

EDITORIAL

The 16S rRNA gene and cyanobacterial taxonomy; current problems and future prospects

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Abstract: Research into cyanobacterial diversity dates back to more than 150 years. Advancement in modern molecular, ultrastructural, ecophysiological and *in vitro* culture techniques broadened our understanding in the cyanobacterial diversity. Molecular data, especially 16S rRNA gene sequence provide basic criteria for present day cyanobacterial taxonomy. As more DNA sequence data become available it came into notice that morphology-based taxonomic classification is unreliable and it could not infer evolutionary relationships. Some strains belonging to the previously assembled taxa which were classified based on traditional morphological distinctness appeared phylogenetically unrelated when their 16S rRNA gene was sequenced. Therefore, this editorial note was written with the objective of highlighting the necessity in revising present system of cyanobacterial classification and importance in establishment of universal criteria for future taxonomic proposals for cyanobacteria. A cyanobacterial phylogenetic tree was reconstructed using past and present 16S rRNA sequence assemblages from the database and from our studies. Phylogenetic tree revealed polyphyletic origin of unicellular order Chroococcales and filamentous order Oscillatoriales. Strains in the genera *Pseudanabaena* from the present study were phylogenetically more distant from rest of the Oscillatoriales in the database and may have independently diverged from the common ancestor at an early stage in the evolution. On the other hand, two *Leptolyngbya* strains from the present study clustered with *Leptolyngbya* accessions from the database, although two strains shared only 89% sequence identity. It appears that those two strains could be distinct species belong to the genera of *Leptolyngbya* and each may have independent evolutionary history. This hypothesis was supported by distinct morphological characters shown in axenic cultures. Present study highlight the importance in understanding that molecular data alone could only provide insights into genetic variability and phylogenetic relatedness, but could not recognize phenotypic variability and their ecological importance and ongoing diversification of strains etc. Thus, construction of an accurate taxonomic classification system requires a 'polyphasic' approach that combines molecular data with phenotypic, biochemical and ecophysiological data. Also it is necessary to revisit all past assemblages of taxa available in the database in order to avoid future taxonomic mislabelling.

Keywords: *Cyanobacteria, Taxonomy, 16S rRNA, Phylogenetic tree, Polyphasic approach*

Cyanobacteria

Cyanobacteria (formerly known as blue green algae) are one of the most attractive organisms on the Earth's biosphere. They are ubiquitous on earth including extreme temperature, light, salinity, pH, desiccation and nutritional availability. Cyanobacteria are prokaryotic organisms. They

resemble bacteria by having the cell wall made up of peptidoglycan and resemble to eukaryotic algae and plants by having oxygenic photosynthetic apparatus with photosystem I and II and chlorophyll *a* as a photosynthetic pigment. Also, cyanobacteria contain additional light-harvesting pigments, phycocyanin and phycoerythrin. Morphologically, cyanobacteria are unicellular, colonial or filamentous. Majority of them are free-living while others associate with

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algae, fungi, bryophytes, pteridophytes and higher plants. Cyanobacteria lack sexual reproduction. Hence, their survival and diversification over billions of years from Precambrian era to present day is thought to be governed by asexual reproduction, horizontal gene transfer, homologous recombination along with rapid acclimation and adaptation to dynamic environmental conditions (Komárek & Kastovsky, 2003; Rudi et al., 1998; Willis & Woodhouse, 2020).

Taxonomy and classification of cyanobacteria

Classification of cyanobacteria is challenging due to their long and complex evolutionary history that had led to the convergence of morphotypes and high levels of cryptic diversity. The existing nomenclature of cyanobacteria was according to either Botanical Code or prokaryotic/bacterial code. The Botanical Code was used since cyanobacteria share similar metabolic features with eukaryotic algae whereas prokaryotic taxonomic scheme of bacteria was used since cyanobacteria are prokaryotic organisms. Over the time bacterial and Botanical nomenclature came into conflict. Therefore, cyanobacterial nomenclature was included under the rules of the International Committee on Systemic Bacteriology which is presently known as International Committee on Systematics of Prokaryotes in the International Committee on Systematic Bacteriology held in 2000 (Labeda, 2000; Tindall, 1999). However, taxa nomenclature in cyanobacteria is still a topic of discussion and there is no consensus on a species concept that can be universally applied to the cyanobacteria (Palinska & Surosz, 2014). Much of the discussions are focused on the criteria for demarcation of a new species.

