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Taxonomic transfer of *Gongrosira burmanica* Skuja (Chaetophorales, Chlorophyceae) to *Ulvella* P. Crouan et H. Crouan (Ulvales, Ulvophyceae) based on morphological observation and phylogenetic analyses

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Abstract: Two green algal specimens, newly recorded from China, were identified as *Gongrosira burmanica* Skuja based on their distinctive morphology. The thallus of these specimens appeared hemispherical, forming cushion–like masses with calcification, and featured erect filaments that were pseudodichotomously branched and partially merged with each other, radiating from the base. Ultrastructural investigation, particularly the observation of pyrenoids penetrated by thylakoid membranes and the absence of plasmodesmata in adjacent cells, provided confirmation that *Gongrosira burmanica* Skuja should be excluded from the order Chaetophorales. Phylogenetic evidence, based on nucleus (18S rDNA) and chloroplast (tufA, rbcL) sequences, clearly indicated that *Gongrosira burmanica* Skuja should be reclassified within the Ulvales (Ulvophyceae) as a new combination species of the genus *Ulvella* P. Crouan et H. Crouan, rather than the Chaetophorales (Chlorophyceae). Further evaluation of the genus *Gongrosira* Kützing is necessary, especially by examining additional specimens, including the type species. It should be noted that the diversity of the Ulvales has been underestimated.

Keywords: Chaetophorales, *Gongrosira* , rbcL, tufA, *Ulvella*, 18S rDNA

Introduction

The genus *Gongrosira* Kützing is a widespread green algal genus and commonly forms a greenish layer or cushion–like masses on stones, gastropods, aquatic macrophytes (e.g., *Potamogeton*, *Myriophyllum*, *Najas*, *Chara*), and other hard surfaces (Wehr et al. 2015). The current understanding of *Gongrosira* Kützing is primarily based on the characteristics of its species. The main distinguishing features of *Gongrosira* Kützing include commonly cushion–like, sometimes plants are heavily incrusted with calcium carbonate crystals, consisting of a prostrate and an erect system of uniseriate filaments; prostrate system is single to multilayered, loose or pseudoparenchymatous and gives rise to the erect filaments whose usually short branches have blunt tips. The cells are cylindrical or somewhat inflated, occasionally with thick and lamellate walls. Each cell contains a prominent parietal chloroplast and one to several pyrenoids. Asexual reproduction occurs through biflagellate zoospores (Printz 1964; Tupa 1974; Wehr et al. 2015; Guiry & Guiry 2023).

The genus *Gongrosira* Kützing was originally established based on the species *Gongrosira sclerococcus* Kützing (Kützing 1843), but its valid name was not determined until Silva (1952). The concept of the genus *Gongrosira* Kützing has evolved primarily through the characteristics contributed by subsequently assigned species, which have been associated with various Chaetophoralean algae (Printz 1964; Bourrelly 1972; Tupa 1974; Sarma 1986), including the Trentepohliaceae (Fritsch 1935; Smith 1950). The genus *Gongrosira* Kützing has been placed in different taxonomic families over time. Initially, it was placed in the Trentepohliaceae by Fritsch in 1935 and Smith in 1950 (Fritsch 1935; Smith 1950). Later, *Gongrosira papuasica* (Borzi) Tupa was placed in the Chlorococcaceae (Watanabe et al. 1992). However, Printz (1964) placed the genus *Gongrosira* Kützing among the Chaetophorales, a classification followed by many phycologists (Hu & Wei 2006; Wehr et al. 2015; Guiry & Guiry 2023). Currently, there are 26 accepted species names and accepted varieties in the database (Guiry & Guiry 2023). However, many species are of doubtful validity, with many of the characters traditionally used to identify species exhibiting considerable overlap (Wehr et al. 2015). Although many *Gongrosira* taxa have been described and different taxonomic status have been assigned, few species have been examined by molecular data. To date, only the *Gongrosira fluminensis* Fritsch is phylogenetically verified to have been transferred into the Ulvophyceae (Liu et al. 2019a).

Gongrosira burmanica Skuja (Skuja 1949; Nakano & Handa 1988) has not been reported in China until now, except for *Gongrosira fluminensis* Fritsch, another species of the genus *Gongrosira* Kützing had been collected in our lab. The aim of present study is to determine the phylogenetic relationship of *Gongrosira burmanica* Skuja through a combination of morphological observation and phylogenetic analyses.

Materials and Methods

Gongrosira burmanica Skuja was collected from the Chishui River, which is located in the National Nature Reserve for rare and endemic fishes in the upper reaches of the Yangtze River, Guizhou Province, China. The voucher specimens described herein were deposited in the Freshwater Algal Herbarium (HBI), Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, Hubei Province, China. Detailed information were listed in Table 1.

For the morphological study, samples were preserved in 4% formalin or in viviality. Microphotographs were captured using an Olympus BX53 light microscope (Olympus Corp., Tokyo, Japan) using the differential interference contrast method. The photographs were taken using an oil immersion objective lens. Natural samples were isolated using an Olympus SZX7 microscope (Olympus Corp., Tokyo, Japan), rinsed with double–distilled H_2O and utilized for genomic DNA extracted and transmission electron microscopy (TEM).

