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Ulvella tongshanensis (Ulvellaceae, Chlorophyta), a new freshwater species from China, and an emended morphological circumscription of the genus *Ulvella*

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Abstract: A new freshwater species of *Ulvella*, *U. tongshanensis* H. Zhu et G. Liu, is described from material collected from rocks under small waterfalls in Hubei Province, China. This unusual species differs from other species in the genus by the macroscopic and upright parenchymatous thalli, and by the particular habitat (most *Ulvella* species occur in marine environments). Phylogenetic analyses of plastid encoded *rbc*L and *tuf*A, and nuclear 18S rDNA sequences, pointed towards the generic placement of *U. tongshanensis* and also showed a close relationship with two other freshwater species, *Ulvella bullata* (Jao) H. Zhu et G. Liu, comb. nov. and *Ulvella prasina* (Jao) H. Zhu et G. Liu, comb. nov. The latter two were previously placed in the genus *Jaoa* and are characterized by disc–shaped to vesicular morphology. Our study once again shows that traditionally used morphological characters are poor indicators for phylogenetic relatedness in morphologically simple algae like the Ulvellaceae. Thus, the morphological circumscription of the genus *Ulvella* is here expanded to include: (1) thalli that are uniseriate in basal and apical parts, and parenchymatous in the middle portion with distinct differentiation of an unbranched dorsal side and a ventral side developing many short branches, and (2) epibiotic or epilithic, disc–shaped to vesicular thalli with a di– or tristromatic structure.

Key words: *Jaoa*, Morphology, New species, Phylogeny, Ulvales, Ulvellaceae, Ulvophyceae

Introduction

The green algal family Ulvellaceae is characterized by prostrate, free–branching filaments, or radiating, compact and coherent filaments forming mono– or polystromatic discs or rosettes. Most species are found in marine or brackish habitats, growing epi– or endophytic, epilithic on various substrates, or endolithic in calcareous substrates (Correa et al. 1987, 1988; Nielsen et al. 2013). A few species are known from freshwater habitats (Zhu et al. 2013; Mareš et al. 2014).

The family was resurrected and emended by O'Kelly & Floyd (1983) to include six genera: *Acrochaete*, *Endophyton*, *Endocladia*, *Ochlochaete*, *Pringsheimiella*, and *Ulvella*. Based on phylogenetic analysis of *tuf*A sequences and morphological observations of cultured plants, Nielsen et al. (2013) transferred most species of *Acrochaete*, *Endophyton*, *Ochlochaete*, *Entocladia*, *Pringsheimiella* to *Ulvella*, and emended and erected a large genus *Ulvella* . As currently circumscribed, *Ulvella* is characterized by irregularly branched, uniseriate filaments, that form open branched tufts, or mono– or polystromatic disc–shaped thalli (Rinkel et al. 2012; Nielsen et al. 2013). The freshwater genus *Jaoa* K.C.Fan (1964), which was formally placed in a separate family, Jaoaceae Fan, has also been shown to belong to the Ulvellaceae based on phylogenetic analysis of 18S rDNA and *rbc*L sequences (Zhu et al. 2013; Mareš et al. 2014). The only two species described are characterized by disc–shaped or hollow, vesicular thalli, up to 6 cm in diameter (Jao 1941, 1947; Zhu et al. 2013). The two species in the genus are morphologically distinguished by either having a distromatic (*J. prasina*) or tristromatic (*J. bullata*) thallus (Zhu et al. 2013).

In this paper, we report on a new green algal species, collected on rocks under a small waterfall in

Mt. Jiugong near Tongshan Couty, Hubei province. The new species is placed in the genus *Ulvella*, mainly based on results from our molecular phylogenetic analysis of three markers (plastid encoded *rbc*L and *tuf*A, and the nuclear 18S rDNA). We also propose to transfer the only two species of the genus *Jaoa* (*J. prasina* and *J. bullata*) to *Ulvella*. Our results call for an expansion of the natural morphological circumscription of the genus *Ulvella*, and in extension of the family Ulvellaceae.

Materials and Methods

Algal samples were collected in Jiugong Moutain Nature Conservation area (29º40'N, 114º25'E), Tongshan County, Hubei Province on rocks under a small waterfall in June, 2013. Samples were kept cool and transferred to the laboratory on the same day. Samples were rinsed in PBS buffer (pH = 7.0) several times to remove epiphytes and sediment. Then, the alga was inoculated on BG–11 medium (Allen 1968) with 1.5% agar in plastic Petri dishes under a constant light source of 50 $\mu mol.m^{-2}.s^{-1}$ and a temperature of 20 °C. To obtain unialgal culture, clean filaments were cut and transferred to new medium several times. The culture strain was deposited in the Freshwater Algae Culture Collection, Institute of Hydrobiology, Chinese Academy of Sciences (FACHB) under accession No. FACHB–1780.

To observe the asexual and sexual reproduction, the old and young filaments were added to nitrogen–deficient BBM (Bischoff & Bold 1963) and BG–11 medium to induce their reproduction. Morphological observations of both natural and cultured plants were performed with a Leica DM5000B microscope. Micrographs were taken with a Leica DFC320 digital camera.

