

Article

Taxonomic Discussion on Cyanobacterial Systematics at Family Level, with Special Regards to Phormidiaceae by Using the Strains of Chinese Newly Recorded Genera *Ancylothrrix* and *Potamolinea*

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Abstract: In the past decades, the taxonomic status of the cyanobacterial family Phormidiaceae has always been chaotic and problematic. In this study, filamentous cyanobacteria were investigated in the east of China, and twenty strains isolated from different locations of Zhejiang Province were characterized. Using the polyphasic approach combining morphological, molecular and phylogenetic features, these strains were grouped within the members of the genera *Ancylothrrix* and *Potamolinea*, the newly recorded genera of cyanobacteria in China. Based on the collected taxonomic information of the family Phormidiaceae, cyanobacterial systematics at family level were further discussed. This study provided a simple and efficient example to perform the phylogenetic evaluation for the monophyly and rationality of currently used families of cyanobacteria by using the regional strains based on the polyphasic approach.

Keywords: cyanobacteria; Phormidiaceae; newly recorded genera; polyphasic approach



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1. Introduction

The cyanobacterial taxonomic system has encountered rapid and radical revisions during the last decades [1–4]. Such revisions, through intensive usage of the polyphasic approach combining morphological, ecophysiological, biochemical and molecular characters, led to the establishment of a large number of new genera and species [5–8]. The updated cyanobacterial taxonomy summarized by Komárek et al. (2014) proposed an eight orders, 46 families and 202 genera system, in which the eight-ordered frame was obtained by phylogenomic analyses. This new system further presented a detailed and effective guideline to characterize the cyanobacterial genera and provided evaluation of the present status of all the 202 cyanobacterial genera mainly based on the availability of molecular data. However, the monophyly at the family level in this system was not discussed even though all the families were described in this system, and such an evaluation on the status of all the families are highly dependent on premised work on the cyanobacterial genera. In order to achieve the ideal taxonomic system of cyanobacteria at all categories, the polyphasic approach should also be used in the studies on the cyanobacterial families, especially some important ones.

The family Phormidiaceae, separated from the family Oscillatoriaceae, was created by Anagnostidis and Komárek (1988) in their monograph titled “modern approach to the classification system of cyanophytes 3-Oscillatoriales”. With *Phormidium* as the type

genus, Phormidiaceae was further divided into three subfamilies as Phormidioideae, Microcoleoideae and Spirulinoideae, including many important genera such as *Planktothrix*, *Trichodesmium*, *Microcoleus*, *Spirulina* and *Arthrospira*. In “system of cyanoprokaryotes (cyanobacteria) state in 2004, Hoffman et al. (2005) indicated that the higher-level cyanobacterial classification system did not reflect the evolutionary history of the cyanobacteria and needed to be revised, and they retained the family Phormidiaceae in Oscillatoriales, Oscillatoriothricaceae in their revised system. Ten years later, the family Phormidiaceae was not included in the updated taxonomic system of cyanobacteria proposed by Komárek et al. (2014) since the authors indicated that the type species of *Phormidium* (*P. lucidum*) was considered to correspond to the family Oscillatoriothricaceae. Later, several taxonomic studies with descriptions of new cyanobacterial genera/species clearly belonging to the family Phormidiaceae were published [9,10]. Moreover, many cyanobacterial studies still retained the name of Phormidiaceae [11–14]. In particular, AlgaeBase (<https://www.algaebase.org/>, accessed on 12 April 2022) currently recognizes the existence of the family Phormidiaceae with a total of five extant genera, including *Ammassolinea* [15], *Ancylothrix* [9], *Cephalothrix* [16], *Potamolinea* [10] and *Pseudoscillatoria* [17], with reliable molecular data, and the genus *Phormidium* was not included in Phormidiaceae in the AlgaeBase. Such conflicting points led to certain confusion about the existence or non-existence of the family Phormidiaceae. Therefore, it is much needed to evaluate the family Phormidiaceae with regards to its existence and phylogeny by using the polyphasic approach.

During an investigation on filamentous cyanobacteria in China, twenty strains from three cyanobacterial samples were isolated at different localities of Zhejiang Province. Phylogenetic analyses showed that these strains were very closely related to the genera *Ancylothrix* and *Potamolinea*, both of which were newly recorded genera of cyanobacteria in China. The present study focused on a taxonomic discussion of the cyanobacterial systematics at the family level, with the aim to explore the relationship among different families by using the strains studies in this study. The results and discussion are expected to help the evaluation of the existence of Phormidiaceae as a family in Oscillatoriales.

2. Materials and Methods

2.1. Cyanobacterial Collection and Cultivation

Twenty cyanobacterial strains studied in this work were isolated from two localities in Zhejiang Province of China. Detailed information about the habitat of studied strains is summarized in Table 1. Benthic mat samples near to riverbank and soil samples (Figure 1) were scraped using a tweezer or scraper and live samples were washed thoroughly with sterile water before isolation. A single trichome from the pretreated samples were isolated by lab-mad Pasteur pipette under a 40 times magnification dissecting microscope (Carl Zeiss STEMI 508, Jena, Germany) and then moved to 24-well plates containing sterilized liquid CT medium for the unialgal culture [18]. After two or three weeks, the blue-green and uncontaminated cultures, after checking, were transferred into screw-capped tubes containing 10 mL of the same medium. These strains were kept at 25 °C under a 12 h:12 h light–dark cycle with a photon flux density of 35 $\mu\text{mol m}^{-2} \text{s}^{-1}$. These strains were archived in the Algae Culture Collection of the college of life and environmental science, Wenzhou University, Wenzhou, China, under the strain number in Table 1.

Table 1. Locality and habitat where the cyanobacterial strains in this study were collected.

Strains	Habitat	Locality	Latitude (N)	Longitude (E)
WZU 0009, 0011, 0013, 0014	Benthos	Feiyun River Basin	27°66'82"	120°05'64"
WZU 0153, 0154	Soil	Dahongyan Scenic Area	28°81'28"	119°66'22"
WZU 0155-0168	Benthos	Niutou Mountain National Forest Park	28°66'76"	119°49'78"