Genomic DNA extraction was carried out using an Axygen DNeasy Plant Kit (Axygen Biotechnology, Hangzhou, China) following the manufacturer's instructions. Approximately 15 mg of filaments were added to 1 ml of 0.5 mm glass beads and 350 µl of phosphate buffer solution (PBS, pH 7.0). The algal cells were lysed by bead beating at 5000 × g for 2 minutes using a mini–beadbeater (Model 3110BX, Biospec Products, Bartlesville, OK, USA). For the amplification of the 18S rDNA sequence, PCR amplification was performed according to Marin et al. (2003). The primer and amplification procedures for rbcL sequence followed those of Zechman et al. (2003) and for tufA followed those of Famà et al. (2002). The PCR products were excised and cloned into a pMD18–T vector, which was then transferred into *E. coli* competent cells DH5α (Takara Bio Inc., Otsu, Shiga, Japan). Twenty clones each of 18S rDNA, rbcL and tufA sequence were sent to WuHan Tsingke BioTech Co., Ltd. (WuHan, China) for sequencing. Universal sequencing primers were M13F and M13F (Vieira & Messing 1982). ContigExpress Project (Invitrogen, Grand Island, New York USA) was used to edit low–quality regions and assemble the partial sequences.

For the transmission electron microscopy, the algal samples were fixed by immersing them in a mixture of 2% glutaraldehyde and 0.05 M phosphate buffer at 5 °C for 2 hours. They were then post–fixed in a solution of 1% osmium tetroxide and 0.05 M phosphate buffer at 5 °C for 2 hours, followed by overnight post–fixation at 5 °C in 1% uranyl acetate and methanol. After the fixation steps, the samples were dehydrated through an ethanol series to remove water content. Subsequently, the dehydrated samples were embedded in Spurr medium that contained propyleneoxide. Ultrathin sections of the embedded samples were cut using a Leica UC7 ultramicrotome (Leica, Wetzlar, Germany). The sections were then post–stained with uranyl acetate and bismuth oxynitrate. Finally, the sections were examined using a Hitachi HT–7700 transmission electron microscope (Hitachi, Tokyo, Japan) operating at an acceleration voltage of 80 kV. This allowed for high–resolution imaging and analysis of the ultrastructure of the algal samples.

Sequences for phylogenetic analyses were downloaded from GenBank (http://www.ncbi.nlm.nih.gov/) based on a BLAST search. All sequences were initially aligned using mafft7.2 (Katoh & Standley 2013). Manual editing and adjustments of the alignments were performed using MEGA6 (Tamura et al. 2013). jModelTest (Darriba et al. 2012) was used to select the evolutionary best–fit substitution models, identifying the GTR+I+Γ as the most appropriate model for all three genes (18S rDNA, rbcL, and tufA). Phylogenetic trees were constructed using both maximum likelihood (ML) and Bayesian methods. The RAxML8.1.20 software (Stamatakis 2014) was used for ML analysis, and MrBayes3.2.6 software (Ronquist et al. 2012) was used for Bayesian analysis. For ML analysis, bootstrap analyses with 1000 replicates were performed to estimate statistical reliability. Bayesian analyses were performed with 2×10^6 generations of Markov chain Monte Carlo iterations and the trees were sampled every 1×10^3 generations. Stationary distribution was assumed to be reached when the average standard deviation of split frequencies between two

Table 1. Collection information and GenBank accession numbers newly obtained in this study.

Strain	Isolator	Date of collection	Locality	GenBank accession numbers		
				18S rDNA	tufA	rbcL
Gongrosira burmanica CS20236801	B.W. Liu	March 2023	Guizhou province, China; on the stones in the Chishui river; freshwater 28°36'56.95" N, 105°46'50.28" E	OR101810	OR114679	OR114681
Gongrosira burmanica CS20236802	B.W. Liu	March 2023	Guizhou province, China; on the stones in the Chishui river, freshwater 28°37'05.58" N, 105°46'27.06" E	OR101811	OR114680	OR114682

runs was lower than 0.01. The initial 25% of calculated trees were discarded as burn–in, and the remaining samples were used to construct a Bayesian consensus tree and infer posterior probabilities. The resulting phylogenetic trees were edited using Figtree 1.4.2 (http://tree.bio.ed. ac.uk/software/figtree/).

