Protein fingerprinting may serve as a complementary tool for the phylogenetic classification of heterocystous (Nostoc, Anabaena, Cylindrospermum, Aulosira and Tolypothrix) Cyanobacteria

S Mishra, P Bhargava, R Rai, Y Mishra, T Zotta, E Parente, L Rai

Citation

S Mishra, P Bhargava, R Rai, Y Mishra, T Zotta, E Parente, L Rai. *Protein fingerprinting may serve as a complementary tool for the phylogenetic classification of heterocystous (Nostoc, Anabaena, Cylindrospermum, Aulosira and Tolypothrix) Cyanobacteria*. The Internet Journal of Microbiology. 2008 Volume 7 Number 2.

Abstract

A combination of morphology, SDS-PAGE, 16S rRNA gene and the fuzzy approach has been used for the first time to attest the phylogenetic affiliation of the cyanobacterial species such as Nostoc, Anabaena, Cylindrospermum, Tolypothrix and Aulosira. The idea was to ascertain as how far SDS-PAGE analysis and morphological taxonomy work coherently and serve as a reliable tool, compatible with 16S rRNA gene based classification of cyanobacteria. Intermingling of Nostoc and Anabaena species in SDS-PAGE and 16S rRNA gene indicates that the two taxa are not clearly separated at the genetic level and may be polyphyletic in origin. In contrast to this, clustering of Tolypothrix and Aulosira species in the same clade attests the taxonomic coherence of these species. However, Cylindrospermum showed greater phylogenetic relatedness with Anabaena strain in all phylogenetic analyses. Despite a few minor incongruities, protein fingerprint depicted greater similarity with 16S rRNA gene as compared to morphological taxonomy. Thus protein fingerprinting developed using fuzzy approach appears to be more compatible with 16S rRNA gene analysis than morphological taxonomy in discriminating cyanobacteria up to the species level.

INTRODUCTION

Cyanobacteria are the most widespread, photosynthetic prokaryotes with remarkable degrees of morphological and developmental diversity. Among the heterocystous cyanobacteria, Nostocalean strains are considered to be the most important component of the N_2-fixing community in nutrient poor, arid and semiarid soils world wide (Doods et al., 1995; Potts, 2000; Wynn-Williams, 2000; Bhatnagar and Bhatnagar, 2005).

Cyanobacteria have been traditionally classified on the basis of their morphological and physiological characteristics (Gietler, 1932; Desikachary, 1959). However, morphology of the strains may change depending on environmental conditions and the diversity of the strains can be altered by selective culture conditions (Palinska et al., 1996). For example the strains of Nodularia were found to loose gas vesicles (Lehtimäki et al., 2000) and Aphanizomenon flos-aquae formed colony (Gugger et al., 2002) in their culture conditions. Komárek & Anagnostidis (1989) have estimated that more than 50% of the strains in culture collections are misidentified. To overcome these limitations, molecular method based on the use of small subunit rRNA gene has been proposed (Woese, 1987). The conservative nature of 16S rRNA gene, its universal distribution and the vast availability of sequence information in public databases (Genbank, EMBL, DDBJ and RDP) make it the marker of choice for taxonomical studies (Weisburg et al., 1991). The classification based on 16S rRNA gene analysis has been often compared with morphological identification and suffers from several limitations. Palinska et al., (1996) demonstrated that the divergence of the genera Synechococcus, Synechocystis, Merismopedia and Eucapsis based on morphological characters was not reflected in genetic diversity. Thus there is a need to develop another marker, which can complement the 16S rRNA gene based phylogeny in a more reliable manner as compared to morphology.

SDS-PAGE whole cell protein pattern analysis has emerged as a powerful tool for bacterial identification having the advantages of being fairly fast and easy and, when performed under highly standardized conditions, it offers a better taxonomic resolution at species or subspecies level

(Vandamme et al., 1996; Derbyshire and Whitton, 1968; Kersters and De Ley, 1975; Elliot et al., 1993). The SDS-PAGE protein profiles analysis has been successfully used to characterize various species of Lactobacillus and Leuconostoc (Perez et al., 2000), Staphylococcus (Berber et al., 2003) and thermophillic bacteria (Liu et al., 2006). However, the use of this technique in the characterization of cyanobacteria is still debatable. Palinska et al., (1996) used it for the first time to characterize Merismopedia and suggested it to be an efficient method to complement the 16S rRNA gene data. On the contrary, Lyra et al., (1997) employed SDS-PAGE fingerprint for the characterization of unicellular and filamentous cyanobacteria but did not find it suitable for phylogenetic assessment up to the species level. However, these limitations can be overcome by using computing facility for storage and processing of large datasets.

The logistic weighting function is a procedure which transforms complex electrophoretic patterns consisting of two vectors of data (molecular weight and band intensities) with varying length into a single vector of fixed length consisting of band intensities accumulated in classes of molecular weight. This data reduction by logistic weighting function provides better cluster pattern as compared to commercial software like diversity database. This approach has already been utilized in case of bacteria (Piraino et al., 2002). Since this approach has never been used before to study cyanobacteria, this paper is the first to employ the reduction of electrophoretic data using logistic weighting function to assess the phylogenetic position of cyanobacteria and to evaluate if the SDS-PAGE identification can be comparable with 16S rRNA gene based classification. The present study is the first attempt to characterize 21 newly sequenced strains of cyanobacteria by harmonizing the approaches of SDS-PAGE fingerprints, 16S rRNA gene and morphology along with bioinformatic tools such as WINCLADA and fuzzy.

