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DIVERSITY ANALYSIS OF GEOGRAPHICALLY ISOLATED STRAINS OF *SPIRULINA* AND RELATED CYANOBACTERIAL GENERA

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ABSTRACT:

Cyanobacterial strains from the genus *Spirulina* and related genera (Arthrospira*, Lyngbya, Oscillatoria and Phormidium*) were procured from the culture collection of CCUBGA, IARI, New Delhi, India. These strains were examined for their phylogenetic relatedness using Randomly Amplified Polymorphic DNA (RAPD) PCR involving twenty 10 mer random primers and ten dual primer combinations. A total of 355 polymorphic DNA bands ranging from 220bp-3800bp were obtained in PCR reactions with single oligos. However, ten set of dual primer combinations generated 225 polymorphic bands ranging from 150bp-5000bp, which revealed a wide range of variability amongst the selected strains. Combined analysis of single and multiplex primer combinations showed a maximum similarity coefficient of about 0.865 amongst two strains of *Arthrospira* (Ar-1 and Ar-2); which also possessed very close proteins content (110.30µg/ml and 119.49µg/ml, respectively). Primers used in RAPD analysis showed unique bands ranging from 0-6 with single primer and from 0-7 with dual primers. The study undertaken revealed extensive evidence for the applicability of RAPD technique in the diversity analysis of *Spirulina* and related genera.

KEY WORDS: Diversity, Spirulina.

INTRODUCTION:

Cyanobacteria are one of the largest and diverse groups of prokaryotic micro-organisms which exhibit oxygenic photosynthesis and contain chlorophyll-'a' and accessory pigments like carotenoids and phycobiliproteins (Castenholz, 2001). This group forms one of the eleven major bacterial phyla and is classified as class Oxyphotobacteria of division I (Gracilicutes) of the kingdom Prokaryotae. Traditionally, members of this group are

classified by using Botanical and Bacteriological Taxonomic Codes (Komarek, 1991; Rippka et al., 1979). However, the authors of both the classification systems have emphasized that the current classification of cyanobacteria is temporary owing to inadequate genetic information and that major revisions will necessarily occur in future (Castenholz, 2001; Komarek, 2003).

Morphological variation within this phylum is greater than in any of the other bacterial kingdom. The morpho-physiological and biochemical parameters are not sufficiently diverse to constitute useful taxonomic criteria and taxonomy based on morphological, nutritional or developmental features does not always correlate with the evolutionary relationships. Moreover, morphological parameters are considerably altered due to the selective culturing conditions (Doers and Parker, 1998; Woese, 1987).

In the last ten years, molecular biology has provided useful tools to acquire genotype information at different levels extending from strain typing to the study of phylogenetic relationships. Regarding cyanobacteria, the research has essentially focused on the investigation of the phylogenetic relationships among the major branches of this phylum (Wilmotte, 1994). However, the classification of cyanobacteria and its revision are complicated by the presence of species based solely on morphology without any genetic information and by the sequences of cyanobacterial species in databases without morphological descriptions (Wilmotte and Herdman, 2001). Analysis of photosynthetic pigments, isoenzyme variations or differentiated cell cultures may also be misleading because of variable expression of cyanobacterial gene products (Rippka et al., 1979). Therefore, there is a need to improve taxonomic criteria by supplementing the information on the basis of molecular characterization.

Random Amplified Polymorphic DNA (RAPD) allows the detection of multilocus genetic variation using short primers of arbitrary sequence (Welsh and McClelland, 1990; Williams et al., 1990). This molecular technique is very easy to perform and requires no prior knowledge of the genomes under investigation. The technique has been used for the analysis of diversity within germplasm populations, phylogenetic relationships, and identification to the strain level (Welsh and McClelland, 1990). Study undertaken has revealed extensive evidence for the applicability of RAPD in cyanobacterial taxonomy, and clearly demonstrated superior discriminative power of RAPD towards differentiation of geographically unrelated *Calothrix* strains (Shalini et al., 2008).

In some cases, single oligonucleotides (oligo) of 10-34 base pairs have been used for RAPD-PCR reaction. Nishihara et al. (1997) have used RAPD analysis to discriminate genotypes of

Microcystis. However, multiplex RAPD-PCR with two 10-mer random oligonucleotides in a single PCR was particularly useful for strain differentiation of cyanobacterial isolates because it increased the number of informative genetic markers (Neilan, 1995). This method increase stringency for amplification reaction and provide a highly reproducible RAPD pattern for each strain analysed. The increased reproducibility provided by this method is primarily a result of the multiplexing of random oligonucleotide primers.

