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EXPERIMENTAL ARTICLES

Morphological Characterization and Molecular Fingerprinting of *Nostoc* Strains by Multiplex RAPD[1]

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Abstract—Morphological parameters studied for the twenty selected *Nostoc* strains were mostly found to be consistent with the earlier reports. But the shape of akinetes observed in this study was a little deviation from the existing descriptions and heterocyst frequency was also found to be different in different strains in spite of growing in the same nitrogen free media. Multiplex RAPD produced reproducible and completely polymorphic amplification profiles for all the strains including some strain specific unique bands which are intended to be useful for identification of those strains. At least one to a maximum of two unique bands was produced by different dual primer combinations. For ten strains out of twenty, strain specific bands were found to be generated. Cluster analysis revealed a vast heterogeneity among these *Nostoc* strains and no specific clustering based on geographical origin was found except a few strains. It was also observed that morphological data may not necessarily correspond to the genetic data in most of the cases. CCC92 (*Nostoc muscorum*) and CCC48 (*Nostoc punctiforme*) showed a high degree of similarity which was well supported by high bootstrap value. The level of similarity of the strains ranged from 0.15 to 0.94. Cluster analysis based on multiplex RAPD showed a good fit revealing the discriminatory power of this technique.

Keywords: cyanobacteria, *Nostoc,* morphology, molecular fingerprinting, phylogeny, RAPD

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Cyanobacteria are gram-negative photoautotrophic bacteria which due to their oxygenic nature of photosynthesis are thought to be responsible for the transition from the early anaerobic environment to the present aerobic one. The variability in their physiological, morphological and developmental characteristics makes them one of the largest groups of photosynthetic prokaryotes. Another important feature of many cyanobacteria is their ability to fix atmospheric nitrogen both under free living and symbiotic condition, which made them potent to be used as biofertilizer especially in rice crop. *Nostoc,* a genus belonging to the family *Nostocaceae* of order *Nostocales* is a filamentous cyanobacteria with intercalary heterocysts and akinetes formed centrifugally in series between the heterocysts. The genus *Nostoc* is represented by roughly 60 recognizable (plus many unclear ones) morphospecies and strain [1]. It has tremendous potential in environmental management as soil conditioners, biomonitors of soil fertility, water quality etc. This apart, *Nostoc* has also been used as human food, source of restriction enzymes, growth promoting substances, amino acids etc. Even some *Nostoc* species have been reported to produce certain novel secondary metabolites with antimicrobial activity and anti-cancerous property [2]. There has been a considerable amount of literature available on the genus *Nostoc* and most of the earlier literature is in Chinese. The existing traditional floras of this genus have been described by a number of workers from different parts of the world [3–6].

Identification system for cyanobacteria is generally based on morphology and developmental characteristics. But these keys are often confusing as they vary with the growth and cultural conditions [7]. Further, analyses of photosynthetic pigment content, isozyme variation or differentiated cell culture may also be misleading because of the variable expression of cyanobacterial gene products in the culture [8, 9]. Such constraints imposed by the conventional methods have led to the use of molecular tools as an alternative and affirmative way for discriminating this group of organisms. Analysis of rDNA sequence diversity [10–13], restriction analysis of amplified 16S rDNA [14], presence of STRR and LTRR [15–17], Random amplified polymorphic DNA technique [18–20] have been widely used for identification and revealing the phylogenetic relationship of a number of cyanobacteria. The molecular (phylogenetic) data should be accepted as a basic criterion. However, the correct classification is impossible without the careful combination of genetic data with morphological diversity and variation, ecological and ecophysiological characteristics, ultra structural studies, and without the correct application of conve-

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nient formal prescriptions for designation of taxa and strains (nomenclatoric rules). Recently, this combined methodology is usually presented as "polyphasic approach".

Random Amplified Polymorphic DNA (RAPD) technique allows the detection of multilocus genetic variation using short primers of arbitrary sequence [21, 22]. This marker system is very easy to perform and do not require any prior knowledge of the genome under investigation. Multiplex RAPD PCR (using pair wise combination of primers) has been proved to increase the discriminatory power of PCR technology because it increases the informative genetic markers [18]. In the present study twenty different *Nostoc* strains from diverse geographical origin were morphologically characterized and RAPD study was also carried out with pair wise combinations of primers to reveal their phylogenetic relationship and genetic diversity.

MATERIALS AND METHODS

Cyanobacterial cultures and their maintenance. Twenty axenic strains of *Nostoc* from diverse geographical origin were chosen from the culture collection of Centre for Conservation and Utilisation of Blue Green Algae (CCUBGA), IARI, New Delhi-12, India (Table 1). Cultures were maintained in chemically defined nitrogen free BG-11 media [39] at 28 ± 2°C under a light intensity of 52–55 μmol photon m^{-2} s^{-1} and L : D cycles of 16 : 8 hours.

Microscopic analysis of the strains. The strains were viewed under an Olympus CX 40 light microscope (Plate 1); vegetative cells, heterocysts and akinetes were described using the keys provided by Desikachary [3].

RAPD. DNA extraction. Genomic DNA was extracted from 1 mL suspension (50–60 mg fresh biomass) of exponentially growing cultures by using *N*-cetyl-*N*,*N*,*N*-trimethylammonium bromide (CTAB) method [24] after minor modifications. Quantity and purity of DNA was estimated by comparing with known standards in ethidium bromide stained 0.8% agarose (Vivantis) gel.

Primers. DNA samples were subjected to amplification using twelve decamer primers (synthesized from Sigma-Aldrich) with GC content of 60–80% (Table 2).

