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Phylogenomics, divergence time estimation and trait evolution provide a new look into the Gracilariales (Rhodophyta)

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ABSTRACT

The Gracilariales is a highly diverse, widely distributed order of red algae (Rhodophyta) that forms a well-supported clade. Aside from their ecological importance, species of Gracilariales provide important sources of agarans and possess bioactive compounds with medicinal and pharmaceutical use. Recent phylogenetic analyses from a small number of genes have greatly advanced our knowledge of evolutionary relationships in this clade, yet several key nodes were not especially well resolved. We assembled a phylogenomic data set containing 79 nuclear genes, 195 plastid genes, and 24 mitochondrial genes from species representing all three major Gracilariales lineages, including: *Melanthalia*, *Gracilariopsis*, and *Gracilaria sensu lato*. This data set leads to a fully-resolved phylogeny of Gracilariales, which is highly-consistent across genomic compartments. In agreement with previous findings, *Melanthalia obtusata* was sister to a clade including *Gracilaria s.l.* and *Gracilariopsis*, which were each resolved as well-supported clades. Our results also clarified the long-standing uncertainty about relationships in *Gracilaria s.l.*, not resolved in single and multi-genes approaches. We further characterized the divergence time, organellar genome architecture, and morphological trait evolution in Gracilarales to better facilitate its taxonomic treatment. *Gracilariopsis* and *Gracilaria s.l.* are comparable taxonomic ranks, based on the overlapping time range of their divergence. The genomic structure of plastid and mitochondria is highly conserved within each clade but differs slightly among these clades in gene contents. For example, the plastid gene *pet*P is lost in *Gracilaria s.l.* and the mitochondrial gene *trn*H is in different positions in the genome of *Gracilariopsis* and *Gracilaria s.l.* Our analyses of ancestral character evolution provide evidence that the main characters used to delimitate genera in Gracilariales, such as spermatangia type and features of the cystocarp's anatomy, overlap in subclades of *Gracilaria s.l.* We discuss the taxonomy of Gracilariales in light of these results and propose an objective and practical classification, which is in agreement with the criteria of monophyly, exclusive characters, predictability and nomenclatural stability.

1. Introduction

Gracilariales is a well-supported red algae clade that is nested within Rhodymeniophycidae (Florideophyceae, Rhodophyta) (Verbruggen et al., 2010). Species in this clade include economically important crops, which provide sources of agarans and bioactive compounds with potential pharmaceutical application (Andriani et al., 2016). The order is characterized by its unique reproductive ontogeny, including the absence of auxiliary cells before fertilization, and the gonimoblast not incorporated to the fusion cell during development (Fredericq and Hommersand, 1989). The application of widely used nuclear (Bellorin et al., 2002) and organellar markers (Gurgel and Fredericq, 2004; Lyra

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et al., 2015) in this clade has greatly advanced our knowledge of infrafamilial relationships. Subsequent expanded plastid and mitochondrial gene sampling also resolved deep nodes of the phylogeny (Iha et al., 2018), though with limited taxon sampling. The current morphological circumscriptions of supraspecific taxa (Gurgel et al., 2018) are primarily based on characters that have been demonstrated to be paraphyletic across subclades (Bellorin et al., 2002; Lyra et al., 2015; Lyra et al., 2021).

The controversy regarding taxonomic schemes in Gracilariaceae is primarily focused on the acceptance of *Gracilaria* Greville *sensu stricto* or *sensu lato*, the latter including *Hydropuntia* Montagne. Here, the key morphological character distinguishing *Gracilaria* from *Hydropuntia* is spermatangia type. *Hydropuntia* is characterized by deep and coalescing (*henriquesiana*) spermatangia conceptacles, while *Gracilaria* has shallow (*textorii*) or deep and non-coalescent spermatangial crypts (*verrucosa*) (Fig. S1). Gurgel and Fredericq (2004) previously reinstated the genus *Hydropuntia*, segregating it from *Gracilaria*. Lyra et al. (2015), however, demonstrated that *Hydropuntia* was not monophyletic and that the *verrucosa* spermatangia type is likely an early developmental stage of the *henriquesiana* spermatangia type as previously proposed (Abbott et al., 1991). Both kinds of spermatangia are present in *Gracilaria domingensis* (Kützing) Sonder ex Dickie and *G. baiana* G.M. Lyra, C.F.D. Gurgel, M.C. Oliveira & J.M.C. Nunes (Lyra et al., 2015; Lyra et al., 2016; Lyra et al., 2021).

The currently accepted classification in Gracilariaceae (Gurgel et al., 2018; Guiry and Guiry, 2021) was based on a *rbc*L phylogeny, which recognizes 237 species divided into two subfamilies, two tribes, seven genera, and four subgenera. *Hydropuntia* was again reestablished and, in order to resolve the polyphyly of *Gracilaria*, two new genera, *Agarophyton* Gurgel, J.N.Norris & Fredericq and *Crassiphycus* Guiry, J.N. Norris, Fredericq & Gurgel (Guiry et al., 2018), were segregated from it. The subfamily Gracilarioideae Stizenberger includes two tribes (Gracilarieae Willkomm and Gracilariopsideae Gurgel, J.N.Norris & Fredericq) and comprises more than 90% of the species in the family, distributed in five genera: *Agarophyton*, *Crassiphycus*, *Gracilaria*, *Gracilariopsis*, and *Hydropuntia*, with distributions concentrated in tropical to subtropical areas. The subfamily Melanthalioideae Gurgel, Fredericq & J.N.Norris includes the genera *Curdiea* Harvey and *Melanthalia* Montagne, with most species endemic to Oceania (Gurgel et al., 2018). However, clear diagnostic features for the new or reestablished taxa were not presented, and the descriptions provided were not stable across all the included species (Lyra et al., 2021). The relationships among the clades *Agarophyton*, *Crassiphycus*, *Gracilaria* and *Hydropuntia* remained unsupported in the previous single or multi genes phylogenies, which hindered the understanding of character distribution and the identification of apomorphies in these clades.

Analyses of character evolution have been useful to assess the utility of traits for delineating major taxonomic groups in Rhodophyta (Diaz-Tapia et al., 2019), and can potentially shed light on evolutionary trends in Gracilariales. A recent study using plastid and mitochondrial genomic data of a limited dataset of Gracilariaceae species (Iha et al., 2018) demonstrated the utility of a larger number of genes to increase support in deeper nodes of phylogenies, and also highlighted the importance of macrostructural genomic characters for better delimiting clades of Gracilariaceae. Recently, divergence time analyses were employed to specifically address Gracilariales evolutionary history (Gurgel et al., 2020), suggesting that most of the diversification in *Gracilaria s.l.* occurred during the late Cretaceous and early Paleogene, while *Gracilariopsis* diversified more recently. However, these results were not used to evaluate whether the taxonomic classification of Gracilariales includes comparable ranks considering the divergence time of taxa. The standardization of time of divergence for the recognition of taxonomic ranks has been advocated as a relevant approach for assigning taxonomic ranks across different groups of organisms (Avise and Johns, 1999).

To accomplish the goals of i) understanding the evolution of Gracilariales; ii) better circumscribing supraspecific taxa; and iii) adopting a classification system that reflects its history, we present here three fully-supported phylogenies of Gracilariales, inferred from plastid and mitochondrial genomes, and the largest set of nuclear genes to date. Our dataset and analyses, which also include a calibrated phylogeny, comparison of organellar genomes architecture and analyses of trait evolution, represent the most complete effort to date for a single red algal order. Based on our solid genomic analyses, we propose a taxonomic scheme that respects the criteria of monophyly, character exclusivity (both on morphological and molecular levels) and divergence time across clades, attending also to the principles of nomenclatural stability and practicality.

2. Material and methods

2.1. DNA extractions and library preparation

Taxa included in our sampling represent the main lineages in which the recent circumscription of *Gracilaria s.s.* and the proposition of new genera, infra and suprageneric taxa in Gracilariaceae (Gurgel et al., 2018) were based. Our samples were obtained from silica dried tissues or herbarium material, including the type specimens of *Gracilaria crassissima* (P.Crouan & H.Crouan) P.Crouan & H.Crouan (holotype: NY966805), *Gracilaria pachydermatica* Setchell & Gardner (type: NY3685560) and *Gracilaria textorii* (Suringar) De Toni (isotype: NY937576) (Data S1). We obtained samples of *Curdiea coriaceae* (J.D. Hooker & Harvey) J.Agardh and *C. crassa* A.J.K.Millar, but the DNA library for both samples failed and we could not obtain the genome data for this genus. We extracted DNA using Maxwell® 16 DNA Purification Kit (Promega, Mannheim, Germany). The resultant DNA was quantified on the Qubit® 3.0 Fluorometer using the Qubit® dsDNA HS Assay Kit (Thermo Fisher Scientific Inc, Waltham, USA). The quality of extracted DNA was verified on Agilent 2100 Bioanalyzer with DNA High Sensitivity chip. We first sheared the genomic DNA to 550 bp using the Covaris S-series (Woburn, MA). We then followed (Bentley et al., 2008) to prepare DNA libraries for samples with at least 1 μg of DNA using the TruSeq® DNA PCR-free LT sample preparation kit (Illumina, San Diego, USA). For samples with lower DNA inputs (at least 7 ng of DNA), we used the KAPA HyperPlus Library Preparation Kit (Kapa Biosystems, Wilmington, USA) for library preparation instead. We used Real-Time PCR (BioRad CFX96 Touch, BioRad Laboratories, Hercule, USA) with the NEBNext Library Quant Kit (New England Biolabs, Ipswich, USA) to verify the final concentrations of the libraries. Finally, we sequenced libraries on the Illumina NextSeq 500 (Illumina, Inc.) with 150 bp paired-ended runs at the FAS Center for Systems Biology at Harvard University.

