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# Description of two new species of *Nostoc* from China based on the polyphasic approach

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**Abstract:** The present study described two new *Nostoc* species, *Nostoc favosum* (CHAB5709, CHAB5713, and CHAB5714) and *Nostoc mirabile* (CHAB5756 and CHAB5784) based on the polyphasic approach combining morphological, genetic and ecological characteristics. Five investigated strains were found to possess morphological features of the *Nostoc* genus. Results showed that the 16S rRNA gene sequences of these five strains displayed ≥ 95%, and ≤ 98% similarity to the genus *Nostoc*. The 16S rRNA gene phylogenetic analysis inferred using bayesian inference, maximum–likelihood and neighbour–joining methods placed these five strains on two separate nodes away from other *Nostoc* species. The 16S–23S rRNA internal transcribed spacer (ITS) secondary structure of two new species showed a unique pattern of D1–D1′, Box–B and V3 helix, which distinguished them from other *Nostoc* species. And the two species were further established by percent dissimilarity of ITS between another *Nostoc* species.

**Key words:** 16S rRNA gene, 16S–23S ITS, new species, morphology, polyphasic approach, taxonomy, *Nostoc*

## Introduction

Cyanobacteria are a morphologically and ecologically diverse group of photoprokaryotes found around the globe (Graham et al. 2008). The traditionally classification of cyanobacteria was mainly based on morphological characteristics (Komárek 2003; Taton et al. 2003, 2006; Johansen & Casamatta 2005; Turicchia et al. 2009; Genuário et al. 2013). But many cyanobacterial taxa are difficult to identify due to the lack of clear morphological characteristics and the vague or overly broad concept of modern genera. In recent decades, however, additional applications of molecular data using 16S rRNA gene sequences have greatly improved the classification and systematics of cyanobacteria (Robertson et al. 2001; Komárek 2006; Fiore et al. 2001; Silva et al. 2014; Bravakos et al. 2016). This progress in taxonomic research resulted in the description of many new cyanobacterial genera, which are based on phylogenetic analyses. However, there still exist several unsolved problems in genera established after molecular sequencing (eg. identification of new taxa by ecologists, who do not use molecular analyses for determination of cyanobacteria in nature) (Komárek 2020). In this situation, Komárek (2020) proposed that modern classification must be applied consistently the polyphasic approach with the phylogenetic classification as the basis, but it is necessary to combine and add all cytomorphological and ecological important data, and adequate use of nomenclature prescriptions.

Genus *Nostoc*, differed from other heterocytous cyanobacteria in that the trichomes with heterocytes and akinetes are embedded in colonial common mucilage. Despite being first reported in the 19th century with type species *Nostoc commune* Vaucher ex Bornet et Flahault (Bornet & Flahault 1888), its taxonomy remains complicated and problematic. To date, over 100 species of *Nostoc* have been described worldwide (Guiry & Guiry 2021), most of these species have not yet been sequenced and have rarely been reported in the literature. Also this taxonomically complicated genus require nomenclatural revision (Komárek 2013). Many of described *Nostoc* species may belong to other genera, but is waiting for the taxonomical revision (such as *Nostoc ellipsosporum* V, *Nostoc spongiaeforme* Ind42, *Nostoc verrucosum* KU005, *Nostoc insulare*

SAG 54.79, *Nostoc carneum* IAM M–35 and *Nostoc piscinale* CENA21 listed in NCBI database). In addition, *Nostoc* has repeatedly been proven to be genetically heterogeneous and polyphyletic (Hrouzek et al. 2005, 2013; Rajaniemi et al. 2005a; Papaefthimiou et al. 2008; Lukešová et al. 2009; Silva et al. 2014). With the advent of polyphasic approaches, some *Nostoc*–like genera have been separated from *Nostoc* in an attempt to make this genus less polyphyletic, including *Mojavia* (Řeháková et al. 2007), *Desmonostoc* (Hrouzek et al. 2013), *Halotia* (Genuário et al. 2015), *Aliinostoc* (Bagchi et al. 2017), *Komarekiella* (Hentschke et al. 2017) and *Desikacharya* (Saraf et al. 2019). A subsequent recent study that isolated several strains from rocky mountain in China exhibit *Nostoc*–like appearance. The investigation showed molecular distinction and the observed cyanobacteria have been assigned with new names as *Minunostoc*, *Compactonostoc*, *Purpureonostoc* and *Violetonostoc* (Cai et al. 2019a, 2019b, 2020a, 2020b).

The study of terrestrial *Nostoc*–like cyanobacteria in China was limited till recent years, during a broader survey on Chinese terrestrial *Nostoc*–like cyanobacteria, we have found and described several new genera and species in the family Nostocaceae (Cai et al. 2018, 2019a, 2019b, 2020a, 2020b). In this study, we isolated morphologically distinct heterocytous cyanobacteria from seldomly sampled regions in China, such as Baishan city in Jilin province and Shennongjia Forestry District in Hubei province. A polyphasic approach was employed to intensively study five selected strains (CHAB5709, CHAB5713, and CHAB5714, CHAB5756 and CHAB5784). Morphological features were observed and studied at different stage of their life cycle. Morphologically, we concluded them as *Nostoc*, and molecular analysis confirmed its position in *Nostoc* sensu stricto. Moreover, phylogeny of 16S rRNA gene, the secondary structure through sequencing of internally transcribed spacer (ITS) between 16S rRNA and 23S rRNA of the ribosomal genes, and percent dissimilarity of ITS were also determined herein to estimate their exact taxonomic status. Overall, the analyses of morphology and molecular data revealed that these five strains did not match any described species of *Nostoc*. These results allowed us to describe two new *Nostoc* species, as *Nostoc favosum* and *Nostoc mirabile*.

## Materials and Methods

**Sampling, isolation and culturing of strains.** Samples used in this study were isolated from two different localities of China having different climatic and geographical conditions. The strains CHAB5709, CHAB5713 and CHAB5714 were isolated from the edge of a stream (42°51.92'N, 127°78.94'E) in Baishan city, Jilin province, temperate climate in China. The strains CHAB 5756 and CHAB 5784 were collected from wet rocks in Shennongjia Forestry District (31°44.62'N, 110°30.92'E), located near the western border of Hubei province, a subtropical monsoon climate in China. Unialgal filaments from the cyanobacterial samples were isolated by lab–made Pasteur pipette under microscope (Olympus C31, Japan) and then cultured in screw capped tubes containing 6 ml of BG11 medium. All isolates were subsequently cultivated at 25 °C under a 12:12 h (light: dark) cycle with a photon flux density of 35 $\mu mol.m^{-2}.s^{-1}$ from white fluorescent lamps. The living culture were maintained in the Chinese Harmful Algae Biology (CHAB) culture collection of the Institute of Hydrobiology, China. And dry materials of strains were obtained by freeze–drying at –40 °C and stored at the Freshwater Algal Herbarium (HBI), Institute of Hydrobiology, Chinese Academy of Science, Wuhan, China.

