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Morphological and Molecular Phylogenetic Analysis of a *Lemanea* Specimen (Batrachospermales, Rhodophyta) from China

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Abstract: The genus *Lemanea* is an evolutionally derived lineage of freshwater Rhodophyta which has a rare distribution. Morphological characterization and molecular phylogenetic analyses were conducted for a *Lemanea* specimen collected from Guilin, China, in this study. The results based on morphological observation and molecular evidence, including *rbc*L and SSU sequences, showed it was a new record of *L. manipurensis* Ganesan, West, Zuccarello et Rout in China. The specimen in this study was morphologically characterized by branches that were positioned in the lower part of the thallus, hair cells absent around the outer cortex, ellipsoidal and single carpospores at the top of the gonimoblast filaments and spermatangia formed in continuous wide rings on the thallus nodes. By comparing the morphology of specimens collected from Guilin, China, and the holotype specimen in India, it was found that the spermatangial shapes were variable among different populations. It was found that the presence or absence of hair cells around the outer cortex was not a reliable characteristic for the identification of the genus *Lemanea* based on a comparison between *L. manipurensis* collected in Guangxi and in India and another four *Lemanea* species previously recorded in China. The results of this study provided molecular evidence and a theoretical basis for molecular phylogenetic research on the genus *Lemanea* and enriched the species diversity and geographical distribution of *Lemanea* in China.

Keywords: freshwater Rhodophyta; *Lemanea*; new record; morphological characteristics; molecular phylogenetics

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1. Introduction

Lemanea Bory (1808) belongs to the Batrachospermales, Nemaliophycidae, Florideophyceae, Eurhodophytina. It is characterized by tufts of cartilaginous, tubular, pseudoparenchymatous gametophytic thalli, lacking cortical filaments, around a central, uniseriate axis. The outer cortex closely abuts T- or L-shaped ray cells. The thallus color is blue-green to olive when young, becoming rusty-brown to black at maturity [1]. This genus is the most derived lineage in the Batrachospermales, which is exclusively distributed in freshwater [2]. It is widely distributed in fast streams and rivers with typically high current velocities as an important constituent in aquatic plant ecosystems. There are currently 16 species currently accepted in Algaebase based on traditional morphological identification [3]. Molecular evidence is accessible for only six species: *Lemanea borealis* Atkinson, *L. fluviatilis* (L.) C. Agardh, *L. fucina* Bory, *L. manipurensis* Ganesan, JA West, Zuccarello, J Rout, *L. occidentalis* ML Vis and KM Müller and *L. condensata* Israelson [4]. Species identification for this genus is based on characteristics including thallus height, branch density and spermatangial morphology [5]. The systematics of *Lemanea* are controversial due to its simple and plastic morphology, and the lack of comparative anatomical and reproductive characteristics [6–8]. In recent years, it was found that species identified by morphology

were not in the same clade identified using molecular evidence [9]. The morphology of *Lemanea* varies with the season and water conditions including salinity, temperature and flow velocity [10,11]. The systematics and diversity of *Lemanea* based only on traditional morphology were not reliable and thus needed revision using molecular methods. With the rapid development of molecular technologies, identification and phylogenetic analyses of Rhodophyta based on gene sequences became common [12–14], except for a few reports for *Lemanea* [15]. Li analyzed the phylogeny of Batrachospermales based on *rbc*L sequences using *Lemanea* specimens from the USA and Canada, and concluded that the Lemaneaceae were a derived lineage in this order [16]. Ganesan collected specimens from India and analyzed the morphology, reproductive characteristics and molecular phylogeny of *L. australis* Atkinson, *L. catenata* Kützing, *L. fluviatilis*, *L. mamillosa* Kützing and *L. torulosa*, and described a new species, *L. manipurensis* [12].

