

Programa de Pós-Graduação em Biodiversidade e Evolução
Universidade Federal da Bahia

**Estudos filogenômicos e morfológicos integrados em
Gigartinales: resolvendo o clado mais desafiador em
Rhodophyta**

Edilene Maria dos Santos Pestana

Salvador

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resolvendo o clado mais desafiador em Rhodophyta**

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Edilene Maria dos Santos Pestana

Orientador(a): José Marcos de Castro Nunes

Tese de Doutorado submetida ao Programa de Pós-Graduação em Biodiversidade e Evolução da Universidade Federal da Bahia como parte dos requisitos necessários à obtenção do título de Doutor na área de Biodiversidade e Evolução.

Aprovada por:

Em: 29 de novembro de 2024.

Prof(a). Dr(a).: José Marcos de Castro Nunes

Prof(a). Dr(a).: Carlos Frederico Deluqui Gurgel

Prof(a). Dr(a).: Mutue Toyota Fujii

Prof(a). Dr(a).: Joel Campos de Paula

Prof(a). Dr(a).: Daniela Milstein

Orientador(a): José Marcos de Castro Nunes

Co-orientador(a): Goia de Mattos Lyra

*“My mission in life is not merely to survive,
but to thrive; and to do so with some
passion, some compassion, some humor,
and some style”*

Maya Angelou

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RESUMO

Gigartinales é uma das ordens mais ricas em espécies dentro das Rhodophyta, com grande importância ecológica e econômica. Ecologicamente, contribui para a formação de habitats, alimentação de organismos marinhos e participação nos ciclos biogeoquímicos. Economicamente, fornece compostos bioativos utilizados nas indústrias alimentícia, cosmética e farmacêutica. Atualmente, são reconhecidas 951 espécies em 36 famílias. No entanto, os caracteres morfológicos utilizados para delimitação de táxons apresentam ampla variação e não são exclusivos deste clado, indicando que sua circunscrição atual não reflete adequadamente sua história evolutiva. Além disso, as relações filogenéticas entre linhagens apresentam baixo suporte, especialmente em ramos ancestrais. Com base nesse histórico taxonômico complexo, este estudo teve como objetivo circunscrever Gigartinales utilizando evidências morfológicas e moleculares, propondo um esquema taxonômico que reflita sua evolução. Foram sequenciadas 57 espécies de Gigartinales e Peyssonneliales, a partir de espécimes históricos e amostras frescas preservadas em sílica, gerando 37 genomas mitocondriais e 40 plastidiais completos. Esses genomas forneceram 28 genes mitocondriais e 215 plastidiais, utilizados para a construção de filogenias. Além disso, foram obtidas 248 sequências do gene nuclear LSU do GenBank e três novas sequências foram geradas neste estudo. As análises de máxima verossimilhança foram conduzidas no CIPRES Science Gateway, enquanto alinhamentos para análise de sintenia foram realizados no Mauve, com inferência de ganho e perda de genes pela parcimônia de Dollo, usando COUNT. Cinco caracteres foram selecionados para análises de evolução: hábito, forma do talo, tipo de crescimento, formação de procarpo e tipo de divisão dos tetrasporângios. Dados de ocorrência de Gigartinales, provenientes do GBIF e Algaebase, foram utilizados para análises biogeográficas, e a correlação entre essas bases foi testada pelo coeficiente de Pearson. Os resultados mostraram que Gigartinales, com suporte de 100% nas filogenias organelares, inclui todas as espécies de Peyssonneliales, representando os principais gêneros da família Peyssonneliaceae. As filogenias organelares indicaram inconsistências no posicionamento de Gigartinaceae, Phylophoraceae e Phacelocarpaceae, enquanto a topologia da árvore nuclear foi congruente com a árvore plastidial. Essas filogenias genômicas forneceram uma base sólida para interpretar eventos moleculares, como perdas, ganhos e translocações de genes, além da evolução de caracteres morfológicos. A análise biogeográfica revelou *hotspots* de riqueza de espécies na costa pacífica dos EUA, no sul da Austrália e na Europa. O teste de correlação de Pearson indicou uma relação positiva

significativa entre os dados do GBIF e Algaebase. Métricas de diversidade filogenética mostraram estruturas filogenéticas distintas em comunidades de Gigartinales, mesmo com similar riqueza de espécies. Os resultados destacam a necessidade de maior amostragem genética e uso de filogenias bem resolvidas para investigar relações entre linhagens de algas vermelhas. Estudos adicionais sobre coleções de macroalgas e metadados digitais também são essenciais para compreender os efeitos ecológicos e vieses de amostragem nos padrões de diversidade filogenética.

Palavras-chave: Algas vermelhas; Biogeografia; Genômica; Sistemática

ABSTRACT

Gigartinales is one of the most species-rich orders within Rhodophyta, with significant ecological and economic importance. Ecologically, it contributes to habitat formation, serves as a food source for marine organisms, and participates in ocean biogeochemical cycles. Economically, it provides bioactive compounds used in the food, cosmetic, and pharmaceutical industries. Currently, 951 species distributed across 36 families are recognized. However, the morphological traits used to delimit taxa exhibit broad variation and are not exclusive to this clade, suggesting that the current circumscription of Gigartinales does not adequately reflect its evolutionary history. Furthermore, phylogenetic relationships among lineages show low support, especially in ancestral branches. Based on this complex taxonomic history, this study aimed to circumscribe Gigartinales using morphological and molecular evidence, proposing a taxonomic framework that reflects its evolution. A total of 57 species of Gigartinales and Peyssonneliales were sequenced, using specimens from historical collections and fresh samples preserved in silica gel. This yielded 37 complete mitochondrial genomes and 40 complete plastid genomes, providing 28 mitochondrial and 215 plastid genes used for phylogenetic construction. Additionally, 248 nuclear LSU gene (28S) sequences were obtained from GenBank, with three new LSU sequences generated in this study. Maximum likelihood analyses were conducted via the CIPRES Science Gateway, while alignments for synteny analyses were performed in Mauve, with gene gain and loss inferred using Dollo parsimony in COUNT. Five traits were selected for evolutionary analyses: habit, thallus form, growth type, procarp formation, and type of tetrasporangial division. Occurrence data for Gigartinales, sourced from GBIF and Algaebase, were used

for biogeographic analyses, with the correlation between these databases tested using Pearson's correlation coefficient. The results revealed that Gigartinales, with 100% support in organellar phylogenies, includes all sampled species of Peyssonneliales, representing the major genera of the family Peyssonneliaceae. Organellar phylogenies indicated inconsistencies in the placement of Gigartinaceae, Phylophoraceae, and Phacelocarpaceae, while the topology of the nuclear tree was congruent with the plastidial tree. These genomic phylogenies provided a robust framework for interpreting molecular events such as gene losses, gains, translocations, and inversions, as well as the evolution of morphological traits. Biogeographic analysis identified species richness hotspots along the Pacific coast of the United States, southern Australia, and Europe. Pearson's correlation test revealed a significant positive relationship between GBIF and Algaebase data. Phylogenetic diversity metrics indicated distinct phylogenetic structures in Gigartinales communities despite similar species richness. These findings underscore the need for broader genetic sampling and well-resolved phylogenies to investigate relationships among red algal lineages. Furthermore, additional studies on macroalgal collections and digital metadata are essential to clarify ecological contributions and sampling biases in phylogenetic diversity patterns.

Keywords: Biogeography; Genomics; Red Algae; Systematics

INTRODUÇÃO GERAL

O Filo Rhodophyta concentra as algas vermelhas e pode ser sido a primeira linhagem algal a divergir no clado Archaeplastida (GAWRYLUK *et al.*, 2019), com fóssil conhecido datado em cerca de 1,2 bilhão de anos (BUTTERFIELD, 2000). Datações com base em métodos diferentes estimam a divergência de Rhodophyta entre 825 milhões (DOUZERY *et al.*, 2004) e 1,5 bilhão de anos (YOON *et al.*, 2004). Atualmente, Rhodophyta apresenta aproximadamente 7.400 espécies distribuídas em cerca de 600 gêneros, sendo o filo mais diverso dentre as macroalgas (GUIRY & GUIRY, 2024). A filogenia mais ampla de Rhodophyta, tanto em número de amostras quanto em número de genes construída até o momento (VERBRUGGEN *et al.*, 2010), revela que as relações entre as linhagens não são bem compreendidas, apresentando baixo suporte principalmente em ramos ancestrais. Os autores apontam que, dentre as 33 ordens representadas na filogenia, Gigartinales foi uma das duas ordens que se mostraram polifiléticas, com linhagens distribuídas em pelo menos quatro clados distantemente relacionados.

Revisão histórica da classificação de Gigartinales

Gigartinales foi proposta por Schmitz (1892), e classificada aa subclasse Florideae (Classe Florideophyceae), com base na ontogenia do cistocarpo, especialmente no que se refere ao seu local de formação e à função da célula auxiliar. A classificação de Schmitz (1892) foi posteriormente aprimorada por Kylin (1956), quando Florideae passou a incluir 6 ordens: Nemalionales, Gelidiales, Gigartinales, Cryptonemiales, Rhodymeniales e Ceramiales. Dentre as ordens propostas por Kylin, a taxonomia de Cryptonemiales e Gigartinales se mostraram particularmente desafiadoras, sendo a utilização da localização da célula auxiliar como caráter diagnóstico questionada ao longo das quatro décadas que se seguiram (SEARLES, 1968; KRAFT & ROBINS, 1985; HOMMERSAND & FREDERICQ, 1990). Kraft & Robins (1985) propuseram a unificação das duas ordens em uma única, nascendo o conceito de Gigartinales *sensu lato*. Enquanto Cryptonemiales encontra-se resolvida como sinônimo de Halymeniales (GUIRY & GUIRY, 2024), Gigartinales ainda apresenta conceito pouco claro e abarca clados distantemente relacionados (VERBRUGGEN *et al.*, 2010). Gigartinales possui 967 espécies aceitas, distribuídas em 36 famílias, sendo as mais representativas Kallymeniaceae com 202 espécies, Gigartinaceae com 135 e Phyllophoraceae com 132 (GUIRY & GUIRY, 2024).

Importância ecológica e econômica

A maioria dos representantes de Gigartinales apresenta distribuição cosmopolita e possui grande importância ecológica na produção primária, na criação de habitats e servindo como fonte de alimentos para a vida marinha (LEE, 2008). A ordem também é conhecida por sua enorme importância econômica, pois suas espécies são fonte de carragenana, polissacarídeos acumulados na porção externa das paredes celulares. As carragenanas comerciais são normalmente divididas em três tipos principais: kappa (κ) -, iota (ι) - e lambda (λ) -carragenana, sendo a κ e a ι mais amplamente utilizada na indústria alimentícia como agente espessante, agente gelificante, agente de suspensão e agente estabilizante para o endurecimento produtos lácteos (LEE, 2008; PEREIRA *et al.*, 2009; PEREIRA *et al.*, 2013). Na indústria cosmética e farmacêutica, as carragenanas são utilizadas para os mesmos fins e ainda têm sido objeto de estudos promissores, como a utilização *in vitro* de extratos de *Solieria filiformis* (Kützinger) P.W.Gabrielson como agentes antivirais naturais contra o vírus causador de herpes (*Herpes simplex* tipo 1) (ANA *et al.*, 2021) e como fonte de antioxidantes, a exemplo de *Eucheuma gelatinum* (Esper) J.Agardh (HA *et al.*, 2022). Um outro estudo demonstrou a ação protetora das carragenanas contra a neurodegeneração dopaminérgica *in vitro* (LIU *et al.*, 2015).

Kappaphycus Doty (Solieriaceae) é uma das algas vermelhas mais importantes economicamente, pois a ampla gama de aplicações da κ -carragenana lhe confere alto valor de mercado alto (PEREIRA *et al.*, 2015). Seu cultivo é realizado em águas tropicais e subtropicais, principalmente na Ásia. Os principais produtores são a Indonésia e as Filipinas, país onde foi implementado o primeiro cultivo bem sucedido de *Kappaphycus*. Países da América Latina, incluindo o Brasil, entraram muito recentemente nessa área e têm desempenhado um papel importante no cultivo de algas marinhas e na extração de carragenanas devido às condições ambientais favoráveis (VALDERRAMA *et al.*, 2013).

Caracterização morfológica

Espécies de Gigartinales apresentam caracteres morfológicos e estruturais muito variados: talos com formas diversas, podendo ser cilíndricos (Fig. 1A) ou achatados (Fig. 1B); podem apresentar constrições; podem ser delgados ou espessos; podem apresentar nervuras; o crescimento pode ser uniaxial, a partir da divisão de uma única célula apical, com filamento central único (Fig. 1C) ou multiaxial, a partir da divisão de várias células, com vários

filamentos centrais (Fig. 1D); e a organização do talo pode ser pseudoparenquimatosa (Fig. 1E) ou pletenquimatosa (Fig. 1F). Quanto aos caracteres reprodutivos, apresentam histórico de vida haplodiplôntico trifásico, predominantemente isomórfico. A transferência do zigoto se dá por conexão de células ou filamentos ou por fusão direta. O carposporófito (Fig. 1G) geralmente apresenta uma célula de fusão e extensos gonimoblastos estéreis, sendo alguns compostos principalmente de carposporângios. Podem ser procárpico, célula auxiliar e carposporófito podem ou não ocorrer no mesmo ramo, e os tetrasporângios são divididos de forma cruciada ou zonada (Fig. 1H) (MIN-THEIN & WOMERSLEY, 1976; HOMMERSAND & FREDERICQ, 1990; SAUNDERS *et al.* 2017; HUISMAN, 2018; GUIRY & GUIRY, 2024).

Tais caracteres, entretanto, não são exclusivos desta ordem e apresentam uma ampla variação de estados, de forma que a circunscrição atual de Gigartinales não é bem definida e o monofiletismo do grupo não foi estabelecido. É preciso avaliar a circunscrição morfológica da ordem num contexto evolutivo e avaliar caracteres úteis para a delimitação de táxons em vários níveis subordinais.

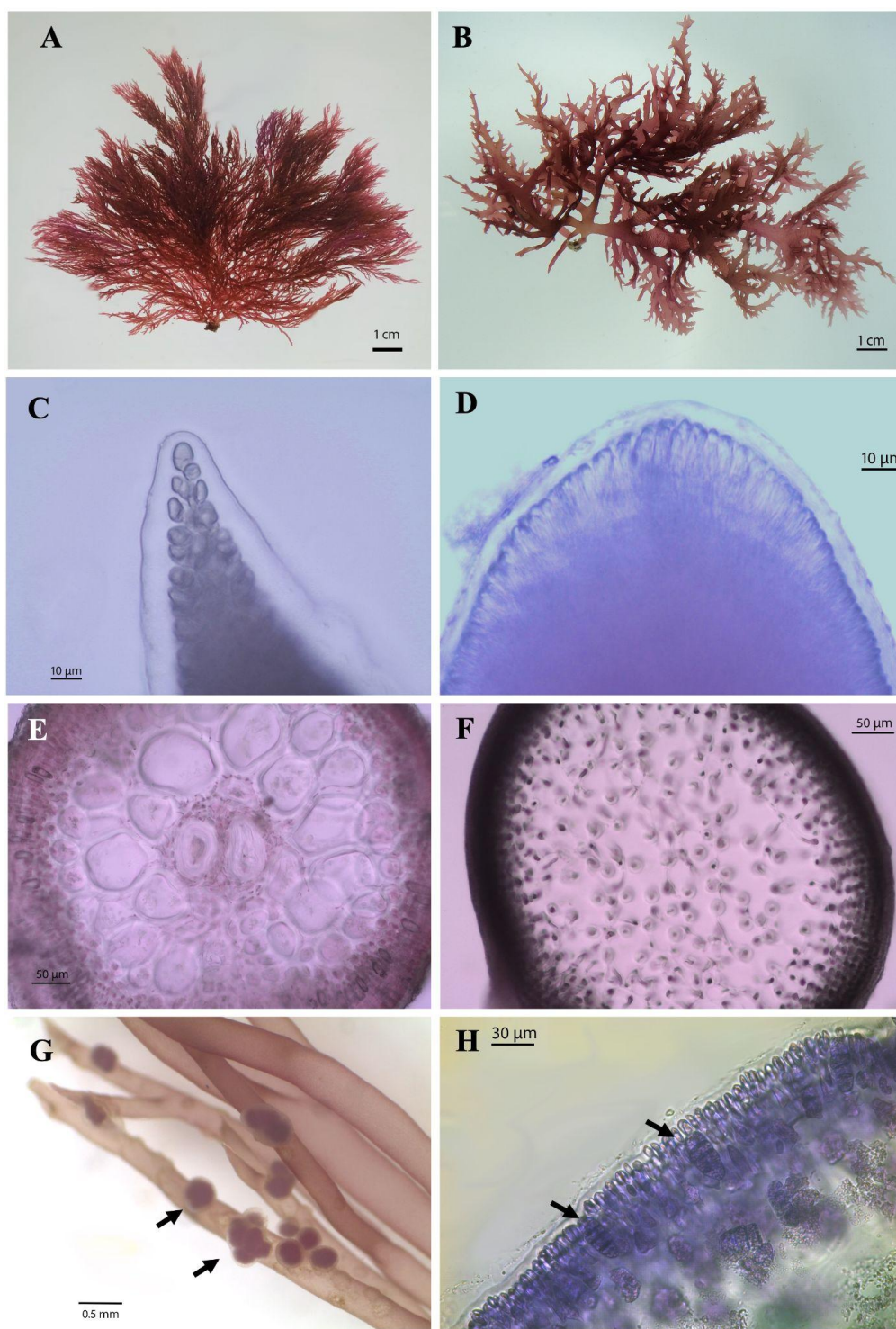


Figura 1. Características gerais de Gigartinales. **A.** Talo cilíndrico. **B.** Talo achatado. **C.** Crescimento uniaxial do talo. **D.** Crescimento multiaxial do talo em vista longitudinal. **E.** Organização pseudoparenquimatosa do talo em vista transversal. **F.** Organização pletenquimatosa do talo em vista transversal. **G.** Carposporófitos crescendo sobre o gametófito (setas). **H.** Tetrasporângios zonados (setas).

Taxonomia molecular

A taxonomia morfológica, embora imprescindível, nem sempre é suficiente em alguns grupos de macroalgas, principalmente quando possuem morfologia e anatomia simples, somada a uma grande plasticidade fenotípica (OLIVEIRA & MILSTEIN, 2010). Nas últimas duas décadas, dados de sequências de DNA trouxeram acurácia adicional para a sistemática, levando à intensificação do uso de técnicas moleculares para o estudo de macroalgas vermelhas (CASSANO, 2009; VERBRUGGEN, 2010; LYRA *et al.*, 2015, 2016; JESUS *et al.*, 2016, 2018; PESTANA *et al.*, 2020, 2021). Estudos com abordagem molecular em Gigartinales têm levado à proposição de novas espécies (HUISMAN *et al.*, 2016; JESUS *et al.*, 2023), gêneros (BÁRBARA *et al.*, 2013; RODRÍGUEZ-PRIETO *et al.*, 2013, 2014; KRAFT & SAUNDERS, 2014; SAUNDERS *et al.*, 2017) e famílias (SAUNDERS *et al.*, 2004; RODRÍGUEZ-PRIETO *et al.*, 2013; DIXON *et al.*, 2015). Evidências morfológicas e moleculares, permitiram que alguns táxons classificados em Gigartinales fossem elevados ao *status* de ordem: Saunders & Kraft (1994) reconheceram Plocamiaceae como clado irmão de Gigartinales, propondo Plocamiales com base em estudos filogenéticos (SSU), combinando dados moleculares e análises das estruturas reprodutivas

A filogenia de Rhodophyta apresentada por Verbruggen *et al.* (2010) apresenta Gigartinales distribuída em quatro clados distantemente relacionados: *core* Gigartinales, *Atractophora* P.Crouan & H.Crouan, Calosiphoniaceae e Peyssonneliaceae, sendo que o clado *core* Gigartinales inclui a espécie tipo, *Gigartina pistillata* (S. G. Gmelin) Stackhouse, com base na qual Gigartinaceae foi descrita. Kravesky *et al.* (2009) propuseram a criação da ordem Peyssonneliales baseada em Peyssonneliaceae, utilizando dados morfológicos e moleculares (*rbcL* e LSU rDNA); Saunders *et al.* (2016) propuseram a ordem Atractophorales, incluindo a família Atractophoraceae, baseada no gênero *Atractophora*, a partir de uma análise multigene (SSU, LSU, EF2, *rbcL*, COI-5P). Apenas Calosiphoniaceae (que tem *Calosiphonia* P.Crouan & H.Crouan como gênero tipo) se encontra sem tratamento taxonômico apropriado e que reflita a sua história evolutiva. Entretanto, estudos recentes têm demonstrado que outros gêneros de Calosiphoniaceae estão distantemente relacionados ao clado *core* Gigartinales. Espécies de *Schimitzia* P.C.Silva, por exemplo, estão bem suportadas como proximamente relacionadas a espécies de *Neoabbottiella* Perstenko,

gênero atualmente classificado em Halymeniaceae (Halymeniales), mas que vem sendo percebido como *incertae sedis* (Skriptsova et al. 2024).

Apesar dos trabalhos incluindo biologia molecular representarem um avanço na taxonomia e sistemática de Gigartinales, as informações acerca da ordem encontram-se fragmentadas e Gigartinales permanece sendo um desafio dentre as algas vermelhas. Abordagens taxonômicas integradas, com filogenias em nível genômico, análise de tempo de divergência e análise de evolução de caracteres têm se mostrado úteis na resolução de clados em Rhodophyta (PAIANO *et al.*, 2018; LYRA *et al.*, 2021; JESUS *et al.*, 2023). Análises de arquitetura de genomas organelares de *Mastocarpus papillatus* (C. Agardh) Kützinger (Phyllophoraceae) (SISSINI *et al.*, 2016), *Kappaphycus alvarezii* (Doty) L. M. Liao (LIU *et al.*, 2019) e do gênero *Hypnea* (JESUS *et al.*, 2023) revelaram o potencial da utilização de sintenia gênica na sistemática molecular de Gigartinales, assim como em outros clados de algas vermelhas (IHA *et al.*, 2018; LYRA *et al.*, 2021).

Aspectos biogeográficos

A distribuição dos seres vivos não é aleatória e é possível perceber padrões de ocorrência resultantes de eventos da história geológica da Terra, geralmente aplicados a categorias taxonômicas mais elevadas, como filos, ordens e famílias (NELSON & PLATNICK, 1981; HUMPHRIES & PARENTI, 1999; CRISCI *et al.*, 2000, MORRONE, 2009) e como resultado de fatores ecológicos em menor escala de tempo, como temperatura, salinidade e disponibilidade de recursos, observados em estudos com foco em gêneros e espécies (NELSON & PLATNICK, 1981; COX & MOORE, 1993; MORRONE, 2009). Os estudos em biogeografia buscam identificar e descrever esses padrões, gerando hipóteses e teorias sobre os eventos que levaram à distribuição observada. Mais comumente, é possível encontrar estudos envolvendo animais e plantas terrestres com hipóteses bem estabelecidas, distribuições bem definidas e até mesmo identificando as barreiras geográficas que levaram à especiação (FLOETER *et al.*, 2009; MIRANDA & MARQUES, 2011).

Quando se trata de biogeografia ecológica, a identificação de barreiras geográficas que determinam a cladogênese e distribuição de grupos é um desafio. Pioneiro em propor uma compartimentação da biota marinha, Dana (1852) utilizou isóclinas para estabelecer seu sistema e até hoje a temperatura é considerada um dos principais fatores primários que regulam a distribuição das espécies em escala global, tornando-se senso comum a existência de floras e faunas características das regiões polares, tropicais e temperadas. Além da

temperatura, pressão, salinidade e luz podem ser importantes variáveis ambientais que limitam a distribuição em gradiente horizontal e vertical (PIELOU, 1979).

A partir dos poucos estudos disponíveis sobre biogeografia de Rhodophyta, é razoável concluir que a maioria das linhagens de Rhodophyta se originou no Mesozóico e pode ser dividida em dois grupos: i) aquelas que evoluíram do Mar de Thetys e ii) aquelas que evoluíram ao longo do perímetro exterior da Pangea (Hommersand 1981). Em Gigartinales apresenta famílias que se enquadram em ambas as categorias. Famílias pertencentes ao complexo Solieriaceae parecem ser originárias do Mar de Thetys (Fredericq *et al.* 1999), enquanto que membros de Gigartinaceae e Kallymeniaceae se encaixam na segunda hipótese (HOMMERSAND, 1981; HOMMERSAND *et al.*, 1994). Contudo, os padrões de distribuição das espécies de Gigartinales é ainda mais desconhecido do que o conhecimento sobre as relações filogenéticas da ordem.

OBJETIVOS

Objetivo geral

Circunscrever o clado Gigartinales (Rhodophyta) com base em evidências morfológicas, moleculares e biogeográficas, propondo um esquema taxonômico para a ordem que reflita a sua história evolutiva.

Objetivos específicos

- Construir e contrastar filogenias de Gigartinales baseadas em genes provenientes de genomas distintos (nuclear, plastidial e mitocondrial), através de amostragem densa de táxons em super matrizes que incluam todos os dados disponíveis no GenBank para o grupo, bem como o conjunto de dados genômicos inéditos, melhorando significativamente a resolução filogenética e acurácia.
- Identificar apomorfias dos clados e subclados em Gigartinales por meio de análise de evolução de caracteres morfológicos e arquitetura de genomas organelares.
- Analisar os metadados públicos de espécimes de Gigartinales e verificar os seus potenciais usos para a identificação de padrões biogeográficos para o grupo estudado.
- Gerar dados que irão contribuir para a ampliação de bancos de dados de taxonomia e biogeografia.

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CAPÍTULO 1

Título: Peyssonneliaceae: a clade in the mega diverse, and now monophyletic, Gigartinales

Autores: Edilene Maria dos Santos Pestana, Goia de Mattos Lyra, Hongrui Zhang & José Marcos de Castro Nunes

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Peyssonneliaceae: a clade in the mega diverse, and now monophyletic, Gigartinales

Edilene Maria dos Santos Pestana^{1,2}, Goia de Mattos Lyra^{1,3}, Hongrui Zhang⁴, José Marcos de Castro Nunes^{1,2}

¹*Programa de Pós-Graduação em Biodiversidade e Evolução (PPG BioEvo), Instituto de Biologia, Universidade Federal da Bahia, Rua Barão de Jeremoabo, s/n, Salvador, Bahia 40170-115, Brazil*

²*Laboratório de Algas Marinhas (LAMAR), Instituto de Biologia, Universidade Federal da Bahia, Rua Barão de Jeremoabo, s/n, Salvador, Bahia 40170-115, Brazil*

³*Departamento de Biologia Vegetal, Universidade do Estado do Rio de Janeiro, R. São Francisco Xavier, 524 - Maracanã, Rio de Janeiro - RJ, 20550-013, Brazil*

⁴*Texas A&M University, 400 Bizzell St, College Station, Texas 77843, USA*

Running title: *Peyssonneliaceae: a clade in Gigartinales*

Author for correspondence: edimpestana@gmail.com, +55 (71) 3283-6598

Abstract

Gigartinales is a historically polyphyletic group of red algae, comprising a large diversity of species, several of which with great ecological and economic relevance. Peyssonneliaceae, a clade of red encrusting algae distributed worldwide, was initially placed in Cryptonemiales, later classified in Gigartinales and was recently elevated to order status as Peyssonneliales. Includes 147 species in 17 currently accepted genera. Gene-based analyses have been used to support the segregation of Peyssonneliaceae from Gigartinales, showing a distant relationship between them. Here we include new organellar genomes from species of Peyssonneliaceae in phylogenetic analyses of two genomic datasets (mitochondrial and plastid) of Rhodophyta to contribute to the elucidation of its evolutionary history. Our results show that Peyssonneliaceae is placed within Gigartinales forming a full-supported clade in both organellar phylogenies. Therefore, we present an updated and broader concept of

Gigartinales, including Peyssonneliaceae. We also segregate Calosiphonaceae from the order, based on available evidence, finally rendering Gigartinales monophyletic. This study contributes to the understanding of relationships among Rhodophyta, evidencing the effectiveness of the genomic approach in inferring these phylogenetic relationships and providing a better understanding of challenging groups as Gigartinales.

