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EXPERIMENTAL ARTICLES

# Fingerprinting and Phylogeny of Some Heterocystous Cyanobacteria Using Short Tandemly Repeated Repetitive and Highly Iterated Palindrome Sequences[1]

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**Abstract**—The presence of repeated DNA, *viz.* short tandemly repeated repetitive (STRR) and highly iterated palindrome (HIP) sequences was used as a typing technique for assessing genetic variability and phylogenetic relatedness of heterocystous cyanobacteria. Primers analogous to the STRR and HIP sequences were used to generate specific fingerprints for the twelve heterocystous cyanobacterial strains and a dendrogram was constructed. STRRmod and HIPTG primers revealed 100% polymorphism and yielded almost identical patterns. *Anabaena* sp. PCC 7120 clustered with *Nostoc muscorum* with both primers. Primer STRRmod supported the heterogeneity between *Nostoc* and *Anabaena* but HIPTG placed these two genera distinctly apart. STRRmod and HIPTG revealed that the members of the two orders were intermixed and thus suggesting a monophyletic origin of heterocystous cyanobacteria.

*Keywords*: repetitive DNA sequences, heterocystous cyanobacteria, typing, STRRmod, HIP

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Cyanobacteria are a morphologically diverse group of organisms ranging from unicellular to filamentous forms. These oxygenic, photoautotrophic prokaryotes are among the most ancient organisms inhabiting the Earth's environment [1, 2]. Cyanobacteria exhibit remarkable diversity in their morphological, developmental and physiological behavior [3]. Nevertheless, in the course of evolution, cyanobacteria have developed some unique physiological and morphological features to respond to the changes in environmental parameters such as light, temperature, pH and nutrients [4]. As a result of these adaptations, they are present in nearly all the ecosystems [1, 5].

The taxonomic status of the cyanobacteria was determined based extensively on their morphological and physiological features. It was observed, however, that the morphology and physiology of environmental isolates was altered in laboratory conditions [6]. In the recent times the development of both bioinformatics and molecular techniques made it possible to mine the genome of the microorganisms for identifying unique sequences that can be used to identify the microorganisms both among and within a group [7–11]. Although 16S rRNA gene has been extensively used in taxonomic and phylogenetic studies involving all the bacterial phyla as a standard marker, due to its conservative nature it does not always provide adequate discriminatory power for identification of cyanobacteria, even when the species are physiologically distinct [12, 13]. Similarly, a number of authors demonstrated the usefulness of other markers; the 16S–23S internal transcribed spacer, phycocyanin-encoding PC–IGS region [14], the house keeping gene (*rpoC1*) [15], and the *nifH* [11, 16] and *nifD* genes [17] have been widely used to detect genetic relatedness and molecular phylogeny within the cyanobacterial lineage.

While repetitive sequences have been increasingly identified in prokaryotic genomes but their function, maintenance and occurrence still remain obscure. The widespread distribution and sufficient conservation of these repetitive sequences within the genome have been used as a supplant technique for the identification of species or strains, as well as in diversity analysis [18, 19]. Ever since their discovery, short tandemly repeated repetitive sequences (STRR sequences) have been widely used to differentiate between environmental cyanobacterial isolates. These heptanuclotide repeat sequences have been identified in several cyanobacterial genera and species, mostly in the case of heterocystous cyanobacteria, *viz. Calothrix* sp. and also some non-heterocystous cyanobacteria [20, 21]. Highly Iterated Palindrome sequence commonly referred to as HIP1 is a repetitive eight- base sequence (5'-GCGATCGC-3'), well represented in the cyanobacterial genome and is hypothesized to be a recombination hotspot [19, 22, 23]. HIP1 sequencing has also

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been used as an alternative methodology for DNA fingerprinting of cyanobacteria [19]. From time to time various researches have been performed in order to characterize cyanobacteria based on the repetitive sequences [24–26]. Up to now, however, no reports have been available on the comparative study of the cyanobacterial strains belonging to the two different orders, i.e. *Nostocales* (Section IV) and *Stigonematales* (section V) using the STRR and HIP sequences. Therefore, in the present communication we attempted to explore the efficacy of the advanced methodology, i.e., DNA fingerprinting based on repetitive sequences for unambiguous differentiation of the cyanobacterial strains and also for diversity assessment.

