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RESEARCH ARTICLE

Phylogenetic relationships among seven freshwater red algal genera in the Batrachospermaceae (Batrachospermales, Rhodophyta) using complete chloroplast genome data

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Abstract

Systematics studies within the freshwater red algal order Batrachospermales have used only one to a few genes to infer evolutionary relationships. The phylogenetic trees presented in these studies recovered monophyletic genera with strong support, but the relationships among genera were often not well supported. Chloroplast genome sequencing may provide the necessary data to obtain a fully resolved, strongly supported phylogeny. In order to test the efficacy of this approach, we focused on a subclade of seven genera and sequenced chloroplast genomes from 10 species. Utilizing new and previously published data, a phylogeny was constructed from 132 chloroplast-encoded genes. The relationships among all seven genera had strong statistical support. The phylogeny and gross morphology of genera showed concordance. *Lemanea* and *Paralemanea* with tube-like pseudoparenchymatous gametophytes formed a clade, and five genera—*Batrachospermum, Lympha, Sirodotia, Tuomeya*, and *Volatus*—with beaded gametophytes formed a second clade. Within the clade of five genera, *Batrachospermum* with pedicellate carposporophytes was sister to the other three genera with axial carposporophytes and *Sirodotia* with prostrate carposporophytes along the axis. The results highlight the potential for chloroplast genome data to clarify evolutionary relationships in the Batrachospermales, offering a promising approach for future studies aimed at understanding the broader evolutionary history of this group.

KEYWORDS
Batrachospermum, chloroplast genome, *Lemanea*, *Lympha*, morphology, *Paralemanea*, phylogenomics, *rbc*L, *Sirodotia*, *Tuomeya*, *Volatus*

Abbreviations: BHO, Floyd Bartley Herbarium at Ohio University; BS, bootstrap support; CTAB, cetyltrimethylammonium bromide; IR, inverted repeat; LCB, locally collinear block; LSC, large single copy; LSU, large subunit; ML, maximum likelihood; *rbc*L, large subunit ribulose-1,5-bisphosphate carboxylase/oxygenase gene; SSC, small single copy.

INTRODUCTION

The Rhodophyta are primarily marine, but there are freshwater representatives distributed throughout its seven classes. The Batrachospermales are one of three orders that are composed of strictly freshwater taxa that primarily inhabit flowing waters (Vis & Necchi Jr., 2021). The order has a global distribution, being recorded from all continents except Antarctica (Necchi Jr., 2016). Batrachospermales contains 23 genera, with some genera being only known from a single continent (i.e., *Tuomeya* in North America), whereas other genera are more widespread (e.g., *Sirodotia* has been recorded from Africa, Asia, Australia, and Europe, as well as North and South America; Vis & Necchi Jr., 2021; Jayalakshmi & John, 2023; Fischer et al., 2024; Necchi Jr et al., 2024). Why some taxa have an expansive distribution while others are more restricted is relatively unknown, but it is most likely related to life-history traits that allow for long-distance dispersal and establishment in suitable habitats as well as flexible physiology allowing some genera to thrive under a variety of conditions.

Members of the Batrachospermales have a macroscopic haploid gametophyte and two diminutive diploid stages, the *chantransia* and carposporophyte (Sheath, 1984). The chantransia undergoes vegetative meiosis, resulting in the gametophyte, and after fertilization, the carposporophyte remains attached to the gametophyte (Sheath, 1984). The carposporophyte releases diploid carpospores that germinate into new chantransia (Sheath, 1984). All characteristics for morphological identification are derived from the gametophyte and configuration of the carposporophyte. The gross morphology and thallus construction of the gametophytes can be bead-like without an outer cortex, bead-like and pseudoparenchymatous with an outer cortex, or tube-like and pseudoparenchymatous with an outer cortex. Likewise, the position and size of the carposporophytes as well as characteristics of the carpogonium (female gamete) are often diagnostic for species and genera (Vis & Necchi Jr., 2021).

In addition to using morphology for systematic studies, DNA sequence data have been employed to test evolutionary relationships within the Batrachospermales. The first study using DNA sequence data showed that *Batrachospermum* was paraphyletic (Vis et al., 1998). The subsequent taxonomic revisions in the order have focused on recognizing monophyletic genera typically by raising sections of *Batrachospermum* that were well-defined based on morphological characters to the generic level and describing the species diversity within each newly circumscribed genus. For example, the sections *Virescentia*, *Macrospora*, and *Turfosa* were raised to genus level as *Virescentia, Montagnia*, and *Paludicola*, respectively (Necchi Jr et al., 2018, 2019; Vis, Lee, et al., 2020). In addition, several molecular phylogenetic studies have revealed cryptic diversity that has led to the description of genera, including *Lympha*, *Notohesperus*, and *Volatus*, which superficially resemble other genera in the order (Chapuis et al., 2017; Evans et al., 2017; Vis & Necchi Jr., 2021).

Typically, molecular systematics studies of the order Batrachospermales have used one to three genes to infer evolutionary relationships within this group. These studies have been successful in providing support for the establishment of genera and relationships among species within each genus; however, strongly supported phylogenetic relationships among genera have been elusive. In the Rhodophyta, multigene datasets of two to three genes have been unable to resolve relationships among red algal classes (Le Gall & Saunders, 2007; Yoon et al., 2006), and a nine-gene data set did not resolve relationships among orders within the subclass Nemaliophycidae (Lam et al., 2016). To resolve these deeper level relationships within the Rhodophyta, Verbruggen et al. (2010) recommended using high-throughput genomic data for lineages that were difficult to resolve.

