Nuno Tavares Martins

Phylogeography and diversity of the genus *Colpomenia* (Ectocarpales, Phaeophyceae) in Brazil and Australia

Filogeografia e diversidade do gênero *Colpomenia* (Ectocarpales, Phaeophyceae) no Brasil e Austrália

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Passarinhos
Soltos a voar dispostos
Achar um ninho
Nem que seja no peito um do outro

de Oliveira & Levy

To be a scientist is to be naive. We are so focused on our search for truth, we fail to consider how few actually want us to find it. But it is always there, whether we see it or not, whether we choose to or not. The truth doesn't care about our needs or wants. It doesn't care about our governments, our ideologies, our religions.

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“O pôr do sol é a prova de que não importa o que aconteça,
cada dia pode terminar lindo.”
Autor desconhecido

À minha mãe Sandra Regina de Oliveira Tavares

In memoriam

Summary

Sumário

Abstract

Colpomenia (Endlicher) Derbès & Solier is a marine brown macroalgal genus with a cosmopolitan distribution, characterized by an anatomically simple, hollow sacciform, and vesicular thallus with smooth to rough appearance often morphologically plastic, and frequently lack reproductive structures. The objectives were to perform phylogeographic and phylogenetic studies in species from Brazil and Australia. *Colpomenia cox*3 DNA sequences identified the presence of *C. sinuosa* complex, containing the true *C. sinuosa*, plus four cryptic lineages in Brazil. *Cox*1 sequences from Australia identified three occurring species: *C. sinuosa, C. claytoniae* and *C. peregrina*. Relatively high intraspecific divergence was identified within *C. claytoniae* and *C. peregrina,* which might correspond to cryptic species. In addition, *cox*1 and *rbc*L-S sequences from Australia documented for the first time the presence of *Mikrosyphar zosterae* in the southern hemisphere and as an endophyte in *Colpomenia* spp. and *Leathesia marina*. A review of phylogeographic studies in Brazil revealed the split of the South Equatorial Current in two at Cape São Roque (4º S) as the most concordant phylogeographic pattern promoting genetic diversification along the Brazilian coast. For *C. sinuosa* population in Brazil, however, the Vitória-Trindade seamount chain represents the region where the largest shift in genetic discontinuity was observed. Along the Australian temperate coast, phylogeographic analyses evidenced that *C. sinuosa* is highly influenced and connected by oceanic currents, especially the Leeuwin and the East Australian Currents. Further studies, including whole genome approaches, will elucidate if the *Colpomenia* cryptic lineages correspond to new species and whether the phylogeographic patterns we observed are also imprinted across the species' entire genetic makeup.

Key-words: Australia, Brazil, *Colpomenia,* DNA barcode, genetic structure, marine barriers, phylogenetic diversity, phylogeography, population genetics, species delimitation.

Resumo

Colpomenia (Endlicher) Derbès & Solier é uma macroalga marinha parda com distribuição cosmopolita, caracterizada por um talo anatomicamente simples, saciforme oco, superfície lisa à rugosa, morfologicamente plástico e geralmente carecendo de estruturas reprodutivas. Definir espécies em *Colpomenia* é uma tarefa desafiadora, de modo que ferramentas moleculares são amplamente usadas para o estudo taxonômico e evolutivo. Os objetivos foram realizar análises filogeográficas e de diversidade em *Colpomenia* do Brasil e da Austrália. Sequências de *cox*3 do Brasil identificaram um complexo de espécies em *C. sinuosa*, contendo a verdadeira *C. sinuosa* e quatro linhagens crípticas. Sequências de *cox*1 da Austrália, identificaram três espécies: *C. sinuosa*, *C. claytoniae* e *C. peregrina*. Divergência genética intraespecífica relativamente alta em *C. claytoniae* e *C. peregrina* indicam a possibilidade de espécies crípticas. Além disso, *cox*1 e *rbc*L-S da Austrália identificaram *Mikrosyphar zosterae* no hemisfério sul e como endófita de *Colpomenia* spp. e *Leathesia marina* pela primeira vez. A revisão de artigos filogeográficos do Brasil identificou a divisão da Corrente Equatorial Sul no Cabo São Roque (4º S) como o principal evento geográfico promovendo diversidade ao longo da costa brasileira. Para *C. sinuosa*, no entanto, a cadeia de Vitória e Trindade (20.5º S) se é a principal barreira ao fluxo gênico da costa brasileira. Na costa australiana, as análises filogeográficas evidenciaram que *C. sinuosa* é amplamente influenciada e conectada pelas correntes oceânicas, especialmente corrente de Leeuwin e do Leste da Austrália. Estudos futuros, incluindo estudos genômicos, devem elucidar se as linhagens crípticas de fato correspondem a novas espécies e o papel dos eventos geográficos promovendo diversidade.

Palavras-chave: Austrália, barreiras marinhas, Brasil, *Colpomenia*, delimitação de espécies, diversidade filogenética, DNA barcode, estrutura genética, filogeografia, genética populacional.

General introduction

Introdução geral

Background

Phylogeography is the study of the geography of genetic lineages and the processes that promote similarities between phylogenetic and geographic structure (Avise 1998). Phylogeography also investigates genetic differences between populations within a single species, understanding the historical and spatial genetic fragmentation between populations, which lastly results in speciation. Thus, Phylogeography attempts to identify extant and extinct biotic and abiotic processes which might have contributed to the phylogeographic patterns observed today.

Pleistocene glaciations represent climatic processes that played important roles in shaping physical barriers to gene flow, influencing the evolution of whole biotas, both in land and in marine habitats (Rocha 2003; Ludt and Rocha 2015). The most recent glacial maxima occurred between 20,000 and 17,000 years ago (Barrows et al. 2002). During this period, the sea level was 70-130 meters lower than present, which changed oceanic currents, seawater temperature and continental shelf extension (Lambeck and Chappell 2001). These affected gene flow between species' populations for a long period. Therefore, Pleistocene glaciations are often regarded as the main vicariant barrier constructer, promoting speciation (Rocha 2003; Ludt and Rocha 2015).

In Brazil, glaciations were important in promoting marine biodiversity, especially due to the emersion of the Vitória-Trindade seamount chain (Pinheiro et al. 2017, 2018). Geographically, the Vitória-Trindade seamount chain (latitude 20.5º S) encompasses the Abrolhos archipelago and six relatively shallow seamount (10-110 m) which extends from the continental shelf to volcanic island of Trindade (20,5º S - 29,3º W). The seamounts are roughly 250 km apart to each other and most of them were emerged during Pleistocene. The seamount emersion caused changes in marine currents flow, especially the Brazil Current, influencing water-mediated gene flow. The evolutionary imprint of oceanographic and climatic processes during glaciations involving the Vitória-Trindade seamount chain

split the Brazilian coastline in two biogeographic sections: (a) a norther possibly subtropical and warmer bioregion, under the effect of a weaker Brazil Current; and (b) a southern possibly temperate and colder bioregion, under the effect of a stronger Malvinas Current. During interglacial periods the Vitória-Trindade seamount chain region can be considered an extinct physical barrier to gene flow, whose effects are detected in extant populations, even after the barrier was dismantled. The barrier attributed to the Vitória-Trindade seamount chain region was proposed to explain extant genetic discontinuities in several marine organisms (Lazoski et al. 2011; Hurtado et al. 2016; Paiva et al. 2019) and macroalgae such as *Crassiphycus caudatus* (J.Agardh) Gurgel, J.N.Norris & Fredericq (Ayres-Ostrock et al. 2019) and *Hypnea pseudomusciformis* Nauer, Cassano & M.C.Oliveira (Nauer et al. 2019). In addition, the region which extends from Espírito Santo to Bahia is proposed as a refugium during Pleistocene glaciations for terrestrial (Carnaval et al. 2009) and marine organisms (Nunes et al. 2008; Pinheiro et al. 2017, 2018; Peluso et al. 2018), including red macroalgae (Ayres-Ostrock et al. 2019). Regions of refugia provide shelter from environmental stressors or advantages in biotic interactions, allowing local populations to long persist, resulting in avoiding species extinctions, playing role in acting as bases for the recolonization of more unstable areas and the generation of more diverse areas (Carnaval et al. 2009).

In Australia, several studies have tested and described the presence of genetic discontinuity in marine populations, several of them shaped by Pleistocene glaciations as well (Waters et al. 2005; Teske et al. 2017). Most studies detected genetic structure that closely match the marine biogeographical provinces of Bennett and Pope (1953) proposed on the basis of community and species distributions: the Flindersian, Maugean and Peronian biogeographic provinces. The concordance between intraspecific (= genetic, phylogeographic discontinuities) and inter-/supra-specific (= ecological, biogeographic discontinuities) patterns have been described in different parts of the world and seems to

be the norm rather than the exception for Australia (Benzie 1999; Waters and Roy 2003; Waters et al. 2010). In Australia, relatively few studies have tested the presence of genetic structure among coastal marine macroalgal populations. Most have focused on large brown algae (kelps) with transoceanic dispersal capabilities. Yet, phylogeographic studies of ubiquitous non-kelp marine macroalgae with poor dispersal capabilities are revealing the presence of genetically highly structured populations at different geographical scales, including the identification of a plethora of cryptic species (Vieira et al. 2014; Leliaert et al. 2018).

Worldwide, fewer studies were carried out targeting the genetic diversity and structure of marine macroalgae, compared to marine animals (Beheregaray 2008). Marine macroalgae are the ecological foundation for the majority of coastal marine ecosystems (Dayton and Tegner 1984), acting as primary producers (Littler and Arnold 1982; Bruno et al. 2006), providing habitat, food, shelter (Seed and O'Connor 1981) and represent the preferred strata for recruitment of several organisms (Botero and Atema 1982). Besides the recognized ecological importance of marine macroalgae, its studies have been largely neglected in comparison to other organisms (Diaz-Pulido et al. 2007).

To date, the genus *Colpomenia* (Endlicher) Derbès & Solier (Scytosiphonaceae, Ectocarpales) encompasses ten taxonomically accepted species (Guiry and Guiry 2021). *Colpomenia sinuosa* (Mertens ex Roth) Derbès & Solier, the type species, is the most widely distributed species of the genus (Lee et al. 2013). *Colpomenia* life history is heteromorphic haplodiplobiontic, with erect yellowish to pale brown, convoluted, bladder-like macroscopic gametophytic thalli alternating with a nearly-microscopic filamentous tufty sporophytic thalli (Freitas Toste et al. 2003). Anatomically, the macrothallus of *C. sinuosa* is relatively simple, hollow sacciform, with vesicular and membranous thallus ranging from smooth to rough appearance, growing isolated or in clumps, with reproductive plurilocular structures organized in sori (Freitas Toste et al. 2003; Lee et al. 2013; Song et al. 2019).

In Brazil, *C. sinuosa* is reported as the only occurring species (Flora do Brasil 2020; Guiry and Guiry 2021), distributed from Ceará (northeastern Brazil) to Rio Grande do Sul (southern Brazil) (Flora do Brasil 2020). *Colpomenia sinuosa* is considered abundant throughout the year, forming a continuous coverage in some rocky shores (Széchy and Cordeiro-Marino 1991; Nunes and Paula 2004; Ouriques and Cordeiro-Marino 2004). In Australia, three *Colpomenia* species are reported: *C. sinuosa*, *C. peregrina* Sauvageau, and *C. claytoniae* S.M.Boo, K.M.Lee, G.Y.Cho & W.Nelson (Guiry and Guiry 2021). Within Australia *C. sinuosa* is also the most geographically widespread (Womersley 1987; Lee et al. 2013; Martins et al. 2021). Morphological variation between *Colpomenia* species in Australia proposed by Womersley (1967) were tested by Clayton (1975) who recognized *C. sinuosa* and *C. peregrina* as distinct species by circumscribing them using morphometric and statistical analyses. However, Australian species remain difficult to delimitate on morphological basis, considering that fertile material is necessary (Womersley 1987) and identification based only on vegetative characters is considered almost impossible (Kraft 2009)

In several marine macroalgal species, including *Colpomenia spp.*, species delimitation remains based on the analysis of vegetative and reproductive characters. However, thallus morphology is often relatively simple, phenotypically plastic, and lack reproductive structures. Therefore, molecular techniques, including DNA *barcode* approaches, have emerged as successful methods to delimitate macroalgae species (Saunders 2005; McDevit and Saunders 2009). Molecular techniques are revealing that macroalgal species generally comprise complexes of cryptic and quasi-cryptic species. Some molecular studies have detected cryptic diversity and possible cryptic species-complexes within *Colpomenia*, such as within *C. sinuosa* (Cho et al. 2009; Lee et al. 2013), *C. peregrina* (McDevit and Saunders 2009; Lee et al. 2014) and *C. claytoniae* (Martins et al. 2021).

In *Colpomenia sinuosa* three major lineages, subdivided into several sub-lineages were described using *cox*3 e *rbc*L DNA sequences of 134 specimens collected across 18 countries, revealing rampant cryptic diversity (Lee et al. 2013). The Brazilian sequences grouped in three distinct lineages with sequences from several geographic regions, even though all the sequences are from the same beach (Rasa Beach, Búzios, Rio de Janeiro) (Lee et al. 2013). Similarly, samples collected in Australia grouped in four distinct lineages (Lee et al. 2013). The existence of high genetic diversity in Brazilian and Australian samples of *C. sinuosa* suggest that: (1) the genus diversity in Brazil and Australia is underestimated; (2) more species might be described after molecular studies; and (3) cryptic and introduced species might be unraveled on further studies.

The *Colpomenia* sacciform habit can trap air within its hollow thalli during low tide or when exposed to high-energy waves, increasing positive buoyancy, conferring floatability, allowing detached thalli to drift and hence disperse long distances (Blackler 1967; Mathieson et al. 2016). The floatability of some *Colpomenia* species might explain their worldwide distribution but very limited information exists on how this floatability impacts genetic connectivity, isolation, diversity and structuring at different spatial scales.

Thus, *C. sinuosa* is a great candidate for studies involving evolutionary patterns, dispersal capacity over barriers to gene flow and the distributional patterns in the marine environment. Phylogeographic represents an important tool to study evolutionary processes that give rise to and help maintain diversity. Furthermore, the *C. sinuosa* thallus allows the occurrence of associated biota (e.g. epiphytes and epizoic) that can raft and disperse along floating macroalgae. Thus, the study of the dispersion and population connectivity of *C. sinuosa* also contributes to a better understanding to the dispersal and population connectivity of associated biota.

Objectives

The objective of this study was to perform phylogeographic and phylogenetic comparisons between species and populations of the genus *Colpomenia* from the Brazilian and Australian coasts. The results provide subsidies to: better understand macroalgal diversity, improve informed decision making regarding the identification of marine conservation zones and the application of marine conservation strategies (e.g. recolonization of impacted areas), and identify sources of distinct genetic diversity for potential economic applications. The molecular dataset allowed us to test various phylogeographic hypotheses relevant to genetic connectivity models in marine environments, as well as to improve our understanding of the speciation process. Our results identified historical and population events that determine the genetic structure of the current *C. sinuosa* populations in Brazil and Australia. These events are often shared among different species and, therefore, are associated with the genetic structuring and differentiation of other marine organism populations.

Hypothesis

This study aimed to answer two main scientific questions:

(1) which is the current *Colpomenia* diversity in Brazil, and Australia?

(2) Are there genetic structure among *C. sinuosa* populations in Brazil and Australia?

For the first question, it was expected the total number of *Colpomenia* species to be underestimated in both countries, with the possibility to detect the occurrence of cryptic diversity, considering that molecular techniques are showing that ubiquitous macroalgal species often comprise complexes of cryptic and quasi-cryptic species (Zuccarello and West 2003; Vieira et al. 2014; Leliaert et al. 2018). In addition, previous studies have reported that cryptic lineages within *Colpomenia* species could represent new species,

such as in *C. sinuosa* (Cho et al. 2009; Lee et al. 2013), *C. peregrina* (McDevit and Saunders 2009; Lee et al. 2014), and *C. claytoniae* (Martins et al. 2021).

For the second question, we expected to understand which processes or features, and other non-physical speciation processes, imprinted the largest effect on the differentiation and evolution in the *C. sinuosa* in Brazil and Australia. In Brazil, we wanted to identify which putative physical barriers to gene flow (as depicted in the literature) were the most important shaping genetic structure. The Vitoria-Trindade seamount chain in particular was suggested as a barrier to gene flow for two marine red macroalgae: *Hypnea pseudomusciformis* (Nauer et al. 2019) and *Crassiphycus caudatus* (Ayres-Ostrock et al. 2019). Would it also be the main region showing genetic discontinuities among continuously distributed *C. sinuosa* populations in Brazil? In the case of Australia, previous phylogeographic studies on marine brown macroalgae evidenced that the distribution patterns are often correlated to Bennett and Pope (1953) biogeographical provinces. These provinces were proposed on the basis of community and species distributions. Therefore, we tested whether the genetic structure of *C. sinuosa* populations would follow this pattern.

Specific objectives

• Detect and describe the presence of genetic structure among populations of species of the genus *Colpomenia* in Brazil and Australia;

• Quantify the genetic diversity levels among populations of species of the *Colpomenia* genus in Brazil and Australia;

• Identify the major evolutionary processes resulting the observed phylogeographic patterns;

• Reveal and describe possible new, cryptic and introduced species.

Chapters' structure

To answer the proposed questions, the thesis was organized in six chapters.

At **Chapter 1**, a comprehensive literature review of Brazil's marine phylogeography was performed (submitted to *Molecular Ecology*). This review aimed to identify regions of spatial phylogeograhic concordance along the Brazilian coast, besides to infer the relevance of historical and contemporary processes shaping the observed genetic structure patterns.

Chapter 2 aimed to assess the *Colpomenia* diversity in Brazil on the basis of detailed morphological observation and molecular analyses (i.e. phylogenetic and species delineation methods). In addition, we aimed to establish if *C. sinuosa* is the only occurring species or if there are several species, including cryptic diversity, along the whole distribution in Brazil.

After reviewing the literature and understanding the geographical processes promoting biodiversity in Brazil (Chapter 1), and studying *Colpomenia* spp. diversity in Brazil (Chapter 2), the phylogeographic analysis could be properly performed on *Colpomenia sinuosa* populations along the Brazilian coast. Thus, in **Chapter 3** (accepted for publication at *Journal of Phycology*) we identify areas of maximum and minimum genetic diversity, test for presence of genetic structure, and attempt to identify which allopatric, sympatric and parapatric processes played a role in the formation and maintenance of genetic structure among *C. sinuosa* along the Brazilian coast.

During a preliminary analysis of *Colpomenia* spp. molecular data from temperate Australia, our results revealed the presence of an endophyte occurring in *Colpomenia* spp.. In **Chapter 4** we study, identify, and characterize this endophyte, *Mikrosyphar zosterae*. This study represents the first report of this epi- endophytic macroalgal species in the southern hemisphere and also on a *Colpomenia* species.

After studying and excluding putative contaminated DNA sequences (endophyte sequences) from our molecular datasets, we focused on the analysis of *Colpomenia* species from Australia. Therefore, the aim of **Chapter 5** was to analyze the *Colpomenia* species from southern and southeastern Australia using DNA barcoding and single-marker species-delimitation methods.

After properly characterizing the *Colpomenia* species biodiversity in Australia, it was possible to perform phylogeographic analysis on *C. sinuosa*. In **Chapter 6,** we performed a phylogeographic analysis on *C. sinuosa* from Australia using a dual-marker approach.

After the six chapters, we expected to answer most, if not all, proposed questions and improve our knowledge on *Colpomenia* species diversity in Brazil and Australia, as well as to understand the main agents shaping biodiversity in both localities.

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Chapter 1

Brazilian Marine Phylogeography: a literature synthesis and data analysis

Filogeografia Marinha Brasileira: uma síntese da literatura e análise de dados

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Abstract: In the last 30 years a plethora of phylogeography studies were published targeting Brazilian marine species. These studies identify several physical and ecological processes as drivers of allopatric, sympatric and parapatric genetic differentiation and speciation. Examples of physical barriers include the split of the South Equatorial Current, the mouth of major rivers such as the Amazon, São Francisco, and Doce river, and coastal upwelling. Examples of ecological speciation include adaptation to differences in substrata and resource use, and reproductive biology. This study provides the first synthesis on Brazilian marine phylogeography literature. We used published data to build datasets and generalized additive models to identify spatial phylogeographic concordance. Our results recognized four phylogeographic regions within the Brazilian coast and identified Cape São Roque as the main multi-taxa multi-marker physical barriers to gene flow. Cape São Roque is associated with the split of the South Equatorial Current, and known to present high environmental heterogeneity promoting ecological differentiation. Vitória-Trindade seamount chain and Cape Santa Marta are also evidenced as two major regions concordant genetic breaks. The Vitória-Trindade seamount chain is likely promoted greater genetic differentiation during periods of glacial maxima, and, currently, may contribute to maintain phylogeographic concordance. Cape Santa Marta, however, is the winter northern limit of the Rio da Plata plume and the intermittent influence of the Malvinas Current. This study represents the first literature synthesis of Brazil's marine phylogeography and provides the recognition of four phylogeographic regions based on a novel explicit quantitative approach to comparative phylogeography.

Key-words: barriers, Brazil, gene flow, genetic breaks, marine, phylogeography, review

Resumo: Nos últimos 30 anos, diversos estudos filogeográficos focaram espécies marinhas brasileiras. Esses estudos identificaram vários processos físicos e ecológicos como promotores de diferenciação genética e especiação, sendo alopátrica, simpátrica e parapátrica. Exemplos de barreiras físicas incluem a divisão da Corrente Sul Equatorial, a foz de grandes rios, como o Amazonas, São Francisco e Rio Doce, e ressurgências costeiras. Exemplos de especiação ecológica incluem adaptação a diferentes substratos, diferentes usos de recursos e biologia reprodutiva. Este estudo fornece a primeira síntese da literatura filogeográfica marinha brasileira. Usamos dados publicados para construir um conjunto de dados e modelos aditivos generalizados para identificar concordância filogeográfica espacial. Nossos resultados reconheceram quatro regiões filogeográficas dentro da costa brasileira e identificaram o Cabo São Roque como a principal barreira física ao fluxo gênico entre diversos táxons e diversos marcadores moleculares. O Cabo São Roque está associado à divisão da Corrente Sul Equatorial e é conhecido por apresentar elevada heterogeneidade ambiental que promove diferenciação ecológica. A cadeia de montes submarinos de Vitória-Trindade e o Cabo Santa Marta também são evidenciados como duas importantes regiões de concordância de quebras genéticas. A cadeia de montes submarinos Vitória-Trindade provavelmente promoveu a diferenciação genética durante os períodos de máximos glaciais e, atualmente, pode contribuir para a manutenção da concordância filogeográfica. O Cabo Santa Marta, entretanto, é o limite norte da pluma do Rio da Plata durante o inverno e possui influência intermitente da Corrente das Malvinas. Este estudo representa a primeira síntese da literatura da filogeografia marinha do Brasil e fornece o reconhecimento de quatro regiões filogeográficas com base em uma nova abordagem quantitativa explícita de filogeografia comparada.

Palavras-chave: barreiras, Brasil, filogeografia, fluxo gênico, marinho, quebras genéticas,

revisão

Introduction

The identification of shared patterns of phylogeographic structure, also known as phylogeographic concordance, is a fundamental cornerstone in phylogeographic studies (Avise, 1998, 2000). To date, three kinds of phylogeographic concordance can be identified with increasing level of biological complexity. First, the simplest form of phylogeographic concordance, referred to as '*genetic concordance*', is the congruence of phylogeographic signatures among different markers, or genomes, within the same species (e.g. cytoplasmic versus nuclear markers). The second and more complex scenario involves multiple species and is referred to as '*taxonomic concordance*' or shared patterns of phylogeographic structure among different taxa. The third and most complex scenario is '*biogeographic*' or '*spatial concordance*' where spatially overlapping genetic breaks among populations from different markers and taxa are observed, often co-occurring between traditionally recognized biogeographic provinces (Avise, 1998, 2000).

Patterns of phylogeographic spatial concordance are usually formed by and therefore inform the presence of extinct and extant drivers of both intraspecific genetic structuring and interspecific biogeographic patterns. As such, phylogeographic spatial concordance represents a powerful tool to recognize regions of historical, ecological, biogeographic and (phylo-) genetic significance, with practical consequences to the design of protected area systems, conservation programs, the development of responsible approaches to the management of natural land and seascapes, and to identify areas where more studies are needed (Arbogast & Kenagy, 2001; Avise, 2000; Dawson, 2013; Hickerson et al., 2010). Phylogeographic concordance across widespread areas are often detected by employing comparative methods across multiple published studies (Soltis, Morris, McLachlan, Manos, & Soltis, 2006; Teske, Von Der Heyden, McQuaid, & Barker, 2011) because it is essentially cost and time prohibitive to detect phylogeographic concordance by generating new data for hundreds of species, using multiple markers, and

sampling across vast geographic areas (Bradshaw, Brook, Gardner, Bickford, & Whiteman, 2010).

Identifying spatial phylogeographic concordance relies on the detection of genetic breaks among populations of co-distributed taxa. However the concept of what constitutes a ‘genetic break’ is fickle and wide-ranging. In the phylogeographic context, Avise et al. (1987) defined “genetic break” as genotype differences by many mutational steps. In such a broad definition, genetic differences among populations could be small or large depending on the studied taxa, genetic marker or molecular technique used. In the marine environment, genetic breaks are created and maintained across multiple spatial scales by microevolutionary, historical, ecological, demographic or a range of abiotic processes such as shifts in climate, oceanography, and geological processes (Bowen, Rocha, Toonen, & Karl, 2013). For example, diverging or convergent currents as well as shifts in upwelling and climatic regimes along a coast can exert similar effects, differentiating coastal diversity on either sides with similar consequences for both intraspecific and interspecific biogeographic patterns (Barshis et al., 2011; Gaylord & Gaines, 2000; Hare, Guenther, & Fagan, 2005; Haye et al., 2014). Although the identification and mapping of genetic breaks are easy to detected and quantify using a range of molecular tools, the ecological and physical processes responsible for their origin and maintenance are often not easy to interpret (McGovern, Keever, Saski, Hart, & Marko, 2010). While allopatric isolation promoting genetic differentiation between populations caused by physical barriers to gene flow has been demonstrated many times over for terrestrial and marine species (Bowen, Rocha, Toonen, & Karl, 2013) opportunities for allopatric isolation in the marine environment are considered scarcer. However, genetic breaks in aquatic can be formed and maintained even in the presence of gene flow, between sympatric or parapatric populations, along ecological and climate boundaries (Bowen et al., 2016, 2013; Kocher, 2004).

To date, multi-taxa comparative molecular-based phylogeographic studies aimed at detecting widespread spatial phylogeographic concordance across hundreds of km include those executed for USA terrestrial and marine biotas (Shafer, Cullingham, Côté, & Coltman, 2010; Soltis et al., 2006), South American terrestrial biota (Turchetto-Zolet, Pinheiro, Salgueiro, & Palma-Silva, 2013), south Europe terrestrial biota (Feliner, 2011; Taberlet, Fumagalli, Wust-Saucy, & Cosson, 1998), Australian terrestrial and marine biota (Byrne, 2008; Teske, Sandoval-Castillo, Waters, & Beheregaray, 2017), African ungulates (Lorenzen, Heller, & Siegismund, 2012) and those that targeted a specific group of organisms (e.g., Bowen & Karl, 2007; Duncan, Martin, Bowen, & De Couet, 2006; Rocha, 2003; Satler & Carstens, 2016). In the marine environment, shallow-water species in particular often share genetic breaks at specific geological features or unique geographical locations that are usually associated to changes in oceanography, climate, substrate composition, or other physical feature (Hu & Fraser, 2016). Many of such geographic features also mark species distributional limits and hence are associated to boundaries between biogeographic provinces. The concordance between phylogeographic structure (genetic breaks) and biogeographic structure (species distribution breaks) suggest that both patterns are driven by shared physical and historical factors (Avise, 1998; Bowen et al., 2016).

Brazil possesses one of the longest north-south tropical coastlines in the world, with approximately 8,000 km, spanning 37 degrees of latitude (from 4° North to 33° South), and is under the influence of several oceanographic, climatic, geological and ecological processes identified as drivers of biogeographic structuring (Floeter et al., 2008; Miloslavich et al., 2011). In the last three decades a plethora of single-species marine phylogeographic studies testing for the presence of genetic breaks along the Brazilian coast have been published. Yet, to date no comprehensive comparative marine phylogeographic study has been conducted in Brazil to help identify shared patterns of

phylogenetic structure along the coast and the likely reasons for their origin and maintenance. Most Brazilian marine 'phylogeographic' reviews so far focused on either single species, a small number of sister taxa (e.g. fishes Pinheiro et al., 2017; *Symbiodinium* spp. Picciani, de Lossio e Seiblitz, de Paiva, e Castro, & Zilberberg, 2016; *Millepora* spp. de Souza et al., 2017), or relied on species distributional data alone instead of molecular data (e.g. Floeter et al., 2008; Pinheiro et al., 2018; Rocha et al., 2005). Thus, published comparative studies on Brazilian marine biogeography to date provide a macroevolutionary assessment rather than a phylogeographic or microevolutionary viewpoint, even though both disciplines are inexorably interconnected. Consequently, a phylogeographic concordance analyses of all molecular studies so far publish for Brazilian marine biota could recognize regions of historical, ecological, biogeographic significance tuned to the genetic component.

The first two Brazilian marine phylogeographic studies were based on allozymes (Aron & Solé-Cava, 1991; Russo & Solé-Cava, 1991) and did not identify genetic structure among *Botryllus niger* (an ascidian) populations collected between Espírito Santo and Rio de Janeiro, or among *Bunodosoma caissarum* (an anemone) populations sampled across multiples sites within Rio de Janeiro State. Since then a large number of articles using different markers, targeting different spatial scales, applying different sampling designs, and looking at a wide range of phylogenetically distant taxa showed the presence of genetically structured populations (see references below). As a result, several physical features acting as putative barriers to gene flow have been proposed to explain the presence of genetic discontinuities in continuously distributed species along the Brazilian coast. Chiefly among them, we have: (a) freshwater and sediment plumes discharged from the mouth of major rivers such as the Amazon, São Francisco, Paraguaçu, Jequitinhonha, Doce, and Paraíba do Sul rivers (da Silva, Marceniuk, Sales, & Araripe, 2016; Floeter et al., 2008; Machado et al., 2017); (b) the split of the South Equatorial Current (SEC) in two

opposing boundary currents between latitudes latitude 4º and 10º S, giving rise to the southward Brazil Current and the northward North Brazil Current (Bezerra et al., 2018; Cortinhas et al., 2016); (c) the Cabo Frio coastal upwelling system at latitude 23º S (Cortinhas et al., 2016; Hurtado et al., 2016) and; (d) the Cassino beach, the longest stretch of sandy beach in the world located in southern Brazil between latitudes 29º and 32º S (Nauer, Gurgel, Ayres-Ostrock, Plastino, & Oliveira, 2019; Trovant et al., 2016).

In several phylogeographic studies, genetic discontinuities coincide with coastal features, supporting their recognition as barriers to gene flow (e.g. Hurtado et al., 2016; Lazoski, Gusmão, Boudry, & Solé-Cava, 2011; Paiva, Mutaquilha, Coutinho, & Santos, 2019). However, sometimes phylogeographic structure does not match any known barrier to gen flow (e.g. Carmo et al., 2019; Rodrigues et al., 2014; Secchi, Wang, Murray, Rocha-Campos, & White, 1998). Furthermore, genetic breaks can occur even in the absence of ecological differentiation, as a result of idiosyncratic lineage sorting and stochastic coalescent processes working on non-recombining markers, a phenomenon particularly observed in cytoplasmic markers (Kuo & Avise, 2005). Extinct barriers to gene flow can also produce genetic discontinuities that perpetuate over time and can be observed in today's populations, even in the absence of extant barriers. One probable example of such case in the Brazilian coastline is the Vitoria-Trindade seamount chain (at latitude 20.5º S, Fig. 1A), which geographically includes the Abrolhos Arquipelago and the associated coral reef system - the largest in Brazil (Pinheiro et al., 2017). During Quaternary's glacial maxima, the drop in sea level emerged large eastward inflected areas of the Brazilian continental shelf, including seamount, splitting the continent's coastline into two sections: (a) a warmer, possibly subtropical, northern bioregion influenced by a weaker Brazil Current; and (b) a colder subtropical, possibly warm-temperate southern bioregion influenced by a stronger Malvinas Current. This barrier has been proposed to explain genetic discontinuities of several marine taxa such as mollusks (*Crassostrea* spp.:

Lazoski et al., 2011), crustaceans (*Excirolana braziliensis*: Hurtado et al., 2016), polychaetes (*Perinereis* spp.: Paiva et al., 2019), and red macroalgae (*Crassiphycus caudatus*: Ayres-Ostrock et al., 2019; *Hypnea pseudomusciformis*: Nauer et al., 2019).

Marine biogeographic regionalization and the identification of biogeographic provinces based on shifts in species composition (beta-diversity) have also been described along the Brazilian coast. The boundary between provinces are usually marked by abiotic processes often considered drivers of phylogeographic structure as well. Among well-known marine biogeographic eco-regionalizations that subdivide the Brazilian coast into different provinces, there are those proposed by Briggs (1974), Sullivan & Bustamante (1999), and Spalding et al. (2007), all of which recognized the Cabo Frio upwelling region as boundary between distinct bioregions. Floeter et al. (2008), however, recognized the Vitória-Trindade seamount chain as a major marine biogeographic boundary based on fishes species distribution (see also Peluso et al., 2018). The extent at which how boundaries among biogeographic provinces coincide with phylogeographic regions along the Brazilian coast have not yet been fully assessed, particularly across multiple phylogenetically-distant taxa. Consequently, we still do not know which processes or features, including the proposed barriers to gene flow mentioned above, and other non-physical speciation processes, imprinted the largest effect on the differentiation and evolution in the Brazil's marine biota. Therefore, the objectives of this study were (a) to provide a comprehensive literature synthesis of Brazil's marine phylogeography; (b) to perform a comparative multi-taxon phylogeographic analyses to identify regions of high spatial phylogeograhic concordance, and (c) infer the relative importance of historical and contemporary processes on the observed patterns of genetic structure along the Brazilian marine coast. Phylogeographic Regions within the Brazilian province are proposed. Comparative phylogeography has traditionally being executed by identifying concordant patterns without explicit quantifying the degree of concordance (or discordance). This

limitation reduces the ability of the discipline to produce predictive models capable to identify areas of greater or lower concordance. This study also provides the first quantitative measure of phylogeographic concordance in the marine environment. Our generalized model helped us to identify which barriers to gene flow are responsible for the greatest imprint on coastal marine total phylogeographic concordance along the Brazilian coast.

Materials and methods

Literature survey

The database used for this synthesis was compiled from searches in the Web of Science® (Institute of Scientific Information, Thomson Scientific). Web of Science is an online academic database from ISI Web of Knowledge® that provides access to information about indexed research journals worldwide. We searched all Web of Science databases for scientific articles published from 1987 (regarded as the birth of phylogeography; Avise, 1987) to 2019 using the following keywords and Boolean command combinations: population* genetic* OR phylogeo*; AND Brazil*; AND ocean* OR sea* OR island OR reef OR rocky shore OR benthic OR marine OR coast*. Preliminary results identified 1380 articles, several of them outside the target parameters. Thus, we used the following secondary filters to further sort phylogeographic articles addressing specifically marine species that occur in Brazil: NOT “Atlantic forest” OR rainforest* OR freshwater. After applying secondary filters, 1170 articles remained.

A third-level filtering was performed by hand, visually scanning each article (i.e. title, abstract, hypotheses, material and methods, or results), and excluding those with the following characteristics: review articles; conference abstracts; technique articles; articles that studied viruses and bacteria (marine or otherwise) and human diseases; articles that presented only one sampled population in Brazil; articles that were purely taxonomic;

articles that involved only populations located in offshore oceanic islands (i.e. São Pedro - São Paulo Archipelago, Trindade seamount chain Island, Rocas Atoll, Fernando de Noronha Archipelago, and Rio Grande seamount); articles that involved non-native Brazilian marine species; articles that despite claiming they implemented phylogeographic analyses, did not use molecular data to test for the presence of phylogeographic structure among populations; and articles addressing taxa that live in close proximity to the marine environment but do not depend in any way of the marine ecosystems to exist. Thus, this study focused on empirical, molecular data-driven phylogeographic articles that minimally addressed the Brazilian's continental coastline and native marine biota, including marine birds and plant mangrove species. We included marine birds and plant mangrove species since their life are closely related to the marine habitat (i.e. feeding, reproduction, seawater temperature, dispersion, among others).

All selected articles were fully read. A database was built based on information extracted from the articles and included: year of publication; species taxonomy assigned to 16 categories (Ascidiacea, Aves, Cetacea, Cnidaria, Crustacea, Echinodermata, Fishes, Kinorhynch, Mollusca, Nemertea, Otariidae, Plantae, Platyhelminthes, Polychaeta, Porifera, and Testudinata); number of analyzed taxa per article; sample site locations (city, state and geopolitical zone name, coordinates); genetic markers used (technique, genome and name of used genes); presence or absence and number of genetic discontinuities reported, locations from where genetic discontinuities were reported; classifications of species habitat (as benthic, pelagic, or aerial) and functional forms (invertebrates, vertebrates, or plants). Basic statistics were calculated from this database using Microsoft Excel tools.

In this study we refer to *article*(*s*) each and every publication or unique reference, and we refer to '*study*(*-ies*)' each species with phylogeographic information found within

each article. Therefore, some articles were comprised of only one study, while multi-taxon articles contained two or more studies (taxa).

Dataset preparation

A data matrix comprised by all sample sites reported in all *studies* was built from the database described above. For each study captured in our database, sites where sampled populations occurred were logged in the data matrix as 0's. Sites located between sampled populations where genetic breaks were reported by the authors of those articles were logged as 1's. We considered a loose definition of what a genetic break is and accepted the varied implicit or explicit definitions adopted by each article (e.g., F_{ST} and spinoffs, reciprocal monophyly, percent divergence, AMOVA). If the authors claimed the existence of genetic discontinuity in any given place and showed empirical evidence to support such claim, we recorded that discontinuity in our data matrix. Thus, whenever panmixia was detected, geographic range between genetically homogeneous populations were marked as a string of 0's between sample sites. From this data matrix, we built a frequency data matrix by calculating the number of all studies reporting a genetic break in each site, normalized by the total number of reported studies per site, regardless whether genetic breaks were found or not (Table S1).

A large normalized genetic break frequency value in a given site indicates that a large number of studies identified that site as a location of high probability of occurrence of processes contributing to genetic differentiation either in that site or on both sides of that site (i.e. analogous to limits between biogeographic provinces). Consequently, sites with high values of genetic break frequency can be considered locations of high phylogeographic concordance. Conversely, a small value means either absence of genetic breaks for most populations and species studied in a particular site (= area of high genetic

connectivity). The frequency of occurrence of genetic breaks not only quantify but also describes how one of the most important concepts in comparative phylogeography is distributed in space (genealogical concordance, aspect ‘iii’ in Avise, 1998, or type III concordance in Avise, 2000).

