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Phylogenetic analysis of *Rhizoclonium* (Cladophoraceae, Cladophorales), and the description of *Rhizoclonium subtile sp. nov.* from China

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Abstract

The genus *Rhizoclonium* (Cladophoraceae, Cladophorales) accommodates uniserial, unbranched filamentous algae, closely related to *Cladophora* and *Chaetomorpha*. Its taxonomy has been problematic for a long time due to the lack of diagnostic morphological characters. To clarify the species diversity and taxonomic relationships of this genus, we collected and analyzed thirteen freshwater *Rhizoclonium* specimens from China. The morphological traits of these specimens were observed and described in detail. Three nuclear gene markers small subunit ribosomal DNA (SSU), large subunit ribosomal DNA (LSU) and internal transcribed spacer 2 (ITS2) sequences were analyzed to elucidate their phylogenetic relationships. The results revealed that there were at least fifteen molecular species assignable to *Rhizoclonium* and our thirteen specimens were distributed in four clades. On the basis of morphological and molecular evidence we propose the new species, *R. subtile sp. nov*.

Keywords: *Rhizoclonium*, phylogeny, SSU, LSU, ITS2, *R. subtile*

Introduction

The genus *Rhizoclonium* (Cladophorales, Chlorophyta) was established by Kützing (1843) and *R. riparium* (Roth 1806: 216) Harvey (1849: 238) is regarded as the type species (Leliaert & Boedeker 2007). Members of *Rhizoclonium* are common inhabitants of marine, brackish and freshwater environments. The genus is generally characterized by uniserial, unbranched filaments, with cylindrical cells and rhizoidal structures (Nienhuis 1975, John 2011, Ichihara *et al*. 2013). Its species usually float in shallow-water or grow attached to various substrata, such as the surface of rocks or mud (van den Hoek & Chihara 2000). Recent morphological and phylogenetic analyses of *Rhizoclonium* have revealed greater species diversity as well as broadened its circumscription. For example, *R. pachydermum* Kjellmann (1877: 55) forms scattered true branches, mainly concentrated in the basal region (Zhao *et al*. 2014); *R. ramosum* Zhao et Liu (2016: 14) possesses true branches extending the entire length of the thallus; and endemic *Rhizoclonium* spp. from Lake Baikal, show morphological affinities with other genera within the Cladophorales (Boedeker *et al*. 2018). Therefore, true branches are not the key character that distinguishes the genus *Cladophora* Kützing (1843: 262) from *Rhizoclonium* (Zhao *et al*. 2014, 2016). Cell diameter, length/cell diameter (L/D) ratio, nuclear number and the presence of rhizoidal laterals (= lateral rhizoids) were considered helpful to identify *Rhizoclonium* species (Ichihara *et al*. 2013, Zhao *et al*. 2016).

More than 60 *Rhizoclonium* species names have been described (Guiry & Guiry 2018), but diagnostic features are not well defined and hence species are poorly circumscribed. No sequence data are available for any of the many, old type specimens, and this hinders the identification and classification of specimens further. At the same time, the reliability of some species names provided by some workers is controversial (Tautz *et al*. 2003, De Clerck *et al*. 2013, Verbruggen 2014, Montecinos *et al*. 2017, Leliaert & De Clerck 2017), and an increasing number of *Rhizoclonium*

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studies fail to link DNA sequences to available taxon names (Boedeker *et al*. 2018). Currently, molecular phylogenetic studies have indicated rampant cryptic diversity in *Rhizoclonium* with the increased taxon sampling (Leliaert & Boedeker 2007, Ichihara *et al*. 2013). The genus *Rhizoclonium* encompasses at least nine, genetically very divergent lineages based on nuclear-encoded small subunit (SSU) and partial large subunit (LSU) rDNA sequences (Boedeker *et al*. 2016). The internal transcribed spacer of the rRNA cistron (ITS), a variable species-specific marker, might be useful in detecting even more hidden diversity (Famá *et al*. 2000, 2002).

The aim of this study is to infer the phylogenetic relationships and species diversity within *Rhizoclonium* based on SSU rDNA, LSU rDNA and the internal transcribed spacer 2 (ITS2) sequences from freshwater *Rhizoclonium* from China. Our specimen data along with 139 Cladophoracean sequences from GenBank, were used for this evolutionary analysis. Furthermore, *Rhizoclonium* morphological criteria and evolution were also discussed in this study, to help better understand the genus.

TABLE 1. Specimens of members of the *Rhizoclonium* genus used in this study with collection data (voucher information, location, date of collection) and GenBank accession numbers.

Clade	Voucher No.	Habitat and Location	Collection Date	Longitude and Latitude	GenBank No.		
					SSU	LSU	ITS
1	HB1404	On cement with water, Wuhan University, Hubei Province, China	Jan–2014	30.59N 114.31E	KM892871	KM892875	KU904770
1	HAN1302	On the rock surface, Yanoda tropical rain forest, Hainan Province, China	Dec–2013	18.25N 109.51E	KP683359	KP683366	KU904798
1	HAN1303	On the rock surface, Yanoda tropical rain forest, Hainan Province, China	Dec–2013	18.25N 109.51E	KP683360	-	KU904799
1	HAN1304	On the rock surface, Yanoda tropical rain forest, Hainan Province, China	Dec–2013	18.25N 109.51E	KM892870	KM892874	KU904800
3	HB1415	On the wall near a lake, Wuhan city, Hubei Province, China	Jun–2014	30.70N 114.12E	-	KU904736	MH756601
3	HB1417	In an artificial pond, Enshi city, Hubei Province, China	Jul–2014	30.30N 109.50E	KU904648	-	-
3	HUN1409	On water-logged ground, Hengshan Mountain in Hunan Province, China	Mar–2014	27.23N 112.87E	KP683361	KP683369	KU904781
3	HUN1426	Attached on a rope, submerged in water, Changde city, Hunan Province, China	Apr–2014	27.99N 112.75E	-	-	KU904785
5	HB1412	On the step wall near a lake, Wuhan city, Hubei Province, China	Jun–2014	31.10N 114.47E	MH754748	KU904735	MH756600
5	HUN1437	On water-logged ground, Zhangjiajie National Forest Park, Hunan Province, China	Nov–2014	29.33N 110.45E	KU904676	KU904744	MH756602
7	CQ1401	Attached on stones of a pool, Chongqing city, China	Mar–2014	29.97N 106.28E	KP683358	KP683365	KU904795
7	HEN1505	On the wall of a small water ditch, Luoyang city, Henan Province, China	Apr–2015	34.42N 112.43E	KU904695	-	KU904791
7	HEN1506	Floating in a narrow ditch, Luoyang city, Henan Province, China	Apr–2015	34.42N 112.43E	KU904696	KU904752	KU904792

"-" missing data.

Material & Methods

Sample collection:—Thirteen *Rhizoclonium* specimens were collected as part of this survey of Cladophoraceae biodiversity in various freshwater locations from China (See Table 1 in the supporting information for detailed collection data). Specimen CQ1401 was sampled from the Chongqing municipality in March 2014; HB1404, HB1412, HB1415 and HB1417 from Hubei Province; HAN1302, HAN1303 and HAN1304 from Sanya city, Hainan Province, growing on the rock surface; HEN1505 and HEN1506 from Luoyang city, Henan Province; HUN1409, HUN1426 and HUN1437 from Hunan Province. The detailed information of these samples is listed in Table 1.

