

Neochroococcus gongqingensis gen. et sp. nov., a new member of coccoid cyanobacteria from a watercourse, Eastern China

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Abstract: The taxonomy of coccoid cyanobacteria has been largely revised in recent years. In this study, a novel coccoid cyanobacterial strain was isolated from a watercourse at the Poyang Lake Model Research Base, Jiangxi province, Eastern China. A polyphasic approach combining morphological and molecular testing was used to characterize this strain referred to as CHAB 4018. Regarding colonial form and cellular spatial arrangement, this strain was morphologically similar to strains of the genus *Eucapsis*. The maximum 16S rRNA gene sequence similarity of this strain to the currently described cyanobacteria genera was 93.40%, exceeding the cutoff for genus delimitation in bacteriology. Furthermore, a phylogenetic tree based on 16S rRNA gene sequences indicated that strain CHAB 4018 formed a unique clade in the family Chroococcaceae and was phylogenetically close to the recently established genus *Cryptococcus* but distant from the *Chroococcus* ‘sensu stricto’ clade and from *Eucapsis*. Thus, a novel coccoid cyanobacterial genus with a new species is here described as *Neochroococcus gongqingensis*. A large phylogenetic tree using more strains suggested phylogenetic intermixture of *Chroococcus*-like and *Eucapsis*-like cyanobacteria, suggesting the need for further studies on the phylogeny and taxonomy of coccoid cyanobacteria.

Key words: Coccoid cyanobacteria, *Neochroococcus gongqingensis*, *Eucapsis*, Polyphasic, Taxonomy

INTRODUCTION

The taxonomic system of cyanobacteria was considerably revised during the past decades after molecular biological methods became common standard, and primary usage of the polyphasic approach of research on cyanobacterial taxonomy led to substantial revisions and establishment of numerous new genera and species to resolve phylogenetically monophyletic categories (KOMÁREK et al. 2014; KOMÁREK 2016). Along with this revision process, nostocacean cyanobacteria have largely encountered a considerable rearrangement of taxonomic units. In contrast, information from polyphasic studies on the coccoid type of cyanobacteria was relatively poor.

The genus *Eucapsis* Clements et Shantz, which is considered one of the most typical genera of coccoid cyanobacteria, was well characterized by its spherical cells arranged into regular cubic colonies, and its colorless, hyaline mucilage typically containing 8–16 but

sometimes up to 512 cells was also described (CLEMENS & SHANTZ 1909; KOMÁREK & ANAGNOSTIDIS 1998). At present, Algaebase contains 13 species of *Eucapsis* which are generally accepted as separate taxa, whereas during the 20th century, only two main species (*E. alpina* Clements et Shantz and *E. minor* (Skuja) Elenkin) were commonly referred to (KOMÁREK & ANAGNOSTIDIS 1998). Progress has been made on several coccoid genera but *Eucapsis* was not among them, only two 16S rRNA gene sequences of approximately 1400 bp are available which do not provide any taxonomic support (*Eucapsis minor* SAG 14.99 and *Eucapsis* sp. 019). During phylogenetic analysis examining the variability of *Chroococcus* morphotypes, KOMÁRKOVÁ et al. (2010) transferred two *Chroococcus* strains with small cells into a cluster representing *Eucapsis*. Furthermore, KOMÁREK et al. (2016) defined the *Eucapsis* cluster by morphological and phylogenetic points of view in which six strains including *Eucapsis minor* SAG 14.99, *Eucapsis* sp. 019, three *Chroococcus* strains with small cells, and

Aphanothece hegewaldii SAG 253.80, and they found phylogenetic evidence of the genus *Eucapsis* being more closely related to the family Merismopediaceae than to Chroococcaceae. This systematic also supports the latest system of KOMÁREK et al. (2014) who proposed that *Eucapsis* belongs to the family Merismopediaceae. However, as described by KOMÁREK et al. (2016), using polyphasic criteria, the classification of *Eucapsis* remains unclear. The scarcity of strains belonging to *Eucapsis* and absence of strains of its type species are the main problems limiting further polyphasic research. Therefore, more strains of *Eucapsis* and morphologically similar genera are urgently required.

In the current study, a novel coccoid cyanobacterial strain was isolated from a watercourse in the city of Gongqing, Jiangxi province, China, and was found to form cubic colonies with cells dividing regularly in three perpendicular planes, resembling colonies of the genus *Eucapsis*. 16S rRNA gene sequence homology and phylogenetic analyses indicated that this strain represents a novel genus of the family Chroococcaceae. The new genus *Neochroococcus* and its type species *Neochroococcus gongqingensis* are described here.

MATERIALS AND METHODS

Sampling and cultivation. A water sample was collected using a plankton net in June 2018 from a watercourse at the Poyang Lake Model Research Base in the city of Gongqing city, Jiangxi Province, China. Single *Eucapsis*-like colonies were isolated using the Pasteur pipette washing method under 40 times microscope (Olympus CKX31, Japan), and were then cultured in 24-well plates containing sterilized liquid MA medium (WATANABE & HIROKI 1997). In two weeks, the content of wells containing blue-green colonies was transferred into screw-capped tubes containing 10 mL MA medium, thereby isolating a strain exhibiting cubic colonies. The isolated strain was maintained at 25 °C under a 12:12 h light:dark cycle of white fluorescent light with a photon flux density of 35 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. The strain was archived in the culture collection of the Collection of Harmful Algae Biology laboratory (CHAB) of the Institute of Hydrobiology, Wuhan, China, under the strain number CHAB 4018.

