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Genetic Diversity and Molecular Phylogeny of Cyanobacteria from Sri Lanka Based on 16S rRNA Gene

R.P. Wanigatunge[1], D.N. Magana-Arachchi[1†], N.V. Chandrasekharan[2], S.A. Kulasooriya[1]

[1]*Institute of Fundamental Studies, Hantana Road, Kandy 20000, Sri Lanka*
[2]*Department of Chemistry, University of Colombo, Colombo 00300, Sri Lanka*

ABSTRACT

The diversity of cyanobacteria in Sri Lanka was studied in different water reservoirs, paddy fields, brackish water and tsunami affected areas using light microcopy, 16S rRNA sequences, followed by phylogenetic analysis. Based on light microscopy, 24 genera were identified from environmental samples belonging to the orders *Chroococcales*, *Oscillatoriales*, *Pleurocapsales* and *Nostocales*. In cultures, 33 genera were identified from all five cyanobacterial orders, including *Stigonematales*. Based on 16S rRNA gene sequences and their morphology, two isolates were identified up to species level, 72 to genus level, one isolate up to family and 11 up to order level. Twelve isolates couldn't be assigned to any taxonomic level. The results of 16S rRNA gene sequences along with the phylogenetic analysis indicated that some cyanobacterial isolates could be accommodated to genus or order level. The 16S rRNA sequence analysis data in this study confirmed that order *Nostocales* and order *Pleurocapsales* cyanobacteria are monophyletic while orders *Chroococcales*, *Oscillatoriales* and *Stigonematales* cyanobacteria are polyphyletic. Polyphasic approach including the combination of light microscopy, cultures and the analysis of 16S rRNA gene sequences provide a promising approach to ascertain the diversity of cyanobacteria in different habitats.

Keywords: 16S rRNA gene, Boom's method, Neighbour joining tree, Phylogeny

1. Introduction

Cyanobacteria are the only known prokaryotes capable of oxygenic photosynthesis, and represent one of the oldest and the most widespread phylogenetic groups of bacteria [1]. They can be found in virtually all ecosystems on Earth including freshwater lakes and rivers, oceans, hot springs and deserts, ranging from the hottest to the cold dry valleys of Antarctic [2]. Cyanobacteria play key roles in global carbon and nitrogen cycles and are also considered as a promising resource for biofuels and variety of biotechnological applications [3]. Therefore, it is very important to have an accurate understanding of cyanobacterial diversity in order to explore their hidden wealth and potential for human welfare.

Traditionally, the classification of cyanobacteria has been formulated under botanical code based on phenotypic properties, but morphological features can often vary with changing environmental conditions [4] and also similar morphological features have arisen convergently in unrelated organisms which share the same environment [5]. Also culturing of morphotypes only provides little information on the composition of communities because of selective culturing conditions [6]. Therefore, much more effort has been directed towards assessing the cyanobacterial diversity using molecular data. 16S rRNA gene sequencing provide valuable criterion for the identification of cyanobacteria up to genus level [7]. A sequence similarity limit of 95% was established as being intergeneric between cyanobacterial genera [8]. However, it is essential to combine genetic data with morphological diversity and variation, ecological and ecophysiological characters and ultrastructural information to construct comprehensive phylogenetic system for cyanobacteria [9].

Sri Lanka has a wide range of topographic and climatic variation and this contributes to its high level of biodiversity. The study conducted by Abeywickrama et al. [10] described about 170 species of cyanobacteria belonging to 45 genera. According to a study of Richerson et al. [11], cyanobacterial biodiversity is declining and some toxigenic cyanobacterial species are becoming dominant due to the anthropogenic activities. Therefore it is important to investigate cyanobacterial biodiversity for formulating strategies for conservation of economically significant species. Most of the identification of cyanobacterial diversity in Sri Lanka has relied on morphological description. These classical approaches identified *Microcystis aeruginosa* and *Spirulina* species as the major bloom forming cyanobacteria in the Beira Lake [12]. Previous mor-

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† Corresponding author
E-mail: dmaganaarachchi@gmail.com
Tel: +94-812232002 Fax: +94-812232131

phological studies have also revealed the presence of dense thick blooms of *M. aeruginosa* from the Kotmale reservoir ("Kotmale Bloom") in 1991, Mahaweli basin [13] and Kuda Sithulpahuwa in the Menik Ganga basin [14]. More detailed investigations conducted by researchers [15] revealed the predominance of toxic cyanobacteria in freshwater bodies in Sri Lanka. A few studies have applied 16S rRNA gene sequences in studying the cyanobacterial diversity in some reservoirs in Sri Lanka [16, 17]. However, the molecular data available for cyanobacteria are still scarce in Sri Lanka. Thus, the present study was specifically focused on determining the cyanobacterial composition in different localities in Sri Lanka by classical culturing and culture-independent techniques along with the partial sequences of 16S rRNA gene. Furthermore, attempt was made to employ 16S rRNA gene to explore phylogenetic relationships among cyanobacteria belonging to five orders of cyanobacteria recognized by Castenholz and Waterbury [18] (corresponding to sections I- V of Boone and Castenholz [19]) isolated from different localities.

2. Materials and Methods

2.1. Sampling and Sample Sites

In order to comprehend the cyanobacterial diversity in different localities, sampling sites were selected to represent wet, intermediate, dry and arid climatic zones and also which covered the areas of the country including urban, rural and agricultural regions. Water samples were collected into sterilized brown glass bottles or sample collecting bottles from selected freshwater reservoirs, lakes, rivers, and brackish water of Sri Lanka (Fig. 1(a)). In addition soil samples from paddy fields were also collected (Fig. 1(b)). Sediment samples from water bodies were also collected into sterile brown glass containers (2.5 L) using a hand corer (Wildco 2424-B; USA). The samples used for molecular analysis were stored at -20°C. Cyanobacterial diversity in tsunami affected areas was also conducted using previously collected soil and water samples mixed with tsunami waves in 2004 (Fig. 1(c)).

2.2. Isolation and Culture Conditions

Isolation and cultivation of cyanobacteria were performed using BG11 [20], $BG11_0$ [21] and ASN111 medium. Samples were grown in both solid and liquid medium. Cultures were incubated in an incubator (Biogene, India) at 28 ± 2°C with fluorescent light with intensity of 4.8×10^{-4} $cm^{-2}W - 5.9 \times 10^{-4}$ $cm^{-2}W$ at a 16:8-h D/L cycle.

2.3 Standard Cyanobacterial Strains

The standard strains, *Lyngbya* (PCC 8937) a filamentous strain and *Microcystis aeruginosa* (PCC 7941) a unicellular strain were obtained from the Pasteur Culture Collection (PCC), France were used in this study.

2.4. Microscopic Observation

The morphological classification of the environmental and cultured samples was performed using observable characters under a compound light microscope (BH2; Olympus, Japan); (400–1,000×). The taxonomic assignments of morphotypes were based on the descriptions of Desikachary [22], Waterbury and Stanier [23], Komárek and Anagnostidis [24], Holt et al. [25], Komárek and Anagnostidis [26], Boone and Castenholz [19] and Komárek and Anagnostidis [27].