MATERIALS AND METHODS

STRAINS, MEDIA AND CULTURE CONDITIONS

Twenty-one strains used in the study were isolated from paddy fields of Eastern Uttar Pradesh. Anabaena sp. PCC7120 was chosen as reference strain. Strains were maintained and cultured in BG-11 medium (Hughes et al., 1958) buffered with Tris/HCl (pH 7.5) under continuous cool white fluorescent light 72 µmol photon m^{-2} s^{-1} PAR irradiance with a photoperiod of 14:10 h (light: dark) at 24 ± 2°C. Cultures were manually shaken 2 to 3 times daily.

MORPHOLOGICAL ASSESSMENT AND PHYLOGENIC TREE CONSTRUCTION

Morphological characterization of 21cyanobacterial strains were conducted using trinocular microscope (Kyowa Getner, Opto-Plan, 2KT, Japan, Model No. 90116) equipped with high resolution Nikon digital camera (model no. DXm 1200). Morphological characters were described according to traditional criteria (Desikachary, 1959; Geitler, 1932). All the (16) morphological characters used to prepare a matrix for WINCLADA are listed in Table 1 and described in detail in Appendix 1.

Among the characters only the presence and absence of sheath and hormogones were codified as binary data, while the remainings were codified as multivariate data. Composite coding was used in preference to binary coding in order to minimize the occurrence of inapplicable or missing entries. Unknown determinations of the true state or missing data, are represented in the matrix by a "?". Primary causes of missing data include character states being not visible in the material available. The order of appearance of the characters in the matrix is functional facilitating future data addition.

The phylogenetic tree was constructed using WINCLADA version 1.00.008 (Nixon, 1999). The maximum parsimony analysis of above mentioned characters was done selecting Arthrospira maxima as an outgroup. The data matrix was analysed using WINCLADA applying the following search parameters for Nona's heuristic search: maximum trees to keep (hold) =100, number of replications (mult* N) =10, starting trees per rep (hold/) =10. A multiple tree bisection-reconnection (TBR) plus TBR (mult* max*) search strategy was employed. All the characters were weighted equally. The distribution of each morphological character and different character states of the isolates was then analyzed to get the most consensus phylogenetic tree through bootstrapping by using consensus compromise following majority fools option.

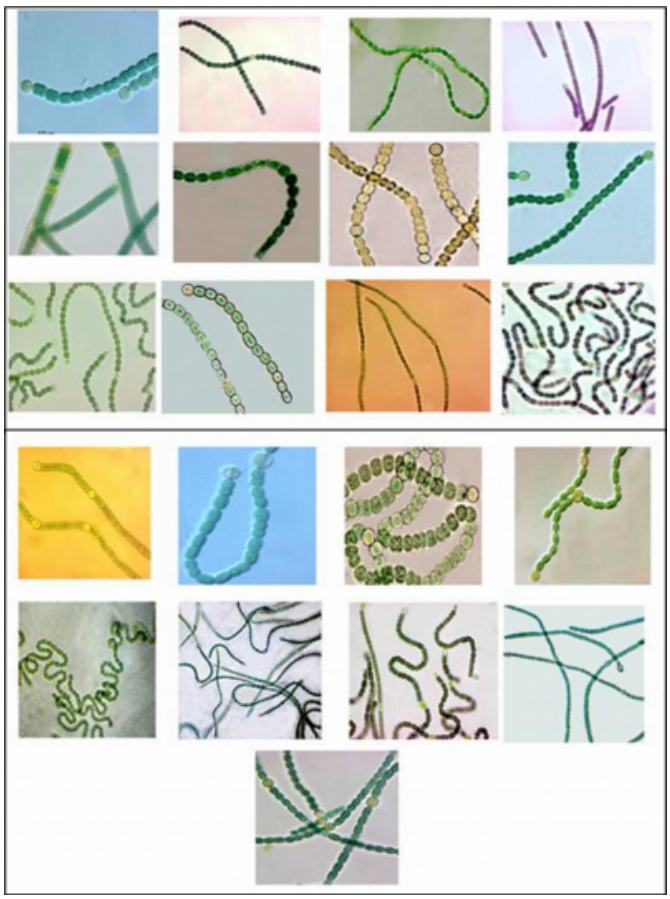
Figure 1

Table 1: Distribution of 16 morphological characters among exempler species. Character states are scored as 0-9, ? (Indeterminate). Refer to Appendix 1 for character descriptions

Taxa	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Arthrospira maxima	0	0	(0,1)	1	?	(0,2)	?	0	0	0	0	0	0	0	0	1
Anabaena doliolum	0	0	(0,8)	0	0	(0,1)	2	2	1	1	3	1	(1,2)	0	0	?
Anabaena oryzae	3	0	(0,5)	0	0	(0,1)	0	1	5	2	3	(1,3)	4	0	0	?
Aulosira fertilissima	0	1	(0,5)	(1,2)	?	1	5	2	1	2	5	(1,9)	2	0	8	?
Anabaena anomala	0	0	(0,2,8)	1	(0,4)	1	2	1	1	3	5	?	?	0	0	?
Cylindrospermum muscicola	1	1	0	0	(1,2)	0	0	1	2	1	3	4	(2,3)	0	0	?
Tolypothrix tenuis	(0,5)	1	5	(1,2)	(2,8)	(1,2)	?	(2,3)	(1,3)	3	?	?	?	6	5	1
Nostoc spongiaeforme	(0,9)	1	(1,2,5)	0	(0,1)	1	7	2	5	2	2	9	2	0	0	?
Nostoc piscinale	(0,2)	1	(2,5,6)	(0,1)	1	1	?	2	5	2	2	3	2	0	0	?
Anabaena torulosa	0	0	(0,5)	0	(0,8)	1	2	2	(1,2)	1	3	2	(2,3)	0	0	?
Nostoc ellipsosporum	4	1	(2,5,6)	0	1	(1,2)	?	2	5	1	2	(1,2)	2	0	0	?
Nostoc rivulare	(0,2)	1	(2,5,6)	1	(0,4)	1	?	2	5	1	2	6	2	0	0	?
Nostoc species LCR21	(0,4)	1	(2,5)	(0,1)	(4,5)	1	?	1	5	4	0	0	0	0	0	?
Nostoc species LCR17	1	1	(3,5)	0	(0,1)	1	?	1	5	1	2	3	2	0	0	?
Nostoc punctiforme	5	1	(6,7)	0	(0,5)	1	?	(1,2)	5	1	2	3	2	0	0	?
Anabaena fertilissima	(0,9)	0	0	(0,1)	1	0	0	2	(1,2)	1	3	3	3	0	0	?
Nostoc linckia	(0,9)	1	(2,5)	0	0	1	?	1	5	1	2	3	2	0	0	?
Nostoc paludosum	6	1	(2,4)	0	0	1	?	2	5	1	2	4	2	0	0	?
Nostoc carneum	4	1	(2,5,6)	0	(1,3)	(2,3)	?	2	5	1	2	(1,4)	2	0	0	?
Anabaena constricta	4	0	0	0	0	0	?	1	5	1	3	3	2	0	0	?
Anabaena sp. PCC7120	0	0	3	(0,1)	1	(0,1)	2	(1,2)	(1,2)	2	3	7	2	0	0	?
Nostoc speciesLCR12	4	1	(2,5)	0	0	1	?	(2,3)	5	5	5	(1,4)	2	0	0	?