The genus Lemanea is distributed widely in North America and Europe [17–23], whereas it is rarely reported in Asia, except India and Israel [12,24,25]. It has also been reported rarely in China. Jao reported two new species of Lemanea from China in 1941 [26]. Xie reported four species in China including L. *sinensia* Jao from Yunnan, *L. crassa* Xie et Shi and *L. simplex* Jao from Chongqing, and *L. ramose* Xie et Shi from Fujian [27]. Additionally, the distribution of Lemanea in Jialing River, Lancang River and Mengxing River was also reported in previous studies [28–30]. To date, the reports of Lemanea in China have all been based on morphological observations and lack molecular evidence. Thus, they are all regarded as doubtful species [4]. As an important lineage in Rhodophyta, the species diversity and distribution of Lemanea in China needs to be explored further, and their molecular sequences and phylogenetics need to be studied.

A *Lemanea* specimen was collected in Guilin, China. Morphological characterization and molecular phylogenetic analysis were conducted on this material as part of a survey of freshwater red algae in China, adding to the species and distribution diversity of this genus.

2. Materials and Methods

The *Lemanea* specimen was collected from Lingchuan, Guilin, China (25°42′ N, 110°32′ E) growing on stones in a clean stream (Figure 1). It was encoded as GLYZC. The sample was transferred to the laboratory as soon as possible and washed with sterilized water carefully to remove the epiphytes. The portion of the samples used for morphological observation was fixed in a centrifuge tube with a 4% formalin solution. The samples used for DNA extraction were preserved in a silica desiccant. For morphological observation, both fresh and formalin-preserved thalli were examined under a BX-51 Olympus microscope equipped with a charge-coupled device (DP72; Olympus, Tokyo, Japan) for photographing.

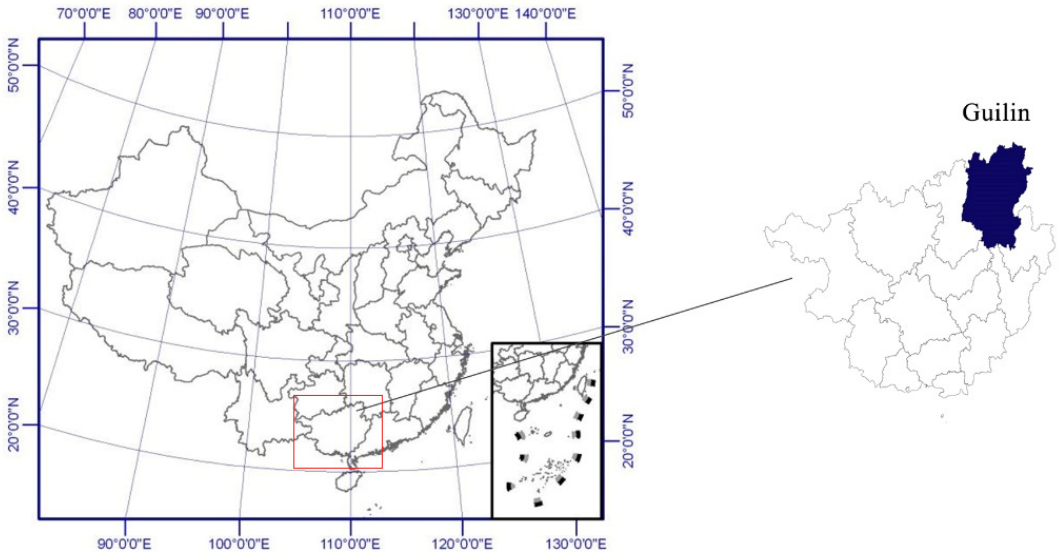

Figure 1. Map showing approximate location of the sample investigated in this study.

Total DNA was extracted from the desiccated thalli following the protocol described by Saunders with modifications as described by Vis and Sheath [31,32]. Polymerase chain reaction (PCR) amplifications were performed using the primers F577 and FR753 for *rbc*L [33], and G01 and G07 for SSU [34]. The PCR products were purified using a SanPrep column DNA gel purification kit (Sangon, Shanghai, China) and then were sent to BGI Tech Corporation (Beijing, China) for sequencing on an ABI 3730XL sequencer. Both amplification primers were used to determine the sequences. The sequences were manually inspected with Sequencher Version 4.14 (http://sequencher.software.informer.com/, accessed on 1 May 2022). The DNA sequence data generated from this study have been deposited in GenBank (accession numbers: MT297643 for *rbc*L and MT294296 for SSU).