Keywords: Genomics, Mitogenome, Peyssonneliales, Plastome, Taxonomy

Introduction

Gigartinales is speciose and historically polyphyletic order of red algae. In the most complete red algae phylogeny to date (Verbruggen *et al.*, 2010), Gigartinales was spread in at least four distantly related lineages, some of which were later segregated and reclassified [e.g., Atractophorales Maggs, L.Le Gall, Filloramo & G.W.Saunders (Saunders *et al.*, 2016); Peyssonneliales Kravesky, Fredericq & J.N.Norris (Kravesky *et al.*, 2009)]. Peyssonneliaceae Denizot, the single family in the Peyssonneliales, is a diverse clade of prostrate crustose red algae, several of which with calcified thallus involved in the formation of coral reef structures, particularly in the tropics. Peyssonneliales was proposed based on molecular (*rbcL* and LSU rDNA) and morphological data, including types from the genera *Peyssonnelia* Decaisne and *Sonderophycus* Denizot (1968) (as *Sonderopelta* Womersley & Sinkora, 1981). Currently, 147 species are reported in the literature, distributed in 17 genera (Guiry & Guiry, 2024).

The taxonomic history of the family has been challenging researchers for nearly three centuries. The first representative of Peyssonneliaceae, *Fucus squamarius* S.G. Gmelin, was described by Gmelin (1768) in his book *Historia fucorum*, in which species were grouped based on characters such as “internally porous cartilaginous frond” and “flat stem cartilaginous”. In his monograph, Zanardini (1841) described the family Squamariaceae (“Squamariae”), based on scale-shaped growth (Latin: squama = scale), designating *F. squamarius* as the type species under the name *Squamaria vulgaris* Zanardini. The family comprised the genera *Hidelbrandia* Zanardini and *Zanardinia* Zanardini, both presenting flat thallus attached to the substrate. However, the name *Squamaria* Zanardini was a homonym of *Squamaria* Ludwig (1757). Simultaneously, Decaisne described *Peyssonnelia squamaria* (S.G.Gmelin) Decaisne ex J.Agardh (1842), classifying it in the family Choristosporeae Descaisne. J. Agardh (1842) recognized the genus *Peyssonnelia* (1841) as legitimate. *Peyssonnelia* remained in Squamariaceae until Denizot (1968), in the book *Les*

algues floridées encroutantes (à l'exclusion des Corallinacées), recognized the illegitimacy of the name, as it was based on *Squamaria* Zanardini. The author proposed the family Peyssonneliaceae, including the genera *Peyssonnelia* (with 48 species) and *Cruoriella* P.Crouan et H.Crouan (1859). Denizot also suggested that the genus *Polysrara* Heydrich (1905) could be included in the family.

Schmitz (1889, 1892) and Schmitz & Hauptfleisch (1897) created a classification system for red algae based on the development of reproductive structures, mainly the site of formation and the function of the auxiliary cell in cystocarp ontogeny. Peyssonneliaceae (still as Squamariaceae) was originally classified in Cryptonemiales F. Schmitz (1892) (Florideae, Rhodophyta) due to the lack of formation of a procarp, with the auxiliary cells being dispersed throughout the thallus. Schmitz's classification was later improved by Kylin (1956), with Florideae including 6 orders: Nemalionales, Gelidiales, Gigartinales, Cryptonemiales, Rhodymeniales and Ceramiales, with Peyssonneliaceae remaining in Cryptonemiales (Krayesky *et al.*, 2009). However, the use of the location of the auxiliary cell as a taxonomic character for delimiting Cryptonemiales and Gigartinales was questioned (Searles, 1968; Kraft & Robins, 1985; Fredericq & Hommersand, 1990), and Kraft & Robins (1985) proposed the unification of the two orders into one, giving rise to the concept of Gigartinales *sensu lato*.

Throughout the 20th century, some authors indicated that Peyssonneliaceae should be classified in Corallinales (Funk, 1927; Hylander, 1928; Feldmann, 1931; Silva & Johansen, 1986), based on the calcification of the thallus. The taxonomic position of Peyssonneliaceae within Gigartinales *sensu lato* was also questioned when the first molecular data became available. Phylogenies based on plastid (Fredericq *et al.*, 1996) or nuclear (Harper & Saunders, 2001; Le Gall & Saunders, 2007) genes separately concluded that Peyssonneliaceae is monophyletic and not nested in any order of Florideophyceae, with the placement of Peyssonneliaceae species varying across the studies.

Until the formal proposition of Peyssonneliales (Krayesky *et al.*, 2009), its species had been represented in phylogenies based on single or multigenes. Verbruggen *et al.* (2010) used a data mining approach and built the largest phylogeny of Rhodophyta so far, both in number of genes (14 *loci*) and species. In this phylogeny, although not robustly supported, Peyssonneliaceae is in a sister relationship with Gelidiales, in a clade also including Gracilariales. These results indicated that Peyssonneliaceae was probably not closely related to any other clade of the polyphyletic Gigartinales F.Schmitz or to Halymeniales G.W.Saunders & Kraft (which now includes former Cryptonemiales species), as proposed

by previous authors. The most recent taxonomic study of Peyssonneliaceae (Pestana *et al.* 2021) was also based on two datasets of single genes and did not investigate the placement of the family in the red algae tree of life. Genomic data are helping to better understand relationships in several clades of red algae (Yang *et al.*, 2016; Nan *et al.*, 2017; Lyra *et al.*, 2021; Jesus *et al.*, 2023). Here we investigated the phylogenetic placement of Peyssonneliaceae, including sequences of several species in a broader genomic dataset of Rhodophyta., which bases our proposal of a classification that better reflects the evolutionary history of the family.

Material and Methods

We selected samples from 6 specimens of Peyssonneliaceae collected in Brazil (Table S1), representing three genera, for organellar genome sequencing (Table S1). DNA had been previously extracted [using 20–30 mg of tissue with extraction kits NucleoSpin[®] Plant II (MachereyNagel Duren, Germany) or PureLink[®] Plant (Invitrogen, Waltham, MA, USA), according to the protocols of the suppliers] and sequenced for single markers. Selected samples' sequences were nested in Peyssonneliaceae in broadly sampled COI-5P and *rcbL* phylogenies of the family (Pestana *et al.*, 2021). In our analyses, we included genomes available in GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>, searched September 2023 – Figure S1 and S2) from the main Rhodophyta lineages, and also three species of Chlorophyta as outgroup.

Sequencing, assembling and annotation

Libraries and sequencing were developed at the FAS Center for Systems Biology at Harvard University. Libraries were prepared with the KAPA HyperPlus Library Preparation Kit (Kapa Biosystems, Wilmington, USA) with 8 min of incubation during fragmentation 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. Average sizes of DNA fragments were verified with the Agilent 2100 Bioanalyzer with the DNA High Sensitivity chip. Real-Time PCR (BioRad CFX96 Touch, BioRad Laboratories, Hercules, USA) with the NEBNext Library Quant Kit (New England Biolabs, Ipswich, USA) was used to verify the final concentrations of libraries and libraries' pool. Sequencing was performed on the Illumina HiSeq v4 High-Output (Illumina, Inc.) with 250 bp paired-end runs.

Raw Illumina reads' quality was analyzed in FastQC (Krueger *et al.*, 2011). Trimmomatic v0.036 (Bolger *et al.*, 2014) was used to clean and trim low-quality reads and bases. Plastomes and mitogenomes were assembled de novo using the PhyloHerb pipeline (Cai *et al.*, 2022). Bandage v.0.8.1 (Wick *et al.*, 2015) was used to select the complete or incomplete contigs by importing the files created by PhyloHerb. We 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* Stackhouse as reference.

Phylogenetic analyses

We extracted genes from mitogenomes and from plastomes (see genes from newly generated genomes in Table S1), generated alignments in MAFFT (Katoh and Standley, 2013), and trimmed unreliable regions using trimAl (Capella-Gutierrez *et al.*, 2009). The evolutionary model GTR+GAMMA was employed for Maximum Likelihood (ML) phylogenetic analyses, performed as a single partition on the CIPRES Science Gateway (Miller *et al.*, 2011). ML analyses were conducted using RAxML (version 8; Stamatakis, 2014), run through CIPRES to leverage computational resources. A total of 1,000 rapid bootstrap replicates were executed to assess node support and increase tree reliability. The resulting ML trees were visualized and edited in FigTree v1.4.3 (Rambaut, 2017) to enhance interpretability.

Results

We obtained 6 mitogenomes (4 complete and 2 incomplete) from representatives of Peyssonneliaceae: *Peyssonelia* sp., *Agissea inamoena* (Pilger) Pestana, Lyra, Cassano & J.M.C. Nunes, *Ramicrosta fujiana* Pestana, G.N.Santos, Cassano & J.M.C.Nunes, *Ramicrosta paradoxa* Pestana, G.N.Santos, Cassano & J.M.C.Nunes, *Sonderophycus* sp. We also obtained three incomplete plastomes from these samples (*R. fujiana*, *R. paradoxa* and *Sonderophycus* sp.). These were added to 143 and 125 published Rhodophyta mitochondrial and plastid genomes, respectively (Figure S1).

Complete mitogenomes' sizes varied from 25,338bp to 29,463bp. The incomplete mitogenome of *Brasilophycus similis* Pestana, G.N.Santos, Cassano & J.M.C.Nunes was larger (31,587) than the other sequenced genomes. Mitochondrial alignment length was

16,445 bp, composed of 28 genes and plastid alignment was 125,545 bp long, composed of 215 genes. Our mitochondrial phylogeny (Figure 1) placed Peyssonneliaceae, a monophyletic clade, within Gigartinales (represented by 16 species in 8 genera; see complete tree in Figure S1) with full support. Peyssonneliaceae was sister to a fully supported clade comprising Endocladiaceae, Gigartinaceae and Phyllophoraceae. This clade, including Peyssonneliaceae, was fully supported as sister to a fully supported clade comprising Solieriaceae, Caulacanthaceae and Cystocloniaceae. Solieriaceae, Caulacanthaceae and Cystocloniaceae.

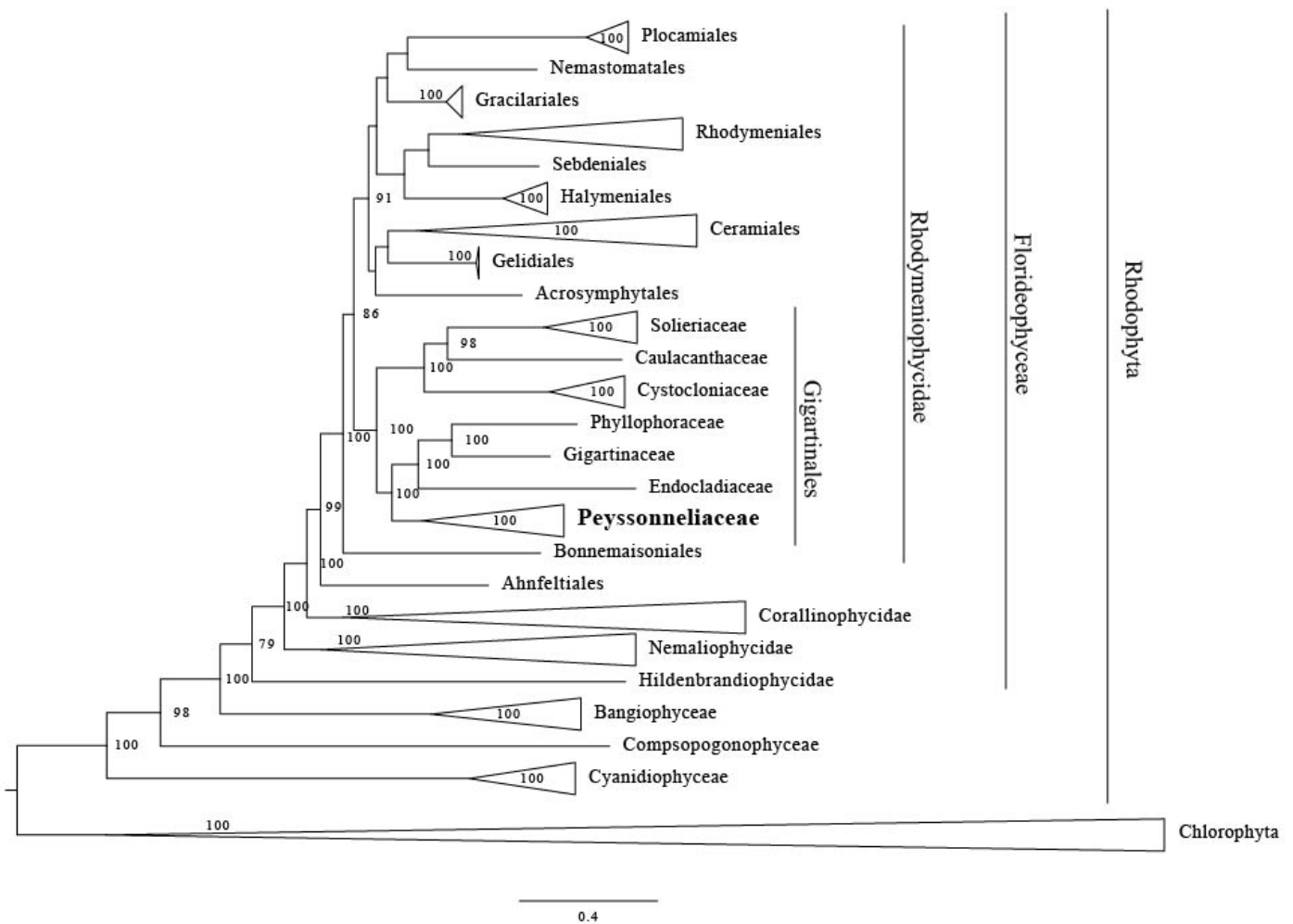


Figure1. Maximum likelihood phylogeny obtained from 28 mitochondrial genes. Numbers at nodes indicate bootstrap replicates. Bootstrap support values are shown above 75%.

Our plastid phylogeny (Figure 2) was fully supported across Florideophyceae clades. Peyssonneliaceae was also recovered as monophyletic and nested in Gigartinales (represented by three species in three genera; see complete tree in Figure S2). It was sister

to a clade comprising Solieriaceae, Gigartinaceae and Phyllophoraceae. We, therefore, transfer Peyssonneliaceae Denizot to Gigartinales F.Schmitz, rendering Peyssonneliales Krayesky an invalid name and update the description of Gigartinales.

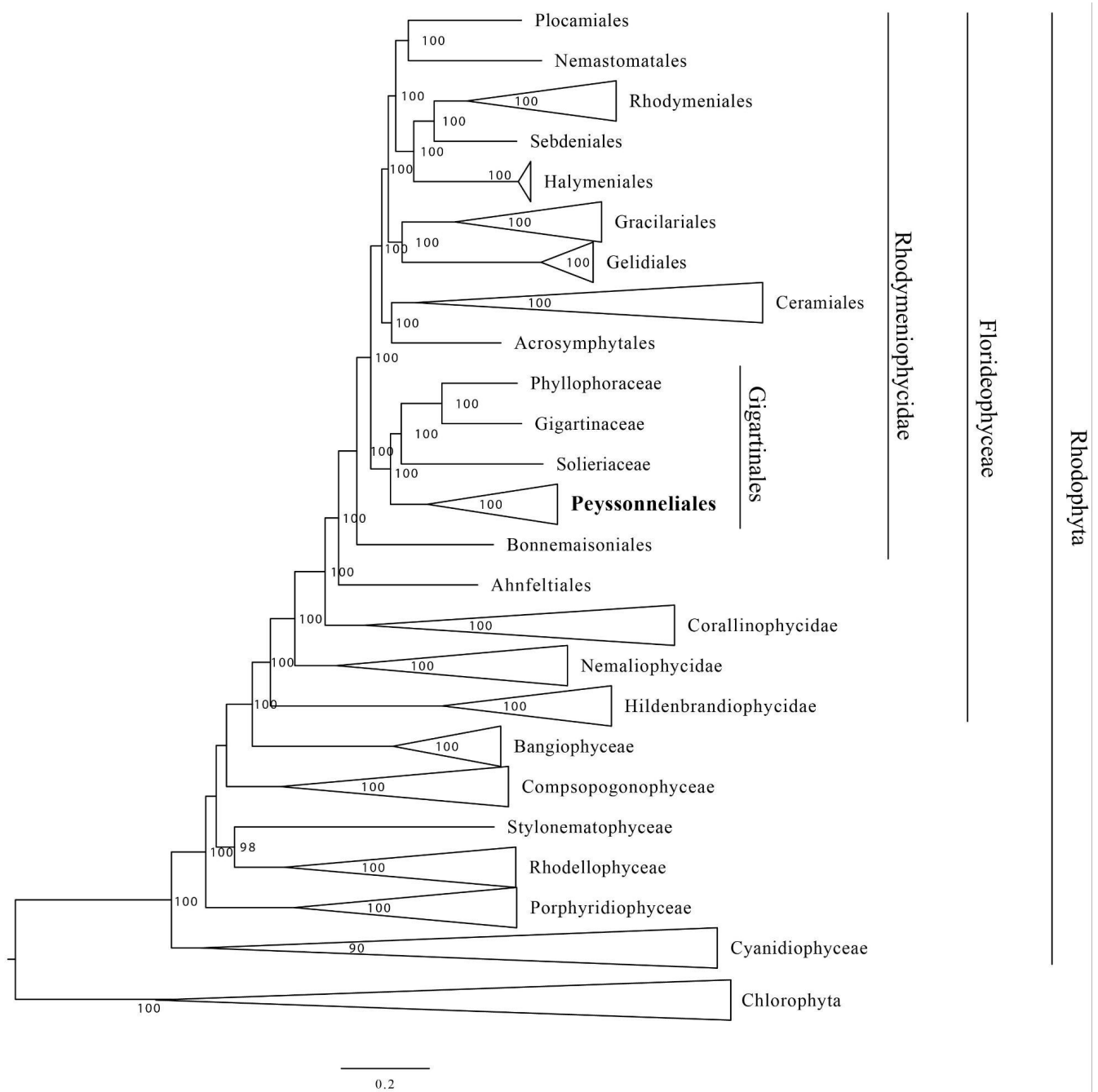


Figure 2. Maximum likelihood phylogeny obtained from 215 plastidial genes. Numbers at nodes indicate bootstrap replicates. Bootstrap support values are shown above 75%.

Taxonomic Treatment

Gigartinales F. Schmitz (1892) emend. E.M.S. Pestana, Lyra, and J.M.C. Nunes

Description—Thallus of various forms. Calcified or not. Structure uniaxial or multiaxial. Procarpic or not. Zygote transfer by connecting cells or filaments or by direct fusion. Carposporophyte usually with a fusion cell and extensive sterile gonimoblasts, some composed mostly of carposporangia. Tetrasporangia cruciately or zonately divided.

Comment: This description was based on Huisman (2018), the most recent and complete description of Gigartinales. Here we add the feature of calcification.

Type: Gigartinaceae Bory de St. Vincent, J.B. (1828). Botanique, Cryptogamie. In: *Voyage autour du monde, exécuté par ordre du Roi, sur la corvette de Sa Majesté, la Coquille, pendant les années 1822, 1823, 1824 et 1825*. (Duperrey, L.I. Eds), pp. 97-200. Paris: Bertrand.

Type genus: *Gigartina* Stackhouse, J. (1809). Tentamen marino-cryptogamicum, ordinem novum; in genera et species distributum, in Classe XXIVta Linnaei sistens. *Mémoires de la Société Imperiale des Naturalistes de Moscou* 2: [50]-97.

Heterotypic synonym: Peyssonneliales Kravesky, Fredericq & J.N.Norris. (2009). A new order of red algae based on the Peyssonneliaceae, with an evaluation of the ordinal classification of the Florideophyceae (Rhodophyta). *Proceedings of the Biological Society of Washington* 122(3): 364-391.

Included families: Acrotylaceae F.Schmitz, Areschougiaceae J.Agardh, Blinksiaceae Hollenberg & I.A.Abbott, Caulacanthaceae Kützing, Chondriellaceae Levring, Chondrymeniaceae Rodríguez-Prieto, Sartoni, Showe M.Lin & Hommersand, Clavicloniaceae Kraft & G.W.Saunders, Corynocystaceae Kraft, Crossocarpaceae Perestenko, Cruoriaceae Kylin, Cubiculosporaceae Kraft, Cystoclioniaceae Kützing, Dicranemataceae Kylin, Dumontiaceae Bory, Endocladiaceae Kylin, Etheliaceae K.R.Dixon, C.W.Schneider & G.W.Saunders, Furcellariaceae Greville, Gainiaceae

R.L.Moe, Gigartinaceae Bory, Gloiosiphoniaceae F.Schmitz, Haemeschariaceae Wilce & Maggs, Kallymeniaceae Kylin, Mychodeaceae Kylin, Mychodeophyllaceae Kraft, Nizymeniaceae Womersley, Peyssonneliaceae Denizot, Phacelocarpaceae Searles, Phyllophoraceae Willkomm, Placentophoraceae Rodríguez-Prieto, G.Sartoni, S.-M.Lin & Hommersand ex Dumilag, W.A.Nelson & Kraft, Polyidaceae Kylin, Ptilocladopsidaceae Rodríguez-Prieto, Freshwater & Hommersand, Rhizophyllidaceae Ardissona, Rissoellaceae Kylin, Schmitziellaceae Guiry, Garbary & G.W.Saunders, Solieriaceae J.Agardh, Sphaerococcaceae Dumortier, Tichocarpaceae Kylin.

Discussion

Gigartinales is a megadiverse order, currently with 963 species classified in 38 families, only behind Ceramiales in number of species in Rhodophyta (Guiry & Guiry, 2024). Species in Gigartinales present varied gross morphology with habit erect or prostrate, thallus cylindrical or flattened, and uniaxial or multiaxial growth (Min-Thein & Womersley, 1976; Fredericq *et al.*, 1999; Soares & Fujii, 2020; Guiry & Guiry, 2024). With the inclusion of Peyssonneliaceae in Gigartinales, the order now also includes species with calcified thallus. Thallus calcification in Peyssonneliaceae is constituted by aragonite crystals, different from the calcite ones present in coralline algae (James *et al.*, 1988). Our findings confirm the taxonomic decision by Kraft & Robins (1985), which included Peyssonneliaceae in Gigartinales, even though these authors transferred the whole Cryptonemiales lineage (now Halymeniales), which is now known to have evolved independently from Gigartinales.

Currently, there is one mitochondrial and one plastid genome of Peyssonneliaceae available: the mitogenome of *Riquetophycus* sp. was robustly nested in Gigartinales (Yang *et al.*, 2015); the plastid genome of *Riquetophycus* sp. was fully supported as sister to *Chondrus crispus*, also in Gigartinales (Lee *et al.*, 2016). These two genomes were placed within Peyssonneliaceae in our study, which includes sequences previously placed as closely related to *Peyssonnelia squamaria* (S.G.Gmelin) Decaisne ex J.Agardh (Pestana *et al.*, 2020; Pestana *et al.*, 2021), type species of *Peyssonnelia*, based on which Peyssonneliaceae was described. Members of Peyssonneliaceae share characters with some gigartinean representatives: i) a crustose habit is also present in the families Etheliaceae, Polyidaceae and Rhizophyllidaceae, and in the genus *Rhodopeltis* Harvey (Dumontiaceae); ii) nemathecial reproductive structures are also observed in Polyidaceae, Rhizophyllidaceae, and

Rhodopeltis. However, the development of carpogonial branches and auxiliary cell branches differ among them. In Polyidaceae, Rhizophyllidaceae and *Rhodopeltis*, the branches originate from a surface cell, while in Peyssonneliaceae they originate laterally, from an intercalary cortical cell (Krayesky *et al.*, 2009).

Our plastid phylogeny placed Peyssonneliaceae as the first lineage to diverge in Gigartinales, with Solieriaceae closely related to Gigartinaceae and Phyllophoraceae. However, our mitochondrial phylogeny recovered Peyssonneliaceae as closely related to Gigartinaceae and Phyllophoraceae (and Endocladiaceae), while Solieriaceae was closely related to Caulachantaceae and Cystocloniaceae. As both trees are nearly fully supported, our results indicate a potential conflict between the topologies, regarding inner relationships in Gigartinales. A broader sampling in Gigartinales may be useful to further investigate the existence of a conflict, and a well-supported nuclear phylogeny is also important to better understand the evolutionary history of Gigartinales, from different sources of evidence. Studies based on nuclear genes found different relationships among families in Gigartinales (Ragan *et al.*, 1994; Harper & Saunders, 2001; Withall & Saunders, 2006; Le Gall & Saunders, 2007; Hu *et al.*, 2007). Nevertheless, the available phylogenies were not robustly supported or did not include species of the families involved in the mentioned potential conflict.

The remaining lineage that rendered Gigartinales polyphyletic was Calosiphonaceae (Verbruggen *et al.*, 2010), which has *Calosiphonia* P.Crouan & H.Crouan as type genus. Although there are no available sequences of species of *Calosiphonia*, recent studies have shown that other genera of Calosiphoniaceae are distantly related to the core Gigartinales clade. Species of *Schimitzia* P.C. Silva, for example, are well supported as closely related to species of *Neoabbottiella* Perestenko, a genus currently classified in Halymeniaceae (Halymeniales), but which has been perceived as *incertae sedis* (Skriptsova *et al.*, 2024). Based on the robust multigene phylogeny available (Skriptsova *et al.*, 2024), we segregated Calosiphonaceae from Gigartinales, which is now a monophyletic order. Sequences from the type species of *Calosiphonia* are needed to properly classify Calosiphonaceae.

Conclusion

Our robustly supported phylogenies resolved two old problems in the red algae tree of life: the monophyly of Gigartinales, and the placement of Peyssonneliaceae, an interesting clade, with species presenting thallus' calcification from aragonite crystals. This rare

character in Rhodophyta is also present in Nemaliales, one of the earliest divergent clades in the class Florideophyceae (Yang *et al.*, 2016). Our results show the importance of investing in broad gene sampling as well as well-sampled phylogenies across red algae for discussing relationships among its main lineages. Furthermore, we draw attention to the need of further studies in Gigartinales, including morphological analyses, sequencing of nuclear regions and dated phylogenies to provide a better understanding of the relationship among inner clades and the evolution of characters in the order. Furthermore, sequencing of type species is a challenging, but crucial investment for fundamenting proper taxonomic decisions in red algae.

Author Contributions

EP, GL, and JMCN conceived and designed the study. EP collected the biological material and conducted morphological analyses. EP, GL and JMCN performed laboratory analyses. HZ assembled organellar genomes. EP and GL generated phylogenetic analyses, discussed and interpreted the data. EP and GL wrote the manuscript with contributions and revision from other co-authors. All authors contributed to the article and approved the submitted version.

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Supplementary material



Figure S1. Complete phylogeny obtained from 28 mitochondrial genes. Numbers at nodes indicate bootstrap replicates. Bootstrap support values are shown above 75%. Samples generated in this study are in bold.

