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A study on the differentiation of filamentous cyanobacterial isolates using DNA fingerprinting approach

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The present study investigates the implication of DNA fingerprinting in discrimination of class isolates collected from a spread of habitats in south west Khasi Hills district of Meghalaya, India. Five totally different primers i.e., M13, enterbacterial repetitive intergenic consensus (ERIC), short tandem repetitive repeats (STRRs) 1A & B and long tandem repetitive repeats (LTRRs), derived from repetitive sequences gift within the ordering of true bacteria, were utilized. The generated knowledge was then evaluated for fingerprinting exploitation procedure and unweighted pairgroup method of arithmetic average (UPGMA) based dendrograms. Clear and variable stripe patterns were obtained for every of the targeted genomic regions used, LTRR being the sole exception. The stripe patterns delineate the existence of 2 basic distinct populations of true bacteria among the 19 isolates. The UPGMA cluster analyses in addition, disclosed the presence of numerous lineages among the isolates. On examination the resolution capability of the four genomic regions studied, it had been seen that, with the exception of LTRR, all the opposite regions hold smart resolution and therefore the ability to differentiate between numerous class populations. The results of the current study indicate the incidence of genetic variants among the 19 morphologically identical isolates of filiform cyanobacteria. Additionally, the study additionally explicates the quality of DNA fingerprinting supplemented with UPGMA cluster analysis as a speedy, economical and value effective tool for differentiating among morphologically identical populations.

Keywords: Cyanobacteria; genetic diversity; DNA fingerprinting; UPGMA cluster analysis

Introduction

The phylum cyanobacteria represents the largest sub-groups of Gram negative microorganisms characterized by the ability to perform photosynthesis. Besides being oxyphototrophic, some of the cyanobacteria are also endowed with the capability of fixing atmospheric nitrogen[1-6]. An additional aspect of cyanobacteria being a subject of research interest lies in the fact that they are amongst the ancient evolutionary lineages of phototrophs believed to have originated during the early Pre-cambrian super eon of the geological time scale (approx 2.6-3.5 Bya)[7-8]. With immense potential, cyanobacteria offer diverse applications in bioremediation, biofertilizers, bio-monitoring of soil fertility and water quality[9-12].

Conventional approaches for identification of cyanobacteria have mostly relied on morphological characters; which, however, tends to suffer from uncertainties and is often misleading[13-17]. Furthermore, assessment of genetic diversity among cyanobacterial population has also been problematic owing to their small size and non distinctive phenotypic characteristics (Rasmussen and Svenning). With the advancement in the field of molecular biology, a number of techniques have been made available for identification, delineation and studying genetic diversity of cyanobacteria. In this context, 16S rRNA sequence analysis, restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD) and amplified ribosomal DNA restriction analysis (ARDRA) analyses have been successfully employed for molecular characterization, identification and resolving taxonomic and phylogenetic issues among the various cyanobacterial taxa[18-26]. With regard to investigation of genetic diversity, PCR based DNA finger printing have been considered as a valuable tool[27-29]. Furthermore, in comparison to the other available techniques, such as 16S rRNA and whole genome sequence analysis, which are commonly used for assessment of microbial diversity, DNA finger printing is a cost effective technique and can be routinely used. In this context, the repetitive sequences, which constitute an important part of

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the prokaryotic genome, have been successfully employed to generate finger print profile for various bacterial[30] as well as cyanobacterial groups (Nilsson *et al*, Rynearson and Ambrust). Though the functions of these repetitive sequences have not been established yet, however, their conserved nature and widespread distribution in the genome makes them very useful in differentiation and discrimination of closely related species and strains of microorganisms including cyanobacteria. M13, ERIC, STRRs and LTRRs represent some of the most widely used repetitive genomic regions for finger printing studies in cyanobacteria[31-33].

Thus, the present study aimed to discriminate 19 filamentous cyanobacterial isolates collected from different pockets and habitats of south west Khasi Hills, Meghalaya, India, using DNA finger printing of five different phenetic markers (M13, ERIC, STRR-1A and 1B and LTRR). In addition, there resolving potential of each of the markers used was also evaluated.

Materials and Methods

Study Area

The south west Khasi Hills district, Meghalaya, India, with an area of 1,341 Km^2 is located at 25.3259° N, 91.2506° E. Cyanobacterial samples were collected from variety of habitats in and around 7 pockets of south west Khasi Hills. The details of the collection sites with the global positioning system (GPS) coordinates are listed below (Table 1).

Isolation, Purification and Cultivation of Cyanobacteria

The cultures were initially grown in BG11 medium or solid medium (with 1.5% agar) in an air conditioned chamber at 24 $\pm$ 2°C with a photon fluence rate of 50 µmol m^{-2} s^{-1} and control illumination of 12 hours light and 12 hours dark following standard protocol of Rippka *et al*[7]. Axenic cultures of cyanobacterial isolates were obtained from processed samples by serial dilution technique and direct streak plate method which were then inoculated into sterile selective culture medium and used for the further studies.