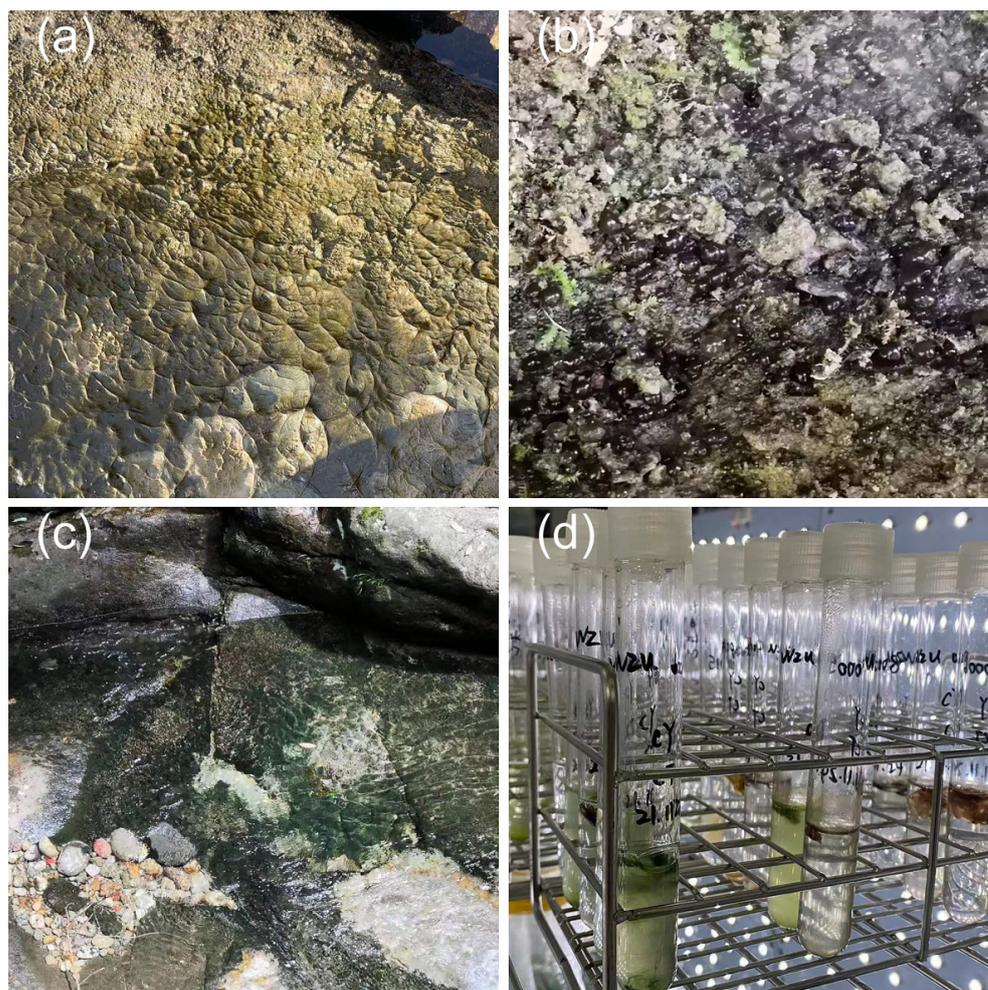


Figure 1. The habitats of studied strains. (a) Benthic mat samples near to riverbank including *Phormidium*-like sp. 1 strains. (b) Soil samples including *Phormidium*-like sp. 2 strains. (c) Benthic mat samples at the bottom of the streams including *Phormidium*-like sp. 3 strains. (d) Macroscopic view of all WZU strains.

2.2. Morphological Observation

All the live cultures were examined with a LEICA DM2000 LED microscope (Leica, Germany). Microphotographs were produced using a LEICA DMC 5400 digital camera (Leica, Germany) photomicrographic system attached to the microscope and the images were analyzed using Leica Application Suite X 3.7.4 software. Measurements of unialgal cellular morphology were made from digital images taken under 1000 times magnification and the mean width of vegetative cells of more than 60 filaments were measured.

2.3. DNA Extraction and PCR Amplification

A certain amount of filament was collected and was washed three times using sterile phosphorus-free sterilized liquid CT medium to avoid contamination with other bacteria. Total genomic DNA from the strains were extracted using the modified cetyltrimethylammonium bromide (CTAB) method [19]. The PCR primers, PA [20] and B23S [21], were chosen for obtaining segments containing 16S ribosomal RNA gene and the associated 16S–23S internal transcribed spacer (ITS) region. The total PCR reaction volume of 50 μL comprised 1 μL of template DNA, each primer (10 $\mu\text{mol L}^{-1}$) for 1 μL , 22 μL sterile water and 25 μL $2\times$ Taq Plus Master Mix (Dye Plus) (Vazyme Biotech Co., Ltd., Nanjing, China). PCR amplification was performed using a SimpliAmpTM Thermal Cycler (Waltham, MA,

USA) with a PCR profile of an initial denaturation at 95 °C for 5 min, 31 cycles of 30 s at 95 °C, 30 s at 55 °C, 2 min at 72 °C and then a final 10 min elongation step at 72 °C.

PCR products were purified using a TIANgel Midi Purification kit (Tiangen Biotech Co., Ltd., Beijing, China) and were then cloned using pClone007 Versatile Simple Vector Kit (Beijing Tsingke Biotech CO., Ltd., Beijing, China). The target gene fragment was inserted into *Escherichia coli* DH5 α cells for replication, and then the inserted fragment was sequenced bidirectionally by using the standard primers M13F (5'-GTA-AAA-CGA-CGG-CCA-GT-3') and M13R (5'-GTC-ATA-GCT-GTT-TCC-TG-3'). Clones including the target fragment were sequenced by the Wuhan Tianyi Huayu Gene Technology (Wuhan, China). All sequences obtained in this study were submitted to the NCBI GenBank database after being checked and under following accession numbers in Tables 2 and 3.

Table 2. Analyses on ITS of 16S–23S region for *Ancylothrux* strains.

Strain	GenBank	Complete ITS (nt)	D1–D1' Helix (nt)	tRNA ^{Ile}	tRNA ^{Ala}	Box–B Helix (nt)	Box–A Helix (nt)	D4	V3 Helix (nt)	D5
WZU 0009	OL742575	441	57	+	+	39	12	7	43	19
WZU 0011	OL742572	441	57	+	+	39	12	7	43	19
WZU 0013	OL742573	445	59	+	+	39	12	7	43	19
WZU 0014	OL742574	441	57	+	+	40	12	7	43	19
7PC	KT819196	437	57	+	+	40	12	7	43	19
8PC	KT819197	438	57	+	+	40	12	7	43	19
9PC	KT819198	436	56	+	+	40	12	7	43	19
10PC	KT819199	515	54	+	+	38	12	7	54	22
11PC	KT819200	364	63	+	–	39	12	7	12	17
12PC	KT819201	409	66	+	–	33	11	8	44	16
13PC	KT819202	516	54	+	+	38	12	7	54	21
WZU 0153	OM237453	511	54	+	+	36	12	7	54	22
WZU 0154	OM237454	511	54	+	+	36	12	7	54	22

Table 3. Analyses on ITS of 16S–23S region for *Potamolinea aeruginosa* strains.

Strains	GenBank	Complete ITS (nt)	D1–D1' Helix (nt)	tRNA ^{Ile}	tRNA ^{Ala}	Box–B Helix (nt)	Box–A Helix (nt)	D4	V3 Helix (nt)	D5
1PC	KX001786	372	63	+	–	49	12	7	30	22
2PC	KX001787	372	63	+	–	49	12	7	30	22
38PC	KX001788	372	63	+	–	48	12	7	30	22
WZU 0155	OM237439	394	61	+	–	49	12	7	54	22
WZU 0156	OM237440	394	61	+	–	49	12	7	54	22
WZU 0157	OM237441	394	61	+	–	49	12	7	54	22
WZU 0158	OM237442	394	61	+	–	49	12	7	54	22
WZU 0159	OM237443	394	61	+	–	49	12	7	54	22
WZU 0160	OM237444	394	61	+	–	49	12	7	54	22
WZU 0161	OM237445	394	61	+	–	49	11	8	54	22
WZU 0162	OM237446	394	61	+	–	49	12	7	54	22
WZU 0163	OM237447	394	61	+	–	49	12	7	54	22
WZU 0164	OM237448	394	61	+	–	49	12	7	54	22
WZU 0165	OM237449	394	61	+	–	49	12	7	54	22
WZU 0166	OM237450	394	61	+	–	49	12	7	54	22
WZU 0167	OM237451	394	61	+	–	49	12	7	54	22
WZU 0168	OM237452	394	61	+	–	49	12	7	54	22

