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Research Article

Molecular and morpho-physiological analyses revealed inter- and intra-generic diversity of filamentous cyanobacteria from saline/alkaline soils

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Abstract – Cyanobacteria play important roles in soil fertility and soil productivity. Diverse filamentous cyanobacteria due to their ability to add nitrogen and organic carbon to soil and to maintain the physical properties of soil are undoubtedly one of the important groups of cyanobacteria. The present study aimed to understand the diversity of filamentous cyanobacteria (both heterocystous and non-heterocystous) obtained from saline/alkaline soils of Uttar Pradesh, India. Fuzzy cluster analyses of 47 filamentous cyanobacteria based on their cellular dimensions showed high inter-generic diversity among heterocystous cyanobacteria while in non-heterocystous cyanobacteria both inter- and intra-generic diversity was observed. The results of the present study indicated that the dimensions of vegetative cells and heterocysts can be useful for the identification and differentiation of cyanobacteria belonging to *Hapalosiphon* and *Lyngbya.* Physiological characterization also revealed a great deal of variation (5-15%) in salinity tolerance. Multiplex HIP fingerprinting revealed a higher genetic diversity in heterocystous (12-76% similarity) than in non-heterocystous cyanobacteria (13-90% similarity). Heterocystous cyanobacteria like *Aulosira laxa* O. Kirchner ex Bornet & Flahault, *Hapalosiphon* sp. and *Nostoc* sp. showed high intra-generic variability while among the non-heterocystous ones, *Lyngbya* was found to be genetically highly heterogeneous. The results of the present study highlight the importance of morpho-physiological and genetic analyses in deciphering the diversity of filamentous cyanobacteria for the understanding of their population structure, ecology and adaptations.

Keywords: cell size, cyanobacteria, diversity, fuzzy clustering, Highly Iterated Palindrome (HIP) fingerprinting

Introduction

Cyanobacteria or blue green algae (BGA) are a group of oxygenic photoautotrophic prokaryotes that were responsible for the first significant increase in atmospheric oxygen (Gould et al. 2008). These diazotrophic photosynthetic microorganisms are distributed in diverse ecological habitats ranging from hot to cold springs, marine to fresh water, pristine to degraded soils, oligotrophic to hypereutrophic environments (Bhatnagar and Bhatnagar 2005, Bagul et al. 2018). Cyanobacteria are a morphologically diverse group comprising both unicellular and colonial (including filamentous) forms. Cyanobacteria are not only abundant in the soil, but also play important roles in soil fertility and soil productivity (Rossi et al. 2017). Filamentous cyanobacteria, due to their ability to add nitrogen and organic carbon to soil and to improve soil physical properties, are undoubtedly one of the important groups of cyanobacteria. Due to their filamentous nature and abundant production of polysaccharides in many species, they can significantly help in soil aggregation (Chakdar et al. 2012). It is well known that filamentous cyanobacteria are associated with biological soil crusts (BSC) the origin of which is due to entrapment of minerals and sand particles by cyanobacterial filaments (Garcia-Pichel et al. 2001). These BSCs are ecologically very important in semi-arid to arid areas as they serve as microhabitats (Zhang 2005). Filamentous cyanobacteria have also been evaluated for their potential role in salinity stress amelioration (Li et al. 2019).

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Despite their ecological significance and application for the amelioration of degraded soils, studies on the diversity of cyanobacteria from saline/alkaline/sodic soils are scanty in India. To our understanding, lack of detailed diversity studies from different habitats, routine sub-culturing based isolation and morphometry based techniques leading to the identification of a few predominant genera are some of the reasons that most of the application-based studies are centred around a few genera like *Anabaena* or *Nostoc*. In India, the area under salt-affected soils is about 6.73 million (m) ha with the states of Gujarat (2.23 m ha), Uttar Pradesh (1.37 m ha), Maharashtra (0.61 m ha), West Bengal (0.44 m ha) and Rajasthan (0.38 m ha) together accounting for almost 75% of saline and sodic soils in the country (Sharma and Singh 2015). Understanding of the genetic as well as the functional variability of the cyanobacterial population of saline sodic soils can help to establish a very broad base of genetic resources for their further exploitation. Diversity of cyanobacteria is generally studied in terms of the variabilities in cellular features (e.g. biometric characteristics of vegetative cells, heterocysts, hormogonia and akinetes; branching pattern, presence or absence of sheaths etc.) or by the use of molecular techniques. Undoubtedly, molecular analyses of diversity are more accurate and trustworthy, as morphological features are prone to environmental fluctuations (Doers and Parker 1988, Kato 1991, Chakdar and Pabbi 2012). A number of molecular tools PCR-RFLP, RAPD, finger printing based on repetitive elements have been used to study inter- and intra-generic molecular diversity of cyanobacteria (Mazel et al. 1990, Neilan et al. 2003, Prabina et al. 2005, Ezhilarasi and Anand 2010, Akoijam and Singh 2011, Chakdar and Pabbi 2012, Chakdar and Pabbi 2017).

Highly Iterated Palindrome (HIP1), a repetitive eight base sequence (5'-GCGATCGC-3'), was first identified in a cadmium-tolerant strain of *Synechococcus* PCC 6301 at the borders of a gene deletion (Gupta et al. 1993). It has been speculated that there are no comparable sequences to HIP1 in other organisms, that they are only known to occur in cyanobacteria and can also be used to fingerprint organisms (Smith et al. 1998). These repetitive sequences have been used to study several cyanobacterial taxa (Lyra et al. 2005, Prasanna et al. 2006, Selvakumar and Gopalaswamy 2008, Chakdar and Pabbi 2012, Singh et al. 2014, Shokraei et al. 2019). The conserved nature along with genome-wide distribution of the repeats and reproducibility has made them ideal tools for biodiversity studies (Selvakumar and Gopalaswamy 2008).

Looking at the insufficient information available about the diversity of filamentous cyanobacteria from saline sodic soils of India, in the present study we analyzed the diversity of 47 filamentous cyanobacteria isolated from saline/alkaline soils of eastern Uttar Pradesh, India. Here we have used HIP fingerprinting as well as fuzzy clustering based on cellular features and empirically compared the techniques to determine whether they can really be correlated.

