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Validation of Selected Oscillatoriales from Various Indian Coasts through Phenetic and 16S rRNA Gene

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A B S T R A C T

K e y w o r d s Cyanobacteria; Oscillatoriales; Habitat; Morphology; phylogeny; 16S rRNA **Article Info** *Accepted:* 25 June 2016 *Available Online:* 10 July 2016	Eight strains of filamentous, non heterocystous cyanobacteria of order Oscillatoriales from diverse marine habitats belonging to coastal areas of India had been categorized using phenotypic and phylogenetic characteristics. The study included observations of morphological features under light microscope and the molecular phylogenetic analysis of the 16S rRNA gene. The phylogenetic relatedness of the investigated strains corroborated well with morphology based taxonomic groupings. The 16S rRNA gene analysis revealed distinct clusters and strengthens the results obtained by traditional morphological methods. A combination of genetic and phenotypic study resolved their taxonomic ambiguities and aids to the precise identification of the chosen filamentous strains.

Introduction

Cyanobacteria constitute one of the most ecologically diverse groups of organisms inhabiting marine, freshwater and terrestrial habitats (Pandey, 2013). Cyanobacteria are oxygenic photosynthetic prokaryotes with enormous morphological diversity and have developed wide ecological tolerance to temperature, light, salinity, alkalinity and UV irradiation (Peter *et al.*, 2015). Traditional classification of cyanobacteria was based on comparisons of morphological features (Geitler, 1932; Desikachary, 1959; Rippka *et al.*, 1979).

The variability in morphological topographies is an integral measure of the diversity and divergence processes in cyanobacteria of all ecosystems over the world (Komarek, 2010). Since several phenotypic features of many cyanobacteria are influenced by the different environmental or culture conditions, the taxonomic classification is extremely difficult (Komarek and Anagnostidis, 1998). A number of the morphological characters such as gas vesicles (Lehtimaki *et al.*, 2000), unicellular to filamentous transition (Shih *et al.*, 2013), mucilaginous colony formation

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(Rippka *et al*., 1979) was apparently arisen and/or been lost several times during the long-term laboratory cultivation, which complicates the identification of cyanobacteria. Thus, the major problem of morphology-based taxonomic assessment lies in their morphological plasticity and the limitation of light microscopy.

Consequently, the classification of cyanobacteria progressed from morphological approach to the most fascinating, updated phylogenetic approach ensuring the prompt identification and description of cyanobacterial taxa. The assessment of the phylogeny of organisms through gene sequence analysis has increased dramatically since the advent of PCR and automated sequencing.

Molecular (phylogenetic) data provide a basic criterion for taxonomic classification and had a great impact on every level of cyanobacterial taxonomy. A number of molecular marker genes have been used to delineate several cyanobacterial taxa. The 16S rRNA gene is an informational macromolecule that plays a central role in inferring phylogenetic relationships and in identification of cyanobacteria (Neilan *et al*., 1997; Nubel *et al*., 1997).

The 16S rRNA gene is reliable, extensively accepted, gold standard molecular marker because of its universal, structurally and functionally conserved nature (Neilan *et al*., 1997).

Currently, the vast availability of this gene sequences in the database aids to find closer relative phylogenetic species. Hence, the study is focused on both phenetic and phylogenetic approach of classification among the chosen filamentous cyanobacteria of order Oscillatoriales available in the National Facility for Marine Cyanobacteria (NFMC) to clarify their systematics.

Materials and Methods

Sources of organisms

Eight marine filamentous, non heterocystous strains isolated from coastal areas of India were selected for the phylogenetic relationship from the repository of the National Facility for Marine Cyanobacteria (NFMC), Bharathidasan University, Tiruchirappalli, Tamil Nadu, India.

Growth and maintenance

The axenic cultures of the chosen marine filamentous strains were maintained and grown in ASN III marine synthetic medium (Rippka *et al*., 1979) with the illumination of white fluorescent light (14:10 h light/dark) at an intensity 2.20 Wm^{-2}. During incubation, the cultures were frequently observed under the light microscope for mono- specificity of the cultures. Morphological assessments, DNA extraction were performed from the exponential growth phase cultures.

Morphological Identification

The morphology of cells and filaments was studied using Leica inverted microscope DMI 3000B and images were documented with Leica digital camera DFC425C. The cell dimensions were measured by Leica application suite software. The morphological identification of strains was based on description proposed by Desikachary (1959), Komarek and Anagnostidis (2005). Taxonomic designations of the studied strains were revalidated in accordance with information gathered during the research.