Modelling

For modeling analyses, we used the normalized dataset described above. Due to the smaller number of studies and hence larger number of missing data from sampling sites at Brazil’s northernmost and southernmost locations, those sites were excluded from the analysis. Consequently, the spatial range included used in mathematical modelling varied between 01° 24' N and 31° 22' S. We considered the existence of at least three studies per site to perform our analyses (n = 3), encompassing 201 of the 211 sites included in the original dataset. Initial data exploration showed that the response of frequency distribution of genetic break concordances to latitude was non-linear. Hence, generalized additive models (*gam*) were used with latitude as a smooth effect. First, we modelled all data combined and then individually by taxa with the greatest number of available data (fishes, crustaceans, mollusks and cnidarians). We used the *gam* subroutine available in the *mgcv* package (Wood, 2017) implemented in R (R Core Team, 2021). We fitted the *gam* using Gaussian distributions including for logistic regression. We also used a smooth term with a cubic regression or cyclic cubic regression spline (for fishes and crustacean data) to represent latitudinal variation. Several *gam* models were tested varying the smooth term parameters: *fx* (fix the degrees of freedom on a regression spline model), the *K* (dimension of the basis used to represent the smooth term) and the *bs* (smoothing term). The optimal model was selected using the *gam.check* tool (Wood, 2017). Model validation was assessed using the generalized cross-validation (GCV) index, the Unbiased Risk Estimation (UBRE), the "*starry-sky*" patterns in the residuals versus linear prediction graph, and the linear relationship between response parameter and the fitted graph. The

Effective Degree of Freedom (EDF) was used to test if *gam* was a valid method to analyze our datasets (i.e. EDF > 2). Model quality was also assessed by comparing adjusted R^2 and the explained deviance.

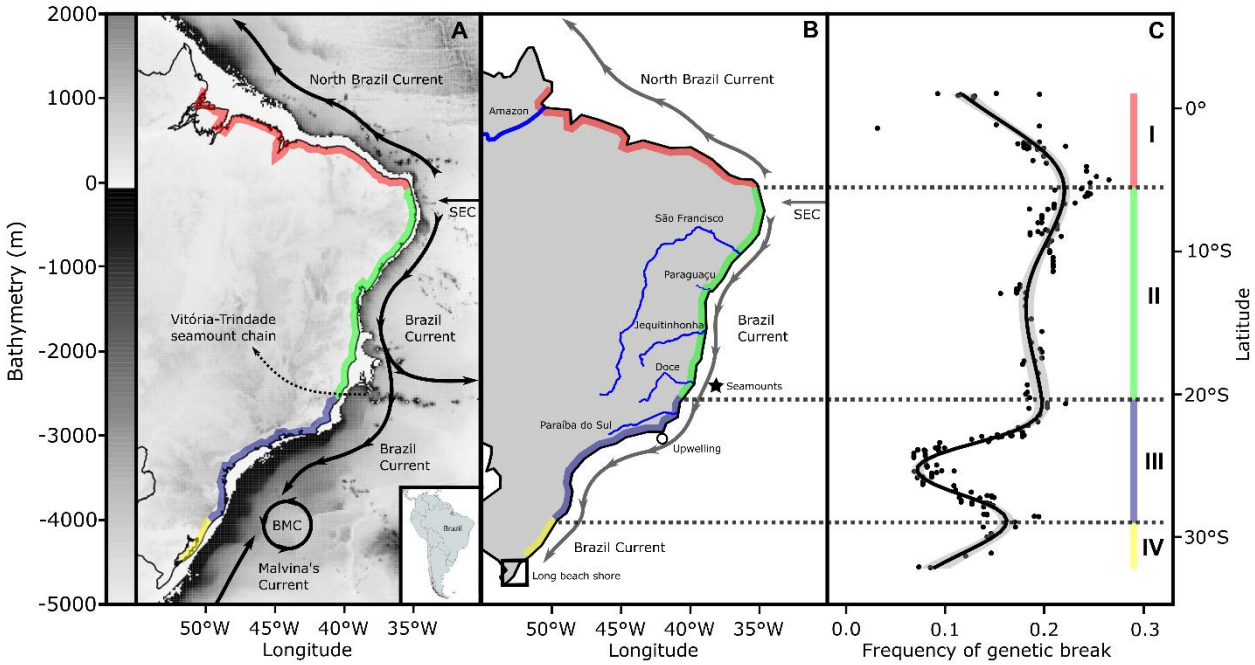

Figure 1. Maps of the Brazilian coast. Different colors indicate different Phylogeographic Regions (I-IV) recognized in this study. SEC = South Equatorial Current. BMC = Brazil-Malvinas Confluence. (A) Past coastline during Pleistocene glacial maxima with sea level 100 meters lower than present time. Paleocurrents according to Clauzet et al. (2007) and Stramma & England (1999). (B) Present day coastline. Major rivers marked in blue. ★ = Location of the Vitória-Trindade seamount chain. ○ = Cabo Frio upwelling system. ◘ = Cassino beach, the longest sandy beach in the southern hemisphere. (C) Generalized additive model of the frequency of putative genetic breaks between populations of marine species per latitude. Data collected from published literature between 1987 and 2019. Roman numbers stand for each of the four Phylogeographic Regions recognized by the model.

Sites of high genetic break concordance identified in the *gam* results were tested using analysis of variances (ANOVA) with type-3 sum of squares. The input data was the proportion of genetic break at the peak of maxima and four surrounding areas as replicates, accounting five sites per *gam* peak. Four peaks were chosen for analysis: the

three peaks of maxima identified in the *gam* results (Natal: 5º 46' S, Vitória: 20º 18' S, and Cape Santa Marta: 30º 36' S) in addition to Cabo Frio upwelling region (22º 57' S). The latter is often regarded as an important barrier to gene flow (Peluso et al., 2018) and a border between Spalding et al. (2007) ecoregions. Normality and homogeneity assumptions of variances were tested using Shapiro-Wilk and modified robust Brown-Forsythe Levene-type test, respectively (Zar, 1999). Tuckey was used as the post hoc test. Analyses were done in R (R Core Team, 2021) using *aov* function in *car* package, and adopting an alpha of 0.05.

Results

Literature survey

The final number of articles was 159, representing 195 studies, covering 211 sites. The northern and southernmost sampling locations were Oiapoque (latitude: 4° 13' N) and Chuí (latitude: 33° 44' S), respectively, spreading across ~7,000 km, representing nearly the entire Brazilian coast. Articles comprised of only one study represented 80% of the total, followed by articles composed by two (11%), three (6%), and four (2%) studies. Only one article presented 6 studies of which only 3 were used based on the second-level filtering criteria listed above (Nunes, Norris, & Knowlton, 2011). The oldest articles were published in 1991 (two articles) and the newest in 2019 (12 articles). Since 1991 the number of publications per year increased, particularly after the year 2001 (four articles/year) when a steady increase is noticed, culminating in the publication of at least 10 articles per year after 2014 (Fig. 2). The five journals with the highest number of articles were listed in Table 1.

Markers

Ten different molecular techniques were reported in the literature: allozymes, DNA sequencing, ddRAD, RAPD, ISSR, AFLP and RFLP, cariology, SNP and microsatellites (Fig. 3A). DNA sequences obtained via automated Sanger sequencing were the most commonly used type of molecular data (71% studies). Among DNA sequence markers, a total of 38 different genes were used; 16, 19 and 3 encoded in the mitochondrial, nuclear and chloroplast genomes, respectively. DNA sequencing of mitochondrial genes was by far the most used technique among all markers and across all studies (80%), followed by the DNA sequencing of nuclear (18%) and chloroplast-encoded markers (2%). Within the mitochondrial genome, the D-loop region and the COI DNA barcode marker were the most used marker (34% each), followed by the cytochrome B gene (15%). The number of articles using microsatellites or allozymes were similar (12% and 10%, respectively). The first allozyme study was published in 1991 (Aron & Solé-Cava, 1991; Russo & Solé-Cava, 1991) and the latest in 2018 (Pazoto, Ventura, Duarte, & Silva, 2018). Only four articles (6%) targeted autotrophic organisms, two terrestrial vascular plants (mangroves) and two red macroalgae, and two of them used chloroplast-encoded markers (Ayres-Ostrock et al., 2019; Mori, Zucchi, Sampaio, & Souza, 2015). Only one more recent article used the SNP technique (Siccha-Ramirez et al., 2018).

Table 1. Top five journals with the largest number of empirical articles containing phylogeographic data on Brazilian marine species.

Journal	Number of articles
PLoS ONE	12
Journal of Experimental Marine Biology and Ecology	11
Genetics and Molecular Biology	8
Marine Biology	8
Molecular Ecology	6
Total number of Journals	74

Geographic coverage, taxonomic coverage, habitats diversity & functional forms

Brazil has 17 geopolitical states along its coastline and they were all included in this synthesis (Table 2). The geopolitical state with the largest number of articles reporting at least one sample site inside its borders was the Rio de Janeiro (48%, 93 studies), followed by São Paulo (46%, 89 studies) and Bahia (44%, 86 studies). The least sampled states were Piauí (4%, eight studies), Sergipe and Amapá (6%, 12 studies). Only 16 high-ranked taxonomic groups were identified in all articles (Fig. 3B). The state with the largest number of studied taxa was São Paulo with 14 taxa followed by Rio de Janeiro (13 taxa) and Santa Catarina (12 taxa). The states with the smallest number of sampled taxa were Amapá and Alagoas with only 4 taxa each (Table 2).

Three taxa presented sample sites located within only one state: otaries (Rio Grande do Sul: Artico et al., 2010), echinoderms (Rio de Janeiro: Calderón, Ventura, Turon, & Lessios, 2010; Duarte, Ventura, & Silva, 2016; Pazoto et al., 2018; Wangensteen, Turon, Pérez-Portela, & Palacín, 2012), and Kinorhynchs (São Paulo: Randsø, Domenico, Herranz, Lorenzen, & Sørensen, 2018). Together with flatworms (= Platyhelminthes, Marigo et al., 2015), phylogeographic studies targeting these taxa concentrated their sampling in the southern Brazil (> 21° S). Fishes and mollusks presented the most comprehensive and spatially widespread sampling across Brazil (100% states), followed by crustacean (16/17 states), and plants (14/17 states). Fishes were the most studied taxon encompassing 32% of all studies, followed by crustaceans (21%) and mollusks (10%) (Fig. 3B). Of all studied taxa, 49% were invertebrates, followed by 47% of vertebrates, and 4% plants, including algae (Fig. 3C). Regarding habitat, 63% studies targeted benthic species, 34% pelagic, and 3% aerial (i.e. coastal and marine birds) (Fig. 3D).

Table 2. Number of studies per Brazilian geopolitical states per taxonomic group extracted from scientific articles published between 1987-2019. See Figure S1 for names of the geopolitical states.

Region	North		Northeast									Southeast			South		
State	AP	PA	MA	PI	CE	RN	PB	PE	AL	SE	BA	ES	RJ	SP	PR	SC	RS
Ascidiacea	0	0	0	0	0	0	0	0	0	0	0	2	4	4	1	3	0
Aves	1	2	1	1	0	0	0	0	0	0	2	0	3	4	1	4	0
Cetacea	0	2	0	0	3	1	0	0	0	0	5	3	5	2	0	4	4
Cnidaria	0	0	1	0	3	2	7	5	4	0	8	6	6	3	0	3	0
Crustacea	4	10	9	1	11	8	0	12	7	5	19	11	20	22	8	18	13
Echinodermata	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0
Fishes	4	17	8	1	15	26	6	12	1	3	29	19	28	23	10	14	11
Kinorhynch	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
Mollusca	3	6	1	1	6	5	6	8	2	1	8	1	10	12	5	5	2
Nemertea	0	0	0	0	0	0	0	0	0	0	3	2	2	4	0	2	0
Otariidae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Plantae	0	6	4	3	4	3	1	4	0	1	4	2	2	4	3	3	0
Platyhelminthes	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1
Polychaeta	0	0	0	0	3	0	2	3	0	0	1	3	2	3	2	0	0
Porifera	0	0	0	0	0	0	0	1	0	0	0	0	2	1	0	1	0
Testudinata	0	0	0	0	2	1	0	0	0	2	5	4	3	3	1	4	3
Total	12	43	24	7	47	46	22	45	14	12	84	53	91	87	32	62	35

Models

In the data matrix, the site with the highest value of genetic break concordances was Galinhos at 05° 03' S, 36° 45' W, north of Rio Grande do Norte State (27%). However, the g*am* result identified Natal city (05° 46’ S) in Rio Grande do Norte as the region with the highest value of genetic break concordance in the entire Brazilian coast (Fig. 1C). In our *gam* results, sites with the greatest values of genetic break frequency were recognized as three distinct peaks of maxima. These peaks coincided with the locations where processes known to act as barriers to gene flow along the Brazilian coast occur: the split of the SEC (at latitude 5º S), the Vitória-Trindade seamount chain (20.5º S), and Cape Santa Marta (28º S) (Figs. 1A-C). ANOVA detected significant differences in the average amount of genetic break frequencies among the three *gam* peaks ($F_{3,16}$ = 28.6, $p < 0.005$, Fig. 4). Pairwise tests showed that Natal site was the region with the greatest values of genetic break concordance along the Brazilian coast (25% ± 1.2 s.d.). The second location of highest phylogeographic concordance was Vitória and Cape Santa Marta with similar values (20% ± 1.1 and 17.4% ± 2.3, respectively). Cabo Frio upwelling sites represented the lowest relative peak of genetic break concordance (17% ± 1.2) but was not significantly different from Cape Santa Marta.

Locations between the three *gam* peaks were identified as distinct Phylogeographic Regions I to IV (Fig. 1A-C). Phylogeographic Region I (0° S – 6° S) was located in the northern Brazil, from the mouth of the Amazon river to Natal city. Region II (6º S – 20.5º S) showed a homogenous distribution of relative higher values of genetic break concordances stretching between two of the main barriers to gene flow recognized along the Brazilian coast: the split of the SEC and the Vitória-Trindade seamount chain. Region III (20.5° S - 28° S) was located between the Vitória-Trindade seamount chain region and Cape Santa Marta. Region III exhibited an inverted bell-shaped distribution with the lowest

genetic break concordance values. Region IV (28º S – 31º S) was located in Brazil's southernmost region and was characterized by a sharp decrease in genetic breaks.

Individual *gam* for the four most studied taxa (fishes, crustaceans, mollusks, and cnidarians) produced patterns of genetic break frequency distribution similar to those reported in the total analysis, although slight differences were noted (Figs. 5A-D). Fishes, the most data-rich taxon (Fig. 5A), presented the I-IV region pattern reported in the total analysis, however, the main peak of maxima was observed at the Vitória-Trindade seamount chain, followed by a second peak at Cape São Roque (SEC split). Latitudes, or a string of adjacent sites, with values of genetic break frequency distribution equal to 0% are interpreted as regions of widespread panmixia, and this was the case for fish populations sampled between Paraná (Laranjeiras Bay: 25° 24' S) and northern Santa Catarina (Itajaí: 26° 54' S) (Fig. 5A). Lastly, Cape Santa Marta also seems to be a strong barrier to gene flow for fishes.

Even though the crustacean *gam* presented comparable topology to those obtained from the other three taxa and the total evidence *gam*, this taxon showed slightly unique pattern of genetic break frequency distribution (Fig. 5B). The greatest concordant genetic break frequency occurred near Cape Santa Marta, between Phylogeographic Regions III and IV. The north and northeastern Brazilian coast showed a somewhat homogenous distribution in genetic break frequency for crustacean populations extending from Pará (01° 24' S) to Espírito Santo (20° 40' S) (Fig. 5B). South of latitude 20° 40' S, one sharp peak was encompass both the Vitória-Trindade seamount chain region (20.5° S) and the Cabo Frio upwelling region (22° 57' S), and is followed by a region of sharp decline (Fig. 5B).

For mollusks and cnidarians, recorded genetic breaks are absent west of Belém (01° 27' S, 48° 29' W) and Fortaleza (03° 43' S, 38° 31' W), respectively, and south of Florianópolis (27° 36' S) for both groups (Figs. 5C and 5D). *Gam* results for mollusks and

cnidarians presented several similarities. Mollusks and cnidarians showed the greatest values of genetic break frequencies in northernmost latitudes (north of Natal, 5º 46' S) with maximum values reaching 50% and 75%, respectively. For mollusks, the maximum genetic break concordance occurred west of Fortaleza (3º 43' S, 38º 31' W), all the way to Maranhão state (1º 18' S, 44º 56' W) (Fig. 5C). For cnidarians, the region of maximum break frequency values occurred north of João Pessoa (07º 06' S, 34º 49' W) (Fig. 5D), peaking at Fortaleza (03° 43' S, 38° 31' W). Mollusks presented an average of 21% genetic break concordances spread across a large portion of the Brazilian coast, from latitude 4° S to 25° S, similarly to Region II in the total dataset results (Fig. 1C). Cnidarians also presented a Region II-like distribution between latitudes 10° S and 20° S. Interestingly, *gam* results from all four taxa showed a region of minimum values (Figs. 5A-D) that tends to coincided with the minima observed in the total evidence *gam* between latitudes 20° S and 25° S (= Region III). This region of minima was always followed by an increase in genetic break frequency farther south (Regions III-IV).

Discussion

Literature synthesis

In Brazil, the first two marine phylogeographic studies were published in 1991 and the yearly number of published studies have increased ever since. The years 2016 and 2018 presented the highest number of Brazilian marine phylogeographic publications to date (15 articles). Considering the 16 taxonomic groups assessed in this study, fishes have been the most studied taxon, accounting for 33% of all publications. This is more than the second and third most studied taxa combined (crustaceans 21%, mollusks 9%). A conspicuous publication bias towards fish phylogeographic studies is observed in the literature globally. Beheregaray (2008) reviewed all phylogeographic articles published between 1987 and 2006 and fishes were ranked second, after mammals, as the taxonomic

group with the largest number of publications without much difference whether they were freshwater (45%) or marine species (55%). Turchetto-Zolet et al. (2013) reviewed all phylogeographic articles published on South America terrestrial and freshwater biota between 1987-2011 and reported fishes as the second ranked taxonomic group (13%) together with overall invertebrates, and second only to plants (17%). This bias towards fishes is probably due to their economic importance as human food and ecotourism, relative easier taxonomic identification, simple life-cycle and ploidy, the availability of well-suited genetic markers, and probably, the existence of a large number of hired expertise in research institutions. Differently, otaries, Kinorhynchs and flatworms presented very localized sampling and a small publication number.

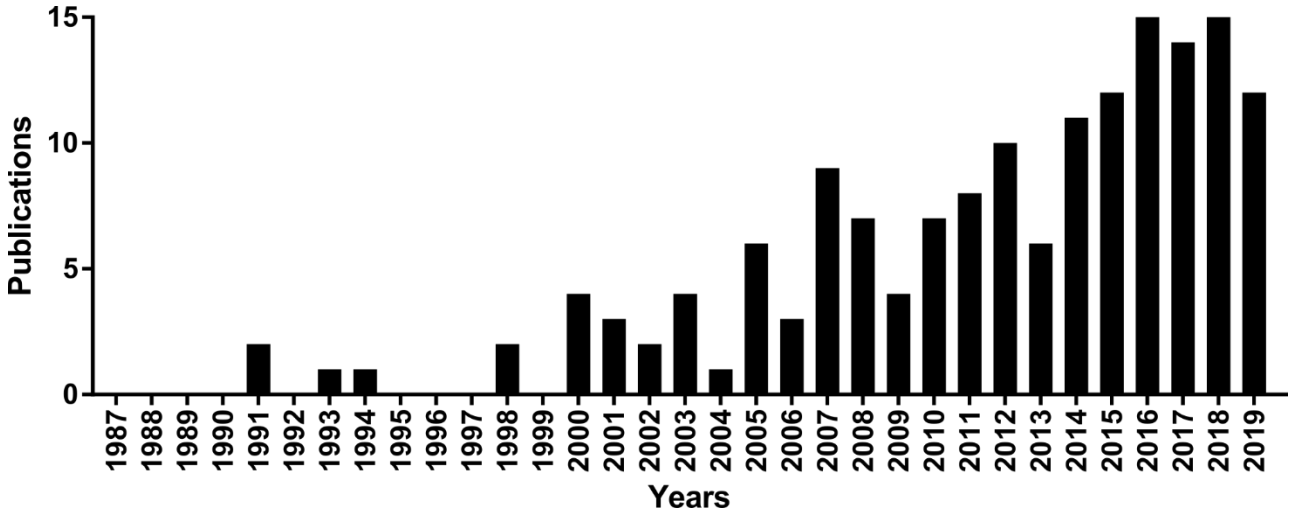

Figure 2. Number of molecular-driven marine phylogeography papers published between 1987 and 2019 with data able to test for the presence of genetic structure among populations of Brazilian marine species.

The number of marine vertebrate and invertebrate studies in Brazil were quite even, 47% and 49%, respectively. This is opposite to what was recorded by Beheregaray (2008) and Turchetto-Zolet et al. (2013) who observed a bias towards vertebrate studies (57%

and 70% respectively). The number of marine plants and algae phylogeographic studies in Brazil were quite underrepresented (4%) as they are for terrestrial and freshwater species in South America (1% algae in Turchetto-Zolet et al., 2013), terrestrial and marine plants in eastern North America (11% in Soltis et al., 2006) and world widely (2% reported in Beheregaray 2008). In the past, the disparity between the number of phylogeographic studies between heterotrophic x autotrophic species was attributed in part to the faster mutation rates observed in animal markers (mitochondrial genome) compared to markers available for plant studies (Soltis et al., 2006). An increase in the number of studies addressing macroalgal phylogeography would improve our understating of population genetic histories and marine phylogeography considering they are poorly-dispersers (Kinlan & Gaines, 2003), potentially being able to identify a larger number of local and regional barriers, and concordant phylogeographic patterns.

The first study using genotypic data addressing the phylogeography of a Brazilian marine species was published in 1998 (Secchi et al., 1998), 11 years after the Avise's seminal 1987 publication. Before 1998, the only six phylogeographic studies (= four articles) addressing Brazilian marine biota used phenotypic (isozyme) data. In Brazil mitochondrial genotypic data started being utilized first in the form of fragment-based methods (e.g. Secchi et al., 1998). Between 2007-2010, RAPD, AFLP, RFLP and similar fragment-based techniques played an important role increasing the number of publications. Microsatellites remain a poorly used technique with only 29 studies (12%) published since 2001 (Beheregaray & Sunnucks, 2001). A similar pattern was observed for genome-wide techniques, such as SNPs, where only one marine study was published to date (Siccha-Ramirez et al., 2018). In the world, genome wide SNP and high-throughput DNA sequencing techniques are becoming the powerhouse in phylogeographic studies (e.g. ddRadseq, Peterson, Weber, Kay, Fisher, & Hoekstra, 2012).

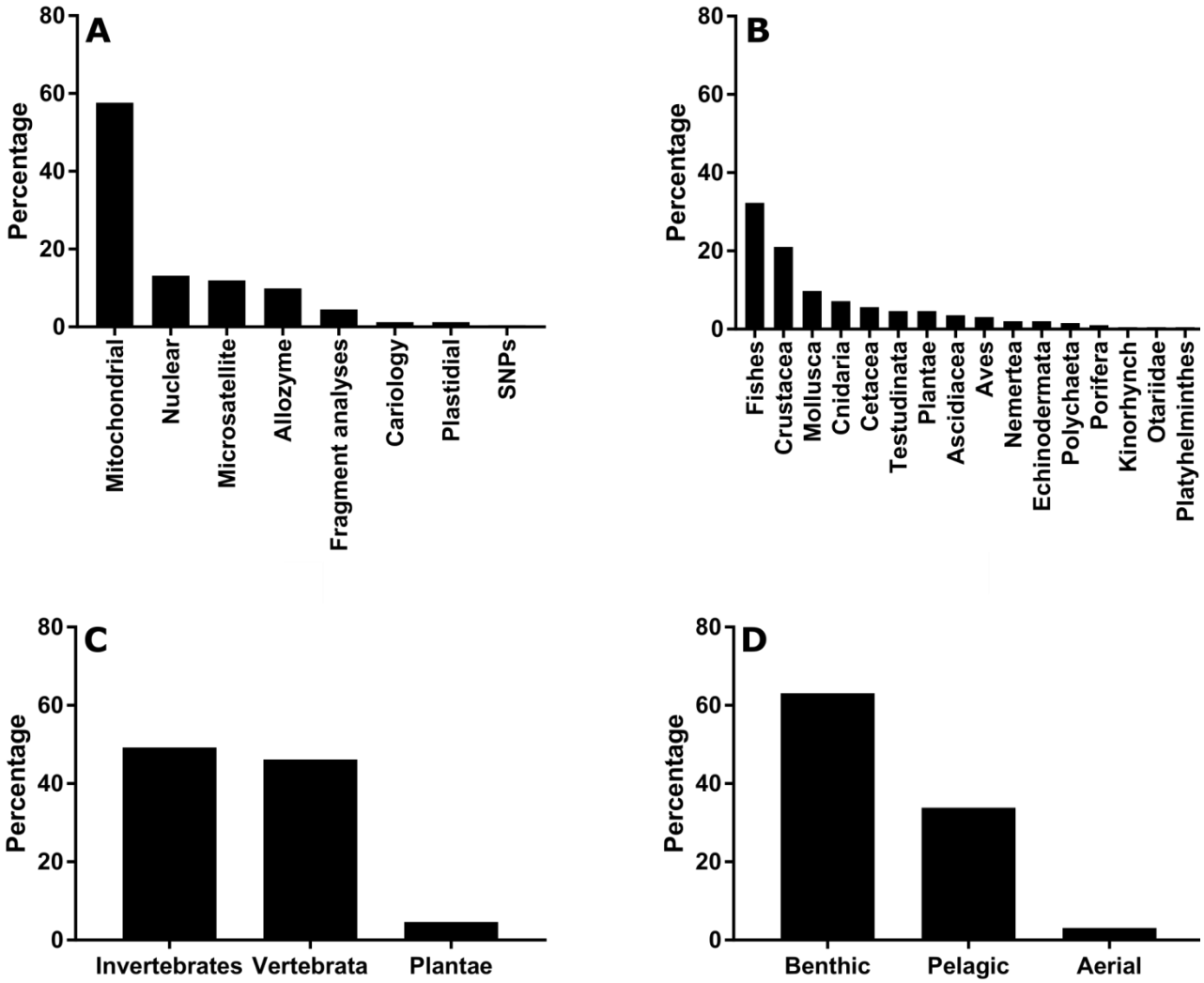

Figure 3. Proportion of marine phylogeography papers published between 1987 and 2019 with data able to test for the presence of genetic structure among populations of Brazilian marine according to (A) types of genetic markers (fragment analysis stands for ddRAD, RAPD, ISSR, AFLP, and RFLP techniques), (B) targeted taxonomic groups, (C) functional forms, and (D) marine habitat.

The two most well sampled and phylogeographically well studied geopolitical states were Rio de Janeiro and São Paulo, which happens to be the two richest states and the ones that hold the largest number of research universities in the country, followed by Bahia. The relationship between number of phylogeographic studies and economic affluence is a well-known worldwide pattern in the phylogeographic literature (reported in

Beheregaray, 2008; Turchetto-Zolet et al., 2013). The least studied regions in Brazil are those located in the extreme northern and southern reaches of the country, where urban infrastructure is reduced and accessibility challenging. Particularly the northernmost reaches, along the Amazonian Rainforest coastline, towards longitude 48° 29' 25" W, is where nearly nothing is known about the phylogeographic structure of most coastal marine species. The Amazon river plume is considered a strong barrier to gene flow for many coastal and shallow benthic species (Rocha, 2003; Rocha, Craig, & Bowen, 2007), but studies in which the Amazon river explains genetic discontinuities, usually compare northern versus southern hemisphere populations (i.e. Caribbean versus northeastern Brazil populations) sampled thousands of kilometers apart across the western Atlantic Ocean (Cóndor-Luján et al., 2021; T. O. de Souza et al., 2015; Liedke, Pinheiro, Floeter, & Bernardi, 2020; Volk, Konvalina, Floeter, Ferreira, & Hoffman, 2021). Still, for some species including deep-water reef species, the Amazon river plume is considered either a porous barrier to gene flow or a porous barrier to species dispersal (Floeter et al., 2008; Rocha, 2003; Rocha et al., 2005).

Generalized additive model

No single physical phenomenon with the power to act as a barrier to gene flow and drive genetic brakes is universal across entire biotas. Nevertheless, our *gam* and ANOVA results recognized Cape São Roque as the region with largest cross-taxa cross marker spatial phylogeographic concordance along the Brazilian coast, and represents the separation between Phylogeographic Regions I and II.

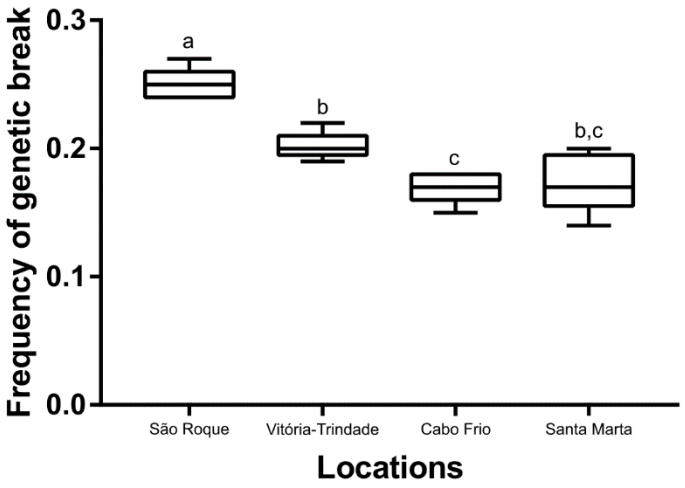

Figure 4. Box plot of the values of genetic break frequencies found at the five sites closest to three peaks identified in the generalized additive model depicted in Figure 1C, plus a forth site closest to Cabo Frio upwelling region. The line within the boxes marks the median. The boundary of the boxes indicates the 25th and 75th percentiles. Vertical line indicate the 10th and 90th percentiles. Different letters above the boxes indicate statistically significant differences ($p < 0.05$).

Phylogeographic Region I (1º N – 5º S) is characterized by North Brazil Current and hence offshore transport within this current is considered higher (Peterson & Stramma, 1991). Cape São Roque is where the South Equatorial Current, which crosses the Atlantic Ocean between 5 – 10° S, bifurcates into two nearshore boundary currents, the northward North Brazil Current and the southward Brazil Current (Molinari, 1983; Peterson & Stramma, 1991). In Brazil, the split of the SEC at or around Cape São Roque was first recognized as a barrier to gene flow for the crustacean *Panulirus argus* (Diniz, Maclean, Ogawa, Cintra, & Bentzen, 2005). Since then several other molecular studies endorsed the interaction between Cape São Roque and the split fo the SEC as a barrier to gene flow, including another crustacean *Micropogonias furnieri* (Puchnick-Legat & Levy, 2006), six fish species (Bezerra et al., 2018; Cortinhas et al., 2016; da Silva et al., 2016; Mendonça, Oliveira, Gadig, & Foresti, 2011; Montes et al., 2018; Santos, Hrbek, Farias,

Schneider, & Sampaio, 2006), mangrove species (*Rhizophora* spp.: Francisco, Mori, Alves, Tambarussi, & de Souza, 2018; Pil et al., 2011), a turtle (*Eretmochelys imbricata:* Proietti et al., 2014), and three hermatypic corals: *Mussismilia hispida*, *Favia gravida*, and *Siderastrea radians* (Peluso et al. 2018, Nunes et al. 2011).

The split of nearshore boundary currents has been recognized as a common vicariant biogeographic process promoting genetic discontinuities in continuously distributed marine species in other parts of the world as well, including species with high dispersal capabilities (Avise, 2000). Examples include the oyster *Crassostera virginica* and the red alga *Gracilaria tikvahiae* distributed north and south of Cape Canaveral where the northward Gulf Stream is deflected offshore and southward costal currents arrive from northern latitudes (Gurgel, Fredencq, & Norris, 2004; Reeb & Avise, 1990), and *Durvillaea antarctica* kelp populations distributed north and south of Taitao Peninsula at latitude ~50º S in Chile where occurs the splits of the Antarctic Circumpolar Current into the Humboldt Current and the Cape Horn current (Fraser, Thiel, Spencer, & Waters, 2010). With respect to Cape São Roque, however, our findings do not match previous biogeographic studies such as the coastal marine realms, ecoregions and provinces recognized by Spalding et al. (2007), including Brazilian reef fish biogeography (Floeter et al., 2008) and subprovinces (Pinheiro et al., 2018).

Phylogeographic Region II (5° S – 20.5° S) presents a constant relative high levels of genetic breaks averaging 20%. Region II is influenced in its entirety by the first half of the Brazil Current. The Brazil Current and its connectivity capabilities are considered weak compared to other boundary currents because most of the water mass from the SEC is diverted northward into the North Brazil Current (Peterson & Stramma, 1991). Traditionally, physical barriers within this region are considered weak, porous, intermittent or species-specific (da Silva et al., 2016). Other processes are used to explain the origin and maintenance of relative high levels of genetic breaks in this region. Within Region II a

range of other potential physical barriers are traditionally associated with the mouth of large rivers such as the São Francisco, the Paraguaçu, Jequitinhonha, and the Doce and Pleistocene refugia (Ayres-Ostrock et al., 2019). River-associated vicariance along the Brazilian eastern coast was first proposed for terrestrial lizards (Pellegrino et al., 2005) and then discussed for marine taxa, such as the mollusk *Anomalocardia brasiliana* (Arruda, Beasley, Vallinoto, Marques-Silva, & Tagliaro, 2009) and the fish *Rhizoprionodon porosus* (Mendonça et al., 2011).

Within Phylogeographic Region II the São Francisco river (which corresponds to one of the largest river basins in South America) is likely a major filter helping define biogeographic subprovincial boundaries of reef fishes (Pinheiro et al., 2018). Freshwater and sediment-rich discharge at the mouth of the São Francisco river mouth alter costal water turbidity and salinity, inhibiting the development of major coral reefs at Sergipe and Alagoas states (da Silva et al., 2016). The reduction in coral reefs allows tides to penetrate further into estuaries, contributing to the establishment of local mangroves, changing the coastal ecosystem and increasing coastal heterogeneity (da Silva et al., 2016). Therefore, across Region II a mosaic of different habitats are formed encompassing coral reefs, sedimentary reefs, mangroves, seagrass beds and the inflow of distinctive freshwater masses (da Silva et al., 2016). Region II shows relatively high values of genetic break frequencies without an apparent relevant physical barrier to gene flow. It is very likely that sympatric or parapratric ecological diversification is playing major role in this region. Sympatric and parapatric genetic differentiation along ecological boundaries, and latitudinal and environmental gradients in marine and freshwater habitats, particularly in tropical regions, are common (Bowen et al., 2013; Nanninga, Saenz-Agudelo, Manica, & Berumen, 2014). Genetic breaks form without the occurrence of physical barriers to gene flow usually in highly heterogeneous or diverse habitats (e.g. DiBattista et al., 2012; Winkelmann, Genner, Takahashi, & Rüber, 2014). As such, higher levels of genetic break

frequencies along Phylogeographic Region II seems to be the result of the combination of different kinds of hard, soft or intermittent physical barriers, including sympatric and parapatric adaptations to ecological differences within and between adjacent areas, a complex scenario with generative circumstances for the birth of genetic differentiation in the sea (Bowen et al., 2013).

At the southern end of Region II, between latitudes 16° S and 20.5° S, the continental shelf becomes shallower and wider to form the Abrolhos Archipelago Basin and eastward Vitória-Trindade seamount chain. Six relatively shallow (10–110 m) 250 km apart seamount characterizes this chain, stretching from mainland until Trindade Island (20º 31' S, 29º 19' W). This region splits the Brazilian coastline into two sections, especially during past periods Quaternary's glacial maxima, when the sea level was 110 m lower than the present day (Peterson & Stramma, 1991; Stramma & England, 1999) and the tropical-temperate transition zone was closer to the equator. The emersion of wider offshore reefs and islands areas in the Abrolhos Arquipelago and the tops of Vitoria-Trindade seamount chain caused strong changes in the flow of the Brazil Current, helping deflect offshore part of its flow. This unique interaction between shelf geomorphology, changes in oceanography, and the presence of a norther sharper tropical–temperate transition zone during periods of Pleistocene glacial maxima is considered a major intermittent barriers of gene flow for several marine organisms, such as mollusks (*Crassostrea* spp.: Lazoski et al., 2011), crustaceans (*Excirolana braziliensis*: Hurtado et al., 2016),polychaete (*Perinereis* spp.: Paiva et al., 2019), and coral (*Mussismilia braziliensis*: Menezes, Sobral-Souza, Silva, & Solferini, 2020). Our results agree with these observations. The genetic discontinuities between populations sampled north and south of the Abrolhos Basin and the Vitória-Trindade seamount region most likely originated in the Pleistocene and remained structured to this day, even after the barrier is no longer active, with the help of other physical and ecological processes.

Region III (20.5° S – 28° S latitude) ranges from the Vitória-Trindade seamount chain to Cape Santa Marta representing a region with the lowest values of genetic break frequencies. The Cabo Frio seasonal upwelling system in the north of the Rio de Janeiro (around 22° S) (Valentin, Andre, & Jacob, 1987) is situated within Region III and has been considered a popular barrier hypothesis for coastal benthic marine species (Peluso et al., 2018). The upwelling occurs with great intensity during austral summer. It is a consequence of seasonal northeast winds, abrupt change in the continental shelf shape and slope, resulting in the upwelling of the South Atlantic Central Water (Valentin et al., 1987). The Cabo Frio upwelling system could act as a barrier to gene flow in two ways: a) physical/physiological barriers due to conspicuous shifts in sea temperatures, or b) local selection limiting recruitment of certain genotypes (Peluso et al., 2018). We detected the presence of a weak sign of phylogeographic concordance in this region. Few studies support the existence of a hard barrier to gene flow in this area: a fish species (*Atherinella brasiliensis*: Cortinhas et al., 2016), two crustaceans (*Excirolana braziliensis*: Hurtado et al., 2016; *Litopenaeus schmitti*: Maggioni, Rogers, & Maclean, 2003), and a cetacean (*Tursiops truncatus*: de Oliveira et al., 2019). Macroecological studies however recognize this region as transition zone among biogeographic provinces (Boschi, 2000; Briggs, 1974; Floeter et al., 2008; Hempel & Sherman, 2003; Palacio, 1982; Spalding et al., 2007). Consequently, the Cabo Frio upwelling system, might be acting more as a genetic filter rather than a barrier to gene flow for the majority of marine organisms, an inference that is supported by empirical molecular data from corals (*Mussismilia hispida* and *Siderastrea stellate*, Peluso et al., 2018)) and some fish species (Affonso & Galetti, 2007; Santos et al., 2006).