For molecular analysis, the specimen collected in nature and the algal cultures of the novel alga, and cultures of *Jaoa prasina* Fan (obtained from Freshwater Algae Culture Collection, Institute of Hydrobiology, under the accession No. FACHB–1441) were rinsed several times in Phosphate Buffered Saline (PBS, pH = 7.0) and then harvested by centrifugation. Cell walls were broken with mini beads in a bead–beater (3110BX, Biospec Products, Bartlesville, USA). Total DNA was extracted using a MagSi Plant DNA Kit (Omega bio–teck, Doraville, USA).

Polymerase chain reaction amplifications were performed using primers *rbc*LQ and *rbc*LB for *rbc*L (Zechman 2003), *tuf*AF and *tuf*AR for *tuf*A (Famà et al. 2002), and universal 18S rDNA primers (Honda et al. 1999). The PCR cycling conditions were as follows: 5 min initial denaturation at 94°C, 34 cycles of denaturation at 94°C for 1 min, annealing at 56 °C (18S rDNA sequence), 52 °C (*rbc*L sequence) and 53 °C (*tuf*A sequence) for 50 s respectively, and extension at 72 °C for 80 s (18S rDNA sequence), 1 min (*rbc*L sequence and *tuf*A sequence), and a final extension of 5 min at 72°C. The products were purified with a Qiaquick Gel Extraction kit (Qiagen Duesseldorf, Germany) according to the manufacturer's instructions. The products of purification were preserved at 4 °C. The purified products were sent to Sangon Biotech Inc., China, for sequencing. Sequences were deposited in GenBank under accession numbers KM226205 to KM226211.

The natural collection and culture shared identical 18S rDNA, *rbc*L and *tuf*A sequences, which indicate that they are the same individual. Sequences selected based on a BLAST search, along with putative relatives and a broader selection of green algae were downloaded from GenBank (http://www.ncbi.nlm.nih.gov/). The sequences (56 18S rDNA, 14 *rbc*L, and 49 *tuf*A sequences) were initially aligned by ClustalX (v. 1.83) (Thompson et al. 1997), and refined manually using Seaview (v. 4.32) (Gouy et al. 2010). Mutational saturation was evaluated in the variable positions of the three alignments by plotting pairwise distances against model–corrected distances using Tamura and Nei (1993) and Kimura (1980) models estimated in MEGA (v.5.0) (Tamura et al. 2011). The result showed that neither transitions nor transversions have reached saturation. Phylogenies were estimated using maximum likelihood (ML) in PAUP4.0* (v.4.0 beta) (Swofford 1998) and Bayesian inference (BI) in MrBayes (v. 3.1.2) (Huelsenbeck et al. 2001). Modeltest (v.3.06) (Posada & Crandal 1998) was executed to select the best–fit evolutionary model under the Hiearchial Likelihood Ratio Tests (hLRTs) and Akaike Information Criterion (AIC). The best–fit models were GTR+I+G for 18S rDNA and *rbc*L, and GTR+I for *tuf*A. For ML analysis, a heuristic search option with random addition of sequences (10 replicates) and the tree bisection and reconnection branch–swapping algorithm were used for tree searching. Bootstrap analysis with 1000 replicates of the dataset for ML was performed to estimate statistical reliability. A Bayesian Markov Chain Monte Carlo analysis was run with four Markov chains (three heated chain, one cold) for 20 million generations with tree sampling every 10000 generations. The stationary distribution was assumed when the average standard deviations of split frequencies between the two runs was lower than 0.01. The first 25% trees were discarded as burn–in, and the remaining samples were used to construct a consensus tree and infer posterior probabilities. The resulting phylogenetic trees were edited using Figtree1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/).

Results

***Ulvella tongshanensis* H. Zhu et G. Liu sp. nov.**

Description: Freshwater alga, forming dark, olive–green, dense, hairy masses, about 1–2 mm long, on the surface of moist rocks (Figs 1 and 2). Thalli usually composed of several to dozens of filamentous or elongate parenchymatous structures (Figs 3–5). Young thalli consisting of uniseriate filaments (Figs 6–7); mature thalli uniseriate at basal and apical parts, and parenchymatous in the middle (Figs 8–12). Thallus attached to the substratum by a small, basal cell (Fig. 10, arrow). Vegetative cells cylindrical in basal and apical parts, 11–16 µm in diameter, 16–34 µm long, width/length ratio 0.5–2.0, and globular or irregular in middle part, 17–26 µm in diameter. Vegetative cell walls 0.8–2.8 µm thick. Chloroplasts sheet–like, filling the entire periphery of vegetative cells, with 0–4 obvious pyrenoids. Distinct differentiation of dorsal and ventral surface of mature plants with numerous short branches protruding on the ventral surface (Figs 9 and 14, black arrows), and no branch formation on the dorsal surface. Reproductive structures, such as

sporangia and gametangia, were not observed.

