Phylogenetically distant clade of Nostoc-like taxa with the description of Minunostoc gen. nov. and Minunostoc cylindricum sp. nov

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Abstract: Two cyanobacterial strains morphologically identified to the genus Nostoc were isolated from a wet rocky wall in a mid-subtropical region in China, and they were taxonomically and phylogenetically characterized based on the polyphasic approach combining morphological and genetic characteristics. 16S rRNA gene sequence analysis showed that the two strains containing six clones were all >99.6% similar to each other, but had < 94.3% similarities to the existing cyanobacterial genera. The phylogenies based on 16S rRNA and rpoC1 gene sequences indicated that their sequences grouped into a unique and robust cluster with high bootstrap values. This unique cluster was separated from the clade of the ‘Nostoc sensu stricto’ and the respective clades formed by the morphologically similar genera Mojavia, Desmonostoc, Aliinostoc, Komarekiella and Halotia. The 16S–23S rRNA ITS secondary structure of the both strains exhibited the unique pattern of D1–D1’, Box–B and V3 helix, distinguishing it from the other heterocytous genera. Such a clear cluster leads to the establishment of Minunostoc gen. nov., with the type species as Minunostoc cylindricum sp. nov.

Key words: Cyanobacteria, Minunostoc, New genus, Polyphasic approach, Taxonomy

INTRODUCTION

Cyanobacteria are one of the most important and the oldest primary producers capable of oxygenic photosynthesis, found in nearly all environments from polar to tropical areas (Whitton & Potts 2000). Given the ubiquity of the distribution and the enormous importance of cyanobacteria in the biosphere, creation of a clear and comprehensible taxonomic system of cyanobacteria is highly needed. Cyanobacteria have been traditionally classified using morphological criteria, however this approach showed to be potentially problematic owing to high phenotypic plasticity occurring in the same species or at different growth stages under culture (Casamatta et al. 2003; Dvořák et al. 2012; Hašler et al. 2012; Shalygin et al. 2017). The systematics and taxonomy of cyanobacteria have been undergoing substantial changes by employing molecular markers, most notable as the 16S rRNA gene and Internal Transcribed Spacer (ITS) regions (Johansen & Casamatta 2005; Hašler et al. 2012; Dvořák et al. 2014; Hašler et al. 2014). As a result, some filamentous cyanobacterial groups are found to be polyphyletic and sometimes intermixed with unicellular forms (Lyra et al. 2001; Hoffmann et al. 2005; Rajaniemi et al. 2005a), strongly implying the need for taxonomic revision. Therefore, phylogenetic analyses based on DNA sequences have become the basis for restructuring cyanobacterial systematics at different categories, and led to the establishment of new groups, such as new families including Tolypothrichaceae and Godleyaceae (Hauser et al. 2014), Aphanizomenonaceae (Komárek et al. 2014), Dapisostemonaceae (Hentschke et al. 2016), Oculatellaceae and Trichocoleaceae (Maï et al. 2018). Furthermore, a variety of novel cyanobacterial genera, particularly heterocytous groups, were proposed, such as the Dolichospermum (Wacklin et al. 2009), Sphaerospermopsis (Zapomělová et al. 2009), Chrysosporum (Zapomělová et al. 2012), Cyanocoloniella (Kástovský et al. 2014), Roholtiella (Bohunicka et al. 2015) and Dactylothamnos (Komárek et al. 2015).