Results

Morphological observations

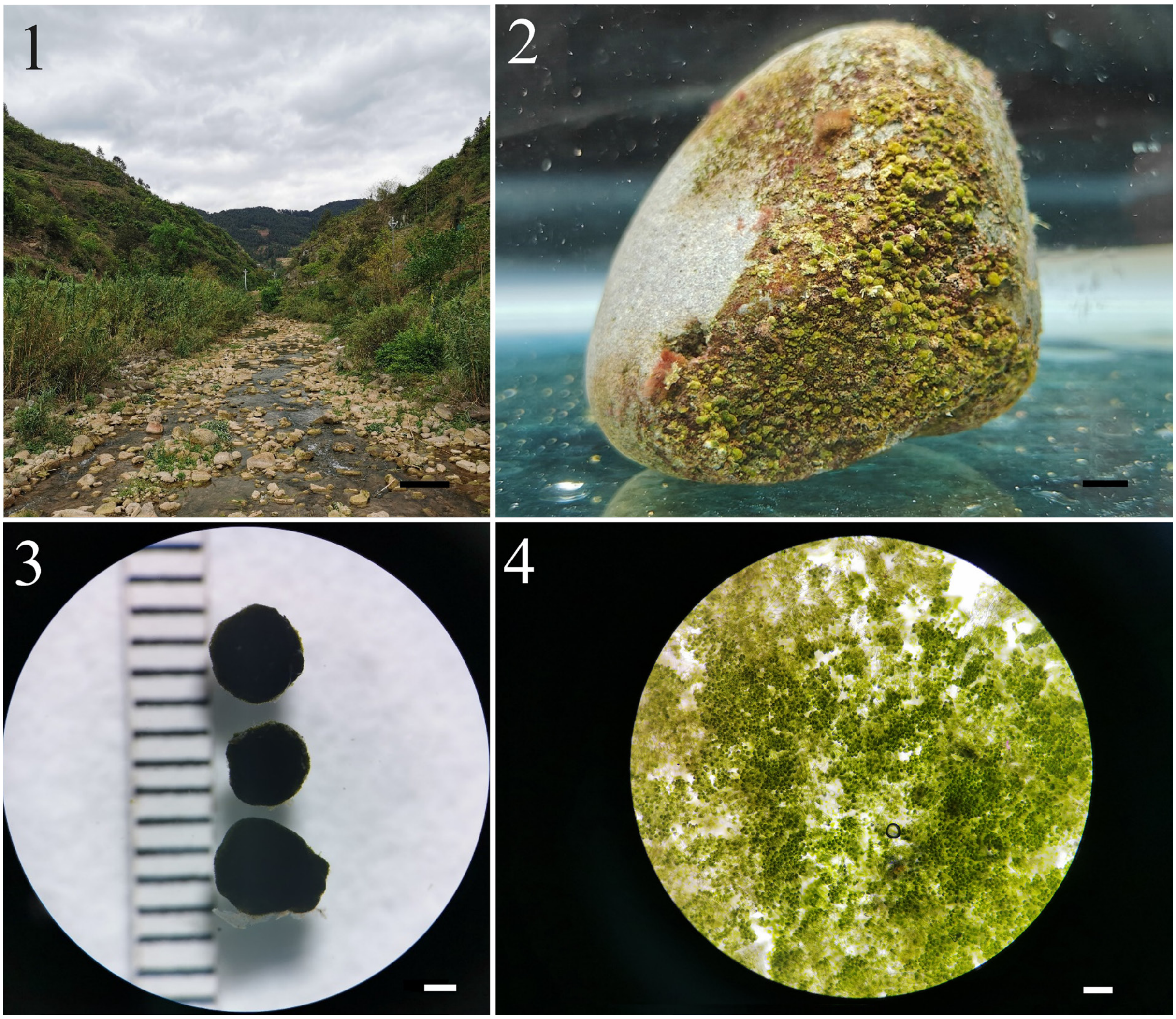
Gongrosira burmanica **Skuja (Skuja 1949)**

Thallus heavily incrusted with calcium carbonate crystals formed black hemispherical plants on stones in a stream (Figs 1, 2). Thallus were hemispherical to cushion–shaped up to 2–5 mm with many algal filaments (Figs 3, 4). Upright threads partly radiating from the base, partly more parallel upwards (Fig. 5), pseudodichotomous, rarely more unilaterally or truly dichotomously branched (Figs 6, 7, 8). The apex cell sometimes divided almost longitudinally and usually, however, the direction of division was skewed and one separated cell was slightly smaller which developed into a side branch (Fig. 6). Hair or hair–like formations were completely absent. In lower parts, some of the cells had already died off and were empty. Zoosporangia arose terminally from terminal cells, were oval and only slightly larger than the vegetative segments (Fig. 7). Erect filaments merged with each other. Cells were cylindrical or subcylindrical, and not constricted or slightly constricted. Cells 15–25 µm wide, length 0.5–1.5 times longer than width. Parietal chloroplast was ring–shaped with 2–5 pyrenoids (Fig. 8).

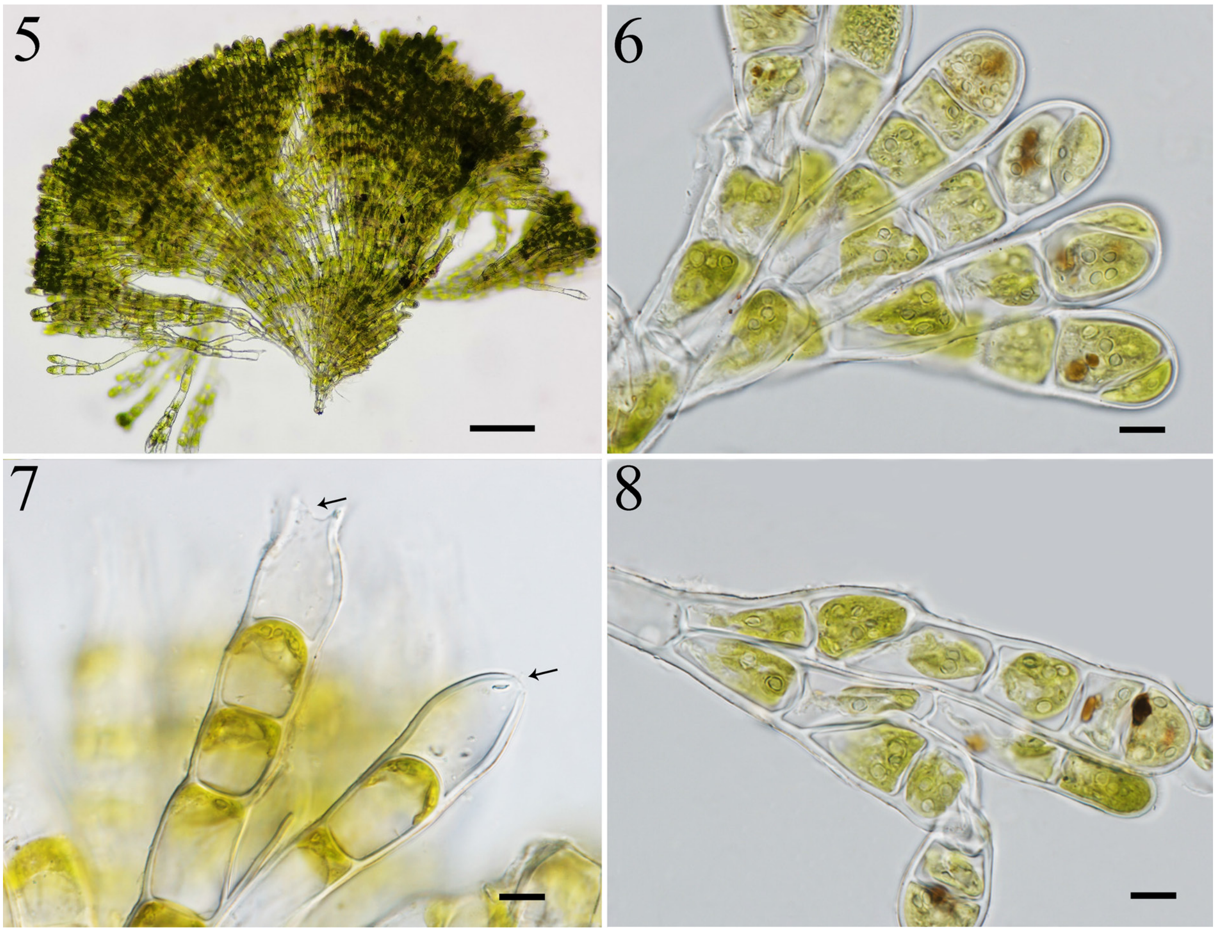
Ultrastructure also revealed single cell was with multiple pyrenoids (Fig. 9). Each pyrenoid was traversed by two thylakoid membranes (Fig. 10). One nucleus was in each cell and adjacent cells were without the plasmodesmata (Fig. 11). Cell wall was thick and multilayered (Fig. 12).