The lowest values of genetic break frequency in the Brazilian coast were observed within Region III. Two processes might explain this pattern. First, the Brazil Current south from 24°S actually intensifies its flow at a rate of about 5% per 100 km (Gordon &

Greengrove, 1986), possibly increasing levels of costal connectivity, and hence panmixia. Secondly, many sympatric tropical marine species in the area tend to retain new mutations and show low levels of genetic diversity, which is a signature of shared past history of generalized low effective population sizes caused by bottlenecks or founder events (Grant, 1998). Within Region III, several studies report this particular genetic pattern such as fishes (da Silva et al., 2016; Machado et al., 2017; Santos et al., 2006), macroalgae (*Crassiphycus caudatus:* Ayres-Ostrock et al., 2019; *Hypnea pseudomusciformis*: Nauer et al., 2019), marine vascular plants (*Rhizophora* spp.: Francisco et al., 2018), a cnidarian (*Mussismilia hispida*: Peluso et al., 2018), nemerteans (*Ototyphlonemertes* spp.: Andrade, Norenburg, & Solferini, 2011), polychaetes (*Perinereis* spp.:Paiva et al., 2019), and a sea turtle (*Caretta caretta*: Reis et al., 2010). This concordance is most likely the result of demographic expansion of tropical populations within Region III promoted by the gradual warming after glacial maxima. Recent spatially and demographically expanded populations into new areas tend to show not only low genetic diversity but also lack of genetic structure (= founder effect). We found only three studies with opposite results to this pattern: two fish studies (*Galeocerdo cuvier*: Carmo et al., 2019; *Macrodon atricauda*: Rodrigues et al., 2014), and a dolphin study (*Pontoporia blainvillei*: Secchi et al., 1998). These three species are not exclusively tropical species, they occur in higher latitudes, and do well and present higher genetic diversity in cold temperate waters. Therefore, it is possible that these three species might have been less affected during glaciation periods compared to tropical and subtropical species that currently inhabit southern Brazil (Regions III and IV). Thus, the two processes described above are not antagonistic but might have been acting together to the observed phylogeographic concordance.

Within Region III, the two sites with the lowest genetic break values in this region coincided with the two largest local freshwater estuaries, the Paranaguá (25° 28’ S) and the Guaratuba (25° 52' S) estuaries (6.94% and 7.25%, respectively). Both bays represent

in fact two large subtropical estuarine systems that reaches the coast through tidal channels characterized by extensive sand beaches, salt marshes, and small shallow rocky shores (Lana, Marone, Lopes, & Machado, 2001). Most importantly, the coastline of both bays are dominated by mangrove swamps (Lana et al., 2001; Marone et al., 2006). Large estuaries are expected to act as physical barriers to gene flow because they often lack large areas of consolidate substrate (upon which richer benthic biotas develop, e.g. rocky and reefs) and disrupt coastal marine environmental conditions by dumping large quantities of freshwater, nutrients, sediments, influencing tides and coastal currents. Instead, our results suggest that these two estuaries represent areas of higher genetic connectivity among adjacent costal marine populations. At the same time, the Paraná state coastline has been scantily studied area with respect to phylogeographic studies and our results could be a effect of poor sampling. Further high resolution population genetic studies across Region III are necessary to elucidate the presence or absence of high levels of phylogeographic concordance in the area.

Low levels of genetic break frequencies in Region III are interrupted at Cape Santa Marta where the third highest peak in our *gam* result marks the beginning of Phylogeographic Region IV (28° S – 32° S). Cape Santa Marta presents a series of particular geographic, climatic, and oceanographic features that identifies this cape as a potential barrier (Campos, Möller, Piola, & Palma, 2013). Firstly, strong variations in seasonal wind field affect the composition of local water masses and their circulation promoting the occurrence of a less known summer coastal upwelling. In Brazil, coastal upwelling cause not only significant drops in water temperature but also increases in nutrient and salinity concentration. Meanwhile, presently and in the winter, the Malvinas Current increases its influence on the Brazilian coast helping the Brazil Coastal Current transport low salinity waters as well as terrigenous material from the Rio de la Plata drainage basin and the Patos Estuary outflow northward, all the way up to Cape Santa

Marta (Campos et al., 2013; Piola, Matano, Palma, Möller, & Campos, 2005). As such Cape Santa Marta might act as a barrier by deflecting the local winter influence of the Malvina's Current offshore (Campos et al., 2013; Peterson & Stramma, 1991).

The Brazil-Malvinas Confluence has been proposed a physical barrier to gene flow (Cortinhas et al., 2016; Vasconcellos, Lima, Bonhomme, Vianna, & Solé-Cava, 2015). The northern limit of the Brazil-Malvinas Confluence moves seasonally from 30 – 35° S during the winter to 40° S – 46° S during the summer (Clauzet, Wainer, Lazar, Brady, & Otto-Bliesner, 2007; Peterson & Stramma, 1991). Currently, the Brazil-Malvinas Confluence is outside our study area. However, during Pleistocene glaciations, the northern limit of Brazil-Malvinas Confluence might have shifted northward towards Cape Santa Marta, possibly further promoting genetic breaks in the region. Regardless where the Brazil-Malvinas Confluence occurs, a dramatically steep gradient in sea-surface temperature is found, reaching 1° C/km (which in itself can act as a barrier) and both currents are deflected from the continental margin to flow offshore in a south-eastward direction (Peterson & Stramma, 1991).

Cape Santa Marta surrounding areas that present high values of genetic break frequencies could also be under the influence of the ecological speciation as the coast becomes permeated with several consecutive small estuaries intercalated with open sea areas. These features can act as barriers, specially to fish species (Beheregaray & Levy, 2000; Rodrigues et al., 2014), cetaceans (Costa et al., 2015; Fruet et al., 2014; Secchi et al., 1998), and crustaceans (Weber & Levy, 2000). Geographically close estuaries can be very different from each other in terms of salinity, temperature, water quality and food stocks. Thus, populations can use different estuaries for either reproduction or foraging, promoting local adaptations. Geographically close populations using different estuaries can present genetic discontinuity, even in the presence of gene flow, due to differences in estuaries usage and ecology driven speciation (Rocha et al., 2005; Weber & Levy, 2000).

Capes, sharp shifts in boundary current direction, and changes in oceanic masses are often identified as barriers by creating oceanographic discontinuities (e.g. Gurgel et al., 2004; Jennings, Shank, Mullineaux, & Halanych, 2009; Teske, Von Der Heyden, McQuaid, & Barker, 2011). Biogeographically, Cape Santa Marta region (~ 30º S) marks the shift between the end of the Brazilian biogeographic tropical province and the beginning of the Argentinian 'warm-temperate' provinces according to Briggs & Bowen (2013). In addition, this area also coincides with the southern-end of the biogeographical Brazilian Marine Province, identified by the end of Santa Catarina rocky reefs (Aued et al., 2018; Briggs & Bowen, 2013; Floeter et al., 2001). Lastly, the southern reaches of Region IV coincides with the north end of the Rio Grande do Sul state, a region that include the world's longest beach, Cassino beach, with 220 kilometers of unconsolidated sandy substratum. Long stretches of sandy beaches have been considered putative barriers for hard bottom marine species (Cruz et al., 2021; Mmonwa, Barker, McQuaid, & Teske, 2021; Nauer et al., 2019; Trovant et al., 2016). However, we could not test if Cassino beach can be considered a barrier because this region is located at the geographic extreme of our study area.

Phylogeography has originated from biogeography, considering that geographic regions are identified by concordant patterns in populations composition (Bowen et al., 2016). Two main approaches have been used to delimit biogeographical regions, oceanographical/geological features (Luiz et al., 2012; Spalding et al., 2007) and species composition (Floeter et al., 2001, 2008; Targino & Gomes, 2020). These two approaches often lack in precision when analyzing sub-provinces within main provinces. This occurs because there is a lack of major barriers in the marine environment and the fact that species composition recalls very old events. By analyzing genetic discontinuities between populations of a same species and using several distinct species, we have identified locations with large frequencies of genetic breaks, which coincide with geographic features. In this study, we also proposed four phylogeographic regions within the Brazil.

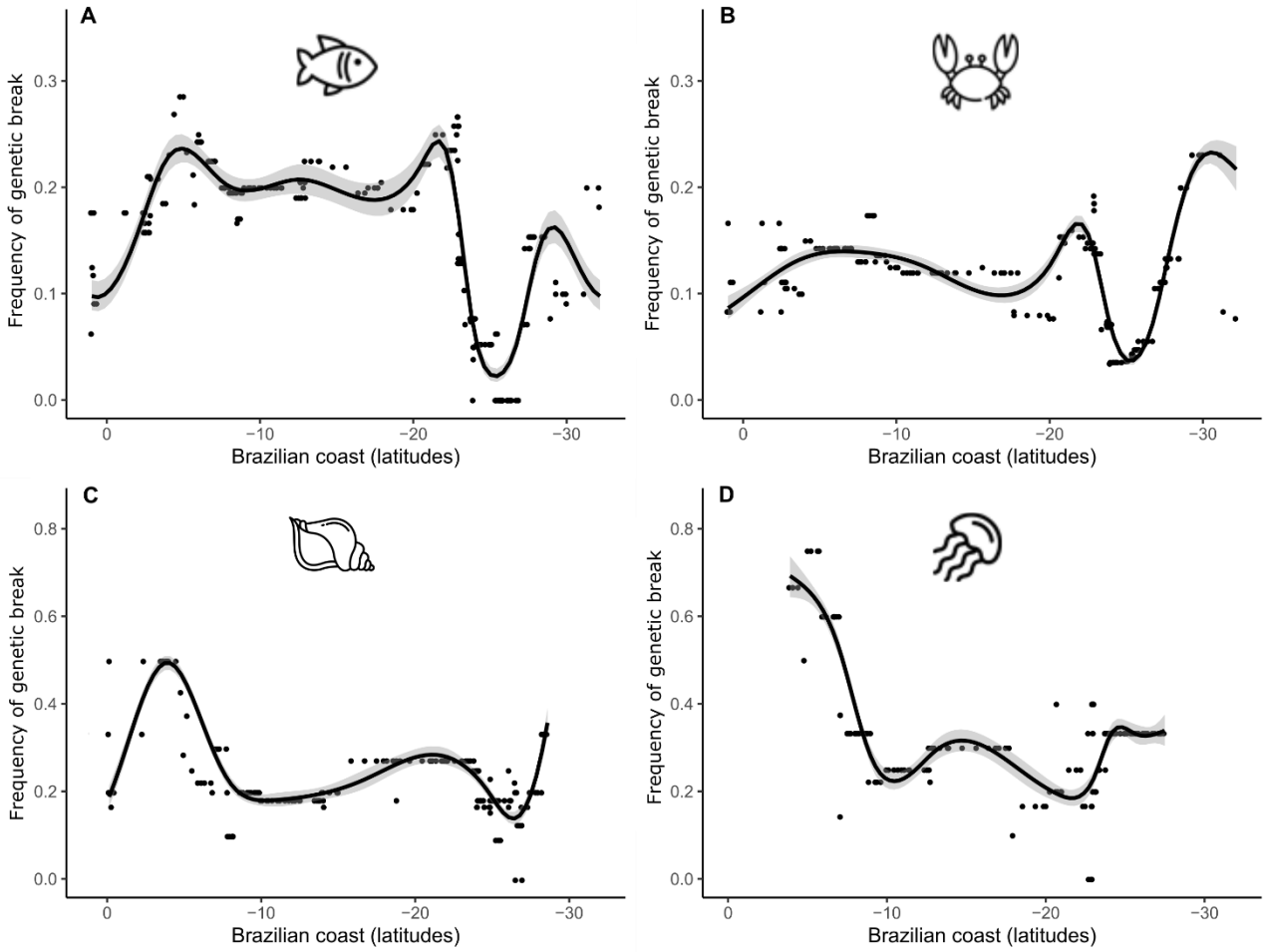

Figure 5. Generalized additive models for the frequency of genetic breaks as a function of latitude for populations of different marine taxa recorded along the Brazilian coast. Data collected from phylogeography papers published between 1991 and 2019 with data able to test for the presence of genetic structure. (A) Fishes, (B) Crustaceans, (C) Mollusks, and (D) Cnidarians. Y-axis labels are not standardized. Icons represent each taxonomic group and were obtained from flaticon.com.

Fishes are the only taxa so far where the processes promoting genetic discontinuity along the Brazilian coast seem to be equally strong at both, Cape São Roque and the Vitória-Trindade seamount chain regions, and to a lesser extent at Cape Santa Marta. For crustaceans, the Vitória-Trindade seamount region does not seem to be a major barrier,

as observed for other taxa. The main crustacean barrier to gene flow occurs near Cape Santa Marta whereas the Cape São Roque represents a more noticeable barrier for mollusks. For the cnidarians there is a strong barriers at northernmost sampled zone and two secondary peaks: one at Abrolhos Reef System (18º S) and another at Santos city (23º S). The cnidarian data should be interpreted with care, considering the lower number of data (n > 3).

Concluding remarks

Brazilian phylogeographic studies are still in their infancy when compared to others biomes and areas of the world. To date, no phylogeographic review has been conducted focusing on Brazilian marine organisms. This study aimed to review the literature, identify the regions of highest spatial phylogeographic concordance and recognize the processes responsible for their origin and maintenance along the Brazilian coast. In this study, Cape São Roque the region where the South Equatorial Current splits into two was the location that presented the largest values of cross-taxa cross-marker genetic discontinuities. These results suggest that this feature represents a convergence of extant and extinct geographic, oceanographic, historical, climatic and ecological processes that makes the most significant driver of genetic differentiation among continuously distributed species along the Brazilian coast. Vitória-Trindade seamount chain represents the second most important region of genetic discontinuities along the Brazilian coast, especially to fishes. The Cabo Frio seasonal upwelling system, although frequently pointed in literature as potential strong barrier, presented lower levels of phylogeographic concordance. On the other hand, the meeting of the Brazil-Malvinas Current confluence, Cape Santa Marta, and the string of estuaries in southern Brazil turned out to be a region of higher levels of genetic breaks as well, representing other major drivers of genetic breaks.

Due to the lack of phylogeographic studies sampling at both extremes of the Brazilian coast, we could not test the effects of the Amazon river outflow and the Rio Grande do Sul long extensions of unconsolidated benthic subtract correspond to major barriers to gene flow for multiple taxa. Future phylogeographic publications will help improve the datasets and consequently the model produced in this study, allowing more spatially refined predictions of areas of high phylogeographic concordance, and the recognition of the likely processes responsible for the origin.

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Chapter 2

Colpomenia sinuosa (Ectocarpales, Phaeophyceae) hidden lineages along the Brazilian coast

Colpomenia sinuosa (Ectocarpales, Phaeophyceae) com linhagens ocultas ao longo da costa brasileira

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Running title: *Colpomenia sinuosa* diversity in Brazil

Abstract

Identification of *Colpomenia* species has been traditionally based on vegetative and reproductive characteristics of the macrothallus. However, *Colpomenia* gametophytic macrothalli are extremely simple, often morphologically plastic, and frequently lack reproductive structures, what makes morphological identification difficult. In Brazil, *C. sinuosa* is morphologically described as the only occurring species. Its distribution ranges from Ceará (northeastern Brazil) to Rio Grande do Sul (southern Brazil), throughout the whole year and forming a continuous coverage in some rocky shores. In the world, at least three *C. sinuosa* lineages are described, two of which occurring in Brazil. We have morphologically and molecularly (*cox*3) investigated the *Colpomenia* diversity along the Brazilian coast. Species delimitation methods and genetic divergence analyses suggest the presence of five hidden lineages within *C. sinuosa* in the world, considering that four occurs in Brazil, including the true *C. sinuosa*. However, no morphological differences could be observed between lineages and then, despite the high divergences between the clades, we have decided not to describe new species. We recommend further studies to test different approaches, such as mating compatibility and Next-Generation Sequencing (e.g. phylogenomics).

Keywords: barcode, Brazil, *Colpomenia sinuosa, cox*3, genetic variation, hidden lineages, Phaeophyceae, phylogenetic diversity, species delimitation methods

Resumo: A identificação de espécies de *Colpomenia* é tradicionalmente baseada nas características vegetativas e reprodutivas do talo. No entanto, os talos de *Colpomenia* são extremamente simples, muitas vezes morfologicamente plásticos e, frequentemente, carecem de estruturas reprodutivas, o que dificulta a identificação morfológica. No Brasil, *C. sinuosa* é a única espécie morfologicamente descrita. Sua distribuição ocorre do Ceará (nordeste do Brasil) ao Rio Grande do Sul (sul do Brasil), durante todo o ano e formando uma cobertura contínua em alguns costões rochosos. No mundo, são descritas pelo menos três linhagens de *C. sinuosa*, duas das quais ocorrem no Brasil. Investigamos a diversidade morfológica e molecular (*cox*3) de *Colpomenia* ao longo da costa brasileira. Os métodos de delimitação de espécies e as análises de divergência genética sugerem a presença de cinco linhagens de *C. sinuosa* no mundo, sendo que quatro ocorrem no Brasil, incluindo a verdadeira *C. sinuosa*. No entanto, não foram observadas diferenças morfológicas entre as linhagens e, então, apesar das altas divergências entre os clados, decidimos não descrever novas espécies. Recomendamos mais estudos para testar diferentes abordagens, como compatibilidade de cruzamento e sequenciamento de próxima geração (por exemplo, filogenômica).

Palavras-Chave: *barcode*, Brasil, *Colpomenia sinuosa*, *cox*3, diversidade filogenética, linhagens ocultas, métodos de delimitação de espécies, Phaeophyceae, variação genética

Introduction

Colpomenia (Endlicher) Derbès & Solier is a marine brown genus with a cosmopolitan distribution, characterized by an anatomically simple, hollow sacciform, vesicular and membranous thallus with smooth to rough appearance growing isolated or in clumps, with reproductive plurilocular structures organized in sori (Cormaci et al. 2012, Song et al. 2019). The life history of *Colpomenia* is heteromorphic and diplobiontic with conspicuous vesicular gametophytes (macrothalli) and inconspicuous, environmentally cryptic, filamentous or pulvinated sporophytes (microthalli) (Freitas Toste et al. 2003).

Colpomenia species are important primary producers and potentially economically valuable. *Colpomenia* species have been proposed as bioindicators (Guerra-García et al. 2006), as model-organism in photosynthesis (Forster and Dring 1994) and in heavy metal uptake studies (Cirik et al. 2012, Zarei and Niad 2020), a food item for amphipods (Poore and Steinberg 1999) and as feed for farmed animals and for human consumption (Wong et al. 1999, Rohani-Ghadikolaei et al. 2012, Tabarsa et al. 2012). *Colpomenia* spp. natural extracts revealed pharmaceutical properties with antimicrobial (Demirel et al. 2009, Salem et al. 2019), antifungal (Mohy El-Din and Mohyeldin 2018), antioxidant (Martins et al. 2013) and antitumoral activities (Khanavi et al. 2010). *Colpomenia* species have also being proposed as primary product for ethanol (Hamouda et al. 2018) and biogas production (El Asri et al. 2017), and useful in the dye textile industry (Azeem et al. 2019).

Identification of *Colpomenia* species has been traditionally based on vegetative and reproductive characteristics of the macrothallus. However, *Colpomenia* gametophytic macrothalli are extremely simple, often morphologically plastic, and frequently lack reproductive structures, making morphological identification difficult. A recent study of DNA barcoding from Australian samples supported the species separation, showing that the diagnostic morphological characters used to identify *Colpomenia* species in Australia are reliable (Martins et al. 2021). Morphological variation between putative *Colpomenia*

species in Australia proposed by Womersley (1967) were tested by Clayton (1975) who recognized *C. sinuosa* (Mertens ex Roth) Derbès & Solier and *C. peregrina* Sauvageau as distinct species by circumscribing them using morphometric and statistical analyses. The number of medullary cell layers, sori shape, and presence or absence of cuticle on the plurilocular sori were the only statistically significant different anatomical features reliably capable to distinguish *C. peregrina* from *C. sinuosa* in Australia (Clayton 1975). However, several other morphological diagnostic characters are still used to distinguish *C. sinuosa, C. peregrina* and *C. claytoniae* S.M.Boo, K.M.Lee, G.Y.Cho & W.Nelson, such as: thallus color, shape and diameter, surface configuration, wall thickness, phaeophycean hair origin, number of cortex and medulla cells, length of plurilocular structures, and length of paraphyses relative to plurilocular structures (Song et al. 2019). Morphological variation of some of these characters, especially in thallus shape, were observed within *C. sinuosa* from several regions along the Brazilian coast (Semir 1977, Széchy and Cordeiro-Marino 1991, Nunes and Paula 2004, Ouriques and Cordeiro-Marino 2004), as well as for several countries (Lee et al. 2013), and also in *C. peregrina* (Lee et al. 2014) and in *C. claytoniae* (Boo et al. 2011).

Some molecular studies have detected cryptic diversity and possible species complex within *Colpomenia* species, such as *C. sinuosa* (Cho et al. 2009, Lee et al. 2013), *C. peregrina* (McDevit and Saunders 2009, Lee et al. 2014) and, *C. claytoniae* (Martins et al. 2021). In a worldwide approach, the existence of two major global lineages were proposed within *C. sinuosa*: a northern and a southern hemisphere lineages (Cho et al. 2009). In a later worldwide approach, Lee et al. (2013) described a more complex diversity within *C. sinuosa*, consisting of three major lineages with several sub-lineages. However, the lack of any morphological differences among the three major groups led the authors to consider the three genetic lineages as cryptic diversity. Two of these major lineages were described to occur in Brazil (specifically in Búzios, Rio de Janeiro, southeastern Brazil).

For *C. peregrina*, McDevit and Saunders (2009) detected three lineages occurring in Canada. Later, in another worldwide study, Lee et al. (2014) identified four major *C. peregrina* lineages. Lastly, Martins et al. (2021) detected three major *C. claytoniae* lineages occurring in Australia. All these studies suggested the occurrence of possible cryptic species within *Colpomenia* species. For example, McDevit and Saunders (2009) detected the genetic divergence among *C. peregrina* COI-5P lineages (1.73%) to be higher than intraspecific divergence (~0.46%), and lower than the intrageneric divergence (~3.04%) observed for several brown macroalgal species, suggesting cryptic speciation.

Morphological diagnosis among *Colpomenia* cryptic complex species and in other macroalgae cases frequently makes defining species problematic (McDevit and Saunders 2009, Lee et al. 2013, Martins et al. 2021) or even impossible based on morphological characters alone (Saunders 2005, Vieira et al. 2014, Leliaert et al. 2018, Song et al. 2019). Molecular techniques are revealing that ubiquitous macroalgal species often comprise complexes of cryptic and quasi-cryptic species (Zuccarello and West 2003, Verbruggen et al. 2005, Vieira et al. 2014, Nauer et al. 2015, 2019, Ximenes et al. 2017, Leliaert et al. 2018, Pestana et al. 2020). The genus *Colpomenia* currently has ten taxonomically accepted species (Guiry and Guiry 2021). To date, only seven out of these ten have been worldwide molecularly characterized using different markers: *C. claytoniae*, *C. ecuticulata* M.J.Parsons, *C. expansa* (D.A.Saunders) Y.-P.Lee, *C. peregrina*, *C. ramosa* W.R.Taylor, *C. sinuosa*, the generic type, and *C. tuberculata* D.A.Saunders (GenBank, accessed on October 2021).

Among *Colpomenia* species, *C. sinuosa* is one of most conspicuous member of intertidal and shallow subtidal rocky shores and reef systems, traditionally recognized as a nearly ubiquitous macroalgae, being recorded all over the tropical and temperate world (Guiry and Guiry 2021). In Brazil, *C. sinuosa* is reported as the only occurring species of the genus (Flora do Brasil 2020, Guiry and Guiry 2021), occurring from Ceará

(northeastern Brazil) to Rio Grande do Sul (southern Brazil) (Flora do Brasil 2020). *Colpomenia sinuosa* is characterized by 1 to 15 cm spherical bladder-like to convoluted (cerebriform), vesicular, hollow and membranous thallus that can grow isolated or in clumps (Freitas Toste et al. 2003, Cormaci et al. 2012, Lee et al. 2013, Guimarães et al. 2016, Song et al. 2019). In Brazil, *C. sinuosa* is characterized as abundant throughout the whole year, forming a continuous coverage in some rocky shore (Széchy and Cordeiro-Marino 1991, Nunes and Paula 2004, Ouriques and Cordeiro-Marino 2004). The surface configuration (smooth vs. rough) was tested as two different species by Semir (1977), but his culture experiments failed to distinguish them. However, smooth surface is more common in juvenile and sheltered sites, and rough surface is more common in strong hydrodynamic sites, even though both can co-occur (Semir 1977, Széchy and Cordeiro-Marino 1991, Ouriques and Cordeiro-Marino 2004). Life-history studies in culture on *C. sinuosa* and *Chnoospora minima* (Hering) Papenfuss from Brazilian material were carried out by Fernandes (2007); however, the development of the whole life history for *C. sinuosa* failed in culture. To date, only one study has performed molecular identification of *C. sinuosa* from Brazil (Lee et al. 2013), who have detected two distinct lineages and possibly two cryptic species; however, this study was limited to few specimens from a single beach (Rasa, Búzios, Rio de Janeiro).

Due to *Colpomenia* ecological and economical importance, it is crucial to recognize and characterize genetically distinct populations that could be holding significant biodiversity. However, biodiversity studies within *C. sinuosa* are lacking in Brazil. Thus, in this study our aim was to use detailed morphological observations and molecular analyses to assess the *Colpomenia* diversity in Brazil and to establish if *C. sinuosa* is the only occurring species or if there are many species, including cryptic diversity throughout its whole distribution in Brazil.

Material and methods

Taxon sampling

A total of 246 specimens of *Colpomenia sinuosa* were collected from 15 populations sampled along the Brazilian coast, ranging from Ceará state (latitude ~4° S) to Santa Catarina state (latitude ~27° S) between 2016 and 2019 (Table S1). Specimens were located at least 10 cm apart, to avoid collecting organisms in the same clumps, which were growing either epilithically, epiphytically or drifted. All sampled specimens were cleaned from epiphytes, rinsed in local seawater, and then desiccated in silica gel.

Morphological studies

Samples were fixed in formalin 4% for morphological analysis from portions from the same individual separated for molecular analysis. The material was analyzed in detail under Stemi 305 EDU stereomicroscope (Zeiss, Göttingen, Germany) and Primo Star optical microscope (Zeiss, Göttingen, Germany). Photographic documentation of whole specimens was done with a Sony Cyber Short W570 digital camera (Tokyo, Japan) representing the most common thallus habit and the morphological variations; vegetative and reproductive microscopic diagnostic characters were made with the Sony Cyber Short W570 manually coupled to a Primo Star optical microscope. Transverse hand sections were obtained with a razor blade and stained with 0.5% aqueous aniline blue and acidified with 1 N HCl. For each specimen studied, a minimum of 10 measurements of each morphometric character were made. A total of nine individuals from the true *C. sinuosa* Lineage and three for each of the remaining Lineages were examined. Measurements are given as length × diameter. Vouchers were deposited in the herbarium of University of São Paulo (SPF), Brazil.

DNA extraction, PCR and sequence alignment

Dried specimens were once again cleaned from potential remaining epiphytes under a Stemi SV 6-Zeiss stereomicroscope (Zeiss, Göttingen, Germany) in the laboratory prior to DNA extraction. For DNA extraction, subsamples of the silica gel dried samples were ground to a fine powder with a mortar and pestle. DNA was extracted using NucleoSpin® Plant II (Macherey-Nagel GmbH & Co, Germany) according to the manufacturer's instructions. The extracted DNA was stored at -20° C until PCR was performed. For *cox*3 gene amplification we used primers F49 and R20 described in Boo et al. (2010). *Cox*3 PCR reactions were conducted in a total volume of 25 µL under one of two protocols: i) 1.0 – 10.0 µg of genomic DNA, 0.2 mM each of deoxynucleotide triphosphate (dNTP), 0.2 mM of each primer, 1X PCR Green Buffer minus Mg (Promega Corp., Madison, WI, EUA), 3 mM of $MgCl_2$, 0.5 µg of BSA, and 0.625 units of Taq polymerase (Promega Corp., Madison, WI, EUA); or ii) 0.001 – 1.0 µg of genomic DNA, 0.4 mM each of deoxynucleotide triphosphate (dNTP), 0.4 mM of each primer, 1X PCR Green Buffer minus Mg (Promega Corp., Madison, WI, EUA), 3 mM of $MgCl_2$, 0.3 M of filtered betaine, and 1.25 units of Taq polymerase (Promega Corp., Madison, WI, EUA). PCR cycle parameters followed Boo et al. (2011).

All the PCR were carried out on a Techne TC-4000 thermocycler (Bibby Scientific Ltd., Staffordshire, UK). PCR products were purified using column GFX TM PCR DNA and Gel Band Purification Kit (GE Healthcare, Pittsburgh, PA, USA) according to the manufacturers' protocol. Sequencing of 10–40 ng purified PCR product was performed using the same primers as those used for PCR amplifications and BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Capillary separation was performed on a 3100 ABI PRISM TM automatic DNA sequencer (Applied Biosystems, Carlsbad, CA, USA). All sequences were manually aligned using BioEdit (Hall 1999). Inconsistencies in sequences were reviewed by checking the electropherograms.

Phylogenetic analysis

A maximum likelihood (ML) tree, Bayesian inference (BI) and pairwise genetic distance matrixes were performed for the *cox*3 marker, applying HKY+I+G evolutionary model, selected under the Akaike information criterion (AIC) implemented on the IQ-Tree web server (accessed at iqtree.cibiv.univie.ac.at, Trifinopoulos et al. 2016). ML was performed with 1,000 bootstrap replicates using IQ-Tree v.1.4.3 on the IQ-Tree web portal. BI trees were estimated employing birth and death tree model prior available in BEAST (ver. 2.6.0, see www.beast2.org/; Bouckaert et al. 2019). The MCMC chain lengths were 100 million generations, sampling every 1000 generations, and 10 initialization attempts. We used a 10% burn-in value. MCMC chain convergence was assessed when all parameters reached effective sample size (ESS) values above 200 in Tracer (ver. 1.7, see www.beast2.org/tracer-2/; Rambaut et al. 2018). TreeAnnotator (ver. 2.6.0, see www.beast2.org/treeannotator/) was used to identify the most credible tree. Distance matrixes were built in PAUP* 4.0a167 (Swofford 2014).

Species delimitation methods

Species delimitation methods (SDM) used different approaches of five major techniques: automatic barcode gap discovery (ABGD, Puillandre *et al.* 2012), statistical parsimony network (SPN, Templeton *et al.* 1992), the Poisson tree processes (PTP, Zhang *et al.* 2013), the general mixed yule coalescent model (GMYC, Pons *et al.* 2006), and the assemble species by automatic partitioning (ASAP, Puillandre et al. 2021).

The ABGD analyses were performed with the online implementation (bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html) using distance matrix as input. ABGD analysis was run with the following parameters: 0.001 minimum intraspecific distance (pmin), 0.1 maximum intraspecific distance (pmax), 10 steps, and 1.5 relative gap width. ABGD initial and recursive partition approaches were considered. SPNs were built with

TCS 1.2.1 (Clement et al. 2000) using 95% and 99% confidence limits as haplotype connection limits. PTP analysis used a ML tree constructed as described below but excluding outgroup sequences. PTP was performed with the online implementation (http://species.h-its.org) under the following parameters: 100,000 MCMC generations, thinning = 100, and burn-in = 0.1. Only the PTP maximum likelihood approach was considered. The ASAP analyses were performed with the online implementation (https://bioinfo.mnhn.fr/abi/public/asap/#) using the distance matrix as input. ASAP analysis were run splitting groups below 0.01 probability and adopting the lowest score, p-value and W values.

For GMYC analyses, four Bayesian ultrametric trees were estimated employing distinct tree model priors available in BEAST 2.6.0 (Bouckaert et al. 2019): the Yule 'birth-only' speciation model (Bouckaert et al. 2019), the birth & death speciation model (BD), the coalescent with constant population size model (CCP), and the coalescent with exponential population growth model (CEP). Every GMYC ultrametric tree was constructed using following the same MCMC parameters as the Bayesian tree constructed for phylogenetic analysis described above. GMYC single and multiple threshold approaches were executed using the package *splits* in R (R Core Team 2021). In summary, we used 16 variations of species delimitation methods: two ABGD (initial and recursive), two SPN (95% and 99% confidence limits), one PTP (maximum likelihood version), eight GMYC (single and multiple threshold approaches of four distinct tree priors), and three ASAP (lowest ASAP-score, p-value and W values). The consensus of the species delimitation methods was obtained using the R package *BarcodingR*, which applies the majority rule (Zhang et al. 2017). In this study, we consider 'Group' as the *BarcodingR* consensus output, and 'Lineage' as our interpretation after combining all the generated data.

Results

Molecular analysis

A total of 246 newly generated *cox*3 sequences were obtained (Table S1), consisting of 13 haplotypes. The *cox*3 alignment for phylogenetic analysis comprised 153 sequences, including 139 *Colpomenia* spp. sequences downloaded from GenBank, 13 newly generated haplotypes sequences, and *Ectocarpus siliculosus* (Dillwyn) Lyngbye (MK045263) as outgroup (Table S2). There are *cox*3 sequences in GenBank from seven currently taxonomically accepted species: *C. claytoniae*, *C. ecuticulata*, *C. expansa*, *C. peregrina*, *C. ramosa*, *C. sinuosa*, and *C. tuberculata*. All of which resulted in high supported clades (BS >99 and PP > 0.99, Fig. 1). Our analyses resolved the fully-supported *C. sinuosa* clade as sister to *C. claytoniae*, *C. expansa* and *C. peregrina* clades with high support (BS = 95 and PP = 0.97). The fully-supported *C. tuberculata* and *C. ramosa* clade was recovered with a sister relationship with the *C. ecuticulata* clade with moderate to high support (BS = 87 and PP = 1). The relationship between the *C. tuberculata-C. ramosa-C. ecuticulata* clade with the remaining clades was moderately supported only for BI (PP = 0.90) (Fig. 1). Only three species have more than two haplotypes to estimate intraspecific variation: *C. claytoniae* (0.00 – 5.41%), *C. peregrina* (0.00 – 4.37%), and *C. sinuosa* (0.00 – 9.10%) (Table 1). All the newly generated sequences haplotypes formed a high support clade with *C. sinuosa* sequences from GenBank (BS > 99 and PP > 1). The lowest interspecific variation occurred between *C. claytoniae* and *C. peregrina* (5.25%), whereas the highest was observed between *C. sinuosa* and *C. tuberculata* (30.96%).

The *cox*3 alignment used for barcode analysis, however, comprised 283 DNA sequences, including all the 246 newly generated sequences, 39 *Colpomenia sinuosa* sequences downloaded from GenBank (Fig. 2). This alignment comprised only the *C. sinuosa* sequences and was 460 bp long, had 101 informative sites, 120 variable sites, and 63 haplotypes. When applying the species delimitation methods to this alignment,

different numbers of primary species hypothesis (PSH) were generated. The consensus contained eight Groups, in which the newly generated sequences fit in four of them (Fig. 2).

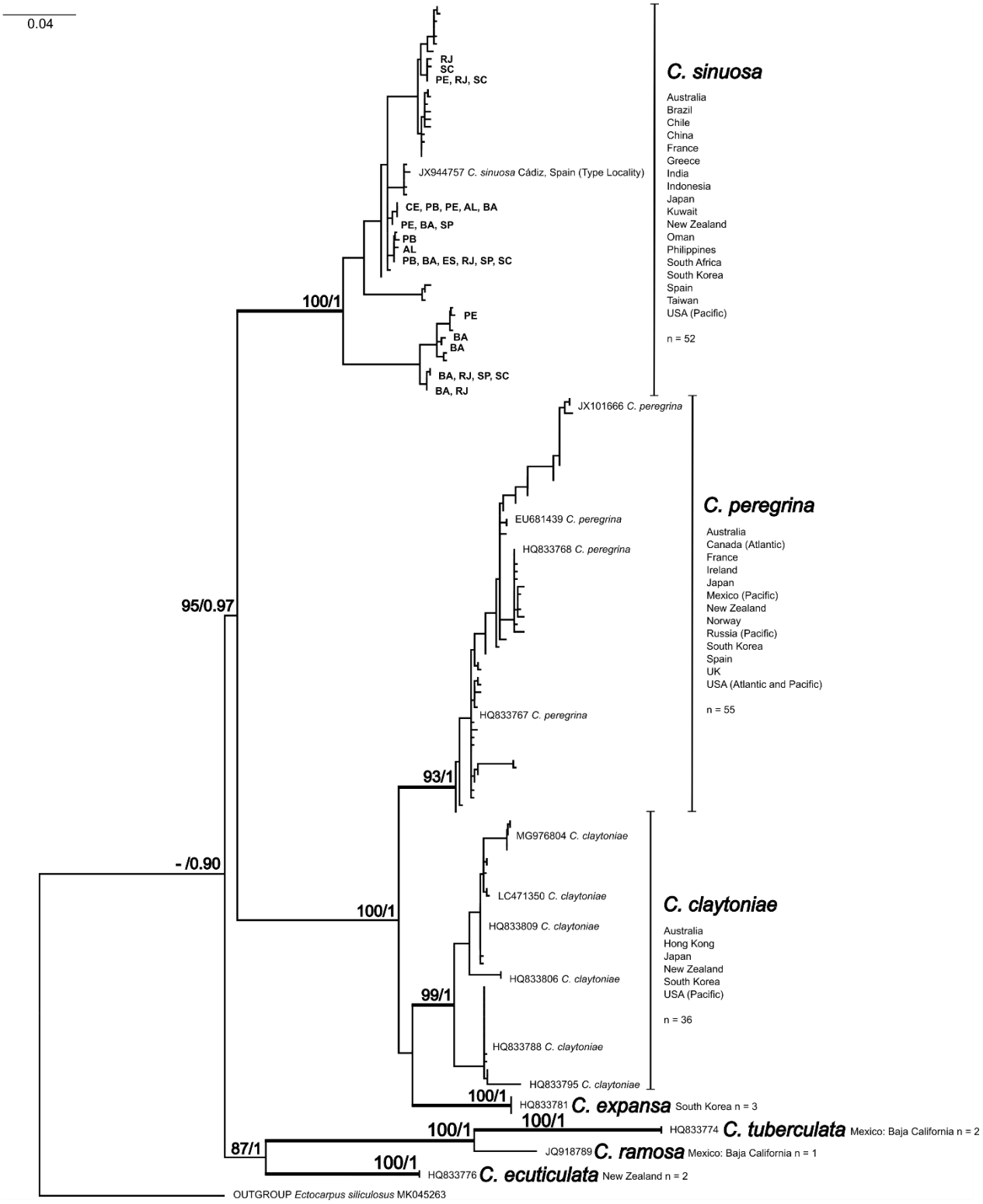


Figure 1. Maximum likelihood (ML) tree of *Colpomenia sinuosa* sequences downloaded from GenBank in addition to newly generated sequences. Bootstrap values (BP) and posterior probabilities (PP) are shown at the nodes as ML/PP. – indicates lack of support or values under 70.

