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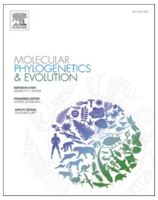

Phylogenomics and taxon-rich phylogenies of new and historical specimens shed light on the systematics of *Hypnea* (Cystocloniaceae, Rhodophyta)

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ABSTRACT

Cystocloniacae is a highly diverse family of Rhodophyta, including species of ecological and economic importance, whose phylogeny remains largely unresolved. Species delimitation is unclear, particularly in the most speciose genus, *Hypnea*, and cryptic diversity has been revealed by recent molecular assessments, especially in the tropics. Here, we carried out the first phylogenomic investigation of Cystocloniaceae, focused on the genus *Hypnea*, inferred from chloroplast and mitochondrial genomes including taxa sampled from new and historical collections. In this work, molecular synapomorphies (gene losses, InDels and gene inversions) were identified to better characterize clades in our congruent organellar phylogenies. We also present taxon-rich phylogenies based on plastid and mitochondrial markers. Molecular and morphological comparisons of historic collections with contemporary specimens revealed the need for taxonomic updates in *Hypnea*, the synonymization of *H. marchantiae* to a later heterotypic synonym of *H. cervicornis* and the description of three new species: *H. davisiana* sp. nov., *H. djamilae* sp. nov. and *H. evaristoae* sp. nov.

1. Introduction

In taxonomically challenging clades of Rhodophyta, morphotype concepts can be variable and not always associated with monophyletic groups (Lyra et al., 2016; Jesus et al., 2019b; Pestana et al., 2021; Santos et al., 2022). Along these lines, molecular-assisted taxonomy has revealed surprising amounts of cryptic diversity in the genus *Hypnea* J.V. Lamouroux (Nauer et al., 2014, 2015, 2016, 2019; Jesus et al., 2016, 2019a; Huisman et al., 2021; Kundu and Bast, 2021). *Hypnea* has received particular attention due to its wide geographic distribution, remarkable species diversity, and economic value (Yokoya et al., 2020). Some species are largely used in the food and cosmetics industries (Knutsen et al., 1995), while others show fungicidal (Melo et al., 1997), antiviral (Mendes et al., 2012) anti-inflammatory (Bitencourt, 2008), and other pharmacological (Rahila et al., 2010) properties.

In the first systematic approach of *Hypnea*, J.Agardh (1852) proposed three infrageneric sections (*Pulvinatae*, *Spinuligerae* and *Virgatae*) characterized by the external morphology of the thallus. A recent phylogenetic study based on three plastid and mitochondrial genes revealed that these sections were polyphyletic and the result of convergent evolution, rejecting this taxonomic arrangement (Jesus et al., 2019b). This approach significantly improved resolution and support in several nodes of the tree compared to previous multigene analyses (Geraldino et al., 2010), even if some uncertainties remained. The latest phylogenetic and taxonomic revisions of *Hypnea* demonstrated the importance of the study of type specimens and topotypes for resolving taxonomic issues

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(Jesus et al., 2019b; Nauer et al., 2019). Currently, only 21 of the 64 taxonomically accepted species have sequences from the holotype or type locality (Yokoya et al., 2020; Guiry and Guiry, 2022).

Even with the recent investments in molecular data and greater sampling intensity (Geraldino et al., 2009, 2010; Jesus et al., 2016, 2019a,b; Nauer et al., 2014, 2015, 2016, 2019; Kang and Nam, 2018; Cabrera et al., 2020; Huisman et al., 2021; Kundu and Bast, 2021), *Hypnea* remains a source of several taxonomic problems, with challenging species delimitation (Price et al., 1992; Jesus et al., 2016, 2019a, b). This has been pronounced not only in the most species-rich genus of Cystocloniaceae, but also in the family as a whole, as molecular efforts have been limited to a few genes so far (Hommersand and Fredericq, 2003; Won et al., 2016; Yang and Kim, 2017; Hoffman et al., 2018; Soares and Fujii, 2020; Tonicelli et al., 2021). Despite its economic and ecological importance, and taxonomic uncertainties, only two species of Cystocloniaceae are currently represented in genomic datasets to date.

The high-throughput sequencing (HTS) made the acquisition of genomes more accessible, generating very large datasets at a reasonable cost (Oliveira et al., 2018). Phylogenomic studies based on organellar genomes have demonstrated its potential to elucidate relevant aspects of red algal evolution, providing support for phylogenies (Yang et al., 2015; Muñoz-Gómez et al., 2017; Nan et al., 2017) including those of difficult algal clades, which have been long-standing taxonomic conundrums, such as Rhodomelaceae (Díaz-Tapia et al., 2017) and Gracilariales (Iha et al., 2018; Lyra et al., 2021). In this context, organellar genomes can generate robust and well-supported phylogenies at different taxonomic levels (Boo et al., 2016; Díaz-Tapia et al., 2017; Ng et al., 2017; Iha et al., 2018; Lyra et al., 2021). Repositories of biodiversity, historical collections have been proven to be valuable and accessible sources of genetic information (Hughey and Gabrielson, 2012; Bakker, 2017), as many new species described being over 50 years old (Bebber et al., 2010). Thereby, herbarium genomics represents a rising field, with significant contributions to systematic, taxonomic, ecological, and evolutionary studies (Bakker, 2017; Davis, 2022).

In this study, we performed two approaches: character-rich phylogenies, based on complete mitochondrial and plastid genomes; and taxon-rich phylogenies, based on single genes from all three genomic compartments. Our dataset includes the first organellar phylogenomic approach for the family, using genomes obtained from new and historical specimens, whose ages ranged from 99 to 127 years. We also mined sequences produced so far from public databases and combined them with our sampling in taxon-rich phylogenies. The main objectives of this study were: (1) to characterize the genome's architecture of members of the Cystocloniaceae family, particularly of the genus *Hypnea*; (2) to explore molecular apomorphic events (gene losses and inversions) to particular lineages, which could provide insights into the clade's delimitation and diversification in *Hypnea*; (3) to understand the relationships among *Hypnea* species and its close relatives by phylogenomics; and (4) to investigate potential hidden diversity and the existence of misapplied *Hypnea* names from historical and modern collections.

2. Material and methods

2.1. Taxon and gene sampling and morphological studies

We selected 51 Cystocloniaceae specimens from recent or historical collections for morphological studies and organellar sequencing, including type and topotype material. Morphological observations were performed as described by Jesus et al. (2015, 2016). Freshly collected specimens were pressed on herbarium sheets and deposited in the Herbarium Alexandre Leal Costa (ALCB) of the Biology Institute of the Universidade Federal da Bahia or at the Herbarium of the Universidade de São Paulo (SPF), Brazil. Abbreviations follow Index Herbariorum (Thiers, 2022). Nomenclature and respective synonyms followed AlgaeBase (Guiry and Guiry, 2022). For new taxa, nomenclature followed the International Code of Nomenclature for algae, fungi, and plants (Turland et al., 2018).

Our final dataset for the phylogenomic data-rich approach comprised 22 specimens of Cystocloniaceae, corresponding to three genera and 18 species, including 13 *Hypnea* species sampled worldwide (Table S1). This sampling covers a great variety of morphologies and the main lineages in the genus *Hypnea* (Jesus et al., 2019b). Furthermore, we obtained genomes from three other Cystocloniaceae species (two *Calliblepharis* Kützing and one *Cystoclonium* Kützing) and two species of Caulacanthaceae (Gigartinales) to be used as outgroups. In addition, organellar genomes of *H. cervicornis* J.Agardh (Xia et al., 2022), *H. pannosa* J.Agardh (Díaz-Tapia et al., 2019), *Kappaphycus alvarezii* (Doty) L.M. Liao (Li et al., 2018; Liu et al., 2019) and *Chondrus crispus* Stackhouse (Burger et al., 1999; Janouškovec et al., 2013), available in GenBank (https://www.ncbi.nlm.nih.gov/genbank/, searched December 2022 – Table 1), were included in our analyses.

