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RESEARCH ARTICLE

Plastid genome structure and phylogenomics of the freshwater red algal order Batrachospermales (Rhodophyta)

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Abstract

For the freshwater red algal order Batrachospermales, the number of plastid genomes available is relatively small compared to the number of genera. Fully assembled plastid genomes can provide insights into plastid evolution and crucial data for phylogenetic reconstruction. In the present study, 18 plastid genomes were generated for a total of 40 plastid genomes from 38 species representing 18 of the 23 genera. The greatly expanded dataset allowed for comparison of the plastid genome structural characteristics with the other orders in the Nemaliophycidae and inference of the phylogenetic relationships of the genera within the order. Results showed the plastid genomes had either one or two RNA operons, and this variation could be intrageneric. All plastid genomes had the *chl*B gene with an intron like all Nemaliophycidae but lacked the *apc*F gene present in all Nemaliophycidae. The loss of the *pbs*A gene was variable in the Batrachospermales and the Nemaliophycidae. Phylogenetic analysis using a 126-gene concatenated dataset produced a fully supported Batrachospermales. In addition, generally high support for the relationships among the genera resulted in the most robust phylogeny to date. Nevertheless, the phylogeny also highlighted that potentially more data will be needed to resolve the relationship among sections of *Nothocladus* and other related genera. Overall, the Batrachospermalean genera were split into two well-supported lineages, which had been noted in other studies using plastid and mitochondrial genomes. However, we lack a combination of characters to distinguish these two lineages, as the morphological characters to describe taxa are shared between them.

KEYWORDS
Batrachospermum, introns, morphology, phylogenomics, plastid genome

INTRODUCTION

The number of organellar genomes, both plastid and mitochondrial, available for the Rhodophyta has been steadily growing, primarily due to the increased ease and decreased cost of high-throughput sequencing. The increased data available from organellar genomes have been valuable for phylogenetic inference at the higher taxonomic levels (phyla and classes) in red algae (Janouškovec et al., 2013; Lee et al., 2015,

Abbreviations: bs, bootstrap; gDNA, genomic DNA; LCB, locally collinear block; ML, maximum likelihood; nt, nucleotides.

2016; Muñoz-Gómez et al., 2017; Yang et al., 2016). Additionally, studies at lower taxonomic levels (orders and families) of red algae have also advanced due to increased data provided by organellar genome sequencing (Costa et al., 2016; da Lemes Silva et al., 2025; Díaz-Tapia et al., 2019; Iha et al., 2018; Lee et al., 2018).

Complete plastid genome data have been used in an array of studies focused on phylogeny, structural rearrangements, and evolution of red algae (Borg et al., 2023; Costa et al., 2016; Lee et al., 2016; San et al., 2025; van Beveren et al., 2022). The conserved structure of plastid genomes in red algae along with their predominantly uniparental inheritance have made plastids the main targets for understanding the evolutionary relationships of Rhodophyta (Costa et al., 2016; Janouškovec et al., 2013). In addition, red algal plastid genomes are particularly interesting because they contain the largest gene complement among all known plastids, are compactly organized, and are slow evolving compared with other plastid-containing lineages (Janouškovec et al., 2013).

Red algae have plastid genomes that vary greatly in size, especially in the early divergent subphyla. The plastid genome sizes typically range from 150,000 nucleotides (nt) in *Cyanidioschyzon merolae* (Cyanidiophyceae) to 259,000 nt in *Porphyridium sordidum* (Porphyridiophyceae; van Beveren et al., 2022). Notably, some members of Proteorhodophytina have the largest plastid genomes sequenced to date, from 205,000 to 1,127,000 nt (Muñoz-Gómez et al., 2017). However, the highly morphologically diverse and species-rich class Florideophyceae of the Eurhodophytina have plastid genomes with a much narrower size reported range (167,000–195,000 nt; Lee et al., 2016; van Beveren et al., 2022).

The number of protein-coding genes in red algae is also the highest among algal groups (184–235; Lee et al., 2016; van Beveren et al., 2022). Plastid genomes of red algae not only have the highest gene content among eukaryotes with primary plastids (Archaeplastida), but their plastid genomes are also the most conserved (Janouškovec et al., 2013; Lee et al., 2016). Plastid genomes are highly suitable for phylogenomic studies, as they are present in multiple copies per cell, making sequences easily obtained from genomic DNA extractions. Additionally, they appear to contain sufficient phylogenetic signal to resolve relationships across various taxonomic levels (Costa et al., 2016; Iha et al., 2018; Muñoz-Gómez et al., 2017). Studies using multiple genes or phylogenomic data have shown that addressing individual orders of Nemaliophycidae can elucidate relationships within and between closely related groups (Costa et al., 2016; Díaz-Tapia et al., 2019; Lam et al., 2016; Paiano et al., 2017).

The strictly freshwater orders Batrachospermales, Thoreales, and Balbianiales are classified within the subclass Nemaliophycidae (Lam et al., 2016; Vis & Necchi, 2021). The order Batrachospermales (Rhodophyta) was previously divided into three families (Batrachospermaceae, Lemaneaceae, and Psilosiphonaceae, Pueschel & Cole, 1982; Sheath et al., 1996) but is currently treated as having a single family, Batrachospermaceae (Entwisle et al., 2009), with 23 genera currently recognized (Jayalakshmi & John, 2023; Necchi Jr et al., 2024; Vis & Necchi, 2021). The Batrachospermales is the most diverse group in terms of number of taxa, morphology, and reproductive characters among the exclusively freshwater orders of red algae (Entwisle et al., 2009; Kumano, 2002; Lam et al., 2016; Vis & Necchi, 2021). Although the order has been well supported in all phylogenetic analyses (Entwisle et al., 2009; Lam et al., 2016; Paiano et al., 2017; Vis & Necchi, 2021), the infraordinal classification is not yet well supported. Phylogenetic analyses have revealed two lineages containing the same set of genera in single gene (Entwisle et al., 2009), multigene (Lam et al., 2016), and (most recently) in genomic analyses (da Lemes Silva et al., 2025; Paiano et al., 2017, 2018). These two lineages may have taxonomic significance in the infraordinal rank, potentially allowing for the recognition of more than one family. A robust phylogeny, however, and an analysis of character evolution are needed to assess morphological features that can be potentially applied to recognize each clade as a family.