PCR amplification. Amplification reactions were carried out with ten dual primer combinations as described in Table 3. The standard, optimized PCR was performed in a total volume of 20 μL containing 1× TAE buffer with 15 mM $MgCl_2$, 10 mM of deoxynucleotide triphosphate (dATP, dTTP, dGTP, dCTP), 10 pM each of the two primers, 1 U *Taq* DNA polymerase (Bangalore Genei Ltd., India) and 90 ng of template DNA. Thermal cycling was achieved in a Master Cycler Gradient (Eppendorf) as per the following programme: initial denaturation at 94°C for 4 min; 30 cycles with (i) denaturation at 94°C for 1 min, (ii) annealing at 34°C for 1 min, (iii) extension at 72°C for 2 min and (iv) final extension at 72°C for 5 min.

Electrophoretic analysis. PCR products were resolved along with a molecular weight marker (GeneRuler 1 Kb, Fermentas, United States) on 1.5% agarose gel in 1× Tris-acetate-EDTA buffer stained with ethidium bromide solution (0.5 mg/mL). These were visualized under UV light and gel photographs were scanned through Gel Doc System (MiniBis Bioimaging System, United States) and the amplification product sizes were evaluated using software AlphaEaseFC (FluorChem 5500) (Alfa Innotech Corporation).

Statistical analysis. Pair wise genetic similarities among the genotypes under study were determined using Jaccard's coefficient [25], $J = N11/(N11 + N10 + N01)$, where N11 is the number of bands present in both individuals i and j, N10 is the number of bands present in the individual i and N01 is the number of bands present in the individual j. Cluster analyses were carried out on similarity estimates using the unweighted pair-group method with arithmetic average (UPGMA) using NTSYS-pc, version 1.80 [26]. The confidence limit of the clustering was checked by calculating the Bootstrap value using the Winboot software. Bootstrap values more than 70% correspond to a probability of more than 95% that true phylogeny have been found [27]. To test the goodness of fit of a clustering to the set of RAPD data, cophenetic correlation coefficient or cophenetic value was estimated using COPH and MXCOMP options in NTSYS-pc program. The degree of fit was interpreted subjectively—$0.9 \le r$ is very good fit, $0.8 \le r \le 0.9$ is good fit, $0.7 \le r \le 0.8$ is poor fit and $r < 0.7$ is very poor fit.

RESULTS AND DISCUSSION

The shape of vegetative cells varied from barrel shaped or cylindrical to ovoid. The dimensions of heterocysts were also similarly large in CCC125 and CCC150 which also have larger vegetative cells. The width of the heterocysts varied from 1.97 to 6.08 μm in different cultures studied while the length varied from 2.48 to 11.20 μm (Table 4). The maximum width of heterocysts was observed in CCC89 which incidentally had the maximum length (11.20 μm). Shape of heterocysts ranged from ovoid or spherical to ellipsoidal. Heterocysts were found to be terminal as well as intercalary in all the *Nostoc* strains. Frequency of heterocysts ranged from 1.9% (CCC131) to 14.8% (CCC92). Strain CCC 125 which had larger vegetative cells and heterocyst also showed a heterocyst frequency of 11% (Table 4). Shape of the akinetes found in this study was varying from barrel or cylindrical to spherical or ovoid. CCC125 (*N. linckia*) had the maximum width (9.83 μm) among all the *Nostoc* strains while CCC131 had the maximum length (11.3 μm).

Table 1. List of *Nostoc* strains used in the study

Sl. no.	Strain no.	Name	Geographical origin and location
1	CCC42	*Nostoc* sp.	Jammu & Kashmir (34°10′N & 75°00′N), India
2	CCC92	*Nostoc muscorum*	Kannur (11°52′N, 75°25′E), Kerala, India
3	CCC48	*Nostoc punctiforme*	Baharaich (27°35′N, 81°36′E), Uttar Pradesh, India
4	CCC94	*Nostoc carneum*	Palghat (10°46′N, 76°42′E), Kerala, India
5	CCC184	*Nostoc* sp.	Calicut (11°15′N, 75°49′E), Kerala, India
6	CCC150	*Nostoc* sp.	New Delhi (28°4′N, 77°09′E), Delhi, India
7	CCC282	*Nostoc* sp.	"
8	CCC100	*Nostoc* sp.	Kannur (11°52′N, 75°25′E), Kerala, India
9	CCC62	*Nostoc linckia*	Baharaich (27°35′N, 81°36′E), Uttar Pradesh, India
10	CCC89	*Nostoc commune*	Nimpith (21°54′N, 88°20′E), West Bengal, India
11	CCC133	*Nostoc paludosum*	New Delhi (28°4′N, 77°09′E), Delhi, India
12	CCC90	*Nostoc picsinale*	Nimpith (21°54′N, 88°20′E), West Bengal, India
13	CCC131	*Nostoc paludosum*	New Delhi (28°4′N, 77°09′E), Delhi, India
14	CCC63	*Nostoc paludosum*	Baharaich (27°35′N, 81°36′E), Uttar Pradesh, India
15	CCC125	*Nostoc linckia*	New Delhi (28°4′N, 77°09′E), Delhi, India
16	CCC139	*Nostoc* sp.	"
17	CCC88	*Nostoc verrucosum*	Nimpith (21°54′N, 88°20′E), West Bengal, India
18	CCC110	*Nostoc spongiaeforme*	Cochin (9°58′N, 76°17′E), Kerala, India
19	CCC151	*Nostoc* sp.	New Delhi (28°4′N, 77°09′E), Delhi, India
20	CCC130	*Nostoc punctiforme*	"