Morphological and ultrastructural characterization. Morphological observation of CHAB 4018 at different growth phases was carried out using a Nikon Eclipse 80i microscope (Nikon, Japan). Microphotographs were produced using a DS-Ri1 digital camera (Nikon, Japan) photomicrographic system and were analyzed using NIS-Elements D 3.2 software (Nikon). Morphometric characteristics of unialgal cells were recorded using digital images recorded at 400-fold magnification, and the mean cellular size was measured in more than 100 cells. For transmission electron microscopy (TEM), cells of the strain were fixed using 2.5% glutaraldehyde in 0.1 M phosphate buffer at a pH 7.2 and 4 °C for three days. The samples were then washed using 0.1 M phosphate buffer after which they were post-fixed using 1% osmium tetroxide for 2 h. Subsequently, the samples were washed again using 0.1 M phosphate buffer

to remove osmium tetroxide after which they were dehydrated using a sequential ethanol gradient (30%, 50%, 70%, 90%, and 100%) and then embedded in Spurr's resin (SPURR 1969). The embedded specimens were trimmed using an ultra-microtome (Leica Ultracut UCT, Austria) with a razor blade and were then sectioned into 80-nm slices. Uranyl acetate (2%) and lead citrate were used to stain the sections. The specimens were examined using an HT7700 (Japan) transmission electron microscope under 80 kV on Hitachi TEM system control (Hitachi, Japan).

DNA extraction and PCR amplification. The strain cultures were collected by filtration using a Millipore filter (3.0 μm aperture, Merck Millipore, Darmstadt, Germany) and were washed three times using sterile phosphorus-free MA medium to avoid contamination with other bacteria. The remaining biomass was then collected in several clean EP tubes. Total genomic DNA was extracted using a modified cetyltrimethylammonium bromide (CTAB) method (NEILAN et al. 1995). The PCR primers PA (EDWARDS et al. 1989) and B23S (GKELIS et al. 2005) were used to amplify a 16S rRNA gene fragment. The total PCR reaction volume of 50 μl comprised 100 ng genomic DNA, 1 μl of each primer (10 $\mu\text{mol}\cdot\text{l}^{-1}$), 22 μl sterile water, and 25 μl 2 \times PCR mix with Taq polymerase (Beijing Tsingke Biotech Co., Ltd., Beijing, China). PCR amplification was performed using a BIO-RAD Thermal Cycler (Bio-Rad, Hercules, California USA) with a thermocycling regime of 94 °C for 3 min followed by 34 cycles 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min and a final elongation step of 72 °C for 5 min. PCR products were purified using a QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and a TSINGKE DNA Gel Extraction Kit (Beijing Tsingke Biotech Co., Ltd., Beijing, China) and were then cloned using a pMDTM18-T vector (TaKaRa, TaKaRa BioInc., Otsu, Japan). Clones including the target fragment were sequenced bidirectionally using an ABI 3730 Automated Sequencer (PerkinElmer, Waltham, Massachusetts USA).

Phylogenetic analyses. The produced 16S rRNA sequences of CHAB 4018 were BLAST-searched using the NCBI online tool, and reference sequences with high similarities were retrieved from GenBank to reconstruct the strain's molecular phylogeny. All downloaded sequences were edited using MAFFT v7.312 software (KATO et al. 2013) after multiple sequence alignment, and a matrix of 88 sequences with 1164 nucleotide sites was produced. For phylogenetic analysis of the 16S rRNA gene, alignments were analyzed using the standard selection nucleic acid substitution model (GTR+I+G) based on the Akaike Information Criterion (AIC) for Bayesian analysis (BI) and maximum likelihood analysis (ML). Selected parameters were individually estimated using MrBayes v3.2.6 (RONQUIST et al. 2012) and PhyML 3.0 (GUINDON et al. 2010). The Kimura-2 model was selected with 1,000 bootstrap replicates using MEGA software v7.0 to perform neighbor joining (NJ) analysis (KUMAR et al. 2016). ML and Bayesian phylogenetic trees were visualized using FigTree v1.4.3, and all phylogenetic trees were edited using Tree View 1.6.6 software (PAGE 1996). A similarity matrix of the 16S rRNA sequences was established using MEGA v7.0 to calculate p-distance with pairwise deletion of gaps.

Analyses of secondary structures of ITS between 16S and 23S rRNA genes. Presence of tRNA gene sequences was tested using the tRNAscan-SE 2.0 web server (LOWE & CHAN 2016). The 16S-23S rRNA ITS secondary structures of the D1-D1' helix of *Neochroococcus gongqingensis* CHAB 4018 and other

closed genera were determined using the Mfold 3.2 web server (ZUKER 2003). The 16S–23S rRNA sequences produced in this study were deposited in the GenBank database under the accession numbers MT011390, MT011391 and MT011392.

RESULTS

Neochroococcus R. Geng et G. Yu gen. nov.

