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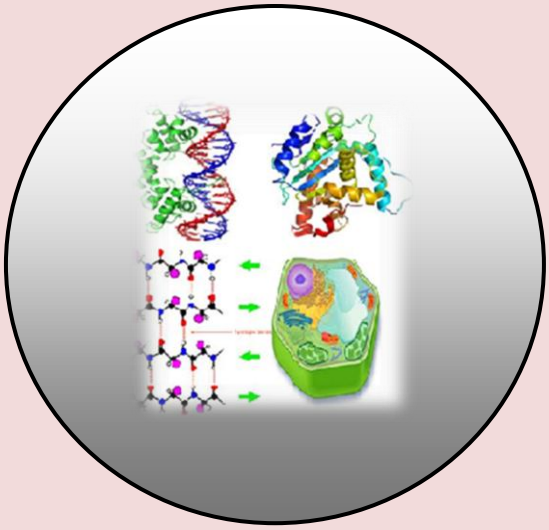

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RESEARCH PAPER

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Isolation and Molecular Characterization of Blue-Green Algae (Cyanobacteria) from Drang Salt Mine Mandi, Himachal Pradesh, India

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ABSTRACT

In the present studies cyanobacterial species were isolated from agricultural fields adjoining the Drang salt mine, District Mandi, Himachal Pradesh following standard culturing and purification techniques. The species were identified morphologically as well as molecularly. Three cyanobacterial species were Leptolyngbya boryana, Cylindrospermum muscicola and Phormidium sp. DNA extractions were made from isolated cells and then extracted DNA were amplified by using PCR. Cyanobacteria specific primers were used to amplify 16S rRNA. Phylogenetic analyses were done by using computer software. According to species identification by sequence analysis, it was observed that molecular data supports morphological systematics.

Keywords: Cyanobacteria, Salt mine, 16S rRNA and Molecular characterization.

INTRODUCTION

Cyanobacteria are unique gram negative, unicellular and multicellular photosynthetic autotrophs (Singh et al., 2011). Their main photosynthetic pigments are chlorophyll a, carotenoids (carotenes and xanthophylls) along with phycobili proteins and phycocyanins. The presence of these phycobili proteins and mucilage, the colour of cyanobacteria may range from dirty yellow to various shades of blue green color. Phycoerythrin pigments may also be present in some cyanobacterial species which imparts red colourization (Altuner, 2010). Some members are also endowed with specialized cells known as heterocyst - thick walled modified cells, which are the site of nitrogen fixation by nitrogenase enzyme (Singh et al., 2011). Morphological characters are basis for identification and classification of cyanobacteria. However, these characters may be changed according to different environmental conditions.

Selecting elective culture conditions may help in inhibiting strain diversity in cultures. The above reasons make it necessary to develop the molecular techniques for identification of cyanobacteria (Lyra et al., 2001).

Identification of microorganisms and evaluation of relationship among them is done by using marker gene 16S rRNA gene. 16S rRNA gene contains many species specific evolutionary conserved sequences. The identification at species level is possible by using PCR for amplification of required sequence. Molecular identification of *Geitlerinema* strain PCC 9452 (*Microcoleus* species strain 10 MFX) and *Oscillatoria limnetica* have been shown by amplification of 16S rRNA genes (Boyer et al., 2001). *Cylindrospermum* was differentiated from Nostocaceae family because of the presence of terminal heterocyst and paraheterocystic akinetes. 45 taxa of this genus were identified (Guiry and Guiry, 2014, Johnsen et al., 2014). The present study is aimed to isolate, purify and identify cyanobacteria using molecular techniques from Drang salt mine site of District Mandi, Himachal Pradesh, India.

MATERIAL AND METHODS

Sample Collection

Soil samples were collected from Drang salt mine area. Samples were collected from mining site in the summer season in the month of May. Different locations were selected and samples were collected for each site. A sterilized trowel (70 % alcohol) was used for scrapping the surface soil. The soil was diged up to different depths i.e. 10 cm, 15 cm, 20 cm below the surface. The samples were collected and transferred to laboratory in a good quality, air tight and clean plastic bags for analysis. The soil was cleaned, air dried, crushed, homogenized and sieved, and then stored in air tight bags for analysis.

