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Occurrence of true branches in *Rhizoclonium* (Cladophorales, Ulvophyceae) and the reinstatement of *Rhizoclonium pachydermum* Kjellman

ZHI-JUAN ZHAO[1,2], HUAN ZHU[1,2], ZHENG-YU HU[1] & GUO-XIANG LIU[1*]

[1]*Key Laboratory of Algal Biology, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan 430072, People's Republic of China*

[2]*University of Chinese Academy of Sciences, Beijing 100039, People's Republic of China*

** Corresponding author (liugx@ihb.ac.cn)*

Abstract

The phylogenetic position of the freshwater green alga *Rhizoclonium pachydermum* (Ulvophyceae: Cladophorales) was investigated using nuclear 18S rRNA gene and internal transcribed spacer 2 (ITS2) sequences. This alga has been referred to as *Cladophora pachyderma*. Based on its morphology, it was formerly classified in the section *Affines* in the genus *Cladophora*. However, this classification was not supported by the current phylogenetic analyses, where *Rhizoclonium pachydermum* formed a well-supported clade with other *Rhizoclonium* species. We consider that *Rhizoclonium* possesses real branches and the most important criteria that characterize the genus are: long unbranched filaments only with rhizoid branches, or only branched at the basal region of the thallus; and cylindrical cells with few or limited numbers of nuclei.

Key words: 18S rDNA, Cladophorales, ITS2, phylogeny, *Rhizoclonium pachydermum*, taxonomy

Introduction

Rhizoclonium pachydermum Kjellman (1877: 55) is a filamentous green alga (Chlorophyta: Cladophoraceae) that usually grows on the wet surfaces of rocks, the walls of wells, and the upper or lower sides of stones, being a fairly shade-loving species (van den Hoek 1963). This alga was established by Kjellman (1877) originally based on a sample he collected on the west coast of Novaya Zemlya. The alga has been recorded from Germany, Sweden, West Greenland, China, and other countries (Jao 1947, Kann 1947, van den Hoek 1963, Christensen 1991, Liu & Hu 1999). Brand (1909) transferred it to the genus *Cladophora* Kützing (1843: 262) because it had real branches and a disc-like holdfast. Since then, *R. pachydermum* Kjellman has been called *Cladophora pachyderma* (Kjellman) Brand (1908: 72) (Hoek 1963, Liu & Hu 1999).

The genus *Cladophora* is a rather heterogeneous assemblage of species, and one of the most species-rich genera among the green macroalgae (van den Hoek 1982, 1984, van den Hoek and Chihara 2000). Van den Hoek (1963) placed *C. pachyderma* in the first group of *Cladophora*: Section *Affines* Brand (1909:70). This section is often characterized as follows: i) long filaments, which grow via frequent intercalary cell divisions; ii) scattered branches, concentrated in the basal region of the plants, inserts laterally, often deflecting the axes over a wide angle; iii) attachment by a disc-like holdfast formed by the lower cell wall of the basal cell; and iv) cells relatively short, length/width ratio mostly 1–2 (van den Hoek 1963).

The two species in this group were *Cl. basiramosa* Schmidle in Wittrock, Nordsted & Lagerheim (1896: 13–14, fasc. 26, no. 1225) (Schmidle 1897) and *Cl. pachyderma*. Van den Hoek supplemented Kjellman's illustrations with some new drawings, which agreed with the taxonomic opinion of Brand. However, he also noted that both algae were very similar. In his opinion, the lack of moniliform chains of zooidangia and the very different ecology appeared to justify the perhaps provisional separation of *Cl. pachyderma* from *Cl. basiramosa* Schmidle. When Christensen (1991) re-examined Kjellman's type material, he found a thin *Oedogonium* and some diatoms that proved to be freshwater species, as well as representatives of the freshwater genera *Gomphonema* and *Eunotia*.

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Thus, Christensen regarded *R. pachydermum* as a freshwater species and *Cl. basiramosa* Schmidle was probably only a synonym of *Cl. pachyderma.* Liu & Hu (1999) described *Cladophora pachydermum* Brand and observed chains of sporangia. Liu considered that the algal morphology may change readily depending on the environment and there was probably just one species in the Section *Affines* (Liu & Hu 1999).

Other than morphological, no phylogenetic studies of *R. pachydermum* have been conducted based on nucleotide sequences. In this study, therefore, we performed molecular phylogenetic analyses using nuclear 18S rDNA and internal transcribed spacer 2 (ITS2) gene sequences to determine the position of *R. pachydermum* in Cladophorales and the relationships between *R. pachydermum*, *Cladophora* species, and some other genera. Two freshwater samples of *Rhizoclonium* Kützing (1843: 261) were also included to evaluate the characters of the genus *Rhizoclonium*.

Materials and methods

Taxon sampling and morphological analysis:—Specimens of *R. pachydermum* HB1210 were sampled from the Institute of Hydrobiology, Chinese Academy of Sciences, Hubei Province, on the wall of a small water ditch during December 2012. *Rhizoclonium riparium* (Roth 1806: 216) Harvey (1849: 238) HB1302 and *R. hieroglyphicum* (Agardh 1827: 236) Kützing (1845: 206) HB1303 were observed at the same time and were also sampled from the Institute of Hydrobiology, Chinese Academy of Sciences, Hubei Province. Morphological and phylogenetic investigations were performed using these three specimens, for which sequence data are available (Table 1).