We also produced an additional nuclear phylogeny, using the 5.8S rRNA gene and the internal transcribed spacer regions (ITS1 and ITS2), obtained for the same set of samples used in the organellar phylogenomic analyzes (23 sequences from 16 Cystocloniaceae species). Nuclear sequences were obtained by mapping the raw Illumina reads to a reference (*Hypnea flexicaulis*, GenBank accession: EU240850) in Geneious Prime 2019 (Ripma et al., 2014). Aiming to investigate the phylogenetic relationships of *Hypnea* species we built a broadly sampled phylogeny based on the plastidial *rbc*L, which is the most represented gene region of the genus in GenBank to date (eg. Yamagishi and Masuda, 2000; Geraldino et al., 2010; Jesus et al., 2015, 2016, 2019a,b; Kang and Nam, 2018; Nauer et al., 2014, 2015, 2016, 2019). Our taxon-rich *rbc*L phylogeny was based on 38 taxa, covering the main lineages of the genus. Because this study includes historical herbarium specimens and possible taxonomic novelties, we further extracted COI-5P and UPA regions from historical herbarium collections and compared them with contemporary specimens' sequences available to confirm its identification.

2.2. DNA extraction, sequencing, assembling and annotation

Newly sampled specimens were preserved in silica gel desiccant. We grinded 20–40 mg of thalli in liquid nitrogen and immediately proceeded with genomic DNA extraction using a modified version of the cetyl trimethyl ammonium bromide (CTAB) procedure (Doyle and Doyle, 1987). Genomic DNA from herbarium specimens was extracted using the Maxwell® 16 DNA Purification Kit (Promega, Mannheim, Germany). We used the Qubit® 3.0 Fluorometer with the Qubit® dsDNA HS Assay Kit (Thermo Fisher Scientific Inc, Waltham, USA) for total DNA quantification, and Agilent 2100 Bioanalyzer with the DNA High Sensitivity chip for verifying DNA quality.

Libraries and sequencing were developed at the FAS Center for Systems Biology at Harvard University. Libraries were prepared using the KAPA HyperPlus Library Preparation Kit (Kapa Biosystems, Wilmington, USA) with 8 min of incubation during the fragmentation step and using Illumina adapters and recommendations (dilutions ratios) for bead size selection for fragments of 550 bp. Libraries concentrations were verified with Qubit® 3.0 Fluorometer using the Qubit® dsDNA HS Assay Kit , and average sizes of the DNA fragments were verified with the Agilent 2100 Bioanalyzer with the DNA High Sensitivity chip. 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 and of the libraries' pool. Libraries were sequenced on the Illumina HiSeq v4 High-Output (Illumina, Inc.) with 250 bp paired-end runs.

Raw Illumina reads' quality was analyzed in FastQC (Andrews, 2011). We used Trimmomatic v0.036 (Bolger et al., 2014) to clean and trim low-quality reads and bases. Both plastid and mitochondrial genomes were assembled *de novo* using the PhyloHerb pipeline (Cai et al., 2022). Bandage v.0.8.1 (Wick et al., 2015), a program for visualizing the

Table 1
General information of complete mitochondrial and chloroplast genomes of species analyzed in this study.

Species	GenBank	Voucher	Total genome size (bp)	GC content (%)	Total number of genes	Protein coding genes	tRNA genes	rRNA genes	N° Introns	Other RNA	Reference
Mitochondria											
Hypnea brasiliensis P.B. Jesus, Nauer & J.M.C. Nunes	OM066868	ALCB 110358	25,170	26.7	50	24	24	2	1	–	This study
Hypnea caraibica Nauer, Cassano & M.C. Oliveira	OM066855	ALCB 118380	25,183	28.2	50	24	24	2	1	–	This study
Hypnea cervicornis J. Agardh	MZ682023		25,060	27.04	50	24	24	2	1	–	Xia et al. (2022)
Hypnea cornuta (Kützing) J. Agardh	OM066867	ALCB 118374	25,129	29.4	50	24	24	2	1	–	This study
Hypnea cryptica P.B. Jesus, Nauer & J.M.C. Nunes	OM066854	ALCB 110424	25,190	29.3	50	24	24	2	1	–	This study
Hypnea edeniana Nauer, Cassano & M.C. Oliveira	OM066860	–	25,073	28.1	50	24	24	2	1	–	This study
Hypnea flava Nauer, Cassano & M.C. Oliveira	OM066858	SPF 57477	25,121	28.0	50	24	24	2	1	–	This study
Hypnea marchantiae Setchell & N.L. Gardner (=*H. cervicornis* J. Agardh)	OM066865	FH 01159049	25,061	27.1	50	24	24	2	1	–	This study
Hypnea musciformis (Wulfen) J.V. Lamouroux	OM066856	SPF 57594	25,082	28.3	50	24	24	2	1	–	This study
Hypnea nidifica J. Agardh (=*H. davisiana* P.B. Jesus, Lyra, J.M.C. Nunes & M.C. Oliveira sp. nov)	OM066864	FH 01159057	25,110	28.4	50	24	24	2	1	–	This study
Hypnea nidulans Setchell	OM066863	FH 01159066	25,082	27.0	50	24	24	2	1	–	This study
Hypnea pseudomusciformis Nauer, Cassano & M.C. Oliveira	OM066857	SPF 57,526	25,093	28.3	50	24	24	2	1	–	This study
Hypnea spinella (C.Agardh) Kützing	OM066859	SPF 57446	25,203	28.0	50	24	24	2	1		
Hypnea wynnei Nauer, Cassano & M.C.Oliveira	OM066852	SPF 57515	25,126	26.5	50	24	24	2	1	–	This study
Calliblepharis jolyi E.C. Oliveira	OM066853	–	25,119	25.5	50	24	24	2	1	–	This study
Calliblepharis jubata (Goodenough & Woodward) Kützing	OM066866	–	25,066	24.5	50	24	24	2	1	–	This study
Caulacanthus ustulatus (Mertens ex Turner) Kützing	OM066861	FH 00789248	25,810	29.1	50	24	24	2	1	–	This study
Catenella caespitosa (Withering) L.M. Irvine	OM066862	FH 00789173	25,174	32.9	50	24	24	2	1	–	This study
Kappaphycus alvarezii (Doty) L.M. Liao	KU885455	–	25,198	29.9	50	24	24	2	1	–	Liang et al. (2016)
Chondrus crispus Stackhouse	Z47547	–	25,836	27.9	59	36	23	3	1	–	Leblanc et al. (1995)
Chloroplast											
Hypnea brasiliensis P.B. Jesus, Nauer & J.M.C. Nunes	OL964145	ALCB 110358	173,928	27.8	235	200	30	3	1	2	This study
Hypnea caraibica Nauer, Cassano & M.C. Oliveira	OL964153	ALCB 118380	173,580	27.9	235	200	30	3	1	2	This study
Hypnea cervicornis J. Agardh	MZ682024	–	176,446	28.09	230	194	30	3	1	2	Xia et al. (2022)
Hypnea cornuta (Kützing) J. Agardh	OL964147	ALCB 118374	173,251	28.4	233	198	30	3	1	2	This study
Hypnea cryptica P.B. Jesus & J.M.C. Nunes	OL964148	ALCB 110424	173,464	28.4	233	198	30	3	1	2	This study
Hypnea edeniana Nauer, Cassano & M.C. Oliveira	OL964142	–	173,918	27.8	234	199	30	3	1	2	This study
Hypnea flava Nauer, Cassano & M.C. Oliveira	OL964143	SPF 57477	174,573	27.9	234	199	30	3	1	2	This study
Hypnea musciformis (Wulfen) J.V. Lamouroux	OL964154	SPF 57594	173,520	27.9	235	200	30	3	1	2	This study
Hypnea nidifica J. Agardh (=*H. davisiana* P.B. Jesus, Lyra, J.M.C. Nunes & M.C. Oliveira)	OL964140	FH 01159057	173,169	28.0	235	200	30	3	1	2	This study
Hypnea nidulans Setchell	OL964141	FH 01159066	173,835	27.8	234	199	30	3	1	2	This study

(*continued on next page*)

Table 1 (*continued*)