Cyanobacteria Isolation

Indigenous cyanobacterial strains were isolated from native soils of Drang salt mine and surrounding area using BG-11 medium. All the glass wares were cleaned thoroughly, dried and sterilized in hot air oven at 180 ^{O}C for six hours before use. The pH was adjusted to 7.5 before autoclaving the medium. The medium used for algal isolation was solidified using 1.5 % agar. The medium was sterilized at 15 lb/in pressure for 15 minutes in an autoclave. The autoclaved medium was cooled down to 45-47 ^{O}C to reduce condensation of moisture during solidification of agar. 30 ml of this medium was poured in petri dishes, pre-sterilized at 180 ^{O}C taking precautions to avoid contamination by whipping the moisture from outside the flask and keeping the flask in flame while pouring the medium. After pouring the agar medium the petri dishes were allowed to cool down so that the medium got solidified. Serial dilution was done. From each dilution series, 0.1 ml of the diluted suspension was inoculated into petri plates containing the agar medium. These poured plates with their bottom sides up were incubated at 27±3 ^{O}C under continuous illumination using cool white fluorescent light (3000 lux) for ten days. After ten days the algal colonies developed were isolated and identified by preparing temporary mounts following (Desikachary, 1959). The species going to be isolated were purified by repeated procedures of streaking and culturing until only one strain remained.

Molecular Identification

DNA Extraction and Amplification

The genomic DNA from pure isolates was extracted according to (Fawley and Fawley 2004). Consensus primers were used to amplify 600 bp 16S rDNA using Taq DNA Polymerase. The PCR reaction mix (50 microliter) consist of 100ng of each primer, 1.0 µl of dNTP mix (2.5mM each), 1X of Taq Buffer A (10X), 3 units of Taq Polymerase enzyme, 20ng of genomic DNA and the final volume was adjusted to 50 µl with double distilled water. The PCR thermocycler was used to amplify the reaction comprises of 94 ^{O}C for 5 min followed by 35 cycles at 94 ^{O}C for 30 sec, 55 ^{O}C 30 sec and annealing temperature for 1 min at 72 ^{O}C followed by final extension temperature at 72 ^{O}C for 7 min. this amplified PCR product was stored at -20 ^{O}C for further use. 5 µl of PCR amplified solution was analyzed on 1 % agrose gel along with step up ^{TM} 500 bp DNA ladder and visualized under UV trans illuminator. DNA fragments were purified using PCR purifying kit.

Sequencing and Phylogeny

The amplified DNA was submitted for sequencing to Genei Laboratories Private limited (Bengaluru, Karnataka, India). Obtained sequences were submitted at US National Centre for Biotechnology Information (NCBI) database for BLAST matching. The sequence data was aligned and analyzed for finding the closest homologous microbes. Construction of phylogenetic tree was done using clustral Omega tool and MEGA 6 software.

RESULTS

Morphological Observations

According to morphological characters observed under light microscope three different species were observed. These species belong to 3 different orders viz Synechococcales, Nostocales and Oscillatoriales of class cyanophyceae. In order Synechococcales, *Leptolyngbya boryana* was observed and in Oscillatoriales, *Phormidium* sp. was observed while in Nostocales *Cylindrospermum muscicola* was observed. The identifying features of the observed species are summarized below.

***Leptolyngbya boryana*:** Filament is green in colour. Apical cells are round and blunt without calyptra. Heterocysts are absent. Akinetes are also absent. Trichome single in firm sheath, mostly colourless, yellow to brown in colour (Figure 1a).

***Cylindrospermum muscicola*:** Thallus is mucilagenous, dark green in colour. Cells are long and cylindrical. Heterocysts are oblong with nodules at both the constrictions and may be terminal or intercalary. Spores are oval in shape and brown coloured. Epispore is smooth (Figure 1b).

***Phormidium* sp.:** Filament is dark green in colour. Filament is straight and granulated at the joints. Cells are broader than longer. Apical cell is longer with hyaline and colorless sheath. Floccose biomass (Figure 1c).