Although a number of workers have studied the genus *Nostoc*, the intrageneric taxonomy of this species still seems complicated [3, 6, 9, 28]. McGuire [14] in a study on the morphological classification of *Nostoc* species, using numerical taxonomy, concluded that several species of *Nostoc* could be distinguished on the basis of 30 morphological characteristics. He proposed that the most useful were the size and shape of akinetes, vegetative cells, and heterocysts, color and luster of plant mass, veined plant mass surface, margin fimbriate, and shape of plant mass in nature. Caudales and Wells [29] mentioned about the "ambiguous concept of sheath surrounding the trichome", and concluded that this character is unreliable. According to De Philippis et al. this feature needs more investigation. They studied 40 *Nostoc* strains from Pasteur Culture Collection, of which only 25 strains showed a significant sheath or slime, and two more strains released polysaccharides to the media (these sheaths are formed by polysaccharides) [30]. Moreover, all hormogonia observed lacked these capsules but can sometimes be surrounded by a viscous slime [30, 31]. In addition, only in some species, hormogonia are released from the vegetative trichome [31]. Komarek has mentioned the following features which may be used as key for identification of the genus *Nostoc* (i) Trichomes

Table 2. Sequence information of the primers used in the study

No.	Primer name	Sequence (5'–3')	% G+C
1	HIP TG	GCGATCGCTG	70
2	HIP GC	GCGATCGCGC	80
3	HIP AT	GCGATCGCAT	60
4	CRA 22	CCGCAGCCAA	70
5	CRA 23	GCGATCCCCA	70
6	CRA 25	AACGCGCAAC	60
7	CRA 26	GTGGATGCGA	60
8	OPA 08	GTGACGTAGG	60
9	OPA11	CAATCGCCGT	60
10	OPA 13	CAGCACCCAC	70
11	OPF 05	CCGAATTCCC	60
12	OPAH 02	CACTTCCGCT	60

isopolar, of the same width along the whole length, (ii) apical cells morphologically indifferent from other cells, (iii) cells cylindrical, barrel-shaped up to almost spherical (forming moniliform trichomes; variability of cell-size and shape sometimes distinct in one and the same species) (iv) heterocysts solitary, terminal or intercalary, (v) akinetes arise apo-heterocytic, oval, little larger than cells; almost all cells between heterocysts change successively in akinetes towards heterocysts [32]. In the present study, the morphological and cultural characteristics of the different strains of *Nostoc* are mostly consistent with the descriptions given by Komarek & Anagnostidis [33] and Desikachary [3]. Size of akinetes, little larger than the vegetative cells is consistent with the currently available literature but the shape of the akinetes which is reported to be ovoid as in case of the type species i.e. *N. commune* [33], it was noticed that akinete shape was consistent with the reports only in case of one strain—CCC 89 (*N. commune*) and in the rest of the nineteen strains, it was barrel shaped or cylindrical or spherical. It was also interesting to note that the heterocyst frequency which is dependent on the level of nitrogen in the medium was different in all the strains grown in the nitrogen free BG-11 media. Thus, it seems that different strains may have differential capacity to develop a distinct range of heterocysts in terms of numbers. Further studies are still needed for these two morphological features which may prove useful for more clear-cut distinction of different *Nostoc* species based on morphological features. But morphological features which are actually a function of environment and growth conditions, sometimes make identification and other related analysis confusing and non-reproducible. Hence, molecular analyses have been increasingly used to characterize and deduce phylogenetic relationships in cyanobacteria. Simple and reproducible fingerprints of any complex genome can be generated using single arbitrarily chosen primers and the polymerase chain reaction [21, 22, 34]. Sequence polymorphisms detected by genomic fingerprinting can be mapped genetically or used in phylogenetic and population studies. The multiplex RAPD-PCR was chosen because of its greater degree of reproducibility, stringency for the differentiation of isolates to the strain level and the inference of phylogeny. A total of ten double primer reactions were chosen to generate RAPD patterns and

Table 3. Size range of the PCR products generated by ten primer combinations

No.	Primer name	Total no. of fragments	No. of polymorphic bands	Size range (kb)
1	HIP TG + HIP AT	49	13	0.250–2.000
2	HIP TG + HIP GC	104	14	0.200–1.700
3	HIP AT + HIP GC	84	15	0.256–1.700
4	HIP GC + CRA 22	67	14	0.160–1.421
5	OPA 13 + CRA 26	50	11	0.150–2.533
6	CRA 25 + OPF 05	59	17	0.240–2.942
7	HIP TG + OPAH 02	35	10	0.200–2.300
8	OPA 08 + HIP AT	71	17	0.120–2.043
9	HIP GC + CRA 23	115	16	0.100–1.984
10	CRA22 + OPA11	90	15	0.280–2.500

Table 4. Different morphological parameters studied for the twenty *Nostoc* strains

