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ORIGINAL ARTICLE

A comparative study reveals the higher resolution of RAPD over ARDRA for analyzing diversity of *Nostoc* strains

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Abstract *Nostoc* is a diverse genus of filamentous cyanobacteria with tremendous potential for agricultural and industrial applications. Morphometric methods and routine 16S rDNA-based identification undermines the genetic diversity and impedes strain-level differentiation. A comparative study to deduce the discriminatory power of random amplified polymorphic DNA (RAPD) and amplified ribosomal DNA restriction analysis (ARDRA) for analyzing the genetic diversity of 20 *Nostoc* strains of diverse geographical origin was carried out. The RAPD primer used in the study generated 100% polymorphic profile. HIP TG primer produced the highest number of bands and fragments. Five primers, viz. OPA 08, OPA 11, HIP GC, OPAH 02 and OPF 05 could produce unique bands for 11 strains. Cluster analysis using the RAPD profile showed 12.5–25% similarity among the strains. Following in silico restriction analysis, two restriction enzymes, viz. *Hae*III and *Hin*fI were selected for ARDRA. However, clustering based on the restriction pattern showed 22.5–100% similarity. Results of the present study clearly indicate higher resolution of RAPD which can be reliably used for strain-level differentiation of *Nostoc* strains.

Keywords *Nostoc* · RAPD · ARDRA · Genetic diversity · Discriminatory power

Introduction

Cyanobacteria or blue-green algae (BGA) are a group of photosynthetic prokaryotes that have colonized the surface of the earth for nearly 3 billion years and are considered to be predecessors of modern day chloroplast (Haselkorn 1978). The variability in their physiological, morphological and developmental characteristics make them one of the largest group of photosynthetic prokaryotes in the planet (Komarek 1991). Historically, these organisms were classified as blue-green algae according to the Botanical Code but Rippka et al. (1979) created the bacteriological classification and their scheme was adopted in Bergey's Manual of Bacteriological Systematics (Boone and Castenholz 2001), the recognized authority on bacteriological classification. The bacteriological approach is based on genetic and phenotypic information about the cyanobacteria present in pure cultures (axenic strains) (Castenholz 2001). Among different molecular techniques, 16S rDNA sequence analysis is the most popular till date. The conserved nature, variation in an orderly fashion across phylogenetic boundaries and lack of lateral gene transfer make 16S rDNA a suitable chronometer for taxonomic and phylogenetic studies. Widespread sequencing of ribosomal RNA genes (Bruno et al. 2012; Řeháková et al. 2014; Keshari et al. 2015, 2016; Sciuto and Moro 2016) has added potentially significant information to the evolutionary phylogenetics of several cyanobacteria. Ribosomal RNA gene sequences or their polymorphisms are playing a pivotal role in studying molecular systematics and phylogenies of cyanobacteria by serving genetic markers for

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cyanobacterial species (Gordon and Giovannoni 1996). Due to its genetic stability, domain structure with conserved and variable regions and its high copy number, the 16S rDNA has also been a tool in resolving taxonomic issues among cyanobacteria especially the strains belonging to a single clade (Palinska et al. 1996; Otsuka et al. 1998). Despite all its advantages, the 16S rRNA gene sequence analysis is more specific for assigning genus and moreover the sequencing of the 16S rRNA gene is time consuming, particularly, classification of a large number of strains is required. Other molecular tools like RAPD (Casamatta et al. 2003; Shalini et al. 2008; Singh 2008), ERIC-PCR (Rasmussen and Svenning 1998; Lyra et al. 2001; Valerio et al. 2005; Bruno et al. 2006), ARDRA (Margheri et al. 2003; Valério et al. 2009), etc., may be more useful for such purposes.

Nostoc is a diverse genus of simple cyanobacteria belonging to the order Nostocales and family Nostocaceae. These are common in both aquatic and terrestrial habitats and found in fresh water, soils and extremely cold and arid habitats as well. *Nostoc* has tremendous potential in environmental management as soil conditioners, biofertilizers, biomonitors of soil fertility, water quality, etc. This apart, *Nostoc* has also been used as human food, source of restriction endonucleases (NspCI), growth promoting substances, amino acids, etc. But for proper exploitation of genus *Nostoc*, it is necessary to work out suitable approaches for identification and to explore the diversity of this genus.

In this study, we present a comparative analysis of diversity using RAPD and ARDRA for 20 *Nostoc* strains isolated from different geographical regions of India.

Materials and methods

Cyanobacterial cultures and their maintenance

Twenty strains of *Nostoc* of 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 (Stanier et al. 1971) at 28 ± 2 °C under a light intensity of 52–55 μmol photon m^{-2} s^{-1} and L:D cycles of 16 h:8 h.