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

Species	Location and collector	Collection date	Voucher no.	GenBank accession no.	
				SSU	ITS
R. pachydermum	Attached on the wall of a small water ditch, Institute of Hydrobiology, Chinese Academy of Sciences, Hubei Province, China; Guo-Xiang LIU	December 2012	HB1210	KC691292	KC791761
R. riparium	Floating on the wet land surface, Institute of Hydrobiology, Chinese Academy of Sciences, Hubei Province, China; Guo-Xiang LIU	January 2013	HB1302	KC791762	KC914570
R. hieroglyphicum	in water, Institute of Hydrobiology, Chinese Academy of Sciences, Hubei Province, China; Guo-Xiang LIU	January 2013	HB1303	KC791763	KC791764

Natural samples were isolated using an Olympus SZX7 microscope and rinsed with double-distilled H_2O. The algae were grown in culture dishes on sterilized BG11 medium solidified with 1.2% agar under a constant light source at 30–50 μmol m^{-2} s^{-1} and at a temperature of 25 °C. The medium was renewed every 2–3 weeks until sufficient biomass (> 0.5 g fresh mass) was obtained for DNA extraction. These cultures can be obtained on request from the Freshwater Algae Culture Collection, Institute of Hydrobiology, Chinese Academy of Science (FACHB) under accession numbers HB1210, HB1302, and HB1303.

Observations were made under differential interference contrast and epifluorescence microscopy using a Leica DM5000B microscope. Micrographs were captured using a Leica DFC320 digital camera. To observe the nuclei, specimens were stained with 0.01% GelRed and observed by microscope (DM5000B; Leica, Wetzlar, Germany).

Molecular phylogenetic analyses:—The algal cultures were rinsed three times with ddH_2O and harvested by centrifugation. The algal cells were broken with mini beads in a beadbeater (3110BX, Biospec Products, Bartlesville, USA) and the total DNA was extracted using the CTAB method (Doyle & Dickson 1987).

PCR amplifications were performed as described by Boedeker (2012), where the complete nuclear-encoded small subunit (SSU) RNA gene was amplified using the primer pairs SR1-SS11H and SSU897-18SC2 (Leliaert *et*

al. 2007). The PCR reactions were conducted using TaKaRa Ex-Taq Polymerase (TaKaRa, Japan). The PCR cycling conditions were as follows: initial denaturation at 94 °C for 3 min; 35 denaturation cycles at 94 °C for 1 min, primer annealing at 55 °C for 1 min, and extension at 72 °C for 1 min 30 s; with a final extension at 72 °C for 3 min. The sequence of ITS2 regions were amplified with the primer CladoITS-9F and CladoITS-7R, as presented in Hayakawa *et al.* (2012). The ITS2 PCR amplications started with 1 min at 94 °C, followed by 30 cycles of 10 s at 98 °C, 30 s at 65 °C, 2 min at 68 °C, ending with a final hold of 10 min at 72 °C. All PCR products were visualized by staining with ethidium bromide after electrophoresis on a 1% agarose gel. The products were purified with an AxyPrep DNA Gel Extraction Kit (AXYGEN) and sent to Sangon Biotech Inc., China, for sequencing. The sequences were assembled using Seqman (Swindell & Plasteer 1997) and deposited in GenBank under the accession numbers shown in Table 1.

The molecular phylogenetic analyses were based on the nuclear-encoded SSU and ITS2 sequences. The sequences of putative relatives were downloaded from GenBank. The sequences were aligned using the default parameters in CLUSTALX 1.83 and adjusted manually with BioEdit version 5.0.9 (Hall 1999). The homology of sites and the accuracy of the alignments were determined by examining the secondary structure, which was calculated using RNA structure 4.11 (Mathews *et al*. 1999) based on a predicted secondary structure model (Luo *et al*. 2006). The final alignment of 18S rDNA sequences comprised a matrix of 37 sequences, including *Ulothrix zonata* (Weber & Mohr 1804: 97) Kützing (1843: 251) and *Ulva fasciata* Delile (1813: 297) as outgroup taxon, while the alignment of ITS2 sequences included 12 taxa with *Ulothrix zonata* as an outgroup.

The SSU (37 sequences) and ITS2 (12 sequences) datasets were analyzed separately. Phylogenetic trees were constructed using maximum likelihood (ML) in PAUP 4.0* (version 4.0 beta, Swofford 2003) and Bayesian inference (BI) in MrBayes (version 3.1.2, Huelsenbeck & Ronquist 2001). The ModelTest 3.7 (Posada & Crandall 1998) program was used to explore the sequence evolution model that fitted the dataset best based on the hierarchical likelihood ratio test (hLRT, Huelsenbeck & Crandall 1997). Bootstrapping was carried out with 100 replicates using a heuristic search strategy. The best-fit models for the 18S rDNA and ITS2 datasets were GTR+I+G and K80+G, respectively. All Bayesian Markov Chain Monte Carlo (MCMC) analyses were run with seven Markov chains (six heated chains, one cold) for $5 \cdot 10^6$ generations, where one tree was kept every 1000 generations. Each analysis reached stationarity (average standard deviation of split frequencies between runs < 0.01) well before the end of the run. A burn-in sample of 1250 trees was removed before calculating the majority rule consensus trees in MrBayes. We obtained posterior probability values for the branching patterns in the BI trees and the bootstrap values for the ML trees.

Results

Rhizoclonium pachydermum Kjellmann (1877: 55–56)

Synonyms: *Cladophora pachyderma* (Kjellmann) Brand (1909: 62–72); *Cladophora basiramosa* Schmidle in Wittrock, Nordsted & Lagerheim (1896: 13–14); *Cladophora basicladioides* Jao (1947: 265–267)

Type:—RUSSIA: Novaja Zeml'a, Malye Karmakuly, *Kjellmann, 26/6/1875*.