2.5. Genomic DNA Extraction

DNA extractions were carried out for cultured samples and environmental samples using standard techniques and nucleic acids were purified by Boom's method [28] using silica particles and guanidium isothiocyanate.

2.6. PCR Amplification of 16S rRNA Gene

The 16S primers, CYA359F and CYA781Ra/ CYA781Rb [29] were used for amplifying and sequencing the 16S rRNA gene for subsequent phylogenetic analyses.

2.7. Sequencing

PCR products were cleaned with GenElute™ Gel Extraction Kit

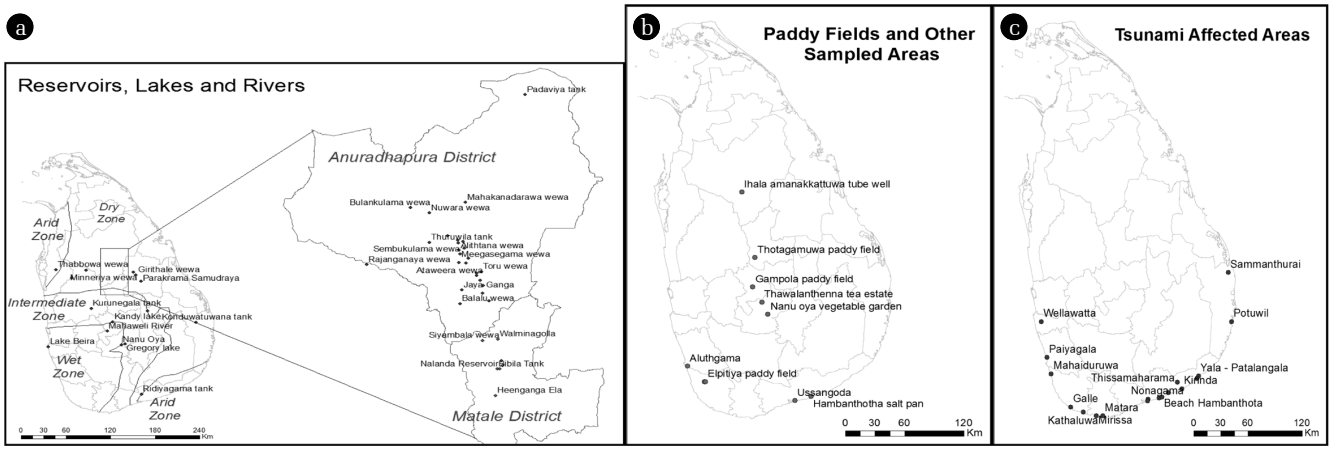

Fig. 1. (a) The map of the geographical locations of sampling sites of freshwater reservoirs, lakes and rivers, (b) The map of the geographical locations of sampling sites of paddy fields and other sampled areas, (c) The map of the geographical locations of sampling sites of tsunami affected areas.

(Sigma, Chemical Co., USA) according to the manufacturer's instructions and then directly sequenced at a commercial facility (Macrogen Inc., South Korea).

2.8. Phylogenetic Sequence Analysis

All the sequence data obtained were analyzed using DNA sequencing software programme BioEdit 7.0.9 [30]. To corroborate the cyanobacterial origin of sequenced samples, a BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) [31] was performed using the program "blastn" against the "Nucleotide collection (nr/nt)" database. Sequences generated in this study were deposited in GenBank at National Center for Biotechnology Information (NCBI) database.

Phylogenetic trees were constructed for cyanobacterial 16S rRNA gene sequences of cyanobacteria reported in this study and cyanobacterial reference sequences obtained from GenBank at NCBI to elucidate the taxonomic positions of cyanobacteria belonging to five orders and unclassified cyanobacterial isolates. 16S rRNA gene sequences were aligned using the ClustalW multiple sequence alignment tools in Version 4.0 of MEGA ANALYSIS software [32]. Pair-wise distances were calculated using the Jukes-Cantor method [33] and phylogenetic trees were constructed for the samples using the neighbour-joining [34] method using p-distance. Bootstrap analyses were performed with 1,000 replicates and only bootstrap percentages above 50 were shown at the branch nodes of phylogenetic distance trees. *Gloeobacter violaceus* PCC 7421 strain (NR_074282) was used as out-group taxon.

3. Results and Discussion

The work presented here was initiated to characterize the cyanobacterial composition of the selected natural habitats throughout Sri Lanka using a phylogenetic approach together with morphological data. The selected water reservoirs are frequently used by people for drinking, bathing, recreational activities, fishing and some are the main water sources for irrigation. Thus, there's a high risk of human health from exposure to cyanobacteria and their toxins during their routing life style. Better understanding of cyanobacterial composition of water reservoirs will enable to make decisions on surface-water management and drinking water treatment and thereby minimizing human health risks.

3.1. Isolation and Morphological Identification of Cyanobacteria

Considering the distribution of cyanobacterial morphotypes, 24 genera was observed in environmental samples representing the orders of Chroococcales, Oscillatoriales, Pleurocapsales and Nostocales. Genus *Microcystis, Chroococcus, Synechocystis, Synechococcus, Gleocapsa, Merismopedia, Coelosphaerium, Xenococcus, Dermocarpa, Chroococcidiopsis, Oscillatoria, Leptolyngbya, Lyngbya, Phormidium, Plectonema, Spirulina, Limnothrix, Pseudanabaena, Anabaena, Scytonema, Nostoc, Cylindrospermopsis, Anabaenopsis* and *Calothrix* were dominant in environmental samples collected from different sites. In cultured isolates, 33 cyanobacterial genera were observed representing all five cyanobacterial orders, including the order Stigonematales. In addition to the genera observed in environmental samples genus *Gloeothece, Aphanocapsa, Aphanothece, Dermocarpa, Planktolyngbya, Tolypothrix, Hapalosiphon, Westiellopsis, Nodularia, Schizothrix, Chlorogloeopsis* and *Fischerella* were conspicuous in cultured samples.

Results obtained from the present study were comparable with the study conducted by Abeywickrama et al. [10] with some deviations. Though Abeywickrama et al. [10] had recorded 45 cyanobacterial genera, out of those 45 only 26 could be observed in the present study. However eight additional genera namely *Gloeothece, Chroococcidiopsis, Leptolyngbya, Limnothrix, Planktolyngbya, Chlorogloeopsis, Nodularia* and *Cylindrospermopsis* were identified which were not described in the study of Abeywickrama et al. [10].

According to the results obtained from present study, the highest diversity of cyanobacteria was recorded from dry zone reservoirs. This result was also supported by a previous study of Perera et al. [35]. The lowest cyanobacterial diversity from the study was from arid zone water reservoirs but according to Perera et al. [35] the lowest cyanobacterial diversity was seen in the intermediate zone. The low cyanobacterial diversity seen in arid zone might be due to the low number of sampling sites used for the particular zone.