PCR amplification of 16S rRNA gene region

Total genomic DNA was extracted from the selected strains using the xanthogenate nucleic acid isolation protocol (Tillett and

Neilan, 2000). To generate the complete sequence of 16S rRNA, the gene was amplified by splitting it into three regions and amplified using different set of primers. Amplification of I region included the forward primer 16S1R-AGA GTT TGA TCC TGG CTC AG and reverse primer 16S740R- TCT ACGCAT TTCACCGCT AC described by Seo and Yokota (2003), II region included the cyanobacterial specific primer CYA-359 F-GGG GAA TTT TCC GCA ATG GG (forward) and equimolar ratio of reverse primer CYA-781 (A) R-GAC TAC TGG GGT ATC TAA TCC CAT T and CYA-781(B) R-GAC TAC AGG GGT ATC TAA TCC CTT T described by Nubel *et al* (1997) and III region included the forward primer CYA-359 F-GGG GAA TTT TCC GCA ATG GG (Nubel *et al*., 1997) and reverse primer 16S-1494 R-GTA CGG CTA CCT TGT TAC GAC (Tillet *et al*., 2001). Amplification was carried out in Eppendorf mastercycler Pro S using GoTaq colorless master mix (Promega, U.S), 0.3µM of each primer and 100 ng of template DNA in a 100 µl reaction. The cycling profile included an initial denaturation of 94 °C for 5 min followed by 30 cycles of denaturation at 94 ºC for 1 min, annealing (I region-55.5 °C, II region-60°C and III region-56.4°C for 1 min) and extension at 72 ºC for 1 min and a final extension at 72 ºC for 7 min. 10µl of the amplified products of expected fragments of corresponding regions were subjected to 1.5 % agarose gel electrophoresis stained with ethidium bromide are visualized under the UV trans-illuminator.

DNA sequencing

The amplified PCR products from 16S rRNA were purified using a QIAquick PCR purification kit (Qiagen, Hilden, Germany) to remove the excess primer and subsequently used as template in sequencing

reactions. The primers employed for PCR amplifications were used to determine the sequences on both strands using the DNA cycle sequencing reaction kit. The DNA sequences were obtained with an ABI 310 automated sequencer using the chain-termination method with big-dye terminators (Eurofins Genomics India PVT Ltd). For the sequence data, automated base calls were checked by manual inspection of the electropherograms of both forward and reverse sequences. The base call conflicts were resolved by alignment and comparison of both strands using DNA baser software v 4.3 (DNA baser). Assembled 16S rRNA gene sequences were submitted to *find chimera* program of the DECIPHER (Wright *et al*., 2012) which can be directly accessible through a webpage - http://decipher.cee.wisc.edu/FindChimeras.html.

Phylogenetic analysis

The 16S rRNA gene sequences of filamentous cyanobacteria chosen in this study were blasted against Genbank nucleotide sequences. The related representative Genbank sequences of each generic groups and the best match from Blastn analysis were chosen as the reference strains. Multiple sequence alignment was done by Clustal X (Thompson *et al*.,1997) with default parameters. The best-fitting nucleotide substitution model was fixed by testing 24 different models for each dataset in MEGA software version 6 (Tamura *et al*., 2013). Phylogenetic tree was generated from this alignment using maximum likelihood analysis in MEGA 6. Bayesian information criterion was employed to achieve the most appropriate substitution model for maximum likelihood. The substitution model used in phylogenetic analysis of 16S rRNA was Kimura 2 parameter model. Gaps and missing data were treated as complete

deletion. In all the cases, bootstrap resampling using 1000 replications was performed to assess the statistical significance of the tree branches.

Results and Discussion

Morphological Identification

The taxonomy of oscillatorian taxa is challenging due to the lack of conspicuous traits (Anagnostidis and Komarek, 1988; Komarek and Anagnostidis, 2005). Eight filamentous strains of Oscillatoriales from the repository belonging to coastal regions of India were identified morphologically and categorized under the genera as *Spirulina*, *Oxynema*, *Phormidium* and *Pseudanabaena* (Komarek and Anagnostidis 2005; Chatchawan *et al*., 2012). The geographical origins and habitat of the organisms applied in this study are listed in Table 1 and Fig. 1 depicts their light micro photographs.