Description: Colonies microscopic, usually small, regular cubic, with 8–16 cells, more or less grouped together; sometimes large colonies composed of sub-colonies; colonies olive–green or red–brown, surrounded by mucilaginous envelopes; mucilage fine, homogeneous and colorless; cells usually spherical or hemispherical after division, with granular content, blue–green, grey–green, olive–green, yellowish, or red–brown; cells arranged three–dimensionally in perpendicular rows and divide regularly in three perpendicular planes in subsequent generations; thylakoids irregularly arranged.

Type species: *Neochroococcus gongqingensis* R. Geng et G. Yu sp. nov.

Etymology: The name of new genus “*Neochroococcus*” was chosen because it was more closely related to *Chroococcus* ‘sensu stricto’ than to *Eucapsis*.

Diagnosis: Using light microscopy, this species appears morphologically similar to the genus *Eucapsis* regarding colonial form and cellular spatial arrangement. However, the phylogenetic tree produced from 16S rRNA gene sequences indicated that this species held a unique position in the family Chroococcaceae and was close to the recently established genus *Cryptococcus* but separated from the *Chroococcus* ‘sensu stricto’ clade and *Eucapsis*. In addition, the 16S rRNA sequence of this strain showed low similarity with those of *Eucapsis* spp. and of the genus *Cryptococcus*, and significant differences in the secondary structure of the 16S–23S ITS region also supported the establishment of this new cyanobacterial genus.

Neochroococcus gongqingensis R. Geng et G. Yu sp. nov. (Fig. 1)

Description: Colonies are microscopic, small, more or less regular cubic, typically with 8–16 cells, sometimes grouped together, large colonies composed of subcolonies. Colonies are surrounded with mucilage, but single cells are not. Mucilage fine, homogeneous, colorless, not stratified or diffluent; cells hemispherical to rounded, sometimes oval, with a diameter of 8.44–(10.25)–12.32 µm; content granular, blue–green, grey–green, or olive–green in growth phase, and yellowish, yellow or red–brown in decline phase; cells arranged three–dimensionally in perpendicular rows and divided regularly in three perpendicular planes in the subsequent generation, more often forming cube colony morphology of eight cells. Thylakoids are irregularly arranged at the periphery

of cells (Fig. 2). Ecology: this species is planktic and inhabits mesotrophic water.

Designated holotype: Dry material of strain CHAB 4018 was stored at the Freshwater Algal Herbarium (HBI), Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, China, with specimen number as JXGQ201806.

Reference strain: The living culture of strain CHAB 4018 was archived at the Collection of Harmful Algae Biology (CHAB), Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, Hubei Province, China.

Type locality: In a watercourse at the Poyang Lake Model Research Base in the city of Gongqing, Jiangxi province, China. (22th June 2018, site 29°12'59.44"N, 115°49'55.86"E).

Etymology: *gongqingensis* comes from Latin “gongqing”, which refers to the Gongqing city where the strain was collected.

Habitat: Free–living in freshwater channels.

Molecular and phylogenetic analyses

After calculating p–distance with pairwise deletion of gaps using MEGA software v7.0, a 16S rRNA similarity matrix was produced using 88 sequences downloaded from GenBank. The 16S rRNA sequences of *Neochroococcus gongqingensis* CHAB 4018 shared 99.65%–99.91% similarity among all three clones, and the three clones showed maximum similarity as 93.40% with the closest coccoid genera (similarities with *Gomphosphaeria*, *Inacoccus*, *Stanieria*, and *Chroococcopsis* were 93.13%–93.40%, 92.96%–93.31%, 92.84%–93.10%, and 92.75%–93.02%, respectively; similarities with *Cyanothece*, *Cryptococcus*, *Chondrocystis*, *Aphanothece*, *Gloeothece*, *Chroococcus*, and *Limnococcus* were 92.24%–92.85%, 88.62%–92.77%, 92.16%–92.42%, 92.14%–92.41%, 88.69%–92.14%, 90.48%–92.07%, and 91.79%–92.06%, respectively; similarities with *Gloeocapsopsis*, *Synechocystis*, *Eucapsis*, and *Merismopedia* were 90.89%–91.96%, 91.73%–91.80%, 91.17%–91.72%, and 83.19%–91.70%, respectively); thus at less than 95% similarity which is considered the threshold of bacterial intergeneric similarity, this strain probably represents a new cyanobacterial genus (Table 1).

The 16S rRNA NJ, ML, and BI phylogenetic trees (Fig. 3) showed that the three clones of CHAB 4018 clones clustered as a unique clade (cluster A), which was supported by NJ, ML, and BI calculations with high bootstrap values of 99%, 100%, and 0.90 respectively; this clade was close to the genera *Cryptococcus* (cluster C) and *Inacoccus* (cluster B) but distant from *Chroococcus* (cluster D) and *Eucapsis* (cluster E). In this phylogenetic tree, cluster D included *Chroococcus minutus*, *Chroococcus virescens*, *Chroococcus turgidus*, and *Chroococcus subviolaceus*, and together they formed the *Chroococcus* ‘sensu stricto’ clade.

Analyses of ITS secondary structures

The obtained 16S–23S ITS regions of *N. gongqingensis* CHAB 4018 together with the sequences downloaded

Table 1. Sequence similarity comparison of the 16S rRNA gene between *Neochroococcus gongqingensis* CHAB 4018 and its closed genera. Similarity = [1-(p-distance)]*100.