Light Microscopy Study

As preliminary investigation, the axenic cyanobacterial cultures were examined under the light microscope at 40X magnification (Radical RXL4B) and the cell form (unicellular or filamentous) along with the presence or absence of heterocyst was studied.

DNA Extraction and PCR Amplification

The cultures at exponential phase were harvested and used for extraction of genomic DNA using commercially available kit (Wizard® genomic DNA purification kit, Promega) following manufacturer's protocol. For DNA finger printing analysis, different primer sets corresponding to tandemly repetitive sequences was used for PCR amplification. The primer sets included M13[34], ERIC (Versalovic *et al*), STRR 1A and 1B and LTRR 1 and 2 (Rasmussen and

Table 1 — List of cyanobacterial isolates collected along with the site of collection and geographical coordinates

Sl. No.	Collection site	Isolates	Code	Habitat	Geographical coordinates
1	Tyrsad	Isolate 1	Cyan_Tyr1	Bare rocks	25.40˚N; 91.66˚E
		Isolate 2	Cyan_Tyr2	Swamp	
		Isolate 3	Cyan_Tyr3	Pond	
2	Umlangmar	Isolate 4	Cyan_Umlang1	Swamp	25.38˚N; 91.65˚E
		Isolate 5	Cyan_Umlang2	Pond	
		Isolate 6	Cyan_Umlang3	Soil	
		Isolate 7	Cyan_Umlang4	Bare rocks	
3	Weiloi	Isolate 8	Cyan_Wei1	Swamp	25.36˚N; 91.61˚E
		Isolate 9	Cyan_Wei2	Soil	
		Isolate 10	Cyan_Wei3	Pond	
4	Sawsymper	Isolate 11	Cyan_Saw1	Pond	25.39˚N; 91.60˚E
		Isolate 12	Cyan_Saw2	Soil	
5	Ponkung	Isolate 13	Cyan_Pon1	Bare rocks	25.38˚N; 91.58˚E
		Isolate 14	Cyan_Pon2	Swamp	
		Isolate 15	Cyan_Pon3	Pond	
6	Umjarain	Isolate 16	Cyan_Umjar1	Bare rocks	25.40˚N; 91.54˚E
		Isolate 17	Cyan_Umjar2	Swamp	
		Isolate 18	Cyan_Umjar3	Soil	
7	Jakrem	Isolate 19	Cyan_Jak1	Hotspring	25.39˚N; 91.50˚E

Svenning). Amplification was performed in a 25 µL reaction volume in PCR thermal cycler (Takara). The reactions conditions were 1 cycle of initial denaturation at 95°C for 5 min, followed by 36 cycles of denaturation at 94°C for 1 min; annealing at 48°C (M13 and STRR 1A), 43°C (for ERIC), 50°C (STRR 1B) and 38°C (LTRR 1 and 2); elongation at 65°C for 5 min (STRR 1A, STRR 1B and LTRR 1 and 2) and at 72°C (M13 and ERIC); final elongation consisted of 1 cycle at their respective elongation temperatures for 7 min. The details of primers used for PCR amplification are listed in Table 2. The resulting amplicons were then separated on 1.5% (w/v) agarose gel and visualized under Gel Doc™ EZ Imager, Bio-Rad.

DNA Fingerprint Analysis

For genetic diversity, the finger printing patterns obtained via PCR were analyzed using bioinformatic tool GelJ v2. The GelJ software is a java based application which is used for analyzing finger printing gel images. The software computes the similarity matrix which is used for generation of dendrogram employing clustering algorithms implemented in Weka[35]. The samples are then compared and grouped based on the banding patterns.

Results

Each of the 19 cyanobacterial isolates collected from different habitats were found to be filamentous and showed the presence of specialized cells, heterocyst, as seen under the microscope. Consequently, the isolates seemed in distinguishable from each other and thus, discrimination based on the morphology was not of much assistance (Fig. 1, a-c). The results of molecular analyses however, revealed a completely different picture. Genomic DNA was successfully extracted from 19 cyanobacterial cultures; the primer sets (Table 2) showed successful amplification for all the targeted genomic regions i.e., M13, ERIC, STRR-1 A and 1B, and LTRR. The PCR amplicons yielded multiple bands of variable lengths for each of the genetic markers used (Fig. 2-6).