Figure 2

Figure 1. Microphotographs illustrating the morphological appearance of



(1) Anabaena doliolum LCR1, (2) Cylindrospermum muscicola LCR6, (3) Nostoc species LCR21,(4) Tolypothrix tenuis LCR7, (5) Aulosira fertilissima LCR4, (6) Anabaena anomala LCR5,(7) Nostoc paludosum LCR16, (8) Nostoc spongiaeforme LCR10, (9 Anabaena oryzae LCR2, (10), Anabaena constricta LCR23, (11) Nostoc piscinale LCR22, (12) Nostoc species LCR17, (13) Anabaena fertilisssima LCR23, (14) Nostoc species LCR12, (15) Anabaena toruolsa LCR24, (16) Nostoc ellipsosporum LCR14, (17) Nostoc carneum LCR19, (18) Nostoc linckia LCR11, (19) Nostoc punctiforme LCR15, (20) Anabaena sp. PCC7120, (21)) Nostoc rivulare LCR13

PROTEIN EXTRACTION AND SDS-PAGE ANALYSIS

Protein extraction was performed using a modified protocol of Wagener et al., (2002) as detailed in Bhargava et al.,(2008). SDS-PAGE was performed in 12% polyacrylamide gel according to Sambrook & Russel (2001). All chemicals were of Sigma grade (Sigma chemical Co., USA) unless and

otherwise stated. 15 µg of protein was loaded in the wells for each sample along with the Sigma wide range molecular weight marker in last lane. Gels were initially run at 15 mA (stacking gel run) and successively at 25 mA (resolving gel run) in a vertical elctrophoretic apparatus (Bio-Rad, USA). Gels were stained in Coomassie brilliant blue R-250 and destained in destaining solution. Gel images were recorded using the Chemi Doc EQ gel documentation system (Bio-Rad, USA)

SDS-PAGE DATA PROCESSING AND STATISTICAL ANALYSIS

The image analysis was performed using the Quantity one software (Bio-Rad, USA). The protein fingerprinting patterns were compared using the information on apparent molecular masses of bands, band intensity, normalized quantity, trace intensity, and Gauss intensity.

SDS-PAGE patterns were processed as described by Piraino et al., (2002), using a logarithmic transformation of molecular weight (log kDa). Classes (16; class width = 0.078 log kDa) were obtained in the range from 5 kDa (starting class) to 75 kDa (last class); flat range (FR) around the class centre and the membership in the flat range (MFR) were 30% and 99%, respectively, in all cases. Hierarchical cluster analysis (Unweighted Pair Group Method Using Average Linkage, UPGMA) was carried out on the matrix of similarity data, calculated by Pearson product-moment correlation of the logistic weighted SDS-PAGE patterns. Statistical analysis was performed using Systat 11.0 for Windows (Systat Software Inc., Richmond, CA, USA).

GENOMIC DNA EXTRACTION, 16S RRNA GENE AMPLIFICATION AND SEQUENCING

Total genomic DNA was extracted by the phenol free method of Srivastava et al., (2007). The amplification of 16S rRNA gene was performed using cyanospecific primers CYA106F (forward) and CYA781R (reverse) (Nübel et al., 1997). These primers were synthesized from Sigma Chemical Co. (USA). PCR reaction mixture (25 µl) contained 100 ng of genomic DNA, 2.5 µl of 10X PCR buffer with 15 mM $MgCl_2$, 200 µM dNTPs, 10 pmol of each primer and 0.2 U Taq DNA polymerase (Bangalore Genei, India). PCR amplification was perfrormed in a iCycler (Bio-Rad, USA) using the following temperature program: initial denaturation for 3 min at 94°C followed by 35 incubation cycles each consisting of 1.5 min denaturation at 94°C, 1 min annealing at 50°C, 2 min elongation at 72°C and a final 5 min elongation at 72°C. All amplifications were done in triplicates to ensure the reproducibility of results. The amplification products were resolved on 1. 2 % agarose gel and stained with ethidium bromide (1 µg ml^{-1}). PCR products (500 ng) were lyophilized in a Speed Vac concentrator, Model-SPD111V (Thermo Electron Corporation, USA). These lyophilized PCR products were subjected to purification and sequencing on commercial basis (Macrogen Inc., Korea). Partial 16S rRNA gene sequences of the isolates were deposited in the GenBank database with accession numbers EF066606- EF066611 and EU446006-EU4460021.