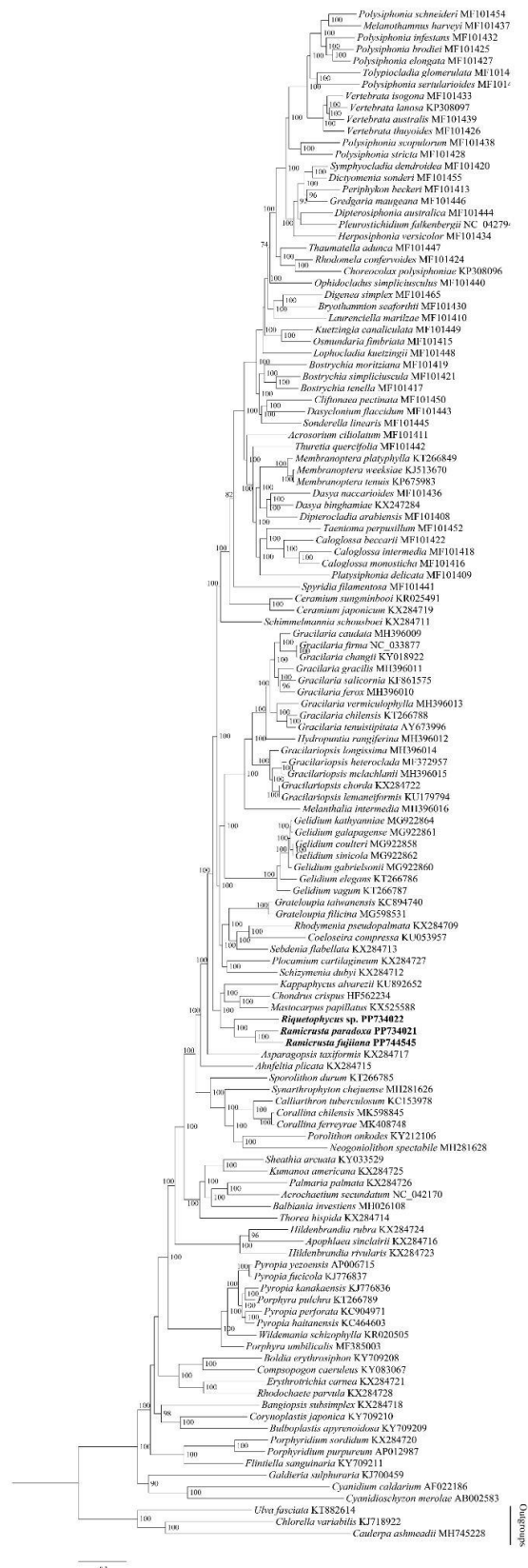


Figure S2. Complete phylogeny obtained from 215 plastidial genes. Numbers at nodes indicate bootstrap replicates. Bootstrap support values are shown above 75%. Samples generated in this study are in bold.

Table S1. Organellar genomes length and gene content of the six Peyssonneliaceae samples.

Identification	<i>Ramicrostata paradoxa</i>	<i>Ramicrostata fujiiana</i>	<i>Brasilophycus similis</i>	<i>Riquetophycus sp.</i>	<i>Agissea inamoena</i>	<i>Peyssonnelia sp.</i>
Collection locality	Ponta do Mutá, Maraú, Bahia, Brazil	Ponta do Mutá, Maraú, Bahia, Brazil	Jauá Beach, Camaçari, Bahia, Brazil	Jauá Beach, Camaçari, Bahia, Brazil	Jauá Beach, Camaçari, Bahia, Brazil	Jauá Beach, Camaçari, Bahia, Brazil
Collection date	27-Sep-2015	27-Sep-2015	27-May-2017	28-May-2017	28-May-2017	28-May-2017
Specimen	type	type	type	non-type	non-type	non-type
GenBank Access Code (mitogenomes)	OP537217	OP537218	PP734019	OP537219	OP537220	PP734020
Mitogenome	complete	complete	incomplete	complete	complete	incomplete
Mitogenome size	27.738	25.858	31.587	25.338	29.463	24.764
Genes/mitogenomes	atp4	atp4	atp4	atp4	atp4	atp4
	atp6	atp6	atp9	atp6	atp6	atp6
	atp8	atp8	cob	atp8	atp8	atp8
	atp9	atp9	cox1	atp9	atp9	atp9
	cob	cob	cox2	cob	cob	cob
	cox1	cox1	cox3	cox1	cox1	cox1
	cox2	cox2	nad1	cox2	cox2	cox2
	cox3	cox3	nad2	cox3	cox3	cox3
	nad1	nad1	nad3	nad1	nad1	nad1
	nad2	nad2	nad4L	nad2	nad2	nad2
	nad3	nad3	nad5	nad3	nad3	nad4
	nad4	nad4	nad6	nad4	nad4	nad5
	nad4L	nad4L	rpl16	nad4L	nad4L	nad6
	nad5	nad5	rpl20	nad5	nad5	rpl16
	nad6	nad6	rps3	nad6	nad6	rpl20
	rpl16	rpl16	rps11	rpl16	rpl16	rps3
	rpl20	rpl20	rps12	rps3	rpl20	rps12

Table S1. Organellar genomes length and gene content of the six Peyssonneliaceae samples.

	rps3	rps3	sdh2	rps11	rps3	sdh2
	rps11	rps11	sdh3	rps12	rps11	sdh3
	rps12	rps12	sdh4	sdh2	rps12	tatC
	sdh2	sdh2	tatC	sdh3	sdh2	
	sdh3	sdh3		sdh4	sdh3	
	sdh4	sdh4		tatC	sdh4	
	tatC	tatC			tatC	
GenBank Access Code (plastomes)	PP734021	PP744545		PP734022		
Plastome	incomplete	incomplete		incomplete		
Plastome size	170.291	174.414		178.930		
Genes / Plastomes	accD	accA		accA		
	acpP	accB		accB		
	acsF	accD		accD		
	apcA	acpP		acpP		
	apcB	apcA		apcA		
	apcD	apcB		apcB		
	apcE	apcD		apcD		
	apcF	apcE		apcE		
	argB	argB		apcF		
	atpA	atpA		argB		
	atpB	atpB		atpA		
	atpD	atpD		atpB		
	atpE	atpE		atpD		

Table S1. Organellar genomes length and gene content of the six Peyssonneliaceae samples.

atpF	atpF	atpE
atpG	atpG	atpF
atpH	atpH	atpG
atpI	atpI	atpH
carA	bas1	atpI
cbbX	carA	bas1
cemA	cbbX	carA
chlI	ccs1	cbbX
clpC	ccsA	ccs1
cpcA	cemA	ccsA
cpcB	chlI	cemA
cpcG	clpC	chlI
dfr	cpcA	clpC
dnaK	cpcB	cpcA
fabH	dfr	cpcB
ftsB	dnaB	cpcG
ftsH	dnaK	cpeA
gltB	dsbD	cpeB
groEL	fabH	dfr
ilvB	ftsB	dnaB
ilvH	ftsH	dnaK
infC	gltB	dsbD

Table S1. Organellar genomes length and gene content of the six Peyssonneliaceae samples.

lysR	groEL	fabH
odpA	ilvB	ftsB
odpB	ilvH	ftsH
ompR	infB	gltB
Orf34	infC	groEL
Orf36	lysR	ilvB
pbsA	moeB	ilvH
petB	ntcA	infB
petD	ompR	infC
petF	orf109	lysR
petG	orf110	moeB
petL	orf115	nblA
petM	orf155	ntcA
pgmA	orf156	odpA
preA	orf197	ompR
psaB	orf476	orf115
psaD	orf719	orf118
psaE	pbsA	orf145
psaF	petA	orf163
psaI	petB	orf193
psaJ	petD	orf249
psaL	petF	orf270
psaM	petG	orf493
psb30	petJ	orf743
psbA	petL	pbsA

Table S1. Organellar genomes length and gene content of the six Peyssonneliaceae samples.

psbB	petM	petA
psbC	petN	petB
psbD	pgmA	petD
psbE	preA	petF
psbF	psaA	petG
psbH	psaB	petJ
psbI	psaC	petL
psbJ	psaD	petM
psbK	psaE	petN
psbL	psaF	pgmA
psbN	psaI	preA
psbT	psaJ	psaA
psbV	psaK	psaB
psbW	psaL	psaC
psbZ	psaM	psaD
rbcL	psbA	psaE
rbcS	psbB	psaF
rne	psbC	psaI
rpl1	psbD	psaJ
rpl2	psbE	psaK
rpl3	psbF	psaL
rpl4	psbH	psaM
rpl5	psbI	psbA
rpl6	psbJ	psbB
rpl12	psbK	psbC
rpl13	psbL	psbD

Table S1. Organellar genomes length and gene content of the six Peyssonneliaceae samples.

rpl14	psbN	psbE
rpl16	psbT	psbF
rpl18	psbV	psbH
rpl19	psbW	psbI
rpl20	psbX	psbJ
rpl21	psbY	psbK
rpl22	psbZ	psbL
rpl23	rbcL	psbN
rpl24	rbcS	psbT
rpl27	rne	psbV
rpl28	rpl1	psbW
rpl31	rpl2	psbX
rpl33	rpl3	psbY
rpl36	rpl4	psbZ
rpoA	rpl5	rbcL
rpoB	rpl6	rbcS
rpoC1	rpl9	rne
rpoZ	rpl11	rpl1
rps1	rpl12	rpl2
rps2	rpl13	rpl3
rps3	rpl14	rpl4
rps4	rpl16	rpl5
rps5	rpl18	rpl6
rps6	rpl19	rpl9
rps7	rpl20	rpl11
rps8	rpl21	rpl12

Table S1. Organellar genomes length and gene content of the six Peyssonneliaceae samples.

rps9	rpl23	rpl13
rps10	rpl24	rpl14
rps11	rpl27	rpl16
rps12	rpl28	rpl18
rps13	rpl29	rpl19
rps14	rpl31	rpl20
rps16	rpl32	rpl21
rps17	rpl33	rpl23
rps18	rpl34	rpl24
rps19	rpl35	rpl27
secA	rpl36	rpl28
secY	rpoA	rpl29
sufB	rpoB	rpl31
sufC	rpoC1	rpl32
syh	rpoC2	rpl33
tatC	rps1	rpl34
thiG	rps2	rpl35
thiS	rps3	rpl36
tilS	rps4	rpoA
trpA	rps5	rpoB
trpG	rps6	rpoC1
trxA	rps7	rpoC2
tsf	rps8	rps1
tufA	rps9	rps2
ycf3	rps10	rps3
ycf4	rps11	rps4

Table S1. Organellar genomes length and gene content of the six Peyssonneliaceae samples.

ycf19	rps12	rps5
ycf20	rps13	rps6
ycf21	rps14	rps7
ycf29	rps16	rps8
ycf33	rps17	rps9
ycf34	rps18	rps10
ycf39	rps19	rps11
ycf45	rps20	rps12
ycf52	secA	rps13
ycf53	secY	rps14
ycf54	sufB	rps16
ycf55	sufC	rps17
ycf60	syfB	rps18
ycf63	syh	rps19
ycf65	tatC	rps20
	thiG	secA
	thiS	secG
	trpA	secY
	trpG	sufB
	trxA	sufC
	tsf	syfB
	tufA	syh
	ycf3	tatC
	ycf4	thiG
	ycf59	thiS
	ycf61	trpA

Table S1. Organellar genomes length and gene content of the six Peyssonneliaceae samples.

	ycf62	trpG
	ycf65	trxA
		tsf
		tufA
		ycf3
		ycf4
		ycf59
		ycf61
		ycf62
		ycf65

CAPÍTULO 2

Título: Phylogenomics, organellar genome architecture and character evolution in Gigartinales (Rhodophyta)

Autores: Edilene Maria dos Santos Pestana, Hongrui Zhang, Rogério Katsuhito Barbosa Maruyama, Cíntia Iha, José Marcos de Castro Nunes, Goia de Mattos Lyra

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**Phylogenomics, organellar genome architecture and character evolution in
Gigartinales (Rhodophyta)**

*Edilene Maria dos Santos Pestana^{1,2}, Hongrui Zhang³, Rogério Katsuhito Barbosa
Maruyama^{1,2}, Cintia Iha⁴, José Marcos de Castro Nunes^{1,2}, Goia de Mattos Lyra^{1,5}*

¹*Programa de Pós-Graduação em Biodiversidade e Evolução (PPG BioEvo), Instituto de
Biologia, Universidade Federal da Bahia, Rua Barão de Jeremoabo, s/n, Salvador, Bahia
40170-115, Brazil*

²*Laboratório de Algas Marinhas (LAMAR), Instituto de Biologia, Universidade Federal da
Bahia, Rua Barão de Jeremoabo, s/n, Salvador, Bahia 40170-115, Brazil*

³*Texas A&M University, 400 Bizzell St, College Station, Texas 77843, EUA*

⁴*Australian National Algae Culture Collection, CSIRO, Castray Esplanade, Hobart,
Tasmania 7000, Australia*

⁵*Departamento de Biologia Vegetal, Universidade do Estado do Rio de Janeiro, R. São
Francisco Xavier, 524 - Maracanã, Rio de Janeiro - RJ, 20550-013, Brazil*

Running title: *Phylogenomics of Gigartinales*

Author for correspondence: edimpestanda@gmail.com, +55 (71) 3283-6598

Abstract

Gigartinales is one of the most diverse orders of Rhodophyta and has great ecological importance, constituting habitats, a source of food for other marine organisms and participating in the biogeochemical cycles of the oceans. In addition, its species have economic importance, producing bioactive compounds that are used in the food, cosmetic and pharmaceutical industries. Little is known about the evolutionary history of Gigartinales, which is represented by only a few species in the broadest phylogenetic efforts. Some families and genera have unclear boundaries, and are based on characters that are not exclusive. Our robust genomic level phylogenies based on plastid and mitochondrial genes, provided a solid framework to understand phylogenetic relationships in Gigartinales. We identified molecular events, such as genes losses, gains, inversions and translocations that

characterize lineages and provided a better understanding of how morphological characters are distributed across the Gigartinales clade.

Key-words: Gene synteny, mitogenomes, plastomes, phylogenomics, red algae.

Introduction

Rhodophyta is one of the oldest clades in the Plant Kingdom, with fossil records from around 1.2 to 1.5 billion years (Nan *et al.*, 2017, Yang *et al.*, 2016). The broadest phylogeny of Rhodophyta constructed to date (Verbruggen *et al.*, 2010), including 33 orders, reveals that relationships between main lineages are not well understood, lacking support especially in ancestral branches. The core Gigartinales clade encompassed two of the five most weakly supported areas of the tree (Verbruggen *et al.*, 2010). Although the order was polyphyletic in the phylogeny presented by Verbruggen *et al.* (2010), more recent taxonomic studies segregated distantly related lineages from it [e.g., Atractophorales Maggs, L.Le Gall, Filloramo & G.W.Saunders (Saunders *et al.*, 2016)] or found robust support to reintegrate others [e.g., Peyssonneliaceae Denizot (Pestana *et al.*, 2024)] to the Gigartinales clade. Gigartinales currently includes 1106 species, distributed in 37 families, the most diverse in number of species being Kallymeniaceae (202), Peyssonneliaceae (147), Gigartinaceae (135) and Phyllophoraceae (132) (Guiry & Guiry, 2024; Pestana *et al.*, 2024). Most representatives of Gigartinales have a cosmopolitan distribution and have great ecological importance in primary production, in providing habitats for other species, and serving as a food source for marine life (Lee, 2008; Guiry & Guiry, 2024). Several species of Gigartinales also have economic importance, as they are sources of carrageenans, a polysaccharide produced in the cell wall, widely used in the food, cosmetic and pharmaceutical industry as a thickening, gelling, suspending and stabilizing agent for dairy products (Lee, 2008; Pereira *et al.*, 2015). Carrageenan neuroprotective properties have been the subject of studies, as they seem to protect the body against dopaminergic neurodegeneration (Liu *et al.*, 2015).

Morphological characterization, although essential for taxonomy, is not always sufficient to distinguish some macroalgae groups (Oliveira & Milstein, 2010). Vegetative characters in Gigartinales are highly variable (Calderon & Boo, 2016; Saunders *et al.*, 2016; Huisman, 2018; Nauer *et al.*, 2019; Soares & Fujii, 2020) and traditionally used morphological characters are not exclusive to this clade, which presents a wide range of states. Genomic level data, particularly from organelles, in integrative taxonomic approaches

has been increasingly used and useful in providing resolution to systematics in various clades of the red algae tree of life (Yang *et al.*, 2015; Yang *et al.*, 2016; Díaz-Tapia *et al.*, 2017; Muñoz-Gómez *et al.*, 2017; Nan *et al.*, 2017; Paiano *et al.*, 2017; Díaz-Tapia *et al.*, 2019; Lyra *et al.*, 2021). It furnishes robust frameworks for the interpretation of character evolution in clades with challenging taxonomy [e.g., Ceramiales (Díaz-Tapia *et al.*, 2019), and Gracilariales (Lyra *et al.*, 2021)], contributing to the establishment of useful characters to recognize taxa on various levels. Organellar genomes also reveal molecular events that further characterize clades in different algae groups [e.g.: brown algae (Starko *et al.*, 2021); red algae, such as Gracilariaceae (Iha *et al.*, 2018; Lyra *et al.*, 2021), and Cystocloniaceae (Jesus *et al.*, 2023)].

Lee *et al.* (2016) provided the most complete overview of the plastome's structure in Rhodophyta, identifying three types (R1, R2 and R3) of general structure, mostly related to the number and position of the rRNA operons and to the direction of variable regions. Only two sequences of Gigartinales, representing two species, were included in the analyses, and they presented different structural types (R1 and R2), demonstrating the potential for variability in such a diverse clade. Gigartinales' genomes have been published and incorporated in datasets of studies at different taxonomic levels (Janouškovec *et al.*, 2013; Sissini *et al.*, 2016; Liu *et al.*, 2019; Zhang *et al.*, 2020). Pestana *et al.* (2024) generated 6 new mitogenomes and 3 new plastomes of Peyssonneliaceae, which provided the grounds for the inclusion of this family in Gigartinales, ultimately rendering the order monophyletic. Jesus *et al.* (2023) generated 17 new mitogenomes and 17 plastomes from 17 and 16 species of Cystocloniaceae, respectively, improving the resolution of *Hypnea* J.V.Lamouroux and providing a new circumscription of the Cystocloniaceae. They analyzed genomes' structural variation across the family, and described three new species.

We present here a pioneering initiative in constructing broad genomics datasets, with main lineages in Gigartinales represented, from different parts of the globe and prioritizing type specimens. We describe morphological and structural genomic traits across families, interpreting characters in light of robustly supported organellar phylogenies.

Material and methods

Fifty-one samples were obtained from herbarium specimens deposited at the Farlow Herbarium (FH, Harvard University) and the New York Botanical Garden (NYBG), including 17 type specimens that have been sequenced for the first time (Table S1).

DNA extractions, libraries preparation and sequencing

We extracted total genomic DNA from 0.01g of tissue using the Maxwell® 16 DNA Purification Kit (Promega Corporation, Inc., Madison, WI, USA). Libraries were prepared at the Faculty of Arts and Science (FAS) Center for Systems Biology at Harvard University, MA, USA, using quarter reactions of the KAPA HyperPlus Library Preparation Kit (Kapa Biosystems, Inc., MA, USA) with 8 minutes of incubation during the fragmentation step and using Illumina adapters and recommendations (dilutions ratios) for bead size selection for 550bp. Libraries initial concentrations were verified with the Qubit® 3.0 Fluorometer, using the Qubit® dsDNA HS Assay Kit (Invitrogen, Carlsbad, CA, USA), and average sizes of DNA fragments were verified with the High Sensitivity HSD1000 ScreenTape Assay in the 2200 TapeStation (Agilent Technologies, Inc., Waldbronn, Germany). We used Real-Time PCR (BioRad CFX96 Touch, BioRad Laboratories, Hercules, USA) with the NEBNext Library Quant Kit (New England Biolabs, Ipswich, USA) for verifying the final concentration of the libraries' pool. All libraries were diluted into 1.0 nM, pooled together and sequenced with the Illumina HiSeq v4 High-Output on Genome Analyzer II (Illumina, Inc., San Diego, CA, USA), with 250 bp paired-end runs.

Genomes assembly and annotation

Plastid and mitochondrial genomes were *de novo* assembled using the PhyloHerb pipeline (Cai *et al.*, 2022), and annotated using MFannot (https://megasun.bch.umontreal.ca/cgibin/dev_mfa/mfannotInterface.pl). We also performed manual inspections and corrections in Geneious Prime 2019 (Dotmatics, Bishop's Stortford, UK), based on reference genomes: i) Mitochondrial - *Caulacanthus okamurae* Yamada (MT193839), *Kappaphycus alvarezii* (Doty) Doty ex P.C.Silva (MT032181); and *Sarcopeltis skottsbergi* (Setchell & N.L.Gardner) Hommersand, Hughey, Leister & P.W.Gabrielson (KU885455); ii) Plastidial - *Chondrus crispus* Stackhouse (HF562234) and *Mastocarpus papillatus* (C.Agardh) Kützinger (KX525588). Genes from newly generated and

published genomes were extracted using a local python script, which identified genes' annotations. Gene alignments from each organelle were performed using MAFFT v.7 (Katoh & Standley, 2013). Alignments were trimmed in trimAl (Capella-Gutiérrez *et al.*, 2009) and concatenated into one supermatrix per organelle. 284 sequences of the nuclear gene LSU (28S) were obtained from GenBank. Additional LSU sequences were obtained for 3 of our samples by mapping our raw reads against reference sequences from closely related taxa on Geneious Prime 2019 (Ripma *et al.*, 2014). The LSU dataset was aligned in Geneious.

Phylogenetic analyses

Maximum Likelihood (ML) analyses for our datasets (mitochondrial, plastidial, and LSU) were performed on CIPRES Science Gateway v.3.3 (<https://www.phylo.org/>), using the tree inference in XSEDE RAxML v.8.2.0 (Stamatakis, 2014). The evolutionary model GTR+GAMMA was employed for Maximum Likelihood (ML) phylogenetic analyses

Analyses of coalescence and gene concordance

To verify the existence of incongruence among gene trees, we generated rooted and unrooted ML trees for each single gene alignment in our mitochondrial and plastid datasets, using IQTree (Minh *et al.*, 2020) via a local python script. We used the unrooted gene trees to generate ASTRAL trees for the mitochondrial and plastid datasets, using PhyloSuite (Zhang *et al.*, 2020), which obtained the species tree from gene trees using wASTRAL-unweighted v1.10.2.1 (Zhang & Mirarab, 2022) by optimizing the objective function of ASTRAL (Zhang *et al.*, 2018). Analyses of basic concordance were applied, using PhyParts (Smith *et al.*, 2015), for each set of rooted gene trees, and using respective ASTRAL trees (mitochondrial or plastid) as references.

Gene synteny analyses

The dataset for synteny analyses was composed exclusively of complete genomes. Mitochondrial and chloroplast genomes were linearized with starting positions standardized at the small subunit ribosomal RNA gene (rns) (following Iha *et al.*, 2018), a gene situated at the beginning of a large and conserved region in both organellar genomes. Alignments for each dataset were performed in Mauve v. 2.4.0, using a progressive Mauve algorithm (Darling *et al.*, 2011) with default settings, automatically calculating seed weights and minimum locally collinear blocks (LCB) to score and compare genetic synteny between species within genomes. We inferred orthogroups for each genome dataset using

OrthoFinder v. 2.3.1 (Emms & Kelly, 2019), from protein sequences translated from coding sequences (CDS) extracted from our genomes in Geneious Prime 2019. The gene gain and loss was inferred by Dollo parsimony, using COUNT (Csűös, 2010).

Analyses of character evolution

We constructed a table of five traditionally used morphological characters for each of the species included in our plastid phylogeny (Table S2), based on literature data, original descriptions and the most recent studies available. We included vegetative and reproductive characters, selecting those for which it was possible to find information for the largest number of samples: i) Growth type; ii) Habit; iii) Thallus shape; iv) Procarp; v) Type of tetrasporangium division. For each analysis, species with unknown morphological character state were removed from the phylogenetic tree using the `drop.tip` function in R v3.6.0 (R CoreTeam, 2018). We inferred the ancestral status of each trait, based on our plastid phylogeny, using the R package `Phytools` v0.7.70 (Revell, 2012). For each trait, we determined the best model for trait evolution based on the AIC criterion. We used the function `fitMk` to evaluate the fitness of three models including equal-rate, symmetrical rate and all-rates-different (ARD) once the chosen traits do not exhibit polymorphism. The ancestral status reconstruction was carried out using the `make.simmap` function in `Phytools` with 100 simulations.

Results

Genome structure

GC content of mitogenomes was 28.4% on average (min = 24.8% in *Callophyllis flabellulata* Setchell, max = 40.1% in *Rissoella verruculosa* (Bertoloni) J.Agardh); length was 28,002 bp on average. *Sonderophycus* sp. Had the shortest (25,338 bp) and *Callophyllis gardneri* Setchell had the longest genome (32,659 bp). Mitochondrial genomes were similar in gene content, ranging from 21 to 27 protein-coding genes, 19 to 27 tRNAs, and presenting three rRNAs (*rrl*, *rrs*, *rrn5*) (Table 1). Regarding chloroplast genomes, GC content was 28.7% on average (min = 27.4% in *C. gardneri*, *Callophyllis odonthalioides* Setchell and *Opuntiella californica* (Farlow) Kylin, max = 31.8% in *R. verruculosa*); sequence length was 181,001 bp on average. *R. verruculosa* had the shortest (174,459 bp) and *Mastocarpus stellatus* (Stackhouse) Guiry the longest genome (186,883 bp). Plastomes comprised 198 to 206 protein genes, and presented 3 rRNAs (*rrl*, *rrs*, *rrn5*), and 30 tRNAs. The exceptions

were *M. stellatus* and *Gymnogongrus griffithsiae* (Turner) C. Martius with 29 tRNAs each (Table 1).

Table 1. General information of mitochondrial and chloroplast genomes of Gigartinales. Bold genomes were sequenced in this study. *Indicate incomplete genomes.