Despite recent advances in the study of organellar genomes, plastid genome data for Batrachospermales remain limited. Paiano et al. (2017) generated seven plastid genomes of taxa within the order, but each was related distantly to the other. Two other studies produced a single plastid genome each, and there were a few others without publications, bringing the total to 12 available in GenBank (Cho et al., 2018; Evans & Vis, 2020; Nan et al., 2017; Sayers et al., 2020). Crowell et al. (2025) recently expanded this dataset by focusing on seven closely related genera, demonstrating that whole plastid genome data can represent phylogenetic relationships among these taxa with high statistical support. In addition, genera with similar morphological characters were shown to be the most closely related (Crowell et al., 2025).

Given the well-supported phylogeny among a small number of genera of previous studies, a more expansive approach including the majority of the genera in the Batrachospermales was undertaken. The research goals were as follows: to document the structural characteristics of the plastid genomes in the Batrachospermales for comparison with the other red algal orders in the Nemaliophycidae and to infer the phylogenetic relationships of the genera using taxon sampling that represents a broad range of vegetative and reproductive morphology within the Batrachospermales.

MATERIALS AND METHODS

Taxon sampling

A total of 18 plastid genomes were generated from 11 genera including seven genera without previously published data (Table S1). When added to previously published data for the order, the data set included 40 plastid genomes for 38 species from 18 genera, including genera representative of the wide variety of vegetative and reproductive characters of the Batrachospermales (Table 1). Each live specimen was divided into two portions: (i) immediately placed in silica gel for DNA sequencing and (ii) preserved via liquid or pressed on herbarium paper and deposited in recognized herbaria (Thiers, 2025; Table S1) for vouchering. Specimens were identified based on current diagnostic characters using recent taxonomic literature (Vis & Necchi, 2021). In addition, the morphological identifications were confirmed by comparing *rbc*L gene sequences with those in GenBank (Sayers et al., 2020).

DNA extraction and sequencing

Three methods for extraction and sequencing were used. For 11 specimens, genomic DNA (gDNA) was extracted using the Qiagen Magattract High Molecular Weight Kit (Qiagen, Hilden, Germany), following the manufacturer's protocol. The gDNA was quantified using a Qubit 2.0 fluorometer (ThermoFisher Scientific, Waltham, Massachusetts, United States) and the Quant-it dsDNA High-Sensitivity Assay kit. Quality checking of gDNA was conducted in a TapeStation 4200 system (Agilent, Santa Clara, California, United States). Libraries were prepared with the Illumina DNA prep protocol, and sequencing was performed on an Illumina NextSeq 2000 platform (Illumina, San Diego, California, United States) with paired-end sequencing producing 2 × 150 bp reads. For *Montagnia australis*, *Paludicola communis*, and *Virescentia viride-americana*, the tissue was ground in liquid nitrogen, and gDNA was extracted using the Nucleospin Plant II kit (Macherey–Nagel, Düren, Germany), following the manufacturer's protocol. The gDNA quantity and quality was assessed using a Qubit 3.0 fluorometer (ThermoFisher Scientific, Waltham, Massachusetts, United States). Libraries of 200–450 nt fragments were completed using the New England BioLabs NEBNext Ultra II FS DNA Library Kit following the protocol for inputs ≤100 ng and were sequenced using the Illumina MiSeq platform (Illumina, San Diego, California, United States) with paired-end sequences producing 2 × 150 bp reads. For *Nothocladus kraftii*, *N. ranulifer*, *N. theaquus*, and *Notohesperus serendipidus*, the material was homogenized in a bead-beater, and a modified CTAB extraction protocol with polyvinylpyrrolidone-10 was utilized (Schenk et al., 2023). The gDNA quantity and quality were assessed using a NanoDrop ND-1000 spectrophotometer (ThermoFisher Scientific, Waltham, Massachusetts, United States). Sequencing of these samples was completed by SeqCenter (Pittsburgh, Pennsylvania, United States) on the Illumina NovaSeq X Plus using libraries of 280 nt fragments, generating paired-end sequences of 2 × 150 bp reads with an Illumina DNA Prep protocol.

Plastid genome assembling and annotation

For 14 samples, the quality of trimmed Illumina reads was checked with FastQC (v0.12.1; Andrews, 2016). Illumina raw reads were trimmed with Trimmomatic version 0.39 (Bolger et al., 2014) to remove adapters and low-quality reads, with the following parameters: ILLUMINACLIP: adapters.fa: 2:30:10; SLIDINGWINDOW: 4:20; MINLEN: 36. After trimming, the clean reads were used for the de novo plastid genome assembly. Three assemblers were used depending on the species. NOVOPlasty version 4.3.1 (Dierckxsens et al., 2017) was used with the *rbc*L gene sequence from closely related genera as the seed sequence for assembly, with a k-mer size of 29 and an insert size of 300. GetOrganelle version 1.5.1c (Jin et al., 2020) was run with k-mers 21, 45, 65, 85, and 105, along with the parameters -R 100 and -F other_pt, using closely related plastid genomes as seed references. SPAdes version 3.15.5 (Nurk et al., 2017; Prjibelski et al., 2020) was also employed. The resulting scaffolds from SPAdes were sorted using tBLASTn (e-value: 1e-10) against a database of plastid genomes from the same genus or, when unavailable, from closely related genera within Batrachospermales, to identify plastid genome-related scaffolds. Plastid genome assemblies obtained with GetOrganelle and SPAdes were visualized and manually inspected using Bandage version 0.8.1 (Wick et al., 2015).

For *Nothocladus kraftii*, *N. ranulifer*, *N. theaquus*, and *Notohesperus serendipidus*, paired-end reads were trimmed using the trim ends function in Geneious Prime 2024.0.7 with default parameters, and the first 15 million were used for a de novo assembly using low-sensitivity parameters in Geneious Prime 2024.0.7 (Biomatters, www.geneious.com). The plastid genome of *Batrachospermum gelatinosum* was used as a reference to identify contigs containing plastid sequences. These plastid contigs were trimmed and de novo assembled. To close gaps in the assembled sequence, contigs were extended by iteratively mapping reads with low sensitivity and a minimum mapping quality of 30 until complete plastid genome sequences could be circularized.