ALIGNMENT AND PHYLOGENETIC ANALYSIS

The 16S rRNA gene sequences of the twenty-one local isolates were multiple aligned using CLUSTAL X (1.83) (Thompson et al., 1997) and manually corrected by using Jalview. A phylogenetic analysis was performed using MEGA 4.0 platform (molecular evolutionary genetics analysis) (Tamura et al., 2007) an analytical package. With the aligned sequences Maximum-parsimony analysis in conjuction with a maximum mini Branch -&-bound option was carried out. To evaluate the robustness of branches in the inferred tree the bootstrap resampling of Felsenstein was employed using 111 random seed and 100 replicates and a consensus tree was generated.

RESULTS AND DISCUSSION

Cyanobacterial taxonomy has remained problematic for many years because of its dependence on morphological and ecological characters (Geitler, 1932; Desikachary, 1959). Taxonomic revisions are required to be done by a multidisciplinary approach including molecular, morphological, physiological, cytological and ecological (Suda et al., 2002; Hoffmann and Gugger, 2003; Komárek and Kaštovsk, 2003; Rajaniemi et al., 2005). In view of the above polyphasic approaches are now getting wider acceptability by taxonomists world over. This paper deals with phylogenetic reconstructions to check the consistency of the taxonomic assignment of the strains. In this study the grouping and closeness of strains in all the analyses have been discussed rather than the number of clades and the clustering pattern of taxa in the individual trees.

MORPHOLOGY BASED PHYLOGENETIC TREE

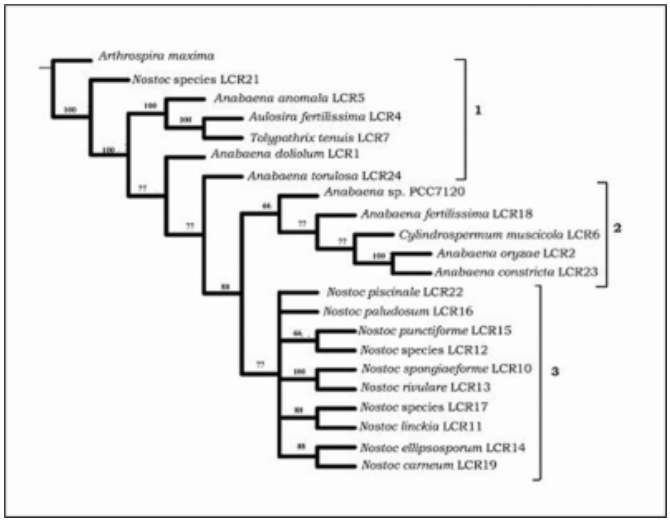
The cluster analysis carried out on the morphological characters grouped the strains in three major clusters (Fig. 2). Cluster 1 harbours Nostoc species LCR21, Anabaena anomala LCR5, Aulosira fertilissima LCR4, Tolypothrix

tenuis LCR7 with basal clade of Anabaena doliolum LCR1 and Anabaena torulosa LCR24. The clustering of Anabaena anomala LCR5, Anabaena doliolum LCR1 and Anabaena torulosa LCR24 is characterized by barrel shaped, 2-15µm wide cells and flattened end spores formed centrifugally adjacent to the heterocyst. Moreover, the clustering together of Aulosira and Tolypothrix can be ascribed on the grounds of the closeness in cell dimensions (5-15µm), cell shape (quadrangular) and most importantly, beginning of false branching in one and the developed branch in the other case (Fig. 1). On the other hand clade 2 is composed of reference strain Anabaena sp. PCC7120, Anabaena fertilissima LCR18, Cylindrospermum muscicola LCR6, Anabaena oryzae LCR2 and Anabaena constricta LCR23. This grouping can be ascribed to the common type of vegetative cell shape i.e. cylindrical, conica,l terminal heterocyst and akinetes positioned as adjacent to the heterocyst.

Further, cluster 3 emerged as a pure Nostoc clade composed of Nostoc piscinale LCR22, Nostoc paludosum LCR16, Nostoc punctiforme LCR15, Nostoc species LCR12, Nostoc spongiaeforme LCR10, Nostoc rivulare LCR13, Nostoc species LCR17, Nostoc linckia LCR11, Nostoc ellipsosporum LCR14, Nostoc carneum LCR19. This clade is characterized by their entangled wave like or tightly coiled aggregation, presence of prominent/diffluent sheath, short spherical/ovoid cells, predominant intercalary heterocyst and akinetes position distant from heterocyst. Probably these characteristic features make their grouping consistent. Contrary to the above, Nostoc species LCR21 did not fall within the Nostoc cluster. This could be attributed to the absence of akinetes in Nostoc species LCR21.

Figure 3

Figure 2: Maximum-Parsimony based phylogram of morphological data showing the clustering of , , , and