Based on previous research, additional sequence data are needed to make progress in understanding evolutionary relationships among genera within the Batrachospermales. Some studies of marine red algae have employed chloroplast genome sequencing to concatenate many genes, resulting in well-supported phylogenies (Costa et al., 2016; Díaz-Tapia et al., 2019). For example, a systematic study of the order Nemaliales used 195 chloroplast-encoded genes to present a fully resolved phylogeny, confirming the monophyly of six families and inferring relationships at the generic level (Costa et al., 2016). For the Ceramiales, 206 chloroplast-encoded genes were used to resolve relationships among genera and families, producing a well-supported phylogeny of this order (Díaz-Tapia et al., 2019). The resolution of relationships at deep nodes within the tree allowed for the examination of thallus structure evolution and transitions between different life-history strategies, as well as other important biological characteristics (Díaz-Tapia et al., 2019).

The whole chloroplast genome approach offers vital data for improving the resolution of phylogenetic relationships among genera in the Batrachospermales, providing a clearer picture of evolutionary patterns as captured by chloroplast–encoded genes. Currently, 10 chloroplast genomes are available, but these existing data are insufficient for establishing a comprehensive phylogenetic tree. A well-supported phylogeny is crucial for exploring evolutionary questions, such as mapping morphological and reproductive traits to understand dispersal and diversification. Before investigating all 23 genera of the order, focusing on a highly supported subclade of seven genera—*Batrachospermum*, *Lemanea*, *Lympha*, *Paralemanea*, *Sirodotia*,

Tuomeya, and *Volatus*—will help evaluate the effectiveness of chloroplast genomes in resolving inter-generic relationships. This clade captures a range of thallus constructions and morphological diversity representative of the entire order, setting the stage for future studies on character evolution once a robust phylogeny is established. Thus, this study aimed to assess the utility of chloroplast genomic data for inferring relationships among these seven genera and to evaluate key taxonomic characters based on the resulting phylogeny.

MATERIALS AND METHODS

Taxon selection, collection, and identification

A well-supported subclade of seven genera in the Batrachospermales was selected for investigation (Figure S1). Within each focal genus, the number of currently recognized species is as follows: *Batrachospermum* (6), *Lemanea* (8), *Lympha* (1), *Paralemanea* (9), *Sirodotia* (9), *Tuomeya* (1), and *Volatus* (3; Vis & Necchi Jr., 2021). After assessing the chloroplast genomes already available for these genera, we supplemented the data to have at least two species per genus, when possible. Specimens for 10 species from North America were obtained (Table S1). All specimens were desiccated in silica and stored at –20°C. Voucher specimens were deposited in the Floyd Bartley Herbarium at Ohio University (BHO). Specimens were identified using diagnostic morphological characters from the literature (Vis & Necchi Jr., 2021 and references therein), and morphological identifications were verified by comparing the percent sequence identity of the *rbc*L gene to sequences in GenBank following Benson et al. (2014).

DNA preparation and sequencing

Total genomic DNA was extracted using two methods (Table S2). Tissue was either ground in liquid nitrogen and the Nucleospin Plat II kit (Macherey–Nagel, Düren, Germany) was used following the manufacturer's protocol, with the addition of an extended lysis step of 2 hours for *Lemanea* or *Paralemanea* species, or the material was homogenized in a bead-beater and a modified CTAB extraction protocol with Polyvinylpyrrolidone-10 was utilized (Schenk et al., 2023). DNA quantity and quality were assessed using a NanoDrop ND-1000 spectrophotometer (ThermoFisher Scientific, Waltham, Massachusetts, United States), Qubit 3 fluorometer (ThermoFisher Scientific, Waltham, Massachusetts, United States), or Agilent 4150 TapeStation (Agilent, Santa Clara, California, United States).

DNA sequencing was performed on one of the following Illumina platforms: NovaSeq, MiSeq, or NovaSeq X Plus (Table S2). For sequencing on the NovaSeq platform, the Ohio University Genomics Facility (Athens, Ohio, United States) constructed libraries of 350 nucleotide fragments using a Nextera XT DNA Library Prep kit (Illumina, Inc., San Diego, California, United States). For sequencing on the Illumina MiSeq platform, libraries of 200–450 nucleotide fragments were constructed using the NEBNext Ultra II FS DNA Library Kit (New England BioLabs, Ipswich, Massachusetts) following the protocol for inputs of ≤100 ng. Sequencing on the Illumina NovaSeq X Plus was performed by SeqCenter (Pittsburgh, Pennsylvania, United States) using libraries of 280 nucleotide fragments constructed with the Illumina DNA Prep kit (Illumina, Inc., San Diego, California, United States).

Chloroplast genome assembly and annotation

For each sequencing method, the resulting reads were trimmed, paired, and the first 15 million paired reads were used in an initial de novo assembly using low sensitivity parameters in Geneious Prime 2024.0.7 (Biomatters, geneious.com). The coding sequences from the published chloroplast genome of *Batrachospermum qujingense* (MN905507 as *Batrachospermum* sp.; Fang et al., 2020) were used as a reference to identify contigs containing chloroplast genome sequence fragments for *B. gelatinosum*. The consensus sequences from contigs containing chloroplast genome fragments were trimmed and assembled. To close subsequent gaps, sequences were extended by iteratively mapping paired-end reads with low sensitivity and a minimum mapping quality of 30 until complete chloroplast genome sequences were obtained. For two samples, *Paralemanea grandis* and *Volatus personatus*, complete chloroplast genomes could not be obtained.

Inverted repeats (IR) were identified using the Find Repeats function in Geneious Prime with a minimum repeat length of 100. Once annotated, the large single copy (LSC) and small single copy (SSC) of each genome were annotated. All rRNA and protein-coding genes were extracted from the published chloroplast genome of *Batrachospermum qujingense* (MN905507) and used to annotate *Batrachospermum gelatinosum* with the Annotate From function in Geneious Prime. Positions of the start and stop codons for all protein-coding genes were confirmed with the Find ORFs function in Geneious Prime and refined as needed. All tRNA genes were annotated using tRNAscan-SE (Lowe & Chan, 2016). Subsequently, the annotated genome of *B. gelatinosum* was used to annotate the remaining genomes.

The progressiveMauve algorithm (Darling et al., 2010) was implemented in Geneious Prime 2024.0.7 using the full alignment option, automatically

calculated seed weights, and automatically calculated minimum locally collinear blocks (LCBs) score to assess synteny among complete chloroplast genomes.

