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Maricoleus vaginatus gen. et sp. nov. (Oculatellaceae, Synechococcales), a novel cyanobacterium isolated from a marine ecosystem in China

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Abstract: Cyanobacteria are widely distributed prokaryotes that play important roles as primary producers in diverse ecosystems. However, taxonomic studies focusing on cyanobacteria from marine ecosystems are rarer than those dealing with cyanobacteria from freshwater environments. In this study, a strain of *Leptolyngbya*–like filaments was isolated from colonies attached to the aquatic weed *Acorus calamus* in Xincun Port, Hainan Province, China. The strain (named WZU 0102) was examined using the polyphasic approach, i.e. the combination of morphology, ultrastructure, phylogeny of 16S rRNA gene and ITS secondary structures. Although this newly isolated strain was found to be morphologically similar to members of the genus *Leptolyngbya* based on light microscopic observations, phylogenetic analysis revealed the strain to be distant from *Leptolyngbya* sensu stricto, forming an independent branch with certain taxonomically ambiguous *Leptolyngbya*–like strains in the constructed phylogenetic trees. The analyses of ITS secondary structures also distinguished the WZU 0102 strain from other related genera. On the basis of these findings, a novel genus *Maricoleus* with type species *Maricoleus vaginatus* is established and described herein. Moreover, two similar *Leptolyngbya*–like strains isolated in Brazil are re–identified as *Maricoleus vaginatus*. Our findings in this study not only expand the currently limited taxonomic coverage of marine cyanobacteria but also contribute to a taxonomic revision of *Leptolyngbya*–like cyanobacteria.

Keywords: cyanobacteria, *Maricoleus*, morphology, 16S rRNA gene, 16S–23S, ITS, taxonomy

Introduction

The cyanobacterial taxonomy and species identification are important tools to understand the biodiversity of cyanobacteria, particularly with respect to the further understanding of the composition and function of cyanobacterial communities in natural biotopes (Debnath et al. 2017). In this regard, the use of molecular methods to solve the classification problems and to estimate the diversity of cyanobacteria has been widely accepted by taxonomists (Garcia–Pichel et al. 2001). The polyphasic approach, combining morphological, ecological and molecular features, has been extensively applied to describe novel taxa of cyanobacteria, and by adopting this approach, cyanobacterial taxonomic system has undergone notable revisions in the past few decades (Komárek et al. 2014; Mareš 2018; Komárek et al. 2020). Currently, however, the taxonomic status of many cyanobacterial genera and species remains unclear. In particular, it is difficult to identify monophyletic groups in a large number of cyanobacterial genera; thus, many groups are still in need of further revision at the genus level, or even at higher categories.

The genus *Leptolyngbya* Anagnostidis et Komárek, a typical polyphyletic group (Chakraborty et al. 2019), is also considered to be the largest, most highly diversified, and heterogeneous genus. In the past cyanobacterial taxonomic systems, type species *Leptolyngbya*

boryana (Gomont) Anagnostidis et Komárek was initially described as *Plectonema boryanum* Gomont, and subsequently reclassified as *Lyngbya pseudoramosa* Hoffmann in 1986 before eventually being established as *Leptolyngbya boryana* by Anagnostidis and Komárek in 1988 (Gomont 1899; Hoffmann 1986; Anagnostidis & Komárek 1988). According to information collated in the Algaebase Database (https://www.algaebase.org/, accessed on 1 December 2022), there are currently 162 recorded species of *Leptolyngbya*, of which 136 species have been taxonomically accepted (Guiry & Guiry 2022). The typical morphological characteristics of this genus are the formation of long thin filaments of isopolar cells 0.5–3.5 μm in width surrounded by a thin but firm colorless sheath. These cylindrical trichomes comprise cylindrical cells that are neither constricted or constricted at the cross–walls, and rarely show false branching (Anagnostidis & Komárek 1988). The species in this genus are often confused with those of other filamentous genera with similar morphological characteristics, such as *Pseudanabaena* Lauterborn and *Limnothrix* Meffert, thereby posing identification difficulties when undertaking field investigations.

The genus *Leptolyngbya* has undergone successive revisions concomitant with ongoing revisions of the cyanobacterial taxonomic system. Since molecular evidences had indicated the filamentous taxa in the orders Pseudanabaenales and Synechococcales often co–occur (Osorio–Santos et al. 2014), the order Pseudanabaenales was lowered to the family level Pseudanabaenaceae (order Synechococcales) in order to establish a monophyletic group (Komárek et al. 2014). Moreover, the taxonomic position of the genus *Leptolyngbya* has been raised to the level of family by Komárek (Komárek et al. 2014). Thus, as designated in the most recent classification system, the genus *Leptolyngbya* belongs to the family Leptolyngbyaceae in the order Synechococcales, and *Leptolyngbya* has been established as the type genus of the family (Komárek et al. 2014). In recent years, taxonomic studies based on the polyphasic approach have resulted in an increasing number of species separated from the genus *Leptolyngbya* such as *Nodosilinea* Perkerson et Casamatta (Perkerson et al. 2011), or other *Leptolyngbya*–like taxa morphological similar to *Leptolyngbya* were described as novel genera, including *Phormidesmis* Turicchia, Ventura, Komárková et Komárek (Turicchia et al. 2009), *Plectolyngbya* Taton, Wilmotte, Smarda, Elster et Komárek (Taton et al. 2011), *Oculatella* Zammit, Billi et Albertano (Zammit et al. 2012), *Haloleptolyngbya* Dadheech, Mahmoud, Kotut et Krienitz (Dadheech et al. 2012), *Pantanalinema* Vaz et Fiore (Vaz et al. 2015), *Alkalinema* Vaz et Fiore (Vaz et al. 2015), *Scytolyngbya* Song, Jiang et Li (Song et al. 2015), *Chroakolemma* Becerra–Absalón et Johansen (Becerra–Absalon et al. 2018), *Albertania* Zammit (Zammit 2018) and *Leptothoe* Konstantinou et Gkelis (Konstantinou et al. 2019). Consequently, it can be anticipated that further taxonomic research focusing on *Leptolyngbya*–like cyanobacteria will enhance the classification system of both Leptolyngbyaceae as well as the whole order Synechococcales. Hence, in order to clarify phylogenetic relationships within the aforementioned cyanobacterial groups, it is considered highly desirable to undertake further morphological, ecological, and molecular studies of the *Leptolyngbya*–like species.

In this study, we isolated a marine cyanobacterium, with morphology similar to those of species in the genus *Leptolyngbya*, from filamentous colonies attached to the aquatic weed *Acorus calamus* L., Sp. Pl. 1: 324 (1753) in Xincun Port, Hainan Province, China. The 16S rRNA gene analyses revealed that this marine strain occupies a phylogenetic position notably distinct from that of *Leptolyngbya.* Moreover, analyses of the secondary structure of the ITS region provided convincing evidence that the ITS of this marine strain has unique secondary structures, thereby supporting the establishment of a novel marine filamentous genus for this strain. We herein describe a novel marine cyanobacterial genus named *Maricoleus*.