The sequences obtained in this study and the sequence data obtained by nucleotide blasting downloaded from GenBank were assembled in Clustal-X 2.0 [35]. Ambiguous bases on both ends were deleted to produce an identical length alignment. Two datasets were used for the phylogenetic analyses: the *rbc*L dataset included 42 taxa and 1 *Tuomeya* as an outgroup, and the SSU dataset included 13 taxa and 2 *Balbiania* taxa as outgroups. The outgroup taxa were selected according to previous studies [10,12]. The pairwise genetic distances between each specimen were computed in MEGA 5.0 [36]. The optimal substitution model for each marker was estimated using Modeltest [37]. Maximum likelihood trees were built using PHYML software [38,39]. The bootstrap analysis was conducted using 1000 replicates. Additionally, Bayesian inference was performed in Mr. Bayes version 3.1.2 [40]. A Markov chain Monte Carlo (MCMC) was initiated in the Bayesian inference and run for 5,000,000 generations; the trees were sampled every 1000 generations. A consensus tree was summarized after 20% trees of burn-in. The resulting phylogenetic trees were edited using Figtree v1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/, accessed on 1 May 2022).

3. Results

3.1. Morphological Characterization

The specimen was green to purple-brown in color, sessile, unbranched in the base of the thallus, and sparsely and unregularly branched in the middle part. The branch was up to 10 cm in height and tapered toward apices (Figure 2a,b). The nodes were obviously inflated and 110–276 μm in diameter. The spermatangia were positioned at the outer cortex and formed a circular ring around the nodes (Figure 2c,d). The thallus was pseudoparenchymatous and the outer cortex consisted of two or three layers, and the cells of the outermost layer were irregular in shape and hairless (Figure 2e). The central axis was uniseriate and lacked cortical filaments (Figure 2f). Carposporophytes were growing from the outer cortex to the central cavity, with short and sparsely branched gonimoblast filaments (Figure 2g). The ellipsoidal carposporangium was at the top and single, 41–52 μm in length and 12–25 μm in diameter (Figure 2h). Morphological comparisons between specimens collected in this study and those reported previously in China and India are listed in Table 1.

3.2. Molecular Analysis

The length of the *rbc*L fragment used for phylogenetic analysis in this study was 996 bp, of which 274 bp (27.51%) were variable sites and 221 bp (22.19%) were informative for parsimony. The length of the SSU marker used for phylogenetic analysis in this study was 932 bp, of which 177 bp (18.99%) were variable sites and 98 bp (10.52%) were parsimony sites. The *p*-distance of the *rbc*L gene between GLYZC and the *L. manipurensis* specimen from India was 0.01 (vs. 0.08–0.09 between GLYZC and other species in the phylogenetic tree). The *p*-distances of the SSU gene between GLYZC and the other Lemaneaceae species were 0–0.01 (vs. 0.01–0.04 between GLYZC and Batrachospermaceae species).