2.2. Nuclear genome assembly and annotation

Raw Illumina reads were gently trimmed with Trimmomatic (parameters: ILLUMINACLIP:IlluminaContaminants.fa:2:30:10 SLIDINGWINDOW:4:10 MINLEN:36) (Bolger et al., 2014). 21mers frequencies were counted using Jellyfish (v.2.2.10) (Marcais and Kingsford, 2011) and submitted to Genomescope v1 (Vurture et al., 2017) to obtain estimates of genome size, repeat content, and ploidy. Trimmed reads were assembled with Megahit (v.1.1.1) (Li et al., 2016), using the default setting. As the tissue samples were collected from the wild and red algae are a known habitat for a variety of organisms, we anticipated that the libraries would be contaminated with exogenous DNA. Thus, we relied on some bioinformatic methods to decontaminate the libraries *in silico*. Our expectation was that the majority of each library would be endogenous DNA with a unique distribution of GC content. Following assembly, we calculated 27mer frequencies and GC content in both the reads and their corresponding assemblies using KAT v2.1.1 (the K-mer Analysis Toolkit) (Mapleson et al., 2017). KAT plots were visually inspected for high-multiplicity clusters to establish boundaries for the

unique portion of the nuclear genomes. We determined filtering thresholds for GC content and 27mer frequency for the 22 genomic libraries displaying clear differentiation at the expected coverage and filtered the assemblies to retain contigs with mean values within the boundaries. Our strategy for filtering out exogenous contigs based on 27mer frequency and GC content worked well for 22 of the assemblies. However, filtering thresholds could not be established for the 11 of the assemblies, as there were no observable boundaries between the 27mer distributions and/or GC content in the plots. This was the case for the libraries sequenced at shallower depth and was likely due to overlap in coverage and/or GC content between contaminant and target DNA. Thus, we also devised a simple taxonomically informed approach for removing contaminated contigs from the 11 assemblies that could not be filtered using the first approach. We assumed that the 22 assemblies that we could successfully filter using the first approach retained only low levels of contamination, that any remaining contaminants still present in those assemblies were unlikely to be shared, and that endogenous contigs in the 11 unfiltered assemblies would share sequence similarity to contigs in the 22 filtered assemblies. Unique taxon identifiers were added to the headers of the contaminant-free contigs for the 22 filtered assemblies and were then concatenated into a single fasta file. A Blast database was constructed from this set of sequences. Next, we aligned all contigs from each of the 11 genome assemblies (queries) that could not be filtered in the first approach to the database of 22 filtered assemblies (targets). We examined the blastn alignments for taxonomic support favoring inclusion. Contigs from the query assemblies were retained if blastn alignments with bit scores of at least 100 were found for a minimum of three taxa in the target database; contigs that did not meet this threshold were removed prior to further analysis.

We annotated the assemblies using three lines of evidence. 12,550 EST accessions from Gracilariales (txid31468) were downloaded from Genbank and aligned to the assemblies using the splice-aware GMAP v5 program (Wu et al., 2016). 27,255 protein accessions from Rhodophyta (txid2763) were downloaded from Genbank. Genome assemblies were aligned to the protein database using Diamond blastx in the –more-sensitive mode. Splice-aware alignments were made using the AAT pipeline r03052011 (Huang et al., 1997) for contig/protein pairs identified by Diamond v0.8.17 (Buchfink et al., 2015). *Ab initio* gene predictions were made with Augustus v3.2.2 (Stanke et al., 2004), using the built-in training for *Galdieria* Merola. Evidence from the ESTs, proteins, and *ab initio* predictions were assigned respective weights of 3, 2, 1, and used as input to Evidence Modeler v1.1.1 (Haas et al., 2008) to produce the final gene annotations.

2.3. Organellar genomes assembly and annotation

We used Trimmomatic to clean and trim low-quality reads and bases from raw read data and BBnorm (Bushnell, 2019) to normalized raw reads to 100x coverage for the DNA libraries with exceptionally high coverage. We assembled the genomes with SPAdes (Bankevich et al., 2012) setting different k-mer sizes (i.e., 22, 33, 55, 77, 99, and 127) and "careful" flag. Mitochondrial and chloroplast assembled contigs were identified using BLASTn (Altschul et al., 1990) searches against a custom-built database containing published organellar genomes from Gracilariaceae. Most of the organellar genomes were identified in a single contig. The genomes split in more than one contig were reassembled using the *de novo* assembly plugin in Geneious to create a consensus contig. We mapped filtered reads against the contigs using Bowtie 2 (Langmead and Salzberg, 2012) to confirm the assembly quality. We confirmed the completeness of the organellar genomes by comparing the architecture and gene synteny with the published Gracilariales genome.

Both mitochondrial and chloroplast genomes were annotated using MFannot (https://megasun.bch.umontreal.ca/cgi-bin/mfannot/mfannotInterface.pl) to find coding sequences (CDS) and ARAGORN (http://www.ansikte.se/ARAGORN/) and RNAweasel (https://megas

un.bch.umontreal.ca/cgi-bin/RNAweasel/RNAweaselInterface.pl) to find RNA and intron sequences. Large and Small ribosomal RNA were checked using the SILVA rRNA database (Quast et al., 2013). We also performed manual inspections and corrections looking for open-reading frames (ORFs) using the ORF finder plugin available in Geneious 9.1.8 (Biomatters, Auckland, New Zealand). Doubtful ORFs were verified using BLASTx on the GenBank website.

2.4. Phylogenetic analyses

For our nuclear dataset, we prepared protein and the corresponding coding sequences (CDS) for every gene annotation. To avoid including un-collapsed alleles, alternative annotated isoforms, and very recently duplicated genes, we clustered CDSs at 98.5% identity using CD-HIT v4.6.6 (Li and Godzik, 2006) for each species, selecting the longest representative for further analyses. Gene clusters were determined by OrthoMCL (Li et al., 2003). Protein pairs were identified via an all-by-all Blast alignment. Orthology and paralogy were predicted using the InParanoid algorithm (Remm et al., 2001) as implemented in OrthAgogue (Ekseth et al., 2014). Homologous clusters were then grouped using the MCL graph-clustering algorithm mcl v14-137 (van Dongen and Abreu-Goodger, 2012).

Gene trees were computed for clusters that: 1) included at least four species with high-quality sequencing libraries, 2) included at least 100 amino acids for each sequence, 3) had a mean of less than five homologous sequences per species, and 4) had a median of less than two sequences per species. Protein sequences were aligned with Muscle v3.8.1551 (Edgar, 2004). High entropy sites in the alignment were identified with TrimAl v1.02 (Capella-Gutierrez et al., 2009). Protein alignments were back translated to codons, masking those identified as high entropy. We retained sequences covering at least 70% of the alignment and confirmed that the alignment still contained at least four species with high-quality sequencing libraries. Aiming to minimize biased missing data and generate robust species tree reconstructions, we selected a subset of 79 genes with at least 27 species (73% taxon completeness) and also requested *Gracilaria cervicornis* to be present in these genes. The specific requirement for the presence of *Gracilaria cervicornis* avoided excessive biased missing data in this species because we found it to be represented in only 2.2% of all genes. Nuclear gene alignments and individual trees are available at https://figshare.com/s/94bd4c3456b5d40c061f. A phylogeny was calculated for each gene cluster using RAxML v8.2.9 (Stamatakis, 2014) under the GTRGAMMA model with 100 bootstraps. All ortholog trees were used for further analyses if the pruning resulted in more than one. We then extracted orthologous sequences from the cluster and repeated the procedure, from filtering through phylogeny building, for each orthologous set. Gene trees were used to build the species tree using the ASTRAL-II coalescent method (Mirarab and Warnow, 2015) (Fig. 1).