2.4. Phylogenetic Analyses

The obtained 16S rRNA gene sequences of this study were compared by using the online tool nucleotide BLAST and the highly similarly sequences of 16S rRNA gene were downloaded from the NCBI GenBank database and used for phylogenetic analyses. All downloaded sequences and obtained sequences were aligned using MAFFT v7.463 [22] and

cut both ends neatly by using BioEdit v7.0.9 [23]. Sequences of *Gloeobaacter violaceus* and *Gloeobaacter kilaueensis* were used as outgroups. After this, multiple sequence alignment finally formed a data matrix of 108 sequences with 1071 nucleotide sites. The nucleic acid substitution model (GTR + F + R4) was selected for the optimal maximum likelihood analysis (ML) and the other model (GTR + F + 4) was selected for the optimal Bayesian analysis (BI). Both BI and ML analysis were conducted on the basis of Akaike Information Criterion (AIC) in PhyloSuite v1.2.2 molecular phylogenetic platform [24]. The specific operation parameters of substitution models were individually estimated by IQ-TREE v2.1.3 [25,26] and MrBayes v3.2.7 [27]. For the ML analysis, a total of 1000 bootstrap replications were made under standard option. BI analysis comprised two parallel runs for 10,000,000 generations, sampling every 100th generation, in which the initial 25% of sampled data were discarded as burn-in. Neighbor-Joining (NJ) analyses were computed using the Kimura 2-parameter model with 1000 bootstrap replications in MEGA 11 [28]. Phylogenetic trees were visualized in FigTree v1.4.3 (<http://tree.bio.ed.ac.uk/software/figtree/>, accessed on 22 March 2022), and all obtained were edited by TreeView 1.6.6 software [29]. Genetic distances of similarity matrices of the 16S rRNA gene sequences were estimated using the Kimura 2-parameter model in MEGA 11 to calculate p-distance with pairwise deletion of gaps.

2.5. Analyses of 16S–23S Internal Transcribed Spacer (ITS)

The presence of tRNA gene sequences were tested with the tRNAscan-SE2.0 web server [30]. The conserved hypothetical secondary structures were constructed using S-fold web-based software [31] and adjusted in the Adobe illustrator 2020 software.

3. Results

3.1. Morphological Description

Diagnosis: The twenty strains isolated in this study were similar to *Phormidium* in morphological and ecological characteristics, and the phylogenetic analysis showed that these strains were close to the species of the genera *Ancylothrix* (WZU 0009, WZU 0011, WZU 0013, WZU 0014, WZU 0153, WZU 0154) and *Potamolinea* (WZU 0155–WZU 0168). According to their morphological and ecological differences, these strains were divided into three different species.

3.1.1. *Phormidium*-like sp. 1

Description: In the natural environment, thallus is macroscopic, with dark-green filaments forming benthic mats on submerged substrata. Filaments are bright-green or dark-green. Sheaths are very thin, colorless and hyaline. Trichomes are cylindrical, 4.6–7.1 µm wide, 3.3–6.0 µm long, shorter than wide to isodiametric, with a single filament growing, contracting slightly at the crosswalls, and the ends of filaments gradually become sharp and undirected left and right curved. Most of the apical cells are conical-rounded and narrowed, without calyptra (Figure 2). Reproduction is by fragmentation of trichomes at necridic cells. They are without heterocytes, aerotopes and akinetes.

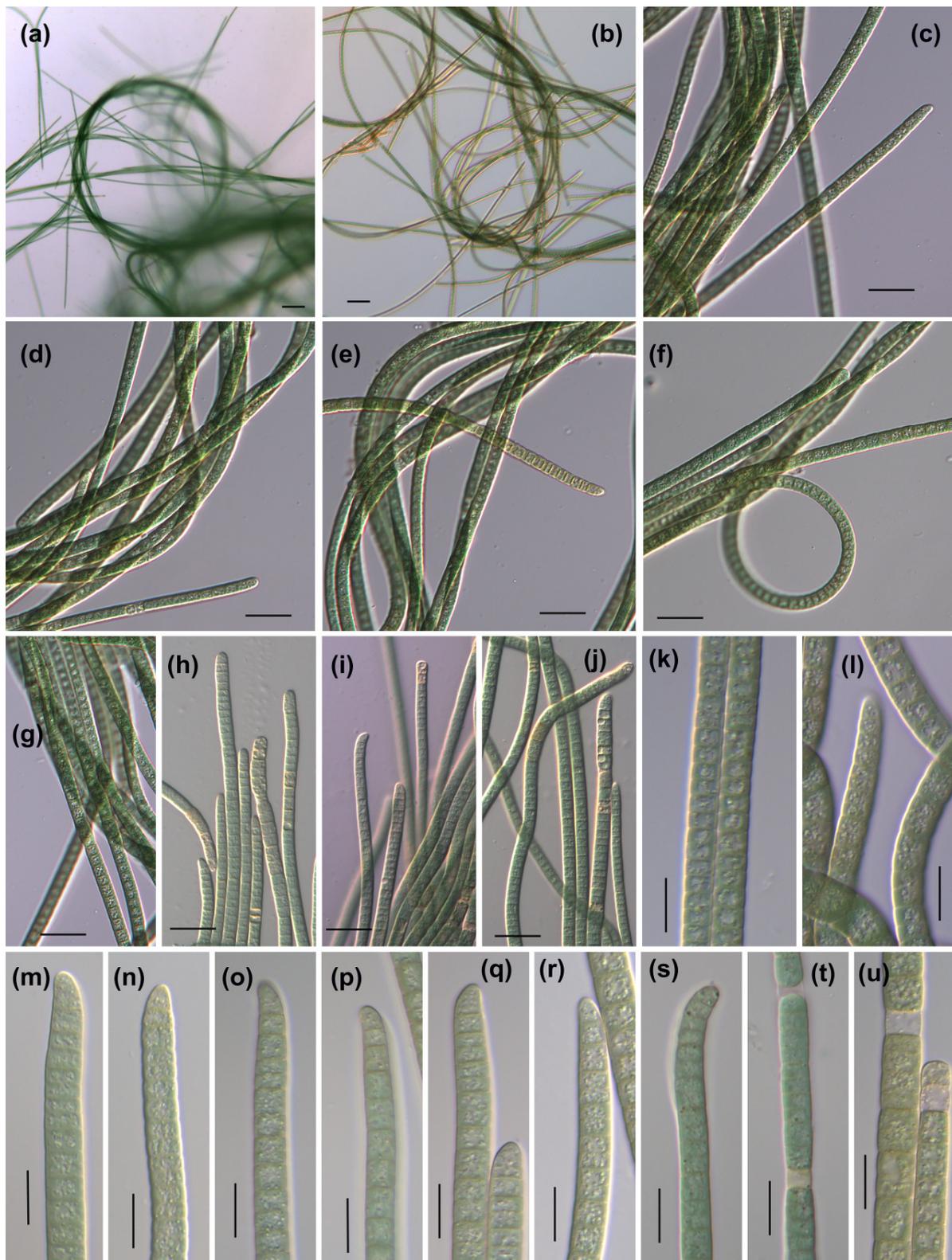


Figure 2. Light microscopy of *Phormidium*-like sp. 1 strains: (a–f) The filaments are densely entangled with each other; (g–j) different shapes of filaments, some apical cells rounded, some apical cells conical-rounded and narrowed; (k,l) trichomes slightly constricted at the crosswalls; (m–s) details of the conical-rounded and narrowed apical cells; (t,u) details of the thin and colorless sheath. Scale bars: (a,b) = 50 μm , (c–j) = 20 μm , (k–u) = 10 μm .

Habitat: River benthos near to riverbank.

Reference strains: WZU 0009, WZU 0010, WZU 0013, WZU 0014.

3.1.2. *Phormidium*-like sp. 2

Description: Thallus is macroscopic forming bright-green compact biofilms on a xerophytic environment. Sheaths are very thin, colorless and hyaline. Through observation, sheaths are more obvious than *Ancylothrix rivularis* in laboratory culture. Filaments are olive-green or dark-green. Trichomes are cylindrical, 3.8–6.1 μm wide and 2.9–6.4 μm long, shorter than wide to isodiametric, with a single filament growing, contracting slightly at the crosswalls and the ends of filaments gradually become sharp and undirected left and right curved. Most of the apical cells are conical-rounded and narrowed, a small number of apical cells are rounded, without calyptra (Figure 3). Reproduction is by fragmentation of trichomes at necridic cells. Heterocytes, aerotopes and akinetes were not observed under culture conditions.