Sl. no.	Strains no.	Shape of vegetative cells	Heterocysts					Akinetes		
			shape	Size (μm)		position	frequency (%)	shape	size (μm)	
				length	breadth				length	breadth
1	CCC42	B	S	3.44–3.86	2.75–3.08	T&I	2.50	B	3.65–4.32	3.23–3.55
2	CCC92	B	E	3.85–5.40	3.33–3.68	T&I	14.80	B	3.23–5.12	3.41–4.05
3	CCC48	C	S	4.05–5.64	3.13–4.18	T&I	6.80	C	5.56–6.85	3.13–3.95
4	CCC94	E	E	4.21–5.94	3.76–5.39	T&I	6.40	B	6.28–8.50	4.05–5.89
5	CCC184	B	O	3.60–6.36	3.85–5.39	T&I	11.30	B	3.85–6.10	3.85–4.77
6	CCC150	C	O	4.39–6.73	4.43–5.73	T&I	9.00	S	5.00–6.47	4.48–5.82
7	CCC282	B	S	2.91–5.69	2.72–4.82	T&I	10.00	B	3.61–4.84	2.91–3.82
8	CCC100	O	O	2.87–4.69	2.41–4.26	T&I	11.10	B	4.17–7.23	3.41–4.28
9	CCC62	B	O	3.05–4.21	1.97–3.67	T&I	7.90	B	2.87–4.28	2.61–3.40
10	CCC89	C	O	5.50–11.20	4.32–6.08	T&I	6.20	O	7.87–8.69	3.92–4.62
11	CCC133	C	O	3.23–5.10	2.56–4.54	T&I	7.2	C	3.41–5.67	2.83–3.26
12	CCC90	C	O	3.06–4.56	3.11–3.36	T&I	7.0	C	4.93–5.81	2.60–3.23
13	CCC131	C	B	5.39–7.88	3.97–6.01	T & I	1.9	C	6.61–11.3	3.00–4.48
14	CCC63	B	E	4.25–8.07	4.39–5.47	T&I	8.2	B	5.73–6.38	4.60–5.82
15	CCC125	B	O	6.38–7.38	3.58–5.97	T&I	11.0	B	5.02–10.9	5.54–9.83
16	CCC139	B	S	3.06–4.17	2.60–3.58	T&I	4.3	S	3.40–5.00	3.63–5.00
17	CCC88	C	E	4.05–7.47	2.72–5.39	T&I	6.0	C	6.17–7.28	2.72–3.40
18	CCC110	B	E	3.05–5.12	2.60–3.72	T&I	6.0	B	3.80–4.81	2.96–4.26
19	CCC151	B	S	2.48–3.69	2.16–3.68	T&I	11.1	S	4.13–5.60	4.11–4.84
20	CCC130	C	O	2.87–6.10	2.60–5.55	T&I	3.52	B	3.85–5.25	3.20–3.72

B: Barrel, C: Cylindrical, E: Ellipsoidal, O: Ovoid, S: Spherical; T: Terminal, I: Intercalary.

diagnostic fingerprints for twenty strains of *Nostoc*. The criteria for choosing these primers were the generally accepted concept towards oligonucleotides of high G+C content [13]. Further, the primers based on the HIP sequence were also used as an octameric palindromic sequence known as HIP 1 has been shown to be present in the chromosomal DNA of many species of cyanobacteria as highly repetitious interspersed sequence [35]. Each of the ten dual primer combinations reproducibly yielded a distinct set of products when used to prime the same cyanobacterial genomic DNA template. Primers which produced consistently even product intensities throughout a pattern were favoured because of high reproducibility of the markers. Multiplex reactions produced a total of six hundred and twenty four distinct polymorphic fragments of molecular size ranging from 0.100 to 2.942 kb (Table 3, Figs. 2, 3). Number of fragments produced by each dual primer combination in multiplex PCR ranged from 35 (HIP TG + OPAH 02) to 115 (HIP GC + CRA 23). The number of polymorphic bands ranged from 10 (HIP TG + OPAH 02) to 17 (CRA 25 + OPF 05 and OPA 08 + HIP AT).

Majority of the amplification products are in the form of strong and well defined bands ranging from 100 to 2942 bp. The PCR based DNA amplification fingerprinting using short oligonucleotides for production of characteristic banding patterns has been widely used to generate fingerprints for identification and discrimination of microbial strains. The technique

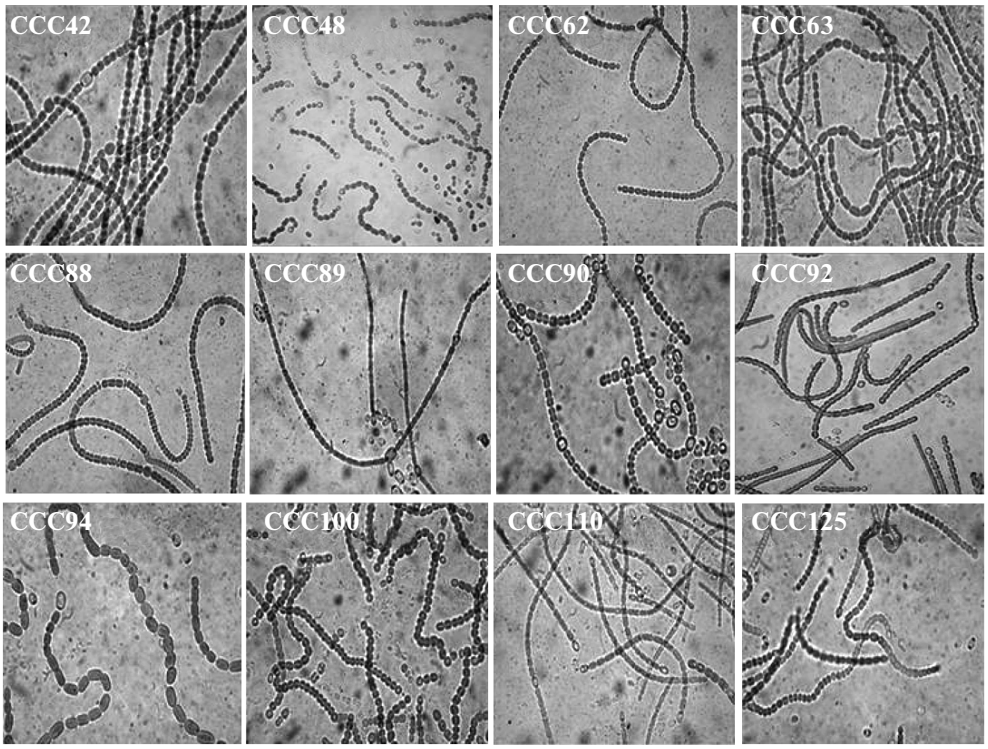

Fig. 1. Microphotographs of the *Nostoc* strains studied.