Materials and Methods

Isolation and cultivation. The novel cyanobacterium characterized in this study was isolated in October 2013 from filamentous colonies attached to the aquatic weed *Acorus calamus* in Xincun Port, Hainan Province, China. Unialgal filaments from the pretreated samples were isolated using a lab–made Pasteur pipette under a ×40 magnification dissecting microscope (Carl Zelss Stemi 508, Germany) and then transferred to 24–well plates containing sterilized modified liquid BG–11 medium (Rippka et al. 1979) diluted to 50% with seawater for cyanobacterial enrichment. Having cultured the filaments for 3 weeks, verified uncontaminated cyanobacterial cultures were transferred to screw–capped tubes containing 10 ml of the same medium. Thereafter, the strain was maintained at 25 °C under a 12h:12h light:dark cycle with a photon flux density of 30 $\mu mol.m^{-2}.s^{-1}$. The living culture of this novel cyanobacteria, named as WZU 0102, is currently maintained in Wenzhou University, Wenzhou, Zhejiang Province, China, and the dry material of this strain (specimen No. WZUH–ZLHN201301) is stored in the Herbarium of Wenzhou University (WZUH).

Morphological and ultrastructural characterization. Morphological observations of strain WZU 0102 were carried out using a LEICA DM2000 LED microscope (Leica, Germany). Microphotographs of the strain were taken using a LEICA DMC 5400 digital camera photomicrographic system (Leica, Germany) attached to the microscope, and the images were analyzed using Leica Application Suite X 3.7.4 software (Leica, Germany). Measurements of unialgal cellular morphology were performed using digital images obtained at ×1000 magnification. The average values of the length and width of filaments and vegetative cells were calculated from measurements taken from more than 100 individuals.

The ultrastructure of strain WZU 0102 was examined by transmission electron microscope (TEM) according to the description by Geng et al. (Geng et al. 2021). Fresh samples of the isolated strain were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 3 days at 4 °C. The fixed samples were then thoroughly washed with 0.1 M phosphate

buffer and post–fixed with 1% osmium tetroxide for 2 h at room temperature. Following the removal of osmium tetroxide with 0.1 M phosphate buffer, samples were dehydrated in a sequential ethanol gradient (30%, 50%, 70%, 90%, and 100%) and thereafter embedded in Spurr's resin (Spurr 1969). The sections were stained using Uranyl acetate (2%) and lead citrate, and the stained specimens were examined using an HT7700 (Hitachi, Japan) transmission electron microscope under 80 kV.

DNA extraction and PCR amplification. Fresh material of strain WZU 0102 was collected and washed three or four times with sterilized modified liquid BG–11 medium to prevent bacterial contamination. 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 (Lepère et al. 2000) were chosen for obtaining segments containing 16S ribosomal RNA gene and the associated 16S–23S internal transcribed spacer (ITS) region. The 50–µl PCR reaction volumes comprised 1 µl of template DNA, 1 µl of each primer (10 µmol.l^{-1}), 25 µl of 2× Taq Plus Master Mix (Dye Plus) (Vazyme Biotech Co., Ltd, Nanjing, China), and 22 µl of sterile water. PCR amplification was performed using a SimpliAmp™ Thermal Cycler (California, USA) with an amplification profile comprising an initial denaturation at 95 °C for 5 min; 35 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 2 min; and a final elongation step at 72 °C for 5 min. The resulting PCR products were purified using a TIANgel Midi Purification kit (Tiangen Biotech Co., Ltd, Beijing, China), cloned using a pClone007 Versatile Simple Vector Kit (Beijng Tsingke Biotech CO., Ltd., Beijing, China), and then inserted into *Escherichia coli* trans5α cells. Positive clones including the target fragment were sequenced using an ABI 3730 Automated Sequencer (PerkinElmer, Waltham, Massachusetts USA) with the standard plasmid primers RV–M (5'–AGCGGATAACAATTTCACACAGGA–3') and M13–47 (5'–AGGGTTTTCCCAGTCACG–3'). The contigs thus obtained were assembled and edited using Sequencher 4.10.1 and submitted to the GenBank database. At least three valid clones of isolated strain were obtained.

Phylogenetic analyses. The 16S rRNA gene sequences obtained from strain WZU 0102 were initially compared with accessions in the NCBI database using Basic Local Alignment Search Tool, and reference sequences with high similarities were downloaded from the GenBank database to establish a molecular phylogeny. All sequences thus obtained were edited using MAFFT v.450 software (Katoh & Standley 2013), after which we generated a matrix of 124 sequences. On the basis of the Akaike Information Criterion in ModelFinder (Kalyaanamoorthy et al. 2017), the standard selection nucleic acid substitution model (GTR+I+G) was selected as the best fitting model for Bayesian inference (BI) and maximum likelihood (ML) analyses. For ML analysis, selected parameters were estimated using IQ–TREE v1.5.6 (Lam–Tung et al. 2015), and a total of 10000 bootstrap replicates were run under the standard option. For BI analysis, particular parameters were estimated by MrBayes v3.2.6 (Ronquist et al. 2012) as follows: two runs of eight Markov chains were run for 2700000 generations, sampling at every 100 generations, with the initial 25% of the sampled data being discarded as burn–in. To perform neighbor–joining (NJ) analysis, we selected the Kimura–2 model with 1000 bootstrap replicates using MEGA software v7.0 (Kumar et al. 2016). All phylogenetic trees were viewed and edited in FigTree v1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/), and p–distances with pairwise deletion of gaps were established using MEGA software v7.0.

Analyses of the 16S–23S Internal Transcribed Spacer (ITS). The tRNAscan–SE 2.0 web server (Lowe & Chan 2016) was used to screen for the presence of tRNA gene sequences, and secondary structures of the D1–D1', Box–B, and V3 helices of the 16S–23S rRNA ITS in strain WZU 0102 and related species were determined using RNAstructure software (version 5.6). The relevant sequences obtained in this study have been deposited in GenBank database with accession numbers OL614991, OL614992, OL614993 and OL614994. Naming of this novel genus is in accordance with the International Code of Nomenclature of algae, fungi, and plants (ICN).

Results

***Maricoleus* R. Geng, Y. Cheng et R. Li gen. nov.**
Description: Thallus blue–green or bright green, turning yellow when old, macroscopic, typically forming benthic masses. Filaments long, straight or slightly entangled, with rare false branching, facultatively surrounded by sheaths. Sheaths layered, colorless, rarely with more than one trichome present, falling off when old. Trichomes isopolar, cylindrical, not attenuated toward ends, not or slightly constricted at the cross–walls. Vegetative cells cylindrical, isodiametric, slightly longer than wide. Thylakoids parietally arranged. Apical cells rounded, sometimes swollen. Reproduction via motile hormogonia formed by necridia.

Type species: *Maricoleus vaginatus* R. Geng, Y. Cheng et R. Li sp. nov.
Etymology: The name of the new genus "*Maricoleus*" was chosen because this genus was isolated from a marine ecosystem and its filaments were usually surrounded by sheaths.

***Maricoleus vaginatus* R. Geng, Y. Cheng et R. Li sp. nov. (Fig. 1)**
Description: Thallus blue–green or bright green, turning yellow when old, macroscopic, typically forming benthic masses. Filaments long, straight or slightly entangled, with rare false branching, facultatively surrounded by sheaths. Sheaths layered, colorless, rarely with more than one trichome present, occasionally widened, detaching when old, and always open at the apex. Trichomes isopolar, cylindrical, not attenuated towards ends, not or slightly constricted on cross–walls. Vegetative cells blue–green or green, typically cylindrical, isodiametric, barrel–like, with granular content and visible parietal chromatoplasm; 1.42–(2.97)–5.34 µm long, 1.54–(2.57)–4.22 µm wide in mature trichomes, and 1.32–(1.95)–2.68 µm long, 1.38–(1.63)–1.93 µm wide in young trichomes. Thylakoids parietally arranged (Fig. 2). Apical cells rounded, sometimes swollen. Reproduction via motile hormogonia or trichome breakage. Heterocytes and akinetes absent.