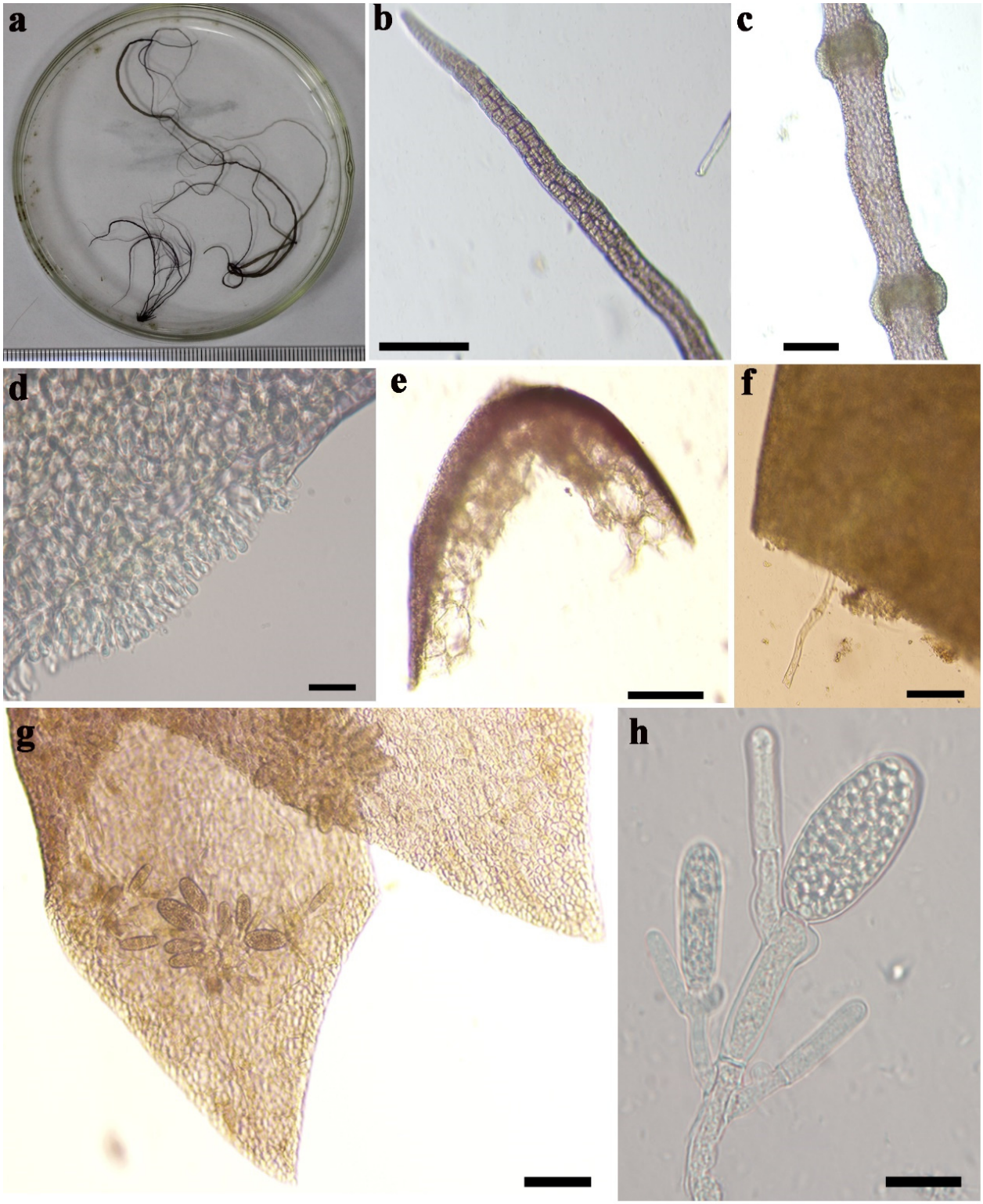

Figure 2. Morphological characteristics of *Lemanea* collected in Guilin, China. (**a**) Morphology of the entire thallus. (**b**) Axes narrow toward the apices. (**c**) Spermatangium growing continuously on the thallus nodes. (**d**) Cross section of the spermatangium. (**e**) Cells of the outer cortex. (**f**) Naked axial filaments with no surrounding cortical filaments. (**g**) Carposporophyte projecting into the center of the thallus. (**h**) Single carpospores terminal on the carposporophyte branches. Scale bar: (**b**,**c**,**e**,**f**) 100 μm; (**d**,**g**,**h**) 20 μm.

Table 1. Morphological comparisons of *Lemanea* species distributed in China and India.