The present study reports the work conducted in *Spirulina* and related cyanobacterial genera using RAPD-PCR technology to evaluate their genetic diversity. The *Spirulina* is cultivated and sold as health food, animal feed, source of food additives and fine chemicals (Borowitzka, 1988). The total DNA restriction pattern analysis has been used to study the genotypic diversity and typing of cyanobacterial strains which are attributed to *Arthrospira maxima* or to *A. platensis* by comparison with the original description of these and by additional morphological criteria (Tomaselli et al., 1996). The identification of this genus is problematic and conflicting specifications have been published (Tomaselli, 1997). Since these organisms are known in the biotechnology field, therefore, their taxonomic status and the tools to readily identify specific strains are needed for a safer and more productive use.

MATERIALS AND METHODS:

Culture procurement and maintenance and proteins estimation

Cyanobacterial strains from the genus *Spirulina* and related genera (*Arthrospira, Lyngbya, Oscillatoria* and *Phormidium*) were procured from the culture collection of CCUBGA, IARI, New Delhi, India (Table 1). The strains of *Spirulina* and *Arthrospira* were grown and maintained in chemically defined modified CFTRI medium (Venkataraman et al., 1995) with a pH of 9.0 at 20±2^{o}C under diffused light intensity of 35-40 µmole photon/m^{2}/s and light and dark cycles of 16:8 hours. However, the non-heterocystous strains of *Lyngbya*, *Oscillatoria* and *Phormidium* were grown in nitrogen enriched chemically defined BG-11 medium (Stanier et al., 1971) with a pH of 7.5 at 28±2^{o}C temperature under a light intensity of 52-55µmole photon/m^{2}/s and light and dark cycles of 16:8 hours. These strains were studied for their morphological characters based upon the keys given by Desikachary (1959) and were axenised by repeated subculturing, streaking, antibiotic treatment and washing with sterilized water (Kaushik, 1987). These cultures were inoculated at an optical density of 0.1 (750 nm) in 50 ml of fresh medium; and thoroughly homogenized suspensions were drawn during exponential phase of growth for DNA extraction and for estimation of total soluble protein (Lowry et al., 1951). The data recorded were subjected to statistical analysis using M-

STATC package according to factorial CRD. Duncan's Multiple Range Test (DMRT) was employed to compare the mean performance of strains for specific parameters.

RAPD study

DNA extraction

Approximately 50-70 mg axenic biomasses of exponentially growing cultures were taken for extraction of Genomic DNA using DNeasy Tissue Kit Manufacturer's Protocol (Qiagen, Cat No.69504). The quantification of DNA was done by comparison with known standards in ethidium bromide stained 0.8% agarose (Pronadisa, Europe) gels against 1 kb molecular weight marker (Fermentas, USA) for one hour at 75 volts and visualized under UV light.

Primers

The DNA samples were subjected to amplification by RAPD technique using twenty decamer oligonucleotides having GC content of 60-80%. These primers were procured from Integrated DNA Technologies and their sequences are listed in Table 2. These random primers were also used in pairs for getting the diagnostic fingerprints and thus a total of ten combinations using different primers were made. The suitability of these primer combinations was scored on the basis of intensity and distribution of bands. These primers exhibited reproducible banding profiles with a sufficient number of bands to be discriminating for each strain of *Spirulina* and related genera.

PCR amplification

Twenty oligonucleotide primers and ten dual primer combinations were used respectively for single primer and multiplex (dual primer) RAPD-PCR. The polymerase chain reaction was carried out in a final volume of 20µl, having 1xTAE buffer containing 15mM $MgCl_2$, 10mM of deoxynucleotide tri-phosphates (dATP, dCTP,dGTP and dTTP), 10 pico moles each of single primer, one U of *Taq* DNA polymerase (Bangalore Genei Ltd., India) and 100 ng of genomic DNA. Amplification was achieved in a Master Cycler Gradient (Eppendorf) programmed for 40 cycles with initial denaturation (94^{o}C for 4 min) followed by 45 cycles composed of denaturation (94^{o}C for 1 min), annealing (34^{o}C for 1 min) and extension (72^{o}C for 2 min); followed by a final extension of 5 min at 72^{o}C; and cooling at 4^{o}C. After single primers provided sufficient DNA fingerprints at the annealing temperature of 34^{o}C, multiplex RAPD was performed by combining dual primers in equimolar ratio (10 pico moles each of two oligos). Amplification conditions for multiplex RAPD-PCR were same as that for single primer, except the number of amplification cycles was reduced to 40.