Genomic 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

Table 1 List of *Nosto*c strains used in the study

Sl. no.	Strain no.	Name	Geographical origin and location
1	CCC 42	*Nostoc* sp.	Jammu and Kashmir (34°10′N and 75°00′N), India
2	CCC 92	*Nostoc muscorum*	Kannur (11°52′N, 75°25E), Kerala, India
3	CCC 48	*Nostoc punctiforme*	Baharaich (27°35′N, 81°36′E), Uttar Pradesh, India
4	CCC 94	*Nostoc carneum*	Palghat (10°46′N, 76°42E), Kerala, India
5	CCC 184	*Nostoc* sp.	Calicut (11°15′N, 75°49E), Kerala, India
6	CCC 150	*Nostoc* sp.	IARI (28°4′N, 77°09′E) fields, India
7	CCC 282	*Nostoc* sp.	IARI (28°4′N, 77°09′E) fields, India
8	CCC 100	*Nostoc* sp.	Kannur (11°52′N, 75°25 E), Kerala, India
9	CCC 62	*Nostoc linckia*	Baharaich (27°35′N, 81°36′E), Uttar Pradesh, India
10	CCC 89	*Nostoc commune*	VIB, Nimpith (21°54′N, 88°20′E), West Bengal, India
11	CCC 133	*Nostoc paludosum*	IARI (28°4′N, 77°09′E) fields, India
12	CCC 90	*Nostoc picsinale*	VIB, Nimpith (21°54′N, 88°20′E), West Bengal, India
13	CCC 131	*Nostoc paludosum*	IARI (28°4′N, 77°09′E) fields, India
14	CCC 63	*Nostoc paludosum*	Baharaich (27°35′N, 81°36′E), Uttar Pradesh, India
15	CCC 125	*Nostoc linckia*	IARI (28°4′N, 77°09′E) fields, India
16	CCC 139	*Nostoc* sp.	IARI (28°4′N, 77°09′E) fields, India
17	CCC 88	*Nostoc verrucosum*	VIB, Nimpith (21°54′N, 88°20′E), West Bengal, India
18	CCC 110	*Nostoc spongiaeforme*	Cochin (9°58′N, 76°17E), Kerala, India
19	CCC 151	*Nostoc* sp.	IARI (28°4′N, 77°09′E) fields, India
20	CCC 130	*Nostoc punctiforme*	IARI (28°4′N, 77°09′E) fields, India

In the third column latitude and longitude of the place are given in the parenthesis

IARI Indian Agricultural Research Institute, *VIB* Vivekananda Institute of Biotechnology

(CTAB) method (Rogers and Bendish 1998) after minor modifications. Quantity and purity of DNA was estimated by comparing with known standards in ethidium bromide stained 0.8% agarose (Vivantis, USA) gel.

Random amplification of polymorphic DNA (RAPD)

Primers DNA samples were subjected to amplification using 12 decamer primers (synthesized from Sigma-Aldrich) with GC content varying from 60 to 80% (Table 2).

PCR amplification Amplification reactions were carried out with the aforementioned oligonucleotide primers. The standard, optimized PCR was performed in a total volume of 20 µl containing 1X TAE buffer with 15 mM $MgCl_2$, 10 mM of dNTP (dATP, dTTP, dGTP, dCTP), 10 pM each of single primer, 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) according to the following program: initial denaturation at 94 °C for 4 min; 30 cycles with steps of denaturation at 94 °C for 1 min, annealing at 34 °C for 1 min, extension at 72 °C for 2 min followed by a final extension at 72 °C for 5 min. PCR products were resolved along with a molecular weight marker (GeneRuler 1 Kb, Fermentas, USA) on 1.5% agarose gel in 1X Tris–acetate–EDTA (TAE) 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, USA) and the amplification product sizes were determined using software AlphaEaseFC (FluorChem 5500) (Alfa Innotech Corporation, USA).

Amplified ribosomal DNA restriction analysis (ARDRA)

Amplification of 16S rRNA gene of cyanobacteria 16S rRNA gene fragment was amplified with universal primers FD1: 5′-AGAGTTTGATCCTGGCTCAG-3′ and RP2: 5′-ACGGCTACCTTGTTACGACTT-3′ (Weisburg et al. 1991; Lyra et al. 1997). The polymerase chain reaction was carried out in a final volume of 25 µl, having 1X TAE buffer containing 2 mM $MgCl_2$, 10 mM deoxynucleotide triphosphates (dATP, dCTP, dGTP and dTTP), 16S primers (FD1 and RP2) 2.5 pmol each and 1 U of *Taq* DNA polymerase (Fermentas, USA) and 50 ng of genomic DNA. Amplification was achieved in a Master Cycler Gradient (Eppendorf) programmed for initial denaturation (94 °C for 5 min) followed by 35 cycles, composed of denaturation (94 °C for 30 s), primer annealing (64 °C for 45 s), extension (72 °C for 2 min) followed by a final extension of 5 min at 72 °C and subsequent cooling at 4 °C temperature. Amplified PCR product was separated along with a molecular weight marker (GeneRuler, 1 kb, Fermentas, USA) by electrophoresis on 1.5% agarose (Vivantis, USA) gel run in 1X TAE (Tris–acetate–EDTA) buffer, stained with ethidium bromide for a period of 1 h at 75 V. These were visualized under UV light and gel photographs were scanned through Gel Doc System (MiniBis Bioimaging System, USA) and the amplification product sizes were evaluated using software AlphaEaseFC (FluorChem 5500) (Alfa Innotech Corporation, USA).