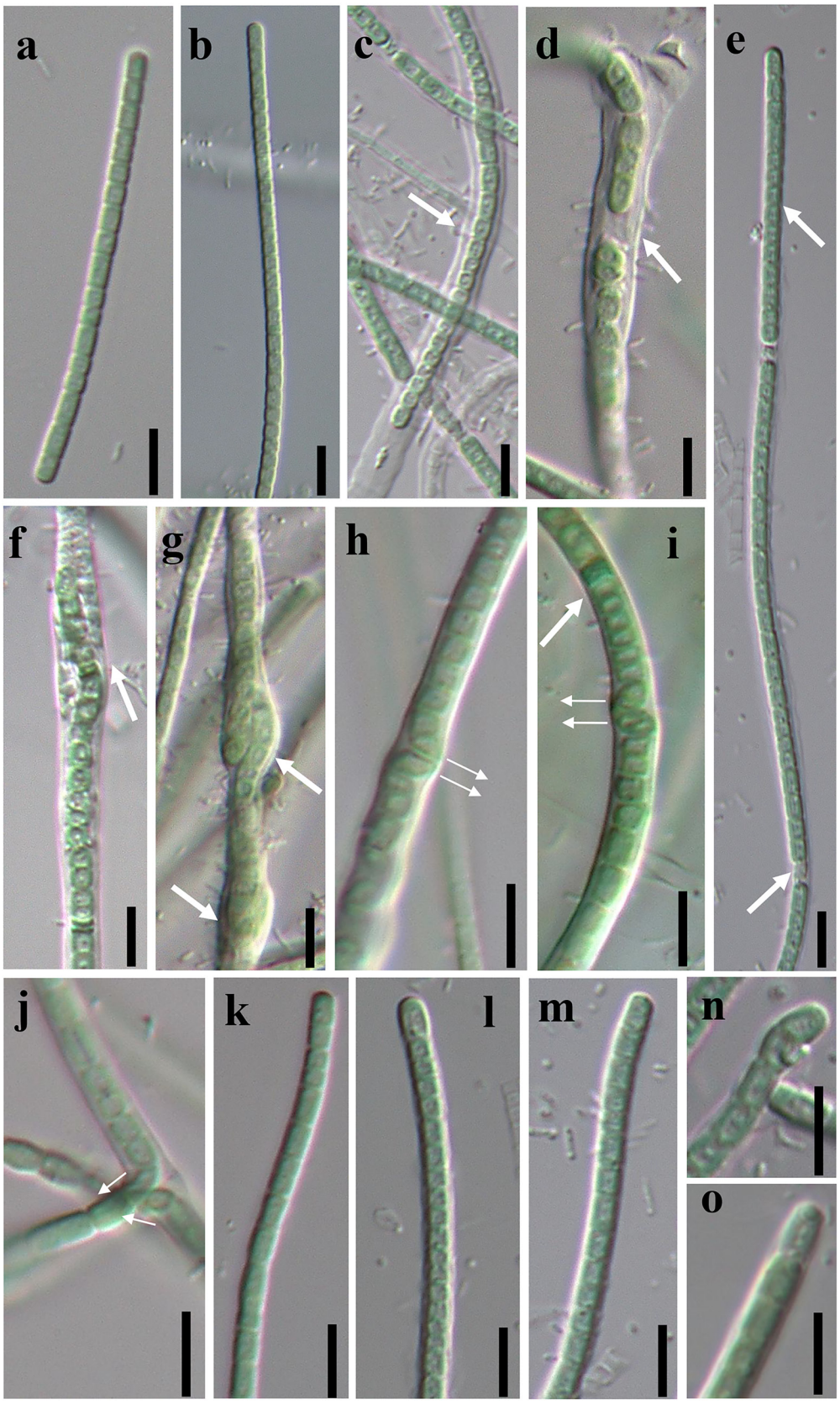

Fig. 1. Light microscopy of *Maricoleus vaginatus* WZU 0102: (a, b) filaments without sheaths; (c, d) filaments with colourless and widened sheaths (Arrows indicate sheaths); (e) filaments with colourless sheaths, hormogonia and necridia (Arrows indicate hormogonia and necridia); (f, g) more than one trichome coiling in a common sheath (Arrows indicate multiple trichomes); (h–j) formation of false branching (Thick arrow indicates necridia, and thin arrows indicate the formation of false branching); (k–o) the details of apical cells. Scale bars 5 μm.

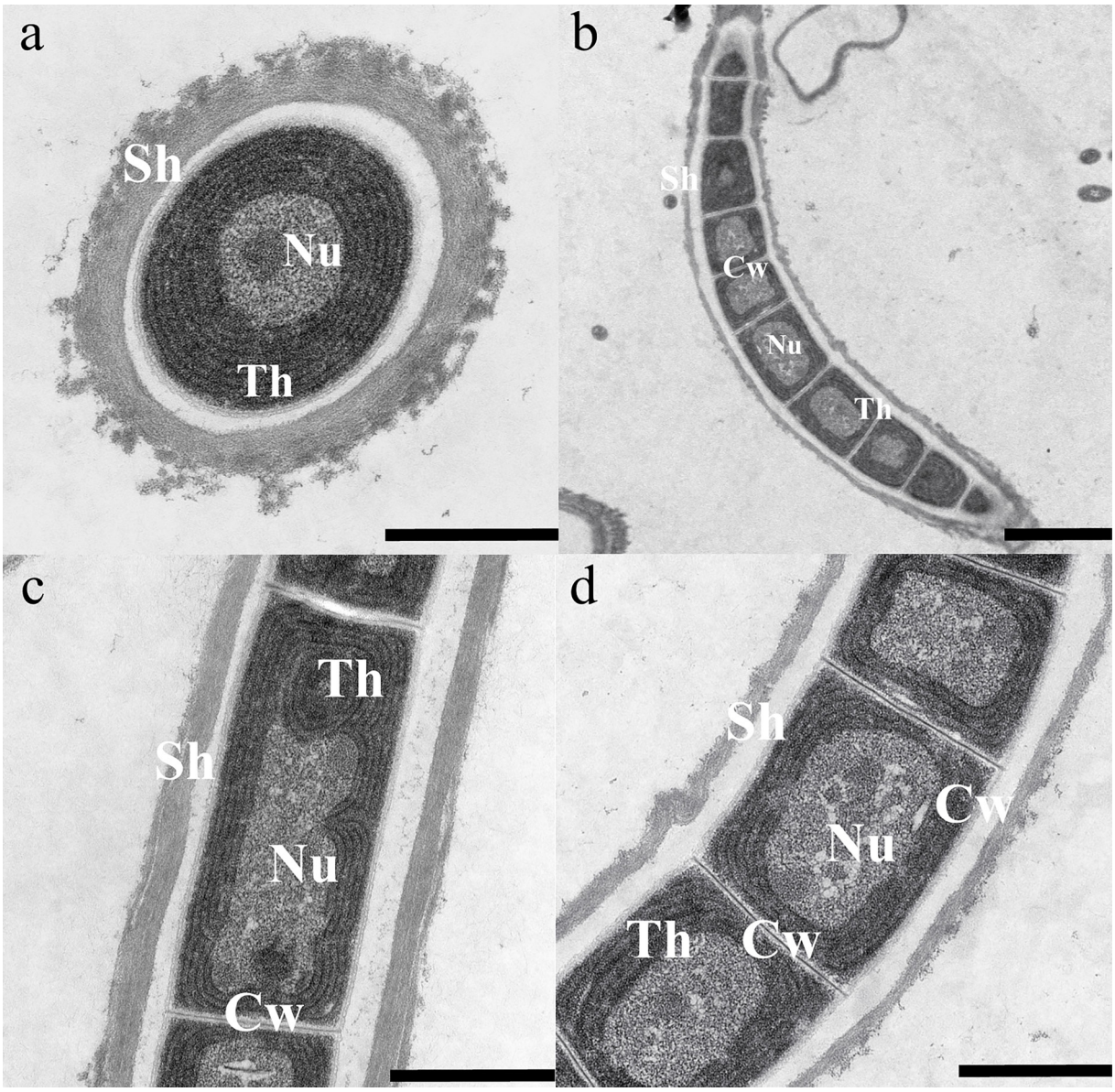

Fig. 2. Ultrastructure of *Maricoleus vaginatus* WZU 0102: (Cw) cell wall; (Th) thylakoids; (Nu) nucleoplasm; (Sh) sheath. Scale bars 1 μm.