Although great improvements have been made in the modern classification of cyanobacteria, there still exist some groups whose taxonomic revisions are needed. The Nostocaceae is among one of the most morphologically complex cyanobacteria, and this family includes unbranched heterocytous cyanobacteria with the ability to
produce akinetes. Several genera, *Anabaena, Trichormus* and *Wollea*, are arguably polyphyletic, and partial taxonomic revision in these genera involved combination and separation among some taxa (Rajaniemi et al. 2005a; Kozhevnikov & Kozhevnikova 2011, Kust et al. 2015; Kust et al. 2018). *Nostoc*, the type genus of this family, is probably the most problematic one since it contains widespread distributed cyanobacterial types. Phylogenetic results have shown that *Nostoc* is polyphyletic (Rajaniemi et al. 2005a, b). Recently, several novel genera, *Majavia* (Réháková et al. 2007), *Desmonostoc* (Hrouzek et al. 2013), *Halotia* (Genuário et al. 2015), *Komarekiella* (Hentschke et al. 2017), *Aliciostoc* (Bagchi et al. 2017) and *Compactonostoc* (Cai et al. 2018), which morphologically appears to be *Nostoc*-like, have been proposed and separated from the ‘*Nostoc sensu stricto*’ clade based on the phylogenetic analysis of the 16S rRNA gene sequences. Over 300 species of the genus *Nostoc* have been described and many of which are not identifiable according to modern taxonomic system and need further investigation and revision. It is expected that more new taxa will be proposed from this polyphyletic genus in the near future (Komárek et al. 2014).

The purpose of this study is to present two novel strains of cyanobacteria isolated from soil habitat from a mid–subtropical region, China. These two novel strains were morphologically similar to *Nostoc*, however, molecular analyses on the 16S rRNA gene, 16–23S internal transcribed spacer (ITS) region and rpoC1 gene revealed these two strains to form a separate clade from *Nostoc*. Accordingly, we described here a new genus of filamentous cyanobacteria, *Minunostoc*.

**Material and Methods**

**Sampling and cultivation.** Samples were collected in October 2014 from the Small Seven–hole Scenic Area (25°15'34.79", 107°42'46.72"E), Guizhou province, China. Visible growths of cyanobacteria on a wet rocky wall were collected with the ladle. Using the micropipette washing method to isolate the heterocystous filaments into unialgal cultures, two strains of *Nostoc*-like cyanobacteria were obtained and cultured in screw–capped tubes containing BG11 medium. The strains were kept at 25 °C under a 12h: 12h LD cycle with a photon flux density of 30 μmol.m−2.s−1 from white fluorescent lamps. The formation of heterocysts in the strains was evaluated in the laboratory. The strains were maintained in the Harmful Algae Biology Herbarium (CHAB) of the Institute of Hydrobiology, China, and coded as CHAB 5843 and CHAB 5844–1 respectively.

**Morphological characterization.** Morphological observation was carried out using a Nikon Eclipse 80i microscope (Nikon, Japan). Trichome size was measured from ≥50 individuals using a Nikon eclipse 80i light microscope with DS–Ri1 digital camera (Nikon, Japan). The image was analyzed using the NIS–Elements D 3.2.

**DNA extraction and PCR amplification.** Unialgal cultures were harvested and the total genomic DNA was extracted using the modified cetyltrimethylammonium bromide (CTAB) method adopted by Neilan et al. (1995). The primers PA and B23S (Edwards et al. 1989; Gkakis et al. 2005) were used for amplification of 16S rRNA gene, the primers 322 and 340 (Iteeman et al. 2000) were used to obtain 16S–23S internal transcribed spacer (ITS), and primers rpoC1–F (5′-GATTGGGAAATGYYCATTYGGYYAA–3′) and rpoC1–R (5′-CAGCCTTGGTCTTCCCACA–3′) were used for amplification of rpoC1 gene. PCR was performed in an MJ Mini Personal Thermal Cycler (Bio–Rad, Hercules, California USA), and the amplification program was carried out as follows: one cycle of 3 min at 94 °C; 30 cycles of 30 s at 94 °C, 30 s at 58 °C (30 s at 55 °C for ITS and rpoC1), and 1 min at 72 °C (30s for ITS and 20s for rpoC1) and then a final 5 min elongation step at 72 °C. The PCR amplification products were purified by QIAquick PCR purification columns (Qiagen, Germany) according to manufacturer’s manual and then cloned into the pMDTM18–T vector (TaKaRa, TaKaRa BioInc., Otsu, Japan). The cloning procedure employed by Sambrook & Russell (2001) was performed. Sequencing was carried out using an ABI 3730 Automated Sequencer (PerkinElmer, Waltham, Massachusetts USA).