Traditionally cyanobacteria were grouped into taxa based on their morphological traits. Later, with the advent of molecular biological techniques and high throughput sequencing technologies phycologists tend to use molecular data in cyanobacterial taxonomy and nomenclature. As a result, some members in the previously assembled taxa based on morphological traits appeared phylogenetically unrelated (Gugger & Hoffmann, 2004; Schirmer et al., 2011). They formed polyphyletic species,

genera and higher taxonomic categories (Komárek et al., 2014). Polyphyly is an indication of the taxonomic mislabeling of many taxa and necessitates revisiting into the early taxonomic grouping for a taxonomic revision. Discovery and availability of such information may undervalue previously published textbooks, book chapters, identification guides and keys, journal articles and presentations on cyanobacteria. However, fear of future revision should not prevent more DNA sequencing and phylogenetic studies. Therefore, aim of writing this editorial note is to discuss the importance of having accurate taxonomic classification through a combinatorial (polyphasic) approach that could involve morphological traits and molecular data.

Reconstruction of phylogenetic tree

The use of small subunit ribosomal RNA (rRNA) gene sequences in classification has revolutionized prokaryotic classification since 1970s. Currently, it has become the most sequenced gene having thousands of sequences deposited in public databases. Among the rRNAs, 16S rRNA sequence identity is the currently accepted “Gold-standard” in prokaryotic systematics since it’s presence in all prokaryotic genomes and possession of highly conserved 5’ and 3’ end regions. Those features enable application of ‘universal’ primers and obtaining a near complete gene sequence. The 16S rRNA sequence identity has also been used in cyanobacteria taxonomy. However, applicability of universal primers designed for bacterial 16S rRNA for cyanobacteria does not always give positive results due to low sequence homology in 16S rRNA between bacteria and cyanobacteria (Lane et al., 1985). Therefore, for the sequencing of cyanobacteria 16S rRNA, cyanobacteria-specific primers have been designed (Nübel et al., 1997).

In order to determine phylogenetic affiliations of cyanobacteria, a phylogenetic tree was reconstructed for 16S rRNA gene for cyanobacteria using at least 20 sequences of complete or near complete 16S rRNA sequences from order Chroococcales, Nostocales, Oscillatoriales and 4 sequences from Stigonematales from the NCBI/GenBank. In addition, 7 partial 16S rRNA sequences from axenic cultures

isolated from Lunugamvehera reservoir, Sri Lanka in March 2020 were also included. The 16S rRNA from axenic cultures were amplified by using the primers for 16S rRNA of cyanobacteria, CYA106F, CYA359F, CYA781R(a) and CYA781R(b) (Nübel et al., 1997). Their species or genus names were determined by conducting sequence similarity search (BLAST) in the NCBI database. A name was assigned by considering total score, query cover and E value and percentage identity. All sequences showed >99% sequence identity to existing accessions in the database. They were named as *Leptolyngbya*-LW1 (MW288946), *Leptolyngbya*-LW2 (MW288942), *Phormidium*-LW1 (MW288943), *Geitlerinema*-LW1 (MW288944), *Pseudanabaena lonchoides*-LW1 (MW288940), *Pseudanabaena*-LW2 (MW288948) and *Nostoc humifusum* (MW288939). Phylogenetic trees were constructed from the distance matrix data using the neighbor-joining (NJ) method. In order to evaluate the robustness of branches in the tree bootstrap resampling was conducted with 1000 replicates.

Assignment of an appropriate taxonomic order for each of the selected 78 sequences (both from the database and sequences from this study) for the construction of phylogenetic tree was a difficult task due to the existing inconsistency in cyanobacterial taxonomy. Therefore, taxonomic classification in the widely used and widely cited AlgaeVision (<http://algaevision.myspecies.info/>) web portal curated by the British National History Museum and The British Phycological Society was adopted.