Apparently, it was observed that, of the five markers used, ERIC-PCR generated highly complex finger print profile with 4-8 bands for each of the isolates, whereas, M13 and STRRs-PCR showed relatively simpler patterns and with fewer (2-4) bands (Table 3). In either of the cases, the bands were sharp and well resolved. The LTRR-PCR on the other hand was not able to produce a pronounced banding pattern. Based on these banding profiles,

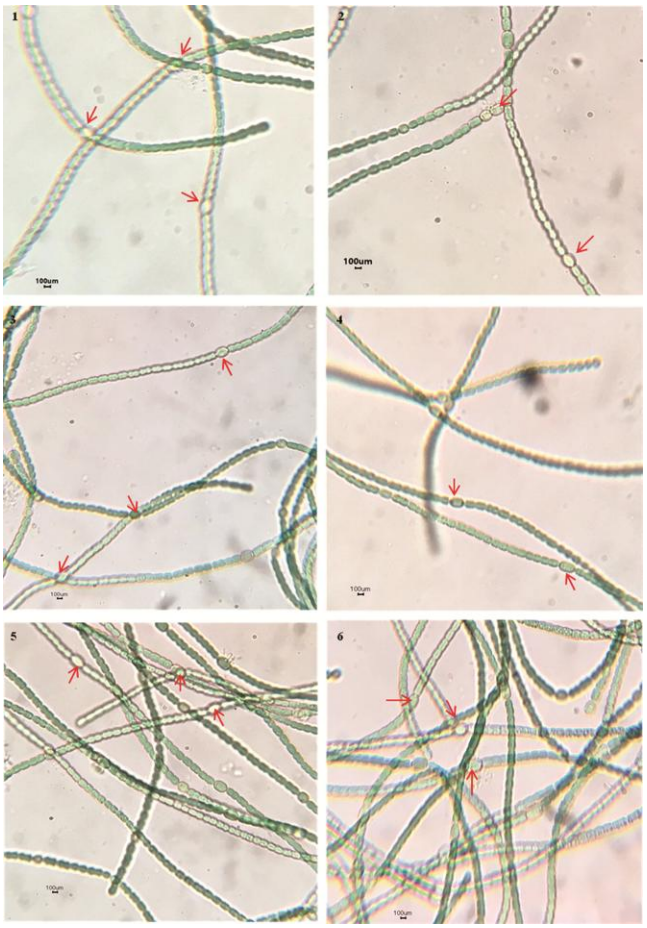


Fig. 1a — Micropictographs of the cyanobacterial isolates 1-6. Heterocysts in the filaments are indicated by arrow in red

Table 2 — Primers used for generating finger printing profiles along with sequence details and annealing temperatures

Sl. No.	Primer	Primer sequence	References
1	M13	5'-GAGGGTGGCGGTTCT-3'	Meyer *et al*., (1993)[34]
2	ERIC	5'-ATGTAAGCTCCTGGGGATTCAC-3'	Versalovic *et al*.,(1991)[30]
		5'-AAGTAAGTGACTGGGGTGAGCG-3'	
3	STRR 1A	5'-CCARTCCCCARTCCCC-3'	
4	STRR 1B	5'-GGGGAYTGGGGAYTGG-3'	Rasmussen & Svenning, (1998)[1]
5	LTRR 1 & 2	5'-GGATTTTTGTTAGTTAAAAC-3'	
		5'-CTATCAGGGATTGAAAG-3'	

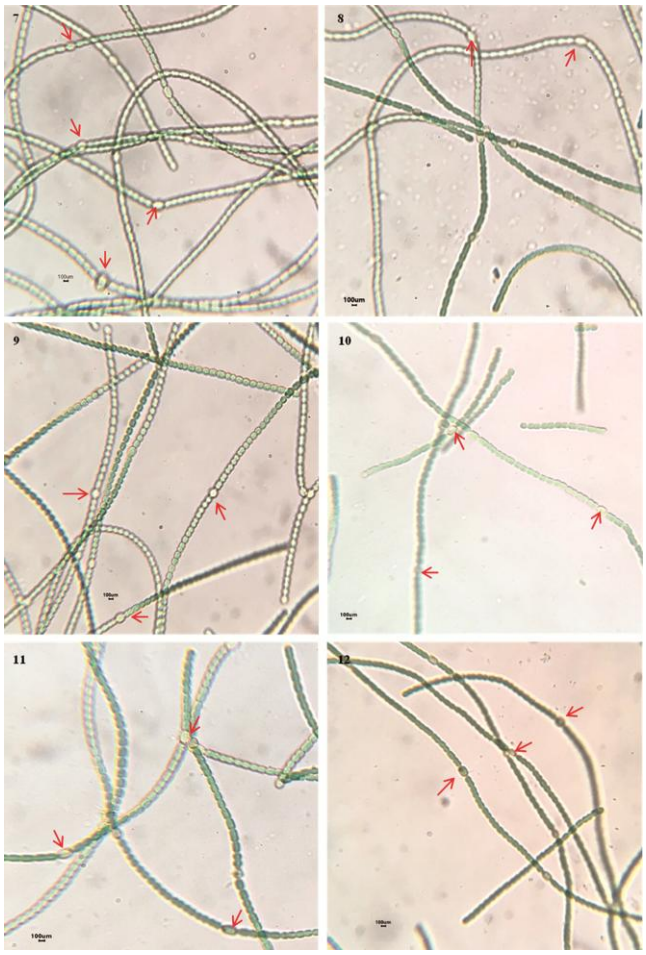
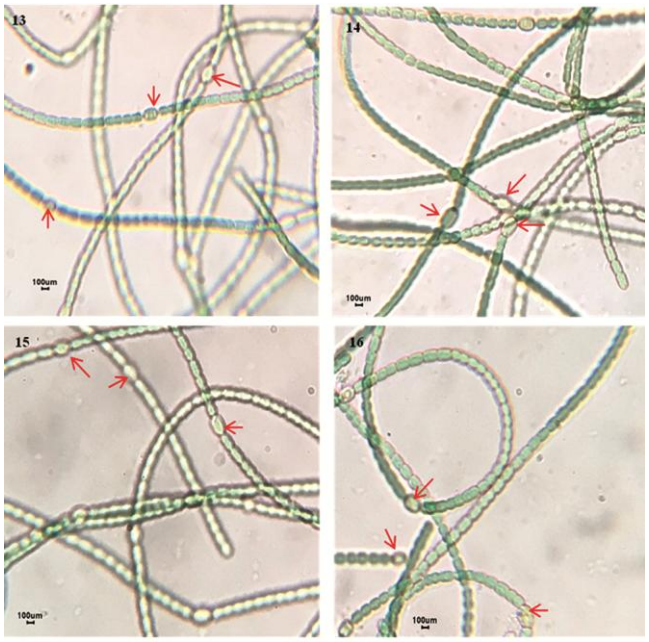