Electrophoresis

The amplified products for both the single primer and multiplex RAPD-PCR were separated along with a molecular weight marker (1 kb, Fermentas, USA) by electrophoresis on 1.5% agarose gels run in 1xTAE (Tris Acetate EDTA) buffer, stained with ethidium bromide for a period of 2.5 hours at 75 volts. These amplified products were visualized under UV light and gel photographs were scanned through Gel Doc System (MiniBis Bioimaging System, USA) and their sizes were evaluated using Software Quantity One (Biorad, USA).

Statistical analysis

The presence or absence of a band at any position on the gel was used to construct the binary matrix for RAPD markers. The relationships amongst the strains were verified using the presence or absence (scored as, 1 and 0 respectively) of bands with random primers used either singly or in multiplex reaction. Pair wise genetic similarities among the genotypes under study were determined using Jaccard's Coefficient (Jaccard, 1908) on the basis of SIMQUAL software. Cluster analyses were carried out on similarity estimates following the Unweighted Pair Group Method with Arithmetic Average (UPGMA) using NTSYS-pc, version 1.80 (Rohlf, 1995).

RESULTS:

Proteins estimation

The total soluble proteins content (µg/ml) differed significantly amongst the strains of *Spirulina* and related genera ranging from the lowest of 40.33 µg/ml in *Phormidium tenue* (Ph-1) to the highest of 157.20 µg/ml in *Spirulina sp.* (Sp-8) followed by the strains of *Spirulina maxima* (Sp-5) and *S. lonar* (Sp-6). However, the other related strains of *Lyngbya, Oscillatoria* and *Phormidium* possessed comparatively small quantities of the total soluble proteins (Table 1).

RAPD-PCR analysis (single primer and multiplex reactions)

A total of 20 single primer reactions and 10 dual primer reactions were chosen to generate RAPD patterns and diagnostic fingerprints for strains of *Spirulina* and related genera. The primers and their combinations, which produced consistently even product intensities throughout were favoured due to high reproducibility of the markers. RAPD-PCR analyses with single and multiplex primers were particularly useful for strain differentiation as it increased the number of informative genetic markers. The number of polymorphic bands ranged from a minimum of 9 (P-10) to the maximum value of 28 (OPA-11) and a total of 355 distinct polymorphic DNA bands ranging from 220 bp to 3800 bp were produced in PCR reaction with single oligos. However, a combination of ten sets of dual primer combination generated distinct polymorphic bands from a minimum of 13 (CRA26+P-100; OPA11+P-3

primer combinations) to the maximum value of 32 (OPD-02+HipGC primer combinations). Dual primer combinations generated 225 distinct polymorphic DNA bands ranging from 150 bp to 5000bp which revealed a wide range of variability amongst the strains examined. None of the primers either used singly or in dual combination revealed any monomorphic band in all the strains examined. The total number of fragments obtained for all the strains under study was minimum with the primer P-3 (59) and was maximum with the primer OPD-16 (123), when analysed with single oligos. However, the multiplex reactions showed least number of fragments with the combination CRA26+P-100 (68) and maximum with the primer combination OPA13+MM (151).