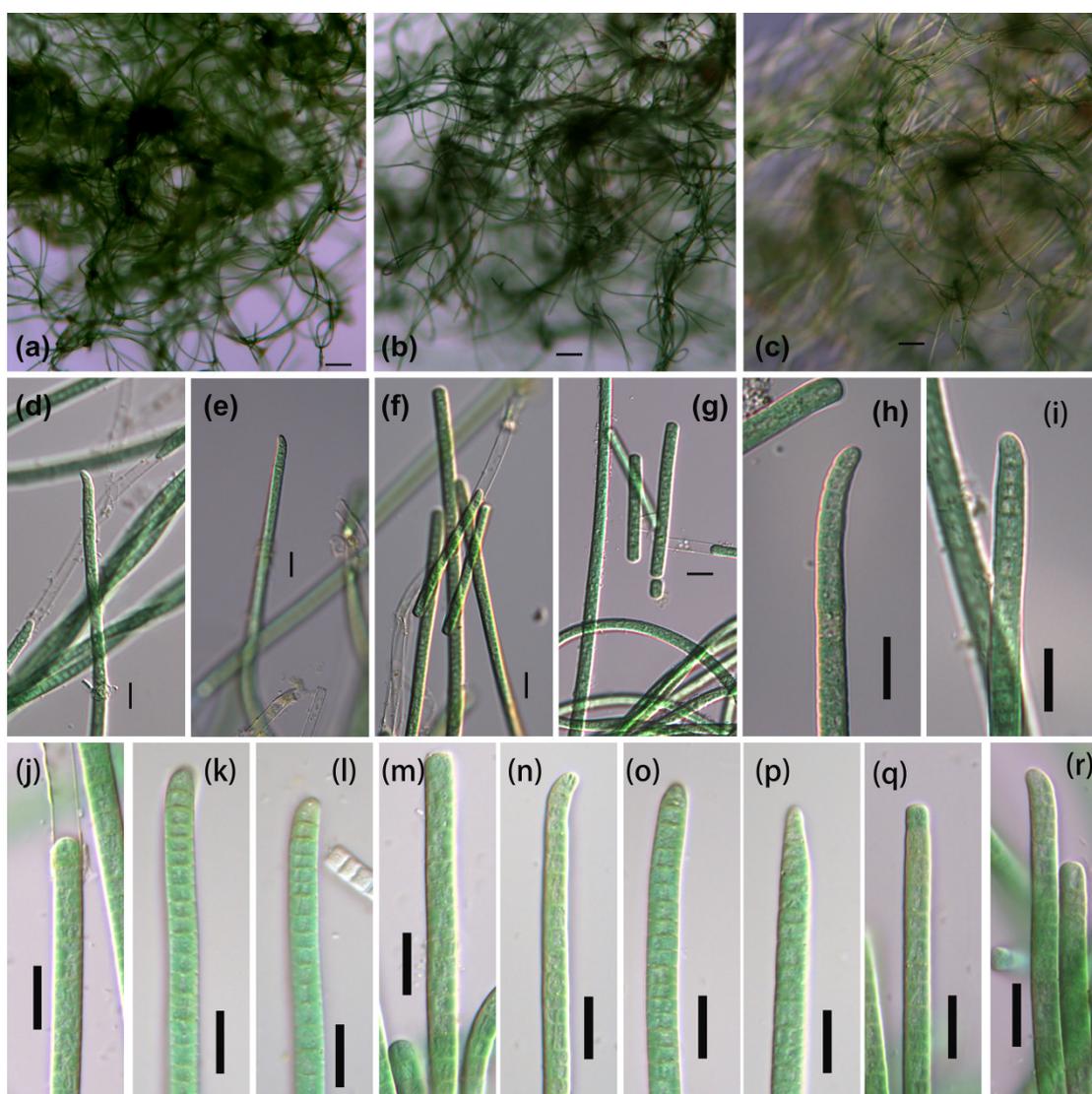


Figure 3. Light microscopy of *Phormidium*-like sp. 2 strains: (a–c) Represent twisted or entwined filaments; (d–g) trichomes wrapped in the sheath; (h,i) trichomes slightly constricted at the crosswalls; (j–r) the details of various diversiform of apical cells. Scale bars: (a–c) = 50 μm, (d–r) = 10 μm.

Habitat: Attached on rock surfaces.

Reference strains: WZU 0153, WZU 0154.

3.1.3. *Phormidium*-like sp. 3

Description: Thallus is attached to the substrate, dark-green. Trichomes are densely entangled with each other. Sheaths are hyaline, colorless and thin. Trichomes are cylindrical and vegetative cells are usually cylindrical, 6.8–10.6 μm wide and 4.5–7.8 μm long, shorter than wide. Trichome has a single filament growing, contracting slightly at the crosswalls, and the ends of filaments gradually become sharp (Figure 4). Most of the apical cells are rounded, a small number of the apical cells are conical-rounded and narrowed, without calyptra. Reproduction is by the disintegration of trichomes at the necridic cells. Heterocytes, aerotopes and akinetes were not observed.