Phylogenetic analyses

For analyses performed in this study, three alignments were generated: (1) the 18S rDNA alignment containing 68 taxa



Figs 1–4. *Gongrosira burmanica* CS202368: (1) the natural environment of *Gongrosira burmanica*; (2) growing on the stones; (3) the outline of the thallus; (4) the thallus with many algal filaments. Scale bars 1m (1), 1 cm (2) and 1 mm (3, 4).



Figs 5–8. Morphology of *Gongrosira burmanica* CS202368: (5) fully developed upright system; (6) the morphology of the upper cells; (7) empty zoosporangia; (8) the type of branching. Scale bars 500 µm (5) and 10 µm (6, 7, 8).

with 1639 bp of representatives of the core Chlorophyta, (2) the tufA alignment containing 89 taxa with 822 bp of representatives of the Ulvophyceae, and (3) the rbcL alignment containing 29 taxa with 1095 bp of representatives of the Ulvales. Sequence names retrieved from GenBank had been checked for their current taxonomic name according to Škaloud et al. (2018). Phylogenetic analyses of the 18S rDNA (Fig. 13), tufA (Fig. 14) and rbcL (Fig. 15) sequences were used to determine the unambiguous phylogenetic placement of the *Gongrosira burmanica* Skuja within the order Ulvales, Ulvophyceae. Phylogenetic trees of 18S rDNA showed that *Gongrosira burmanica* Skuja was the member of the Ulvellaceae as the closest relative with the *Ulvella shanxiensis* H. Su, F.R. Nan, J.P. Lv, Q. Liu, S.L. Xie et J. Feng (Su et al. 2018) with a high support value (BP/PP, 100/1.00). The phylogenetic tree of tufA showed a similar scenario with that from 18S rDNA. *Gongrosira burmanica* Skuja as a separate clade formed a discrete clade with the *Ulvella tongshanensis* H. Zhu, F. Leliaert, Z.J. Zhao, S. Xia, Z.Y. Hu et G.X. Liu (Zhu et al. 2015). In the phylogenetic tree of rbcL, however, *Gongrosira burmanica* Skuja as a separate clade formed a discrete clade with the *Ulvella prasina* (Jao) H. Zhu, S. Xia, Q. Zhang, G.X. Liu et Z.Y. Hu (Zhu et al. 2015) instead of *Ulvella tongshanensis* H. Zhu, F. Leliaert, Z.J. Zhao, S. Xia, Z.Y. Hu et G.X. Liu.

