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Article

# Taxonomic interpretation of non-heterocystous Cyanobacteria (Oscillatoriales) from eastern India with particular emphasis on *Lyngbya Plectonema* complex

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## ABSTRACT

Filamentous non-heterocystous cyanobacterial taxa from 8 genera were collected from different ecological niches like high altitudes, plains and estuaries of eastern India. The systematic accounts of 23 taxa were investigated with a polyphasic approach considering morpho taxonomy, cultural behavior, and molecular phylogenetic analysis with 16S and 16S-23S Internal Transcribed Spacer (ITS) regions as molecular markers. The collected taxa were from the families Oscillatoriaceae, Phormidiaceae and Pseudanabaenaceae with 8 representative genera viz. *Lyngbya*, *Plectonema*, *Oscillatoria*, *Limnothrix*, *Leptolyngbya*, *Planktothrix*, *Desertifilum* and *Phormidium*. The 16S-23S ITS region-based molecular characterization of 13 species from Oscillatoriaceae, 6 species from Phormidiaceae, and 4 species from Pseudanabaenaceae were found to be congruent with earlier phylogenetic studies using other markers. Phylogenetic tree analysis revealed habitat-specific clustering of ITS sequences of the investigated taxa. The 16S molecular marker-based phylogenetic analysis, along with cultural studies of *the Lyngbya-Plectonema* clade, highlighted the need for morphotaxonomic reconsideration of *Lyngbya birgei* and *Plectonema tomasinianum* related to the formation of false branching. The present study affirmed that 98% sequence similarity in the ITS region can be considered as a threshold percentage for conspecificity determination in the *Lyngbya* genus.

**Keywords:** Cyanobacteria; ITS; Oscillatoriaceae; Phormidiaceae; Phylogenetic tree; Pseudanabaenaceae.

## INTRODUCTION

The filamentous cyanobacteria without heterocyst and akinetes are generally grouped together in the order Oscillatoriales [1]. This large group has simple trachomatous members with or without the sheath. Besides different morphological traits like sheath morphology, cell shape, size and cross-wall constriction, a number of other characteristics were considered from time to time for proper identification of cyanobacterial taxa like nucleoid structure [2–4], fimbriae [5], membrane system architecture [6], composition of cell wall external structure [7,8], consequences of cyanophage infection [9] and polyphosphate bodies [10–12]. Monumental taxonomic works have been done to classify the group according to their morphological perspectives [13,14]. Both non-heterocystous and heterocystous genera were classified under the same order, Nostocales, where non-heterocystous filamentous cyanobacteria were grouped into the family Oscillatoriaceae with a total of 15 genera and 250 species [14]. The genera *Lyngbya*, *Phormidium*, and *Oscillatoria* dominated the group, having 65, 45, and 76 species, respectively. Relying solely on morphological traits, few misidentifications at the generic level were

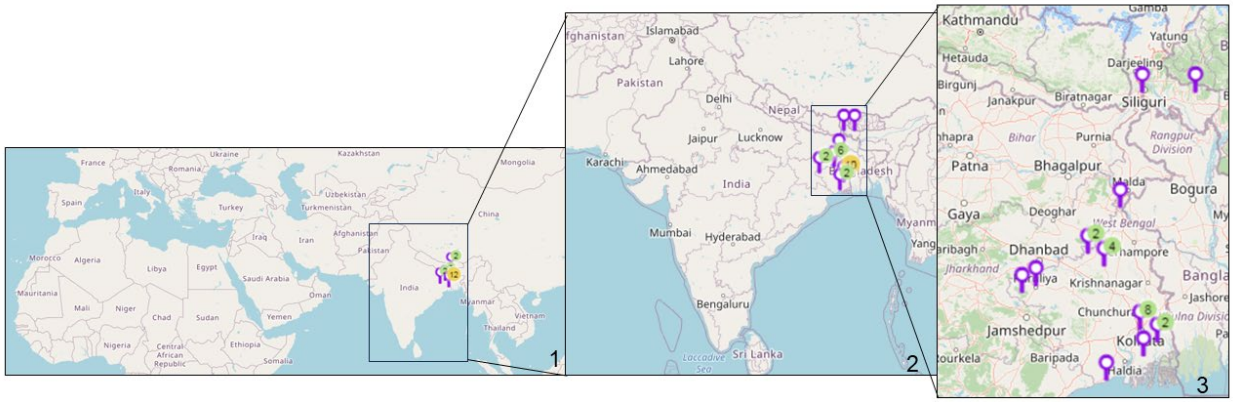
detected in classical taxonomy after the intervention of molecular methods along with ultrastructure of thylakoids and cultural behavior studies.[1] Based on this, several genera like *Lyngbya*, *Oscillatoria, Leptolyngbya,* and *Phormidium* were reconsidered and split into many new genera to reduce confusion. However, the genus *Oscillatoria* is still understudied compared to others [15]. For cyanobacteria, 16S rDNA, 16S-23S ITS region, phycocyanin gene, *if* D, *psb*A, *rbcL*X, and *rpo*C1 are considered the significant molecular markers used for the determination of phylogenetic relationship in taxonomically complex group [16–20]. Though 16S rDNA is widely used for oscillations, the reliability of 16S rDNA has been restricted to higher taxonomic groups and has not been proven efficient at the sub-generic level because of its conserved nature.[21] In that context, the intergenic spacer region between the 16S-23S region was investigated to study the variation at the species level. The heterogeneities were noticed mainly in the size, secondary structure, and, most importantly, insertion and deletion of tRNA genes in multiple operons [22]. ITS region was less utilized in population genetics and molecular systematic studies for these variations.

Phylogenetic relationships using intergenic spacer sequences can be resolved by targeting the same operon in different species with the help of operon-specific primers. The first sequencing of the intergenic spacer region of *Microcystis* sp. was done to resolve phylogenetic relationships at population level [23]. Boyer et al. (2001)[24] compared the variations in the ITS region for the first time in five heterocystous genera. The ITS region should always be handled accurately to avoid complications associated with multiple non-identical operons. However, problems may arise if sequence data is assigned to morphologically misidentified samples. The presence of several cryptic species in *Microcoleus* was reported by using 16S and ITS sequence data [22]. Instead of directly constructing a phylogenetic tree, secondary structures of the ITS region should also be used for better resolution at the molecular level. Due to a few earlier trials worldwide, no such threshold similarity percentage of the ITS region is fixed for concluding conspecificity in the order Oscillatoriales.

## MATERIALS AND METHODS

### Sample collection

Cyanobacterial samples were collected from 23 sites in Eastern India (Figs 1-3). The latitude and longitude of the collection sites are represented in Table 1. The sites were located in northern hilly areas, suburb areas, lateritic soil areas, hot spring habitats, riverine plains, and also from mangrove regions, i.e., intertidal areas. The collection areas included aquatic habitats, moist soils and tree barks.




**Figs 1-3: Map of sampling locations of Cyanobacteria in India. Fig. 1. Location sites are indicated. Fig. 2. Enlarged view of India showing the collection sites. Fig. 3. Enlarged view of eastern India showing the collection sites. (Map courtesy- Mapline, mapping software).**

Immediate field vouchers were prepared at the sampling site using 4% formaldehyde in 25×50mm screw cap bottles (Tarson). The vouchers were deposited in the Calcutta University Herbarium for Algae (CUH/AL). The voucher numbers were assigned and are represented in Table 2. The remaining samples were cleaned thoroughly in double distilled water 2-3 times to remove debris from filaments. A portion of cleaned filaments was soaked in blotting paper, and approximately 100 mg of dried filaments were kept at -20ºC for molecular characterization purposes.

| Site number | Names of collection sites | Latitude | Longitude |
|---|---|---|---|
| Site 1 | Canning, South 24Parganas | 22°19'58.62" N | 88° 41' 12.624" E |
| Site 2 | Canning, South 24Parganas | 22° 19' 59.628" N | 88° 41' 11.364" E |
| Site 3 | Bakreswar, Birbhum | 23° 52' 42.924" N | 87° 22' 7.68" E |
| Site 4 | Behala, Kolkata | 22° 29' 21.912" N | 88° 18' 55.908" E |
| Site 5 | Dankuni, Howrah | 22° 38' 5.532" N | 88° 18' 8.748" E |
| Site 6 | Dankuni, Howrah | 22° 38' 11.364" N | 88° 18' 18.792" E |
| Site 7 | Kasba, Kolkata | 22° 31' 19.38" N | 88° 23' 18.96" E |
| Site 8 | Saheb bandh, Purulia | 23° 19' 20.424" N | 86° 22' 19.884" E |
| Site 9 | Ballygunge, Kolkata | 22° 31' 38.496" N | 88° 21' 50.904" E |
| Site 10 | Hasimara, Alipurduar | 26° 40' 23.916" N | 89° 24' 22.212" E |
| Site 11 | Mahananda river, Siliguri | 26° 39' 31.716" N | 88° 24' 23.616" E |
| Site 12 | Mahananda river, Maldah | 24° 40' 45.48" N | 87° 58' 3.18" E |
| Site 13 | Hedua, Kolkata | 22° 35' 14.82" N | 88° 22' 7.608" E |
| Site 14 | Dhakuria, Kolkata | 22° 30' 43.38" N | 88° 21' 35.784" E |
| Site 15 | Bhubandanga, Birbhum | 23° 40' 28.92" N | 87° 41' 17.88" E |
| Site 16 | Ajay river, Birbhm | 23° 36' 55.764" N | 87° 41' 47.292" E |
| Site 17 | Kopai river, Birbhum | 23° 41' 16.224" N | 87° 38' 53.628" E |
| Site 18 | Kopai river, Birbhum | 23° 41' 19.968" N | 87° 38' 53.844" E |
| Site 19 | Baghmundi, Purulia | 23° 11' 33.324" N | 86° 6' 4.176" E |
| Site 20 | Mathurapur, South 24Parga-nas | 22° 5' 38.364" N | 88° 25' 5.268" E |
| Site 21 | Mandarmani, Purba Medini-pur | 21° 39' 49.5" N | 87° 42' 21.996" E |
| Site 22 | Bakreswar, Birbhum | 23° 52' 47.46" N | 87° 22' 2.712" E |
| Site 23 | Duff street, Kolkata | 22° 35' 14.964" N | 88° 22' 13.008" E |

**Table 1: Sample collection sites, along with their latitudes and longitudes.**

## Morphological characterization

The slides were prepared, and morphological details were studied using Carl Zeiss Axiostar microscope with 40X and 100X objective lenses. Photomicrographs were taken using Canon T2-T2 1, 6x SLR 426115. Morphological identifications were performed using Komarek and Anagnostidis (2005) and AlgaeBase [25].