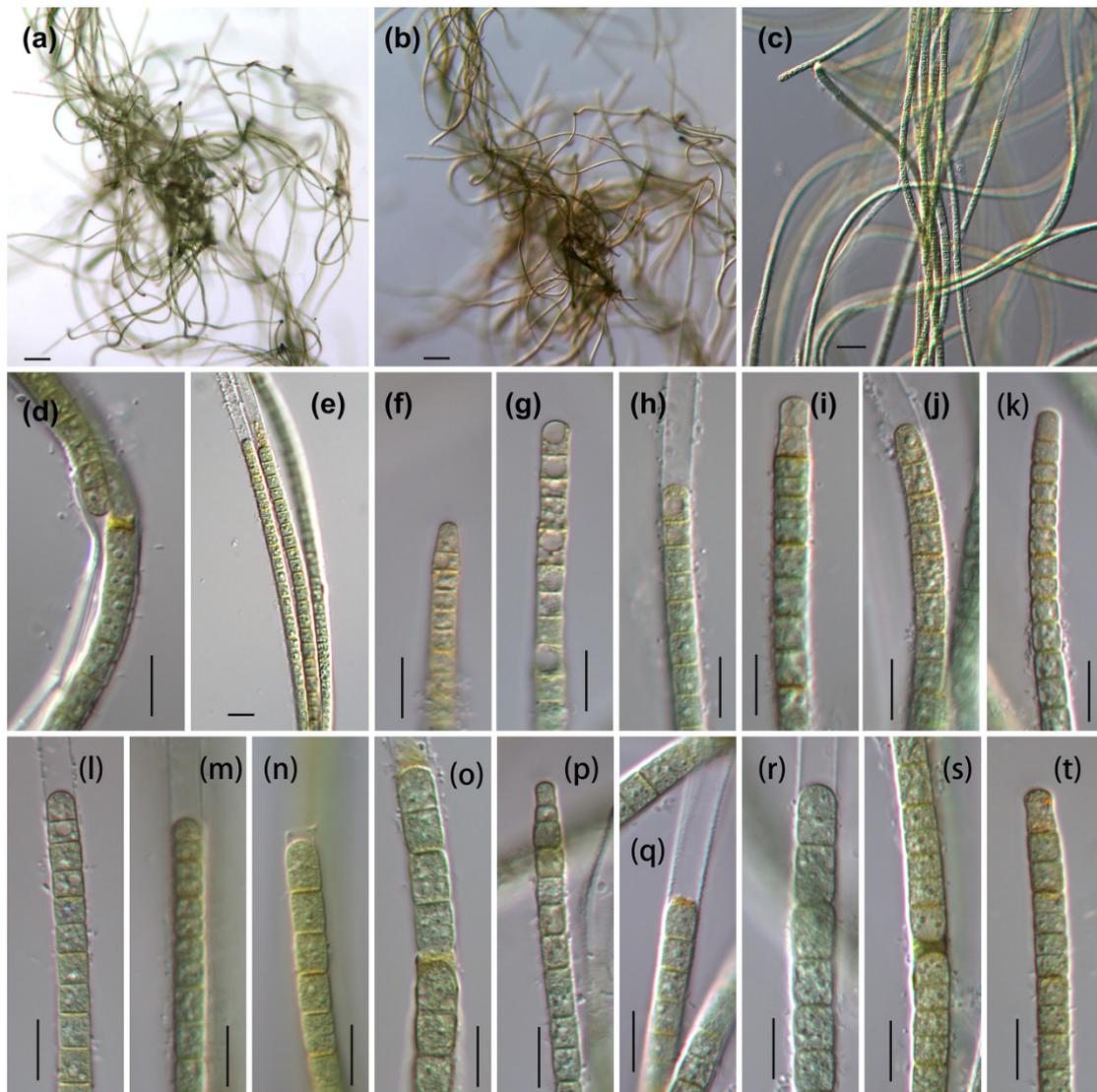


Figure 4. Light microscopy of *Phormidium*-like sp. 3 strains: (a–c) Numerous trichomes are densely entangled with each other; (d,e) detail of the sheath attached to filaments; (f–n,p–r,t) the details of various diversiform of apical cells; (o,s) detail of biconcave necridic cells. Scale bars: (a,b) = 50 μm , (c,e) = 20 μm , (d,f–t) = 10 μm .

Habitat: At the bottom of the stream.

Reference strains: WZU 0155–WZU 0168.

3.2. Molecular and Phylogenetic Analyses

The phylogenetic analyses of the 16S rRNA gene confirmed that the studied strains were a well supported clade among genera *Ancylothrrix* and *Potamolinea*. In total, 108 sequences from the order Oscillatoriales were used based on NJ, ML and BI methods to construct the 16S rRNA gene phylogenetic trees (Figure 5) in order to better understand the strains from Phormidiaceae in the present and investigate their relationships in the order Oscillatoriales, including in the families Coleofasciculaceae, Desertifilaceae, Microcoleaceae and Oscillatoriaceae and all genus from Phormidiaceae. In this study, six strains (WZU 0009, 0011, 0013, 0014, 0153, 0154) with the strains of two species, *Ancylothrrix rivularis* and *Ancylothrrix terrestris*, were placed in a clade based on the phylogenetic tree with high bootstrap values of 99%/100%/1.00 supported by the NJ/ML/BI approaches. Pairwise p-distance analysis of the 16S rRNA gene can also be used as evidence in phylogenetic comparison [32]. The 16S rRNA gene similarities between *Phormidium*-like sp. 1 and *Ancylothrrix rivularis* strains, and *Phormidium*-like sp. 2 and *Ancylothrrix terrestris* strains were all >99%, higher than 97%—the threshold of bacterial genus classification (Table 4). Phylogenetic analyses placed the other fourteen WZU strains (0155–0168) with *Potamolinea aerugineocaerulea* in a robust clade; all the species from species *Potamolinea aerugineocaerulea* shared 98.13%–100% similarities, also higher than the threshold of bacterial species classification (Table 5). Therefore, based on phylogenetic analysis, we determined that *Phormidium*-like sp. 1–3 represent the three species *Ancylothrrix rivularis*, *Ancylothrrix terrestris* and *Potamolinea aerugineocaerulea*, respectively.

Table 4. Comparison of the 16S rRNA gene sequence similarity among all *Ancylothrrix* strains.

Strains	1	2	3	4	5	6	7	8	9	10	11	12
1. WZU 0009												
2. WZU 0011	99.74											
3. WZU 0013	99.66	99.57										
4. WZU 0014	99.91	99.83	99.74									
5. 7PC	99.66	99.57	99.66	99.74								
6. 8PC	99.57	99.48	99.57	99.66	99.91							
7. 9PC	99.74	99.66	99.74	99.83	99.91	99.83						
8. 10PC	95.94	95.84	96.13	96.03	96.12	96.03	96.39					
9. 11PC	96.03	95.94	96.13	96.12	96.22	96.12	96.39	100.00				
10. 12PC	95.85	95.76	95.87	95.94	96.04	95.14	96.13	99.57	99.65			
11. 13PC	95.55	95.46	95.79	95.64	95.74	95.65	96.04	99.48	99.48	99.22		
12. WZU 0153	95.74	95.66	95.75	95.84	96.13	96.03	96.03	99.83	99.83	99.39	99.31	
13. WZU 0154	95.93	95.84	95.75	96.03	96.12	96.03	96.03	100.00	100.00	99.57	99.48	99.83

Table 5. Comparison of the 16S rRNA gene sequence similarity among all *Potamolinea aerugineocaerulea* strains.

Strains	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1. <i>P. aerugineocaerulea</i> 1PC																
2. <i>P. aerugineocaerulea</i> 2PC	99.93															
3. <i>P. aerugineocaerulea</i> 38PC	99.46	99.52														
4. WZU 0155	98.41	98.48	98.69													
5. WZU 0156	98.41	98.48	98.69	100												
6. WZU 0157	98.41	98.48	98.69	100	100											
7. WZU 0158	98.41	98.48	98.69	100	100	100										
8. WZU 0159	98.41	98.48	98.69	100	100	100	100									
9. WZU 0160	98.41	98.48	98.69	100	100	100	100	100								
10. WZU 0161	98.41	98.48	98.69	100	100	100	100	100	100							
11. WZU 0162	98.41	98.48	98.69	100	100	100	100	100	100	100						
12. WZU 0163	98.41	98.48	98.69	100	100	100	100	100	100	100	100					
13. WZU 0164	98.41	98.48	98.69	100	100	100	100	100	100	100	100	100				
14. WZU 0165	98.20	98.27	98.49	99.53	99.53	99.53	99.53	99.53	100	100	100	100	100			
15. WZU 0166	98.13	98.20	98.42	99.46	99.46	99.46	99.46	99.46	99.46	99.46	99.46	99.46	99.46	99.46	99.93	
16. WZU 0167	98.20	98.27	98.49	99.53	99.53	99.53	99.53	99.53	99.53	99.53	99.53	99.53	99.53	99.53	100	99.93
17. WZU 0168	98.41	98.48	98.69	100	100	100	100	100	100	100	100	100	100	99.53	99.46	99.53