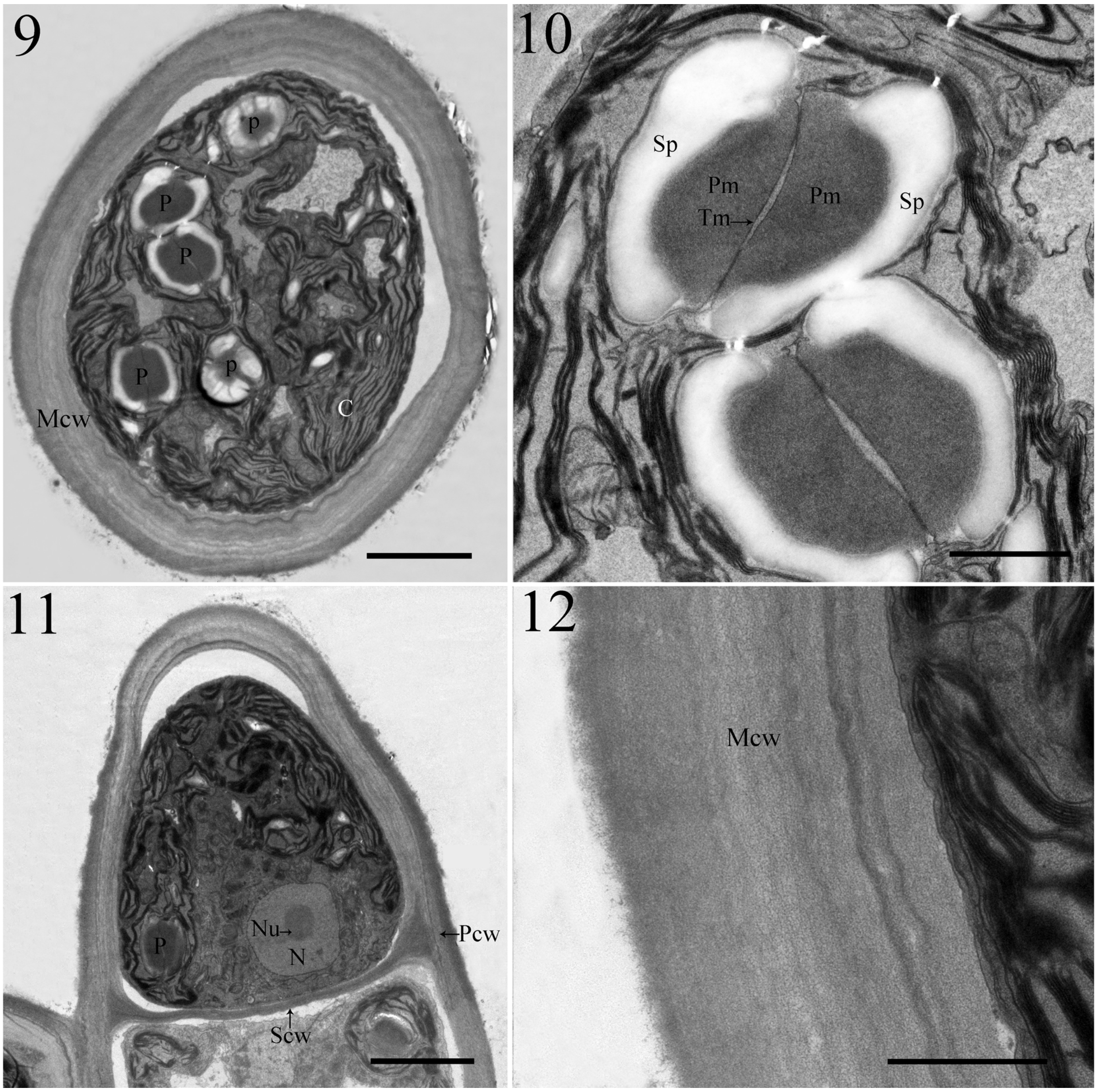
Taxonomic treatment

Ulvella burmanica (Skuja) B.W. Liu, Q.Y. Dai, Y.K. Liu, H. Zhu et G.X. Liu comb. nov. (Figs 1–12)

Basionym: *Gongrosira burmanica* H. Skuja (Skuja 1949, 74, pl. I–XIII, figs 4–9).

Description: Thallus were hemispherical with calcification formed cushionlike masses on stones in a stream. Erect filaments merged with each other. Upright threads partly radiating from the base, partly more parallel upwards and pseudodichotomously branched. The apex cell sometimes divided almost longitudinally and usually, however, the direction of division was skewed and one separated cell was slightly smaller which developed into a side branch. In lower parts, some of the cells had already died off and were empty. Zoosporangia arose terminally from terminal cells, were oval and only slightly larger than the vegetative segments. Cells were cylindrical or subcylindrical, and not constricted or slightly constricted. Cells 15–25 µm wide, length 0.5–1.5 times longer than width. Parietal chloroplast was ring–shaped with 2–5 pyrenoids.

Voucher specimen: Formaldehyde–fixed material was stored at the Freshwater Algal Herbarium (HBI), Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan,



Figs 9–12. Ultrastructure of *Gongrosira burmanica* CS202368: (9) the number of pyrenoids; (10) pyrenoid with two traversing thylakoid membranes; (11) adjacent cell wall; (12) multilayered cell wall. (C) chloroplast; (Mcw) multilayered cell wall; (N) nucleus; (Nu) nucleolus; (P) pyrenoid; (Pm) pyrenoid matrix; (Pcw) primary cell wall; (Scw) secondary cell wall; (Sp) starch plate; (Tm) thylakoid membranes. Scale bars 5 µm (9, 11) and 1 µm (10, 12).

Hubei Province, China., as specimen No. CS20236801 and CS20236802.

Distribution: So far, the species has been recorded only in Myanmar, Japan and China. In the field, this alga was found during winter to early summer and many colonies of black hemispherical of this species grew on stones in rivers.

DISCUSSION

Species of the genus *Gongrosira* Kützing can be distinguished based on various characteristics such as cell dimensions, the relative development of erect and prostrate system, nature of branching, position of sporangia, and presence or absence of lime encrustation. Among these species, *Gongrosira burmanica* Skuja stands out due to its pseudodichotomous branching, the number of pyrenoids per cell and calcification (Skuja 1949; Printz 1964). Our alga agreed well with the description by Skuja (1949) and Printz (1964). Therefore, we identified our alga as *Gongrosira burmanica* Skuja. What was slightly different was the size of cells and the number of pyrenoids. The cell size difference might attributed to its out of full development and phenotypic plasticity. The difference in pyrenoids number might be caused by the compactness of the chloroplast of apical cells, making it difficult to accurately determine the exact number of pyrenoids under a light microscope.