Fig. 1b — Micropictographs of the cyanobacterial isolates 7-12. Heterocysts in the filaments are indicated by arrow in red



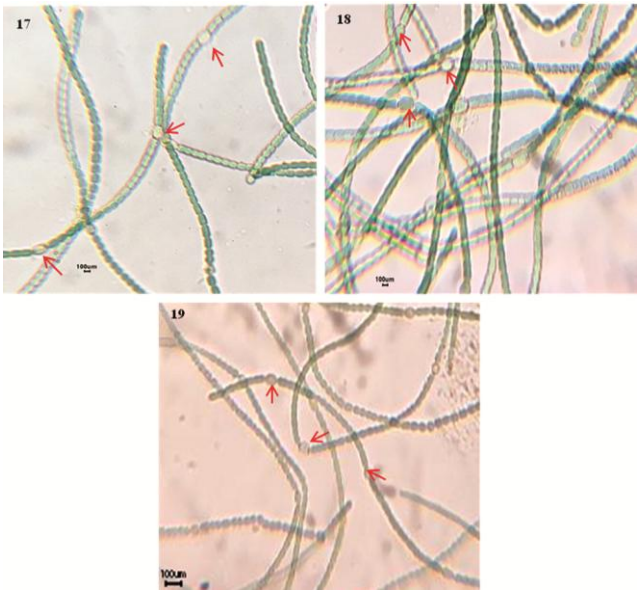


Fig. 1c — Micropictographs of the cyanobacterial isolates 13-19. Heterocysts in the filaments are indicated by arrow in red

DNA fingerprints were generated and used for discrimination of the isolates. From the finger print profiles it can be clearly seen that the 19 cyanobacterial isolates showed two distinct types of banding pattern throughout the analyses. The isolates Cyan_Tyr2, Cyan_Tyr3, Cyan_Umlang2, Cyan_Umlang4, Cyan_Wei1, Cyan_Wei2, Cyan_Wei3, Cyan_Saw2, Cyan_Pon1, Cyan_Pon2, Cyan_Umjar1, Cyam_Umjar2, Cyan_Umjar3 and Cyan_Jak1 showed similar banding pattern and thus formed a group. The second group included the isolates Cyan_Tyr1, Cyan_Umlang1, Cyan_Umlang3, Cyan_Saw1 and Cyan_Pon3 which also revealed identical banding patterns. Thus, the existence of two groups was prominent from the finger print profiles of all the genomic regions.

The difference in the banding pattern was further studied using UPGMA cluster analysis, for which, the finger print profiles were translated into dendrograms. The dendrograms clustered the cyanobacterial isolates into different factions taking into account the similarity in banding patterns. The overall topology of the dendrograms in all the phenetic markers revealed noticeable homology and basically divided the tree into two distinct lineages (referred to as major and minor cluster). In concurrence with the finger print results, 14 isolates (i.e., isolates Cyan_Tyr2, Cyan_Tyr3, Cyan_Umlang2, Cyan_Umlang 4, Cyan_Wei1, Cyan_Wei2, Cyan_Wei3, Cyan_Saw2, Cyan_Pon1, Cyan_Pon2, Cyan_Umjar1, Cyam_Umjar2,