For all plastid genomes, annotation was performed using MFannot (Beck & Lang, 2010) or Geneious Prime

TABLE 1 Character states of genera of Batrachospermales within the two lineages shown in the phylogenetic analyses.

		Carpogonial branch		Carpogonium		Carposporophyte			
Genus	**Thallus construction**	**Length[a]**	**Orientation**	**Relative length[b]**	**Symmetry**	**Arrangement**	**Origin**	**Position**	**Size[c]**
Lineage 1									
Batrachospermum	Bead-like, no outer cortex	Long	Straight	Short	Symmetric	Compact	External	Pedunculate	Small
Lemanea	Pseudoparenchymatous, with outer cortex	Short	Straight	Short	Putatively symmetric	Compact	Inside thallus	Inside thallus	Small
Lympha	Bead-like, no outer cortex	Short	Straight	Short	Symmetric	Compact	External	Axial	Large
Nocturama	Bead-like, no outer cortex	Predominantly long	Straight	Short	Symmetric	Compact	External	Pedunculate	Small
Nothocladus section *Australasicus*	Bead-like, no outer cortex	Short or long	Straight	Short or long	Symmetric	Compact or diffuse	External	Pedunculate	Small or large
Nothocladus section *Kraftii*	Bead-like, no outer cortex	Long	Straight	Short	Symmetric	Compact	External	Pedunculate	Small
Nothocladus section *Theaquus*	Bead-like, no outer cortex	Short	Straight	Short or long	Symmetric	Compact	External	Axial	Large
Paralemanea	Pseudoparenchymatous, with outer cortex	Short	Straight	Short	Putatively symmetric	Compact	Inside thallus	Inside thallus	Small
Sheathia	Bead-like, no outer cortex	Long	Straight	Short	Symmetric	Compact	External	Pedunculate	Small
Sirodotia	Bead-like, no outer cortex	Short	Straight	Short or long	Asymmetric	Diffuse	External	N/A	N/A
Torularia	Bead-like, no outer cortex	Short	Straight	Short	Symmetric	Compact	External	Axial	Large
Tuomeya	Pseudoparenchymatous, with outer cortex	Short	Contorted or curved	Short	Asymmetric	Compact	Inside thallus	Axial	Large
Volatus	Bead-like, no outer cortex	Short	Contorted or curved	Short or long	Asymmetric	Compact	External	Axial	Large
Lineage 2									
Acarposporophycos	Bead-like, no outer cortex	Short	Straight	Short	Symmetric	N/A	N/A	N/A	N/A
Kumanoa	Bead-like, no outer cortex	Short	Contorted or curved	Short or long	Asymmetric	Compact	External	Axial	Large
Montagnia	Bead-like, no outer cortex	Short	Straight	Short	Symmetric	Compact	External	Pedunculate	Small
Notohesperus	Bead-like, no outer cortex	Short	Straight	Short	Symmetric	Compact	External	Axial	Large
Paludicola	Bead-like, no outer cortex	Short	Straight	Long	Symmetric	Compact	External	Axial	Large
Psilosiphon	Pseudoparenchymatous, with outer cortex	–	–	–	–	–	–	–	–
Virescentia	Bead-like, no outer cortex	Short	Straight	Long	Symmetric	Compact	External	Axial	Large

Note: Characteristics not present in a genus are represented by "N/A" (not applicable), and if unknown, represented by "–."

[a]Carpogonial branch: short, less than one-third of the whorl diameter; long, greater than or equal to one-third of the whorl diameter; short carpogonial branches are typically <40 μm in length, whereas long carpogonial branches are typically ≥40 μm in length.

[b]Carpogonium relative length: short, less than 1.5 times shorter than the carpogonial branch; long, greater than or equal to two times longer than the carpogonial branch. Short carpogonia are typically ≤35 μm in length, whereas long carpogonia are typically >35 μm in length.

[c]Carposporophyte size: small, less than half of the whorl diameter; large, greater than half of the whorl diameter.

2024.0.7. Annotations generated in MFannot were carefully reviewed and manually refined in Geneious Prime 2024.0.7 using the Find ORFs function. For plastid genomes annotated directly in Geneious Prime, all rRNA and protein-coding genes were identified by extracting sequences from the published plastid genome of *Batrachospermum gelatinosum* (PX060844, Crowell et al., 2025). A second RNA operon, when present, was identified using the find repeats function in Geneious Prime with a minimum repeat length of 100 nt. If a second RNA operon was present, the large single copy (LSC) and small single copy (SSC) regions were annotated. The tRNAs were identified with tRNAscan-SE version 2.0.7 annotator in GeSeq with default parameters (Chan & Lowe, 2019). Plastid genome maps were generated in Geneious Prime 2024.0.7 (Biomatters, www.geneious.com).

To assess the plastid genome structure and gene content within the Batrachospermales, the plastid genome annotations for the 20 previously published plastids were reviewed and updated as needed. In GenBank there were plastid data available for members of the Acrochaetiales, Balbianiales, Nemaliales, Palmariales, and Thoreales that could have been used as a comparison with the Batrachospermales. Representative plastid genomes from these orders were also reviewed and updated for comparison (Table 2 and Table S2). Synteny among Batrachospermales plastid genomes was investigated via whole plastid genome alignment with the progressiveMauve algorithm version 2.4.0 (Darling et al., 2010) within Geneious Prime using automatically calculated seed weights and automated calculation of locally collinear block (LCB) scores.

Phylogenomic analyses

A total of 40 Batrachospermales sequences were used in the phylogenetic analyses. These plastid genomes included two each for *Kumanoa mahlacensis* and *Sheathia dispersa* and one each for 38 species representing 18 genera. For the outgroup, the following plastid genomes were used: *Acrochaetium subsecundum* (MH026107), *Balbiania investiens* (MH026108), *Nemalion* sp. (LT622871), *Palmaria palmata* (KX284726), and *Thorea hispida* (KX284714; Table 2). We used five outgroup taxa for two reasons: (1) preliminary analyses indicated that using more taxa increased branch support, and (2) the order that is most closely related to the Batrachospermales is unknown, thus having representatives of the five other orders within Nemaliophycidae with data available seemed most appropriate.