SDS-PAGE BASED PHYLOGENETIC TREE

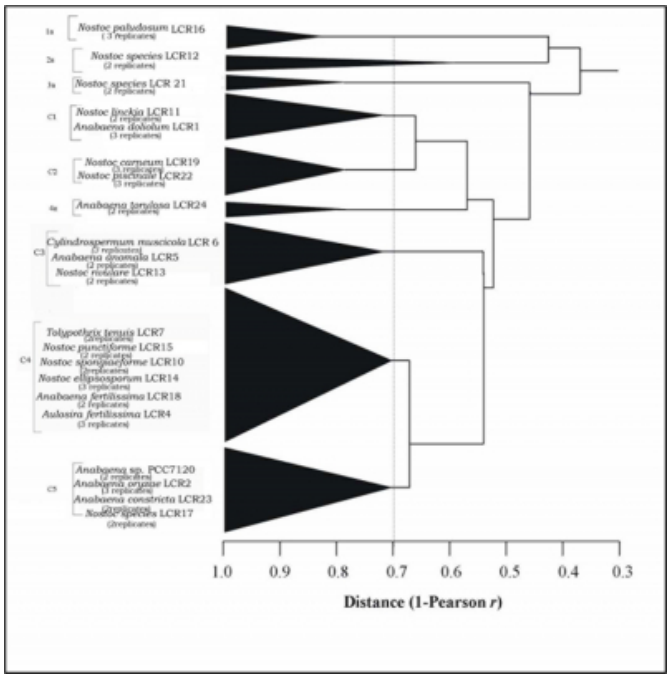
The hierarchical cluster analysis (Unweighted Pair Group Method Using Average Linkage, UPGMA) carried out using the logistic weighted SDS-PAGE data set generated five major clusters at a similarity level (Pearson product-moment coefficient) of 0.7 (Fig. 3). Only four strains (position 1a, 2a, 3a and 4a) failed to group in any major cluster because their SDS-PAGE profile was different from the other strains. Most of the strain replicates grouped together at a similarity level ranging from 0.99 to 0.85 showing that the analysis was reproducible. Cluster C1 and C2, in the upper section of the dendrogram, were very near. Cluster C1 grouped Nostoc linckia LCR11 and Anabaena doliolum LCR1 while cluster C2 included the replicates of the species of Nostoc carneum LCR19 and Nostoc piscinale LCR22. On the other hand clusters C3, C4 and C5 in the lower section of the dendrogram grouped most of the isolates. Cluster C3 included the strains Cylindrospermum muscicola LCR6, Anabaena anomala LCR5 and Nostoc rivulare LCR13. The largest cluster C4 grouped most of the Nostoc species such as Nostoc punctiforme LCR15, Nostoc spongiaeforme LCR10, Nostoc ellipsosporum LCR14, Anabaena fertilissima LCR18 and the strains of the species Tolypothrix tenuis LCR7 and Aulosira fertilissima LCR4. The nearest group of cluster C4 was cluster C5 that included

the strains of Anabaena sp. PCC7120, Anabaena oryzae LCR2, Anabaena constricta LCR23 and Nostoc species LCR17. SDS–PAGE analysis confirmed the high similarity level of the species Nostoc punctiforme LCR15, Nostoc

spongiaeforme LCR10 and Anabaena fertilissima LCR18 (Cluster C4). However, the species of Nostoc rivulare LCR5, Tolypothrix tenuis LCR7, Aulosira fertilissima LCR4 and Anabaena sp. PCC 7120 included in the nearest cluster C3, C4 and C5 possessed a good level of similarity.

Figure 4

Figure 3: Dendrogram showing the similarity relationship among the SDS-PAGE patterns of whole cell proteins of cyanobacterial strains. Similarities were calculated as Pearson product-moment correlations among the logistic weighted patterns and clustering was performed by using the UPGMA algorithm



16S RRNA GENE BASED PHYLOGENETIC TREE

In contrast to SDS-PAGE analysis the deepest nodes of the phylogenetic tree with only partial support by the bootstrap values separated the twenty-one strains in two large clades (Fig. 4). Clade 1 that included clusters I and II corresponded to Nostoc and Anabaena with representatives of the species Nostoc spongiaeforme LCR10, Nostoc linckia LCR 11, Nostoc rivulare LCR13 and Anabaena torulosa, Anabaena constricta Nostoc species LCR21, Nostoc species LCR12, Nostoc species LCR17 and Anabaena sp. PCC 7120, while clade 2 consisted of clusters III and IV.

The large cluster I, included maximum number of nostocalean strains except few like Nostoc carneum, Nostoc piscinale, Nostoc punctiforme, Nostoc paludosum and Nostoc ellipsosporum are included in cluster II and III. These nostocalean strains belonging to cluster 1 contained in this clade also showed morphological resemblance with Nostoc strains in terms of entangled filamentous aggregation and predominant intercalary heterocyst. The frequent occurrence of these strains within cluster I and II could be due to distinct morphological features like entangled trichome aggregation, cylindrical/barrel-shaped cells, terminal and intercalary/terminal or intercalary heterocyst and akinete position (adjacent/distant) with respect to heterocyst

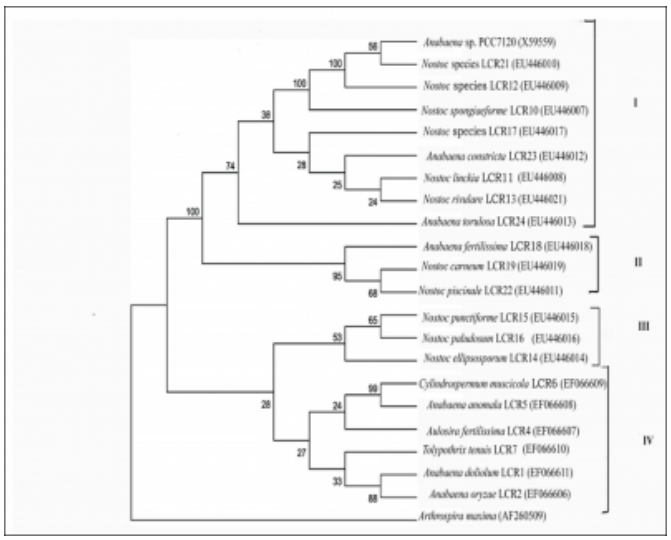
Thus the 16S rRNA gene data supports the grouping of the species indicated that Nostoc and Anabaena are not clearly separated at genetic level and may have their common origin. This further suggests that the Nostoc strains were heterogenous and seemed to form monophyletic cluster containing more than one genus.