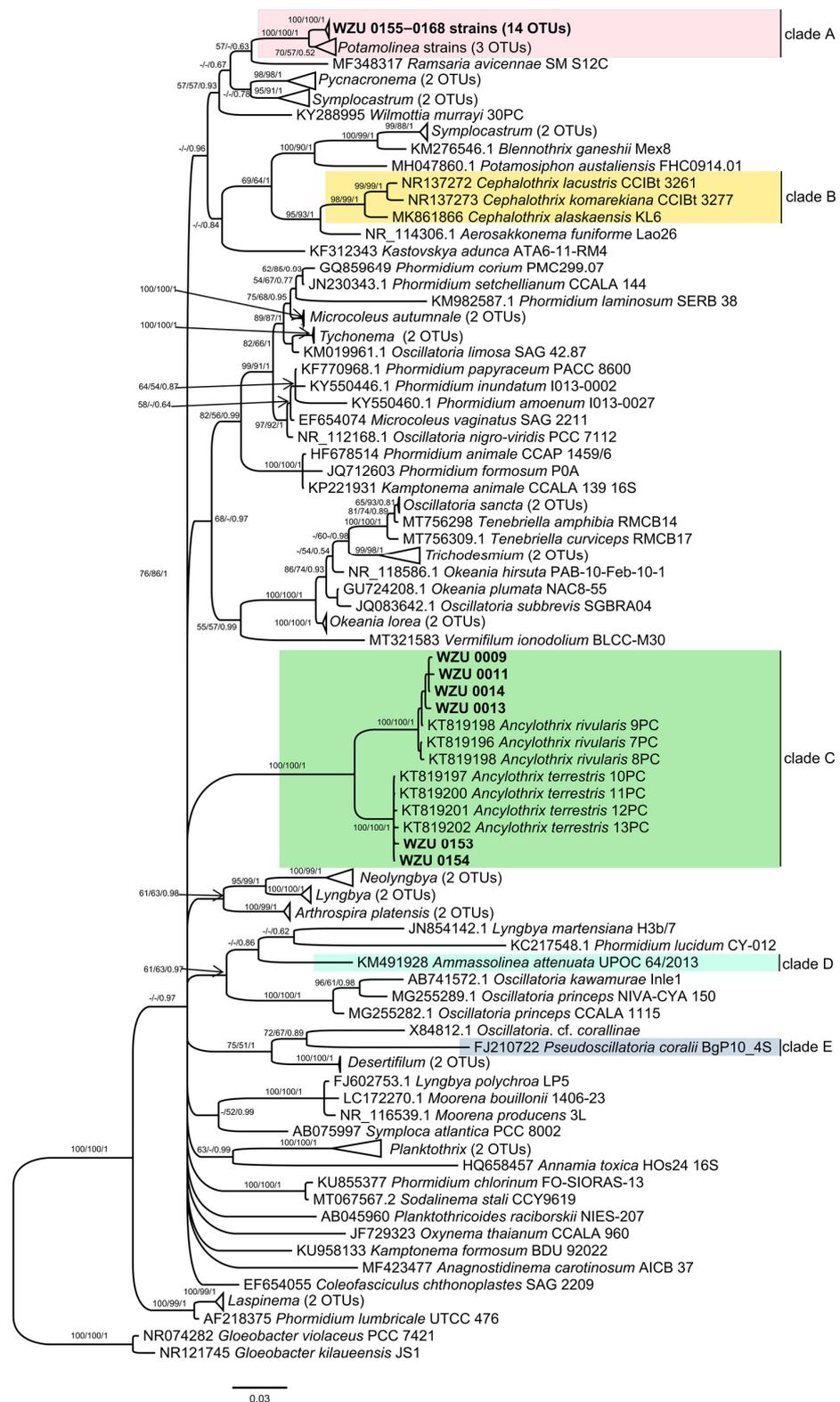


Figure 5. Bayesian inference (BI) phylogenetic tree of WZU strains based on 16S rRNA gene sequences. Bootstrap values greater than 50% are shown on the BI tree for NJ/ML methods and Bayesian posterior probabilities, clade A–E represent *Potamolinea*, *Cephalothrix*, *Ancylothrix*, *Ammassolinea*, *Pseudoscillatoria*. Bar, 0.03 substitutions per nucleotide position.

3.3. Analyses of ITS between 16S and 23S rRNA Gene and Secondary Structures

The 16S–23S ITS secondary structures were compared among *Ancylothrux* and *Potamolinea aerugineocaerulea* in this study. The ITS region can be used as a tool to distinguish closely related organisms [33,34]. Specific information of the various parts of the 16S–23S ITS region is summarized in Tables 2 and 3.

For genus *Ancylothrux*, all sequences of the six strains contained both tRNA^{Ile} and tRNA^{Ala}. Compared with the original seven strains, the D1–D1', Box–B and V3 helix (nt) of all thirteen strains exhibited eight (Figure 6), six (Figure 7) and seven (Figure 8) different types. For genus *Potamolinea*, all strains contained only tRNA^{Ile} without tRNA^{Ala}, and regardless of whether D1–D1', Box–B or V3 helices, WZU strains showed their uniqueness (Figure 9).

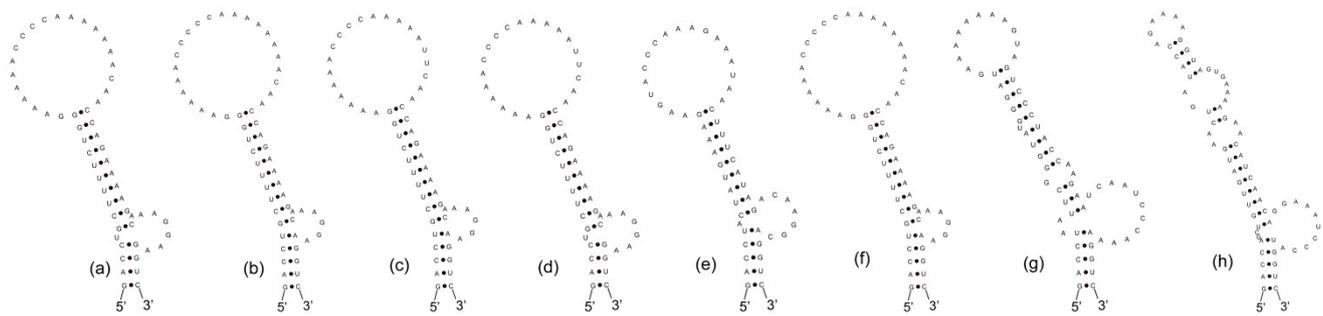


Figure 6. D1–D1' helix of *Ancylothrux* strains: (a) *Ancylothrux rivularis* WZU 0009, 0011, 0014; (b) *Ancylothrux rivularis* WZU 0013; (c) *Ancylothrux rivularis* 7PC, 8PC; (d) *Ancylothrux rivularis* 9PC; (e) *Ancylothrux terrestris* 10PC, WZU 0153, WZU 0154; (f) *Ancylothrux terrestris* 13PC; (g) *Ancylothrux terrestris* 11PC; (h) *Ancylothrux terrestris* 12PC.

