the *nif*H gene based tree (Fig. 4) the major clusters were A and B. The cluster A consisted of the sub-clusters A1, A2 and A3. The sub-cluster A1 consisted of the strains *Nostoc*, *Fischerella*, *Scytonema*, *Anabaena*, *Tolypothrix* and *Cylindrospermum*. The sub-cluster A2 consisted of the genus *Nostoc* and *Cylindrospermum*. The sub-cluster A3 had representatives of the genus *Mastigocladus*, *Cylindrospermum* and *Westiellopsis*. The cluster B consisted only of the members of the order Nostocales with *Nostoc* and *Anabaena* being the prominent genera.

Principal Component Analysis

The PCA analysis performed revealed a scattered plot with strains being placed irrespective of their phylogenetic affinities. *Anabaena spherica* CG13, *Anabaena doliolum* CG12 and *Anabaena oryzae* CG14 separated distantly from the rest of the cyanobacterial strains. *Mastigocladus laminosus* CG22 and *Cylindrospermum stagnale* CG18 were placed in an intermediate position in the plot. Rest of all the cyanobacterial strains paired with each other at close distances irrespective of their taxonomic status (Fig. 2).

Discussion

The area sampled were different paddy fields of Chhattisgarh, India which are poorly studied. So,

our primary aim was to assess the diversity and molecular phylogeny among the Nostocalean and Stigonematalean strains. This attempt could possibly be helpful in resolution of taxonomic and phylogenetic issues of heterocystous cyanobacterial.

Phylogenetic Analyses

The phylogenetic analyses of the 16S rRNA and *nif*H gene sequences did provide some crucial insights into the genetic diversity and the phylogenetic relatedness among the heterocystous cyanobacteria of the paddy fields. *Nostoc* strains exhibited the maximum genetic heterogeneity in both the trees, thus concaving with earlier reports[6,29]. Also, the *Anabaena* strains once again showed conflicting affiliations in both intra-generic and inter-generic perspectives. The constant pairing of *Anabaena oryzae* with *Nostoc khannae* again ignited the issue of genetic proximity of the *Nostoc* and *Anabaena* genus. Overall, it was still evident that the genus *Nostoc* and *Anabaena* were having the maximum heterogeneity amongst all the studied genera[6,29-30]. But, the debate over the separation or merger of the genus *Nostoc* and *Anabaena* still remained indecisive[6,31]. The genus *Cylindrospermum* which had three representatives in the present study was found to be diverging even within itself with *Cylindrospermum muscicola* parting ways with the other two isolates viz., *Cylindrospermum* sp. and *Cylindrospermum stagnale*. This divergence of *Cylindrospermum muscicola* and *Cylindrospermum stagnale* found in our study was found to be incoherent with the other studies of these strains[6,29]. This also indicated towards the possible effect of environmental conditions on the genetic diversity of heterocystous cyanobacteria. The false branching strain *Scytonema bohnerii* that we studied showed a pairing tendency with the reference strain from India, thus indicating towards the possible genetic relatedness of these false branching forms. The strain *Tolypothrix tenuis* showed affinities towards *Anabaena*, *Nostoc* and *Scytonema*, thus making the clustering tendency of this genus obscure and uncertain. The true branching strain *Mastigocladus laminosus* paired with the gene bank reference strain of the similar type but also showed affiliation at a loose level (bootstrap value of 65) with gene bank reference strains of the genus *Nostoc*. The other two stigonematalean members in our study *Westiellopsis* sp. and *Fischerella muscicola* paired with each other with good bootstrap values in the 16S rRNA tree but aligned very far from each other in the *nif*H tree. Thus the true branching cyanobacteria also, showed prominent polyphyletic roots along with significant intermixing with the unbranched Nostocales order[6,16,29,32,33].

Thus, our phylogenetic analyses using the 16S rRNA gene and the *nif*H gene as a molecular marker failed to delineate the order Nostocales and the Stigonematales (as per the traditional classificatory system) from each other. This study also proved that although the 16S rRNA gene can be a good identification marker for heterocystous cyanobacteria but its use for assessing phylogenetic relationships is limited possibly because of its low resolution in delineating closely related cyanobacteria of same species[13,34]. Further, it is also recommended that improved resolution and coverage could be obtained using a polyphasic approach in sync with multi-locus molecular studies. The scattered placement of the members of the orders Nostocales and Stigonematales reignited the complex debate over the taxonomic proximity of these two orders. Due to phenotypic plasticity and environmental constraints, cyanobacterial taxonomy is still a matter of debate[7,15,29,30]. Hence, the PCA plot obtained has clearly reflected the above mentioned attributes. The orders Nostocales and Stigonematales are intermixed and the trends obtained in the PCA plot conclave with these findings. This plot gains importance because of it having been constructed on the basis of morphological characters. Hence, it is evident that these two orders are intermixed even when studied on a morphological scale[24]. Thus the PCA plot very firmly corroborates with our phylogenetic findings.

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Conflict of interest

The authors declare that they have no conflict of interest.

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