The genus *Spirulina* are most frequently assigned to one of a few species, *Spirulina subsalsa* Oersted, *Spirulina labyrinthiformis* Gomont and *Spirulina major* Kutzing based on trichome diameter and coil shape regardless of their habitat of origin. Subsequently, halotolerant, euryhaline *Spirulina* sp. with tightly coiled trichomes from hypersaline waters was reclassified as *Halospirulina tapeticola* (Nubel *et al*., 2000). The strain BDU 141021 isolated from stones of Marina Park and water sports complex of Andaman Island was initially assigned as *Spirulina subsalsa*.

The traditional identification approach of the genera *Oscillatoria/Phormidium/Lyngbya* is mainly established on the occurrence of sheaths and does not concur with molecular data; therefore it has to be drastically re-evaluated (Komarek, 2003). The main morphological and taxonomic markers of

species belonging to the genus *Phormidium* used are: the shapes of cells and filaments, types of end cells and facultative presence of sheaths. The taxonomy of *Phormidium* is still unclear (Chatchawan *et al*., 2012), and therefore it's separation into eight groups based on modern criteria was proposed by Komarek and Anagnostidis (2005) and they had validly described 163 species of *Phormidium* which are reclassified and relocated from the old traditional genera *Oscillatoria* and *Lyngbya*, based on various morphological characters. *Oxynema* is a new genus separated from the traditional *Oscillatoria* and *Phormidium* genera attributing by the main distinguishing features of cylindrical trichomes growing often in fasciculate mats, narrowed ends with bent, elongated and distinctly pointed terminal cells, without calyptra. The sheaths are lacking or facultative and diffluent (Chatchawan *et al*., 2012). Of the eight strains, four strains namely BDU 10251, 70493, 92071and 120161 exhibited rather uniform basic morphological characters and were adjudged to the genus *Oxynema*.

According to the conventional approach, strain BDU 46241 of the present study was determined previously as *Phormidium tenue* (Desikachary 1959). The characteristic morphological features observed in BDU 46241 strain were pinkish to brownish red thallus, trichomes straight or somewhat flexous or attenuated towards the apex, 1-2 μm wide, 2.5-5μm long, slightly constricted at the ungranulated cross walls, apical cells rounded or –conical-rounded bent acute without calyptras. A large number of oscillatorialean species with trichomes up to 3 μm wide exhibiting little or no motility of the Geitlerian genera: *Phormidium, Plectonema* and *Lyngbya* was included and created into a new genus to *Leptolyngbya* (Anagnostidis and Komarek, 1988; Marquardt and palinska, 2007). Thus,

Phormidium tenue had been revalidated as *Leptolyngbya tenuis* mainly based on their trichome size (Komarek and Anagnostidis, 2005).

Two BDU strains 41121 and 70492 were originally determined as *Oscillatoria minnesotensis* according to the classification system of Desikachary (1959). The characteristic key features defined for *Oscillatoria minnesotensis* were 2-5 µm width; 2-4 µm long homogeneous cell content, apical cell rounded and *Pseudanabaena catenata* were 2µm broad; 3 µm long, cells cylindrical, truncated at both ends, blue green, sometimes somewhat brownish (Desikachary, 1959). According to Komarek and Anagnostidis (2005), the morphological features described for *Pseudanabaena catenata* are cells cylindrical truncate at both ends, 2-6 µm long; cell content homogeneous or clearly differentiated in chromatic and centroplasma without aerotopes (or facultatively with apical gas vesicles and aerotopes; apical cell rounded or slightly conical truncated, without aerotopes; trichome straight, rarely

variously bent, flexible, 1.2-2(2.2) µm wide, strongly constricted at the thickened, hyaline, translucent and ungranulated cross-walls, not attenuated at the ends. In present study based advanced light microscopic analysis of both BDU 41121 and 70492 bedelineate clearly that the strains possess cylindrical cells of similar size truncated at both end of cells with granular cell content, clearly differentiated chromatic and centroplasma make it fit to characteristic feature of *Pseudanabaena sp.* In order to overcome some of these problems and to permit identification of cyanobacteria at the genetic level, phylogenetic analysis of 16S rRNA gene sequence have been applied to confirm their generic entity of the investigated strains.