Morphological observations:—Thalli dark green. Filaments long and almost unbranched, growing by intercalary cell divisions (Figs. 1A–1B). Attached by simple basal rhizoids, often forming a disciform holdfast at the lower end (Figs. 1A–1C). Branches concentrated in the basal region, laterally inserted, and often deflected from the axis. No secondary branch system. Cells were cylindrical and thick-walled (Figs. 2A–2C). Diameter of branches = 22.5–30.0 μm, L/D (length/diameter) = 1.3–4.5 (n = 60); main filaments = 37.5–50.0 μm, average length = 65.7 μm and L/D = 1.0–2.7 (n = 60). Diameter of apical cells = 25–30 μm (n = 65), diameter of basal cells = approximately 25–50 μm (n = 65). Two to eight nuclei per cell. A pore halfway along the zooidangia. Cell structure: Each cell had 1–4 nuclei (Figs. 1D, 2E).

Rhizoclonium pachydermum grows on the wet surfaces of rocks, the wall of wells, etc. It can be seen throughout the year, but usually grows well in a cool, damp environment. We also re-observed the samples cited in Jao (1947) and Liu & Hu (1999). The former was collected by Jao in 1938 in Yangshuo, Guangxi, and the latter by Liu in 1992 in Suizhou, Hubei. The branches in the basal region can be observed in these materials, although *Cl. pachydermum* collected by Liu showed fewer branches, and the branched in Jao's specimen are more abundant.

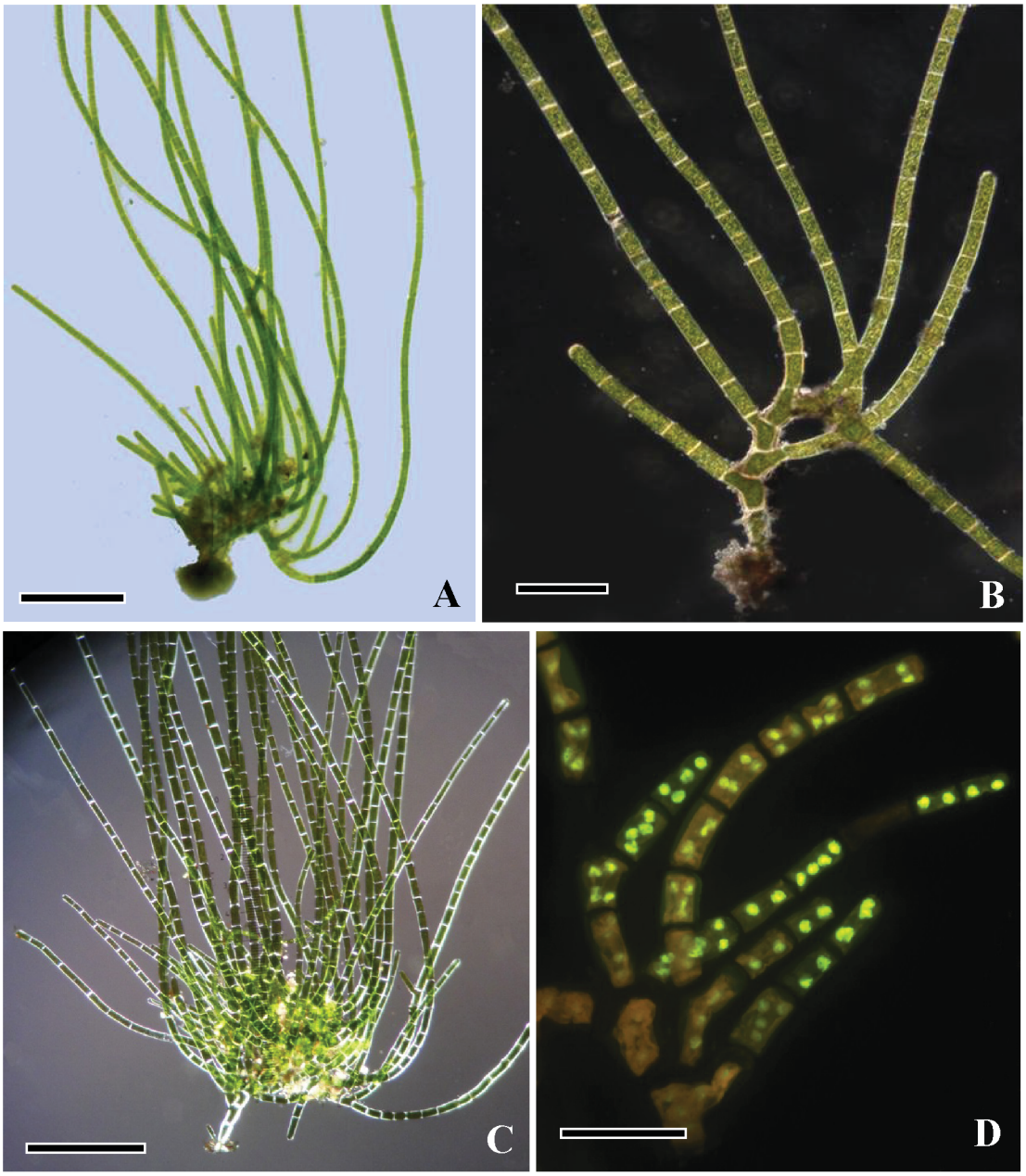

FIGURE 1. *Rhizoclonium pachydermum*. A. Entire filament. Note the long, almost unbranched filaments. B. Basal region. Note the basal branches and the holdfast. C. Long, almost unbranched filaments. D. Showing the number of nuclei. Scale bars: A, C = 500 μm B, D = 100 μm.