We combined available mitochondrial and chloroplast genomes of Gracilariaceae from GenBank with our newly sequenced genomes to generate concatenated protein sequence alignments for mitochondrial and chloroplast genomes, respectively. The resultant matrices contained 24 mitochondrial genes from 37 species (42 specimens; GenBank accession number MZ336076-MZ336099) and 195 plastid genes from 37 species (48 specimens, with 32 new ones; GenBank accession number MZ336044-MZ336075), respectively (Data S1, Fig. 2). These alignments were subsequently generated by MUSCLE v3.8.1551. We performed maximum likelihood (ML) analyses using IQ-TREE 1.6.12 (Nguyen et al., 2015) with 1,000 replicates of ultrafast bootstrap (Minh et al., 2013). The best alignment partition scheme was determined by PartitionFinder (Lanfear et al., 2012) was implemented in IQ-TREE (-m TEST-MERGE). *Rhodymenia pseudopalmata* (J.V.Lamouroux) P.C.Silva was selected as the outgroup for rooting purposes (Kim et al., 2014; Lee et al., 2016). Here, we applied a threshold of 75% bootstrap support to define well-supported clades, which is higher than the widely used 70% bootstrap percentage cut-off associated with 95% confidence that the clade is
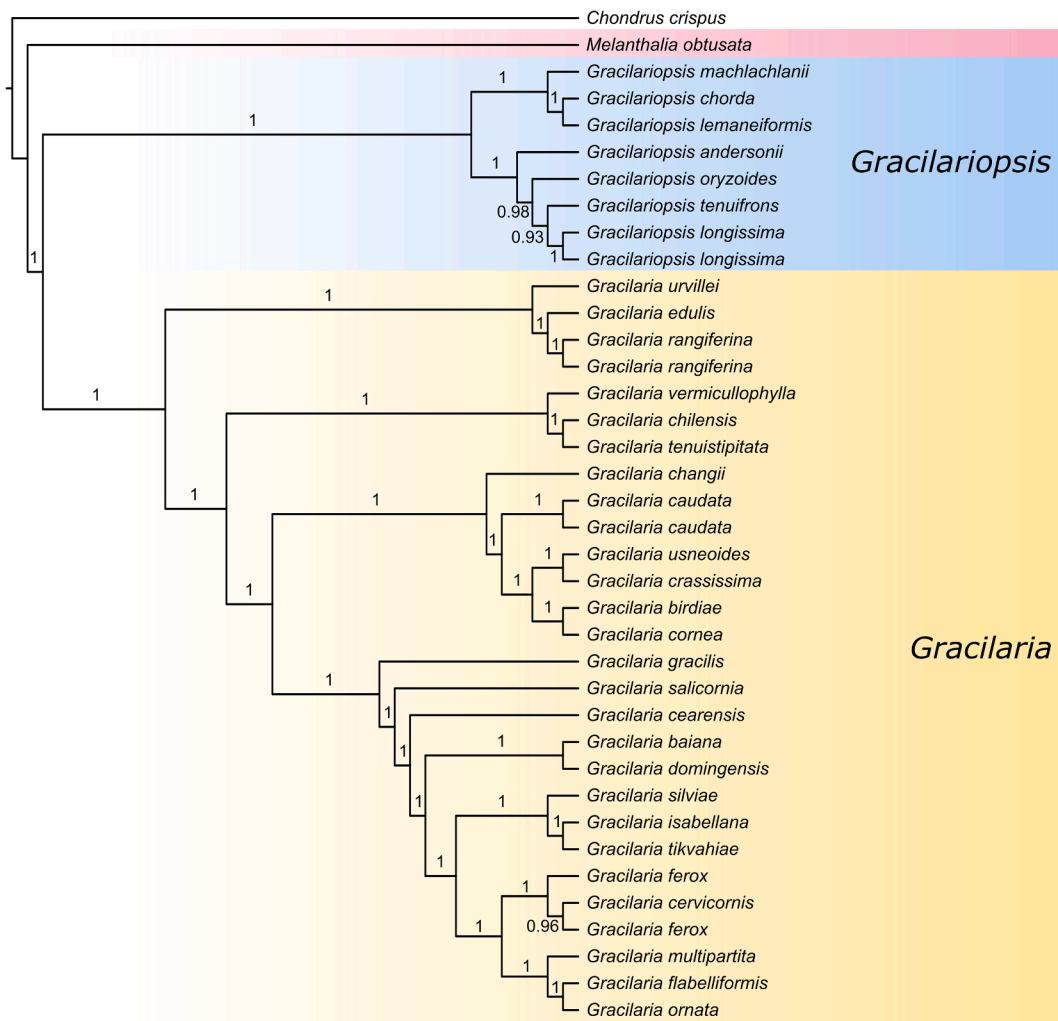
Fig. 1. ASTRAL tree with 79 nuclear genes. Bootstrap support values are indicated on the branches. Main lineages are highlighted in different colors: *Melanthalia* in pink, *Gracilariopsis* in blue, and *Gracilaria sensu lato* in yellow. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

likely real (Hillis and Bull, 1993).

2.5. Divergence time estimation

Our divergence time estimation analysis used 195 chloroplast protein data from 50 samples. We chose the chloroplast dataset because the ML phylogenetic tree is almost fully supported, it has a larger gene content matrix compared to the mitochondrial dataset, and it presents adequate phylogenetic signal (Janouškovec et al., 2013). We performed a time-calibrated Bayesian analysis with PhyloBayes v4.1 (Lartillot and Philippe, 2004) using a fixed topology from the ML analysis described above. Age estimates from four points in the red algal phylogeny presented by (Nan et al., 2017) were used for calibration. These calibration points include the constrained ages of the most recent common ancestor (mrca) of Gracilariales and *Rhodymenia pseudopalmata* at 180–350 million years ago (mya); the mrca of *Gracilariopsis* and *Gracilaria* at 125–220 mya; the mrca in *Gracilaria sensu lato* at 90–172 mya, and the mrca of *Gracilaria chilensis* and *Gracilaria tenuistipitata* was 35–60 mya. Divergence time estimation of Gracilariales was conducted under the log-normal autocorrelated relaxed clock using these constraints to generate a relative molecular dating for *Gracilariopsis*, *Gracilaria sensu lato* and the four genera proposed by (Gurgel et al., 2018).

2.6. Morphological character evolution

We chose five characters that have been previously discussed in the literature for ancestral trait reconstruction: i) thallus shape; ii) spermatangia type; and, in the cystocarp, iii) organization of gonimoblasts and carposporangial chains; iv) presence and position of traversing filaments (also known as nutritive tubular cells) and v) presence of inner pericarp (Table S1, Fig. S1). For the thallus shape category, we grouped the "compressed" and "flattened" thalli in the trait "not-cylindrical", because it is hard to set the threshold between the two shapes.

We constructed a selected ML phylogenetic tree using chloroplast protein data with 30 species representing the currently accepted genera in Gracilariales and information in the literature on the chosen morphological characters. When the morphological character was unknown in a species, we removed this species from the tree using the drop.tip function in R v3.6.0 (R CoreTeam, 2018). The phylogeny was built using IQ-TREE and Phylobayes as described above. The ancestral status of each trait was inferred using the R package Phytools v0.7.70 (Revell, 2012). For each trait, we first determined the best model for trait evolution using AIC criterion. For traits that do not exhibit polymorphism, we used the function fitMk to evaluate the fitness of three models including equal-rate, symmetrical rate and all-rates-different (ARD). For traits that exhibit polymorphic status, which include spermatangial type and traversing filaments, we used the fitpolyMk function
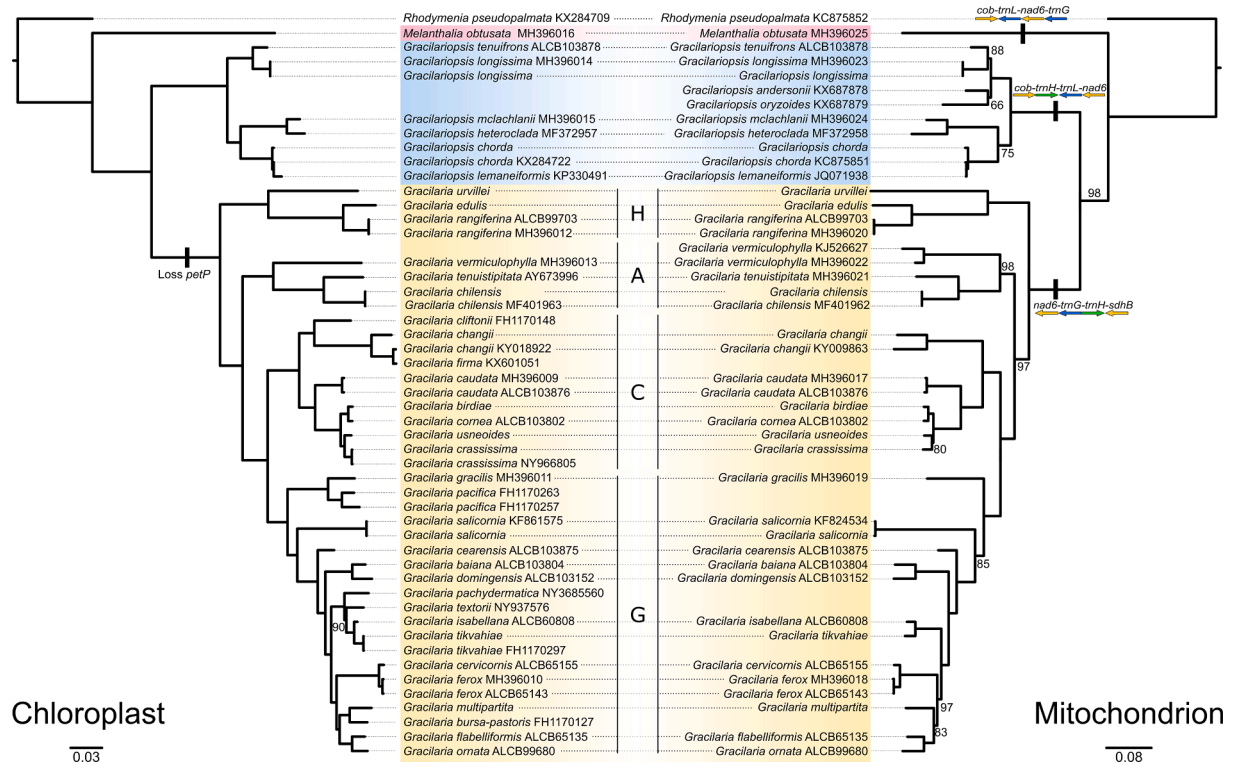
Fig. 2. Organellar phylogenetic trees. On the right, the maximum likelihood (ML) tree is based on 24 concatenated mitochondrial protein sequences. On the left, ML tree is based on 195 concatenated plastid protein sequences. All branches are 100% Bootstrap supported, except when other values are indicated. Main lineages are highlighted in different colors: *Melanthalia* in pink, *Gracilariopsis* in blue, and *Gracilaria sensu lato* in yellow. Genome's architecture features are plotted on the trees. In the chloroplast, the loss of the *petP* gene is indicated in the clade *Gracilaria s.l.* clade. In the mitochondrion, the different positions of *trn*H, between *trn*G and *sdh*B in *Gracilaria s.l.*, and between *cob* and *trn*L in *Gracilariopsis* are indicated. The *trn*H gene is absent in *Melanthalia obtusata*. In the mid section, the genera proposed by Gurgel et al. 2018 are indicated by letters, *Hydropuntia* (H), *Agarophyton* (A), *Crassiphycus* (C) and *Gracilaria s.s.* (G). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