Holotype here designated: Dry material of *Maricoleus vaginatus* WZU 0102 was preserved in the Herbarium of Wenzhou University (WZUH), Wenzhou, Zhejiang Province, China, as specimen No. WZUH–ZLHN201301.
Reference strain: WZU 0102.
Type locality: In Xincun port, Hainan Province, China. (October 2013, 18°25'0.81" N, 109°58'58.25" E).
Etymology: The specific name "*vaginatus*" was derived from the sheaths, because the ensheathed trichomes dominated in the culture over the naked ones.
Habitat: Masses attached to emergent plants or floating in sea water.

Diagnosis: The studied species is morphologically similar to the species of the *Leptolyngbya*–like genera, but its filaments are with rare false branching, vegetative cells are longer than wide and usually surrounded by colorless sheaths, and apical cells are rounded, sometimes swollen. Phylogenetic analyses revealed that this strain clusters in an independent clade close to the genera of Oculatellaceae, distant from the *Leptolyngbya* sensu stricto branch. However, the 16S rRNA sequence of studied strain indicated low similarity with those of Oculatellaceae genera. The significant differences in the ITS secondary structures also supported the establishment of this novel genus.

Molecular and phylogenetic analyses
In this study, we obtained four 16S rRNA gene clones (1494 bp) of *M. vaginatus* WZU 0102, among which there was 99.79% sequence similarity. Based on NJ, ML and Bayesian approaches, 142 sequences (138 reference sequences downloaded from NCBI database) were used to construct the respective 16S rRNA gene phylogenetic trees. The integrated tree clearly showed that the *M. vaginatus* strains (clade A) and uncultured

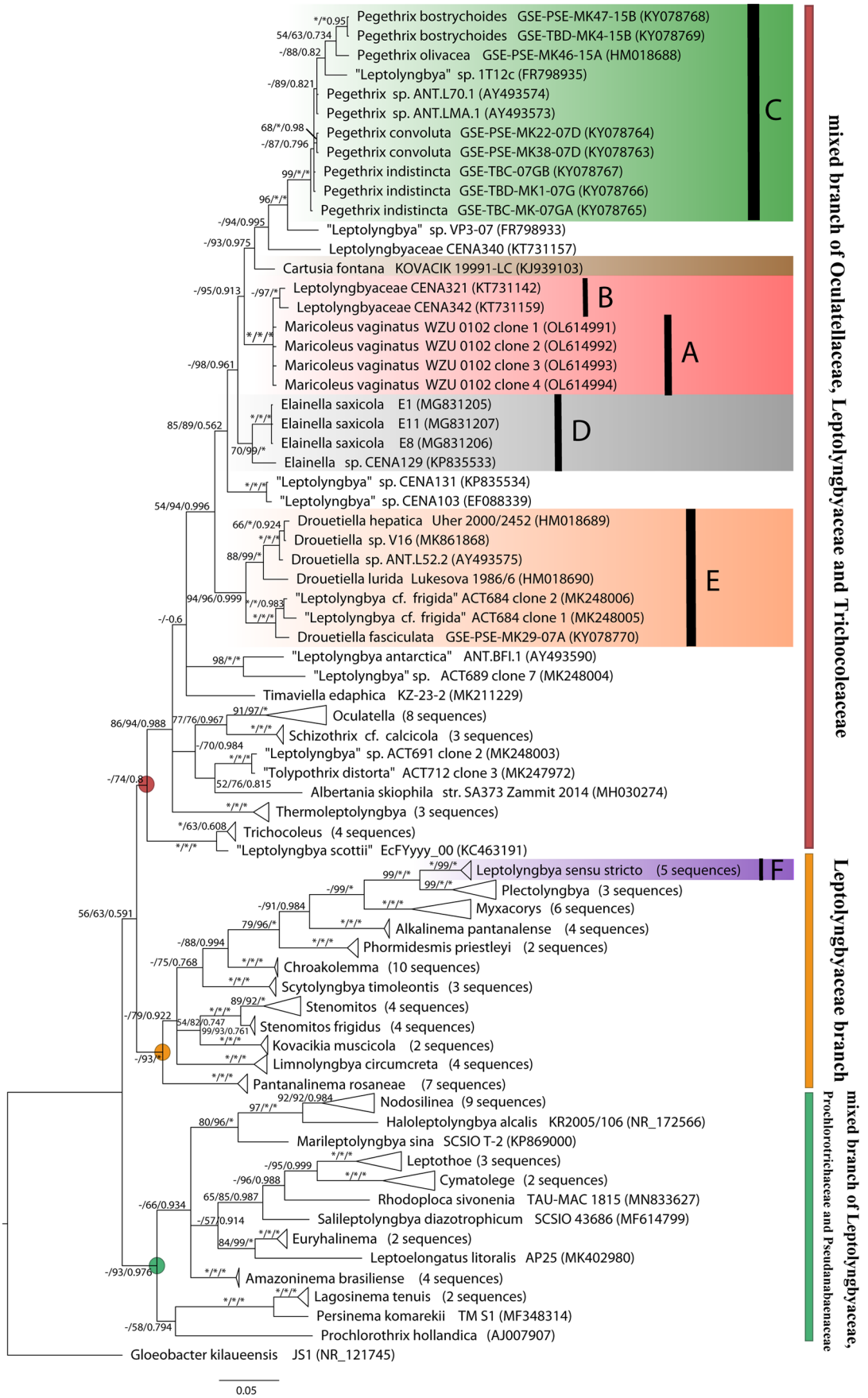

Fig. 3. Phylogenetic tree of 16S rRNA gene sequences with Bayesian inference (BI) analysis. Bootstrap values of NJ/ML/BI methods greater than 50% are showed. "*" indicates bootstrap values of 100 in ML and NJ and BI posterior probabilities of 1.00. This novel strain of this study indicates in bold. Bar, 0.05.

Table 1. Sequence similarity comparison of the 16S rRNA gene between *Maricoleus vaginatus* strains and other related taxa (Minimum–Maximum). Similarity= [1–(p–distance)]*100.

