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EXPERIMENTAL ARTICLES

Morphological and Molecular Characterization of *Calothrix* Isolates Obtained from Diverse Environments in India[1]

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Abstract—Thirty cyanobacterial strains of *Calothrix* (family *Rivulariaceae*) isolated from diverse geographical regions of India were analyzed using morphological and molecular approaches. Most of the isolates were planktonic while some grew benthically. Significant differences were observed with regard to the shape and size of the vegetative cells, heterocysts, and akinetes. Analyses of molecular polymorphisms using Restriction Fragment Length Polymorphisms (RPLP) of 16S rRNA genes with the reference strain led to unambiguous differentiation of the isolates as well as understanding of their genetic relationships.

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Cyanobacteria (blue-green algae) constitute one of the largest groups of photosynthetic prokaryotes which occupies a wide range of terrestrial and aquatic environments. Due to their capacity for oxygenic photosynthesis and the ability of some cyanobacteria to fix atmospheric nitrogen, these organisms are unique among prokaryotes [1]. *Calothrix*, a typical component of the subsurface littoral community, is a well-documented cyanobacterial genus. These benthic organisms are among the most easily recognizable cyanobacterial genera [2]. In accepted morphological and ecological manuals, identification of the genus *Calothrix* is based on the "Presence of uniform trichomes, absence of sheath or presence of more or less diffluent sheath forming free or floccose or soft mucilaginous thallus. Unbranched or falsely branched; with a distinct meristematic zone and trichothallic growth, heterocysts present or absent, if present then basal sometimes intercalary and hormogones present; spores present or absent, if present may be single or in series" [3]. Characteristics of vegetative cells, heterocysts, and akinetes are changeable and vary considerably due to phenotypic changes under different environmental conditions and or selective culturing conditions [4]. Current cyanobacterial taxonomy is based primarily on observed morphological characteristics, which are often confusing and do not discriminate between different isolates. The taxonomically important characteristics vary so drastically that reliable identification of species becomes difficult or even impossible [5]. A number of new valuable molecular biological tools for taxonomic purposes have been developed which could be efficiently applied for checking the genetic purity of isolates in cyanobacterial cultures.

Restriction Fragment Length Polymorphisms (RFLPs) have been used extensively as an efficient DNA fingerprinting method to identify symbiotic cyanobacteria like *Anabaena azollae* [6] and *Nostoc* spp. from cycads [7]. Nelissen et al. [8] developed a cyanobacterial-specific oligonucleotide probe which enabled identification of the PCR products from 16S rRNA gene. Molecular techniques such as RFLP and 16S rRNA gene sequencing, have also been employed in phylogenetic analysis and characterization of cyanobacterial diversity [9]. Gugger et al. reported that the morphologically distinct cyanobacterial genera *Anabaena* and *Aphanizomenon* exhibit high degree of similarity in 16S rRNA [10–12]. 16S rRNA gene sequencing has extended the knowledge regarding the phylogeny of microcystin and nodularin-producing planktonic *Microcystis* and *Nodularia* strains [13]. Furthermore, strains belonging to the genus *Synechococcus* were found to be highly divergent and are widely scattered across the evolutionary tree [14].

Although 16S ribosomal gene analysis has been a method of choice for deducing phylogenies and establishing evolutionary relationships, morphological data have always been applied together with molecular data to derive meaningful inferences about a more accurate determination of the taxonomic status of cyanobacteria.

The aim of this study was to correlate the morphological parameters with genetic diversity amongst *Cal-*

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Table 1. List of *Calothrix* strains