**Morphological characterization.** All live cultures were examined with a Nikon Eclipse 80i microscope (Nikon, Tokyo, Japan) using its differential interference contrast microscopy. Microphotographs were analyzed with Nikon software NIS–Elements 3.2D (Nikon, Tokyo, Japan). Morphometric characteristics included length and width of vegetative cells, heterocytes and akinetes, as well as the size of macrocolonies and microcolonies (n > 50) were described using the Nikon Eclipse 80i microscope equipped with a Nikon DS–Ri1 digital camera.

**Molecular analysis.** Total genomic DNA was isolated from liquid cultures of unialgal cyanobacterial strains using Clarke's (Clarke 2009) method. The primers PA (Edwards et al. 1989) and B23S (Gkelis et al. 2005) were used to amplify the 16S rRNA gene. Primers 322 and 340 (Iteman et al. 2000) were used to obtain the 16S–23S ITS region. The PCR reaction contained 1 µl of genomic DNA (100 ng $ML^{-1}$), 0.5 µl of each primer (10 $\mu mol.l^{-1}$), 8 µl of sterile water and 10 µl of 2× PCR mix with Taq polymerase (Cat TSE001, Beijing Tsingke BiotechCo., Ltd., Beijing, China), in a final volume of 20 µl. PCR was performed in an MJ Mini Personal Thermal Cycler (Bio–Rad, Hercules, California USA), and the PCR cycle had initial denaturation at 94 °C for 3 min, followed by 34 cycles of 94 °C for 30 s, 58 °C for 30s (30 s at 55 °C for ITS), 72 °C for 1 min (30s for ITS), and a final 5 min elongation step at 72 °C. The PCR amplification products were purified using TSINGKE DNA Gel Extraction Kit (Cat GE0101–200, Beijing Tsingke Biotech Co., Ltd., Beijing, China) and then cloned into the pMDTM18–T vector (TaKaRa Bio Inc., Otsu, Japan) using the procedure of Sambrook & Russell (2001). All sequencing was carried out by the ABI 3730 Automated Sequencer (PerkinElmer, Waltham, MA, USA). The 16S rRNA and 16S–23S ITS gene sequences of the cyanobacterial strains CHAB5709, 5713, 5714, 5756 and 5784, isolated in this study were deposited in the NCBI GenBank database under accession numbers: MW649141, MW649142, MW649809, MW649810, MW649811, MW652791, MW652792, MW652793, MW652794, and MW652795.

**Phylogenetic analysis.** 16S rRNA gene sequences obtained in this study and those representing main groups of heterocytous cyanobacteria retrieved from GenBank were used for phylogenetic analyses with in total 184 sequences. Sequences using MAFFT v7.312 (Katoh & Standley 2013) with auto–selected strategy FFT–NS–I (with default parameters) and visually checked in mega v.7.0.14 (Kumar et al. 2016). The 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 were run via MEGA software X (Kumar et al. 2018). The BI was calculated with MrBayes

v3.2.6 (Ronquist et al. 2012) in the CIPRES Science Gateway V.3.3 (Miller et al. 2015, http://www.phylo.org/), in the BI analyses, two runs of eight Markov chains were executed for 8 million generations, sampling every 100 generations, with 25% of the sampled trees discarded as burn–in (the average standard deviation of split frequencies is 0.003). In the ML analyses, a total of 10000 bootstrap replicates were conducted to evaluate the relative support for branches by performing ultrafast bootstrap on IQ–TREE web server (Trifinopoulos et al. 2016). All phylogenetic tree consensus files were visualized in FigTree, v1.4.3 (Rambaut 2016) with *Chroococcidiopsis thermalis* PCC7203 as the outgroup. Calculation of p–distance with pairwise deletion of gaps was done with MEGA software v.7.0.14 (Kumar et al. 2016) and used to calculate sequence identity [100 × (1 – p)] for 16S rRNA data.

**16S–23S secondary structure analysis.** 16S–23S rRNA secondary structures of D1–D1′, Box–B, and V3 helices were determined using "RNAstructure", ver. 5.6 (Mathews Lab 2013). The sequences containing both $tRNA^{Ile}$ and $tRNA^{Ala}$ were used for all ITS analyses (except for *Nostoc neudorfense* ARC8, which has no $tRNA^{Ile}$ and $tRNA^{Ala}$). Percent dissimilarity based on 16S–23S ITS was calculated based on p–distance.

## Results

***Nostoc favosum* F. Cai et R. Li sp. nov. (Fig. 1)**
**Description**: Colonies spherical, start yellow green, and become to gray green. Filaments released by the colony rupture, covered with or without sheath, and the filaments densely entangled to form young colony. Within the colony, the filaments segmented into several small groups, and the small groups showing compartmentalization of mucilage, later to form small spherical colony. Consequently, the older colony consist of aggregations of microcolonies, just like a beehive. Eventually the older colony broken to release these small colonies. Sheath thick, colorless. Vegetative cells short barrel shaped to subspherical, or oblong, 2.7–3.1–3.8 μm long and 2.7–4.4–4.6 μm wide. Heterocytes subspherical, with a diameter of 2.8–4.3–4.9 μm. Akinetes not observed during years of cultivation.

**Reference strains:** The living culture were deposited in Collection of Harmful Algae Biology (CHAB), Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, Hubei Province, China as strain CHAB5709, CHAB5713 and CHAB5714.
**Type locality:** Isolated from the edge of a stream in Jilin province, China (42°51.92'N, 127°78.94'E).
**Holotype here designated:** Dry material of the strain CHAB5709 was stored at the Freshwater Algal Herbarium (HBI), Institute of Hydrobiology, Chinese Academy of Science, Wuhan, China, as specimen No. JLCN201401.
**Etymology**: *favosum* = "favose", refers to typical morphological characteristics of this species.
**Habitat:** Free–living on edge of a stream in the soil.