A conservative approach was taken and only genes that were present in all taxa or only missing in one taxon were considered for phylogenetic analyses. Genes missing in a single taxon were retained for analyses to maximize data from plastid genomes while still minimizing the risk of bias in the phylogenetic inference. Alignment of the individual genes was conducted in Geneious Prime using the MUSCLE v5.1 algorithm (Edgar, 2022).

Phylogenetic analyses were conducted using the genes that readily aligned, including 118 genes present in all taxa and eight genes that were present in 44 of the 45 taxa. These 126 genes were concatenated using Alignment Manipulation and Summary (AMAS; Borowiec, 2016). The final alignment was 89,919 nt in length. The best-fit model, GTR + F + R6, was determined for the concatenated alignment using ModelFinder in IQ-Tree version 2.3.5 (Kalyaanamoorthy et al., 2017; Minh et al., 2020). A maximum likelihood (ML) tree with 1000 ultrafast bootstraps (Hoang et al., 2018) was generated with the concatenated alignment partitioned by gene and codon in IQ-Tree 2.3.5.

Morphological character assessment

Morphological characters for the 18 genera under investigation in the Batrachospermales were compiled from the literature (Vis & Necchi, 2021, and references therein). Within the Batrachospermales, the gametophyte thallus structure; the carpogonia and carpogonial branch morphology and size; and the carposporophyte structure, size, and placement were considered as taxonomically important. Typically, a suite of these characters distinguishes the genera from one another.

RESULTS

DNA extraction and sequencing

Three extraction and sequencing methods were used to produce the plastid genomes in this study. For species sequenced on Illumina NextSeq 2000 platform, the numbers of paired-end reads were as follows: *Acarposporophycos brasiliensis* (8,450,584), *Kumanoa capensis* (54,966,038), *K. equisetoidea* (13,746,414), *K. procarpa* (15,863,342), *Nocturama novamundensis* (22,122,012), *Paludicola aquanigra* (4,602,468), *P. diamantinensis* (7,988,020), *Psilosiphon scoparius* (17,193,174), *Sheathia dispersa* (17,792,160), *S. involuta* (19,356,674), and *Torularia puiggariana* (52,463,408). For species sequenced on Illumina MiSeq platform, the number of paired reads is as follows: *Montagnia australis* (14,616,660), *P. communis* (15,265,102), and *Virescentia viride-americana* (11,111,410). For samples sequenced on an Illumina NovaSeq X Plus, the number of reads was as follows:

TABLE 2 Overview of plastid genome characteristics generated in this study (in bold) and previously published for members of the Batrachospermales and other Nemaliophycidae orders.

Species	Plastid genome size (nt)	% GC content	Protein-coding genes	YCF	ORF	tRNA	RNA operon	Intron	Coverage (mean)	GenBank accession number	References
Batrachospermales											
Acarposporophycos brasiliensis	183,356	30.6	196	18	5	31	1	1	50	PX373572	This study
Batrachospermum gelatinosum	188,711	29.5	201	20	6	32	2	1	855	PX060844	Crowell et al. (2025)
B. naiadis	188,526	29.9	201	20	6	32	2	1	2390	PX060845	Crowell et al. (2025)
B. qujingense[a,b]	184,902	29	201	20	6	29	2[c]	1	–	MN905507	Unpublished
Kumanoa ambigua[a]	182,932	28.2	201	21	6	31	1	1	345	MG252485	Paiano et al. (2018)
K. americana[a]	184,025	29.3	201	21	6	31	1	1	215	KX284725	Cho et al. (2018)
K. capensis	184,267	29.2	201	20	7	32	1	2	103	PV564056	This study
K. equisetoidea	184,008	29.3	202	21	7	31	1	2	352	PX516260	This study
K. mahlacensis[a]	181,368	29.8	199	21	6	31	1	1	176	MG252486	Paiano et al. (2018)
K. mahlacensis	183,893	29.6	199	19	7	31	1	1	–	MK641509	Unpublished
K. procarpa	184,368	29.4	199	19	6	31	1	2	448	PX109641	This study
Lemanea borealis	188,030	30.8	200	19	6	32	2	1	614	PX060846	Crowell et al. (2025)
L. occidentalis	188,242	30.8	200	19	6	32	2	1	2275	PX060847	Crowell et al. (2025)
Lympha mucosa[a,d]	≥189,825	29.5	198	19	5	32	2	2	146	MN509464	Evans and Vis (2020)
Montagnia australis	179,420	28.4	193	17	5	30	1	1	65	PX147381	This study
M. macrospora	176,626	28.1	192	18	6	33	1	1	478	MG252483	Paiano et al. (2018)
Nocturama novamundensis	185,426	28.2	200	21	5	31	1	2	40	PX171394	This study
Nothocladus kraftii	184,530	28.8	200	19	6	30	1	1	999	PX672968	This study
N. ranulifer	183,398	29.3	199	20	5	30	1	1	3875	PX672969	This study
N. theaquus	187,490	29.1	199	20	6	31	2	1	12,810	PX672970	This study
Notohesperus serendipidus	183,582	28.3	193	19	4	30	1	1	19,480	PX672971	This study
Paludicola aquanigra	179,217	28.5	194	18	4	31	1	2	310	PX373573	This study
P. communis	183,091	28.6	197	20	5	30	1	1	966	PX373574	This study

(Continues)

TABLE 2 (Continued)