**Phylogenetic analysis.** 16S rRNA and rpoC1 gene sequences obtained from *Minunostoc* and those related sequences of cyanobacterial strains cited from GenBank were used for phylogenetic analyses. All the sequences were aligned using CLUSTAL X version 2.0 (Larkin et al. 2007). The final phylogenetic trees were constructed using neighbor–joining (NJ), maximum likelihood (ML), and Bayesian inference (BI). The NJ analysis using Kimura–2 model upon default parameters with 1000 bootstrap replicates was run via MEGA software v7.0 (Kumar et al. 2016), the ML algorithms were performed using PAUP V4.0b10 (Swofford 2003) and Bayesian inference were analyzed with MrBayes 3.2.2 (Ronquist et al. 2012). The ModelTest 3.06 (Posada and Crandall, 1998) program was used to explore the sequence evolution model that fitted the dataset based on Akaike information criterion (AIC). The best–fit models, selected for the ML and BI analyses of the 16S rRNA gene and rpoC1 gene were GTR+I+G, the obtained consensus phylogenetic tree was edited with Tree View 1.6.6 (Page 1996). *Chroococcidiopsis thermals* PCC73023 and *Oscillatoria tenuis* CCAP 1459/4 were chosen as the outgroup taxon for two genes respectively. The obtained nucleotide sequences have been deposited in the GenBank database with accession numbers MH918062–MH918063, MK045294–MK045297, MH919979–MH919980 and MH992098–MH992099.

**RESULTS**

**Minunostoc F. Cai et R. Li gen. nov.**

**Description:** Colonies macroscopic, irregularly clustering, gelatinous, free–living on wet rocky wall, blue–green. Filaments flexuous, freely entangled, sheath usually present, colourless. Vegetative cells barrel–shaped, and more or less longer than wide. Heterocytes and akinetes not known.
Type species: *Minunostoc cylindricum* F. Cai et R. Li

**Etymology:** The name of genus ‘*Minunostoc*’ was chosen due to the small cylindrical cells.

*Minunostoc cylindricum* F. Cai et R. Li sp. nov. (Fig. 1)

**Description:** Colonies are macroscopic, gelatinous, irregularly clustered, free–living on wet rocky wall in the form of irregular, amorphous, blue–green gelatinous clusters. Filaments flexuous, freely entangled. Sheath usually present, colourless. Vegetative cells barrel–shaped, 3.1–(4.3)–5.4 μm long, 2.5–(3.2)–3.8 μm wide, length: width ratio 1.3. Heterocytes and akinetes were not observed during 3 years of observation. In the BG11 and BG110 medium, the novel strains were never shown to form heterocysts. The strains did not grow in the BG110 medium, since the cultures started to bleach after 4 days of incubation in this medium.

**Holotype here designated:** Harmful Algae Biology Herbarium (CHAB) of the Institute of Hydrobiology, Wuhan, China. CHAB5843 and CHAB5844–1.

**Reference strains:** CHAB 5843, CHAB 5844–1.

**Type locality:** Small Seven–hole Scenic Area, Guizhou province, China (25°15′34.79″N, 107°42′46.72″E).

**Habitat:** Free–living on wet rocky wall.

**Etymology:** *cylindricum*, refereeing to the cylindrical cells of the strain.

**Molecular and phylogeny analysis**

The evolutionary distance based on the 16S rRNA gene sequences from 97 cyanobacterial taxa including *Nostoc* CHAB5843 and CHAB5844–1 resulted in the formation of 14 clusters (Fig. 2). Among these clusters, two strains shared 99.6–99.9% similarity with each other (Table 1), but all six clones had < 94.3% similarities with the closest genera (*Nostoc* 93.5%–94.1%, *Desmonostoc* 94.1%–94.2%, *Komarekiella* 94.2%–94.3%, *Halotia* 92.5%–92.6%, *Compstonostoc* 93.8%–93.9%, and *Aliinostoc* 92.5%–92.6%).