The most conservative SDM evidenced three PSH (ABGD initial, ASAP W, and SPN 95%), where the least conservative was ASAP (considering ASAP-score) which evidenced 12 PSH (Fig. 2). All SDM results, without exception, identified Group 6 as a distinct group (Fig. 2), even though these sequences were neither collected nor sequenced in this study. Three *C. sinuosa cox*3 sequences from type locality (near Cádiz, Spain; JX944752, 54, and 55), formed a well-supported inner clade (BS = 98 and PP = 1) with a sequence from France (JX944757), showing low divergence (0.00 – 0.44%, Fig. 2, Table 2). The sequences of *C. sinuosa* from type locality were resolved as a group with the majority of the sequences from Brazil in five of the SDM, with genetic divergence ranging from 0.00 to 1.84% (Fig. 2, Table 2). The divergence among *C. sinuosa* sequences from type locality to the remaining seven Groups (excluding the authentic *Colpomenia sinuosa* Group) ranged from 2.79 to 9.10.

Table 1. Genetic divergence values (%) of *Colpomenia* spp. for the *cox*3 marker. Lower triangle shows minimum and upper triangle maximum divergence. Diagonal shows minimum and maximum intraspecific divergences. The *cox*3 alignment for matrix comprised 152 sequences, including 139 *Colpomenia* spp. sequences downloaded from GenBank, 13 newly generated haplotypes sequences.

	C. claytoniae	*C. ecuticulata*	*C. expansa*	*C. peregrina*	*C. ramosa*	*C. sinuosa*	*C. tuberculata*
C. claytoniae	0.00 - 5.41	21.20	9.66	9.86	25.55	19.08	30.32
C. ecuticulata	16.52	0.00	18.72	19.87	19.80	20.15	27.71
C. expansa	6.97	18.72	0.00	8.55	24.92	15.85	27.67
C. peregrina	5.23	15.25	5.82	0.00 - 4.37	23.47	16.69	28.11
C. ramosa	23.35	19.80	24.92	18.03	NA	22.89	14.98
C. sinuosa	14.78	14.49	13.68	10.40	19.54	0.00 - 9.10	30.96
C. tuberculata	27.00	27.71	27.67	22.28	14.98	26.39	0.00

Morphological analysis

Description

Figures 3-10

Thallus globular with smooth surface (sacciform) or rough surface (cerebriform), solitary or in clumps, hollow, membranous in texture, brownish-yellow or greenish-brown, 2.0–5.0 cm in diameter (Figs 3, 4). Fixation by inconspicuous rhizoids originating from the outermost cortical cells, 5.0–7.5 µm in diameter. In surface view, cortical cells are polygonal and irregularly arranged. Phaeophycean hairs, multicellular, forming tufts immersed in depressions of the thallus; each hair filament originates from an outer cortical cell (Figs 5, 6). In transverse section, cortical region consisting of 1-2 layers of pigmented small and quadratic cells, 5.0–7.5 × 5.0–7.5 µm; medullary region consisting of 2–4 layers of colorless large and irregularly polyhedral cells, become gradually larger towards the thallus center, 37.5–112.5 µm diameter (Figs 5, 7-9). Plurilocular structures cylindrical, uni- or biseriate, tiny, 10–30 × 2.5–5.0 µm (Figs 9, 10), intertwined by paraphyses, and grouped in irregular sori covered by a hyaline cuticle (Fig. 7). Sori presenting unicellular cylindrical or clubbed paraphyses usually longer than plurilocular structures (Figs 8, 9).

Habitat. Epilithic from mid intertidal to shallow subtidal zone at 3m in protected to highly exposed wave exposure, also growing in tide pools or in reef formation, collected in the protected reef region. Epiphytic on *Sargassum* (C.Agardh). Drifted.

Examined material: BRAZIL. PARAÍBA: João Pessoa, Praia Caribessa, *Araújo P*, 16.XII.2017 (Lineage 1: CS0057). **PERNAMBUCO: Goiana, Ponta de Pedras,** *Rodrigues S, Carvalho MFO*, 14.VI.2018 (Lineage 1: CS0200; Lineage 4: CS0196). **Tamandaré, Praia dos Carneiros,** *Fujii MT*, 24.II.2018 (Lineage 2: CS0114). **ALAGOAS: Maceió, Praia de Pajuçara,** *Carneiro VAR*, 09.XI.2018 (Lineage 1: CS0320). **BAHIA: Salvador, Praia de Stella Maris,** *Santos GN*, 31.I.2018 (Lineage 5: CS0262, CS0263). **Ilha de Itaparica, Praia da Penha,** *Santos GN*, 01.II.2018 (Lineage 4: CS0277). **Barra Grande, Ponta do Mutá,** *Pessôa AC,* 18.II.2018 (Lineage 4: CS0066). **Abrolhos, Ilha Siriba,** *Martins NT*, 17.X.2018 (Lineage 1: CS0302, CS0304). **ESPÍRITO SANTO: Marataízes, Praia de Castelhanos,** *Harb T, Oliveira W, Chow F*, 30.IV.2018 (Lineage 1: CS0195). **RIO DE JANEIRO: Armação dos Búzios, Praia Rasa,** Martins NT, Cassano V, 18.III.2018 (Lineage 1: CS0122). **Arraial do Cabo, Praia do Forno,** *Ayres-Ostrock L*, 30.XI.2017 (Lineage 2: CS0021, CS0022).

SÃO PAULO: Ubatuba, Ilha de Anchieta, *Martins NT*, 20.VIII.2017 (Lineage 1: CS0003; Lineage 5: CS0001). **SANTA CATARINA: Bombinhas, Praia de Bombinhas,** *Ayres-Ostrock L*, 13.X.2016 (Lineage 1: CS0131; Lineage 2: CS0132).

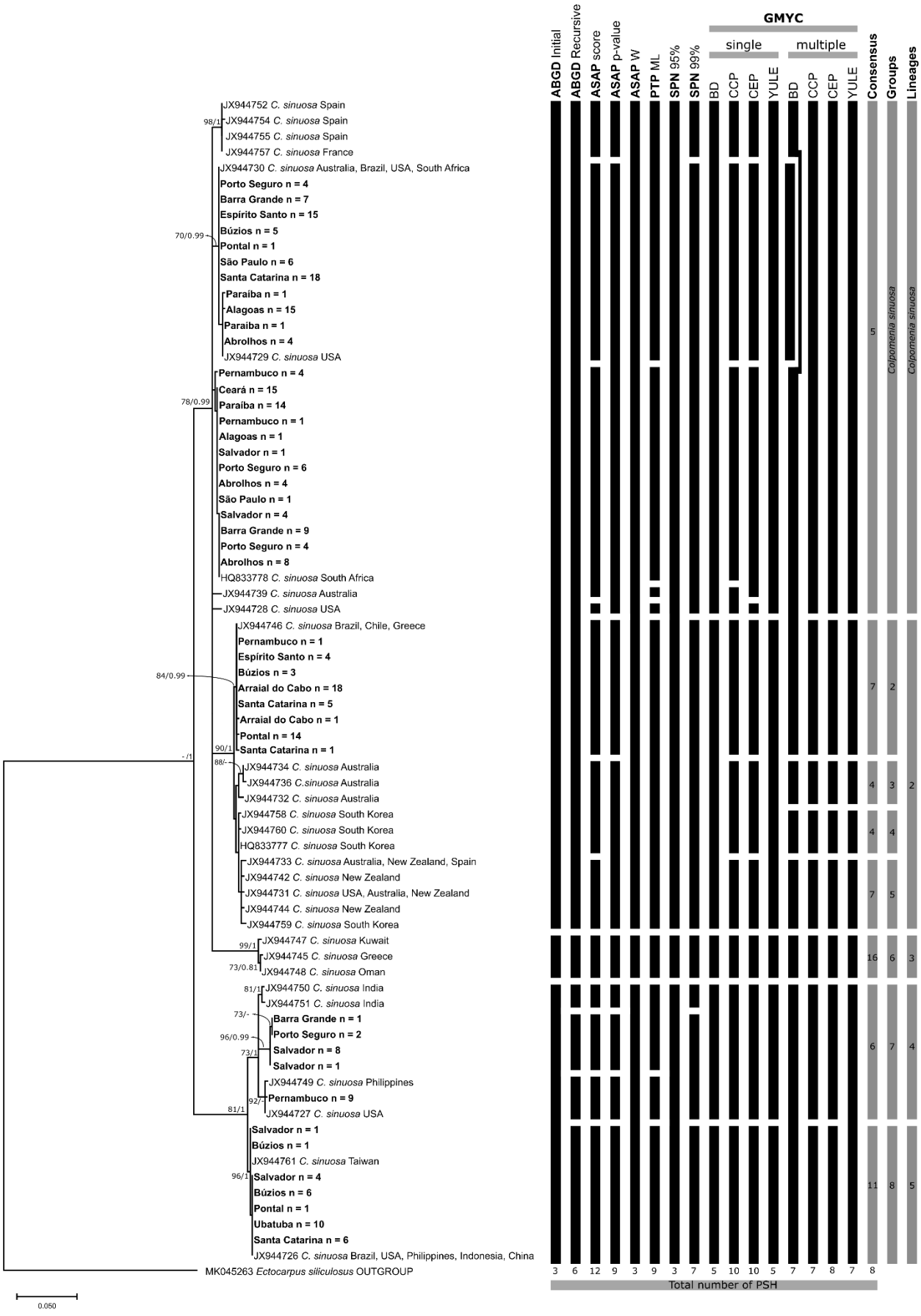

ABGD Initial
ABGD Recursive
ASAP score
ASAP p-value
ASAP W
PTP ML
SPN 95%
SPN 99%
GMYC
single
multiple
BD
CCP
CEP
YULE
BD
CCP
CEP
YULE
Consensus
Groups
Lineages
JX944752 C. sinuosa Spain
JX944754 C. sinuosa Spain
JX944755 C. sinuosa Spain
JX944757 C. sinuosa France
JX944730 C. sinuosa Australia, Brazil, USA, South Africa
Porto Seguro n = 4
Barra Grande n = 7
Espírito Santo n = 15
Búzios n = 5
Pontal n = 1
São Paulo n = 6
Santa Catarina n = 18
Paraiba n = 1
Alagoas n = 15
Paraiba n = 1
Abrolhos n = 4
JX944729 C. sinuosa USA
Pernambuco n = 4
Ceará n = 15
Paraíba n = 14
Pernambuco n = 1
Alagoas n = 1
Salvador n = 1
Porto Seguro n = 6
Abrolhos n = 4
São Paulo n = 1
Salvador n = 4
Barra Grande n = 9
Porto Seguro n = 4
Abrolhos n = 8
HQ833778 C. sinuosa South Africa
JX944739 C. sinuosa Australia
JX944728 C. sinuosa USA
JX944746 C. sinuosa Brazil, Chile, Greece
Pernambuco n = 1
Espírito Santo n = 4
Búzios n = 3
Arraial do Cabo n = 18
Santa Catarina n = 5
Arraial do Cabo n = 1
Pontal n = 14
Santa Catarina n = 1
JX944734 C. sinuosa Australia
JX944736 C.sinuosa Australia
JX944732 C. sinuosa Australia
JX944758 C. sinuosa South Korea
JX944760 C. sinuosa South Korea
HQ833777 C. sinuosa South Korea
JX944733 C. sinuosa Australia, New Zealand, Spain
JX944742 C. sinuosa New Zealand
JX944731 C. sinuosa USA, Australia, New Zealand
JX944744 C. sinuosa New Zealand
JX944759 C. sinuosa South Korea
JX944747 C. sinuosa Kuwait
JX944745 C. sinuosa Greece
JX944748 C. sinuosa Oman
JX944750 C. sinuosa India
JX944751 C. sinuosa India
Barra Grande n = 1
Porto Seguro n = 2
Salvador n = 8
Salvador n = 1
JX944749 C. sinuosa Philippines
Pernambuco n = 9
JX944727 C. sinuosa USA
Salvador n = 1
Búzios n = 1
JX944761 C. sinuosa Taiwan
Salvador n = 4
Búzios n = 6
Pontal n = 1
Ubatuba n = 10
Santa Catarina n = 6
JX944726 C. sinuosa Brazil, USA, Philippines, Indonesia, China
MK045263 Ectocarpus siliculosus OUTGROUP
70/0.99
98/1
78/0.99
84/0.99
90/1
88/-
- /1
99/1
73/0.81
81/1
73/-
96/0.99
73/1
92/-
81/1
96/1
Colpomenia sinuosa
Colpomenia sinuosa
3 6 12 9 3 9 3 7 5 10 10 5 7 7 8 7 8
Total number of PSH
0.050

Figure 2. Maximum likelihood (ML) analysis for *cox*3 DNA sequences of *Colpomenia sinuosa* from Brazil and downloaded sequences from GenBank, and all results of single-marker species-delimitation methods. Bootstrap values (BP) and posterior probabilities (PP) are shown at the nodes as ML/PP. – indicates lack of support or values under 70. Samples generated in this study are in bold. Codes following sequence names refer to GenBank accession numbers. Black vertical bars represent each of the barcode species delimitation methods applied. Consensus votes, *BarcordingR* output vertical grey bars represent Groups and different Lineages.

Discussion

Based on molecular and morphological studies on the diversity of *Colpomenia* along the Brazilian coast, we have identified only one occurring species, *C. sinuosa* with four cryptic lineages with a single evolutionary origin. Phylogenetically, the topology we obtained was similar as those previously obtained, such as for *cox*3 (Green et al. 2012), *cox*3 + *atp*6 (Lee et al. 2014, Song et al. 2019), and *cox*1 (Martins et al. 2021). Even though some interspecific relationships appears to be poorly resolved, the tree topology of the species described so far seems to be well-established.

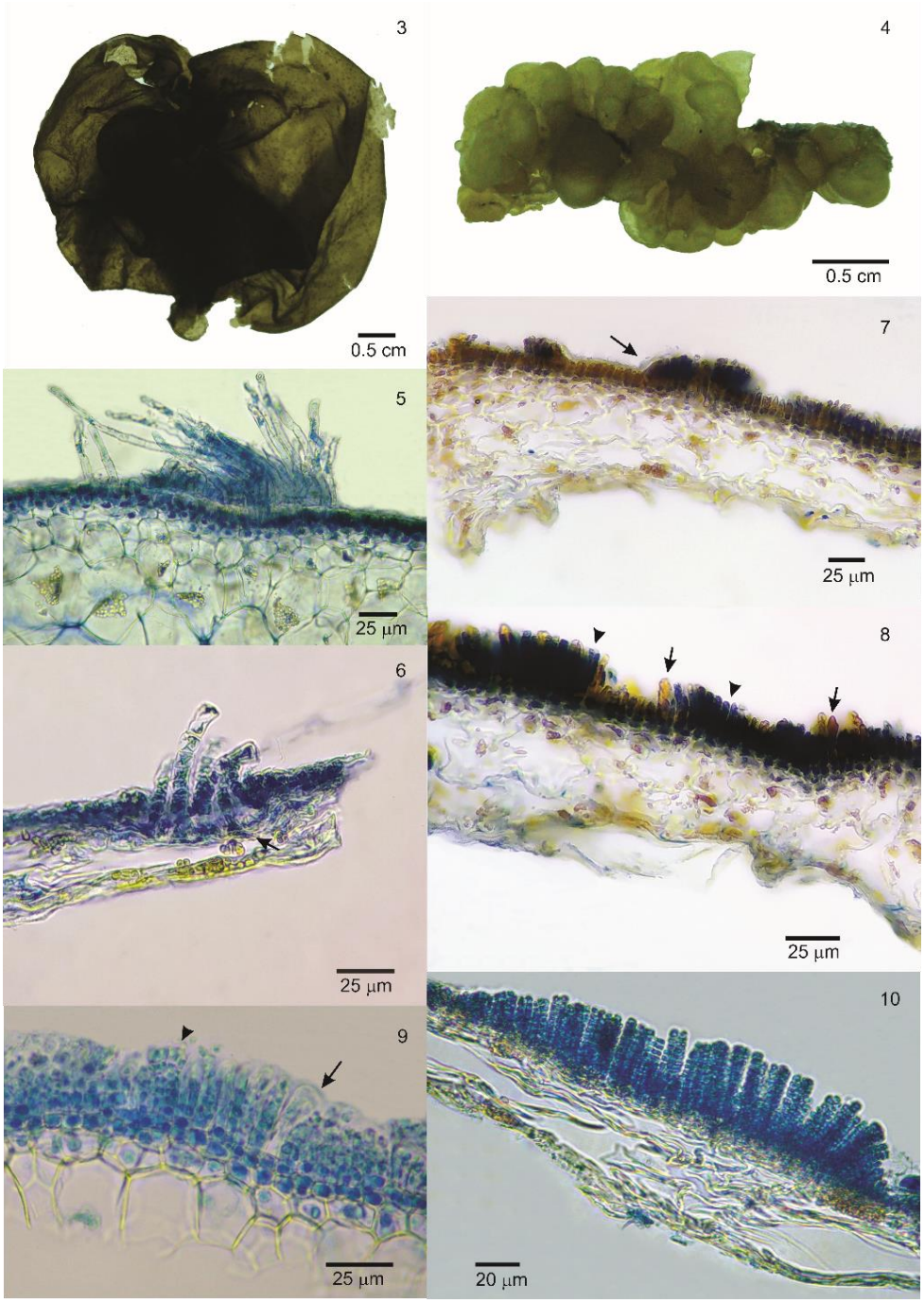
The *Colpomenia sinuosa* sequences from type locality evidenced lowest genetic divergence to Lineage 2 (2.53%) and highest when compared to the remaining Lineages 3, 4 and 5 (4.67, 7.43 and 7.11%, respectively). These divergences are similar to the maximum *cox*3 intraspecific divergence detected in *C. claytoniae* (4.6%, Boo et al. 2011), *C. peregrina* (3.7%, Lee et al. 2014) and *C. sinuosa* (7.4%, Lee et al. 2013) and the interspecific divergence between *C. claytoniae* and *C. peregrina* (5.23%). The intraspecific divergence detected by these authors are similar to the intraspecific divergence we obtained, but for *C. sinuosa* varied from 7.4% (Lee et al. 2013) to 9.1% (this study). Therefore, the obtained intraspecific variation for *C. sinuosa* was higher than the data in

literature. The genetic divergence between *C. sinuosa* sequences from type locality was low when compared to sequences from Group 1 (up to 1.84%), therefore this Group correspond to the true *C. sinuosa* Lineage. Our SDM methods detected eight distinct Groups of *C. sinuosa*. Thus, when analyzing the votes attributed by *BarcodingR* package in R software and the genetic divergence (up to 1.57%), we decided to combine Groups 2, 3, 4 and 5 as a single Lineage (i.e., Lineage 2). Therefore, based on SDM and genetic divergence, each Lineage seems to represent a molecular cryptic species.

The largest individuals collected in this study was 5 cm diameter. This is larger than individuals from Espírito Santo (3cm, Gomes et al. 1989). However, this is smaller than the majority of the studies performed along the Brazilian coast (> 10cm, Széchy and Cordeiro-Marino 1991, Nunes and Paula 2004), including another study from Espírito Santo (up to 7cm, Crispino 2000), and several regions around de world (up to 15cm, Song et al. 2019). The plants analyzed in this study correspond morphologically to several descriptions made for the Brazilian coast (Semir 1977, Széchy 1986, Gomes et al. 1989, Széchy and Cordeiro-Marino 1991, Crispino 2000, Nunes and Paula 2004, Ouriques and Cordeiro-Marino 2004), besides some small and slightly differences. For example, we have observed up to 4 cells layers at the medullary region, while these authors report up to 5 (-7) seven cells layers. In addition, we detected slightly smaller medullary and cortex cells, however, this might be an artefact considering that the majority of our specimens were desiccated and later rehydrated. Lastly, our plurilocular structures were also smaller, considering we observed up to 30 µm long while reported to be up to 43 µm long in several studies along the Brazilian coast (Semir 1977, Gomes et al. 1989, Széchy and Cordeiro-Marino 1991, Crispino 2000, Nunes and Paula 2004).

Table 2. Genetic divergence values (%) of *Colpomenia sinuosa*. for the *cox*3 marker. Lower triangle shows minimum and upper triangle maximum divergence. Diagonal shows minimum and maximum intraspecific divergences. The *cox*3 alignment for matrix included all the 246 newly generated sequences and 38 *Colpomenia sinuosa* sequences downloaded from GenBank, totaling 284. TL = *C. sinuosa* type locality sequences. CS = *C. sinuosa* sequences, including sequences from type locality. L2, L3, L4 and L5 stands for different lineages within *C. sinuosa*.

	TL	CS	L2	L3	L4	L5
TL	0.00 - 0.44	1.84	3.57	5.25	9.10	7.71
CS	0.89	0.00 - 1.84	3.57	5.25	9.10	7.71
L2	2.53	1.81	0.00 - 1.57	6.07	8.26	6.96
L3	4.67	4.14	4.32	0.00 - 0.67	8.95	7.92
L4	7.43	6.20	6.34	7.59	0.00 - 1.35	2.32
L5	7.11	5.29	6.03	7.32	1.61	0.00 - 0.22



Figures 3-10. *Colpomenia sinuosa* thallus from the Brazilian coast (3) smooth, (4) rough. (5) Transverse section of the thallus showing medullary and cortical regions with tuft of phaeophycean hairs. (6) Detail of phaeophycean hairs emerging from cortex depression; each arising from a cortical cell (arrow). (7) Partial cuticle covering the plurilocular structures sorus (arrow). (8 and 9) Plurilocular sctructures (arrowheads) and paraphyses (arrows). (10) Several plurilocular structures.

Along the Brazilian coast, the existence of a single *Colpomenia* species is tested for long time, since Semir (1977), who refuted the separation of *C. sinuosa* into two distinct species on the base of the presence of smooth or rough thallus by laboratory-culture experiments. Differences in the surface configuration of the thalli were commonly attributed to the different degree of wave exposure. However, co-occurrence of both smooth and rough surface configuration in the same collection sites were observed, and reinforce the Semir's results, although rough surface is more common in exposed to strong wave action sites (Semir 1977, Széchy and Cordeiro-Marino 1991, Ouriques and Cordeiro-Marino 2004). Lee et al. (2013) identified a wide-ranging of morphologically variable *C. sinuosa* lineages worldwide, without a clear distinction among lineages, and considered intraspecific cryptic diversity. Our results were similar to Lee et al. (2013), since the analyzed morphological characteristics overlapped in the different lineages of *C. sinuosa* from the Brazilian coast.

In Brazil, the true *C. sinuosa* Lineage is the most widely distributed, occurring in every state and the only collecting site which was absent is Arraial do Cabo (Rio de Janeiro), where all the 19 sequences correspond to two haplotypes within Lineage 2. *Colpomenia sinuosa* was observed as the most widely distributed species in Australia (Womersley 1987, Martins et al. 2021), China (Song et al. 2019) and in the world as a whole (Lee et al. 2013). The remaining lineages evidenced restricted distribution or abundance. Lineage 2, occurs mostly south of the Vitória-Trindade seamount chain (n = 47) and one single sequence occurring northerly (Pernambuco). Lineage 2 encompasses sequences from Brazil and sequences from Spain, Australia, New Zealand, South Korea, and USA. Therefore, this lineage appears to be a sub-tropical/temperate adapted, although has been indicated as anthropogenically introduced to the Atlantic (i.e. Brazil, Spain and Greece) and Chile (Lee et al. 2013). Lineage 5, such as Lineage 2, also evidences a tendency to occur in cold waters, considering that the majority occurs

southern the Vitória-Trindade seamount chain (n = 24) but there are five sequences from Salvador (Bahia) and encompasses sequences from Taiwan, China, Philippines, Indonesia and USA. Lineage 4, however, represents a more tropical trend, although restricted to 21 sequences, occurs in Bahia and Pernambuco states and GenBank sequences from India, Philippines, and USA. Lineage 3 does not occur in Brazil, only occurring in Kuwait and Greece. Besides worldwide distribution of Lineages 4 and 5, Lee et al. (2013) discuss these might have naturally occurred due to intrinsic genetic structure.

Cryptic diversity appears to be common in several *Colpomenia* species (Cho et al. 2009, McDevit and Saunders 2009, Lee et al. 2013, 2014, Martins et al. 2021). The occurrence of convergent evolution, parallelisms and character losses seems to be uncoupling genetic and morphological differentiation (Fowler-Walker et al. 2006, Leliaert et al. 2018). Therefore, several studies are failing to delimitate *Colpomenia* cryptic lineages. We have collected in Praia Rasa, Búzios (Rio de Janeiro, Brazil) and identified three different lineages occurring at this beach (Lineages 1, 2 and 4) as well as previously Lee et al. (2013) identified three distinct lineages collected at this same beach. Fernandes (2007) has also collected at this beach and failed to obtain the full life history of *C. sinuosa* in laboratory culture. Molecular identification of the specimens was not carried out by the author. It is possible and reliable to obtain every stages of *C. sinuosa* life history in laboratory (Freitas Toste et al. 2003). Therefore, the failure in obtaining fertile offspring by Fernandes (2007) might have occurred due to the co-occurrence of cryptic lineages that might have been studied and might be incompatible. However, if this has indeed occurred, remains to be tested. Considering that morphological evidences are failing to support the species delimitation in *Colpomenia* spp. cryptic lineages, performing crossing experiments testing the biological concept of species (i.e. mating compatibility) might help to elucidate these quagmires, and define whether different cryptic lineages correspond to different species.

This study has extensively investigated the *Colpomenia sinuosa* along the Brazilian coast. By applying SDM and genetic divergence, we have identified five *C. sinuosa* cryptic lineages in the world, four of which occurring in Brazil. Our intraspecific topology is similar to Lee et al. (2013), however, we interpreted the data as five lineages instead of three groups with sub-groups. Due to the lack of morphological differences, we have decided not to describe new species, similar decisions upon similar genetic divergence were taken by Lee et al. (2013) and Song et al. (2019). Phylogenetic analyses using single gene or concatenated analyses with few genes are failing to determine if intraspecific cryptic lineages within *Colpomenia* species indeed correspond to different species. Therefore, we recommend further studies to test different approaches, such as mating compatibility and Next-Generation Sequencing (e.g. phylogenomics). Considering the data obtained so far, we cannot tell apart the observed diversity correspond to a single or several species hidden in cryptic lineages within *Colpomenia sinuosa* species complex.

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Table S1. List of *Colpomenia* spp. collection sites along the Brazilian coast and their respective meta-data.

Site	State	City	Beach	N	Date	Collectors	Coordinates
1	Ceará	Icapuí	Ponta Grossa	15	01/09/2019	Ximenes CF, Ximenes PV, Ribeiro ALN	-4.627507, -37.503233
2	Paraíba	João Pessoa	Caribessa	16	16/02/2017	Araújo P	-7.077192, -34.828710
3	Pernambuco	Tamandaré	Carneiros	5	24/02/2018	Fujii MT	-8.713937, -35.083415
3	Pernambuco	Goiana	Ponta de Pedras	10	14/06/2018	Rodrigues S, Carvalho MFO	-7.633987, -34.812187
5	Alagoas	Maceió	Pajuçara	11	22/09/2018	Carvalho N	-9.664033, -35.703786
5	Alagoas	Maceió	Pajuçara	5	09/11/2018	Carneiro VAR	-9.664033, -35.703786
6	Bahia	Salvador	Stella Maris	9	31/01/2018	Santos GN	-12.948912, -38.340989
6	Bahia	Ilha de Itaparica	Penha	8	01/02/2018	Santos GN	-12.985258, -38.616709
6	Bahia	Salvador	Farol de Itapuã	2	24/02/2018	Pessôa AC	-12.956171, -38.352413
7	Bahia	Barra Grande	Ponta do Mutá	6	18/02/2018	Pessôa AC	-13.880233, -38.947824
7	Bahia	Barra Grande	Taipu de Fora	11	18/02/2018	Pessôa AC	-13.941611, -38.927220
8	Bahia	Porto Seguro	Mucugê	6	10/01/2018	Oliveira VP	-16.497755, -39.068043
8	Bahia	Porto Seguro	Parracho	10	15/10/2018	Martins NT	-16.507448, -39.070453

Table S1. Continuing

9	Bahia	Abrolhos	Ilha Siriba	16	17/10/2018	Martins NT	-17.969832, -38.710340
10	Espírito Santo	Itaipava	Itaoca	4	24/02/2017	Fujii MT	-20.904446, -40.776809
10	Espírito Santo	Anchieta	Parati	15	30/04/2018	Harb T, Oliveira W, Chow F	-20.839695, -40.627023
11	Rio de Janeiro	Búzios	Rasa	15	18/03/2018	Martins NT, Cassano V	-22.733842, -41.957532
12	Rio de Janeiro	Arraial do Cabo	Forno	19	30/11/2017	Ayres-Ostrock L	-22.968147, -42.015837
13	Rio de Janeiro	Rio de Janeiro	Pontal	16	12/10/2019	Martins NT	-23.033656, -43.470521
14	São Paulo	Ubatuba	Ilha Anchieta	9	20/08/2017	Martins NT	-23.536789, -45.062898
14	São Paulo	Ubatuba	Vermelha do Sul	8	22/08/2017	Martins NT	-23.512250, -45.171682
15	Santa Catarina	Bombinhas	Bombinhas	6	13/10/2016	Ayres-Ostrock L	-27.147530, -48.483007
15	Santa Catarina	Bombinhas	Bombinhas	9	16/10/2019	Martins NT, Gurgel CFD	-27.147530, -48.483007
15	Santa Catarina	Florianópolis	Armação	15	04/04/2018	Gurgel CFD	-27.748995, -48.500020

Table S2. Details of the taxa and molecular data used in this study.

Reference	Code	Species	Country
Boo et al 2011	HQ833810	*Colpomenia claytoniae*	Australia
Boo et al 2011	HQ833802	*Colpomenia claytoniae*	Hong Kong
Boo et al 2011	HQ833804	*Colpomenia claytoniae*	Hong Kong
Boo et al 2011	HQ833805	*Colpomenia claytoniae*	Hong Kong
Boo et al 2011	HQ833806	*Colpomenia claytoniae*	Hong Kong
Boo et al 2011	HQ833782	*Colpomenia claytoniae*	Japan
Hanyuda et al 2019	LC471350	*Colpomenia claytoniae*	Japan
Boo et al 2011	HQ833798	*Colpomenia claytoniae*	New Zealand
Boo et al 2011	HQ833807	*Colpomenia claytoniae*	New Zealand
Boo et al 2011	HQ833808	*Colpomenia claytoniae*	New Zealand
Boo et al 2011	HQ833809	*Colpomenia claytoniae*	New Zealand
Boo et al 2011	HQ833801	*Colpomenia claytoniae*	South Africa
Boo et al 2011	HQ833783	*Colpomenia claytoniae*	South Korea
Boo et al 2011	HQ833784	*Colpomenia claytoniae*	South Korea
Boo et al 2011	HQ833785	*Colpomenia claytoniae*	South Korea
Boo et al 2011	HQ833786	*Colpomenia claytoniae*	South Korea
Boo et al 2011	HQ833787	*Colpomenia claytoniae*	South Korea
Boo et al 2011	HQ833788	*Colpomenia claytoniae*	South Korea
Boo et al 2011	HQ833789	*Colpomenia claytoniae*	South Korea
Boo et al 2011	HQ833790	*Colpomenia claytoniae*	South Korea
Boo et al 2011	HQ833791	*Colpomenia claytoniae*	South Korea
Boo et al 2011	HQ833792	*Colpomenia claytoniae*	South Korea
Boo et al 2011	HQ833793	*Colpomenia claytoniae*	South Korea
Boo et al 2011	HQ833794	*Colpomenia claytoniae*	South Korea
Boo et al 2011	HQ833795	*Colpomenia claytoniae*	South Korea
Boo et al 2011	HQ833796	*Colpomenia claytoniae*	South Korea
Boo et al 2011	HQ833797	*Colpomenia claytoniae*	South Korea
Boo et al 2011	HQ833799	*Colpomenia claytoniae*	South Korea
Boo et al 2011	HQ833800	*Colpomenia claytoniae*	South Korea
Boo et al 2011	HQ833803	*Colpomenia claytoniae*	South Korea
Boo et al 2011	HQ833814	*Colpomenia claytoniae*	South Korea
Boo et al 2011	HQ833811	*Colpomenia claytoniae*	USA: California
Boo et al 2011	HQ833812	*Colpomenia claytoniae*	USA: California
Boo et al 2011	HQ833813	*Colpomenia claytoniae*	USA: California
Not specified	MG976804	*Colpomenia claytoniae*	USA: California
Not specified	MH350895	*Colpomenia claytoniae*	USA: California
Boo et al 2011	HQ833775	*Colpomenia ecuticulata*	New Zealand
Boo et al 2011	HQ833776	*Colpomenia ecuticulata*	New Zealand
Boo et al 2011	HQ833779	*Colpomenia expansa*	South Korea
Boo et al 2011	HQ833780	*Colpomenia expansa*	South Korea
Boo et al 2011	HQ833781	*Colpomenia expansa*	South Korea
Boo et al 2011	HQ833768	*Colpomenia peregrina*	Australia
Green et al 2012	JX101661	*Colpomenia peregrina*	Canada: Nova Scotia
Green et al 2012	JX101662	*Colpomenia peregrina*	Canada: Nova Scotia
Green et al 2012	JX101663	*Colpomenia peregrina*	Canada: Nova Scotia

Green et al 2012	JX101664	*Colpomenia peregrina*	Canada: Nova Scotia
Silberfeld et al 2010	EU681439	*Colpomenia peregrina*	France
Boo et al 2011	HQ833767	*Colpomenia peregrina*	Korea
Lee et al 2014	JX027338	*Colpomenia peregrina*	Not specified
Lee et al 2014	JX027339	*Colpomenia peregrina*	Not specified
Lee et al 2014	JX027340	*Colpomenia peregrina*	Not specified
Lee et al 2014	JX027341	*Colpomenia peregrina*	Not specified
Lee et al 2014	JX027342	*Colpomenia peregrina*	Not specified
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Lee et al 2014	JX027362	*Colpomenia peregrina*	Not specified
Lee et al 2014	JX027363	*Colpomenia peregrina*	Not specified
Lee et al 2014	JX027364	*Colpomenia peregrina*	Not specified
Lee et al 2014	JX027365	*Colpomenia peregrina*	Not specified
Lee et al 2014	JX027366	*Colpomenia peregrina*	Not specified
Lee et al 2014	JX027367	*Colpomenia peregrina*	Not specified
Lee et al 2014	JX027368	*Colpomenia peregrina*	Not specified
Lee et al 2014	JX027369	*Colpomenia peregrina*	Not specified
Lee et al 2014	JX027370	*Colpomenia peregrina*	Not specified
Lee et al 2014	JX027371	*Colpomenia peregrina*	Not specified
Lee et al 2014	JX027372	*Colpomenia peregrina*	Not specified
Lee et al 2014	JX027373	*Colpomenia peregrina*	Not specified
Lee et al 2014	JX027374	*Colpomenia peregrina*	Not specified
Lee et al 2014	JX027375	*Colpomenia peregrina*	Not specified
Green et al 2012	JX101665	*Colpomenia peregrina*	USA: Maine
Green et al 2012	JX101666	*Colpomenia peregrina*	USA: Maine
Green et al 2012	JX101667	*Colpomenia peregrina*	USA: Maine
Green et al 2012	JX101668	*Colpomenia peregrina*	USA: Maine
Green et al 2012	JX101672	*Colpomenia peregrina*	USA: Massachusetts
Green et al 2012	JX101673	*Colpomenia peregrina*	USA: Massachusetts
Green et al 2012	JX101674	*Colpomenia peregrina*	USA: Massachusetts

Green et al 2012	JX101675	*Colpomenia peregrina*	USA: Massachusetts
Green et al 2012	JX101669	*Colpomenia peregrina*	USA: New Hampshire
Green et al 2012	JX101670	*Colpomenia peregrina*	USA: New Hampshire
Green et al 2012	JX101671	*Colpomenia peregrina*	USA: New Hampshire
Lee et al 2012	JQ918789	*Colpomenia ramosa*	Mexico: Baja California
Lee et al 2013	JX944732	*Colpomenia sinuosa*	Australia
Lee et al 2013	JX944733	*Colpomenia sinuosa*	Australia
Lee et al 2013	JX944734	*Colpomenia sinuosa*	Australia
Lee et al 2013	JX944735	*Colpomenia sinuosa*	Australia
Lee et al 2013	JX944736	*Colpomenia sinuosa*	Australia
Lee et al 2013	JX944737	*Colpomenia sinuosa*	Australia
Lee et al 2013	JX944738	*Colpomenia sinuosa*	Australia
Lee et al 2013	JX944739	*Colpomenia sinuosa*	Australia
Lee et al 2013	JX944740	*Colpomenia sinuosa*	Australia
Lee et al 2013	JX944741	*Colpomenia sinuosa*	Australia
Lee et al 2013	JX944756	*Colpomenia sinuosa*	France
Lee et al 2013	JX944757	*Colpomenia sinuosa*	France
Lee et al 2013	JX944745	*Colpomenia sinuosa*	Greece
Lee et al 2013	JX944746	*Colpomenia sinuosa*	Greece
Lee et al 2013	JX944750	*Colpomenia sinuosa*	India
Lee et al 2013	JX944751	*Colpomenia sinuosa*	India
Lee et al 2013	JX944747	*Colpomenia sinuosa*	Kuwait
Lee et al 2013	JX944742	*Colpomenia sinuosa*	New Zealand
Lee et al 2013	JX944743	*Colpomenia sinuosa*	New Zealand
Lee et al 2013	JX944744	*Colpomenia sinuosa*	New Zealand
Lee et al 2013	JX944748	*Colpomenia sinuosa*	Oman
Lee et al 2013	JX944749	*Colpomenia sinuosa*	Philippines
Lee et al 2013	HQ833778	*Colpomenia sinuosa*	South Africa
Boo et al 2011	HQ833778	*Colpomenia sinuosa*	South Africa
Lee et al 2013	HQ833777	*Colpomenia sinuosa*	South Korea
Boo et al 2011	HQ833777	*Colpomenia sinuosa*	South Korea
Lee et al 2013	JX944758	*Colpomenia sinuosa*	South Korea
Lee et al 2013	JX944759	*Colpomenia sinuosa*	South Korea
Lee et al 2013	JX944760	*Colpomenia sinuosa*	South Korea
Lee et al 2013	JX944752	*Colpomenia sinuosa*	Spain
Lee et al 2013	JX944753	*Colpomenia sinuosa*	Spain
Lee et al 2013	JX944754	*Colpomenia sinuosa*	Spain
Lee et al 2013	JX944755	*Colpomenia sinuosa*	Spain
Lee et al 2013	JX944761	*Colpomenia sinuosa*	Taiwan
Lee et al 2013	JX944725	*Colpomenia sinuosa*	USA
Lee et al 2013	JX944726	*Colpomenia sinuosa*	USA
Lee et al 2013	JX944727	*Colpomenia sinuosa*	USA
Lee et al 2013	JX944728	*Colpomenia sinuosa*	USA
Lee et al 2013	JX944729	*Colpomenia sinuosa*	USA
Lee et al 2013	JX944730	*Colpomenia sinuosa*	USA
Lee et al 2013	JX944731	*Colpomenia sinuosa*	USA
Boo et al 2011	HQ833773	*Colpomenia tuberculata*	Mexico: Baja California
Boo et al 2011	HQ833774	*Colpomenia tuberculata*	Mexico: Baja California