Strains	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1. <i>Neochroococcus gongqingensis</i> CHAB 4018 clone 1																					
2. <i>Neochroococcus gongqingensis</i> CHAB 4018 clone 2	99.65																				
3. <i>Neochroococcus gongqingensis</i> CHAB 4018 clone 3	99.91	99.74																			
4. <i>Chondrocystis</i> sp. ANT.L59B.1	92.33	92.16	92.42																		
5. <i>Chroococcus turgidus</i> AICB61	92.14	92.05	92.23	91.43																	
6. <i>Cyanothece</i> sp. WH 8904	92.76	92.59	92.85	92.14	92.40																
7. <i>Gloeothece membranacea</i> PCC 6501	92.06	91.88	92.14	91.97	91.33	92.50															
8. <i>Gonphosphaeria aponina</i> SAG 52.96	93.40	93.13	93.40	93.22	91.43	92.50	92.59														
9. <i>Limnocooccus limneticus</i> Svet06	91.97	91.79	92.06	93.37	90.96	91.50	91.14	93.37													
10. <i>Stamieria cyanosphaera</i> PCC 7437	93.02	92.84	93.10	92.92	92.83	93.46	92.03	92.22	92.10												
11. <i>Aphanothece sacrum</i> FPU1	92.32	92.14	92.41	92.49	91.59	95.24	93.02	92.86	91.23	92.74											
12. <i>Chroococcus subviolaceus</i> CCIB3506	91.55	91.55	91.64	91.71	96.38	91.78	90.99	91.28	90.46	91.60	91.00										
13. <i>Gloeocephopsis crepidinum</i> BDU 20121	91.96	91.61	91.87	91.42	91.77	92.06	91.34	91.17	90.43	93.73	91.79	90.46									
14. <i>Eucapsis</i> sp. 019	91.62	91.62	91.70	90.45	91.05	92.76	91.95	90.29	90.18	90.80	91.96	90.64	89.48								
15. <i>Chroococcopsis gigantea</i> SAG 12.99	92.93	92.75	93.02	92.29	91.22	91.95	90.88	91.95	90.97	93.89	91.60	90.53	91.62	91.33							
16. <i>Chroococcus turgidus</i> CCIB3508	91.98	91.98	92.07	91.18	99.29	91.96	91.17	91.63	91.07	92.12	91.61	96.48	91.08	91.16	91.23						
17. <i>Cryptococcus brasiliense</i> CCIB3410	92.68	92.50	92.77	91.09	91.87	91.42	91.25	91.09	90.88	92.04	91.16	91.27	91.51	91.06	92.83	91.71					
18. <i>Inacoccus carmineus</i> CCIB3475	93.22	93.05	93.31	93.92	91.78	93.02	92.05	93.04	91.97	92.65	92.76	92.33	91.60	90.99	93.36	91.62	92.59				
19. <i>Aphanothece hegewaldii</i> SAG 253.80	91.63	91.63	91.72	90.20	90.89	92.77	91.78	90.13	90.02	90.81	91.98	90.48	89.50	99.82	91.34	91.00	91.08	91.01			
20. <i>Eucapsis minor</i> SAG 14.99	91.35	91.17	91.44	90.54	89.46	91.70	91.95	90.20	90.01	91.33	91.53	89.05	90.11	92.76	90.89	89.66	90.00	90.91	92.95		
21. <i>Merismopedia punctata</i> PMC260.06	91.62	91.44	91.70	90.55	89.99	92.23	91.87	90.92	90.26	91.22	92.23	89.40	89.48	89.82	89.81	89.75	89.83	90.46	89.75	91.42	
22. <i>Synechocystis</i> sp. KSU-AQIQ-1	91.71	91.53	91.80	90.38	89.91	91.70	92.14	91.01	90.35	91.23	92.23	89.85	90.11	90.89	89.82	89.85	89.13	90.73	90.91	91.87	95.06

Table 2. Analyses on ITS of 16S–23S region for *Neochroococcus gongqingensis* CHAB 4018.

Organisms	GenBank	ITS total length (nt)	D1-D1' helix length (nt)	D1-D1' helix in Fig. 4	D2 region	tRNA ^{le}	Box B helix length (nt)	Box A spacer
<i>Neochroococcus gongqingensis</i> CHAB 4018	MT011390	291	57	A	CTATAAAAAA	+	–	GCACCTTGAAAA
<i>Cryptococcus komarkovaeum</i> CCALA 054	MF072346	226	59	B	CTTTCAAATTA	+	–	GCACCTTGAAAA
<i>Cryptococcus brasiliense</i> CCIBt 3410	MF072345	345	56	C	CTTTCAAACTT	+	–	GCACCTTGAAAA
<i>Inacoccus carmineus</i> CCIBt 3418	MF072348	445	64	D	CTTTCAAATTA	+	35	GCACCTTGAAAA
<i>Inacoccus carmineus</i> CCIBt 3475	MF072349	446	63	E	CTTTCAAATTA	+	35	GCACCTTGAAAA

from NCBI were used to reconstruct the ITS secondary structure. The analyses indicated that the ITS of the 16S–23S region of all CHAB 4018 clones contained only one tRNA^{le} at a total length of 291 bp (Table 2). The D1–D1' helix as the most conservative structure differed largely between CHAB 4018 and other taxa used in this study with the D1–D1' helix of CHAB 4018 showing a larger loop below one small loop and one small unidirectional bulge (Fig. 4).