Genome	Size (bp)	GC content (%)	CDS	tRNA	rRNA
Mitogenomes					
<i>Agissea inamoena</i> *	29,463	26	25	23	3
<i>Betaphycus gelatinus</i>	25,275	29.7	25	24	3
<i>Brasilophycus similis</i>	31,587	28.3	22	24	3
<i>Callophyllis acrocarpa</i>	29,464	25.5	24	22	3
<i>Callophyllis crenulata</i>	29,508	25.5	24	22	3
<i>Callophyllis edentata</i>	29,418	25.5	24	22	3
<i>Callophyllis flabellulata</i>	32,192	24.8	24	22	3
<i>Callophyllis gardneri</i>	32,648	25.8	26	22	3
<i>Callophyllis gardneri</i>	32,659	25.3	25	23	3
<i>Callophyllis odonthalioides</i>	32,644	25.8	26	22	3
<i>Callophyllis stenophylla</i>	29,402	26.1	24	22	3
<i>Catenella caespitosa</i>	25,173	32.9	24	23	3
<i>Caulacanthus ustulatus</i>	26,026	29.1	23	23	3
<i>Chondracanthus serratus</i>	25,959	27.5	25	23	3
<i>Chondracanthus serratus</i>	25,943	27.5	25	23	3
<i>Chondracanthus spinosus</i>	26,057	27.3	25	23	3
<i>Chondrus crispus</i>	25,694	27.9	26	22	3
<i>Chondrus crispus</i>	25,835	27.9	25	23	3
<i>Cirrulicarpus carolinensis</i>	31,843	28	27	23	3
<i>Ethelia umbricola</i>	26,658	28.7	25	21	3
<i>Eucheuma denticulatum</i>	25,327	30.3	24	24	3
<i>Gloiopeltis furcata</i>	25,636	37.4	24	23	3
<i>Gymnogongrus griffithsiae</i>	25,812	29.4	25	22	3
<i>Hypnea marchantiae</i>	25,061	27.1	23	23	3
<i>Hypnea nidifica</i>	25,082	27	23	23	3
<i>Hypnea nidulans</i>	25,110	28.4	23	23	3

<i>Iridaea ciliata</i>	26,466	28.5	25	24	3
<i>Iridaea cordata</i>	25,703	29	25	22	3
<i>Kappaphycus alvarezii</i>	25,198	29.9	24	23	3
<i>Kappaphycus striatus</i>	25,242	30.1	24	24	3
<i>Mastocarpus cristatus</i>	25,838	32.7	24	23	3
<i>Mastocarpus latissimus</i>	26,208	31.6	26	23	3
<i>Mastocarpus papillatus</i>	26,132	35.1	25	23	3
<i>Mastocarpus papillatus</i>	25,906	35	26	22	3
<i>Mastocarpus stellatus</i>	25,826	31.1	23	22	3
<i>Mazzaella leptorhynchos</i>	25,684	28.8	26	22	3
<i>Meredithia crenata</i>	31,338	27.6	24	23	3
<i>Ochtodes crockeri</i>	28,903	30.6	24	21	3
<i>Opuntia californica</i>	26,717	25.3	24	19	3
<i>Peyssonnelia</i> sp. *	24,764	27.6	27	19	3
<i>Phacelocarpus tortuosus</i>	28,639	28.5	24	22	3
<i>Phacelocarpus tristichus</i>	29,050	28.9	24	23	3
<i>Ramicrusta fujiana</i>	25,858	28.7	24	23	3
<i>Ramicrusta paradoxa</i>	27,738	28.3	24	23	3
<i>Riquetophycus</i> sp.	25,338	27.3	23	29	3
<i>Riquetophycus</i> sp.	26,351	25.7	24	21	3
<i>Rissoella verruculosa</i>	26,302	40.1	23	22	3
<i>Sarcopeltis skottsbergii</i>	25,908	28.5	24	22	3
<i>Sarcopeltis skottsbergii</i>	25,908	28.5	24	22	3
Plastomes					
<i>Betaphycus gelatinus</i>	178,394	28.9	204	30	3
<i>Callibrephepharis</i> sp.	176,735	27.5	199	30	3
<i>Callophyllis acrocarpa</i>	181,367	27.5	202	30	3
<i>Callophyllis edentata</i>	181,368	27.5	202	30	3
<i>Callophyllis gardneri</i>	182,246	27.4	202	30	3
<i>Callophyllis gardneri</i>	182,021	27.4	202	30	3
<i>Callophyllis odonthalioides</i>	182,295	27.4	202	30	3
<i>Catenella caespitosa</i>	177,036	31	204	30	3

<i>Caulacanthus okamurae</i>	173,516	30.1	201	30	3
<i>Caulacanthus ustulatus</i>	173,755	29.4	199	30	3
<i>Chondracanthus serratus</i>	175,591	28.7	202	30	3
<i>Chondracanthus spinosus</i>	175,539	28.7	202	30	3
<i>Chondrus crispus</i>	180,102	28.7	204	30	3
<i>Chondrus crispus</i>	180,086	28.7	204	30	3
<i>Cyprosiphonia woodii</i>	180,927	28.7	202	30	3
<i>Dicranema revolutum</i>	179,808	30.2	199	30	3
<i>Eucheuma denticulatum</i>	177,003	29.6	205	30	3
<i>Gymnogongrus griffithsiae</i>	186,364	28.6	206	29	3
<i>Hypnea brasiliensis</i>	173,928	27.8	200	30	3
<i>Hypnea caraibica</i>	173,580	27.9	200	30	3
<i>Hypnea cornuta</i>	173,251	28.4	198	30	3
<i>Hypnea cryptica</i>	173,464	28.4	198	30	3
<i>Hypnea edeniana</i>	173,918	27.8	199	30	3
<i>Hypnea flava</i>	174,573	27.9	199	30	3
<i>Hypnea musciformis</i>	173,520	27.9	200	30	3
<i>Hypnea nidifica</i>	173,169	28	200	30	3
<i>Hypnea nidulans</i>	173,835	27.8	199	30	3
<i>Hypnea pannosa</i>	173,112	27.7	200	30	3
<i>Hypnea pseudomusciformis</i>	173,589	27.9	199	30	3
<i>Hypnea pseudomusciformis</i>	173,626	27.9	199	30	3
<i>Hypnea pseudomusciformis</i>	173,616	27.9	199	30	3
<i>Hypnea</i> sp.	177,247	29.3	200	30	3
<i>Hypnea wynei</i>	175,748	27.7	200	30	3
<i>Iridaea cordata</i>	180,605	29	204	30	3
<i>Kappaphycus striatus</i>	176,763	29.3	202	30	3
<i>Leiomenia cribrosa</i>	182,766	28	204	30	3
<i>Mastocarpus cristatus</i>	184,971	29.1	204	30	3
<i>Mastocarpus papillatus</i>	184,381	29.1	204	30	3
<i>Mastocarpus papillatus</i>	184,382	29.1	204	30	3
<i>Mastocarpus stellatus</i>	186,883	29	205	29	3
<i>Mazzaella leptorhynchus</i>	179,805	29	204	30	3

<i>Meredithia crenata</i>	181,268	29.5	201	30	3
<i>Ochtodes crockeri</i>	181,808	29.5	206	30	3
<i>Opuntiella californica</i>	183,605	27.4	202	30	3
<i>Phacelocarpus tortuosus</i>	179,903	29.1	203	30	3
<i>Ramicrusta fujiana</i> *	174,414	29.4	202	30	3
<i>Ramicrusta paradoxa</i> *	170,291	29.8	202	30	3
<i>Riquetophycus</i> sp.	180,384	29.8	202	30	3
<i>Riquetophycus</i> sp.*	178,930	29.8	202	30	3
<i>Rissoella verruculosa</i>	174,459	33.1	200	30	3
<i>Sarcopeltis skottsbergii</i>	179,603	29	206	30	3
<i>Sarcothalia radula</i>	175,511	28.7	202	30	3

Phylogenomic analyses

We obtained (complete or incomplete) organellar genomes from 62 species: 47 mitochondrial genomes and 45 plastid genomes, from which 28 and 215 genes, respectively, were extracted and used for phylogenetic analyses. Our mitochondrial, plastid and nuclear datasets comprised 12 (33%), 13 (36%) and 29 (80%) families of Gigartinales, respectively. Both organellar phylogenies recovered a fully supported clade including all sampled species of Gigartinales, and relationships between families were also well supported (>80% BP).

In the mitochondrial phylogeny (Fig. 1), Gigartinaceae and Phyllophoraceae were placed in a clade (100% BP), which is in a sister relationship (100% BP) with a clade (96% BP) comprising Kallymeniaceae, Rhizophyllidaceae, Etheliaceae, Polyidaceae, Peyssonneliaceae, Phacelocarpaceae and Endocladiaceae.

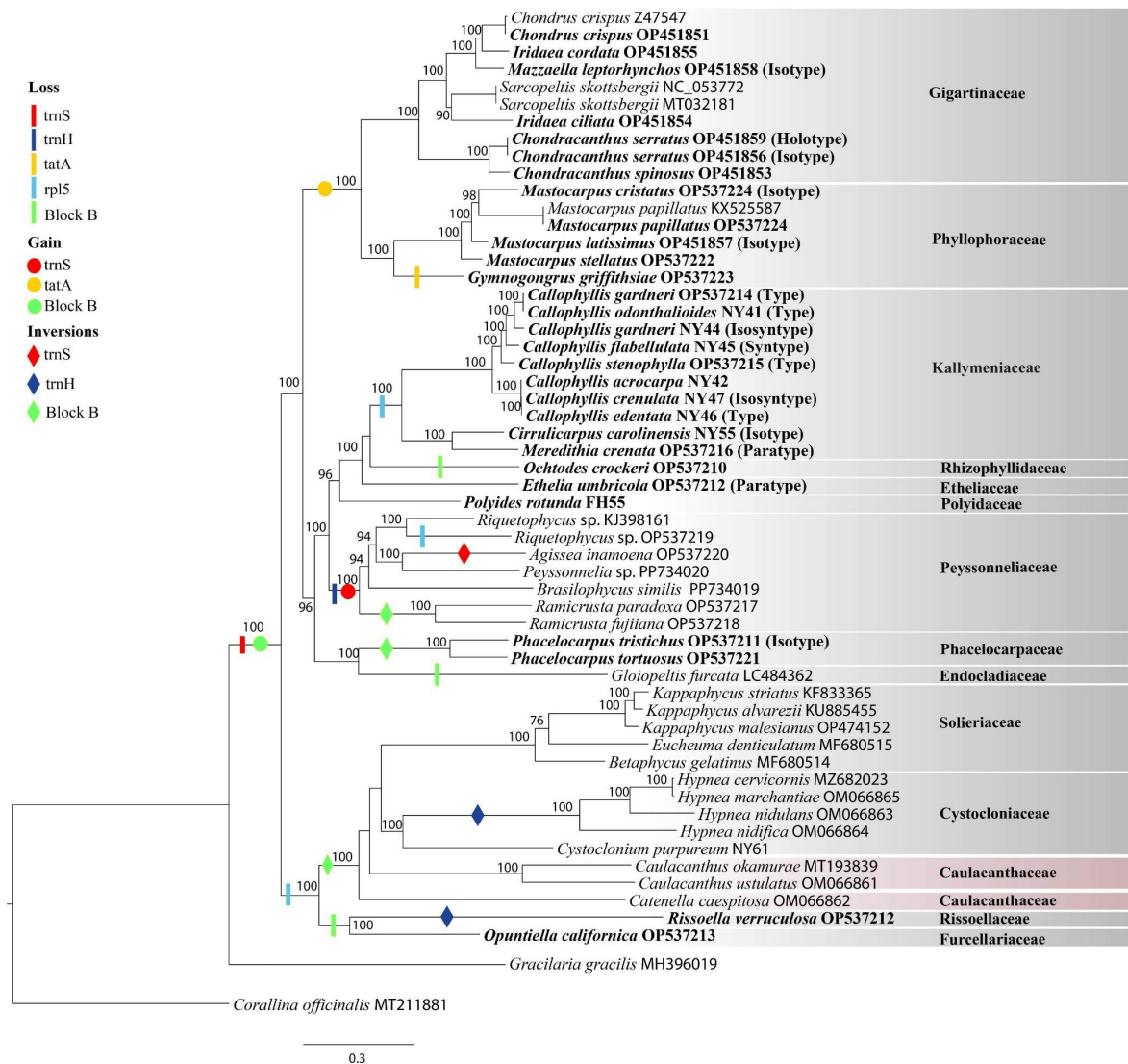


Figure 1. Maximum likelihood phylogeny based on 28 mitochondrial genes. Numbers at nodes indicate bootstrap support values (BP), shown when $> 75\%$. Names in bold represent sequences generated in this study. Sequences from type specimens are indicated in parenthesis, specifying the kind of type. Genome's architecture features are plotted on the tree, according to the legend. Monophyletic families are highlighted in gray and polyphyletic families are highlighted in red.

In contrast, in our plastid phylogeny (Fig. 2), the sister clades (100% BP) Gigartinaeae and Phyllophoraceae were sister (100% BP) to a fully supported clade including Rissoellaceae, Furcellariaceae, Cystocloniaceae, Solieriaceae, Dicranemataceae, and the polyphyletic Caulacanthaceae, revealing a conflict regarding inner relationships in Gigartinales.

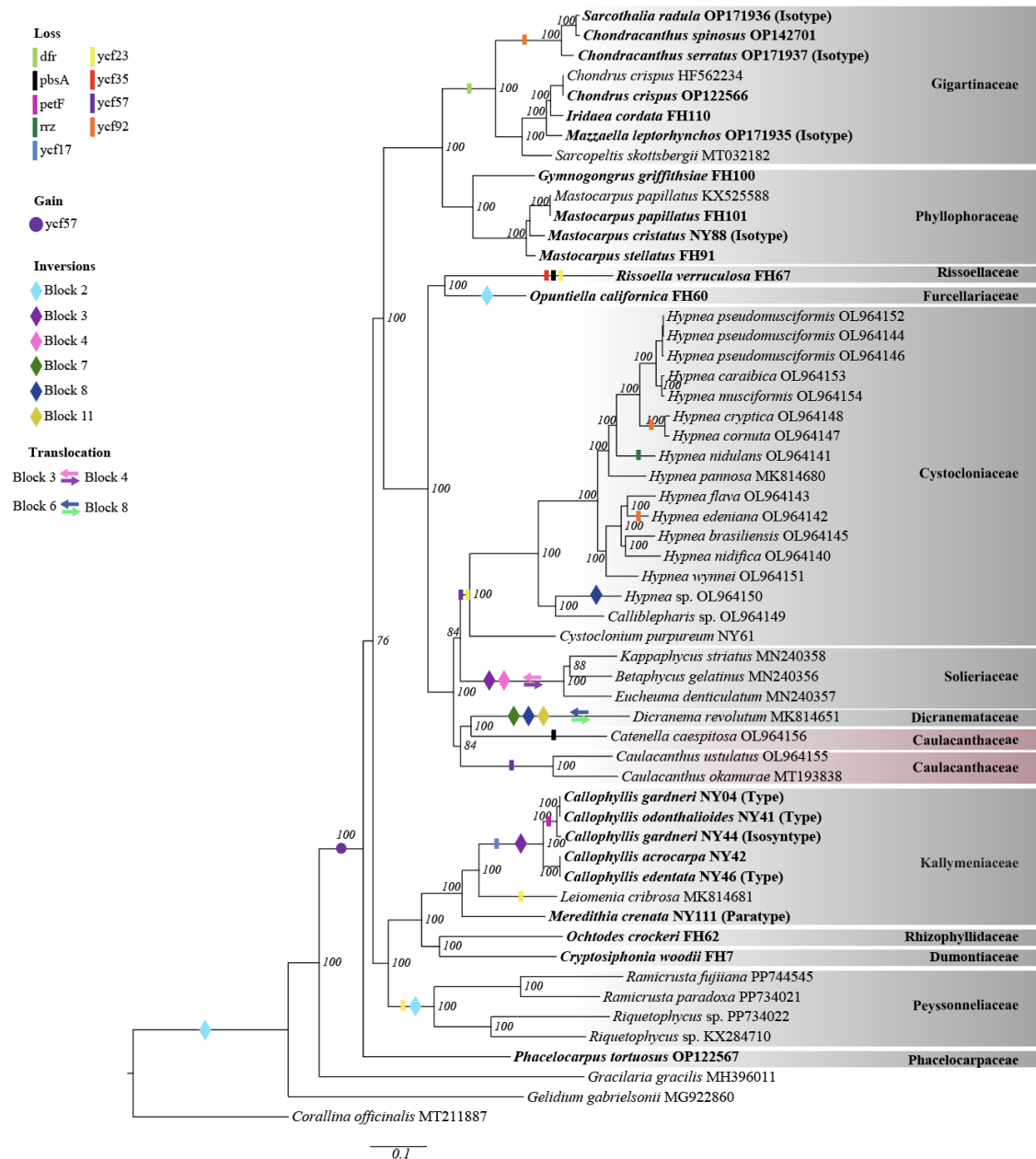


Figure 2. Maximum likelihood phylogeny based on 215 plastidial genes. Numbers at nodes indicate bootstrap support values (BP), shown when > 75%. Names in bold represent sequences generated in this study. Sequences from type specimens are indicated in parenthesis, specifying the kind of type. Genome's architecture features are plotted on the tree, according to the legend. Monophyletic families are highlighted in gray and polyphyletic families are highlighted in red.

Our nuclear tree's topology (Fig. 3), although based on different species sampling, is mostly consistent with the plastid one, placing Gigartinaceae and Phylloporaceae closer (87% BP, Fig. 3) to Dicranemataceae, Solieriaceae, Cystocloniaceae and Furcellariaceae, than to Kallymeniaceae, Peyssonneliaceae or Endocladiaceae.

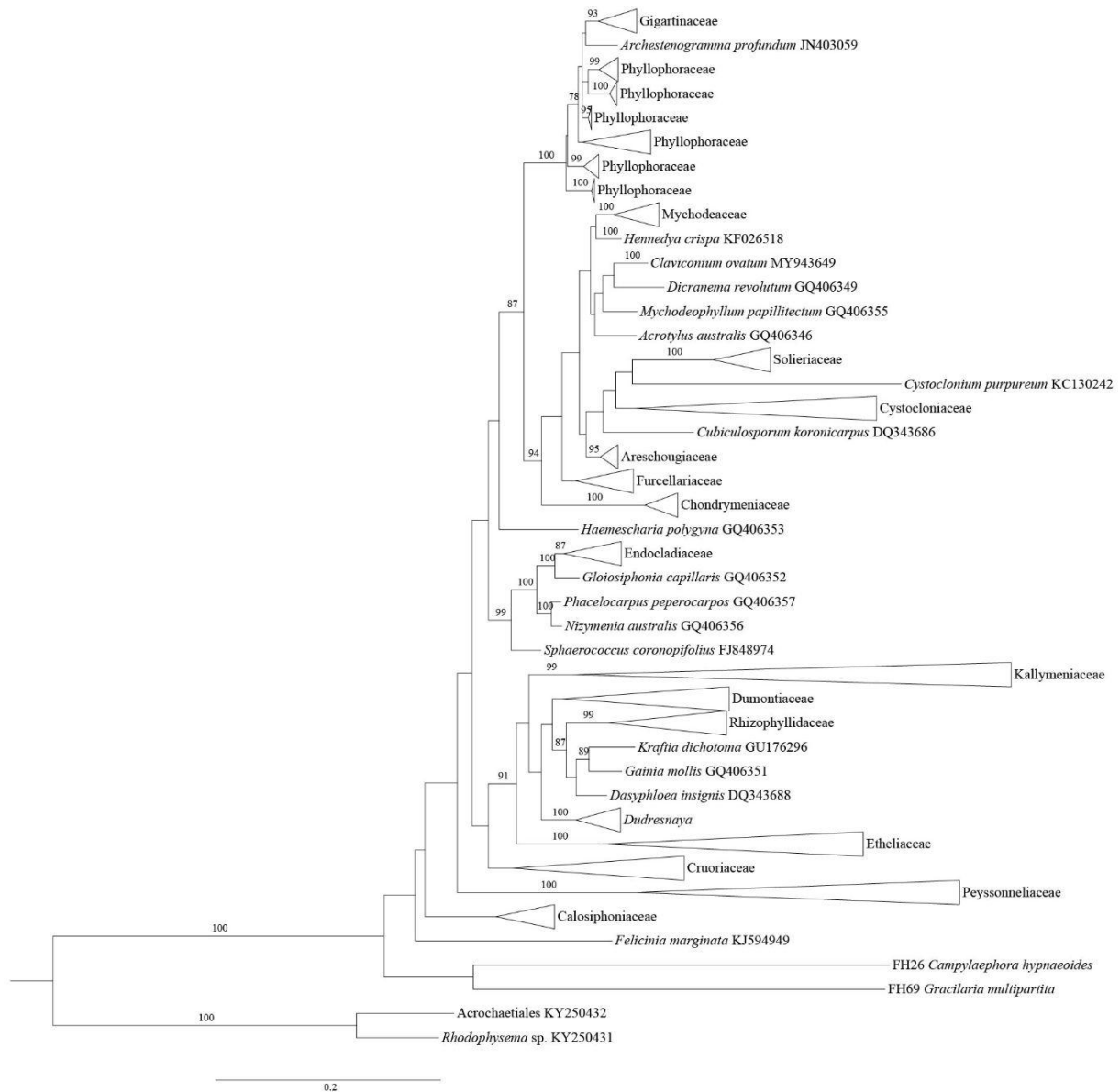


Figure 3. Maximum likelihood phylogeny obtained from nuclear LSU rDNA. Numbers at nodes indicate bootstrap support values (BP), shown when > 75%.

The placement of Phacelocarpaceae may be also conflicting between organellar trees, but differences in species sampling may be taken into consideration. Phacelocarpaceae was sister to Endocladiaceae (100% BP) in the mitochondrial phylogeny, both placed in the clade that is sister to Gigartinaceae and Phylloporaceae. In our plastid phylogeny

Phacelocarpaceae was the earlier diverging lineage in the order, being sister to all the other families in Gigartinales. However, the clade that comprises the other families (except for Phacelocarpaceae) is not robustly supported (76%BP).

ASTRAL trees and gene discordance in the organellar datasets

The mitochondrial ASTRAL tree (Fig. S1) had overall weaker support than the mitochondrial concatenate tree (Fig.1), although the topologies were highly congruent. The only different placement was the sequence of *Catenella caespitosa* (Withering) L.M.Irvine, which was placed in the unsupported Solieriaceae clade. The relationship of Gigartinaceae + Phyllophoraceae with the clade comprising Kallymeniaceae, Rhizophyllidaceae, Etheliaceae, Polyidaceae, Peyssonneliaceae, Phacelocarpaceae and Endocladiaceae, had full bootstrap support. However, from 27 mitochondrial genes, only six (atp6, cob, rns, nad4, nad6, ymf39) were concordant with this placement (Fig. 4). Proportions of mitochondrial genes that support each clade present on the reference tree (ASTRAL tree, Fig. S1) are presented on pie charts plotted for each node in Fig. 4. The plastid ASTRAL tree (Fig. S2) was robustly supported overall, and its topology was largely congruent with the plastid concatenate tree (Fig. 2). The only exception was the placement of *Phacelocarpus tortuosus* Endlicher & Diesing, which was the first lineage to diverge in the order in the concatenate tree (Fig. 2), while in the ASTRAL tree, the first lineage to diverged was the clade comprising Kallymeniaceae, Rhizophyllidaceae, Dumontiaceae and Peyssonneliaceae. The relationship of Gigartinaceae + Phyllophoraceae with the clade including Rissoelaceae, Furcellariaceae, Cystocloniaceae, Solieriaceae, Dicranemataceae, and the polyphyletic Caulachantaceae was weak bootstrap support (0.72BP), but only 31 (of 208) plastid genes were concordant with this placement (Fig. 5). Proportions of plastid genes that support each clade present on the reference tree (ASTRAL tree, Fig. S2) are presented on pie charts plotted for each node in Fig. 5.

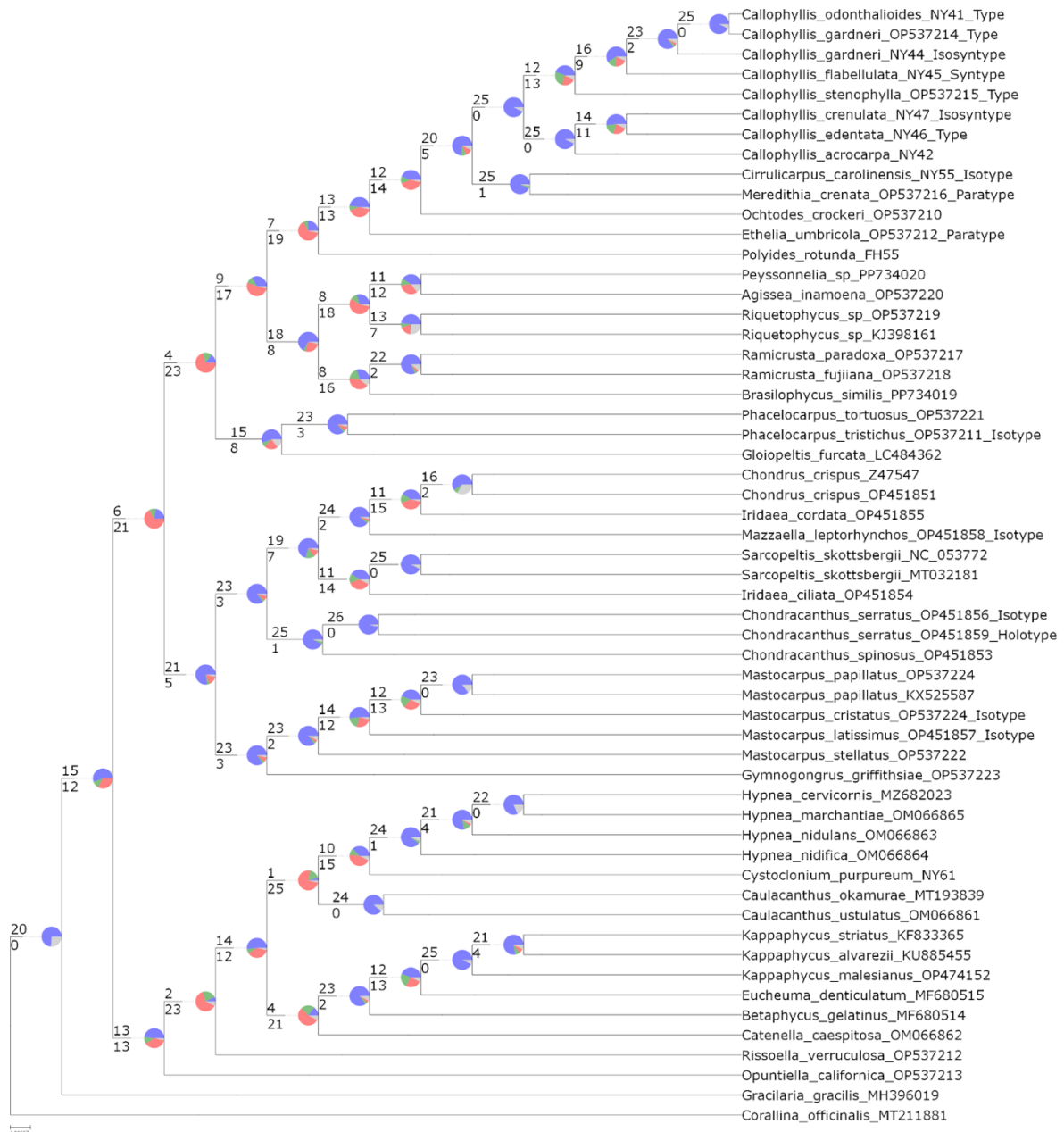


Figure 4. ASTRAL (species tree) topology based on the mitochondrial dataset of Gigartinales (27 genes), with summary of conflicting and concordant genes. For each branch, the top number indicates the number of genes concordant with the species tree at that node, and the bottom number indicates the number of genes in conflict with that clade in the species tree. The pie charts at each node present the proportion of genes that support that clade (blue), the proportion that support the main alternative for that clade (green), the proportion that support the remaining alternatives (red), and the proportion that present less than 50% bootstrap to support or not (conflict) this clade (gray).