***Nostoc mirabile* F. Cai et R. Li sp. nov. (Fig. 2)**
**Description**: Thallus macroscopic in nature and in culture. In culture, forming macroscopic masses on test tube bottom in liquid media. On agar, macroscopic colonies, as aggregation of spherical colonies with distinct mucilage, which remain distinct throughout the whole life cycle. Macrocolonies dark green. Filaments with no sheath released from the colony, and later form distinct, colorless sheath, and terminal heterocyte outside of the sheath. Then the trichomes irregular, tightly entangled in the sheath, to form young long filamentous colony, the heterocytes appeared at the end of the colony. The sheath outside of the colony, shrank to separate the filamentous colony into smaller colonies. Later these smaller colonies connected by heterocyte, and eventually formed spherical colonies. Vegetative cells elliptic or spherical, 2.8–3.4–3.7 μm long and 3.0–3.5–3.9 μm wide. Heterocytes larger than vegetative cells, short barrel shaped that protrude from the population, subspherical within the colony. 3.3–3.9–4.2 μm long, 3.6–4.1–4.8 μm wide. Akinetes not observed during years of cultivation.

**Reference strains:** CHAB5756, CHAB5784, deposited in Collection of Harmful Algae Biology (CHAB), Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, Hubei Province, China.
**Type locality:** Isolated from wet rocks in Shennongjia Forestry District, Hubei province (31°44.62'N, 110°30.92'E).
**Holotype here designated:** Dry material of the strain CHAB5784 was stored at the Freshwater Algal Herbarium (HBI), Institute of Hydrobiology, Chinese Academy of Science, Wuhan, China, as specimen No. HBCN201402.
**Etymology**: *mirabile*, referring to unique morphological characteristics of this species.
**Habitat:** Free–living on wet rocky substrates.

**Molecular and phylogenetic analysis**
The 16S rRNA gene sequences of the studied strains were obtained and evaluated with BLAST analyses in NCBI. The evolutionary distance matrix based on 16S rRNA gene showed that three *Nostoc favosum* strains shared 99.3%–99.7% similarity with each other, but they shared 96%–98% similarities with other *Nostoc* species. Two *Nostoc mirabile* strains showed sequence similarity of 99.4% to each other, while they showed 95%–97% to other *Nostoc* species (Table 1). The 16S rRNA phylogenetic trees of ML, NJ and BI were constructed with 184 nucleotide sequences including the studied strains, the nearly complete 16S rRNA gene sequences after pairwise alignment was approximately 1166 bp. Bayesian tree showed that the studied *Nostoc* strains, together with other *Nostoc* species, formed a single cluster, and this unique cluster was supported by NJ, ML, BI approaches with high bootstrap values of 100%, 100%, and 1.00, respectively (Fig. 3).

**16S–23S ITS region**
The percent dissimilarity among aligned ITS sequences

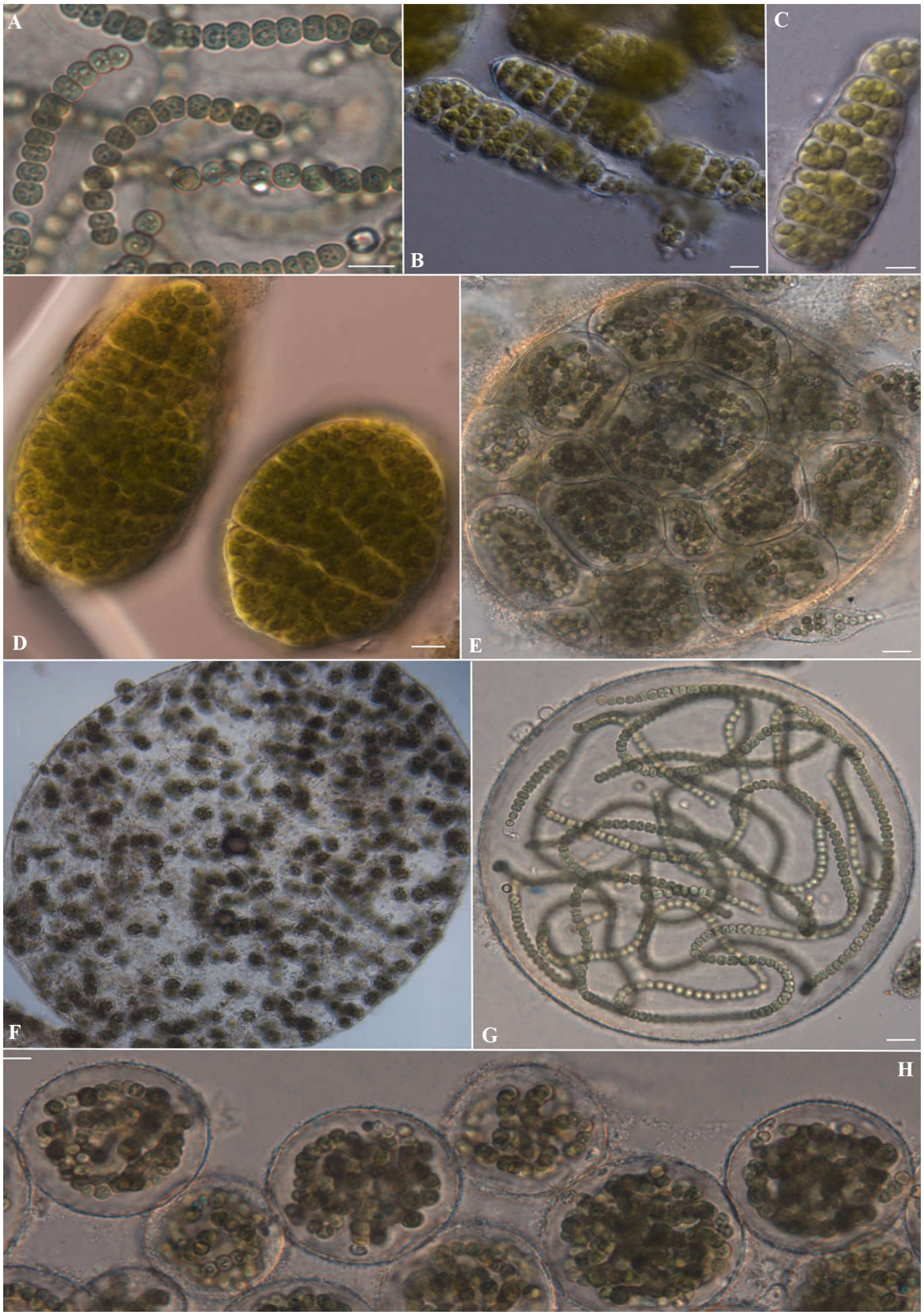


Fig. 1. Micrographs of *Nostoc favosum* under the light microscopy (LM): (A) free–living trichomes with or without heterocytes; (B–D) young colony with densely entangled filaments; (E–F) older colony with a honeycomb–like structure is composed of a number of smaller spherical microcolonies; (G–H) smaller spherical microcolonies released from the older colony. Scale bar 10 µm.