## MATERIALS AND METHODS

**Cyanobacterial strains.** Different heterocystous cyanobacterial strains (*Calothrix brevissima* HM573459, *Nostoc spongiaeforme* HM573463, *Nostoc muscorum* HM573462, *Westiellopsis* sp. HM573464, *Cylindrospermum musicola* HM573454, *Nostoc calcicola* HM573461, *Anabaena* sp. 1 HM573457, *Anabaena doliolum* HM573455, *Scytonema bohnerii* HM573459, and *Anabaena oryze* HM573456) were isolated from the agricultural fields and local ponds of Banaras Hindu University, Varanasi, India. *Hapalosiphon welwitschii* HM573460 and *Anabaena* sp. PCC 7120 HM573458 were obtained from Centre for Conservation and Utilization of Blue Green Algae, Indian Agricultural Research Institute, PUSA, New Delhi and from Professor C.P. Wolk, Laboratory of Plant Research, Michigan State University, East Lansing, USA respectively. The twelve cyanobacterial strains were grown in 250 mL Erlenmeyer flasks containing 150 mL of BG–11° medium, pH 7.4 in a culture room $27 \pm 2$°C and an average light intensity of 25 $\mu E\ m^{-2}\ s^{-1}$ at the surface with light/dark rhythm of 14 : 10 h [27]. Exponentially grown cyanobacterial strains were harvested and used for the following investigations.

**DNA extraction and PCR analysis.** Fresh algal biomass (8 days old) was harvested by centrifugation. The pellet obtained was washed twice in sterile Milli Q water and a blob was taken for DNA extraction. The DNA of twelve heterocystous cyanobacterial strains was isolated by using bacterial genomic extraction kit (Himedia, MB505) following the manufacturer's protocol.

For PCR amplification, several short repetitive sequences (STRR and HIP primers) were tested; successful results were obtained with the primer sets STRRmod (5'-GCGCCCCAATCC-3') [28] and HIPTG (5'-GCGATCGCTG-3') [19]. All the reactions were carried out in a 25 μL volume reaction mixture containing primer (50 pmol $\mu L^{-1}$) deoxynucleoside triphosphate (1.25 mM), 3.0 μL of template DNA (50 ng $\mu L^{-1}$), 1 U *Taq* polymerase (Bangalore Genei, India), 10× PCR buffer. The thermal profile consisted of initial denaturation for 6 min at 95°C, 35 cycles of 1 min, at 94°C, annealing at 56°C for 1 min, and 5 min extension at 65°C. A final extension step was performed at 65°C for 16 minute and holding at 4°C. Gel electrophoresis was performed on a 1.5% agarose gel to visualize the amplified products along with a 100 bp plus gene ruler (Bangalore Genei, India). The gel was stained with ethidium bromide and visualized under a gel documentation unit (Bio-Rad, United States) using Quantity One software.

**Data analyses.** The presence or absence of distinct and reproducible bands in each of the individual DNA fingerprinting pattern generated by STRRmod and HIP PCR profiles were converted into binary data, and the pooled binary data were used to construct a composite dendrogram. The software NTSYS-pc, version 1.80 [29] was used to calculate the Jaccard similarity index and construct the dendrogram using the Unweighted Pair-Group Method with Arithmetic Average (UPGMA). PCR reactions and electrophoresis were repeated at least thrice for each primer to ascertain the reproducibility of the banding patterns.

## RESULTS

**DNA fingerprinting based on STRRmod and HIP primers.** In order to explore the genetic variability among the 12 heterocystous cyanobacterial strains DNA fingerprinting based on repetitive sequences was performed using two specific primers *viz.* STRRmod and HIPTG. HIPTG and STRRmod primers generated polymorphic profiles indicating the conservation and distribution pattern of these repeat sequences in the cyanobacterial genome and also revealed 100% polymorphism among the twelve cyanobacterial strains.