Mignerot et al 2019	MK045263	*Ectocarpus siliculosus*	Italy
This study	CS0396	*Colpomenia sinuosa*	Brazil: Ceará
This study	CS0398	*Colpomenia sinuosa*	Brazil: Ceará
This study	CS0401	*Colpomenia sinuosa*	Brazil: Ceará
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This study	CS0116	*Colpomenia sinuosa*	Brazil: Rio de Janeiro, Búzios
This study	CS0117	*Colpomenia sinuosa*	Brazil: Rio de Janeiro, Búzios
This study	CS0118	*Colpomenia sinuosa*	Brazil: Rio de Janeiro, Búzios
This study	CS0119	*Colpomenia sinuosa*	Brazil: Rio de Janeiro, Búzios
This study	CS0120	*Colpomenia sinuosa*	Brazil: Rio de Janeiro, Búzios
This study	CS0121	*Colpomenia sinuosa*	Brazil: Rio de Janeiro, Búzios
This study	CS0122	*Colpomenia sinuosa*	Brazil: Rio de Janeiro, Búzios
This study	CS0124	*Colpomenia sinuosa*	Brazil: Rio de Janeiro, Búzios
This study	CS0125	*Colpomenia sinuosa*	Brazil: Rio de Janeiro, Búzios
This study	CS0126	*Colpomenia sinuosa*	Brazil: Rio de Janeiro, Búzios
This study	CS0127	*Colpomenia sinuosa*	Brazil: Rio de Janeiro, Búzios
This study	CS0128	*Colpomenia sinuosa*	Brazil: Rio de Janeiro, Búzios
This study	CS0129	*Colpomenia sinuosa*	Brazil: Rio de Janeiro, Búzios
This study	CS0130	*Colpomenia sinuosa*	Brazil: Rio de Janeiro, Búzios
This study	CS0417	*Colpomenia sinuosa*	Brazil: Rio de Janeiro, Búzios
This study	CS0021	*Colpomenia sinuosa*	Brazil: Rio de Janeiro, Arraial do Cabo
This study	CS0022	*Colpomenia sinuosa*	Brazil: Rio de Janeiro, Arraial do Cabo
This study	CS0023	*Colpomenia sinuosa*	Brazil: Rio de Janeiro, Arraial do Cabo
This study	CS0021	*Colpomenia sinuosa*	Brazil: Rio de Janeiro, Arraial do Cabo
This study	CS0025	*Colpomenia sinuosa*	Brazil: Rio de Janeiro, Arraial do Cabo
This study	CS0026	*Colpomenia sinuosa*	Brazil: Rio de Janeiro, Arraial do Cabo
This study	CS0027	*Colpomenia sinuosa*	Brazil: Rio de Janeiro, Arraial do Cabo
This study	CS0028	*Colpomenia sinuosa*	Brazil: Rio de Janeiro, Arraial do Cabo
This study	CS0030	*Colpomenia sinuosa*	Brazil: Rio de Janeiro, Arraial do Cabo
This study	CS0031	*Colpomenia sinuosa*	Brazil: Rio de Janeiro, Arraial do Cabo
This study	CS0032	*Colpomenia sinuosa*	Brazil: Rio de Janeiro, Arraial do Cabo
This study	CS0033	*Colpomenia sinuosa*	Brazil: Rio de Janeiro, Arraial do Cabo
This study	CS0034	*Colpomenia sinuosa*	Brazil: Rio de Janeiro, Arraial do Cabo
This study	CS0035	*Colpomenia sinuosa*	Brazil: Rio de Janeiro, Arraial do Cabo
This study	CS0036	*Colpomenia sinuosa*	Brazil: Rio de Janeiro, Arraial do Cabo
This study	CS0037	*Colpomenia sinuosa*	Brazil: Rio de Janeiro, Arraial do Cabo
This study	CS0038	*Colpomenia sinuosa*	Brazil: Rio de Janeiro, Arraial do Cabo
This study	CS0039	*Colpomenia sinuosa*	Brazil: Rio de Janeiro, Arraial do Cabo
This study	CS0040	*Colpomenia sinuosa*	Brazil: Rio de Janeiro, Arraial do Cabo
This study	CS0446	*Colpomenia sinuosa*	Brazil: Rio de Janeiro, Rio de Janeiro
This study	CS0447	*Colpomenia sinuosa*	Brazil: Rio de Janeiro, Rio de Janeiro
This study	CS0448	*Colpomenia sinuosa*	Brazil: Rio de Janeiro, Rio de Janeiro
This study	CS0449	*Colpomenia sinuosa*	Brazil: Rio de Janeiro, Rio de Janeiro
This study	CS0450	*Colpomenia sinuosa*	Brazil: Rio de Janeiro, Rio de Janeiro
This study	CS0452	*Colpomenia sinuosa*	Brazil: Rio de Janeiro, Rio de Janeiro
This study	CS0453	*Colpomenia sinuosa*	Brazil: Rio de Janeiro, Rio de Janeiro
This study	CS0451	*Colpomenia sinuosa*	Brazil: Rio de Janeiro, Rio de Janeiro
This study	CS0455	*Colpomenia sinuosa*	Brazil: Rio de Janeiro, Rio de Janeiro

This study	CS0456	*Colpomenia sinuosa*	Brazil: Rio de Janeiro, Rio de Janeiro
This study	CS0457	*Colpomenia sinuosa*	Brazil: Rio de Janeiro, Rio de Janeiro
This study	CS0460	*Colpomenia sinuosa*	Brazil: Rio de Janeiro, Rio de Janeiro
This study	CS0461	*Colpomenia sinuosa*	Brazil: Rio de Janeiro, Rio de Janeiro
This study	CS0462	*Colpomenia sinuosa*	Brazil: Rio de Janeiro, Rio de Janeiro
This study	CS0463	*Colpomenia sinuosa*	Brazil: Rio de Janeiro, Rio de Janeiro
This study	CS0465	*Colpomenia sinuosa*	Brazil: Rio de Janeiro, Rio de Janeiro
This study	CS0001	*Colpomenia sinuosa*	Brazil: São Paulo
This study	CS0002	*Colpomenia sinuosa*	Brazil: São Paulo
This study	CS0003	*Colpomenia sinuosa*	Brazil: São Paulo
This study	CS0004	*Colpomenia sinuosa*	Brazil: São Paulo
This study	CS0006	*Colpomenia sinuosa*	Brazil: São Paulo
This study	CS0007	*Colpomenia sinuosa*	Brazil: São Paulo
This study	CS0008	*Colpomenia sinuosa*	Brazil: São Paulo
This study	CS0009	*Colpomenia sinuosa*	Brazil: São Paulo
This study	CS0010	*Colpomenia sinuosa*	Brazil: São Paulo
This study	CS0012	*Colpomenia sinuosa*	Brazil: São Paulo
This study	CS0013	*Colpomenia sinuosa*	Brazil: São Paulo
This study	CS0014	*Colpomenia sinuosa*	Brazil: São Paulo
This study	CS0016	*Colpomenia sinuosa*	Brazil: São Paulo
This study	CS0017	*Colpomenia sinuosa*	Brazil: São Paulo
This study	CS0018	*Colpomenia sinuosa*	Brazil: São Paulo
This study	CS0019	*Colpomenia sinuosa*	Brazil: São Paulo
This study	CS0020	*Colpomenia sinuosa*	Brazil: São Paulo
This study	CS0131	*Colpomenia sinuosa*	Brazil: Santa Catarina, Bombinhas
This study	CS0132	*Colpomenia sinuosa*	Brazil: Santa Catarina, Bombinhas
This study	CS0133	*Colpomenia sinuosa*	Brazil: Santa Catarina, Bombinhas
This study	CS0134	*Colpomenia sinuosa*	Brazil: Santa Catarina, Bombinhas
This study	CS0135	*Colpomenia sinuosa*	Brazil: Santa Catarina, Bombinhas
This study	CS0361	*Colpomenia sinuosa*	Brazil: Santa Catarina, Bombinhas
This study	CS0362	*Colpomenia sinuosa*	Brazil: Santa Catarina, Bombinhas
This study	CS0491	*Colpomenia sinuosa*	Brazil: Santa Catarina, Bombinhas
This study	CS0492	*Colpomenia sinuosa*	Brazil: Santa Catarina, Bombinhas
This study	CS0493	*Colpomenia sinuosa*	Brazil: Santa Catarina, Bombinhas
This study	CS0494	*Colpomenia sinuosa*	Brazil: Santa Catarina, Bombinhas
This study	CS0495	*Colpomenia sinuosa*	Brazil: Santa Catarina, Bombinhas
This study	CS0496	*Colpomenia sinuosa*	Brazil: Santa Catarina, Bombinhas
This study	CS0497	*Colpomenia sinuosa*	Brazil: Santa Catarina, Bombinhas
This study	CS0498	*Colpomenia sinuosa*	Brazil: Santa Catarina, Bombinhas
This study	CS0246	*Colpomenia sinuosa*	Brazil: Santa Catarina, Florianópolis
This study	CS0247	*Colpomenia sinuosa*	Brazil: Santa Catarina, Florianópolis
This study	CS0249	*Colpomenia sinuosa*	Brazil: Santa Catarina, Florianópolis
This study	CS0250	*Colpomenia sinuosa*	Brazil: Santa Catarina, Florianópolis
This study	CS0251	*Colpomenia sinuosa*	Brazil: Santa Catarina, Florianópolis
This study	CS0252	*Colpomenia sinuosa*	Brazil: Santa Catarina, Florianópolis
This study	CS0253	*Colpomenia sinuosa*	Brazil: Santa Catarina, Florianópolis
This study	CS0254	*Colpomenia sinuosa*	Brazil: Santa Catarina, Florianópolis
This study	CS0255	*Colpomenia sinuosa*	Brazil: Santa Catarina, Florianópolis

This study	CS0256	*Colpomenia sinuosa*	Brazil: Santa Catarina, Florianópolis
This study	CS0257	*Colpomenia sinuosa*	Brazil: Santa Catarina, Florianópolis
This study	CS0258	*Colpomenia sinuosa*	Brazil: Santa Catarina, Florianópolis
This study	CS0259	*Colpomenia sinuosa*	Brazil: Santa Catarina, Florianópolis
This study	CS0260	*Colpomenia sinuosa*	Brazil: Santa Catarina, Florianópolis
This study	CS0524	*Colpomenia sinuosa*	Brazil: Santa Catarina, Florianópolis

Chapter 3

Phylogeography of *Colpomenia sinuosa* (Ectocarpales, Phaeophyceae) along the Brazilian coast

Filogeografia de *Colpomenia sinuosa* (Ectocarpales, Phaeophyceae) ao longo da costa brasileira

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Running title: *Colpomenia* phylogeography in Brazil

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Abstract

Colpomenia sinuosa is a brown macroalgal species-complex. Three major *C. sinuosa* phylogenetic lineages, subdivided into eight subgroups, have been identified based on *cox*3 DNA sequences from worldwide collections. To date molecular data from Brazilian *C. sinuosa* populations have been limited to 10 specimens collected in a single locality although the species occurs along the entire Brazilian coast. Consequently, knowledge on population genetic diversity and spatial genetic structuring along most of the Brazilian coastline is nonexistent. To fulfil this gap in knowledge we performed a phylogeographic analysis of *C. sinuosa* populations in Brazil. The highly variable *cox*3 marker was sequenced for 148 individuals collected in 12 localities in Brazil. Results identified two genetically distinct population groups (north vs. south) separated at 20.5º S latitude. Genetic diversity in northern populations is 14.6 and 15.5 times greater than southern populations in terms of haplotype and nucleotide diversity, respectively. Among northern populations, the Bahia state holds the largest genetic diversity. The southern populations had lower genetic diversity and no internal genetic sub-structure suggesting past bottlenecks followed by recent colonization from northern haplotypes. Our results reinforce the crucial importance of historical and extant allopatric, parapatric and sympatric processes driving marine macroalgal evolution in the Southwestern Atlantic Ocean.

Key-words: Atlantic Ocean, Brazil, *Colpomenia*, *cox*3, Ectocarpales, genetic structure, marine barriers, phylogeography, population genetics, Vitória-Trindade seamount chain

Resumo

Colpomenia sinuosa é um complexo de espécies de macroalgas pardas, sendo descritas três linhagens filogenéticas, subdivididas em oito subgrupos, a partir de sequências de *cox*3 de todo o globo. No entanto, dados moleculares de *C. sinuosa* de populações brasileiras estão limitados a 10 espécimes coletados numa única localidade. Consequentemente, o conhecimento da diversidade genética populacional e da estrutura genética espacial é inexistente ao longo do litoral brasileiro. Dessa forma, nós realizamos análises filogeográficas de populações de *C. sinuosa* no Brasil. O marcador genético *cox*3 foi sequenciado para 148 indivíduos coletados em 12 localidades brasileiras. Os resultados identificaram dois grupos populacionais (norte vs. sul) separados na latitude 20,5º S. A diversidade genética das populações ao norte é 14,6 e 15,5 vezes maiores do que as populações ao sul em termos de diversidade de haplótipos e nucleotídeos, respectivamente. Entre as populações do norte, o estado da Bahia deteve a maior diversidade genética. As populações do sul evidenciaram menor diversidade genética e nenhuma subestrutura genética interna, sugerindo eventos de gargalo anteriores, seguidos por colonização recente de haplótipos do norte. Nossos resultados não indicam introduções recentes de haplótipos exóticos no Brasil e reforçam a importância crucial de processos alopátricos, parapátricos e simpátricos passados e existentes que impulsionam a evolução das macroalgas marinhas no sudoeste do Oceano Atlântico

Palavras-chave: barreiras marinhas, Brasil, cadeia de Vitória-Trindade, *Colpomenia*, *cox*3, Ectocarpales, estrutura genética, filogeografia, genética populacional, Oceano Atlântico,

Introduction

The marine brown macroalga *Colpomenia sinuosa* (Mertens ex Roth) Derbès & Solier is a common and conspicuous member of tropical and temperate intertidal reef habitats, worldwide (Lipkin 2002, Guiry and Guiry 2021). *C. sinuosa* life history is heteromorphic haplodiplobiontic, with erect yellowish to pale brown, convoluted, bladder-like macroscopic gametophytic thalli alternating with a nearly-microscopic filamentous tufty sporophytic thalli (Freitas Toste et al. 2003). *C. sinuosa* gametophytes can grow isolated or in clumps, epiphytic or epilithic, and present plurilocular reproductive structures organized in punctate sori (Freitas Toste et al. 2003, Cormaci et al. 2012, Lee et al. 2013). The gametophyte bladder-like habit can trap air within its hollow thalli during low tide or when exposed to high-energy waves, increasing positive buoyancy, conferring floatability, allowing detached thalli to drift and hence disperse long distances (Blackler 1967, Mathieson et al. 2016). No information exists on sporophytes, whether they can grow epiphytically or epilithically, either on consolidated or floating substrates.

Colpomenia sinuosa is the most globally widespread of the 10 described *Colpomenia* species. *C. sinuosa* is also one of the seven *Colpomenia* species that have been molecularly characterized using different markers (GenBank, accessed on April 2021). Cho et al. (2009) published the first molecular-assisted spatially broader assessment of *C. sinuosa* populations using *rbc*L and ITS DNA sequences of 18 specimens collected across six countries (Australia, Japan, New Zealand, South Africa, South Korea, and Spain). They identified the existence of two major *C. sinuosa* lineages: a northern and a southern hemisphere clade. Later, Lee et al. (2013) using *cox*3 e *rbc*L DNA sequences of 134 *C. sinuosa* specimens collected across 18 countries, recognized the existence of three major lineages, subdivided into several sub-lineages, revealing rampant cryptic diversity. In addition, Lee et al. (2013) reported complex trans-oceanic dispersal routes, intricate evolutionary histories, and probable human-based introductions, which

might have recently shuffled some phylogeographic patterns. Similarly to other once regarded broadly distributed single species such as *Lobophora variegata* (J.V.Lamouroux) Womersley ex E.C.Oliveira (Vieira et al. 2014), *Portieria hornemannii* (Lyngbye) P.C.Silva (Leliaert et al. 2018), and *Hypnea musciformis* (Nauer et al. 2019). Cho et al. (2009) and Lee et al. (2013) showed that *C. sinuosa* is a good model species for phylogeographic studies.

In Brazil *C. sinuosa* is the only *Colpomenia* species reported along its ~8000 km coastline. Molecular-assisted *C. sinuosa* biodiversity studies in Brazil are lacking and to date there are only two studies addressing seaweed phylogeography in Brazil, Nauer et al. (2019) for *Hypnea pseudomusciformis* and Ayres-Ostrock et al. (2019) for *Crassiphycus caudatus*. Marine phylogeographic studies in Brazil, using macroalgae as model organisms or otherwise, hypothesize the influence of distinct processes acting as barriers to gene flow along the Brazilian coast, such as: (1) estuaries or the mouth of major rivers such as the Doce river, the São Francisco river, and the Amazon river (Floeter et al. 2008, da Silva et al. 2016, Machado et al. 2017); (2) the split of the South Equatorial Current into two at Cape Seixas – the easternmost point in the South American continent (Cortinhas et al. 2016, Bezerra et al. 2018); (3) the offshore extension of the continental shelf leading to the Abrolhos Archipelago and the Vitória-Trindade seamount chain (Lazoski et al. 2011, Hurtado et al. 2016, Pinheiro et al. 2017, Ayres-Ostrock et al. 2019, Nauer et al. 2019, Paiva et al. 2019); (4) southern Bahia state as a climate refuge during glaciation maxima (Ayres-Ostrock et al. 2019, Menezes et al. 2020); (5) the Cape Frio coastal upwelling (Cortinhas et al. 2016, Hurtado et al. 2016); (6) the Cape Santa Marta coastal upwelling (Secchi et al. 1998, Beheregaray and Levy 2000, Campos et al. 2013, Fruet et al. 2014, Costa et al. 2015); (7) the southern Brazil Bight separating a tropical from a subtropical marine climate zone (Horta et al. 2001); and (8) the Cassino Beach, the longest stretch of sandy beaches in the southern hemisphere, located between latitudes 32º S and 34º S

(Trovant et al. 2016, Nauer et al. 2019). A plethora of studies are still needed to determine how these processes have driven macroalgae – and other marine species – genetic diversity, structuring, differentiation and speciation along the Brazilian coast. Furthermore, there is a global lack of knowledge regarding marine macroalgal phylogeography compared to marine animals (Diaz-Pulido et al. 2007, Beheregaray 2008).

Therefore, the objective of this study was to perform a phylogeographic analysis of *Colpomenia sinuosa* populations along the Brazilian coast to (i) identify areas of maximum and minimum genetic diversity, (ii) test for presence of genetic structure; (3) identify allopatric, sympatric and parapatric processes responsible for the formation and maintenance of genetic structure; and (iv) perform a comparative phylogeographic analysis between our results and the literature. Our results, when integrated with other marine phylogeographic studies, help to identify patterns of phylogeographic concordance that are crucial information for: identifying shared evolutionary histories among co-distributed taxa, identifying areas of biogeographic interest; and for informed decision making in nature conservation and the management of marine renewable resources.

Material and methods

Taxon sampling

Colpomenia sinuosa specimens of were collected from 12 populations distributed along the Brazilian coast, ranging from Ceará (latitude ~4º S) to Santa Catarina (latitude ~27º S) (Table 1 and S1). Specimens were located at least 10 cm apart, to avoid resampling the same specimen when growing in clumps. All sampled specimens were cleaned from epiphytes, rinsed in local seawater, and then desiccated in silica gel. Dried specimens were once again cleaned from potential remaining epiphytes under a Stemi SV 6-Zeiss stereomicroscope (Zeiss, Göttingen, Germany) in the laboratory prior to DNA extraction.

DNA extraction, PCR and sequence alignment

*Cox*3 gene was chosen because it presents good phylogeographic signal in a large scale phylogeographic study of *C. sinuosa* (Lee et al. 2013), in addition, single-marker approach is powerful enough to characterize phylogeographic patterns (Krueger-Hadfield et al. 2021). For DNA extraction, subsamples of the silica gel dried samples were ground to a fine powder with a mortar and pestle. DNA was extracted using NucleoSpin® Plant II (Macherey-Nagel GmbH & Co, Germany) according to the manufacturer's instructions. The extracted DNA was stored at -20º C until PCR was performed. For gene amplification we used *cox*3 primers F49 and R20 described in Boo et al. (2010). PCR reactions were conducted in a total volume of 25 µL under one of two protocols: i) 1.0 – 10.0 µg of genomic DNA, 0.2 mM each of deoxynucleotide triphosphate (dNTP), 0.2 mM of each primer, 1X PCR Green Buffer minus Mg (Promega Corp., Madison, WI, EUA), 3 mM of $MgCl_2$, 0.5 µg of BSA, and 0.625 units of Taq polymerase (Promega Corp., Madison, WI, EUA); or ii) 0.001 – 1.0 µg of genomic DNA, 0.4 mM each of deoxynucleotide triphosphate (dNTP), 0.4 mM of each primer, 1X PCR Green Buffer minus Mg (Promega Corp., Madison, WI, EUA), 3 mM of $MgCl_2$, 0.3 M of filtered betaine, and 1.25 units of Taq polymerase (Promega Corp., Madison, WI, EUA). PCR cycle parameters followed Boo et al. (2011) and were carried out on a Techne TC-4000 thermocycler (Bibby Scientific Ltd., Staffordshire, UK). PCR products were purified using column GFX TM PCR DNA and Gel Band Purification Kit (GE Healthcare, Pittsburgh, PA, USA) according to the manufacturers' protocol. Sequencing of 10–40 ng purified PCR product was performed using the same primers as those used for PCR amplifications and BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Capillary separation was performed on a 3100 ABI PRISM TM automatic DNA sequencer (Applied Biosystems, Carlsbad, CA, USA). All sequences were manually aligned using BioEdit (Hall 1999). Inconsistencies in sequences were reviewed by checking the electropherograms.

Sequences of unique haplotypes were deposited in the NCBI GenBank with accession numbers MW981282 to MW981286 (Table S2).

Table 1. Compilation results of *Colpomenia sinuosa* phylogeographic data along the Brazilian coast, individually population, north vs. south phylogroups and total populations. Where N = sequences, h = haplotypes, S = polymorphic sites, m = number of mutations, k = average number of nucleotide differences, Hd = haplotype diversity, and Pi = nucleotide diversity. Tajima's D and Fu & Li's D and F. Bold represents statistically significant results ($p < 0.05$). N/A means not applicable. Haplotypes represents total number of C1, C2, C3, C4 and C5 haplotypes sequences.

Sites	Populations	N	h	S	m	k	Hd	Pi	Tajima's D	Fu & Li's D	Fu & Li's F	Haplotypes				
1	Ceará	15	1	0	0	0.00000	0.00000	0.00000	N/A	N/A	N/A	15	0	0	0	0
2	Paraíba	16	3	5	5	1.05833	0.24167	0.00230	-0.98088	0.45883	0.08156	14	0	1	0	1
3	Pernambuco	5	2	1	1	0.40000	0.40000	0.00087	-0.81650	-0.8165	-0.77152	1	4	0	0	0
4	Alagoas	16	2	5	5	0.62500	0.12500	0.00136	**-1.92860**	**-2.56338**	**-2.74209**	1	0	0	15	0
5	Salvador	5	2	1	1	0.40000	0.40000	0.00087	-0.81650	-0.8165	-0.77152	4	1	0	0	0
6	Barra Grande	16	2	4	4	2.10000	0.52500	0.00457	**2.32158**	1.14136	**1.66782**	9	0	7	0	0
7	Porto Seguro	14	3	4	4	1.75824	0.70330	0.00382	1.31061	1.16427	1.36997	4	6	4	0	0
8	Abrolhos	16	3	4	4	1.73333	0.66667	0.00377	1.36998	1.14136	1.37661	8	4	4	0	0
9	Espírito Santo	15	1	0	0	0.00000	0.00000	0.00000	N/A	N/A	N/A	0	0	15	0	0

Table 1. Continuing

10	Rio de Janeiro	5	1	0	0	0.00000	0.00000	0.00000	N/A	N/A	N/A	0	0	5	0	0
11	São Paulo	7	2	3	3	0.85714	0.28571	0.00186	-1.35841	-1.42725	-1.52246	0	1	6	0	0
12	Santa Catarina	14	1	0	0	0.00000	0.00000	0.00000	N/A	N/A	N/A	0	0	18	0	0
	North	103	5	6	6	2.06929	0.64400	0.00450	1.77977	0.15711	0.82431	56	15	16	15	1
	South	45	2	3	3	0.13333	0.04400	0.00029	-1.70627	**-2.95719**	**-3.00540**	0	1	44	0	0
	Total	148	5	6	6	2.17963	0.67503	0.00474	**2.16734**	0.08465	0.93922	C1	C2	C3	C4	C5

Population genetic and spatial structure

Haplotype diversity (Hd), nucleotide (Pi) diversity (Nei 1987), average number of nucleotide differences (k), total number of mutations (m), number of haplotypes (h), and number of segregating sites (S) were calculated for each population using DNAsp 6 (Rozas et al. 2017). Statistical parsimony network was built using TCS 1.21 (Clement et al. 2000) with a 95% connection limit.

The presence of genetic isolation by distance was tested for individual allele frequencies via Moran's *I* test corrected to spatial autocorrelation across distance (Moran 1950) using the function *Moran.I* available in the *ape* package (Paradis and Schliep 2019) in the R software (R Core Team 2021), adopting an alpha = 0.05. Twelve spatial classes corresponding to each population, or geographic location, were incorporated as latitude and longitude.

Hypotheses of population subdivision along the Brazilian coast, and the presence of putative barriers to gene flow between them, were tested using permutational multivariate analysis of variance (PERMANOVA) (Anderson et al. 2008) conducted as an add-on module to Primer v.6 (PRIMER-E Ltd., Plymouth, UK), based on a dataset comprised by haplotype frequencies per population. We tested four hypotheses of barriers to gene flow: i) the split of the South Equatorial Current into two at latitude 5.5º S (CE x PE -SC); ii) the mouth of the São Francisco river at latitude 10º S (CE-AL x Salvador-SC); iii) the Vitória-Trindade seamount chain at latitude 20.5º S (CE-Abrolhos x ES-SC); and iv) the Cabo Frio seasonal upwelling system at latitude 23º S (CE-RJ x SP-SC). Another putative barrier to gene flow is the estuary represented by the mouth of the Doce river at latitude 19º S. Because this estuary is too close to the Vitória-Trindade seamount chain, in this study they were confounded to a single test.

The degree of genetic differentiation between populations was determined using F_{ST} pair-wise differences (Nei 1973) implemented in Arlequin 3.5 (Excoffier and Lischer 2010) with 3000 permutations, 0.05 significance level, and using number of different alleles.

Coalescent inferences of demographic history

The coalescent age between haplotypes was based on a Bayesian ultrametric tree (chronogram) estimated using Beast 2.5 (Bouckaert et al. 2019). MCMC chain length was 10 million generations long, saving a tree every 1,000 generations, discarding the first 10,000 generations as the burn-in, all priors variation set to *exponential* model and with the Birth & Death speciation model in effect. The time-calibrated Bayesian tree was estimated using three calibration points (in years), the mean Ectocarpales age, mean Laminariales age, and the coalescent point between Ectocarpales and Laminariales extracted from the time-calibrated multi-marker study of Silberfeld et al. (2010). The alignment used for the ultrametric tree contained all Ectocarpales *cox*3 sequences used by Silberfeld- et al. (2010) downloaded from GenBank and all haplotypes sequenced in this study (Fig. 1). Best-fit model of sequence evolution was determined using Bayesian Information Criterion (BIC) implemented in JModelTest 2.1.6 (Guindon and Gascuel 2003, Darriba et al. 2012) available in the Cipres 3.3 online gateway (Miller et al. 2010).

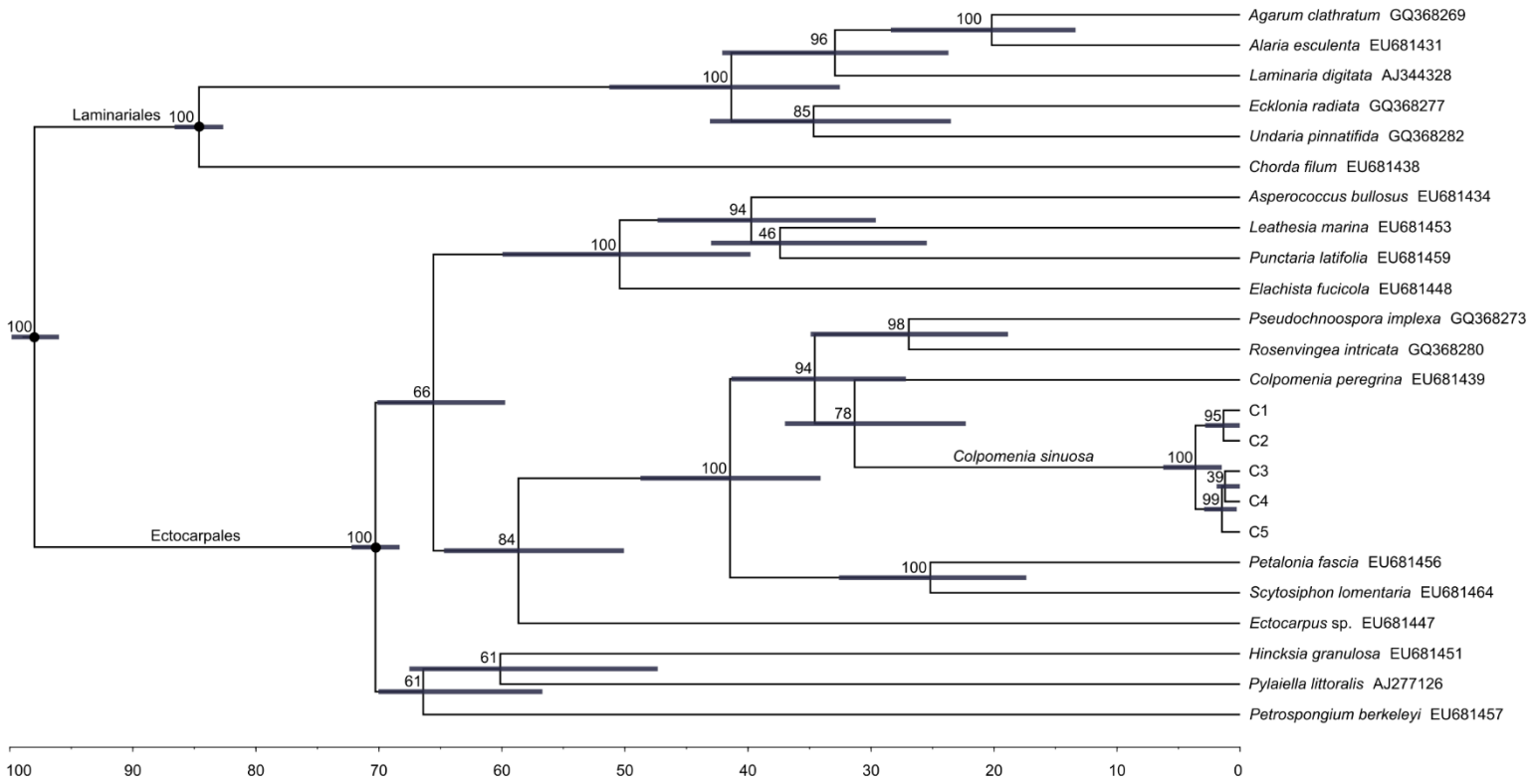

Figure 1. *Cox*3 Bayesian ultrametric tree for *Colpomenia sinuosa* haplotypes generated in this study (C1 – C5) and Ectocarpales sequences generated by Silberfeld et al. (2010) and downloaded from GenBank. Time calibration was based on the mean Ectocarpales, Laminariales and Ectocarpales-Laminariales coalescence age as estimated by Silberfeld et al. (2010), represented by a circle (●). Time scale in million years before present. Grey bars represent 95% HPD. Node numbers are posterior probabilities.

To reconstruct demographic changes over time, historical demographic dynamics of *C. sinuosa* populations were inferred using Bayesian Skyline Plots (BSP) implemented in Beast 2.5 and Tracer 1.7.1 (Rambaut et al. 2018). This coalescence-based approach uses standard MCMC sampling procedures to evaluate posterior probability distribution of effective population size during intervals under a GTR substitution model (Drummond 2005). We used all the newly generated *cox*3 DNA sequences to test the overall population history. MCMC chain length was 10 million generations long, saving a tree every 1,000 generations, discarding the first 10,000 generations as the burn-in.

The mutation rate in substitutions per site per year (s/s/y) was calculated based on sequence divergence values (described in Avise et al. 1998, Weir and Schuluter 2008) and the mean Ectocarpales age (Silberfeld et al. 2010). Maximum likelihood corrected pairwise sequence divergences using the best fit evolutionary model identified in JModeltest2 were calculated using PAUP* 4.0a167 (Swofford 2014). We also applied statistical tests of neutrality for DNA polymorphism to assess shifts in demographic history using Tajima's D (Tajima 1989), Fu & Li's D and F tests (Fu and Li 1993) implemented in DNAsp 6.

Results

Newly generated dataset

We generated a total of 148 new *cox*3 DNA sequences. The alignment was 460 bp long and resulted in five haplotypes, C1-C5, with six variable sites (Table 1). Intraspecific genetic variation among Brazilian *C. sinuosa* specimens ranged between 0.00% and 1.02%, while differences between Brazilian and *C. sinuosa* specimens from the type locality varied between 1.02% and 1.28%.

Genetic diversity

The statistical parsimony network revealed two haplotype groups separated by 3 mutations (Fig. 2), one comprised of specimens from mid and northeastern Brazil (C1 and C2, in color) and another comprised mainly by specimens from mid and southern Brazil (C3 and C5, C4 being the exception). Moran's *I* test revealed the presence of significant spatial autocorrelation along the Brazilian coast with decreasing haplotypic similarity with increasing geographical isolation, particularly at scales farther than 3,000 km ($r = 0.5430$, $p < 0.001$).

The presence of significant spatial autocorrelation, a form of spatial genetic structuring, was also supported by the PERMANOVA analyses, which detected the presence of significant genetic structure between populations located north and south of the Vitória-Trindade seamount chain region (PERMANOVA, Pseudo-$F_{1,9} = 13.246$, $p < 0.01$, Permutations = 999). PERMANOVA results identified two *C. sinuosa* phylogroups along the Brazilian coast, the north and the south Brazil phylogroups (Fig. 3) separated by the region that encompass the Vitória-Trindade seamounts and the Doce river. Amongst all the PERMANOVA testing different barriers to gene flow hypothesis, this was the only significant result. The south Brazil phylogroup is composed by four populations (SC-ES) characterized by the dominance of haplotype C3 (and the presence of C2 in the São Paulo population). The north Brazil phylogroup is composed by eight northernmost populations and the presence of all five haplotypes (Fig. 3). Three significantly different subgroups were detected within the north Brazil phylogroup: one subgroup comprised by the Alagoas population (almost entirely composed of haplotype C4); a second subgroup encompassing Ceará, Paraíba, and Salvador populations (characterized by the high frequency of haplotypes C1 and C2); and a third subgroup formed by the Pernambuco, Salvador, Barra Grande, Porto Seguro, and Abrolhos populations which represents a transition zone between south and north Brazil phylogroups (Fig. 3 and Table 1). Similar results were

observed in from F_{ST} (Table 2) estimates where significant genetic differentiation was detected between populations south and north of the Vitória-Trindade seamounts (F_{ST}: 0.243 – 1.000, $p < 0.05$). Non-significant F_{ST} values were observed among south Brazil phylogroup populations suggesting panmixia within the South Brazil Bight (F_{ST}: 0.000 – 0.149, $p > 0.05$). Larger F_{ST} values, some of which were extremely high, were observed among populations within the north Brazil phylogroup (F_{ST}: 0.000 – 0.931, $p < 0.05$ for F_{ST} values above 0.170).

Southern Bahia populations presented the highest values of genetic diversity: haplotype diversity (Porto Seguro); nucleotide diversity (Barra Grande), and average number of nucleotide differences (Barra Grande) (Table 1). Four populations were genetically fixed, resulting in no internal genetic diversity: Ceará (C1), Espírito Santo (C3), Rio de Janeiro (C3), and Santa Catarina (C3) (Table 1). Populations that evidenced larger number of polymorphic sites and total number of mutations were Alagoas and Paraíba (Table 1). The south Brazil phylogroup presented lower genetic diversity across all measured parameters (Hd, Pi, k, S and m) compared to the north Brazil phylogroup (Table 1).