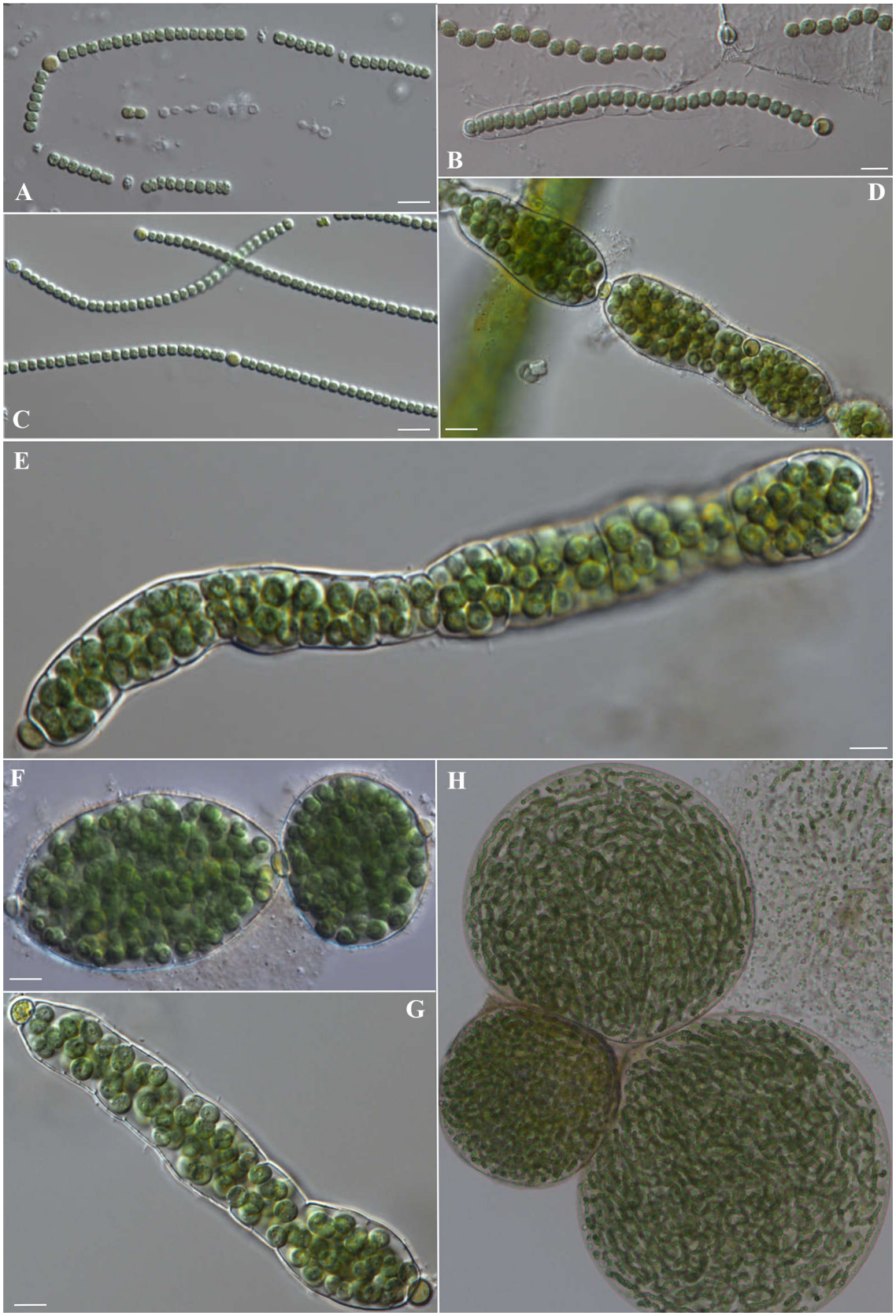


Fig. 2. Micrographs of *Nostoc mirabile* under the light microscopy (LM): (A–C) filaments with internal and terminal heterocytes; (E, G) filamentous young colony, showing heterocytes appeared at the end of the colony; (D, F) filamentous young colonies, connected by heterocytes; (H) aggregation of spherical colonies with distinct mucilaginous. Scale bar 10 μm.