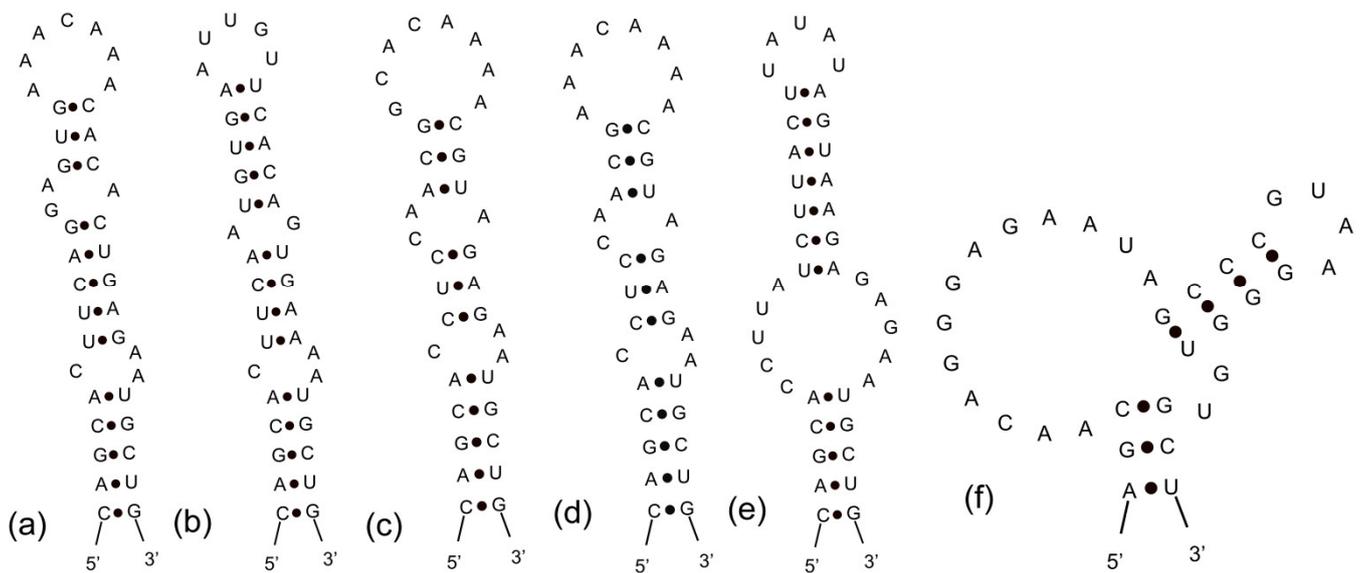


Figure 7. Box–B helix of *Ancylothrux* strains: (a) *Ancylothrux rivularis* WZU 0009, 0011, 0013, 0014; (b) *Ancylothrux rivularis* 7PC, 8PC, 9PC; (c) *Ancylothrux terrestris* 10PC, 13PC; (d) *Ancylothrux terrestris* WZU 0153, 0154; (e) *Ancylothrux terrestris* 11PC; (f) *Ancylothrux terrestris* 12PC.

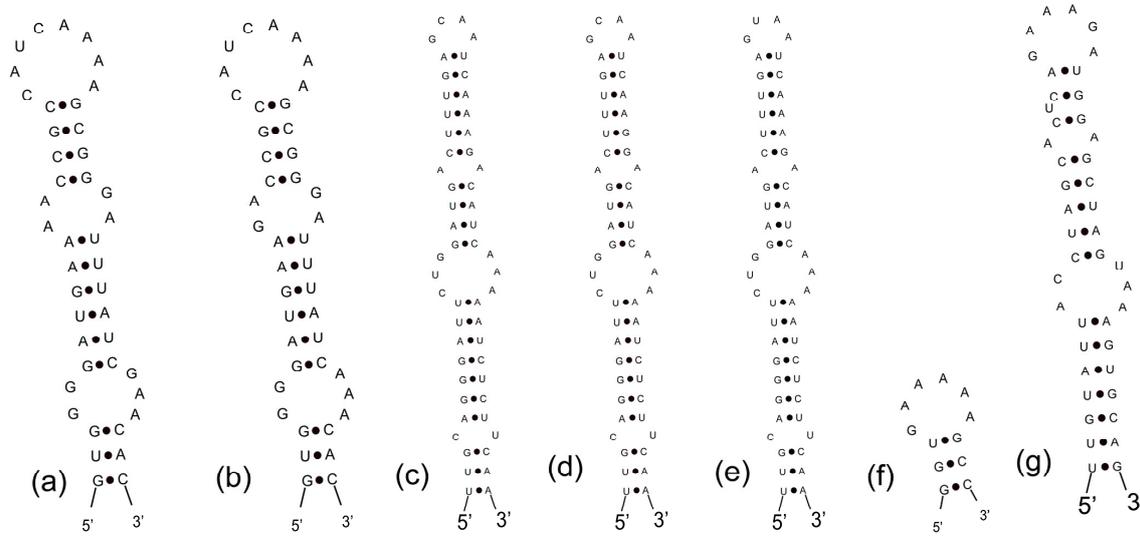


Figure 8. V3 helix of *Ancylothrrix* strains: (a) *Ancylothrrix rivularis* WZU 0009; (b) *Ancylothrrix rivularis* WZU 0011, 0013, 0014 and *Ancylothrrix rivularis* 7PC, 8PC, 9PC; (c) *Ancylothrrix terrestris* 10PC; (d) *Ancylothrrix terrestris* 13PC; (e) *Ancylothrrix terrestris* WZU 0153, 0154; (f) *Ancylothrrix terrestris* 11PC; (g) *Ancylothrrix terrestris* 12PC.

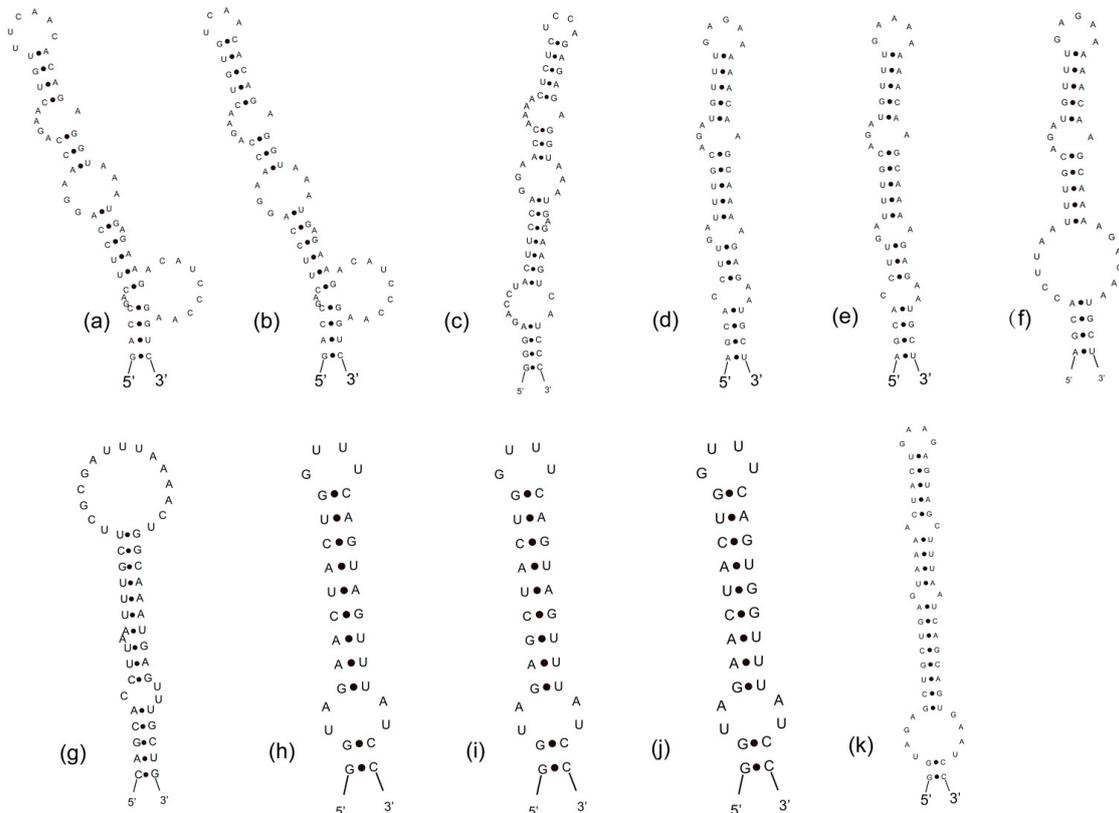


Figure 9. D1–D1' helix of *Potamolinea aerugineocaerulea*: (a) *Potamolinea aerugineocaerulea* 1PC, 2PC; (b) *Potamolinea aerugineocaerulea* 38PC; (c) *Potamolinea aerugineocaerulea* WZU strains. Box–B helix of *Potamolinea aerugineocaerulea*: (d) *Potamolinea aerugineocaerulea* 1PC; (e) *Potamolinea aerugineocaerulea* 2PC; (f) *Potamolinea aerugineocaerulea* 38PC; (g) *Potamolinea aerugineocaerulea* WZU strains. V3 helix of *Potamolinea aerugineocaerulea*: (h) *Potamolinea aerugineocaerulea* 1PC; (i) *Potamolinea aerugineocaerulea* 2PC; (j) *Potamolinea aerugineocaerulea* 38PC; (k) *Potamolinea aerugineocaerulea* WZU strains.