has been successfully used to fingerprint and differentiate cyanobacteria as well [20, 35, 36]. Genomic DNA of symbiotic and free living forms of cyanobacterial isolates were amplified by eleven decamer primers which resulted in fingerprint characteristic to each culture [19]. Similarly, molecular polymorphism was analyzed in a set of *Calothrix* strains using primers based on repetitive sequences in the genome [20]. In the present study, all the primer combinations produced varying number of products for the strains of *Nostoc* and all the primers showed 100% polymorphism. Perumal et al. carried out a study on phylogenetic analyses of cyanobacteria using RAPD and reported that the bands attained employing seven different primers for twelve strains were polymorphic [37]. Similarly, multiplex RAPD-PCR has also proved to be a useful technique for strain differentiation of cyanobacterial isolates because it increased the informative genetic markers. Combination of two primers in a single RAPD reaction has been successfully used to analyze the polymorphism and phylogeny of toxigenic cyanobacteria [18]. Multiplex RAPD analyses, using ten dual primer combinations have been used to reveal phylogenetic relatedness of *Spirulina* and related genera [38].

Multiplex RAPD PCR of twenty *Nostoc* strains produced ten unique bands ranging from 0.147 to 1.984 kb for ten strains. It was however, interesting to note that eight out of twelve primers used in the study produced one or more unique bands of specific molecular size. The occurrence of unique bands has been used successfully to differentiate closely related cyanobacterial strains [37] and it is worthwhile to mention that production of unique bands within the same genera i.e. *Nostoc*, can be helpful in developing specific identification profile for particular strains. The presence of unique bands ranged from one to two in different strains of *Nostoc* with selected primers. It is also important that only one or two unique bands were produced making it easy to develop specific and quick strain specific profiles for their identification.

The dendrogram based on multiplex combinations consisting of ten pairs of primers grouped the strains into two main clusters of seventeen and three strains respectively (Fig. 4). The cluster I was further subdivided into four subclusters of six, four, four and three strains. Although no specific clustering could be observed based on geographical origin of the strains but a few strains belonging to the same geographical area could be found clustered together. For example, CCC133 (*N. paludosum*), CCC131 (*N. paludosum*) and CCC125 (*N. linckia*) isolated from IARI research fields (New Delhi, India) were found in the same cluster. Similarly, CCC90 (*N. piscinale*) and CCC89 (*N. commune*) from Nimpith (West Bengal, India) were also grouped in one subcluster. CCC92 (*N. muscorum*) and CCC48 (*N. punctiforme*) were found in the same cluster showing a similarity coefficient of more than 0.85. Values obtained for cophenetic correlation indicated a good fit for the clustering analysis. A boot-

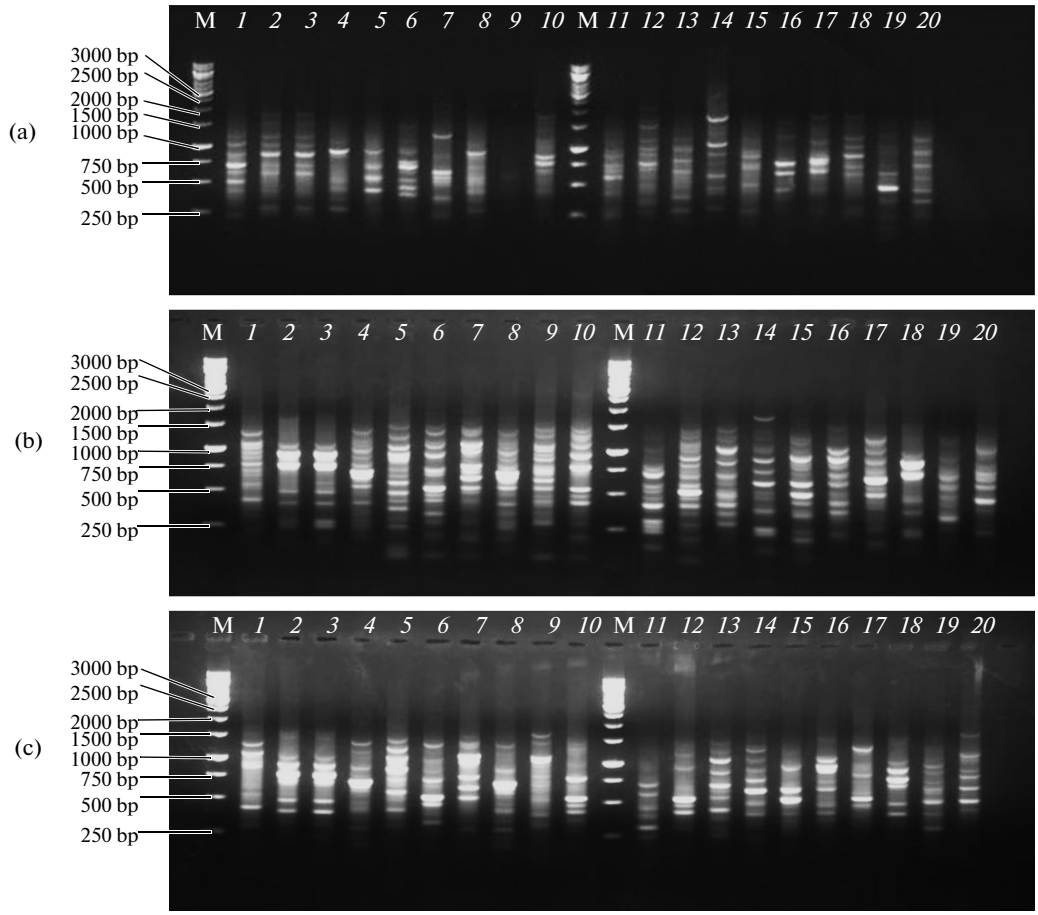

Fig. 2. RAPD PCR fingerprinting of the twenty *Nostoc* strains using dual primer combinations: (a)—HIP AT + HIP TG, (b)—HIP YG + HIP GC, (c)—HIP AT + HIP GC.