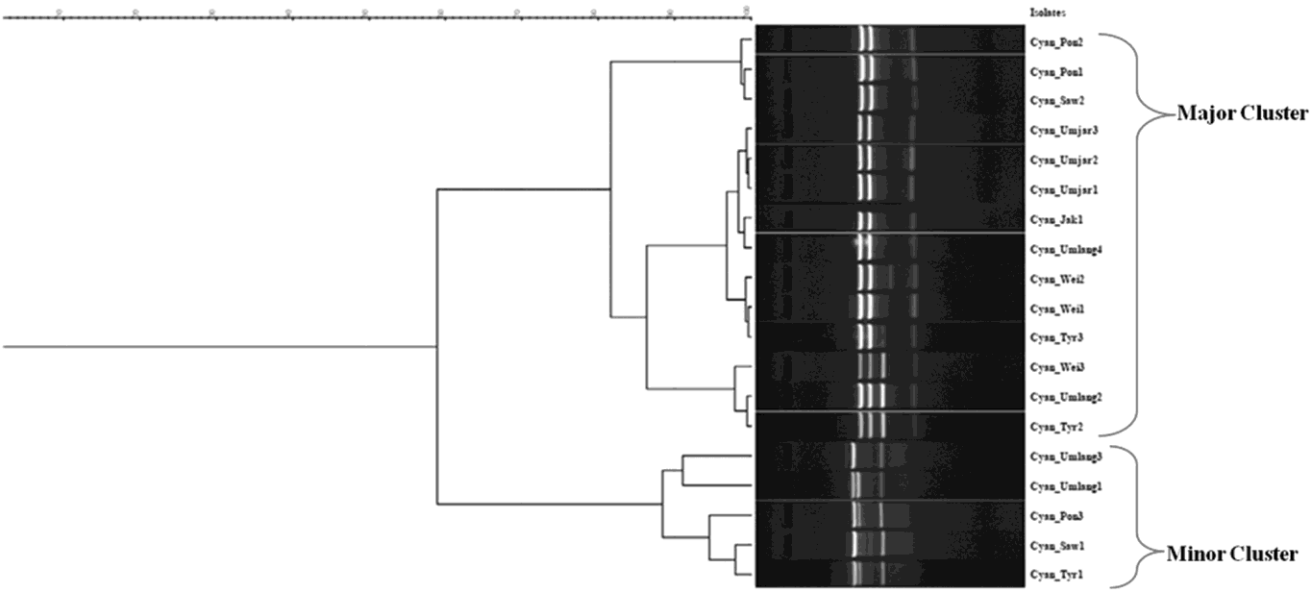


Fig. 2 — UPGMA dendrogram and PCR fingerprinting pattern of the 19 cyanobacterial culture obtained from M13 primer

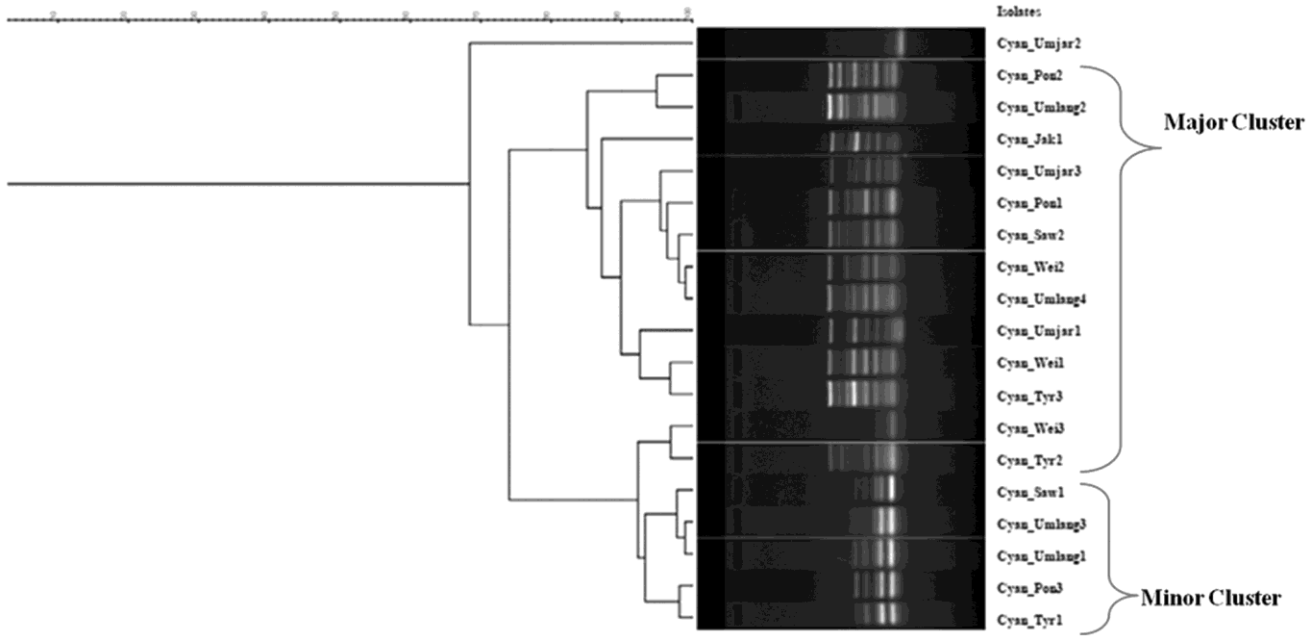


Fig. 3 — UPGMA dendrogram and PCR fingerprinting pattern of the 19 cyanobacterial culture obtained from ERIC primer