Culture observations: Clear morphological differences of vegetative thalli were observed between thalli from natural habitats and plants grown under laboratory culture conditions. Plants in culture grew slowly after inoculation. Two types of cultural morphology were observed. The first type was characterized by short filaments consisting of a vegetative cells with a similar morphology as thalli from natural habitat: robust cells with length/width ratio 0.4–1.5. Branches at this stage were short and consisted of 2–5 cells (Figs 15–16). The second type was characterized by long lateral branches consisting of slender cells, 5–11 µm wide and 26–72 µm long, growing prostrate and irregularly on solid culture medium (Figs 17–18). The centeral part of the thalli consisted of many globular cells (Fig. 17, arrowhead). Initiation of reproduction failed on BBM or BG–11 medium. *Acrochaete*–type hairs were observed in thalli grown in nitrogen–deficient medium (Figs 19–20, arrowheads), but not in thalli grown in regular BBM and BG–11 medium.

Etymology: The species epithet refers to the Holotype locality (Tongshan County).
Type locality: Jiugong Moutain Nature Conservation area (29º40'N, 114º25'E), Tongshan County, Hubei Province, China; on wet stones under a small waterfall.
Iconotype: Fig. 9.
Holotype: HB1306 (HBI), collected by Huan Zhu, Guo–Xiang Liu and Zhi–Juan Zhao, 28 June 2013; deposited in the Freshwater Algal Herbarium (HBI), Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, Hubei Province, China.
Authentic strain: FACHB–1780.

Distribution: So far, the plant has been found only in Jiugong Mountain Nature Conservation area. It grows mingled with *Cladophora* species on the wet surface of rocks and wood under waterfalls.

Phylogenetic analyses
The 18S rDNA alignment included 56 Chlorophyta sequences (including 15 Ulvellaceae sequences), and consisted of 1638 characters, of which 821 (50.1%) were variable and 582 (35.5%) were parsimony informative. The *rbc*L alignment included 14 Ulvales sequences (5 Ulvellaceae sequences) and 1266 characters, of which 258 (20.4%) sites were variable and 145 (11.5%) were parsimony informative. The *tuf*A alignment included 49 Ulvales sequences (42 Ulvellaceae sequences) and 772 characters, of which 322 (41.7%) were variable and 237 (30.7%) were parsimony informative sites.

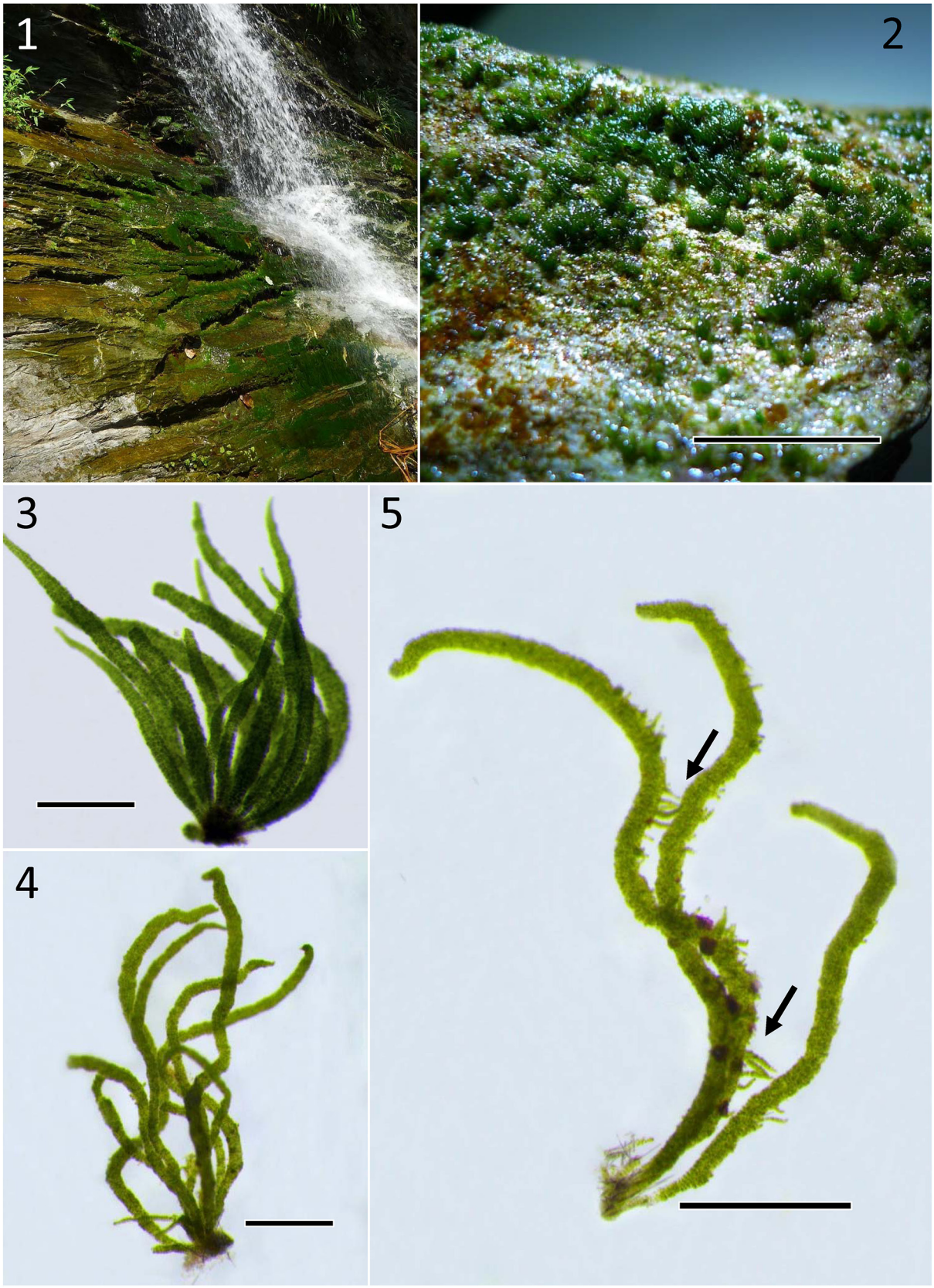
Maximum likelihood trees based on the 18S rDNA, *rbc*L and *tuf*A alignments, with indication of ML bootstrap support and Bayesian posterior probabilities, are presented in Figs 21–23. The topology of our *tuf*A phylogeny (Fig. 22) was consistent with previous studies (Nielsen et al. 2013, 2014). Phylogenetic analyses of the tree markers recovered a highly supported *Ulvella* (= Ulvellaceae) clade (*sensu* Nielsen et al. 2013), and unambiguously placed our new species within that clade. Although phylogenetic relationships within the *Ulvella* clade are generally weakly supported, the phylogenies of the three markers indicated a close relationship between *Ulvella tongshanensis*, *Ulvella* (*Jaoa*) *prasina* and *Ulvella* (*Jaoa*) *bullata*.
The phylogenetic placement of the two *Jaoa* species (including the type, *J. prasina*) warrants the transfer to *Ulvella*.