Selection of restriction enzymes Initially seven restriction enzymes, viz. *Eco*RI, *Eco*RV, *Hind*III, *Hae*I, *Hae*III, *Hin*fI and *Pst*I were selected, but to make sure which of these may be more suitable for analyzing the diversity,

Table 2 Details of the primers and size of amplification products

No.	Primer name	Sequence (5′–3′)	Size range of fragments (kb)	Total no. of fragments	Total no. of bands	No. of polymorphic bands	% polymorphism
1	CRA 22	CCGCAGCCAA	0.309–3.042	69	15	15	100
2	CRA 23	GCGATCCCCA	0.236–2.169	51	12	12	100
3	CRA 25	AACGCGCAC	0.222–2.113	68	16	16	100
4	CRA 26	GTGGATGCGA	0.176–3.43	51	14	14	100
5	OPA 08	GTGACGTAGG	0.374–2.985	46	15	15	100
6	OPA 11	CAATCGCCGT	0.281–3.610	37	10	10	100
7	OPA 13	CAGCACCCAC	0.261–2.593	74	12	12	100
8	HIP AT	GCGATCGCAT	0.272–1.687	33	10	10	100
9	HIP TG	GCGATCGCTG	0.222–2.460	107	19	19	100
10	HIP GC	GCGATCGCGC	0.318–2.128	56	16	16	100
11	OPAH 02	CACTTCCGCT	0.394–2.490	34	12	12	100
12	OPF 05	CCGAATTCCC	0.315–2.259	59	15	15	100
Total				685	166	166	

10 16S rDNA sequences of different *Nostoc* strains were downloaded from the EMBL database (Table 3) and in silico restriction digestion was carried out using the Cleaver software (Jarman 2006). Analyzing the restriction fragments, it was found that there was no recognition site for *Hae*I and *Hind*III (Table 3) and among the rest of the five enzymes, *Hin*fI and *Hae*III were comparatively performing better than the others to produce differential restriction fragmentation pattern for the selected sequences (Table 3). So, finally *Hin*fI and *Hae*III were selected for restriction digestion of the 16S rRNA gene.

Restriction digestion of amplified 16S rRNA gene 8 µl of amplified PCR products were digested overnight at 37 °C with 5 U of each of the restriction enzymes namely *Hae*III and *Hin*fI, procured from New England Biolabs (Rasmussen and Svenning 2001). Digested products were separated along with a molecular weight marker (100 bp ladder, Vivantis, USA) by electrophoresis on 3% agarose (Vivantis, USA) gel run in 1X TAE buffer, stained with ethidium bromide for a period of 3 h at 75 V and gel photographs were visualized under UV light and gel photographs were scanned through Gel Doc System (MiniBis Bioimaging System, USA) and the amplification product sizes were evaluated using software AlphaEaseFC (FluorChem 5500) (Alfa Innotech Corporation, USA).

Statistical analysis and dendrogram construction

Fingerprints generated by RAPD and ARDRA from different cyanobacterial strains were compared and all bands were scored. The presence or absence of fragments was converted into binary data. Pairwise genetic similarities among the genotypes under study were determined using Jaccard's coefficient (Jaccard 1908), $J = N11/(N11 + N10 + N01)$, 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 (Rohlf 1995).

Results

RAPD

Each of the RAPD primers reproducibly yielded a distinct set of products when used for prime amplification reactions from the cyanobacterial genomic DNA template. The profiles were generated on the agarose gel using one primer to prime the cyanobacterial genomic DNA from each of the 20 *Nostoc* strains tested (Fig. 1). The analysis of the profile

Table 3 In silico restriction analysis of 16S rRNA gene sequences downloaded from EMBL database