to determine the best model. For "spermatangial type", we ordered the trait transitions as "superficial → textorii → verrucosa → henriquesiana". For "presence and position of traversing filaments", we assessed three alternative transition matrices: "absent → connected to the inner pericarp → connected to the outer pericarp", "absent → connected to the outer pericarp → connected to the inner pericarp", and unordered (Table S1). Finally, the ancestral status reconstruction was carried out using the make.simmap function in Phytools with 100 simulations.

3. Results

3.1. Whole genome sequencing and annotation of nuclear genes

We assembled the nuclear genomes for 35 individuals (Data S1) to the contig level. The mean assembly size is 209,971,090+/-171,104,661 bp. The mean number of contigs per assembly is 410,030+/-493,754 with a mean n50 of 1,197+/-989 bp. The high number of contigs and low n50s are unexpected given that the libraries were prepared from dried tissue, highly contaminated, and sequenced with short reads. While the nuclear assemblies are generally highly fragmented, regions containing protein-coding genes assembled well enough for robust phylogenetic analysis. We annotated 10,947+/-6,172 protein-coding nuclear genes per assembly on average, with a mean length of 342+/-69 amino acids per gene. As a proxy, we count genes as complete if they begin with a methionine and end with a stop codon. On average, 75+/-12% of annotated genes are complete.

3.2. Organellar genome features

The mitochondrial genomes from various Gracilariales lineages were similar in length, GC content and gene content (Data S1). These mitochondrial genomes were found to be 25,340–27,466 bp in length and 25.4–29.1% in GC content. They typically contain between 25 and 27 protein coding genes, three ribosomal genes (*rrl*, *rrs* and *rrn*5), and an intron in the *trn*I gene. Most mitochondrial genomes presented between 22 and 25 tRNA, but *G. edulis* and *G. urvillei* presented 27 and 31 tRNA genes, respectively (Data S1).

Similarly, the length and GC content of the chloroplast genomes were also found to be conserved in *Gracilaria* and *Gracilariopsis*. We obtained fully circularized plastid sequences for all samples except *Gracilaria pacifica* I.A.Abbott (herbarium voucher FH1170263). These plastid genomes were 178,239–190,895 bp in length and had 27.2–29.3% GC contents. They included between 202 and 216 protein coding genes, 31 tRNA (including an intron in *trn*M*)* and three rRNA genes. However, both *G. cornea* and *G. birdiae* lack the *rrn*5 gene (Data S1).

3.3. Phylogenies and divergence time estimation

We obtained three highly consistent and well-supported phylogenies inferred from 79 nuclear genes, 24 mitochondrial genes, and 195 plastid genes, respectively (Figs. 1 and 2). *Melanthalia obtusata* (Labillardière) J. Agardh was placed as sister to the two other major clades in Gracilariaceae, comprising species of *Gracilariopsis* and species currently classified in *Gracilaria* (subgenera *Gracilaria* and *Corallopsis* Gurgel, J.N. Norris et Fredericq), *Hydropuntia* [subgenera *Hydropuntia* and *Polycavernosa* (C.F. Chang et B.-M. Xia) Gurgel, J.N.Norris et Fredericq], *Agarophyton* Gurgel, J.N.Norris et Fredericq and *Crassiphycus* Guiry, J.N.

Norris, Fredericq & Gurgel (as *Crassa* Gurgel, J.N. Norris et Fredericq). All these groups were strongly supported in all phylogenies from different genomes, except for the subgenera *Corallopsis*, which includes *G. salicornia*, *G. gracilis,* and *G. pacifica*, do not form a monophyletic clade in any of our phylogenomics analyses (Fig. 2). The conflicts between our phylogenies involve *Gracilaria ferox*, which was not monophyletic in the nuclear tree, while our organellar trees resolved this species as monophyletic. Also, the parasitic species *Gracilariopsis oryzoides* was sister to *Gp. andersonii* in the mitochondrial tree, but closely related to *Gp. tenuifrons* and *Gp. longissima* in the nuclear tree. Our results resolved the hitherto unclear relationships among inner clades of *Gracilaria s.l.*, placing *Hydropuntia* as its earliest diverging lineage. The *Agarophyton* clade is well-supported as closely related to the sister clades *Crassiphycus* and *Gracilaria s.s.*

The molecular dating analysis (Fig. 3) from the chloroplast protein dataset showed the split between *Gracilariopsis* and *Gracilaria s.l.* during middle Jurassic ~ 169 Mya (199–152 Mya). The range between the mrca of *Gracilariopsis* and *Gracilaria s.l.* lineages overlap, and the origin of each clade occurred between the late Jurassic and the very early Cretaceous, ~160 Mya (187–141 Mya) and ~ 143 Mya (167–129 Mya), respectively. The four currently accepted genera *Gracilaria*, *Hydropuntia*, *Agarophyton* and *Crassiphycus* have similar ages dating from the middle of Cretaceous, ~120 Mya (140–105 Mya), ~111 Mya (132–90 Mya), ~115 Mya (131–102 Mya), and ~ 111 Mya (131–92 Mya), respectively (Fig. 3).

3.4. Organellar genomes architecture

Mitochondrial and plastid genomes were highly conserved in gene synteny and the architecture of the organellar genomes were consistent
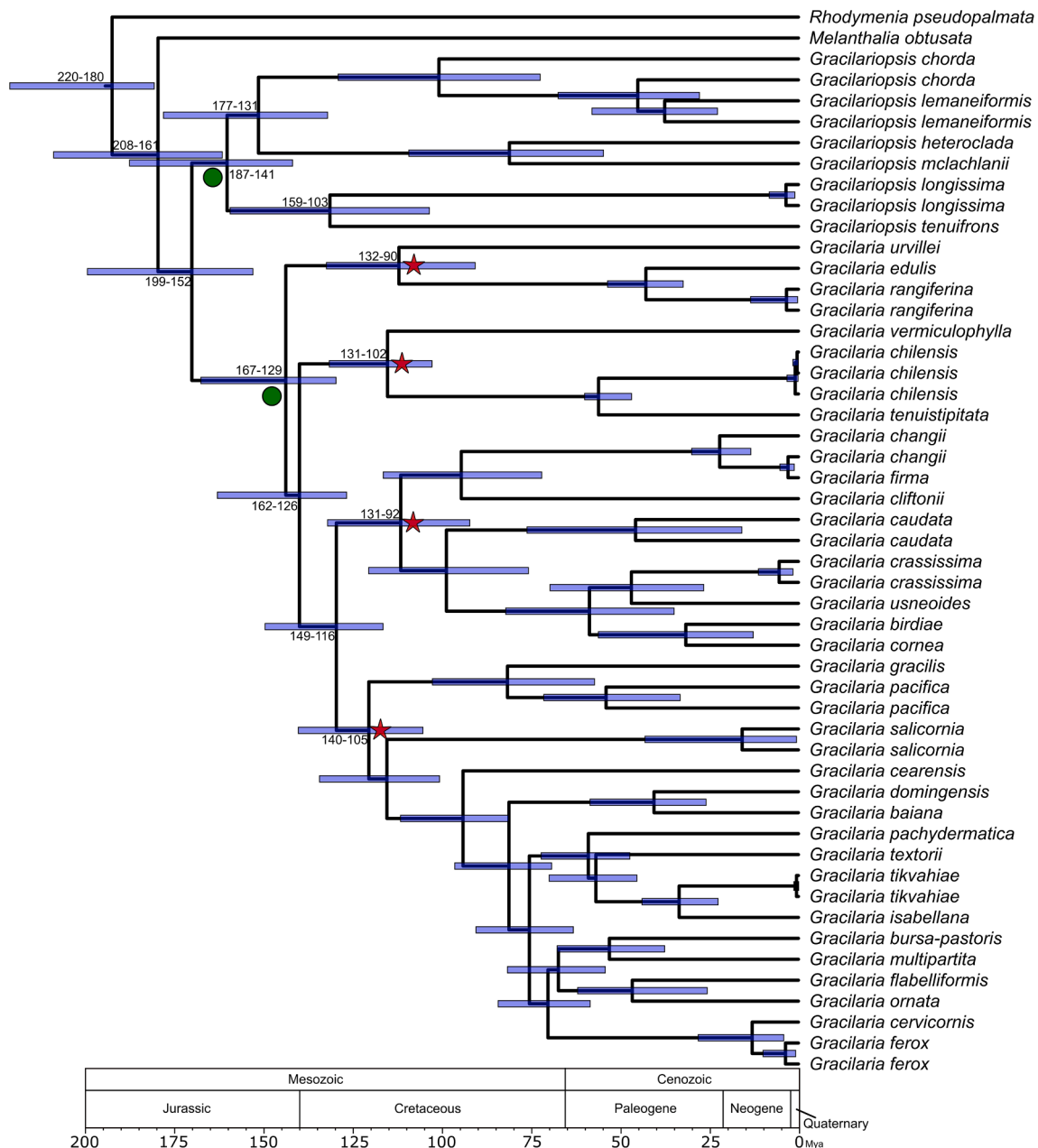
Fig. 3. Bayesian divergence time estimation tree. The scale date is in million years ago (mya). Error bars on node ages are indicated in blue horizontal bars. The green circles indicate the origin of the *Gracilariopsis* and the *Gracilaria sensu lato* lineages. Each red star indicates the origin of the lineages in *Gracilaria sensu lato*, which have been described as genera by Gurgel et al. (2018), from top to bottom: *Hydropuntia*, *Agarophyton*, *Crassiphycus*, and *Gracilaria s.s.* (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