DISCUSSION

The polyphasic approach considers morphological, molecular phylogenetic, cytomorphological, and ecological characteristics and is the most appropriate method for

characterizing cyanobacterial taxa so as to further resolve their taxonomy (COMTE et al. 2007; SCIUTO et al. 2011; DVOŘÁK et al. 2015; KOMÁREK 2018). Previous studies repeatedly indicated that coccoid cyanobacteria are particularly heterogeneous and belong to various phylogenetic lineages (KOMÁREK et al. 2011). According to the latest cyanobacterial system proposed by KOMÁREK et al. (2014), coccoid cyanobacteria comprise five orders (Gloeobacterales, Synechococcales, Pleurocapsales, Chroococciopsidales and Chroococcales). One considerable novelty of this new system is that the family Chroococcaceae including the genus *Chroococcus* and the family Merismopediaceae containing the genus *Eucapsis* belong to Chroococcales and Synechococcales respectively, suggesting substantial phylogenetic distance between these two families.

In the current study, we aimed to extend a polyphasic

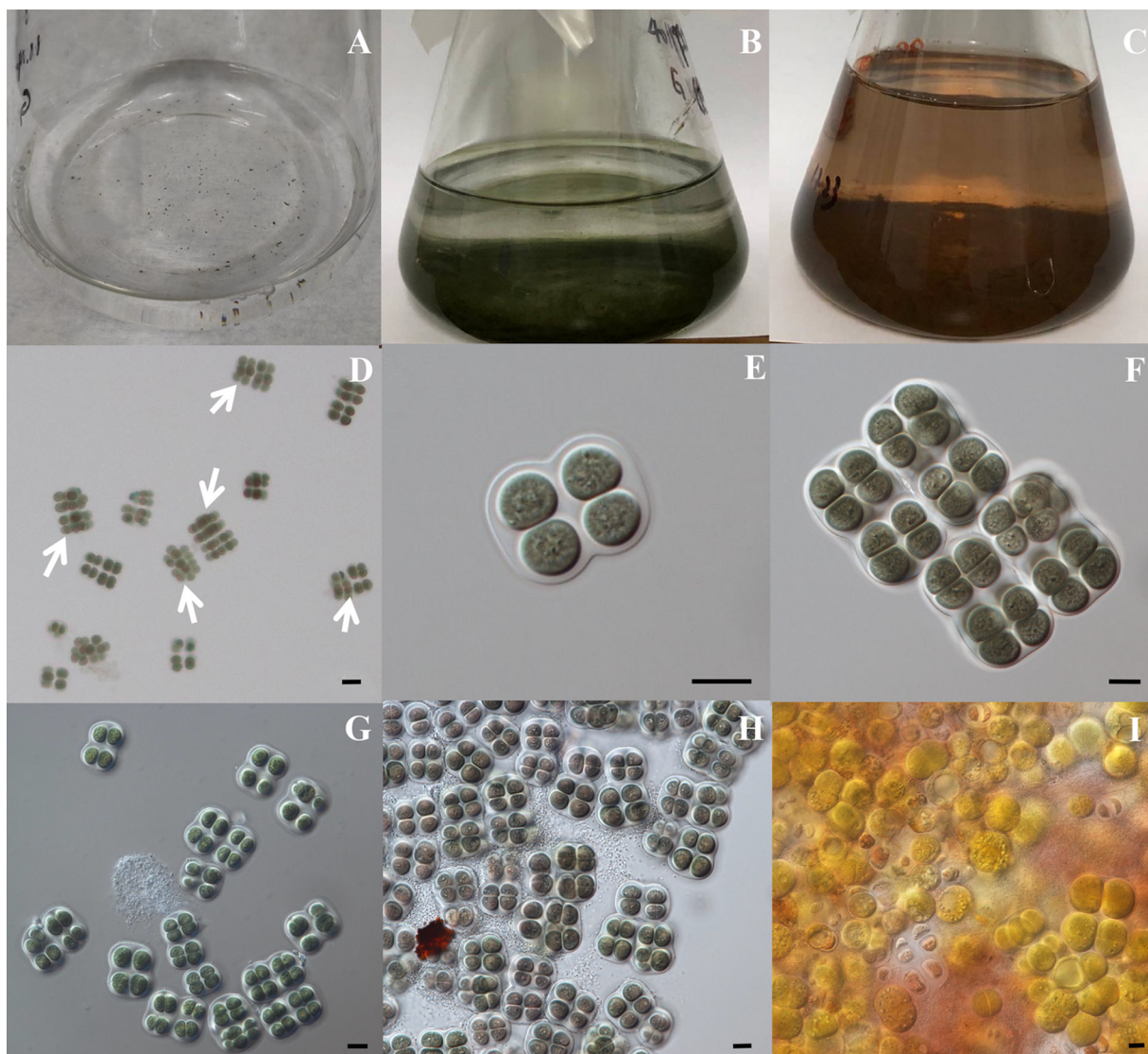


Fig. 1. Light microscopy of *Neochroococcus gongqingensis* CHAB 4018: (A–C) cells of *Neochroococcus gongqingensis* CHAB 4018 in the growth, stability and decline phase in liquid MA medium; (D) arrows indicate the cube colony morphology of CHAB 4018; (E) CHAB 4018 cells with a sheath; (F–G) CHAB 4018 colonies under different growth conditions; (H) Cells of CHAB 4018 began to turn red; (I) CHAB 4018 colonies of 7-month-old. Scale bars 20 μ m (D), 10 μ m (E–I).