Based on 16S rRNA gene sequences from 97 cyanobacterial taxa including *Nostoc* CHAB5843 and CHAB5844–1 in this study, the phylogenetic trees were constructed using the NJ, ML, and Bayesian methods. The ML tree showed that two strains formed a distinctive lineage, clearly contrast to other taxa without this structure.

In the 16S rRNA gene phylogeny, the phylogenetic trees were constructed using the NJ, ML, and Bayesian approaches with high bootstrap values of 100%, 100%, and 1.00 respectively. Consistent with the phylogenetic trees, all novel strains had a pronounced, terminal loop (12 bp, Fig. 4N). Moreover, *Minunostoc* had two unidirectional bulges at the base of the stem, contrast to other taxa without this structure.

**Discussion**

Analyses on the secondary structures of D1–D1’ helix (Fig. 4) revealed similar arrangements in several genera. Compared with other taxa, *Minunostoc* CHAB5843 and CHAB5844–1 possessed a unique structure. All other taxa exhibited smaller terminal loops (3–6 bp, Fig. 4A–4M), whereas *Minunostoc* had a pronounced, terminal loop (12 bp, Fig. 4N). Moreover, *Minunostoc* had two unidirectional bulges at the base of the stem.

Shown in Fig. 5, the Box–B helix of *Minunostoc* consisted of 4 bp (GGAA) helix in the base of the stem, followed by a 5:3 base bilateral bulge, and then further followed by a 1:2 base bilateral bulge, and the terminal loop contained 3 bp bases (Fig. 5N), which was different from other taxa.

Although basal portion of V3 helix was conserved in sequences, the V3 of *Minunostoc* was also quite distinct from the V3 helix in other taxa (Fig. 6). In details, the V3 helix of *Minunostoc* consisted of very long stems (10 bp), followed by a 1:2 base bilateral bulge, and further followed by a 5 bp terminal loop (Fig. 6N).
Table 1. Comparison of the 16S rRNA gene sequence similarity among *Minunostoc* and its related taxa.

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Fig. 1. Micrographs of *Minunostoc cylindricum* under the light microscopy (LM). (A) general view of microcolonies; (B, C, G) colonies wrapped by diffuent mucilaginous sheath; (D, E, H) single trichomes; (F) single trichomes with sheath. Scale bars 10μm.
and H were phylogenetically divided. Cluster F consisted of sequences from the recently described genus Halotia, with its type species Halotia branconii isolated from biofilms in Antarctica and having tolerance of 1–10% NaCl. Cluster C consisted of two sequences from the genus Mojavia. Cluster G also contained sequences from the recently described genus Aliinostoc, which morphologically appears to be Nostoc–like, but phylogenetically separated from Nostoc sensu stricto. Cluster E contained the newly established genus Komarekiella. The core Nostoc cluster including the type species Nostoc commune was placed in cluster A, supported by bootstrap values of 98%, 88% and 1.00 (NJ, ML and Bayesian analyses). The sequences from the strains of the novel genus Minunostoc were placed in a unique cluster H, outside of the core Nostoc clade by NJ, ML and Bayesian analyses. 16S rRNA gene sequence similarities between Minunostoc and the closet genera were shown as less than 94.3%, below the bacterial genus cut–off in which a similarity limit of 95% was recommended as being intergeneric (Wayne et al. 1987; Stackebrandt & Goebel 1994; Stackebrandt & Ebers 2006). The rpoC1 sequence–based phylogeny was the same as that from the 16S rRNA gene sequences since Minunostoc strains were grouped into a single clade separating from the Nostoc groups.