**Molecular characterization**

The stored samples were subjected to genomic DNA extraction following phenol-chloroform extraction method [26]. Extracted DNA was stored in Tris-EDTA buffer at -20ºC. Partial 16S rDNA and 16S-23S ITS regions were amplified using suitable primers (Table 2) [27,28]. The amplifications were carried out in a thermal cycler machine (Biorad T100 PCR thermal cycler). The PCR cycle conditions were initial denaturation for 5 min at 94ºC, 35 cycles of 94ºC for 30 s, 45 s at 52ºC for ITS region and 58.5ºC for 16S rDNA region, 1 min at 72ºC, and a final extension step for 10 min at 72ºC. Using a gel documentation system, the PCR products were visualized in 1% agarose gel (Vilber). The PCR products were subjected to purification and Sanger sequencing through a commercial sequencing service (Syngex India).

**Phylogenetic analysis**

In order to construct a phylogenetic tree, close relatives of the taxa were searched using the robust NCBI database using BLASTn queries (http://blast.ncbi.nlm.nih.gov). The collected sequences were visually inspected and then manually edited using BioEdit sequence alignment editor version 7.0.9.0 (Tom Hall, Ibis Biosciences, Carlsbad, USA). The sequences were submitted to NCBI, and accession numbers were assigned (Table 3, Table 4). The phylogenetic tree constructions were carried out in Molecular Evolutionary Genetics Analysis (MEGA) (version 7) [29]. The multi-sequence comparison by log expectation (MUSCLE) program aligned the sequences. The best evolutionary model for the used dataset was selected based on the lowest BIC value calculated. The tree was constructed by the maximum likelihood analysis (ML) using the model K2 + G and the neighbor-joining analysis (NJ) based on sequence differences with uniform rates in 1,000 bootstrap replications. The phylogenetic tree obtained was visualized using Tree Graph 2 [30].

The secondary structures of the 16S-23S internal transcribed spacer (ITS) were determined using Mfold version 2.3 with default parameters 31. According to Iteman et al. (2002) 32, different conserved and variable regions were identified.

| Primer name | Sequence | References |
|---|---|---|
| ITSCYA236F | 5′CTGGTTCRAGTCCAGGAT3′ | 27 |
| ITSCYA225R | 5′TGCAGTTKTCAAGGTTCT3′ | 27 |
| CYA106F | 5′CGG ACG GGT GAG TAA CGC GTG A3′ | 28 |
| CYA781R | 5′GAC TAC TGG GGT ATC TAA TCC CAT T3′ | 28 |

**Table 2: Primers used for amplification and sequencing.**

**Cultural behavior studies**

The selected samples were thoroughly cleaned in sterile double distilled water and cultured in BG11 medium ($NaNO_3$-1.5 g $L^{-1}$; $MgSO_4.7H_2O$-0.075 g $L^{-1}$; $CaCl_2.2H_2O$-0.036 g $L^{-1}$; $Na_2CO_3$ 0.02 g $L^{-1}$; $K_2HPO_4$- 0.04 g $L^{-1}$) at 20°C under cool fluorescent lights at 20–30 µmol photons $m^{-2}$ $s^{-1}$ with 16:8 h light: dark. All the cultures were kept for 15 days, and morphology was studied after subsequent time intervals. Slides were prepared, and photomicrographs were taken under 40X and 100X objective lenses using the Carl Zeiss Axiostar microscope attached with Canon T2-T2 1, 6x SLR 426115.

## RESULTS

Twenty-three samples were identified based on morphological characters and grouped under 8 genera, namely *Leptolyngbya*, *Planktothrix*, *Oscillatoria*, *Lyngbya*, *Phormidium*, *Limnothrix*, *Plectonema,* and *Desertifilum*. Among them, *Lyngbya* was found in rivers of the northern uphill and lateritic soil regions and the Gangetic plain associated with the coastal region. Genus *Oscillatoria* was collected from northern hilly aquatic habitats and the regions surrounding populated cities, whereas *Plectonema* was recorded from the aquatic habitats of lateritic soil area*s. Leptolyngbya* was found in the hot spring habitat and brackish mangrove region, whereas *Limnothrix* was detected only from the hot spring. Three genera like, *Planktothrix, Phormidium,* and *Desertifilum,* were solely found in suburban areas (Table 3). Photomicrographs of collected samples are represented in Figs 4-16 and 17-28. A total of 11 species were identified namely *Leptolyngbya valderiana* (Gomont) Anagnostidis et Komarek, *Limnothrix vacuolifera* (Skuja) Komárek ex G.McGregor, *Planktothrix pseudagardhii* Suda, *Phormidium autumnale* [Agardh] Trevisan ex Gomont, *Phormidium formosum* (Bory ex Gomont) Anagnostidis and Komárek, *Lyngbya birgei* G.M. Smith, *Lyngbya aestuarii* Liebman ex Gomont, *Lyngbya semiplena* J.Agardh ex Gomont, *Plectonema tomasinianum* Bornet ex Gomont, *Oscillatoria princeps* Vaucher ex Gomont and *Oscillatoria sancta* Kutzing ex Gomont. Taxonomic descriptions are provided in supplementary material.