investigation to coccoid cyanobacteria by using our newly isolated strain from a subtropical freshwater channel in the city of Gongqing. Using light microscopy, strain CHAB 4018 showed cubic colonies within an envelope containing up to eight cells, which is a relatively typical colonial form of the genus *Eucapsis*. The cell size of this strain was within the range of the type species *E. alpina*; however, each cubic colony of strain CHAB 4018 comprised fewer cells than those of *E. alpina*, and strain CHAB 4018 is planktic in mesotrophic water, whereas *E. alpina* occurs predominantly in littoral areas of mesotrophic swamps (KOMÁREK et al. 2016). The novel strain was also similar to *Chroococcus prescottii* regarding cellular morphology and cubic colonial form; however, the latter typically produces smaller cells (5–8 μm in diameter) and sarcinoid clusters. Further morphological observations revealed a change in cell color from green to red in all cultivations of this new strain (Fig. 2H), which differs from the species *Inacoccus* CCIBT 3475 which produces red-colored sheaths (GAMA et al. 2019) and from *Eucapsis* spp. and *Chroococcus* spp. Comparison of the 16S rRNA gene sequences showed maximum similarity of 93.40% of strain CHAB 4018 with previously identified cyanobacterial taxa, which is below the threshold value for bacterial genus and species delimitation as proposed by WAYNE et al. (1987), STACKEBRANDT & GOEBEL (1994) and STACKEBRANDT & EBERS (2006). Therefore, strain CHAB 4018 most likely represents a novel cyanobacterial genus. The

ITS secondary structure including differences in the D1–D1' helix also supported that this taxon belongs to a new genus (Fig. 4). Phylogenetic tree based on the 16S rRNA gene sequences further showed that the strain CHAB 4018 was separated from the *Chroococcus* 'sensu stricto' clade (cluster D) and the recently defined *Eucapsis* mentioned above. Strain CHAB 4018 was further shown to be more closely related with *Chroococcus* 'sensu stricto' than with *Eucapsis*. Recent taxonomic revisions of *Chroococcus*-like cyanobacteria led to substantial modifications and new insight into the taxonomy of the genus *Chroococcus*, and phylogenetic results produced from *Chroococcus* strains indicated that some morphological features used for discrimination of *Chroococcus* species depend on environmental factors (KOMÁRKOVÁ et al. 2010). Following polyphasic taxonomic investigations of *Chroococcus*, KOMÁRKOVÁ et al. (2010) and KOVÁČIK et al. (2011) defined one particular clade of *Chroococcus* strains as the real clade for the genus (*Chroococcus* 'sensu stricto'). GAMA et al. (2019) examined *Chroococcus*-like strains from the Brazilian Atlantic Forest, and their results led to the establishment of two new genera *Inacoccus* and *Cryptococcus* which are related to *Chroococcus*, and they proposed the large phylogenetic clade *Chroococcus* 'sensu lato' to include the clades *Chroococcus* 'sensu stricto', *Inacoccus*, *Cryptococcus*, and *Limnococcus* (Fig. 1, GAMA et al. 2019). The genus *Limnococcus* was described as an independent genus after it was transferred from *Chroococcus limneticus* by