Material and methods

Procurement and maintenance of cultures

Forty-seven strains of cyanobacteria isolated from saline/alkaline habitats of eastern part of Uttar Pradesh, India were procured from the National Agriculturally Important Microbial Culture Collection (NAIMCC), ICAR-National Bureau of Agriculturally Important Microorganisms (ICAR-NBAIM), India (On-line Suppl. Tab. 1). Cultures were maintained in chemically defined BG-11 media with and without nitrogen source for non-heterocystous and heterocystous cyanobacterial isolates respectively at 28 ± 2 °C under a light intensity of 52–55 μmol photon m^{-2} s^{-1} and L:D cycles of 16:8 h (Stanier et al. 1971).

Morphometric characterization of cyanobacterial isolates

Freshly raised cyanobacterial strains were viewed under 400× and 1000× magnification under a light microscope (Olympus, Japan) (On-line Suppl. Fig. 1) to check the purity and morphological identity according to the keys provided by Desikachary (1959). The length and width of vegetative cells and heterocysts were determined by analysing the captured images using Prog Res Capture Pro 2.6 software (JENOPTIK, Germany) under 400× magnification using a fluorescent microscope (Olympus BX41, Japan). For each cyanobacteria, five vegetative cells and heterocysts (only for heterocystous cyanobacteria) of different filaments were measured. The mean values for length and width of vegetative cells and heterocysts were used for clustering.

Physiological characterization of isolates for tolerance to salinity and alkalinity

The ability of the cyanobacterial isolates to tolerate alkalinity and salinity were checked by growing them on BG-11 N^+/N^- agar with varying pH (8, 9 and 10) and salinity (5, 10 and 15% (w/v) NaCl concentrations. One mL of freshly grown cyanobacterial culture suspension was taken in sterile microcentrifuge tube (2 mL) and centrifuged at 10000 rpm for 10 min. Pellets were washed with autoclaved distilled water for 2-3 times. Sterile glass beads were added to the culture tube containing cell pellets and 1 mL of autoclaved distilled water was added to it. Cells were vortexed for 30 seconds to homogenise the filamentous cells into suspension. 40 μL of this suspension was spotted on BG-11 medium agar plates of different pH and salt concentrations. The culture plate was incubated for 10 days under conditions as described in the section Procurement and maintenance of cultures. After incubation, plates were observed for growth and results were recorded. Presence of growth was recorded as "1" and absence was recorded as "0". This binary data was further used for clustering following the procedures detailed in sub-section Genetic diversity analyses in the section Statistical analyses.

Genomic DNA extraction

Genomic DNA was extracted from 1 mL (50–60 mg fresh biomass) of exponentially grown cyanobacterial strains by using Nucleo-pore® gDNA Fungal/Bacterial kit (Genetix Biotech Asia Pvt. Ltd) following the manufacturer's protocol with a few modifications. Quantity and purity of DNA was estimated by comparison with known standards in ethidium bromide stained 0.8% agarose (Vivantis) gel.

Multiplex Highly Iterated Palindrome (HIP) fingerprinting

Primers

DNA samples were subjected to amplification using HIP based primers viz. HIP AT, HIP TG and HIP GC in dual combinations. The details of primers sequence used in this study are: (1) HIP AT: 5'-GCGATCGCAT-3' (2) HIP TG: 5'-GCGATCGCTG-3' and (3) HIP GC: 5'-GCGATCGC-GC-3' with 60%, 70% and 80% GC content respectively.

PCR amplification

The standard, optimized PCR was performed in a total volume of 50 µL containing 25 µL of 2X Go Taq Green Master mix (Promega), dual combination of HIP primers with 10 pM each of single primer and 90 ng of template DNA (Chakdar and Pabbi 2012). Thermal cycling was achieved in a Master Cycler Gradient (peQSTAR, Germany) as described earlier by Chakdar and Pabbi (2012). PCR products were resolved along with a molecular weight marker (Promega 1 Kb DNA ladder) on 2.0% agarose gel for 6 hours (50 volts) in 1X Tris–Borate–EDTA (TBE) buffer and stained with ethidium bromide solution (1 µg/mL). These were visualized under UV light and gel photographs were captured through gel documentation system (Universal Hood II, BIO-RAD, USA) and the amplification product sizes were determined using the software FluorChem 5500 (Alfa Innotech Corporation, USA). The bands, ranging from 300 to 3000 bp, were scored for diversity analyses. The banding pattern was scored as "1" for presence and "0" for absence of a band. This binary (0, 1) matrix was used for genetic diversity analyses.

Statistical analysis

Morphological diversity analyses

Fuzzy C-Means (FCM) clustering technique is a soft clustering algorithm proposed by Bezdek (1973, 1981). Unlike K-means clustering algorithm in which each data object is the member of only one cluster, a data object is the member of all clusters with varying degrees of fuzzy membership between 0 and 1 in FCM clustering algorithm. Hence, the data objects closer to the centers of clusters have higher degrees of membership than objects scattered in the borders of clusters. For morphological diversity analysis, FCM clustering algorithm was executed. Fitness of the clustering was measured using Dunn's fuzziness coefficient and fuzzy silhouette index. Dunn's fuzziness coefficient is a goodness-of-fit criterion for fuzzy clustering that measures how close the fuzzy solution is to the corresponding hard solution. A higher value of Dunn's fuzziness coefficient indicates good clustering. Dunn's fuzziness coefficient is generally normalized so that it varies from 0 (completely fuzzy) to 1 (hard cluster). The fuzzy silhouette index is another performance evaluation criterion of FCM clustering; it lies between 0 to 1, where 0 indicates completely fuzzy clustering and 1 indicates completely crisp or hard clustering.

Here, FCM clustering was performed for the data generated from morphometry. Before performing the FCM clustering, principal component analysis (PCA) was carried out to reduce the dimension as PCA is a dimension-reduction technique. Besides, by projecting the data into a lower-dimensional space, PCA can help eliminate noise and irrelevant features, making the underlying structure of the data more apparent for clustering. Further, reducing dimensions through PCA can help visualize the data, allowing us to assess the clustering results and the distribution of data points in a more understandable way. Separate clustering was carried out for heterocystous and non-heterocystous cyanobacteria. PCA and FCM cluster analyses were implemented using R version 3.6.3 (2020-02-29), Platform: x86_64-w64-mingw32/x64 (64-bit).