Strains and EMBL accession numbers of 16S rRNA gene	Fragments (in bp) generated by different hexacutter and tetracutter restriction enzymes						
	*Eco*RI	*Eco*RV	*Hae*I	*Hae*III	*Hind*III	*Hin*fI	*Pst*I
ENA|AB088405|AB088405.2 *Nostoc commune* strain: M-13	699,594,151	1444	1444	457,389,274,187,137	1444	394,334,251,186,123,85,71	1290,154
ENA|AB093490|AB093490.1 *Nostoc entophytum* IAM M-267	699,592,151	1442	1442	457,389,272,187,137	1442	844,194,186,135,83	1288,154
ENA|AB101003|AB101003.1 *Nostoc commune*	699,594,151	1444	1444	457,389,274,187,137	1444	394,334,251,194,186,85	1290,154
ENA|AB245144|AB245144.1 *Nostoc* cf. *verrucosum*	700,594,83	1377	1377	458,389,274,187,69	1377	645,335,194,118,85	1291,86
ENA|AB251859|AB251859.1 *Nostoc commune*	699,594,151	1444	1444	457,389,274,187,137	1444	394,334,251,194,186,85	1290,154
ENA|AB251860|AB251860.1 *Nostoc commune*	699,450,151	1444	1300	457,389,187,137,130	1300	394,334,251,186,135	1146,154
ENA|AB251862|AB251862.1 *Nostoc commune*	699,594,151	1444	1444	457,389,274,187,137	1444	394,334,251,186,123,71,44,41	1290,154
ENA|AB251863|AB251863.1 *Nostoc commune*	699,594,151	1444	1444	457,389,274,187,137	1444	394,334,251,194,186,85	1290,154
ENA|AB251864|AB251864.1 *Nostoc commune*	699,597,151	1447	1447	457,389,277,187,137	1447	394,334,251,197,186,85	1293,154
ENA|AB511947|AB511947.1 *Nostoc verrucosum*	700,594,151	1445	1445	458,389,274,187,137	1445	645,335,194,186,85	1291,154

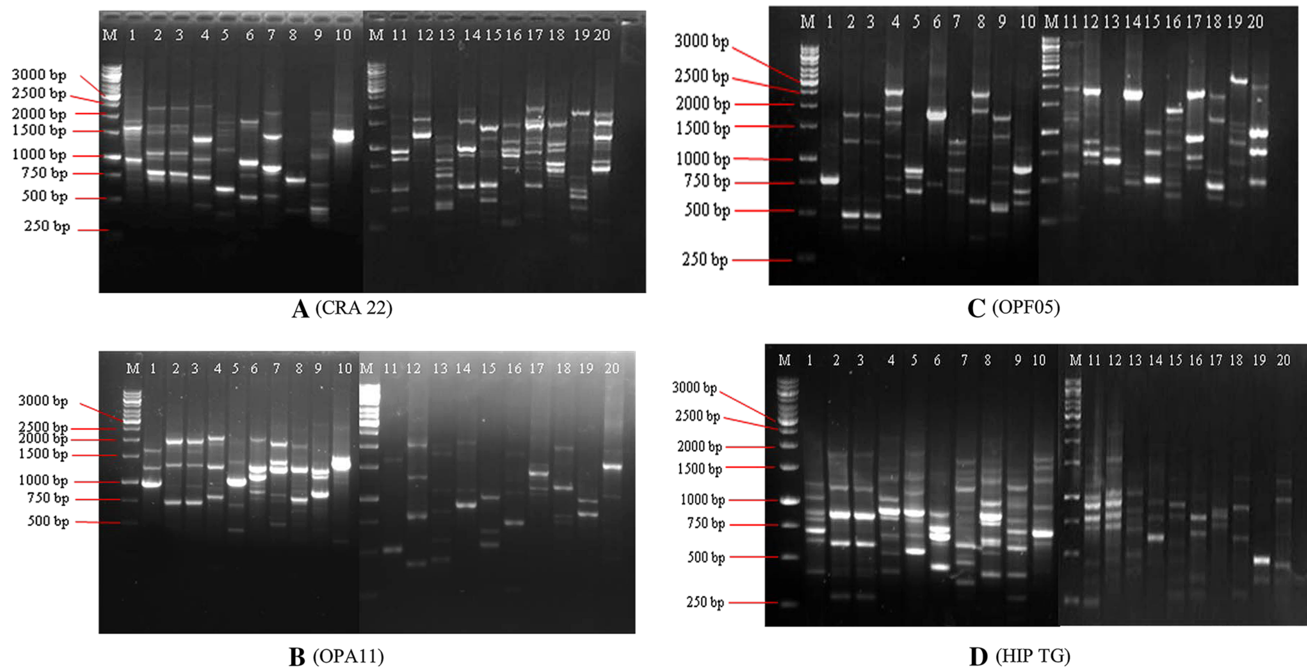

Fig. 1 RAPD profile of the studied *Nostoc* strains generated using: **a** CRA22, **b** OPA11, **c** OPF05, **d** HIP TG. *Lane 1* CCC42. *Lane 6* CCC150. *Lane 11* CCC133. *Lane 16* CCC139. *Lane 2* CCC 92. *Lane 7* CCC282. *Lane 12* CCC90. *Lane 17* CCC88. *Lane 3* CCC48. *Lane 8* CCC100. *Lane 13* CCC131. *Lane 18* CCC110. *Lane 4* CCC94. *Lane 9* CCC62. *Lane 14* CCC63. *Lane 19* CCC151. *Lane 5* CCC184. *Lane 10* CCC89. *Lane 15* CCC125. *Lane 20* CCC130