Lane *1* = CCC42. Lane *6* = CCC150. Lane *11* = CCC 33. Lane *16* = CCC139.
Lane *2* = CCC 2. Lane *7* = CCC282. Lane *12* = CCC90. Lane *17* = CCC88.
Lane *3* = CCC48. Lane *8* = CCC100. Lane *13* = CCC 31. Lane *18* = CCC110.
Lane *4* = CCC94. Lane *9* = CCC62. Lane *14* = CCC63. Lane *19* = CCC51.
Lane *5* = CCC184. Lane *10* = CCC89. Lane *15* = CCC125. Lane *20* = CCC 30.

strap value of 100% was observed in case of CCC92 and CCC48 clustered together and a bootstrap value of 99% was obtained for another cluster consisting of CCC94 and CCC100. But bootstrap values for all other clusters were low, indicating very low confidence regarding their clustering (Fig. 3).

Genetic relatedness among the *Nostoc* strains based on the binary data indicated a high degree of divergence between *Nostoc* strains isolated from different geographical locations of India. The level of similarity of the strains obtained was in the range of 0.15 to 0.94. It was also observed that major cluster represents a mixed group indicating a considerable polymorphism between the strains. Genomic fingerprinting for cyanobacteria belonging to different genera has been carried out using arbitrarily primed PCR and a matrix of pairwise combination of primers [18, 20, 35]. A recent study reported a high degree of variability among the *Calothrix* strains where a single primer PCR reaction showed a maximum of 60% similarity and the similarity of 58% with dual primer PCR reaction [20]. In the present study also, it has been observed that the strains showing a good similarity coefficient were clustered together irrespective of the dual primer combinations. Most of the other studies have used a mixed population of cyanobacteria representing different genera belonging to a particular geographic origin or based on their characteristic of being toxigenic or free living and symbiotic etc. but studies with same genera are limited. This may provide a better understanding of the genetic diversity within a genus and will be helpful in designing the marker for the specific strains as well as their further discrimination based on nitrogen fixing ability.

The strains of *Nostoc* could be clearly distinguished and the dendrograms based on multiplex RAPD indicated higher goodness of the fit of clusters (r = 0.85896). Moreover, the multiplex generated good

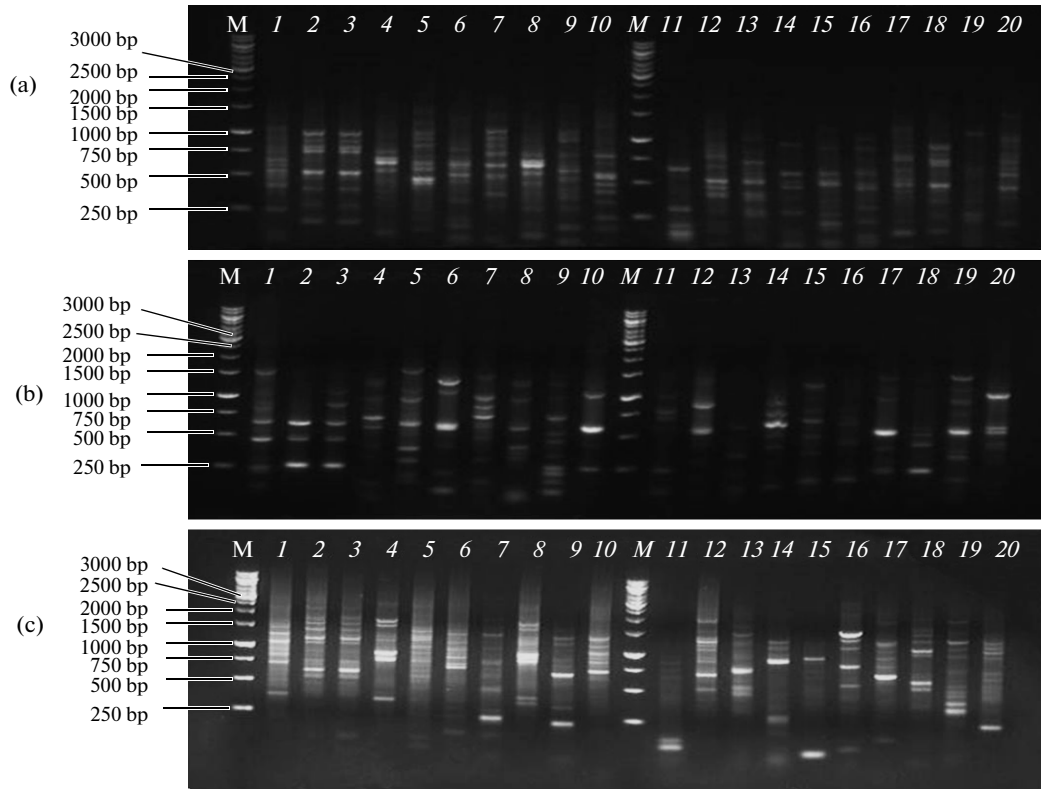

Fig. 3. RAPD PCR fingerprinting of the twenty *Nostoc* strains using dual primer combinations: (a)—HIP GC + CRA 22, (b)—OPA 08 + HIP AT, (c)—OPA 08 + HIP AT.