Phylogenomic analyses

Genes from 10 chloroplast genomes generated in this study, along with nine from GenBank, were used for phylogenetic analyses (Table 1). The ingroup consisted of 14 species as follows: *Batrachospermum gelatinosum, B. naiadis, B. qujingense* (MN905507, Fang et al., 2020), *Lemanea borealis, L. occidentalis, Lympha mucosa* (MN509464, Evans & Vis, 2020), *Paralemanea blumii, P. grandis, Paralemanea* sp. (MG252487, Paiano et al., 2018), *Sirodotia delicatuliformis* (MG252489, Paiano et al., 2018), *S. suecica, Tuomeya americana, Volatus carionii*, and *V. personatus*. Five previously published chloroplast genomes in the Batrachospermales were used as outgroups, as follows: *Kumanoa ambigua* (MG252485), *K. americana* (NC031178), *K. mahlacensis* (MG252486), *Sheathia longipedicellata* (KY033529 as *B. arcuatum*), and *Virescentia viride-brasiliense* (MG252484) (Cho et al., 2018; Nan et al., 2017; Paiano et al., 2018). For the previously published genomes, annotations were confirmed or updated (Table S3). A conservative approach was taken, and only genes present in all 19 taxa were assessed for phylogenetic analyses. Individual genes were aligned in Geneious Prime using the MUSCLE v5.1 algorithm (Edgar, 2022).

Phylogenetic analyses were conducted using (1) the *rbc*L gene only, which was the gene relied upon in previous systematics studies, and (2) the chloroplast-encoded genes present in all taxa and readily able to be aligned. The 132 chloroplast-encoded genes were concatenated using AMAS (Alignment Manipulation and Summary; Borowiec, 2016). The best-fit model was determined for the *rbc*L gene alone and for the concatenated alignment using ModelFinder in IQ-Tree version 2.3.5 (Kalyaanamoorthy et al., 2017). For the *rbc*L gene dataset, the TIM2+I+G4+F model was selected, and for the concatenated alignment, the GTR+F+R4 model was the best fit. A maximum likelihood (ML) tree with 1000 ultrafast bootstraps (Hoang et al., 2018) was generated for the *rbc*L gene and the concatenated alignment partitioned by gene and codon in IQTree 2.3.5, using the respective models (Minh et al., 2020).

Morphological character assessment

For the seven genera, morphological characters were compiled from the literature (Vis & Necchi Jr, 2021 and references therein). Among these genera and the Batrachospermales in general, the gross morphology of the gametophyte, features of the carpogonial branch, and the structure, size, and placement of the carposporophytes are taxonomically important. These characters were photo-documented using a SC100 camera (Olympus America Inc., Lake Seneca, New York, United States) on a BX-40 compound microscope (Olympus America Inc.) and SZ40 dissecting scope (Olympus America Inc.).

RESULTS

DNA extraction and sequencing

Two extraction and three sequencing methods were used to produce the chloroplast genomes in this study. For species sequenced on Illumina NovaSeq, the numbers of paired-end reads were as follows: *Batrachospermum gelatinosum* (100789654), *B. naiadis* (88410074), *Lemanea borealis* (108373364), *L. occidentalis* (103455680), *Paralemanea grandis* (69368188), and *Sirodotia suecica* (103392072). For *Volatus personatus* sequenced with Illumina MiSeq, the number of paired-end reads was 74804172. For samples sequenced on an Illumina NovaSeq X Plus, the numbers of reads were as follows: *Paralemanea blumii* (147852680), *Tuomeya americana* (90781494), and *V. carionii* (90639644).

Chloroplast structure

Eight complete and two partial chloroplast genomes were generated, and each had a quadripartite structure containing an LSC region, a SSC region, and two IR regions (Figures S2–S9). Genome sizes ranged from 187,783 bp (*Paralemanea blumii*) to 189,523 bp (*Tuomeya americana*) for the complete genomes, and an estimated 97% of the partial genomes were obtained (Table 1). The number of protein coding genes ranged from 200 to 201, and G+C content ranged from 28.6% (*Volatus personatus*) to 30.8% (*Lemanea* spp. and *P. blumii*). The number of tRNA genes ranged from 28 (*Paralemanea* spp.) to 32 (Table 1, Figures S2–S9). The complete chloroplast genomes and the two partial genomes all contained a single intron in the *chl*B gene.

Two incomplete chloroplast genomes, *Paralemanea grandis* and *Volatus personatus*, were generated, with each containing a single gap. In *P. grandis*, the gap was between *rpl*21 and *rnz*. In *V. personatus*, the gap was between *glt*B and *rpo*Z. All protein-coding genes present in other taxa within these genera were identified, with no missing protein-coding genes from the two incomplete genomes.

Whole-genome alignments revealed the absence of rearrangements among the complete chloroplast genomes. A single collinear block was predicted with the MAUVE algorithm for whole-genome alignments (Figure S10), illustrating the conserved structure and collinearity among these chloroplast genomes.

TABLE 1 Overview of chloroplast genomes used in this study.