Genetic diversity analyses

Pairwise genetic similarities among the genotypes under study were determined using Jaccard's coefficient (Jaccard 1908), $J = N_{11}/(N_{11} + N_{10} + N_{01})$, where N_{11} is the number of bands present in both individuals i and j, N_{10} is the number of bands present in the individual i and N_{01} 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 mean (UPGMA) using NTSYS pc, version 1.80 (Shalini et al. 2008, 2009). Bootstrap value was determined using Winboot software and the confidence limit of the clustering was also checked. 1000 replicates were used for bootstrap analyses. To test the goodness of fit of a clustering to the set of RAPD data, cophenetic correlation coefficient or cophenetic value was estimated using the COPH and MXCOMP options in NTSYS pc program as described by Chakdar and Pabbi (2012). The degree of fit was interpreted subjectively as $0.9 \leq r$ is very good fit, $0.8 \leq r \leq 0.9$ is good fit, $0.7 \leq r \leq 0.8$ is poor fit and $r < 0.7$ is very poor fit.

Results

Measurements of cellular dimensions

The mean length of the vegetative cells of heterocystous cyanobacteria ranged from 3.17 to 10.67 µm while width ranged from 3.08 to 6.05 µm. *Hapalosiphon* sp. K23 (10.67 µm) and *Nostoc* sp. K60 (3.17 µm) were recorded as having the highest and lowest cell lengths respectively. Incidentally, *Nostoc* sp. K60 also showed the lowest (3.17 µm) vegetative cell width while the highest cell width was recorded in *Hapalosiphon* sp. K76. The average length and width of heterocyst ranged from 3.87-13.12 µm and 3.67-9.69 µm respec-

tively. The highest heterocyst cell length and width were recorded in *Hapalosiphon* sp. K28 (13.12 µm) and *Nostoc* sp. K67 (9.69 µm) respectively. On the other hand, the lowest heterocyst cell length and width were recorded in *Aulosira laxa* K53 (3.86 µm) and *Toxopsis calypsus* K49 (3.70 µm) respectively. In the case of non-heterocystous cyanobacteria, the mean cell length and width ranged from 2.87-9.95 µm and 1.9-9.0 µm respectively. *Lyngbya hieronymusii* K95 was recorded with the highest cell length (9.95 µm) and width (9.0 µm) while *Desertifilum* sp. K4 (2.87 µm) and *Phormidium* sp. K6 (2.8 µm) showed the lowest cell length and width, respectively.

Fuzzy clustering based on cell size

For heterocystous and non-heterocystous cyanobacteria, separate clustering was carried out. Analyses of the heterocystous cyanobacteria showed 3 distinct clusters (Fig. 1A) where 78.51% variation was explained by two principal coordinates or Dimensions (Dim 1: -0.386*LV-0.502*WV-0.511*LH-**0.581*WH**; Dim 2: **0.891*LV**+0.011*WV-0.339*LH-0.303*WH). The fitness of the clustering was good as indicated by Dunn's fuzziness coefficient (0.687) and fuzzy silhouette index (0.775) which however also indicated that few of the isolates could be clustered in more than one group.

Isolates of *Hapalosiphon* spp. *viz. Hapalosiphon* sp. K23, *Hapalosiphon* sp. K28, *Hapalosiphon* sp. K32, *Hapalosiphon* sp. K50, *Hapalosiphon* sp. K62, *Hapalosiphon* sp. K76 and *Hapalosiphon* sp. K99) were distributed in Cluster 1 and 2. *Hapalosiphon* sp. K23 showed ~31% probability of being placed in Cluster 2 while *Hapalosiphon* sp. K50 showed ~26% probability of being placed in Cluster 3 (On-line Suppl. Tab. 2). All isolates of *A. laxa*, *T. calypsus*, *Scytonema* sp. and *Nostoc* spp. *viz. Nostoc* sp. K51, *Nostoc* sp. K56, *Nostoc* sp. K60 and *Nostoc* sp. K68 (except *Nostoc* sp. K67, which