Strains	Name	Source of isolation	Designation (as used in text)
CCC14	*Calothrix* sp.	Sam, Rajasthan, India	Ca_1
CCC18	*Calothrix* sp.	Bhuj ,Gujarat, India	Ca_2
CCC43	*Calothrix* sp.	Jammu & Kashmir, India	Ca_3
CCC65	*Calothrix brevissima*	Baharaich, Uttaranchal, India	Ca_4
CCC76	*Calothrix wembaerensis*	Baharaich, Uttaranchal, India	Ca_5
CCC77	*Calothrix* sp.	Baharaich, Uttaranchal, India	Ca_6
CCC124	*Calothrix* sp.	Tellicherry, Kerala, India	Ca_7
CCC142	*Calothrix* sp.	IARI, New Delhi, India	Ca_8
CCC144	*Calothrix* sp.	IARI, New Delhi, India	Ca_9
CCC145	*Calothrix* sp.	IARI, New Delhi, India	Ca_{10}
CCC146	*Calothrix* sp.	IARI, New Delhi, India	Ca_{11}
CCC155	*Calothrix* sp.	IARI, New Delhi, India	Ca_{12}
CCC166	*Calothrix* sp.	IARI, New Delhi, India	Ca_{13}
CCC168	*Calothrix* sp.	New Jaipaigudi,West Bengal, India	Ca_{14}
CCC178	*Calothrix* sp.	Vikasnagar, West Bengal, India	Ca_{15}
UTEX379	*Calothrix membranacea*	Dr. Alexy A. Vepritskiy, USA,	Ca_{16}
CCC217	*Calothrix* sp.	Managlore, Karnataka, India	Ca_{17}
CCC222	*Calothrix* sp.	North Goa, India	Ca_{18}
CCC224	*Calothrix* sp.	South Goa, India	Ca_{19}
CCC240	*Calothrix* sp.	North Goa, India	Ca_{20}
CCC261	*Calothrix* sp.	IARI, New Delhi, India	Ca_{21}
CCC262	*Calothrix* sp.	IARI, New Delhi, India	Ca_{22}
CCC269	*Calothrix* sp.	IARI, New Delhi, India	Ca_{23}
CCC270	*Calothrix* sp.	IARI, New Delhi, India	Ca_{24}
CCC293	*Calothrix* sp.	Sam, Rajasthan, India	Ca_{25}
CCC326	*Calothrix* sp.	IARI, New Delhi, India	Ca_{26}
CCC330	*Calothrix marchica*	Cuttack, Orrisa, India	Ca_{27}
CCC336	*Calothrix javanica*	Cuttack, Orrisa, India	Ca_{28}
CCC351	*Calothrix* sp.	Cuttack, Orrisa, India	Ca_{29}
CCC377	*Calothrix scytonemicola*	IARI, New Delhi, India	Ca_{30}

Note: CCC (Cyanobacterial Culture Collection, Centre for Conservation and Utilization of Blue-Green Algae (CCUBGA), IARI, New Delhi, India) UTEX (Culture Collection of Algae at the University of Texas, Austin.

othrix isolates from different geographical origins using RFLP of PCR-amplified 16S rRNA genes.

MATERIALS AND METHODS

Cyanobacterial strains. Thirty cyanobacterial isolates from the genus *Calothrix* including the reference strain (UTEX 379, *Calothrix membranacea*) were obtained from the culture collection of Centre for Conservation and Utilization of Blue green Algae (CCUBGA), Indian Agricultural Research Institute (IARI), India (Table 1). The reference strain from The Culture Collection of Algae, University of Texas, Austin, United States, designated in our study as Ca_{16}, was kindly provided by Dr. A.A. Vepritskiy (Darrin Fresh Water Institute, RPI, Bolton Landing, NY, USA). The isolates were grown and maintained in chemically defined nitrogen-free BG-11 medium at 28 ± 2°C

under illumination (52–55 μmol quanta m^{-2} s^{-1}; the light and dark periods were 16 and 8 h, respectively [15].

The algological purity of the isolates was confirmed by repeated subculturing, antibiotic treatment, and washing with sterilized water. The exponential phase cultures (15 days old) were homogenized (to break clumps and obtain uniform suspensions) and the samples were taken in triplicate for further analyses.

Cultural characteristics and microscopic analysis of the strains. Morphology of the selected set of *Calothrix* isolates was studied at different stages of growth under the conditions described above. Solid media was prepared by addition of 1.2–1.5% agar and autoclaved before use. After fourteen days of incubation, the cultures were subjected to streaking on agarized BG-11 medium for obtaining discrete colonies under uniform cultivation conditions. These colonies were picked up and inoculated in 50-ml flasks containing 20 ml BG-11 medium and incubated till the exponential phase (14 days). The isolates were examined under a Microphot-FX light microscope (Nikon); cell number per filament, shape and size of vegetative cells, heterocysts, and akinetes were described using the keys provided by Desikachary [3].