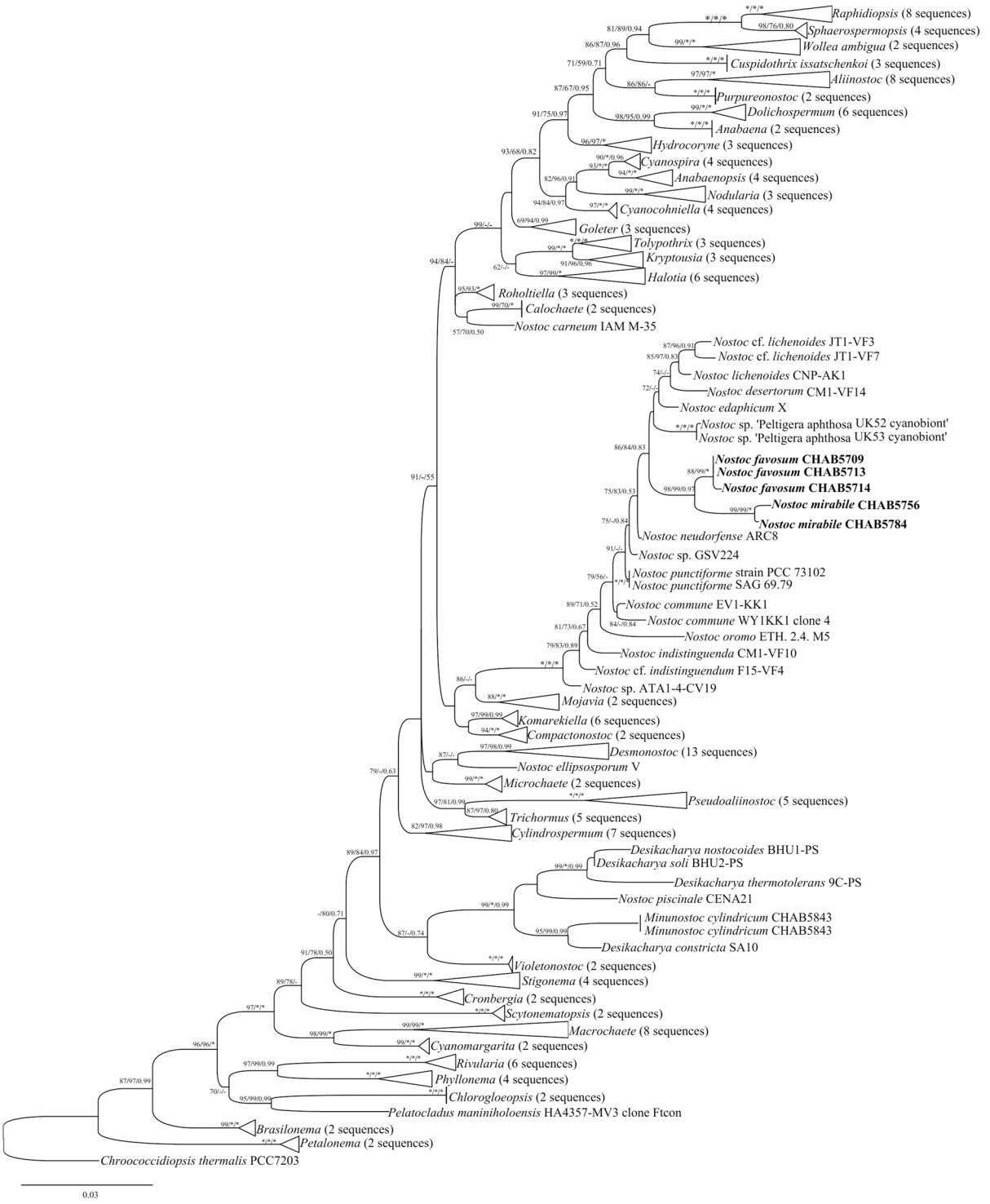


Fig. 3. Bayesian tree (BI) phylogenetic tree based on 16S rDNA sequences (1166bp) of the studied strains and other cyanobacterial strains. Bootstrap values greater than 50% with NJ/ ML/Mrbayes methods are indicated on the tree, and the asterisks at the nodes mean 100. The novel species are in bold font.

between our strains and the other *Nostoc* species for which ITS sequence with both tRNA genes was available, varied from 7.5%–16.6%. Percent dissimilarity of *Nostoc favosum* to other species is in the range of 11.4%–14.6%, and three *Nostoc favosum* strains shared 0.2% dissimilarity with each other. Percent dissimilarity of *Nostoc mirabile* to other species is in the range from 11.2% to 16.6%, and two *Nostoc mirabile* strains shared 0% dissimilarity with each other (Table 2).

The secondary structures of conserved ITS domains in *Nostoc* are highly similar (Figs 4–6), particularly in the D1–D1′ helices which were structurally identical and had the similar patterns. *Nostoc favosum* and *Nostoc mirabile* showed different nucleotides in the

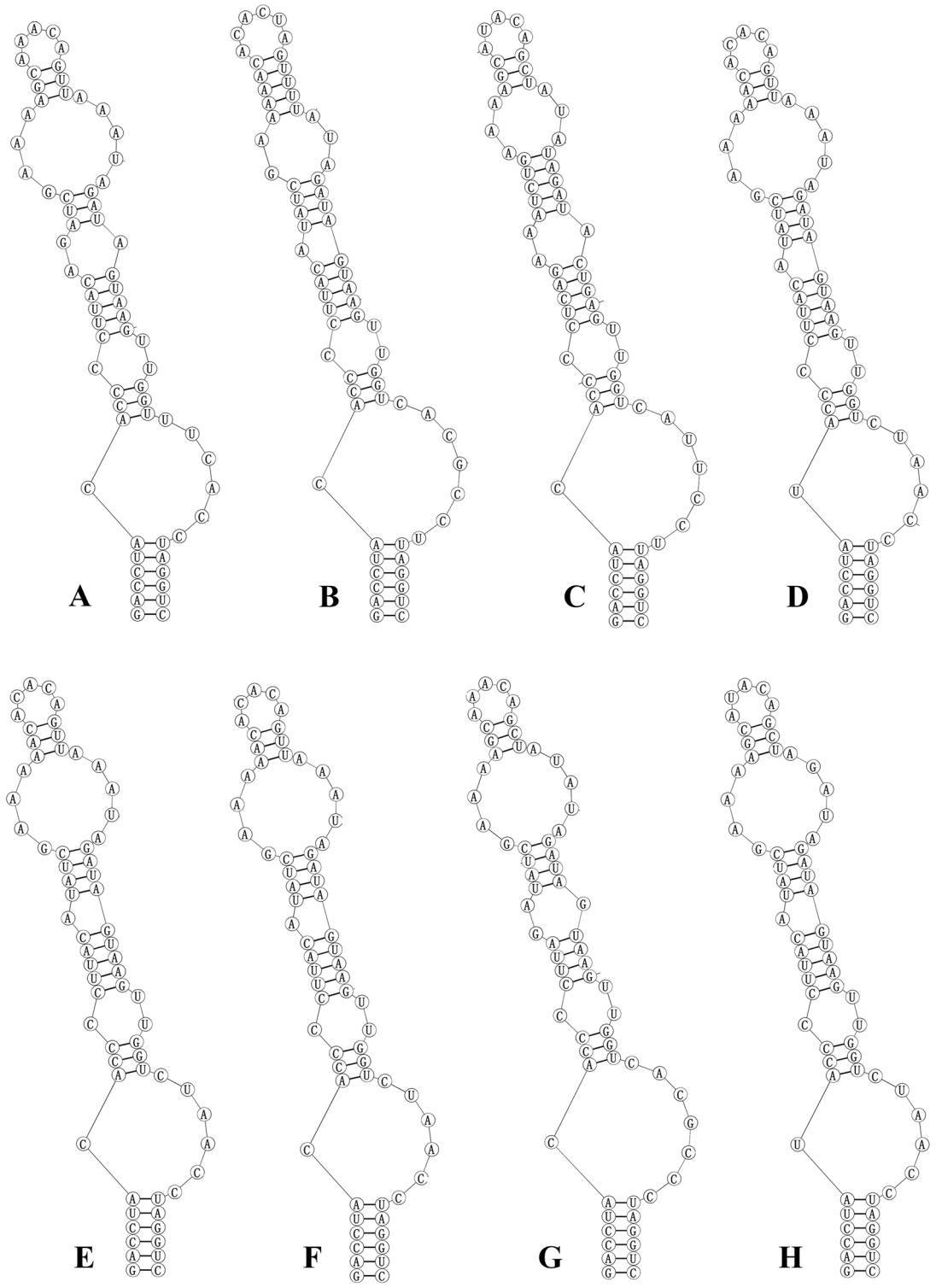


Fig. 4. Secondary structure of D1–D1' helix: (A) *Nostoc mirabile* CHAB5784, CHAB 5756; (B) *Nostoc favosum* CHAB5709, CHAB5713, CHAB5714; (C) *Nostoc commune* WY1–KK1; (D) *Nostoc punctiforme* PCC73102; (E) *Nostoc desertorum* CM1–VF14; (F) *Nostoc indistinguendum* CM1–VF10; (G) *Nostoc lichenoides* CNP–AK1; (H) *Nostoc oromo* ETH2.4.M5.