showed that each primer varied in the total number of fragments or total number of bands generated. A total of 685 DNA fragments were generated by all the single primers used. Among these a maximum of 107 fragments (Table 2) were generated by HIP TG primer followed by 74 fragments by primer OPA 13, whereas the least number of fragments, i.e., 33 were generated by HIP AT closely followed by OPAH 02 which generated 34 fragments. Similarly, the maximum numbers of bands, i.e., 19 were produced by HIP TG, whereas HIP AT produced only ten bands. The size of the fragments ranged from 0.176 to 3.430 kb. It was interesting to note that all the primers used produced 100% polymorphic bands. None of the primers produced monomorphic bands in RAPD study using single-primer reactions. On further analysis of the genetic profile generated by each primer, quite a few unique bands were observed with some primers. The number of the unique bands varied from one to two for different primers. A maximum of two unique bands were observed with five primers, viz. OPA 08, OPA 11, HIP GC, OPAH 02 and OPF 05 (Table 4). The molecular weight of these unique bands varied from 340 bp (by OPA 11 for CCC 282) to 2154 bp (by CRA 23 for CCC63). When the clustering was done using the data generated from all the 12 RAPD primers, the strains were divided into two main clusters containing 14 and 6 strains, respectively (Fig. 2). The strains showed high level of genetic divergence amongst themselves and each isolate was quite distinct from the other with varying degree of similarity which was in the range of 0.125 to about 0.25 except that *Nostoc punctiforme* (CCC 48) and *Nostoc muscorum* (CCC 92) showed a similarity coefficient of 0.70. Cluster analysis based on RAPD data did not show any clustering based on geographical origin. The analysis has revealed that all the *Nostoc* strains used in this study are quite distinct with some strains sharing more closeness to one another with a varying degree of similarity which changed with the set of primers used. Overall it was observed that strains CCC 48 (*Nostoc punctiforme*) and CCC 92 (*Nostoc muscorum*) showed high degree of similarity under different set of primers.

ARDRA

As expected after selecting the restriction enzymes through in silico analysis, *Hae*III and *Hin*fI produced differential pattern of restriction fragments for all the 20 *Nostoc* strains studied. *Hae*III produced a total of 58 fragments ranging from 143 to 925 bp in size while *Hin*fI produced 57 fragments ranging from 152 to 1030 bp in size (Table 5). Dendrogram (Fig. 3) based on ARDRA data for all the 20 *Nostoc* strains revealed two major clusters, viz. one (MC I) with only *N. spongiaeforme* (CCC110) from Cochin, Kerala, and other one (MC II) with rest of the 19 strains of

Table 4 Unique bands obtained with single-primer RAPD-PCR in *Nostoc* strains

Sl no.	Strain no.	CRA 22	CRA 23	CRA 25	CRA 26	OPA 08	OPA 11	OPA 13	HIP AT	HIP TG	HIP GC	OPAH 02	OPF 05
1	CCC42												
2	CCC92												
3	CCC48												
4	CCC94												1600 bp
5	CCC184								1700 bp				
6	CCC150						856 bp						
7	CCC282									340 bp	517 bp		
8	CCC100											1500 bp	
9	CCC62											380 bp	
10	CCC89					2027 bp	325 bp						
11	CCC133					2107 bp							
12	CCC90												
13	CCC131												
14	CCC63		2154 bp										
15	CCC125												
16	CCC139												1356 bp
17	CCC88												
18	CCC110												
19	CCC151												
20	CCC130										1233 bp		