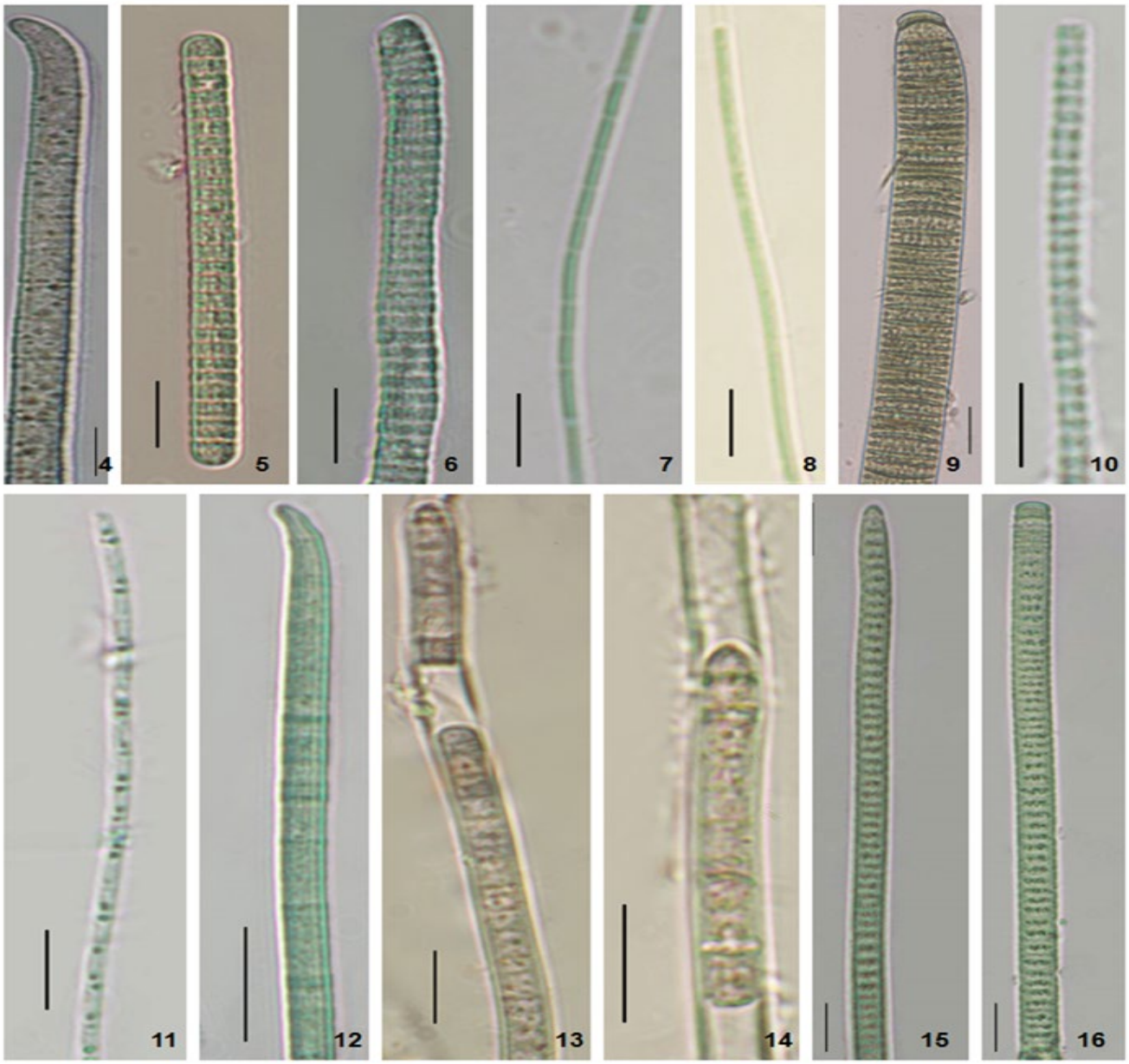
| Specimen (as deposited in NCBI GenBank) | Specimen (Morphologically) | Voucher Numbers | Habitat | GenBank Accession numbers |
|---|---|---|---|---|
| *Leptolyngbya* sp. RPL2 | *L. valderiana* | CUH/AL/MW/CYANO-174 | Mangrove (Brackish water) | MN640091 |
| *Leptolyngbya* sp. RPL1 | *L. valderiana* | CUH/AL/MW/CYANO-172 | Mangrove (Brackish Water) | MN640090 |
| *Leptolyngbya* sp. BKR | *Leptolyngbya* sp. | CUH/AL/FW/CYANO-103 | Hotspring | MW238348 |
| *Limnothrix* sp. BKR | *Limnothrix vacuolifera* | CUH/AL/FW/CYANO-104 | Hotspring | MW393862 |
| *Planktothrix* sp. DNK | *P. pseudagardhii* | CUH/AL/FW/CYANO-98 | Suburb areas | MW238345 |
| *Planktothrix* sp. DNK2 | *P. pseudagardhii* | CUH/AL/FW/CYANO-96 | Suburb areas | MW366802 |
| *Planktothrix* sp. S41 | *Planktothrix* sp. | CUH/AL/FW/CYANO-270 | Suburb areas | MW393863 |
| *Phormidium* sp. AKS1 | *P. formosum* | CUH/AL/FW/CYANO-248 | Suburb areas | MN817999 |
| *Desertifilum* sp. RPL | *Desertifilum* sp. | CUH/AL/FW/CYANO-260 | Suburb areas | MW366801 |
| *Phormidium* sp. RPL | *P. autumnale* | CUH/AL/FW/CYANO-94 | Suburb areas | MW238346 |
| *Plectonema* sp. KAS | *P. tomasinianum* | CUH/AL/FW/CYANO-138 | River (Lateritic soil area) | MN814325 |
| *Plectonema* sp. KOP | *P. tomasinianum* | CUH/AL/FW/CYANO-121 | River (Lateritic soil area) | MN814326 |
| *Plectonema* sp. AJY | *P. tomasinianum* | CUH/AL/FW/CYANO-127 | River (Lateritic soil area) | MN814324 |
| *Lyngbya* sp. NB | *L. birgei* | CUH/AL/FW/CYANO-74 | River (Northern Hilly area) | MT258376 |
| *Lyngbya* sp. MHN | *L. birgei* | CUH/AL/FW/CYANO-161 | River | MN818000 |
| *Lyngbya* sp. SUND | *L. aestuarii* | CUH/AL/MW/CYANO-136 | Mangrove (Brackish water) | MN817998 |
| *Lyngbya* sp. MANI | *L. semiplena* | CUH/AL/MW/CYANO-246 | Coastal region | MN630163 |
| *Lyngbya* sp. RAB | *L. birgei* | CUH/AL/FW/CYANO-134 | Freshwater | MN638845 |
| *Lyngbya* sp. PRLA | *L. birgei* | CUH/AL/FW/CYANO-124 | River (Lateritic soil area) | MK224842 |

| | | | | |
|---|---|---|---|---|
| *Lyngbya* sp. HDA | *L. birgei* | CUH/AL/FW/CYANO-120 | Freshwater Pond | MN817997 |
| *Lyngbya* sp. SHNTN | *L. birgei* | CUH/AL/FW/CYANO-111 | River (Lateritic soil area) | MN630164 |
| *Oscillatoria* sp. MND | *O. princeps* | CUH/AL/FW/CYANO-72 | River (Northern Hilly area) | MW238347 |
| *Oscillatoria* sp. | *O. Sancta* | CUH/AL/FW/CYANO-89 | Suburb areas | MN630162 |

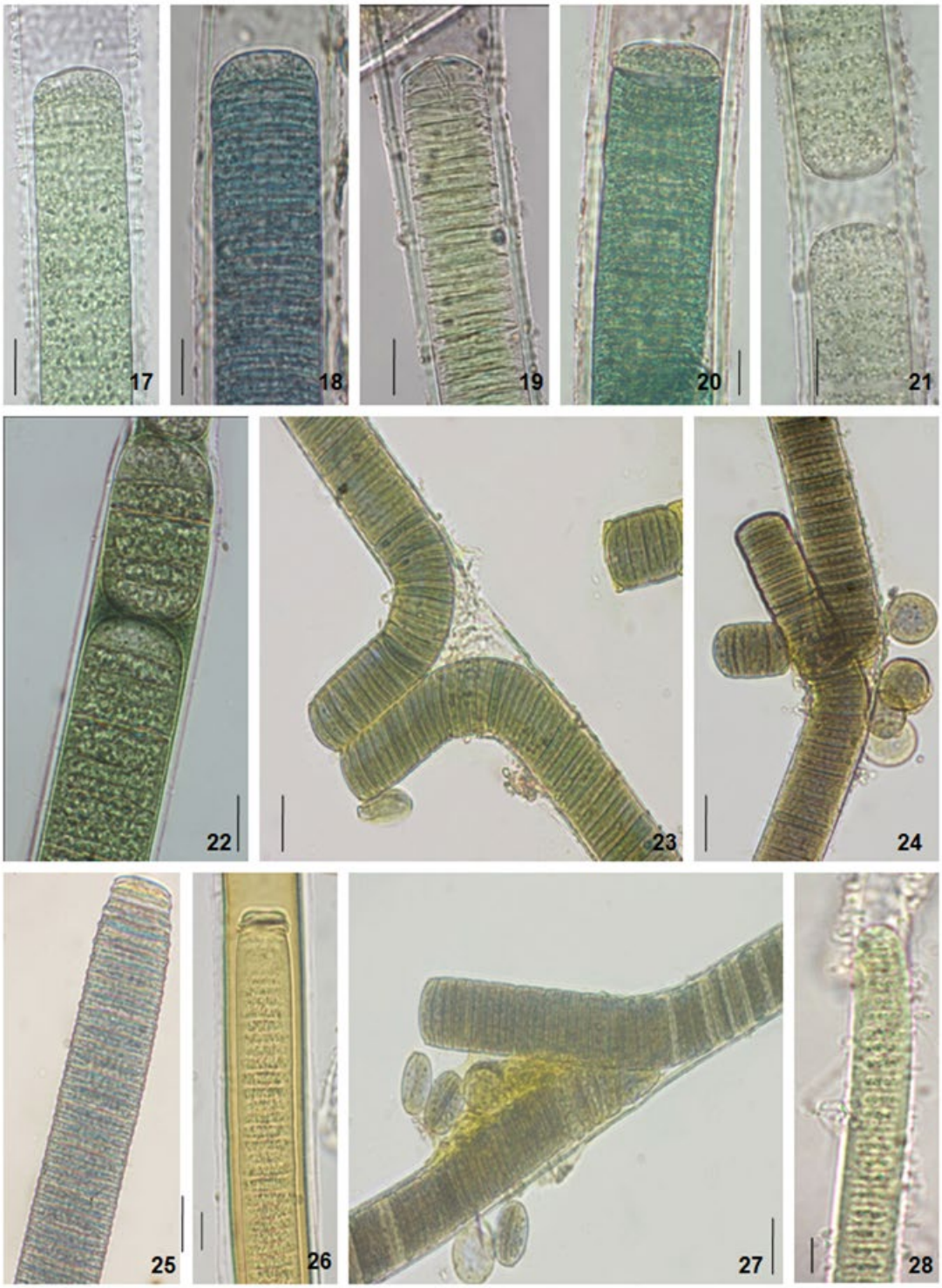
**Table 3: Lists specimens with their voucher numbers and GenBank accession numbers of 16S-23S ITS sequences.**

| **Specimen (as deposited in NCBI GenBank)** | **Specimen (Morphologically)** | **Voucher Numbers** | **Habitat** | **GenBank Accession numbers** |
|---|---|---|---|---|
| *Plectonema* sp. KAS | *P. tomasinianum* | CUH/AL/FW/CYANO-138 | River (Lateritic soil area) | MT192753 |
| *Plectonema* sp. KPY | *P. tomasinianum* | CUH/AL/FW/CYANO-121 | River (Lateritic soil area) | MT192750 |
| *Plectonema* sp. AJY | *P. tomasinianum* | CUH/AL/FW/CYANO-127 | River (Lateritic soil area) | MT192748 |
| *Lyngbya* sp. NB | *L. birgei* | CUH/AL/FW/CYANO-74 | River (Northern Hilly area) | MT192720 |
| *Lyngbya* sp. MHN | *L. birgei* | CUH/AL/FW/CYANO-161 | River | MT192718 |
| *Lyngbya* sp. RAB | *L. birgei* | CUH/AL/FW/CYANO-134 | Freshwater | MT254899 |
| *Lyngbya* sp. PRLA | *L. birgei* | CUH/AL/FW/CYANO-124 | River (Lateritic soil area) | MT254898 |
| *Lyngbya* sp. HDA | *L. birgei* | CUH/AL/FW/CYANO-120 | Freshwater pond | MT254994 |
| *Lyngbya* sp. SHNTN | *L. birgei* | CUH/AL/FW/CYANO-111 | River (Lateritic soil area) | MW362745 |