	GLYZC	***L. manipurensis***	***L. sinica***	***L. crassa***	***L. ramosa***	***L. simplex***
Thallus height/cm	10–13	10	9–16	9–13	1–3	2
Branch	No branch at the base, scarcely branched at the lower part	Sparse, irregular branching in the lowermost region of the erect shoots	Opposite, alternate or forked branches	No branch	Densely branched, especially in the upper part	No branch
Stalk	No obvious stalk	No obvious stalk	Slender stalk	Slender stalk	No obvious stalk	No obvious stalk,
Node	Obviously expanded	Expanded	Obviously expanded	Obviously expanded	Obviously expanded or not	irregularly corrugated constriction
Carposporophyte	Ellipsoidal, single, 41–52 μm in length, 12–25 μm in diameter	Ellipsoidal, single, 80–120 μm in length, 30–50 μm in diameter	Columnar obovate, single, 75–95 μm in length, 30–50 μm in diameter	Columnar obovate, oval or dumbbell shape; single; 90–120 μm in length; 50–80 μm in diameter	Oval, single, 80–100 μm in length, 25–60 μm in diameter	Columnar obovate, single, 110–140 μm in length, 50–70 μm in diameter
Spermatangium	Continuous wide rings on the nodes	Isolated irregular patches, sometimes completely covering the axis	Continuous wide rings on the nodes on the upper part; incomplete rings on the lower part	In isolated patches, usually 5–6 obvious protrusions	In isolated patches, usually 6–8, or incomplete rings	In isolated patches, irregular, usually 2–4 formed rings
Reference	This study	[12]	[27]	[27]	[27]	[27]

All three methods including maximum likelihood, neighbor joining and Bayesian inference produced similar topologies. Therefore, only the Bayesian trees for *rbc*L and SSU are shown in Figures 3 and 4, respectively, with all the supporting values included on the nodes. In *rbc*L phylogenetic tree (Figure 3), there were two main branches consisting of the genera *Lemanea* and *Paralemanea*, with high supporting values of 0.98/87.2/93 and 1.00/100/100., respectively The GLYZC specimen collected in this study clustered with *L. manipurensis* from India, supported by 1.00/100/100. In the SSU phylogenetic tree (Figure 4), there was only one accessible sequence of the genus *Lemanea* in the GenBank database (AF026051 for *L. fluviatilis*). The GLYZC formed a sister relationship with AF026051 but with low values. Three *Paralemanea* sequences formed a monophyletic clade supported by 1.00/91.5/95.

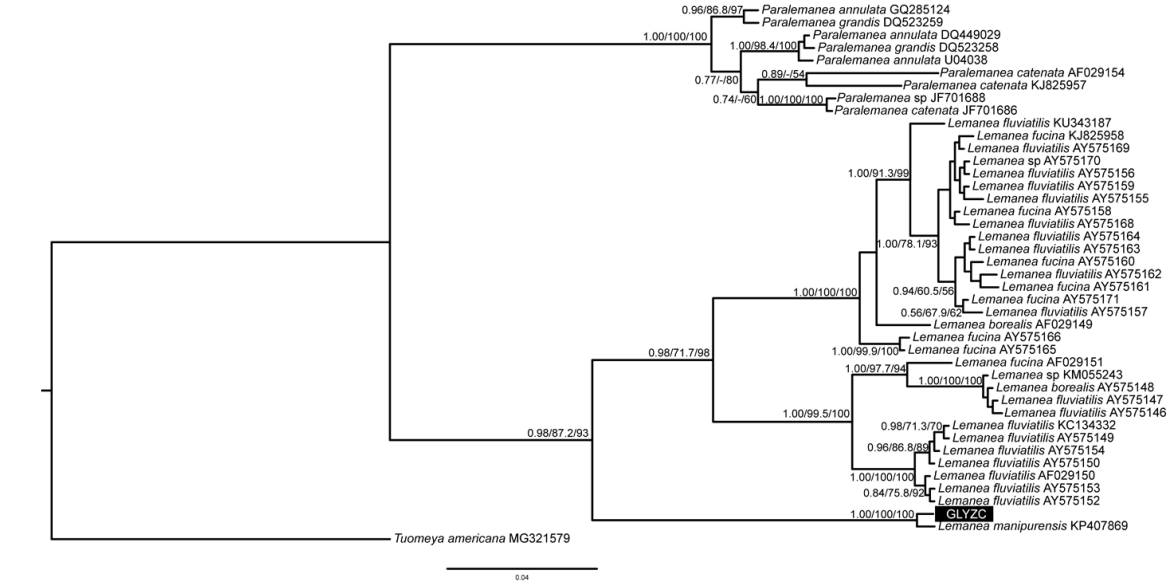

Figure 3. The phylogenetic tree constructed from *rbc*L sequences. The numbers on the branch nodes represent the Bayesian posterior probabilities, maximum likelihood bootstrap tree support values andneighbor joining bootstrap tree support values. Supporting values below 50% are noted with "-". The *Lemanea* specimen collected in this study is indicated by a black box.