***Ulvella prasina* (Jao) H. Zhu et G. Liu comb. nov.**
Basionym: *Coelodiscus prasinus* Jao, Studies on the fresh–water algae of China IX. Coelodiscaceae. Sinensia, 12: 291–298, 1941.
Synonym: *Jaoa prasina* (Jao) Fan, Acta Phytotax. Sinica, 9 (1): 101, 1964.

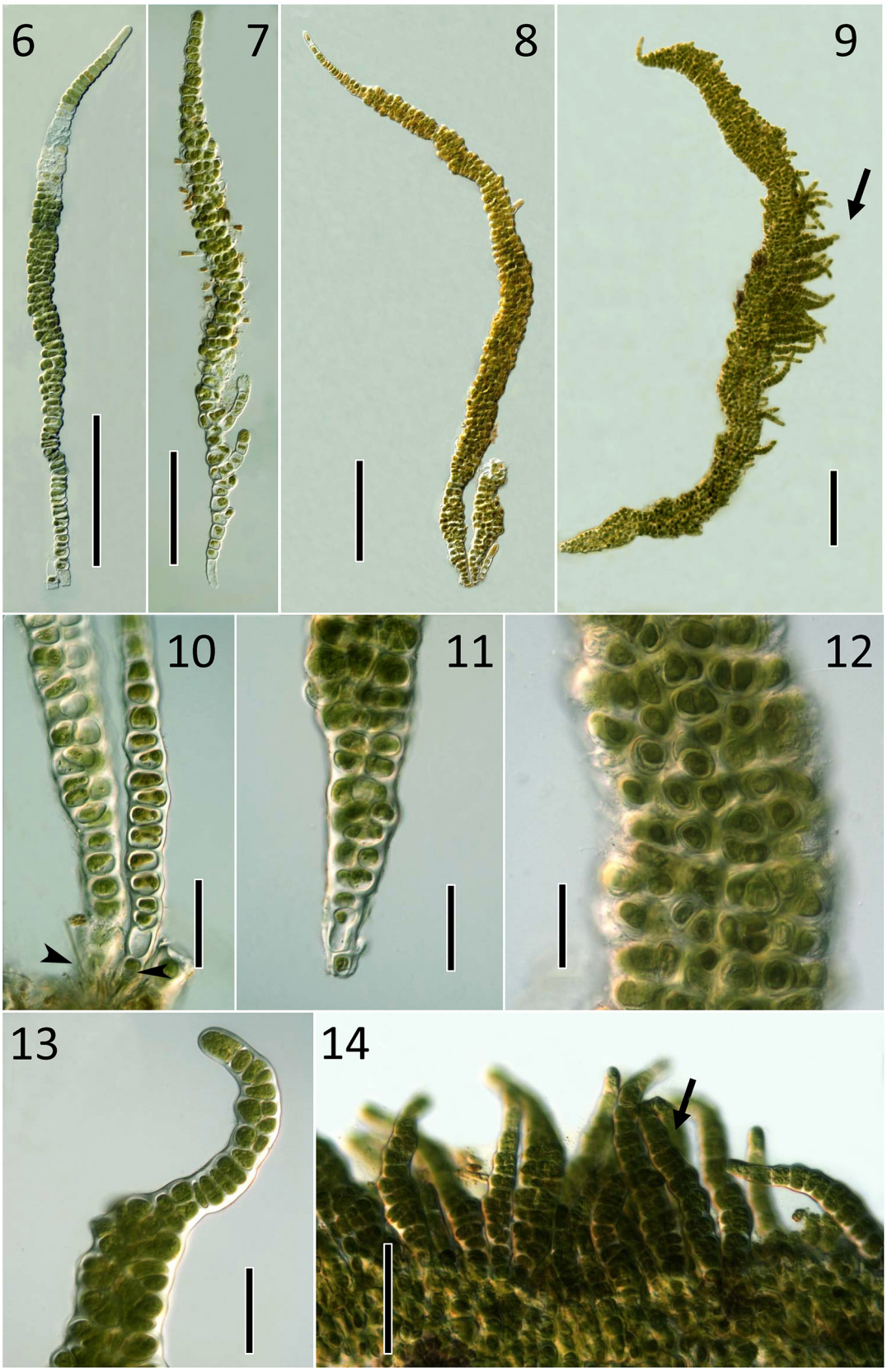
***Ulvella bullata* (Jao) H. Zhu et G. Liu, comb. nov.**
Basionym: *Coelodiscus bullatus* Jao, Bot. Bull. Acad. Sinica, 1: 255–256, f.1, 1947.
Synonym: *Jaoa bullata* (Jao) Fan, Acta Phytotax. Sinica, 9 (1): 101, 1964.