The small cluster II and III are composed of Nostoc strains. The placement of Anabaena fertilissima within Nostoc cluster II may be due to a lack of genetic distance between the Nostoc and Anabaena strains. This is in accordance with the reported higher degree of genetic relatedness among heterocystous species relative to other cyanobacteria (Lachance M., 1981). The intermingling of Anabaena with Nostoc and other strains like Cylindrospermum and Tolypothrix deciphers lack of correlation between sequence and morphology and suggested polyphyletic origin. In conclusion, the lack of congruency of phylogenies based on morphological criteria may indicate a close genetic relationship in the genera Nostoc and Anabaena.

The lowest clade of the phylogenetic tree corresponded to the wide radiation including the genera Anabaena, Cylindrospermum, Aulosira and Tolypothrix where Aulosira and Tolypothrix grouped together depicting their phenotypic similarity (Geitler, 1932) and Cylindrospermum muscicola and Anabaena anomala were clustered together on the basis of sequence relatedness. However, this clustering displays low sequence similarity and low bootstrap support which may be due to the small numbers of characters supporting each node, further indicating them as closely related strains that have not diverged broadly. This cluster deserves a complete characterization and possibly the addition of more strains for a better interpretation.

Figure 5

Figure 4: Majority rule consensus tree of 16S rRNA gene sequences by using Maximum-parsimony method



All the three approaches were used together not only to find out the congruencies /incongruencies of the three systems in classifying the cyanobacterial isolates but also to check as how far the polyphasic approach is reliable as compared to single approach. Cluster C1 grouped Nostoc linckia LCR11 and Anabaena doliolum LCR1 together. The 16S rRNA gene maximum-parsimony tree not only puts them in distinct clades but indicates morphological delimitation as well. Thus it can be anticipated that the two organisms have divergent evolutionary relationships. Their SDS-PAGE brings them in one clade because they have been acquired from the same culture conditions and hence analogous protein profile. The above observation is in agreement with the reports (Tomitani et al., 2006; Prasanna et al., 2005; Henson et al., 2004; Gugger et al., 2002) that traditional techniques for identification and systematics of cyanobacteria based on morphological traits suffer from various limitations such as environmental pressure, as phenotypic characters are prone to environmental changes. Cluster C2 included the replicates of the species of Nostoc carneum LCR19 and Nostoc piscinale LCR22. This is well attested by high sequence similarity in 16S rRNA gene and high bootstrap values. The two strains appear close morphotypes. Thus the congruence of genotypic and phenotypic data attested the taxonomic coherence of these species. Cluster C3 encompassed Cylindrospermum muscicola LCR6, Anabaena anomala LCR5 and Nostoc rivulare LCR13. Their clustering pattern is in accordance with 16S rRNA gene based MP tree except Nostoc rivulare LCR13 harbouring clade3; however, all the three strains are separated into different clades on morphological grounds, like straight/entangled aggregation, barrel or cylindrical cell morphology, attenuated/conical apical cell, akinete position etc. Thus morphological clustering of species was not supported by 16S rRNA gene sequences. The clade is represented by a phylogenetically coherent but morphologically diverse group of strains.

It may be thus proposed that the strains were phylogenetically linked and are of same genetic make up but appear quite different under different physiological conditions as a result of differential gene expression. Morphological differences suggest that they might have undergone different environmental pressures.

The grouping of Cylindrospermum muscicola and Anabaena anomala was consistently observed in all phylogenetic analyses either SDS-PAGE or 16S rRNA gene analysis. A larger number of strains would be necessary to understand whether they represent a new phylogenetic unit.

The coherent positioning of Aulosira and Tolypothrix at genetic level corresponds to their traditional phenotypic traits and suggested that sometime clear traditional phenotypes can be found that correspond to the genotypes. However, an ambiguous morphology can often be observed which may correspond to the genetic diversity (Whitton, 1987).

The largest cluster C4 was quite congruent with the cluster 3 of 16S rRNA gene based MP tree. This exclusively harboured the species of Nostoc and Anabaena including, Nostoc punctiforme LCR15, Nostoc spongiaeforme LCR10, Nostoc ellipsosporum LCR14, Anabaene fertilissima LCR18 and strains of the species Tolypothrix tenuis LCR7, Aulosira fertilissima LCR4 suggesting that Nostoc strains were heterogeneous. They seemed to form a monophyletic cluster containing more than one genus. Intermingling of Anabaena species indicates that the two taxa are not clearly separated and distinguished at genetic level and may be polyphyletic in origin. It is worth mentioning here that apical cell shape emerged as a diacritical parameter for more clear distinction of Nostoc and Anabaena in morphological tree. Although in the above case a good agreement existed between phenotypic and genotypic features regarding Tolypothrix tenuis LCR7 and Aulosira fertilissima LCR4. They are clustered together in cluster 3 with different strains of Nostoc and Anabaena in MP tree. This strongly indicates their independent evolution than that of other nostocalean

strains. The SDS-PAGE, 16S rRNA gene and morphology based classification displayed high similarity level for these species, Thus phenotypic characteristics of this and other strains reflect incongruency with 16S rRNA gene sequences demonstrating that the genotypic plasticity of the heterocystous, entangled/coiled forms, consequently illustrated the adequacy of trichome aggregation, heterocyst and akinete position for Anabeana species characterization.

The species Anabaena oryzae LCR2 and Anabaena constricta LCR23 are close together both in morphological and SDS-PAGE dendrogram showing a low level of similarity with Nostoc species. This is in accordance with the DNA-DNA hybridization data of Lachance (1981) and hybridization patterns with repetitive DNA (STRR) sequences (Mazel et al., 1990). The diversity between Anabaena strains has also been previously demonstrated (Prasanna et al., 2005).