Microscopic observations of samples collected from Lake Beira and Lake Kandy revealed the presence of *Microcystis* species as dominant cyanobacterial genera. Further, these observations were supported by the microscopic observations of Silva and Samaradiwakara [36] and Hirimburegama [12], who reported the occurrence of cyanobacteria in waters of the Kandy Lake and Lake Beira. The diversity of cyanobacterial species in present study showed comparable observations to previous studies conducted by Magana-Arachchi et al. [16, 17], Magana-Arachchi and Liyanage [37] and Perera et al. [38].

According to observations, the genus *Cylindrospermopsis* was recorded only from dry zone lakes namely Kala wewa, Balalu wewa, Nachchaduwa wewa, Parakrama Samudraya, Minneriya wewa, Girithale wewa, Nuwara wewa, Padaviya tank and Jaya Ganga. However in another study, during the driest period in 2011, for the first time *C. raciborskii* was recorded from Lake Gregory which is located in the upland wet zone of Sri Lanka [35]. Therefore, more eco-physiological studies are required to explore the distribution of *C. raciborskii* in Sri Lanka.

Cyanobacteria are the dominant microbial inhabitants of rice fields [39]. Considering the cyanobacterial diversity in paddy fields the present study frequently recorded the genera *Microcystis, Gleocapsa, Gloeothece, Leptolyngbya, Phormidium, Plectonema Pseudanabaena, Anabaena* and *Calothrix* representing three cyanobacterial orders namely, order Chroococcales, Oscillatoriales and Nostocales. These observations were well supported by the previous studies of other researchers [40-42].

Considering the cyanobacterial diversity of salt pans of Hambanthota, genus *Synechococcus, Lyngbya, Leptolyngbya, Oscillatoria, Phormidium, Schizothrix* and *Nostoc* were recorded as dominant cyanobacteria. There have been no previous records of cyanobacterial diversity in salt pans of Sri Lanka, but the results obtained in this study was comparable with previous studies conducted in other countries [43-45].

3.2. Identification of Cyanobacterial Isolates using 16S rRNA Gene

Amplification of a part of the 16S rRNA gene using the known

cyanobacterial specific oligonucleotide primers CYA359F and CYA781Rb and / CYA781Ra yielded the unique fragment of ~450 bp. Analysis of DNA samples obtained from environmental and cultured samples gave positive amplification with both CYA781Ra and CYA781Rb reverse primers and revealed the presence of cyanobacteria in tested water reservoirs, lakes, rivers, brackish waters, paddy fields and tsunami affected areas. These cyanobacterial specific primers have been successfully used for studying diversity and dynamics of populations in different ecosystems [46, 47]. Based on recommendation given by Boutte et al. [48] the cyanobacterial specific reverse primers CYA781Ra and CYA781Rb were used separately in the study to determine the cyanobacterial community composition in mixed cultures and environmental water samples.

PCR products that showed significant reproducibility after duplicate analysis with CYA359F and CYA781Rb or CYA781Ra were selected for further assessment. In order to assess the genetic diversity of cyanobacteria (n= 98), partial sequencing of the 16S rRNA gene was carried out. The obtained sequences showed similarity to previously reported cyanobacterial sequences ranging from 93% to 100% (Supplementary Table 1).

Among the identified isolates, 18 isolates were belonging to order Chroococcales, 49 to order Oscillatoriales, ten to order Pleurocapsales, five isolates to order Nostocales and three isolates were belonging to the order Stigonematales. Based on these results, order Oscillatoriales cyanobacteria could be considered as the versatile species since they were distributed throughout the studied areas. Based on 16S rRNA gene sequences and with their morphological features, two isolates were identified up to species level, 72 isolates up to genus level, one isolate up to family and 11 isolates up to order level. Twelve isolates could not be assigned to any taxonomic level. Three unclassified cyanobacteria (EU310425, EU310423 and EU283868) did not show any sequence similarity to previously recorded sequences of known isolates recorded either from Sri Lanka or from other parts of the world. Therefore, the 16S rRNA sequences of such species were deposited in GenBank either as filamentous cyanobacterial species or unicellular cyanobacterial species taking into account their morphology. These cyanobacterial isolates could be considered as novel cyanobacterial genera. Nine other unclassified cyanobacterial isolates (GU967422, GU300773, GU815988, GU368104, GU815983, KF650428, GU594038, GU594037 and GU594033) showed sequence similarity ranging from 94%-100% previously recorded cyanobacteria. But either considering their morphology or 16S rRNA gene sequences these isolates could not be assigned to any taxonomic level. Unicellular cyanobacterium DPW7 (GU967422) showed 97% sequence similarity with previously recorded *Schizothrix arenaria* HA4233-MV5 (JN385286.1). But morphology of this isolate did not match with genus *Schizothrix* and therefore it was deposited in NCBI database as a unicellular cyanobacterium DPW7. Further, unicellular cyanobacterium N2a (GU368104) isolated from Lake Gregory demonstrated 94% sequence similarity to *Oscillatoria acuminata* NTDM04 (GU812860.1). However morphological features did not correspond to genus *Oscillatoria* and could not be assigned into an exact genus. Therefore further studies with complete 16S rRNA sequences are necessary to resolve the phylogenetic positions of these cyanobacterial species.

In this study the genus *Chroococcidiopsis* was isolated from different localities. However there were no previous records about this genus from Sri Lanka except one record [49] or could have been misidentified as different genera or was unexamined.

The 16S rRNA results obtained from the present study clearly recognized that there is a significant diversity in cyanobacteria in the country and some of these cyanobacterial isolates have not been characterized previously.

3.3. Phylogeny of Order Chroococcales Cyanobacteria

The order Chroococcales isolates were separated into nine distinct clusters (1-9) and one separate branch of *Cyanothece* sp. TW1 (GU815989) (A) (Fig. 2).

Most of the taxa of order Chroococcales, whose partial 16S rRNA gene sequences were aligned in this study, were homogenously clustered. However some *Microcystis* species and Chroococcales cyanobacterial isolates were heterogeneously clustered. Previous 16S rRNA studies have shown that polyphyletic nature of orders Chroococcales cyanobacteria [50]. According to the phylogenetic analysis results *M. aeruginosa* bl (EF051239) isolated from Lake Beira clustered with *M. wesenbergii* VN212 (AB666077) without any evolutionary distance but with a very low bootstrap value. Previous studies too have shown that there is no phylogenetic resolution among species of *Microcystis* within a clade [51].

Five, order Chroococcales cyanobacterial isolates obtained from present study, clustered in cluster 4. Based on 16S partial sequences, Chroococcales cyanobacterium YR5 (EU310421), Chroococcales cyanobacterium K14 (EU283867), Chroococcales cyanobacterium N1a (GU368107) and Chroococcales cyanobacterium N16 (GU368109) could be assigned only to the order Chroococcales, without characterizing them up to the genus level. The utility of the 16S rRNA sequence was limited in defining of genera within order Chroococcales. Even though it was difficult to determine the taxonomic and phylogenetic position among the species based on the 16S rRNA gene sequences, *Merismopedia* Ku1 isolated from present study formed monophyletic relationship with other *Merismopedia* species, thereby confirming it as *Merismopedia glauca*.