Cyan_Umjar3 and Cyan_Jak1) formed a single cluster which further segregated into distinct and smaller sub clusters. The grouping of isolates in the major clade did not show any sort of regularity across the phenetic markers studied. The minor cluster on the other hand, encompassing 5 isolates (i.e., Cyan_Tyr1, Cyan_Umlang1, Cyan_Umlang3, Cyan_Saw1 and Cyan_Pon3) showed consistency throughout and formed a single clade (Fig. 2-6). It is also worth mentioning that the dendrogram obtained from the finger print data of ERIC revealed a slightly different result. Firstly, isolate 17 or Cyan_Umjar formed an isolated clade, which in other fingerprint profiles is seen to cluster in the major clade. Secondly, as mentioned earlier, the minor clade in the rest of the phenetic markers showed the assemblage of five isolates. However, in case of ERIC, two other isolates (Cyan_Wei3 and Cyan_Tyr2) are also seen clustered along with the five isolates.

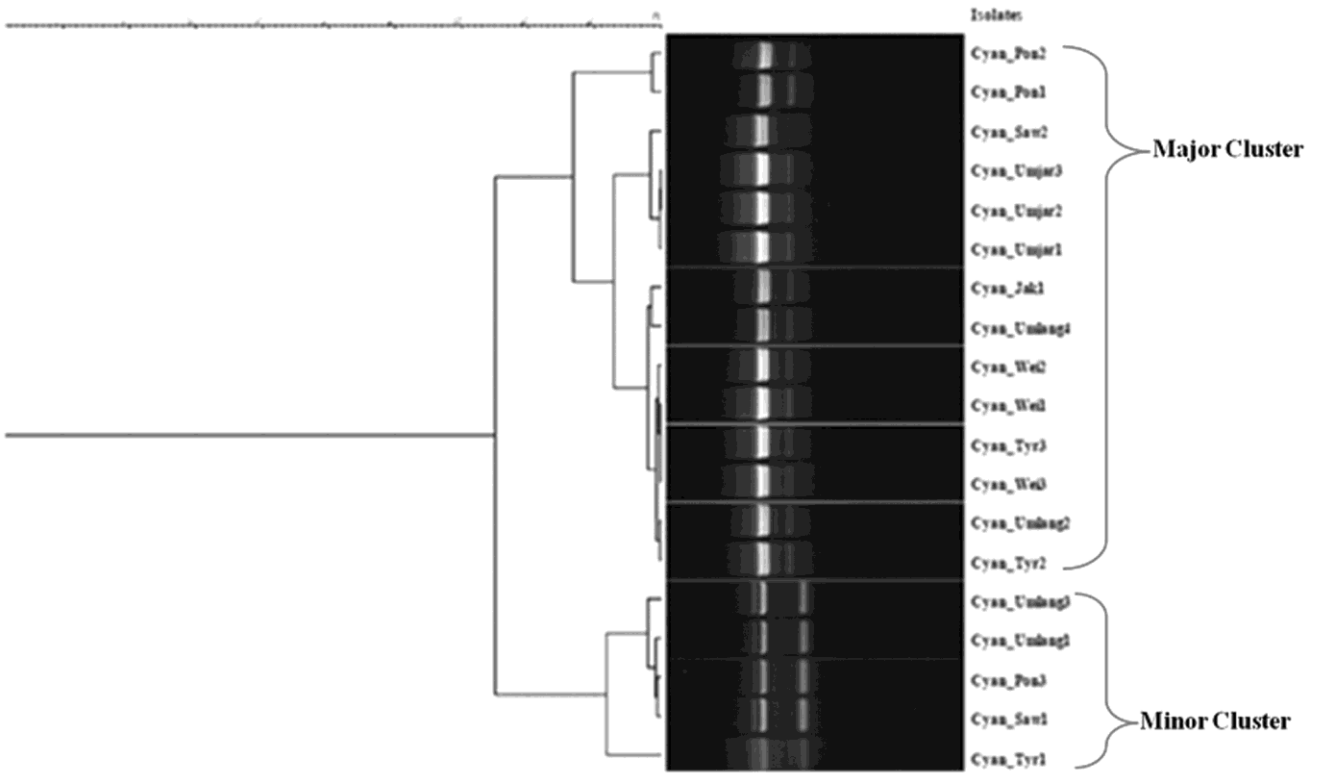


Fig. 4 — UPGMA dendrogram and PCR fingerprinting pattern of the 19 cyanobacterial culture obtained from STRR-1A primer