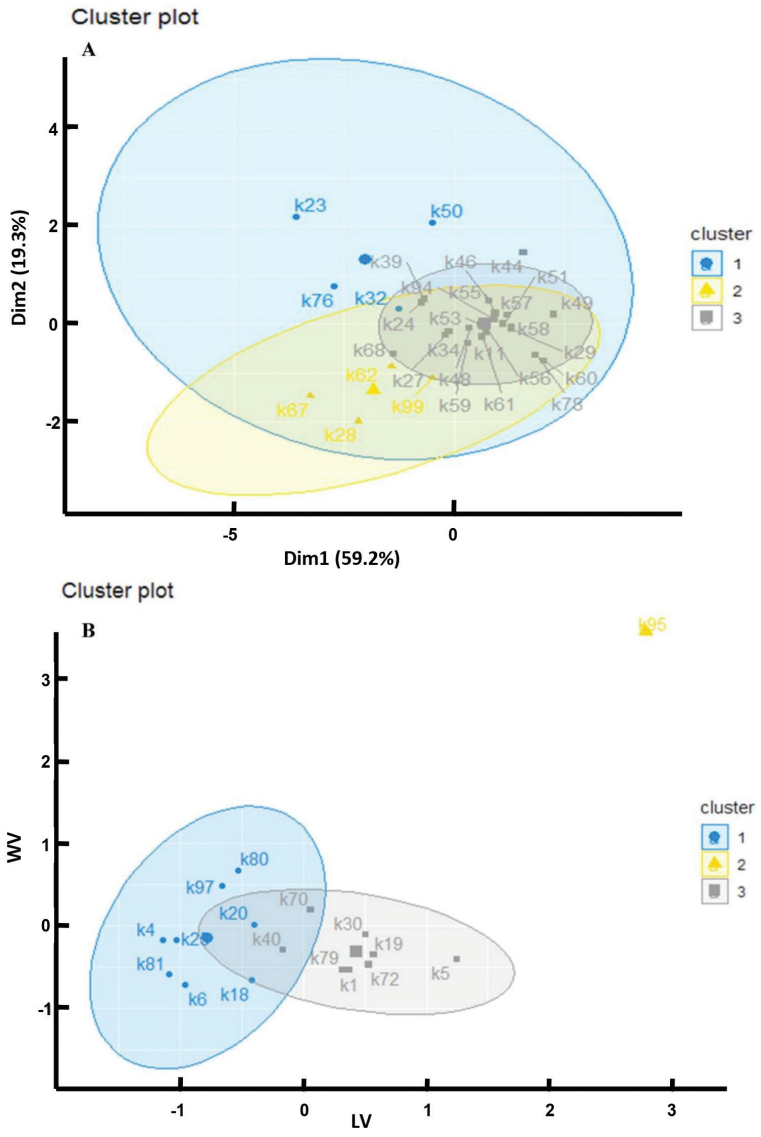

Fig. 1. Fuzzy cluster plot of heterocystous cyanobacteria (A) and non-heterocystous cyanobacteria (B). The fuzzy cluster plot was constructed using the cellular dimension of vegetative cells and heterocysts of heterocystous cyanobacteria. Whereas, length (LV) and width of vegetative (WV) cell were used for non-heterocystous cyanobacteria. In case of clustering of heterocystous cyanobacteria, Dimension (Dim) 1 and 2 were the most important principal coordinates explaining 59.17% and 19.33% variation. In Dim1, width of heterocyst contributed maximum variation while in dimension 2, length of vegetative cell had the major contribution.

Tab. 1. Final cluster prototypes for heterocystous and non-heterocystous cyanobacteria. LV – length of vegetative cell, WV – width of vegetative cell, LH – length of heterocyst, WH – width of heterocyst, NA – not applicable.

I. Heterocystous cyanobacteria					
	LV	WV	LH	WH	Remarks
Cluster 1	7.167261	5.36375	7.61384	6.494746	Large vegetative cells and medium sized heterocysts
Cluster 2	4.956879	4.76203	9.432821	6.941643	Small to medium sized vegetative cells and large heterocysts
Cluster 3	4.531235	4.297334	4.943985	4.835359	Small vegetative cells and small heterocysts
II. Non-heterocystous cyanobacteria					
Cluster 1	3.507888	2.794574	NA	NA	Large vegetative cells
Cluster 2	9.937472	8.983922	NA	NA	Small to medium sized vegetative cells
Cluster 3	5.76292	2.515487	NA	NA	Small vegetative cells

was placed in Cluster 2) were placed in Cluster 3. However, two isolates of *A. laxa* K24, *A. laxa* K39, *Nostoc* sp. K68 showed probabilities of being placed in Cluster 2 (On-line Suppl. Tab. 2). Isolates placed in Cluster 1 showed close proximity among themselves while isolates placed in Cluster 2 and 3 showed high variability. The results related to cluster prototype presented in Tab. 1 showed that clusters had significant variations with respect to cellular dimensions among themselves indicating high inter-generic variations as clustering was related to their generic identity.

In the case of non-heterocystous cyanobacteria also, three clusters were formed where Cluster 2 contained only one isolate *L. hieronymusii* K95 (Fig. 1B). Dunn's fuzziness coefficient (0.796) and fuzzy silhouette index (0.684) showed the good fit of the clustering; however, the fitness indices also indicated that a few of the isolates could be clustered in more than one group. Isolates of *Desertifilum* sp., *Lyngbya* sp. and *Phormidium* sp. grouped both in Cluster 1 and Cluster 3 while *Alkalinema pantanalense* K25 and isolates of *Halomicronema* sp. were placed exclusively in Cluster 1 and

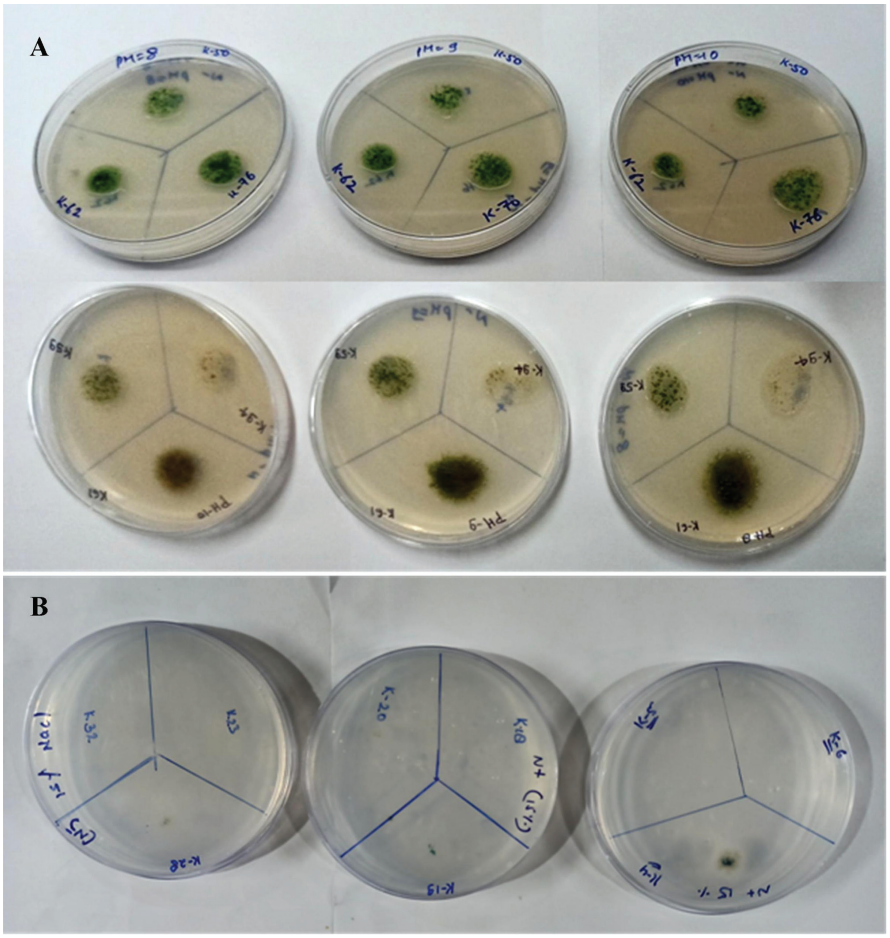

Fig. 2. Representative image depicting growth of heterocystous/non-heterocystous cyanobacteria on BG11 N^+/N^- agar plate amended to different alkalinity (i.e., pH 8, 9 and 10) (A) and salinity (i.e., 5, 10 and 15% NaCl concentration) (B).