through the evolutionary history of Gracilariales (Fig. 2). Species of *Gracilaria s.l.* lost the cytochrome b6f complex subunit P gene (*pet*P) in the chloroplast genome, while this gene is present in *Gracilariopsis* and *Melanthalia obtusata*. In the mitochondrial genome, the transfer RNA for histidine gene (*trn*H) is located in different positions: between *cob* and *trn*L genes in *Gracilariopsis* and between *trn*G and *sdh*B in *Gracilaria sensu lato*. This gene is absent in *M. obtusata* (Fig. 2).

3.5. Trait evolution

Our trait evolution analyses indicated that ARD model with ordered transition states was suitable for spermatangial type analysis and traversing filaments with the transition stated ordered as "absent → connected to the inner pericarp → connected to the outer pericarp". For the non-polymorphic traits, equal-rates and symmetric models were equally suitable for both thallus shape and the organization of gonimoblasts and carposporangial chains, and ARD was the best model for
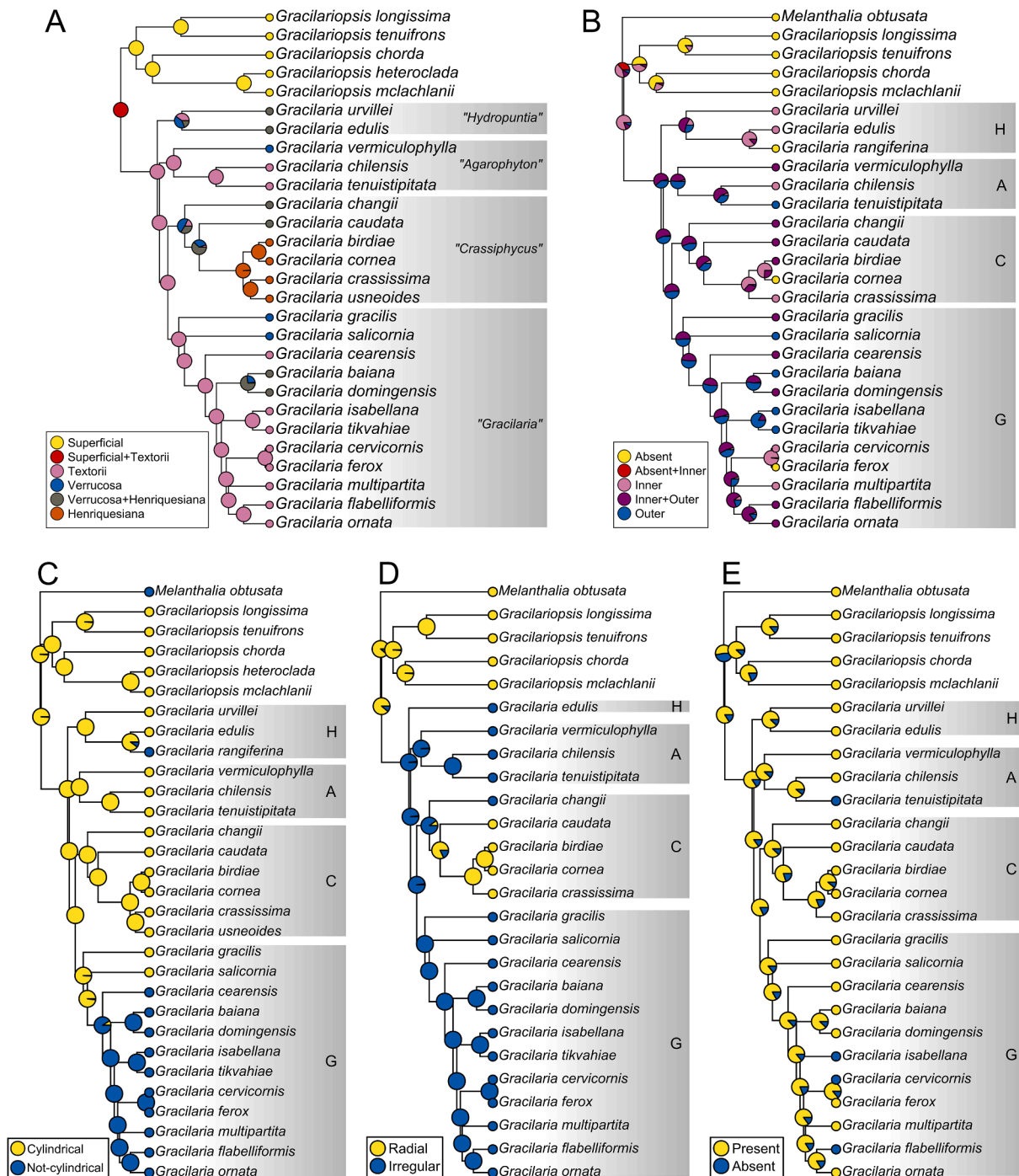
Fig. 4. Trait evolution in Gracilariales. A. Spermatangial type. B. Presence and position of traversing filaments (also known as nutritive tubular cells). C. Thallus shape. D. Organization of gonimoblasts and carposporangial chains in the cystocarp. E. Presence or absence of inner pericarp in the cystocarp. The pie charts at the nodes indicate the proportion of the ancestral character reconstruction based on the best model defined for each trait. The genera proposed by Gurgel et al. (2018) *Hydropuntia* (H), *Agarophyton* (A), *Crassiphycus* (C) and *Gracilaria s.s.* (G) are highlighted in grey. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

presence of the inner pericarp in the cystocarp.

The ancestral character estimation for spermatangial type indicated that the mrca between *Gracilariopsis* and *Gracilaria s.l.* probably presented both superficial and *textorii* spermatangial type (Fig. 4A). After *Gracilariopsis* diverged from *Gracilaria s.l.,* the spermatangial type transitions to superficial, which is seen in the extant species. The ancestor of *Gracilaria s.l.* had the *textorii* type spermatangia. The deeper spermatangia (*verrucosa* and *henriquesiana*) that are present in species of *Hydropuntia, Agarophyton, Crassiphycus* and *Gracilaria s.s.* arose independently several times. All the transition events that gave rise to the *henriquesiana* type were accompanied by the *verrucosa* type, which is observed in the mrca of *Hydropuntia, Crassiphycus, Gracilaria baiana* and *G. domingensis*. The exclusive presence of the *henriquesiana* type of spermatangia was observed only in a subclade of *Crassiphycus* comprising *G. birdiae, G. cornea, G. crassissima* and *G. usneoides*. *Gracilaria changii* and *G. caudata,* also placed in *Crassiphycus,* present both *henriquesiana* and *verrucosa* types (Fig. 4A). Genera adopted in the classification by Gurgel et al. (2018) are plotted on Fig. 4 to facilitate the evaluation of the support of these categories in light of our analyses of trait evolution.

We cannot determine if the traversing filaments (Fig. 4B) were present in the mrca of Gracilariales. Based on our model, this feature was independently lost several times. It was probably lost in the ancestral of *Gracilariopsis* and three times independently in *Hydropuntia, Crassiphycus,* and *Gracilaria s.s.* (Fig. 4B). The trait is not consistent throughout the lineages of *Gracilaria s.l.*

The most recent common ancestor of Gracilariales probably presented a cylindrical thallus (Fig. 4C). The non-cylindrical shape arose at least three times during the evolution: in *Melanthalia obtusata, Gracilaria rangiferina* and in part of the *Gracilaria s.l.* clade (Fig. 4C).

The organization of gonimoblasts and carposporangial chains were probably radial in the origin of Gracilariales and this state remained in *Melanthalia* and *Gracilariopsis* (Fig. 4D). The transition to irregular organization occurred in the ancestral of *Gracilaria s.l.* and this state returned to radial in the sister group of *G. changii,* the subclade *Crassiphycus*.

The presence of the inner pericarp in the cystocarp is the dominant state in Gracilariales (Fig. 4E). However, it was independently lost in *G. tenuistipitata, G. isabellana, G. cervicornis,* and *G. flabelliformis*.