PCR amplification of the twelve heterocystous cyanobacterial strains using the HIPTG primer revealed nine distinct banding profiles out of which five generated profiles were unique *viz. Scytonema bohnerii*, *Westiellopsis* sp., *Nostoc calcicola*, *Hapalosiphon welwitschii* and *Nostoc spongiaeforme* (Fig. 1). The PCR product ranged between 250 and 3500 bp. The heterocystous cyanobacteria, such as *Anabaena* sp. PCC 7120, *Cylindrospermum musicola*, *Nostoc muscorum, Anabaena* sp. 1 and *Anabaena doliolum* produced the maximum number of bands (from 16 to 22) with the HIPTG primer during amplification. The banding pattern of *Anabaena* sp. PCC 7120 and *Nostoc muscorum* was found to be identical. Likewise, *Anabaena* sp. 1 and *Anabaena doliolum* also showed similar banding patterns.

STRRmod generated multiple bands of varying length i.e., 350–4000 bp in each of the test strains (Fig. 2). Eight distinct banding profiles were observed in this case, out of which six profiles were unique in *Scytonema bohnerii*, *Westiellopsis* sp., *Nostoc calcicola*, *Hapalosiphon welwitschii*, *Calothrix brevissima* and *Nostoc spongiaeforme*. *Cylindrospermum musicola* and