PCR and restriction enzyme analysis of 16S rRNA genes. Genomic DNA was extracted from 50–70 mg of axenic cyanobacterial biomass by using the N-cetyl-N,N,N-trimethylammonium bromide (CTAB) method with minor modifications [16]. The 1500 bp fragments of 16S rRNA genes were amplified with universal primers FD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and RP2 (5'-ACGGCTACCTTGTTACGACTT-3') [17] according to the modified reaction protocol [18]. PCR was performed in a total volume of 25 μl containing 50 ng of template DNA, 1.5 mM 10 × *Taq* Buffer, primers(0.5 μM each), dNTPs (25 mM each), and 1U of *Taq* DNA polymerase (Bangalore Genei Ltd.). Amplification was carried out in a Programmable Master Cycler Thermal Controller (Eppendorf, Germany), with the following temperature profile: 1 cycle at 94°C for 5 min; 35 cycles of 94°C for 30 s; 64°C for 45 s; and 72°C for 2 min; 1 cycle at 72°C for 5 min; and final step at 4°C. The PCR products were analyzed in horizontal 1% (wt/vol) agarose (Promega) gel in TAE buffer (20 mM *Tris*-acetate, 0.5 mM EDTA, pH 8.0).

Amplified PCR products (5–10 μl) were digested overnight at 37°C with 5 U of one of the following enzymes: *Dpn*I, *Hae*III, *Rsa*I, *Mse*I, and *Eco*RI (New England Biolabs) [19]. Restriction fragments (10 μl) were analyzed by horizontal electrophoresis in 3.0% agarose (Sisco Research Laboratories Pvt. Ltd.) gel in 1× TAE buffer (0.5M EDTA, 1 M Tris-acetate, pH 8.0) and electrophoresed at 75 V for 3 h and visualized by ethidium bromide (0.5 μg/ml). The molecular mass standard was a λ 100-bp ladder (Fermentas). The patterns of the restriction fragments were visualized on a UV transilluminator; the images were obtained using a Gel Doc System (MiniBis Bioimaging System, United States) and the amplification product sizes were evaluated using the Quantity one software package (Biorad, USA).

Statistical analysis. Fingerprints generated from different cyanobacterial isolates were compared and all bands were scored in decreasing order of their molecular weights for each DNA sample. The presence or absence of particular DNA fragments was converted into binary data and the pairwise genetic similarities among the genotypes under study were determined using the Jaccard's coefficient [20]. Cluster analyses were carried out on similarity estimates using the unweighted pair-group method with arithmetic average (UPGMA) using NTSYS-pc, version 1.80 [21].

RESULTS AND DISCUSSION

Distribution of cyanobacteria was examined in water-deficient regions of Rajasthan, India, namely, Achrol, Jaiselmer, Manwar, and Pokharan. Common cyanobacterial genera like *Calothrix*, *Phormidium*, *Oscillatoria*, *Nostoc*, *Anabaena*, *Westillopsis*, and *Chlorogloeopis* were isolated from arid zones samples [22]. Many cyanobacterial isolates exhibit high nitrogen-fixing potential and have been the favorite organisms for genetic manipulation. However, the identification of the genus *Calothrix* is complex as some morphological features vary and may not be always observable in culture [23]. Our investigation implies that both the cultural characteristics and molecular markers participate in creation of diversity, which is basically genotypic and certainly not limited to 16S rDNA.

Morphological characterization. Most of the *Calothrix* isolates were planktonic except Ca_9, Ca_{14} and Ca_{17} which grew at the bottom of the flask. Most of the isolates possessed dark-green coloured thallus. A number of isolates grew as floccose masses while some grew as benthic and the strains Ca_3 and Ca_4 formed evenly distributed suspensions. The trichomes grew as densely entangled/distinctly or slightly constricted colonies except in isolates of Ca_6, Ca_{24} and Ca_{27} where trichomes were irregularly straight or bent. Trichomes of the reference isolate (Ca_{16}) were densely entangled, planktonic, with floccose growth. Desikachary [24] stressed that cultural studies are essential to study the pattern of blue-green algae, which exhibit a wide range of variability.

All the *Calothrix* isolates were studied microscopically in terms of size and shape of vegetative cells, heterocysts and akinetes; position of heterocysts in filaments and their frequency were determined. Using these parameters, the identification and purity of the culture were confirmed as per the keys given by Desikachary [3]. The morphological parameters of the selected *Calothrix* isolates determined by microscopic examination are presented in Table 2.