Species	GenBank	Voucher	Total genome size (bp)	GC content (%)	Total number of genes	Protein coding genes	tRNA genes	rRNA genes	N° Introns	Other RNA	Reference
Hypnea pseudomusciformis Nauer, Cassano & M.C. Oliveira	OL964152	ALCB 114369	173,616	27.9	234	199	30	3	1	2	This study
Hypnea pseudomusciformis Nauer, Cassano & M.C. Oliveira	OL964146	ALCB 110417	173,589	27.9	234	199	30	3	1	2	This study
Hypnea pseudomusciformis Nauer, Cassano & M.C. Oliveira	OL964144	SPF 57526	173,626	27.9	234	199	30	3	1	2	This study
Hypnea wynnei Nauer, Cassano & M.C. Oliveira	OL964151	SPF 57515	175,748	27.7	235	200	30	3	1	2	This study
Calliblepharis jolyi E.C. Oliveira	OL964150	–	177,247	29.3	235	200	30	3	1	2	This study
Calliblepharis jubata (Goodenough & Woodward) Kützing	OL964149	–	176,735	27.5	234	199	30	3	1	2	This study
Caulacanthus ustulatus (Mertens ex Turner) Kützing	OL964155	FH 00789248	173,755	29.4	233	199	30	3	1	1	This study
Catenella caespitosa (Withering) L.M. Irvine	OL964156	FH 00789173	177,036	31.0	237	204	30	3	1	0	This study
Kappaphycus alvarezii (Doty) L.M.Liao	KU892652	–	178,205	29.6	234	201	29	3	1	1	Liu et al. (2019)
Chondrus crispus Stackhouse	HF562234	–	180,086	28.7	240	204	30	3	1	3	Janouškovec et al. (2013)

assembly graphs, was then used to select the complete or incomplete contigs of plastomes and mitogenomes by importing the files created by PhyloHerb. Both plastomes and mitogenomes were annotated using MFannot (https://megasun.bch.umontreal.ca/cgi-bin/dev_mfa/mfannotInterface.pl) to find coding sequences (CDS). We also annotated the genomes using Geneious Prime 2019 (Ripma et al., 2014) based on available genome references. We performed manual inspections and corrections, searching for open-reading frames (ORFs) using the ORF finder plugin available in Geneious Prime 2019 (Ripma et al., 2014), with *Chondrus crispus* as reference.

2.3. Phylogenetic analyses

Genes from both complete and incomplete organellar genomes were included in the phylogenomic analyses. We extracted 26 genes from 20 complete and four incomplete mitogenomes and 199 genes from 20 complete and five incomplete plastomes (Table S2 and S3) and performed alignments in MAFFT (Katoh and Standley, 2013). The unreliable regions were trimmed in trimAl (Capella-Gutiérrez et al., 2009) and then concatenated into one data matrix. The best evolutionary model of each dataset was assessed with jModeltest 2.1.7 (Darriba et al., 2012), and the GTR + I + G was selected for all datasets, and both ML and Bayesian analyses were performed as a single partition on the CIPRES web portal (Miller et al., 2010). We performed maximum likelihood (ML) analyses on the Randomized Accelerated ML (RAxML) web server (version 7.0.4; Stamatakis, 2006) with 1,000 rapid bootstrap replicates. Bayesian inference (BI) analyses were carried out in MrBayes on XSEDE v.3.2.6 (Ronquist et al., 2012). Each Bayesian analysis comprised two independent runs of 5 million generations with four parallel Monte Carlo Markov chains (MCMC), sampling every 1,000 generations. A 25 % burn-in was discarded once convergence was reached. Both ML and BI trees and the branch support values were visualized in FigTree v.1.4.3 (Rambaut, 2017).

The *rbc*L taxon-rich phylogeny was inferred using ML and BI, as described above. For COI-5P and UPA datasets, clustering trees were performed in MEGA 11 software (Tamura et al., 2021) using the neighbor-joining (NJ) algorithm based on Kimura two-parameter corrected distances (Kimura, 1980) with 2,000 bootstrap replicates. To assess the level of variation in all datasets, estimates of divergence values within and among species and genera were computed using the MEGA 11. We applied a threshold of 90 % Bayesian posterior probability (PP) or 75 % non-parametric bootstrap support in our ML and NJ analyses, which were plotted in our trees, to consider a clade as at least moderately supported. This threshold is higher than the widely used 70 % bootstrap percentage cut-off often associated with 95 % confidence that the clade is likely real (Hillis and Bull, 1993).

2.4. Genomic comparison and InDel analyses

Collinear analyses were carried out using complete and circularized plastomes and mitogenomes, including species in the outgroup (Table S1). We standardized the start position of all complete genomes to assess synteny, following Iha et al. (2018): i) at the small subunit ribosomal RNA gene (*rrs*) for plastomes; and ii) at the Asparagine tRNA gene (*trnN*) for mitogenomes. Organellar genomes were aligned using a progressive Mauve algorithm in Mauve v.2.3.1 (Darling et al., 2004) to identify the putative presence of structural variation among species. The conserved segments of sequences free from any internal rearrangements, also known as Locally Collinear Blocks (LCBs), identified by MAUVE, were color-coded.

To investigate the occurrence of other genomic structural variants along organellar genomes, we mapped all insertions/deletions (InDels) from Cystocloniaceae species using as reference the newly sequenced organellar genomes of *Caulacanthus ustulatus* (Turner) Kützing (Caulacanthaceae). For each dataset, an alignment file was generated containing 15 complete plastomes or mitogenomes of Cystocloniaceae and the reference genome, respectively. These alignment files were then used to search InDels in each organellar genome using a custom script, and shared InDels from Cystocloniaceae and major internal clades were extracted by BEDTools (Quinlan and Hall, 2010).

We searched for plasmid-derived sequences (PDS) in all complete organellar genomes, including the genomes downloaded from GenBank, using 10 published red algae plasmids available. The PDSs were searched using tBLASTx with 52 plasmid CDS queries against a database of Gracilariaceae organellar genomes (Iha et al., 2018). We also used BLASTn with complete plasmid sequences against the genomes database (-task blastn). For both searches, we considered only hits with an e-value less than 1.0e^{-20}.

3. Results

3.1. Comparative genome structure and content

Table 1 summarizes the composition and structure of the complete organellar genomes analyzed here, including 13 species of Cystocloniaceae (11 of which are classified in *Hypnea* and two in *Calliblepharis*), two species of Caulacanthaceae, one species of Solieriaceae and one species of Gigartinaceae. All mitochondrial genomes were highly congruent in CG content, length and number of genes. CG content was 27.5 % on average in Cystocloniaceae, ranging from 24.5 % in *C. jubata* to 29.4 % in *H. cornuta.* Lengths ranged from 25,061 bp in *H. marchantiae* to 25,203 bp in *H. spinella*, an average of 25,122 bp. Cystocloniaceae mitogenomes contain a set of 50 genes, comprising 2 rRNAs (*rrl* and *rrs*), 24 tRNAs and 24 protein-coding genes. Genomes of other Gigartinales presented the same gene content as Cystocloniaceae species, except for *Chondrus crispus,* which presented three rRNAs (*rrl, rrs* and *rrn5*) and 23 tRNAs (Table 1 and Table S4). All mitogenomes analyzed had a Group II intron in the *trnI* tRNA gene ranging from 466 bp in *C. jubata* to 478 bp in *C. jolyi* .

Chloroplast genomes also were very similar in CG content, length and number of genes (Table 1 and Table S5). CG content was 28.0 % on average in Cystocloniaceae, ranging from 27.7 % in *Hypnea wynnei* to 29.3 % in *Calliblepharis jolyi.* Lengths ranged from 173,169 bp in *H. marchantiae* to 177,247 bp in *C. jolyi*, an average of 174,253 bp. The gene content of Cystocloniaceae plastomes encoded a total of 233–235 genes, including 198–200 protein-coding genes, 30 tRNAs, three rRNAs (*rrl, rrs* and *rrn5*), 1 misc_RNA coding for RNase P RNA (*rnpB* gene) and one transfer-messenger RNA (tmRNA *ssrA* gene). The sole difference in gene content was the absence of the protein-coding genes *rnz* in *H. nidulans*, and *ycf20* in *C. jubata*. Among the other Gigartinales, the plastome of *Caulacanthus ustulatus* had the smallest length (173,755 bp) and number of protein-coding genes (199), while *Chondrus crispus* had the largest (180,086 bp of length, and 204 protein-coding genes). The CG content ranged from 28.7 % in *C. crispus* to 31.0 % in *Catenella caespitosa*. All outgroups differed from Cystocloniaceae species mainly by the number of special RNAs: one misc_RNA in *C. ustulatus* (*rnpB*), *Catenella caespitosa* (*rnpB*), and *Kappaphycus alvarezii* (*ssrA*); and three in *C. crispus* (two misc_RNAs: *ffs* and *rnpB*; and one tmRNA: *ssrA*). A Group II intron was found in the *trn*Me (cat) tRNA gene in all chloroplast genomes of the analyzed Gigartinales' species, ranging from 1,353 bp in *H. nidifica* to 1,979 bp in *C. jolyi.*