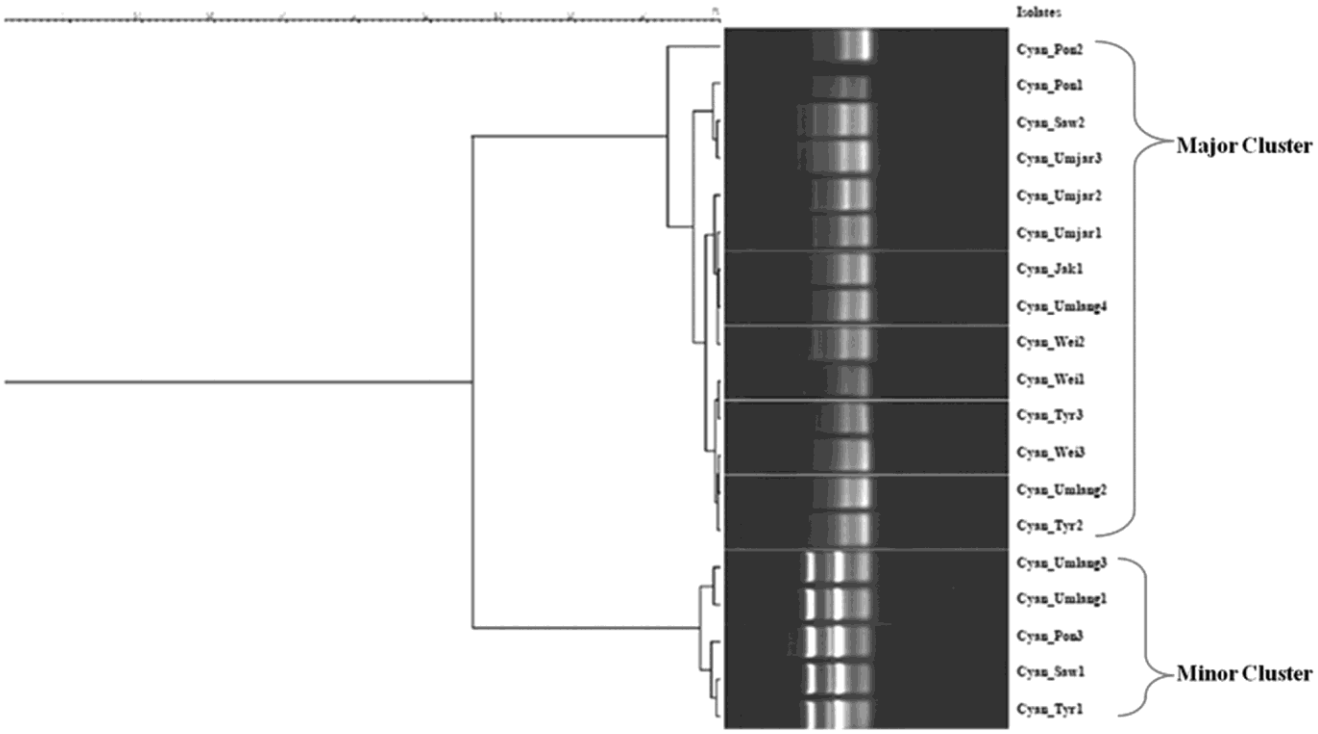


Fig. 5 — UPGMA dendrogram and PCR fingerprinting pattern of the 19 cyanobacterial culture obtained from STRR-1B primer