**Table 4: List of freshwater *Lyngbya* and *Plectonema* samples with their voucher numbers and GenBank accession numbers of 16S sequences**




**Figs 4-16: Photomicrographs of the specimens. Fig. 4. *Phormidium formosum* (*Phormidium* sp. AKS1). Fig. 5. *Planktothrix pseudagardhii* (*Planktothrix* sp. DNK). Fig. 6. *Planktothrix pseudagardhii* (*Planktothrix* sp. DNK2). Fig. 7. *Leptolyngbya valderiana* (*Leptolyngbya* sp. RPL1). Fig. 8. *Leptolyngbya valderiana* (*Leptolyngbya* sp. RPL2). Fig. 9. *Oscillatoria sancta* (*Oscillatoria* sp.). Fig. 10. *Leptolyngbya* sp. (*Leptolyngbya* sp. BKR). Fig. 11. *Limnothrix vacuolifera* (*Limnothrix* sp. BKR). Fig. 12. *Desertifilum* sp. (*Desertifilum* sp. RPL) Fig. 13-14. *Phormidium autumnale* (*Phormidium* sp. RPL). Fig. 15-16. *Planktothrix* sp. (*Planktothrix* sp. S41). Scale Bar- 5 µm.**

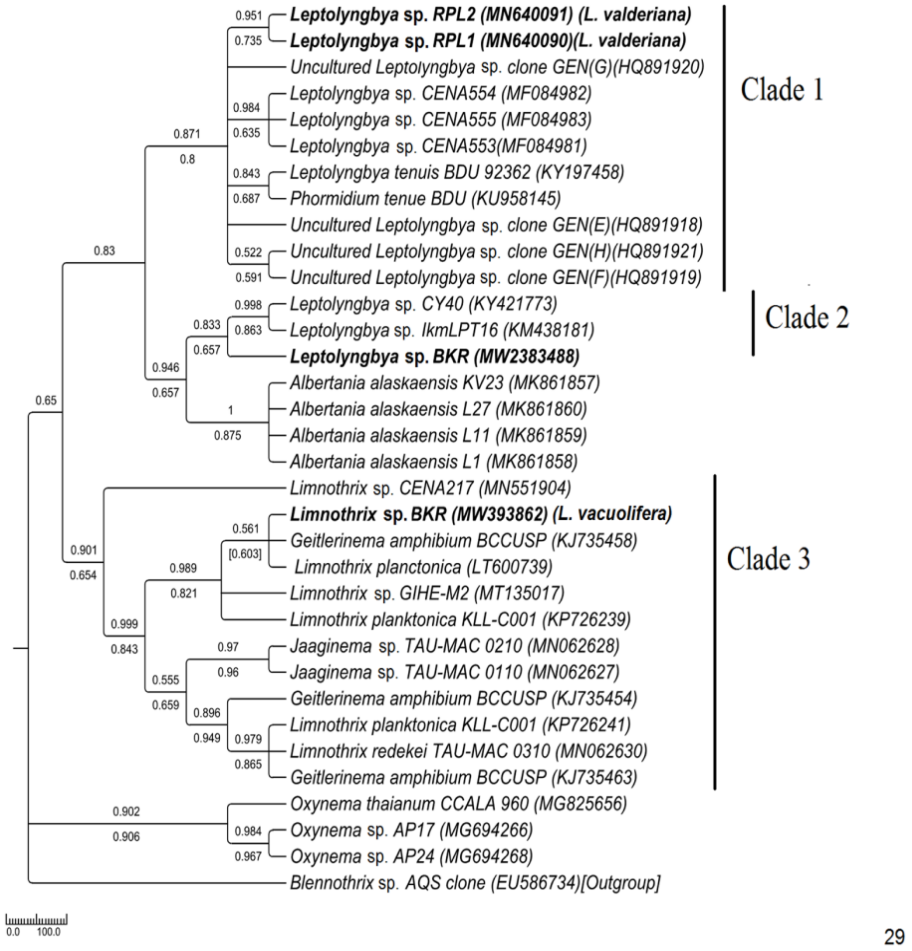



**Figs 17-28: Photomicrographs of the specimens. Fig. 17. *Lyngbya birgei* (*Lyngbya* sp. PRLA). Fig. 18. *Lyngbya birgei* (*Lyngbya* sp. NB). Fig. 19. *Lyngbya birgei* (*Lyngbya* sp. SHNTN). Fig. 20. *Lyngbya birgei* (*Lyngbya* sp. RAB). Fig. 21. *Lyngbya birgei* (*Lyngbya* sp. MHN). Fig. 22. *Lyngbya birgei* (*Lyngbya* sp. HDA). Fig. 23. *Plectonema tomasinianum* (*Plectonema* sp. KOP). Fig. 24. *Plectonema tomasinianum* (*Plectonema* sp. AJY). Fig. 25. *Oscillatoria princeps* (*Oscillatoria* sp. MND). Fig. 26. *Lyngbya aestuarii* (*Lyngbya* sp. SUND). Fig. 27. *Plectonema tomasinianum* (*Plectonema* sp. KAS). Fig. 28. *Lyngbya semiplena* (*Lyngbya* sp. MANI). Scale Bar- 5 µm.**

**A phylogenetic study using 16S-23S ITS region**

The genera were grouped into three families, namely Pseudanabaenaceae, Oscillatoriaceae, and Phormidiaceae, on the basis of morphology. The phylogenetic trees were constructed within each family. The ITS length was around 250bp except for the MW238347 *Oscillatoria* sp. MND (morphologically identified as *O. princeps*) was almost 450bp. The similarity matrices of the sequences are represented in Supplementary Tables 1-6.

The Pseudanabaenaceae tree was constructed from a total of 34 sequences of ITS, including 4 from our study and 30 from the NCBI database (Fig. 29). The samples were resolved into 3 distinct clades with moderate to high bootstrap support (NJ/ML) values. The sequence similarities were represented in supplementary table 1. In clade 1 brackish water taxa MN640090 *Leptolyngbya* sp. RPL1(morphologically *L. valderiana*) and MN640091 *Leptolyngbya* sp. RPL2 (morphologically *L. valderiana*) formed a monophyletic clade with high bootstrap support with other saline *Leptolyngbya* sequences of the NCBI database (HQ891920, MF084983, MF084982, MF084981) and showed polyphyly with the experimental MW2383488 *Leptolyngbya* sp. BKR (morphologically *Leptolyngbya* sp.) from Hotspring. The MW2383488 *Leptolyngbya* sp. BKR formed monophyly with a hot-spring KM438181 *Leptolyngbya* sp. of Greece (clade 2). The sequences of *Albertania*, which has recently been established as a new genus from *Leptolyngbya,* showed similarity with the *Leptolyngbya* sp. BKR and shared sister clade with the experimental taxa. Our experimental MW393862 *Limnothrix* sp. BKR (morphologically *Limnothrix vacuolifera*) sequence was well separated from the *Leptolyngbya* clades and formed a monophyletic clade with *Limnothrix*, *Geitlerinema*, and *Jaaginema* having high bootstrap values (clade 3).




29

**Figure 29: Phylogenetic tree of ITS sequences based on neighbor-joining and the maximum likelihood of the family Pseudanabaenaceae. Bootstrap values (NJ/ML) for nodes >50% were shown in the tree. Genbank accession numbers are provided.**

The phylogenetic tree of Phormidiaceae was built up with 29 NCBI sequences along with 6 sequences of the current study (Fig. 30). Samples appeared to be divided into four clades of moderate to high bootstrap values—experimental MW238346 *Phormidium* sp. RPL (morphologically *Phormidium autumnale*) was placed in clade 1 with moderate to high bootstrap support and showed (90-91%) sequence similarity with its neighboring sister clades containing *Microcoleus* and *Phormidium autumnale* samples—the MN817999 *Phormidium* sp. AKS (morphologically identified as *Phormidium formosum*) was found to be very close to *the Planktothrix* clade (clade2) (having 60-70% similar sequences) and shown monophyly with the same instead of *the Phormidium* clade. The three *Planktothrix* samples were separated into two sub-clades within clade 2 and showed paraphyletic origin regardless of their morphological similarity. Experimental MW238345 *Planktothrix* sp. DNK (morphologically *Planktothrix pseudagardhii*) and MW393863 *Planktothrix* sp. S41 sequences were 91% similar and formed monophyletic clades with high bootstrap support with other *Planktothrix* sequences of the NCBI database (Supplementary Table 2)—another MW366802 *Planktothrix* sp. DNK2 (morphologically *Planktothrix pseudogardhii*) was separated and formed a distinct clade with *Planktothrix* samples from the NCBI database. MW366801 *Desertifilum* sp. RPL was found to form a monophyletic lineage with 99% similar *Desertifilum* sequences of NCBI databases consistent with their morphology and separated from clade 1 and clade 2.