The present study reveals that the phenotypically diverse genera are closely related at their genetic level and represent their realistic phylogenetic relationship at the class and up to the genera level. It is also suggested that the clustering of strains in SDS-PAGE and 16S rRNA gene analyses is congruent and illustrates complementarity. Despite a few incongruities the clustering pattern of taxa in SDS-PAGE and 16S rRNA gene analyses are quite complementary to each other. The SDS-PAGE and 16S rRNA gene based phylogenetic analysis affirm the heterogeneity but monophyletic origin of nostocalean strains. Merging of Anabaena strains with Nostoc indicates their common origin. However, their taxonomic placement seems debatable. For the nostocales it appears that the assignment of clades may be premature due to the limited number of taxa presently available for phylogenetic analysis but high bootstrap values strongly point towards its monophyletism. There were few strains like Cylindrospermum muscicola LCR6, Tolypothrix tenuis LCR7, and Aulosira fertilissima LCR4 taken as a single strain in the study and these least number of strains is not adequate to explore their phylogenetic relationship evidently. Hence large number of strains must be isolated and employed in the study to assess their phylogenetic relationships more precisely.

In conclusion, single-dimensional SDS-PAGE phylogeny may be used as a complementary tool for the 16S rRNA gene based analysis to identify the nitrogen-fixing species at the molecular level. This shows immense importance of the classical cyanobacterial taxonomic systems. Molecular methods should be used in parallel or as a supplement to the phenotypic characterization but never replace it otherwise the more precise and probably more reliable molecular data would produce confusing results concerning the occurrence of cyanobacterial genera and species in nature.

Appendix 1. Morphological character descriptions, arranged in an order conducive to minimal handling of cyanobacterial taxa during character scoring.

ACKNOWLEDGEMENTS

Lal Chand Rai is thankful to ICAR, New Delhi for financial support. Poonam Bhargava is thankful to UGC for the award to SRF and Rashmi Rai and Yogesh Mishra to CSIR for JRF and SRF respectively. We are also thankful to Head and Program Coordinator, CAS in Botany, BHU for facilities.

References

1. Allen MB, Arnon DI (1955) Studies on nitrogen fixing blue-green algae. I. Growth and nitrogen fixation by Anabaena cylindrica Lemm. Plant Physiol 30: 366-372.
2. Berber I, Cokmus C, Atalan E (2003) Characterization of Staphylococcus species by SDS-PAGE of whole cell and extracellular proteins. Microbiol 72: 54-59.
3. Bhatnagar A, Bhatnagar M (2005) Microbial diversity in desert ecosystems. Curr Sci 89: 91-100.
4. Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantity of proteins utilizing the principle of protein dye binding. Anal Biochem 72: 248-254.
5. Derbyshire E, Whitton BA (1968) A disc electrophoretic study of proteins of blue-green algae. Phytochem 7: 355-1360.
6. Desikachary TV (1959) Cyanophyta, part I and II. New Delhi: Indian Council of Agricultural Research.
7. Doods WK, Gudder DA, Mollenhauer D (1995) The ecology of Nostoc. J Phycol 31:2-18
8. Elliot JA, Facklam RR (1993) Identification of Leuconostoc spp. by analysis of soluble whole cell protein patterns. J Clin Microbiol 31: 1030-1033.
9. Geitler L (1932) Cyanophyceae. In Kryptogamenflora von Deutschland, Österreich und der Schweiz, vol.14. Edited by L. Rabenhorst. Leipzig: Akademische Verlagsgesellschaft.
10. Gugger M, Lyra C, Henrikson P, Coutè A, Humbert JF, Sivonen K (2002) Phylogenetic comparison of the cyanobacterial genera Anabaena and Aphanizomenon. Int J Syst Evol Microbiol 52: 1867-1880.
11. Henson BJ, Hesselbrock SM, Watson LE, Barnum SR. (2004) Molecular phylogeny of the heterocystous cyanobacteria (subsections IV and V) based on nifD. Int. J Syst Evol Microbiol 54: 493-497.
12. Hoffmann L, Gugger M. (2003) Morphological and molecular characterisation of a stigonematalean cyanobacterium isolated from spanish cave. Algol Stud 109:259-65.
13. Hughes EO, Gorham PR, Zehndera A (1958) Toxicity of unialgal culture of Microcystis aeruginosa. Can J Microbiol 4: 225-236.
14. Kersters K, Ley J De (1975) Identification and grouping of bacteria by numerical analysis of their electrophoretic protein patterns. J Gen Microbiol 87: 333-342.
15. Koma´rek J, Anagnostidis K (1989) Modern approach to