Unique bands

Different primers used in RAPD analysis showed unique bands ranging from 0 to 6 with single primer and from 0 to 7 with dual primer combinations. The primers CRA25 and CRA26 produced single distinct band of 2700bp and 400bp with *Oscillatoria* sp. (Os-1 and Os-2). HipGC showed three unique bands of 2300bp, 1700bp and 300bp with only *Spirulina platensis* (Sp-3), whereas primers HipTA, HipTG, MM and OPA10 showed single band of 450bp in *Lyngbya* sp. (Ly-2), 1800bp in *Spirulina platensis* (Sp-3), 500bp in *Phormidium*(Ph-1) and 475 bp in *Spirulina maxima* (Sp-5). Other primers OPA13 and OPD-18 produced distinct band of 1500bp and 2300bp in *Oscillatoria* sp. OPD-02 showed maximum of six unique bands of molecular size 2900bp and 2600bp in *Spirulina platensis* (Sp-1), 1200bp in *Arthrospira* (Ar-2), 3100bp and 350bp in *Oscillatoria* sp. (Os-1); and 1700bp in *Phormidium* sp. (Ph-1). Primer P-10 did not reveal any unique band in the present study. None of the primers when used singly resulted in any distinct band in the two strains of *Spirulina platensis* (Sp-4 and Sp-7). *Spirulina platensis* mutant (Sp-7) did not produce any unique band with any primer either used singly or in combinations.

Five dual primer combinations produced single distinct band of 2400bp, 500bp, 225bp, 250bp and 2100bp in strains of *Spirulina*, *Oscillatoria* and *Phormidium*. On the other hand, few combinations exhibited more number of unique bands and the largest number of seven unique bands were recorded with OPD-02+HipGC primer combination, followed by six unique bands with the primer combination OPA10+ HipTG. The dual primer combinations of CRA22+P-10, OPA13+MM and OPD-16 + HipCA did not reveal any unique band in the present study. RAPD profiles with single and dual oligonucleotides showing comparatively higher numbers of unique bands has been represented (Fig.1).

Measurement of genetic relatedness

RAPD-PCR profiles with twenty single primers and ten dual primer combinations were used to generate fingerprints for the genomic DNA of *Spirulina* and related genera. Numerical analysis of PCR profiles clustered by UPGMA enabled the plotting of dendrograms constructed from amplicons generated by single and dual primer combinations. The computer aided analysis of RAPD fingerprints indicated great heterogeneity with similarity coefficient ranging from 0.53 to 0.90 with random primers. Clustering analysis, based on the computation of data set with single primer and dual primer combinations, revealed two main clusters; with *Lyngbya* (Ly-1, Ly-2), *Oscillatoria* (Os-1, Os-2) and *Phormidium* (Ph-1, Ph-2) grouped together in one cluster and the remaining ten strains of *Spirulina* and *Arthrospira* were grouped into the second cluster (Fig. 2).

Two *Arthrospira* strains (Ar-1 and Ar-2) depicted highest similarity in the data analysis. These two strains also depicted closer proteins contents of 110.02 µg/ml and 119.49 µg/ml, respectively; which were higher than the total soluble proteins contents shown by other non-heterocystous, filamentous cyanobacterial genera like *Lyngbya*, *Oscillatoria* and *Phormidium*. Overall analysis indicated that there was more closeness amongst the *Spirulina* and *Arthrospira* strains and the rest of the related strains were distinctly placed.

DISCUSSION:

In the present investigation, single primer and multiplex RAPD-PCR were used to assess the diversity relationships amongst *Spirulina* and related cyanobacterial genera; and to compare the performance of RAPD-PCR involving single primer and multiplexing of primers. A total of twenty single primer reactions and ten dual primer reactions were chosen to generate RAPD profiles. The results indicated that the primers chosen on the basis of large number of primers tested were informative enough to differentiate strains of *Spirulina* and related genera. Hip-TG primer constructed on the basis of conserved sequences is reported to be frequently occurring in large number of cyanobacterial strains (Robinson et al., 1995) and is reported to be useful in distinguishing several species of cyanobacteria (Smith et al., 1998). Most of the RAPD methods use short primers (10 mer), however, it must be emphasized that combination of longer primers (18 mer) was used in RAPD technique earlier (Welsh and McClelland, 1990). The use of 5-mer or dual arbitrary priming with longer primers has also been suggested (Caetano-Anolles et al., 1991; Neilan, 1995). Holt and Cote (1998) developed an RAPD-PCR that incorporated longer primers (24 mer) designed from conserved region of dextran scarce genes.