Phylogenetic analysis of 16S rRNA gene sequence

Before the availability of molecular data, the two filamentous orders, Nostocales and Stigonematales were solely differentiated by a distinct morphological trait in Nostocales; formation of heterocysts. The reconstructed phylogenetic tree (Figure 1) revealed that all strains in the orders of Nostocales (I) and Stigonematales (II) were monophyletic, which implies that two taxa and all their descendants were originated from a recent common ancestor. Previous phylogenetic analyses have also revealed monophyletic origin of these two orders (Ishida et al.,

2001). Inclusion of more recently published accessions together with some earlier accession in the database in the present phylogenetic tree did not change previously observed monophyletic origin of the two orders. These results imply that morphology-based identification and differentiation of strains in the order Nostocales and Stigonematales appears to be consistent with the molecular phylogeny.

Previously it has been shown that the unicellular order Chroococcales and filamentous order Oscillatoriales are polyphyletic (Ishida et al., 2001) which means strains in Chroococcales and Oscillatoriales were not descended from common ancestors. The phylogenetic relations depicted in the reconstructed phylogenetic tree also supports polyphyletic nature of the two orders. Chroococcales formed two clusters. One containing the majority of strains (III) and the other strains formed a small cluster (IV). Interestingly, two Oscillatorial strains, *Leptolyngbya* and *Schizothrix* also clustered with Chroococcales (III and IV respectively). Those may have probably diverged recently in the evolution and provide evidence for the morphological diversification from unicellular to filamentous form or vice versa.

Oscillatoriales formed four clusters (V-VIII). Strains from the present study separated into two clusters (VII and VIII). *Leptolyngbya*-LW1 and -LW2 are in the same cluster with *Leptolyngbya* from the database. In contrast, *Pseudanabaena*-LW1 and LW2 did not cluster with strains of *Pseudanabaena* from the database. *Pseudanabaena*-LW1 clustered with *Phormidium* and *Geitlerinema* whereas *Pseudanabaena*-LW2 seemed to be phylogenetically more distant from rest of the Oscillatoriales and may have independently diverged from the common ancestor at an early stage in the evolution.

The distance matrix of strains from the present study showed that *Leptolyngbya*-LW1 and -LW2 shared 88.8% and *Pseudanabaena*-LW1 and LW2 shared 83.4%. Therefore, they share less than 97.5% identity with each other. In 1994, Stackbrandt et al. (Stackebrandt & Goebel, 1994) proposed that if the 16S rRNA sequence identity is less than 97.5%, such sequences could be recognized as separate species if any phenotypic separation exists. Accordingly,

Leptolyngbya-LW1 and -LW2 and *Pseudanabaena*-LW1 and LW2 could be distinct species belong to the genera of *Leptolyngbya* and *Pseudanabaena* respectively and each may have independent evolutionary history (Johansen & Casamatta, 2005).

In order to confirm the above assumption, morphological and culture characteristics were carefully investigated to see whether any phenotypic distinction could be identified.