Natural samples were preserved in 10% formalin and alcohol solution, respectively. All specimens were deposited in the Freshwater Algal Herbarium (HBI), Institute of Hydrobiology, Chinese Academy of Science, under the accession numbers: CQ1401, HB1404, HB1412, HB1415, HB1417, HAN1302, HAN1303, HAN1304, HEN1505, HEN1506, HUN1409, HUN1426 and HUN1437.

Morphological observations:—We observed and measured morphological characters including cell sizes, length/diameter (L/D) ratio, basal rhizoids, lateral rhizoids, filament bending using a Leica DM5000B microscope. The micrographs were captured using a Leica DFC320 digital camera. Nuclei were stained with 0.01% GelRed and were observed using microscopy (DM5000B; Leica, Wetzlar, Germany). Comparison of morphological features among several *Rhizoclonium* clades is listed in Table 3.

DNA extraction, amplification and phylogenetic analyses:—Genomic DNA was extracted from materials using the DNeasy Plant Mini Kit (QIAGEN, Valencia, CA, USA) following the manfacturer's protocol and was stored at -20 °C.

Molecular phylogenetic analyses were based on SSU, LSU and ITS2 sequences. Primer information for these three molecular markers is listed in Table 2. The SSU rDNA gene was amplified using a combination of the primers SR1-SS11H and SSU897-18SC2 (Leliaert & Boedeker 2007). The LSU sequence was amplified using the universal primers C1 forward and D2 reverse (Hassouna *et al*. 1984, Leliaert *et al*. 2003). The ITS region was amplified using the universal primers ITS-9F and ITS-7R (Hayakawa *et al*. 2012).

TABLE 2. Summary of oligonucleotide primers used for polymerase chain reaction (PCR) and sequencing.

Target gene	Primer	Primer sequence(5'–3')	Direction	Reference
18S rDNA	SR1	5'-TACCTGGTTGATCCTGCCAG-3'	Forward	Leliaert & Boedeker (2007)
18S rDNA	SS11H	5'-CCTTTAAGTTTCAGCCTTGCGACC-3'	Reverse	Leliaert & Boedeker (2007)
18S rDNA	SSU897	5' GGTGAAATTCTTGGATTTGCGAAAGACG-3'	Forward	Leliaert & Boedeker (2007)
18S rDNA	18SC2	5'-TCCGCAGGTTCACCTACGGAG-3'	Reverse	Leliaert & Boedeker (2007)
28S rDNA	C1	5'-ACCCGCTGAATTTAAGCATAT-3'	Forward	Hassouna *et al*. (1984); Leliaert *et al*. (2003)
28S rDNA	D2	5'-TCCGTGTTTCAAGACGG-3'	Reverse	Hassouna *et al*. (1984); Leliaert *et al*. (2003)
ITS2	ITS-9	5'-CCGCCCGTCGCTCCTACCGATTGGGTGTG-3'	Forward	Hayakawa *et al*. (2012)
ITS2	ITS-7	5'-TCCCTTTTCGCTCGCCGTTACTA-3'	Reverse	Hayakawa *et al*. (2012)

The polymerase chain reaction (PCR) of SSU started with one initial denaturation step of 3 min at 94 °C, followed by 35 cycles of denaturation for 1 min at 94 °C, annealing for 1 min at 55 °C, and a final extension for 1 min 30 s at 72 °C. The LSU PCR amplifications started with 5 min at 94 °C, followed by 31 cycles of 30 s at 94 °C, 30 s at 57 °C, and 30 s at 72 °C, ending with a final hold of 5 min at 72 °C. Amplification of the ITS rDNA was obtained after 1 min at 94°C, followed by 30 cycles of 10 s at 98 °C, 30 s at 65 °C, and 2 min at 68 °C, with a final extension of 72 °C for 10 min. PCR products were visualized by staining with ethidium bromide after electrophoresis on a 1.5% agarose gel and then sent to Tsingke Biotech Co. Ltd, China, for sequencing, resulting in 11 SSU sequences, 9 LSU sequences and 12 ITS sequences.

Additional SSU, LSU and ITS sequences of Cladophorales for the molecular phylogenetic analyses were obtained from the National Center for Biotechnology Information (NCBI) database. The ITS2 regions were delimited using the ITS2 database (http://its2.bioapps.biozentrum.uni-wuerzburg.de/; searched on 20 September 2018, Koetschan *et al*. 2009) with a maximum E-value of 0.1. Sequences were refined manually with BioEdit version 5.0.9 (Hall 1999) and sequence matrices for phylogenetic analysis were aligned using MAFFT 7.0 (Katoh & Standley 2013). Highly variable regions that could not be aligned unambiguously were excluded to improve alignment, resulting in 1,656 positions for the SSU rDNA dataset, 589 positions for the LSU rDNA dataset, and 245 positions for the ITS2 dataset. The pairwise distance among the taxa was obtained using MEGA 7.0 (Kumar *et al*. 2016, see Tables 5–7).

TABLE 3. Comparison of morphological features among several *Rhizoclonium* clades.

Clade/Species name	Cell diameter (μm)	L/ D ratio	Nuclear number	Basal rhizoids	Lateral rhizoids	Real branches	Reference
Clade 1 (= *R. subtile*)	17.5–47.1	0.9–5	3–9	No	No	No	This study
Clade 2 (= *R. pachydermum*)	37.5–50	1–2.7	1–4	Basal rhizoids, sometimes forming holdfasts	Lateral rhizoids	True branches, only in the basal region	Zhao *et al*. (2014); Van den Hoek (1963)
Clade 3	10–32	2–10	1–4	Basal rhizoids, with a transparent, rounded tip, one or more cells in length	No lateral rhizoids observed.	No	This study; Zhao *et al*. (2014)
Clade 5	17.1–24.8	2–6	1, 2 or 4	Basal rhizoids up to 1–3 cell in length	Short lateral rhizoids , rarely, non-septate, like spinose projection	No	This study
Clade 6 (= *R. ramosum*)	15.3–52.8	1.3–5.8	4–17	Basal rhizoids	Lateral rhizoids. sparse and irregular, usually containing one to a few cells	True branches	Zhao *et al*. (2016)
Clade 7	14.6–22.8	1.2–4.0	1, 2 or 4	Basal rhizoids	Lateral rhizoids with several cells, frequently	no	This study

The SSU (68 sequences), LSU (76 sequences) and ITS2 (27 sequences) datasets were analyzed separately. Alignment files were carried out using RaxML (Stamatakis *et al*. 2005) in PAUP 4.0* (version 4.0 beta, Swofford 2003). Model parameters for RaxML analyses were determined from hierarchical likelihood ratio tests (hLRT, Huelsenbeck & Crandall 1997) using Modeltest (version 3.7, Posada & Crandall 1998). Based on the Akaike information criteria, the best-fit evolutionary model: TVM+G for SSU, GTR+I+G for LSU, SYM+I+G for ITS2 were selected. Bootstrapping for ML was carried out with 100 replicates, respectively. In addition, phylogenetic constructions were also performed using Bayesian inference (BI) in MrBayes (version 3.2, Ronquist *et al*. 2012). Posterior probabilities were calculated using a Markov chain Monte Carlo (MCMC) analyses, run with seven Markov chains (six heated, one cold) for 3×10^6, 4×10^6, 2×10^6 generations with sampling every 1,000 generations for SSU, LSU and ITS2, respectively. Topologies were summarized using the sumt command, with the first quarter discarded as burn-in. A stationary distribution was reached when an average standard deviation of the split frequencies between two parallel runs was ≤ 0.01. The first 25% of the generations were discarded as burn-in before calculating the majority-rule consensus trees in MrBayes.