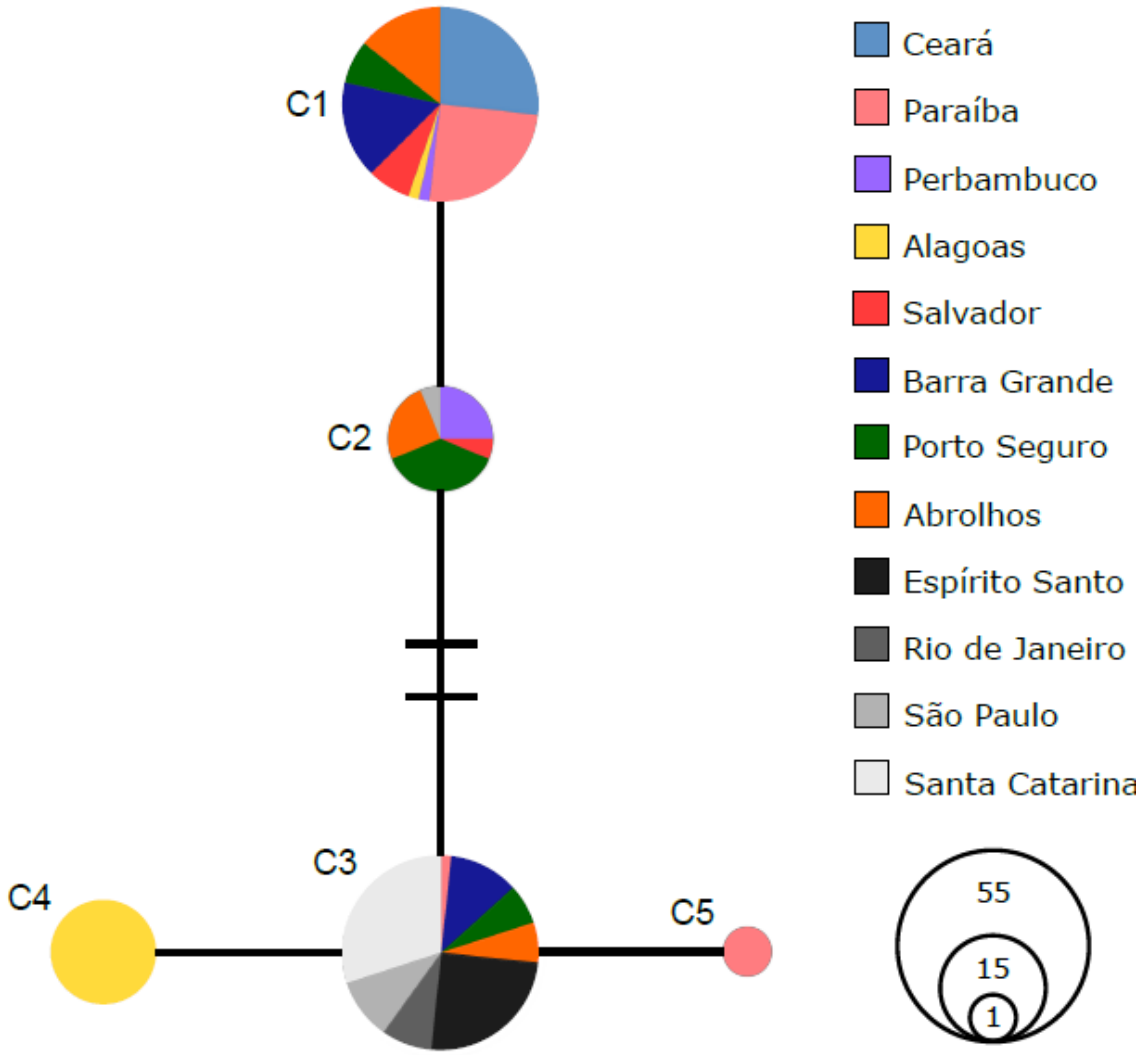

Figure 2. Statistical parsimony network (95% interval confidence) of *Colpomenia sinuosa* *cox*3 sequences obtained from organisms collected at 12 sites along the Brazilian coast. Size of the circle indicate the minimum number of sequences. Eight northernmost populations are represented in colors, while four southernmost populations are represented in grey scale. North and south populations are genetically different according to PERMANOVA results and are geographically separated by the Vitória-Trindade seamount chain.

The estimated evolutionary clock was 1.5 x 10^{-9} s/s/y. The oldest diversification among Brazilian *C. sinuosa* haplotypes occurred ~3.63 Mya (HPD: 1.49 to 6.21 Mya), when the C1 and C2 clade diverged from the C3, C4 and C5 (Figs. 1, 2 and 4). C1

diverged from C2 around 1.35 Mya (HPD: 0.03 to 2.81 Mya). C3 diverged from C4 around 1.23 Mya (HPD: 0.03 to 1.88 Mya). C5 diverged from the C3-C4 MRCA around 1.48 Mya (HPD: 0.28 to 2.89 Mya). BSP detected demographic expansion starting approximately 10,000 years before present, after a long period of constant demography that lasted for as much as 450 thousand years. The same pattern was observed in the north Brazil phylogroup with the exception that the demographic expansion started 50,000 years ago instead of 10,000 y.a. A subtle slow yet constant population increase in the last 50,000 years was suggested for the south Brazil phylogroup, followed by a minor sharper expansion in the last 2,000 years (Fig. 5).

Neutrality tests detected recent population expansion after a bottleneck (or the occurrence of selective sweeps) in the Alagoas population, and balancing selection or sudden population contraction in the Barra Grande population (Table 1). Analyzing separately, the north Brazil phylogroup was in demographic stability, while the south Brazil phylogroup showed significant signs of recent population expansion after a bottleneck or selective sweeps (Table 1).

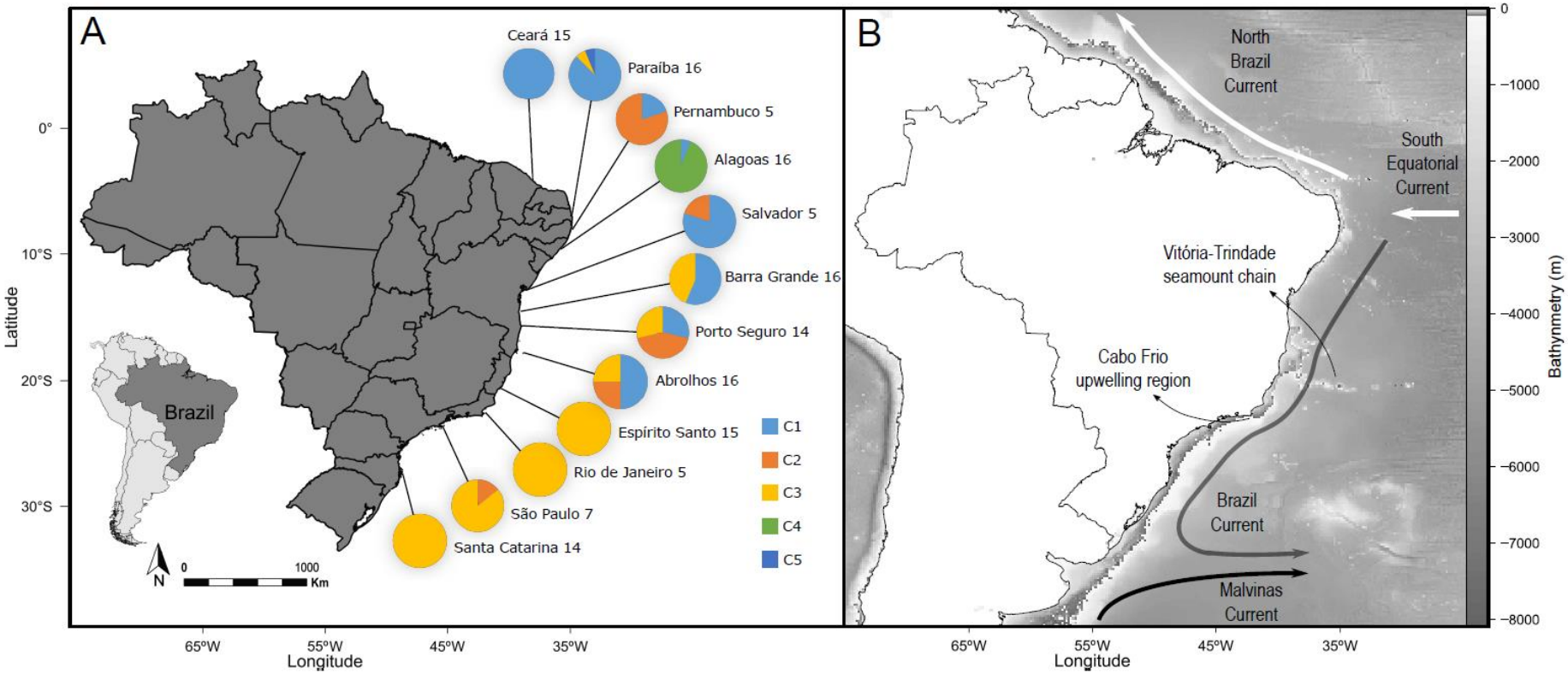

Figure 3. (A) *Colpomenia sinuosa cox*3 DNA sequence haplotype composition within twelve populations sampled along the Brazilian coast. Numbers after population names represent sample size (n). Five haplotypes were found: C1, C2, C3, C4 and C5. (B) Map of the Brazilian coast, showing current (solid contour) and past (pixelated contour) coastline, the latter showing sea level 100 meters lower than present time. Extant boundary currents according to Peterson and Stramma (1991).

Discussion

We detected the presence of significant genetic structure among *Colpomenia sinuosa* populations along the Brazilian coast. Two major phylogroups are evident and split the Brazilian coast into two phylogeographic areas, a northern genetically diverse tropical phylogroup (north Brazil phylogroup), and a southern genetically poor subtropical phylogroup (south Brazil phylogroup). Our molecular clock results suggest these two regions started diverging ~3.63 mya, in the late Pliocene (early Piacenzian), a period of global mean temperature 2–3 ºC higher and sea levels ~20 meters higher than the present time. The Piacenzian was the last age before the Quaternary glaciations (De La Vega et al. 2020).

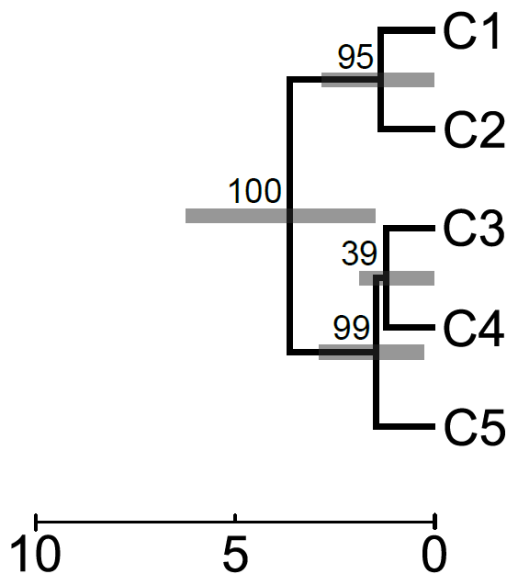

Figure 4. *Cox*3 Bayesian ultrametric tree for *Colpomenia sinuosa* haplotypes generated in this study (C1 – C5). Time scale in million years before present. Grey bars represent 95% HPD. Time calibration based on the mean Ectocarpales age estimated by Silberfeld et al. (2010). All posterior probabilities are shown above the node.

The region where the genetic discontinuity between the north and south Brazil phylogroups occur is located around latitude 20.5˚ S. This region gathers a range of

unique physical, geological, climatic, ecological, oceanographic and historical drivers of genetic isolation and differentiation that, together have shaped Brazil's marine phylogeography and biogeography (Pinheiro et al. 2017, Ayres-Ostrock et al. 2019, Volk et al. 2021). First and foremost, latitude 20.5˚S is part of the transition between tropical and subtropical marine zones (Machado et al. 2017). Differences in seawater temperature, and patterns of ocean circulation, promote prominent changes in environmental conditions, which can be responsible for population genetic differences as a result of adaptations to local thermal conditions. Climate-wise, the shift between tropical and subtropical zones near latitudes 20.5˚S along the Brazilian coast has been recognized as a barrier to gene flow for fish species (Santos et al. 2006, Machado et al. 2017) and marine macroalgae (Ayres-Ostrock et al. 2019, Nauer et al. 2019). Geologically, this region presents the largest shallow offshore expansion of the continental shelf in the Brazilian coast (the Abrolhos bank), which is accompanied by the Vitória-Trindade seamount chain extending nearly perpendicular to the coastline (Fig. 3). While in the present time the weak Brazil Current flows southward along the Brazilian coast, undisturbed by the Abrolhos Bank-Vitória-Trindade seamount chain, during periods of glaciation maxima and lower sea level, the Brazil Current flow might have been disturbed and partially diverted offshore, compromising alongshore genetic connectivity. The region around latitude 20.5˚ S is also where the mouth of the Doce river is located. The Doce river basin drains a terrestrial area of approximately 86.715 km^2 and discharges an annual average of ~ 985.12 m^3/s of freshwater into coastal waters (historical estimates based on models considering the absence of human impact and a fully forested basin, Lyra and Rigo 2019). Latitude 20.5˚S is also near the region where a major shift in marine substrate habitat occurs in Brazil. Biogenic reefs (including calcareous and sandstone sedimentary rocks) dominate the benthos at north of this region while igneous rocky reefs characterize benthic habitats south of this region (Floeter et al. 2001, Pinheiro et al. 2017). All these factors, whether

working in isolation or in tandem, help to explain the formation of *Colpomenia sinuosa* north and south phylogroups.

The recognition of latitude 20.5˚S region, which includes The Vitória-Trindade seamount chain, the Doce river estuary and the Abrolhos bank, is the area of greatest genetic discontinuity observed among continuously distributed *C. sinuosa* populations along the Brazilian coast, agrees with phylogeographic patterns described for several other marine species such as mollusks (Lazoski et al. 2011), crustaceans (Hurtado et al. 2016) and annelids (Paiva et al. 2019). However, the mechanisms on how such drivers of genetic isolation operate are quite different. While the Doce river has been considered an extant putative hard or semi-permeable (= soft) barrier gene flow, the Vitoria-Trindade seamount chain is considered an old intermittent barrier driving genetic isolation during periods of glacial maxima (Pinheiro et al. 2017, Menezes et al. 2020). Unfortunately, our results were not able to estimate which of them exerted the largest historical influence driving *C. sinuosa* phylogeographic structure.

Table 2. F_{ST} pairwise differences. Numbers in bold indicate significant differences ($p < 0.05$).

	1	2	3	4	5	6	7	8	9	10	11	12
1. Ceará	0.000											
2. Paraíba	0.028	0.000										
3. Pernambuco	**0.876**	**0.652**	0.000									
4. Alagoas	**0.931**	**0.806**	**0.802**	0.000								
5. Salvador	0.241	0.000	0.412	**0.794**	0.000							
6. Barra Grande	**0.390**	**0.202**	**0.457**	**0.663**	0.136	0.000						
7. Porto Seguro	**0.518**	**0.364**	0.042	**0.591**	0.152	0.142	0.000					
8. Abrolhos	**0.323**	**0.169**	0.194	**0.591**	0.000	0.022	0.000	0.000				
9. Espírito Santo	**1.000**	**0.867**	**0.903**	**0.935**	**0.903**	**0.523**	**0.518**	**0.546**	0.000			
10. Rio de Janeiro	**1.000**	**0.807**	**0.800**	**0.903**	**0.800**	**0.387**	**0.369**	**0.411**	0.000	0.000		
11. São Paulo	**0.910**	**0.729**	**0.622**	**0.824**	**0.656**	**0.314**	**0.243**	**0.316**	0.118	0.000	0.000	
12. Santa Catarina	**1.000**	**0.878**	**0.915**	**0.941**	**0.915**	**0.550**	**0.547**	**0.572**	0.000	0.000	0.149	0.000

In the marine environment, shallow-water benthic species often show genetic discontinuities and spatial phylogeographic concordance at locations with unique geological, oceanographic, and climatic features (Avise 2000, Hu and Fraser 2016). Many of physical and ecological drives of genetic structure often mark concordant limits in species distribution, and hence delimit borders between biogeographic provinces as well. Latitude 20.5˚S physical and ecological features split the Brazilian coast not only into two *C. sinuosa* phylogeographic groups but between two biogeographic provinces, or Large Marine Ecosystems according to (Hempel and Sherman 2003, Sherman et al. 2005). The concordance between phylogeographic structure (genetic breaks) and biogeographic structure (species distribution breaks) suggest that both patterns are determined by shared physical and historical factors (Avise 1998, 2000, Bowen et al. 2016).

Populations south of latitude 20.5˚S (= south phylogroup) occupy a long stretch of coastline (~1,200 Km), however they presented lower levels of genetic diversity and genetic sub-structure, which are signs of sudden population expansion after a demographic bottleneck (Grant 1998). Even though Bayesian Skyline plots observed demographic expansion on both north and south phylogroup population, statistically significant neutrality tests were only observed for the south phylogroup. This result suggests that southern Brazil populations as a whole experienced a demographic expansion in recent geological time. C3 is by far the most frequent and often exclusive haplotype in southern (subtropical) populations. C3 also occurs in the fringe populations between south and north Brazil phylogroups suggesting that this is a cold-adapted tropical haplotype that was selected to recolonize southern latitudes as the planet warmed after each glacial period. Our results and data interpretation agree with a plethora of other Brazilian marine phylogeography studies. For example, the expansion and colonization of southern Brazil by tropical marine species after the last glaciation has been reported for a large range of marine taxa such as red macroalgae (Ayres-Ostrock et al. 2019, Nauer et

al. 2019), mangrove vascular plants (Francisco et al. 2018), a cnidarian (Peluso et al. 2018), a sea turtle (Reis et al. 2010), fish species (Santos et al. 2006, da Silva et al. 2016, Machado et al. 2017), an annelid (Paiva et al. 2019), and nemerteans (Andrade et al. 2011).

In this study, the Cabo Frio upwelling region (latitude 23ºS) was not identified as a barriers to gene flow for *C. sinuosa* populations. The Cabo Frio upwelling system is expected to act as a barrier to gene flow for coastal benthic marine organisms (Peluso et al. 2018) due to the sudden shift in sea temperatures during the summer seasons (Valentin et al. 1987). This upwelling has been recognized as an effective barrier to gene flow for some marine species such as fishes (Cortinhas et al. 2016), crustaceans (Maggioni et al. 2003, Hurtado et al. 2016), and cetaceans (de Oliveira et al. 2019). However, the Cabo Frio upwelling has not yet been observed as a relevant barrier to gene flow for marine macroalgae (Ayres-Ostrock et al. 2019, Nauer et al. 2019), including this study.

Some degree of *C. sinuosa* population sub-structure was detected within the north Brazil phylogroup. The three genetically richest *C. sinuosa* populations are located within southern Bahia. Southern Bahia populations (= Abrolhos, Porto Seguro, Barra Grande, and Salvador) are more genetically diverse (Hd = 0.525 – 0.703) than populations north of Salvador (Hd = 0.000 – 0.400). This region is the genetic transition zone between *C. sinuosa* south and north phylogroups. Southern Bahia is a biodiversity hotspot for both terrestrial and marine species, acting as a historical glacial refugium during the Pleistocene (Carnaval et al. 2009, Lazoski et al. 2011, Hurtado et al. 2016, Paiva et al. 2019, Menezes et al. 2020). The southern Bahia refugium theory as a driver of genetic diversity and structure has also been proposed as an explanation for the presence of phylogeographic structure observed in another marine macroalgae, the agarophyte *Crassyphycus caudatus* (Ayres-Ostrock et al. 2019).

Another process that helps explain the larger levels of genetic diversity found in southern Bahia populations refers to the riverine hypothesis. Southern Bahia populations might have been affected by Pleistocene geological climate instability, which contributed to fluctuations of freshwater discharge into the ocean. Past and current freshwater discharge at Bahia's hydrographic basin produced a plethora of large number of river mouths and larger estuaries which can act as impermeable and semipermeable barriers to gene flow (Pellegrino et al. 2005). Comparatively, hydrographic basins in locations adjacent to southern Bahia state generate smaller high-flow rivers (Matos et al. 2007). The riverine theory as a driver of genetic diversity and structure has been proposed for terrestrial (Pellegrino et al. 2005) and marine organisms, such as fishes (*Bagre bagre* Linnaeus (1766); da Silva et al. 2016), and mollusks (*Anomalocardia brasiliana* Gmelin (1791); Arruda et al. 2009). River mouths and their estuaries as intermittent barriers to gene flow, together with the range of other processes described above, likely helped to drive current patterns of genetic diversity and structure in southern Bahia *C. sinuosa* populations.

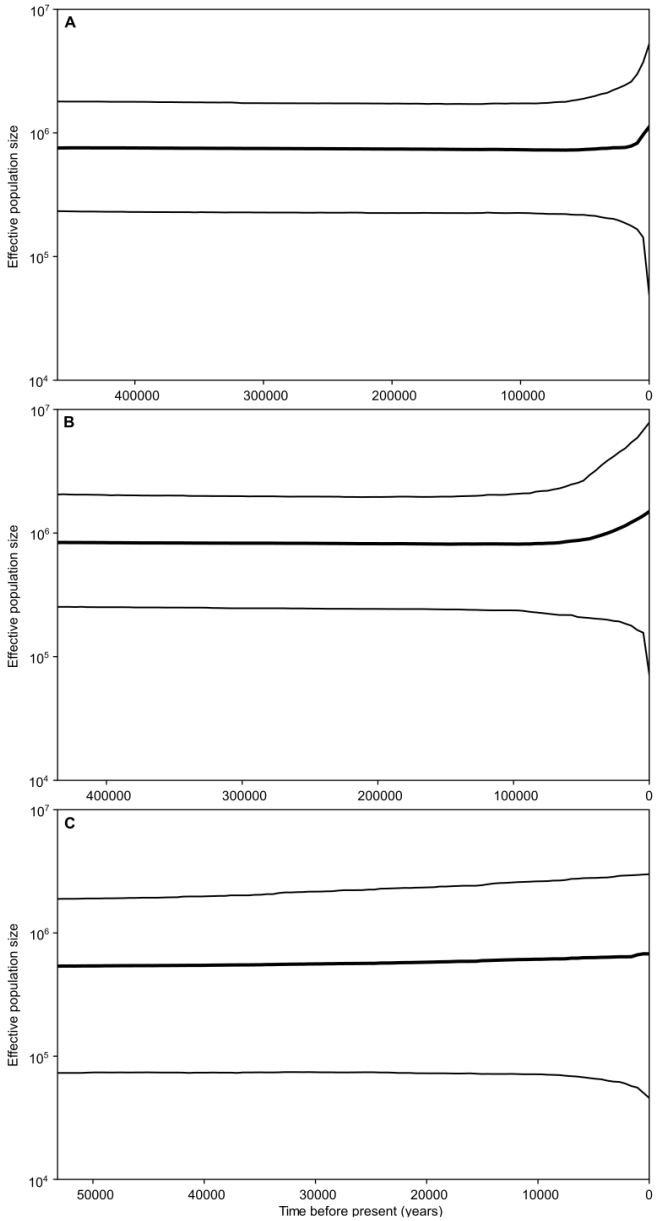

Figure 5. Bayesian skyline plot of C*olpomenia sinuosa cox*3 DNA sequences for specimens collected across 12 sites along the Brazilian coast. The thick solid line represents mean effective population size (Ne) based on *cox*3 mutation rate ($1.9x10^{-9}$ s/s/y) per year before present day. Upper and lower thin solid lines represent the 95% confidence limits. A = all 12 populations, B = northernmost eight populations, and C = southernmost four populations. North and south populations are genetically different according to PERMANOVA results and are geographically separated by the Vitória-Trindade seamount chain.

The uniqueness of the Alagoas population with nearly no genetic diversity and the dominance of C4 suggests recent bottleneck, strong selective sweep, or recent colonization event from single or a small founding population. In some regions, *C. sinuosa* populations are known to suddenly appear and disappear, producing highly dynamic demographics. During their disappearance, it remains unknown whether *C. sinuosa* specimens persist in the benthic community in a cryptic condition, such as tiny crusts, inconspicuous tuft forms (= diploid phase), or as unicellular propagules. Our results suggest recent colonization from a haplotype yet not detected in other locations, or recent macro-thalli population expansion from a local reduced genetic stock. Further investigations, increasing sampling size and implementing BSP could elucidate if this bottleneck have occurred on recent years (probably due to anthropogenic actions) or during the last million years.

Three out of five *C. sinuosa* haplotypes identified in this study are new (C2, C4 and C5) compared to all other ~37 *cox*3 DNA haplotypes available in GenBank. C3 has been reported in Hawaii (JX944729 and JX944730, Lee et al. 2013), South Africa, Australia (Perth WA) and Brazil (JX944730, Lee et al. 2013). C1 has been reported in Hawaii (JX944725, Lee et al. 2013) and South Africa (HQ833778, Boo et al. 2011). The tropical C1 and the subtropical C3 are not only the most widely distributed *C. sinuosa* haplotypes in the world (Lee et al. 2013) but were also the two most geographically widespread haplotypes along the Brazilian coast. Using worldwide samples, Lee et al. (2013) divided *C. sinuosa* into three clades. C1 and C3 belong to an exclusive tropical-subtropical clade. This clade was likewise identified as an ancestral lineage. The overall lower genetic diversity detected in this study compared to Lee et al. (2013) suggests that the Brazilian populations are not the origin of *C. sinuosa* populations in the world.

We did not detect evidences to support the presence of human-based *C. sinuosa* introductions in Brazil. The occurrence of *C. sinuosa* populations in Brazil is older than

would be expected if anthropogenic introduction had occurred. Our results are opposite to previous reports supporting human-driven *Colpomenia* spp. introductions in several parts of the world (Parsons 1982, Cho et al. 2005, Norris 2010, Boo et al. 2011), including Brazil (Lee et al. 2013). Our Rio de Janeiro population is the exact same location sampled by Lee et al. (2013): Praia Rasa, Búzios city. We did not recover either in the Rio de Janeiro population or in any other Brazilian population the two haplotypes reported by Lee et al. (2013) for Brazil (JX944726 and JX944746). Instead, we found only the C3 haplotype in the Rio de Janeiro. Consequently, we were not able to cast further light on the status of Lee et al. (2013) Brazilian *C. sinuosa cox*3 sequences. Future sampling, particularly around Rio de Janeiro, should help to test Lee et al. (2013) hypothesis on the origin of *C. sinuosa*, or other *Colpomenia* species, in Brazil. Our results, however, do not corroborate Lee et al. (2013) suggestion but point to a natural origin of *C. sinuosa* in Brazil that is as older as the origin of the human species in the planet, ~ 3.8 mya.

In conclusion, our study demonstrates the existence of one major genetic break along the Brazilian coastline, represented by the Vitória Trindade seamount chain (20.5º S), resulting in two divergent *C. sinuosa* phylogroups (north vs. south). The widespread sampling along the Brazilian coast allowed us to produce a more detailed understanding of *C. sinuosa* genetic diversity and population structure. The combination of past oceanographic, geological and climatic variations alongside with extant coastal processes explain the evolution and diversification of *C. sinuosa* in Brazil. This study also demonstrates the impact of a complex region characterized by the interaction of changes in climate over geological time (glaciations), the Brazil Current, the Abrolhos basin, the Doce river and the Victoria-Trindade seamount chain driving genetic structuring, and potentially recent speciation, along the Brazilian coast. Acknowledging the existence of multiple phylogeographical lineages is important not only for understand recent historical processes shaping genetic diversity in tropical regions, but also as subsidies for

conservation and the management of natural marine resources. Future studies using other genetic markers and a more spatially dense sampling design will contribute to elucidate further biogeographic and phylogeographic patterns in Brazilian *Colpomenia* populations.

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Supplemental material

Table S1. List of *Colpomenia sinuosa* collection sites along the Brazilian coast and their respective meta-data.

Site	State	City	Beach	N	Date	Collectors	GPS Coordinates
1	Ceará	Icapuí	Ponta Grossa	15	01/09/2019	Ximenes CF, Ximenes PV and Ribeiro ALN	-4.627507, -37.503233
2	Paraíba	João Pessoa	Caribessa	16	16/02/2017	Araújo P	-7.077192, -34.828710
3	Pernambuco	Tamandaré	Carneiros	5	24/02/2018	Fujii MT	-8.713937, -35.083415
4	Alagoas	Maceió	Pajuçara	5	09/11/2018	Carneiro VAR	-9.664033, -35.703786
4	Alagoas	Maceió	Pajuçara	11	22/09/2018	Carvalho N	-9.664033, -35.703786
5	Bahia	Salvador	Farol de Itapuã	2	24/02/2018	Pessôa AC	-12.956171, -38.352413
5	Bahia	Salvador	Stella Maris	1	31/01/2018	Santos GN	-12.948912, -38.340989
5	Bahia	Ilha de Itaparica	Penha	2	01/02/2018	Santos GN	-12.985258, -38.616709
6	Bahia	Barra Grande	Ponta do Mutá	5	18/02/2018	Pessôa AC	-13.880233, -38.947824
6	Bahia	Barra Grande	Taipu de Fora	11	18/02/2018	Pessôa AC	-13.941611, -38.927220
7	Bahia	Porto Seguro	Mucugê	5	10/01/2018	Oliveira VP	-16.497755, -39.068043
7	Bahia	Porto Seguro	Parracho	9	15/10/2018	Martins NT	-16.507448, -39.070453

Table S1. Continuing

8	Bahia	Abrolhos	Ilha Siriba	16	17/10/2018	Martins NT	-17.969832, -38.710340
9	Espírito Santo	Marataízes	Castelhanos	15	30/04/2018	Harb T, Oliveira W and Chow F	-20.839695, -40.627023
10	Rio de Janeiro	Búzios	Rasa	5	18/03/2018	Martins NT and Cassano V	-22.733842, -41.957532
11	São Paulo	Ubatuba	Ilha Anchieta	5	20/08/2017	Martins NT	-23.536789, -45.062898
11	São Paulo	Ubatuba	Vermelha do Sul	2	22/08/2017	Martins NT	-23.512250, -45.171682
12	Santa Catarina	Bombinhas	Bombinhas	2	13/10/2016	Ayres-Ostrock L	-27.147530, -48.483007
12	Santa Catarina	Bombinhas	Bombinhas	2	16/10/2019	Martins NT and Gurgel CFD	-27.147530, -48.483007
12	Santa Catarina	Florianópolis	Armação	10	04/04/2018	Gurgel CFD	-27.748995, -48.500020

Table S2. GenBank depositing sequences metadata.

Haplotype	GenBank code	State	City	Beach	Date	Collector	GPS Coordinates
C1	MW981282	BA	Porto Seguro	Parracho	15 Dec 2018	Martins, NT	-16.507448, -39.070453
C2	MW981283	BA	Porto Seguro	Parracho	15 Dec 2018	Martins, NT	-16.507448, -39.070453
C3	MW981284	BA	Porto Seguro	Parracho	15 Dec 2018	Martins, NT	-16.507448, -39.070453
C4	MW981285	PB	João Pessoa	Caribessa	16 Dec 2017	Araújo, P	-7.077192, -34.828710
C5	MW981286	AL	Maceió	Pajuçara	09 Nov 2018	Carneiro, VAR	-9.664033, -35.703786

Chapter 4

First record of *Mikrosyphar zosterae* (Chordariaceae, Phaeophyceae) in the southern hemisphere and as an endophyte in the brown algal genera *Leathesia* and *Colpomenia*

Primeiro registro de *Mikrosyphar zosterae* (Chordariaceae, Phaeophyceae) no hemisfério sul e como endófita das algas pardas *Leathesia* e *Colpomenia*

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Running title: First record of *Mikrosyphar zosterae*

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Abstract: Several brown filamentous algae, in particular to the family Chordariaceae, are known to occur as endophytic to marine algae. Among them, the genus *Mikrosyphar* is relatively understudied. *Mikrosyphar* specimens are tiny, consisting of uniseriate and branched prostrate filaments isolated or aggregated in a pseudoparenchyma, forming brown patches on its hosts. During an investigation of molecular genetics in *Leathesia marina* and *Colpomenia* spp. in temperate Australia, we identified the presence of *Mikrosyphar zosterae* as endophyte in both genera using *rbc*L-*rbc*S and COI-5P DNA sequences. This is the first time the endophytic *M. zosterae* is reported having as hosts the macroalgae *L. marina* and *Colpomenia* spp. and occurring in the southern hemisphere. Our endophyte sequences matches with low genetic divergence the reference *M. zosterae* DNA sequences obtained from isolated unialgal cultured material and hence the true *M. zosterae*. We have confirmed the identification of 12 *Leathesia marina* as hosts through COI-5P DNA sequencing. In contrast, the occurrence of *Mikrosyphar* inside *Colpomenia* seems to be less common, since we have detected only two *Colpomenia* spp. (*C. claytoniae* and *C. sinuosa*) hosting endophytes in our broad sampling in Australia. Further investigations will help to clarify whether this endophyte also occurs on other Australian marine species and in other regions of the world, especially where *Leathesia* is already reported. In addition, laboratory experiments would elucidate if this relationship is harmful or not to the host.

Key-words: Australia, brown filamentous algae, COI-5P, *Colpomenia*, endophyte, *Leathesia, Mikrosyphar, rbc*L-*rbc*S.

Resumo: Diversas algas pardas filamentosas, em particular da família Chordariaceae, são conhecidas como endofíticas de algas marinhas. Entre elas, o gênero *Mikrosyphar* é relativamente pouco estudado. Os espécimes de *Mikrosyphar* são diminutos, consistindo de filamentos prostrados unisseriados e ramificados isolados ou agregados em um pseudoparênquima, formando manchas marrons em seus hospedeiros. Durante uma investigação da genética molecular em *Leathesia marina* e *Colpomenia* spp. na região temperada da Austrália, identificamos a presença de *Mikrosyphar zosterae* como endófita em ambos os gêneros usando as sequências de *rbc*L-*rbc*S e COI-5P. Esta é a primeira vez que a endofíta *M. zosterae* é encontrada em *L. marina* e *Colpomenia spp*. no hemisfério sul. Nossas sequências da endófita possuem baixa divergência genética com as sequências de DNA referência de *M. zosterae* obtidas de material isolado em cultura unialgácea e, portanto, correspondem a verdadeira *M. zosterae*. Confirmamos a identificação de 12 indivíduos de *Leathesia marina* como hospedeiros, através do sequenciamento de COI-5P. No entanto, a ocorrência de *Mikrosyphar* em *Colpomenia* parece ser menos comum, uma vez que detectamos apenas dois indivíduos de *Colpomenia* spp. (*C.* claytoniae e *C.* sinuosa) hospedando endófitas em nossa ampla amostragem na Austrália. Outras investigações ajudarão a esclarecer se esta endófita também ocorre em outras espécies marinhas australianas e em outras regiões do mundo, especialmente onde a *Leathesia* é relatada. Além disso, experimentos de laboratório poderiam elucidar se essa relação é prejudicial ou não ao hospedeiro.

Palavras-chave: algas pardas filamentosas, Austrália, COI-5P, *Colpomenia*, endófita, *Leathesia*, *Mikrosyphar*, *rbc*L-*rbc*S.

Several filamentous endophytic genera are assigned to the Phaeophyceae, in particular to the family Chordariaceae (Cormaci *et al.* 2012). Brown endophytic filamentous algae are known to cause infections in host marine algae (Schoenrock *et al.* 2013; Ogandaga *et al.* 2016, 2017; Gao *et al.* 2019). Acting as pathogens, they may cause morphological, physiological and ecological changes in the host alga such as production of galls and wart-like spots, changes in metabolism and growth rates, and changes in survivorship and reproduction (Schoenrock *et al.* 2013; Ogandaga *et al.* 2016, 2017; Gao *et al.* 2019). Among brown algal filamentous endophytes, the genus *Mikrosyphar* Kuckuck is relatively understudied. To date, only *Mikrosyphar zosterae* Kuckuck (1895: 177) was studied thoroughly (Ogandaga *et al.* 2016, 2017).

Besides *Mikrosyphar zosterae*, four other taxonomically accepted species currently assigned to the genus are: *M. pachymeniae* Lindauer (1960: 165), *M. polysiphoniae* Kuckuck (1897: 381), *M. porphyrae* Kuckuck (1897: 355), and *M. sphacelariae* Levring (1974: 27) (Guiry & Guiry 2021). These *Mikrosyphar* species' names are closely related to the respective host from where they were first collected, observed and described. *Mikrosyphar* specimens are tiny, consisting of uniseriate and branched prostrate filaments isolated or aggregated in a pseudoparenchyma, forming brown patches on its hosts (Cormaci *et al.* 2012; Ogandaga *et al.* 2017). The endophytic thallus is usually located under the cell wall of the hosts' cortical cells (in morphologically simpler hosts), or can reach deeper into the interspatial region between cortical cells, or the upper medullary cell layers in hosts with more complex thalli. *Mikrosyphar* species have been reported growing on red macroalgae (e.g. *Polysiphonia* Greville, *Pachymenia* J.Agardh, *Porphyra* C.Agardh, and *Chondrus* Stackhouse), brown macroalgae (e.g. *Sphacelaria* Lyngbye), and seagrasses (e.g. *Spartina* Schreber and *Zostera* Linnaeus) (Ogandaga *et al.* 2017; Guiry & Guiry 2021). *Mikrosyphar* species occurs in temperate regions of eastern Canada and USA, Europe, South Korea, Australia, and New Zealand (Guiry & Guiry 2021).

Mikrosyphar zosterae was first reported in Germany, Baltic Sea, its type locality (Guiry & Guiry 2021), and later reported for eastern Canada and USA, Europe, and South Korea (Ogandaga *et al.* 2017; Guiry & Guiry 2021). *Mikrosyphar zosterae* was reported for the first time on the red seaweed *Chondrus ocellatus* Holmes (1896: 252) in South Korea (Ogandaga *et al.* 2016) where it can cause warts-like spots and degenerative lesions.

Ogandaga *et al.* (2016, 2017) isolated *M. zosterae* from *Chondrus ocellatus* collected in Korea, and cultivated it in unialgal cultures. They also, for the first time, molecularly identified isolated unialgal *M. zosterae* specimens using DNA sequencing and described the *M. zosterae* ontogenetic development reporting different developmental morphological types such as heterotrichous, pseudoparenchymatous and monostromatic prostrate thalli. *Mikrosyphar zosterae* DNA sequences were obtained for the chloroplast-encoded RuBisCo spacer region (GenBank accession number: KU569308) (Ogandaga *et al.* 2016, 2017). The morphological, molecular and culture evidence suggest that Ogandaga *et al.* (2016, 2017) obtained *M. zosterae*, and hence these two studies became the current foundational references to *M. zosterae* knowledge in the world.

Two ecologically relevant Ectocarpales species are the saccate genera *Leathesia* S.F.Gray (Chordariaceae) and *Colpomenia* (Endlicher) Derbès & Solier (Scytosiphonaceae). *Leathesia* encompasses 14 widely distributed species (Guiry & Guiry 2021), occurring in cold waters from subtropical to temperate regions (Oates 1989). The life-history of *Leathesia marina* (Lyngbye 1819: 193) Decaisne (1842: 370) is well known and exhibits four different *in vitro* morphologies: two morphotypes of diploid phases occurring as either globular macrothalli or globular microthalli, and two haploid phases, occurring as branched erect microthalli or branched prostrate microthalli. The genus *Colpomenia* currently has ten taxonomically accepted species distributing from tropical to temperate waters (Guiry & Guiry 2021). *Colpomenia* is characterized by solitary or gregarious habit, hollow vesicular, cerebriform thallus, yellowish-brown color, with smooth

to rough appearance and plurilocular structures in punctate sori (Toste *et al.* 2003). The life-history of *Colpomenia sinuosa* (Mert. ex Roth 1806: 327) Derbès & Solier in Castagne (1851: 95) is heteromorphic diplobiont with vesicular gametophytes and filamentous or pulvinated sporophytes (Toste *et al.* 2003).

During an investigation of molecular genetics in *Leathesia marina* and *Colpomenia* spp. in temperate Australia, we identified the presence of *Mikrosyphar zosterae* as endophyte in both genera using *rbc*L-*rbc*S and COI-5P DNA sequences. In this study, we reported the endophytic *M. zosterae* having as hosts the macroalgae *L. marina* and *Colpomenia* spp. in Australia, southern hemisphere, for the first time.