4. Discussion

In the absence of molecular phylogenetic data, generic delimitation in Gracilariaceae had been based primarily on the morphology and ontogeny of reproductive structures, including cystocarp and spermatangial features (Fredericq and Hommersand, 1989). In the last 30 years, DNA markers such as SSUrDNA, ITS, *rbc*L and COI-5P have greatly advanced our knowledge of the evolutionary history of Gracilariaceae. However, uncertainties remained in the phylogeny on the account of limited gene and taxon sampling. Our results provide fully resolved phylogenies of the Gracilariales across all three genomic compartments (Figs. 1 and 2). The topologies of our trees bring novelties to previous phylogenies based on smaller datasets (Bellorin et al., 2002; Gurgel and Fredericq, 2004; Gurgel et al., 2018; Iha et al., 2018; Lyra et al., 2015; Lyra et al., 2021), and our analyses strongly support (greater than 75%) all major clades, which represent the main lineages of Gracilariales. Unfortunately, we could not obtain a larger dataset of the subfamily Melanthalioideae, which is represented here only by *Melanthalia obtusata*. According to previous single gene phylogenies, *Curdiea* is placed as a sister-group to *Melanthalia* (Gurgel et al., 2018; Lyra et al., 2021). Future studies with genomic data of *Curdiea* species and other *Melanthalia* representants will be essential to better understand the evolution of this lineage and its relationship with other Gracilariales clades.

A major contribution of our results is the resolution of the relationships in *Gracilaria s.l.,* which have been uncertain to date. The relationships among the currently accepted *Hydropuntia, Agarophyton,*

Crassiphycus and *Gracilaria s.s.* clades were not well-supported in previous phylogenies (Lyra et al., 2015) or only values of posterior probability were presented (Gurgel et al., 2020), which consistently overestimate support (Simmons et al., 2004). Our results resolve the *Hydropuntia* clade as sister to all other clades in *Gracilaria s.l.,* while *Agarophyton* is sister to the clade including *Crassiphycus* plus *Gracilaria s. s.* This finding is relevant to the understanding of character evolution in Gracilariales and, therefore, to the definition of taxa in accordance with well-supported evidence of the clades' evolutionary history.

Conflicts among our phylogenies were observed i) regarding the placement of *Gracilaria ferox,* which was recovered as paraphyletic in the nuclear tree; and ii) in the placement of the parasitic species *Gracilariopsis oryzoides,* sister to *Gp. andersonii* in the mitochondrial tree and closely related to the sister species *Gp. tenuifrons* and *Gp. longissima* in the nuclear tree. Incongruent phylogenetic signals between mitochondrial, plastid, and nuclear genomes have similarly been observed in other groups of red algae (Broom et al., 2008; Lee et al., 2018). The molecular evolutionary processes involved in the source of this conflict between nuclear and organellar genomes require further investigation, which is beyond the scope of the present study. In light of our highly congruent phylogenies, we investigated various sources of morphological and molecular evidence to better circumscribe genera of Gracilariaceae, including genomic architecture, comparison of time divergence of Gracilariaceae subclades, and trait evolution of characters historically used to circumscribe genera.

4.1. Organellar genome architecture

Comparative genomics has been increasingly used for taxonomic purposes in many different plant groups (Asaf et al., 2019; Farwagi et al., 2015; Khan et al., 2020; Turmel et al., 2017). Although the genomes were highly conserved in gene synteny across the Gracilariales, losses and differences in gene positions marked the evolutionary history among the *Melanthalia, Gracilariopsis* and *Gracilaria s.l.* (Iha et al., 2018). The plastid genomes of *Melanthalia obtusata* and members of the *Gracilariopsis* clade present the gene coding for the cytochrome b6f complex subunit P gene (*pet*P), which was lost in *Gracilaria s.l.* In the mitochondrial genome, the transfer RNA for histidine gene (*trnH*), absent in *M. obtusata,* is differently positioned in *Gracilariopsis* and *Gracilaria s.l.* (Fig. 2). Differences in genome architecture among inner lineages of these three clades were not observed, including the taxa described by Gurgel et al. (2018). Therefore, the results of our analyses of plastid and mitochondrial architecture did not support the division of *Gracilaria s.l.* into more than one genus.

4.2. Divergence time estimation for the standardization of taxonomic ranks

The hierarchical classification of organisms is important for scientific communication. Classification systems are not absolute and can have a high degree of arbitrariness. Efforts have been employed, however, in the development of methods to standardize the delineation of taxonomic ranks across the tree of life (Avise and Johns, 1999; Avise and Liu, 2011; Giribet et al., 2016; Kraichak et al., 2017). The temporal banding approach is one such method, which assigns taxonomic ranks based on similar ages of the nodes in a phylogenetic tree (Avise and Johns, 1999).

The divergence time estimation presented here (Fig. 3) brings insights to the understanding of the Gracilariales evolutionary history and, consequently, to the debate around its classification. Our calibrated tree reveals that the clades *Gracilariopsis* and *Gracilaria s.l.* have similar ages, in opposition to recent findings (Gurgel et al., 2020), in which analysis was performed based only with *rbc*L and presents a much later origin of *Gracilariopsis*. The age of the cladogenesis between *Gracilariopsis* and *Gracilaria s.l.* (~169 Mya) agreed with a previous divergence time estimation phylogram for Rhodophyta based on a chloroplast dataset (171 Mya; Nan et al., 2017), earlier than the 126 Mya (139–117 Mya)

reported by Gurgel et al. (2020). Our results also differ from the previous divergence times analysis of Gracilariales (Gurgel et al., 2020) regarding the ages of the currently accepted genera *Hydopuntia, Agarophyton, Crassiphycus,* and *Gracilaria s.s.* We found their origin in mid Cretaceous (between 120 and 110 Mya), while the previous analysis had shown the origin of these clades during late Cretaceous and Paleogene (between 71 and 32 Mya; Gurgel et al., 2020).

Based on the temporal banding approach, our results indicate that *Gracilariopsis* and *Gracilaria s.l.* are comparable taxonomic ranks. *Gracilaria s.l* is considerably more diverse than *Gracilariopsis*, and our results indicate a clear difference in the speed of evolutionary events leading to diversification in both clades.

4.3. Morphological trait evolution

Our results indicate that the genera classification proposed by Gurgel et al. (2018) are monophyletic, but *Hydropuntia, Gracilaria, Agarophyton* and *Crassiphycus* are not clearly circumscribed. Spermatangia types are not useful for distinguishing taxonomic categories above the species level in *Gracilaria s.l.*, as our analyses of character evolution revealed. The most recent common ancestor of *Gracilaria s.l.* probably presented only the shallow, *textorii* type of spermatangia, while the deeper spermatangial conceptacles arose independently several times in these lineages. It is interesting that all the transition events that gave rise to the *henriquesiana* type also gave rise to the *verrucosa* type, indicating that the *henriquesiana* type may be a modification of the *verrucosa* type or a later stage of development of the same structure, as Abbott et al. (1991) suggested. *Gracilaria baiana* and *G. domingensis*, for example, possess both *verrucosa* and *henriquesiana* type of spermatangia and are nested in the *Gracilaria s.s.* clade, characterized by shallow, *textorii* type spermatangia according to (Gurgel et al., 2018).

Spermatangia types, *textorii, henriquesiana* or *verrucosa*, are used as diagnostic features of *Agarophyton, Crassiphycus* and *Hydropuntia*, as well as features of cystocarps, such as presence of inner pericarp, presence and position of traversing filaments, or organization of gonimoblast and carposporangial chains (Gurgel et al., 2018; Gurgel et al., 2020). The results of our analyses of ancestral character reconstruction (Fig. 4A, B, D and E) demonstrate that each of these reproductive characters overlap among the taxa proposed or reinstated by (Gurgel et al., 2018).

Vegetative characters, such as thallus shape, also overlap among *Gracilaria s.l.* clades and among *Gracilariopsis* species (Fig. 4C). *Melanthalia obtusata*, sister to all the other Gracilariales clades in our phylogenies, presents non-cylindrical thallus, mostly compressed, while *Gracilariopsis* mostly includes species with cylindrical thallus, except for *Gracilariopsis silvana* (Gurgel et al., 2003), not included in our analyses. Even in the *Gracilaria s.s.* clade, which mostly includes species with flattened thalli, cylindrical thalli can be observed (e.g., *Gracilaria microcarpa* Dreckmann, Núñez-Resendíz & Sentíes, not included in our analyses; Dreckmann et al., 2018).

5. Conclusion

Results of our analyses, which included genomic architecture, comparison of time divergence of Gracilariales subclades, and trait evolution of characters historically used to circumscribe genera, strongly support *Gracilaria s.l.* as a well-delimited genus, while the alternative and currently accepted proposal for supraspecific taxa delimitation is not supported under the criteria we adopted. Classification systems need to reflect aspects of the evolutionary history of the group, including the amount of change and relative time of branching, being also useful in several different ways, such as predictability, mnemonic ease, therefore being functional and convenient for science practice (Stevens, 1985). The definition of exclusive characters, or exclusive combinations of characters, demands the comprehension of plesiomorphic and apomorphic states in a given clade. Granting taxonomic status to a clade in accordance with a clear circumscription that covers all its members

supports both the criteria of predictability and stability. Several species of *Gracilariopsis* and *Gracilaria s.l.* are highly important for the aquaculture, food, pharmaceutical and biotechnological industries. Therefore, the application of functional taxonomic names is essential for the maintenance of these applied fields.