Nucleotide sequences and accession numbers

The 16S rRNA gene sequences obtained from the present study were submitted to Genbank and their accession numbers were KX452360–KX452367

Table.1 The geographical origins and habitat of the organisms applied in this study.

Organisms	Geographical origin and Habitat
Halospirulina tapeticola BDU 141021	On stones, Marina Park and water sports complex, Andaman
Oxynema thaianum BDU 10251	Backwater/Stagnant on shore, Vedaranyam, Tamil Nadu
Oxynema thaianum BDU 70493	Stagnant sea water, Kovalam Beach, Chennai, Bay of Bengal region.
Oxynema thaianum BDU 91992	On the sides of boat, Thevara, Kochi, Arabian sea region.
Oxynema thaianum BDU 120161	On muddy soil, Arambol salt pan, Goa coast, Arabian sea.
Leptolyngbya tenuis BDU 46241	On submerged rocks, Actual sea, Mandabam, Gulf of Mannar
Pseudanabaena catenata BDU 41121	On Coral rock, Actual sea, Kurusadai Island, Gulf of Mannar
Pseudanabaena catenata BDU 70492	Stagnant sea water, Kovalam beach, Chennai, Bay of Bengal region

Fig.1 Bright field photomicrographs: a:*Halospirulina tapeticola* BDU 141021,
b:*Oxynema thaianum* BDU 10251, c:*Oxynema thaianum* BDU 70493, d:*Oxynema thaianum*
BDU 91992, e:*Oxynema thaianum* BDU 120161, f:*Phormidium tenue* BDU 46241, g:
Pseudanabaena catenata BDU 41121 h: *Pseudanabaena catenata* BDU 70492.
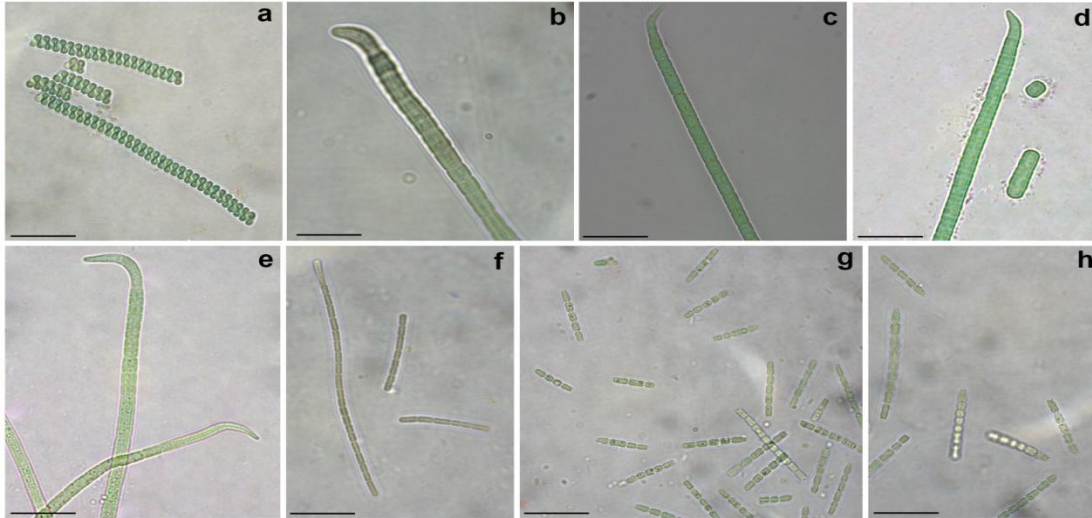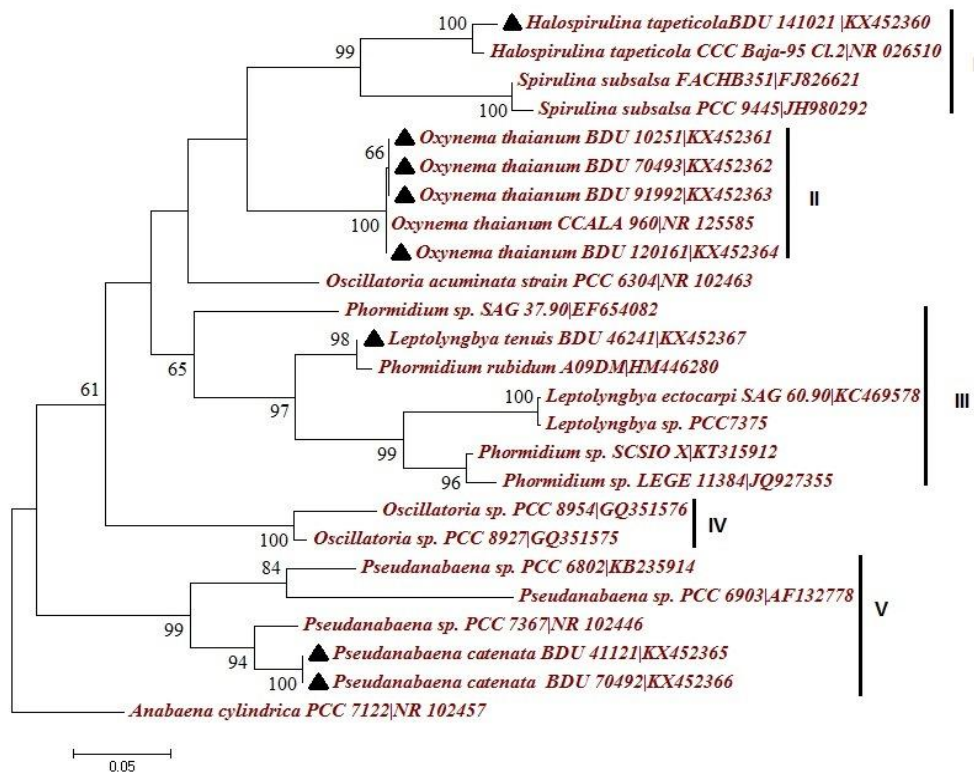Scale Bar – 10µm