Cyanothece sp. TW1 (GU815989) formed a single branch (A) with a 0.02001 evolutionary divergence from *Synechococcus* species in cluster 7 suggesting that this isolate is more similar to *Synechococcus* species. However partial 16S rRNA gene sequence of *Cyanothece* sp. TW1 (GU815989) showed 99% sequence similarity to *Cyanothece* sp. PCC 7425 (CP001344).

3.4. Phylogeny of Order Oscillatoriales Cyanobacteria

The order Oscillatoriales isolates were separated into seven distinct clusters (1-7) and two separate branches of *Leptolyngbya* sp. J4 (EU310428) (A) and uncultured *Oscillatoria* sp. clone N5b (KF650426) (B) (Fig. 3). *Leptolyngbya* sp. J4 (EU310428) formed a single branch (A) with 0.4934 evolutionary divergence from *Leptolyngbya* species in cluster 1 and 2 suggesting that this isolate could be belonging to a different *Leptolyngbya* species. Uncultured Oscillatoria sp. clone N5b (KF650426) formed a single branch

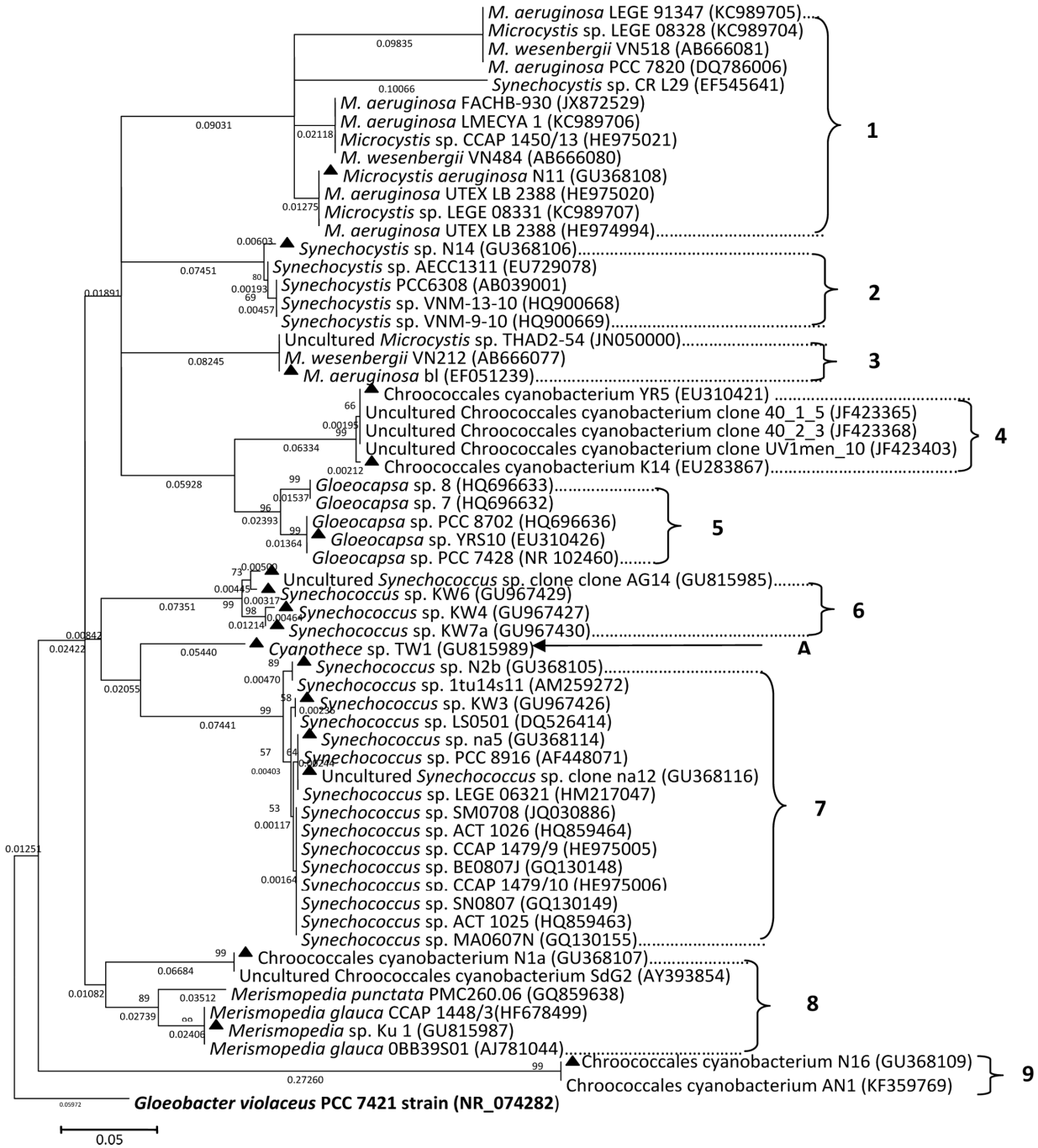

Fig. 2. Topology of 16S rRNA gene sequences of order Chroococcales isolates obtained from this study and closely related sequences from the National Center for Biotechnology Information (NCBI) database, constructed by means of neighbour-joining method. Bootstrap values above 50% calculated from 1,000 re-sampling were placed on the node. The scale bar represents five base substitutions for 100 nucleotide positions. The obtained sequences in this study are indicated by black triangle and an out group taxon is in bold font.

(B) with an evolutionary divergence of 0.20887 from other order *Oscillatoriales* cyanobacteria. Therefore this isolate should be represented in a different *Oscillatoria* species.

Association between morphology and phylogenetic position of the isolated Oscillatoriales cyanobacterial strains in this study were well supported with previous literature [9]. Komárek [9] showed that most of the traditional genera in order Oscillatoriales cyanobacteria have proven to be heterogeneous and consequently in need of revision. According to the phylogenetic analysis, the polyphyletic nature of order Oscillatoriales cyanobacteria including