Sequence and Phylogenetic Analysis

The phylogenetic tree obtained by using neighbor joining method from analysis of 16S rRNA sequence showed the distinction of genus at high accuracy (Figure 2). The species morphologically identified in our study was placed in expected correct clades by this tree. Genbank accession numbers of cyanobacteria species are shown in Table 3.

DISCUSSION

Since 19th century, taxonomy and classification of cyanobacteria species have been done according to their cell morphology. However, the species identified on the basis of morphological traits are not always correct enough and various genus need major revisions. Recently researchers are focusing on the importance of molecular characterization of cyanobacteria for reliable results (Wehr and Sheath, 2003, Hasler et al., 2012). Recent approaches on taxonomy of cyanobacteria are multidisciplinary which includes combination of morphology and ecology coupled with molecular characterization. Therefore, the species which are identified by molecular methods must be in accordance with phenotypic and ecological data (Hoffman et al., 2005, Komarek et al., 2010, Komarkova et al., 2010). Cyanobacteria species *Leptolyngbya boryana, Cylindrospermum muscicola* and *Phormidium* sp. have been taxonomically identified using both morphological and molecular characterizations. The genus and respective species of these cyanobacteria are mistaken morphologically to each other but it was clear from molecular characterization using 16S rRNA gene sequencing (Hasler et al., 2012). For exploring phylogenetic relationships among cyanobacteria, analysis of the 16S rRNA gene sequence has proved a better method among molecular methods (Zepomelova et al., 2010). Molecular characterization of cyanobacteria isolated from rice fields of Uttar Pradesh has been investigated (Srivastava et al., 2009) and observed that six PCR fragments (*A. doliolum, A. anomala, A. oryzae* and *A. variabilis)* showed high similarity with *Anabaena* (96-99 %), four with *Nostoc* (94-98 %), two with *Aulosira* (95 % each), *Cylindrospermum* (95-98 %), *Gloeotrichia* (94-98%), *Hapalosiphon* (94-96 %) and *Fischerella* (87 %). Marquardt et al. (2007) have done molecular characterization of 30 strains of cyanobacterial genus *Phormidium* and analyzed 925 bp part of the 16s rRNA gene and internal transcribed spacer (ITS) and yielded three products.

Molecular study of 72 filamentous, heterocystous cyanobacterial strains isolated from Loktak lake, Manipur belonged to *Nostoc*, *Anabaena, Calothrix, Cylindrospermum* and *Mastigocladus* genera. A polyphasic approach was used for characterization which was based on morphological features and PCR based polymorphism which revealed tremendous level of diversity. Three primer targeted regions were used for genetic polymorphism determination out of these short tandemly repeated repetitive STRR1A gave best fingerprint profile of cyanobacterial strains. This profile was used for molecular characterization (Akoijam and Singh, 2011). Johnsen et al. (2014) have studied 26 strains of *Cylindrospermum* using molecular methods using sequence data for 16S rRNA gene and 16S-23S internal transcribed spacer (ITS) region. Phylogenetic analysis revealed three different clades. The clades contained five fundamental species: *C. majus, C. stagnale, C. licheniforme, C. muscicola* and *C. catenatum.* Three other new species were *C. badium, C. moravicum* and *C. pellucidum*. Authors indicated from their investigations that *Cronbergia* is the later synonym of *Cylindrospermum.* Karan et al. (2017) have been conducted a pioneer study on isolation and molecular identification of some blue-green algae from fresh water sites in Tokat province of Turkey. Seven species: *Geitlerinema carotinosum, Nostoc linckia, Oscillatoria limnetica, Chroococcus minutus, Cylindrospermum* sp., *Phormidium* sp. and *Anabaena oryzae* were reported and identified by molecular methods.

Table 1. Conditions for amplification in a PCR.