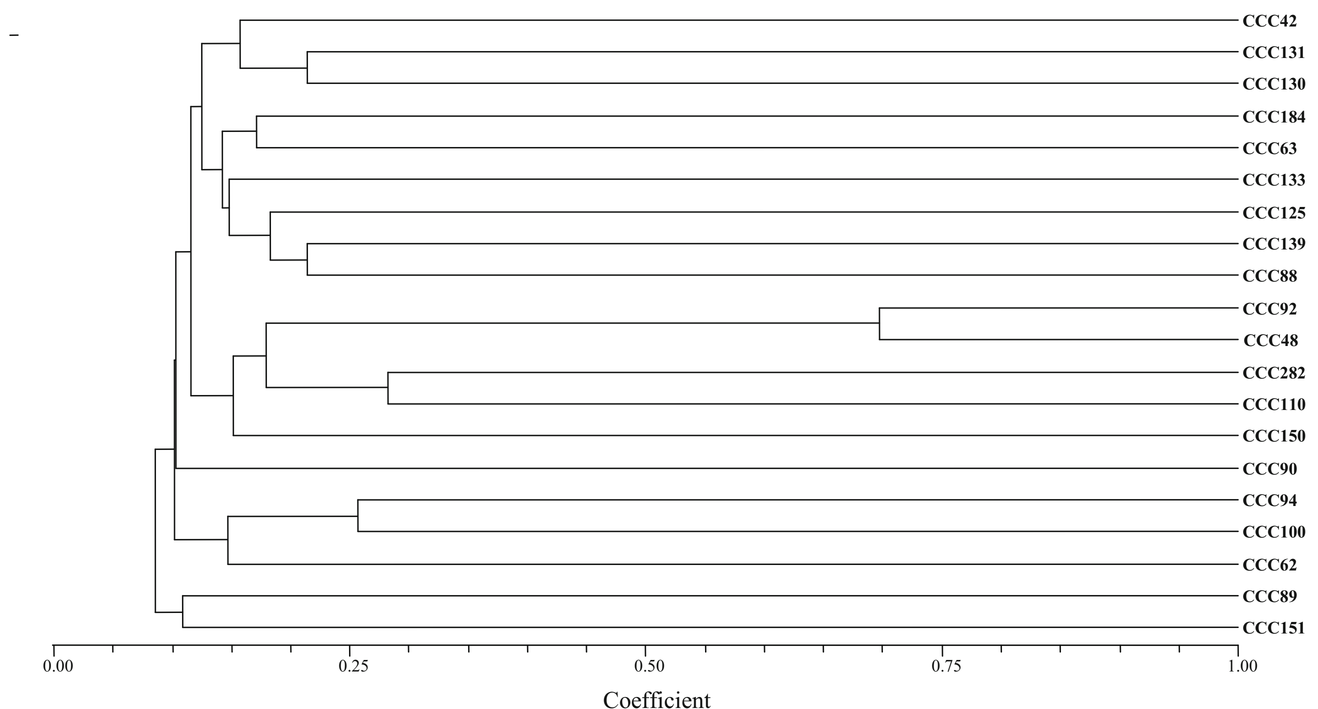

Fig. 2 Dendrogram generated using RAPD profile of the studied *Nostoc* strains. Dendrogram was constructed following the UPGMA method

Nostoc. Degree of similarity among the *Nostoc* strains as revealed by ARDRA ranged from 22.5 to 100%. In MC II, there were two subclusters comprising two strains (SC I: CCC63 and CCC139) and rest of the 17 strains (SC II). In SC II, it was found that CCC42 (*Nostoc* sp. from Jammu and Kashmir) was placed in a completely separate clade and rest of the 16 strains placed in another cluster showed more than 50% similarity among themselves. CCC92 and

Table 5 Restriction fragments produced by digestion with *HaeIII* and *HinfI*

Sl. no.	Cyanobacterial strains	Fragments (bp) generated after restriction digestion	
		*Hae*III	*Hinf*I
1	CCC42	196, 355, 978	169, 1140
2	CCC92	193, 551, 848	137, 163, 925
3	CCC48	190, 567, 873	158, 284, 894
4	CCC94	186, 301,926	158, 284, 991
5	CCC184	185, 301, 925	158, 284, 994
6	CCC150	185, 301, 584	163, 284, 1026
7	CCC282	185, 301, 584	163, 288, 1030
8	CCC100	175, 285, 899	285, 326, 752
9	CCC62	175, 275, 592	169, 294, 610
10	CCC89	180, 286, 560,	175, 285, 610
11	CCC133	160, 239,825	163, 265, 1063
12	CCC90	173, 277, 590	169, 294, 610
13	CCC131	151, 233, 849	460, 1080
14	CCC63	143, 226, 536	188, 294, 778
15	CCC125	148, 240, 779	169, 284, 470
16	CCC139	147, 247, 552	163, 569, 805
17	CCC88	173, 276, 587	163, 292, 610
18	CCC110	825, 395	530, 1010
19	CCC151	152, 269, 637	158, 265, 520
20	CCC130	636, 415	152, 304, 980
Total fragments		58	57

CCC48 were placed in the same clade in SCII with 80% similarity. In SC II, CCC184 (*Nostoc* sp.) and CCC94 (*N. carneum*) were placed in the same clade with 100% similarity. It will be worthy to mention here that both of these strains were geographically related as their origin belongs to Kerala. Similarly, two *Nostoc* strains, viz. CCC150 and CCC282 isolated from IARI fields were also found to be placed in the same clade with 100% similarity. *N. verrucosum* (CCC90) and *N. piscinale* (CCC88) isolated from VIB, Nimpith, West Bengal, also showed 100% similarity and were placed in the same clade despite of their specific designation based on morphological features. In the same clade, *N. linckia* (CCC62) was also placed with 100% similarity although it was obtained from Baharaich, UP, *N. commune* (CCC89) isolated from VIB, Nimpith, also showed almost 90% similarity with the clade comprising CCC88, CCC90 and CCC62. CCC131 (*N. paludosum*) and CCC151 (*Nostoc* sp.) isolated from IARI were also placed in same clade with 67.5% similarity. Not for all the strains, but for some of the strains (8 strains) ARDRA analysis revealed clustering based on geographical origin.