The secondary folding patterns of the 16S–23S ITS were also very different from those of the closely related taxa within the Nostocaceae. D1–D1’, Box–B and V3 helix of Minunostoc were quite unique in comparison with those of Nostoc, Desmonostoc, Mojavia, Halotia, Komarekiella, Aliinostoc and Compactonostoc.
supporting the establishment of this new genus.

The genus Minunostoc will become the seventh genus separating from Nostoc after introduction of the polyphasic approach. However, the unique morphological features in the genus Minunostoc included absence of heterocytes and akinetes, and no small spherical colonies formation along the whole life cycle. By the detailed morphological comparison among Minunostoc and those genera recently separating from Nostoc including Mojavia, Desmonostoc, Halotia, Komarekiella, Alinostoc and Compactonostoc, Minunostoc differs from Mojavia, Halotia, Komarekiella and Compactonostoc since it does not form the aggregated filaments of microcolonies. Komarekiella have the typical type of germination of akinetes, originating a bigger vegetative cell and a smaller heterocyte as its first division. Both Desmonostoc and Minunostoc possess the same type of cell division in tri-

In conclusion, a few but unique morphological features shown in the filamentous cyanobacterial strains examined in this study, plus the strong molecular evidences based on 16S rRNA gene sequence homology, the phylogenetic relationship, and 16S–23S ITS secondary structures, it is strongly recognized that the novel genus of Minunostoc represents a new monophyletic group in the cyanobacterial systematics.

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Reference

Fig. 4. D1–D1’ helix in Minunostoc cylindricum and other heterocytous cyanobacteria: (A) Nostoc commune WY1–KK1; (B) Nostoc lichenoides CNP–AK1; (C) Nostoc punctiforme PCC73102; (D) Nostoc desertorum CM1–VF14; (E) Desmonostoc geniculatum HA4340–LM1; (F) Desmonostoc muscorum CENA18–D1; (G) Mojavia pulchra JT2–VF2; (H) Halotia branconii CENA392; (I) Komarekiella atlantica CCIBT3481; (J) Goleter apudmaren HA4340–LM2; (K) Trichormus anomalus HA4352–LM2; (L) Aliinostoc morphoplasticum NOS; (M) Compactonostoc shennongjiaensis CHAB5781; (N) Minunostoc cylindricum CHAB5843, CHAB5844–1.


Fig. 5. Box–B helix in *Minunostoc cylindricum* and other heterocystous cyanobacteria: (A) *Nostoc commune* WY1–KK1; (B) *Nostoc lichenoides* CNP–AK1; (C) *Nostoc punctiforme* PCC73102; (D) *Nostoc desertorum* CM1–VF14; (E) *Desmonostoc geniculatum* HA4340–LM1; (F) *Desmonostoc muscorum* CENA18–D1; (G) *Mojavia pulchra* JT2–VF2; (H) *Halotia branconii* CENA392; (I) *Komarekiella atlantica* CCIBT 3481; (J) *Goleter apudmare* HA4340–LM2; (K) *Trichormus anomalus* HA4352–LM2; (L) *Aliinostoc morphoplasticum* NOS; (M) *Compactonostoc shennongjiaensis* CHAB5781; (N) *Minunostoc cylindricum* CHAB5843, CHAB5844–1.


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Fig. 6. V3 helix in Minunostoc cylindricum and other heterocytous cyanobacteria: (A) Nostoc lichenoides CNP–AK1; (B) Nostoc commune WY1–KK1; (C) Nostoc punctiforme PCC73102; (D) Nostoc desertorum CM1–VF14; (E) Desmonostoc geniculatum HA4340–LM1; (F) Desmonostoc muscorum CENA18–D1; (G) Mojavia pulchra JT2–VF2; (H) Halotia branconii CENA392; (I) Komarekiella atlantica CCIBT 3481; (J) Goleter apudmare HA4340–LM2; (K) Trichormus anomalus HA4352–LM2; (L) Aliinostoc morphoplasticum NOS; (M) Compactonostoc shennongjiaensis CHAB5781; (N) Minunostoc cylindricum CHAB5843, CHAB5844–1.

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