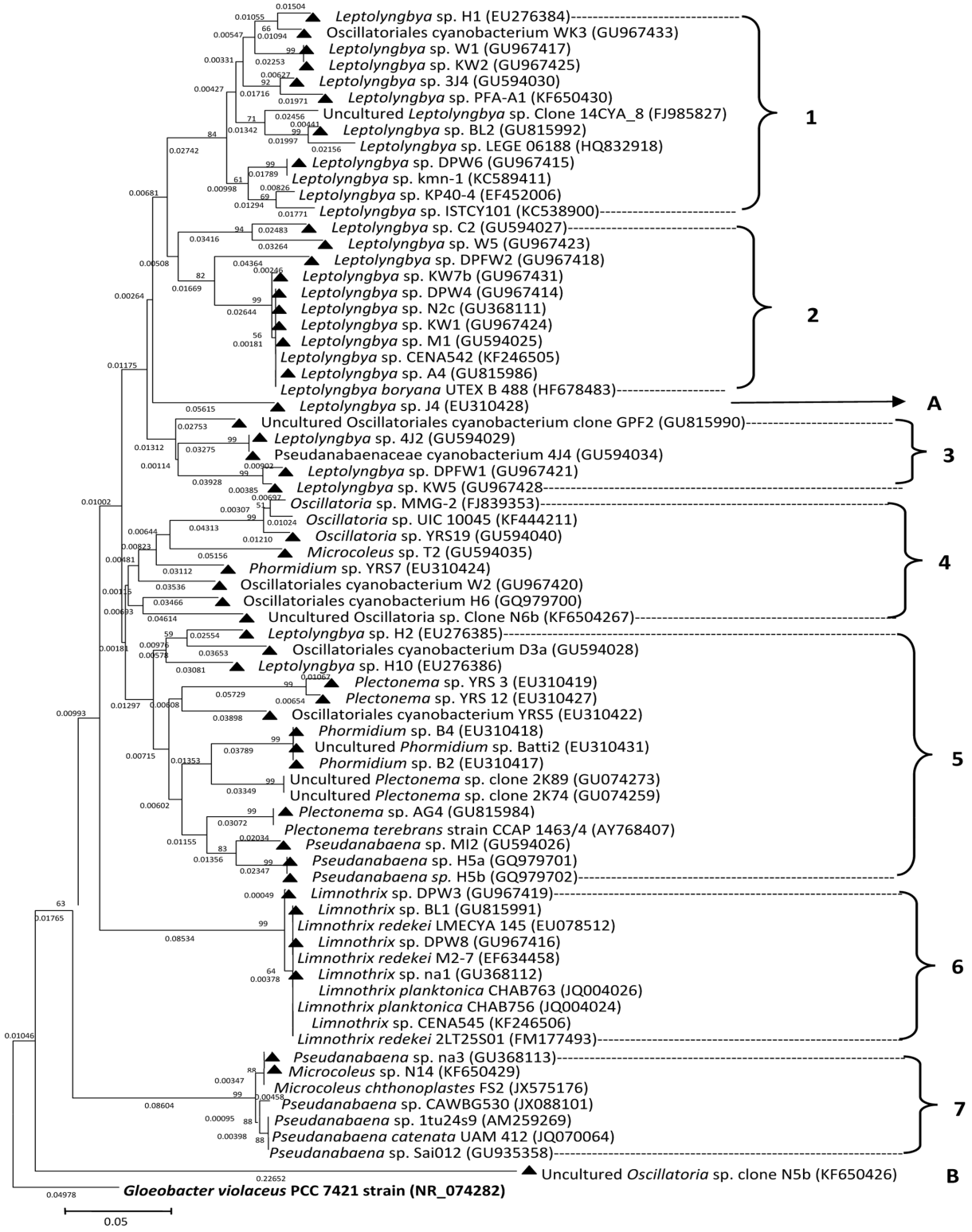

Fig. 3. Topology of 16S rRNA gene sequences of order Oscillatoriales isolates obtained from this study and closely related sequences from the National Center for Biotechnology Information (NCBI) database, constructed by means of neighbour-joining method. Bootstrap values above 50% calculated from 1,000 re-sampling are placed on the node. The scale bar represents five base substitutions for 100 nucleotide positions. The obtained sequences in this study are indicated by black triangle and an out group taxon is in bold font.

genus *Leptolyngbya, Oscillatoria, Pseudanabaena, Plectonema, Microcoleus* and *Phormidium* was confirmed. Previous studies have also shown the polyphyletic nature of order Oscillatoriales cyanobacteria based on the 16S rRNA gene sequences [52, 53].

The partial 16S rRNA gene phylogeny of the order Oscillatoriales allowed the identification of order Oscillatoriales cyanobacterium WK3 as *Leptolyngbya* sp. WK3, order Oscillatoriales cyanobacterium D3a as *Leptolyngbya* sp. D3a, order Oscillatoriales cyanobacterium YRS5 as *Plectonema* sp. YRS5 family Pseudanabaenaceae cyanobacterium 4J4 as *Leptolyngbya* sp. 4J4. Also some cyanobacterial isolates were identified up to species level using 16S rRNA phylogenetic analysis. *Leptolyngbya* species DPW4, N2c, KW1, M1 and A4 were identified as *Leptolyngbya boryana* and *Plectonema* sp. AG4 as *Plectonema terebrans* AG4. However these cyanobacterial isolates were indistinguishable using only morphological characters and each of these groups separated into distinct clusters during phylogenetic analysis. The overall phylogenetic tree generated from order Oscillatoriales sequences suggested that several very similar morphotypes may belong to distinct genera. For instance, Oscillatoriales cyanobacterium D3a showed very similar morphological characters to Oscillatoriales cyanobacterium YRS5. But both were separated into two distinct genera namely *Leptolyngbya* and *Plectonema* during the 16S rRNA gene sequence phylogenetic analysis. The differences between morphotypes and genotypes may result from the variation induced during culturing [54], or differential selection may favor the maintenance of different genotypes in the population. Thus, all the morphological characteristics at the genus level were not always significant. Therefore molecular methods are needed for the classification of this morphologically indistinguishable group.

3.5. Phylogeny of Order Nostocales Cyanobacteria

The order Nostocales isolates were separated into four distinct clusters (1- 4) (Fig. 4). Cluster 1 represents cyanobacterial strains belonging to genus *Anabaena, Nostoc* and *Calothrix* with a 51 % bootstrap value. Cluster 2 consisted of *Anabaena* and *Nostoc* isolates, supported by a bootstrap value 81%. Both cluster 1 and 2 were well supported by a 100% bootstrap value. Cluster 3 consisted of genus *Cylindrospermopsis* and *Raphidiopsis* cyanobacterial isolates and with a 100% bootstrap value. Cluster 4 was consisted of genus *Calothrix* supported with a 100% bootstrap value.

According to the results obtained for the phylogenetic analysis, all isolates showed monophyletic nature of order Nostocales cyanobacteria. Previous studies have also shown that heterocystous cyanobacteria consistently form a monophyletic cluster among cyanobacteria on the basis of their 16S rRNA gene sequences [55].

3.6. Phylogeny of Order Stigonematales Cyanobacteria

The order Stigonematales isolates were separated into four distinct clusters (1-4) (Fig. 5). Cluster 1 composed of cyanobacterial strains belonging to genus *Fischerella, Westiellopsis, Nostochopsis* and *Hapalosiphon* and cluster 2 was composed of genus *Westiellopsis* and *Hapalosiphon*. Both clusters were supported with a 100% bootstrap value.