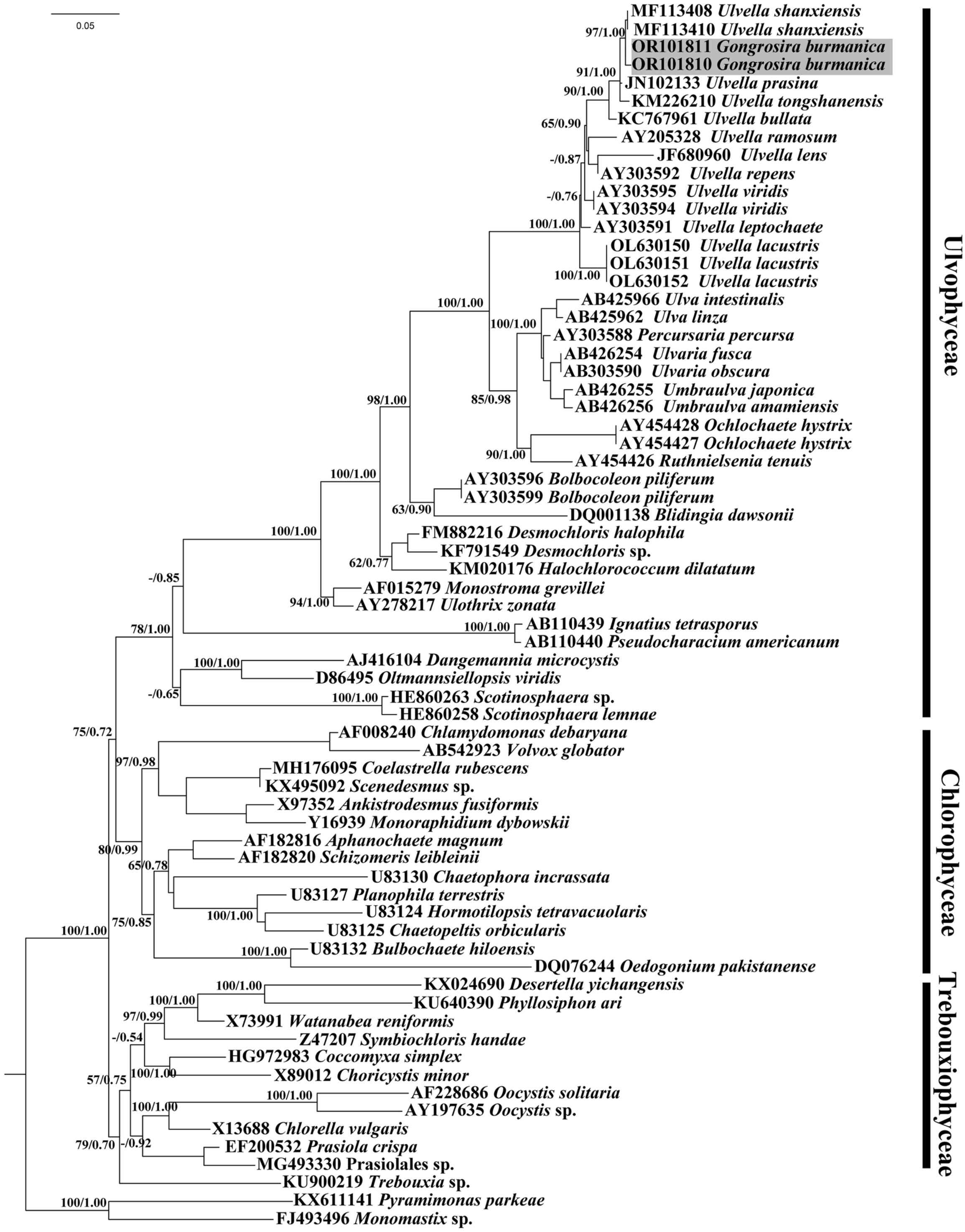


Fig. 13. Phylogenetic tree of the core Chlorophyta constructed by the 18S rDNA sequences. The numbers above on the nodes represent the Bayesian inference posterior probabilities (PP) and bootstrap support values (BP) from maximum likelihood (ML, constructed by RAxML). Values above 0.5 for BI and 50 for ML are shown. The new sequence of this study is shaded in grey.

Ultrastructural analysis revealed that a single cell had two to five pyrenoids instead of one to four by Skuja (1949) and Printz (1964).

This particular species showed similarities to *G. disciformis* Fritsch and *G. jollyi* Sarma, but it differed from them in two main aspects: the presence of pseudodichotomous branches that spread radially from the basal portion of the plant body and its smaller cell size. *Gongrosira burmanica* Skuja was initially discovered in Myanmar and later reported in Japan (Skuja 1949; Nakano & Handa 1988). This was the first recorded instance of this alga being found in China. All records reported the *Gongrosira burmanica* Skuja occurring on stones in rivers. This alga was commonly observed from