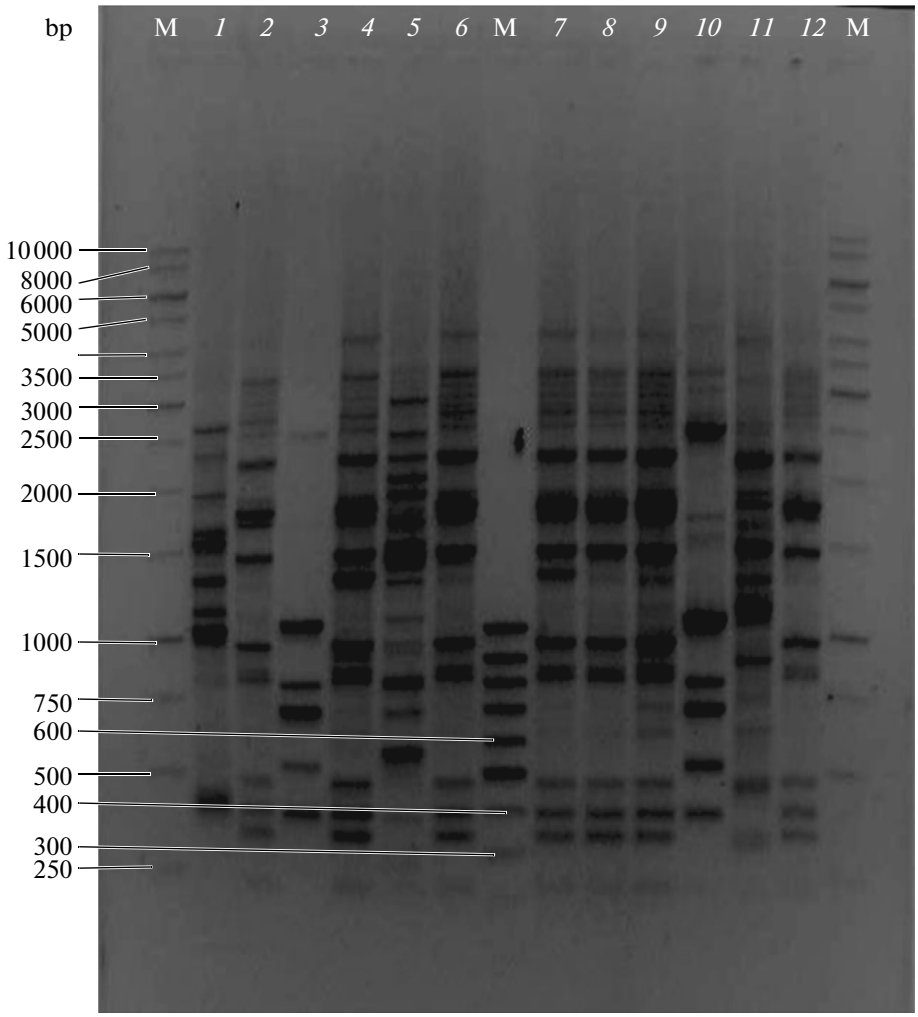


**Fig. 1.** DNA fingerprinting pattern generated in twelve heterocystous cyanobacteria by using HIPTG primer. Lane M represents the molecular markers used. Lane *1–12* represents the cyanobacterial strains as: *1—Scytonema bohnerii, 2—Anabaena oryzae, 3—Westiellopsis* sp., *4—Anabaena* sp. PCC 7120, *5—Nostoc calcicola, 6—Cylindrospermum musicola, 7—Nostoc muscorum, 8—Anabaena* sp. 1, *9—Anabaena doliolum, 10—Hapalosiphon welwitschii, 11—Calothrix brevissima, 12—Nostoc spongiaeforme.*

*Anabaena doliolum* exhibited similar fingerprinting patterns with the primer STRRmod. The maximum number of bands (19 bands) was observed in *Nostoc muscorum*.

**Phylogenetic analyses.** The PCR profiles of the twelve heterocystous cyanobacterial strains using two different primers were used as a diagnostic tool for assessing genetic diversity. Jaccard's pair wise genetic similarity coefficients ranged from 0.14 to 1.00. The HIPTG primer based dendrogram (Fig. 3) revealed two major clusters, i.e., cluster I and cluster II. The cluster I was subdivided into subclusters IA and IB. In the subcluster IB, *Cylindrospermum musicola* and *Anabaena oryzae* formed a tight cluster, while the subcluster IA contained *Calothrix brevissima* as a single genotype. Cluster II was further subdivided into subclusters IIA and IIB. Subcluster IIA comprised of a single heterocystous cyanobacterium, *Scytonema bohnerii*. Subcluster IIB was again divided into $IIB_1$ and $IIB_2$. The subsubcluster $IIB_1$ was represented by *Hapalosiphon welwitschii*. The subsubcluster $IIB_2$ was further subdivided into two distinct subsubclusters. Subsubcluster $IIB_{2a}$ contained *Anabaena* sp. 1 and *Anabaena doliolum*. Sub sub cluster $IIB_{2b}$ was again comprised of two groups, i.e., $IIB_{2b1}$ and $IIB_{2b2}$. The group $IIB_{2b1}$ represented a tight clustering of *Anabaena* sp. PCC 7120 and *Nostoc muscorum*. Similarly, $IIB_{2b2}$ was comprised of *Nostoc spongiaeforme* and *Nostoc calcicola*. *Westiellopsis* sp. was found as an outgroup.

Similarly, the dendrogram based on STRRmod also showed two major clusters, i.e., cluster I and II (Fig. 4). Cluster I contained a single genotype, i.e., *Westiellopsis* sp. Cluster II was subdivided into two sub clusters, i.e., IIA and IIB. Cluster IIA included members of section IV, *viz. Calothrix brevissima* and *Nostoc calcicola*. Cluster IIB was further subdivided into two subclusters $IIB_1$ and $IIB_2$. Subcluster $IIB_1$ comprised