The higher number of fragments was produced by OPD-16, P-100 and OPD-18 which were 123, 115 and 114 respectively; and when OPD-16 and OPD-18 were used in combination

with other primers, the number of fragments enhanced to 145 and 130. However, when the primer P-100 was used in dual combination, the number of fragments reduced to 68. The average number of fragments obtained was more with two primer combinations of CRA25+P-8 and OPD16+HipCA as compared to that when these were used singly. These results were consistent with other studies where on an average, two primer combinations in RAPD reactions amplified 1.3 times as many bands as single primer reactions (Hu et al., 1995). It has also been suggested that even three primers when used separately revealed enough polymorphism to identify species of the symbiotic genus *Anabaena*. The results created a phylogenetic tree with a topology similar to that derived with 22 primers (Saitou and Nei, 1987). On the other hand, a single 10-mer primer proved sufficient for distinguishing among 64 strains of *Helicobacter pylori* (Akopyanz et al., 1992). Interestingly, the overlapping of the fragments was less visible in PCR reactions involving combinations of primers possibly due to the reason that smaller fragments produced under multiplex reaction competed more effectively during extension period against the longer sized fragments produced by single primer reactions. Alternatively, PCR products that use a single primer can produce hairpins during renaturation, putting them at a disadvantage relative to the PCR products made using two primers. Further, it was also seen that pairwise combinations exhibited patterns that differed by more than 50% from those produced by single primer reactions.

Multiplex RAPD-PCR has also been successively used to generate unique and identifying DNA profiles for *Anabaena* and *Microcystis*, which are responsible for the production of nuisance blooms in various freshwater systems, including recreational and drinking water supplies (Neilan, 1995). Different primers used in RAPD analysis showed sufficient number of distinct bands ranging from none to six with single primers and none to seven with dual primer combinations. Primer P-100 in single primer reaction and few dual primer combinations (CRA22 + P-10, OPA13 + MM and OPD-16 + HipCA) does not revealed any unique band. The use of two-primer mixtures may also generate a higher proportion of erroneous polymorphic bands than those produced by the use of single primers (Haldane et al., 1996). The dendrogram developed after cluster analysis based upon RAPD in cyanobacteria indicating a high degree of divergence among the related strains, have also been undertaken by other workers (Jeberlin Prabina et al., 2003).

The maximum closeness was exhibited between the two *Arthrospira* strains (Ar-1 and Ar-2) with single and dual primer combinations. These two strains also exhibit closer total soluble protein content of 110.30µg/ml and 119.49µg/ml. The higher similarity value amongst these

strains was probably due to their isolation from same geographical location (Round, 1981) which may have been influenced by the ease of dispersal, mechanism of transport, effectiveness of carrier and ability to tolerate transport conditions (Kristiansen, 1996a; 1996b). The combined analysis revealed that the four strains of *S. platensis* (Sp-1, Sp-2, Sp-3 and Sp4) grouped together in the same sub-cluster. However, the mutant of *Spirulina platensis* (Sp-7) showed more closeness to the *Spirulina* sp. (Sp-8), obtained from the Loktak Lake (Manipur, India). *Spirulina lonar*, obtained from the Canary Islands (Spain) appeared comparatively more close to the sub-cluster of Sp-7 and Sp-8. An interesting finding is that, all the strains of *Spirulina* and *Arthrospira* showed comparatively higher protein content and were also grouped together in the dendrogram. However within this cluster, the position of different strains of *Spirulina* and *Arthrospira* in the dendrogram cannot be correlated to the total soluble proteins content. The proteins content of cyanobacterial strains is an important physiological character, which is likely to be influenced by the prevailing geographical conditions of the regions from where they have been isolated (Round, 1981). Although, nearly all the populations of cyanobacteria from different locations differ from each other, yet they may stabilize in long-term cultures (Waterbury and Rippka, 1989; Kato and Watanabe, 1993).

It is thus clear that the protein content of these closely related strains only loosely correlate with the results obtained from the RAPD analysis undertaken with random oligonucleotide primers used either alone or in combinations. These strains varied widely in their morphological attributes besides, the biochemical parameters like protein content may also have been influenced by the adaptation of these strains to different environmental conditions. Therefore, the current study does not allow the use of biochemical parameters as a self-explanatory tool in understanding of the phylogenetic relationships of closely related genera. However, sufficient evidence exist for the use of various parameters as a supplementary tool for analysis of results obtained from RAPD-PCR either single primer or combinations of primers.