Two other live samples of *Rhizoclonium* were also observed: filaments of *R. riparium* HB1302 were light green and attached to the wet soil surface. Lateral rhizold present, sharp, and mostly one- to two-celled, chloroplasts of the rhizold sometimes lacking or uneven; diameter of cells = 17.5–25.0 μm and average length = 19.1 μm, L/D = 3.3 ± 0.6 (n = 60); growth by intercalary cell division; cells cylindrical with thin walls (Figs. 3A-3D). Thalli of *R. hieroglyphicum* HB1303 were yellow green to light green, mostly silky; floating; unbranched loosely interwoven filaments, often with a rhizoid at the end but without any rhizoidal branches. Cells cylindrical, almost uniform diameter = 20–25 μm, L/D = 2–10. Each cell of both samples had 1–4 nuclei based on three samples (Figs. 3D, 3H).

Phylogenetic analyses:—The PCR amplicons of the 18S rDNA of *R. pachydermum* comprised about 1700 bp. The base frequencies were found to be homogeneous across the 37 taxa. The overall average pairwise distance was 0.064. The PCR amplicons of the ITS2 sequences of *R. pachydermum* Kjellman comprised approximately 800 bp. The alignment of the isolates and their putative relatives measured about 223 nucleotides in this study. The base frequencies across the 12 taxa were found to be homogeneous. The overall average pairwise distance was 1.105.

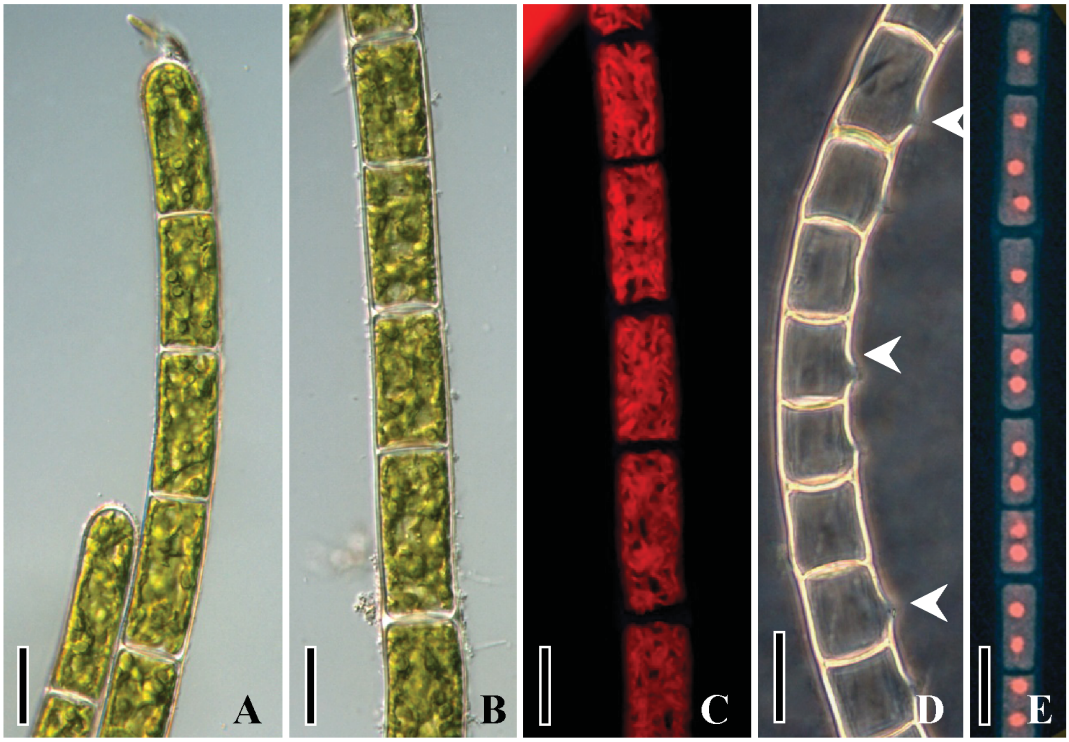

FIGURE 2. *Rhizoclonium pachydermum*. A–B. Gross morphology. C. Autofluorescence showing the reticulated structure of the chloroplasts. D. Gametangia, where the cell is empty after liberating the gametes through a lateral pore. The arrowhead indicates a domed pore. E. Autofluorescence showing the number of nuclei. Scale bars: A–D = 50 µm, E= 100 µm.