Strain/Genus	1	2	3	4	5	6	7	8	9	10	11	12
1. *Maricoleus vaginatus* WZU 0102												
2. Leptolyngbyaceae CENA321/342	99.15–99.36											
3. *Leptolyngbya* sp. 1T12c	95.41	95.2–95.73										
4. *Leptolyngbya* sp. VP3–07	95.41	95.09–95.52	96.69									
5. *Leptolyngbya* sp. CENA131/103	96.05–96.26	95.84–96.48	95.30–95.52	94.77–94.98								
6. *Elainella* sp. CENA129	96.91	96.58–96.61	95.30	94.98	96.69–96.91							
7. Leptolyngbyaceae CENA340	96.37	95.94–96.48	95.73	95.73	94.13–94.34	95.84						
8. *Leptolyngbya boryana* PCC 6306	89.86	89.75–89.97	89.86	89.33	89.65–89.86	89.54	89.43					
9. *Leptolyngbya* cf. *frigida* ACT684	94.56	94.34–94.77	94.24	93.92	94.56–94.77	94.77	93.60	90.07				
10. *Drouetiella* (5 strains)	94.45–95.09	94.34–94.98	94.13–94.66	93.81–94.77	94.88–95.41	94.56–95.20	92.96–93.70	89.33–90.18	96.48–98.83			
11. *Pegethrix* (11 strains)	95.09–95.73	94.66–96.62	97.87–98.93	96.58–97.23	94.45–95.2	94.56–95.20	95.09–96.05	89.22–89.97	93.81–94.13	93.70–94.66		
12. *Cartusia fontana* KOVÁČIK 1999/1–LC	97.23	97.12–97.33	96.26	96.48	96.16–96.37	96.80	96.16	88.79	95.09	94.98–95.52	95.84–96.58	
13. *Elainella saxicola* E1/E8/E11	96.58	96.16–96.58	95.84	95.73	95.84–96.05	97.76	95.73	90.39	95.20	94.45–95.09	95.20–96.05	96.91

Table 2. Characteristics of 16S–23S ITS region for *Maricoleus vaginatus* and other related strains.

Organisms	GenBank	ITS total length (nt)	D1–D1' helix length (nt)	D2 region	$tRNA^{Ile}$	$tRNA^{Ala}$	Box B helix length (nt)	Box A spacer	V3 helix length (nt)
***Maricoleus vaginatus* WZU 0102**	OL614991–94	446	64	CTTTCAAACTAT	+	+	34	GAACCTAGAAAA	19
Pegethrix bostrychoides GSE–PSE–MK47–15B	KY078768	614	87	CTTCCAAACTAT	+	+	36	GAACCTTGAAAA	94
Drouetiella lurida Lukesova 1986/6	HM018690	455	64	CTTCTAAGCTAT	+	+	33	GAACCTTGAAAA	–
Elainella saxicola E1	MG831205	519	62	CTTCTAAGCTAT	+	+	33	GAACCTAGAAAA	19
Timaviella edaphica KZ–23–2	MZ518093	485	67	CTTTCAAGCTAT	+	+	33	GAACCTTGAAAA	58
Thermoleptolyngbya sichuanensis PKUAC–SCTA183	MN315648	782	64	CTTCCAAACTAT	+	+	48	GAACCTTGAAAA	74
Oculatella ucrainica KZ–5–4–1	MG652620	462	64	CTTTCAAACTAT	+	+	34	GAACCATGAAAA	50
Leptolyngbya boryana PCC 6306	EF429290	477	51	CTTCTAAACTAT	+	+	33	GAACCAAGAAAA	21

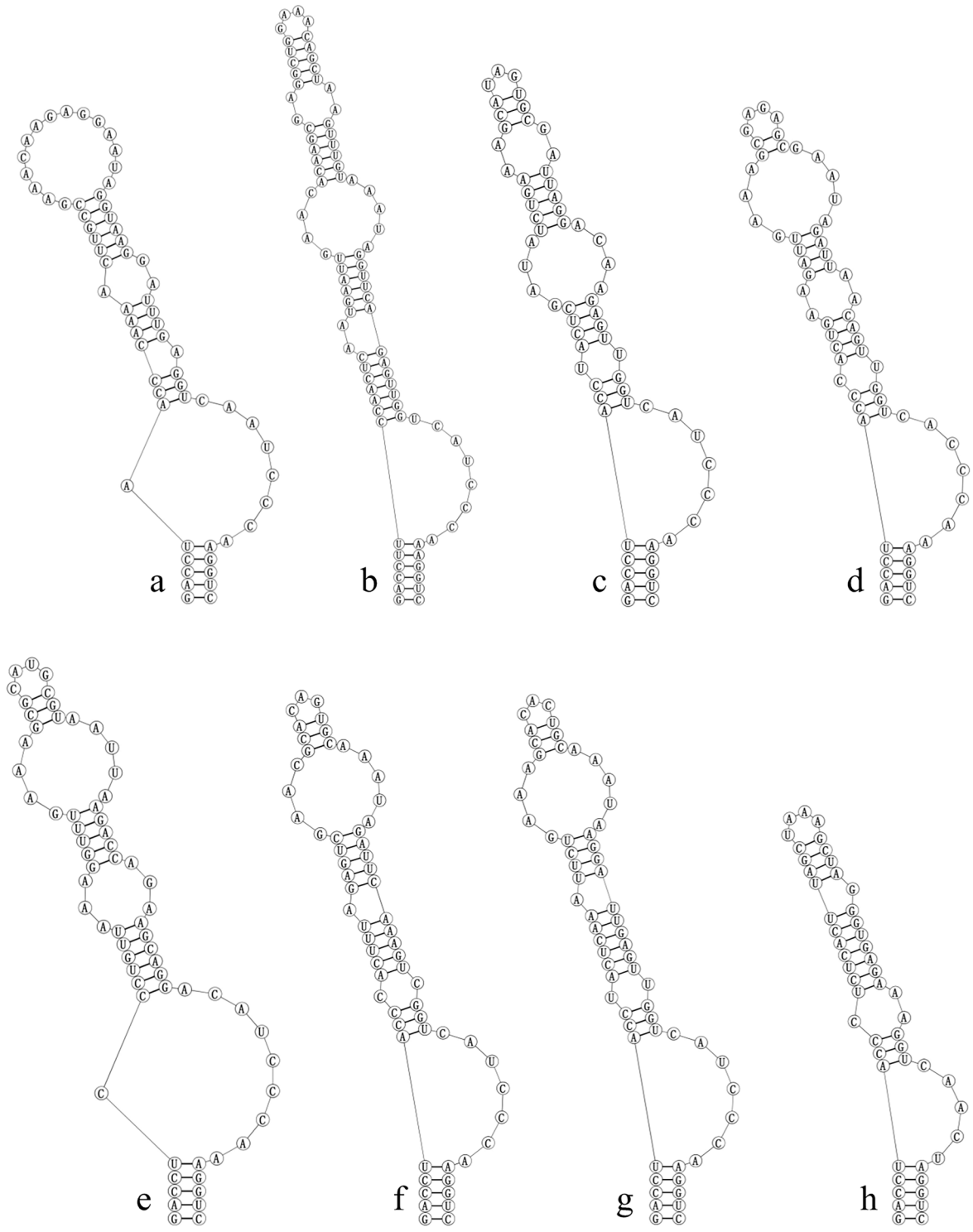

Fig. 4. The secondary structures of D1–D1' helix in *Maricoleus vaginatus* and other related species: (a) *Maricoleus vaginatus* WZU 0102; (b) *Pegethrix bostrychoides* GSE–PSE–MK47–15B; (c) *Drouetiella lurida* Lukesova 1986/6; (d) *Elainella saxicola* E1; (e) *Timaviella edaphica* KZ–23–2 ; (f) *Thermoleptolyngbya sichuanensis* PKUAC–SCTA183; (g) *Oculatella ucrainica* KZ–5–4–1 ; (h) *Leptolyngbya boryana* PCC 6306.