The acceptance of the genus *Gracilaria s.l.* respects the criterion of monophyly and is fundamental on the presence of an exclusive combination of morphological characters, which makes this clade a practical and relevant unit of taxonomic information (Lyra et al., 2021). *Gracilaria s.l.* allows the prediction of features of taxa that may not yet be described, as it bears a set of characters not shared with its sister clade, *Gracilariopsis*. The adoption of a temporal banding approach is far from ideal for taxonomy, but it is useful in the absence of apomorphic morphological features in a single lineage as *Gracilaria s.l.* Although it is very difficult to estimate the population size in the mrca of *Gracilaria s.l.* and *Gracilariopsis*, the earlier origin and low diversification of *Gracilariopsis* compared to *Gracilaria s.l.* would indicate a possible (or multiples) bottleneck during its evolutionary process that constrained the speciation through the time, while *Gracilaria s.l.* fast evolved to differ into internal lineages. Bottleneck effects decrease biodiversity and can fix alleles (Nei et al., 1975). This hypothesis would explain the low biodiversity and the fixation of morphological traits in *Gracilariopsis*, while *Gracilaria s.l.* presents a higher diversity of morphological features (Fig. 4).

6. Taxonomic changes

Based on the findings from our robust analyses, combining phylogenomics and morphological data, we propose a revised taxonomic scheme, which expands the circumscription of the genus *Gracilaria*, accepting the *sensu lato* concept. Nomenclature and respective synonyms followed AlgaeBase (https://www.algaebase.org) (Guiry and Guiry, 2021).

Gracilariales Fredericq and Hommersand, 1989: 225

Gracilariaceae Nägeli, 1847: 240

Gracilaria Greville, 1830: 121

Lectotype species: *Gracilaria compressa* (C.Agardh) Greville, 1830:121; see (Steentoft et al., 1991); basionym: *Sphaerococcus compressus* C. Agardh, 1822: 308. The currently accepted name of the type species is *Gracilaria bursa-pastoris* (S.G. Gmelin) P.C. Silva, 1952: 265; basionym:

Fucus bursa-pastoris S.G. Gmelin, 1768: 121.

Pertinent synonyms: *Agarophyton* Gurgel, J.N.Norris and Fredericq, 2018; *Congracilaria* Yamamoto, 1986; *Corallopsis* Greville, 1830; *Crassiphycus* Guiry, J.N.Norris, Fredericq and Gurgel, 2018; *Gracilariocolax* Weber-van Bosse, 1928; *Hydropuntia* Montagne, 1842.

Type locality: Mediterranean Sea, Cádiz, Iberian Peninsula, southwestern Spain (Gargiulo et al., 1992).

In light of the newly proposed taxonomic scheme, we herein formalize the consequent changes to the names of species formerly placed under synonymized genera.

Gracilaria birdiae E.Plastino and E.C.Oliveira, 2002: 390

Homotypic synonyms: *Crassa birdiae* (E.Plastino and E.C.Oliveira) Gurgel, J.N.Norris and Fredericq, 2018, *nom. inval*; *Crassiphycus birdiae* (E.Plastino and E.C.Oliveira) Gurgel, J.N.Norris and Fredericq in Guiry et al., 2018.

Gracilaria caudata J. Agardh, 1852: 598

Homotypic synonyms: *Ceramianthemum caudatum* (J. Agardh) Kuntze, 1891; *Hydropuntia caudata* (J. Agardh) Gurgel and Fredericq, 2004; *Crassa caudata* (J.Agardh) Gurgel, J.N.Norris and Fredericq in Gurgel et al. 2018, *nom. inval.*; *Crassiphycus caudatus* (J.Agardh) Gurgel, J.N. Norris and Fredericq in Guiry et al., 2018.

Gracilaria cornea J. Agardh, 1852: 598

Homotypic synonyms: *Ceramianthemum corneum* (J. Agardh) Kuntze, 1891; *Hydropuntia cornea* (J.Agardh) M.J.Wynne, 1989; *Crassa cornea* (J. Agardh) Gurgel, J.N.Norris and S.Fredericq in Gurgel et al. 2018, *nom.*

inval.; *Crassiphycus corneus* (J.Agardh) Gurgel, J.N.Norris and Fredericq in Guiry et al., 2018.

Gracilaria changii (B. Xia et I.A. Abbott) I.A. Abbott, J. Zhang and B. Xia, 1991: 23

Basionym: *Polycavernosa changii* B. Xia and I.A. Abbott, 1987: 407.

Homotypic synonyms: *Hydropuntia changii* (B.Xia and I.A.Abbott) M. J.Wynne, 1989; *Crassa changii* (B. Xia and I.A.Abbott) Gurgel, J.N.Norris and Fredericq in Gurgel et al. 2018, *nom. inval.*; *Crassiphycus changii* (B. Xia and I.A.Abbott) Gurgel, J.N.Norris and Fredericq in Guiry et al., 2018.

Gracilaria chilensis C.J.Bird, McLachlan and E.C.Oliveira, 1986: 2928

Homotypic synonyms: *Gracilaria sordida* W.A. Nelson, 1987; *Agarophyton chilensis* (C.J. Bird, McLachlan and E.C.Oliveira) Gurgel, J.N. Norris and Fredericq, 2018.

Heterotypic synonyms: *Gracilaria secundata* f. *compacta* V.May 1948; *Gracilaria secundata* f. *pseudoflagellifera* V.May 1948; *Gracilaria sordida* W.A.Nelson, 1987.

Gracilaria crassissima (P. l. Crouan and H. M. Crouan) P. l. Crouan and H. M. Crouan in Schramm and Mazé, 1866: 46

Basionym: *Plocaria crassissima* P. l. Crouan and H. M. Crouan in Schramm and Mazé, 1865: 20.

Homotypic synonyms: *Polycavernosa crassissima* (P. l. Crouan and H. M. Crouan) Fredericq and J. N. Norris, 1985; *Hydropuntia crassissima* (P. l. Crouan and H. M. Crouan) M. J. Wynne, 1989; *Crassa crassissima* (P. Crouan and H.Crouan) Gurgel, J.N.Norris and Fredericq in Gurgel et al. 2018, *nom. inval.*; *Crassiphycus crassissimus* (P.Crouan and H.Crouan) Gurgel, J.N.Norris and Fredericq in Guiry et al., 2018.

Heterotypic synonym: *Gracilaria horizontalis* F.S. Collins and Hervey, 1917.

Gracilaria edulis (S. G. Gmelin) P.C. Silva, 1952: 293

Basionym: *Fucus edulis* S. G. Gmelin, 1768: 113.

Homotypic synonyms: *Fucus lichenoides* var. *edulis* (Gmelin) Turner, 1808; *Fucus lichenastrum* var. *edulis* (Gmelin) Poiret, 1817; *Hydropuntia edulis* (S.G. Gmelin) Gurgel and Fredericq, 2004; *Fucus coralloides* Poiret, 1808 (*nom. superfl. & illegit.*); *Sphaerococcus lichenoides* var. *tenuis* C. Agardh, 1822 (*nom. superfl. & illegit.*).

Heterotypic synonyms: *Fucus lichenoides* Turner, 1808. (*non* S. G. Gmelin, 1768); *Gigartina lichenoides* J. V. Lamouroux, 1813; *Sphaerococcus lichenoides* (J. V. Lamouroux) C.Agardh, 1817; *Fucus lichenastrum* Poiret, 1817; *Gracilaria lichenoides* (J. V. Lamouroux) Greville, 1830; *Plocaria lichenoides* (J. V. Lamouroux) J.Agardh, 1847; *Ceramianthemum lichenoides* (J. V. Lamouroux) Kuntze, 1891; *Sphaerococcus vieillardii* Kützing, 1863; *Sphaerococcus lemania* Kützing, 1868; *Gracilaria lichenoides* f. *lemania* (Kützing) V.May 1948; *Sphaerococcus setaceus* Kützing, 1868, *illegit* (*non* J. Agardh *ex* Frauenfeld, 1854; *Sphaerococcus spinescens* Kützing, 1868; *Gracilaria spinescens* (Kützing) Agardh, 1876; *Ceramianthemum spinescens* (Kützing) Kuntze, 1891; *Polycavernosa fastigiata* C. F. Chang and B.M. Xia, 1963; *Hydropuntia fastigiata* (Chang and B.M. Xia) M.J. Wynne, 1989; *Gracilaria taenioides* J.Agardh, 1852; *Ceramianthemum taenioides* (J. Agardh) Kuntze, 1891; *Gracilaria lichenoides* f. *taenioides* (J. Agardh) V. May 1948; *Gracilaria bifaria* J. Agardh, 1901.

Gracilaria eucheumatoides Harvey, 1860:331

Homotypic synonym: *Ceramianthemum eucheumatoides* (Harvey) Kuntze, 1891; *Hydropuntia eucheumatoides* (Harvey) Gurgel and Fredericq, 2004.

Gracilaria excavata (Setchell and Gardner) G.M.Lyra, C.Iha, M.C. Oliveira, J.M.C.Nunes *comb. nov.*

Basionym: *Corallopsis excavata* Setchell and Gardner, Proceedings of the California Academy of Science, Fourth Series 12(29), 756, pl. 23: figs. 24, 25; pl. 44 b; 48, 1924.