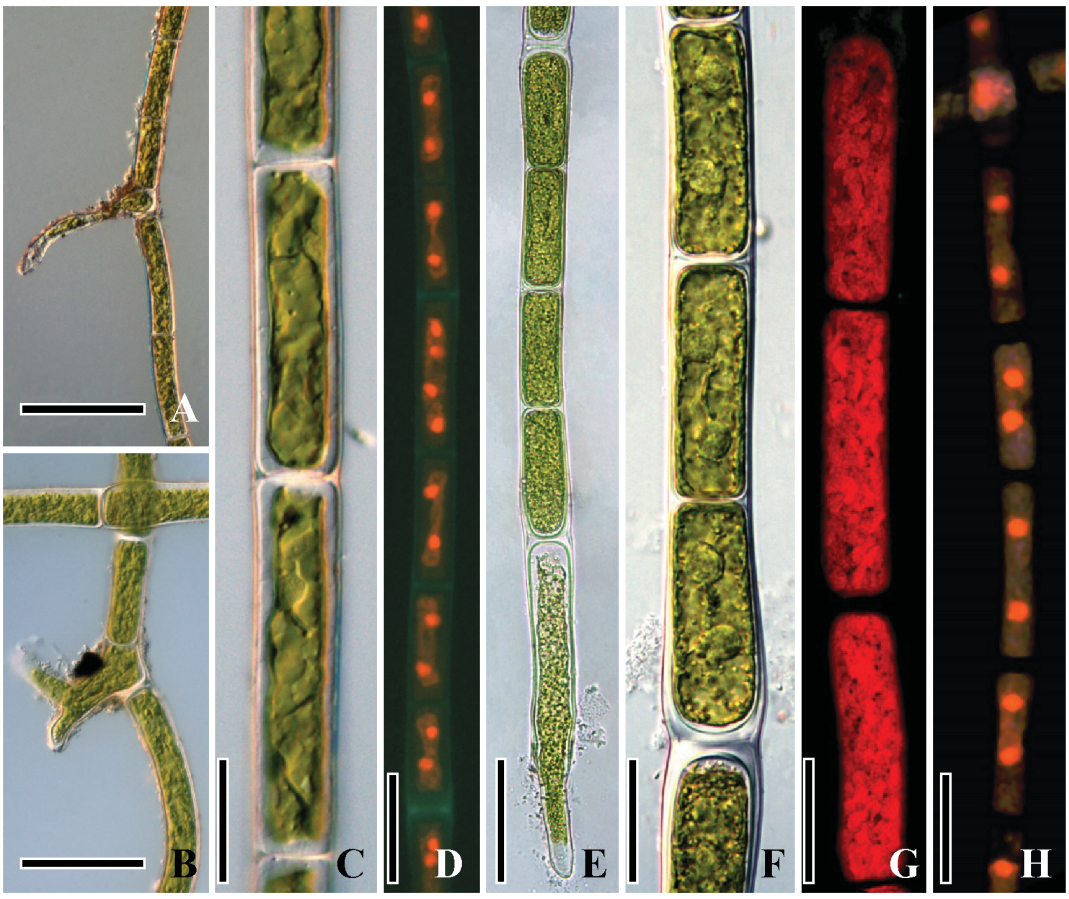

FIGURE 3. A–D. *Rhizoclonium riparium* HB1302. A–B. Filament with rhizoidal cells. C. The reticulated structure of the chloroplasts. D. Autofluorescence showing the number of nuclei. E–H. *Rhizoclonium hieroglyphicum* HB1303. E–F. Structure of cells. G. Autofluorescence showing the reticulated structure of the chloroplasts. H. Autofluorescence showing the number of nuclei. Scale bars: A, B, D, E, G, H = 50 µm. C, F = 25 µm.

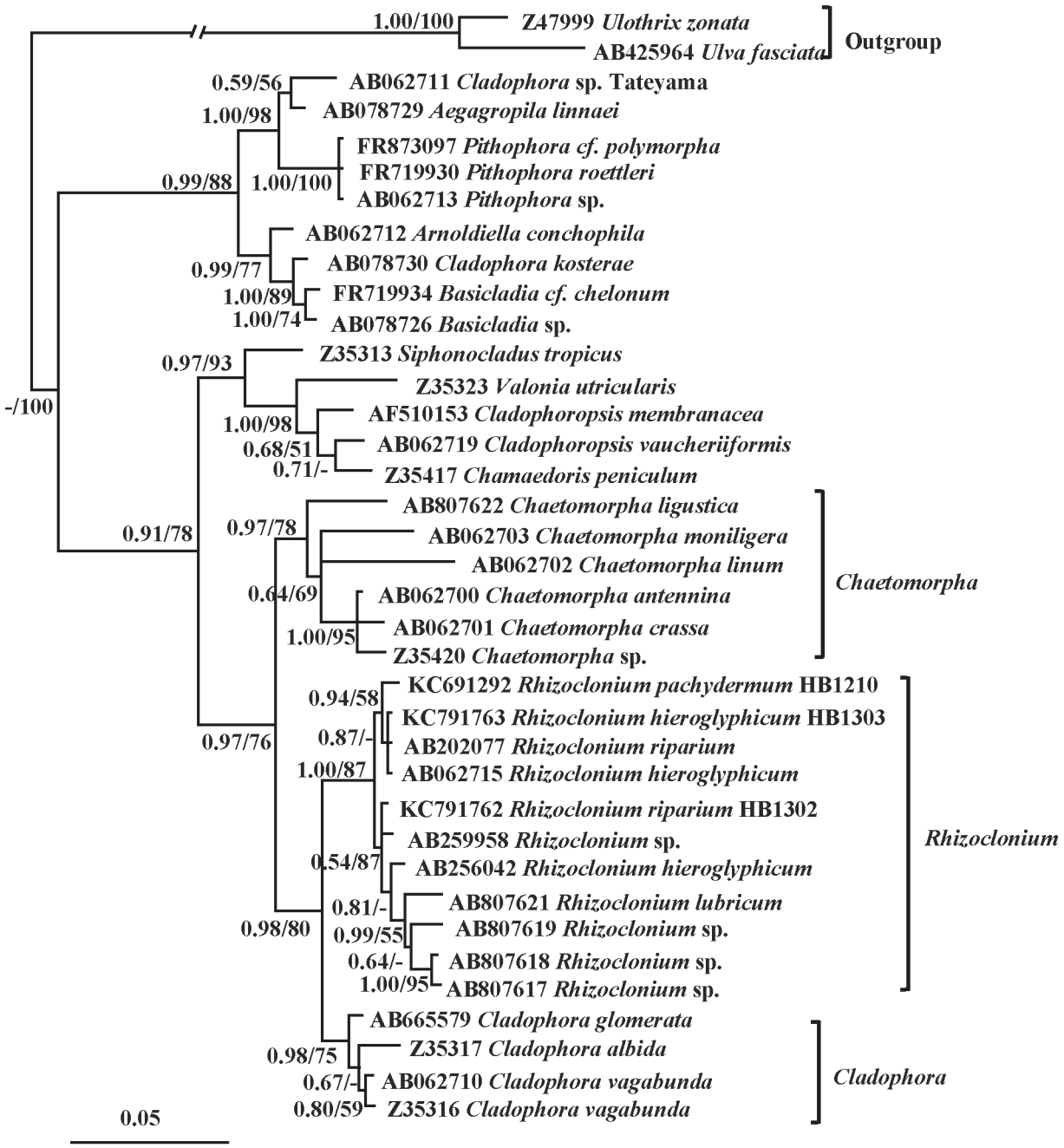

FIGURE 4. Phylogenetic tree of the 18S rDNA sequences from *Rhizoclonium* genus members and relatives. The Bayesian inference (BI) posterior probabilities and bootstrap support based on maximum-likelihood (ML) analyses are shown on the nodes. Values > 0.50 are shown for BI and > 50 for ML.