D1–D1' helix in comparison with other *Nostoc* species (Fig. 4A and 4B).

The Box–B helices of *Nostoc* were all very similar at the basal part of the helix, but the sequences and structure at the end are different, the eight *Nostoc* species were divided into the eight types (Fig. 5). The base stem of the Box–B helix of *Nostoc mirabile* (Fig. 5A) consisted of 5 bp helix, followed by a 3 : 3 base bilateral bulge, and then further followed by a unpaired nucleotide (A) on 3′ side, the terminal loop contained 5 bp bases (GAUGA). The base stem of *Nostoc favosum* (Fig. 5B) consisted of 5 bp helix, and then followed by a 3 : 3 base bilateral bulge, the terminal loop contained 6 bp bases (UUAAUU).

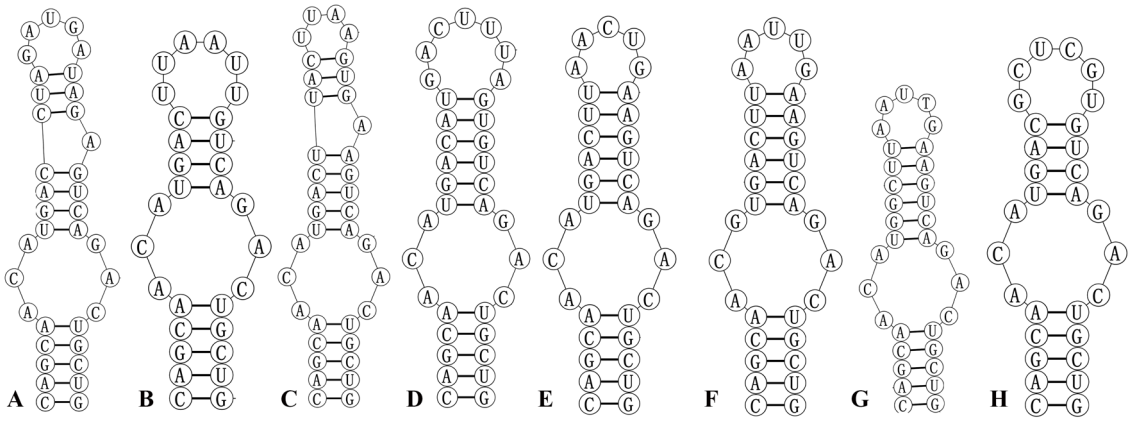


Fig. 5. Secondary structure of Box–B helix: (A) *Nostoc mirabile* CHAB5784, CHAB 5756; (B) *Nostoc favosum* CHAB5709, CHAB5713, CHAB5714; (C) *Nostoc commune* WY1–KK1; (D) *Nostoc punctiforme* PCC73102; (E) *Nostoc desertorum* CM1–VF14; (F) *Nostoc indistinguendum* CM1–VF10; (G) *Nostoc lichenoides* CNP–AK1; (H) *Nostoc oromo* ETH2.4.M5.

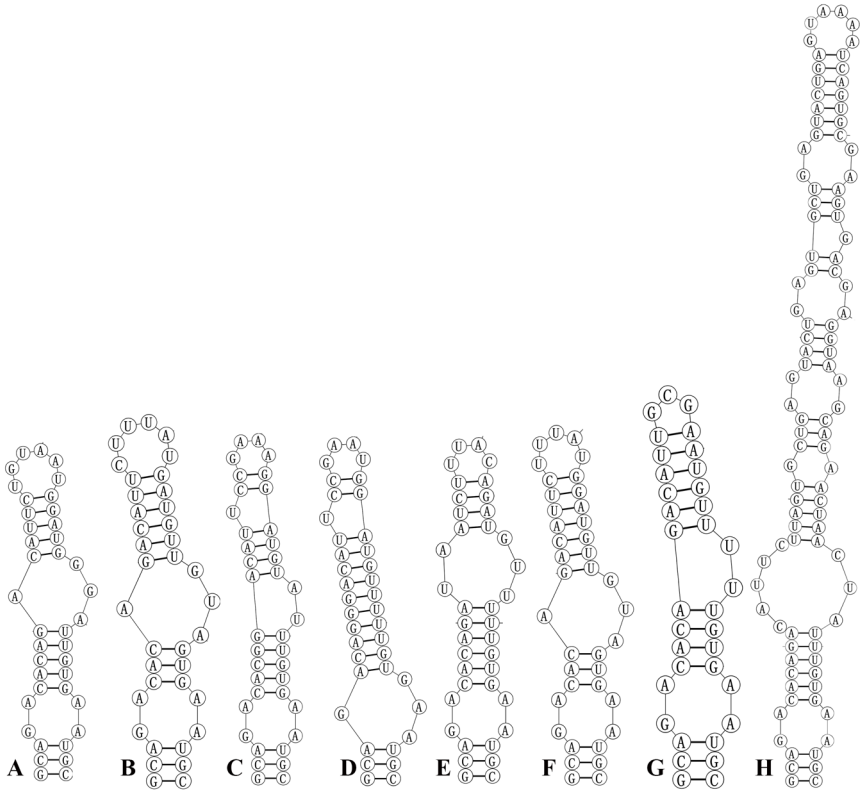


Fig. 6. Secondary structure of V3 helix: (A) *Nostoc mirabile* CHAB5784, CHAB 5756; (B) *Nostoc favosum* CHAB5709, CHAB5713, CHAB5714; (C) *Nostoc commune* WY1–KK1; (D) *Nostoc punctiforme* PCC73102; (E) *Nostoc desertorum* CM1–VF14; (F) *Nostoc indistinguendum* CM1–VF10; (G) *Nostoc oromo* ETH2.4.M5; (H) *Nostoc lichenoides* CNP–AK1.