Cluster 3 respectively. *Halomicronema* sp. K70 had ~32% probability of being placed in Cluster 1 while *Desertifilum* sp. K18 had ~27% probability of being grouped in Cluster 3. *Lyngbya wollei* K80 had ~ 26% probability of being placed in both Cluster 2 and Cluster 3. Similarly, *Halomicronema* sp. K40 had 50% probability of being grouped in both Cluster 1 and Cluster 3 (On-line Suppl. Tab. 3). For non-heterocystous cyanobacteria also, clustering showed a high degree of both inter- and intra-generic variability with respect to cell dimensions (Tab. 1).

Physiological characterization of isolates for tolerance to salinity and alkalinity

All cyanobacterial isolates except *Lyngbya aestuarii* K97 (tolerated pH 8-9) showed tolerance to pH ranging from 8-10 (Fig. 2A). However, great variation was observed in the case of salinity tolerance (Fig. 2B). Among the non-heterocystous cyanobacteria, isolates K4, K18, K19 of *Desertifilum* sp. and *Phormidium* sp. K30 showed tolerance to 15% NaCl (w/v). Only four isolates viz. *Halomicronema* sp. K79, *Desertifilum* sp. K20, *L. aestuarii* K97 and *Leptolyngbya* sp. K72 showed tolerance to 5% NaCl. Among the heterocystous cyanobacteria, *A. laxa* K24, *Hapalosiphon* sp. K28, *Hapalosiphon* sp. K62 and *Scytonema* sp. K55 showed tolerance to 15% NaCl. 16 heterocystous isolates could tolerate NaCl only to 5% while 3 isolates showed tolerance to NaCl only up to 10%.

Cluster analyses of the non-heterocystous isolates showed (Fig. 3A) three distinct clusters viz. a) high salinity & high alkalinity tolerant (04 isolates); b) Low salinity & high alkalinity tolerant (04 isolates) and c) only high alkalinity tolerant (09 isolates). On the other hand, heterocystous cyanobacteria showed (Fig. 3B) four distinct clusters viz. a) high salinity & high alkalinity tolerant (04 isolates); b) moderately high salinity & high alkalinity (03 isolates); c) low salinity & low alkalinity tolerant (09 isolates) and d) only high alkalinity tolerant (14 isolates).

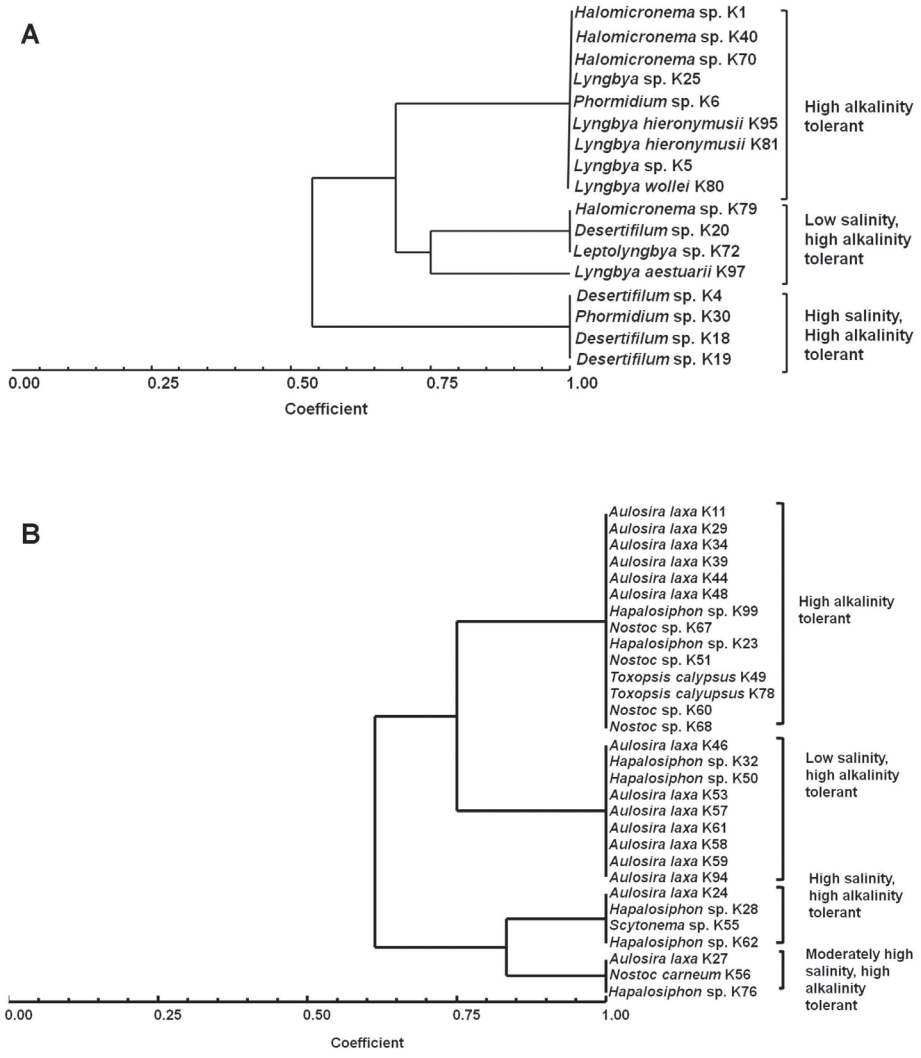

Fig. 3. Dendrogram representing the clustering of filamentous cyanobacteria based on their ability to tolerate variable levels of alkalinity and salinity. A – clustering of non-heterocystous cyanobacteria; B – clustering of heterocystous cyanobacteria. The heterocystous cyanobacteria were clustered in three distinct groups: high alkalinity tolerant; low salinity and high alkalinity tolerant; and high salinity and high alkalinity tolerant. The non-heterocystous cyanobacteria were clustered in four distinct groups: high alkalinity tolerant; low salinity and high alkalinity tolerant; high salinity and high alkalinity tolerant; and moderately high salinity and high alkalinity tolerant.