Species	Plastid genome size (nt)	% GC content	Protein-coding genes	YCF	ORF	tRNA	RNA operon	Intron	Coverage (mean)	GenBank accession number	References
P. diamantinensis	181,653	28.7	194	18	5	31	1	2	137	PX421651	This study
Paralemanea sp.[a]	187,837	31	201	19	6	28	2	1	364	MG252487	Paiano et al. (2018)
Pr. blumii	187,783	30.8	201	20	6	28	2	1	1320	PX060848	Crowell et al. (2025)
Pr. grandis[d]	≥183,183	30.3	201	20	6	28	2	1	210	PX060849	Crowell et al. (2025)
Psilosiphon scoparius	182,178	30.9	197	20	5	31	1	1	70	PX516257	This study
Sheathia dispersa[e]	179,671	29.1	196	18	5	31	1	1	1708	MG252488	Paiano et al. (2018)
S. dispersa	183,683	29.2	198	18	5	30	1	1	361	PX516258	This study
S. involuta	188,048	29.7	198	18	5	32	2	1	874	PX516259	This study
S. longipedicellata[a,f]	187,354	29.9	200	19	5	31	2	1	–	KY033529	Nan et al. (2017)
Sirodotia delicatulaformis[a]	185,489	29.2	200	20	5	32	2[c]	1	1984	MG252489	Paiano et al. (2018)
Si. suecica	186,915	29.6	200	19	6	32	2	1	6536	PX060850	Crowell et al. (2025)
Torularia puiggariana	186,712	29.1	199	20	5	32	2	1	284	PX421652	This study
Tuomeya americana	189,523	29.3	200	20	5	32	2	1	2600	PX060851	Crowell et al. (2025)
Virescentia viride-americana	184,608	28.6	199	20	4	30	1	1	970	PX421653	This study
V. viride-brasiliensis[a]	171,682	28.5	189	17	3	34	1	1	961	MG252484	Paiano et al. (2018)
Volatus carrionii	188,856	29.1	200	19	6	32	2	1	502	PX060852	Crowell et al. (2025)
V. personatus[d]	≥183,580	28.6	200	19	6	29	2[c]	1	1858	PX060853	Crowell et al. (2025)
Acrochaetiales											
Acrochaetium secundatum	192,311	32.0	206	24	6	33	2	2	–	MH026107	Evans et al. (2019)
Balbianiales											
Balbiania investiens	186,778	34	199	21	5	33	2	0	562	MH026108	Evans et al. (2019)

TABLE 2 (Continued)

Species	Plastid genome size (nt)	% GC content	Protein-coding genes	YCF	ORF	tRNA	RNA operon	Intron	Coverage (mean)	GenBank accession number	References
Nemaliales											
Galaxaura rugosa	181,215	29.6	204	24	5	31	1	1	327	LT622865	Costa et al. (2016)
Liagora harveyana	182,933	33.9	204	23	5	31	1	1	3239	LT622869	Costa et al. (2016)
Nemalion sp.	182,930	35.5	202	24	4	31	1	1	3297	LT622871	Costa et al. (2016)
Scinaia undulata	183,795	35.9	206	24	4	31	1	1	1479	LT622873	Costa et al. (2016)
Palmariales											
Palmaria palmata	192,960	33.9	204	23	5	33	2	2	343	KX284726	Cho et al. (2018)
Thoreales											
Thorea hispida	175,193	28.3	193	19	3	30	1	2	1003	KX284714	Cho et al. (2018)

Abbreviations: ORF, open reading frame; YCF, Hypothetical chloroplast open reading frame.

[a]Statistics reflect updated annotations made in this study.

[b]Identification updated from *Batrachospermum* sp. to *B. qujingense* (Fang et al., 2020).

[c]Assumed to be two operons due to at least one RNA gene duplicate.

[d]Incomplete chloroplast genome.

[e]Identification updated from *Sheathia arcuata* to *S. dispersa* (Vis et al., 2020).

[f]Identification updated from *Sheathia arcuata* to *S. longipedicellata* (Vis et al., 2020).

N. kraftii (105,879,318), *N. ranulifer* (125,797,194), *N. theaquus* (160,756,500), and *Notohesperus serendipidus* (196,152,700).

Plastid structure and genome arrangement

The 18 newly sequenced plastid genomes were fully assembled, annotated, and mapped as circular molecules (Figures S1–S18), bringing the total number of Batrachospermales plastid genomes to 40. The structure for all Batrachospermales plastid genomes was similar in size, number of genes, and GC content (Table 2). The Batrachospermales differed by <20 kb in size, ranging from 171,682 nt in *Virescentia viride-brasilensis* to >189,825 nt in *Lympha mucosa* (mean = 184,715 ± 3519). The number of protein-coding genes ranged from 189 in *V. viride-brasilensis* to 202 in *Kumanoa equisetoidea* (mean = 199 ± 3). Similarly, the GC content ranged from 28.1 in *Montagnia macrospora* to 31.0 in *Paralemanea* sp. (mean = 29.3 ± 0.8). The three *Paralemanaea* species had the lowest number of tRNAs (28), and *V. viride-brasilensis* had the highest, with 34 (mean = 31 ± 1.3). Among the Batrachospermales, the presence of one or two RNA operons was variable across taxa (Table 2). Approximately half of the taxa had two RNA operons, while the remainder lacked this feature. In taxa with two RNA operons, the plastid genome had a quadripartite structure. Both the previously published and new plastid genomes of *Sheathia dispersa* had two RNA operons; both plastid genomes of *Kumanoa mahlacensis* had only one RNA operon.

Within the Batrachospermales, taxa contained either one or two introns as well as notable patterns of gene presence or absence among the genera and species (Table 2, Table S2). For all taxa, an intron in the *chl*B gene was present (Figure S19). An intron was also present in a *trn*M gene when that gene was present in the plastid genome (Figure S20). This gene and intron were present in seven species from four genera (Figure 1, Table S2). The *dfr* gene was absent from the three species of *Paludicola*, whereas the *mla*E gene was missing from all sampled species of *Montagnia* ($n = 2$), *Paludicola* ($n = 3$), and *Virescentia* ($n = 2$; Figure 1, Table S2). The *pbs*A gene had the broadest pattern of loss, missing from the monospecific genera *Acarposporophycos*, *Notohesperus*, and *Psilosiphon*, and in *N. ranulifer* and *N. theaquus* as well as all sampled species of *Kumanoa* (6), *Montagnia* (2), and *Paludicola* (3; Figure 1, Table S2). The *his*S gene was missing from *Torularia puiggariana, N. theaquus*, and *Psilosiphon scoparius* (Figure 1, Table S2). Three local collinear blocks were predicted with the MAUVE algorithm for the 40 plastid genomes (Figure S21).