Although many genetic tools exist for phylogenetic analysis, the RAPD-PCR scores over other genetic tools because of their ability to examine genomic variation without prior sequence information, faster analysis, relatively low cost of the technique and requirement of only nanograms of DNA (Williams et al., 1990). This technique greatly increase the number of informative genetic markers, particularly the combinations of 10-mer oligonucleotide primers increased the reproducibility and stringency of the results (Neilan 1995). A

significant increase in the discriminatory power of multiplex RAPD-PCR has also been obtained to discriminate the strains of Bacillus cereus (Ghelardi et al. 2002).

The misidentification of strains very frequently hampers the progress towards achieving a more robust taxonomy. The variations induced during culturing or differential selection may also result in differences between morphotypes and genotypes which may favor maintenance of different genotypes in the population (Otsuka et al. 2001).

Therefore, it is concluded that the cyanobacterial specific primers along with other recent advances in molecular methods and several morphological and biochemical parameters can be of great help in decoding the phylogenetic relationships of *Spirulina* and related genera. Hence, for establishing phylogenetic relationships among closely related cyanobacterial strains of *Spirulina* and related genera, one need to use additional genetic markers along with morphological and biochemical parameters.

It appeared that utilization of two primers combinations in RAPD-PCR did not enhance the similarity coefficient and did not increase the number of polymorphic bands very significantly. However, there was an enhancement in the total number of fragments with dual primer combinations. Also, it is worthwhile to mention that only ten dual primer combinations were used in multiplex RAPD reaction. The multiplex RAPD-PCR was chosen because of its greater degree of reproducibility, stringency for accurate differentiation and influence on diversity analysis. The studies have indicated the sensitivity of RAPD – PCR technique which is derived by using the entire genome as the PCR template provides a taxonomy and phylogeny for the cyanobacteria which are comparable to those achieved by more traditional methods. The differences between morphotypes and genotypes may also result from the variations induced during culturing (Otsuka et al., 2001). Therefore, we need to use additional genetic relationships amongst *Spirulina* and related genera so that the data obtained may provide better understanding of the systematic of the order Oscillatoriales.

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Table 1: Strains of *Spirulina* and related genera and their proteins content

Strains[1]	Taxonomic description	Source/Origin of strains	Designation[2] (as used in text)
CCC 477	*Spirulina platensis*	West Germany	Sp-1 (117.42[12])
CCC 478	*S. platensis*	Israel	Sp-2 (104.43[7])
CCC 479	*S. platensis*	Vietnam	Sp-3 (108.93[9])
CCC 480	*S. platensis*	Mysore (India)	Sp-4 (107.51[8])
CCC 481	*S. maxima*	China	Sp-5 (128.29[15])
CCC 482	*S. lonar*	Israel	Sp-6 (120.74[14])
CCC 483	*S. platensis* (Mutant)	CCUBGA, IARI, New Delhi (India)	Sp-7 (111.02[11])
CCC 538	*Arthrospira* sp.	Canary islands (Spain)	Ar-1 (110.30[10])
CCC 539	*Arthrospira* sp.	Canary islands (Spain)	Ar-2 (119.49[13])
CCC 540	*Spirulina* sp.	Loktak Lake, Manipur (India)	Sp-8 (157.20[16])
CCC 102	*Lyngbya* sp.	Kannur, Kerala (India)	Ly-1 (70.03[4])
CCC 333	*Lyngbya bergei*	Cuttack, Orissa (India)	Ly-2 (70.98[5])
CCC 305	*Oscillatoria* sp.	IARI fields, New Delhi (India)	Os-1 (61.87[3])
CCC 309	*Oscillatoria* sp.	IARI fields, New Delhi (India)	Os-2 (53.09[2])
CCC 27	*Phormidium tenue*	Arunachal Pradesh (India)	Ph-1 (40.33[1])
CCC 104	*Phormidium* sp.	Kannur, Kerala (India)	Ph-2 (76.48[6])