Fig.2 Phylogenetic relationship of marine filamentous cyanobacteria inferred by maximum
likelihood analysis of 16S rRNA gene sequences. Number at nodes indicates the bootstrap
values (1000 replicates) obtained from the analysis. Bootstrap values (≥ 50%) are indicated on
the branches. The investigated strains are marked with a black triangle.

Phylogenetic analysis

Phylogenetic tree was constructed with nearly complete 16S rRNA gene sequence (~1350 base pairs) of 25 organisms (Fig. 2) using *Anabaena cylindrica* PCC 7122 as an out group. 16S rRNA gene formed five main clusters in phylogenetic tree. *Halospirulina tapeticola* BDU 141021 clustered with *Halospirulina tapeticola* CCC Baja-95 Cl.2 of Pacific Ocean origin with highest bootstrap values while *Spirulina subsalsa* reference strains retrieved from the public database appeared as sister taxa. BDU 141021 were morphologically similar to *Halospirulina tapeticola* (Nubel *et al*., 2000) by their similar trichome width of 1.5- 4 μm, gliding motility and tightly coiled trichomes which confirms their generic epithet as *Halospirulina tapeticola*. The genus *Oxynema thaianum* formed a monophyletic group (cluster II) with strains BDU 10251, 70493, 91992, and 120161 isolated from coastal line of Tamil Nadu, Kerala, Konkan coast and the reference *Oxynema thaianum* CCALA 960 isolated from saltern of Thailand despite their distant geographical origin indicating that it is quite conserved on the DNA level besides to rather similar morphological traits. *Leptolyngbya tenuis* BDU 46241 isolated from submerged rocks of Gulf of Mannar clustered with *Phormidium rubidium* A09DM isolated from rocky shore of Gujarat in cluster III containing *Leptolyngbya/Phormidium* reference *strains*. This was in agreement with earlier phylogenetic studies, signifying the non-monophyletic nature of *Leptolyngbya valderiana* (Premanandh *et al*., 2009). *Leptolyngbya tenuis* BDU 46241 possessed pinkish to brownish red thallus and very thin trichomes of 1-2 μm wide, 2.5-5μm long. *Phormidium rubidium* A09DM is a phycoerythrin rich organism (Parmar *et al*., 2011). The clustering of BDU 46241 with *Phormidium rubidium* A09DM may possibly because of their phycoerythrin

nature reflected in 16S rRNA gene. BDU 41121 and 70492 strains previously determined as *Oscillatoria minnesotensis* falls within the clade of the genus *Pseudanabaena* in cluster V with highest bootstrap further authenticates that they belonged to *Pseudanabaena catenata*.

In summary, a morphological description and phylogenetic placement of the eight investigated strains validly confirm their generic designation and evade typical taxonomic complications.

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