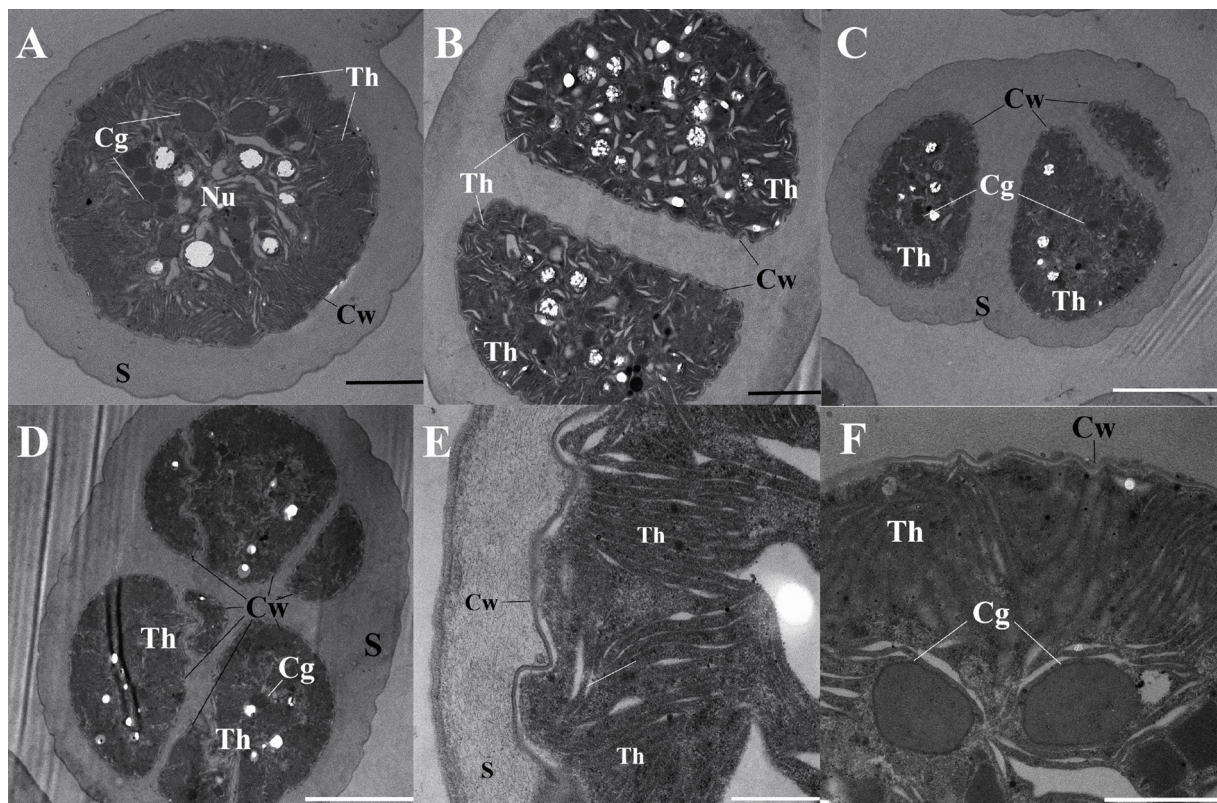


Fig. 2. Ultrastructure of *Neochroococcus gongqingensis* CHAB 4018: (Cw) cell wall; (Cg) cyanophycin granule; (Th) thylakoids; (Nu) nucleoplasm; (S) sheath. Scale bars 2 μm (A–B), 5 μm (C–D), 0.5 μm (E), 1 μm (F).

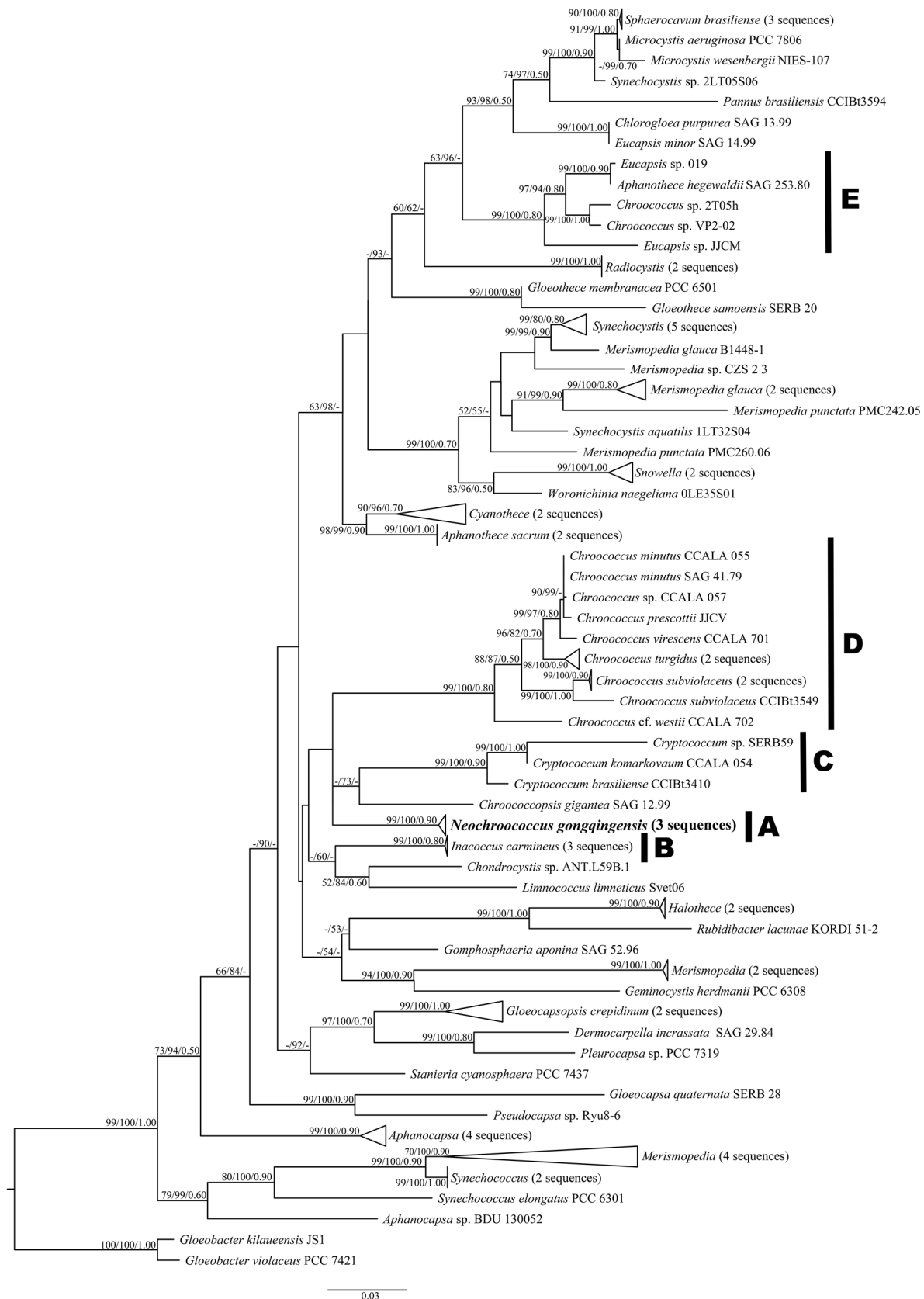


Fig. 3. Phylogenetic tree of 16S rRNA gene sequences (1164bp) derived from maximum-likelihood (ML) analysis. Bootstrap values of NJ / ML / BI methods greater than 50% are showed on the ML tree. The novel coccooid strain of this study is indicated in bold. Bar, 0.06.