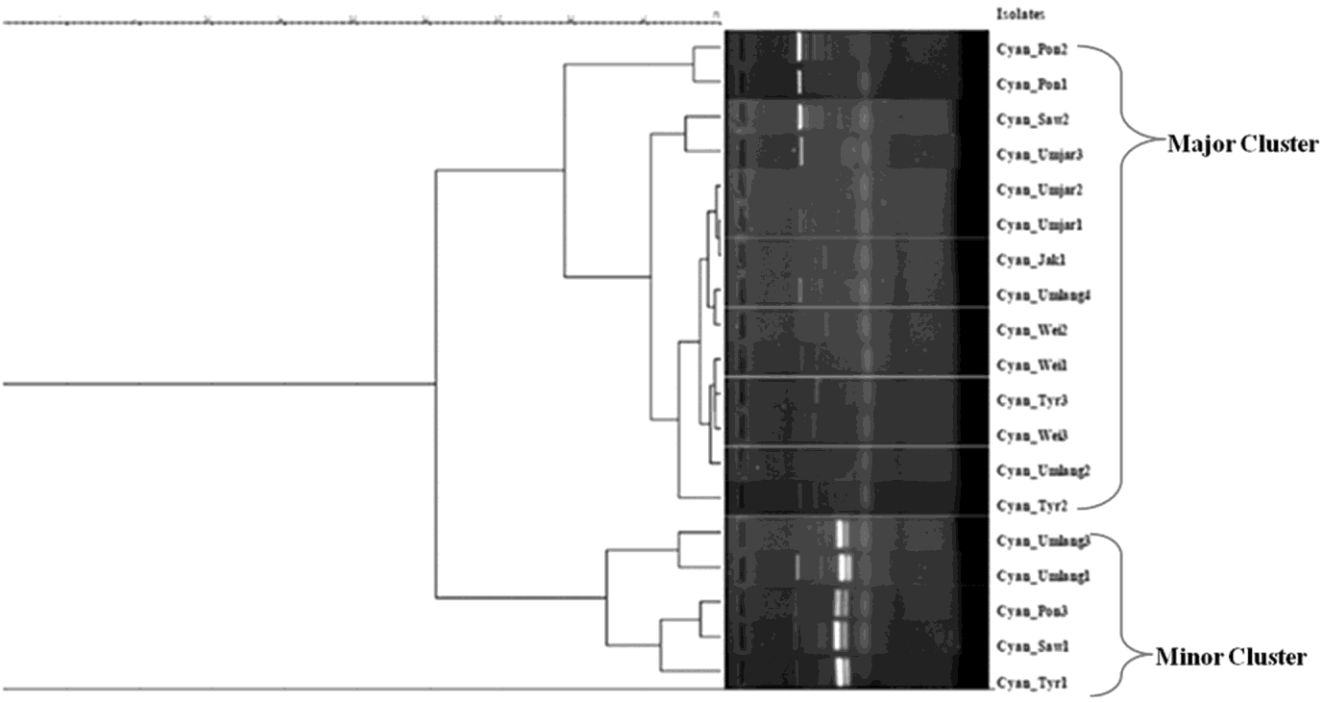


Fig. 6 — UPGMA dendrogram and PCR fingerprinting pattern of the 19 cyanobacterial culture obtained from LTRR primer

Table 3 — Comparative results of the finger printing patterns of the five primer sets used

Primers used	No. of bands/isolate	Molecular lengths range (~kb)
M13	3 - 4	0.3 - 1.5
ERIC	4 - 8	0.07 - 0.8
STRR-1A	3 - 5	0.2 - 0.8
STRR-1B	~3	0.15 - 1.2
LTRR	3 - 5	0.2 - 1.8

Discussion

Molecular profiling plays a vital role in classification and diversity of any group of microorganisms, predominantly in the investigation of untypable strains[36]. Genomic fingerprinting, in comparison to other techniques used for classification and diversity, does not rely on sequence data which tends to be expensive, laborious and demands expertise for data analyses. Additionally, the high levels of detectable polymorphism and the rapidity of the method make this technique an extremely efficient tool for analyzing genetic variations[37]. In the present study, 19 cyanobacterial samples collected from 7 different localities and habitats were analyzed and discriminated based on the finger print profiles. The results of the PCR based DNA finger printing analysis showed segregation of the cyanobacterial isolates into distinct assemblages. PCR amplification of M13, ERIC, STRR-1A and 1B, provided a clue for the existence of genetic variants among the 19 isolates as different banding patterns were observed in the finger print profiles. Although, the banding pattern obtained from LTRR-PCR was not clear enough, it did reveal the presence of distinct cyanobacterial population. The UPGMA cluster analysis also provided a much elaborated indication of diversity among the 19 cyanobacterial isolates. This was evident from the dendrograms which segregated into major and minor clades and even smaller sub-clades, with each sub-clade representing a discrete genetically different population. While, grouping of isolates in the major clade did not show any consistency, the minor clade, interestingly comprised of the same five isolates throughout the analyses, suggestive of the fact that the five isolates probably belong to the same or rather closely related group. Additionally, if we consider the collection sites (rocks, swamp, soil and pond) of these five isolates, it can be seen that there exist no correlation between their habitats, geographic locations and their groupings. This indicates that the five isolates though identical need not necessarily be from the same habitat or geographic location. As such, it is known that cyanobacteria are able to thrive in a variety of conditions and thus similar cyanobacteria may be found in different habitats.

The utility of molecular fingerprinting using the genomic regions such as M13, ERIC, STRRs and LTRRs for inference of genetic diversity and discrimination of cyanobacterial samples has been reported by various authors such as Rasmussen and Svenning.,1998, Liaimer *et al*., 2016, Valerio *et al*., 2009, Lyra *et al*., 1997, Zheng *et al*., 2002, Nilsson *et al*., 2000 , Muralitharan and Thajuddin., 2010, Gnat *et al*., 2015. The same was also evident in our results. Thus, indicating the suitability of these primers in discriminating morphologically similar cyanobacterial population. Though, LTRR has also been commonly used for fingerprinting analyses of cyanobacteria (Rasmussen and Svenning., 1998, Valerio *et al*., 2009), our results did not reveal a satisfactory banding pattern for the same. Thereby, suggesting M13, ERIC or STRRs as better primers for discrimination of identical cyanobacterial isolates. Comparatively, the results also suggested, ERIC to possess best resolving capability among all the phenetic markers used.

To conclude, 19 cyanobacterial isolates collected from range of habitats and localities though appeared similar in morphology, showed differences at the genetic level which was resolved successfully using fingerprinting approach followed by UPGMA cluster analysis. Thus, molecular finger printing supplemented with UPGMA cluster analysis provides a cost effective and an efficient tool for studying diversity. Furthermore, results of the present study provides aptness of the genomic regions (M13, ERIC, STRRs and LTRR) in resolving genetic differences among closely related taxa, particularly in cases where morphological characterization does not offer much assistance.

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