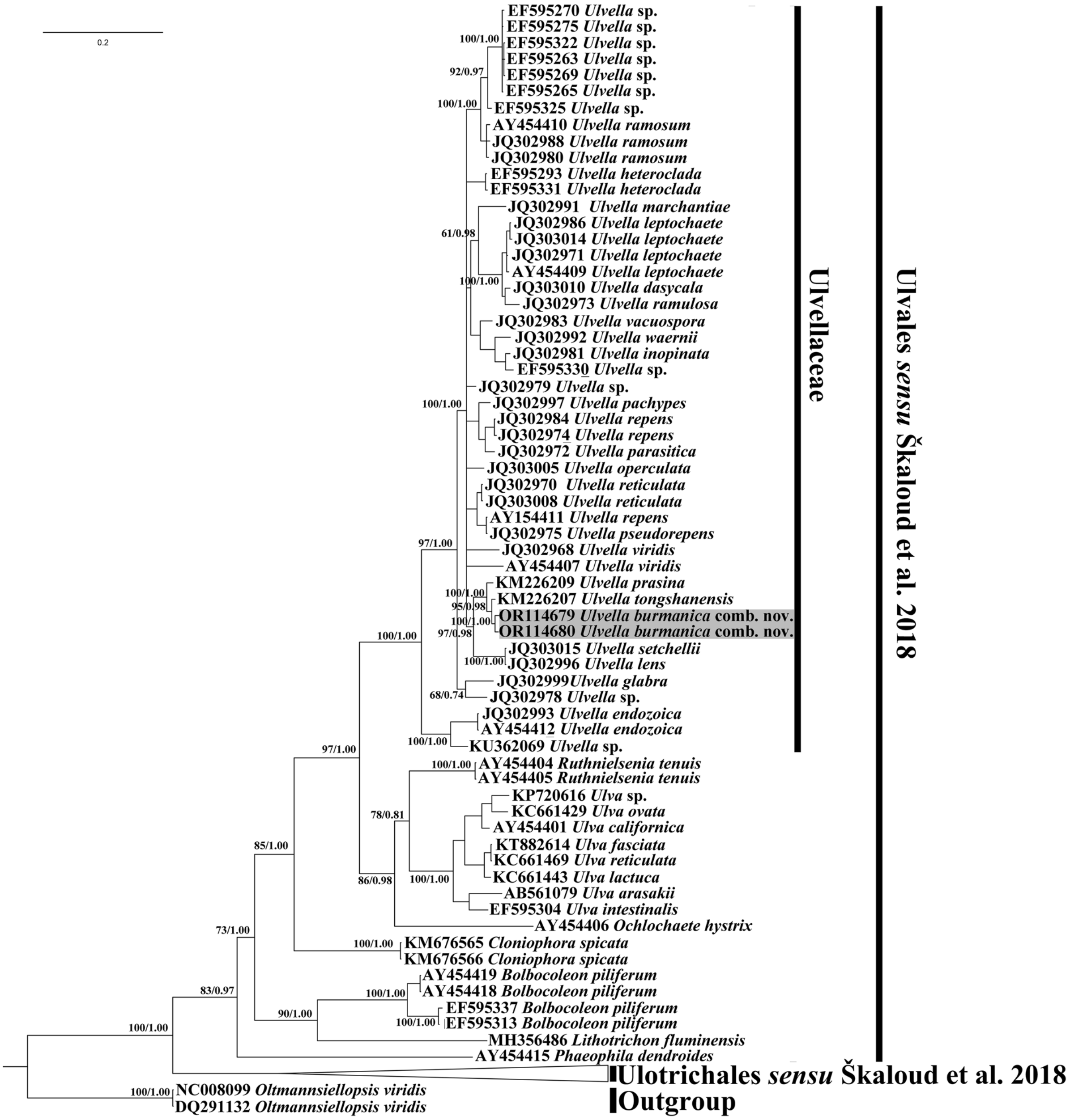


Fig. 14. Phylogenetic tree of the Ulvophyceae constructed by the tufA sequences. The numbers above on the nodes represent the Bayesian inference posterior probabilities (PP) and bootstrap support values (BP) from maximum likelihood (ML, constructed by RAxML). Values above 0.5 for BI and 50 for ML are shown. The new sequence of this study is shaded in grey.

winter to early summer. It was known that the members of *Gongrosira* had biflagellate zoospores (Printz 1964; Tupa 1974) and Skuja (1949) reported the formation of akinete in this alga. However, we and Nakano & Handa (1988) never observed biflagellate zoospores and akinetes during in our specimens.

Examination of the ultrastructural characteristics refused the placement of *Gongrosira burmanica* Skuja into Chaetophorales. In Chaetophorales, cells were interconnected by plasmodesmata (Stewart et al. 1973; John 1984; Melkonian 1990), while *Gongrosira burmanica* Skuja lacked the plasmodesmata, which indicates its exclusion from the Chaetophorales. A similar situation occurred in *G. papuasica* (Borzì) Tupa. An ultrastructural investigation of *G. papuasica* (Borzì) Tupa showed its pyrenoid was penetrated by thylakoid membranes, and the adjacent cells lacked plasmodesmata (Watanabe et al. 1992). This finding further suggested that *G. papuasica* (Borzì) Tupa should also be excluded from the Chaetophorales, which was also confirmed by molecular data. Two sequences (accessions U18503 and DQ015756) of *G. papuasica* (Borzì) Tupa UTEX 1916 deposited in the GenBank database belonged to Chlorophyceae. It was important to note that adequate morphological investigation of *G. papuasica* (Borzì) Tupa UTEX 1916 had been conducted by Johnson