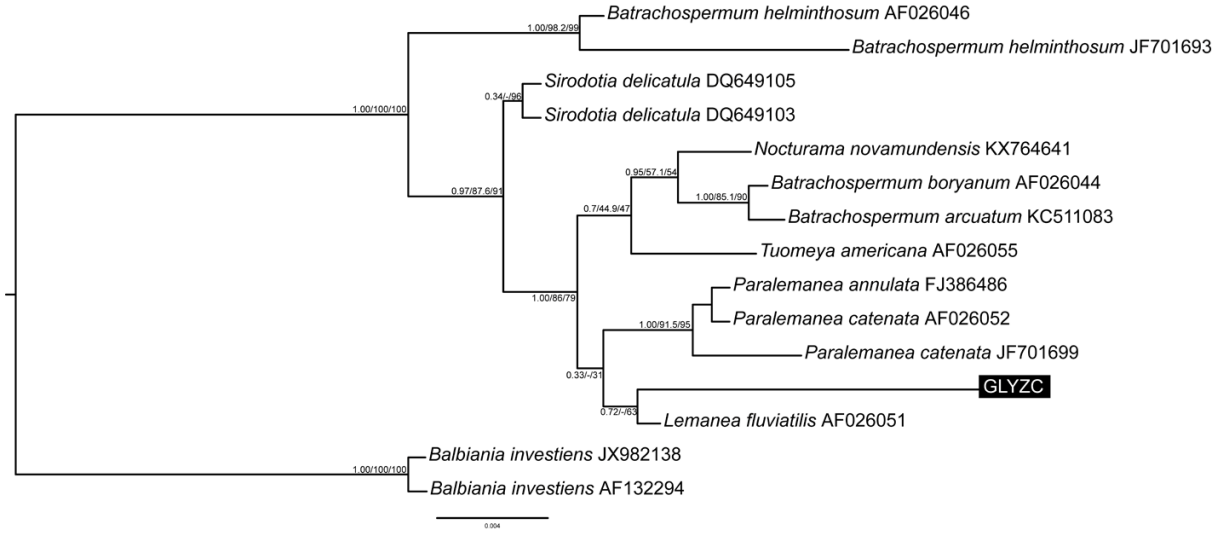

Figure 4. The phylogenetic tree constructed from SSU sequences. The numbers on the branch nodes represent the Bayesian posterior probabilities, maximum likelihood bootstrap tree support values and neighbor joining bootstrap tree support values. Supporting values below 50% are noted with "-". The *Lemanea* specimen collected in this study is indicated by a black box.

4. Discussion

The typical characteristics of the genus *Lemanea* are a thallus growing in tufts with a naked central axis. The morphology of GLYZC collected in this study was consistent with this characteristic. The *rbc*L phylogeny showed a close relationship between GLYZC and *L. manipurensis*, a new species reported in 2015 [12]. *L. manipurensis* was reported in Manipur in northeast India and characterized by a carposporophyte with very short gonimoblast filaments and a single, large, elongate carpospore at the terminal cell of each branch [12]. The morphological characteristics observed in GLYZC collected in this study was consistent with these characters; therefore, GLYZC was identified as a new record of *L. manipurensis* in China. According to the morphological comparison, the *L. manipurensis* distributed

in China was larger in thallus height than the type specimen (10–13 cm for GLYZC vs. 10 cm for *L. manipurensis* in India). The branches were at the lower part of *L. manipurensis* collected in this study and at the base of the thallus in the type specimen. The shapes of the spermatangium of *L. manipurensis* were slightly different between the specimen collected in this study and that distributed in India. The spermatangia were continuous wide rings on the nodes in both young and mature branches in *L. manipurensis* collected in this study, whereas they occurred in isolated, low and indistinct patches in *L. manipurensis* from India. The carposporophytes of *L. manipurensis* collected from China were 41–52 μm in length and 12–25 μm in diameter, which were much smaller than those of *L. manipurensis* from India [12]. Although there are a few morphological discrepancies between GLYZC and the type specimen of *L. manipurensis*, the material from China was identified as *L. manipurensis*, mainly on the basis of the molecular evidence. It can be inferred that the morphological characteristics including the thallus height, the position of the branches, the shape of spermatangia and the size of carposporophytes are variable among different populations within a species. Validation of the reliability of these characteristics for species identification needs observation on more specimens and molecular information in further studies.