Only the Bayesian trees are presented for 18S rDNA and ITS2. Most of the phylogenies reconstructed from the 18S rDNA dataset had strongly defined backbone topologies and well-supported internal clades (Fig. 4). In the 18S rDNA phylogenetic trees, the *Rhizoclonium* species formed a well-supported group (1.00/87 for BI/ML) with an interesting topology. *Rhizoclonium pachydermum* had relatively short phylogenetic distances from other *Rhizoclonium* members. In the ITS2 gene phylogenetic tree, the two groups of *Rhizoclonium* and *Cladophora* were well separated and *R. pachydermum* was definitely included in *Rhizoclonium,* which further supported the 18S rDNA topology (Fig. 5).

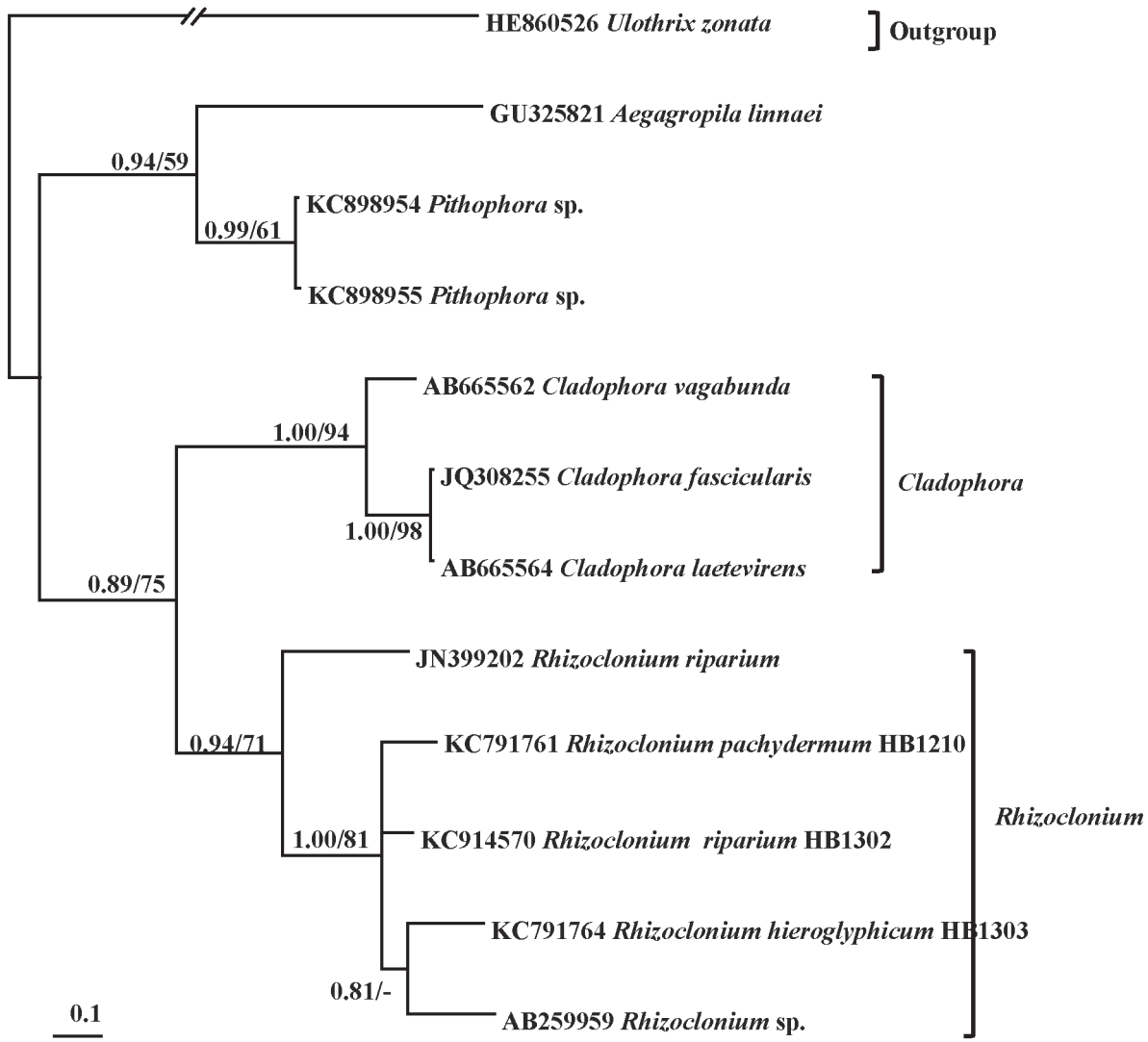

FIGURE 5. Phylogenetic tree of the ITS2 sequences of members of *Rhizoclonium* and *Cladophora*. The Bayesian inference (BI) posterior probabilities and bootstrap support based on maximum-likelihood (ML) analyses are shown on the nodes. Values > 0.50 are shown for BI and > 50 for ML.