The Batrachospermalean plastid genomes and the eight representatives of five orders (Acrochaetiales, Balbianiales, Nemaliales, Palmariales, and Thoreales) in the subclass Nemaliophycidae were generally similar in size and the number of protein-coding genes (Table 2). In terms of gene content, *apc*F was present in the other Nemaliophycidae plastid genomes but absent from the Batrachospermales (Figure 1, Table S2). The *grx* gene was only present in *Acrochaetium secundatum* (Acrochaetiales), *Balbiania investiens* (Balbianiales), and *Palmaria palmata* (Palmariales; Table S2). The genes *ycf*34, *ycf*35, and *ycf*46 were absent from the Batrachospermales and *Bl. investiens* (Balbianiales; Table S2). Of the three genes (*dfr*, *pbs*A, and *mla*E) that showed a pattern among taxa in the Batrachospermales, *dfr* and *mla*E were present in the other Nemaliophycidae taxa (Figure 1, Table S2). The *pbs*A gene was missing in *Thorea hispida* (Thoreales) and *Nemalion* sp., but was present in other members of Nemaliales and Nemaliophycidae (Figure 1, Table S2). Either one or two introns were detected in all the other Nemaliophycidae plastid genomes except *Bl. investiens* (Balbianiales), which had none. The seven plastid genomes had the same intron in the *chl*B gene as in all Batrachospermales. The *trn*M gene was present in *A. secundatum* (Acrochaetiales), *Pl. palmata* (Palmariales), and *T. hispida* (Thoreales), and the intron was detected in those taxa, like in the Batrachospermales taxa (Figure 1, Table S2).

Phylogenomics

The ML tree produced from the concatenated alignment recovered a fully supported Batrachospermales ultrafast bootstrap, bs = 100; (Figure 1). In addition, there was generally high support for the relationship among the genera. There were two well-supported (bs = 100) lineages: the first containing the genera *Batrachospermum*, *Lemanea*, *Lympha*, *Nocturama*, *Nothocladus*, *Paralemanea*, *Sheathia*, *Sirodotia*, *Torularia*, *Tuomeya*, and *Volatus* and the second comprising the genera *Acarposporophycos*, *Kumanoa*, *Montagnia*, *Notohesperus*, *Paludicola*, *Psilosiphon*, and *Virescentia* (Figure 1). Following the interpretation that any ultrafast bootstraps below 95 indicate unsupported relationships (Hoang et al., 2018), there were six branches that were not well supported (bs < 95). The placement of *Tuomeya. americana* as sister to *Batrachospermum* spp. had weak support (bs = 79). The genera *Nothocladus*, *Torularia*, *Nocturama*, and *Sheathia* formed a clade, but that clade was only moderately supported (bs = 90). Two other relationships within that clade were unsupported: *Nocturama* sister to *Sheathia* spp. (bs = 89) and *Torularia* sister to *Nothocladus ranulifer* (bs = 55; Figure 1). Among the genera in the second lineage, the sister relationship of

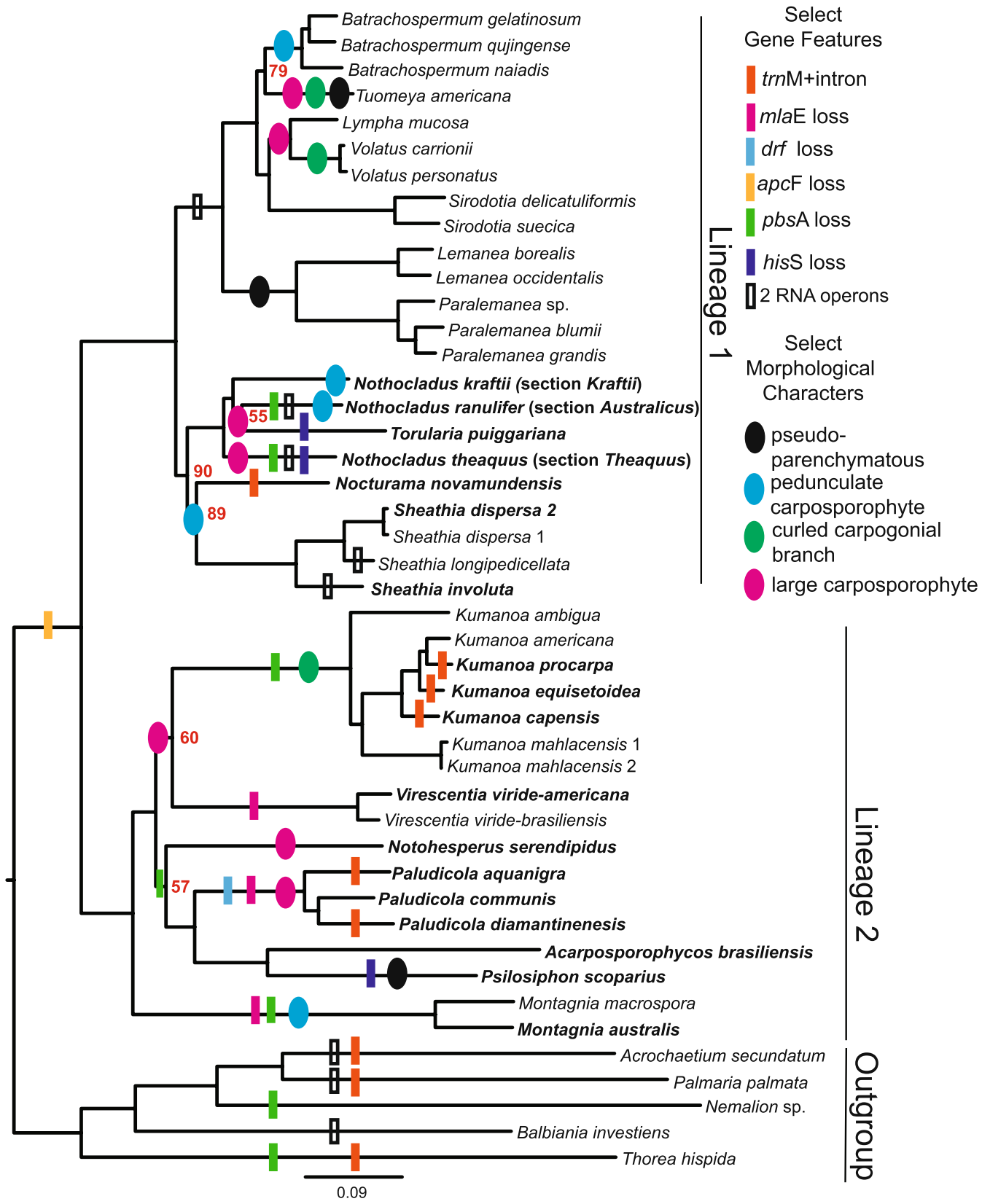

FIGURE 1 Maximum likelihood phylogeny based on the concatenation of 126 plastid genes depicting relationships among genera in the Batrachospermales and five outgroup taxa from the Nemaliophycidae. Newly sequenced taxa in bold. Support values are ultrafast bootstraps (bs); most branches had 100 bs (not shown) and only those <100 bs are provided adjacent to the branch (in red). Most gene features that differed among taxa were added to the phylogeny; not shown are genes only present in the outgroup taxa (*grx*, *ycf*34, *ycf*35, *ycf*45) and the *chl*B gene because it was present in all taxa except *Balbiania investiens*. Select morphological characters were added to the phylogeny to illustrate characters present in both lineage 1 and 2.