KOMÁRKOVÁ et al. (2010), and its distant generic position was verified. *Limnococcus* was further placed in the family Merismopediaceae rather than Chroococcaceae in the latest system of cyanobacterial taxonomy by KOMÁREK et al. (2014); however, phylogenetic support for this placement is lacking so far. Phylogenetic trees produced by KOMÁRKOVÁ et al. (2010) and KOMÁREK et al. (2016) did not clearly support *Limnococcus* to belong to Merismopediaceae. In contrast, the phylogenetic results of the present study suggested that *Eucapsis*-like strain CHAB 4018 is part of the large *Chroococcus* ‘sensu lato’ clade and is more closely related to *Limnococcus* and *Chroococcus* ‘sensu stricto’ than to the *Eucapsis* clade. Consistent with GAMA et al. (2019), our results indicated that *Limnococcus* was more closely related to *Chroococcus* than to *Merismopedia* and *Eucapsis*, which challenges the current family placement of *Limnococcus*. Phylogenetic analyses also showed that *Eucapsis minor* SAG 14.99 did not group with the other five strains of the defined *Eucapsis* clade (Fig. 3 cluster E). Thus, the diversity of *Eucapsis*-like cyanobacteria, even with regard to only a limited number of strains, was higher than expected in the present study.

In conclusion, polyphasic taxonomic assessment of the novel strain *Neochroococcus gongqingensis* CHAB 4018 showed morphological differences, 16S rRNA gene dissimilarity, and differences in the secondary structure of ITS, and its phylogenetic relationship among the examined coccoid cyanobacteria supported the establishment of *Neochroococcus* gen. nov. and *Neochroococcus gongqingensis* sp. nov. *Eucapsis*-like cyanobacteria showed higher diversity than expected. A relatively large phylogenetic tree comprising more strains suggested

phylogenetic intermixture of *Chroococcus*-like and *Eucapsis*-like cyanobacteria which further elucidates the phylogeny and taxonomy of coccoid cyanobacteria.

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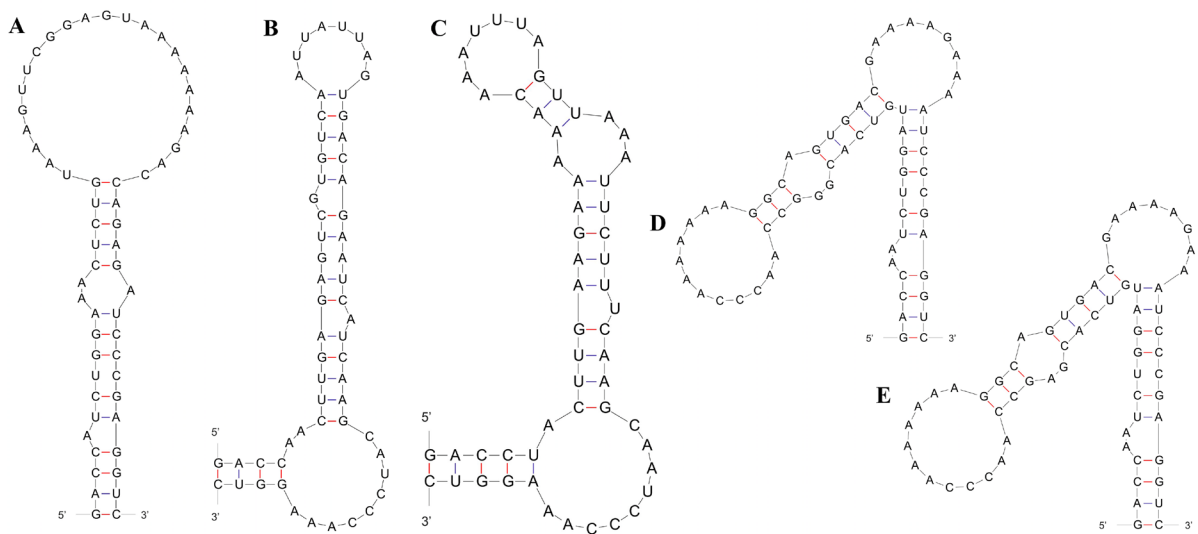


Fig. 4. D1–D1’ helix in *Neochroococcus gongqingensis*, *Cryptococcus* strains and *Inacoccus* strains: (A) *Neochroococcus gongqingensis* CHAB 4018; (B) *Cryptococcus komarkovaum* CICALA 054; (C) *Cryptococcus brasiliense* CCIBt 3410; (D) *Inacoccus carmineus* CCIBt 3418; (E) *Inacoccus carmineus* CCIBt 3475.

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