HIP fingerprinting

All the three primer combinations HIP AT + HIP TG; HIP AT + HIP GC and HIP TG + HIP GC showed 100% polymorphism (Tab. 2, Fig. 4A-C). HIP AT + HIP TG produced a total of 357 fragments ranging from 170-2156 bp. HIP AT + HIP GC set produced 379 fragments ranging from 157-2129 bp. 269 fragments ranging from 103 bp to 2547 bp were generated by HIP TG + HIP GC. For the purpose of scoring, only the fragments ranging from 300-3000 bp were used.

Genetic diversity analyses

Genetic diversity analyses for heterocystous and non-heterocystous cyanobacteria were carried out separately. Genetic similarity among the 17 non-heterocystous cyanobacteria range from ~13-90% with two major clusters. The major cluster 1 comprised all isolates of *Halomicronema*, *Leptolyngbya* sp. K72, *L. wollei* K80, *L. hieronymusii* K81 and K95, *Lyngbya* sp. K5, *Phormidium* sp. K30 and *A. pantanalense* K25 (Fig. 5A). On the other hand, major cluster 2 contained all isolates of *Desertifilum*, *Phormidium* sp. K6,

Tab. 2. Size range of the PCR products generated by three primer combinations.

Sl. No.	Primer combination	Total no. of fragments	% polymorphic bands	Size range (bp)
1.	HIP AT + HIP TG	357	100	170 - 2156
2.	HIP AT + HIP GC	379	100	157 - 2129
3.	HIP TG + HIP GC	269	100	103 - 2547

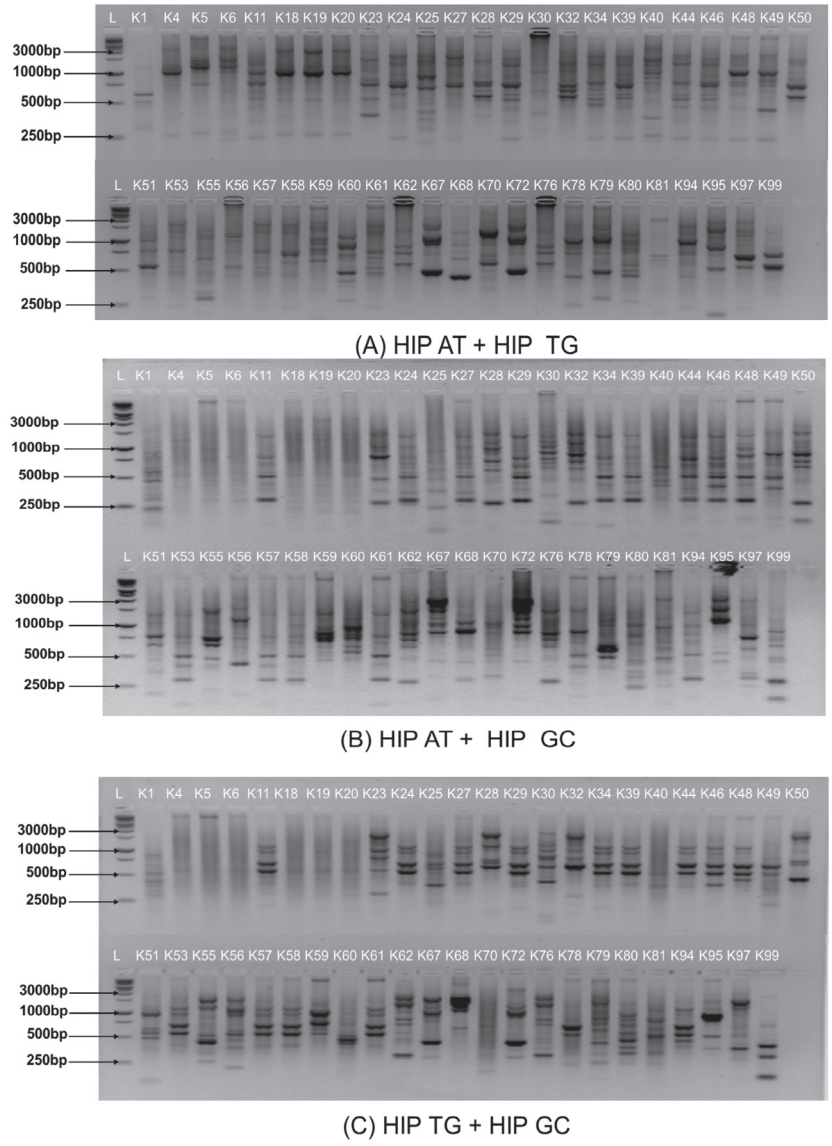

Fig. 4. Gel photograph presenting the HIP (Highly Iterated Palindrome) element based fingerprint of the forty-seven different filamentous heterocystous and non-heterocystous cyanobacterial isolates generated using dual primer combinations: A – HIP AT + HIP TG, B – HIP AT + HIP GC, C – HIP TG + HIP GC. L: 1kb DNA ladder (Promega, G571A).

and *L. aestuarii* K97. *Desertifilum* sp. K18 and K19 showed ~90% similarity indicating that they may be different isolates of the same species. The fitness of the clustering was found to be good (r = 0.87).