Species	% GC content	Protein coding genes	Genome size (nt)	YCF[a]	ORF[b]	tRNA	Intron	Coverage (max; min)	GenBank accession number	References
Ingroup taxa										
Batrachospermum gelatinosum	29.5	201	188,711	20	6	32	1	2887; 5	PX060844	This study
B. naiadis	29.9	201	188,526	20	6	32	1	7025; 4	PX060845	This study
B. qujingense[c,f]	29	201	184,902	20	6	32	1	–	MN905507	Unpublished
Lemanea borealis	30.8	200	188,030	19	6	32	1	1597; 4	PX060846	This study
L. occidentalis	30.8	200	188,242	19	6	32	1	5636; 5	PX060847	This study
Lympha mucosa[c,d]	29.5	198	≥189,825	19	5	32	2	3236; 0	MN509464	Evans and Vis (2020)
Paralemanea sp.[c]	31	199	187,837	20	6	28	1	1934; 1	MG252487	Paiano et al. (2018)
P. blumii	30.8	201	187,783	20	6	28	1	4786; 3	PX060848	This study
P. grandis[d]	30.3	201	≥183,183	20	6	28	1	–	PX060849	This study
Sirodotia delicatulaformis[c]	29.2	200	185,489	20	5	32	1	3485; 460	MG252489	Paiano et al. (2018)
S. suecica	29.6	201	186,915	19	6	32	1	6718; 3	PX060850	This study
Tuomeya americana	29.3	200	189,523	20	5	32	1	1219; 4	PX060851	This study
Volatus carrionii	29.1	200	188,856	19	6	32	1	1216; 4	PX060852	This study
V. personatus[d]	28.6	200	≥183,580	19	6	29	1	–	PX060853	This study
Outgroup taxa										
Kumanoa ambigua[c]	28.2	201	182,932	21	6	31	1	844; 4	MG252485	Paiano et al. (2018)
K. americana[c]	29.3	201	184,025	21	6	31	2	225[e]	NC031178	Cho et al. (2018)
K. mahlacensis[c]	29.8	199	181,368	21	6	31	1	2198; 1	MG252486	Paiano et al. (2018)
Sheathia longipedicellata[c,g]	29.9	200	187,354	18	5	31	1	–	KY033529	Nan et al. (2017)
Virescentia viride-brasiliensis[c]	28.5	189	171,682	17	3	34	1	5343; 1	MG252484	Paiano et al. (2018)

[a]YCF = Hypothetical chloroplast open reading frame.
[b]ORF = Open reading frame.
[c]Statistics reflect updated annotations made in this study.
[d]Incomplete chloroplast genome.
[e]Mean coverage as reported in Cho et al. (2018).
[f]Identification updated from *Batrachospermum* sp. to *B. qujingense* (Fang et al., 2020).
[g]Identification updated from *Sheathia arcuata* to *Sheathia longipedicellata* (Vis, Tiwari, et al., 2020).

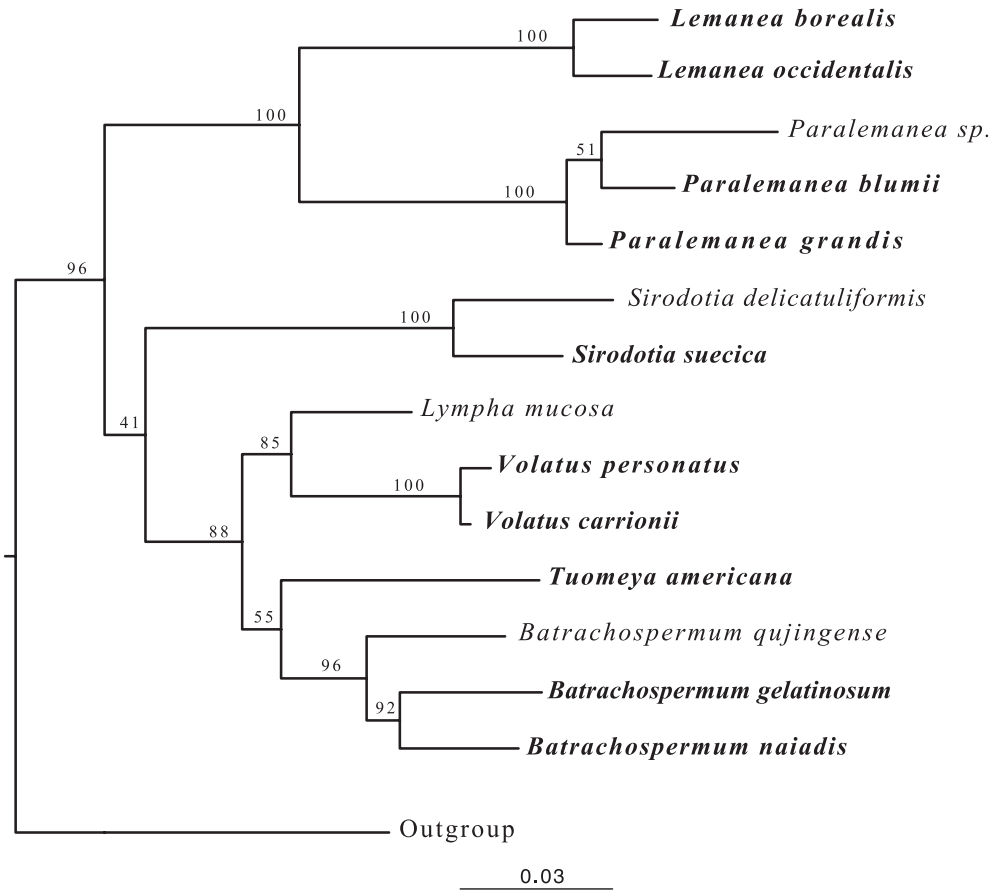


FIGURE 1 Maximum likelihood phylogeny depicting relationships among the seven genera based on the *rbc*L gene alone. Support values are ultrafast bootstraps. Taxa in bold are new data from this study. Outgroup taxa from the Batrachospermales are not shown but provided in Table 1.

Chloroplast gene phylogenies

There were 181 genes in common for all ingroup and outgroup taxa. Of these genes, 49 could not be reliably aligned among all taxa and were excluded from phylogenetic analyses. Therefore, the final data set included 132 protein-coding genes present in all chloroplast genomes for all taxa (Table S3).

For the *rbc*L single-gene analysis, the ML phylogeny showed a well-supported ingroup by ultra-fast bootstrap support (bs; Figure 1). The genera with two or more species showed strong support (bs ≥ 96) as well. The sister relationship of *Lemanea* and *Paralemanea* was well supported. The clade including the remaining five genera received weak support (bs = 41). Within this clade, the relationships among the genera had weak or moderate support; the relationship of *Batrachospermum* spp. to *Tuomeya americana* showed weak support (bs = 55), and the relationship of *Lympha mucosa* to *Volatus* spp. was moderate (bs = 85; Figure 1).