Phylogenetic trees were displayed using TreeView (Page 1996). The final annotation was performed using Adobe Illustrator CS3. The habitats of all sequences were marked on the phylogenetic trees, and dark blue dots were coded to represent samples collected from the marine environment, light blue dots represented brackish water, green dots represented freshwater, and brown dots indicated samples from an unknown habitat or unknown salinity.

TABLE 4. Information of three matrices consisting of *Rhizoclonium* sequences.

Matrix	Taxa	Length	Conserved sites	Variable sites	Parisim-info sites	Estimated base frequencies (A/C/G/T)	Best-fit evolutionary model (Bayesian information criterion)
SSU	68 samples	1656	1405	240	148	0.25/0.22/0.28/0.25	TVM+G
LSU	76 samples	589	349	219	188	0.26/0.22/0.30/0.22	GTR+I+G
ITS2	27 samples	245	68	175	148	0.23/0.23/0.25/0.29	SYM+I+G

Results

Morphological observations:—*Rhizoclonium subtile* Z. ZHAO & G. LIU, *sp. nov*. **(Figs. 1–2, clade 1)**

Description:—Thalli are light green to fresh green, slender, soft and very long (up to 20 cm), usually growing on wet rocks or cement with water (Figs. 1A–1B). Thalli often intertwined into masses, growing in abundance and forming sponge-like cushions with a foamy appearance (Fig. 1B). No true branches. Basal rhizoids, lateral rhizoids and holdfasts lacking (Figs. 2A–2B). Cells are cylindrical and their diameter is uniform (Fig. 2C). Chloroplasts parietal and arranged in a reticulate pattern (Fig. 2D). Long unbranched filaments growing by intercalary cell divisions. The diameter of cells is 17.5–47.1 μm, with a length/diameter (L/D) ratio of 0.9–5. Swollen cells occasionally occurring in the filaments, their diameter up to 70 μm and L/D < 1 (Fig. 2E). Each cell has 3–9 nuclei (Fig. 2F). Pyrenoids are usually distributed in both the internal and peripheral regions of the chloroplast. Under unfavourable conditions cells transform into akinetes, forming club-shaped or barrel-like spores with 2.5–5 μm thick cell walls.

Etymology:—The epithet of the new species refers to the subtle morphological differences exhibited by the thalli, which are long and slender with no true branches, rhizoids, and rhizoidal laterals.

FIGURE 1. The habitat of *Rhizoclonium subtile*. A. The habitat viewed from a distance. Filaments grows near a waterfalls. B. The same habitat in Figure A magnified. Thalli attached on the surface of wet rocks, forming spongy-like or foamy appearance.

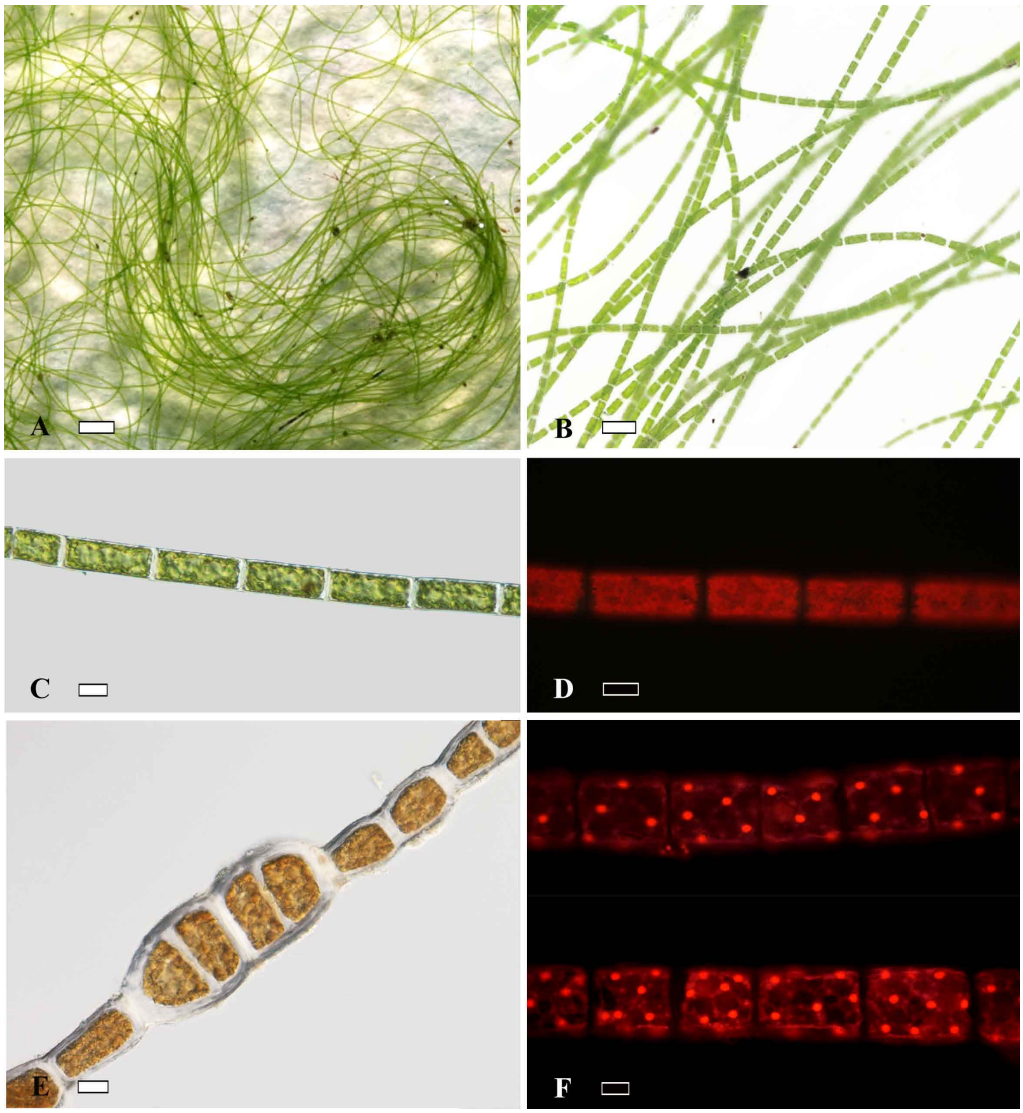

FIGURE 2. *Rhizoclonium subtile*. A–B. Entire filament. Note the long, unbranched filaments. C. Gross morphology. D. Autofluorescence showing the reticulated structure of the chloroplasts. E. Swollen cells occur in the filaments. F. Autofluorescence showing the number of nuclei. Scale bars: A = 500 µm, B= 100 µm, C–F = 20 µm.

Type locality:—HAN1303 (HBI), Sanya city (18°25´N, 109°51´E), Hainan Province, China; collected from the rocks near a waterfall in December 2013.

Holotype:—Deposited as HAN1303 in the Freshwater Algal Herbarium (HBI), Institute of Hydrobiology, Chinese Academy of Science, Wuhan, Hubei Province, China.

Distribution:—Sanya city, Hainan Province; Wuhan city, Hubei Province. Specimen vouchers: HAN1302, HAN1303, HAN1304 and HB1404. It usually grows on the wet rocks. In addition, this species was also reported in Hawaiian freshwater (voucher: ARS07405_00001) and Laos: Mekong river, Don Kon (isolate: H92).