Among the 30 heterocystous cyanobacteria, the genetic similarity ranged from ~12-76%. Two major clusters viz. cluster 1 with 29 cyanobacteria and cluster 2 with only *T. calypsus* K49 were observed (Fig. 5B). In the major cluster 1, all 15 isolates of *Aulosira* and 7 isolates of *Hapalosiphon* formed separate sub-clusters. On the other hand, *Nostoc* spp. showed clustering with *Hapalosiphon* (*Nostoc* sp. K51, *N. carneum* K56, and *Nostoc* sp. K68) as well as *Scytonema* sp. K55 (*Nostoc* sp. K67 and *Nostoc* sp. K60). Among the *A. laxa* isolates clustered together, the genetic similarity ranged from 26-76% while the same was found in a range of 31-57% in the case of *Hapalosiphon* sp. isolates. The degree of fitness of clustering was good (r = 0.89). The result clearly showed a very high intra-generic diversity among the studied filamentous heterocystous cyanobacteria.

In the majority of the cases the clustering of both non-heterocystous and heterocystous cyanobacteria was supported by significant bootstrap values (Fig. 5). More than 70% bootstrap values correspond to > 95% probability that true phylogeny have been found (Hillis and Bull 1993).

Discussion

Filamentous cyanobacteria like *Nostoc*, *Anabaena*, *Phormidium*, *Microcoleus*, *Lyngbya*, *Calothrix* etc. are frequently encountered in BSC (Garcia-Pichel et al. 2001, Zhang 2005). However filamentous cyanobacteria like *Nostoc, Anabaena, Calothrix, Microcoleus, Hapalosiphon, Cylindrospermum, Scytonema, Aulosira, Phormidium* and *Oscillatoria* have been reported to be present not only in BSCs from arid and semi-arid areas but also in alkaline and saline soils (Pandey et al. 2005, Srivastava et al. 2009). The predominance of heterocystous or non-heterocystous cyanobacteria is reported to be governed by factors like pH,

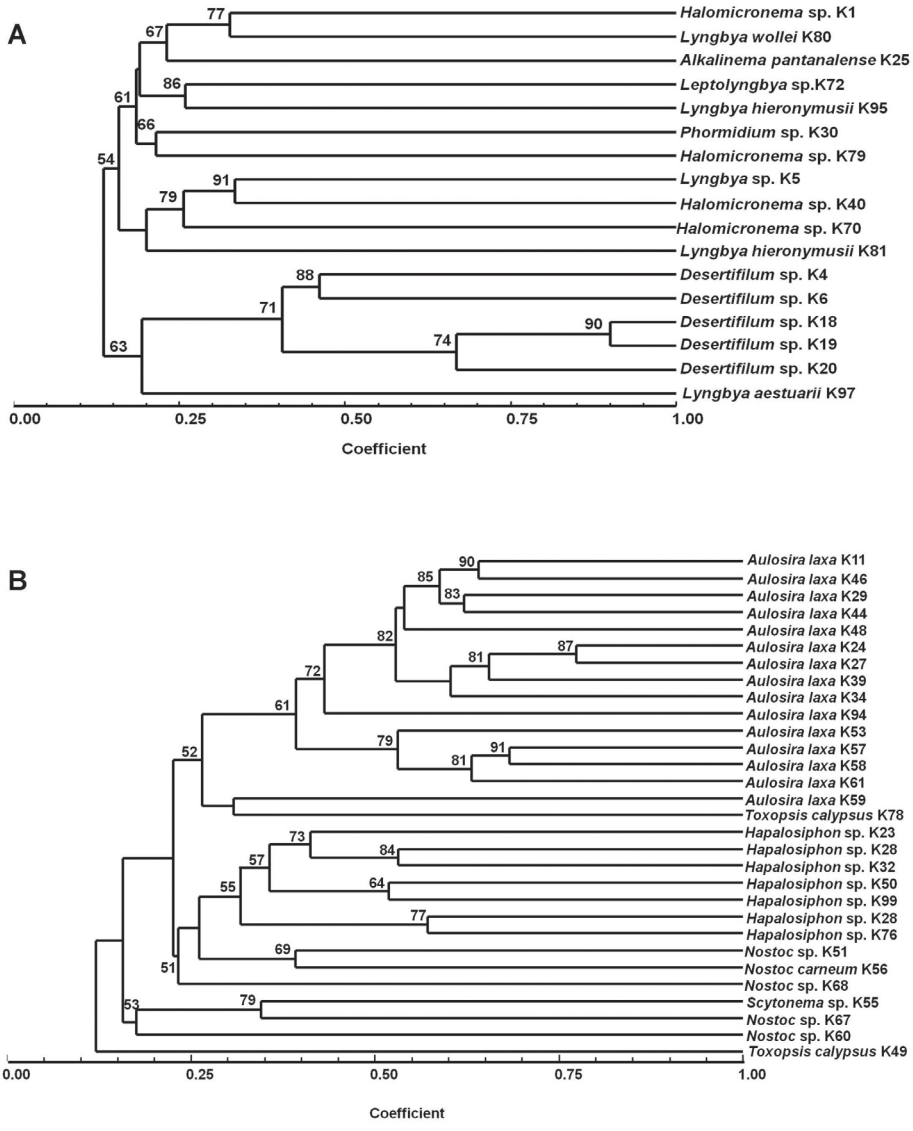

Fig. 5. Dendrogram depicting genetic diversity based on multiplex HIP fingerprinting: A – non-heterocystous cyanobacteria, B – heterocystous cyanobacteria. The values at the nodes are bootstrap values. Bootstrap values > 50 have been presented in the dendrograms.

salinity or nitrogen availability (Srivastava et al. 2009). Many of such filamentous cyanobacteria have been used for the amelioration of salinity stress in various crops, but it is noteworthy that the majority of these cyanobacteria were from four genera viz. *Anabaena*, *Nostoc*, *Hapalosiphon* and *Calothrix* (Li et al. 2019).