Table 2. Morphological characterization (microscopic examination) of cyanobacterial strains of *Calothrix*

Strains	Vegetative cell			Heterocysts					Akinete		
	Shape	Size (mm)		Shape	Size (mm)		Position	Freq. (%)	Shape	Size (mm)	
		Length	Breadth		Length	Breadth				Length	Breadth
Ca_1	Barrel shaped	2.88–5.34	2.14–4.07	Hemispherical	3.11–7.16	3.01–5.26	T	9.30	Conical	6.61–9.93	4.56–5.04
Ca_2	Oblong	2.39–5.68	3.89–4.78	Hemispherical	2.67–4.31	2.20–4.12	T	8.82	Conical	6.45–6.54	3.11–3.74
Ca_3	Elongated & almost rectangular	3.38–6.41	2.61–5.34	Hemispherical	4.84–7.16	5.34–7.48	T	13.51	Cylindrical	6.94–8.03	3.78–4.81
Ca_4	Cylindrical	2.88–5.29	2.67–3.58	Hemispherical	3.85–7.02	5.34–7.18	T	6.15	Conical	6.45–8.54	4.84–7.76
Ca_5	Barrel shaped	3.58–7.40	5.04–7.42	Hemispherical	5.5–8.76	4.31–6.69	T	12.00	Cylindrical	7.65–8.61	5.50–5.97
Ca_6	Elongated & almost rectangular	3.11–6.94	2.67–5.07	Hemispherical	5.34–9.67	5.07–8.44	T&I	13.33	Conical	8.44–9.19	6.43–7.12
Ca_7	Elongated & almost rectangular	3.78–7.55	3.74–4.56	Hemispherical	4.31–5.68	2.67–5.07	T	11.54	Conical	7.49–8.54	6.41–5.98
Ca_8	Cylindrical	4.00–6.02	2.46–4.42	Spherical	5.45–8.76	5.12–7.59	T	6.52	Cylindrical	7.00–6.12	4.31–5.69
Ca_9	Cylindrical	3.42–6.96	3.12–6.25	Hemispherical	3.85–8.84	4.40–7.42	T	17.39	Cylindrical	7.40–8.44	6.80–7.12
Ca_{10}	Cylindrical	4.31–6.25	3.11–5.59	Hemispherical	4.40–6.84	3.78–4.92	T	8.77	Cylindrical	9.99–10.10	4.92–6.13
Ca_{11}	Rectangular	2.88–6.04	1.93–5.58	Spherical	2.67–5.87	1.69–4.92	T	12.50	Cylindrical	6.96–9.65	5.34–6.22
Ca_{12}	Cylindrical	3.11–8.01	2.36–5.97	Hemispherical	5.07–7.16	5.04–6.09	T	8.16	Oval	6.94–12.2	6.23–9.08
Ca_{13}	Rectangular	4.67–9.21	4.12 –6.22	Hemispherical	4.85–8.45	4.10–7.29	T&I	11.54	Spherical	9.93–11.7	9.85–11.0
Ca_{14}	Cylindrical	3.42–9.09	2.96–5.56	Spherical	4.07–7.26	4.4–7.18	T	13.21	Oval	9.33–17.7	5.26–12.3
Ca_{15}	Cylindrical	2.35–4.13	1.8–2.48	Hemispherical	2.3–3.55	1.5–2.44	T	9.65	Ellipsoidal	5.58–7.25	3.83–6.42
Ca_{16}	Rectangular	3.85–6.09	2.67–5.04	Hemispherical	4.07–6.23	4.27–7.02	T	10.23	Oval	5.04–12.0	3.85–7.78
Ca_{17}	Elongated	2.88–5.75	2.10–4.84	Spherical	4.56–5.07	4.27–5.12	T	9.80	Oval	5.07–7.78	3.25–5.78
Ca_{18}	Rectangular	3.85–7.02	3.25–5.04	Spherical	4.27–7.12	3.74–7.49	T	12.32	Oval	6.00–9.55	2.67–7.29
Ca_{19}	Elongated	2.67–5.90	2.53–4.27	Hemispherical	5.34–6.50	4.27–6.12	T	11.21	Oval	5.75–10.30	5.04–8.76
Ca_{20}	Rectangular	2.72–5.97	2.11–5.58	Spherical	3.89–7.16	3.74–5.58	T	9.65	Oval	6.96–9.63	5.34–6.94
Ca_{21}	Rectangular	4.92–8.89	4.40–6.69	Hemispherical	6.94–8.81	5.58–6.52	T	12.22	Cylindrical	10.00–12.12	7.02–7.44
Ca_{22}	Cylindrical	5.37–7.59	2.67–3.78	Hemispherical	4.31–9.12	3.78–6.43	T	10.22	Ellipsoidal	5.78–7.88	4.12–7.12
Ca_{23}	Barrel shaped	4.17–7.55	3.74–5.97	Hemispherical	5.78–7.65	5.68–7.78	T	8.65	Ellipsoidal	6.97–16.8	4.35–8.95
Ca_{24}	Barrel shaped	4.07–7.49	3.20–6.25	Hemispherical	4.12–7.52	4.22–6.99	T	7.54	Ellipsoidal	6.52–10.2	5.26–7.59
Ca_{25}	Barrel shaped	2.88–7.88	2.11–5.75	Hemispherical	4.84–8.01	3.11–6.43	T	9.65	Oval	8.29–14.1	4.40–5.78
Ca_{26}	Cylindrical	3.12–5.69	2.59–5.55	Hemispherical	4.58–6.93	3.26–5.59	T	10.55	Oval	4.52–9.56	3.26–7.66
Ca_{27}	Barrel shaped	3.02–6.61	2.42–6.09	Spherical	3.89–6.50	4.27–5.87	T	12.56	Oval	5.12–9.56	4.44–7.89
Ca_{28}	Barrel shaped	3.00–5.89	2.93–6.23	Spherical	5.26–8.12	4.99–6.23	T	8.77	Ellipsoidal	7.29–10.10	4.42–5.76
Ca_{29}	Cylindrical	4.12–8.74	3.32–6.59	Hemispherical	5.58–7.15	5.48–6.99	T	7.98	Ellipsoidal	5.23–8.29	4.21–6.94
Ca_{30}	Barrel shaped	4.27–8.44	4.12–6.84	Hemispherical	5.58–7.99	3.16–5.59	T&I	9.11	Oval	9.22–14.19	6.23–12.9