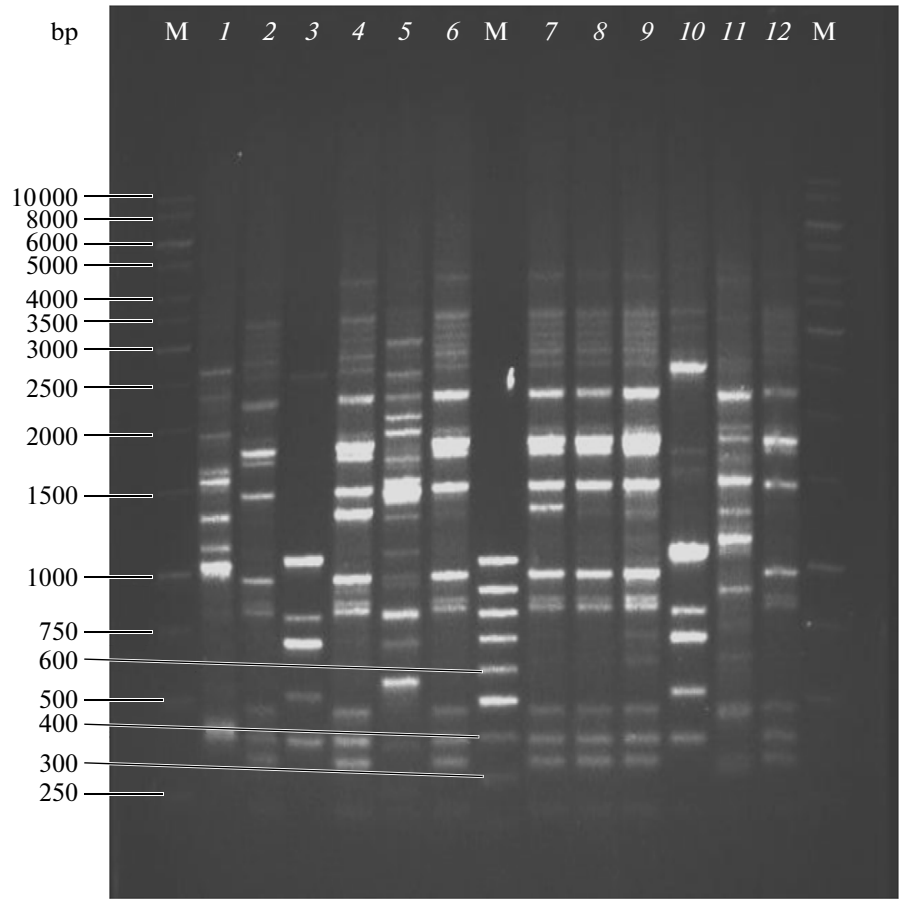


**Fig. 2.** DNA fingerprinting pattern generated in twelve heterocystous cyanobacteria by using STRRmod primer. Lane *1–12* represents the cyanobacterial strains as: *1—Scytonema bohnerii, 2—Anabaena oryzae, 3—Westiellopsis* sp., *4—Anabaena* sp. PCC 7120, *5—Nostoc calcicola, 6—Cylindrospermum musicola, 7—Nostoc muscorum, 8—Anabaena* sp. 1, *9—Anabaena doliolum, 10—Hapalosiphon welwitschii, 11—Calothrix brevissima, 12—Nostoc spongiaeforme.*

of single species *Scytonema bohnerii*. Subcluster $IIB_2$ again formed two major subsubclusters, $IIB_{2a}$ and $IIB_{2b}$. Subsubcluster $IIB_{2a}$ contained three strains (*Anabaena oryzae*, *Nostoc spongiaeforme*, and *Anabaena* sp. 1). Similarly, subsubcluster $IIB_{2b}$ enclosed *Cylindrospermum musicola* and *Anabaena doliolum* in a distinct group and *Anabaena* sp. PCC 7120 and *Nostoc muscorum* in another single tight cluster. *Haplosiphon welwitschii* formed a single group.

## DISCUSSION

Development of molecular methods is essential for the rapid and accurate analysis of heterocystous cyanobacteria and also for elucidating cyanobacterial taxonomy [30]. Thus, the discovery of repetitive sequences has been found to be a valuable tool for studying genetic relatedness within and among the various groups of cyanobacteria. Genomic fingerprinting with REP, ERIC, and BOX primers has been mostly used for intra-specific comparisons of bacteria [23, 31, 32]. The presence of highly iterated palindrome sequences in many cyanobacteria also helped in cyanobacterial typing based on the DNA amplification between the adjacent repeated HIP sequences present in the chromosomal DNA of cyanobacteria [19, 22, 33].