Comparative genomic analyses revealed that mitochondrial and plastid structure and gene content were highly conserved among the analyzed Cystocloniaceae species and its Gigartinalean relatives. Despite the high degree of synteny, some inversion events were detected in both mitochondrial and chloroplast genomes. The Mauve alignment of mitogenomes resulted in two highly similar LCBs (Fig. S1). On the largest LCB (green), the transfer RNA for histidine gene (*trnH*) of all 13 *Hypnea* species was inverted in relation to its sister clade *Calliblepharis* and all outgroups. In the smaller red LCB, a *trnR-trnY* inversion was observed only in *Chondrus crispus*. Collinear analysis of plastomes revealed five main conserved regions (Fig. S2), where two inversions were noted: one in *C. joly,* with approximately 4 kb in the *ycf3-infB* region (blue LCB); and another in *Kappaphycus alvarezii* in the *psaM-ycf21* region, with about 12.6 kb (yellow LCB). Plasmid-derived sequences were not observed in the mitochondrial and plastid genomes of Cystocloniaceae.

3.2. InDels

We mapped all *InDells* against our previously built phylogenies (Fig. 1) to verify possible events supporting each clade. In the mitochondrial genomes, 82 phylogenetically informative events were

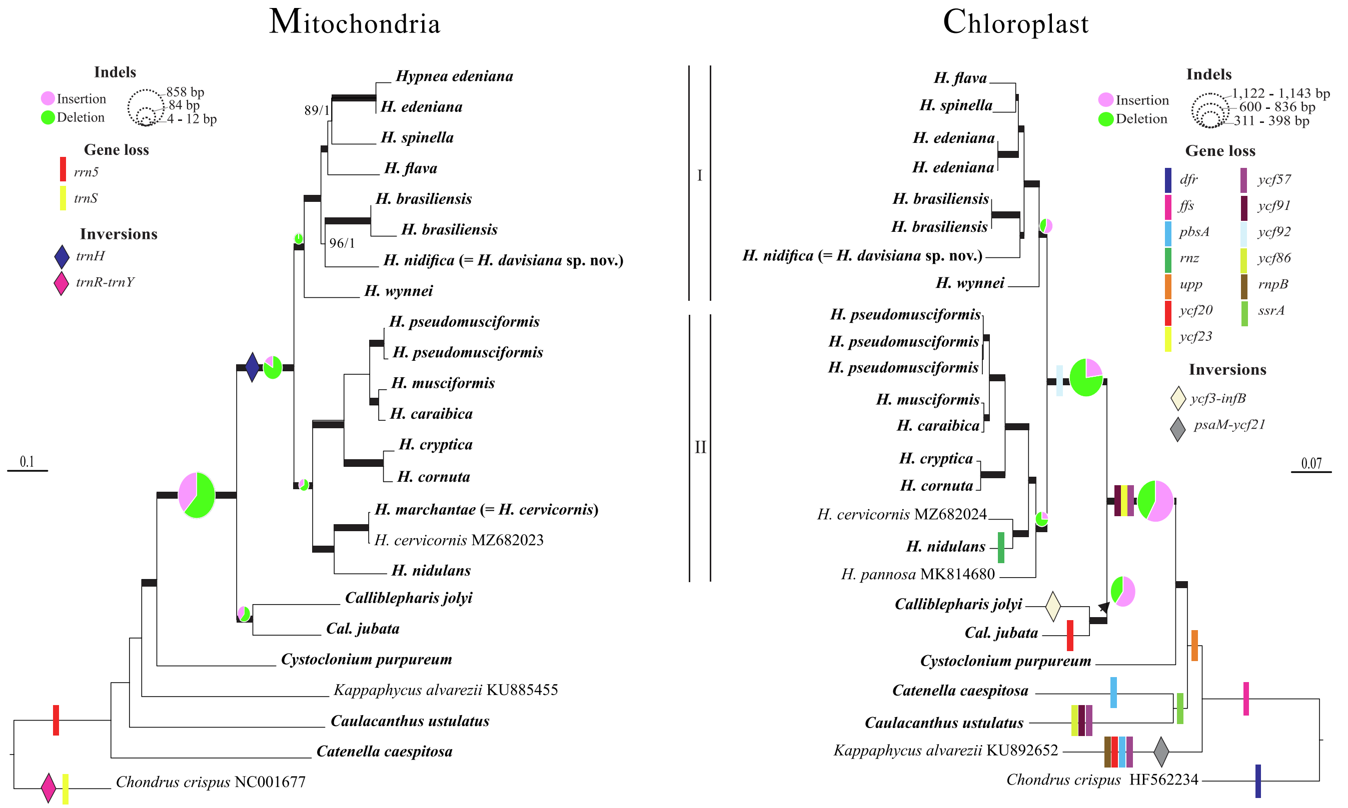

Fig. 1. Maximum likelihood (ML) trees based on concatenated 24 mitochondrial genes (left) and 190 plastid genes (right). Bootstrap (BP) and Bayesian posterior probability (PP) values are presented at nodes. Branches in bold are 100% supported. Genome's architecture features are plotted on the trees (for interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