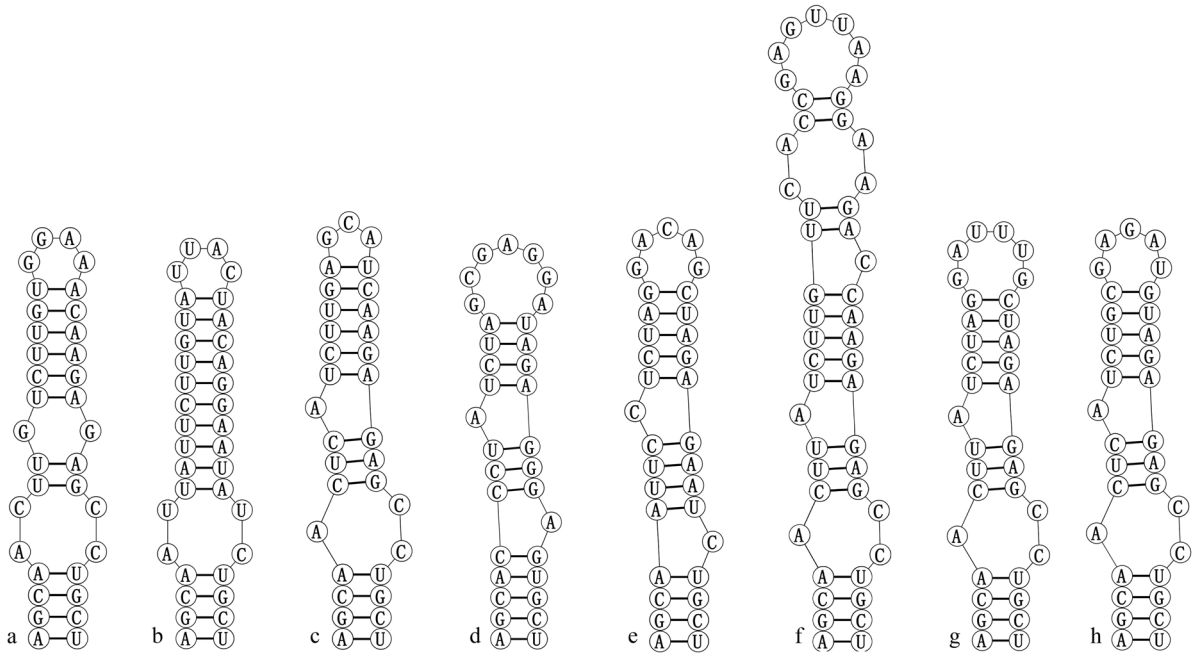

Fig. 5. The secondary structures of Box–B helix in *Maricoleus vaginatus* and other related species: (a) *Maricoleus vaginatus* WZU 0102; (b) *Pegethrix bostrychoides* GSE–PSE–MK47–15B; (c) *Drouetiella lurida* Lukesova 1986/6; (d) *Elainella saxicola* E1; (e) *Timaviella edaphica* KZ–23–2; (f) *Thermoleptolyngbya sichuanensis* PKUAC–SCTA183; (g) *Oculatella ucrainica* KZ–5–4–1; (h) *Leptolyngbya boryana* PCC 6306.

Leptolyngbyaceae CENA strains (clade B) were clustered to form an independent branch (red part in Fig. 3), close to the genera *Cartusia* Mai, J.R.Johansen et Pietrasiak (brown part), *Pegethrix* Mai, J.R.Johansen et Bohunická (clade C, green part), *Elainella* Jahodářová, Dvořák et Hašler (clade D, gray part), and *Drouetiella* Mai, J.R.Johansen et Pietrasiak (clade E, orange part), although distant from the *Leptolyngbya* sensu stricto branch that includes the type species *Leptolyngbya boryana* (clade F, purple part), with high NJ, ML, and BI bootstrap values of 99%, 100%, and 1.00, respectively, and occupies a unique phylogenetic position in the family Oculatellaceae (Fig. 3). Sequences of the *M. vaginatus* and uncultured Leptolyngbyaceae CENA strains were found to have similarities of between 99.15% and 99.36% (Table 1), which was higher than the 97% threshold defining bacterial species classification, thereby indicating the high probability that the uncultured cyanobacterium and *M. vaginatus* WZU 0102 were strains of the same species. In addition, *Cartusia fontana* (Hansgirg) Mai, J.R.Johansen et Pietrasiak (Fig. 3 brown part) and *Elainella saxicola* Jahodářová, Dvořák et Hašler strains (Fig. 3 clade D) were found to show 97.23% and 96.57% sequence similarities with the newly isolated strain, respectively (Table 1). However, although these similarity values are higher than the threshold necessary for bacterial genera classification, these two taxa were observed to have significant morphological differences from *M. vaginatus* (Table 3) and to be located separately in the 16S rRNA phylogenetic tree (Fig. 3). Consequently, these three taxa were identified as different genera. Thus, on the basis of the aforementioned results, we believe that we have sufficiently strong evidence to justify designating *Maricoleus* as a novel cyanobacterial genus in the family Oculatellaceae.

Analyses of ITS secondary structures

The full length of the four ITS clones of *M. vaginatus* WZU 0102 obtained in this study was 446 bp (Table 2), with all sequences containing both tRNA ^{Ile} and tRNA ^{Ala}. Given the unavailability of the ITS sequences of some related strains (like Leptolyngbyaceae CENA321/342, *Cartusia fontana*, *Leptolyngbya* sp. VP3–07, etc.) in the current databases, along with WZU 0102, we selected species of seven relative genera for ITS secondary structure analysis, namely, *Pegethrix bostrychoides* Mai, J.R.Johansen et Bohunická, *Drouetiella lurida* Mai, J.R.Johansen et Pietrasiak, *Elainella saxicola*, *Timaviella edaphica* (Elenkin) Vinogradova et Mikhailyuk, *Thermoleptolyngbya sichuanensis*, *Oculatella ucrainica* Vinogradova et Mikhailyuk, and *Leptolyngbya boryana* (Table 2).

Although the D1–D1' helix is considered to be the most conserved ITS secondary structure in cyanobacteria, we identified seven different structures among the eight assessed species (Fig. 4). In *M. vaginatus* WZU 0102, the D1–D1' helix contains 64 bases, whereas lengths in the seven compared species ranged from 51 to 87 bases (Table 2). With the exception of *P. bostrychoides*, which had a 6 bp (5'–GACCUU–AAGGUC–3') basal stem, the D1–D1' helices of all other strains were found to have a 5 bp (5'–GACCU–AGGUC–3') conserved basal stem. In *M. vaginatus* WZU 0102, the predicted D1–D1' helix