Eighteen individuals of *Leathesia marina* were collected in five Australian localities: Twin Rocks, South Australia (n = 1), Blanket Bay, Victoria (n = 5), Kennett River, Victoria (n = 3), Lorne, Victoria (n = 5), and Bicheno, Tasmania (n = 4). One individual of *Colpomenia sinuosa* and one of *C. claytoniae* S.M.Boo, K.M.Lee, G.Y.Cho & W.Nelson (2011: 160), both from Portsea, Victoria were also collected (Table S1). In the field, all specimens were cleaned from epiphytes, rinsed with local seawater and then stored in silica gel desiccant. Dried specimens were once again cleaned from potential remaining epiphytes under a Stemi SV 6-Zeiss stereomicroscope (Zeiss, Göttingen, Germany). DNA extraction, DNA sequence reactions and automated DNA Sanger sequencing via capillary separation were performed as described in Dixon *et al.* (2012). The plastid *rbc*L-*rbc*S spacer (with partial flanking *rbc*L and *rbc*S sequences) and the mitochondrial COI-5P were amplified according to Mattio *et al.* (2008) and Saunders and McDevit (2012), respectively. All newly generated sequences were deposited to GenBank under accession numbers MW465909-41 (Table S1). Multiple alignments for both *rbc*L-*rbc*S and COI-5P sequences were built using Geneious v.5.5.6 (Kearse *et al.* 2012).

A maximum likelihood (ML) tree with 1,000 bootstrap replicates was performed for the *rbc*L-*rbc*S marker, using TIM3 + F + G4 evolution model, selected under Alkaike

information criterion. ML and model selection analysis were performed using IQ-Tree v.1.4.3 (Nguyen *et al.* 2015) on the IQ-Tree web portal. Neighbor joining (NJ) tree was built for the COI-5P marker using p-distances, and 2,000 bootstrap replicates. NJ tree and pairwise genetic distance matrixes were built in PAUP* 4.0a167 (Swofford 2014). The use of NJ for DNA barcode markers is justified since, with this marker, we are not interested in phylogeny reconstruction but to cluster specimens into operational taxonomic units (Wilson *et al.* 2019).

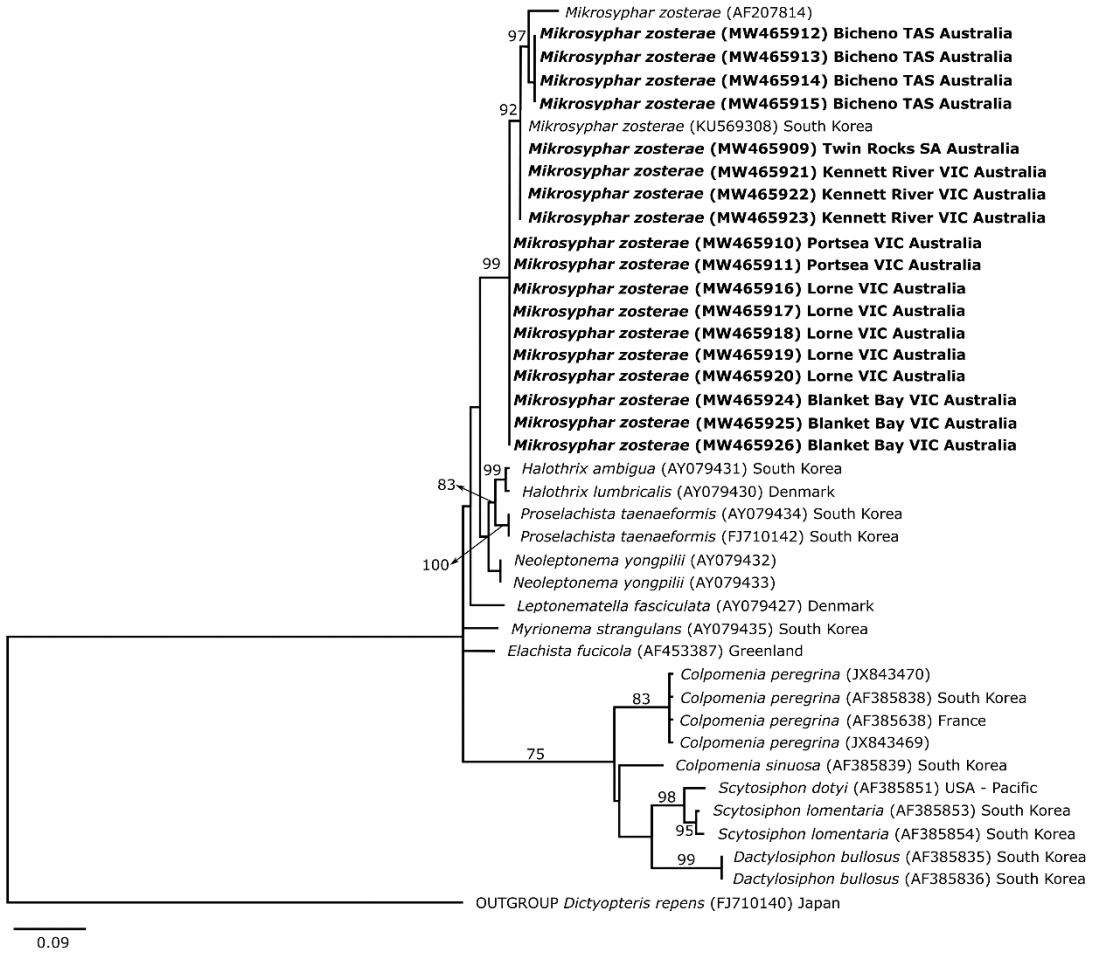

Figure 1. Maximum likelihood (ML) analysis for *rbc*L-*rbc*S spacer DNA sequences of *Mikrosyphar zosterae* and related taxa. Bootstrap values higher than 75 are shown at the nodes. Samples generated in this study are in bold. Codes following sequence names refer to GenBank accession numbers.

For the *rbc*L-*rbc*S analysis, 37 DNA sequences were used in an alignment of 533 bp, including 18 newly generated sequences of *Mikrosyphar zosterae* (16 amplified from the host *Leathesia marina*, one from *Colpomenia claytoniae* and one from *C. sinuosa*). The remaining sequences were downloaded from GenBank (Table S1). *Dictyopteris repens* (Okamura) Børgesen was used as outgroup (Table S1). We generated 13 new COI-5P DNA sequences of *Leathesia marina*, one *C. claytoniae*, and one *C. sinuosa* and built an alignment of 610 bp with 46 sequences, of which 31 were downloaded from GenBank (Table S1). Two Dictyotales were used as outgroups in the COI alignment, *Dictyopteris polypodioides* (DC. in Lamarck & De Candolle 1805: 15) J.V.Lamouroux (1809: 322) and *Dictyopteris hoytii* W.R.Taylor (1960: 229) (Table S1). As there are neither *rbc*L-*rbc*S DNA spacer sequences of *Leathesia* nor COI-5P sequences of *Mikrosyphar* in public databases, we built a matrix using the overlapping *rbc*L-3' end region with an alignment of 200 bp length. This alignment comprised 29 sequences of which 18 corresponded to *Mikrosyphar zosterae* sequences newly generated in this study. The remaining were downloaded from GenBank: one *Mikrosyphar zosterae*, one *Mikrosyphar porphyrae*, and nine *Leathesia marina* (Table S1). This short alignment was analyzed for genetic divergence.

Table 1. Pairwise genetic distances (%) of *Mikrosyphar zosterae* for the *rbc*L-S DNA sequences. Lower triangle shows minimum and upper triangle maximum distance values. Diagonal shows minimum and maximum distances.

	1	2	3
1. *Mikrosyphar zosterae* (this study, n = 18)	0.00 - 1.23	1.10	16.20
2. *M. zosterae* (GenBank, n = 2)	0.00	0.40	19.10
3. *Colpomenia peregrina* (GenBank, n = 3)	14.88	17.35	0.87

Results of the *rbc*L-*rbc*S ML analysis are shown in Fig. 1. All newly generated *M. zosterae* DNA sequences generated in this study formed a high support clade (BS = 99) with the *M. zosterae* reference DNA sequence generated by Ogandaga *et al.* (2016, 2017) in South Korea (KU569308) and from an unspecified site (AF207814). A distinct *rbc*L-*rbc*S haplotype was detected for sequences from Bicheno (Tasmania), which formed a clade with high bootstrap value (BS = 97) with a *M. zosterae* from unspecified site (AF207814). The *rbc*L-*rbc*S genetic distance between the two *M. zosterae* from GenBank was 0.40%. The genetic distances between *M. zosterae* from continental Australia and the two from GenBank ranged from 0.00 to 1.23% (Table 1). The genetic distances between Tasmanian *M. zosterae* specimens and the remaining *M. zosterae* sequences ranged from 0.82 to 1.23%. Until the time of the submission of this study for publication, there were no *rbc*L-*rbc*S DNA sequences of *Leathesia* in public DNA databases (e.g. GenBank).

In our results, *rbc*L-*rbc*S primers amplified the endophyte, *Mikrosyphar,* while COI primers amplified the host: *Leathesia marina, Colpomenia claytoniae* and *Colpomenia sinuosa.* Therefore, our COI-5P NJ results (Fig. 2) grouped the newly generated Australian COI DNA sequences with sequences of either *L. marina* (also as *L. difformis* Areschoug 1847: 376) within a well-supported clade (including EU681412, a sequence from Plouguerneau, Brittany, France, the closest location to the type locality Funen Island, Hindsholm, Denmark), or *Colpomenia claytoniae* and *C. sinuosa*, also well-supported (Fig. 2). Genetic distance between Australian and non-Australian *Leathesia marina* COI sequences was 0.98% (Table 2). Until the time of the submission of this study for publication, there were no COI-5P DNA sequences of *Mikrosyphar* available in public DNA databases (e.g. GenBank). For *Colpomenia*, one newly generated sequence matched the sequence of *C. claytoniae* from Japan with 0.50% genetic divergence, whereas our other sequence matched *C. sinuosa* from Australia and South Korea (n = 8), with 0.33–1.81%

genetic divergence. These divergence values are within the range of intraspecific variation of *C. sinuosa* for COI-5P (0.00–1.80%) (Table 2) and close to *Colpomenia peregrina* Sauvageau (1927: 321) intraspecific variation (1.40–1.70%) (McDevit & Saunders 2017).

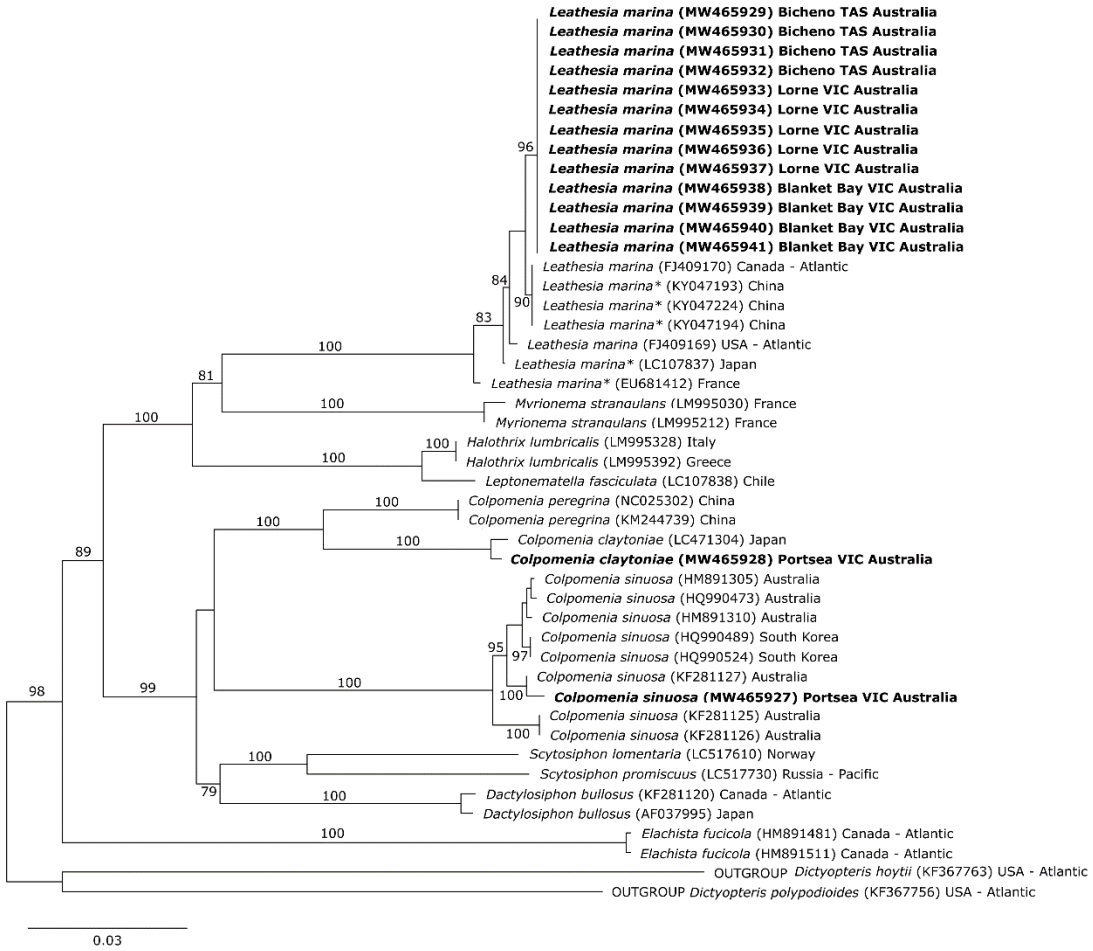

Figure 2. Neighbor joining (NJ) analysis for COI-5P DNA sequences of *Leathesia marina*, *Colpomenia claytoniae* and *Colpomenia sinuosa* and related taxa. Bootstrap values higher than 75 are shown at the nodes. Codes following sequence names refer to GenBank accession numbers. *sequences originally referred to as *Leathesia difformis* Areschoug in GenBank.

Table 2. Pairwise genetic distances (%) of *Leathesia* S.F.Gray and *Colpomenia* (Endlicher) Derbès & Solier species for the COI-5P DNA sequence marker (533 bp). Lower triangle shows minimum and upper triangle maximum distance values. Diagonal shows minimum and maximum distance values. * sequences originally referred to as *Leathesia difformis* Areschoug in GenBank.

	1	2	3	4	5	6	7	8
1. *Leathesia marina* (this study, n = 13)	0.00 - 0.00	16.09	15.44	0.99	16.07	15.46	14.45	0.99
2. *Colpomenia sinuosa* (this study, n = 1)	15.95	NA	11.49	16.26	1.81	11.43	10.51	15.93
3. *C. claytoniae* (this study, n = 1)	15.41	11.49	NA	15.57	11.80	0.50	5.74	14.92
4. *L. marina* (GenBank, n = 7)*	0.16	15.93	14.92	0.00 - 0.98	16.23	15.6	14.59	0.00
5. *C. sinuosa* (GenBank, n = 8)	15.60	0.33	11.15	15.41	0.00 - 1.80	12.08	10.82	15.57
6. *C. claytoniae* (GenBank, n = 1)	15.44	11.43	0.50	14.93	11.07	NA	6.21	14.93
7. *C. peregrina* (GenBank, n = 2)	14.43	10.51	5.74	13.93	10.16	6.21	0.00	13.93
8. *L. marina* (EU681412)*	0.98	15.93	14.92	0.00	15.41	14.93	13.93	NA

Using molecular data, we first confirmed the presence of *Mikrosyphar zosterae* as endophyte in *Leathesia marina* and *Colpomenia* spp. in Australia. This is also the first record of *Mikrosyphar zosterae* in the southern hemisphere. Our endophyte *rbc*L-*rbc*S sequenced matches with low genetic divergence reference DNA sequences generated by Ogandaga *et al.* (2017), who have obtained DNA sequences from South Korea (KU569308) *M. zosterae* isolated unialgal cultured material and hence *M. zosterae*. The genetic divergence found for all *rbc*L-*rbc*S sequences of *M. zosterae* (0.00-1.23%) is lower than the average intraspecific divergence detected for *rbc*L-*rbc*S sequences for other brown filamentous algae, such as *Elachista nipponica* Umezaki (1965: 182) (1.50%) from Japan and Korea (Lee *et al.* 2002), reinforcing that the detected genetic divergence corresponds to intraspecific divergence, showing that our samples contained *M. zosterae.* In this study, phylogenetic analysis of the *rbc*L-3' end alignment also allowed us to identify *rbc*L sequences attributed to *Leathesia marina* in GenBank that correspond to *Mikrosyphar* spp. due to low genetic divergence to our sequences, 0.00–2.34% (Table 3).

We have confirmed the identification of 13 specimens of *Leathesia marina* through COI-5P DNA sequencing, 12 of which provided *Mikrosyphar zosterae rbc*L-*rbc*S DNA sequences. In contrast, the occurrence of *Mikrosyphar* inside *Colpomenia* seems to be less common, since we have detected only two endophytes in our broad sampling of *Colpomenia* in Australia (n = 44, data not shown). The COI-5P intraspecific divergence observed in *L. marina* was low, < 1%, including a sequence collected near the type locality (EU681412). There are no COI-5P sequences from the type localities for both *C. sinuosa* (Spain) and *C. claytoniae* (South Korea) in public databases, however, the intraspecific divergence observed in this study for *C. sinuosa* (0.33–1.81%) and for *C. claytoniae* (0.50%) were low indicating that they belong the same phylogenetic species, respectively. The intraspecific divergence detected in *L. marina*, *C. claytoniae* and *C. sinuosa* were similar to the intraspecific variation observed in a previous study on Colpomenia peregrina

(1.40–1.70%) (McDevit & Saunders 2017), and lower than observed in five distinct genetic species within the *Scytosiphon lomentaria* (Lyngbye 1819: 74) Link (1833: 232) complex (Scytosiphonaceae) from Japan (5.80%) (Kogame et al. 2015).

Table 3. Pairwise genetic distances (%) of *Leathesia* S.F.Gray and *Colpomenia* (Endlicher) Derbès & Solier species for the *rbc*L-3' DNA sequence marker (200 bp). Lower triangle shows minimum and upper triangle maximum distance values. Diagonal shows minimum and maximum distance values.

	1	2	3	4
1. *Mikrosyphar zosterae* (this study, n = 18)	0.00 - 0.58	0.58	3.51	2.34
2. *M. zosterae* (GenBank, n = 1)	0.00	NA	2.92	1.75
3. *M. porphyrae* (GenBank, n = 1)	2.92	2.92	NA	4.68
4. *Leathesia marina* (GenBank, n = 9)	0.00	0.00	0.92	0.00 - 2.34

A previous study observed *Mikrosyphar zosterae* causing damage to their host (i.e. *Chondrus ocellatus*, Ogandaga *et al.* 2016), however, future studies will help to elucidate the ongoing *Mikrosyphar—Leathesia*/*Colpomenia* endophyte–host relationship. We have detected possible misidentification of *Leathesia* sequences deposited in GenBank, which might correspond to *Mikrosyphar*. This misidentification might have occurred due to endophyte contamination. However, because to date there are no available DNA sequences for the same marker for *Leathesia* and *Mikrosyphar*, we could only compare both genera with the *rbc*L-3' end dataset we produced. Further studies and more data are needed to further elucidate the phylogenetic and evolutionary relationships among host and endophyte species.

In summary, the occurrence of *Mikrosyphar* as endophyte in *Leathesia marina* seems to be a common event occurring in southern Australia, and to a lesser extent, in

Colpomenia spp. thalli. Further investigations will help to clarify whether this endophyte also occurs on other Australian marine species and in other regions of the world, especially where *Leathesia* is already reported. In addition, laboratory experiments could elucidate if this relationship is harmful or not to the host.

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TABLE S1. Details of the taxa and molecular data used in this study.

Species	Source	GenBank code	Marker	Collection data
Colpomenia claytoniae	GenBank	KF281129	COI-5P	Western Australia, Australia
Colpomenia claytoniae	GenBank	KF281130	COI-5P	Western Australia, Australia
Colpomenia claytoniae	GenBank	KF281131	COI-5P	Western Australia, Australia
Colpomenia claytoniae	GenBank	KF281132	COI-5P	Western Australia, Australia
Colpomenia claytoniae	GenBank	LC471304	COI-5P	Kagoshima, Japan
Colpomenia expansa	GenBank	HQ990530	COI-5P	Jeju, Korea
Colpomenia peregrina	GenBank	EU681397	COI-5P	Roscoff, France
Colpomenia peregrina	GenBank	HM890984	COI-5P	British Columbia, Canada
Colpomenia peregrina	GenBank	HM891007	COI-5P	British Columbia, Canada
Colpomenia peregrina	GenBank	HM891489	COI-5P	Nova Scotia, Canada
Colpomenia peregrina	GenBank	HM891490	COI-5P	Nova Scotia, Canada
Colpomenia peregrina	GenBank	HM891504	COI-5P	Nova Scotia, Canada
Colpomenia peregrina	GenBank	HM891506	COI-5P	Nova Scotia, Canada
Colpomenia peregrina	GenBank	HM891524	COI-5P	British Columbia, Canada
Colpomenia peregrina	GenBank	KF281124	COI-5P	Massachusetts, USA
Colpomenia peregrina	GenBank	KF281128	COI-5P	British Columbia, Canada
Colpomenia peregrina	GenBank	KM244739	COI-5P	Qingdao, China
Colpomenia peregrina	GenBank	LM995279	COI-5P	Roscoff, France
Colpomenia peregrina	GenBank	NC025302	COI-5P	Qingdao, China
Colpomenia peregrina	GenBank	AF385837	rbcL-S	Seocheon, Korea
Colpomenia peregrina	GenBank	AF385838	rbcL-S	Roscoff, France
Colpomenia peregrina	GenBank	GU252550	rbcL-S	El Tabo, Chile
Colpomenia peregrina	GenBank	JX843461	rbcL-S	Not specified
Colpomenia peregrina	GenBank	JX843462	rbcL-S	Not specified
Colpomenia peregrina	GenBank	JX843463	rbcL-S	Not specified
Colpomenia peregrina	GenBank	JX843464	rbcL-S	Not specified
Colpomenia peregrina	GenBank	JX843465	rbcL-S	Not specified
Colpomenia peregrina	GenBank	JX843466	rbcL-S	Not specified
Colpomenia peregrina	GenBank	JX843467	rbcL-S	Not specified
Colpomenia peregrina	GenBank	JX843468	rbcL-S	Not specified
Colpomenia peregrina	GenBank	JX843469	rbcL-S	Not specified
Colpomenia peregrina	GenBank	JX843470	rbcL-S	Not specified
Colpomenia sinuosa	GenBank	HM891305	COI-5P	Tasmania, Australia
Colpomenia sinuosa	GenBank	HM891310	COI-5P	Tasmania, Australia
Colpomenia sinuosa	GenBank	HQ990473	COI-5P	Tasmania, Australia
Colpomenia sinuosa	GenBank	HQ990489	COI-5P	Jeju, Korea
Colpomenia sinuosa	GenBank	HQ990524	COI-5P	Jeju, Korea
Colpomenia sinuosa	GenBank	KF281125	COI-5P	Western Australia, Australia
Colpomenia sinuosa	GenBank	KF281126	COI-5P	Western Australia, Australia
Colpomenia sinuosa	GenBank	KF281127	COI-5P	Western Australia, Australia
Colpomenia sinuosa	GenBank	AF385839	rbcL-S	Jeju, Korea
Colpomenia sinuosa	GenBank	FJ710143	rbcL-S	Yeosu, Korea
Dactylosiphon bullosus	GenBank	AF037995	COI-5P	Hokkaido, Japan
Dactylosiphon bullosus	GenBank	KF281120	COI-5P	British Columbia, Canada
Dactylosiphon bullosus	GenBank	AF385835	rbcL-S	Guryongpo, Korea

Dactylosiphon bullosus	GenBank	AF385836	rbcL-S	Jeju, Korea
Dictyopteris hoytii	GenBank	KF367763	COI-5P	North Carolina, USA
Dictyopteris polypodioides	GenBank	KF367756	COI-5P	North Carolina, USA
Dictyopteris repens	GenBank	FJ710140	rbcL-S	Ishigaki, Japan
Elachista fucicola	GenBank	HM891481	COI-5P	Prince Edward Island, Canada
Elachista fucicola	GenBank	HM891511	COI-5P	New Brunwick, Canada
Elachista fucicola	GenBank	AF453387	rbcL-S	Godtháb, Greenland
Halothrix ambigua	GenBank	AY079431	rbcL-S	Shinan, Korea
Halothrix lumbricalis	GenBank	LM995328	COI-5P	Napoli, Italy
Halothrix lumbricalis	GenBank	LM995392	COI-5P	Korinthos, Greece
Halothrix lumbricalis	GenBank	AY079430	rbcL-S	Århus Bugt, Denmark
Leathesia marina	GenBank	EU681412	COI-5P	Roscoff, France
Leathesia marina	GenBank	FJ409169	COI-5P	Rhode Island, USA
Leathesia marina	GenBank	FJ409170	COI-5P	British Columbia, Canada
Leathesia marina	GenBank	KY047193	COI-5P	Qingdao, China
Leathesia marina	GenBank	KY047194	COI-5P	Qingdao, China
Leathesia marina	GenBank	KY047224	COI-5P	Qingdao, China
Leathesia marina	GenBank	LC107837	COI-5P	Aomori, Japan
Leathesia marina	GenBank	AB302143	rbcL-S	Akkeshi, Japan
Leathesia marina	GenBank	AB302145	rbcL-S	Shigajima, Japan
Leathesia marina	GenBank	AB302147	rbcL-S	Maasholm, Germany
Leathesia marina	GenBank	AB302149	rbcL-S	Kaikoura, New Zealand
Leathesia marina	GenBank	AB302151	rbcL-S	Oaro, New Zealand
Leathesia marina	GenBank	AB302153	rbcL-S	Katiki Beach, New Zealand
Leathesia marina	GenBank	AY996365	rbcL-S	Pohang, Korea
Leathesia marina	GenBank	LC108037	rbcL-S	Aomori, Japan
Leathesia marina	GenBank	MT185437	rbcL-S	Las Grutas, Argentina
Leptonematella fasciculata	GenBank	LC107838	COI-5P	Chile
Leptonematella fasciculata	GenBank	AY079427	rbcL-S	Møns Klint, Denmark
Mikrosyphar porphyrae	GenBank	AF207806	rbcL-S	Not specified
Mikrosyphar zosterae	GenBank	AF207814	rbcL-S	Not specified
Mikrosyphar zosterae	GenBank	KU569308	rbcL-S	Manripo, Korea
Myrionema strangulans	GenBank	LM995030	COI-5P	Roscoff, France
Myrionema strangulans	GenBank	LM995212	COI-5P	Roscoff, France
Myrionema strangulans	GenBank	AY079435	rbcL-S	Shinan, Korea
Neoleptonema yongpilii	GenBank	AY079432	rbcL-S	Kuman, Korea
Neoleptonema yongpilii	GenBank	AY079433	rbcL-S	Sodol, Korea
Proselachista taeniaeformis	GenBank	AY079434	rbcL-S	Pohang, Korea
Proselachista taeniaeformis	GenBank	FJ710142	rbcL-S	Pohang, Korea
Scytosiphon dotyi	GenBank	AF385851	rbcL-S	California, USA
Scytosiphon lomentaria	GenBank	LC517610	COI-5P	Ona, Norway
Scytosiphon lomentaria	GenBank	AF385853	rbcL-S	Jeju, Korea
Scytosiphon lomentaria	GenBank	AF385854	rbcL-S	Daecheon, Korea
Scytosiphon promiscuus	GenBank	LC517730	COI-5P	Kamchatka, Russia
Colpomenia claytoniae	This study	MW465928	COI-5P	Portsea VIC Australia - 38°19'04.8"S 144°42'48.0"E
Colpomenia sinuosa	This study	MW465927	COI-5P	Portsea VIC Australia - 38°19'04.8"S 144°42'48.0"E

Leathesia marina	This study	MW465929	COI-5P	Bicheno TAS Australia - 41°51'33.9"S 148°17'04.0"E
Leathesia marina	This study	MW465930	COI-5P	Bicheno TAS Australia - 41°51'33.9"S 148°17'04.0"E
Leathesia marina	This study	MW465931	COI-5P	Bicheno TAS Australia - 41°51'33.9"S 148°17'04.0"E
Leathesia marina	This study	MW465932	COI-5P	Bicheno TAS Australia - 41°51'33.9"S 148°17'04.0"E
Leathesia marina	This study	MW465933	COI-5P	Lorne VIC Australia - 38°32'49.2"S 143°59'17.1"E
Leathesia marina	This study	MW465934	COI-5P	Lorne VIC Australia - 38°32'49.2"S 143°59'17.1"E
Leathesia marina	This study	MW465935	COI-5P	Lorne VIC Australia - 38°32'49.2"S 143°59'17.1"E
Leathesia marina	This study	MW465936	COI-5P	Lorne VIC Australia - 38°32'49.2"S 143°59'17.1"E
Leathesia marina	This study	MW465937	COI-5P	Lorne VIC Australia - 38°32'49.2"S 143°59'17.1"E
Leathesia marina	This study	MW465938	COI-5P	Blanket Bay VIC Australia - 38°49'41.4"S 143°35'01.6"E
Leathesia marina	This study	MW465939	COI-5P	Blanket Bay VIC Australia - 38°49'41.4"S 143°35'01.6"E
Leathesia marina	This study	MW465940	COI-5P	Blanket Bay VIC Australia - 38°49'41.4"S 143°35'01.6"E
Leathesia marina	This study	MW465941	COI-5P	Blanket Bay VIC Australia - 38°49'41.4"S 143°35'01.6"E
Mikrosyphar zosterae	This study	MW465909	*rbc*L-S	Twin Rocks SA Australia - 31°27'56.7"S 131°08'08.3"E
Mikrosyphar zosterae	This study	MW465910	*rbc*L-S	Portsea VIC Australia - 38°19'04.8"S 144°42'48.0"E
Mikrosyphar zosterae	This study	MW465911	*rbc*L-S	Portsea VIC Australia - 38°19'04.8"S 144°42'48.0"E
Mikrosyphar zosterae	This study	MW465912	*rbc*L-S	Bicheno TAS Australia - 41°51'33.9"S 148°17'04.0"E
Mikrosyphar zosterae	This study	MW465913	*rbc*L-S	Bicheno TAS Australia - 41°51'33.9"S 148°17'04.0"E
Mikrosyphar zosterae	This study	MW465914	*rbc*L-S	Bicheno TAS Australia - 41°51'33.9"S 148°17'04.0"E
Mikrosyphar zosterae	This study	MW465915	*rbc*L-S	Bicheno TAS Australia - 41°51'33.9"S 148°17'04.0"E
Mikrosyphar zosterae	This study	MW465916	*rbc*L-S	Lorne VIC Australia - 38°32'49.2"S 143°59'17.1"E
Mikrosyphar zosterae	This study	MW465917	*rbc*L-S	Lorne VIC Australia - 38°32'49.2"S 143°59'17.1"E
Mikrosyphar zosterae	This study	MW465918	*rbc*L-S	Lorne VIC Australia - 38°32'49.2"S 143°59'17.1"E
Mikrosyphar zosterae	This study	MW465919	*rbc*L-S	Lorne VIC Australia - 38°32'49.2"S 143°59'17.1"E
Mikrosyphar zosterae	This study	MW465920	*rbc*L-S	Lorne VIC Australia - 38°32'49.2"S 143°59'17.1"E
Mikrosyphar zosterae	This study	MW465921	*rbc*L-S	Kennett River VIC Australia - 38°40'02.2"S 143°51'49.2"E
Mikrosyphar zosterae	This study	MW465922	*rbc*L-S	Kennett River VIC Australia - 38°40'02.2"S

				143°51'49.2"E
Mikrosyphar zosterae	This study	MW465923	*rbc*L-S	Kennett River VIC Australia - 38°40'02.2"S 143°51'49.2"E
Mikrosyphar zosterae	This study	MW465924	*rbc*L-S	Blanket Bay VIC Australia - 38°49'41.4"S 143°35'01.6"E
Mikrosyphar zosterae	This study	MW465925	*rbc*L-S	Blanket Bay VIC Australia - 38°49'41.4"S 143°35'01.6"E
Mikrosyphar zosterae	This study	MW465926	*rbc*L-S	Blanket Bay VIC Australia - 38°49'41.4"S 143°35'01.6"E

Chapter 5

Colpomenia species from south and southeastern Australia (Ectocarpales, Phaeophyceae): a DNA barcoding approach

Espécies de *Colpomenia* do sul e sudeste da Austrália (Ectocarpales, Phaeophyceae): uma abordagem de DNA *barcoding*

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Running title: DNA Barcode of *Colpomenia* from Australia

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Abstract

Defining species in the brown algal genus *Colpomenia* is a challenging endeavour because of their morphological similarity, overlapping phenotypic variation, the absence of conspicuous diagnostic characters, and often lack of reproductive structures crucial for their identification. Thus, the use of molecular tools has become widely used to study *Colpomenia* taxonomy and evolution. The following four *Colpomenia* species are described along the Australian coast: *C. claytoniae* M.Boo, K.M.Lee, G.Y.Cho & W.Nelson, *C. ecuticulata* M.J.Parsons, *C. peregrina* Sauvageau, and *C. sinuosa* (Mertens ex Roth) Derbès & Solier. The objective of this study was to assess the diversity of *Colpomenia* species in southern and south-eastern Australia by using DNA barcoding techniques and single-marker species delimitation methods. We generated 44 new COI-5P DNA sequences from nine different populations across three Australian states (South Australia, Victoria and Tasmania), and applied 13 variations of four species delimitation methods (ABDG, SPN, PTP, GMYC). Our results recognized three *Colpomenia* species in the region, namely, *C. sinuosa*, *C. claytoniae*, and *C. peregrina*. *Colpomenia sinuosa* is the most widely distributed species in Australia. *Colpomenia peregrina* and *C. claytoniae* presented high levels of intraspecific genetic divergence. We did not find *C. ecuticulata*, although it has been previously reported from nearby our sampling area.

Key-words: Australia, DNA barcode, COI-5P, *Colpomenia, cox*1, macroalgae, Phaeophyceae, species delimitation, seaweed.

Resumo

A definição de espécies do gênero de algas pardas *Colpomenia* é uma tarefa desafiadora devido à sua similaridade morfológica, variação fenotípica, ausência de caracteres diagnósticos conspícuos e, frequentemente, falta de estruturas reprodutivas que são cruciais para sua identificação. Dessa forma, ferramentas moleculares têm se tornado amplamente utilizadas para estudar a taxonomia e a evolução de *Colpomenia*. Quatro espécies de *Colpomenia* são descritas ao longo da costa australiana: *C. claytoniae* M.Boo, KMLee, GYCho & W.Nelson, *C. ecuticulata* MJParsons, *C. peregrina* Sauvageau e *C. sinuosa* (Mertens ex Roth) Derbès & Solier. O objetivo deste estudo foi avaliar a diversidade de espécies de *Colpomenia* no sul e sudeste da Austrália usando técnicas de DNA *barcoding* e métodos de delimitação de espécies de marcador único. Geramos 44 novas sequências de DNA COI-5P de nove populações diferentes em três estados australianos (South Australia, Victoria e Tasmania) e aplicamos 13 variações de quatro métodos de delimitação de espécies (ABDG, SPN, PTP, GMYC). Nossos resultados reconheceram três espécies de *Colpomenia* na região: *C. sinuosa*, *C. claytoniae* e *C. peregrina*. *Colpomenia sinuosa* é a espécie mais amplamente distribuída na Austrália. *Colpomenia peregrina* e *C. claytoniae* apresentaram altos níveis de divergência genética intraespecífica. Não encontramos *C. ecuticulata*, embora tenha sido relatada anteriormente nas proximidades da nossa área de amostragem.

Palavras-chave: algas marinhas, Austrália, COI-5P, *Colpomenia*, *cox*1, delimitação de espécies, DNA *barcode*, macroalgas, Phaeophyceae.

Introduction

Colpomenia (Endlicher) Derbès & Solier is a marine brown macroalga with a cosmopolitan distribution, and is a common and conspicuous component of tropical and warm-temperate intertidal rocky shores (Lipkin 2002). The genus is characterised by an anatomically simple, hollow sacciform (cerebriform), vesicular or membranous thallus growing isolated or in clumps, with reproductive plurilocular structures organised in punctate sori (Freitas Toste *et al*. 2003; Cormaci *et al*. 2012; Lee *et al*. 2013). The life history of *Colpomenia* is heteromorphic and diplobiontic, with conspicuous vesicular gametophytes (macrothalli) and inconspicuous, environmentally cryptic, filamentous or pulvinated sporophytes (microthalli; Freitas Toste *et al*. 2003). Species delimitation has traditionally been based on vegetative and reproductive characteristics of the macrothallus. However, *Colpomenia* gametophytic macrothalli are extremely simple, often morphologically plastic, and frequently lack reproductive structures.

The scarcity of morphological characters among *Colpomenia* species and in other macroalgae cases frequently makes defining species problematic, particularly when dealing with potential species complexes (e.g. Lee *et al.* 2013). The resulting taxonomical quagmires are often complicated by the occurrence of homoplasies owing to convergent evolution, parallelisms, character losses or simply by the shear uncoupling between genetic and morphological differentiation (Fowler-Walker *et al.* 2006; Harvey and Goff 2006; Vieira *et al.* 2014; Leliaert *et al.* 2018). In some cases, defining species on the basis of morphological characters is impossible (Saunders 2005; Vieira *et al.* 2014; Leliaert *et al.* 2018; Song *et al.* 2019). Unsurprisingly, the use of molecular tools to define species has become very popular and widely used (Boo *et al.* 2011; Song *et al.* 2019).

Currently, 10 accepted *Colpomenia* species are recognised (see AlgaeBase, M. D. Guiry and G. M. Guiry, www.algaebase.org). Among these, *C. sinuosa* is reportedly widespread on tropical and temperate rocky shores. Molecular techniques are showing

that ubiquitous macroalgal species often comprise complexes of cryptic and quasi-cryptic species (e.g. *Bostrychia radicans* (Montagne) Montagne–*B. moritziana* (Sonder ex Kützing) J.Agardh complex (Zuccarello and West 2003), *Lobophora variegata* (J.V.Lamouroux) Womersley ex E.C.Oliveira (Vieira *et al*. 2014), *Portieria hornemannii* (Lyngbye) P.C.Silva (Leliaert *et al*. 2018), and *Hypnea musciformis* (Wulfen) J.V. Lamouroux complex (Nauer *et al*. 2015, 2019)). Lee *et al*. (2013) used *cox*3 DNA sequences to show that worldwide *C. sinuosa* samples comprise three major clades and at least eight distinct lineages, each of these lineages being potentially a new species. DNA barcoding has emerged as a successful method to identify macroalgal species (McDevit and Saunders 2009; de Jesus *et al*. 2016; Camacho *et al*. 2019). The 5' end of the cytochrome *c* oxidase 1 (COI-5P) has been accepted as a fast and informative DNA barcode for delineating species (Hebert *et al*. 2003). Since then, other genes have been proposed as DNA barcodes (Mattio and Payri 2010; Saunders and Moore 2013); however, the COI-5P remains the most popular barcode for brown and red macroalgae (Leliaert *et al*. 2014). COI-5P DNA sequences have been utilised to resolve brown algal taxonomic problems in genera such as *Saccharina* Stackhouse (McDevit and Saunders 2009), *Planosiphon* McDevit & G.W.Saunders and *Scytosiphon* C.Agardh (McDevit and Saunders 2017).