The family Lemaneaceae consists of the genera *Lemanea* and *Paralemanea*. Based on traditional morphological systematics, the genus *Lemanea* is characterized by the occurrence of hair cells in the outer cortex. However, there were no hair cells in the outer cortex of *L. manipurensis* collected in this study, indicating that hair cells in the outer cortex cannot be considered as a reliable criterion for the genus *Lemanea*. The morphological characteristics for the identification of the genus *Lemanea* were expanded in our study. Up to now, there have been four species reported in China, including *L. sinica*, *L. simplex*, *L. ramosa* and *L. crassa*, all of which are endemic to China and were identified based on morphology with no molecular information. These four species have numerous characteristics in common with each other and *L. manipurensis*, and all five species are from Asia [4]. According to the morphological comparison listed in Table 1, the newly recorded *L. manipurensis* was similar to *L. sinica* and *L. crassa* in thallus height, while the thallus was obviously larger than that of *L. simplex* and *L. ramose* [27]. The position and density of the branches were different between *L. manipurensis* and the other species, with the branches on the lower part in *L. manipurensis*, no branch in *L. crassa* and *L. simplex*, and branches on the base position in *L. sinica* and the upper part in *L. ramose*. The carposporangial size of *L. manipurensis* was smaller than that of the other four species reported in China. Morphological characters including the shape of spermatangium, thallus height and branching are critical for identification of the genus *Lemanea* [5,27]. The shapes of the carposporangium were similar in *L. manipurensis* and *L. sinica* and *L. simplex*. There were large similarities between *L. manipurensis* and *L. sinica*, except for the stalk and branching. Therefore, the molecular sequence of *L. sinica* was needed to validate if they were the same species. This also suggests the necessity to supply molecular sequences of the genus *Lemanea* currently found in China to elucidate the species distinctness.

The *rbc*L gene is a common DNA barcode for phylogenetic analyses in freshwater Rhodophyta at the systematic level including the order, family, genus and species [13–15,41,42]. It is also the most commonly used in molecular phylogenetic analyses of the genus *Lemanea* [10,12,15,43]. The phylogeny constructed from the *rbc*L gene is more reliable and can be used for species identification for the genus *Lemanea*. By comparison, the application of the SSU sequence in *Lemanea* has been infrequent. Accessible SSU sequences in the GenBank database are limited and resulted in the low supporting values of the phylogenetic tree. This suggests the urgent need for molecular phylogenetic analyses of *Lemanea* species worldwide. The first molecular information of *Lemanea* species from China was provided in our study, contributing data and a theoretical basis for phylogenetic analyses of this genus, and supplementing the species and distribution diversity of the genus *Lemanea* in China.

5. Conclusions

Morphological comparisons and molecular analyses both supported the new record of *L. manipurensis* in China, the fifth reported species of the genus *Lemanea* in China. The specimen in this study was morphologically characterized by branches that were positioned in the lower part of the thallus, hair cells absent around the outer cortex, carpospores that were ellipsoidal and single at the top of the gonimoblast filaments and spermatangia that formed continuous wide rings on the thallus nodes. Given the morphological discrepancies between GLYZC and type specimen of *L. manipurensis* from India, the material from China was identified mainly through molecular evidence. By comparing the morphology of specimens collected from Guilin, China, and the holotype specimen in India, it was found that spermatangial shapes are variable among different populations of *L. manipurensis*. The first molecular information of *Lemanea* species from China was provided in our study, providing molecular evidence and a theoretical basis for molecular phylogenetic research on the genus *Lemanea*, and enriching the species diversity and geographical distribution of *Lemanea* in China.

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