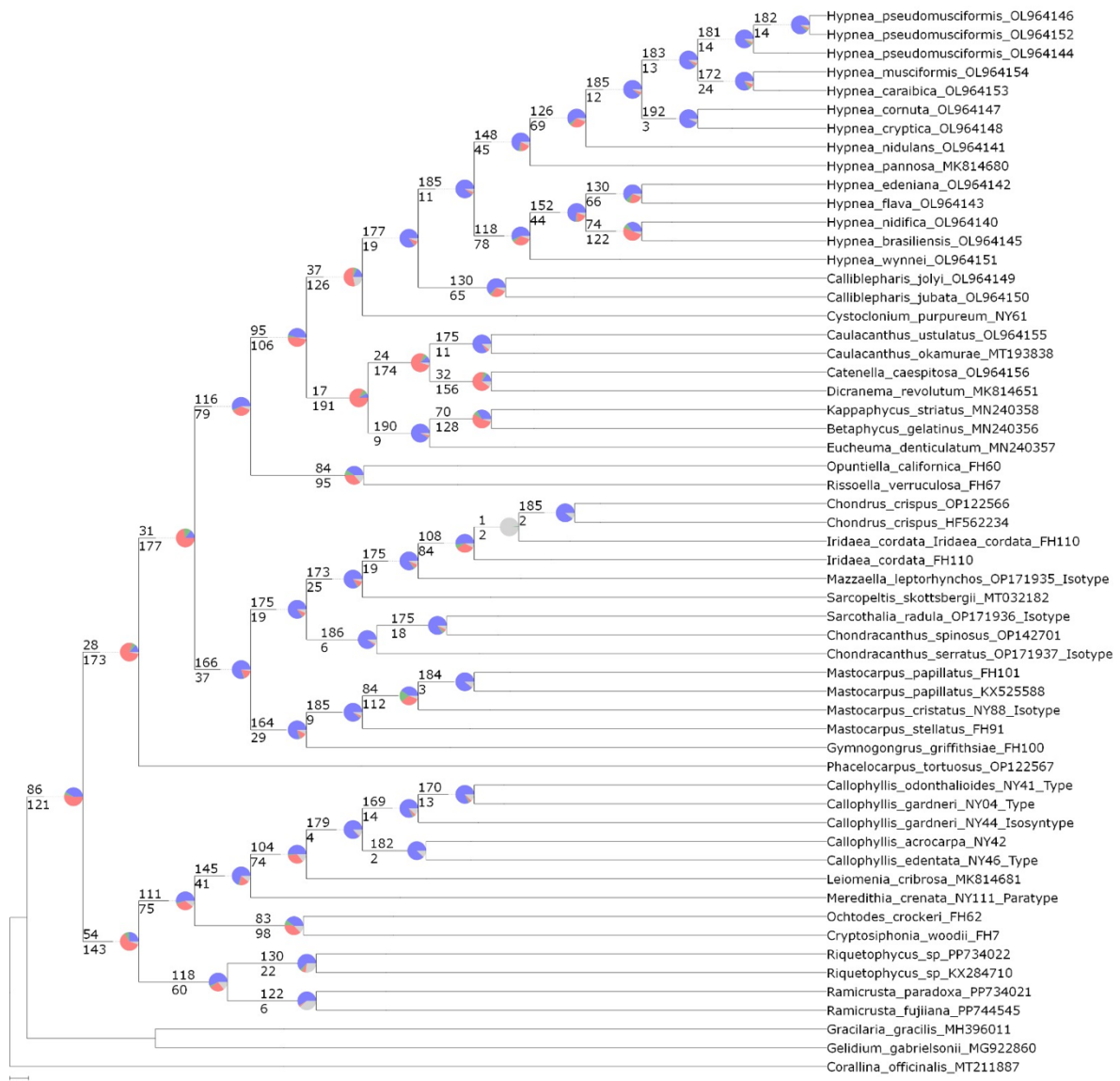


Figure 5. ASTRAL (species tree) topology based on the plastid dataset of Gigartinales (208 genes), with summary of conflicting and concordant genes. For each branch, the top number indicates the number of genes concordant with the species tree at that node, and the bottom number indicates the number of genes in conflict with that clade in the species tree. The pie charts at each node present the proportion of genes that support that clade (blue), the proportion that support the main alternative for that clade (green), the proportion that support the remaining alternatives (red), and the proportion that present less than 50% bootstrap to support or not (conflict) this clade (gray).

Comparative genome structure and content

Mitochondrial genomes are highly conserved, forming one main LCB between rns and trnA (block 1 in Fig. 6), and two smaller LCB: block 2 (trnS-trnR-trnY) and block 3 (from

trnN to rpl20) (Fig. 6). Block 2 was inverted (Fig. 6B) in Caulacanthaceae, Cystocloniaceae, Phacelocarpaceae, Ramicrusta, Solieriaceae and lost (Fig. 6C) in *Rissoella verruculosa*, *Opuntella californica*, *Ochtodes crockeri*, *Gloiopeltis furcata*. The trnH was inverted in species of Cystocloniaceae and in *Rissoella verruculosa*, and was absent in Peyssonneliaceae species. Peyssonneliaceae gained the trnS (Fig. 1), which is placed between cob and trnL, with an inversion in *Agissea inamoena* (Pilger) Pestana, Lyra, Cassano & J.M.C. Nunes (Fig. 1). The gene tatA was gained in the clade comprising the sister families Gigartinaceae and Phylloporaceae, and it was lost in *Gymnogongrus griffithsiae* (Fig. 1). The gene rpl5 was lost in *Sonderophycus* sp., Kallymeniaceae, and in the clade comprising Solieriaceae, Cystocloniaceae, Caulacanthaceae, Rissoellaceae and Furcellariaceae (Fig. 1).

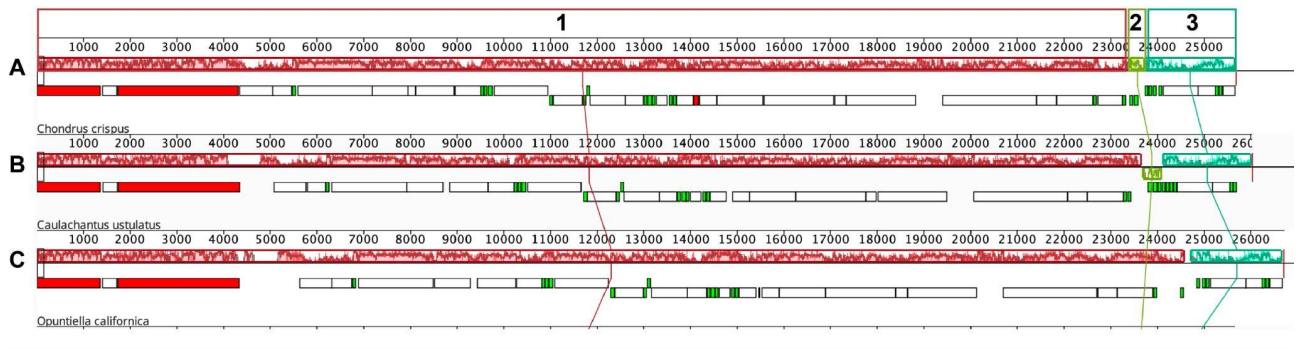


Figure 6. Part of the Progressive MAUVE alignment of mitochondrial genomes, exhibiting three genomes (A-C), which represent the diversity of mitogenome structure in our dataset. A-B. Sequences containing three collinear blocks, 1, 2 and 3. B. Sequence containing inversion in block 2. C. Sequence containing only blocks 1 and 3. The complete MAUVE alignment of mitogenomes is presented on Figure S3.

We identified two structural types of plastome genomes, regarding the number/position of rDNA operons (following Lee *et al.*, 2016): type R1 is present in most of the analyzed species; type R2 is present in *Riquetophycus* sp. And in *Opuntella californica*. The plastid collinearity map formed 11 LCB (Fig. 7) and showed some variation across Gigartinales' families, except for blocks 1, 5, 9 and 10. Block 2 (rps6 to ycf46) was inverted in *Opuntella californica* and Peyssonneliaceae species (Fig. 7B). Block 3 (psaM to psb30) was inverted (Fig. 7C) in species of *Callophyllis* and Solieriaceae. Block 4 (ycf64 to ycf21) was inverted (Fig. 7D) in Solieriaceae species. Block 8 was inverted in *Hypnea* sp. (Fig. 7E). Blocks 7 (ycf41 to rps18), 8 (infB to ycf8), and 11 (psbC to ompR) were inverted in *Dicranema revolutum* (C.Agardh) J.Agardh (Fig. 7F). Translocations were observed

between blocks 3 and 4 (Fig. 7D) in Solieriaceae species, and between blocks 6 (hypothetical protein to psbI) and 8 in *D. revolutum* (Fig. 7F).

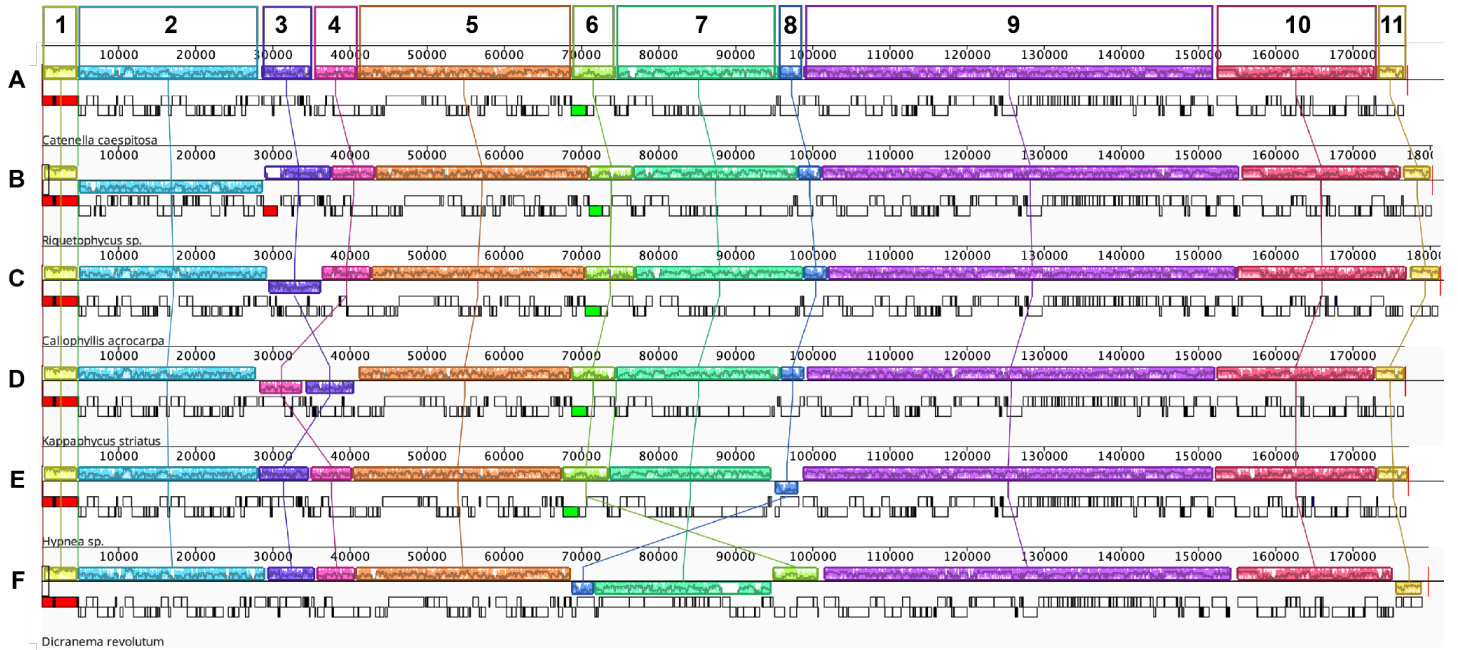


Figure 7. Part of the plastid progressive MAUVE alignment, exhibiting six genomes (A-F), which represent the diversity of plastome structure in our dataset. A-F. Sequences containing 11 collinear blocks. B. Sequence containing inversion in block 2. C. Sequence containing inversion in block 3. D Sequence containing translocation between blocks 3 and 4 and inversion in block 4. E. Sequence containing inversion in block 8. F. Sequence containing translocation between blocks 6 and 8 and inversion in blocks 7 and 11. The complete MAUVE alignment of 73oodie73as is presented on Figure S4.

Gigartinales gained one gene, *ycf57*, and had nine genes lost across the order. The gene *drf* was lost in the clade Gigartinaceae (Fig. 2). Numerous genes' losses were observed in the analyzed such as: i) *pbsA*, lost in *Catenella caespitosa* and *Rissoella verruculosa*; ii) *petF*, lost in *Callophyllis gardneri*; iii) *rrz*, lost in *Hypnea nidulans* Setchell; iv) *ycf17*, lost in *Callophyllis*; v) *ycf23*, lost in *Rissoella verruculosa*, *Leiomenia cribrosa* (Harvey) Huisman & G.W.Saunders, and in the clades Cystocloniaceae and Peyssonneliaceae; vi) *ycf35*, lost in *Rissoella verruculosa*; vii) *ycf57*, lost in *Caulacanthus*; viii) *ycf92*, lost in *Hypnea cryptica* P.B.Jesus & J.M.C.Nunes, *Hypnea cornuta* (Kützinger) J.Agardh, *Hypnea edeniana* F.Nauer, V.Cassano & M.C.Oliveira, *Sarcothalia radula* (Esper) Edyvane & Womersley, *Chondracanthus spinosus* (Kützinger) Guiry, and *Chondracanthus serratus* (N.L.Gardner) Hughey & Hommersand.

Analyses of character evolution

Our trait evolution analyses indicated that the model ARD was suitable for growth type, habit, procarp formation and tetrasporangium division type. Equal-rates and symmetric models were equally suitable for thallus shape.

The most recent common ancestor (mrca) of Gigartinales probably presented a multiaxial form of growing (Fig. 8A). The uniaxial growth type arose at least four times during the evolution: in *Phacelocarpus tortuosus*, *Cryptosiphonia woodii* (J.Agardh) J.Agardh, the ancestor of Caulacanthaceae and in the ancestor of Cystocloniaceae (Fig. 8A). The habit erect is the dominant state in Gigartinales but, probably, the habit prostrate was present in the origin of Gigartinales and was retained by *Leiomenia cribrosa*, *Meredithia crenata* C.W.Schneider, G.W.Saunders & C.E.Lane and the ancestor of Peyssonneliaceae (Fig. 8B).

We cannot determine which thallus shape (Fig. 8C) was present in the mrca of Gigartinales. Peyssonneliaceae, Kallymeniaceae, Furcellariaceae and Rissoelaceae present exclusively flattened thallus, while Dumontiaceae, Rizophyllidaceae, Caulacanthaceae, Solieriaceae and Cystocloniaceae present exclusively cylindrical thallus (Fig. 8C). Gigartinaceae and Phyllophoraceae are quite similar and consistent in terms of the analyzed morphological characters. Only for thallus shape there is overlap of states: while most species in these families present flattened thallus, each family presents one species with cylindrical thallus: *Gymnogongrus griffithsiae* in Phyllophoraceae, and *Chondracanthus serratus* in Gigartinaceae.

The formation of procarp was probably absent in the origin of Gigartinales and this state remained in Peyssonneliaceae, Dumontiaceae, Rizophyllidaceae, Caulacanthaceae and Solieriaceae (Fig. 8D).

The division of tetrasporangia in Gigartinales was probably cruciate in its origin and this state was modified to irregularly cruciate in *Cryptosiphonia woodii* (Fig. 8E). The transition to zonate tetrasporangia occurred in the ancestor of Furcellariaceae, Rissoelaceae, Caulacanthaceae, Solieriaceae and Cystocloniaceae (Fig. 8E). This trait also arose in *Phacelocarpus tortuosus* (Fig. 8E).

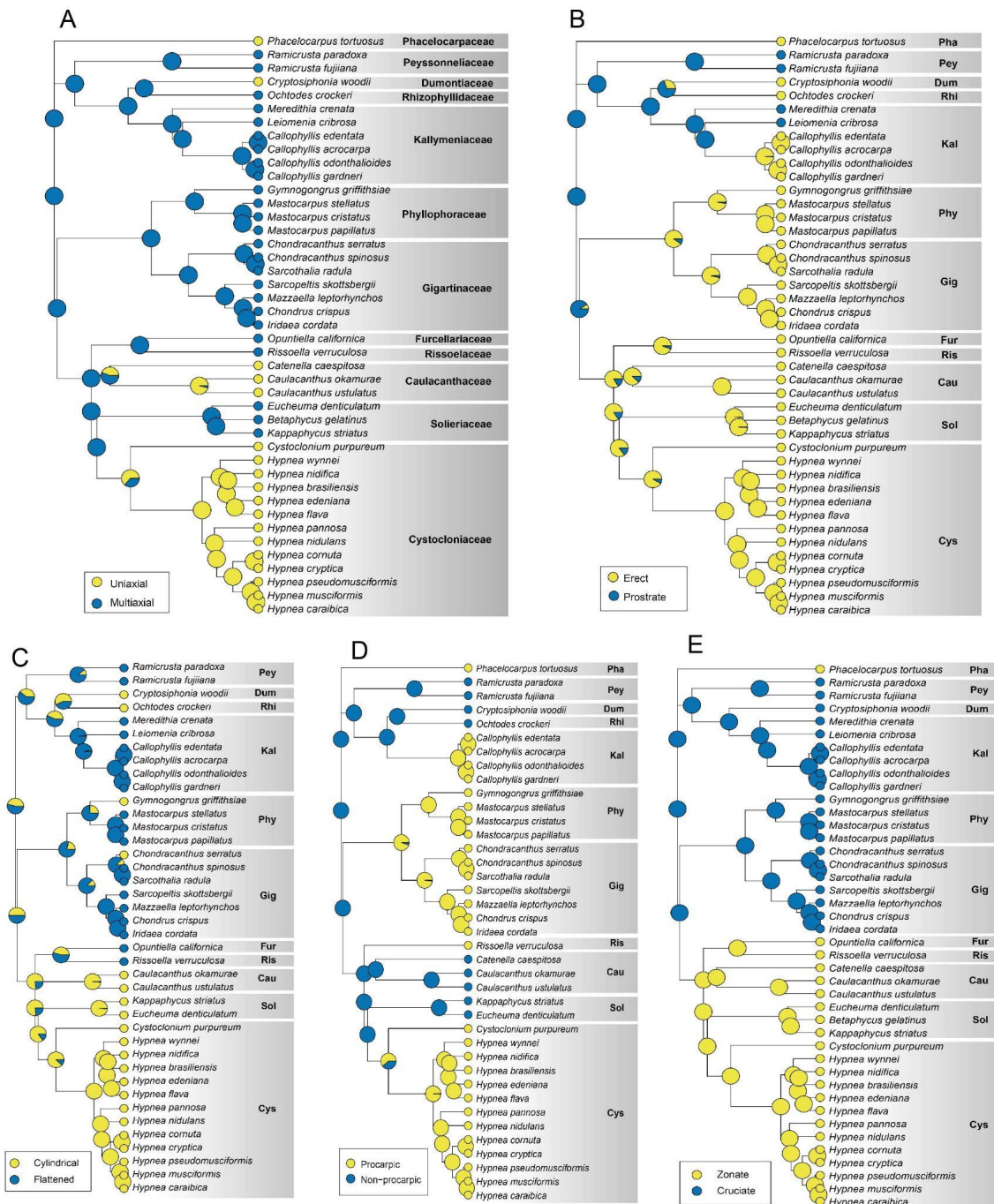


Figure 8. Trait evolution in Gigartinales based on plastid phylogeny. A. Growth type. B. Habit. C. Thallus shape. D. Procarp formation. E. Tetrasporangia division type. Pie charts at nodes indicate the proportion of the ancestral character reconstruction based on the best model defined for each trait. Family's names are highlighted in gray.

Discussion

Phylogenetic relationships among main lineages of Gigartinales

We generated 51 new organellar genomes, increasing the number of Gigartinales genomes previously available on GenBank (29 mitochondrial and 52 plastidial) by more than 75%. Complete organellar genomes from red algae historical collections, especially type materials, are invaluable resources for phylogenetic, systematic and phylogeography studies (Boo, 2016). Our sampling represents all the main lineages of Gigartinales, and included 17 newly sequenced type specimens. Our phylogenies are the first to date to use larger gene content matrices (28 genes for the mitochondrial and 215 genes for the chloroplast) for Gigartinales, and both had very high bootstrap support across the trees.

Both organellar trees recovered the monophyly of Gigartinales and showed congruent topologies, with the exception of the conflicting placement of the clade comprising Gigartinaceae and Phyllophoraceae (Guiry & Garbary, 1990; Hommersand *et al.*, 1994; Saunders *et al.*, 2004; Verbruggen *et al.*, 2010; Dumilag *et al.*, 2019). Verbruggen *et al.* (2010) evidenced the polyphyly of Gigartinales, distributed in four distantly related clades: core Gigartinales, *Atractophora*, Calosiphoniaceae and Peyssonneliaceae. Subsequent studies have focused on the groups outside core Gigartinales and proposed a taxonomic scheme that accommodates them [(Peyssonneliaceae) Krayesky *et al.*, 2009; (Atractophoraceae) Saunders *et al.*, 2016]. More recently, Pestana *et al.* (2024), based on genomic data, placed Peyssonneliaceae back within Gigartinales. The authors also segregated Calosiphoniaceae, a family that includes *Calosiphonia* (3 spp.) and *Schmitzia* (5 spp.), recircumscribing the order, and resolving Gigartinales as monophyletic.

Caulacanthaceae was polyphyletic in both of our phylogenies, indicating that its current taxonomic classification may not accurately reflect its evolutionary history. We obtained the sequence of the type species, *Caulacanthus ustulatus* (Turner) Kützinger, from a specimen collected in the Azores, Portugal. Although the collection is not from its type locality in Cádiz, Spain, our results may indicate that the clade in which this sequence is placed represents the true *Caulacanthus*. However, the sequence for *Catenella caespitosa* was not included in this clade, raising questions about its placement within the family. *Catenella caespitosa* is not the type species for the genus *Catenella*, as that designation belongs to *Catenella opuntia* (Goodenough & Woodward) Greville. This discrepancy highlights the need for further data to clarify the placement of species within

Caulacanthaceae, particularly regarding the positioning of *Catenella* Greville species. Our newly generated genomes from type specimens of several species constitute a relevant source for future taxonomic developments in the taxonomy of inner clades in Gigartinales.

Coalescence analyses reveal gene discordances in organellar genomes

Phylogenomic studies in red algae generally provide trees based on concatenation for organellar genomes (Díaz-Tapia *et al.*, 2017; Paiano *et al.*, 2018; Díaz-Tapia *et al.*, 2019; Lyra *et al.*, 2021; Jesus *et al.*, 2023), while analyses based on coalescence are performed for nuclear data (Lyra *et al.*, 2021), notoriously subject to biological sources of conflicts, such as recombination, incomplete lineage sorting, hybridization, and genes duplications and losses. Walker *et al.* (2019) conducted a pioneer study, finding gene tree conflicts in plastomes of a broad angiosperms' dataset. Little is known about inheritance of organelles in Rhodophyta (Hawkes, 1978; Bouzon *et al.*, 2000; Delivopoulos, 2000; Choi *et al.*, 2008; Wang *et al.*, 2021), and plastid heteroplasmy, which is well documented in angiosperm species (Johnson & Palmer, 1989; Lee *et al.*, 1988; Hansen *et al.*, 2007; Carbonell-Caballero *et al.*, 2015), can't be excluded as a possible source of gene tree conflict in red algae phylogenies based on organellar datasets. Mitochondrial heteroplasmy may be rare in plants (Khachaturyan *et al.*, 2024), but we can't exclude the possibility of biparental inheritance and of conflicting gene tree topologies hidden in mitochondrial 'species trees'.

We applied coalescence analyses to our datasets to verify the existence of incongruence among individual gene trees' topologies, which was motivated by the presence of the conflicting placements of the sister clades Gigartinaceae and Phyllophoraceae in the mitochondrial and plastid concatenated trees (Figs. 1 and 2, respectively). Our results (Figs. 4 and 5) indicated that not all of the genes in each dataset support the topologies of the mitochondrial or plastid 'species trees' [both concatenate (Figs. 1 and 2, respectively) and ASTRAL (Figs. S1 and S2, respectively)]. Further analyses are needed to explore the biological sources of such gene conflicts. Such efforts would ideally include population sampling for each species to permit testing hypotheses, such as the presence of hybridization or incomplete lineage sorting. Our sampling aimed at one individual per species, having limited applications in this sort of investigation. However, our results succeeded in demonstrating that: i) we should be skeptical about the homogeneity of evolutionary histories of each gene in organellar genomes, and ii) coalescence analyses are useful tools for revealing eventual such variations. Future exploratory phylogenomic analyses of both mitochondrial and plastid datasets should evaluate the utility and establish criteria for

filtering genes for higher phylogenetic signal, as Walker *et al.* (2019) indicated for angiosperm phylogenies based on plastomes.

Gene synteny

We identified molecular events, such as gene losses, gains, inversions and translocations that characterize inner clades of Gigartinales.

Mitochondrial genomes. The gain of *tatA* is a shared event among the Gigartinaceae-complex species and the loss of *rpl5* is shared by the Solieriaceae-complex species. The *rrn5* gene was previously considered lost in Cystocloniaceae (Jesus *et al.*, 2023). However, here we were able to annotate this rRNA gene in Cystocloniaceae species. 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. Liu *et al.*, 2019; Zhang *et al.*, 2020).

Plastid structure. We confirmed the presence of two types of plastome structure, as Lee *et al.* (2016) described. The R1 type, previously identified in *Chondrus crispus*, is predominant in Gigartinales, occurring in most of the analyzed species. The R2 type, known in *Riquetophycus* sp., was now also observed in the distantly related *Opuntella californica*, showing that this structural type arose at least two times in Gigartinales' evolution.

We observed the loss of *ycf23* in Cystocloniaceae, *Rissoella verruculosa* (Rissoellaceae), *Leiomenia cribrata* (Kallymeniaceae) and Peyssonneliaceae species. Jesus *et al.* (2023) identified the loss of genes *ycf22* and *ycf23* as apomorphies of Cystocloniaceae. However, the *ycf22* was found in all species studied, including Cystocloniaceae species. We also observed the inversion of the mitochondrial *trnH* in *Hypnea* species, previously identified as an apomorphy of the genus (Jesus *et al.*, 2023).

In terms of rearrangements, it was possible to observe a large number of inversions in plastid genomes, including six whole blocks. *Dicranema revolutum* (Dricranemataceae) had the larger number of inversions, three blocks. Translocations were also observed in plastid genomes, frequently associated with block's inversions. Blocks 3 and 4 were inverted and translocated in Solieriaceae, and blocks 6 and 8 in *Dicranema revolutum*. The rearrangement of the blocks 3 and 4 was first recognized in *Kappaphycus alvarezii*, Solieriaceae (Liu *et al.*, 2019), and later described as a "12.5- kb gene fragment from *psaM* to *ycf21* completely reversed" in three other Solieriaceae species (Zhang *et al.*, 2020). Here we observed that this region is highly variable, presenting different arrangements among the gigartinean families. Our collinearity analysis separated this region in two blocks, 3, from *psaM* to

psb30; and 4, from ycf64 to ycf21. Block 3 was also inverted in *Callophyllis* (Kallymeniaceae). Also, the two blocks are translocated between each other in the Solieriaceae species in comparison to other Gigartinales taxa. Gigartinaceae species presented a reduction in block 3 due to the absence of the dfr gene (~1900pb).

Morphology and traits evolution

Recognition of the characters to be used in the identification and classification of taxa is a relevant service that taxonomists aim to provide to the community (Dayrat, 2005). In macroalgae, vegetative characters, such as shape and growth type, are easier to be observed and help distinguish species by structural organization, but can undergo independent adaptive changes, complicating evolutionary interpretation (Garbary & Gabrielson, 1990). Reproductive characters, like spore type and gonimoblast development, are more conserved and useful for identifying phylogenetic relationships, making them preferred for understanding ancestry among groups (Searles, 1968; Norris, 1971). Combining both types of characters allows us to cross-validate findings and compensate for the limitations of each character type. For this reason, we used both (3 vegetative and 2 reproductive) in our analyses of ancestral characters evolution. The procarpic development of the carposporophyte is central for the proposition of Gigartinales by Schmitz (1892), differentiating this order from the others due to the procarpic nature of its development, associated with the development of the carposporophyte towards the interior of the thallus. Classification based on this diagnostic character was questioned over the four decades that followed (Searles, 1968; Kraft & Robins, 1985; Hommersand & Fredericq, 1990) and nowadays many representatives of Gigartinales are known for having a non-procarpic development.

Morphology and molecular-based studies (Freshwater *et al.*, 1994; Fredericq *et al.*, 1999) have identified two main lineages in Gigartinales: i) the Gigartinaceae-complex, comprising the families Gigartinaceae and Phyllophoraceae and Petrocelidaceae (currently recognized as Phyllophoraceae), characterized by the formation of procap and cruciate tetrasporangia; ii) the Solieriaceae-complex, comprising the families Solieriaceae, Furcellariaceae, Hypneaceae (currently recognized as Cystocloniaceae), Cystocloniaceae and Caulacanthaceae, characterized by cruciate tetrasporangia; regarding the procarp, it can be present or absent, depending on the family. Our organellar phylogenies also recovered these two main lineages, also adding to the Solieriaceae-complex the Rissoellaceae in the

mitochondrial phylogeny, and both Rissoellaceae and Dicranemataceae in the plastid phylogeny.

Concerning the conflict in our organellar trees regarding the placements of Gigartinaceae and Phyllophoraceae, which are sister clades in both phylogenies, our traits evolution analyses reveal that these two families are similar for most of the analyzed traits, and share various traits with both lineages to which they are sister clades in each phylogeny.