Gracilaria firma C.F.Chang and B.M.Xia, 1976: 143

Homotypic synonyms: *Crassa firma* (C.F.Chang and B.-M.Xia) Gurgel, J.N.Norris and Fredericq in Gurgel et al. 2018, *nom. inval*; *Crassiphycus firmus* (C.F.Chang and B.-M.Xia) Gurgel, J.N.Norris and Fredericq in Guiry et al., 2018.

Gracilaria millardetii (Montagne) J.Agardh, 1885: 64

Basionym: *Rhodymenia millardetii* Montagne in Montagne and Millardet, 1862: 9.

Homotypic synonym: *Hydropuntia millardetii* (Montagne) Gurgel, J. N.Norris and Fredericq, 2018.

Gracilaria multifurcata Børgesen, 1953: 42

Homotypic synonyms: *Polycavernosa multifurcata* (Børgesen) Chang and B. Xia, 1963; *Hydropuntia multifurcata* (Børgesen) M.J. Wynne, 1989.

Gracilaria perplexa K. Byrne & Zuccarello, 2002: 302

Homotypic synonym: *Hydropuntia perplexa* (K. Byrne and Zuccarello) Conklin, O'Doherty and A. R. Sherwood, 2014

Gracilaria preissiana (Sonder) Womersley in Min-Thein and Womersley, 1976: 109

Basionym: *Rhodymenia preissiana* Sonder, 1845: 56.

Homotypic synonyms: *Rhodophyllis preissiana* (Sonder) Kützing, 1849; *Calliblepharis preissiana* (Sonder) Harvey, 1859; *Hydropuntia preissiana* (Sonder) Gurgel and Fredericq, 2004.

Heterotypic synonyms: *Calliblepharis pannosa* Harvey, 1855b; *Gracilaria pannosa* (Harvey) J. Agardh, 1885; *Ciliaria pannosa* (Harvey) Kuntze, 1891.

Gracilaria punctata (Okamura) Yamada, 1941: 203

Basionym: *Rhodymenia punctata* Okamura, 1929.

Homotypic synonyms: *Crassa punctata* (Okamura) Gurgel, J.N.Norris and Fredericq in Gurgel et al. 2018, *nom. inval.*; *Crassiphycus punctatus* (Okamura) Gurgel, J.N.Norris and Fredericq in Guiry et al., 2018.

Gracilaria rangiferina (Kützing) Piccone, 1886: 71

Basionym: *Sphaerococcus rangiferinus* Kützing, 1849.

Homotypic synonym: *Hydropuntia rangiferina* (Kützing) Gurgel and Fredericq, 2004.

Heterotypic synonyms: *Gracilaria dentata* J. Agardh, 1852; *Ceramianthemum dentatum* (J. Agardh) O. Kuntze, 1891; *Polycavernosa dentata* (J. Agardh) Lawson and John, 1987; *Hydropuntia dentata* (J. Agardh) Wynne, 1989, *Gracilaria henriquesiana* Hariot, 1908; *Polycavernosa henriquesiana* (Hariot) Chang and Xia, 1963; *Hydropuntia henriquesiana* (Hariot) Wynne, 1989.

Gracilaria secundata Harvey, 1863

Homotypic synonyms: *Crassa secundata* (Harvey) Gurgel, J.N.Norris and Fredericq in Gurgel et al. 2018, *nom. inval.*; *Crassiphycus secundatus* (Harvey) Gurgel, J.N.Norris and Fredericq in Guiry et al., 2018.

Gracilaria tenuistipitata C.F.Chang and B.M.Xia, 1976: 102

Homotypic synonym: *Agarophyton tenuistipitatum* (C.F. Chang and B. M. Xia) Gurgel, J.N.Norris and Fredericq, 2018.

Gracilaria transtasmanica (M.Preuss, N.Muangmai and Zuccarello) G. M.Lyra, C.Iha, J.M.C.Nunes, C.C.Davis *comb. nov.*

Basionym: *Agarophyton transtasmanicum* M.Preuss, N.Muangmai and Zuccarello 2020 in Preuss et al. Phycologia 59(3): 240, figs 3-12, 2020.

Gracilaria truncata Kraft, 1977: 495

Basionym: *Rhodymenia prolifera* Harvey, 1855a: 249.

Homotypic synonyms: *Rhodymenia prolifera* Harvey, 1855b; *Calliblepharis prolifera* (Harvey) J.Agardh, 1876; *Palmaria prolifera* (Harvey) Kuntze, 1891; *Tylotus prolifer* (Harvey) Kylin, 1932.

Heterotypic synonyms: *Crassa truncata* (Kraft) Gurgel, J.N.Norris and Fredericq in Gurgel et al. 2018, *nom. inval.*; *Crassiphycus proliferus* (Harvey) Gurgel, J.N.Norris and Fredericq in Guiry et al., 2018.

Gracilaria tsudae (I.A. Abbott and I. Meneses) I.A. Abbott 1991: 223.

Basionym: *Polycavernosa tsudae* I.A. Abbott and I. Meneses in Meneses and Abbott, 1987: 195.

Homotypic synonym: *Hydropuntia tsudae* (I. A. Abbott and I. Meneses) M. J. Wynne, 1989: 477.

Gracilaria urvillei (Montagne) I.A. Abbott, 1991: 23

Basionym: *Hydropuntia urvillei* Montagne, 1842: 7.

Homotypic synonym: *Hydropuntia urvillei* Montagne, 1842.

Gracilaria usneoides (C. Agardh) J. Agardh, 1852: 595

Basionym: *Sphaerococcus usneoides* C. Agardh, 1822: 333.

Homotypic synonyms: *Laurencia usneoides* (C. Agardh) Kützing, 1849; *Ceramianthemum usneoides* (C. Agardh) Kuntze, 1891; *Hydropuntia*

usneoides (C. Agardh) Gurgel and Fredericq, 2004; *Crassa usneoides* (Mertens ex C.Agardh) Gurgel, J.N.Norris and Fredericq in Gurgel et al. 2018, *nom. inval.*; *Crassiphycus usneoides* (Mertens ex C.Agardh) Gurgel, J.N.Norris and Fredericq in Guiry et al., 2018.

Gracilaria vermiculophylla (Ohmi) Papenfuss, 1967: 101

Basionym: *Gracilariopsis vermiculophylla* Ohmi, 1956: 271.

Homotypic synonym: *Agarophyton vermiculophyllum* (Ohmi) Gurgel, J.N.Norris and Fredericq, 2018.

Gracilariopsis E.Y. Dawson, 1949: 40

Holotype species: *Gracilariopsis sjoestedtii* (Kylin) E.Y. Dawson, 1949: 43; basionym: *Gracilaria sjoestedii* Kylin, 1930: 55. The currently accepted name of the type species is *Gracilariopsis andersonii* (Grunow) E.Y. Dawson, 1949: 43, basionym: *Cordylecladia andersonii* Grunow in Piccone, 1886: 62.

Type locality: Mussel Point, Pacific Grove, California, U.S.A.

Pertinent synonym: Gracilariophila Setchell and Wilson in Wilson, 1910. The synonymy follows Wynne (2019), in his proposal of conserving Gracilariopsis E.Y. Dawson against Gracilariophila Setchell and Wilson, adopting the criteria of stability of nomenclatural practice.

Melanthalia Montagne, 1843: 296

Holotype species: *Melanthalia obtusata* (Labillardière) J. Agardh, 1852: 614.

Fucus obtusatus Labillardière, 1807: 111. The currently accepted name of the type species is *Melanthalia obtusata* (Labillardière) J. Agardh, 1852: 614.

Type locality: Southeast Tasmania (Guiry and Guiry, 2021).

Curdiea Harvey, 1855b: 333

Holotype species: *Curdiea laciniata* Harvey, 1855b: 333. The currently accepted name of the type species is *Curdiea angustata* (Sonder) A.J.K. Millar, 1990: 342; basionym: *Epymenia angustata* Sonder, 1853: 677.

Type locality: Port Fairy, Victoria, Australia (Guiry and Guiry, 2021).

7. Synthesized taxonomic scheme

Gracilariales Fredericq and Hommersand, 1989
Gracilariaceae Nägeli, 1847
Gracilaria Greville, 1830
Gracilariopsis E.Y. Dawson, 1949
Melanthalia Montagne, 1843
Curdiea Harvey, 1855b

CRediT authorship contribution statement

Goia M. Lyra: Conceptualization, Methodology, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Writing – review & editing, Funding acquisition, Project administration. **Cintia Iha:** Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Visualization, Writing – original draft, Writing – review & editing. **Christopher J. Grassa:** Formal analysis, Writing – original draft. **Liming Cai:** Formal analysis, Writing – original draft. **Hongrui Zhang:** Formal analysis. **Christopher Lane:** Resources, Writing – review & editing. **Nicolas Blouin:** Resources. **Mariana C. Oliveira:** Conceptualization, Resources, Writing – original draft, Writing – review & editing, Funding acquisition, Supervision. **José Marcos Castro Nunes:** Conceptualization, Resources, Writing – original draft, Writing – review & editing, Funding acquisition, Supervision. **Charles C. Davis:** Conceptualization, Resources, Writing – original draft, Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ympev.2021.107294.

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