the classification system of Cyanophytes 4 - Nostocales. Arch Hydrobiol Suppl 82: 247–345.
16. Komárek J, Kaštovsk J (2003) Coincidences of structural and molecular characters in evolutionary lines of cyanobacteria. Algol Stud 109:305-25.
17. Lachance M (1981) Genetic relatedness of heterocystous cyanobacteria by deoxyribonucleic acid-deoxyribonucleic acid reassosciation. Int J Syst Bacteriol 31: 139-147.
18. Lehtima¨ki J, Lyra C, Suomalainen S, Sundman P, Rouhiainen L, Paulin L, Salkinoja-Salonen M, Sivonen, K. (2000) Characterization of Nodularia strains, cyanobacteria from brackish waters, by genotypic and phenotypic methods. Int J Syst Evol Microbiol 50: 1043–1053.
19. Liu B, Li H, Wu S, Zhang X, Xie L (2006) A simple and rapid method for the differentiation and identification of thermophillic bacteria. Can J Microbiol 52: 753-758.
20. Lyra C, Hantula J, Vainio E, Rapala J, Rouhiainen L, Sivonen K (1997) Characterization of cyanobacteria by SDS-PAGE of whole- cell proteins and PCR/RFLP of the 16S rRNA gene. Arch Microbiol 168: 176-184.
21. Mazel D, Houmard J, Castets AM, Tandeau de Marsac N (1990) Highly repetitive DNA sequences in cyanobacterial genomes. J Bacteriol 172: 2755-2761.
22. Nixon KC (1999) Winclada (BETA). Published by the author, New York.
23. Nübel U, Garcia-Pichel F, Muyzer G (1997) PCR primers to amplify 16S rDNA genes from cyanobacteria. Appl Environ Microbiol 63: 3327-32.
24. Palinska KA, Liesack W, Rhiel E, Krumbein, WE (1996) Phenotype variability of identical genotype: the need for a combined approach in cyanobacterial taxonomy demonstrated on Merismopedia like isolates. Arch Microbiol 166: 224-233.
25. Perez G, Cardell E, Zarete V (2000) Protein fingerprinting as a complementary analysis to classical phenotyping for the identification of lactic acid bacteria from Tenerife cheese. Lait 80 : 589-600.
26. Piraino P, Ricciardi A, Salzano G, Zotta T, Parente E. (2006) Use of unsupervised and supervised artificial neural networks for the identification of lactic acid bacteria on the basis of SDS-PAGE patterns of whole cell proteins. J Microbiol. Methods 66: 336-346.
27. Potts M (2000) Nostoc. In The Ecology of Cyanobacteria, pp. 465-504, Edited by BA Whitton & M Potts. Dordrecht: Kluwer
28. Prasanna R, Kumar R, Sood A, Prasanna BM, Singh PK (2005) Morphological, physiochemical and molecular characterization of Anabaena strains. Microbiol Res 161: 187-202.
29. Rajaniemi P, Hrouzek P, Kaštovská K, Williame R., Rantala A, Hoffmann L, Komárek J, Sivonen K (2005) Phylogenetic and morphological evaluation of the genera Anabaena, Aphanizomenon, Trichormus and Nostoc (Nostocales, Cyanobacteria). Int J Syst Evol Microbiol 55: 11-26
30. Sambrook J, Russel DW (2001) Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory Press,Cold Spring Harbor, New York.
31. Srivastava AK, Ara A, Bhargava P, Mishra Y, Rai SP, Rai LC (2007) A rapid and cost-effective method of genomic DNA isolation from cyanobacterial culture, mat and soil suitable for genomic finger printing and community analysis. J Appl Phycol 19: 373-382.
32. Suda S, Watanabe MM, Otuska S, Mahakant A, Youngmanitachi W, Noparatnaraporn N, Liu Y, Day JG. (2002) Taxonomic revision of water-bloom-forming species of oscillatorioid cyanobacteria. Int J Syst Evol Microbiol 52: 1577-95.
33. Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol 24:596-1599.
34. Thompson JD, Gibson T, Plewniak J, Jeanmougin F, Higgins DG. (1997) The CLUSTAL_X Windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucl Acid Res 25: 4876-4882.
35. Tomitani A, Knoll AH, Cavanaugh CM, Ohno T (2006) The evolutionary diversification of cyanoacteria: Molecular-phylogenetic and paleontological perspectives. Proc Natl Acad Sci U S A 103: 5442-5447.
36. Vandamme P, Pot B, Gillis M, De Vos P, Kersters K, Swings J (1996) Polyphasic taxonomy, a consensus approach to Bacterial Systematics. Microbiol Rev 60:407-438.
37. Wagner MA, Eschenbrenner M, Honrn TA, Kraycer JA., Mujer CV, Hagius S, Elzer P, Del Vecchio VG (2002) Global analysis of the Brucella melitensis proteome: identification of proteins expressed in laboratory grown culture. Proteomics 2:1047-1060.
38. Weisburg WG, Barns SM, Pelletier DA, Lane DJ (1991) 16S ribosomal DNA amplification for phylogenetic study. J Bacteriol 173: 697-703.
39. Whitton BA. (1987) The biology of Rivulariaceae. In The Cyanobacteria, pp. 513-534. Edited by P.Fay and C.Van Baalen. Amsterdam: Elsevier.
40. Woese CR (1987) Bacterial Evolution. Microbiol Rev 51: 221-271.
41. Wynn-Williams DD (2000) Cyanobacteria in deserts-life at the limit? In The Ecology of Cyanobacteria, pp. 341-366. Edited by BA Whitton, M Potts. Dordrecht: Kluwer.

Author Information

Swati Mishra, M. Sc. Ph. D. (Pursuing)
Centre of Advanced Study in Botany, Banaras Hindu University, Varanasi-221005, India

Poonam Bhargava, M. Sc. Ph.D
Centre of Advanced Study in Botany, Banaras Hindu University, Varanasi-221005, India

Rashmi Rai, M. Sc. Ph.D. (Pursuing)
Centre of Advanced Study in Botany, Banaras Hindu University, Varanasi-221005, India

Yogesh Mishra, M. Sc. Ph. D.
Centre of Advanced Study in Botany, Banaras Hindu University, Varanasi-221005, India

Teresa Zotta, M. Sc. Ph. D.
Dipartimento di Biologia, Difesa e Biotecnologie Agro-forestali Università degli Studi della Basilicata, Viale dell'Ateneo Lucano 10, 85100 Potenza, Italy

Eugenio Parente, M. Sc. Ph. D.
Dipartimento di Biologia, Difesa e Biotecnologie Agro-forestali Università degli Studi della Basilicata, Viale dell'Ateneo Lucano 10, 85100 Potenza, Italy

Lal Chand Rai
Centre of Advanced Study in Botany, Banaras Hindu University, Varanasi-221005, India