Discussion

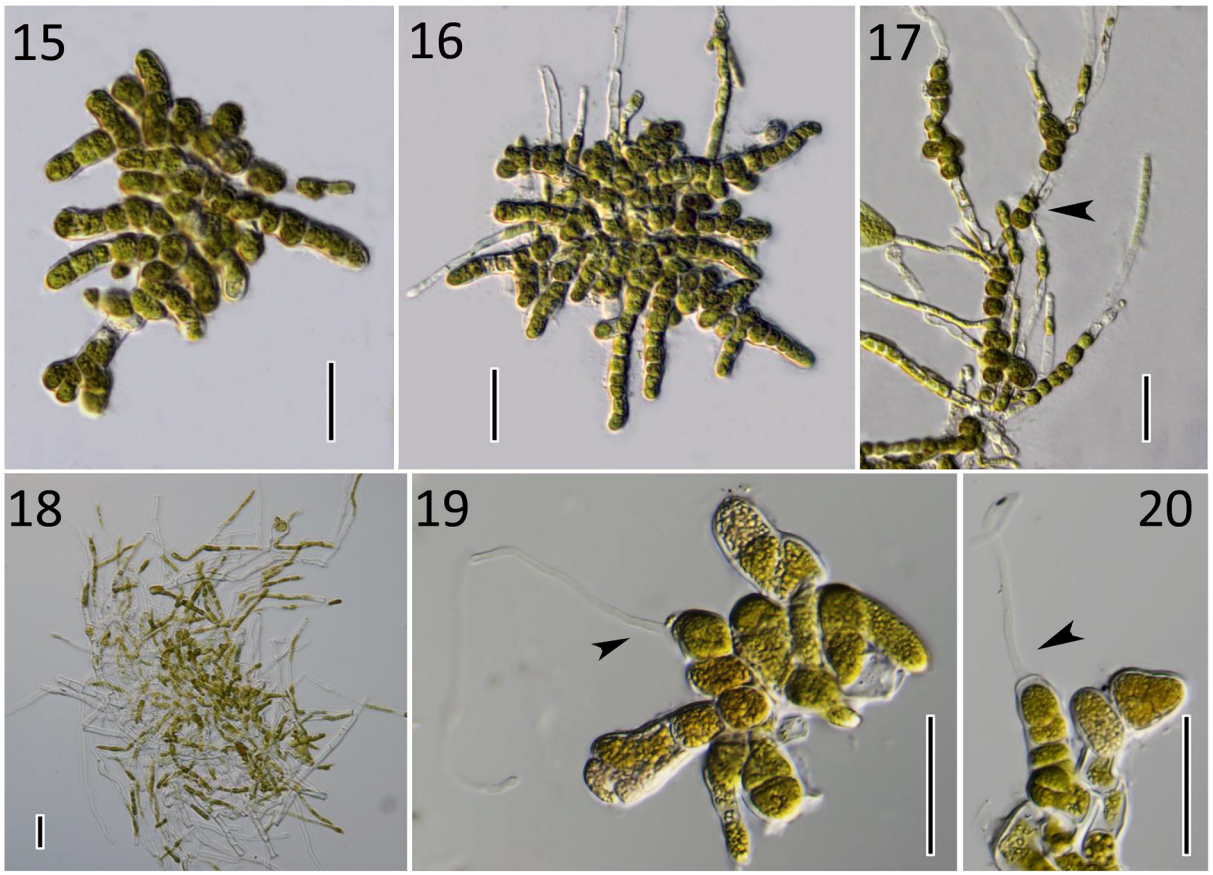
The taxonomy of the Ulvellaceae has recently been reassessed based on morphological, ultrastructural and molecular phylogenetic data (O'Kelly et al. 2004; Carlile et al. 2011; Nielsen et al. 2013; Mareš al. 2014). O'Kelly et al. (2004) transferred the genus *Ochlochaete* from Ulvellaceae to Ulvaceae mainly based on the phylogenetic position of generitype, *Ochlochaete hystrix*. Nielsen et al. (2013) proposed an emendation of a single large genus *Ulvella*, including most species previously placed in genera such as *Acrochaete*, *Endophyton*, *Entocladia*, *Ochlochaete*, *Pringsheimiella* and *Pseudodictyon*. Two species must be mentioned here, *Pseudulvella consociata* and *Smithsoniella earleae*. In the present and previous studies, the two species were placed in Ulvellaceae clade (Fig. 21, boxed with dotted line) (Carlile et al. 2011; Mareš et al. 2014) based on 18S rDNA data. The phylogenetic position of the generitype of *Pseudulvella*, *Pseudulvella americana*, however is clearly a member of the Chaetopeltidales (Sanchez–Puerta et al. 2006) (Fig. 21, boxed with solid line). *Pseudulvella consociate*, however, may have to be transferred to *Ulvella*. *Smithsoniella* is a monotypic genus, and therefore the 18S data hints toward an inclusion of *Smithsoniella* in *Ulvella*. However, because these taxa are not the focus of this study, we refrain from making any formal taxonomic changes.
Our molecular phylogenetic data placed our new species, *Ulvella tongshanensis*, into the genus *Ulvella*, along with two species previously placed in *Jaoa* (*Ulvella bullata* and *U. prasina*). *Ulvella tongshanensis*