Discussion

We evaluated the morphological and ecological characters shared by *R. pachydermum* and similar species. The results indicated that *Cl. basicladioides* (Jao 1947), *Cl. basiramosa* (van den Hoek 1963), *R. pachydermum* (Christensen 1991) and *Cl. pachyderma* (van den Hoek 1963; Liu & Hu 1999) are simply different names for the same species. They show the common features of this alga: attachment growth in freshwater; long, almost unbranched filaments; growth by intercalary cell division; the presence of real branches and branches concentrated in the basal region; formation of a disc-like holdfast at the lower end; thick-walled cylindrical cells, with a L/D ratio of approximately 1–4; and the occasional production of chains of sporangia. In 1991, Christensen found that *R. pachydermum* formed branches of the ordinary *Cladophora* type, which was not observed in the material from nature, which is also found in akinetes, as reported by Schmidle for *Cl. basiramosa*, who also showed that *Cl. pachyderma* was a freshwater species. Compared the morphological characteristics of HB1210 and *Cl. basiramosa* (van den Hoek 1963), we find they hold the same structures: branches laterally inserted, concentrated in the basal region; attachment by a disciform holdfast; cell size, etc. We first identified HB1210 as *Cl. basiramosa*. Christensen (1991) regarded *Cl. basiramosa* was probably only a synonym of *Cl. pachyderma.* Liu & Hu (1999) described *Cl. pachydermum* and considered that there was probably just one species in the Section *Affines* (Liu & Hu 1999), which means *Cl. basiramosa* was the same with *Cl. pachyderma.* We agreed the opinion of Christensen

that *Cl. pachyderma* and *Cl. basiramosa* are simply different names for the same species. Our molecular phylogenetic analysis detect a well-supported *Rhizoclonium* clade different from the *Cladophora* clade, hence we choose to name this alga as *R. pachyderma*, alloing us us to discuss the status of the present classification, particularly *Rhizoclonium* species and their taxonomy, where morphological characters have mostly been used previously for classification.

Concerning the extension of the generic boundary of *Rhizoclonium*, Ichihara e*t al.* (2013) suggested that the *Rhizoclonium* clade shares two morphological features: cells with diameter smaller than 60 μm and with L/D ratio smaller than 3.0. We support this conclusion, but we also think there are still some other features for the *Rhizoclonium* clade. A morphological comparison between our specimens and similar *Rhizoclonium* species is summarized in Table 2, which shows that these species share some common morphological characteristics: thin cylindrical cells (< 60 μm in diameter), multiple chloroplasts that form a reticulum with several pyrenoids, and, importantly, cells containing few or limited numbers of nuclei. In contrast to the general circumscription of *Rhizoclonium* (Kützing 1849, Setchell & Gardner 1920, Chapman 1956, Leliaert & Boedeker 2007), the results showed that: i) the number of nuclei is not always two to four, because some species had up to eight or even > 16; ii) instead of characterizing *Rhizoclonium* with unbranched filaments, the genus can have real branches.

TABLE 2. Comparison of morphological features between *R. pachydermum* and morphologically allied *Rhizoclonium* species.

	Cell diameter(μm)	L/D ratio	Nuclear number	Chloroplast morphology	Rhizoidal branches
R. pachydermum	37.5–50.0 (38.6 ± 1.0)	1 – 2.7 (2.1±0.5)	1–4 (2.3 ± 0.8)	parietal reticulate	real branches, concentrated in the basal region
R. riparium HB1302	17.5–25.0 (19.1 ± 1.0)	2–7 (3.3 ± 0.6)	1–4 (2.1 ± 0.7)	parietal reticulate	frequent (1–2 cells long)
R. hieroglyphicum HB1303	20 – 25 (21.3 ± 0.6)	2–10 (3.4 ± 1.2)	1–4 (2.1 ± 0.8)	parietal reticulate	no rhizoidal branches observed
R. curvatum	40	0.5–1.0	1–4 (Womersley 1984)	dense; Womersley (1984)	frequent (1–2 cells long)
R. riparium	(18–) 25–35 (–70)	1–3 (–6)	1–4 (–8) (Nienhuis 1975)	parietal reticulate	frequent (2–5 cells long)
R. crassipellitum	33–43	1.5–3.0	-	-	the absence of rhizoids (West & West 1897)
R. breve	28.9 ± 1.5	0.7 ± 0.1	4.1 ± 1.1	parietal reticulate	rarely (Ichihara *et al.* 2013)
R. hieroglyphicum	10–32	2–5	2–4 (–8)	reticulate; Elisa (1993)	one or more cells long
R. fontanum	12–22	2–5	-	-	2–3(–6)cells long
R. lubricum	35–50 (mostly 40)	1–2 or 4–6	10–12 (–15) (Scagel 1966)	parietal reticulate or perforated; Scagel (1966)	-

Our study suggests that the presence of few or a limited number of nuclei is an important character of *Rhizoclonium.* Borzi (1883) found 1–4 nuclei, Gay (1891) observed 1–5, and Parodi and Caceres (1993) considered that 2–4 nuclei per cell was a good character for the species *R. hieroglyphicum*. Based on 500 randomly selected cells, Gardavsky (1993) found that the cells of *R. fractiflexum* Gardavsky (1993: 126) could exceptionally contain up to eight nuclei. American specimens (Balakrishnan 1961) were typically found to possess 4–6 nuclei per cell, but up to 16 nuclei. The average number of nuclei of *R. lubricum* collected from the field by Ichihara *et al.* (2013) were 15 ± 2.7, up to 19.1 ± 4.7 in culture materials. This change of number may be associated with the species and habitat. All of these previous studies indicate that the nuclei number of *Rhizoclonium* is low and that it can be

counted. By contrast, *Cladophora* species have numerous nuclei, which generally lie under the chromatophores and close to the cell wall (van den Hoek 1963).