According to the present study, some cyanobacterial isolates were identified up to species level using 16S rRNA phylogenetic analysis. *Chlorogloeopsis* sp. WWS1 was identified as *Chlorogloeopsis fritschii* WWS1. Isolates of order Stigonematales subjected to 16S rRNA phylogenetic analysis showed the polyphyletic nature of this group. A study by Gugger and Hoffmann [55] showed that 16S rRNA data of Stigonematales strains were polyphyletic and could be separated into at least two major groups based on the branching pattern. The first group was characterized by T-branching and the second group by Y-branching.

3.7. Phylogeny of Order Pleurocapsales Cyanobacteria

The order Pleurocapsales isolates were separated into two distinct clusters (1-2) (Fig. 6). According to the present study, order Pleurocapsales cyanobacteria showed a monophyletic nature. However, their polyphyletic nature was discussed in a study by Ishida et al. [56]. But the monophyletic nature of order Pleurocapsales seen in this study might be due to limited taxon sampling. Similar results have been shown for section V cyanobacteria due to limited taxon sampling by others [57].

In 16S rRNA analyses the phylogenetic position of *Chroococcidiopsis* is unresolved. In the present study both *C. thermalis* and *C. cubana* clustered within the same cluster while isolated strains in the present study could not be assigned to particular species. Maximum parsimony analysis places the single small subunit rRNA sequence available for *C. thermalis* strain PCC 7203 (isolated from soil) close to the *Nostoc* group [58] whereas *C. thermalis* clusters with *Microcoleus chthonoplastes* (*Oscillatoria* group) in neighbor-joining analyses. It is anticipated that sequence analyses of additional species of *Chroococcidiopsis* are needed to assess its phylogenetic position among the other unicellular terrestrial cyanobacteria.

3.8. Phylogeny of Unclassified Cyanobacteria

The nine partial 16S rRNA sequences obtained for unclassified cyanobacteria from this study and 52 previously sequenced 16S rRNA sequences representing five orders from NCBI database were used to construct the neighbour-joining tree (Fig. 7).The analyzed isolates were separated into three distinct clusters (1- 3) (Fig. 7) and one branch representing unicellular cyanobacterium K3 (GU594033). Uncultured cyanobacterium AP1 (GU300773) and unicellular cyanobacterium DPW7 (GU967422) clustered with 69% bootstrap value with a 0.02161 evolutionary distance and could be distantly related cyanobacterial genera. Unicellular cyanobacterium K3 (GU594033) formed a single branch with 0.10825 evolutionary distances with cluster 1 and therefore representing different cyanobacterial genera or species.

The 16S rRNA sequences of nine unclassified cyanobacterial isolates were compared with known cyanobacterial sequences in GenBank to deduce their phylogenetic position. According to the results obtained from present study, uncultured cyanobacterium clone Pa1 (GU815988) could be identified up to genus level as *Synechococcus*. Further, uncultured cyanobacterium clone PFB-A2 (KF650428) identified in this study were identified up to genus level as *Leptolyngbya*. Similarly, unicellular cyanobacterium N2a (GU368104) could be categorized up to order level as Chroococcales cyanobacterium. According to the positions of these cyanobacteria in the phylogenetic tree, morphological features could be correlated to the 16S rRNA sequences of above isolates. Therefore

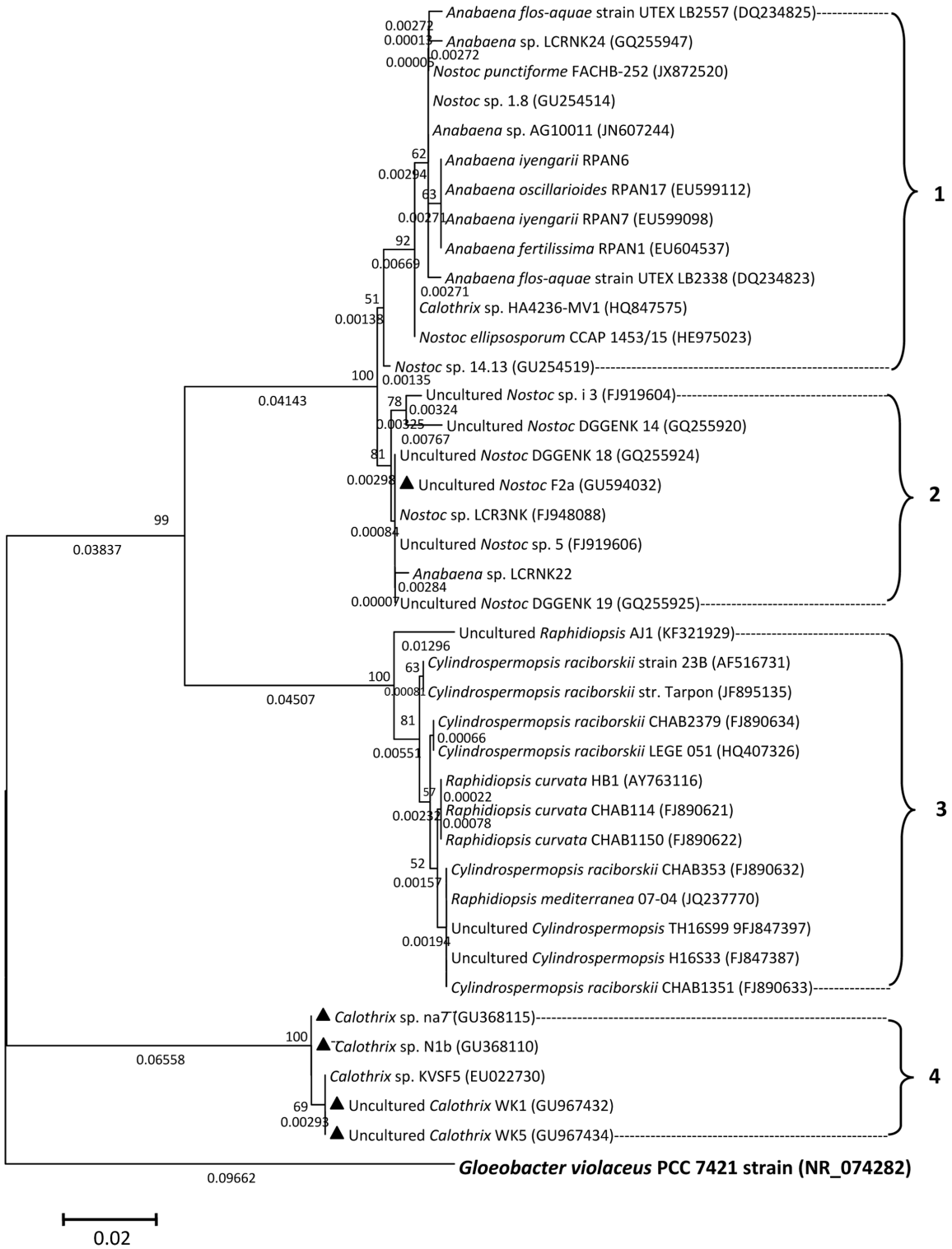

Fig. 4. Topology of 16S rRNA gene sequences of order Nostocales isolates obtained from this study and closely related sequences from the National Center for Biotechnology Information (NCBI) database, constructed by means of neighbour-joining method. Bootstrap values above 50% calculated from 1,000 re-sampling are placed on the node. The scale bar represents two base substitutions for 100 nucleotide positions. The obtained sequences in this study are indicated by black triangle and an out group taxon is in bold font.