Conclusion

The challenges in classifying algal groups arise when the traits used to separate them become ambiguous. Research by Searles (1968) and Norris (1971) suggests that the distinctions between multiaxial versus uniaxial growth or procarpic versus non-procarpic development might be overstated and inconsistent across related species and genera. Other traits, such as inward versus outward gonimoblast development or zonate versus cruciate tetrasporangia, could also lose relevance as taxonomic criteria evolve. Although these characteristics have been useful in classification, they may ultimately be reconsidered as phylogenetic evidence progresses (Kraft, 1973). Our results confirm the ambiguity of such characters and evidence the lack of unique and non-overlapping morphological evidences of independent evolution of lineages, challenging the recognition of genera or families in Gigartinales. However, genomic level data provide such evidence. Therefore, as our results show, it is currently possible to provide a solid service to science in delimiting taxa in Gigartinales, but there is much work to be done to provide the community with the characters to recognize such taxa, as Dayrat (2005) defends, including molecular characters. Our results also demonstrate that we should be cautious about the homogeneity of evolutionary histories across genes in organellar genomes and that coalescence analyses can reveal such variations. Future phylogenomic studies should assess the utility of mitochondrial and plastid datasets and establish criteria for filtering genes to enhance phylogenetic signal

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Supplementary material

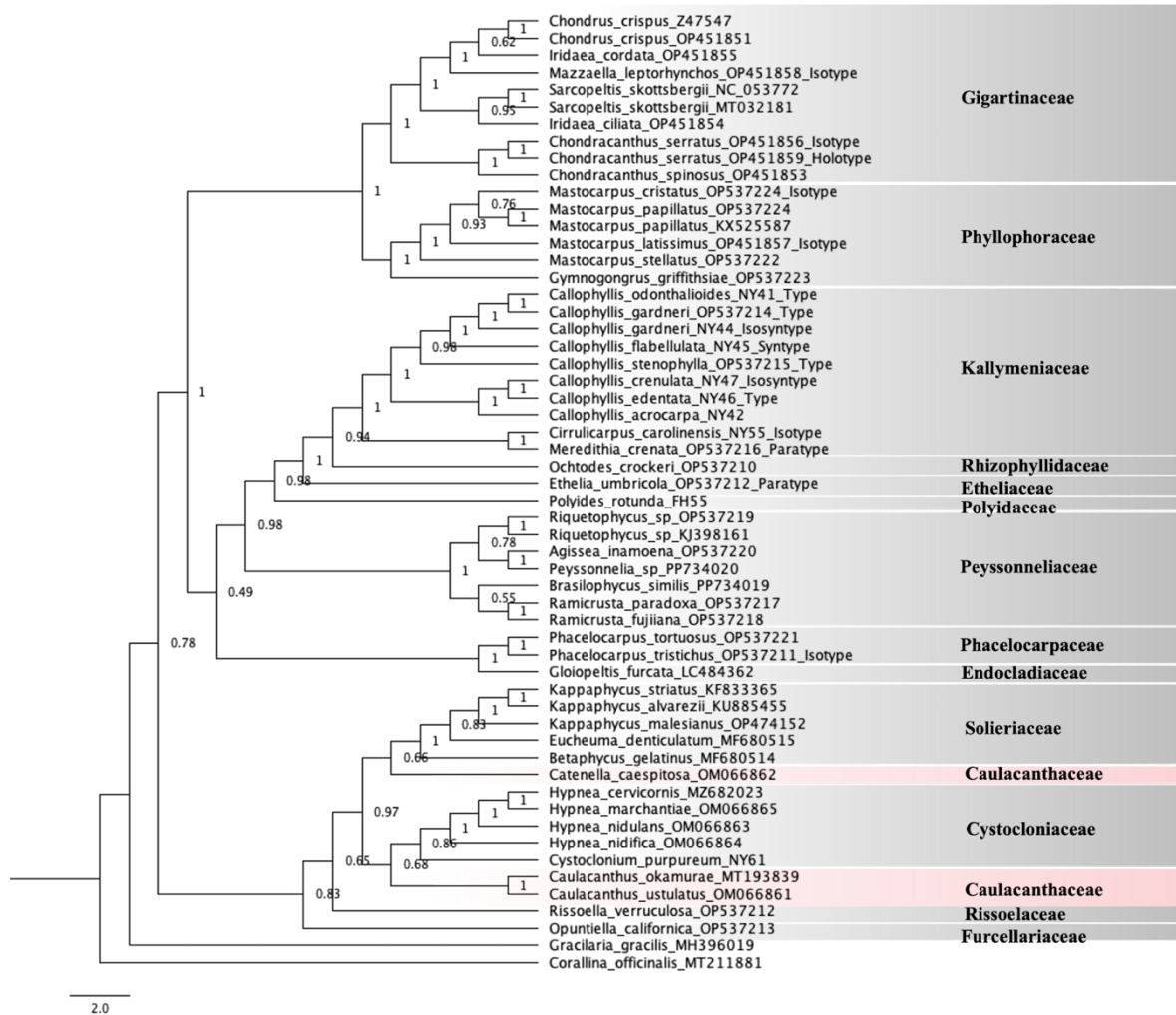


Figure S1. ASTRAL tree for the mitochondrial dataset (27 genes). Bootstrap support values are presented for each node.

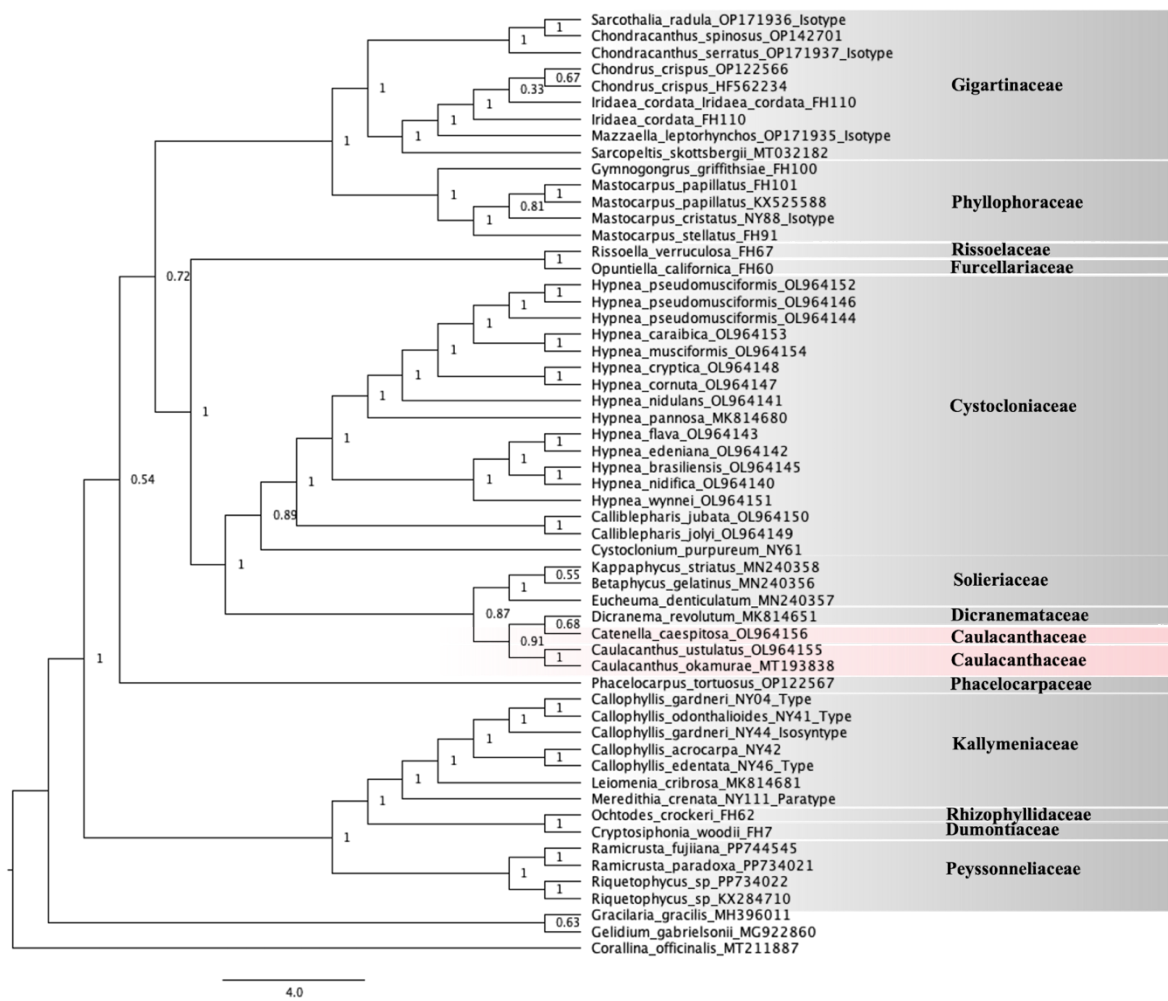


Figure S2. ASTRAL tree for the plastid dataset (208 genes). Bootstrap support values are presented for each node.

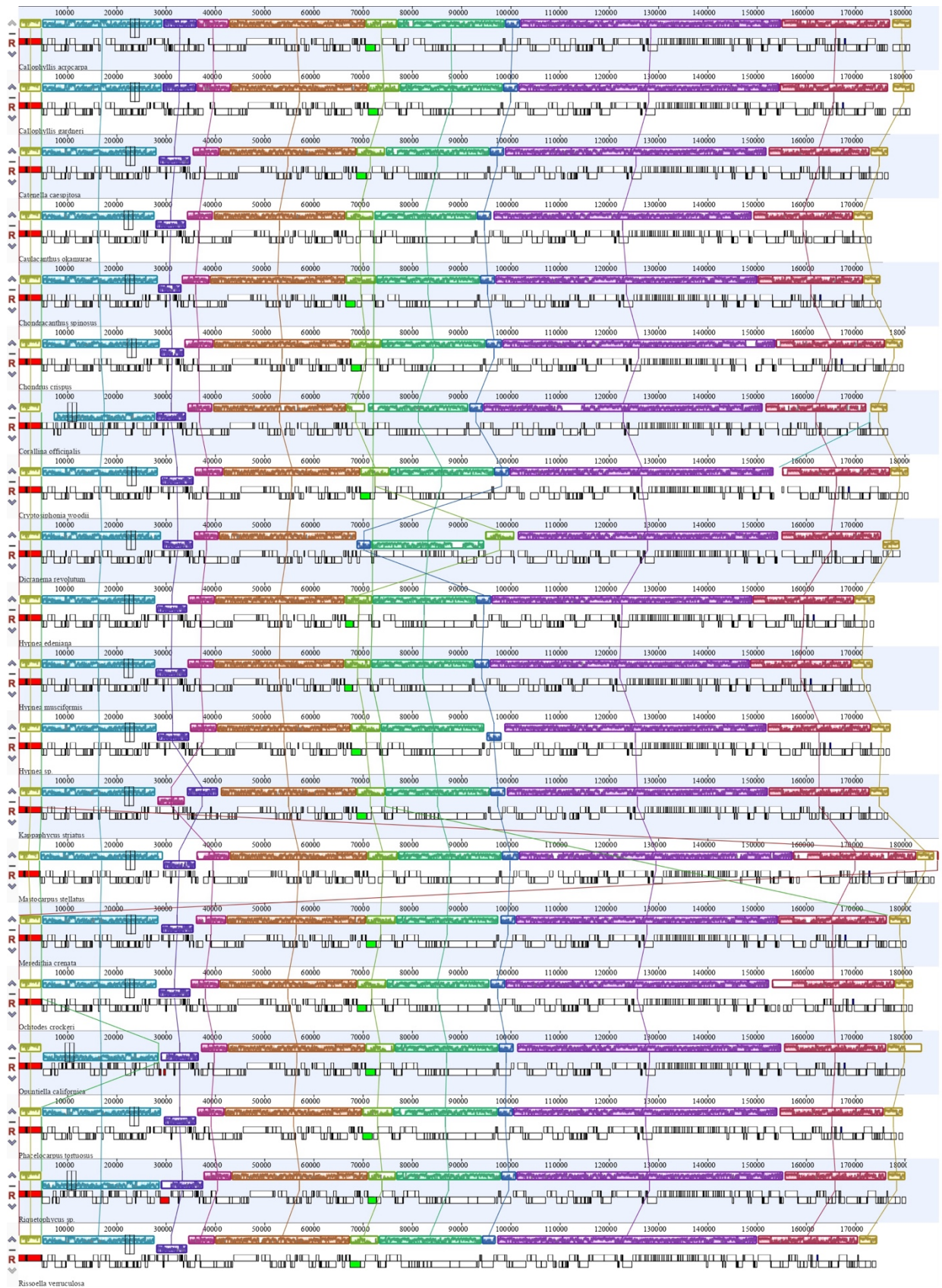


Figure S4. Complete MAUVE alignment of plastomes.

Table S1. Herbarium specimens sequenced in this study.

Specimen identification	Currently accepted name (if different)	Voucher / Barcode	Collector/other identifying notes	Collection date	Locality
<i>Callophyllis acrocarpa</i> Setchell		2136707	N. L. Gardner	November, 1922	United States of America, California, Marin Co.
<i>Callophyllis crenulata</i> Setchell		937464	N. L. Gardner	June 08, 1901	United States of America, Washington, Whidbey Island, Island County
<i>Callophyllis edentata</i> Kylin		937456	J. H. Kylin	July 22, 1924	United States of America, Washington, San Juan Co., Turn Island
<i>Callophyllis flabellulata</i> Setchell		937501	D. Lyall	January, 1859	Canada, British Columbia, Vancouver Island, Esquimalt
<i>Callophyllis gardneri</i> Setchell		03685540			
<i>Callophyllis gardneri</i> Setchell		937463	N. L. Gardner	May, 21 to Apr, 1911	United States of America, California, Los Angeles Co.
<i>Callophyllis odonthalioides</i> Setchell		2136709	N. L. Gardner	December 12, 1923	United States of America, California, Santa Barbara Co.
<i>Callophyllis stenophylla</i> Setchell		2136712	N. L. Gardner	May, 1920	United States of America, California, Marin Co. Duxbury Reef
<i>Chondrus crispus</i> Stackhouse		00788946	R. Thaxter	October 4, 1921	United States, Maine, York County
<i>Cirrularcarpus carolinensis</i> G.I.Hansen		900040	G. I. Hansen	October 10, 1974	United States of America, North Carolina, Onslow
<i>Cryptosiphonia woodii</i> (J.Agardh) J.Agardh		00618734	W. J. Eyerdam	May 26, 1932	United States, Alaska, Alaskan Peninsula, Unalaska Island
<i>Cystoclonium purpurascens</i> f. <i>stellatum</i> Collins	<i>Cystoclonium purpureum</i> (Hudson) Batters	2136554	F. S. Collins	July 11, 1903	United States of America, Maine Cumberland Co., South Harpswell
<i>Ethelia umbricola</i> C.W.Schneider, A.K.Kivela & C.E.Lane		2051409	C. W. Schneider / C. E. Lane	August 20, 2010	Bermuda, Vic. Middle buoy, Eastern Blue Cut channel, due north of Daniel's Head, Somerset Island
<i>Fucus rotundus</i> Hudson	<i>Polyides rotunda</i> (Hudson) Gaillon	00904160	Miss C. E. Clarke	s.d.	United States, Massachusetts, Magnolia
<i>Gigartina asperifolia</i> J.Agardh	<i>Chondracanthus spinosus</i> (Kützinger) Guiry	00907374	Gardner	December, 1908	United States, California, Los Angeles, San Pedro

<i>Gigartina canaliculata</i> f. <i>laxa</i> F.S.Collins	<i>Chondracanthus serratus</i> (N.L.Gardner) Hughey & Hommersand	937546	M. S. Snyder	s.d.	United States of America, California, San Diego
<i>Gigartina dichotoma</i> N.L.Gardner	<i>Mastocarpus latissimus</i> (Harvey) S.C.Lindstrom, Hughey & Martone	900143	N. L. Gardner	November, 1926	United States of America, California, Marin Co., Duxbury Reef, near Bolinas
<i>Gigartina leptorhynchus</i> f. <i>caespitosa</i> E.Y.Dawson	<i>Mazzaella leptorhynchus</i> (J.Agardh) Leister	900164	E. Y. Dawson	March 23-24, 1948	United States of America, California, Los Angeles Co, Santa Catalina Island
<i>Gigartina radula</i> f. <i>typica</i> Setchell	<i>Sarcothalia radula</i> (Esper) Edyvane & Womersley	937548	W. A. Setchell / R. E. Gibbs	December 13, 1898	United States of America, California, Marin Co., Dillon's Beach
<i>Gigartina papillata</i> (C.Agardh) J.Agardh	<i>Mastocarpus papillatus</i> (C.Agardh) Kützing	00907714	Gardner	October, 1932	United States, California, San Francisco County, Fort Point
<i>Gigartina papillata</i> f. <i>cristata</i> Setchell	<i>Mastocarpus cristatus</i> (Setchell) S.C.Lindstrom, Hughey & Martone	937543	W. A. Setchell	May 18, 1897	United States of America, California, Monterey Co., Pyramid Point
<i>Gigartina serrata</i> N.L.Gardner	<i>Chondracanthus serratus</i> (N.L.Gardner) Hughey & Hommersand	937541	N. L. Gardner	July, 1924	Mexico, Baja California, Ensenada
<i>Gymnogongrus griffithsiae</i> (Turner) C.Martius		01171016	F. S. Collins	1885	United States, Massachusetts, Eastham
<i>Iridaea cordata</i> (Turner) Bory		01159354	M. A. Barber	August, 1899	United States, Washington, Port Townsend
<i>Iridophycus ciliatus</i> (Kützing) Setchell & N.L.Gardner	<i>Iridaea ciliata</i> Kützing	01159490	Hassler Expedition	s.d.	Canada, Magdalene Islands
<i>Kallymenia californica</i> Farlow	<i>Opuntella californica</i> (Farlow) Kylin	00903274	N. L. Gardner	September, 1927	United States, California, San Mateo, Moss Beach
<i>Mastocarpus stellatus</i> (Stackhouse) Guiry		00887093	Scott LaGreca	September 30, 2002	United States, Massachusetts, Boston, The Graves Island
<i>Meredithia crenata</i> C.W.Schneider, G.W.Saunders & C.E.Lane		2051413	C. W. Schneider / C. E. Lane	March 31, 2003	Bermuda, Walsingham Pond, vic. Castle Harbour
<i>Ochtodes crockeri</i> Setchell & N.L.Gardner		00903009	Wm. R. Taylor	January 17, 1934	Ecuador, Galapagos Island, Santa Maria Island, Black Beach Anchorage
<i>Phacelocarpus tortuosus</i> Endlicher & Diesing		00903581	E. M. Holmes	1891	Canada, New Brunswick, Cape Colony
<i>Phacelocarpus tristichus</i> J.Agardh		900602	J. C. Melvill	1884	Mauritius
<i>Fucus rotundus</i> Hudson	<i>Polyides rotunda</i> (Hudson) Gaillon	00904160	Miss C. E. Clarke ex Herbarium	s.d.	United States, Massachusetts, Magnolia
<i>Rissoella verruculosa</i> (Bertoloni) J.Agardh		00882214	Debray	May 28, 1890	

Table S2. Characters used in traits evolution analysis.

Species	Habit	Thallus shape	Growth type	Procarp	Tetrasporangium division	References
<i>Betaphycus gelatinus</i>	Erect	-	Multiaxial	-	Zonate	Huisman, 2018
<i>Callophyllis acrocarpa</i>	Erect	Flattened	Multiaxial	Present	Cruciate	Clarkston & Saunders, 2013
<i>Callophyllis edentata</i>	Erect	Flattened	Multiaxial	Present	Cruciate	Clarkston & Saunders, 2013
<i>Callophyllis gardneri</i>	Erect	Flattened	Multiaxial	Present	Cruciate	Clarkston & Saunders, 2013
<i>Callophyllis odonthalioides</i>	Erect	Flattened	Multiaxial	Present	Cruciate	Clarkston & Saunders, 2013
<i>Catenella caespitosa</i>	Erect	Flattened	Uniaxial	Absent	Zonate	Prud'homme van Reine <i>et al</i> , 1983
<i>Caulacanthus okamurae</i>	Erect	Cylindrical	Uniaxial	Absent	Zonate	Petrocelli <i>et al</i> , 2020
<i>Caulacanthus ustulatus</i>	Erect	Cylindrical	Uniaxial	Absent	Zonate	Br��ret, 2007
<i>Chondracanthus serratus</i>	Erect	Cylindrical	Multiaxial	Present	Cruciate	Hughey & Hommersand 2008
<i>Chondracanthus spinosus</i>	Erect	Flattened	Multiaxial	Present	Cruciate	Hughey & Hommersand 2008
<i>Chondrus crispus</i>	Erect	Flattened	Multiaxial	Present	Cruciate	Huisman, 2018
<i>Cryptosiphonia woodii</i>	Erect	Cylindrical	Uniaxial	Absent	Cruciate	Lindstrom, 1988
<i>Cystoclonium purpureum</i>	Erect	Cylindrical	Uniaxial	Present	Zonate	Thrainsson & Hommersand, 2012
<i>Eucheuma denticulatum</i>	Erect	Cylindrical	Multiaxial	Absent	Zonate	Huisman, 2018
<i>Gymnogongrus griffithsiae</i>	Erect	Cylindrical	Multiaxial	Present	Cruciate	Huisman, 2018
<i>Hypnea brasiliensis</i>	Erect	Cylindrical	Uniaxial	Present	Zonate	Jesus <i>et al.</i> , 2016
<i>Hypnea caraibica</i>	Erect	Cylindrical	Uniaxial	Present	Zonate	Huisman, 2018

<i>Hypnea cornuta</i>	Erect	Cylindrical	Uniaxial	Present	Zonate	Jesus <i>et al.</i> , 2019
<i>Hypnea cryptica</i>	Erect	Cylindrical	Uniaxial	Present	Zonate	Nauer <i>et al.</i> , 2019
<i>Hypnea edeniana</i>	Erect	Cylindrical	Uniaxial	Present	Zonate	Nauer <i>et al.</i> , 2019
<i>Hypnea flava</i>	Erect	Cylindrical	Uniaxial	Present	Zonate	Nauer <i>et al.</i> , 2019
<i>Hypnea musciformis</i>	Erect	Cylindrical	Uniaxial	Present	Zonate	Huisman, 2018
<i>Hypnea nidifica</i>	Erect	Cylindrical	Uniaxial	Present	Zonate	Huisman, 2018
<i>Hypnea nidulans</i>	Erect	Cylindrical	Uniaxial	Present	Zonate	Huisman, 2018
<i>Hypnea pannosa</i>	Erect	Cylindrical	Uniaxial	Present	Zonate	Huisman, 2018
<i>Hypnea pseudomusciformis</i>	Erect	Cylindrical	Uniaxial	Present	Zonate	Nauer <i>et al.</i> , 2019
<i>Hypnea wynnei</i>	Erect	Cylindrical	Uniaxial	Present	Zonate	Nauer <i>et al.</i> , 2019
<i>Iridaea cordata</i>	Erect	Flattened	Multiaxial	Present	Cruciate	Huisman, 2018
<i>Kappaphycus striatus</i>	Erect	Cylindrical	Multiaxial	Absent	Zonate	Huisman, 2018
<i>Leiomenia cribrosa</i>	Prostrate	Flattened	Multiaxial	-	Cruciate	Huisman, 2018
<i>Mastocarpus cristatus</i>	Erect	Flattened	Multiaxial	Present	Cruciate	Huisman, 2018
<i>Mastocarpus papillatus</i>	Erect	Flattened	Multiaxial	Present	Cruciate	Huisman, 2018
<i>Mastocarpus stellatus</i>	Erect	Flattened	Multiaxial	Present	Cruciate	Huisman, 2018
<i>Mazzaella leptorhynchus</i>	Erect	Flattened	Multiaxial	Present	Cruciate	Huisman, 2018
<i>Meredithia crenata</i>	Prostrate	Flattened	Multiaxial	-	Cruciate	Huisman, 2018
<i>Ochtodes crockeri</i>	Erect	Cylindrical	Multiaxial	Absent	-	Huisman, 2018
<i>Opuntia californica</i>	Erect	Flattened	Multiaxial	-	Zonate	DeCew <i>et al.</i> , 1992

<i>Phacelocarpus tortuosus</i>	Erect	-	Uniaxial	Present	Zonate	Huisman, 2018
<i>Ramicrusta fujiiana</i>	Prostrate	Flattened	Multiaxial	Absent	Cruciate	Pestana <i>et al.</i> , 2020
<i>Ramicrusta paradoxa</i>	Prostrate	Flattened	Multiaxial	Absent	Cruciate	Pestana <i>et al.</i> , 2020
<i>Rissoella verruculosa</i>	Erect	Flattened	Multiaxial	Present	Zonate	Huisman, 2018
<i>Sarcopeltis skottsbergii</i>	Erect	Flattened	Multiaxial	Present	Cruciate	Huisman, 2018
<i>Sarcothalia radula</i>	Erect	Flattened	Multiaxial	Present	Cruciate	Huisman, 2018

CAPÍTULO 3

Título: Challenges and prospects of available digital metadata for biogeographical assessments in Gigartinales (Rhodophyta)

Autores: Edilene Maria dos Santos Pestana, Goia de Mattos Lyra, Marcos de Castro Nunes, Daniel S. Park

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Challenges and prospects of available digital metadata for biogeographical assessments in Gigartinales (Rhodophyta)

Edilene Maria dos Santos Pestana^{1,2}, Goia de Mattos Lyra^{1,3}, José Marcos de Castro

Nunes^{1,2}, Daniel S. Park⁴

¹Programa de Pós-Graduação em Biodiversidade e Evolução (PPG BioEvo), Instituto de Biologia, Universidade Federal da Bahia, Rua Barão de Jeremoabo, s/n, Salvador, Bahia 40170-115, Brazil

²Laboratório de Algas Marinhas (LAMAR), Instituto de Biologia, Universidade Federal da Bahia, Rua Barão de Jeremoabo, s/n, Salvador, Bahia 40170-115, Brazil

³Departamento de Biologia Vegetal, Universidade do Estado do Rio de Janeiro, R. São Francisco Xavier, 524 - Maracanã, Rio de Janeiro - RJ, 20550-013, Brazil

⁴Department of Biological Sciences, Purdue University, 915 W State St, West Lafayette, Indiana 47907, EUA

Running title: Digital metadata for biogeographical analyses Gigartinales.

Author for correspondence: edimpestana@gmail.com, +55 (71) 3283-6598

Abstract

The order Gigartinales includes a large number of species with great ecological importance, serving as habitats and food for a vast diversity of marine life. Several species are also economically important, being widely used in food and cosmetics industries. However, we lack biogeographical knowledge of the clade, and spatial patterns of diversity have yet to be assessed across global scales. While digitized herbarium data is widely available, most scientific collections focus on land plants. This study applies phylogenetic diversity metrics to digital collections of Gigartinales to identify distribution patterns within the clade. We demonstrate the usefulness of GBIF data for biogeographical research on Gigartinales. Phylogenetic diversity metrics helped distinguish communities with distinct

phylogenetic structures, offering a deeper view of Gigartinales diversity beyond species count. Further investigation into macroalgae collections and digital metadata is essential to separate ecological effects from sampling biases in phylogenetic structure patterns.

Introduction

Gigartinales is one of the most diverse orders in Rhodophyta, currently including 952 species, distributed in 36 families, the most representative being Kallymeniaceae, Gigartinaceae and Phyllophoraceae (Guiry & Guiry, 2024). The order Gigartinales is monophyletic (Pestana *et al.*, 2024) and includes a large number of species with great ecological importance, serving as habitats and food for a vast diversity of marine life (Lee, 2008; Guiry & Guiry, 2024). In addition, some are economically important, being widely used in the food and cosmetics industries (Lee, 2008; Pereira *et al.*, 2015). Over the decades, taxonomy in Gigartinales has been largely based on morphology (Searles, 1968; Kraft & Robins, 1985; Fredericq & Hommersand, 1990), and, more recently, in molecular analyzes (Verbruggen *et al.*, 2010; Saunders *et al.*, 2016; Nauer *et al.*, 2019).