Clade 3: Fig. 3

Description:—Thalli are light green to bright green, uniseriate and unbranched growing attached to substrates or floating. Filaments are sometimes intertwined with each other, forming hair-like masses (Fig. 3A). Cell diameter is 10–32 µm, with a length/diameter (L/D) ratio of 2–5 (sometimes up to 7). Pyrenoids are embedded within the chloroplast. No lateral rhizoids are observed (Fig. 3B). Basal rhizoids are common and consists of 1–3 cells, arising from basal cells (Fig. 3C). The shape of rhizoids is typical and representative of *Rhizoclonium*: becoming gradually thinner, ending with a transparent, chloroplast lacking, rounded tip (Fig. 3C). Cells are cylindrical and uniform, becoming barrel or club-shaped with the changes of the environment (Fig. 3B, 3D). Occasionally, few swollen cells or filaments that are bending can be observed. Chloroplasts are parietal and arranged in a reticulate pattern (Fig. 3E). There are 2 or 4 nuclei per cell (Fig. 3F).

Ecology and distribution:—The plants were found in Hubei and Hunan Provinces and grow in water, attached to substrates or floating. Specimen vouchers: HB1415, HB1417, HUN1409 and HUN1426.

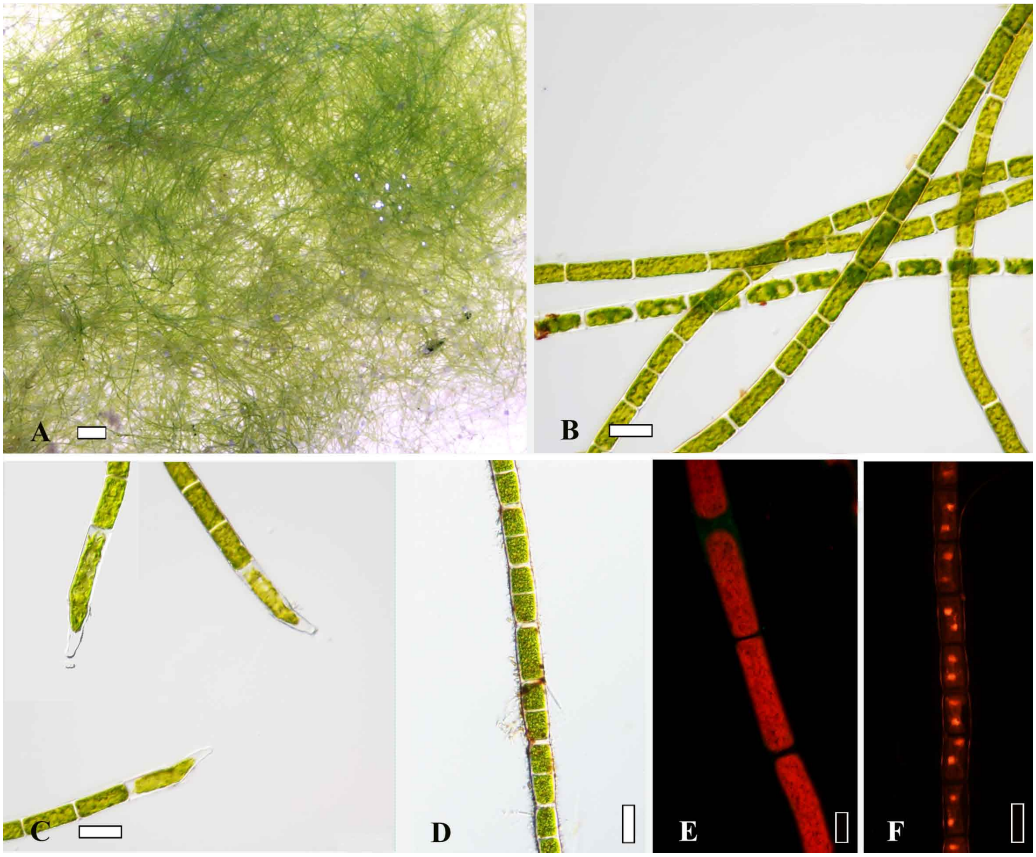

FIGURE 3. Morphological structures of clade 3 samples. A–B. Field and entire filament. C. Filament with rhizoids. D. Gross morphology with vegetative cells. E. Autofluorescence showing the reticulated structure of the chloroplasts. F. Autofluorescence showing the number of nuclei. Scale bars: A = 500 μm, B, C, D, F = 50 μm, E = 20 μm.

Clade 5: Fig. 4

Description:—Thalli bright green to light yellowish green, soft, no true branches, growing on rocks and walls in shallow-water. Cells are cylindrical and uniform (Fig. 4A). Cell diameter is 17.1–24.8 μm, with a length/diameter (L/D) ratio of 2–6. Chloroplasts are arranged in a reticulate pattern. The filaments are long and unbranched and grow by intercalary cell divisions. Filaments are attached to the substratum by basal rhizoids, usually containing one cell, but up to 1–3 cells in length (Figs. 4B–4C). Chloroplasts of basal rhizoids are uneven, usually ending with a transparent tip (Figs. 4B–4C). Short lateral rhizoids are present (but sparse), non-septate, appearing like spinose projections (Fig. 4D). Cells contain 1, 2 or 4 nuclei (Figs. 4E–4F). In some cells, only one nucleus exists and it is located in the middle of the cell (Fig. 4E). Pyrenoids are usually distributed in both the internal and peripheral regions of the chloroplast.

Ecology and distribution:—The plants were found in Wuhan city, Hubei Province; Zhangjiajie National Forest Park, Hunan Province. This species grows on the rocks or walls in shallow-water. Specimen vouchers: HUN1437 and HB1412.

Clade 7: Fig. 5

Description:—Thalli light green, no true branches, growing attached to rocks or stones sometimes forming floating plants (Fig. 5A). Plants grow by intercalary cell division. Cells are cylindrical, nearly uniform in diameter (Fig. 5A). Cell diameter is 14.6–22.8 μm, with a length/diameter (L/D) ratio of 1.2–4; reticulated chloroplasts arranged peripherally in the cytoplasm. Under unfavourable conditions cells are transformed into akinetes, club-shaped or barrel-like cells with thick cell walls (Fig. 5B). Pyrenoids are bilenticular, usually distributed in both the internal and peripheral regions of the chloroplast (Fig. 5C). Each cell contains 1, 2 or 4 nuclei (Fig. 5D). Filaments attach by simple basal rhizoids, which sprout from the basal cells, 2–4 cells in length, chloroplasts lacking (Fig. 5E). Rhizoids lateral, usually containing several cells (occasionally up to 10 cells), frequently formed from the middle to lower part of vegetative cells (Fig. 5A). Chloroplasts of lateral rhizoids are usually complete. Sometimes, the shape of rhizoids is uneven or slightly tapering to the tip.