For the multi-gene analysis, the ML analysis resulted in strong support (bs = 100) for the ingroup clade (Figure 2). In addition, all internal branches received strong support (bs = 95–100). As with the *rbc*L single gene tree, the ingroup was divided into two subclades, one with *Lemanea* and *Paralemanea* and the second with *Batrachospermum*, *Lympha*, *Sirodotia*, *Tuomeya*, and *Volatus*. However, this latter subclade was strongly supported, as were the generic relationships within it. The placement of the genus *Tuomeya* differed between the two analyses, with *Tuomeya* being sister to *Batrachospermum* with weak support (bs = 55) in the *rbc*L gene tree and sister to *Sirodotia*, *Lympha*, and *Volatus* with strong support (bs = 95) in the multigene phylogeny.

Morphological characteristics

The seven genera investigated showed a range in gametophyte thallus form, carpogonial branch morphology, and carposporophyte size and shape (Figure 3). Both *Lemanea* and *Paralemanea* exhibit pseudoparenchymatous tubes with an outer cortex (Figure 3a,f). Like these genera, *Tuomeya* has an outer cortex but is bead-like (Figure 3b,c), and all three show carposporophyte development within the outer cortex (Figure 3f). *Batrachospermum*, *Lympha*, *Sirodotia*, and *Volatus* have whorls of fascicles that are not fused and have a

beads-on-a-string appearance (Figure 3c). Six genera, *Lemanea*, *Lympha*, *Paralemanea*, *Sirodotia*, *Tuomeya*, and *Volatus*, have short carpogonial branches, with *Tuomeya* and *Volatus* having contorted branches (Figure 3d) rather than straight. The carpogonial branch of *Batrachospermum* is long and results in a pedicellate carposporophyte (Figure 3e,h). For *Lympha* and *Volatus*, the carposporophytes are large and close to the main axis of the gametophyte (Figure 3g). Except for *Sirodotia*, the carposporophyte has a spherical, subspherical appearance with the gonimoblast filaments of the carposporophyte radiating from a few central cells and terminating in carposporangia (Figure 3i). *Sirodotia* has a diffuse carposporophyte composed of prostrate gonimoblast filaments along the main axis and produces upright branches terminating in carposporangia (Figure 3j).

The well-resolved multi-gene phylogeny and some of the gross morphological characteristics were concordant (Figures 2 and 3). The phylogeny showed two subclades, one clade containing the two tube-like genera *Lemanea* and *Paralemanea*, and the other