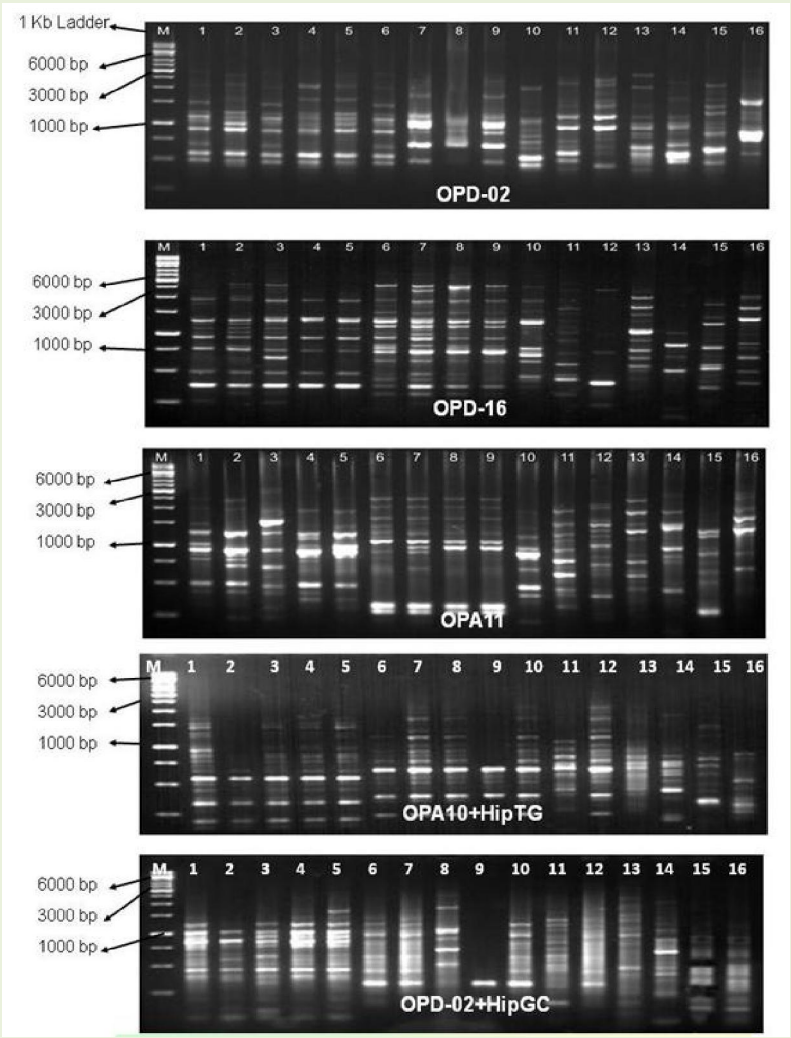
[1]Cyanobacterial Culture Collection (CCC)
[2]Values in parenthesis indicate proteins content (µg/ml); SE(m)± = 6.967, CD (P=0.01) = 27.093; n=3
Note: Superscript denote DMRT ranking in ascending order

Table 2: Sequences of RAPD oligonucleotide primers with PCR products obtained and their size range

S. No.	Primers	Primer sequence (5'-3')	Total no. of fragments	No. of polymorphic products	Size range (bp)
1.	CRA22	CCGCAGCCAA	98	19	240-2200

2.	CRA25	AACGCGCAAC	94	20	260-2500
3.	CRA26	GTGGATGCGA	83	16	240-2000
4.	D-02	GGACCCAACC	61	17	400-2600
5.	HipCA	GCGATCGCCA	86	19	300-1800
6.	HipGC	GCGATCCGCC	68	17	230-2200
7.	HipTG	GCGATCGCTG	102	18	330-2300
8.	MM	TCACGGTGCA	80	15	260-3800
9.	OPA10	GTGATCGCAG	79	16	350-2000
10.	OPA11	CAATCGCCGT	98	28	450-3000
11.	OPA13	CAGCACCCAC	95	17	230-3100
12.	OPD-02	GGACCCAACC	106	24	300-2400
13.	OPD-16	AGGGCGTAAG	123	25	220-2600
14.	OPD-18	GAGAGCCAAC	114	17	260-2200
15.	OPD-20	ACCCGGTCAC	89	18	250-2400
16.	P-2	ACAACTGCTC	60	12	400-2700
17.	P-3	TGACTGACGC	59	11	420-3000
18.	P-8	CCGCAGCCAA	103	18	500-2500
19.	P-10	GCGATCCCCA	64	9	240-2400
20.	P-100	ATCGGGTCCG	115	19	250-3800
21.	CRA22+P-10	CCGCAGCCAA+ GCGATCCCCA	126	18	200-5000
22.	CRA25+P-8	AACGCGCAAC+ CCGCAGCCAA	169	27	160-2400
23.	CRA26+P-100	GTGGATGCGA+ ATCGGGTCCG	68	13	230-1400
24.	OPA10+HipTG	GTGATCGCAG+ GCGATCGCTG	120	29	240-2200
25.	OPA11+P-3	CAATCGCCGT+ TGACTGACGC	110	13	230-2700
26.	OPA13+MM	CAGCACCCAC+	151	20	180-