Kumanoa and *Virescentia* was unsupported (bs = 60), as well as *Notohesperus serendipidus* as sister to *Paludicola, Acarposporophycos*, and *Psilosiphon* (bs = 57).

Morphological characters

Within the Batrachospermales, characteristics related to the gametophyte thallus, carpogonial branch, carpogonium, and carposporophytes may differentiate the genera but may also be shared among genera (Table 1). A pseudoparenchymatous gametophyte thallus was present in *Tuomeya*, *Lemanea*, and *Paralemanea* from lineage 1 and *Psilosiphon* from lineage 2 of the phylogeny (Figure 1). Three genera, *Tuomeya*, *Volatus* (lineage 1), and *Kumanoa* (lineage 2) had curled carpogonial branches. In lineage 1, pedunculate carposporophytes were common, being present in *Batrachospermum*, *Nocturama*, *Sheathia*, and *Nothocladus* (Sections *Kraftii* and *Australicus*) but only present in *Montagnia* from lineage 2. The character state of large carposporophytes was prevalent in both lineages: *Tuomeya*, *Lympha*, *Volatus*, *Nothocladus* (Sections *Australicus* and *Theaquus*), and *Torularia* in lineage 1 and *Kumanoa*, *Virescentia*, *Notohesperus*, and *Paludicola* in lineage 2 (Figure 1).

DISCUSSION

Plastid genome structure

Our analysis of 40 plastid genomes (including 18 newly sequenced) has expanded variations of members of the Batrachospermales for most characteristics, particularly plastid genome size, number of coding genes, and GC content, but these were all within the range reported for other orders of Nemaliophycidae (Table 2; van Beveren et al., 2022). It is noteworthy that the values for these three plastid genome characteristics were mostly close to the lowest ranges for the other orders: 171,682–189,825 in the Batrachospermales versus 181,215–192,960 nt in the other orders of Nemaliophycidae for plastid genome size, 189–202 versus 199–206 for coding genes, and 28.1%–31.0% versus 29.6%–35.9% for GC content. These data support the pattern observed by Paiano et al. (2017) that Batrachospermales plastid genomes tend to be among the smallest and least gene-dense within the Florideophyceae.

The greatly expanded number of plastid genomes in the Batrachospermales has provided corroboration of previous observations about gene losses from the plastid genomes in the order and new insights. Paiano et al. (2017) documented the lack of *apc*F in the plastid genome of eight members of the order, and this finding was confirmed in the current study with 40 plastid genomes. These researchers (Paiano et al., 2017) suggested that this lack may be interpreted as a gene loss or a gene transfer to the nucleus, or potentially, the function was replaced by another type of allophycocyanin (Chang et al., 2015; Glazer et al., 1997). The new data indicate that *apc*F is likely lacking in the order as a whole, but which mechanism might be responsible for its lack is not clear. The gene *apc*F encodes the allophycocyanin beta subunit, a phycobilin component of the light-harvesting proteins characteristic of red algae (Apt & Grossman, 1993). The hypothesis that a new type of allophycocyanin appeared in the Batrachospermales seems most likely, since a new type of phycocyanobilin-containing phycoerythrin was described for several bluish freshwater red algae species (Glazer et al., 1997), including the "Chantransia" stages of members of Batrachospermales. For most other plastid gene losses, it is not yet possible to determine if they represent true losses or transfers to the nucleus. In addition, for these remaining genes, there is little information in the literature regarding their functional relevance. These losses have involved both members of specific gene families (e.g., *ycf*) and individual genes (e.g., *grx*) that play various roles in algal metabolism. When additional data become available for the nuclear genome, a topic we plan to investigate, a definitive answer as to whether these genes have been lost or transferred may be determined.

Within the Batrachospermales, either one or two RNA operons were present in the plastid genomes. Most taxa in lineage 1 had two operons, and all taxa in lineage 2 had one. Of the three *Nothocladus* species sequenced, *N. kraftii* had one operon whereas *N. ranulifer* and *N. theaquus* had two, but the phylogenetic relationships were not well supported. However, the genus *Sheathia* was monophyletic, and the number of operons differed among species, confirming that this trait can be variable within a genus. In species for which two plastid genomes were available (*Sheathia dispersa* and *Kumanoa mahlacensis*), both plastid genomes had the same number of operons, which suggests the presence of one or two RNA operons is consistent within species, even if it varies among taxa.

Duplicated rRNA operons are widespread in red algae, having been reported in four of the seven classes (Lee et al., 2016), with Compsopogonophyceae, Porphyridiophyceae, and Rhodellophyceae being the exceptions. Within the Florideophyceae, duplicated rRNA operons occur in all subclasses except the Ahnfeltiophycidae, whereas in the Nemaliophycidae, they are absent only in the Thoreales and the genus *Nemalion* (Nemaliales; Table 1; Lee et al., 2016). The presence of single inactivated rRNA operon or partially

inactivated rRNA operons in some Florideophycean species suggests independent losses of one operon copy in these taxa (Lee et al., 2016).