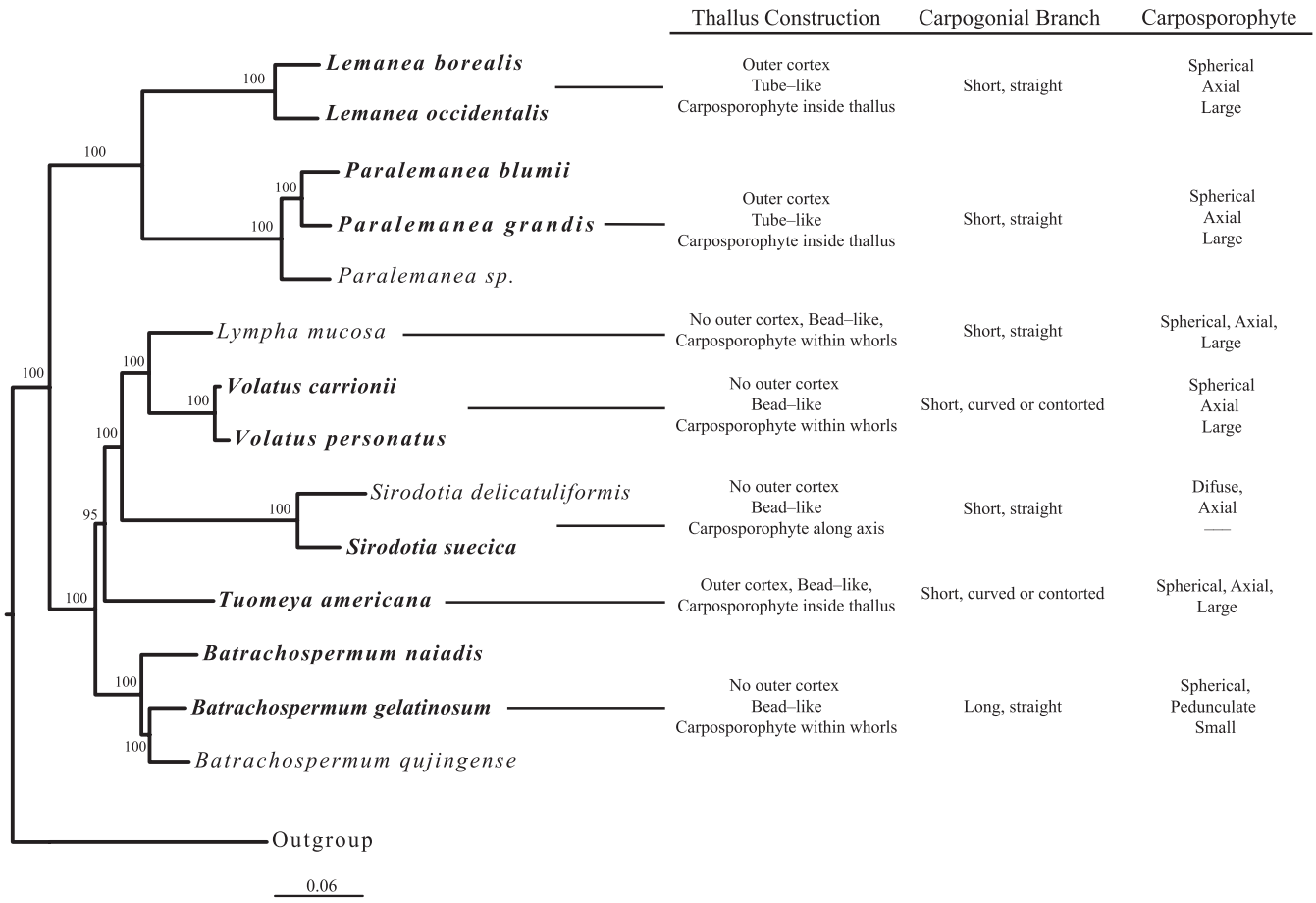
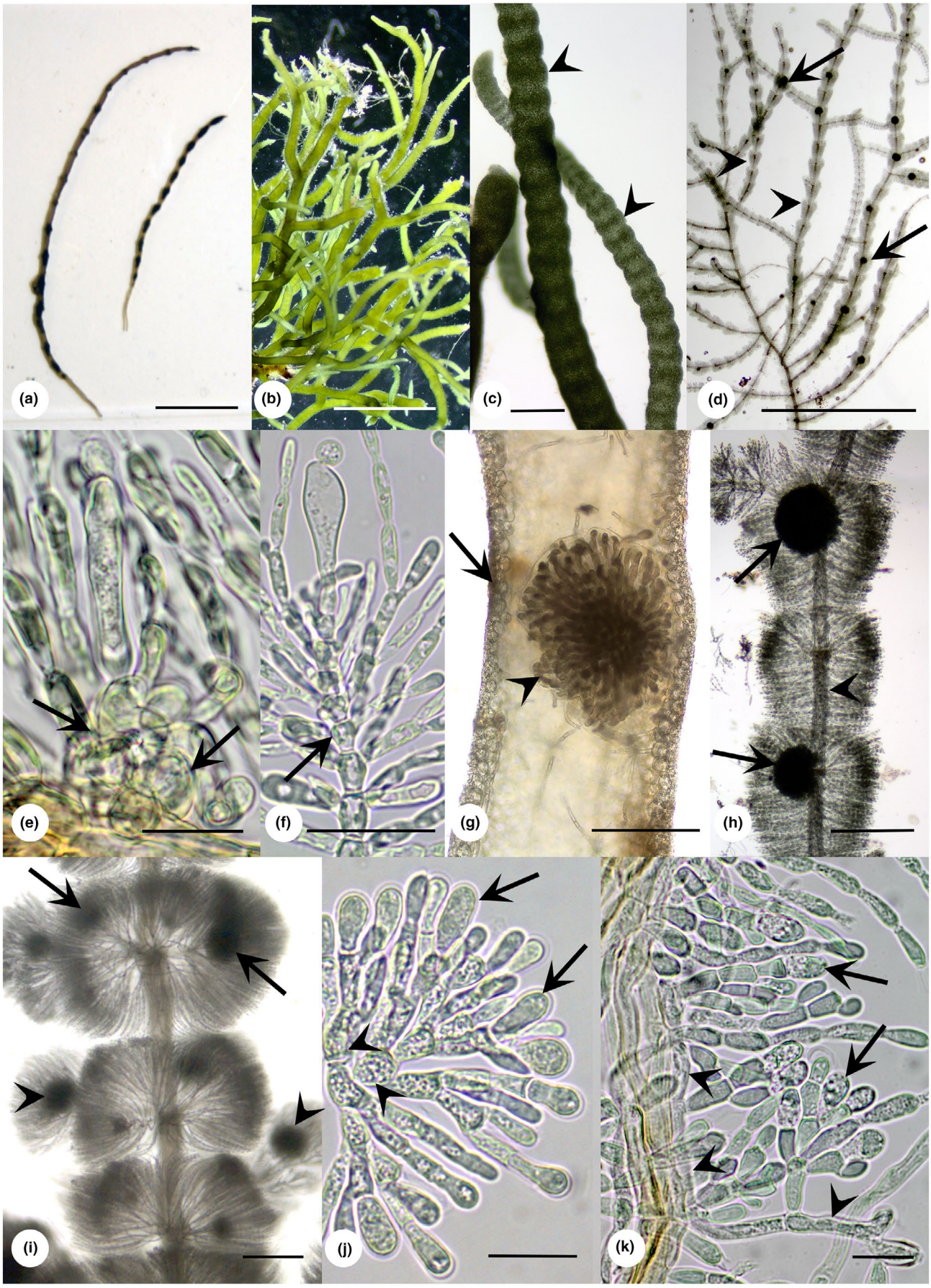


FIGURE 2 Maximum likelihood phylogeny depicting relationships among the seven genera based on the concatenation of 112 chloroplast genes. Support values are ultrafast bootstraps. Taxa in bold are new data from this study. Outgroup taxa from the Batrachospermales are not shown but provided in Table 1. Select morphological characters are shown for each genus.

FIGURE 3 Representative morphological characteristics of genera in this study. (a) habit of *Lemanea occidentalis* showing overall tube-like thallus morphology with outer cortex. (b) habit of *Tuomeya americana* with highly branched thallus. (c) Bead-like whorls (arrowheads) with outer cortex of *T. americana*. (d) habit of *Volatus personatus* having highly branched thallus with whorls of branches (arrowheads) resembling beads on a string and large prominent axial carposporophytes (arrows). (e) *Volatus personatus* with cells forming a coiled carpogonial branch (arrows). (f) *Batrachospermum naiadis* with numerous cells forming a long straight carpogonial branch (arrow). (g) Longitudinal section through *Paralemanea grandis* showing the outer cortex of cells (arrow) and developing large carposporophyte (arrowhead) inside the outer cortex of the thallus. (h) *Volatus personatus* showing large carposporophytes (arrows) closely associated with the main axis (arrowhead). (i) *Batrachospermum naiadis* with small pedunculate carposporophytes (arrows) in the outer part of the whorl and some (arrowhead) protruding from the whorl. (j) *Volatus personatus* with terminal carposporangia (arrows) on branches originating with a small grouping of gonimoblast cells (arrowheads) which results in a spherical carposporophyte. (k) *Sirodotia suecica* with terminal carposporangia (arrows) on upright branches originating from prostrate gonimoblast cells (arrowheads) running along the main axis resulting in a diffuse carposporophyte. Scale bars: 1 cm, a, 0.5 cm b, d, 20 μm c, e, f, 250 μm g–i, 15 μm j, k.