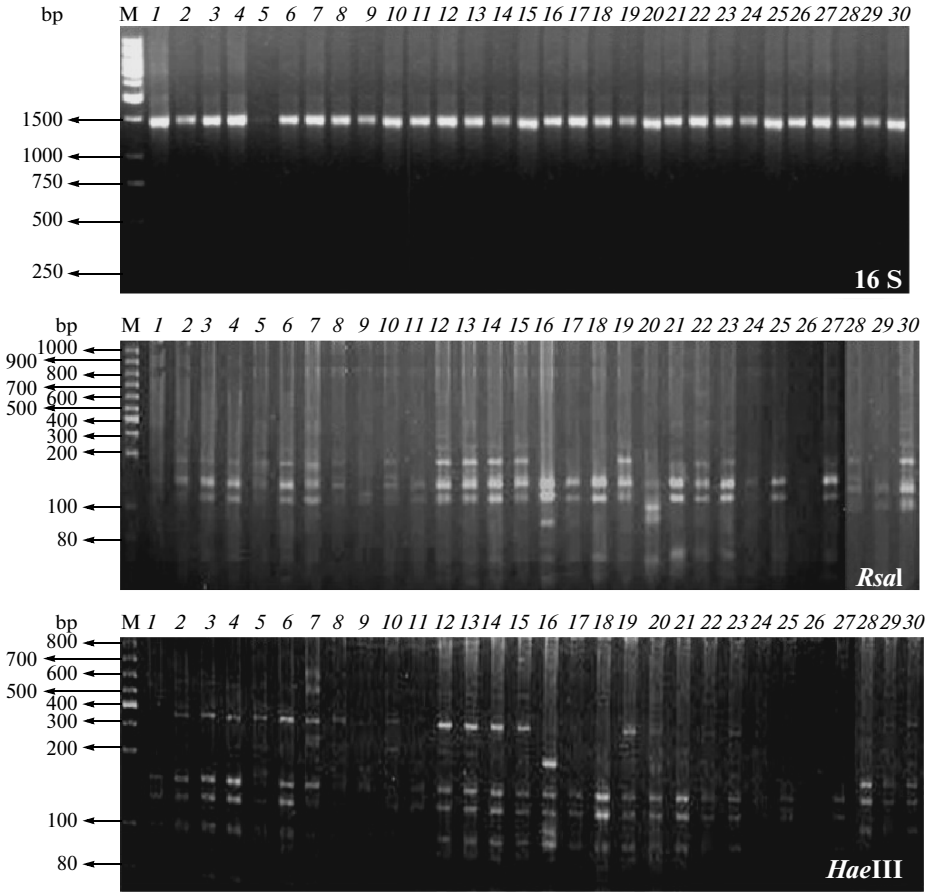

Fig. 1. 16S product and RFLP profile of *Calothrix* strains obtained using enzymes *Rsa*I and *Hae*III.