Figs 1–5. Natural morphology of *Ulvella tongshanensis* sp. nov.: (1–2) Habitat of *U. tongshanensis*, growing epilithic on wet rocks under a waterfall; (3–4) Upright, clustered parenchymatous thalli; (5) Parenchymatous thalli with branches on the ventral surface (arrow). Scale bar 1 cm (1–2), 0.5 mm (3–4), 250 µm (5).



Figs 6–14. Microscopy of *Ulvella tongshanensis* from the field: (6–9) Different stages in vegetative thallus development; (10) Young thallus of *Ulvella tongshanensis* composed of a uniseriate filament (arrow); (11) Basal part of mature thallus; (12) Parenchymatous middle part of thallus; (13) Uniseriate apical filament, becoming parenchymatous below; (14) Parenchymatous middle part with numerous branches (arrow). Scale bar 250 μm (6–9), 50 μm (10–13), 100 μm (14).



Figs. 15–20. *Ulvella tongshanensis* in culture: (15–16) Robust vegetative filaments of *Ulvella tongshanensis* incubated on solid medium (BG–11) after two weeks; (17–18) New narrow filaments consisted of slender cells; (19–20) *Acrochaete*–type hairs of *U. tongshanensis* cultured on nitrogen–deficient medium (arrow). Scale bars 40 μm.

differs from other species in the genus by macroscopic, upright parenchymatous thalli. *Ulvella bullata* and *U. prasina*, are characterized by macroscopic, vesicular thalli, with a distromatic (*U. prasina*) or tristromatic (*U. bullata*) structure (Jao 1941, 1947). In the revision proposed by Nielsen (2013), six natural growth forms of *Ulvella* were described, all including microscopic species with prostrate or upright filaments that are openly branched or with branches appressed to form mono– and polystromatic disc–shaped thalli. Our results add two natural growth forms: (1) thalli composed of uniseriate basal and apical parts, and parenchymatous middle part; mature plants with distinct differentiation of unbranched dorsal surface and ventral surface with many new short branches (*U. tongshanensis* H. Zhu et G. Liu), and (2) macroscopic thalli (up to 6 cm in diameter) epibiotic, attached by rhizoids, disc–shaped or vesicular, with many unequal size folds on the surface, di– or tristromatic (e.g. *Ulvella prasina* (Jao) H. Zhu et G. Liu).

Our 18S rDNA and *rbc*L phylogenetic analyses indicate a closer relationship between *U. tongshanensis* and *U. prasina*, than between the two former *Jaoa* species. This is somewhat surprising from a morphological viewpoint, but once again illustrates how morphological data can be misleading for predicting phylogenetic relationships.

Ulvella prasina and *U. tongshanensis* have distinct morphological differences, further warranting their recognition as distinct species. Cells in *U. prasina* are clearly differentiated in rhizoidal cells and cells forming the vesicular thallus, whereas in *U. tongshanensis*, no such differentiation is observed. The vesicular thallus of *U. prasina* is composed of two cell layers with the inner cells being bigger than the outer cells. Again, this cellular differentiation was not observed in *U. tongshanensis*. *Ulvella prasina* and *U. bullata* grow on the bottom of sluggish streams, ponds or lakes, on rocks and wood (Jao 1941, 1947; Zhu et al. 2013; Mareš et al. 2014). In contrast, *U. tongshanensis* grew on rocky substrate under a waterfall. The obvious difference in their habitats may explain these morphological differences.