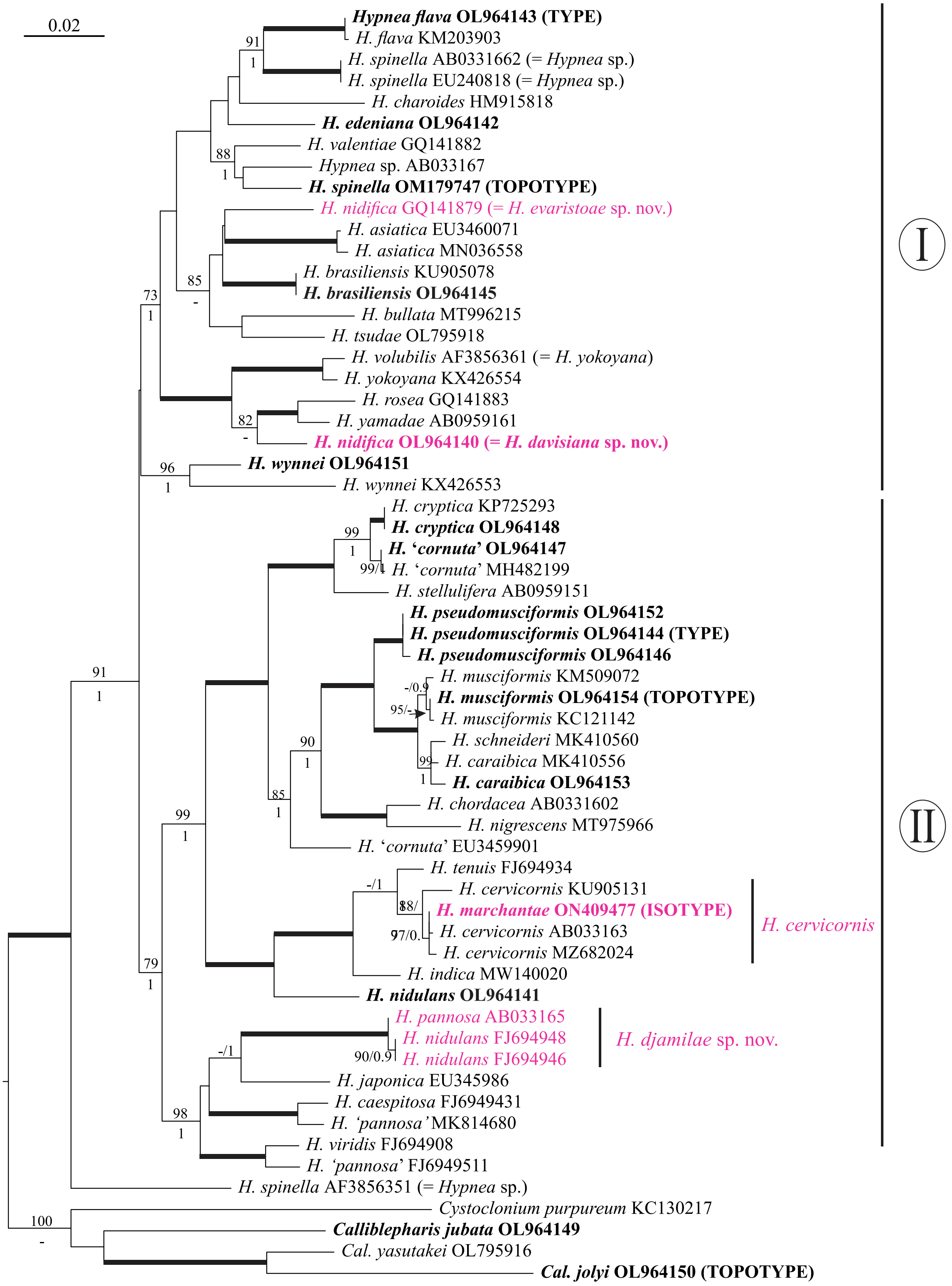

Fig. 2. Maximum likelihood (ML) *rbc*L tree. Bootstrap (BP) and Bayesian posterior probability (PP) values are presented at nodes. Branches in bold are 100% supported. New sequences are in bold and those from new taxonomic arrangements are in pink. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

inferred: 28 insertions and 54 deletions, totalizing 995 bp. Insertions ranged from 1 to 10 bp in length, while deletions ranged from 1 to 716 bp in length; accounting for 98 bp inserted and 897 bp deleted. The Cystocloniaceae was supported by 50 events (19 insertions, 31 deletions), while the genera *Calliblepharis* and *Hypnea* were supported by 13 (5 insertions, 8 deletions) and 12 events (2 insertions, 10 deletions), respectively (Fig. 1 - left, Table S6). In the chloroplast genomes, 555 informative events were observed (260 insertions and 295 deletions – 3,810 bp). Insertions ranged from 1 to 41 bp in length, accounting for 1,706 bp of total insertions; while deletions ranged from 1 to 203 bp in length, totaling 2,104 bp deleted. Of these, 207 events supported the Cystocloniaceae (260 insertions, 295 deletions), while *Calliblepharis* was reinforced by 119 events (72 insertions, 47 deletions) and *Hypnea* by 114 events (26 insertions, 88 deletions). In *Hypnea*, the two large subclades (I and II) were also corroborated by InDels events in both genomes (Fig. 1 - right, Table S6).

3.3. Organellar phylogenomics of Hypnea

We assembled organellar genomes for 22 specimens, including 13 *Hypnea* species and seven species used as outgroups, including the centenary herbarium specimens *Caulacanthus ustulatus* (126 years old) and *Catenella caespitosa* (presumably 176 years old, see Discussion) (Table 1 and Table S4), both deposited at the Farlow Herbarium (Table S1). It was not possible to assemble complete organellar genomes for the archival specimen of *Cystoclonium purpureum* (117 years old), for which 24 mitochondrial and 102 chloroplast genes were obtained. For *Hypnea marchantiae* and *H. spinella* , the complete mitogenomes of both and most chloroplast genes (187 genes) of *H. spinella* were obtained, while plastomes of *H. marchantiae* failed. Genome sequencing statistics of specimens analyzed in this study are presented in Table S2. Phylogenetic relationships among *Hypnea* species based on mitochondrial and plastid genomes recovered identical topologies in both ML and BI approaches within each dataset (Fig. 1). Both organellar phylogenies recovered the family Cystocloniaceae and the genus *Hypnea* (with full support) as monophyletic. *Calliblepharis* and *Hypnea* were resolved as sister clades with full support in all of our analyses. Likewise, *Cystoclonium*, was placed as sister to the other genera.

Inner relationships in *Hypnea* were well-resolved in both phylogenies, with full support for the majority of its subclades. Two main clades were recovered, which could not be associated with any morphological exclusive characters. However, most of the species with prostrate habit were grouped in clade I, while those with erect thallus were clustered in clade II. The exceptions were the presence of *H. nidifica*, with erect thallus, in clade I, and *H. nidulans*, a mat-forming species, in clade II. The clade I comprise *H. wynnei* and the sister clades formed by *H. brasiliensis* + *H. nidifica* and *H. edeniana* + *H. flava* + *H. spinella*. Within this latter subclade some conflicts were identified between the mitogenome and the plastome trees. In the mitochondrial tree (Fig. 1, left), *H. edeniana* was moderately supported as sister to *H. spinella* (ML = 89, PP = 1), and this clade was placed as sister to *H. flava* (ML = 100, PP = 1.0). However, in the chloroplast tree (Fig. 1, right), *H. flava* was placed as sister to *H. spinella*, and this clade was fully supported as sister to *H. edeniana*. Both datasets systematically recovered the clade II with *H. nidulans* as sister to *H. marchantiae/H. cervicornis*, and these as sister to the clades comprising *H. caraibica* + *H. musciformis* + *H. pseudomusciformis* and *H. cornuta* + *H. cryptica*. For *H. pannosa*, only the plastid genome was available and, in this tree, it was placed as sister to all other species in clade II.

The reduced phylogeny from regions of SSU rDNA (ITS1 + 5.8S + ITS2; Fig. S1) included only samples also represented in the organellar genomes' phylogenies. It was overall strongly supported, and its topology was nearly identical to those obtained from our genomic datasets, except for conflicts in the clade I of *Hypnea*.

3.4. Taxon-rich gene phylogenies

Phylogenetic relationships among *Hypnea* species were based on the analysis of the *rbc*L matrix (Fig. 2), as it includes the greatest number of taxa represented in public databases. The *rbc*L phylogeny was consistent with our phylogenomic analyses, recovering *Hypnea* as monophyletic with full support, and splitting it in two large clades (I and II). Although poorly supported, clade I included mainly mat-forming species: *H. asiatica, H. brasiliensis, H. bullata, H. charoides, H. edeniana, H. flava, H. nidifica, H. spinella, H. tsudae, H. valentiae, H. volubilis, H. yokoyana,* and *H. wynnei*. Clade II was moderately supported and mixed species with prostrate and erect thalli: *H. caespitosa, H. caraibica, H. cervicornis, H. chordacea, H. cornuta, H. cryptica, H. indica, H. japonica, H. musciformis, H. nidulans, H. nigrescens, H. pannosa, H. pseudomusciformis, H. schneideri, H. tenuis* and *H. viridis*. Plastid and mitochondrial sequences extracted from historical herbaria specimens (FH and NYBG, Table 1 and Table S4) were compared against those from contemporary specimens in public databases. Sequences of *rbc*L (Fig. 2), COI-5P (Fig. S4) and UPA (Fig. S5) were compared with those from contemporary specimens and confirmed the identity of the archival specimens *Cystoclonium purpureum, Caulacanthus ustulatus* and *Catenella caespitosa*.

Both *rbc*L (Fig. 2) and COI-5P (Fig. S4) trees nested sequences of the isotype specimen of *Hypnea marchantiae* and *H. cervicornis* in single fully-supported clades, consistent with the recognition of a single species (0 to 1.62 % in *rbc*L, and 0 to 3,06 % in COI-5P), which is in agreement with our morphological observations (Fig. 3A, B). In the *rbc*L phylogeny, *H. nidulans* was not monophyletic: our newly generated sequence from a French Polynesia' specimen, Tahiti (close to the type locality), was distantly related to contemporary specimens identified as such (up to 5,97 % of divergence in *rbc*L and up to 12.23 % of divergence in COI-5P). Our specimen was placed as sister to a clade comprising *H. indica*, *H. tenuis* and *H. cervicornis*, while other sequences available in GenBank were placed sister to *H. japonica*. These findings demonstrate the need to review the taxonomic status of the Asian specimens previously identified as *H. nidulans* (Fig. 3I). Also, *H. nidifica* was not monophyletic (Figs. S2, Table S4 and S5), with the historical specimen, sequenced here, being distantly related to the topotype from Hawaii (Fig. S5, HQ421469), and to the specimens from Singapore and India. Our morphological and molecular analyses reinforce the existence of three distinct lineages under the name *H. nidifica* (Fig. 3E-H and J).