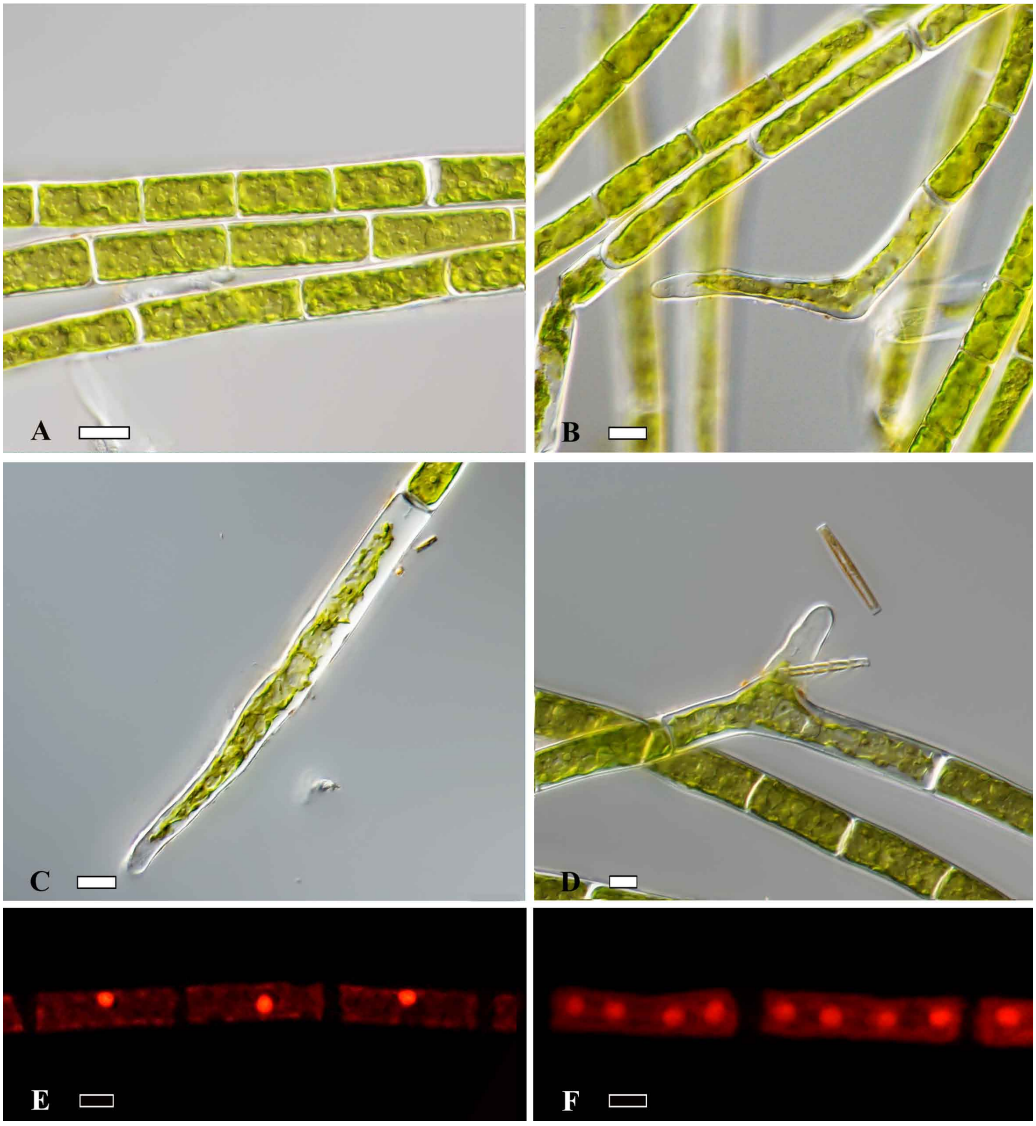

FIGURE 4. Morphological structures of clade 5 samples. A. Gross morphology. B–C. Filament with rhizoids. D. Filament with lateral rhizoids, non-septate and spiked. E–F. Autofluorescence showing the number of nuclei. Scale bars: A–F = 20 μm.

Ecology and distribution:—The plants were found in Chongqing city and Henan Provinces, attached on the walls of small ditches, growing on stones near pools, or floating. Specimen vouchers: CQ1401, HEN1505 and HEN1506.

Phylogenetic analyses:—Each phylogenetic tree constructed using RaxML and BI methods showed consistent topologies, and therefore just the Bayesian trees are presented here (Figs. 6–8). Analyses of SSU and LSU sequences clearly revealed the phylogenetic affinity among the three genera of Cladophoraceae—*Cladophora*, *Rhizoclonium* and *Chaetomorpha* (Kützing 1845): the former two genera showed a closer sister relationship while *Rhizoclonium* occupied a terminal position on the phylogram. The *Rhizoclonium* lineage was divided into different numbers of clades in the three marker inferred trees. Some clades were more divergent positioned long branches, separated by up to 5.8% pairwise genetic distance in the ITS2 phylogeny. The overall average pairwise distance among all *Rhizoclonium* analyzed here was 0.024 (SSU), 0.109 (LSU), 0.407 (ITS2), respectively. Detailed information of data matrices are listed in Tables 5–7. SSU phylogenetic analyses yielded thirteen moderately resolved *Rhizoclonium* clades (Fig. 6). These data indicated that the thirteen specimens from China belonged to four genetically different clades: clade 1 (including specimens HAN1302, HAN1304, HB1404), clade 3 (including specimens HB1417, HUN1409), clade 5 (including specimens HB1412, HUN1437), and clade 7 (including specimens CQ1401, HEN1505, HEN1506). Clades 1–6 clustered in a large clade (1.00/60), clades 8–12 clustered in another large clade (0.95/68), while clade 13 formed the earliest diverging lineage (1.00/100) in the SSU tree. With these additional sequences, the *Rhizoclonium* genus now identifies fifteen main clades in the LSU phylogeny, in which the evolutionary relationship of clades 1–6 (1.00/83), clades 8–15 (except clade 13) (0.91/50), and clade 13 (1.00/100) are supported (Fig. 7). The phylogenetic placement

of clade 7 with other clades was not well supported and the relationships among clades 1–6 were also unstable in three phylogenetic hypotheses. Clade 11 (in SSU) and clade 11* (in LSU) showed similar phylogenetic positions, sister to *R. breve* Ichihara & Miyaji (2013: 408) (= clade 10), but whether these two represent the same taxon can not be determined (* distinguishes them as *incertae sedis*). The same is true for clade 13* (Fig. 8). There were only seven clades (clade 1, clade 2, clade 3, clade 5, clade 6, clade 7 and clade 13*) identified in ITS2 phylogenetic tree based on the limited number of sequences (Fig. 8). In addition, the habitat analysis revealed that clade 1, clade 3, clade 4, clade 5 and clade 13 contained two habitat types: freshwater and marine/brackish environments, as well as clade 7 which included three salinity types (Figs. 6–8). Species names that are deemed reliable or the result of detailed morphological examination are noted in parentheses (including *R. subtile, R. pachydermum* and *R. ramosum*).

FIGURE 5. Morphological structures of clade 7 samples. A. Gross filaments morphology with long lateral rhizoids. B. Akinetes, club-shaped or barrel-like with thick cell walls. C. The arrow indicating the bilenticular pyrenoid observed by the light microscopy. D. Autofluorescence showing the number of nuclei. E. Basal rhizoids with chloroplasts lacking. Scale bars: A–B = 50 µm, C–E = 20 µm.

Discussion

In this study, a total of thirteen freshwater *Rhizoclonium* specimens were examined and determined to represent four clades/species (clade 1, clade 3, clade 5 and clade 7) on the basis of morphology and DNA sequences. *Rhizoclonium subtile* defined clade 1 (representative specimens: HAN1302, HAN1304, HB1404), collected from the surface of rocks or cement with water, and showing distinct morphological and ecological characteristics (Figs. 1–2). This species is characterized by forming long unbranched unserial filaments that typically lack rhizoids and rhizoidal laterals, and that display 3–9 nuclei per cell, and grow in abundance, forming spongy-like or a foamy appearance. The phylogenetic analyses based on three markers strongly supported its distinctiveness and relationship (Clade 1- 1.00/93 for SSU, 0.86/88 for LSU, 0.98/91 for ITS2) to other *Rhizoclonium* species (Figs. 6–8). These data support the recognition of clade 1 as a new species: *R. subtile*.