The present analysis based on the application of the repetitive sequences yielded some unique fingerprinting pattern for the twelve heterocystous cyanobacterial strains (of the section IV and V) with the extent of variation dependent on the primer used. HIPTG primer revealed high level of DNA polymorphism and it further confirmed the utility of HIP primers in discriminating organisms both at inter and intra-specific level. The utility of the HIP primers in assessing the diversity among the cyanobacterial strains of the same species has been reported earlier [19, 26].

Both STRRmod and HIPTG revealed 100% DNA polymorphism in all the 12 heterocystous cyanobacterial strains. STRRmod primer generated a large number of amplicons, but very few products were similar in size among the twelve strains (Fig. 2). The profile pattern generated by the strains *Anabaena* sp. PCC 7120 and *Nostoc muscorum* was found to be very much similar. Therefore, these two species have been placed

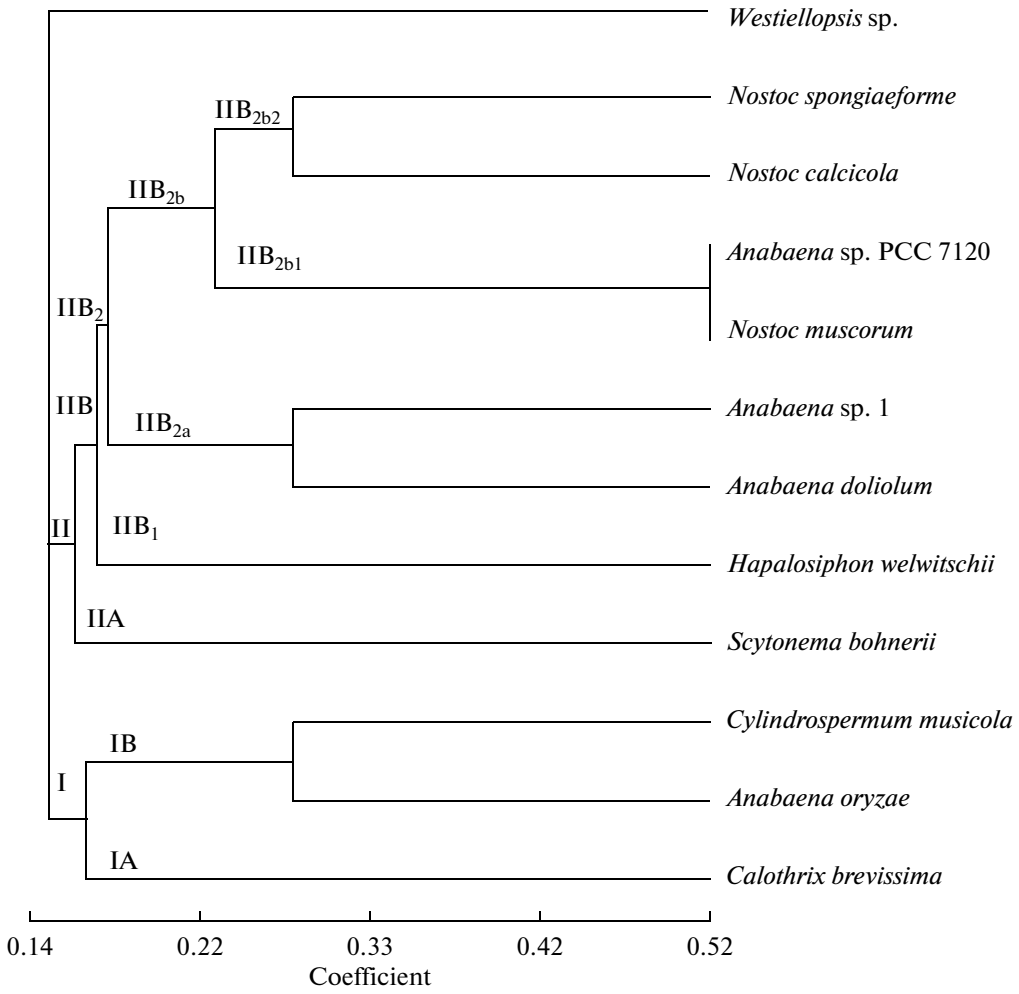


**Fig. 3.** Dendrogram based on HIPTG-PCR fingerprinting profile of twelve heterocystous cyanobacterial strains using Jaccard's similarity coefficient and UPGMA clustering method. The phylogenetic analysis was performed using NTSYS software.