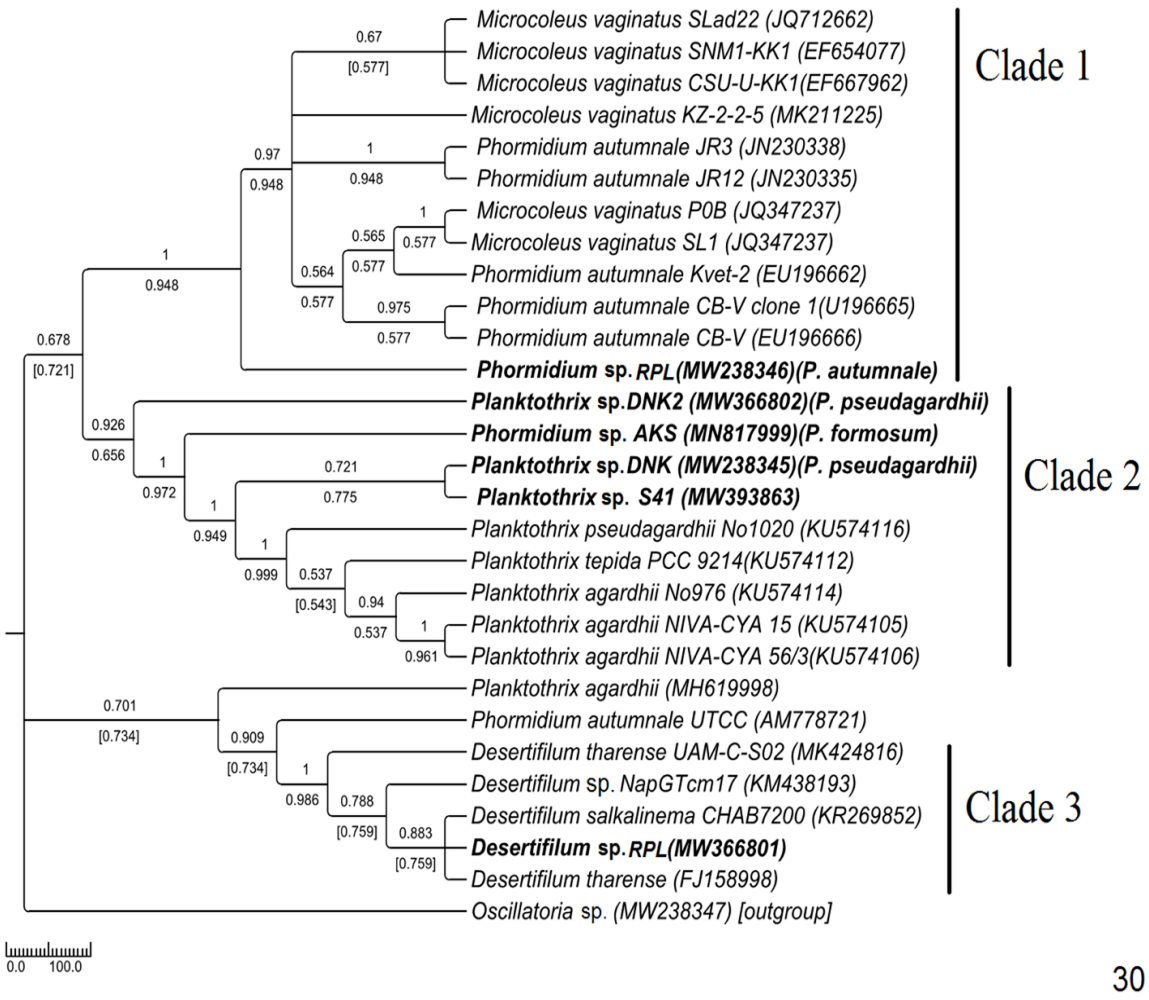



**Figure 30: Phylogenetic tree of ITS sequences based on neighbor-joining and the maximum likelihood of the family Phormidiaceae. Bootstrap values (NJ/ML) for nodes >50% were shown in the tree. Genbank accession numbers are provided.**

Phylogenetic relationships in Oscillatoriaceae were drawn using a total of 37 sequences, out of which 13 sequences were from the present study and 24 from the NCBI database (Fig. 31). Samples were divided into five clades of moderate to high bootstrap values. The experimental sample MN630162 *Oscillatoria* sp. (morphologically *Oscillatoria sancta*) formed a monophyletic clade (clade 2) with other *Oscillatoria sancta* sequences of the NCBI database with high bootstrap values. The sequences were 94-96% similar—another experimental MW238347 *Oscillatoria* sp. MND sequences from hilly regions (morphologically *Oscillatoria princeps*) formed a separate clade (clade 5) having high bootstrap values with *Oscillatoria* sequences collected from the NCBI database. The sequence of this strain's sister clades was 99% similar (Supplementary Table 3). It was well separated from the *Oscillatoria sancta* with 84% sequence similarity. Among the two brackish *Lyngbya* sequences, MN817998 *Lyngbya* sp. SUND (morphologically *L. aestuarii*) showed 81-82% sequence similarity with deposited sequences in NCBI and formed a monophyletic clade (clade 1) with the sequences. The MN630163 *Lyngbya* sp. MANI (morphologically *L. semiplena*) showed 97% similarity with *Neolyngbya irregularis*, which was also marine and formed a monophyletic clade (clade 3) with it (Supplementary Table 4). All the freshwater *Lyngbya* samples collected from high as well as low altitude regions (morphologically *L. birgei*) formed a single monophyletic clade (clade 4), which was separated from the marine *Lyngbya* clades, i.e., clade 1 and clade, 3. This monophyletic clade also contained *Plectonema* sequences along with *Lyngbya*.

Due to less availability of deposited sequences in GenBank, this clade mainly consists of sequences from the present investigation rather than from any online database. Though this clade contains sequences from two different genera, all the sequences were 99-100% similar (Supplementary Table 5). No threshold percentage for species discrimination had been recorded in the case of the 16S-23S ITS region. To attain better resolution in this clade 4, a 16S molecular marker-based phylogenetic tree was built (Fig. 32).

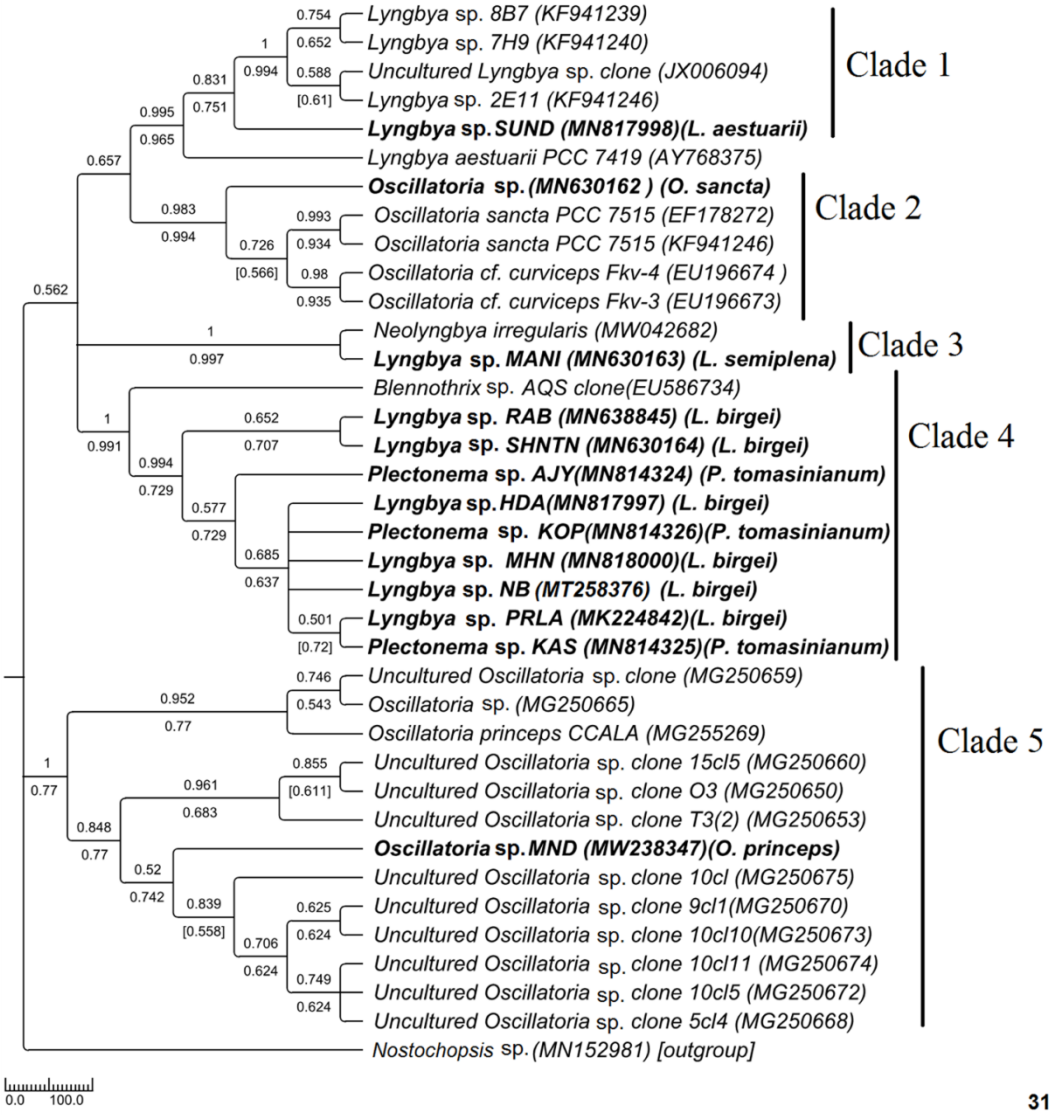



**Figure 31: Phylogenetic tree of ITS sequences based on neighbor-joining and the maximum likelihood of the family Oscillatoriaceae. Bootstrap values (NJ/ML) for nodes >50% were shown in the tree. Genbank accession numbers are provided.**