However, the knowledge on the distribution patterns of gigartinean species is still obscure. A small number of studies focus on family level data (Guiry and Gabary, 1990; Hommersand *et al.*, 1993;1994), and early information on the distribution of species of Gigartinales need to be taken with care, as they may include entities that are no longer classified in this order, such as species in the pantropical Gracilariaceae (Trono *et al.*, 1983; McLachlan & Bird, 1984, 1986), later elevated to the ordinal rank (Fredericq & Hommersand, 1989). Most of the research dedicated to understand biogeographical patterns in Gigartinales are focused on genera [e.g., *Hypnea* (Jesus *et al.*, 2015; 2019); *Chondracanthus* (Bulboa Contador *et al.*, 2020); *Caulacanthus* (Yang & Kim, 2023)] or species [e.g., *Chondrus crispus* (Hu *et al.*, 2010); *Dumontia contorta* (Hoek, 1982).

The state of the distribution of living beings is not random and it is possible to perceive patterns of occurrence resulting from events in the geological history of the earth, generally applied to broader taxa, such as phyla, orders and families (Nelson & Platnick, 1981; Humphries & Parenti, 1999; Crisci *et al.*, 2000; Morrone 2009) and as a result of ecological factors in a shorter time scale, such as temperature, salinity and resource availability, observed in genera and species (Nelson & Platnick, 1981; Cox & Moore, 1993; Morrone, 2009). Studies in biogeography strive to identify and describe these patterns, generating hypotheses and theories about the events that led to the observed distribution.

More commonly, it is possible to find studies involving land animals and plants with well-established hypotheses, well-defined distributions and even identifying the geographic barriers that led to speciation (Floeter *et al.*, 2009; Miranda & Marques, 2011).

From the limited studies available on biogeography of Rhodophyta, it is reasonable to conclude that most lineages of Rhodophyta originated in the Mesozoic and can be divided into two groups: i) those that evolved from the Sea of Thetys and ii) those that evolved along the outer perimeter of the Pangea (Hommersand, 1981). In Gigartinales there are families that fall into both categories. Families belonging to the Solieriaceae complex originated from the Sea of Thetys (Fredericq *et al.*, 1999) and members of Gigartinaceae and Kallymeniaceae fit the second hypothesis (Hommersand, 1981; Hommersand *et al.*, 1994). When it comes to ecological biogeography, the identification of geographic barriers that determine cladogenesis and distribution of groups is challenging. A pioneer in proposing a compartmentalization of marine biota, Dana (1852) used isoclines to establish his system and until today temperature is considered one of the main primary factors that regulate the distribution of species on a global scale, being common sense the existence of floras and faunas characteristic of polar, tropical and temperate regions. In addition to temperature, pressure, salinity and light may be important environmental variables limiting the distribution in both a horizontal and vertical gradient (Pielou, 1979).

Miranda & Marques (2011) pointed out that studies in marine biogeography are mostly ecological in nature and focused on characterizing patterns of geographic distribution and delimiting areas of endemism (e.g. Anderson *et al.*, 2009). Studies in land plants traditionally use metrics not so common in biogeography research in Phycology, such as phylogenetic diversity metrics. The total phylogenetic diversity in a community (PD), represents the total length of the phylogenetic branches that connect all the species present in the community, and is directly related to species richness (SR) (Faith, 1992). However, species richness is not the only determinant. Even with fewer species, if the species in a particular community are evolutionarily more distant from one another, the PD can still be relatively high (Horn, 1996). The metrics of phylogenetic dispersion, MPD and MNTD, are also related, with a stronger correlation between them, indicating an association between overall phylogenetic dispersion (MPD) and the distance between nearest neighbors (MNTD) (Swenson *et al.*, 2012; Erickson *et al.*, 2014). Kembel *et al.* (2010) observed that although the two metrics capture different aspects of phylogenetic structure, they are frequently correlated.

The raw values of phylogenetic diversity metrics provide a direct view of phylogenetic diversity in the studied areas. They are useful for absolute comparisons and can indicate how diverse a community is without considering randomness or sampling effort (Faith, 1992; Tucker *et al.*, 2017). Standardized metrics (SES) are essential for assessing whether the observed phylogenetic diversity is greater or smaller than expected randomly. Additionally, standardized metrics adjust for sampling and distribution biases, providing a more accurate view of the phylogenetic structure of communities (Swenson, 2006; Tucker *et al.*, 2017). These adjustments are critical for interpreting patterns in phylogenetic diversity and understanding the influence of ecological and evolutionary traits on community structure (Siefert *et al.*, 2015).

Given the challenging taxonomic history of Gigartinales, a comprehensive biogeographic study of the order is needed to better understand its evolutionary history. Digitized data from herbarium specimens are increasingly available (Canteiro *et al.*, 2019; Canhos *et al.*, 2022) and used in studies that demand large time and geographical coverages (Heberling, 2021), including the understudied tropics (Davis *et al.*, 2022; Park *et al.*, 2022). However, most of the knowledge on the usage of herbarium species for scientific research is based on collections of land plants (Daru *et al.*, 2017; Paton *et al.*, 2020). Here we apply phylogenetic diversity metrics to digital, publicly available collections of Gigartinales, to better understand general patterns of distribution in this clade.

Material and Methods

Species occurrence data and taxonomic standardization

Occurrence data of Gigartinales were collected from two public open-source databases: i) Algaebase (<http://www.algaebase.org>), a platform based on literature data, was queried and collection information was gathered at country level. ii) Global Biodiversity Information Facility (GBIF; <https://www.gbif.org/> accessed on april 2023) data was obtained using “Gigartinales” and its families as keywords (GBIF download link: <https://api.gbif.org/v1/occurrence/download/request/0105316-230224095556074.zip>).

We standardized the taxonomy of each species by checking for misspellings, synonyms, formatting errors, hybrid names and infraspecific ranks, against the backbone taxonomy from AlgaeBase [<http://www.algaebase.org> (Guiry & Guiry 2024)]. We filtered our GBIF dataset, excluding duplicate specimens, species with less than 10 unique records,

or occurrences lacking: i) coordinate information (or the 0° ones); ii) species name, iii) geographic location.

Correlation test

To investigate if data available on GBIF, which contains coordinates information for collection localities, would reflect the literature distributional status, based on specialists field studies, we compared both datasets. We standardized the occurrence information by “country”, available for both datasets. For occurrences that lacked country names, manual association was performed by matching the provided coordinates from GBIF with the appropriate country. This was done by transforming the occurrence data into a spatial object using the *sf* package (Pebesma, 2018), followed by performing a spatial join with the country shapefile. The shapefile of countries was obtained from the *rnatualearth* package (South, 2017).

Using R software version 4.1.3 (R Core Team, 2022), we assessed the normality assumption for the number of species per country using the Shapiro-Wilk test (Shapiro & Wilk, 1965). Afterwards, we ran the Pearson correlation test (Pearson, 1895) between the number of species per country in each source.

Phylogenetic diversity tests

To evaluate the phylogenetic diversity in marine environments, we analyzed the total phylogenetic diversity (PD), mean phylogenetic distance (MPD) and mean nearest taxon distance (MNTD) for species present within geographical grid cells. To ensure spatial consistency, we divided the global ocean surface into a grid consisting of polygons representing spatial cells based on latitude and longitude, using the R package *dggridR* (McCune & Keitt, 2017) with a spacing of 250. The cell variable was assigned to each occurrence based on the corresponding grid cell number. This standardized grid of polygons was used for further analysis, using also our global specimens dataset from GBIF.

A phylogeny including the species present in our specimens dataset was used to calculate phylogenetic distances. We built a dataset of *rbcL* sequences of Gigartinales' species available in GenBank (<https://www.ncbi.nlm.nih.gov/genbank>). The phylogeny was estimated using Maximum Likelihood analyses (ML), performed on CIPRES Science Gateway v.3.3 (<https://www.phylo.org/>). We used the tree inference and sequence alignment interface in XSEDE RAXML v.8.2.0 (Stamatakis 2014). The evolutionary model GTR+GAMMA was employed for ML phylogenetic analyses Minimal bootstrap supports

of 75% were required to define clades. The *rbcL* tree is presented in Figure S1. The tree was pruned to match the occurrence dataset, using the R package *ape* (Paradis *et al.* 2004). Before running the analyses, verified the correlation between species richness per country obtained from GBIF and the species richness per country represented in the pruned tree.

The phylogenetic diversity metrics were calculated using the R package *picante* (Kembel *et al.* 2010). Values were calculated for each country polygon cell using a species-by-site presence/absence matrix constructed from the GBIF occurrence data. To evaluate whether the raw metrics (PD, MPD and MNTD) were significantly different from a random expectation, we performed null model tests; species identities were randomized 999 times within each cell to generate a distribution of null values. The observed values were then compared to the null distribution, yielding standardized effect size (SES) (ses.PD, ses.MPD and ses.MNTD) values, which adjust metrics to take into consideration sampling and distribution biases. Cells with positive SES values indicate greater phylogenetic diversity than expected by chance, while negative values suggest clustering of related species. Pearson correlation tests between the phylogenetic diversity metrics were conducted in R using the *stats* package (R Core Team, 2024). Standardized effect size values of MPD were plotted into a map to visualize its spatial distribution using the R package *ggplot2* (Wickham, 2016).

Results

Algaebase checklist contained 959 species distributed in 135 countries. The GBIF dataset contained a total of 134,253 occurrence records after filtering and taxonomic standardization, and the checklist included 579 valid species distributed across 185 countries. The final list used in the correlation test was based on the species richness values found in the countries that had data available in both databases, comprising a total of 121 countries.

Our results demonstrated a significant positive correlation ($r = 0.92$, $p < 0.001$) between the data available from the literature (AlgaeBase) and data from specimens (GBIF), regarding species richness per country (Figure 1).

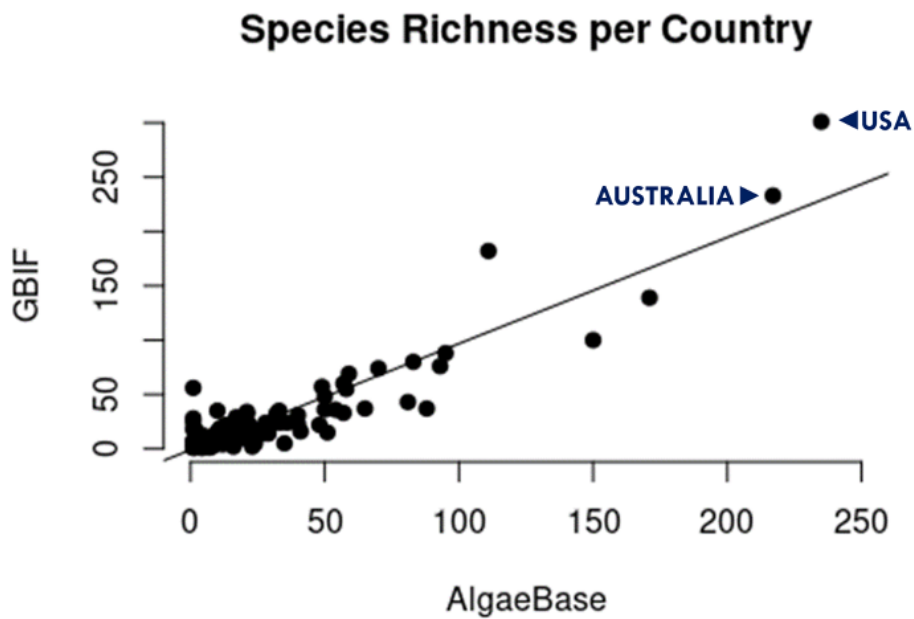


Figure 1. Correlation between species richness per country from Algaebase and GBIF. Each point represents a country.

Richness per country

The USA (Algaebase=235; GBIF=301) and Australia (Algaebase=217; GBIF=233) showed a larger number of species richness in comparison to other countries, followed by Mexico, Japan and Canada in both datasets, although their rankings varied between the two sources. We mapped the species richness distribution across the globe and identified some richness hotspots, such as the pacific coast of the USA, Southern Australia and Europe (Figure 2).

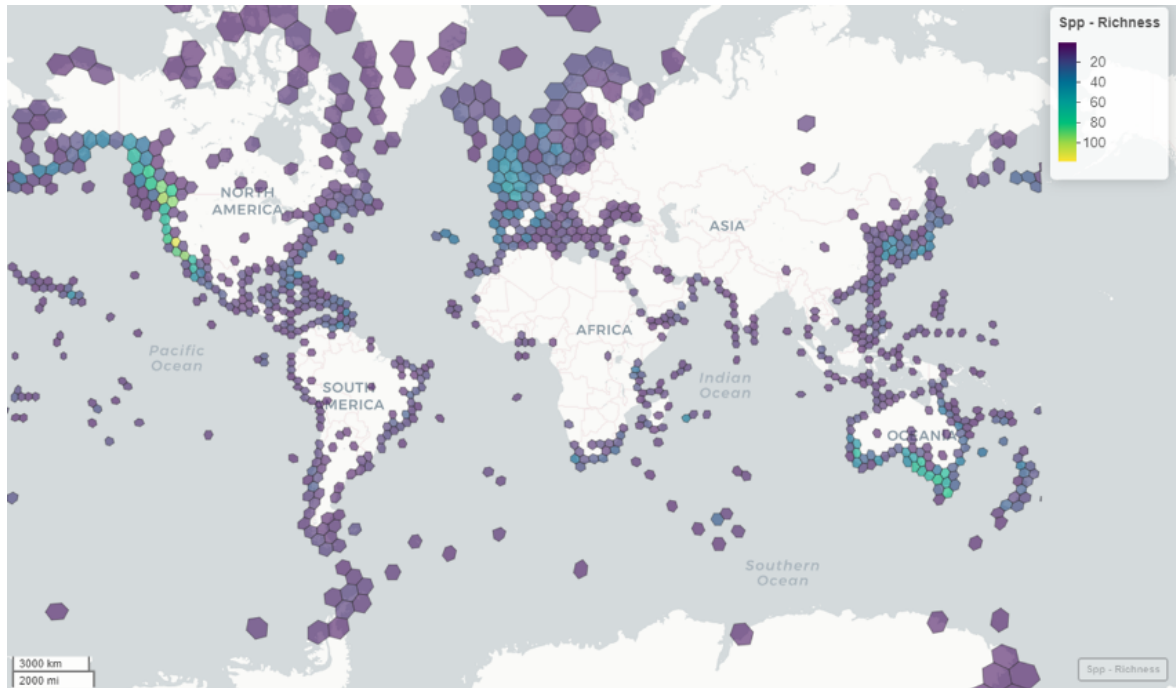


Figure 2. Gigartinales richness distribution across the globe, based on specimens' data (GBIF)

Phylogenetic diversity per country

The Pearson correlation between species richness from GBIF data and the species richness represented in the pruned tree used to conduct the phylogenetic diversity tests is very high ($r = 0.99$, $p < 0.001$).

Species richness (SR) and PD are strongly positively correlated (0.96). MPD and MNTD show a moderate positive correlation (0.50) which suggests that communities with greater overall phylogenetic diversity also exhibit a tendency for their species to be more distantly related to their nearest neighbors. MPD and ses.MPD have a strong correlation (0.70), which means that countries with a high MPD tend to have species that are more phylogenetically spread out than a null model would predict. SR and PD have negative correlations with MNTD and some of the standardized effect size metrics, which suggests that, as species richness and total phylogenetic diversity increase in a region, the phylogenetic relationships among species become closer. There was no correlation between ses.MPD and SR (0.0071) and PD (0.028), which suggests that phylogenetic structure, as indicated by ses.MPD, does not directly depend on species richness or on the total phylogenetic diversity within the countries represented in this study. Phylogenetic diversity metrics correlation is shown in Figure 3.

In our dataset, the variation in used metrics were: i) PD, from 0.21 (in Romenia) to 7.75 (in Australia); ii) MPD, from 0.082 (in Kuwait) to 0.40 (in Hong Kong); iii) MNTD, from 0.050 (in Canada) to 0.37 (in Malta). Most countries showed negative values of standartized metrics, indicating that, in these regions, species are more phylogenetically clustered than expected by chance. Hong Kong showed the highest ses.PD value (2.45) and Canada showed the lowest (-7.7). Standardized effect size of MPD values ranged from -6.55 (in Eritrea) to 3.20 (in Japan), and three countries showed values above 2: Japan, China (3.06) and Korea (2.37) (Fig.4). The overseas territory of France, French Southern and Antarctic Lands showed the higher value of ses.MNTD (1.44), while Canada had the lowest value (-6.95). Phylogenetic diversity metrics results are shown in Table S1.

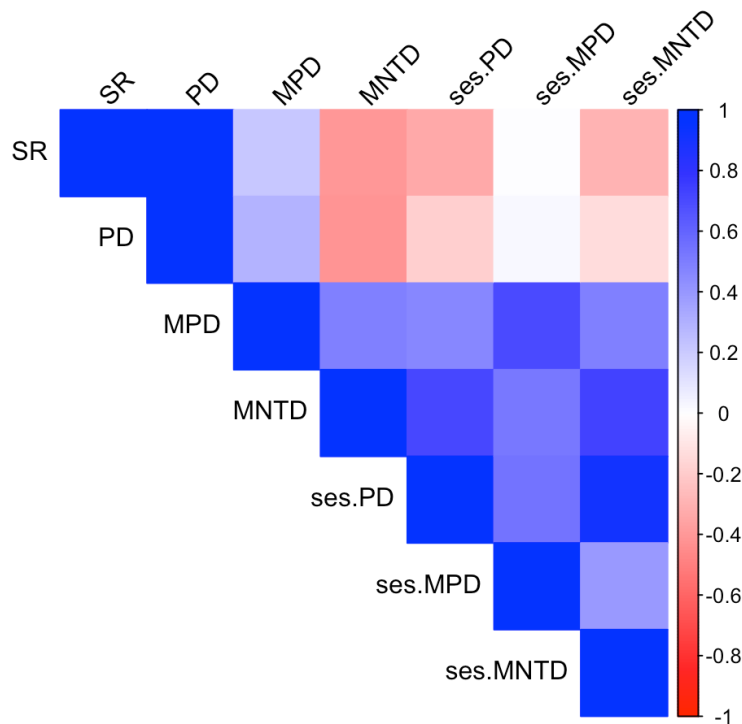


Figure 3. Correlogram between phylogenetic diversity metrics. SR=Species richness; PD=Total phylogenetic diversity; MPD=Mean phylogenetic distance; MNTD=Mean nearest taxon distance. ses.PD, ses.MPD and ses.MNTD=Standardized effect size of each metric. Blue represents positive correlations and red represents negative correlations. Color intensity indicates the degree of correlation between metrics.

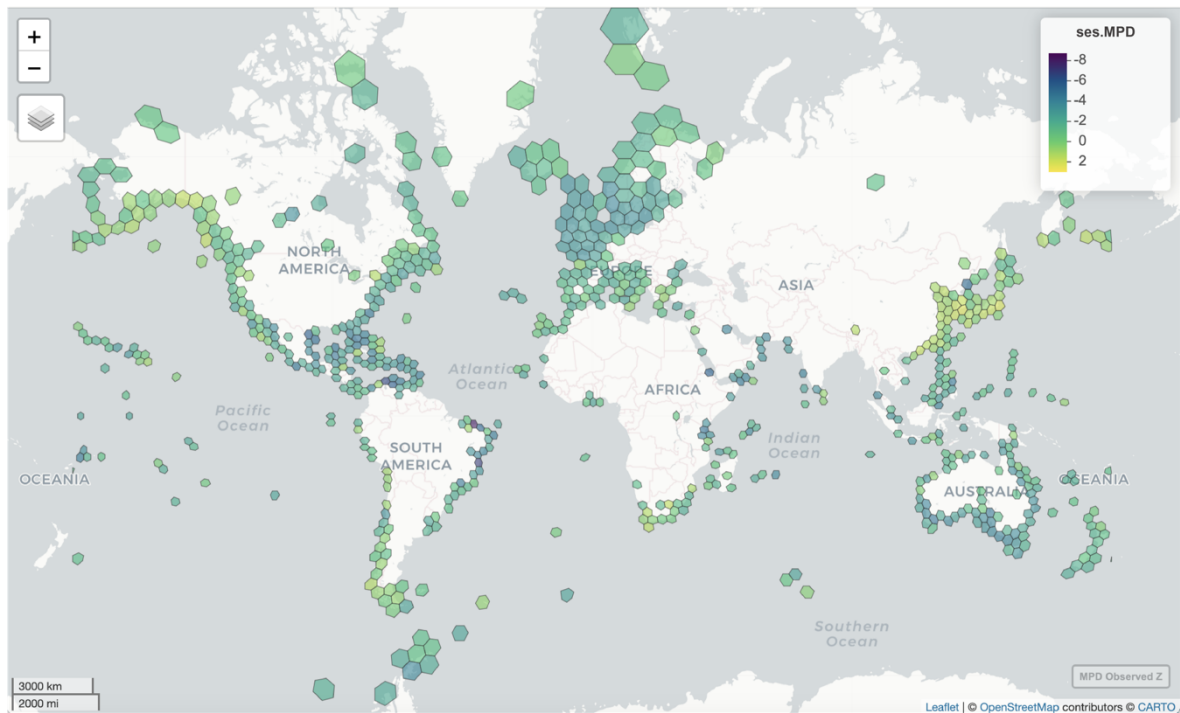


Figure 4. Gigartinales ses.MPD across the globe, based on specimens' data (GBIF).

Discussion

GBIF: a useful resource for biogeographical studies in Gigartinales

Data from GBIF reflects biogeographical information available in Algaebase, which is important as data on species' distribution from Algaebase, while generated by specialists in each group, are not standardized. For some taxa, distribution information is available per country, while for some others it can be presented by continent, or ocean, for example. Therefore, using distribution data from Algaebase is challenging and lacks specificity. Our results provide the phycological community with robust grounds for using GBIF data on biogeographical research on Gigartinales. As GBIF data is not exclusively generated by specialists (data from several Citizen Science initiatives are available), we encourage other researchers to apply such tests to broader algal datasets.

As we compiled GBIF and Algaebase information by country, it is important to consider that the term "country" in our analyses includes both independent nations and territories associated with other nations as separate entities. This approach is advantageous as it reflects the geographic location being referenced (e.g., mainland France vs. French overseas territories; U.S. mainland vs. Puerto Rico), which provides more precise ecological context. Thus, even using "country" as a proxy for locality, rather than precise coordinates,

offers a certain level of geographic resolution. However, large countries, such as the United States or Russia, which span diverse biogeographic regions and are bordered by more than one ocean, may still require additional spatial filtering to capture finer-scale patterns, particularly when examining large and ecologically varied territories.

Our results revealed a strong positive correlation between GBIF occurrences and GenBank *rbcL* sampling, which suggests that GenBank sampling may, to some extent, reflect the occurrence data of Gigartinales for that gene. However, the correspondence of species is not perfect, which can, in some cases, significantly reduce the representation of species in a phylogenetic tree for the studied region. For instance, while there are 139 species recorded for Mexico in GBIF, only 79 species have available *rbcL* sequences.

Species richness x Phylogenetic diversity in Gigartinales

Our results indicate that raw phylogenetic diversity metrics for Gigartinales are consistent with findings observed in other groups for which these tests are widely applied. Several studies demonstrate a strong positive correlation between species richness (SR) and phylogenetic diversity (PD). In our dataset, the United States, Australia and Canada display the highest SR among the analyzed countries, with values of 167, 137 and 119 species, respectively. This high SR is positively correlated with PD, for which these countries also present the highest values (7.60, 7.76 and 5.21). Our data also evidenced that PD does not always scale directly with SR. Australia, despite having fewer species than the United States, has a slightly higher PD, suggesting that evolutionary relatedness among species plays a significant role in shaping PD.

Nevertheless, PD values do not necessarily reflect a community's actual evolutionary diversity or functional uniqueness (Forest *et al.*, 2007). Mean pairwise distance (MPD) and mean nearest taxon distance (MNTD) provide valuable complementary metrics by focusing on the average evolutionary distances among species pairs and nearest neighbors, respectively (Webb *et al.*, 2002). The United States and Australia present a lower MNTD, 0.058 and 0.070, respectively, compared to other countries, indicating closer evolutionary relationships among its species. Standardized metrics, such as ses.MPD, further show that both Australia (-3,41) and Canada (-2,89) exhibit strong phylogenetic clustering, whereas the United States' value near zero (0.23) suggests a more random phylogenetic structure, highlighting the complexity of diversity patterns and the value of multiple metrics in ecological studies (Webb *et al.*, 2002; Mazel *et al.*, 2014).

In contrast, Japan, China and South Korea, with 58, 16 and 40 species respectively, have higher MPD and MNTD in comparison with the USA, Australia and Canada, despite their lower SR. Standardized metrics further refine these observations. Japan, China and Korea's ses.MPD are positive, 3.20, 3.05 and 2.36, respectively, suggesting a more random phylogenetic structure than expected by chance. These results reinforce the importance of using both raw and standardized metrics to capture the complexities of phylogenetic diversity, emphasizing how evolutionary relatedness and species richness interact in shaping community structures (Webb *et al.*, 2002; Mazel *et al.*, 2014).

Locations with lower SR, such as Hong Kong (3 species), Malta (2 species), and the Turks and Caicos Islands (3 species), exhibit high MPD and MNTD values, suggesting a high degree of evolutionary distinctiveness among their species. The standardized values for these locations are all positive, meaning that the observed species are more evolutionarily distinct from each other than would be expected by chance. Such a pattern can indicate that these species may be occupying distinct ecological niches, potentially reducing competition among them, or that they are remnants of lineages that have persisted in isolation (Park *et al.*, 2018). However, Hong Kong, Malta, the Turks and Caicos Islands, and other regions with lower SR present analytical challenges due to potential sampling biases that can lead to inflated measures of phylogenetic diversity. Park *et al.* (2018) emphasize that biases in taxon sampling, where certain clades may be disproportionately represented, can impact metrics like PD and MPD, especially when small community samples include unique evolutionary lineages. These conditions can inflate phylogenetic diversity measures, making low-SR regions appear more diverse than they truly are. Additionally, Rock & Daru (2021) discuss how geographic and taxon sampling biases may elevate estimates of phylogenetic diversity in areas where relictual or distinct taxa are preferentially sampled over more common, closely related species. The positive standardized values across PD, MPD, and MNTD observed here suggest a potential overrepresentation of evolutionarily distinct species, indicating that sampling bias could be contributing to the high phylogenetic diversity in these low-SR countries. This emphasizes the need for balanced sampling approaches to accurately interpret phylogenetic diversity patterns in regions with limited species.

Most countries showed negative values of standardized metrics, indicating that, in these regions, species are more phylogenetically clustered than expected by chance. These patterns of phylogenetic clustering in many countries with different SR and environmental conditions, suggest that Gigartinales species within regions tend to be closely related. This could reflect natural evolutionary processes. In marine environments, such processes can

result from factors like environmental gradients, habitat stability, and geographical barriers, which promote the evolution of closely related species within specific regions (Johansson *et al.*, 2015; Hays *et al.*, 2021). Additionally, geographical barriers, such as ocean currents or isolated marine refugia, can limit gene flow and contribute to the phylogenetic clustering of species over time (Hays *et al.*, 2021). However, the possibility of sampling biases also needs to be carefully considered, as certain regions or taxonomic groups may be disproportionately represented due to the research interests or logistical constraints of the researchers conducting the surveys. For example, in countries like Brazil, high species richness of certain genera, such as *Hypnea*, could stem from concentrated efforts in studying specific groups (Jesus *et al.*, 2015, 2018; Nauer *et al.*, 2019), thus inflating the apparent phylogenetic proximity within the region. Such sampling biases can distort the true picture of biogeographical patterns, making it essential to account for them in future studies. Understanding the extent to which these biases influence the results is critical, as it helps refine interpretations and enhances the reliability of conclusions regarding the relationship between species and their environments.