The vegetative cells ranged from barrel shaped, isodiametric or cylindrical to rectangular. The size of the vegetative cells of the reference strain was 3.85–6.09 × 2.67–5.04 μm. Filaments of Ca_{15} consisted of cylindrical cells (2.35–4.13 × 1.80–2.48 μm) while the maximum cell size (4.97–9.21 × 4.42–6.22 μm) was observed in filaments of Ca_{13}. The frequency of heterocysts varied from 6.15% (Ca_4) to the highest value of 17.39% (Ca_9). Hemispherical heterocysts were yellow, green or hyaline probably due to the disappearance of photosynthetic pigments and were observed in all the isolates examined. Basal hemispherical heterocysts were observed in all isolates except Ca_6, Ca_{13} and Ca_{30} which exhibited intercalary heterocysts and in isolates Ca_2 and Ca_5, two pored heterocysts were also recorded, which was peculiar feature for deciding its entity at species level. A terminal heterocyst occurred at the broad basal end of the trichomes in most of the isolates studied; in a couple of isolates, two-pored heterocysts were also reported [25]. The frequency of heterocyst occurrence was reported to depend on environmental conditions [26].

Akinetes were observed in all isolates at the late exponential stages of growth. The akinetes were generally observed close to the heterocysts. In most of the isolates terminal akinetes were found attached to the heterocysts; isolate Ca_{17}, however, possessed intercalary akinetes. The akinetes were 2–3.5 times longer than the vegetative cells while their width was only slightly greater. The largest akinetes were observed in Ca_{21} (10–12.12 × 7.02–7.44) and the smallest, in Ca_{26} (4.52–9.56 × 3.26–7.66). Akinetes were observed after the exponential phase; they germinated under favorable conditions after a resting period. Development of akinetes in *Calothrix* has been suggested to take place through enlargement and encystment of the cells [27].

RFLP analysis of amplified 16S rRNA genes. Extracted DNA from 30 *Calothrix* isolates was used for 16S rRNA gene amplification using the primers FD1 and RP2. A single amplified product of 1500 bp for 16S rRNA gene was observed in all the isolates examined. The amplified fragments for 16S rRNA gene were digested with restriction enzymes *Dpn*II, *Hae*III, *Rsa*I, *Mse*I, and *Eco*RI (Figs. 1 and 2). The sum of the fragment sizes was approximately 1500 bp in most of the cases examined. The combined data obtained for restriction patterns using five enzymes were used to calculate the similarity matrix which was utilized for cluster analysis for UPGMA dendrogram.