Lane *1* = CCC42. Lane *6* = CCC150. Lane *11* = CCC133. Lane *16* = CCC139.
Lane *2* = CCC92. Lane *7* = CCC282. Lane *12* = CCC90. Lane *17* = CCC88.
Lane *3* = CCC48. Lane *8* = CCC100. Lane *13* = CCC131. Lane *18* = CCC110.
Lane *4* = CCC94. Lane *9* = CCC 62. Lane *14* = CCC63. Lane *19* = CCC151.
Lane *5* = CCC184. Lane *10* = CCC89. Lane *15* = CCC125. Lane *20* = CCC130.

number of unique bands where no unique bands in a number of strains could be generated with single reaction.

It is very clear from the bootstrapping that the strains CCC92 and CCC48 clustered together with a very high similarity coefficient and bootstrap value and are very closely related. This may again be of some significance where morphological studies which have long been considered as a basis of identification of cyanobacteria do have correlation to its molecular make up. But this needs further investigation. Similarly, CCC94 and CCC100 showed a high similarity coefficient and high bootstrap values indicating that they may be related. As in case of most of the nodes the bootstrap value is very low which may be due to the high heterogeneity among the strains tested and suggests increasing the number of genetic markers for further analysis. There has been several studies where one or the other PCR based marker beside RAPD has been used to carry out phylogenetic analysis of cyanobacterial strains including *Nostoc* spp., however, a level of variation will always exist while comaparing the same set of organisms using different molecular methods.

Rajaniemi et al. (2005) reported that within the *Nostoc* cluster; sequences of *N. calcicola*, *N. edaphicum* and *Nostoc* sp. 1tu14s8 shared high 16S rRNA sequence similarity (97.7%) and clustered together with high bootstrap values in all the gene trees [39]. These *Nostoc* strains also shared many common features such as terminal conical heterocysts and narrow, straight hormogonia and were morphologically differentiated from the other studied *Nostoc* strains [40]. The high sequence and morphological similarity suggested that *N. calcicola*, *N. edaphicum* and *Nostoc* sp. 1tu14s8 could be assigned to a single species. In addition, *N. muscorum* and *N. ellipsosporum* were morphologically [40] and genetically more closely related to each other than to the other studied *Nostoc* strains. However, the 16S rRNA gene sequence similarity of these two *Nostoc* strains and *Trichormus azollae* to any other strains was <96.7%, indicating that these strains are not related to each other or to other *Nostoc* strains at the species level. Other workers also suggested revision of the genera *Nostoc* as well as division of genus into a number of genera [41, 42]. Although phylogenetic study based on 16S rDNA was not done in the present

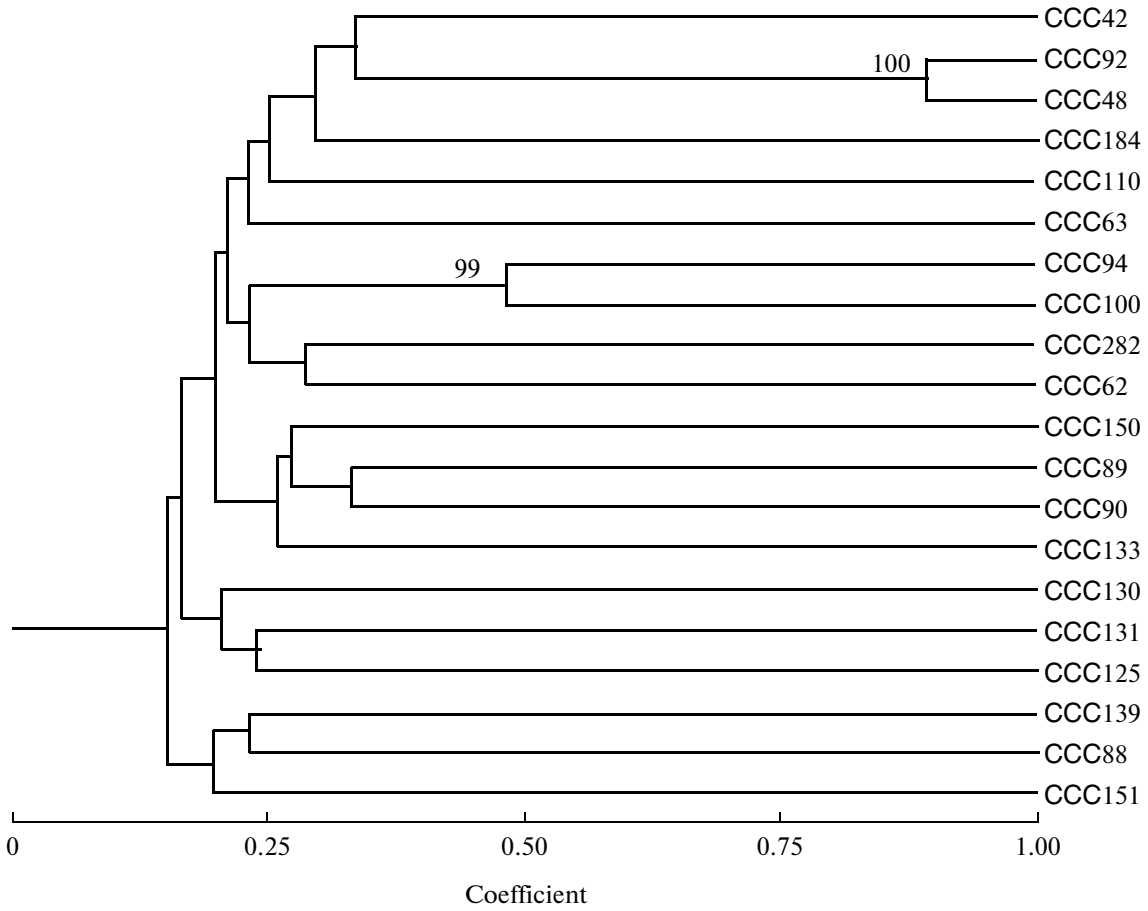

Fig. 4. Dendrogram of *Nostoc* strains based on RAPD PCR profile generated by ten dual primer combinations. Values presented at the nodes indicate bootstrap values (only values >50% are presented here).