3.5. Taxonomic results

From the sequencing of complete organellar genomes of historical herbarium collections, and comparison of extracted gene sequences (COI-5P, *rbc*L and UPA) with those from contemporary specimens in public databases, our results revealed the need for the following taxonomic updates: (i) the reduction of *Hypnea marchantiae* to a later heterotypic synonym of *H. cervicornis* and; (ii) the description of three new species: *Hypnea davisiana* sp. nov., *Hypnea djamilae* sp. nov. and *Hypnea evaristoae* sp. nov.

Hypnea cervicornis J.Agardh (1852, p. 451–452).

Lectotype: Botanical Museum Herbarium, Lund, Sweden (LD 33878!, labeled as *Sphaerococcus spinellus*; Agardh, 1822: 323 *var. laxior*; Haroun and Prud'Homme van Reine, 1993: 122).

Type locality: Bahia State, Brazil "*in litore maris prope Bahian*".

Homotypic synonym: *Hypnophycus cervicornis* (J.Agardh) Kuntze (1891: 900).

Heterotypic synonyms: *Hypnea musciformis* var. *pumila* Harvey (1834: 154); *H. aspera* Kützing (1868: 14); ***H. marchantiae*** Setchell & N. L. Gardner (1924: 759 - synonymy **designated here,** Fig. 3**A, B)**; *H. boergesenii* Tanaka (1941: 233–235); *H. flexicaulis* Yamagishi and Masuda (2000: 28).

GenBank accession of *rbc*L sequence from the topotype (Jesus et al., 2016): KU905169.

Hypnea davisiana sp. nov. P.B. Jesus, Lyra, J.M.C. Nunes & M.C. Oliveira (Fig. 3G, H)

Holotype: Farlow Herbarium (FH 01159057!); French Polynesia, Tahiti, near Faarapa; coll. W.A. Setchell & H.E. Parks n° 5193, 19 Jun 1922; vegetative.

Misapplied names: *Hypnea nidifica* sensu W.A. Setchell & H.E. Parks (1922).

Etymology: Named in honor of Angela Davis, an American black woman, philosopher, and an exemplary activist for the intersectional approach of the black feminist movement.

Diagnosis: *Hypnea davisiana* is similar to *H. brasiliensis* in the prostrate habit, being mainly reddish-pink and by its soft texture; it can be

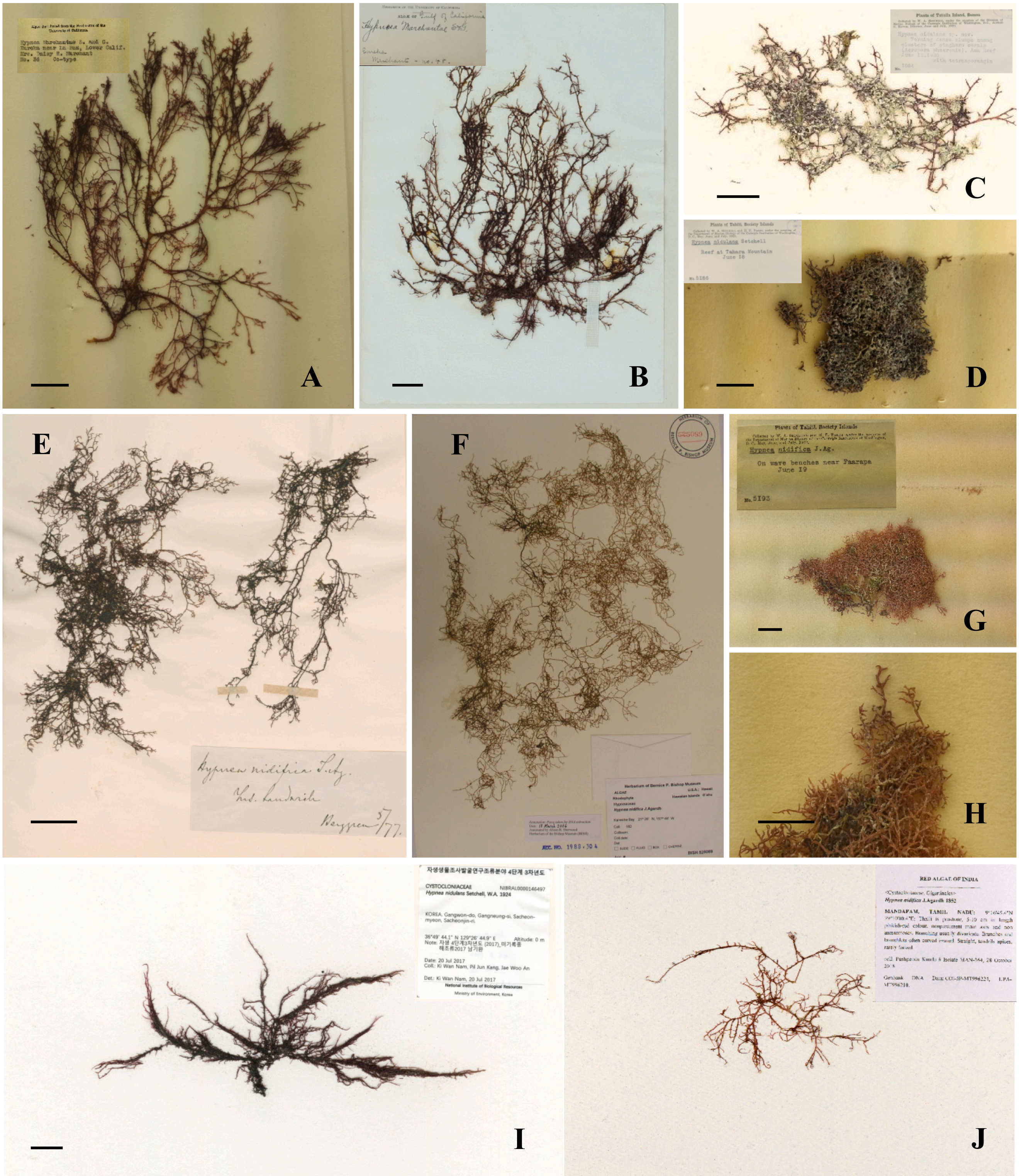

Fig. 3. Historical herbarium specimens analyzed in the present study. (A) Isotype of *Hypnea marchantiae* (FH 01159049!). (B) Holotype of *H. marchantiae* (UC22119!). (C) Holotype of *H. nidulans* (FH 01159065!). (D). *H. nidulans* (FH 01159066!). (E) Holotype of *H. nidifica* (BM000005220!). (F) Contemporary topotype specimen of *H. nidifica* (BISH 525089). (G, H) Holotype of *H. davisiana* sp. nov (FH 01159057!). (I) Holotype of *H. djamilae* sp. nov (NIBRAL0000146497!). (J) Holotype of *H. evaristoae* sp. nov (LWG003054!). Scale bars: A-D, G, I, 1 cm; E, 2 cm; H, 0,0.5 cm.

distinguished from this species by its branchlets, that are much twisted and similar in thickness to branches and principal axes. GenBank accessions from the holotype: mitogenome - OM066864, plastome - OL964140.

Description: Thallus prostate, mainly epiphytic, reddish-brown, forming tangled tufts (Fig. 3G). Thallus delicate, soft in texture, up to 5 cm long, attached to the substratum by many secondary holdfasts formed on all portions of branches and branchlets. Main axis indistinguishable, irregularly and intensely branched, twisted, growing among other algae. Spine-like branchlets straight, alternately to irregular, scattered throughout the thallus. Apices of the branches and branchlets acute, straight, commonly forked (Fig. 3H).

Remarks: Setchell and Parks identified this specimen collected from French Polynesia as *Hypnea nidifica*, to which we sequenced its organellar genomes. Our trees show that this specimen is a distinct lineage than the topotype material of *H. nidifica* from Hawaii (Fig. S5) and they differ morphologically (Fig. 3G vs 3E, F). Therefore, we propose *Hypnea davisiana* sp. nov. for the Polynesian specimen.

Hypnea djamilae sp. nov. P.B. Jesus, Lyra, J.M.C. Nunes & M.C. Oliveira (Fig. 3I)

Holotype: Herbarium of National Institute of Biological Resources, KB (NIBRAL0000146497!); Sacheonjin Beach, Gangneung, Korea; Coll. K. W. Nam, P.J. Kang & J.W. Ahn, 20 Jul 2017.