## Phylogenetic study in Oscillatoriaceae using 16S rDNA region

The average sequence length in the tree was around 700bp. Samples were divided into two clades of moderate to high bootstrap values. This tree topology (Fig. 32) was more or less congruent with a phylogenetic tree with ITS sequence data. The non-availability of the same sequence in the NCBI database for 16S and ITS sequence data resulted in a difference in the topology of the phylogenetic tree. However, the basic topology remained the same, which supported the conspecificity of all freshwater *Lyngbya.* The 16S sequence similarity in *Lyngbya* and *Plectonema* ranged between 99-100%, similar to their similarity in the ITS region (Supplementary Table 6). In the 16S rDNA-based phylogenetic tree, *Plectonema* showed polyphyletic origin. MT192748

*Plectonema* sp. AJY (morphologically *Plectonema tomasinianum*) and MT192750 *Plectonema* sp. KPY (morphologically *Plectonema tomasinianum*) showed 98-99% similarity with *Microseira whole* sequences and formed a monophyletic clade with them, whereas MT192753 *Plectonema* sp. KAS (morphologically *Plectonema tomasinianum*) formed a clade with *Lyngbya* sp. NB has 100% sequence similarity.

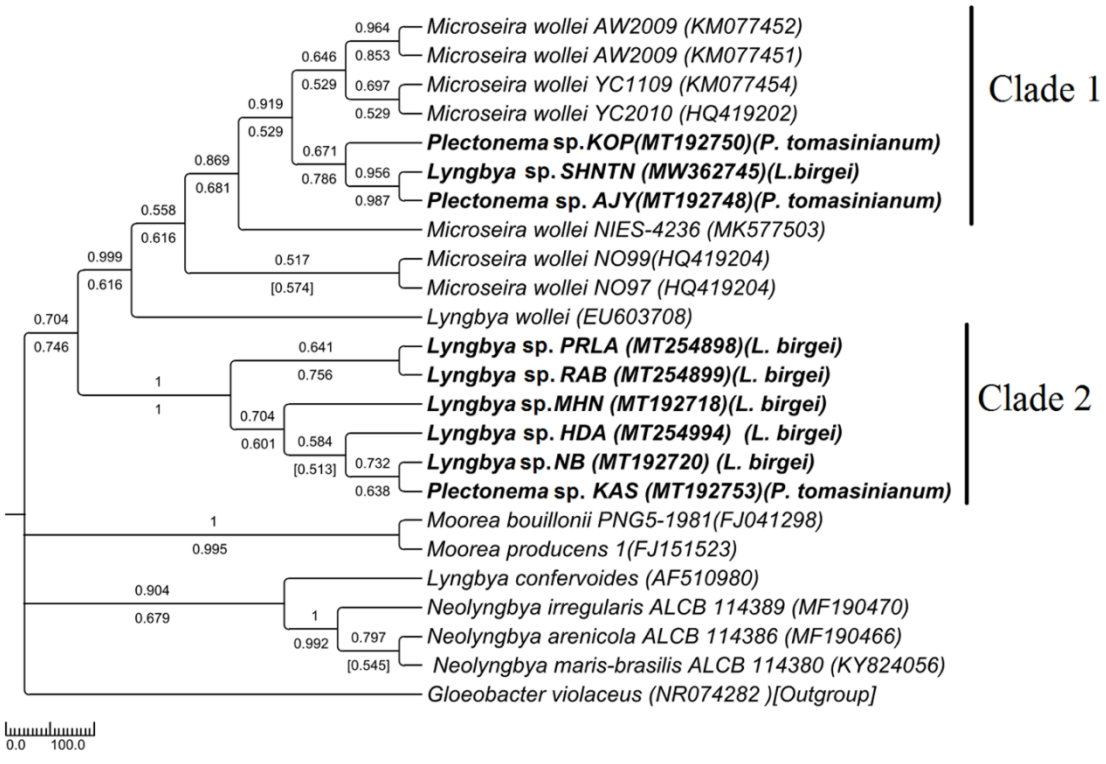



**Figure 32: Phylogenetic tree of 16S sequences based on neighbor-joining and the maximum likelihood of the family Oscillatoriaceae. Bootstrap values (NJ/ML) for nodes >50% are shown in the tree. Genbank accession numbers are provided.**

## Cultural behavior study of *Lyngbya-Plectonema* complex

The cultural behavior of *Plectonema tomasinianum* samples and *Lyngbya birgei* were studied for further clarification. Surprising similarities in different growth stages were recorded among them. On the same note, *Lyngbya* samples formed false branching like *Plectonema* samples during the growth of filaments. The detailed changes are represented in Table 5, Figs 33-42, and Figs 43-57.

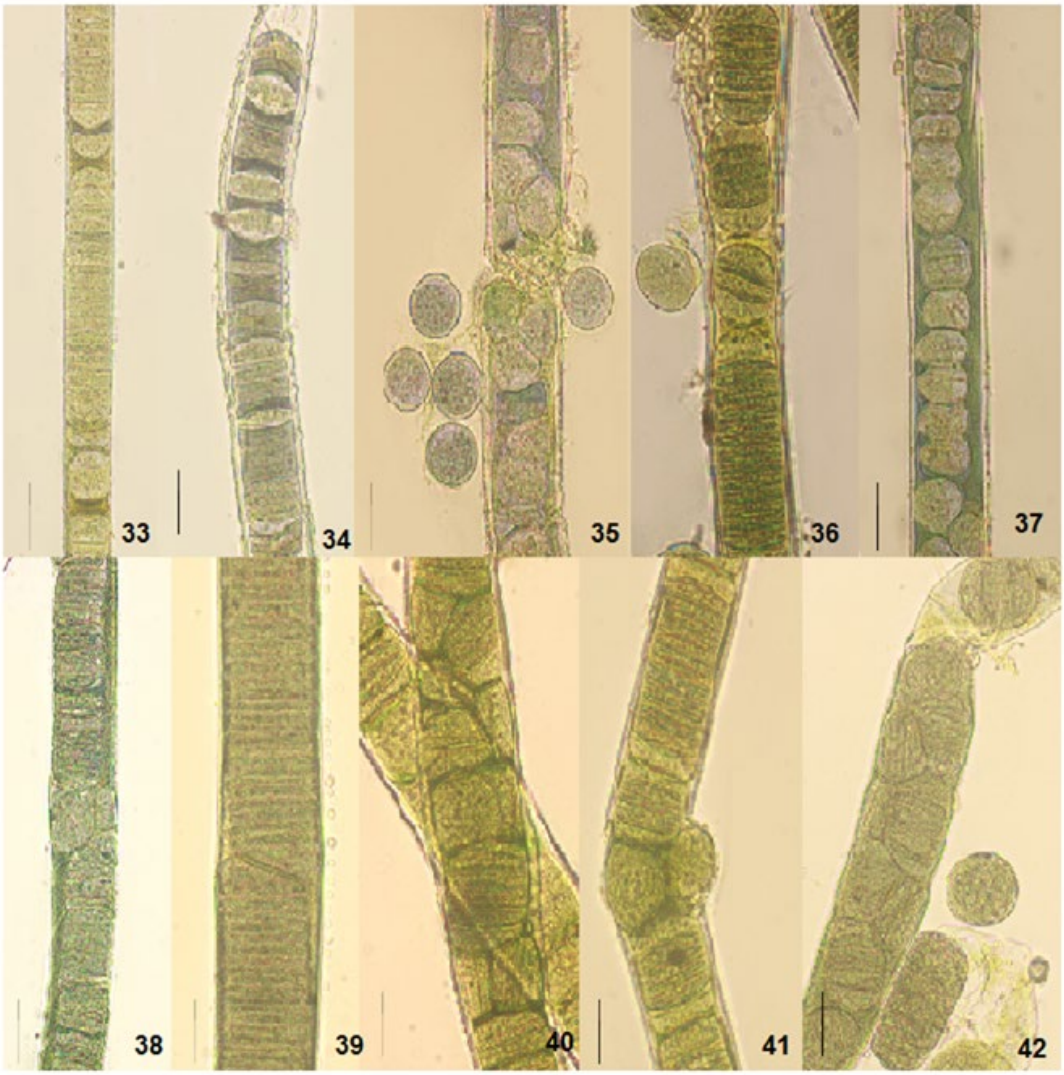



**Fig. 33-42: Light photomicrographs of *Lyngbya birgei* and *Plectonema tomasinianum* filaments at different growth phases in culture show the formation of separation discs and pseudohormogone formation. Fig. 33. Separation disc formation in *Lyngbya* sp. RAB filament. Fig. 34. Separation disc formation in *Lyngbya* sp. HDA filament. Fig. 35. Pseudohormogone formation in *Plectonema* sp. KAS. Fig. 36. Hormogone and pseudohormogone formation in *Plectonema* sp. KPY. Fig. 37. Pseudohormogone formation in *Plectonema* sp. AJY. Fig. 38. Cell division in different planes in *Lyngbya* sp. RAB. Fig. 39. Cell division in different planes in *Lyngbya* sp. HDA. Fig. 40. Cell division in different planes in *Plectonema* sp. KAS. Fig. 41. Cell division in different planes in *Plectonema* sp. KPY. Fig. 42. Cell division in different planes in *Plectonema* sp. AJY. Scale bar- 20 µm.**