Discussion

With the advent of sequencing technologies available with the advantages of minimal costs and less time, 16S rDNA sequencing has become the most powerful tool to identify novel taxa for bacterial domain. Other powerful tools like DNA–DNA hybridization (DDH), G+C contents are also equally important for this purpose as 16S rDNA-based identification does not guarantee species identity (Fox et al. 1992). Hence, 16S rDNA gene sequencing to reveal intrageneric or intraspecific diversity of bacteria, may not always be a tool of choice. PCR-based methods like RAPD, PCR–RFLP, ERIC-PCR, REP-PCR have been widely used for analysis of diversity in wide range of bacteria. RAPD has an advantage over most of the other PCR-based methods in that it covers the whole genome as a random primer can bind anywhere in the genome; however, reproducibility of the RAPD data sometimes makes it inconvenient for use. Careful optimization of the RAPD conditions and use of a sufficient number of primers depending on the number of isolates being studied can make this tool very useful for diversity analysis. On the other hand, the PCR–RFLP of 16S rDNA also has its own advantages and limitations. Choice of restriction enzymes for digestion of the DNA becomes the key factor for the analytical power of this tool.

RAPD has been a widely used tool for analyzing the genetic diversity of various cyanobacterial groups. Casamatta et al. (2003) used RAPD to study the genetic variability in *Phormidium retzii*. In 2008, Shalini and Gupta carried out phylogenetic analysis of 30 *Calothrix* strains using single and multiplex RAPD which showed its superior discriminatory power for analyzing variability. Singh (2008) also used RAPD to analyze the genetic diversity of *Spirulina* and related genera. Genetic relatedness of *Phormidium*-like strains originating from distinct geographical sites was determined using RAPD (Palinska et al. 2011). Arima et al. (2012) showed that RAPD profiles generated through HIP primers can be useful to distinguish among the genotypes of *N. commune*. Similarly, Singh et al. (2014) also showed that HIP-based DNA fingerprinting technique could produce strain-specific and unique banding pattern for heterocystous cyanobacteria belonging to Subsection IV and V. Neilan (1995) used multiplex RAPD for determining genetic heterogeneity and generating unique identifying genetic profile of bloom-forming members of the genera *Anabaena* and *Microcystis*. The RAPD profile generated in the study was able to distinguish up to strain level among all the cyanobacteria studied.

In the present study, degree of heterogeneity revealed by the RAPD was enormous which is clear from the fact that