		TCACGGTGCA			2600
27.	OPD-02+HipGC	GGACCCAACC+ GCGATCCGCC	140	32	150-3000
28.	OPD-16+HipCA	AGGGCGTAAG+ GCGATCGCCA	145	24	230-1800
29.	OPD-18+D-02	GAGAGCCAAC+ GGACCCAACC	130	24	220-3000
30.	OPD-20+P-2	ACCCGGTCAC+ ACAACTGCTC	136	25	230-4600



Lane 1-4 : Sp-1 to Sp-4 (*Spirulina platensis*)
Lane 5 : Sp-5 (*S. maxima*);
Lane 6 : Sp-6 (*S. lonar*)
Lane 7 : Sp-7 (*S. platensis*, mutant);
Lane 8 & 9: Ar-1 and Ar-2 (*Arthrospira* sp.)
Lane 10: Sp-8 (*Spirulina* sp.);
Lane 11: Ly-1 (*Lyngbya* sp.)
Lane 12: Ly-2 (*Lyngbya bergei*);
Lane 13 & 14 : Os-1 and Os-2 (*Oscillatoria* sp.)
Lane 15: Ph-1

(*Phormidium tenue*);

Lane 16: Ph-2 (*Phormidium* sp.)

Fig. 1: RAPD profiles showing comparatively higher number of unique bands

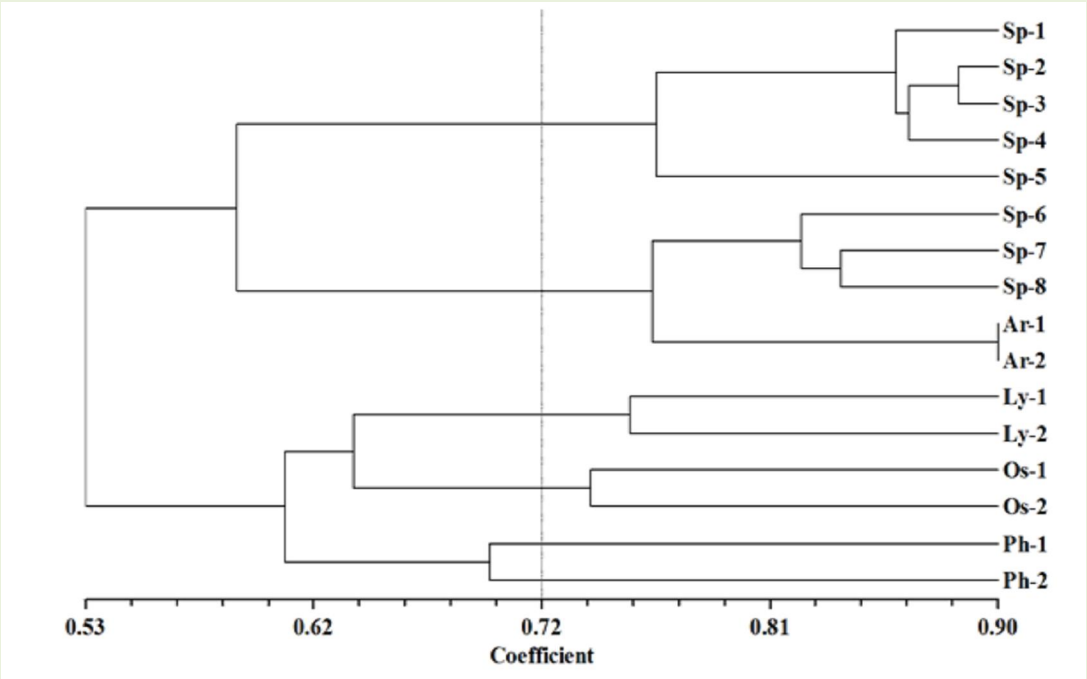


Fig. 2: Clustering analysis of the isolates of *Spirulina* and related genera using twenty single primer and ten dual primer combinations