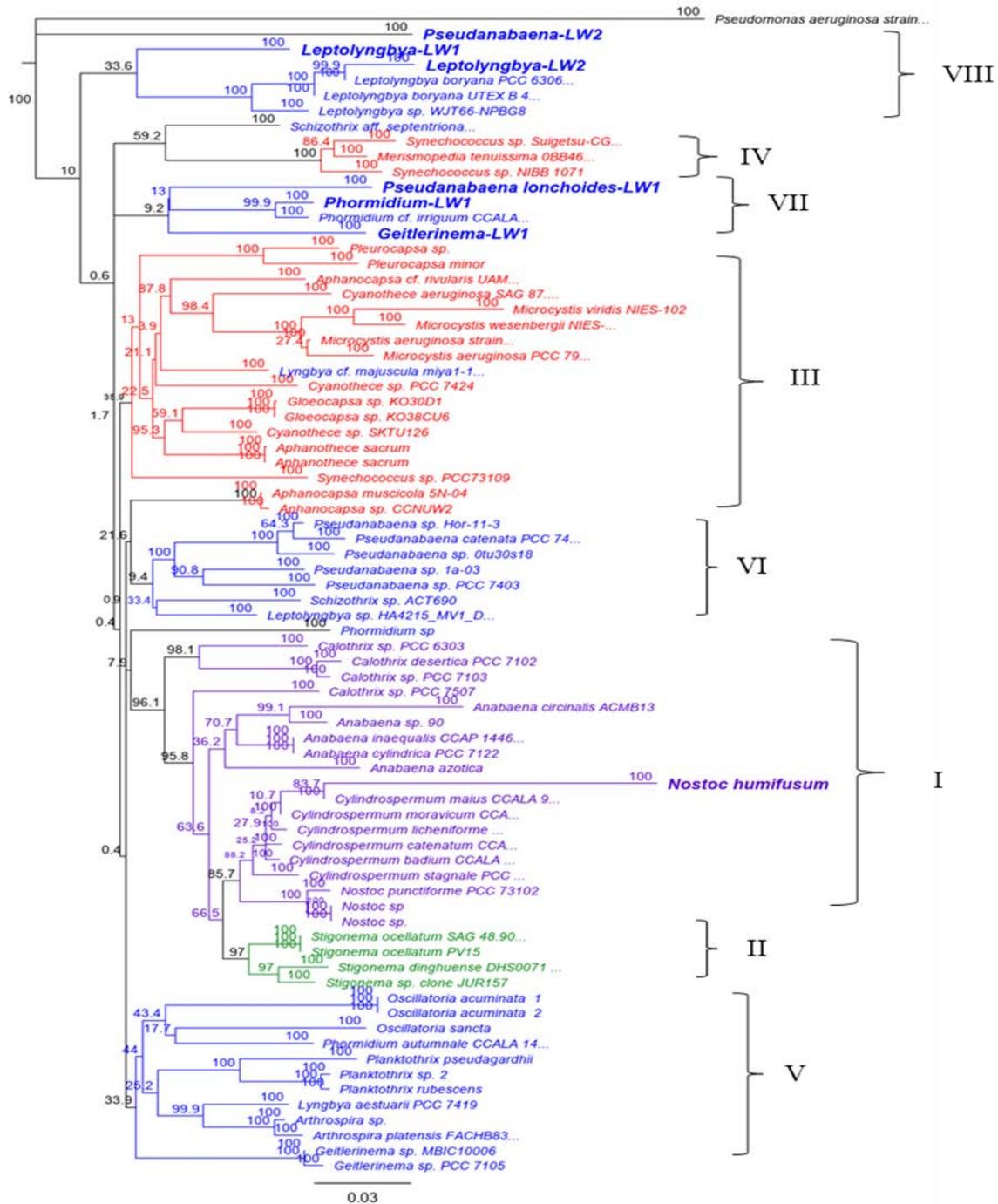


Figure 1: Phylogenetic neighbor-joining tree of 16S rRNA of strains in the cyanobacterial order Chroococcales (red), Nostocales (purple), Oscillatoriales (blue) and Stigonematales (green). *Leptolyngbya*-LW1 and -LW2, *Pseudanabaena lonchoides*-LW1, *Pseudanabaena*-LW2