Distinct difference in morphology between natural and cultural habitats are very common in Ulvellaceae (Rinkel et al. 2012; Nielsen et al. 2013; Nielsen et al. 2014). For reasons unknown, there were two morphological types of *U. tongshanensis* in culture. *Acrochaete*–type hairs were commonly found in cultures grown in nitrogen–deficient medium.

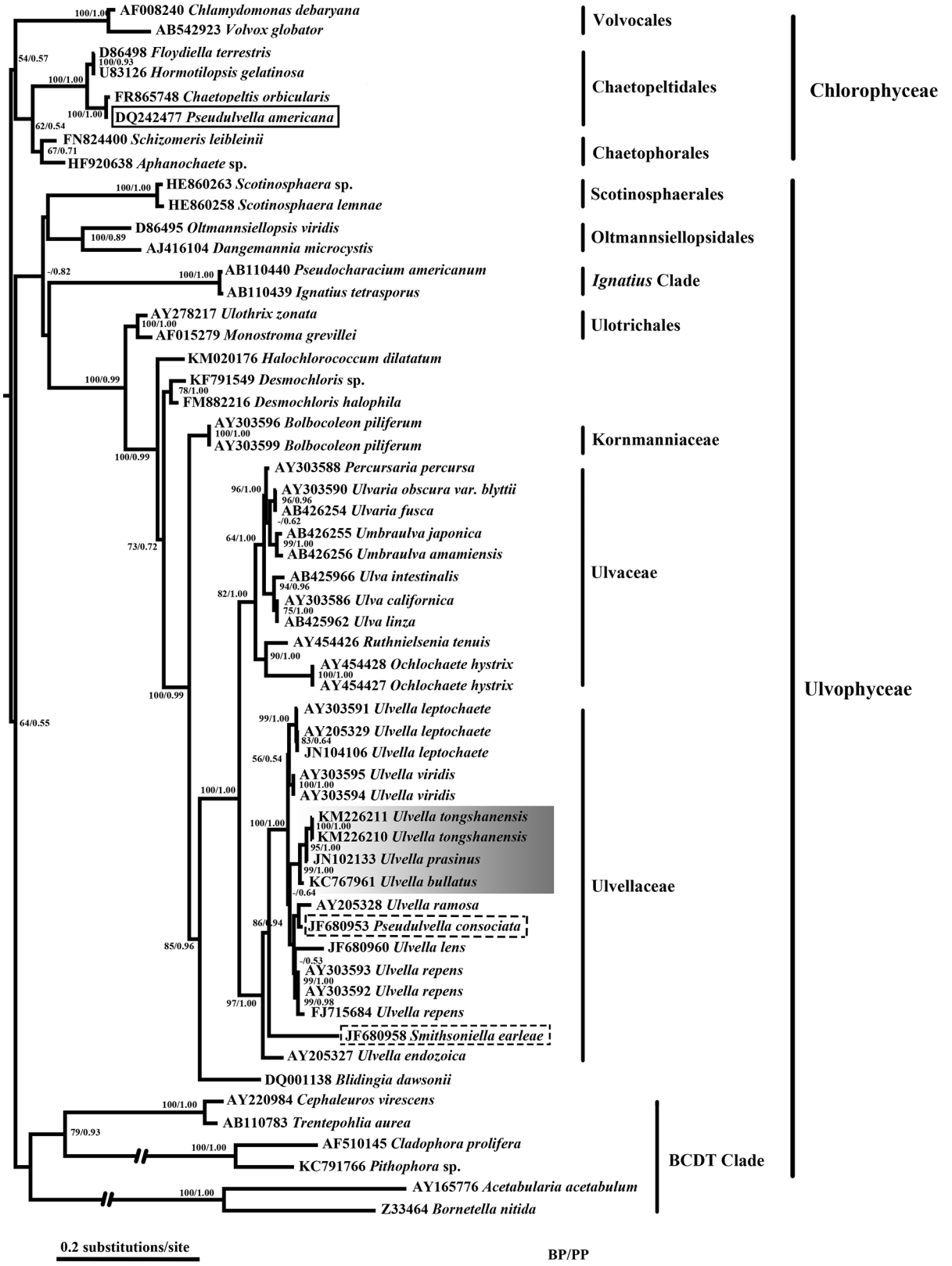


Fig. 21. ML phylogenetic tree based on 18S rDNA sequences from species of Chlorophyta. ML bootstrap values and Bayesian posterior probabilities are shown on each node. Values below 50 or 0.50 are not given. *Pseudulvella americana, Smithsoniella earleae* and *Pseudulvella consociata* are boxed with solid line and dotted line respectively; the new combinations and new species are shaded in grey.