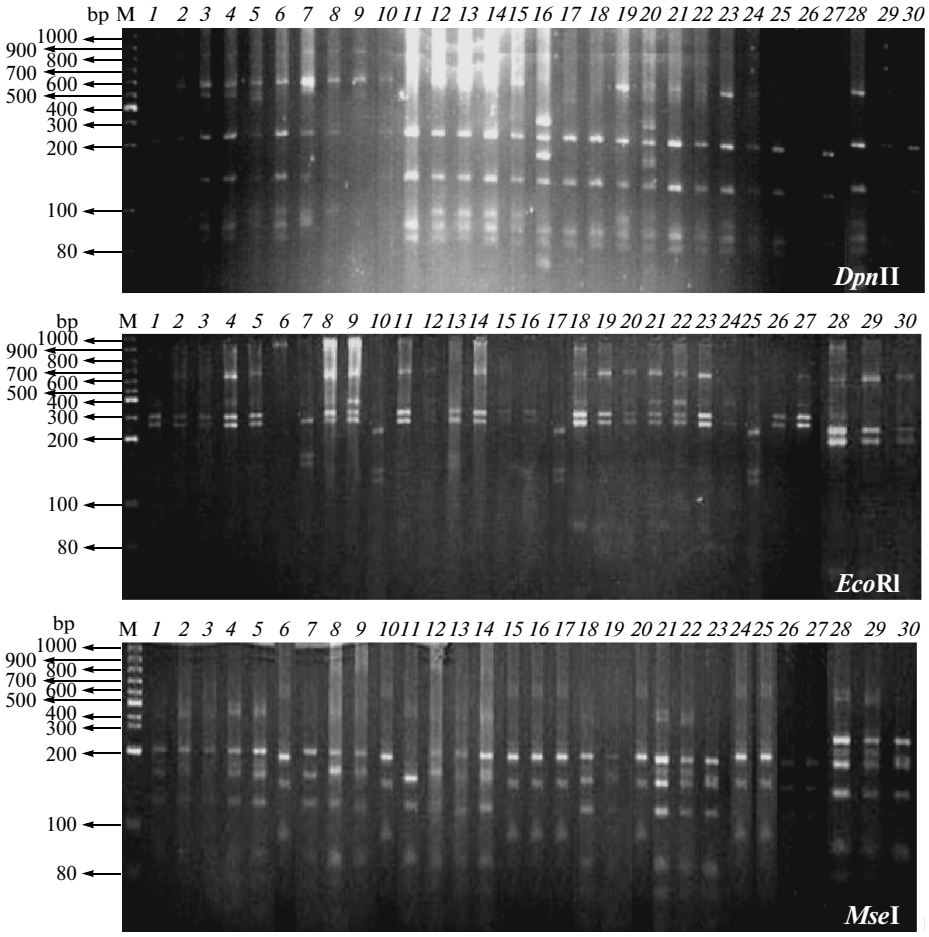

Fig. 2. RFLP profile of *Calothrix* strains obtained using enzymes *Dpn*II, *Eco*RI and *Mse*I.

The 16S rRNA region is known to be highly conserved among eubacteria and analysis of genetic variation within this region has been used in phylogenetic studies of free-living cyanobacteria [28]. Our analysis of RFLP fingerprints indicated a higher level of divergence for the reference isolate Ca_{16} (UTEX 379) which occupied a separate branch in the dendrogram generated. *Calothrix brevissima* (Ca_4) and *C. membranacea* (Ca_5), isolated from Baharaich, Uttaranchal, India were identified as distinct species according to microscopic examination. However, according to the RFLP analyses these isolates occupied the same subcluster with a similarity of 92%. Likewise, other *Calothrix* isolates (Ca_{27}, Ca_{28} and Ca_{30}) were identified on the basis of morphological parameters as *C. marchica, C. javanica*, and *C. scytonemicola*, respectively. In the subcluster C_2, two strains Ca_{28} and Ca_{30} interestingly shared almost identical RFLP patterns and exhibited maximum similarity coefficient of 0.952 (95.2%), indicating a strong genetic resemblance though isolated from different geographical locations. On the other hand, two strains namely Ca_{28} and Ca_{29} which were isolated from same geographical area, shared a similarity coefficient of 0.909 (Fig. 3). These results are in accordance with the investigations of Palinska et al. [29] who have shown that morphological differences are not necessarily revealed at the 16S rRNA gene level. These workers also found out that according to morphological and physiological characteristics, three different species of the cyanobacterial genus *Merismopedia* exhibited 100% similarity at 16S rRNA gene level. On the basis of DNA fingerprinting analyses, it has been suggested that hormogonia-forming species are not the most ancient forms of heterocystous cyanobacteria [17]. Neilan et al. [30] reported heterogeneity in the cluster containing mostly heterocystous planktonic strains of the genera *Anabaena* and *Aphanizomenon* by using PCR-RFLP of the phycocyanin locus with intergeneric spacer. On the other hand, RFLP of 16S rRNA genes provided a better and broader taxonomic application as this method is suitable for all bacteria [18].

The sequencing of 16S rRNA genes is a routine procedure which improve the robustness of phylogeny reconstruction, identification results, and primers evaluations [31]. The 16S rRNA genes from 42 cyanobacterial cultures and environmental samples belong-