Misapplied names: *Hypnea pannosa* sensu Yamagishi and Masuda (2000), *Hypnea nidulans* sensu Geraldino et al. (2010) and Kang and Nam (2018).

Etymology: Named in honor of Djamila Taís Ribeiro dos Santos, a black Brazilian philosopher and an important contemporary voice in defense of blacks and women.

Diagnosis: *Hypnea djamilae* is similar to *H. charoides* in the general aspect, with axes percurrent and branches and branchlets usually upwards; these species can be distinguished by *rbc*L sequence divergence up to 5.7 %.

GenBank accessions from Asian specimens: *rbc*L - FJ694948, COI-5P - FJ694900.

Description: Thallus erect, mainly epilithic, reddish-brown, loosely entangled. Cartilaginous in texture, up to 10 cm long. Main axes percurrent, terete, irregularly, alternately or dichotomously branched. Spine-like branchlets straight, alternately to irregularly scattered throughout the thalli, usually upwards and abruptly curved in the adaxial direction. Apices of the branches and branchlets acute, sometimes curved. In cross-section, the middle portion of axes show a circular and pigmented axial cell, much smaller than the periaxial cells. The axial cell is surrounded by 4–6 periaxial cells, oval to triangular, 1–2 layers of hyaline medullary cells gradually smaller toward the periphery. Lenticular thickenings are usually present. Zonate tetrasporangia.

Remarks: We describe *Hypnea djamilae* sp. nov. in order to accommodate the Asian specimens previously identified as *H. nidulans* and *H. pannosa*. Our anatomical characterization is based on description and images provided by Kang and Nam (2018) for specimens from Korea.

Hypnea evaristoae sp. nov. P.B. Jesus, Lyra, J.M.C. Nunes & M.C. Oliveira (Fig. 3J)

Holotype: Herbarium of National Botanical Research Institute, Lucknow, LWG (LWG003054!); Tamil Nadu, India; Coll. P. Kundu; 28 Oct 2018.

Misapplied names: *Hypnea nidifica* sensu Kundu and Bast (2021).

Etymology: Named in honor of Dra. Maria da Conceição Evaristo de Brito, an important Brazilian writer, literary and educator, who highlights the condition of black women in a society marked by prejudice.

Diagnosis: *Hypnea evaristoae* is similar to *H. nidifica* in the general aspect, with erect intricate growth and lacking a percurrent axis; it can be distinguished from the topotype specimen from Hawaii by sequence divergence up to 2.21 % in UPA. GenBank accessions from the holotype: COI-5P - MT996224, UPA - MT996210.

Description: Thallus erect, intricate-caespitose, reddish in color. Axes percurrent absent, terete, divaricate, irregular to dichotomously branched. Spine-like branchlets straight, at acute angles, sparsely scattered throughout the thalli. Apices of the branches and branchlets acute, straight, sometimes forked or curved. Tetrasporangia in sori at basal portions of the thallus.

Remarks: From the comparison of UPA sequences between the Hawain topotype of *Hypnea nidifica* and voucher specimens from the Indian Ocean identified as such by Kundu and Bast (2021) (Fig. S5), we concluded that they are distinct taxonomic entities and described *H. evaristoae* sp. nov. Our anatomical characterization is based on figures provided by Kundu and Bast (2021) for specimens from India.

4. Discussion

4.1. Comparative genome structure, gene content and InDels

Based on the comparison of composition and structure of whole organellar genomes, it was possible to identify gene losses, *InDels* and inversions that support the inner clades in a phylogenetic context. Both analyses of the mitochondrial and plastid genome architecture supported the monophyly of *Hypnea*. The loss of the plastid genes *ycf23* and *ycf92* constitutes an apomorphy of the family Cystocloniaceae. In terms of rearrangements, the most remarkable finding was the inversion of the *trnH* gene in mitogenomes of all sequenced *Hypnea* species, an apomorphy of the genus. In addition, with respect to insertions/deletions (InDels) 12 phylogenetic informative events were observed in mitogenomes and 114 in plastomes. While relevant in some phycological studies (Borza et al., 2009; Smith et al., 2012; Ren et al., 2020), *InDels* have been more widely explored in approaches aiming at understanding genetic diversity and phenotypic heterogeneity in plants (Saxena et al., 2018; Song et al., 2020).

Currently, there are 124 mitochondrial and 171 plastid genomes of Rhodophyta available in Genbank®. Here, we generated 42 new red algal organellar genomes – 21 mitochondrial and 21 from chloroplasts – thus, increasing the number of available mitogenomes and plastomes by 16.9 % and 12.3 %, respectively. Composition and structure of the complete organellar genomes sequenced here were highly consistent in length and number and set of genes with those of other Florideophyceae (Hagopian et al., 2004; Yang et al., 2015; Lee et al., 2016; Ng et al., 2017; Ho et al., 2018; Iha et al., 2018; Li et al., 2018; Paiano et al., 2018; Liu et al., 2019; Lyra et al., 2021). Some gene losses in the Florideophyceae chloroplast genomes are lineage-specific (Ng et al., 2017), like the ones we observe in Cystocloniaceae (*ycf23* and *ycf92* genes).

Regarding the mitochondrial genomes, it is interesting to note that among all gigartinalean taxa analyzed here - Caulacanthaceae, Cystocloniaceae, Gigartinaceae and Solieriaceae - and previously published (Endocladiaceae, Gigartinaceae, Phyllophoraceae, Solieriaceae - Leblanc et al., 1995; Tablizo and Lluisma, 2014; Sissini et al., 2016; Li et al., 2018; Watanabe et al., 2019), only *Chondrus crispus* (Gigartinaceae) presented three rRNAs. Gene losses are rare in Florideophyceae mitochondrial genomes, being practically restricted to genes encoding ribosomal proteins (Salomaki and Lane, 2017). However, the presence of the 5S rRNA (*rrn5*) gene, an integral component of the large ribosomal subunit, is highly variable in mitochondrial genomes, both within and among different lineages of Rhodophyta (Leblanc et al., 1997; Burger et al., 1999; Yang et al., 2015; Iha et al., 2018; Yuan, 2018; Wang et al., 2019).

The increase in the number of sequenced genomes has revealed that the *rrn5* gene is more widespread than previously thought (Valach et al., 2014), and new annotations for this gene have been made in published genomes (eg. Iha et al., 2018; Lyra et al., 2021). Considering the current distribution of the *rrn5* gene in the analyzed mitochondrial Gigartinalean genomes, it is possible that it had been lost after the divergence of clade Gigartinaceae. If the occurrence of this gene is verified in other Gigartinales, it would mean that it may have been lost several times during the diversification of this clade, denoting the important role for nuclear-encoded proteins in the plant organelles (Sloan et al., 2010; Valach et al., 2014). Confirmation of both hypotheses requires more extensive sequencing of mitochondrial genomes from representatives of the order.

4.2. Phylogenomic analyses in Hypnea

We present the first phylogenomic analyses of the family Cystocloniaceae with focus on the genus *Hypnea*, including samples from new and historical collections in robustly resolved phylogenies based on plastomes and mitogenomes. The full support in all inner nodes of the chloroplast tree reinforces the power of this genome for resolving conflicting nodes in Rhodophyta (Janouškovec et al., 2013; Díaz-Tapia et al., 2017; Iha et al., 2018). Although our phylogenomic analyses included around 25 % of the species in *Hypnea* they had full resolution overall and advanced the evolutionary studies of the genus, previously limited to single or multi-genes analyses (Geraldino et al., 2010; Jesus et al., 2019b).

Hypnea was well-supported as monophyletic and species belonging to the sections proposed by J.Agardh were scattered in two well-separated inner clades, corroborating the findings of Jesus et al. (2019b). Although the traditional concept of J.Agardh's sections cannot be applied in an evolutionary context, we observed that in our phylogenies, species with prostrate and erect thalli were split into two distinct clades of *Hypnea* (clades I and II respectively, Fig. 1). Further phylogenomic sampling is required to improve our understanding of the evolution of thallus growth and the origin of erect and prostrate habits. This approach also could be relevant in the future for advancing in the classification of the Cystocloniaceae, as in several genera whose relationships or monophyly remain unresolved (e.g. *Calliblepharis* and *Meridionella*).