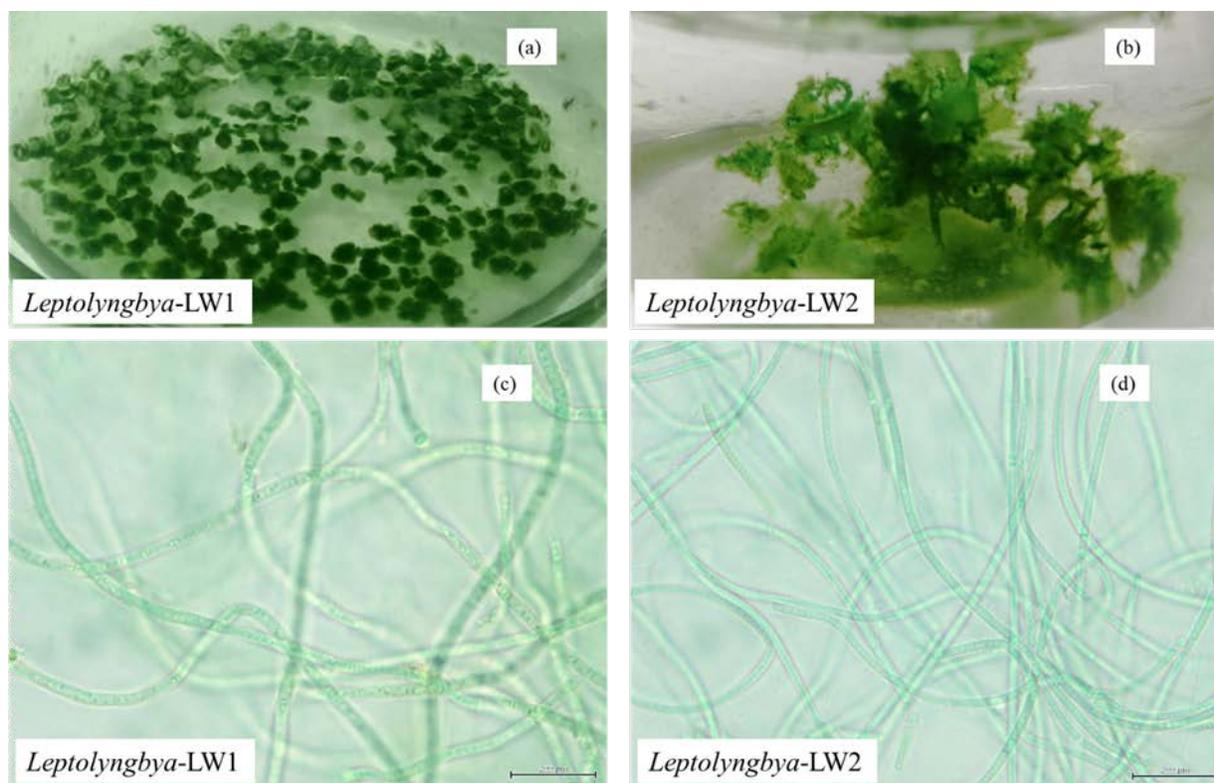


Figure 2: Distinct colony morphology of *Leptolyngbya*-LW1 (a) and -LW2 (b) in 2 months old axenic cultures grown in BG11 medium and their filaments (c) and (d) respectively with 1000x magnification.

Previous studies have shown that both genera are known to be problematic due to i) very small filamentous structure ($<3.5 \mu\text{m}$ width) and difficulty in differentiation in the light microscope ii) polyphyletic nature iii) consisting of strains previously identified as other Oscillatoriales and transferred to the genera of *Leptolyngbya* and *Pseudanabaena* (Abed et al., 2003; Wilmotte & Herdman, 2001). The colony morphology in axenic cultures of *Leptolyngbya*-LW1 and -LW2 showed distinct morphology (Figure 2a and 2b). *Leptolyngbya*-LW1 formed uniform bead-like clumps of many individual colonies whereas *Leptolyngbya*-LW2 formed irregularly shaped thin film of colonies suspended in the culture broth in 2 months old cultures grown in BG11 broth. Distinct culture morphology is an evidence for phenotypic separation (Albrecht et al., 2017; Berrendero et al., 2011). However, any morphological distinction in the filamentous structure could not be identified under the magnification of light microscope (Figure 2c and 2d). The axenic culture morphology and filamentous structure of *Pseudanabaena*-LW1 and LW2 exhibited

uniform features except the visual appearance of the pattern of pigmentation of the colonies (data not shown). The colonies of *Pseudanabaena*-LW1 appeared bluish-green whereas it was dark green in *Pseudanabaena*-LW2. It could probably be due to the ratio of phycocyanin: chlorophyll *a*. Therefore, additional evidences such as ultra-morphological and biochemical distinctness may be required for more accurate delineation of species. One should be careful when using a long laboratory-maintained cultures for taxonomic purposes. Because, during transferring of natural populations into culture media, differential responses can be expected depending on their adaptability/genetic mutations. Thus, growing cyanobacteria in culture media may lead to selection of morphological and ecophysiological modifications within the population which may not represent natural population (Komárek, 2006).

Concluding Remarks

The 16S rRNA gene sequence is the basis of modern classification of cyanobacteria. It is considered as one

of the major advancement in cyanobacteria taxonomy and has substantially replaced traditional morphology-based identification. On the other hand, morphology-based identification requires 'life-long' experience and time consuming. However, morphological diversification is the key feature that determines taxonomic distinction. Molecular data alone can only provide information on genetic variability and phylogenetic affiliations but could not recognize ecological importance of different phenotypes, ongoing diversification of strains etc. Therefore, in addition to the traditional morphological data and modern molecular data, ultrastructural morphological features, ecophysiological and biochemical properties that are combined in a polyphasic approach would support more accurate taxonomic classification of cyanobacteria.

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