together in the constructed dendrogram based on STRRmod sequences (Fig. 4). However, the DNA fingerprint of the two strains obtained using HIPTG sequences produced identical banding patterns and thus both cyanobacteria formed a tight cluster (Figs. 1, 3). Use of both short sequence repeats supports the reclassification of *Anabaena* sp. PCC 7120 as *Nostoc* sp. PCC 7120. This is in accordance with earlier observations [34, 35]. The difference in the clustering pattern of the strains observed with the two different primer sets indicates that various primers have diverse degrees of resolution, so that for obtaining a clearer picture regarding cyanobacterial diversity different primers have to be included in the PCR amplification.

A unique aspect of this investigation is the clustering of the cyanobacterial cultures. STRRmod and HIPTG differentiated between the true branching genera. *Westiellopsis* sp. and *Hapalosiphon welwitschii* and placed them way apart contrary to the phylogeny obtained on the basis of the 16S rRNA, where both formed a tight cluster [10]. The results obtained by using both primers (STRRmod and HIPTG) suggest that there exists a close relationship between *Anabaena* and *Cylindrospermum* strains as they were found to be intermixed. A similar finding has been also reported by Lyra et al. [36]. The application of primer STRRmod confirmed the heterogeneity between *Nostoc* and *Anabaena* [37, 38]. Similarly, use of the primer HIPTG placed the strains of the two genera, *Nostoc* and *Anabaena*, distinctly apart from each other and no intermixing of these two genera was observed except in the case of *Anabaena* sp. PCC 7120 and *Nostoc muscorum* [17]. Thus, the dendrogram constructed on the basis of STRRmod and HIPTG banding patterns supported the polyphyletic origin of the twelve heterocystous cyanobacterial strains and also suggested a close association between the true branching (*Stigonematales*) and the unbranched cyanobacteria (*Nostocales*) (Figs. 3, 4). Our results corroborates the earlier findings based on polyphasic approach [10, 11].