The V3 helices were similar only in the base part and varied in structure and length (Fig. 6). The base stem of *Nostoc mirabile* (Fig. 6A) consisted of 3 bp helix, followed by a 2 : 2 base bilateral bulge, and then further followed by a 1 : 3 base bilateral bulge, the terminal loop contained 6 bp bases (UGUAAU). The base of the stem of *Nostoc favosum* (Fig. 6B) consisted of 3 bp helix, and then followed by two bilateral bulges, the terminal loop contained a 6 bp nucleosides (CUUUAU).

## DISCUSSION

Due to the morphological plasticity, complex life cycle, and huge diversity, the taxonomy of genus *Nostoc* becomes problematic (MOLLENHAUER et al. 1999; KOMÁREK et al. 2014; SINGH et al. 2016). Molecular data showed up that the genetic diversity of the genus exceeded its morphological diversity, which clearly supported its polyphyletic status (RAJANIEMI et al. 2005a, 2005b; ŘEHÁKOVÁ et al. 2007; HROUZEK et al. 2013; GENUÁRIO

et al. 2015; Bagchi et al. 2017; Hentschke et al. 2017). Recent efforts have been made to reevaluate the genus by recognizing *Nostoc* sensu stricto, a clade that includes the type species, *Nostoc commune*, and excludes species that fall outside this clade that bear the name *Nostoc*, but cannot be put in that genus if monophyly must be achieved at the genus level (Řeháková et al. 2007; Hrouzek et al. 2013; Genuário et al. 2015; Bagchi et al. 2017; Hentschke et al. 2017; Saraf et al. 2019; Cai et al. 2019a, 2019b, 2020a, 2020b).

Taxonomical studies have found that molecular evidences, morphological, biogeographical, and ecological data are often consistent, and their combination can better distinguish species with similar morphology. This is the essence of the polyphasic approach, which has been followed when making decisions at the species level (Komárek 2018; Mai et al. 2018). In this study, the species *Nostoc favosum* and *Nostoc mirabile* were characterized on the basis of the polyphasic approach. The phylogenetic analyses in this study, combined with the cut off values for the genus and species delimitation, clearly indicated that investigated strains are member of the genus *Nostoc*. In the 16S rRNA gene phylogenetic tree, *Nostoc favosum* and *Nostoc mirabile* clustered within the *Nostoc* branch at a unique node. This indicated that species *Nostoc favosum* and *Nostoc mirabile* are new members of the genus *Nostoc*. The original identity cut–off value recommended for separation of bacterial species was 97.5% (Stackebrandt & Goebel 1994). However, on the basis of extensive comparison of more bacterial strains, Stackebrandt & Ebers (2006) recommended 98.7–99.0% of 16S rRNA gene sequence similarity as the species threshold. Other research proposed that 98.65% of 16S rRNA gene sequence similarity could be used as a threshold to distinguish two species based on twofold cross–validation statistical test (Kim et al. 2014). The highest 16S rRNA gene sequence similarity between the *Nostoc favosum* strains detected in this study and other *Nostoc* strains (*Nostoc* cf. *lichenoides* JT1–VF3) is 98%. The highest 16S rRNA gene sequence similarity of *Nostoc mirabile* strains with other *Nostoc* strains is 97%, which provided the molecular evidence to support the recognition of the two species we have named.

Up to now, four species, *Desikacharya nostocoides* (the type species of *Desikacharya*), *Desikacharya soli*, *Desikacharya thermotolerans* and *Desikacharya constricta* have been reported under the genus *Desikacharya*, which are considered certain features such as constrictions along with prominent coiling (Kabirnataj et al. 2020). Kabirnataj et al. (2020) discussed that *Minunostoc cylindricum* should be a member of the genus *Desikacharya*, in contrast, the *Desikacharya* species within the lineage, were differentiated into two distinct clades

Table 1. Comparison of the 16S rRNA gene sequence similarity among the studied strains and its related taxa.

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1.*Nostoc favosum* CHAB5709 | ID | | | | | | | | | | | | | |
| 2.*Nostoc favosum* CHAB5713 | 0.999 | ID | | | | | | | | | | | | |
| 3.*Nostoc favosum* CHAB5714 | 0.993 | 0.994 | ID | | | | | | | | | | | |
| 4.*Nostoc mirabile* CHAB5756 | 0.97 | 0.971 | 0.97 | ID | | | | | | | | | | |
| 5.*Nostoc mirabile* CHAB5784 | 0.972 | 0.973 | 0.972 | 0.994 | ID | | | | | | | | | |
| 6.*Nostoc commune* WY1KK1 | 0.975 | 0.976 | 0.973 | 0.957 | 0.959 | ID | | | | | | | | |
| 7.*Nostoc punctiforme* PCC 73102 | 0.977 | 0.978 | 0.977 | 0.963 | 0.965 | 0.983 | ID | | | | | | | |
| 8.*Nostoc neudorfense* ARC8 | 0.975 | 0.976 | 0.975 | 0.961 | 0.962 | 0.976 | 0.99 | ID | | | | | | |
| 9.*Nostoc oromo* ETH. 2.4. M5 | 0.971 | 0.972 | 0.967 | 0.95 | 0.951 | 0.964 | 0.974 | 0.972 | ID | | | | | |
| 10.*Nostoc desertorum* CM1–VF14 | 0.96 | 0.96 | 0.96 | 0.951 | 0.952 | 0.966 | 0.978 | 0.97 | 0.961 | ID | | | | |
| 11.*Nostoc indistinguenda* CM1–VF10 | 0.965 | 0.966 | 0.967 | 0.953 | 0.953 | 0.971 | 0.98 | 0.973 | 0.96 | 0.969 | ID | | | |
| 12.*Nostoc lichenoides* CNP–AK1 | 0.976 | 0.977 | 0.976 | 0.96 | 0.961 | 0.973 | 0.987 | 0.983 | 0.978 | 0.975 | 0.97 | ID | | |
| 13.*Nostoc* cf. *indistinguendum* F15–VF1 | 0.977 | 0.978 | 0.975 | 0.96 | 0.96 | 0.977 | 0.984 | 0.98 | 0.968 | 0.97 | 0.98 | 0.975 | ID | |
| 14.*Nostoc* cf. *lichenoides* JT1–VF3 | 0.98 | 0.98 | 0.978 | 0.961 | 0.963 | 0.974 | 0.982 | 0.979 | 0.975 | 0.971 | 0.966 | 0.988 | 0.98 | ID |
| 15.*Nostoc edaphicum* X | 0.975 | 0.976 | 0.975 | 0.968 | 0.97 | 0.975 | 0.989 | 0.986 | 0.976 | 0.973 | 0.972 | 0.987 | 0.978 | 0.982 |