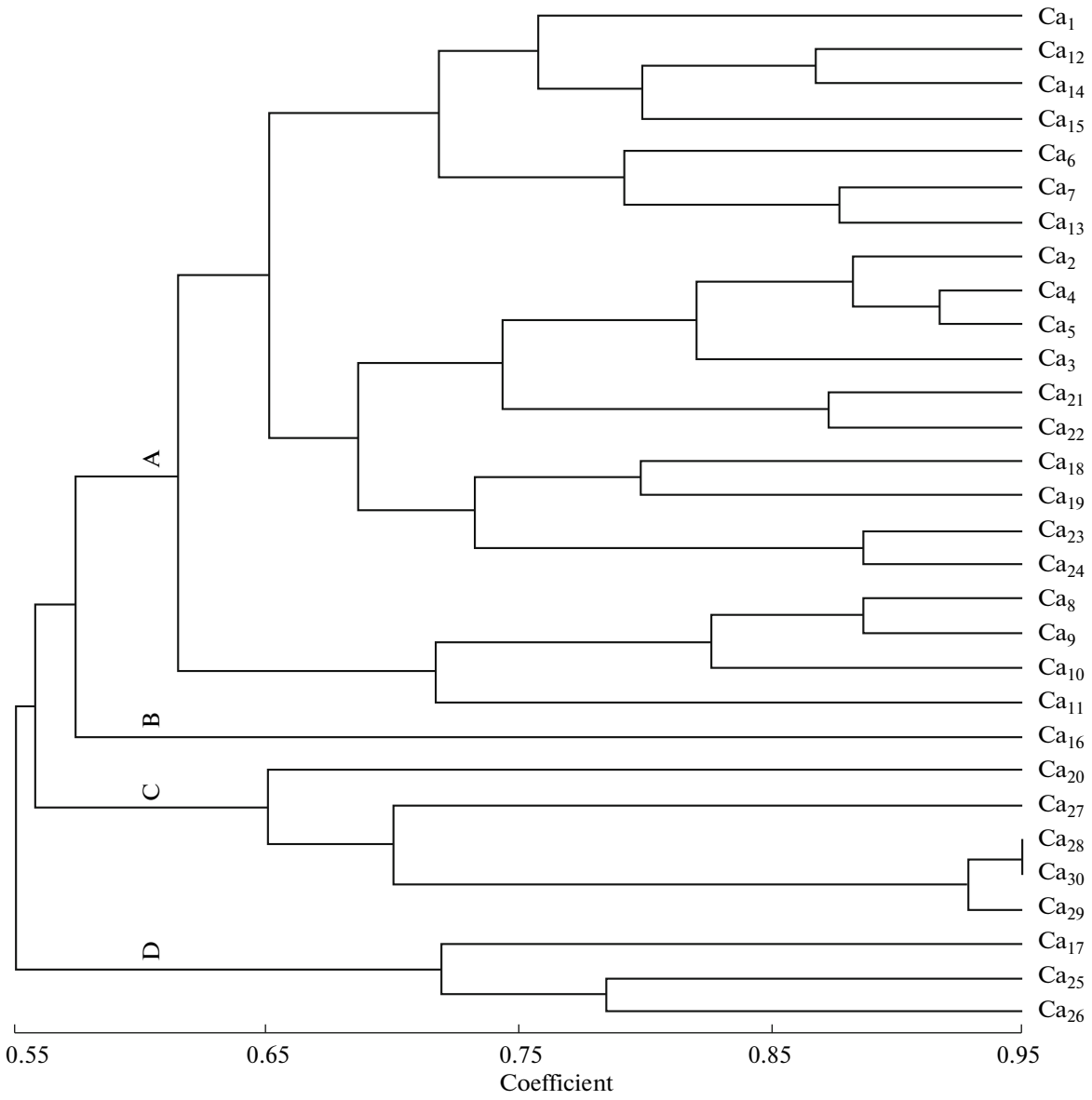

Fig. 3. UPGMA dendrogram of *Calothrix* strains based on RFLP-PCR analysis with five different enzymes.

ing to the genus *Calothrix* and the morphologically similar genera *Rivula-ria*, *Gloeotrichia* and *Tolypothrix* have been sequenced. The evolutionary distances between cyanobacteria, which have been morphologically identified as *Calothrix*, suggest that they belong to at least five different genera [2]. Other molecular biological approaches described which could have been used for the identification, such as single and multiplex randomly amplified polymorphic DNA analysis and the sequence analysis of ITS region of ribosomal RNA operons [32]. Phylogenetic analysis of the genes responsible for secondary metabolites, macromolecules, analysis of ITS between 16S and 23S subunits and repetitive extragenic palindromic fingerprinting may reveal species variations exceeding those observed in 16S rDNA sequences [12]. However, the approach used in the present study involving morphological and molecular procedures for investigating the genetic diversity and ecological significance amongst the isolates of *Calothrix* is powerful and helpful.

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