(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
(i)
(j)
(k)

clade containing the five bead-like genera. *Lympha*, *Sirodotia*, *Tuomeya*, and *Volatus* have short or twisted carpogonial branches with the carposporophyte associated with the axis, whereas *Batrachospermum*, which was sister to these genera, is the only genus with long, straight carpogonial branches resulting in pedunculate carposporophytes (Figure 3h).

DISCUSSION

Our study has contributed to the understanding of chloroplast structure in the Batrachospermales with the addition of eight complete genomes as well as two partial genomes. The complete chloroplast genomes share many similarities with those previously reported for the Batrachospermales and other Florideophyceae (Costa et al., 2016; Díaz-Tapia et al., 2019; Janouškovec et al., 2013; Paiano et al., 2018). These genomes were gene dense and exhibited highly conserved gene order. Chloroplast genome sizes in members of the Batrachospermales (171,722–187,354 bp) were reported to be near the lowest range for the Florideophyceae (171,392–194,153 bp; Costa et al., 2016; Lee et al., 2016 and references therein); however, the *Tuomeya americana* chloroplast genome (189,523 bp) approached the upper range. In the Batrachospermales, the number of protein coding genes ranged from 189 to 201, similar to other Florideophyceae (184–235; Costa et al., 2016; Lee et al., 2016). Genome organization within red algae has been shown to be highly conserved in comparison with the complex history of rearrangements in green algal chloroplast genomes (Costa et al., 2016; Glass et al., 2023; Janouškovec et al., 2013; Lam et al., 2016; Leliaert & Lopez-Bautista, 2015; Melton et al., 2015; Turmel et al., 2015). Organization of chloroplast genomes in the Batrachospermales from data generated in this study as well as in previous studies (Lee et al., 2016; Paiano et al., 2018) has shared this pattern of conservation.

In the eight complete chloroplast genomes and one of the two partial genomes generated in this study, two IR regions containing the complete ribosomal operon (16S, 5S, and 23S rRNA genes) were identified. Likewise, the previously published *Lympha mucosa* chloroplast genome also had this genome architecture (Evans & Vis, 2020). For *Batrachospermum qujingense* (GenBank MN905507) and *Paralemanea* sp. (Paiano et al., 2018), a partial second IR was reported. The chloroplast genome of *Sirodotia delicatuliformis* was the only species reported to contain a single ribosomal operon. This quadripartite structure is known from nearly all primary plastid-containing lineages, including embryophytes, green algae, and red algae (Green, 2011; Lee et al., 2016, and references therein) with few exceptions (i.e., legumes and some zygnematophytes). The extent of this chloroplast genome architectural feature within the whole of Batrachospermales is unknown, but at least *Sheathia longipedicellata* (as *Batrachospermum arcuatum*, Nan et al., 2017) has been reported to have two complete IR regions. From our experience, this region can be challenging to identify, and reexamination of the original raw data or generating new data may yet reveal a typical IR in *S. delicatuliformis*.

The phylogenomics approach employed in this study was successful in recovering strong support for the relationships among seven Batrachospermales genera, and the phylogeny showed concordance with key morphological features. For example, *Lemanea* and *Paralemanea* genera, with tube-like gametophytes, were recovered as monophyletic, and the five bead-like genera were also supported as monophyletic. In previous systematic studies using one or only a few genes, *Lemanea* and *Paralemanea* typically were well supported as sister taxa, but *Sirodotia* was often placed with these taxa with weak support (Vis et al., 1998; Chapuis et al., 2017; Vis & Necchi Jr., 2021 figure 5.1). The placement of *Sirodotia* within the clade of bead-like genera is consistent with the gross morphology. Similarly, *Tuomeya* has been identified as sister to *Batrachospermum* in studies using the *rbc*L gene alone or in combination with one to two other genes, or related to *Lympha* and *Volatus* with weak support (Chapuis et al., 2017; Vis & Necchi Jr., 2021 figure 5.1). The placement in the multigene analysis of *Tuomeya* with *Lympha*, *Sirodotia*, and *Volatus* allied it with genera that have similar morphology, as these four genera all have short carpogonial branches and either one large carposporophyte per whorl or a diffuse carposporophyte (Vis & Necchi Jr., 2021). Therefore, the phylogenetic results presented here are congruent with key morphological features that reflect shared evolutionary histories. Thallus construction, particularly the presence of an outer cortex, does not appear to be monophyletic. This trait has been observed in three genera within this clade (*Lemanea*, *Paralemanea*, and *Tuomeya*) and in two other distantly related genera (*Petrohua* and *Psilosiphon*) within the Batrachospermales (Figure S1; Vis & Necchi Jr., 2021, table 5.1). Although this pattern could reflect convergent evolution to similar ecological conditions as noted in Vis et al. (2007), further investigation is needed to determine these underlying drivers.

In a recent phylogenomic study of the Batrachospermales utilizing mitochondrial genomes, Da Silva et al. (2025) recovered an identical topology among the seven genera examined in this study. However, support values were low at some nodes within the clade (Da Silva et al., 2025; Figure 2). In contrast, the chloroplast genome phylogeny presented in this study recovers the same topology with consistently greater support across nodes (bs ≥95; Figure 2), underscoring the utility of chloroplast genomes for resolving relationships among genera within the Batrachospermales. This increased resolution may reflect the larger size and gene content of chloroplast

genomes in the Batrachospermales, as the current study analyzed 132 chloroplast genes compared to the 17 mitochondrial genes used by Da Silva et al. (2025). The consistent topology recovered from both datasets strengthens confidence in the inferred relationships among these seven genera.