A comparison of morphological features among *Rhizoclonium* clades was summarized in Table 3. Branches, basal rhizoids or rhizoidal laterals showed differences among *Rhizoclonium* species. For example, *R. pachydermum* has true branches and a discoid holdfast, which were not observed in our thirteen samples. Lateral rhizoids are frequent in clade 7, while they are absent in clade 3. Basal rhizoids are characteristic of clade 3, with a rounded tip, and this is different

from that of clade 5. Therefore, rhizoids and lateral rhizoids have reference value for distinguishing *Rhizoclonium* species, at least for clade 3, clade 5 and clade 7. However, in culture experiments, it has been demonstrated that the proliferation and complexity of branches and rhizoids may be influenced by substratum firmness or light (Nienhuis 1975, Christensen 1991, Zhao *et al*. 2016).

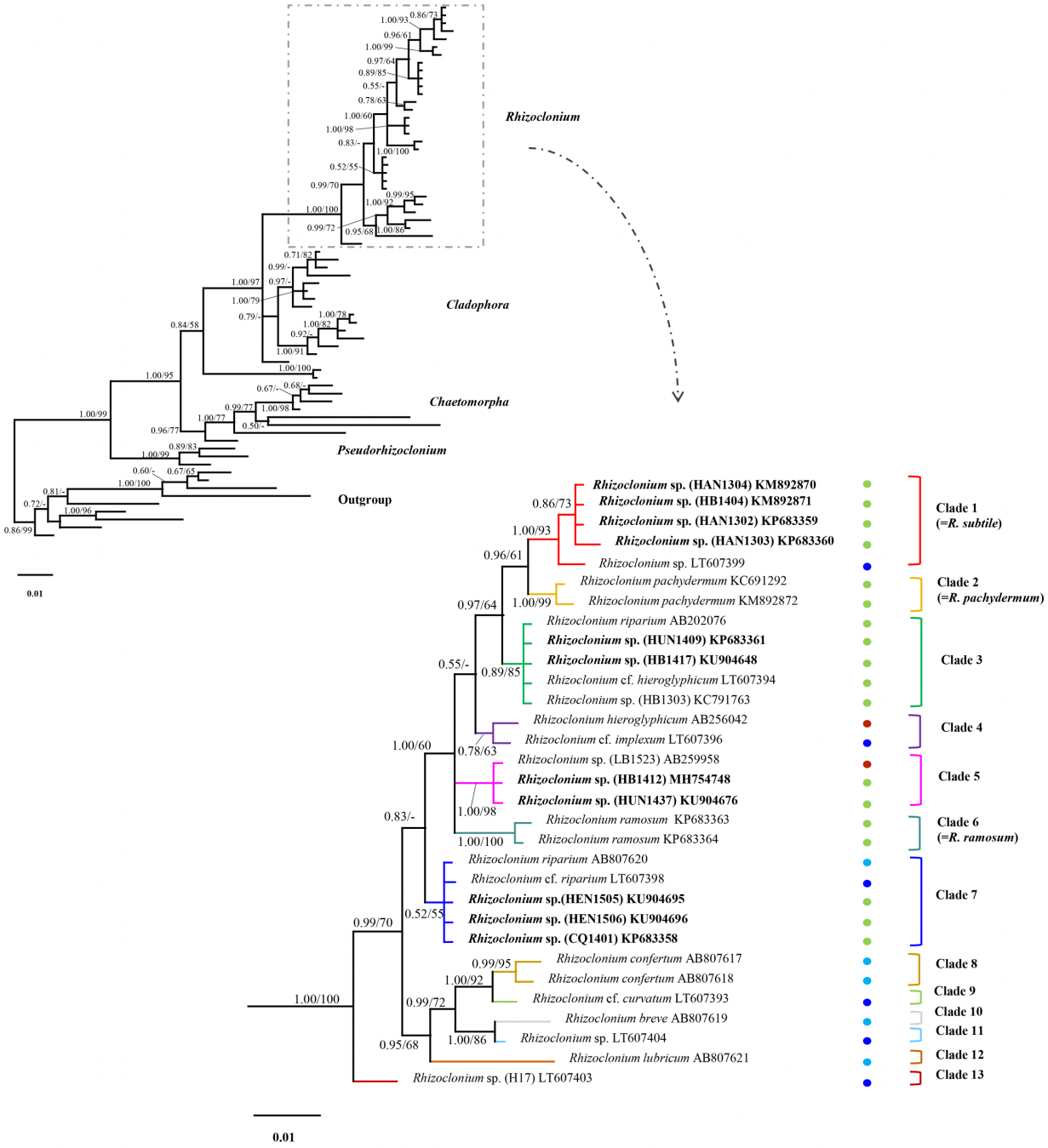

FIGURE 6. (A) Phylogenetic tree of the SSU sequences of members of Cladophoraceae. The posterior probabilities (PP) from Bayesian inference (BI) and RaxML bootstrap values are shown on the nodes. Support values > 0.50 are shown for BI and > 50 for RaxML. (B) Close-up of the genus *Rhizoclonium* marked with dotted lines in (A), with the *Rhizoclonium* lineages for better visualization. *Rhizoclonium* clades are marked with different colors. The starred lineage showed similar phylogenetic positions, but whether they represent the same taxon can not be determined (* distinguishes them as *incertae sedis*). Colored dots represented habitats. Dark blue dots were coded to represent samples collected from marine habitats, light blue dots represented brackish water, green dots represented freshwater, and brown dots indicated samples from an unknown habitat or unknown salinity.

Table 3 showed that there was a high level of cell diameter variation and length-width ratios among *Rhizoclonium* clades. For example, *R. ramosum* and *R. subtile* are different in basal rhizoids, lateral rhizoids, and true branches, but their cell diameter overlaps (former: 15.3–52.8 µm; latter: 17.5–47.1 µm). *Rhizoclonium riparium*, *R. hieroglyphicum*

(Agardh 1827: 236) Kützing (1845: 206), and *R. fractiflexum* Gardavsky (1993: 126) are also statistically similar in cell diameter. Therefore, using cell diameter alone to clarify different species is problematic. In previous studies, pyrenoid morphology was used as another criterion for characterizing families, genera, and species in the Cladophorales (Miyaji 1999, Van Den Hoek & Chihara 2000, Hanyuda *et al*. 2002). However, this character is of limited taxonomic value. Three pyrenoid types, recognized in the *Rhizoclonium* and *Pseudorhizoclonium* genera, also exist in other genera within the Cladophorales, such as in *Aegagropila* Kützing (1843) and *Chaetomorpha* (Miyaji 1999, Boedeker *et al.* 2012, Ichihara & Miyaji 2016, Zhao *et al.* 2016, Ichihara *et al.* 2016). Ichihara *et al*. (2016) demonstrated that pyrenoid morphology may not be a suitable and effective taxonomic character for distinguishing *Rhizoclonium* species. For example, the pyrenoid ultrastructure of *R. riparium* (bilenticular type) is the same as that reported for *R. hieroglyphicum* (Matsuyama *et al*. 1998); the rare zonate pyrenoid bilenticular type occurs in both *R. ramosum* and *R. breve* (Zhao *et al*. 2016). Despite differences in the presence/absence of basal rhizoids or lateral rhizoids, samples from clade 3, clade 5 and clade 7 are morphologically similar to *R. riparium*. There are three clades (clade 1, clade 2 and clade 6) that provided definite species names based on the distinct morphology and molecular data, namely *R. subtile*, *R. pachydermum* and *R. ramosum*, respectively. Other clades (unnamed in this study) will be discussed and assigned names in a subsequent publication after increased taxon sampling.