Numerous gene features varied within the Batrachospermales and among the Batrachospermales and the five other Nemaliophycidae orders available for comparison. Of interest was the loss of the *pbs*A gene, which encodes a heme oxygenase enzyme that is activated under iron limitation (Richaud & Zabulon, 1997). Costa et al. (2016) noted its absence from some members of the Nemaliales, and Cho et al. (2018) reported its absence in *Thorea hispida* (Thoreales) and five species of Batrachospermales. The present study confirmed the loss of this gene in all members of *Kumanoa* sequenced to date (six species, seven plastid genomes); the genera *Montagnia* and *Virescentia*; two species of *Nothocladus* (*N. ranulifer* and *N. theaquus*); and a clade containing *Paludicola*, *Acarposporophycos*, *Notohesperus*, and *Psilosiphon*. Cho et al. (2018) hypothesized that absence of this gene may be linked to freshwater habitats in which iron is not limited, and therefore, the gene may be redundant or unnecessary. With many more plastid genomes now available, it appears to be more difficult to link the absence to freshwater habitats, as the absence of the gene has occurred in about half of the Batrachospermales sequenced to date and was present in the freshwater *Balbiania investiens* (Balbianiales). A *trn*M with an intron was present in the Batrachospermalean *Nocturama novamundensis*; in some species of *Kumanoa* and *Paludicola*; and in the representatives of the Thoreales, Acrochaetiales, and Palmariales. The functional role and the importance of its presence or absence are not clear at this stage.

Phylogeny

The current study has produced the most robust phylogeny of the Batrachospermales to date. Represented on the tree are 18 of the 23 genera currently recognized in the order (Vis & Necchi, 2021). In previous studies, genera were supported as monophyletic, but the relationships among the genera often had low support and could vary depending on genes and taxa sampled (Entwisle et al., 2009; Vis & Necchi, 2021).

Although most branches across the phylogeny were highly supported, *Tuomeya* as sister to *Batrachospermum* received low support. The relationship of this genus to others has been difficult to determine. Recently, da Lemes Silva et al. (2025)—using mitochondrial genomes—showed it as sister to the clade of *Sirodotia*, *Lympha*, and *Volatus* but with no support. Crowell et al. (2025) observed the same placement with higher support. The relationships of the monospecific genus *Tuomeya* may continue to be difficult to resolve, as there are no other species to add to the data set. Nevertheless, incorporating mitochondrial or nuclear genome data alongside plastid genome data in analyses could provide additional insights into the placement of *Tuomeya* within the order and improve support for other currently unsupported branches.

The Australasian genus *Nothocladus* is currently divided into four morphologically diverse sections (Entwisle et al., 2016; Vis & Necchi, 2021). The genus is often paraphyletic in phylogenetic analyses using one to many genes, especially in relation to *Torularia* (Entwisle et al., 2016; Vis & Necchi, 2021). In the current study, three sections (*Kraftii*, *Australicus*, and *Theaquus*) were placed in a clade with *Torularia*, rendering the genus paraphyletic. In addition, the larger clade containing *Nocturama* and *Sheathia* was not highly supported. Future research should focus on sequence data from *Nothocladus* section *Nothocladus* as well as on more species of the other sections, to clarify the relationships among the sections and determine if the sections should be elevated to genera.

Within the Batrachospermales clade, there were two well-supported lineages that potentially might have taxonomic significance. These two lineages have been recovered in other phylogenetic studies using one to multiple genes and organellar genomes (da Lemes Silva et al., 2025; Entwisle et al., 2016; Lam et al., 2016; Paiano et al., 2017, 2018). In the present study, lineage 1 contained 11 genera, and lineage 2 has seven genera. The morphological characters used to describe taxa were shared between the two lineages. In summary, there is no single morphological character or combination of characters that could be applied to define each of these two lineages.

We were able to make comparisons in these gene features among the Batrachospermales, which now have been well sampled, with five other orders in the Nemaliophycidae that had representative plastid genomes. However, there are seven other orders in the subclass (Lam et al., 2016; Nan et al., 2017) that so far have no plastid genome sequenced. Therefore, more plastid genomes will be needed in order to describe general gene features as well as phylogenetic inferences in comparison to other orders in the Nemaliophycidae.

AUTHOR CONTRIBUTIONS

Roseanna M. Crowell: Data curation (equal); formal analysis (equal); methodology (equal); writing – original draft (equal). **Nadia M. Lemes Da Silva:** Data curation (equal); methodology (equal); writing – review and editing (equal). **Monica O. Paiano:** Data curation (equal); methodology (equal); writing – review and editing (equal). **Morgan L. Vis:** Conceptualization (equal); funding acquisition (equal); writing – original draft (equal). **Orlando Necchi Jr.:** Conceptualization (equal); funding acquisition (equal); writing – original draft (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.

Figure S1. Gene map of *Acarposporophycos brasiliensis* plastid genome.

Figure S2. Gene map of *Kumanoa capensis* plastid genome.
Figure S3. Gene map of *Kumanoa equisetoidea* plastid genome.
Figure S4. Gene map of *Kumanoa procarpa* plastid genome.
Figure S5. Gene map of *Montagnia australis* plastid genome.
Figure S6. Gene map of *Nocturama novamundensis* plastid genome.
Figure S7. Gene map of *Nothocladus kraftii* plastid genome.
Figure S8. Gene map of *Nothocladus ranulifer* plastid genome.
Figure S9. Gene map of *Nothocladus theaquus* plastid genome.
Figure S10. Gene map of *Notohesperus serendipidus* plastid genome.
Figure S11. Gene map of *Paludicola aquanigra* plastid genome.
Figure S12. Gene map of *Paludicola communis* plastid genome.
Figure S13. Gene map of *Paludicola diamantinensis* plastid genome.
Figure S14. Gene map of *Psilosiphon scoparius* plastid genome.
Figure S15. Gene map of *Sheathia dispersa* plastid genome.
Figure S16. Gene map of *Sheathia involuta* plastid genome.
Figure S17. Gene map of *Torularia puiggariana* plastid genome.
Figure S18. Gene map of *Virescentia viride-americana* plastid genome.
Figure S19. Depiction of the *chl*B gene with an intron that was present in all 40 Batrachospermales plastid genomes.
Figure S20. Depiction of the *trn*M gene with an intron.
Figure S21. Alignment of the 37 Batrachospermales plastid genomes.
Table S1. Herbarium voucher, collection information, and GenBank accession numbers for plastid genomes generated in the present study.
Table S2. Matrix of plastid genes and taxa for this study. Filled cells indicate gene presence.

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