Conclusion

Our study demonstrates the usefulness of GBIF data for biogeographical research on Gigartinales. Phylogenetic diversity metrics applied to our Gigartinales' dataset helped distinguish communities that, despite SR, differ in their phylogenetic structure, offering a more nuanced view of diversity beyond species count alone. Our results were consistent with those found in the literature for other groups, such as land plants, which demonstrates the viability of using these metrics for algae, specifically Gigartinales. Further investigations into macroalgae herbaria collections and available digital metadata are needed to fully disentangle the contributions of ecological processes versus sampling biases to observed patterns of phylogenetic structure.

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Supplementary material

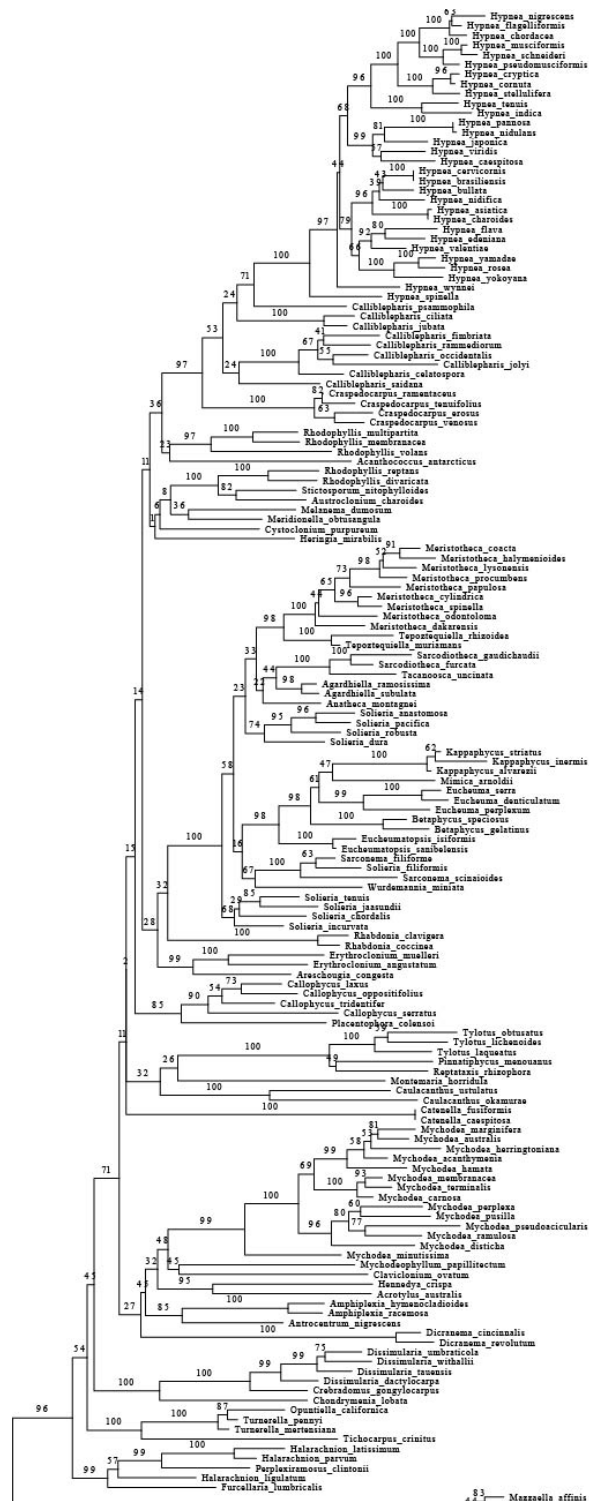


Figure S1. Complete *rbcL* phylogeny used. Numbers at nodes indicate bootstrap replicates. Bootstrap support values are shown above 75%.

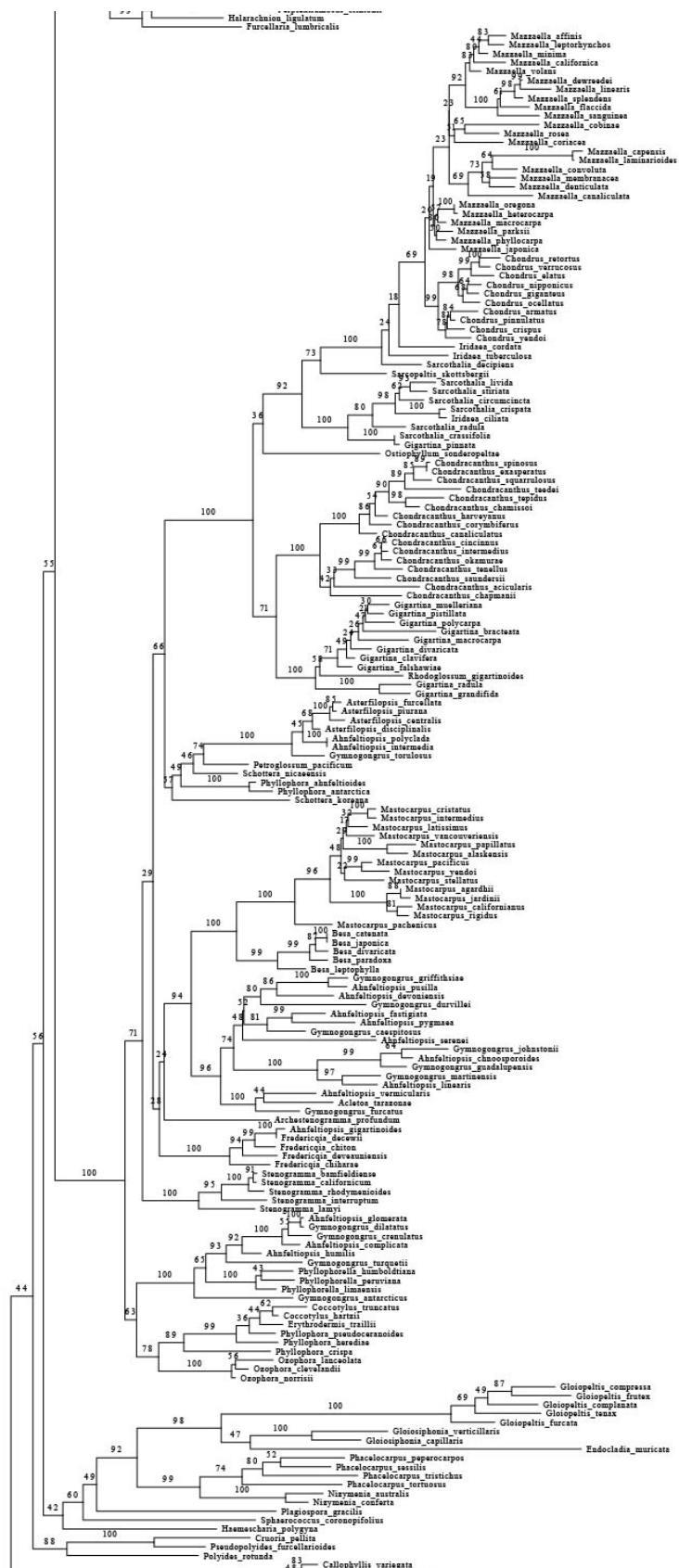


Figure S1. (Continuation) Complete *rbcL* phylogeny used. Numbers at nodes indicate bootstrap replicates. Bootstrap support values are shown above 75%.

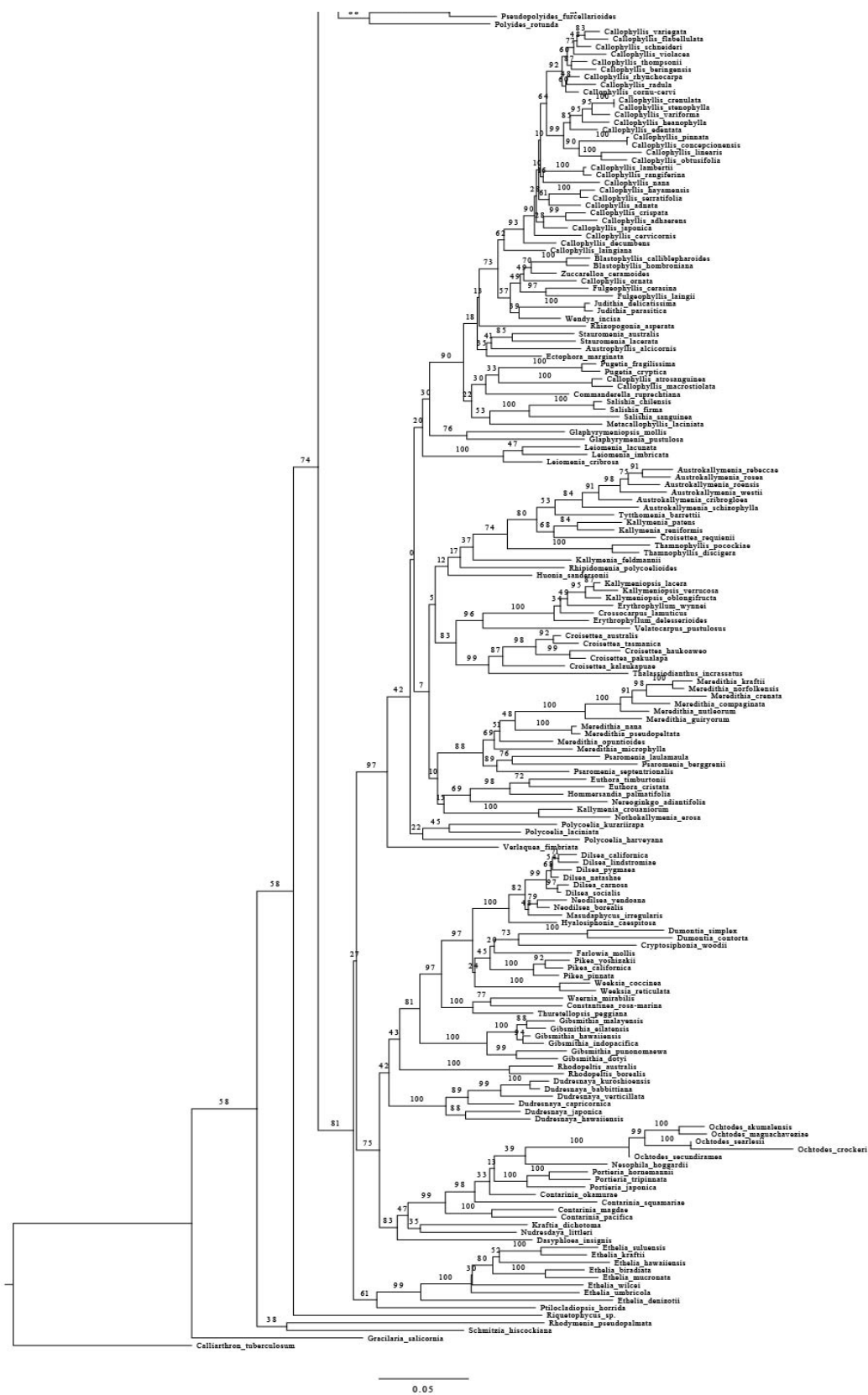


Figure S1. (Continuation) Complete *rbcL* phylogeny used. Numbers at nodes indicate bootstrap replicates. Bootstrap support values are shown above 75%.

Table S1. Phylogenetic diversity metrics results. SR=Species richness; PD=Total phylogenetic diversity; MPD=Mean phylogenetic distance; MNTD=Mean nearest taxon distance. ses.PD, ses.MPD and ses.MNTD=Standardized effect size of each metric.

Country	SR	PD	MPD	MNTD	ses.PD	ses.MPD	ses.MNTD
United States of America	167	7,599	0,305	0,058	-5,955	0,230	-4,230
Australia	137	7,758	0,291	0,070	-0,810	-3,411	-1,738
Canada	119	5,216	0,291	0,050	-7,835	-2,898	-6,956
Mexico	79	4,245	0,312	0,064	-4,940	1,374	-4,429
Japan	58	3,808	0,326	0,079	-2,595	3,197	-2,987
United Kingdom	47	3,715	0,277	0,110	-0,210	-3,525	-0,028
France	46	3,709	0,286	0,113	0,040	-2,335	0,176
South Africa	45	3,715	0,310	0,108	0,311	0,815	-0,343
New Zealand	41	3,197	0,296	0,102	-1,099	-0,931	-1,213
South Korea	40	2,559	0,326	0,071	-4,065	2,369	-4,270
Spain	39	3,226	0,287	0,118	-0,353	-1,986	0,047
Portugal	36	3,174	0,286	0,128	0,355	-1,978	0,733
Ireland	33	2,870	0,270	0,123	-0,286	-3,561	-0,002
Jersey	29	2,554	0,268	0,126	-0,650	-3,563	-0,146
Brazil	29	1,880	0,242	0,082	-4,558	-5,545	-3,303

Chile	27	2,023	0,295	0,086	-3,011	-0,749	-3,172
Isle of Man	27	2,534	0,272	0,138	0,010	-2,949	0,571
Norway	27	2,422	0,262	0,130	-0,644	-3,702	-0,047
Philippines	26	1,752	0,236	0,086	-4,434	-6,108	-3,229
Russia	24	1,812	0,282	0,086	-3,118	-1,766	-3,342
Guernsey	24	2,219	0,270	0,131	-0,652	-2,891	-0,307
Switzerland	24	2,191	0,268	0,129	-0,864	-2,998	-0,397
Tanzania	24	1,655	0,242	0,088	-4,110	-5,198	-3,234
Italy	23	2,192	0,271	0,136	-0,435	-2,767	-0,098
Venezuela	22	1,852	0,277	0,113	-2,143	-2,073	-1,678
French Southern and Antarctic Lands	21	2,258	0,307	0,166	0,970	0,235	1,438
Mauritius	21	1,819	0,276	0,120	-2,033	-2,182	-1,277
Bermuda	20	1,954	0,307	0,133	-0,590	0,215	-0,562
Taiwan	20	1,805	0,293	0,116	-1,530	-0,879	-1,592
Netherlands	20	1,943	0,273	0,132	-0,688	-2,332	-0,711
Indonesia	20	1,494	0,241	0,097	-3,646	-4,711	-2,650
Denmark	19	1,763	0,255	0,135	-1,425	-3,451	-0,595
Madagascar	18	1,529	0,278	0,113	-2,576	-1,851	-1,905
Oman	18	1,425	0,263	0,098	-3,203	-2,743	-2,767

Puerto Rico	18	1,488	0,263	0,105	-2,818	-2,807	-2,324
Norfolk Island	17	1,582	0,298	0,107	-1,671	-0,440	-2,277
Kenya	17	1,379	0,244	0,106	-3,182	-4,176	-2,290
China	16	1,743	0,352	0,118	0,006	3,060	-1,764
Guadeloupe	15	1,378	0,267	0,129	-2,130	-2,216	-1,266
Germany	15	1,494	0,246	0,146	-1,209	-3,631	-0,499
Morocco	14	1,488	0,287	0,151	-0,726	-1,043	-0,403
Panama	14	1,294	0,276	0,121	-2,219	-1,632	-1,756
Ecuador	14	1,183	0,264	0,097	-2,997	-2,512	-2,805
Reunion Island	14	1,255	0,262	0,113	-2,526	-2,659	-2,057
Faroe Islands	14	1,304	0,244	0,133	-2,181	-3,565	-1,220
French Polynesia	13	1,335	0,277	0,147	-1,238	-1,570	-0,730
Iceland	13	1,363	0,256	0,155	-1,060	-2,707	-0,415
Cuba	12	1,158	0,285	0,117	-2,174	-1,013	-2,058
Seychelles	12	1,066	0,236	0,130	-2,837	-3,597	-1,564
Dominican Republic	12	0,984	0,227	0,105	-3,597	-4,143	-2,631
Wallis Futuna	12	1,022	0,217	0,122	-3,252	-4,764	-1,931
Jamaica	12	0,942	0,211	0,098	-3,916	-5,047	-2,893
Yemen	12	0,946	0,209	0,105	-3,787	-5,114	-2,627

Argentina	11	1,245	0,304	0,149	-0,667	0,040	-0,923
Greece	11	1,273	0,290	0,169	-0,459	-0,713	-0,180
Antarctica	11	1,081	0,279	0,134	-2,078	-1,235	-1,511
Martinique	11	1,192	0,276	0,159	-1,178	-1,386	-0,534
New Caledonia	11	0,975	0,262	0,103	-3,023	-2,111	-2,795
Haiti	11	1,003	0,262	0,106	-2,839	-2,216	-2,675
Papua New Guinea	11	1,023	0,259	0,117	-2,564	-2,271	-2,274
Costa Rica	11	1,083	0,250	0,141	-2,096	-2,782	-1,271
India	11	1,093	0,247	0,141	-2,034	-2,795	-1,270
Iran	11	0,947	0,220	0,116	-3,339	-4,370	-2,251
Mozambique	10	1,238	0,314	0,168	0,059	0,501	-0,337
Croatia	10	1,285	0,306	0,200	0,458	0,127	0,835
Peru	10	1,097	0,294	0,116	-1,265	-0,425	-2,364
US Virgin Islands	10	1,128	0,290	0,164	-0,980	-0,695	-0,528
Trinidad and Tobago	10	1,204	0,289	0,172	-0,319	-0,706	-0,251
Bahamas	10	1,027	0,259	0,136	-1,867	-2,152	-1,556
Algeria	9	1,015	0,259	0,168	-1,194	-2,035	-0,539
Tunisia	9	0,887	0,255	0,116	-2,377	-2,351	-2,347
Saint Vincent and the Grenadines	9	0,865	0,238	0,127	-2,762	-3,046	-1,985

Singapore	9	0,753	0,235	0,084	-3,687	-3,141	-3,500
Barbados	9	0,739	0,209	0,094	-3,968	-4,241	-3,136
Colombia	8	1,009	0,307	0,176	-0,398	0,143	-0,477
Malaysia	8	0,974	0,302	0,154	-0,686	-0,073	-1,231
Cape Verde	8	0,941	0,295	0,153	-1,112	-0,341	-1,192
Falkland Islands	8	0,858	0,294	0,126	-1,972	-0,359	-2,103
Fiji	8	0,941	0,289	0,162	-0,990	-0,658	-0,935
Palau	8	0,889	0,277	0,148	-1,575	-1,142	-1,417
Federated States of Micronesia	8	0,762	0,257	0,094	-2,753	-1,897	-3,152
Belize	8	0,833	0,244	0,138	-2,145	-2,573	-1,662
United States Minor Outlying Islands	7	0,708	0,226	0,112	-2,481	-2,866	-2,617
Ghana	7	0,695	0,218	0,116	-2,719	-3,266	-2,543
Eritrea	7	0,512	0,127	0,071	-4,575	-6,554	-3,941
Guam	6	0,662	0,270	0,131	-1,997	-1,158	-2,110
Svalbard and Jan Mayen	6	0,681	0,262	0,139	-1,884	-1,438	-1,895
Mauritania	6	0,754	0,258	0,177	-1,032	-1,559	-0,950
Honduras	6	0,665	0,240	0,143	-1,943	-2,113	-1,801
Greenland	6	0,664	0,241	0,158	-2,036	-2,176	-1,360
Uruguay	6	0,672	0,232	0,136	-1,859	-2,490	-1,974

Mayotte	6	0,616	0,214	0,142	-2,445	-3,066	-1,892
Saudi Arabia	6	0,587	0,209	0,132	-2,866	-3,304	-2,109
Dominica	5	0,732	0,307	0,219	-0,076	0,100	-0,057
Sri Lanka	5	0,754	0,305	0,221	0,198	0,038	-0,073
Northern Mariana Islands	5	0,676	0,274	0,198	-0,674	-0,889	-0,619
Thailand	5	0,652	0,258	0,172	-1,002	-1,308	-1,287
Curacao	5	0,617	0,252	0,167	-1,476	-1,587	-1,265
Saint Maartin	5	0,540	0,211	0,131	-2,486	-2,883	-2,154
Pakistan	5	0,514	0,187	0,137	-2,741	-3,521	-2,166
Antigua and Barbuda	5	0,496	0,179	0,096	-2,891	-3,858	-3,134
Belgium	4	0,633	0,316	0,202	0,084	0,313	-0,692
Saint Christopher and Nevis	4	0,582	0,287	0,198	-0,563	-0,428	-0,830
American Samoa	4	0,570	0,285	0,198	-0,645	-0,474	-0,863
Saint Helena	4	0,562	0,279	0,168	-0,808	-0,624	-1,492
Cocos Islands	4	0,518	0,235	0,194	-1,339	-1,671	-0,884
Bulgaria	4	0,491	0,234	0,191	-1,814	-1,765	-0,974
Vietnam	4	0,499	0,218	0,110	-1,614	-2,087	-2,643
Maldives	4	0,455	0,214	0,134	-2,128	-2,250	-2,247
Egypt	4	0,447	0,211	0,133	-2,285	-2,310	-2,279

Somalia	4	0,537	0,210	0,159	-1,157	-2,421	-1,648
Finland	4	0,426	0,206	0,121	-2,534	-2,543	-2,436
Kuwait	4	0,365	0,083	0,061	-3,241	-5,660	-3,750
Hong Kong	3	0,661	0,407	0,344	2,453	1,964	1,297
Turks and Caicos	3	0,591	0,371	0,306	1,340	1,278	0,699
Saint Lucia	3	0,479	0,296	0,195	-0,290	-0,136	-1,092
Türkiye	3	0,490	0,293	0,258	-0,168	-0,175	-0,102
Democratic Republic of the Congo	3	0,477	0,295	0,248	-0,340	-0,190	-0,236
Slovenia	3	0,475	0,294	0,259	-0,410	-0,220	-0,101
Saint Pierre and Miquelon	3	0,481	0,283	0,269	-0,300	-0,430	0,097
Estonia	3	0,366	0,214	0,203	-2,010	-1,729	-0,981
Saint Barthélemy	3	0,406	0,192	0,139	-1,363	-2,214	-2,083
El Salvador	3	0,401	0,189	0,139	-1,414	-2,226	-2,090
Grenade	3	0,408	0,194	0,140	-1,373	-2,238	-2,049
Malta	2	0,407	0,373	0,373	0,796	0,864	0,885
Heard Island and McDonald Islands	2	0,421	0,364	0,364	0,951	0,793	0,771
Tonga	2	0,397	0,340	0,340	0,536	0,450	0,429
Israel	2	0,374	0,256	0,256	0,178	-0,593	-0,554
Aruba	2	0,353	0,235	0,235	-0,177	-0,868	-0,867

Montenegro	2	0,276	0,225	0,225	-1,525	-0,898	-0,994
Ukraine	2	0,230	0,108	0,108	-2,350	-2,369	-2,455
Guinea-Bissau	2	0,305	0,108	0,108	-1,026	-2,380	-2,432
Cayman Islands	2	0,305	0,095	0,095	-1,112	-2,593	-2,634
Romania	2	0,214	0,091	0,091	-2,705	-2,607	-2,794

Considerações finais

As filogenias robustamente suportadas geradas por esse estudo resolve dois problemas antigos na árvore da vida das algas vermelhas: o monofiletismo de Gigartinales e o posicionamento de Peyssonneliaceae. Noventa e dois genomas organelares foram sequenciados, aumentando significativamente os dados genômicos de Gigartinales disponíveis. Foi possível caracterizar e comparar esses genomas ao longo da ordem e apontar eventos que caracterizam famílias e gêneros. A caracterização morfológica pode se mostrar um desafio na classificação de grupos de algas quando as características usadas para os separar se tornam ambíguas e inconsistentes entre espécies e gêneros relacionados, perdendo relevância à medida que os critérios taxonômicos evoluem. Nossos resultados confirmam a ambiguidade desses caracteres e ressaltam a ausência de evidências morfológicas únicas e não sobrepostas para a evolução independente de linhagens, o que desafia a definição de gêneros ou famílias em Gigartinales. Os dados genômicos fornecem evidências para uma delimitação mais precisa dos táxons. Assim, como demonstrado em nosso estudo, embora seja possível realizar uma delimitação taxonômica sólida com os dados atuais, ainda há muito trabalho a ser feito para fornecer à comunidade científica os caracteres necessários para o reconhecimento adequado desses táxons. Além disso, nossos resultados destacam a importância de uma amostragem genética abrangente e o uso de filogenias bem amostradas para esclarecer as relações entre as principais linhagens de algas vermelhas. Recomendamos a inclusão de sequenciamento de regiões nucleares e a realização de filogenias datadas para melhorar a compreensão das relações internas entre clados, incluindo o sequenciamento de espécies-tipo, que, embora desafiador, é essencial para apoiar decisões taxonômicas robustas em algas vermelhas. Esse estudo também demonstra o valor dos dados do GBIF para a pesquisa de biogeografia em Gigartinales. As métricas de diversidade filogenética aplicadas ao nosso conjunto de dados permitiram distinguir comunidades que, apesar da similaridade na riqueza de espécies, apresentam diferenças significativas na estrutura filogenética, oferecendo uma visão mais refinada da diversidade além da simples contagem de espécies. Esses resultados corroboram achados similares em outros grupos, como plantas terrestres, e demonstram a aplicabilidade dessas métricas para algas vermelhas. Investigações adicionais em coleções de herbários de macroalgas e o uso de metadados digitais são fundamentais para esclarecer as contribuições de processos ecológicos e vieses de amostragem nos padrões observados de estrutura filogenética.



ATA DA SESSÃO PÚBLICA DO COLEGIADO DO PROGRAMA DE PÓS-GRADUAÇÃO EM BIODIVERSIDADE E EVOLUÇÃO - INSTITUTO DE BIOLOGIA - UFBA

**Título de Tese: Estudos filogenômicos e morfológicos integrados em Gigartinales:
resolvendo o clado mais desafiador em Rhodophyta**

Doutoranda: Edilene Maria dos Santos Pestana

Orientador: José Marcos de Castro Nunes

Coorientadora: Goia de Mattos Lyra

De acordo com o regimento geral da UFBA e com o regimento interno deste programa de pós-graduação, foram iniciados os trabalhos da Comissão Examinadora, composta pelos professores **Dr. José Marcos de Castro Nunes** (Presidente), **Dr. Carlos Frederico Deluqui Gurgel**, **Dra. Mutue Toyota Fujii**, **Dr. Joel Campos de Paula** e **Dra. Daniela Milstein**, às **08:00** horas do dia **29 de novembro de 2024**. A doutoranda fez a apresentação oral de sua tese durante 54 minutos. Após o encerramento das arguições, às 12:45h horas, a Comissão Examinadora pronunciou-se pela sua aprovação, conforme parecer em anexo. Esta Ata será assinada pelos membros da Comissão Examinadora e deste Colegiado de curso, para compor o processo de emissão do diploma.

Salvador, 29 de novembro de 2024.

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**Título de Tese: Estudos filogenômicos e morfológicos integrados em Gigartinales:
resolvendo o clado mais desafiador em Rhodophyta**

Doutoranda: **Edilene Maria dos Santos Pestana**

Orientador: **José Marcos de Castro Nunes**

Coorientadora: **Goia de Mattos Lyra**

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PARECER DA BANCA

A Comissão Examinadora considera o trabalho de conclusão de curso:

☒ Aprovado

☐ Reprovado

Os itens abaixo deverão ser modificados conforme sugestão da banca:

☐ Introdução

☐ Revisão Bibliográfica

☐ Metodologia

☐ Resultados Obtidos

☐ Conclusões

Para assinatura do orientador **APENAS APÓS A EFETUAÇÃO DAS MODIFICAÇÕES SUGERIDAS**

Declaro, para fins de homologação, que as modificações, sugeridas pela banca examinadora, acima mencionada, foram cumpridas integralmente.

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Orientador