| No. of days | *Lyngbya* sp. RAB | *Lyngbya* sp. HDA | *Plectonema* sp. KAS | *Plectonema* sp. KPY | *Plectonema* sp. AJY |
|---|---|---|---|---|---|
| 3 | Separation disc formation. | Separation disc formation. | Pseudo- Hormogonia formation. | Hormogonia formation. | Hormogonia formation. |

| | | | | | |
|---|---|---|---|---|---|
| 6 | Hormogonia formation continued, with cell division in different planes. | Diagonal fragmentations were noticed. | Cell division in different planes is initiated. | Cell division in different planes is initiated. | Cell division in different planes was observed with hormone formation. |
| 9 | Diagonal fragmentation followed by growth of trichome in a single sheath | Cell division in different planes, followed by growth of nascent trichomes. | Diagonal cell division followed by fragmentation of trichome. | The nascent trichome remained enclosed in the same sheath as the mother filament. | The nascent trichome remained enclosed in the same sheath as the mother filament. |
| 12 | Nascent filament growing with mother filament within the common sheath. | Nascent filament growing with mother filament within the common sheath. | Nascent filament growing with mother filament within the common sheath. | Nascent filament growing with mother filament within the common sheath. | Nascent filament growing with mother filament within the common sheath. |
| 15 | The nascent filament became mature and developed as a false branch within the same sheath, observed as a criss-cross structure. | False branches developed. | False branches developed. | False branches developed. | False branches developed. |

**Table 5: Variation in growth performances of *Lyngbya* and *Plectonema* in cultural condition.**

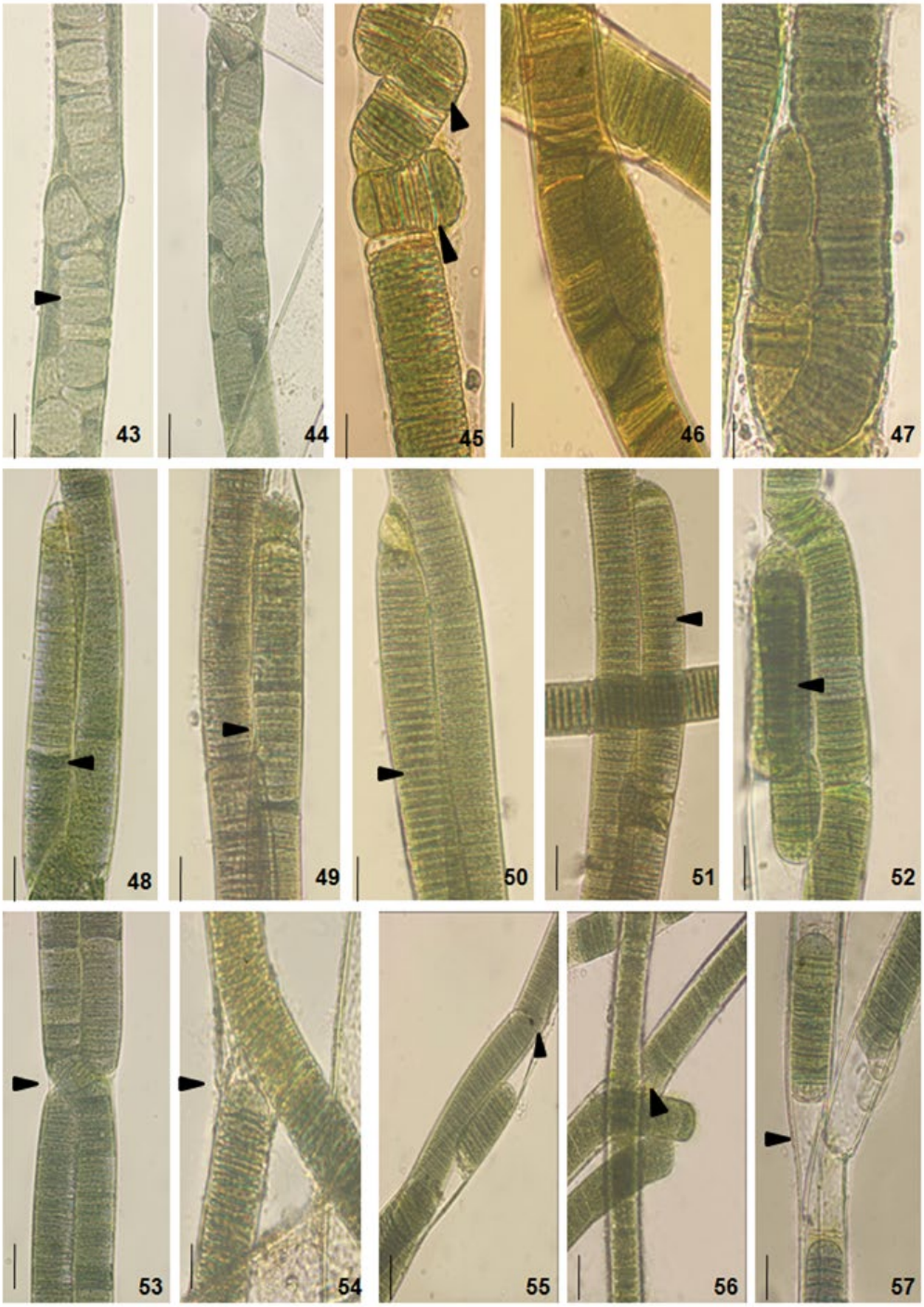



**Fig. 43-57: Light photomicrographs of *Lyngbya birgei* and *Plectonema tomasinianum* filaments at different growth phases in culture showing diagonal fragmentation followed by initiation of nascent trichome and false branch development. Arrows indicate the changes. Fig. 43. Filament of *Lyngbya* sp. RAB. Fig. 44. Filament of *Lyngbya* sp. HDA. Fig. 45. Filament of Plectonema sp. KAS. Fig. 46. Filament of Plectonema sp. KPY. Fig. 47. Filament of *Plectonema* sp. AJY. Fig. 48. Filament of *Lyngbya* sp. RAB. Fig. 49. Filament of *Lyngbya* sp. HDA. Fig. 50. Filament of Plectonema sp. KAS. Fig. 51. Filament of Plectoneme sp. KPY. Fig. 52. Filament of *Plectonema* sp. AJY. Fig. 53. Filament of *Lyngbya* sp. RAB. Fig. 54. Filament of *Lyngbya* sp. HDA. Fig. 55. Filament of Plectoneme sp. KAS. Fig. 56. Filament of Plectonema sp. KPY. Fig. 57. Filament of *Plectonema* sp. AJY. Scale bar- 20 µm.**

**ITS secondary structure analysis**

The 16S-23S ITS regions were subjected to the identification of conserved regions followed by secondary structure analysis for freshwater *Lyngbya* and *Pleconema* samples. The amplified ITS region contained $tRNA^{Ala}$, $tRNA^{Ile}$, $V_2$, Box A and B helices. The lengths of individual helices are represented in Table 6. The secondary structures are provided in Supplementary Figure 1**.** No difference in secondary structures was noticed in all the investigated samples.

## DISCUSSION

The current approaches to cyanobacterial systematics faced considerable challenges in building up conclusive results regarding the congruence of morphological, cultural, and molecular data for phylogenetic interpretation [24]. In the present study, 23 taxa under 8 genera of the order Oscillatoriales were investigated with a polyphasic approach considering morphometry, cultural behavior, and ITS sequence-based molecular data in a need-based manner. As very few ITS sequence data of Oscillatoriales were available in the online database, we tried to incorporate our data as a reference value and constructed phylogenetic trees per the existing NCBI database.

To date, genera of non-heterocystous Oscillatoriales have yet to be well resolved by molecular approach. The *Leptolyngbya* is one of the most abundant and complicated genera of Pseudanabaenaceae due to its simple morphology with genetic variation[1]. A few *Lyngbya*, *Plectonema* and *Phormidium* members with very thin trichomes (0.5-3.5µm wide) were described as a new genus *Leptolyngbya*[33]. The monophyletic assemblage of this genus has not been proved yet. Polyphyletic assemblage based on 16S rDNA analysis was observed in the case of *Pseudanabaena*, *Leptolyngbya*, *Limnothrix*, and *Geitlerinema* [34,35]. In our present study, the polyphyletic origin of *Leptolyngbya* and *Limnothrix,* as well as habitat-specific clustering among species of *Leptolyngbya*, was noticed (Fig. 29). The sequences were clustered with other sequences with similar types of ecological preferences. Cyanobacterial habitat selectivity is a matter of debate to many researchers. The argument regarding endemism [36] or cosmopolitan distribution has existed for a long time[37]. However, present-day research has demonstrated that habitat specificity or nutrient requirement is related to genome size [38,39]. In the current study, genetic diversity among cyanobacteria dwelling in freshwater as well as extreme environments, like marine and hot springs, was studied based on ITS-based phylogenetic analysis for the very first time, which indicated that this molecular maker is capable of discriminating species belonging to different habitats. Morphologically ambiguous identification is inevitable in the family Phormidiaceae of Oscillatoriales due to its misleading nature of identifying criteria. The name of *Phormidium* was considered an invalid name due to more than 100 years of taxonomic confusion[40]. However, Casamatta et al. (2005)[41] did not agree with the fact totally but concluded that generic and subgeneric categorizations in this genus should not essentially correspond to systematic relations. The genus was revised and grouped using morphological and ultrastructural criteria to ease the identification process[1]. The 16S rDNA-based analysis showed a phylogenetically close *Microcoleus vaginatus* and *Phormidium autumnale* assemblage with almost indistinguishable morphological characters[34]. Both the taxa mainly differed in their sheath properties and filament size influenced by environmental factors; therefore, they are inconsistent in culture conditions and 16S rDNA-based phylogenetic study[33]. In the current ITS sequence-based study also, the *Microcoleus* and *Phormidium* formed a monophyletic assemblage that appeared as sister to the experimental genus *Phormidium* (morphologically *P. autumnal*), indicating congruency between 16S and ITS sequence-based interpretations (Fig. 30).