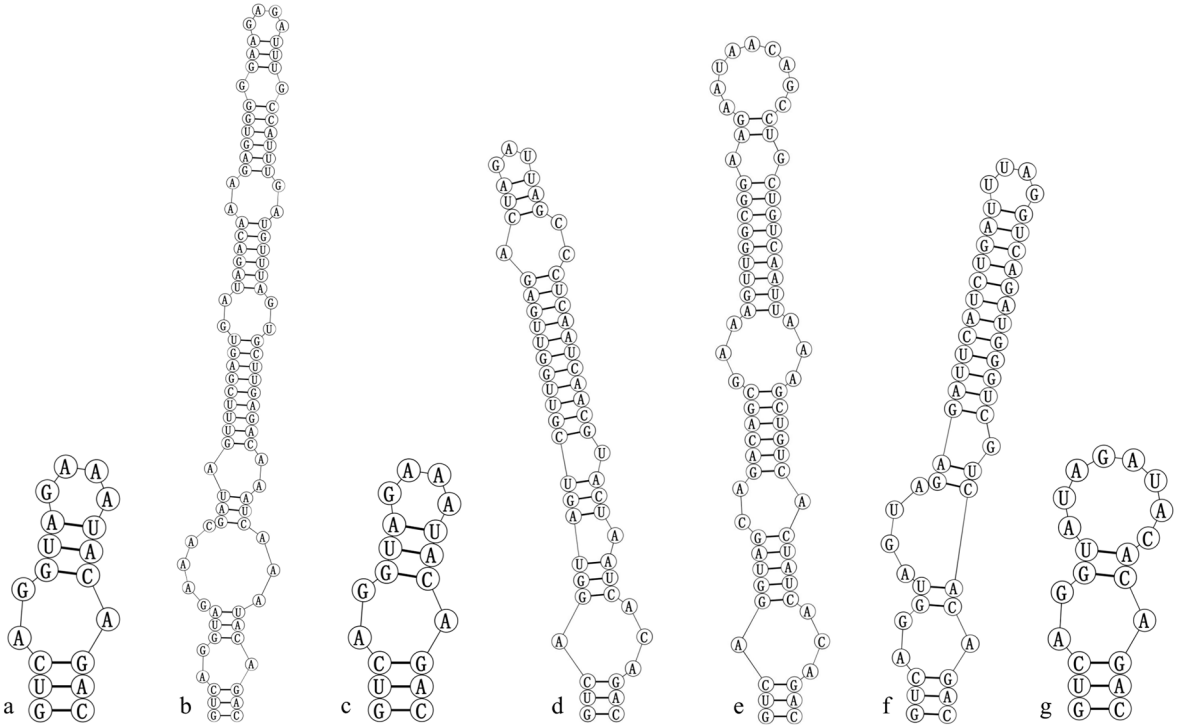

Fig. 6. The secondary structures of V3 helix in *Maricoleus vaginatus* and other related species: (a) *Maricoleus vaginatus* WZU 0102; (b) *Pegethrix bostrychoides* GSE–PSE–MK47–15B; (c) *Elainella saxicola* E1; (d) *Timaviella edaphica* KZ–23–2; (e) *Thermoleptolyngbya sichuanensis* PKUAC–SCTA183; (f) *Oculatella ucrainica* KZ–5–4–1; (g) *Leptolyngbya boryana* PCC 6306.

consists of a 1:8 base bilateral bulge above the basal stem, a 3 bp helix, a single base right unilateral bulge, a 4 bp helix followed by a 1:2 base bilateral bulge, and a 6 bp helix with a terminal loop consisting of 15 bases (Fig. 4a), which differed significantly from the structures established for the other seven species. A specific feature of D1–D1' helix of *M. vaginatus* was the absence of a small, 3–5 base terminal loop which was present in all other species (Fig. 4b–4h).

There were five different types of Box–B helices in this study (Fig. 5), ranging in size from 48 bases in *T. sichuanensis* to 33 bases in *D. lurida*, *E. saxicola*, *T. edaphica,* and *L. boryana*, with that in *M. vaginatus* being 34 bases (Table 2). Although *M. vaginatus* and *P. bostrychoides* were established to have a similar type of Box–B helix, these strains differed with respect to the fact that *M. vaginatus* has an extra 1:1 base bilateral bulge above the 2:2 base bilateral bulge (Fig. 5a and 5b) and also differed slightly in sequence length, base number, and base arrangement in the terminal loop. A further specific feature of *M. vaginatus* was the absence of asymmetric bulges, which were present in the other six species (Fig. 5c–5h).

The V3 helices of studied species were highly variable, with sequence lengths ranging from 19 to 94 bases (Table 2). As the ITS sequence of *D. lurida* is too short to fold into a V3 helix, only the structures of the other seven species are described here (Fig. 6). In our study, *M. vaginatus* and *E. saxicola* had identical V3 helix patterns in terms of base sequence, sequence length, and stem–loop structure, which consists of a 3 bp (5'–GUG–GAC–3') conserved basal stem followed by a 2:1 base bilateral bulge and a further 3 bp helix, and ended with a 4 base terminal loop (Fig. 6a and 6c). The remaining five related species were found to form different stem–loop structure (Fig. 6b, 6d–6g).

DISCUSSION

It is widely acknowledged that morphological characteristics alone do not provide a sufficiently accurate reflection of the evolutionary relationships among cyanobacteria. With the gradual application of electron microscopy and 16S rRNA gene sequence analyses, the cyanobacterial taxonomic system has undergone extensive reconstruction and revision in recent decades (ANAGNOSTIDIS & KOMÁREK 1985; KOMÁREK et al. 2014). Modern cyanobacterial taxonomy seeks to establish monophyletic groups at order, family, genus, and species levels, and in this regard, a polyphasic approach, which combines morphological, ecological, molecular sequence, and phylogenetic analyses, has gradually emerged as the most suitable strategy for characterizing and describing cyanobacterial taxa (ANAGNOSTIDIS & KOMÁREK 1985; CASAMATTA et al. 2005; SIEGESMUND et al. 2008; PERKERSON et al. 2011; KOMÁREK et al. 2014).

In this study, we isolated a marine filamentous cyanobacterial strain from Xincun Port, Hainan Province, the typical morphological features of which indicated an affiliation to the *Leptolyngbya*–like taxa

Table 3. Morphological comparison of *Maricoleus* and other related strains.

	Filaments	False branching	Sheath	Apical cell shape	Pigmentation	Cell size (µm)		Distribution	Habitat
						Long	Wide		
Maricoleus vaginatus WZU 0102	filaments long, straight or slightly entangled	rare	sheaths facultatively existent, colorless, thin, rarely with more than one trichome present, fall off when old, and always open at the apex.	rounded, timesswollen	blue–green, bright green and turn yellow when they are old	1.42–(2.97)–5.34	1.54–(2.57)–4.22	China	sea water
Leptolyngbyaceae CENA321/342	morphological traits similar to those traditionally described for *Leptolyngbya*							Brazil	salt–excreting leaves
Leptolyngbya sp. VP3–07	/	/	/	/	brown–green	/	/	Italy	wet rock surface
Leptolyngbya sp. CENA131	filaments entangled, loosely arranged	/	sheaths colorless (culture conditions)	/	brown–green	2.0–3.5	1.6–2.0	Brazil	reservoir
Leptolyngbya sp. CENA103	loosely arranged, entangled	rare	sheath fine, inconspicuous, hyaline	/	brownish	1.3–(2.2)–3.3	1.6–(2.1)–2.6	Brazil	waste stabilization pool
Leptolyngbya sp. CENA129	filaments entangled, loosely arranged	/	sheaths colorless (culture conditions)	/	green	1.0–3.0	1.0	Brazil	reservoir
Leptolyngbyaceae CENA340	morphological traits similar to those traditionally described for *Leptolyngbya*							Brazil	salt–excreting leaves
Drouetiella	mostly solitary, at times consolidated into fascicles	with infrequent single false branching	sheath clear, thin, and firm, occasionally widened.	cylindrical, untapered, rounded, without calyptra	bright blue green, olive–green, reddish brown, brownish, purplish–brown	*D. lurida*: (2.1) 2.9–3.8 (5.4); *D. fasciculata*: 3.1–4.4 (5.4); *D. hepatica*: (2.2) 3.1–4.5	*D. lurida*: 1.7–2.1; *D. fasciculata*: 1.5–2.4 (3.0); *D. hepatica*: 1.7–3.0	USA, Slovakia	tailings of Cainozoic clay, large seep wall, waterfall and subaerial limestone
Pegethrix	mostly solitary	with infrequent double and single false branching	sheath clear, thin and firm to soft and widened, but never diffluent	rounded, without calyptra	bright blue–green, dark olive–green	*P. bostrychoides*: 1.0–3.0; *P. olivacea*: 1.7–2.6; *P. convoluta*: 1.0–2.5–(3.7); *P. indistincta*: (1.3)–1.7–2.7	*P. bostrychoides*: 1.5–2.5–(3.0); *P. olivacea*: 1.9–2.8; *P. convoluta*: 1.3–2.5 (3.2); *P. indistincta*: 1.9–3.3	USA	sandstone seep wall, small pool, and waterfall
Cartusia fontana KOVÁČIK 1999/1–LC	filaments straight or flexuous, up to 5.4 µm wide in filaments with sheath	without false branching	sheath colorless, firm, usually thin, but occasionally widened	rounded	bright blue–green, becoming olive–green with age	1.0–1.3; 1.3–2.0	1.8–2.7; 2.7–3.5	Slovakia	wall surface
Elainella saxicola	straight, undulate, bent, often with loops	present	sheath colourless, thin and distinct, variable in length	rounded	yellow–green, green, grey–green	1.34–(2.3)–3.76	1.73–(2.27)–2.63	Vietnam	ephemeral water-body in the forest, on granite and sand in waterfall