Steps	Temperature (°C)	Duration (min.)
Initial denaturation	94	5:00
Denaturation	94	0:30
Annealing	55	0:30
Extension	72	1:00
Final extension	72	7:00

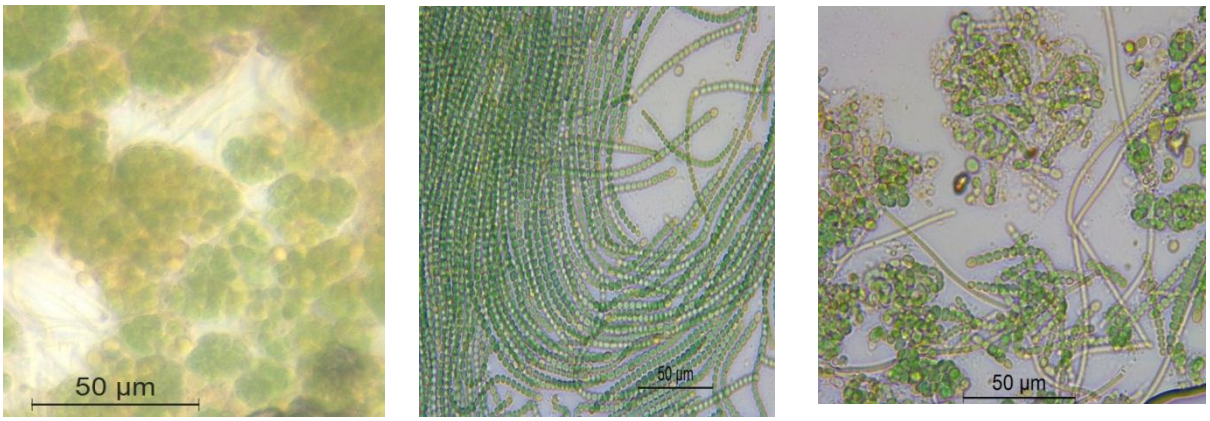
The PCR was carried out for a total of 35 cycles.

Table 2. The systematics information of purified cyanobacteria.

Class	Order	Family	Genus
Cyanophyceae	Synechococcales	Leptolyngbyaceae	*Leptolyngbya*
Cyanophyceae	Nostocales	Nostocaceae	*Cylindrospermum*
Cyanophyceae	Oscillatoriales	Phormidiaceae	*Phormidium*

Table 3. GenBank accession numbers of cyanobacteria species from 16S rRNA sequence.

Sr. No.	Accession number	Species	Strain
1.	MN736181	*Leptolyngbya boryana*	HPUSD11
2.	MN736180	*Cylindrospermum muscicola*	HPUSD12
3.	MN813958	*Phormidium* sp.	HPUSD13



a). b). c).

Figure 1. Light microscopy images of strains a.) *Leptolyngbya boryana* HPUSD11, b.) *Cylindrospermum muscicola* HPUSD12, c.) *Phormidium* sp. HPUSD13.

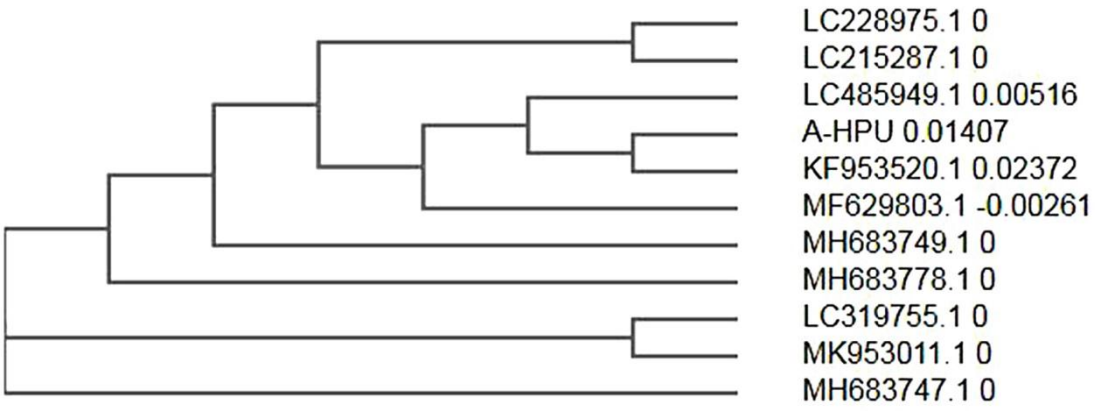


Figure 2. Phylogenetic tree made using neighbor joining method (*Leptolyngbya boryana* HPUSD11).