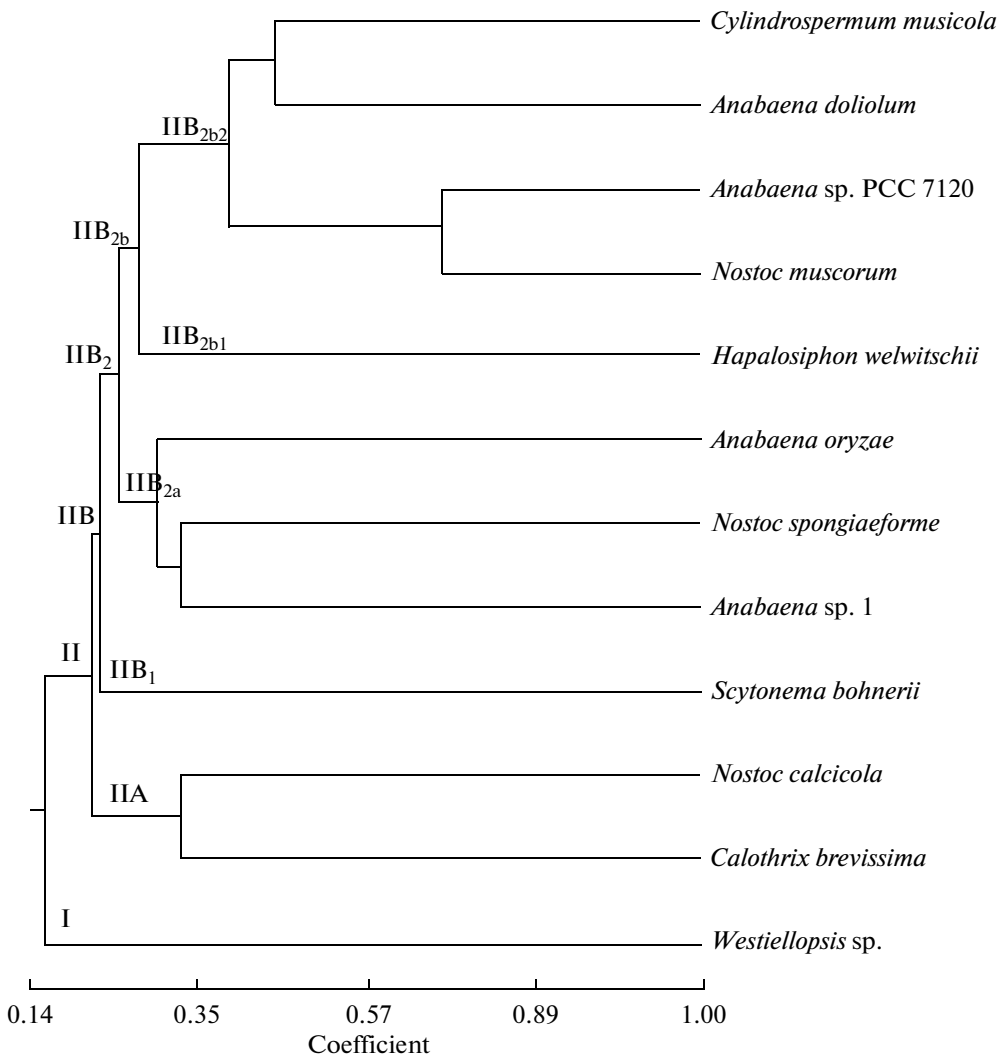


**Fig. 4.** Dendrogram based on STRRmod-PCR fingerprinting profile of twelve heterocystous cyanobacterial strain using Jaccard's similarity coefficient and UPGMA clustering method. The phylogenetic analysis was performed using NTSYS software.

Phylogenetic analyses was also carried out earlier in the same laboratory and on the same heterocystous cyanobacteria based on the 16S rRNA/polyphasic approach [10]. The dendrograms (Figs. 3, 4) obtained in the present investigation were compared to the phylogeny obtained on the basis of the 16S rRNA gene so as to confirm the utilization of tandem repeats as species markers and for diversity assessment. The clustering pattern obtained based on different markers (16S rRNA, STRRmod and HIPTG) suggested ambiguous relationship of *Cylindrospermum musicola* with *Nostoc* and *Anabaena* thus signifying common origin. Likewise, *Scytonema bohnerii* formed a distinct clade in the obtained tree topologies. At the same time, due the conservatism of the 16S rRNA gene its applicability below genus level has been contested [39]. The STRRmod and HIPTG fingerprints discriminated successfully between the two *Anabaena* strains, *Anabaena doliolum* and *Anabaena* sp. 1, which appeared to be conspecific according to the results of the 16S rRNA gene sequencing [10], showing the taxonomic potential of repetitive repeats. Congruency was observed in the phylogeny deduced by using STRRmod, HIPTG, and 16S rRNA gene. The exception was observed in the clustering pattern of the true branching cyanobacteria (order *Stigonematales*). While the 16S rRNA gene sequencing suggested monophyletic origin of the strains *Westeillopsis* sp. and *Hapalosiphon welwitschii*, in the present investigations these were found to be intermixed, thus suggesting polyphyletic origin [40]. Hence, 16S rRNA gene-based analysis is not adequate enough to provide true phylogenies of the cyanobacterial strains. Therefore, we urge to apply varied markers (*viz*. DNA fingerprinting, IGS, ITS) together with 16S rRNA analysis for clear resolution of the taxonomic positions.

From the present investigation it is concluded that (i) the repetitive sequences found in the genomes of

cyanobacteria are highly useful in exploring genomic relationships and typing among the tested strains and can be used as a diagnostic key for cyanobacterial discrimination and identification and (ii) on the basis of the diverse phylogenies it might be extrapolated that the members of the order *Nostocales* and *Stigonematales* have similar origin.

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