(Fig. 1). The cell width of this strain also falls within the range of *Leptolyngbya*, and the parietal arrangement of thylakoids is consistent with that of *Leptolyngbya* (Fig. 2). However, the morphological features characterizing the genus *Leptolyngbya*, and even the family Leptolyngbyaceae, are simple widespread features that are commonly observed in a considerable number of fine filamentous cyanobacterial genera, which makes it impossible to accurately identify the isolated strain as a *Leptolyngbya* taxon based solely on morphological characters. Consequently, a molecular approach is essential for examination and unambiguous identification.

As mentioned in the Results section, the *M. vaginatus* strain (China) and uncultured Leptolyngbyaceae CENA strains (Brazil) were clustered into an independent branch (red part in Fig. 3) of the constructed phylogenetic trees, close to the genera *Cartusia*, *Pegethrix* (clade C), and *Elainella* (clade D), with high NJ, ML, and BI bootstrap values of 99%, 100%, and 1.00, respectively. Notably, this highly supported branch was distant from that containing *Leptolyngbya* sensu stricto (clade F, purple part), indicating that the novel genus *Maricoleus* occupies a unique phylogenetic position in the family Oculatellaceae rather than within the Leptolyngbyaceae (Fig. 3). Although the similarity values obtained for *M. vaginatus* and *C. fontana* and *E. saxicola* were higher than the threshold for bacterial genera classification (Wayne et al. 1987; Stackebrandt & Goebel 1994; Stackebrandt & Ebers 2006), we continue to recognize these as distinct genera, given their conspicuous morphological differences (Table 3) and clearly separated phylogenetic positions (Fig. 3). Therefore, on the basis of morphological characters, phylogenetic relationships, and DNA sequence similarities, we herein treat the clade containing the isolated strain WZU 0102 (Fig. 3 red part) as a novel genus in family Oculatellaceae.

The secondary structure of the 16S–23S rRNA ITS region is considered to be an important criterion for cyanobacterial classification and is often used for inter–generic differentiation (Iteman et al. 2000; Boyer et al. 2002; Johansen & Casamatta 2005; Johansen et al. 2011; Martins et al. 2019). We detected considerable differences in sequence length, base arrangement, and stem–loop structure in the D1–D1', Box–B, and V3 helices of analyzed strains. That is a result of the rearrangement of bases in the ITS sequences of different species which led to the formation of different types of stable secondary structures at the lowest free energy level (Chakraborty et al. 2018). Consistent with their different positions in phylogenetic trees, we detected notable variations in nucleotide sequences and significant differences in stem–loop folding patterns between the WZU 0102 and other related strains (Figs 4–6). *Maricoleus* formed an evolutionary branch independent on other related Leptolyngbyaceae and Oculatellaceae genera. That is consistent with our observations of differences in the ITS secondary structures of these groups. All differences between the isolated strain and species of closely related genera support our decision to establish this strain as a novel genus.

In addition to the taxonomic features of the organisms per se, habitat features can play a contributory role in cyanobacterial classification, given that species isolated from different habitats may have different genetic and evolutionary histories (Nubel et al. 2000; Řehakova et al. 2007; Dadheech et al. 2012; Komárek 2016; Cai et al. 2020). *M. vaginatus* WZU 0102 is a marine filamentous cyanobacterium isolated from Xincun Port in Lingshui County, Hainan Province. Similarly, the re–classified *M. vaginatus* strains CENA321 and CENA342 were isolated from the salt–excreting leaves of *Avicennia schaueriana* in a saline environment along the coastline of São Paulo State, Brazil (Alvarenga et al. 2016). In contrast, the filamentous cyanobacterium *Cartusia fontana* KOVÁČIK 1999/1–LC was isolated from the surface of a wall in Slovakia (Mai et al. 2018), whereas strains of *E. saxicola* were isolated from an ephemeral forest waterbody, on granite, and in sand under a waterfall (Jahodářová et al. 2018).

Currently, as proposed by Mai et al. (2018), the family Leptolyngbyaceae is subdivided into the four monophyletic families Trichocoleaceae, Oculatellaceae, Leptolyngbyaceae, and Prochlorotrichaceae (Mai et al. 2018). However, our findings in the present study revealed that certain strains in Leptolyngbyaceae clustered together with those in the other three families in the phylogenetic tree, rather than forming a monophyletic group (Fig. 3). Although in recent years there have been extensive revisions of the family Leptolyngbyaceae, there remain uncertainties as to the taxonomic status of some organisms classified in the genus *Leptolyngbya*, which although located distant from the *Leptolyngbya sensu stricto* branch, have been incorrectly identified as "*Leptolyngbya* sp." strains (Johansen et al. 2011; Osorio–Santos et al. 2014; Shimura et al. 2015). Numerous strains morphologically similar to *Leptolyngbya* have been sequenced for the 16S rRNA gene but simply noted as "*Leptolyngbya* sp.," although subsequent analyses revealed that these were clearly distant from the *Leptolyngbya* sensu stricto clade. Consequently, these strains should be removed from the genus *Leptolyngbya*, and even the family Leptolyngbyaceae, and transferred to other taxa or established as new genera/species based on polyphasic characterization, as exemplified by the re–classification proposed in the present study. With the increasing exploration of hitherto unsurveyed habitats, it is expected that a growing number of novel cyanobacterial species will be described and determined using the polyphasic approach.

In conclusion, in this study, we propose the establishment of the novel cyanobacterial genus *Maricoleus* based on the polyphasic characterization of a strain isolated from a marine ecosystem in China. Although morphologically similar to *Leptolyngbya*, this strain is phylogenetically closer to genera in the family Oculatellaceae. The type species of this new genus has

been designated *Maricoleus vaginatus*, and two strains of *Leptolyngbya*–like cyanobacteria isolated from Brazil have been re–identified as *Maricoleus vaginatus*. The findings of this study broaden our understanding of the diversity of cyanobacteria in marine ecosystems and highlight the fact that taxonomic criteria based on morphology are gradually losing their original utility. Moreover, our molecular analyses provide evidence to indicate that phenotypic convergence is a general pattern of cyanobacterial macroevolution. Discussions of a larger number of strains and sequences are needed to gain further insights for future revisions of the taxonomy of *Leptolyngbya*–like cyanobacteria.

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