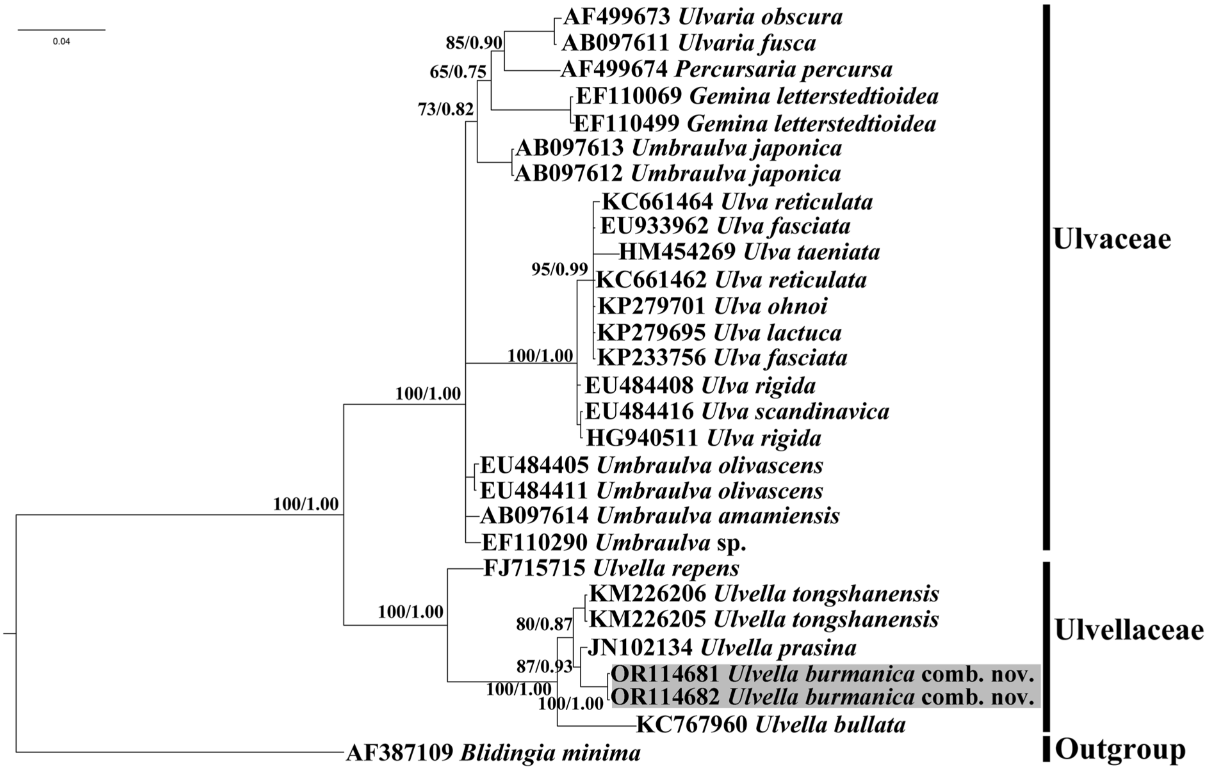


Fig. 15. Phylogenetic tree of the Ulvales constructed by the rbcL sequences. The numbers above on the nodes represent the Bayesian inference posterior probabilities (PP) and bootstrap support values (BP) from maximum likelihood (ML, constructed by RAxML). Values above 0.5 for BI and 50 for ML are shown. The new sequence of this study is shaded in grey.

& John (1992) and Tupa (1974).

The pyrenoid structures of *Gongrosira burmanica* Skuja were more similar to the *Ulvella* spp. (Liu et al. 2019a) and different from those found in Chaetophorales, where thylakoid bands appressed to pyrenoid matrix periphery or penetrated a short distance into the pyrenoid matrix (Stewart et al. 1973; Liu et al. 2019b).

Phylogenetic analyses have revealed that many filamentous and sarcinoid algae previously assigned to the Chlorophyceae or Trebouxiophyceae actually belong to the Ulvophyceae (Ettl & Gärtner 1995; Darienko & Pröschold 2017; Liu et al. 2019a). We provided the first molecular record of the genus *Gongrosira* Kützing (Liu et al. 2019a) and herein. Molecular data including 18S rDNA and the chloroplast encoded tufA and rbcL strongly supported that *Gongrosira burmanica* Skuja was a member of the Ulvales (Ulvophyceae) instead of Chaetophorales (Chlorophyceae). *Gongrosira burmanica* Skuja was the new member of the genus *Ulvella* P. Crouan et H. Crouan within family Ulvellaceae. However, it was important to note that the support values for the internal branch of the genus *Ulvella* P. Crouan et H. Crouan were moderate to low, which could be attributed to the limited samples and DNA sequences available at the time. Recently, several new members have been described in genus *Ulvella* P. Crouan et H. Crouan (Malavasi et al. 2022; Kwon et al. 2022; Yan et al. 2022), indicating that the diversity of the Ulvales is underestimated.

Within the genus *Ulvella* P. Crouan et H. Crouan, *Gongrosira burmanica* Skuja was found to be grouped within a clade that included species exclusively found in China, such as *U. shanxiensis*, *U. tongshanensis*, *U. prasina* and *U. bullata*, which was initially unexpected. It should be noted that the morphology of *Gongrosira burmanica* Skuja clearly differed from *U. shanxiensis* (disc–shaped irregular pocket of cisterna with more or less uneven surface), *U. tongshanensis* (upright, clustered parenchymatous thalli), *U. prasina* (disc–shaped or vesicle–like thalli) and *U. bullata* (disc–shaped or vesicle–like thalli).

Based on the findings of this study, it was evident that *Gongrosira burmanica* Skuja should be reclassified within the Ulvales as a new member of the genus *Ulvella* P. Crouan et H. Crouan, rather than being classified under the Chaetophorales (Chlorophyceae). Therefore, we proposed the transfer of *Gongrosira burmanica* Skuja from Chaetophorales (Chlorophyceae) to Ulvales (Ulvophyceae) as a new combination species, *Ulvella burmanica* (Skuja) B.W. Liu, Q.Y. Dai, Y.K. Liu, H. Zhu et G.X. Liu comb. nov.

It is acknowledged that many species within the genus *Gongrosira* Kützing have uncertain validity, and a few have already been transferred to other genera (Wehr et al. 2015). We first provide the molecular data of the *Gongrosira burmanica* Skuja (herein) and *Gongrosira fluminensis* Fritsch (Liu et al. 2019a), as well as the taxonomic transfer of these species to the order Ulvales. However, it should be noted that neither of these species represents the type species of the genus *Gongrosira* Kützing. As a result, the true phylogenetic position of the genus *Gongrosira* Kützing remains unknown. We refrained from drawing any definitive conclusions regarding the need for redividing the genus *Gongrosira* into smaller groups or integrating it into the order Ulvales as a whole. The obvious phylogenetic position of *Gongrosira burmanica* Skuja (herein) and *Gongrosira fluminensis* Fritsch in the Ulvales, Ulvophyceae could potentially contribute to solving the taxonomic issues of the genus *Gongrosira* Kützing. In the future, comprehensive investigations involving additional specimens, combined with natural morphological and culture–based surveys, as well as molecular analyses, will be necessary to reassess the microfilamentous genus *Gongrosira* and uncover hidden diversity within the Ulvophyceae. These efforts will undoubtedly enhance our understanding of the Ulvophyceae and lead to updates in our taxonomic knowledge.

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