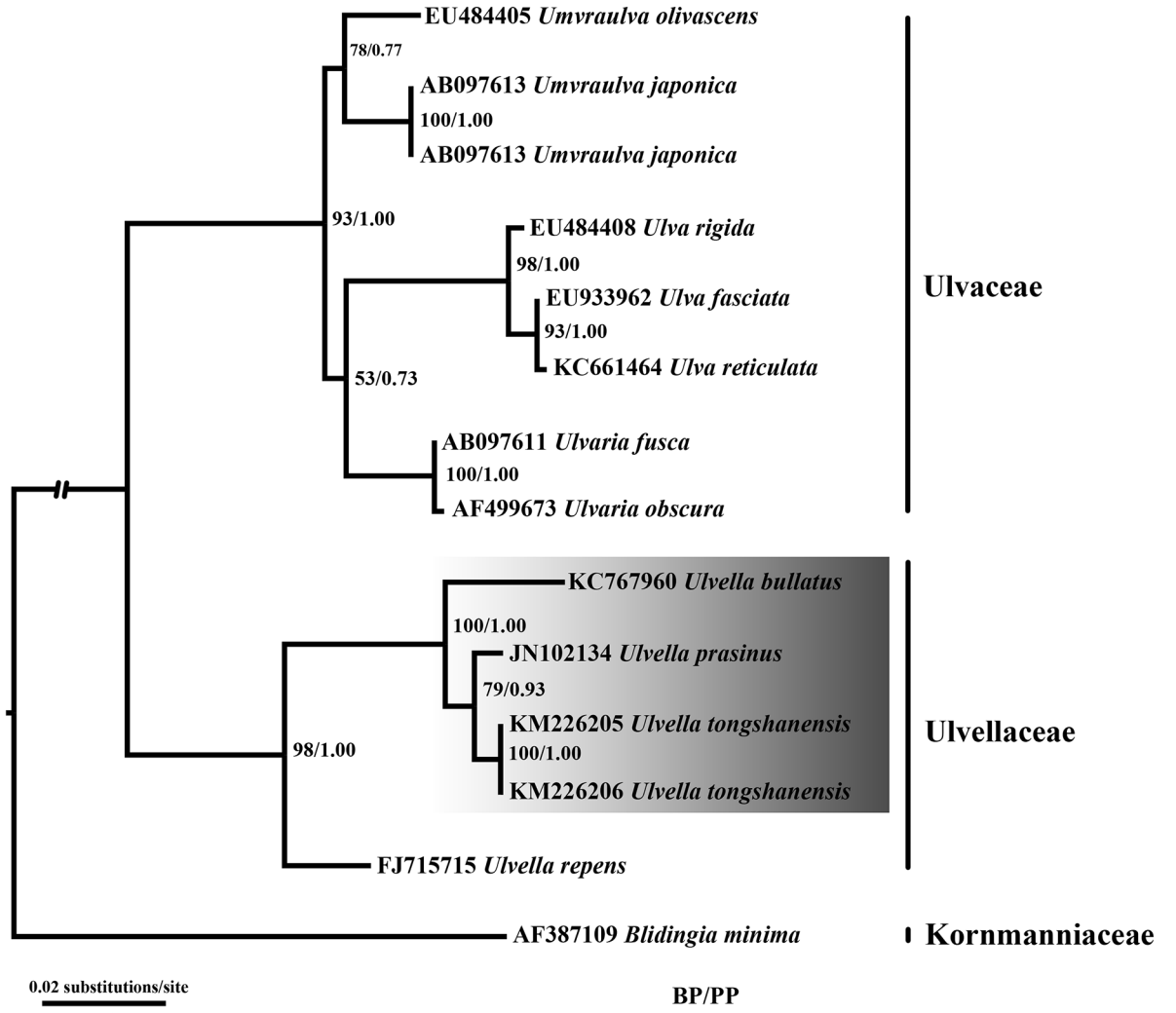


Fig. 22. ML phylogenetic tree based on *rbc*L sequences from species of Ulvales. ML bootstrap values and Bayesian posterior probabilities are shown on each node. Values below 50 or 0.50 are not given. The new combinations and new species are shaded in grey.

As Tan et al. (1999) suggested, various forms of environmental stress may play important roles in morphological switch. In our opinion, nitrogen may function greatly in activating *Acrochaete*–type hairs forming.

Most species in *Ulvella* are found in marine or brackish habitats. Our phylogenetic results showed a single clade of freshwater species (*U. prasina*, *U. bullata* and *U. tongshanensis*), thus indicating a single transition from marine to freshwater habitats in the genus. However, we speculate that there may be more freshwater algae in *Ulvella* still remaining to be discovered, and future investigation on freshwater *Ulvella* is needed. The current molecular and morphological data support our new material as a new species in *Ulvella*. However, more research, such as extensive sampling in different habitats, will be required to assess intraspecific morphological variability in this group of algae.

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Fig. 23. ML phylogenetic tree based on *tuf*A sequences from species of Ulvales. ML bootstrap values and Bayesian posterior probabilities are shown on each node. Values below 50 or 0.50 are not given. The new combinations and new species are shaded in grey.

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