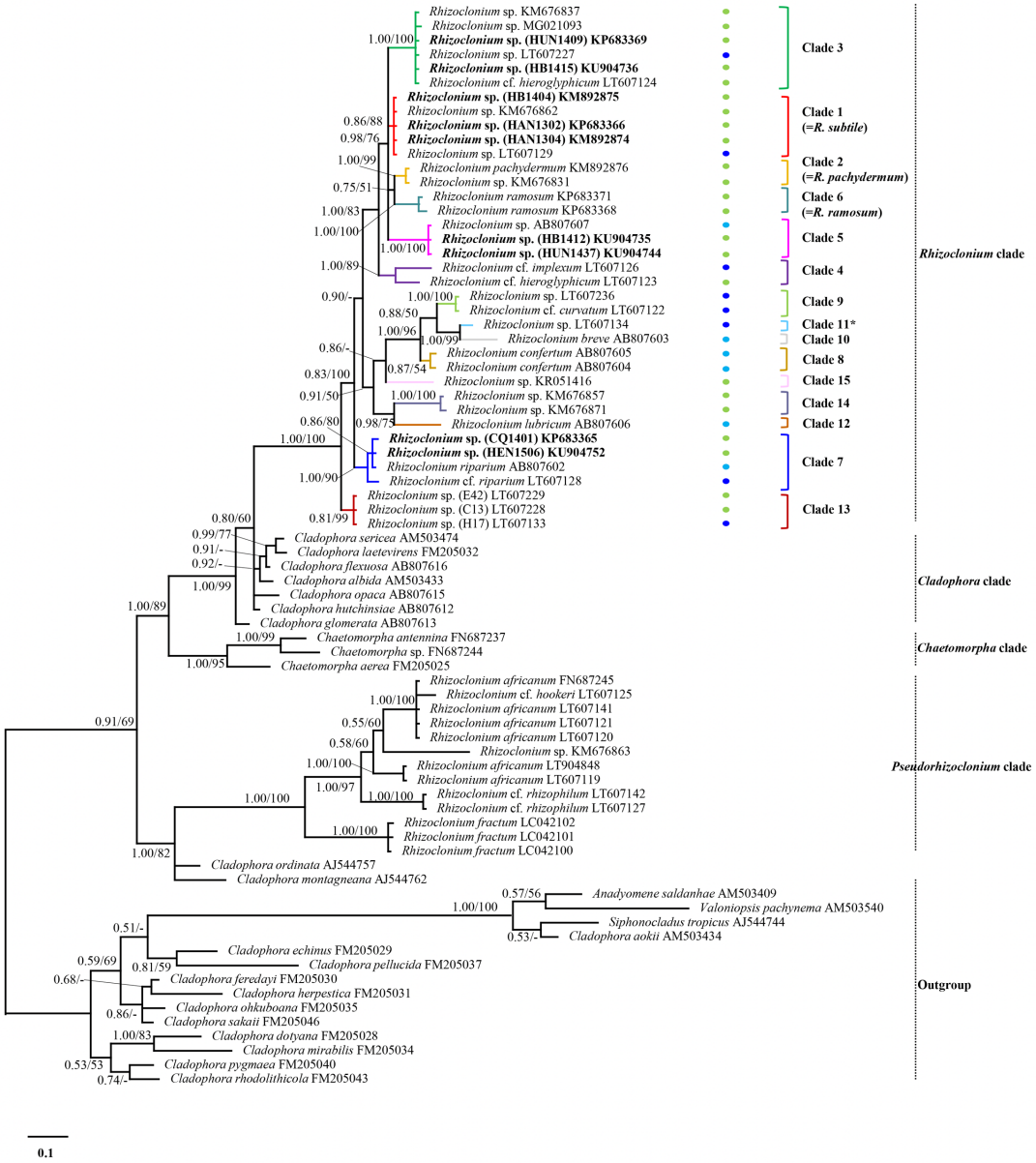

FIGURE 7. Phylogenetic tree of the LSU rDNA sequences of Cladophoraceae. The posterior probabilities (PP) from the Bayesian inference (BI) and RaxML bootstrap values are shown on the nodes. Support values > 0.50 are shown for BI and > 50 for RaxML. *Rhizoclonium* lineages are marked in different colors and habitats of all sequences and labeled with colored dots as in Figure 6.

TABLE 5. Pairwise distance of SSU rDNA sequence among the *Rhizoclonium* taxa in this study.

	Clade 1	Clade 2	Clade 3	Clade 4	Clade 5	Clade 6	Clade 7	Clade 8	Clade 9	Clade 10	Clade 11	Clade 12	Clade 13	Clade 14	Clade 15
Clade 1															
Clade 2	0.004														
Clade 3	0.004	0.003													
Clade 4	0.005	0.005	0.003												
Clade 5	0.006	0.007	0.004	0.004											
Clade 6	0.008	0.008	0.007	0.006	0.007										
Clade 7	0.007	0.007	0.005	0.004	0.004	0.006									
Clade 8	0.011	0.010	0.009	0.008	0.010	0.013	0.008								
Clade 9	0.010	0.008	0.007	0.007	0.009	0.012	0.007	0.003							
Clade 10	0.011	0.010	0.009	0.0080	0.009	0.013	0.008	0.008	0.007						
Clade 11	0.017	0.018	0.017	0.016	0.016	0.019	0.015	0.016	0.016	0.015					
Clade 12	0.015	0.014	0.013	0.011	0.012	0.015	0.010	0.011	0.012	0.012	0.020				
Clade 13	0.009	0.008	0.007	0.006	0.007	0.008	0.004	0.009	0.009	0.009	0.016	0.011			

TABLE 6. Pairwise distance of LSU rDNA sequence among the *Rhizoclonium* taxa in this study.

	Clade 1	Clade 2	Clade 3	Clade 4	Clade 5	Clade 6	Clade 7	Clade 8	Clade 9	Clade 10	Clade 11*	Clade 12	Clade 13	Clade 14	Clade 15
Clade 1															
Clade 2	0.011														
Clade 3	0.019	0.026													
Clade 4	0.032	0.038	0.048												
Clade 5	0.028	0.031	0.041	0.050											
Clade 6	0.022	0.022	0.034	0.046	0.039										
Clade 7	0.028	0.030	0.043	0.042	0.048	0.030									
Clade 8	0.051	0.057	0.051	0.069	0.074	0.055	0.043								
Clade 9	0.066	0.072	0.064	0.077	0.086	0.075	0.055	0.022							
Clade 10	0.060	0.064	0.062	0.082	0.076	0.060	0.055	0.046	0.043						
Clade 11*	0.056	0.060	0.056	0.074	0.076	0.062	0.047	0.032	0.029	0.030					
Clade 12	0.056	0.054	0.059	0.057	0.074	0.058	0.049	0.062	0.062	0.066	0.062				
Clade 13	0.029	0.035	0.043	0.042	0.051	0.043	0.022	0.035	0.048	0.050	0.045	0.045			
Clade 14	0.052	0.057	0.060	0.063	0.077	0.063	0.052	0.059	0.073	0.083	0.067	0.054	0.053		
Clade 15	0.049	0.051	0.055	0.061	0.062	0.063	0.047	0.049	0.058	0.062	0.054	0.050	0.045	0.065	

TABLE 7. Pairwise distance of ITS2 sequence among the *Rhizoclonium* taxa in this study.