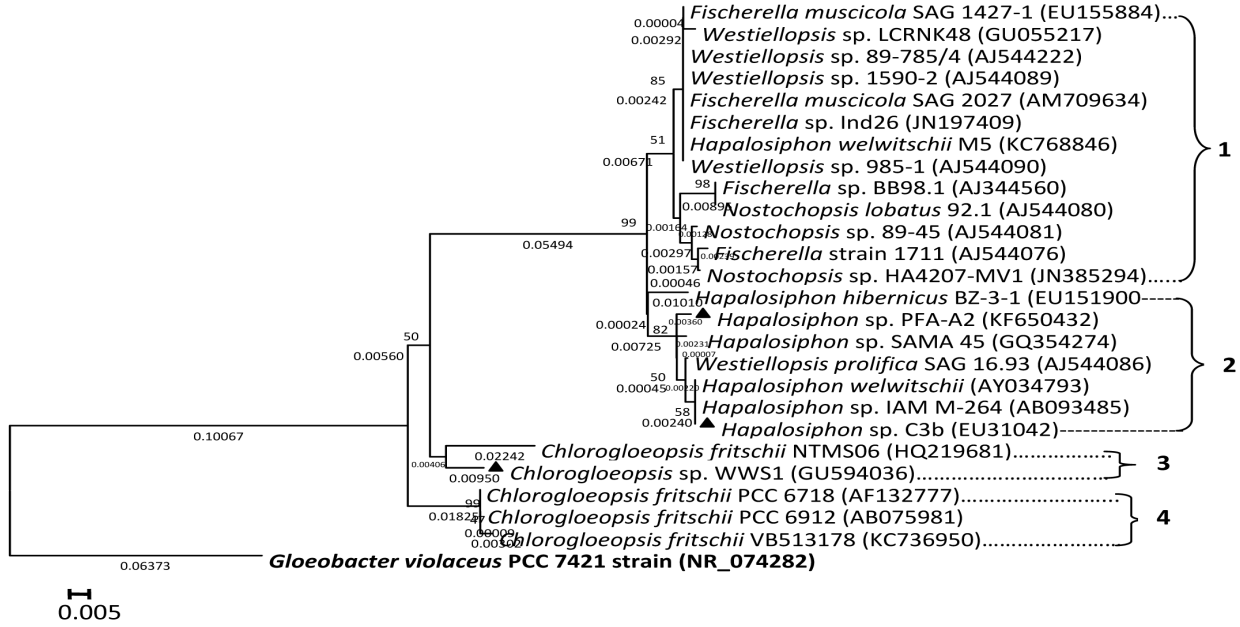

Fig. 5. Topology of 16S rRNA gene sequences of order Stigonematales isolates obtained from this study and closely related sequences from the National Center for Biotechnology Information (NCBI) database, constructed by means of neighbour-joining method. Bootstrap values above 50% calculated from 1,000 re-sampling are placed on the node. The scale bar represents five base substitutions for 100 nucleotide positions. The obtained sequences in this study are indicated by black triangle and an out group taxon is in bold font.

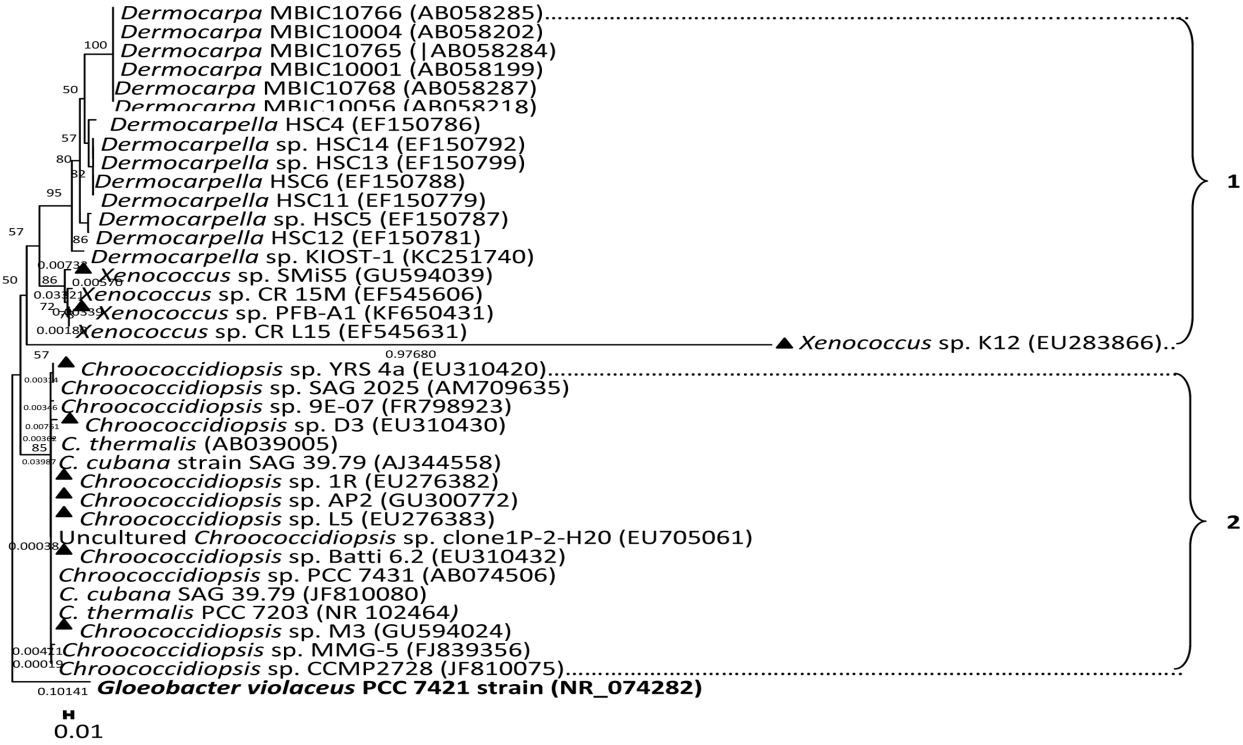

Fig. 6. Topology of 16S rRNA gene sequences of order Pleurocapsales isolates obtained from this study and closely related sequences from the National Center for Biotechnology Information (NCBI) database, constructed by means of neighbour-joining method. Bootstrap values above 50% calculated from 1,000 re-sampling are placed on the node. The scale bar represents one base substitution for 100 nucleotide positions. The obtained sequences in this study are indicated by black triangle and an out group taxon is in bold font.