Cluster analyses of the 47 filamentous cyanobacteria based on cellular dimensions and tolerance to salinity and alkalinity showed high variability among the isolates. Among the heterocystous cyanobacteria, significant morphological variability was observed within the members of the genus *Hapalosiphon* while the other genera like *Aulosira* or *Nostoc* had limited intra-generic variability. In case of non-heterocystous cyanobacteria, inter- and intra-generic morphological variability was higher as all the clusters contained isolates belonging to different genera. Among non-heterocystous cyanobacteria, isolates of *Lyngbya* showed higher morphological variations. Initial classifications of cyanobacteria were solely based on morphology and still an important criterion for identification. However, many times their variation with environmental conditions makes them unsuitable for correct identification. Very few studies, like Mishra et al. (2015), reported that morphological attributes like trichome aggregation, heterocyst shape and akinete shape are stable features and could be used for identification. The results of the present study indicated that dimensions of vegetative cells and heterocysts can be effective for identification and differentiation of cyanobacteria belonging to *Hapalosiphon* and *Lyngbya*. This observation is in compliance with the distinction of various species of *Hapalosiphon* (like *H. welwitschii* West & G.S.West, *H. delicatulus* West & G.S.West, *H. intricatus* West & G.S.West, *H. pumilus* Kirchner ex Bornet & Flahault, etc.) and *Lyngby*a (like *L. chaetomorphae* Iyengar & Desikachary, *L. lachneri* (W.Zimmermann) Geitler, *L. infixa* Frémy, *L. baculum* Gomont, etc.) based on dimensions of filaments or trichomes (Desikachary 1959). Looking at the advances in computational techniques and the development of robust algorithms, it does not seem to be really impossible to use morphological information for understanding actual biological diversity. Heterocystous cyanobacteria showed high intra-generic variability in the case of salinity while all isolates were highly alkali-tolerant. Isolates of *Aulosira* and *Hapalosiphon* were found to have variable tolerance to salinity. However, the variability of salinity tolerance was lower in non-heterocystous than in heterocystous cyanobacteria. Growth and colonization of cyanobacteria are known to be greatly influenced by soil pH and salinity (Pandey et al. 2005, Nayak and Prasanna 2007). Cyanobacterial growth is favoured under neutral to alkaline conditions while acidic conditions may limit the growth of many cyanobacteria (Šesták 2001). In the present study, all the isolates of cyanobacteria studied could also tolerate up to pH 10. It was been reported that the metabolic trade-off between ionic balance and heterocyst formation or diazotrophy may limit the proliferation of heterocystous cyanobacteria under highly saline conditions (Vitousek et al. 2002, Berman-Frank et al. 2003). Srivastava et al. (2009) reported that low salinity favoured the presence of heterocystous cyanobacteria while highly saline soils predominantly harboured non-heterocystous cyanobacteria. Kirkwood et al. (2008) reported that heterocystous cyanobacteria could still persist under saline conditions although it might be suboptimal for growth. As observed in the present study, the varying tolerance of heterocystous cyanobacteria to salinity might be an important fitness trait to proliferate under saline/alkaline soils. Consistently with the reports of Srivastava et al. (2009), *Aulosira* turned out to be highly salinity-adapted (5-15%) heterocystous cyanobacteria. All the isolates of *Desertifilum* sp. showed higher salinity tolerance than the other non-heterocystous cyanobacteria. The salinity tolerance exhibited by the *Desertifilum* isolates in the present study are much beyond the tolerance limit (3%) of all the reported species of the genus *Desertifilum* (Dadheech et al. 2014, Cai et al. 2017).

HIP fingerprinting of heterocystous revealed higher genetic variability than in non-heterocystous cyanobacteria. Clustering of non-heterocystous cyanobacteria based on HIP fingerprinting also showed high intra- and inter-generic variability. Despite being the most commonly used molecular marker for understanding phylogeny and describing novel prokaryotic taxa, the *16S rRNA* gene often cannot resolve intra-generic diversity (Woese 1987, Tindall et al. 2010). Although *16S rRNA* gene contains informative hypervariable regions, it does not have enough divergence to resolve the differences among the closely related members of a genus (Fox et al. 1992, Drugă et al. 2013). Repetitive elements like HIP can be very effective for such purposes due to their abundance throughout the cyanobacterial genomes (Xu et al. 2018). Earlier studies showed that molecular tools like RAPD and HIP fingerprinting can effectively resolve the intra-generic diversity of cyanobacteria like *Nostoc*, *Anabaena*, *Hapalosiphon, Calothrix* etc. (Chakdar and Pabbi 2012, Shukla et al. 2013, Singh et al. 2014). In the present study, the isolates of *A. laxa*, *Hapalosiphon* sp. and *Nostoc* sp. showed distinct clustering with high intra-generic variability indicating that multiplex HIP fingerprinting could effectively differentiate among closely related members of these genera. In the case of non-heterocystous cyanobacteria like *Phormidium* and *Lyngbya 16S rRNA* genes have been reported to be insufficient for intra-generic taxonomic resolution (Marquardt and Palinska 2007, Engene et al. 2010). In the present study, *Lyngbya* was also found to be genetically highly heterogeneous and HIP fingerprinting could distinguish between two *L. hieronymusii* isolates. Furthermore, HIP fingerprinting could effectively distinguish the isolates belonging to *Halomicronema* and *Desertifilum*.

Conclusion

The results of the present study showed the importance of morphological, physiological and genetic analyses to understand the diversity of filamentous cyanobacteria. All these analyses can supplement each other to provide a bet-

ter understanding of the population structure and ecology of these cyanobacteria. The findings indicated that the variation in salinity tolerance of filamentous cyanobacteria along with their inherent alkali tolerance helped to proliferate a heterogeneous population of both heterocystous and non-heterocystous cyanobacteria in saline/alkaline soils. With great genetic and physiological diversity, such cyanobacteria can be a potential biological resource for the reclamation of such degraded soils. However, further in-depth studies are required to understand their actual genetic and physiological potential.

Author contribution statement

H.C. contributed in conceptualization of the work. S.V. carried out the experiments and validated the study. R.V. carried out the morphometric characterization. A.B. contributed to the statistical analysis. S.V. and H.C. investigated the study and prepared the original draft. H.C., S.V., S.Y.B., N.S. and V.M. contributed in writing-reviewing the draft. H.C. and A.K.S. supervised the study and edited the final draft and gave final approval for the publication of this version. All authors read and approved the final manuscript.

Availability of data and material

Gene sequences are available in NCBI, Cultures are available in National Agriculturally Important Microbial Culture Collection (NAIMCC) at ICAR-NBAIM, Mau, Uttar Pradesh, India and other information are available with the corresponding author.

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