with high genetic divergences (less than 95% similarity of 16S rRNA gene), and *Desikacharya* has been shown to be polyphyletic in our analyses. The 16S rRNA gene sequence of *Desikacharya constricta* had less than 95% similarities to the type species *Desikacharya nostocoides* and *Desikacharya soli*, however, it had 96.9% similarity to *Minunostoc cylindricum*. The phylogenetic tree also showed that the sequence of *Desikacharya constricta* clustered within the *Minunostoc* clade. In addition, the 16S rRNA gene sequence similarities between *Minunostoc cylindricum* and other *Desikacharya* strains (except for *Desikacharya constricta*) were shown as less than 95%, below the bacterial genus cut–off (Table S1). Thus, we conclude that *Minunostoc cylindricum* should not belong to the genus *Desikacharya* (below 95% sequences similarities is a strong evidence for separation of genera), and the taxonomic status of *Desikacharya constricta* should be separated from the *Desikacharya* genus, and belonged to the *Minunostoc* genus.

The 16S–23S ITS secondary structure analysis has been demonstrated to be effective tool to differentiate cyanobacteria species (Iteman et al. 2000; Řeháková et al. 2007; Johansen et al. 2011; Bohunická et al. 2015; Berrendero–Gómez et al. 2016; Mareš 2018). In this study, the secondary structures of D1–D1′, Box–B and V3 helices were also analyzed, which enabled our strains to be distinguished from other *Nostoc* species. It is shown here that D1–D1′, Box–B and V3 helices of *Nostoc favosum* and *Nostoc mirabile* strains were quite different from other selected *Nostoc* species. In addition, the percent dissimilarity within the 16S–23S ITS region is increasingly useful for cyanobacterial species delimitation. Percent dissimilarity within the same species has always been less than 4.0%, with an average value below 2.0%, percent dissimilarity between species is typically over 7.0% (Erwin & Thacker 2008; Osorio–Santos et al. 2014; Pietrasiak et al. 2014; Bohunická et al. 2015; Mai et al. 2018; Mesfin et al. 2020). *Nostoc favosum* is distinct from the other *Nostoc* species based on percent dissimilarity of the ITS region, which is > 11.4%, and *Nostoc mirabile* is > 11.2% (Table 2). And dissimilarity in the ITS regions between these two new taxa is well above the 4% level. This fits well within the criteria for species differentiation based on percent dissimilarity of > 4.0%. So, in this study, the percent dissimilarity in the ITS regions strongly supported their separation into two new *Nostoc* species.

Morphologically, the *Nostoc mirabile* strains closely resembles *Nostoc punctiforme* (Hariot 1891, p. 31), having filamentous colony stage, heterocytes appeared at the end of the colony, and densely entangled filaments in colony. *Nostoc mirabile* create macroscopic colonies observable without microscopy, whereas *Nostoc punctiforme* is only microscopic in nature. In addition, the phylogenetic analysis of the 16S rDNA, 16S rRNA gene similarity and the 16S–23S ITS, show that *Nostoc mirabile* and *Nostoc punctiforme* are genetically not closely related. The morphologically closet *Nostoc* species to *Nostoc favosum* in the literature is *Nostoc desertorum* Řeháková et Johansen (2007, p. 488, Figs 2, 27–33), described from sandy desert soil in California. *Nostoc desertorum* shares the characteristic of compartmentalization of colonial mucilage in older colony. *Nostoc favosum* differs in that the filaments segmented into several small groups in young colony, later many small groups grow into spherical colony, surrounded by large spherical sheath. *Nostoc desertorum* can form fairly flat masses in culture, a feature never seen in *Nostoc favosum*. Ecologically, *Nostoc favosum* strains were described from the edge of a stream, humid climate very different from the sandy desert soil habitat in which *Nostoc desertorum* was found.

In summary, two new species of *Nostoc* are separated based on a combination of the 16S rRNA gene–based phylogeny, 16S rRNA gene threshold of 98.65%, the ITS secondary structures, 16S–23S ITS percent similarity, as well as morphological characteristics

Table 2. Percent dissimilarity based on 16S–23S ITS sequence for operons containing both tRNA genes among the studied strains and the closest sister taxa.

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| 1.*Nostoc mirabile* CHAB5756 | | | | | | | | | | |
| 2.*Nostoc mirabile* CHAB5784 | 0 | | | | | | | | | |
| 3.*Nostoc favosum* CHAB5709 | 11.25 | 11.25 | | | | | | | | |
| 4.*Nostoc favosum* CHAB5713 | 11.25 | 11.25 | 0.20 | | | | | | | |
| 5.*Nostoc favosum* CHAB5714 | 11.50 | 11.50 | 0.20 | 0.20 | | | | | | |
| 6.*Nostoc commune* WY1KK1 | 16.38 | 16.38 | 13.86 | 13.86 | 14.11 | | | | | |
| 7.*Nostoc punctiforme* PCC73102 | 16.68 | 16.68 | 12.84 | 12.84 | 12.84 | 10.60 | | | | |
| 8.*Nostoc desertorum* CM1–VF14 | 14.61 | 14.61 | 11.47 | 11.47 | 11.47 | 9.99 | 8.78 | | | |
| 9.*Nostoc indistinguenda* CM1–VF10 | 14.77 | 14.77 | 13.79 | 13.79 | 13.79 | 10.68 | 9.55 | 8.16 | | |
| 10.*Nostoc lichenoides* CNP–AK1 | 14.78 | 14.78 | 14.10 | 14.10 | 14.10 | 10.21 | 9.63 | 10.50 | 9.63 | |
| 11.*Nostoc oromo* ETH.2.4.M5 | 16.12 | 16.12 | 14.40 | 14.40 | 14.65 | 7.59 | 8.64 | 10.79 | 10.20 | 9.44 |

and ecological parameters. In China, the investigation and researches on *Nostoc*–like cyanobacteria from all kinds of environmental conditions have been largely performed, and several new genera and species have been found and published in the family Nostocaceae. The investigation of less studied regions and habitats in China could reveal the diversity of family Nostocaceae and bring new inside into the diversity of cyanobacteria on the world.

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Supplementary material

The following supplementary material is available for this article:

Table S1. Comparison of the 16S rRNA gene sequence similarity among *Desikacharya* and *Minunostoc*.

This material is available as part of the online article (http://fottea.czechphycology.cz/contents)


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