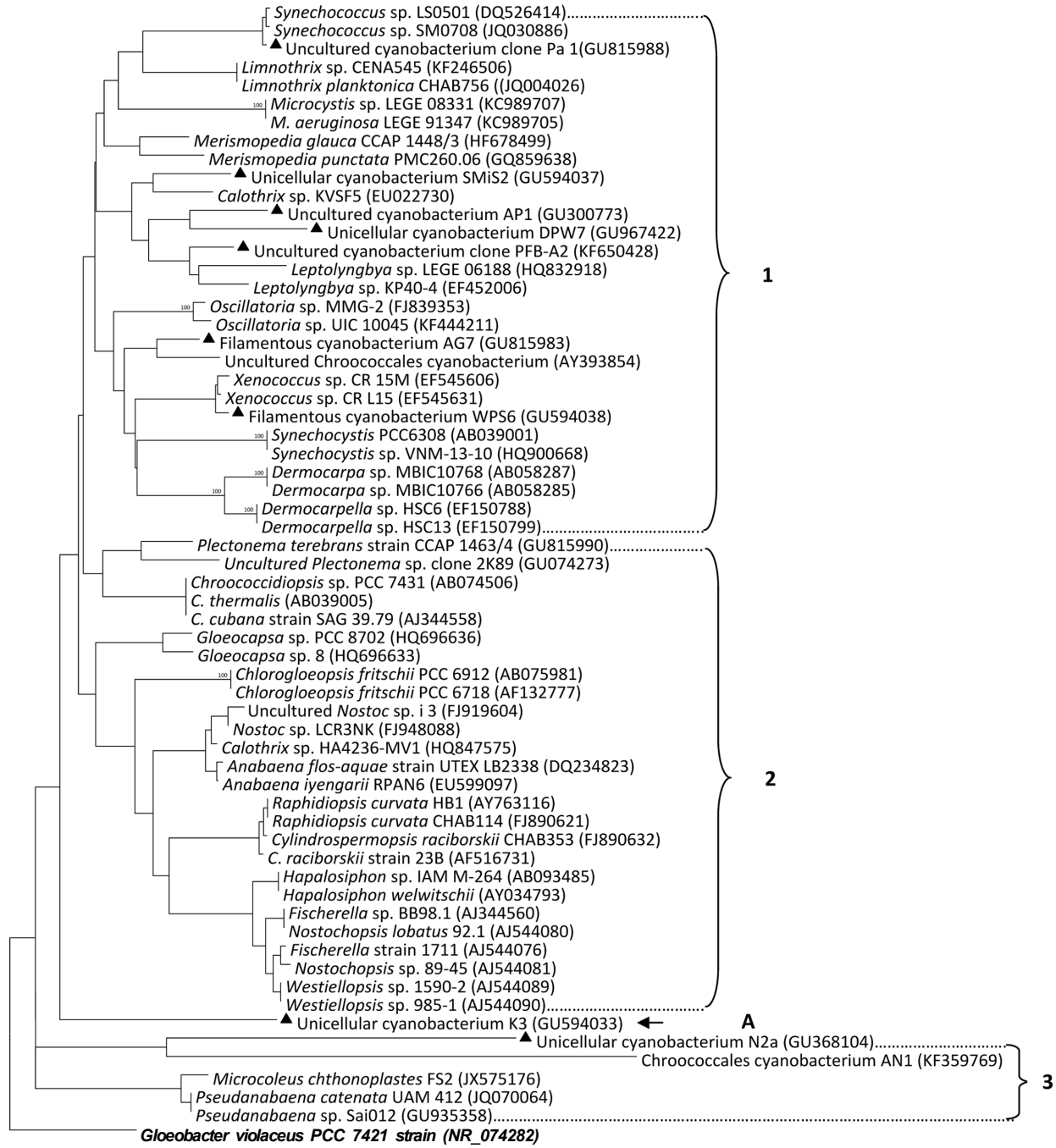

Fig. 7. Topology of 16S rRNA gene sequences of unclassified cyanobacterial isolates obtained from this study and cyanobacterial sequences representing five orders from the National Center for Biotechnology Information (NCBI) database, constructed by means of neighbour-joining method. Bootstrap values above 50% calculated from 1,000 re-sampling are placed on the node. The scale bar represents two base substitutions for 100 nucleotide positions. The obtained sequences in this study are indicated by black triangle and out group taxon is in bold font.

these results confirmed the usefulness of 16S rRNA gene as a valuable tool for identification of cyanobacteria up to order or genus level. Previous studies by several authors have proven this phenomenon. The 16S rRNA gene has also been used for the classification of cyanobacterial strains that belonged to a single clade [51, 59]. However, the taxonomic resolution offered by 16S rRNA genes is insufficient for discrimination of closely related organisms. Consequently, research has increasingly focused on the rRNA 16S to 23S internal transcribed spacer (rRNA-ITS) region. rRNA-ITS sequences are promising tools for comprehensive analysis of cyanobacteria since its greater degree of sequence heterogeneity and availability of considerable number of published data [60].

Some of the results obtained for phylogenetic analysis of unclassified cyanobacteria were incongruent with their morphology. Morphologically identified unicellular cyanobacterium SMiS2 (GU594037) clustered with a *Calothrix* species, filamentous cyanobacterium AG7 clustered with an uncultured Chroococcales cyanobacterium while filamentous cyanobacterium WPS6 (GU594038) clustered with Xenococcus species. These results may be due to misidentification of cultured isolates or altered morphology during growth under different culture conditions [61].

The results obtained from this study have contributed greatly to the knowledge of cyanobacterial diversity in Sri Lanka in terms of morphology and their phylogeny based on 16S rRNA sequences. In addition, the successful isolation of cyanobacteria in culture has also expanded the knowledge of the physiology of these unique organisms, and provides a basis for further work. However, more phylogenetic studies are needed with other molecular marker genes to confirm the phylogenetic position of previously unidentified cyanobacterial isolates.

5. Conclusions

Considering the distribution of cyanobacterial morphotypes, 24 genera was observed in environmental samples representing the orders of Chroococcales, Oscillatoriales, Pleurocapsales and Nostocales. In cultured isolates, 33 genera were observed for all five cyanobacterial orders, including Stigonematales. Ninety eight cyanobacterial isolates were identified using 16S rRNA gene sequences and were deposited in NCBI database. Eighteen isolates belonged to order Chroococcales, 49 to Oscillatoriales, ten to Pleurocapsales, five to Nostocales and three to Stigonematales. The 16S rRNA sequence analysis data in this study confirmed that order Nostocales and order Pleurocapsales cyanobacteria are monophyletic group while orders Chroococcales, order Oscillatoriales and Stigonematales cyanobacteria are polyphyletic.

Conflict of Interest

The authors declare that they have no conflict of interest.

Supplementary information

Supplementary data to this article can be found online at http://eeer.org/journal/view.php?number=665. Table 1. Results of blast analysis of 16S rRNA gene sequences of cyanobacterial isolates.

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