4.3. Taxonomic changes and new species proposition

Our phylogenomic and phylogenetic investigations illuminate several taxonomic insights that need attention, mainly at species taxonomic rank. Plastid and mitochondrial sequences were determined from historical herbarium specimens and compared against those from contemporary specimens in public databases.

Hypnea marchantiae was described by Setchell and Gardner (1924) based on specimens collected by Daisy R. Marchant in 1917 from Eureka, Baja California, Mexico. All of our analyses, which included sequences from the isotype specimen of *H. marchantiae* (FH01159049!, Marchant N° 36, Fig. 3A) and topotype specimens of *H. cervicornis* (Jesus et al., 2016), demonstrated that these species are conspecific (Figs. 1, 2 and 3). Their sequences differed only by 0 to 11 bp (0–0.87 %) in *rbc*L, and 0 to 14 bp (0–3.05 %) in COI-5P, which are considered as intraspecific variation. Furthermore, morphological analyses of isotype and holotype (UC221192!, Marchant N° 48, Fig. 3B) specimens of *H. marchantiae* also agree with the characterization of *H. cervicornis* (Jesus et al., 2016), presenting long thalli with percurrent axes, intensely branched, "antler-like" divaricate branches and bifurcate apices. These morphological similarities may be the cause of the absence of records of specimens of *H. marchantiae* in recent collections (Guiry and Guiry, 2022), even in their type locality. Here, we propose *H. marchantiae* Setchell & N.L.Gardner (1924) as a heterotypic synonym of *H. cervicornis* J.Agardh (1852).

Hypnea nidulans was described from American Samoa as a mat-forming species, usually 5–6 mm in height, forming dense clumps among corals (Setchell, 1924). In this study, the holotype of *H. nidulans* (FH 01159065!, Fig. 3C) could not be sequenced, but a specimen also collected and identified by Setchell from Tahiti in 1922, and housed at the Farlow Herbarium of Harvard University, USA (FH 01159066! Fig. 3D) was successfully sequenced. Both the holotype and the mentioned specimen exhibit the same external morphological features described in the original protologue. The historical specimen was distantly related to contemporary Asian specimens identified as *H. nidulans* from Philippines and Japan in both COI-5P (up to 12.23 % of divergence) and *rbc*L (up to 5,97 % of divergence) analyses (Fig. 3). Our findings demonstrated the need to review the taxonomic status of the Asian specimens previously identified as *H. nidulans.* The determination of sequences of the specimen identified by Setchell was essential for the accurate application of the name *H. nidulans* to contemporary specimens. Kang and Nam (2018) registered the occurrence of *Hypnea nidulans* in Korea based on the similarity of *rbc*L sequences with other Asian specimens (Yamagishi and Masuda, 2000; Geraldino et al., 2010). However, these specimens are morphologically consistent with the *H. charoides*' concept, with percurrent axes and branchlets curved in the adaxial direction. Our results indicated that *H. nidulans* from French Polynesian is a distinct taxonomic entity from contemporary Asian specimens named as such; thus, we described the specimens from Japan (Yamagishi and Masuda, 2000), Philippines (Geraldino et al., 2010) and Korea (Kang and Nam, 2018) as a new species: *Hypnea djamilae*, establishing as holotype the specimen NIBRAL0000146497!, deposited at KB Herbarium, Korea (Fig. 3I).

Another historical specimen collected by W.A. Setchell and H.E. Parks in Tahiti in 1922 (FH01159057!), and identified as *Hypnea nidifica,* was analyzed in this study and compared with its holotype (BM000005220!, Fig. 3E) and contemporary topotype specimens from Hawaii (Sherwood et al., 2010) (Fig. 3F). The Sherwood's specimens are morphologically similar to the holotype in the absence of the percurrent axes and divaricate branching pattern. Despite our voucher specimen sharing indistinguishable main axes with *H. nidifica*, it differs from this species in the prostrate habit, small size and mat-forming growth. Molecularly, the French Polynesian specimen was distantly related to the topotype (UPA sequence - HQ421469, Sherwood et al., 2010) and to the contemporary specimens named as such from Singapore (Geraldino et al., 2010) and India (Kundu and Bast, 2021). Considering all these findings, we propose the new species *Hypnea davisiana* (Fig. 3G,H), establishing as holotype the aforementioned specimen from the Farlow Herbarium (FH 01159057!).

In addition, we describe a third new species, *Hypnea evaristoae* (LWG003054!, Fig. 3J), based on specimens previously identified as *H. nidifica* from the Indian Ocean (Kundu and Bast, 2021). The presence of *H. nidifica* had already been reported by Geraldino et al. (2010 - COI-5P: GQ141879 and *rbc*L: FJ694932) in Singapore and, more recently, by Kundu and Bast (2021 - COI-5P: MT996224 and UPA: MT996210) in India based on molecular data. Although these last specimens are similar in external morphology to the holotype of *Hypnea nidifica*, our molecular analyses of UPA sequences clearly demonstrate that the contemporary specimens from the Indian Ocean differ from topotype and constitute two separate lineages.

Despite the increase of studies focusing on *Hypnea*, particularly in South America, morphological plasticity in the genus is still poorly understood (Jesus et al., 2019b). As well as cryptic species, singletons are common in genus, mainly among species with pulvinate morphology (Nauer et al., 2014, 2015, 2016; Jesus et al., 2019b), and formally describing these taxa is essential to assess the real diversity of this genus. According to Masuda et al. (1997), a critical reassessment of *Hypnea* species delimitation would need to use extensive preserved collections, including those from the type localities. Here, molecular identification of specimens from historical collections uncovered three new species, further demonstrating the importance of herbaria as repositories of biodiversity (Besnard et al., 2018). As also demonstrated by Hughey et al. (2014) and Boo et al. (2016), our data evidenced the importance of centenary herbarium collections, especially type specimens, for the correct application of names to taxonomically complicated groups, such as red algae.

The development of DNA sequencing technologies allowed the use of historical specimens (vouchers) in taxonomic and evolutionary studies by sampling rare species, and even recently extinct taxa (Bakker, 2017; Besnard et al., 2018). Sequences of *rbc*L (Fig. 2), COI-5P (Fig. S4) and UPA (Fig. S5) were compared with those from contemporary specimens and confirmed the identity of the archival specimens *Cystoclonium purpureum, Caulacanthus ustulatus* and *Catenella caespitosa.* In addition to Cystocloniaceae taxa, we sequenced complete organellar genomes from two archival centenary specimens of Caulacanthaceae. The specimen of *Catenella caespitosa* (FH 00789173!) collected by C.W. Nägeli in Torquay, England, is assumed to be 176 years old (Döring and Dressler, 2017), being, to our knowledge, the oldest specimen of Florideophyceae to have its organellar genomes completely sequenced. As stated by Bakker (2017), the fragmented DNA of herbarium specimens does not seem to represent a problem for the application of second-generation sequencing techniques.

5. Conclusion

Our analyses of modern and historical herbarium specimens included a phylogenomic framework and comparisons of composition and structure of the organellar genomes, evidencing relevant molecular synapomorphies (gene losses, InDels and gene inversions) in *Hypnea*. Furthermore, broadly sampled phylogenies in species representation from single nuclear, plastid or mitochondrial markers permitted the accurate identification of specimens based on sequenced type material, further supporting coherent taxonomic decisions. This novel approach for *Hypnea* and Cystocloniaceae improved the resolution and clade support, evidencing lineage divergences.

Funding

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CRediT authorship contribution statement

Priscila Barreto de Jesus: Conceptualization, Methodology, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Writing – review & editing, Funding acquisition, Project administration. **Goia de Mattos Lyra:** Conceptualization, Methodology, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Writing – review & editing, Funding acquisition, Project administration. **Hongrui Zhang:** Formal analysis, Investigation, Data curation, Writing – review & editing. **Mutue Toyota Fujii:** Resources, Writing – review & editing. **Fabio Nauer:** Resources. **José Marcos de Castro Nunes:** Conceptualization, Resources, Writing – review & editing, Funding acquisition. **Charles C. Davis:** Conceptualization, Resources, Funding acquisition, Writing - review & editing. **Mariana Cabral Oliveira:** Conceptualization, Resources, Writing – review & editing, Funding acquisition, 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.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ympev.2023.107752.

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