According to the traditional definition, branches are considered to be the most important character that distinguishes *Cladophora* from the other Cladophoraceae (apart from *Spongomorpha* Kützing (1843: 273)) (Hoek 1963). We consider that real branches may be present in some *Rhizoclonium* species. Nonrhizoidal branches in the basal region have appeared in *Rhizoclonium* species more than once. Gardavsky (1993) reported the occurrence of vegetative branches (as real branches) in *R. fractiflexum. R. lapponicum* Brand (1913: 181) also has branches, which are widely separated from one another, although two or three may be present together occasionally. Prescott (1982) described the coarse branched filaments of *R. hookeri* Kützing (1849: 383). Thus, when we evaluate whether a sample belongs to *Rhizoclonium*, more characters and data need to be considered, and true branches may be present. Therefore, the branched pattern is also an important character that distinguish *Cladophora* from *Rhizoclonium*, since in *Rhizoclonium* unbranch, or scattered branches, concentrate in the basal region of the plants, while in *Cladophora* acropetally-organized branch systems are often observed in terminal parts. Evidently, the genus *Cladophora* also requires a full revision in future.

TABLE 3. Collection information for the specimens in this paper and their GenBank accession numbers.

			GenBank No.	
Taxon	Locality	Voucher, culture	SSU	ITS2
Ingroup				
Cladophora albida	Roscoff, France	A83.3	Z35317	*
Cladophora glomerata	Japan: Fukui, Wakasa, Hasu River	glo-4	AB665579	*
Cladophora vagabunda	Japan: Ishikawa, Shika	-	AB062710	*
Cladophora vagabunda	Roscoff, France	UTEX LB 1465	Z35316	*
Cladophora sp. *Tateyama*	Japan: Toyama, Tateyama	-	AB062711	*
Rhizoclonium pachydermum	Wuhan, Hubei, China	HB1210	KC691292	KC791761
Rhizoclonium riparium	Wuhan, Hubei, China	HB1302	KC791762	KC914570
Rhizoclonium hieroglyphicum	Wuhan, Hubei, China	HB1303	KC791763	KC791764
Rhizoclonium riparium	Japan: Kanagawa, Bay Aburatubo	MRA-1	AB202077	*
Rhizoclonium hieroglyphicum	Japan: Chiba, Yachimata	-	AB062715	*
Rhizoclonium hieroglyphicum	-	CCAP 540/1	AB256042	*
Rhizoclonium sp.	USA	LB1523	AB259958	*
Rhizoclonium sp.	Japan	IK-026	AB807618	*
Rhizoclonium sp.	Japan	SAP114356	AB807617	*
Rhizoclonium sp.	Japan	SAP114350	AB807619	*
Rhizoclonium lubricum	Japan	SAP114360	AB807621	*
Pithophora cf. *polymorp*	USA	L0793291	FR873097	*
Pithophora roettleri	Portugal: Montemor-o-Velho	L0793289	FR719930	*
Aegagropila linnaei	Russia: Sakhalin, Lake Toba	-	AB078729	*
Pithophora sp.	Japan: Hyogo, Kamigori	-	AB062713	*
Basicladia cf. *chelonum*	USA: Missouri	L0793296	FR719934	*
Basicladia sp.	-	UTEX LB 810	AB078726	*
Cladophora kosterae	botanical garden, Paris, France	L0793294, UTEX LB1485	AB078730	*
Arnoldiella conchophila	Japan: Hokkaido, Akan	-	AB062712	*
Cladophoropsis membranacea	Panama: Rio Mar, (Pacific)	BW-01481	AF510153	*

..... continued on the next page

TABLE 3 (continued)

Taxon	Locality	Voucher, culture	GenBank No. SSU	ITS2
Siphonocladus tropicus	-	UTEX LB 2369	Z35313	*
Valonia utricularis	-	VuF	Z35323	*
Cladophoropsis vaucheriiformis	Japan: Okinawa, Gushikawa	-	AB062719	*
Chamaedoris peniculum	-	CMP5	Z35417	*
Chaetomorpha moniligera	Japan:Hokkaido,Otaru	-	AB062703	*
Chaetomorpha antennina	Japan:Shizuoka,Shimoda	-	AB062700	*
Chaetomorpha crassa	Japan:Ishikawa,Shika	-	AB062701	*
Chaetomorpha ligustica	Japan	SAP:114369	AB807622	*
Chaetomorpha linum	Japan:Kochi	-	AB062702	*
Chaetomorpha sp.	-	WC	Z35420	*
Rhizoclonium sp.	USA	LB1523	*	AB259959
Rhizoclonium riparium	China	AST2010021	*	JN399202
Aegagropila linnaei	United Kingdom: Scotland, Loch Watten, Caithness	L0793543	*	GU325821
Pithophora sp.	Wuhan, Hubei, China	HB1204	*	KC898955
Pithophora sp.	Wuhan, Hubei, China	HB1201	*	KC898954
Cladophora vagabunda	Japan:Fukui, Mihama, Lake Kugushi	vag-1	*	AB665562
Cladophora laetevirens	Japan:Fukui, Mihama, Lake Hiruga	lae-1	*	AB665564
Cladophora fascicularis	China: Qingdao	AST2010014	*	JQ308255
Outgroup				
Ulothrix zonata	-	SAG 38.86	Z47999	*
Ulva fasciata	Japan: Kochi, Usa	#1	AB425964	*
Ulothrix zonata	Russia:Irkutsk	WELT:A032277	*	HE860526

"-" missing data.
"*"not used in the phylogenetic trees.

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