The current study examined chloroplast genomes from seven genera in the order Batrachospermales for which 23 genera are currently recognized (Vis & Necchi Jr., 2021). Chloroplast genome data are available for a single species of four additional genera, although there are multiple species recognized in three of these (Cho et al., 2018; Evans & Vis, 2020; Fang et al., 2020; Nan et al., 2017; Paiano et al., 2018). These genera all have gametophytes with the bead-like gross morphology but differ from each other: *Sheathia* has long carpogonial branches resulting in pedunculate carposporophytes, and *Kumanoa* and *Virescentia* have a short, straight, or contorted carpogonial branch with axial carposporophytes (Vis & Necchi Jr., 2021). However, they only represent a small fraction of the remaining genera and the morphological diversity. For example, there are two tube-like genera, *Petrohua* and *Psilosiphon*, that were distantly related to each other in previous two-gene (*rbc*L and LSU) phylogenies, but these two genes were insufficient to resolve strongly supported relationships among them and other Batrachospermales (Vis et al., 2007). We argue that based on the results of the present study, expanding the phylogenomic approach to include all members of the Batrachospermales would likely result in a fully resolved, strongly supported phylogeny. In turn, this robust phylogeny would be essential to the study of the evolution of key morphological and reproductive characters within the order as a whole.

AUTHOR CONTRIBUTIONS

Roseanna M. Crowell: Conceptualization (equal); data curation (equal); formal analysis (lead); funding acquisition (equal); methodology (equal); project administration (lead); writing – original draft (lead). **Kenneth G. Karol:** Formal analysis (equal); methodology (equal); writing – review and editing (equal). **Morgan L. Vis:** Conceptualization (equal); funding acquisition (equal); methodology (equal); supervision (equal); writing – review and editing (equal).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Table S1. Species and collection information of chloroplast genomes generated in the present study.

Table S2. Species, collection information, and sequencing information for chloroplast genomes generated in the present study.

Figure S1. Maximum likelihood tree based on rbcL gene sequences depicting the relationship of the subclade utilized in this study (bold red line) to other taxa within the Batrachospermales. Support values are maximum likelihood bootstraps.

Figure S2. Gene map of *Batrachospermum gelatinosum* chloroplast genome. Genes labeled on the outside of the outer circle are transcribed clockwise, and genes on the inside are transcribed counterclockwise. The asterisk (*) indicates an intron. The inner circle represents the nucleotide content (G/C = dark gray, A/T = light gray). Gene boxes are color-coded by functional groups shown in the key.

Figure S3. Gene map of *Batrachospermum naiadis* chloroplast genome. Genes labeled on the outside of the outer circle are transcribed clockwise, and genes on the inside are transcribed counterclockwise. The asterisk (*) indicates an intron. The inner circle represents the nucleotide content (G/C = dark gray, A/T = light gray). Gene boxes are color-coded by functional groups shown in the key.

Figure S4. Gene map of *Lemanea borealis* chloroplast genome. Genes labeled on the outside of the outer circle are transcribed clockwise, and genes on the inside are transcribed counterclockwise. The asterisk (*) indicates an intron. The inner circle represents the nucleotide content (G/C = dark gray, A/T = light gray). Gene boxes are color-coded by functional groups shown in the key.

Figure S5. Gene map of *Lemanea occidentalis* chloroplast genome. Genes labeled on the outside of the outer circle are transcribed clockwise, and genes on the inside are transcribed counterclockwise. The asterisk (*) indicates an intron. The inner circle represents the nucleotide content (G/C = dark gray, A/T = light gray). Gene boxes are color-coded by functional groups shown in the key.

Figure S6. Gene map of *Paralemanea blumii* chloroplast genome. Genes labeled on the outside of the outer circle are transcribed clockwise, and genes on the inside are transcribed counterclockwise. The asterisk (*) indicates an intron. The inner circle represents the nucleotide content (G/C = dark gray, A/T = light gray). Gene boxes are color-coded by functional groups shown in the key.

Figure S7. Gene map of *Sirodotia suecica* chloroplast genome. Genes labeled on the outside of the outer circle are transcribed clockwise, and genes on the inside are transcribed counterclockwise. The asterisk (*) indicates an intron. The inner circle represents the nucleotide content (G/C = dark gray, A/T = light gray). Gene boxes are color-coded by functional groups shown in the key.

Figure S8. Gene map of *Tuomeya americana* chloroplast genome. Genes labeled on the outside of the outer circle are transcribed clockwise, and genes on the inside are transcribed counterclockwise. The asterisk (*) indicates an intron. The inner circle represents the nucleotide content (G/C = dark gray, A/T = light gray). Gene boxes are color-coded by functional groups shown in the key.

Figure S9. Gene map of *Volatus carionii* chloroplast genome. Genes labeled on the outside of the outer circle are transcribed clockwise, and genes on the inside are transcribed counterclockwise. The asterisk (*) indicates an intron. The inner circle represents the nucleotide content (G/C = dark gray, A/T = light gray). Gene boxes are color-coded by functional groups shown in the key.

Figure S10. Chloroplast genome alignment of the circularized Batrachospermales plastid genomes. A unique locally collinear block (LCB) was recovered, and it is indicated by a corresponding box. Within each LCB, a sequence similarity profile is reported. Annotations are represented by the boxes below the LCB: protein-coding genes and tRNAs are represented by white boxes, while rRNAs genes are represented by filled (shaded) boxes. ORFs are represented by light green boxes. The position of the boxes above or below the line refers to the orientation of the gene.

Table S3. Matrix of species and chloroplast genes used in this study. Open boxes indicate that the gene was not identified. A "1" denotes outgroup taxon. Bolded genes (132) were included in phylogenetic analysis; underlined genes (49) were present in all taxa, but not readily alignable for phylogenetic analyses. Species abbreviations as follows: Bgel: *Batrachospermum gelatinosum*, Bnai: *Batrachospermum naiadis*, Bquj: *Batrachospermum qujingense*, Lbor: *Lemanea borealis*, Locc: *Lemanea occidentalis*, Lmuc: *Lympha*.

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