study but critically analyzing the earlier reports and to get an idea about the phylogenetic relationship among the members of the genus *Nostoc*, twenty one (21) 16S rDNA sequences from EMBL were aligned using MEGA 5 software and phylogenetic tree (Fig. 5) constructed using UPGMA method showed that eight *N. commune* strains were grouped in four clusters. It was noted that *N. commune* strain M13 (Hyogo, Japan; ENA|AB101003) was found in separate cluster from another *N. commune* (Hyogo, Japan; ENA|AB251860) strain and similarity percentage was found to be 97.6% while *N. verrucosum* (ENA|AB245144) showed 100% similarity with *N. commune* (ENA|AB251683). Few strains showed a distance more than 4 (i.e. similarity <96%) which indicated that they may be from different genus. Hence, it is evident that strains from single species may be placed in different clades while two different species actually belong to a single species. Thus, from the present study and also from the study done based on secondary data, it was inferred that strains from same species may be placed in distant clusters while different species may be grouped in the same cluster.

The molecular studies among *Nostoc* strains carried out with the objective to differentiate them from one another has revealed extensive evidence for the applicability and discriminating power of RAPD towards the differentiation of morphologically and geographically unrelated strains. The results have further corroborated that immense intrageneric as well as intraspecific diversity exists within *Nostoc* and a novel RAPD-PCR based classification of the strains belonging to this genus as an alternative and complementary approach to the traditional methods will further aid in the study of cyanobacterial systematics. The use of HIP 1 (an octameric repetitive sequence found in cyanobacteria) fingerprinting has also proved to be useful for studies of the population dynamics of this important group and to evaluate the performance and dominance of bioinoculants over the native cyanobiota. It is true that DNA profiles produced for cyanobacteria provide a rapid and reliable basis for their genetic typing but presently the integration of phenotypic, genotypic and phylogenetic information will be the best approach for a polyphasic taxonomy of the genera *Nostoc* for their correct identification and grouping.

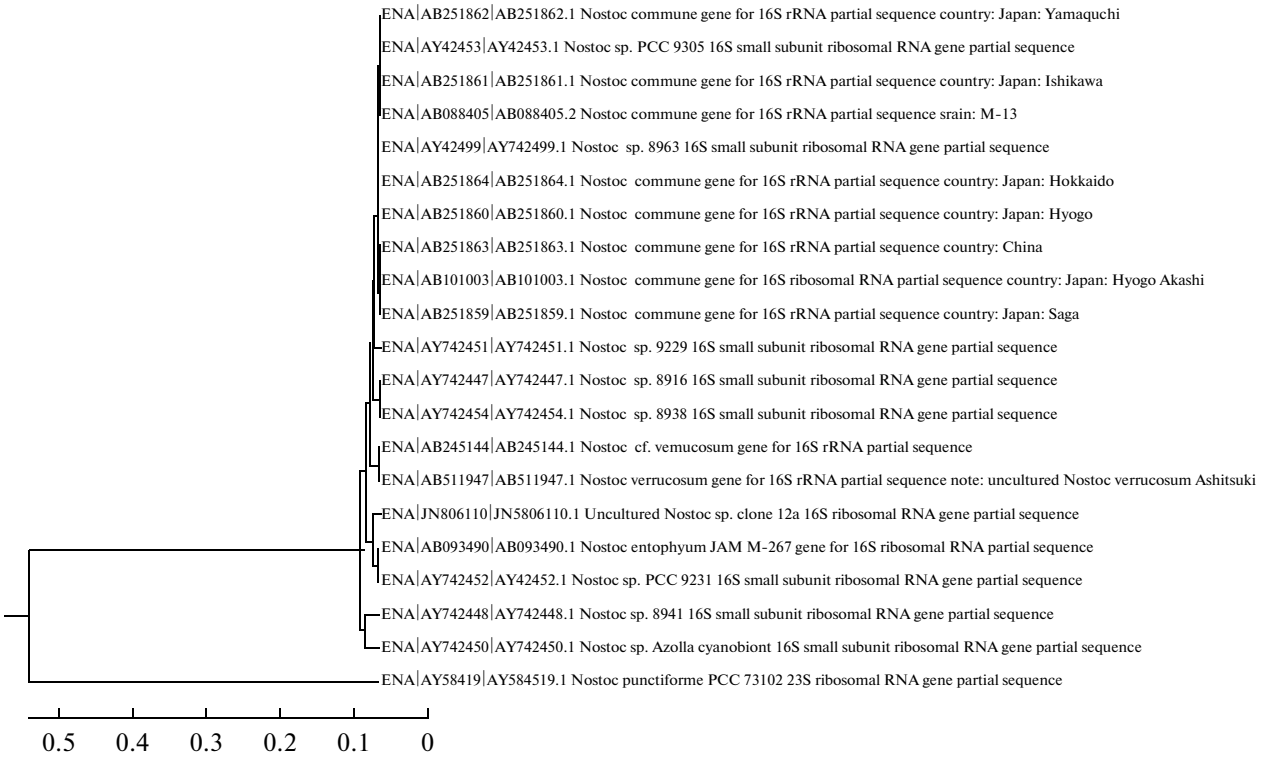

Fig. 5. Phylogenetic tree of 21 *Nostoc* strains generated using the 16S rDNA sequences obtained from EMBL.

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