	Clade 1	Clade 2	Clade 3	Clade 5	Clade 6	Clade 7	Clade 13*
Clade 1							
Clade 2	0.080						
Clade 3	0.249	0.272					
Clade 5	0.316	0.316	0.397				
Clade 6	0.248	0.255	0.376	0.323			
Clade 7	0.493	0.430	0.498	0.546	0.484		
Clade 13*	0.301	0.309	0.272	0.472	0.401	0.581	

Phylogeny and evolution of the genus Rhizoclonium. In this study, we analyzed the genus *Rhizoclonium* using morphological and molecular data. It is difficult to link the molecular species clades to existing names, however DNA sequences can provide useful and direct data to recognize genera and species of Cladophoraceae. The classification of taxa based on molecular sequence data is common practice at the species level as more and more cryptic species are discovered (Bakker *et al*. 1995, van der Strate *et al*. 2002, Leliaert *et al*. 2009c, 2014, Payo *et al*. 2013, De Clerck *et al*. 2013, Muangmai *et al*. 2014, West *et al*. 2014). In the absence of diagnostic characters, Zhu *et al*. (2018) identified eight freshwater *Cladophora* clades based on multiple DNA markers. In this study, fifteen genetically divergent *Rhizoclonium* lineages (clades 1–15) were recognized based on LSU data. The new species *R. subtile* formed an independent branch with significant genetic separation from all published species. Clade 13, located in the basal position of the *Rhizoclonium* clade formed an early-diverging lineage. Subsequently, one large group, represented by clades 8–15 (except clade 13), were well supported. Clades 1–6 exemplified the other large group, of which the relationships were not well-resolved within the group, but appear to have formed a more recent evolutionary radiation.

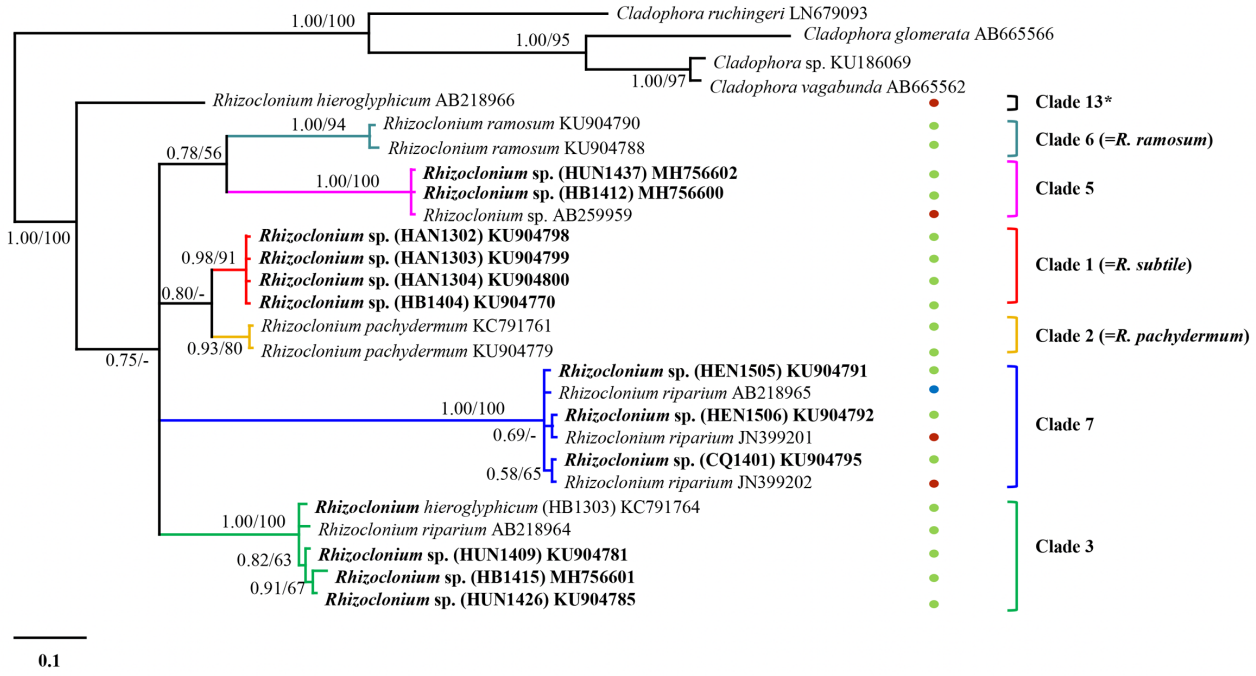

FIGURE 8. Phylogenetic tree of the ITS2 sequences of *Rhizoclonium*. The Bayesian inference (BI) posterior probabilities and bootstrap support values based on RaxML analyses are shown at the nodes. Support values > 0.50 are shown for BI and > 50 for RaxML. *Rhizoclonium* lineages are marked in different colors and habitats of all sequences and labeled with colored dots as in Figure 6.

It is generally accepted that the Cladophorales orginated in the marine environment and the adaptation to the freshwater environment happened more than once (Hanyuda *et al*. 2002). Our study supported this conclusion further: an evolutionary trend from marine to freshwater happened in the *Cladophora* clade, and another migration to freshwater happened in the *Rhizoclonium* clade (Figs. 6–7). The habitat analyses indicated that the evolution of *Rhizoclonium* did not show a clear transition trend from the marine to the freshwater environment. In contrast, marine, brackish and freshwater habitats were frequently mixed in some clades, for instance, the diverse habitat environments of clade 1, clade 3, clade 5, clade 7 and clade 13 (Figs. 6–8). Imai *et al*. (1997) revealed that the same *Rhizoclonium* strain can tolerate salinity gradients from 0.1–34 under culture conditions, suggesting its ability for effective osmoacclimation. For this reason, traditional methods for applying some species names based on the habitat type, as for example *R. hieroglyphicum* used only for some freshwater samples, may not be appropriate. In addition, unbranched and branched *Rhizoclonium* were found in the same clade, which further demonstrated that morphology does not entirely reflect evolution in the Cladophoraceae.

Most previous studies mainly described the morphological features of *Rhizoclonuim* samples and revealed its phylogenetic relationship to *Cladophora*. This study reports molecular phylogenetic analyses on the genus *Rhizoclonium* that are based on multiple markers and a large number of sequences, yielding several novel relationships that were either not supported or not represented in earlier studies. However, the species names of some clades are not resolved as yet. The selection and sequencing of epitypes may be one way to solidify *Rhizoclonium* taxonomy (Leliaert & De Clerck 2017). Further sampling, DNA sequencing and morphological work are needed to sort out the relationships of *Rhizoclonium* in the Cladophoraceae.

Conclusion

We collected thirteen *Rhizoclonium* specimens from China and recognized them as four distinct molecular species. Through analyses of morphological characters and three markers, the biodiversity worldwide of *Rhizoclonium* was increased from nine to fifteen genetic clades, and a new species *R. subtile* was described.

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