The genus *Planktothrix* was placed under Phormidiacae (after separation from *Oscillatoria*) because of their cellular characteristics and support from 16S rDNA sequencing[42]. In our study, the placement of MW238346 *Phormidium* sp. (morphologically *P. formosum*) with *Planktothrix* sequences (Fig. 30) justified polyphyly of *Phormidium* as proved by other authors also[42]. This kind of polyphyly may have resulted due to inadequate deposited sequences, and thus, more ITS sequence-based research should be done to enrich the sequence database. Paraphyly of *Planktothrix* was observed in our study (Fig. 30) as interpreted in earlier studies using 16S rDNA[42]. *Desertifilum* was considered a new genus under Phormidiaceae after being separated from *Microcoleus* and *Phormidium* based on 16S rDNA-based analysis coupled with morphological parameters[43]. In the current study, *Desertifilum* was in a monophyletic lineage consistent with their morphology and separated from the *Microcoleus-Phormidium* clade. The interpretation is congruent with the 16S rDNA-based study by the earlier workers. This is the first report of *Desertifilum* from eastern India.

Polyphyletic nature of *Oscillatoria* was also reflected in this ITS sequence-based study (Fig. 31). In the phylogenetic tree of Oscillatoriaceae, MN630162 *Oscillatoria* sp. (morphologically *O. sancta*) having apical cap was placed in monophyletic lineage with other *O. sancta* sequences with high sequence similarity and good bootstrap support whereas MW238347 *Oscillatoria* sp. MND (morphologically *O. princeps*) formed a well-supported clade with other *Oscillatoria* strains and was distantly placed from *O. Sancta*. As studied by Hasler et al. (2012)[, 34] *Oscillatoria sancta* having thickened apical cap formed well-supported lineage with other calyptrate taxa in 16S rDNA-based study. The origin of calyptra or their role in cyanobacteria has never been investigated[44]. Phylogenetic remoteness, as observed in our study, cannot be disregarded as both the sequences have shared a high level of similarity with their sister sequences having good bootstrap support. The genus *Oscillatoria* was not revised earlier; however, from our observation, it can be concluded that *Oscillatoria* having an apical cap showed a somewhat different evolutionary history from that of *Oscillatoria* without an apical cap. Further detailed investigation is needed in this regard.

For *Lyngbya*, brackish water and marine species were well separated, and their monophyletic assemblage with other ecologically consistent strains supported again that ITS-based sequence clustering can reflect ecological selectivity. Genetic divergence can reflect habitat preferences for ecologically distinct organisms[45]. In the phylogenetic tree of Oscillatoriaceae, freshwater *Lyngbya* sp. (morphologically *L. birgei*) formed a different monophyletic lineage than that of marine *Lyngbya* (Fig. 31). The morphological similarity was also tested at the molecular level because all the samples have shown 99-100% similarity. Surprisingly, *Plectonema* shared a common monophyletic lineage with the *Lyngbya* clade, having 99-100% sequence similarity. Though no threshold percentage value is available in the literature for species-level identification, the high-level sequence similarity of the ITS study may be considered further.

The ITS tree topology was compared with rDNA based phylogenetic tree (Fig. 32), and the topology was almost similar. However, two *Plectonema* samples (MT192748 *Plectonema* sp. AJY and MT192750 *Plectonema* sp. KPY) (morphologically *P. tomasinianum*) and MW362745 *Lyngbya* sp. SHNTN, all collected from rivers flowing over lateritic soil regions, formed a monophyletic clade with *Microseira wollei*, a nascent genus separated from *Lyngbya.* MT192753 *Plectonema* sp. KAS collected from similar habitats and having exactly similar morphology have been placed in *the Lyngbya* clade with 98% similar sequences. As per convention, more than 98% 16SrDNA similarity can be considered the same species[46]. Thus, in our present study, morphological identification of freshwater *Lyngbya* sequences from high to low altitudes became congruent with molecular characterization based on ITS and 16S sequence data. Both morphological and molecular details support that all freshwater *Lyngbya* belong to the same species. However, a problem in identifying

*Plectonema* still needs to be resolved. Secondary structures (Table 6, Supplementary Figure 1) were also investigated, but no differences were noticed among the samples.

*Plectonema* was initially classified under Scytonemataceae[14,47] based on false branching formation, though no heterocyst was found. Genus *Lyngbya* was classified under Oscillatoriaceae by Geitler (1932), where the occasional occurrence of false branching was also noticed. Later on, the observation of other scientists inferred that false branching occurred facultatively in a tiny part of the population of *P. tomasinianum,* and the character was also overcredited, which should not be done because of its inconsistency[48–50]. Scarcity in the *P. tomasinianum* sequence in online databases led to the dilemma that existed for a long. In the present study, false branching in *Lyngbya* culture again confounded the generic discrimination between *Plectonema tomasinianum* and *Lyngya birgei.* Supporting the earlier workers, the current study also emphasized and proposed that this kind of plastic, pleomorphic character of cyanobacteria should not be considered as an autapomorphic character at the genus level.

## CONCLUSIONS

The study marks the first-ever attempt to use ITS-based phylogenetic analysis on Indian non-heterocystous filamentous cyanobacteria. The results of our research demonstrate the impressive discriminatory ability of the 16S-23S ITS region in identifying and distinguishing cyanobacteria from various habitats. Our study highlights the novelty of this approach and its potential for revolutionizing the field of cyanobacterial research. Our research shows that species clustering in the ITS-based phylogenetic tree is habitat-specific. For instance, marine species of *Leptolyngbya* form a monophyletic clade with other marine *Leptolyngbya* sp., while hotspring taxa form a separate monophyletic clade with other hotspring *Leptolyngbya* species. Moreover, the study revealed the polyphyletic nature of *Planktothrix* and *Phormidium* in the phylogenetic tree of Phormidiaceae. The ITS-based species clustering supported earlier 16S rDNA-based studies, highlighting the significance of the ITS region in species identification.

We present a novel approach to differentiate various species of *Lyngbya* by proposing 98% sequence similarity as a threshold percentage of ITS sequence. Furthermore, this study emphasizes the need to re-evaluate the taxonomic status of *Lyngbya birgei* and *Plectonema tomasinianum* as they appear highly similar based on 16S rDNA and ITS sequence-based analysis and cultural behavior study. Our study offers valuable insights into the complexities of identifying these species and calls for more research in this area.

**Supplementary Materials:** The following are available online at www.revistabionatura.com/xxx/s1,

Figure S1: Secondary structures of 16S–23S internal transcribed spacer (ITS) sequences from the *Lyngbya* sp.HDA, *Lyngbya* sp.RAB, *Plectonema* sp. KOP, *Plectonema* sp. KAS and *Plectonema* sp. AJY., Table S1: A partial similarity matrix (P distance) generated using 16S-23S ITS region (Family- Pseudanabaenaceae), Table S2-A partial similarity matrix (P distance) generated using 16S-23S ITS region (Family- Phormidiaceae), Table S3-A partial similarity matrix (P distance) generated using 16S-23S ITS region (Family- Oscillatoriaceae-genus *Oscillatoria*), Table S4-A partial similarity matrix (P distance) generated using 16S-23S ITS region (Family- Oscillatoriaceae-genus *Lyngbya*(marine)), Table S5-A partial similarity matrix (P distance) generated using 16S-23S ITS region (Family- Oscillatoriaceae-genus *Lyngbya-Plectonema*(freshwater)), Table S6-A partial similarity matrix (P distance) generated using 16S region (Family Oscillatoriaceae-genus *Lyngbya-Plectonema*)

**Author Contributions:** "Conceptualization, Sreemanti Banerjee and Ruma Pal.; methodology, Sreemanti Banerjee.; software, Sreemanti Banerjee; validation, Sreemanti Banerjee, Akanksha Singh and Ruma Pal; formal analysis, Sreemanti Banerjee; investigation, Sreemanti Banerjee, Akanksha Singh; resources, Sreemanti

Banerjee; data curation, Sreemanti Banerjee; writing—original draft preparation, Sreemanti Banerjee; writing—review and editing, Ruma Pal; visualization, Sreemanti Banerjee; supervision, Ruma Pal; project administration, Sreemanti Banerjee; funding acquisition, Sreemanti Banerjee and Ruma Pal. All authors have read and agreed to the published version of the manuscript."

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