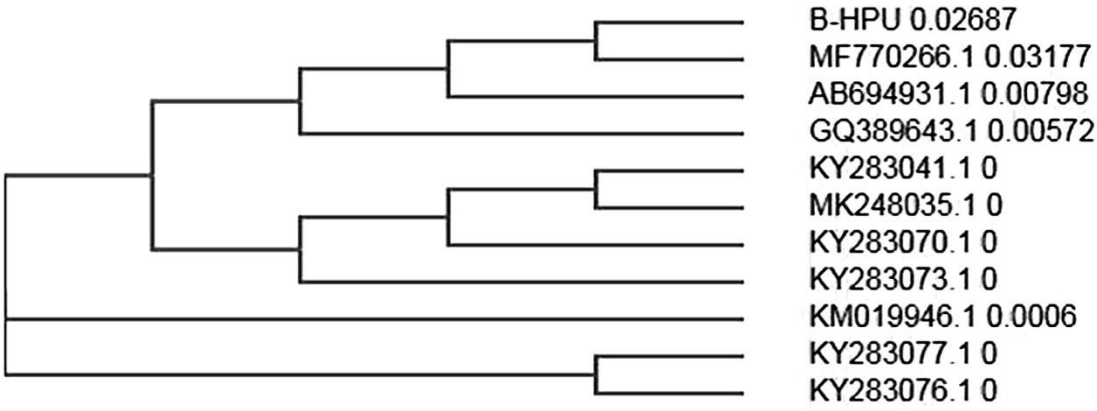


Figure 3. Phylogenetic tree made using neighbor joining method (*Cylindrospermum muscicola* HPUSD12).

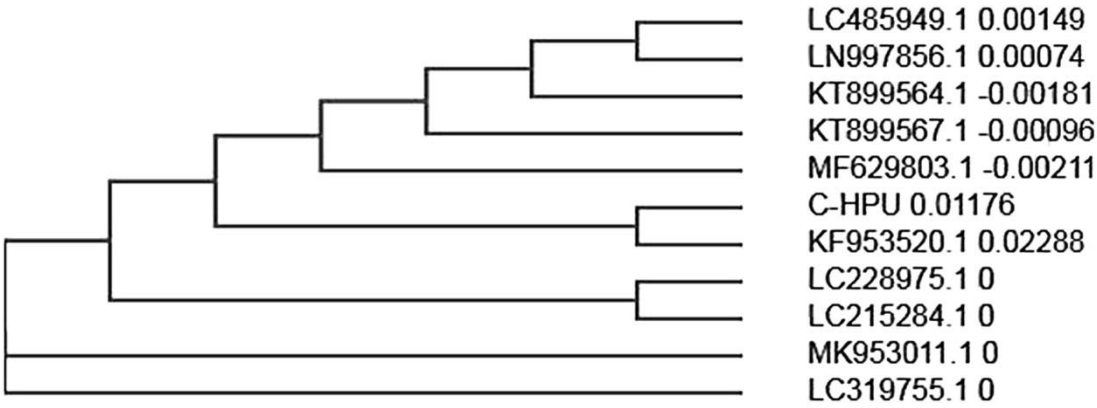


Figure 4. Phylogenetic tree made using Neighbor joining method (*Phormidium* sp. HPUSD13).

CONCLUSION

Our study is important in the way as it fills the gap of molecular characterization of cyanobacteria in literature. In our study isolation, identification and molecular characterization of cyanobacteria from salt mine site of District Mandi of Himachal Pradesh, India was done for the first time. Morphological systematics of the cyanobacterial species were conducted according to their morphological traits. Molecular data was obtained by using molecular characterization methods. In our study, molecular characterization results of species have supported morphological classification of three species. Molecular analysis and morphological description results were in harmony with each other. This may not always happen because sometimes morphological systematic results are not compatible with molecular studies. It may be little bit difficult in this situation to do systematic study. However, when doing systematic classification studies, it become necessary to do molecular characterization of the species for identification of suspicious data. We believe that our study is a valuable addition for identification and classification of cyanobacteria by combining multidisciplinary approaches.

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