The studied D1–D1' helix showed conserved secondary structures in strains from the *Ancylothrix rivularis* clade, and variable secondary structures in strains from the *Ancylothrix terrestris* clade. WZU0009, 0011 and 0014 share the same structure (Figure 6a). For the *Ancylothrix rivularis* clade, the main difference lies in the terminal loop: the number of unpaired bases A and C have a slight difference (Figure 6a–c). For *Ancylothrix terrestris* clade, WZU 0153, 0154 and 10PC, and 13PC share the same structure (Figure 6e,f). 11PC and 12PC have a clear difference to the other four strains. (Figure 6g,h). For the *Potamolinea aerugineocaerulea* clade, the strains in this study showed a clear difference from the three original strains. WZU strains consisted of one 4-bp helix, one 5-bp helix, one small unidirectional bulge, one 2-bp helix, one 3:3 bp base bilateral bulge, one 3-bp helix, one 4:1 unidirectional bulge, one 5-bp helix and one 4-bp apical loop (Figure 9c).

The studied Box–B helix showed higher conserved secondary structures in strains from the *Ancylothrix rivularis* clade than the D1–D1' helix, and WZU strains shared the same structure (Figure 7a); other original strains shared the structure also (Figure 7b). The secondary structures between the two types showed high similarity. There are slight differences in their respective base bilateral bulge. For the *Ancylothrix rivularis* clade, WZU strains share the same structure with 10PC and 13PC; the main difference lies in the terminal loop and the number of unpaired bases had a slight difference (Figure 7c,d). 11PC and 12PC have a clear difference from the other four strains. (Figure 7e,f). For the *Potamolinea aerugineocaerulea* clade, WZU strains had their own unique Box–B helix, consisting of one 5-bp helix, one 1:2 bp base bilateral bulge, one 3-bp helix, one small unidirectional bulge, one 7-bp helix and 15-bp unpaired bases in the terminal loop (Figure 9g).

The studied V3 helix showed the highest conserved secondary structures in strains from the *Ancylothrix rivularis* clade. Except for WZU 0009, all other strains in the *Ancylothrix rivularis* clade shared the same structure (Figure 8b); WZU 0009 had a one 2:3 bp base bilateral bulge (5'-GG-GAA-3') (Figure 8a), while all other strains in the *Ancylothrix rivularis* clade at the same place had a one 2:3 bp base bilateral bulge (5'-GG-AAA-3') (Figure 8b). For the *Ancylothrix rivularis* clade, WZU strains share the same structure with 10PC and 13PC (Figure 8c–e), whereas, in the terminal loop, the unpaired bases arrangement had a slight difference (C-U). For the *Potamolinea aerugineocaerulea* clade, WZU strains have their own unique V3 helix, consisting of one 2-bp helix, one 4:4 bp base bilateral bulge, one 8-bp helix, one 1:1 bp base bilateral bulge, one 4-bp helix, one 1:1 bp base bilateral bulge, one 5-bp helix and 4-bp unpaired bases in the terminal loop, which differ significantly from other strains in the *Potamolinea aerugineocaerulea* clade (Figure 9h–k).

4. Discussion

With the advent of phylogenetic analyses based on molecular sequence data, extensive revision and reconstruction of cyanobacterial classification have been performed in recent years. An ideal cyanobacterial taxonomic system in which all categories should be monophyletic has been repeatedly proposed [2,35,36]. As we described above, the order-level and genus-level classification in the newly revised system of cyanobacteria have been well documented [32]. However, phylogenetic elucidation and evaluation on the monophyly of cyanobacterial groups at a family level have barely been conducted, implying that taxonomic revision of the cyanobacterial families may be enforced in the future. In this study, we aimed to investigate the diversity of oscillatocean cyanobacteria in the east of China. Through isolating filamentous strains from mat-forming habitats, twenty strains with *Phormidium*-like morphologies were successfully obtained. Previous studies on the *Phormidium*-like group based on phenotypic and genotypic data, mostly using 16S rRNA gene sequences, have revealed a wide diversity and heterogeneity, leading to the establishment of several new genera such as *Roseofilum* [37], *Ammassolinea* [15] and *Kamptonema* [38]. DNA sequence-based comparison and phylogenetic analyses in this study revealed these twenty strains as belonging to the two newly proposed genera *Ancylothrix* and *Potamolinea*, respectively. Furthermore, the secondary structures of ITS also supported their taxonomic positions decided by the 16S rRNA gene sequences. Both *Ancylothrix* and

Potamolinea were found in China for the first time, and they were added as new recorded genera of cyanobacteria in China. *Ancylothrix* was newly described by Martins et al. (2016) from samples in southern Brazil and was further found to contain two internal groups: one group represents a species with only freshwater occurrences and the other group with only aerophytic populations, both corresponding to *Ancylothrix rivularis* and *A. terrestris*, respectively. The finding of the genus *Ancylothrix* in the present study with two species and habitats was exactly the same as that by Martins et al. (2016), reflecting the common and stable features of the genus *Ancylothrix* [9]. Similarly, the genus *Potamolinea* was created by Martins and Branco (2016) from ten populations of bottom streams in Brazil, with further description of two species. *Potamolinea aeruginosaerulea*, found in the present study, was very similar to that described by Martin and Branco (2016) in ecological and phylogenetic aspects [10].

The modern taxonomic system of cyanobacteria is superior to the traditional systems because it more closely reflects the phylogeny. However, the delimitation of cyanobacterial family is still unclear. This is indeed the case for the family Phormidiaceae. Continuous changes along the history of Phormidiaceae, from the beginning of the family in 1988 to its disappearance in 2014 by Komárek et al. (2014), and the current retention of this family in many studies, have resulted in much confusion and uncertainty for the cyanobacterial taxonomic system. The genera *Ammassolinea* [15], *Ancylothrix* [9], *Cephalothrix* [16] and *Potamolinea* [10] were founded after Komárek et al. (2014), but the inventors of those new genera only considered the morphological similarity to the polyphyletic genus *Phormidium* without taking into account the fact of the absence of the family Phormidiaceae in Komárek et al. (2014). In order to evaluate the monophyly of the family Phormidiaceae, the phylogenetic tree, based on a large number of 16S rRNA gene sequences including those from type species of both *Oscillatoria* and *Phormidium*, was obtained (Figure 5). As shown in Figure 5, the strains of different *Phormidium* species including the type species, were shown to be diversely and sparsely located at different clades, indicating *Phormidium* as a largely heterogeneous genus. Furthermore, phylogenetic evaluation showed that the five extant genera within the family Phormidiaceae in AlgaeBase did not group together, and were diversely distributed at different clades, corresponding to Oscillatoriaceae, Microcoleaceae and Coleofasciculaceae, respectively (Figure 5). Obviously, the family Phormidiaceae currently used is far from the monophyly based on the phylogenetic evaluation. With the combination of the above results and analyses, the conclusion was drawn that the family Phormidiaceae should not exist in the current taxonomic system of cyanobacteria. The results and conclusion strongly support the taxonomic removal of the family Phormidiaceae in the revised taxonomic system by Komárek et al. (2014), clearly preventing further confusion in determining the taxonomic belonging of *Phormidium*-like cyanobacteria.

The taxonomic revision of cyanobacteria at genus and species levels based on the polyphasic approach have been largely performed and emphasized during the last decades, but there are fewer studies on taxonomic revisions at family level and elucidation for family delimitation. The present study provided a simple and efficient example to perform phylogenetic evaluation for the monophyly and rationality of currently accepted families of cyanobacteria by using the regional strains based on the polyphasic approach.

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