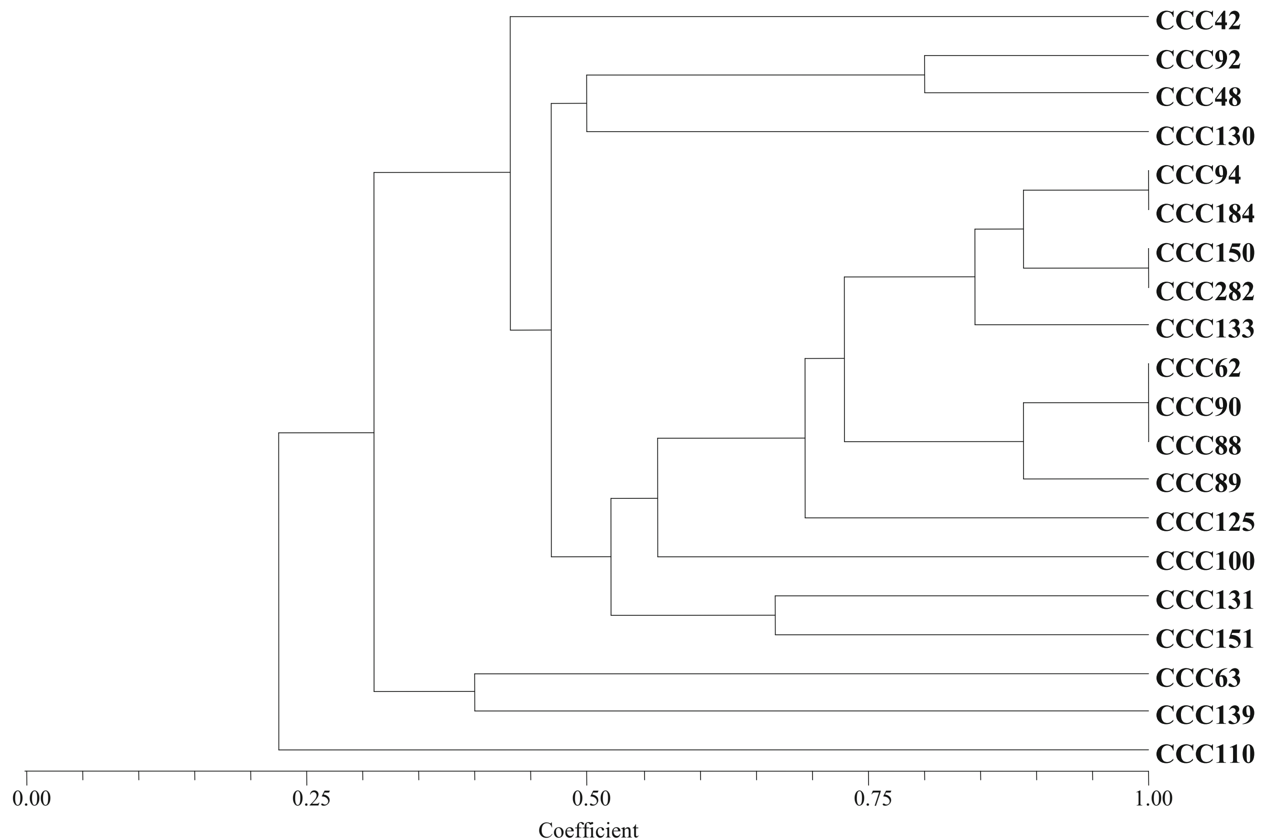

Fig. 3 Dendrogram generated using ARDRA profile of the studied *Nostoc* strains. Dendrogram was constructed following the UPGMA method

in RAPD study, similarity coefficient ranged from 0.125 to 0.25 (except 0.70 for CCC92 and CCC48), while ARDRA showed similarity coefficient in the range of 0.225–1.00 where 13 strains showed more than 50% similarity among themselves. Intra-generic or intra-specific diversity of the studied *Nostoc* strains has been clearly revealed by the RAPD which indicates highly heterogeneous nature of this genus while diversity revealed by ARDRA is very limited. The strains (CCC94 and CCC184; CCC150 and CCC282; CCC62, CCC88 and CCC90), which showed 100% similarity in ARDRA, were distantly related in the dendrogram generated by RAPD. The strains, viz. CCC62 (*N. linckia* from Uttar Pradesh), CCC90 (*N. piscinale* from West Bengal) and CCC88 (*N. verrucosum* from West Bengal) showing 100% similarity point out an important and interesting fact that despite having identical 16S rDNA sequence, the strains may not be clonal which is evident from the different RAPD profiles of these strains. This fact is also supported by the differential specific designation based on the morphology. However, the cultured strains of cyanobacteria often show little similarity to natural populations as some phenotypic traits are apparently not expressed under the controlled conditions. This problem may lead to the misidentification of cultures (Palinska et al. 1996; Wilmotte and Herdman 2001) and can reduce the value of phylogenetic reconstructions and other analyses of evolutionary interrelationships. Although some of the *Nostoc* strains showed clustering based on geographical origin, it was not conclusive enough to infer that the conservativeness of 16S rDNA is influenced by the geographical region or geographically related strains are also phylogenetically related.

An interesting fact was revealed from this study that CCC92 (*N. muscorum*) and CCC48 (*N. punctiforme*) were genetically very closely related as evident from the RAPD-banding profiles. However, ARDRA was able to sufficiently discriminate between these two strains. These two strains were reported morphologically distinct in terms of the shape of their vegetative cells; heterocyst shape, frequency; akinete shape, size and frequency (Chakdar and Pabbi 2012) and they were placed in two different species due to such significant variations in morphological attributes. It appears from the results of the present study that the taxonomic placement of these two strains based on their morphology does not correlate with their genetic relatedness and hence, they appear to be two different strains of the same species; however, it needs to be clarified following a polyphasic approach.

It is clear from the present study that for analyzing the intra-generic or intra-specific diversity of cyanobacteria,

RAPD is a far better tool compared to ARDRA. The strains which may show high similarity or may be identical in ARDRA pattern, are not necessarily the same as they may have significant variations distributed over the genome. Particularly, for a genus like *Nostoc* which is highly diverse in nature, it will not be wise to choose tool like ARDRA which may underestimate the diversity among the different strains. However, other genera of cyanobacterial group may be explored for testing the intrageneric discriminatory power of ARDRA.

In silico tools have been used for ARDRA of bacterial groups like Phytoplasma followed by identification, but only very limited reports (Iteman et al. 2002) are available for its use in cyanobacteria. Wei et al. (2007) were able to classify 800 Phytoplsma isolates into 28 groups by following the in silico approach. A careful choice of REs enabled the use of the ARDRA technique to discriminate among *Lactobacillus*, *Streptococcus* and *Bifidobacterium* at the genus level, but not at species level (Collado and Hernandez 2007). From the present study, we will also suggest to go for in silico ARDRA (can be done by a number of programs) which can really reduce the efforts of the researchers for selecting the appropriate REs for ARDRA. The availability of huge number of 16S rDNA sequences in different databases all over the world along with a number of freely available in silico analytical tools have empowered the researchers to dig out more and more information with minimum effort. Not only time will be saved through this, but also the consumables can be saved.

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Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest.

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