Australia supports one of the most diverse marine macroalgal floras in the world (Phillips 2001) and *Colpomenia* species have been recorded for nearly the entire coastline of Australia (see the Australasian Virtual Herbarium at https://avh.chah.org.au. 2021). Four species are reported for Australia, namely *C. claytoniae* M.Boo, K. M.Lee, G.Y.Cho & W.Nelson, *C. ecuticulata* M.J.Parsons, *C. peregrina* Sauvageau, and *C. sinuosa* (Mertens ex Roth) Derbès & Solier (Clayton 1975; Womersley 1987; Boo *et al*. 2011). No molecular studies have tested whether the current morphology-based perception of Australia's *Colpomenia* taxonomy represents its actual species diversity. Cryptic and cryptogenic

species might be occurring in Australia, which only be revealed can with molecular techniques. The objective of this study was to analyse the saccate brown macroalgal *Colpomenia* species from southern and south-eastern Australia, by using DNA barcoding and single-marker species-delimitation methods.

Material and methods

Study area and collections

In total, 44 *Colpomenia* specimens were collected from February 2010 to April 2012 from nine different locations across the following three Australian states: South Australia (SA), Victoria (Vic.), and Tasmania (Tas.; Table 1). Specimens were collected from intertidal rocks during low tide, or by snorkelling or SCUBA diving in the subtidal. Specimens were cleaned from epiphytes and rinsed in clean seawater before being desiccated on silica gel for preservation and DNA extraction. All specimens, and parts thereof, were deposited in the State Herbarium of South Australia.

Table 1. List of *Colpomenia* collection sites along the Australian coast and their respective meta-data.

Species	State	Beach	GenBank code	N	Date	Coordinates
C. sinuosa	South Australia	Stokes Bay	MZ054831	1	26 Feb 2011	-35.622151, 137.206486
			MZ054832	1	26 Feb 2011	-35.622151, 137.206486
			MZ054836	2	26 Feb 2011	-35.622151, 137.206486
		Vivonne Bay	MZ054833	2	25 Feb 2011	-35.996028, 137.184383
		Hanson Bay	MZ054834	1	25 Feb 2011	-36.017088, 136.853668
			MZ054840	2	25 Feb 2011	-36.017088, 136.853668
			MZ054843	9	25 Feb 2011	-36.017088, 136.853668
		Pennington Bay	MZ054835	1	24 Feb 2011	-35.852518, 137.746999
			MZ054841	2	24 Feb 2011	-35.852518, 137.746999
			MZ054844	7	24 Feb 2011	-35.852518, 137.746999
	Victoria	Kennett River	MZ054837	1	31 Jan 2011	-38.663796, 143.865753
		Portsea	MZ054842	1	27 Feb 2010	-38.318376, 144.713331
		Portsea	MW465927	1	27 Feb 2010	-38.318376, 144.713331
		Mallacoota	MZ054839	3	20 Apr 2012	-37.571377, 149.764745
	Tasmania	North Trial Harbour	MZ054838	5	02 Jan 2011	-41.931604, 145.173418
C. claytoniae	Victoria	Portsea	MW465928	1	27 Feb 2010	-38.318376, 144.713331
	Tasmania	North Trial Harbour	MZ054845	1	02 Jan 2011	-41.931604, 145.173418
C. peregrina	Victoria	Mallacoota	MZ054846	3	20 Apr 2012	-37.571377, 149.764745
			MZ054847	1	20 Apr 2012	-37.571377, 149.764745

Molecular analysis

DNA was extracted from dried thalli pulverised with plastic pestle in liquid nitrogen inside an Eppendorf microtube. DNA was extracted using the Nucleospin Plant II system (Machery-Nagel GmbH & Co, Düren, Germany) with the SDS buffer option or by using the Plant DNeasy Minikit (Qiagen Ltd, Crawley, UK). Both DNA extractions protocols followed the manufacturer's instructions. DNA purification was performed using GeneClean III (Qbiogene, Cambridge, UK).

The mitochondrial COI-5P gene was polymerase chain reaction (PCR) amplified in 25-mL reactions composed of 1X GeneAmp PCR Buffer (Applied Biosystems, Carlsbad, CA, USA), 3 mM of MgCl2, 0.32 M Betaine, 100 mM of each dNTP, 0.2 mM of each forward and reverse primers, 0.72 U of Ampli*Taq* Gold (Applied Biosystems) or *Taq* TI (FisherBiotec, Wembley, WA, Australia), and 1 mL of 1:10 diluted DNA template. A combination of primer sequences listed in Saunders and McDevit (2012) were used to amplified and automated DNA sequence reactions. Amplifications were run on the Palm Cycler (Corbett Research, Sydney, NSW, Australia), with an initial denaturation step at 95ºC for 5–9 min, followed by 30 cycles of 94ºC for 40 s, 45ºC for 40 s and 72ºC for 45 s, terminated by 72ºC for 7 min. PCR products were cleaned using MultiScreen PCR cleanup filter plates (Merck-Millipore, Darmstadt, Germany) attached to a vacuum manifold and commercially sequenced in the Australian Genome Research Facility (AGRF, Adelaide, SA, Australia). All sequences were aligned using Geneious Pro (ver. 5.5.6, see www.geneious.com/; Kearse *et al*. 2012) and manually checked.One sequence per haplotype per collection site was deposited in the NCBI GenBank, with accession numbers MW465927, 28 and MZ054831-47 (Table 1). DNA alignment was constructed using ClustalW (ver. 1.2.2, seewww.clustal.org/clustal2/; Thompson *et al*. 1994) implemented in Geneious Pro (ver. 5.5.6; Kearse *et al*. 2012) and manually corrected afterwards. All

Colpomenia COI-5P DNA sequences currently available in GenBank were downloaded (www.ncbi.nlm.nih.gov/genbank/, accessed May 2021) and included in the alignment.

Species delimitation methods

Single-marker species-delimitation methods (SDM) used different approaches of four major techniques, namely, automatic barcode gap discovery (ABGD, Puillandre *et al*. 2012), statistical parsimony network (SPN, Templeton *et al*. 1992), the Poisson tree processes (PTP, Zhang *et al*. 2013), and the general mixed Yule coalescent model (GMYC, Pons *et al*. 2006).

The ABGD analyses were performed with the online implementation (bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html) using a HKY+I distance matrix as input. ABGD analysis was run with the following parameters: 0.001 minimum intraspecific distance (pmin), 0.1 maximum intraspecific distance (pmax), 10 steps, and 1.5 relative gap width. ABGD initial and recursive partition approaches were considered. SPNs were built with TCS 1.2.1 (Clement *et al*. 2000) by using 95 and 99% confidence limits as haplotype connection limits. PTP analysis used a maximum-likelihood (ML) tree constructed as described below but excluding outgroup sequences. PTP was performed with the online implementation (http://species.h-its.org) under the following parameters: 100 000 MCMC generations, thinning = 100, and burn-in = 0.1. Only the PTP ML approach was considered.

For GMYC analyses, four Bayesian ultrametric trees were estimated employing distinct tree model priors available in BEAST (ver. 2.6.0, see www.beast2.org/; Bouckaert *et al*. 2019): the Yule 'birth-only' speciation model (Bouckaert *et al*. 2019), the birth and death speciation model (BD), the coalescent with constant population-size model (CCP), and the coalescent with exponential population growth model (CEP). Every GMYC ultrametric tree was constructed using the HKY + I evolutionary model identified as the

best model to fit the alignment (without outgroup) under the Bayesian information criterion (BIC) in JModelTest (ver. 2.1.6, see https://github.com/ddarriba/jmodeltest2/; Darriba *et al*. 2012). The MCMC chain lengths were 100 million generations, sampling every 1000 generations, and 10 initialisation attempts. We used a 10% burn-in value. MCMC chain convergence was assessed when all parameters reached effective sample size (ESS) values above 200 in Tracer (ver. 1.7, see www.beast2.org/tracer-2/; Rambaut *et al*. 2018). TreeAnnotator (ver. 2.6.0, see www.beast2.org/treeannotator/) was used to identify the most credible tree. GMYC single and multiple threshold approaches were executed using the package '*splits*' in R (ver. 1.0, see https://splits.r-forge.r-project.org/; Fujisawa and Barraclough 2013). In summary, we used 13 variations of species-delimitation methods, namely, two ABGD (initial and recursive), two SPN (95 and 99% confidence limits), one PTP (ML version) and eight GMYC (single- and multiple-threshold approaches of four distinct tree priors). The consensus of the species-delimitation methods was obtained using the R package '*BarcodingR*' (ver. 1.0-3, see https://cran.r-project.org/web/packages/BarcodingR/) which applies the majority rule (Zhang *et al*. 2017).

Phylogenetic analysis

Maximum likelihood (ML) phylogenetic analysis was performed using the RAxML-HPC2 (ver. 8.0, see https://cme.h-its.org/exelixis/web/software/raxml/; Stamatakis 2014) available in the CIPRES Science Gateway (ver. 3.3, see www.phylo.org/; Miller *et al*. 2010). ML set-up parameters included 1000 alternative runs on distinct starting trees (=maximum currently allowed number of alternative runs in RAxML in CIPRES) and 1000 non-parametric bootstrap (BS) replications. RAxML implements only the GTR+Gamma model of molecular evolution, which cover most DNA alignments (Stamatakis 2014), even though JModelTest identified the HKY + I model as the best model. Preliminary analyses

showed that ML results using either model produced the exact same topologies. ML tree was visualised in FigTree (ver. 1.3.1, A. Rambaut, Institute of Evolutionary Biology, University of Edinburgh, Edinburgh, UK, see https://github.com/rambaut/figtree/releases). Pairwise genetic-distance matrices were built in PAUP* (ver. 4.0a167, see https://paup.phylosolutions.com/; Swofford 2014) by using *p*-distances.

Results

The final COI-5P alignment comprised 71 sequences, including 44 newly generated *Colpomenia* sequences, 27 sequences downloaded from GenBank, and one outgroup (*Ectocarpus* sp. MK045263). The alignment was 521 bp long, had 103 informative sites, 126 variable sites, and 26 haplotypes. When applying the species delimitation methods to this alignment (after outgroup removal), different numbers of primary species hypothesis (PSH) were generated. The consensus contained groups corresponding to three described and well characterised *Colpomenia* species occurring in Australia, namely, *C. sinuosa*, *C. claytoniae*, and *C. peregrina* (Fig. 1).

The majority of the SDM were conservative and four PSH were recovered by ABGD initial, SPN 95%, PTP and all the GMYC approaches using a single threshold. The GMYC multiple-threshold approaches were often less conservative (i.e. five to seven PSH) than were the single-threshold approaches (four PSH). The method that estimated the greatest number of PSH was the SPN 99% with nine PSH.

All SDM results, without exception, identified *C. expansa* as a distinct species, even though this species was neither collected nor sequenced in this study and only one COI-5P DNA sequence is available in GenBank from South Korea (HQ990530). *Colpomenia sinuosa* was resolved as a single PSH in 12 SDM, and as two PSH in the SPN with 99% confidence limit. In total, 9 of 13 SDM results identified *C. claytoniae* as a distinct species.

Last, seven SDM recognised a single PSH within the *C. peregrina* clade, four SDM recognised two PSH, and two SDM recognised three PSH within the *C. peregrina* clade.

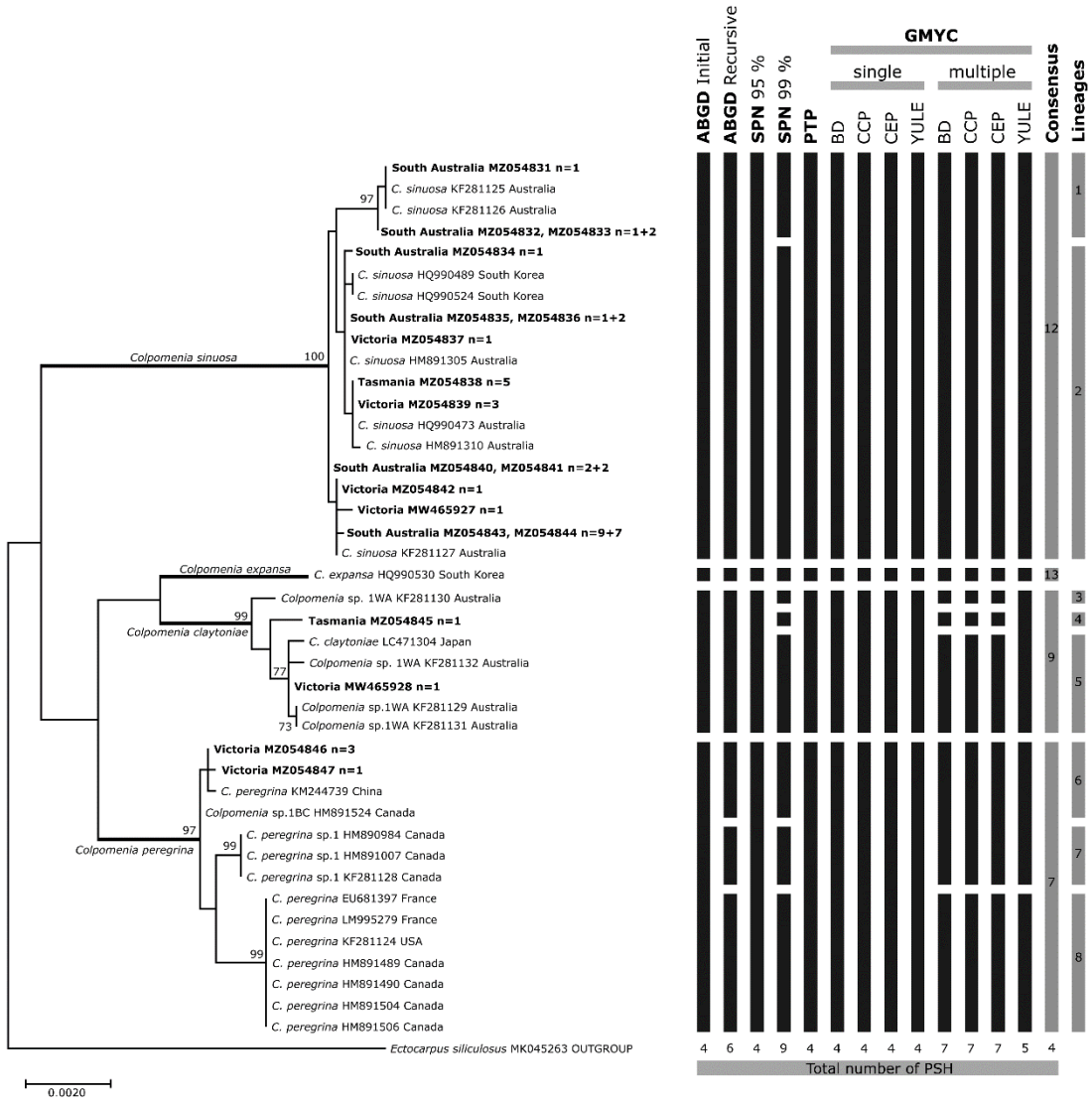

Figure 1. Maximum likelihood (ML) analysis for *cox*1 DNA sequences of *Colpomenia* species from Australia and downloaded sequences from GenBank, and all results of single-marker species-delimitation methods. Bootstrap values higher than 70 are shown at the nodes. Samples generated in this study are in bold. Codes following sequence names refer to GenBank accession numbers. Black vertical bars represent each of the barcode species delimitation methods applied. Consensus votes and different lineages are represented by vertical grey bars.

Genetic distance comparisons were calculated on the basis of the final results of our SDM. The intraspecific variation within *C. sinuosa* ranged from 0.00 to 1.92%, whereas the variation within *C. claytoniae* and *C. peregrina* both ranged from 0.00 to 1.73%. We were unable to estimate intraspecific divergence in *C. expansa* because there is only one sequence available in GenBank.

Discussion

Our molecular analyses confirmed the existence of the following three distinct *Colpomenia* species in south–southeastern Australia: *C. claytoniae*, *C. peregrina*, and *C. sinuosa*. *Colpomenia sinuosa* was the most widely distributed species, occurring in all three states of South Australia, Victoria and Tasmania. Our results are in agreement with historical accounts of *Colpomenia* distribution in Australia that have identified *C. sinuosa* as the most common and widespread *Colpomenia* species in Australia (Womersley 1987). *Colpomenia peregrina* was found only in Mallacoota (Vic.). *Colpomenia claytoniae* was collected in North Trial Harbour (Tas.) and Portsea (Vic.).

Our analyses using the COI-5P DNA sequences (also known as *cox*1) identified three *C. peregrina* lineages, namely Lineages 6, 7 and 8 (Fig. 1). All three *C. peregrina* COI-5P lineages are found in Canada but Lineage 6 is also present in the western Pacific Ocean (Australia and China), Lineage 7 is so far restricted to Canada, and Lineage 8 can also be found in the USA (Massachusetts) and France (Roscoff). Our results mirror Lee *et al*. (2014) who used *cox*3 DNA sequences to study 359 *C. peregrina* specimens in 28 populations sampled around the world. They identified the following four major *C. peregrina* lineages (groups) with a strong phylogeographic signal at continental scales: Groups 1 and 2 distributed across northern Pacific Ocean, Group 3 distributed in the north-

western Pacific Ocean and northern Atlantic Ocean, and Group 4 restricted to north-western and south-western Pacific oceans.

The genetic divergence among *C. peregrina* COI-5P lineages (1.73%) was higher than intraspecific divergence (~0.46%), and lower than the intrageneric divergence (~3.04%) observed for several brown macroalgal species (see McDevit and Saunders 2009), suggesting cryptic speciation. Although the SDM consensus resolved the *C. peregrina* lineages as a single species, SDM results varied. Seven SDM methods (=50 % + 1) suggested a single species; four methods suggested two species (Lineages 6 and 7 different species from Lineage 8); and two methods suggested three species (Lineages 6, 7 and 8 different from each other; Fig. 1). Our results concur with McDevit and Saunders (2017) who noted that diversification within *C. peregrina* populations requires further studies that would benefit from the inclusion of other markers and larger, more widespread, sampling. *Colpomenia peregrina* has a worldwide distribution in temperate seas (Womersley 1967, 1987), including northern Europe (Ireland, England and France; Minchin 1991) and in the Mediterranean (Verlaque *et al*. 2015). European records of *C. peregrina* have been considered anthropogenic introductions from north-eastern Pacific populations (Minchin 1991). However, Lee *et al*. (2014) showed that European populations represent introductions from north-western Pacific populations instead. Lee *et al*. (2014)'s phylogeographic analysis showed that the north-western Pacific Ocean is the centre of origin of *C. peregrina*, northern Atlantic populations represent a recent arrival, and south-western Pacific populations (including Australia) are genetically distinct from both north-western Pacific and northern Atlantic populations.

Several studies have compared molecular species delimitation methods across a range of different taxa and speciation scenarios (Carstens *et al*. 2013; Giarla *et al*. 2014; Luo *et al*. 2018). Performance varies widely among methods (e.g. distance-based, coalescent-based), markers (e.g. mt DNA, cp DNA, nuclear DNA, multi-locus markers),

taxa and presence or absence of distinct micro-evolutionary forces (e.g. admixture, gene flow, population size, selection). As a consequence, a general universal conclusion on which method is the best is not possible (Miralles and Vences 2013; Dellicour and Flot 2015). Each method could be considered a distinct line of evidence showing different aspects of the datasets and the speciation process of independently evolving lineages (White *et al*. 2014). Consequently, the recommendation is the use of multiple methods, multiple markers, well sampled taxa, and an integrative approach that consider other lines of evidence such as morphology, ecology, and biogeography, to produce final delimitations and taxonomic decisions. In this study, SPN 99% and GMYC with multiple thresholds recognised the largest number of primary species hypotheses. SPN set at 99% cut-off limits the number of mutational differences associated with Templeton's 'parsimony' probability of connecting two haplotypes (Templeton *et al*. 1992; Clement *et al*. 2000), isolating them into unconnected networks and, hence, potentially overestimating the number of putative species. GMYC multiple thresholds tend to either recognise the same species delimitation as the single-threshold option or to support a larger number of putative species because it relaxes the cut-off between interspecific (Yule model) and intraspecific (coalescent model) divergence across branch lengths to vary among clades. Although the GMYC multiple-threshold approach is expected to better fit the data, it does not necessarily improve species delimitation compared with the single-threshold option (e.g. Blair and Bryson 2017). Our consensus coincidently recognised the smallest number of PSH in the dataset, agreeing with Carstens *et al*. (2013, p. 4369) who recommend that 'inferences drawn from species delimitation studies should be conservative, for in most contexts it is better to fail to delimit species than it is to falsely delimit entities that do not represent actual evolutionary lineages.'

This study represents the first report of *C. claytoniae* in Tasmania. Our results identified in the *C. claytoniae* clade a pattern similar to the one observed in the *C.*

peregrina clade, namely, three distinct lineages. No COI-5P DNA sequences from *C. claytoniae* (Korea) and *C. sinuosa* (Spain) topotype specimens are available in GenBank. Future worldwide sampling and data analysis, including data from type species or topotype specimens, will possibly separate geographically isolated and genetically distinct populations into different species (e.g. Atlantic versus Pacific species).

We did not sequence *C. ecuticulata* samples, although we collected within the species' reported distribution range (Womersley 1987). *Colpomenia ecuticulata* occurs in southwestern Asia, eastern Asia, New Zealand and Australia (see AlgaeBase, www.algaebase.org). In Australia, *C. ecuticulata* was reported from drift specimens collected in Port MacDonnell, South Australia (Womersley 1987). Possible reason for this omission are as follows: (i) we failed to find and collect this species (i.e. we did not collect at the same beach as did Womersley (1987) or this is a seasonal species); (ii) Womersley (1987) misidentified the South Australian specimens; or (iii) this species was locally extinct at this region. Womersley (1987) had already noticed this species to be rare in Australia, but common in New Zealand. Further studies, including studies on voucher specimens, will elucidate whether *C. ecuticulata* reported in Australia indeed corresponds with *C. ecuticulata* cited elsewhere.

One sequence named '*Colpomenia expansa*' downloaded from GenBank (HQ990530) was recognised in our study as a distinct species. However, very little information is available for this sequence. There is no morphological analysis and the collecting site (South Korea) is very far from type locality (California, USA). Therefore, this sequence is from a *Colpomenia* lineage not found in southern and south-eastern Australia.

Differences in morphological variation among putative *Colpomenia* species in Australia are known since the work of Clayton (1975) who tested Womersley's (1967) taxonomic concepts recognising *C. sinuosa* and *C. peregrina* as distinct species. Clayton (1975) better circumscribed these species using morphometric and statistical analyses.

According to Clayton (1975), the number of medullary cell layers, shape of sori, and presence or absence of cuticle on the plurilocular sori are the only statistically significant different anatomical features reliably capable of distinguishing *C. peregrina* from *C. sinuosa* in Australia. This study supports the morphology based species concepts for *Colpomenia* in both Australia (Womersley 1967, 1987; Clayton 1975) and in other parts of the world (Song *et al*. 2019). Hence, morphological diagnostic characters used thus far to identify *Colpomenia* species in Australia are proving to be reliable.

Colpomenia sinuosa is the most widely distributed species in Australia and the world. Further studies will help elucidate the *Colpomenia* diversity in the entire Australian continent, answer the question whether *C. peregrina* corresponds to a single or multiple cryptic species, and whether there are new or endemic species yet to be described. Our new generated COI-5P sequences will be important for future research in *Colpomenia* diversity or evolution and Australian macroalgae flora.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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Chapter 6

Phylogeography of *Colpomenia sinuosa* (Ectocarpales, Phaeophyceae) in Australia

Filogeografia de *Colpomenia sinuosa* (Ectocarpales, Phaeophyceae) na Austrália

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Running title: *Colpomenia* phylogeography in Australia

Abstract

Marine brown macroalgae are known to present a large variety of thallus morphologies that, once detached from the substrate, allow them to float, drift, and be carried to long-distances by currents. The intertidal genus *Colpomenia*, are characterized by a bladder saccate thalli capable to trap air within its thalli or folds, conferring capability of buoyancy, which allow them to drift. The objectives of this study were to perform a phylogeographic analysis on the widely distributed species *Colpomenia sinuosa* in Australia. We used a dual-marker approach, mtDNA *cox*1 and cpDNA *rbcL*-*rbc*S, on macroalgal samples collected across south and southeastern Australian coast. Spatial genetic structure was detected. The *rbc*L-*rbc*S evidenced population genetic structure influenced by the Leeuwin current (Ningaloo, Perth and Twin Rocks), different from organisms at south and southeastern Australian coasts (Kangaroo Island, Victoria and Tasmania), also different from Heron Island (Queensland) population. The *cox*1 marker, however, detected difference between Maugean (= Kangaroo Island) and Flindersian (= Victoria and Tasmania) marine provinces.

Key-words: Australia, *cox*1, *Colpomenia*, genetic structure, Leeuwin current, macroalgae, marine barriers, phylogeography, population genetics, *rbc*L-*rbc*S

Resumo

Macroalgas marinhas pardas são conhecidas por possuir uma ampla variedade de formas do talo e, que uma vez destacadas do substrato, possuem capacidade de boiar, se dispersarem e serem carregadas por longas distâncias em correntes. O gênero *Colpomenia* é caracterizado por um talo saciforme capaz de reter ar no seu interior ou em suas dobras, conferindo flutuabilidade que lhe permite dispersar. O objetivo desse estudo foi realizar estudos filogeográficos da espécie *Colpomenia sinuosa* distribuída em toda Austrália. Utilizamos uma abordagem de dois marcadores molecular, mtDNA *cox*1 e cpDNA *rbc*L-S, em amostras coletadas em quase toda costa australiana. Os dados de *rbc*L-S evidenciaram estrutura genética populacional ao longo da corrente de Leeuwin (Ningaloo, Perth e Twin Rocks), que são diferentes das populações ao sul e sudeste (Kangaroo Island, Victoria e Tasmania) que, por sua vez, também são diferentes da população de Heron Island (Queensland). O marcador *cox*1, por outro lado, identificou diferença entre as províncias marinhas Maugean (= Kangaroo Island) e Flindersian (= Victoria e Tasmania).

Palavras-chave: Austrália, barreiras marinhas, *Colpomenia*, corrente de Leeuwin, *cox*1, estrutura genética, filogeografia, genética populacional, macroalgas, *rbc*L-*rbc*S.

Introduction

The ecology and evolution of marine benthic macroalgae are highly influenced by their dispersal capabilities. Planktonic and recruitment stages are two important components of macroalgal dispersal (Hedgecock 1986, Caley et al. 1996). Most macroalgal spores have a very limited dispersal capabilities, being disperse over just a few meters (Kinlan and Gaines 2003). Conversely, for some macroalgal species, adult thalli, or parts thereof, may drift over large distances and either release spores or reattach themselves in new localities (Thiel and Gutow 2005, Fraser et al. 2009). Other macroalgal species can grow epiphytically on rafting (macroalgal) material, hitchhiking on the dispersal capabilities of buoyant structures or taxa (Nikula et al. 2010, Macaya et al. 2016, Waters et al. 2018). Consequently, macroalgal thallus morphology and ecology plays a pivotal role in the dispersal of benthic marine biota.

In some species, thallus special adaptations confer the alga the potential to display positive buoyancy, floatability, and hence support long-distance current-mediated dispersal (Fraser et al. 2009, 2020, Durrant et al. 2015). Marine brown macroalgae are known to present a large variety of thallus morphologies that, once detached from the substrate, allow them to float, drift, and be carried to long-distances by currents. Several brown algal species have unique parts of the thallus known as pneumatocyst (= aerocystis) or gas bladders that confer them positive buoyancy (e.g. Fucales: *Sargassum*, *Fucus*. Laminariales: *Macrocystis*, *Nereocystis*). Other brown algae do not possess pneumatocyst, such as the intertidal genus *Colpomenia*, but are characterized by a bladder shaped thalli (= saccate habit) capable to trap air within its thalli or folds during low tides or high-energy wave exposure, increasing positive buoyancy, conferring floatability, and allowing them to drift (Blackler 1967, Mathieson et al. 2016).

Abiotic factors are also major drivers of dispersal, acting as either facilitators or barriers to dispersal, migration and gene flow. Cessation of gene flow promotes population genetic

isolation, differentiation, leading to speciation. Gene flow is often limited by either physical biogeographic barriers promoting allopatric differentiation (Mendonça et al. 2011) or ecological gradients, habitat heterogeneity and discontinuities promoting parapatric and sympatric speciation (Rocha et al. 2005, Rundle and Nosil 2005). With respect to coastal physical barriers to gene flow, several oceanographic and geographic features have been proposed to explain genetic discontinuities in the marine environment such as bluffs, capes, peninsulas, and promontories, as well as boundary currents, long stretches of sandy beaches, the mouth of major rivers, and shifts in climate – in particular, temperature (Defeo and De Alava 1995, Defeo 1996, Wares et al. 2001, Bilton et al. 2002). The interplay between a species autoecology, genetic diversity and abiotic drivers of spatial connectivity determine the evolutionary fate of the species and the phylogeographic patterns we see today.

Several studies have tested and described the presence of genetic discontinuity in marine populations across southern Australia (Waters et al. 2005, Teske et al. 2017, Weber et al. 2017). Most studies detected genetic structure that closely match the marine biogeographical provinces of Bennett and Pope (1953) proposed on the basis of community and species distributions: the Flindersian, Maugean and Peronian biogeographic provinces (Li et al. 2013). The concordance between marine intraspecific (= genetic, phylogeographic discontinuities), and inter- supraspecific (= ecological, biogeographic discontinuities) patterns have been described in different parts of the world and seems to be the norm rather than the exception, e.g. temperate Australia (Waters and Roy 2003, Waters et al. 2010), northern Australian (Benzie 1999) and California (Dawson 2001). Nearly all coastal marine phylogeographic studies not only in southern Australia but worldwide used animals as study organisms (Beheregaray 2008). Consequently, compared to animal studies, there is a lack of knowledge regarding the genetic diversity

and structure of macroalgae, particularly studies involving species found in biodiversity hotspots (Durrant et al. 2015).

Australia holds one of the highest macroalgal species diversity, and highest levels of macroalgal endemism in the world (Phillips 2001). However, relatively fewer studies have tested the presence of genetic structure among coastal Australian marine macroalgal populations. Most studies have focused on large brown algae (kelps and false-kelps) with transoceanic dispersal capabilities such as *Macrocystis pyrifera* (Linnaeus) C.Agardh (Macaya and Zuccarello 2010) and *Durvillea* spp. (Fraser et al. 2009, Fraser and Waters 2013), or have been either taxonomically (e.g. *Ecklonia radiate* (C.Agardh) J.Agardh (Coleman et al. 2009) or geographically limited (Coleman 2013). Yet, phylogeographic studies of ubiquitous non-kelp marine macroalgae are revealing the presence of genetically highly structured populations at different geographical scales, including the identification of a plethora of cryptic species (Vieira et al. 2014, Leliaert et al. 2018). *Colpomenia sinuosa* (Mertens ex Roth) Derbès & Solier is a good example of worldwide distributed macroalgal species (Lipkin 2002) showing signs of extensive internal genetic structure (Lee et al. 2013). Non-kelp Australian macroalgae with complex life-cycles (e.g. heteromorphic haplo-diplobiontic) and distinct dispersal capabilities (e.g. spores release and increased thallus drifting potential), such as *C. sinuosa*, might reveal phylogeographic patterns not seen in other marine organisms. In Australia, *C. sinuosa* is a species reported around the entire country (AVH 2021) representing a good model organism for phylogeographic studies.

Colpomenia sinuosa is a benthic species characterized by 1 to 15 cm membranous, spherical, hollow, bladder-like to convoluted (cerebriform) thallus that can grow isolated or in clumps (Freitas Toste et al. 2003, Cormaci et al. 2012, Lee et al. 2013). The heteromorphic life history alternates between vesicular erect macroscopic gametophytes and a prostrate filamentous, often pulvinate, nearly-microscopic sporophytes (Freitas

Toste et al. 2003). The gametophytes present clusters of phaeophycean hairs and uniseriate plurilocular reproductive organs organized in discrete cortical sori (Womersley 1987, Song et al. 2019). Intraspecific genetic diversity among *C. sinuosa* populations were initially studied by Cho et al. (2009) using ITS DNA sequences. They observed the existence of two major lineages: a northern and a southern hemisphere clade. Later, Lee et al. (2013) applied *cox*3 DNA sequences to study *C. sinuosa* populations from 28 different locations across the world. They observed a more entangled *C. sinuosa* evolutionary history, identifying several trans-oceanic dispersal routes, complex evolutionary scenarios, and probable human-based introductions – the latter might have shuffled the natural patterns of phylogeographic structure.

The objective of this study was to perform a phylogeographic analysis on the widely distributed species *Colpomenia sinuosa* in Australia to test for the presence of phylogeographic structure not yet reported for other marine species. We used a dual-marker approach, the mitochondria-encoded *cox*1 gene and the chloroplast-encoded *rbc*L-*rbc*S spacer on specimens collected across five Australian States: Western Australia, South Australia, Victoria, Tasmania and New South Wales. The DNA barcode *cox*1 marker has been proven to be a highly variable marker in brown algae (McDevit and Saunders 2009). The *rbc*L-*rbc*S spacer is recognized as a variable marker due to the presence of a spacer region between the end of the *rbc*L gene and the beginning of the *rbc*S gene, a region not under the influence of severe natural selection and hence, a place where most mutations do not have a deleterious effect (Kogame et al. 1999). Both markers have been successfully used in marine macroalgal phylogeographic studies (Cheang et al. 2010, Vitales et al. 2019).

Material and methods

Taxon sampling

A total of 85 *Colpomenia sinuosa* specimens were collected across 12 populations spread across five Australian states: Western Australia (n = 7); South Australia (n = 53); Victoria (n = 10); Tasmania (n = 7) and; Queensland (n = 8). Details on sample location are listed in Table 1. All specimens were cleaned from epiphytes, rinsed in clear seawater and stored in silica gel desiccant.

DNA extraction, PCR and sequence alignment

Dried specimens were cleaned from potential remaining epiphytes under a Stemi SV 6-Zeiss stereomicroscope (Zeiss, Göttingen, Germany) prior to DNA extraction. DNA extraction, DNA sequence reactions and automated DNA Sanger sequencing via capillary separation were performed as described in Dixon et al. (2012). Plastid *rbc*L-*rbc*S spacer (with partial flanking *rbc*L and *rbc*S sequences) and the mitochondrial *cox*1 were PCR amplified according to Mattio et al. (2008) and Saunders and McDevit (2012), respectively. Multiple alignments for both *rbc*L-*rbc*S and *cox*1 sequences were built using ClustalW (Thompson et al. 2002) implemented in Geneious v.5.5.6 (Kearse et al. 2012).

Population genetic and spatial structure

Haplotype (Hd) and nucleotide (Pi) diversity (Nei 1987), average number of nucleotide diversity (k), total number of mutations (m), number of haplotypes (h) and number of segregating sites (S) were calculated using DNAsp v6 (Rozas et al. 2017). Statistical parsimony network was built using TCS v1.21 (Clement et al. 2000) with a 95% connection limit.

Table 1. List of *Colpomenia sinuosa* collection sites along the Australian coast and their respective meta-data.

Marker	State	City	N	Coordinates
*rbc*L-*rbc*S	WA	Ningaloo	3	-22.722168480872256, 113.67671449005164
		Perth	5	-32.05725034147189, 115.74098239516472
	SA	Twin Rocks	13	-31.468575883171326, 131.1300012427883
		Pennington Bay KI	11	-35.85239239859285, 137.7467677677549
		Stokes Bay KI	8	-35.63019172530397, 137.16928119559148
		Vivonne Bay KI	7	-35.99642600549606, 137.18420159586515
	VIC	Portsea	5	-38.3186481916373, 144.7131824714528
		Mallacoota	3	-37.572826185482434, 149.76727887657452
	TAS	North Trial Harbour	5	-41.93174736879291, 145.17262986463095
		Bicheno	2	-41.87822913659781, 148.31540768382146
	QLD	Heron Island	8	-23.442530448260488, 151.9144060556764
*cox*1	SA	Hanson Bay KI	11	-36.01764176008528, 136.85406665865196
		Pennington Bay KI	9	-35.85239239859285, 137.7467677677549
		Stokes Bay KI	5	-35.63019172530397, 137.16928119559148
		Vivonne Bay KI	2	-35.99642600549606, 137.18420159586515
	VIC	Portsea	3	-38.3186481916373, 144.7131824714528
		Mallacoota	3	-37.572826185482434, 149.76727887657452
	TAS	North Trial Harbour	5	-41.93174736879291, 145.17262986463095

Hypotheses of population subdivision along the Australian coast, and the presence of putative barriers to gene flow between them, were tested using permutational multivariate analysis of variance (PERMANOVA) (Anderson et al. 2008) conducted as an add-on module to Primer v.6 (PRIMER-E Ltd., Plymouth, UK), based on a dataset comprised by haplotype frequency per population. We obtained a spatially wider sampling coverage for the *rbc*L-*rbc*S marker. Therefore, using the *rbc*L-*rbc*S we tested the presence of genetic structure broadly, across Australia's five marine biogeographical provinces: i) Damperian; ii) Flindersian; iii) Maugean; iv) Peronian; and v) Solanderian (Bennett and Pope 1953). For the *cox*1 markers, however, we obtained a more concentrated sampling

within a narrower spatial scale, focusing on the south and southeastern Australian coasts. Thus, with *cox*1 we tested the presence of genetic structure within only three biogeographical provinces: i) Flindersian; ii) Maugean; and iii) Peronian.

The degree of genetic differentiation between populations was determined using F_{ST} pair-wise differences (Nei 1973) implemented in Arlequin v3.5 (Excoffier and Lischer 2010) with 3000 permutations, 0.05 significance level, and using number of different alleles. We tested for demographic equilibrium using Tajima's D (Tajima 1989), Fu & Li's D and F tests (Fu and Li 1993) implemented in DNAsp.

Results

DNA fragments characteristics

From the 85 collected specimens we were able to generate 70 high quality *rbc*L-*rbc*S spacer DNA sequences from 11 populations spread ~7,000 Km apart. A 6 bp insertion was observed within the *rbc*L-*rbc*S intergenic spacer. We assumed this insertion to be a single mutational step because insertions of several nucleotides bases could occur in a single event. Therefore, the final alignment was 545 bp long and resulted in four haplotypes (R1-R4) with six variable nucleotide positions (Table 2). A total of 38 high quality *cox*1 DNA sequences were obtained from 7 populations spread ~1,500 Km apart. The alignment was 582 bp long and resulted in nine haplotypes (C1-C9) with 15 variable nucleotide positions (Table 3). Intraspecific genetic variation among Australian *C. sinuosa* specimens ranged between 0.0% and 1.1% for *rbc*L-*rbc*S and from 0.0% to 1.9% for *cox*1

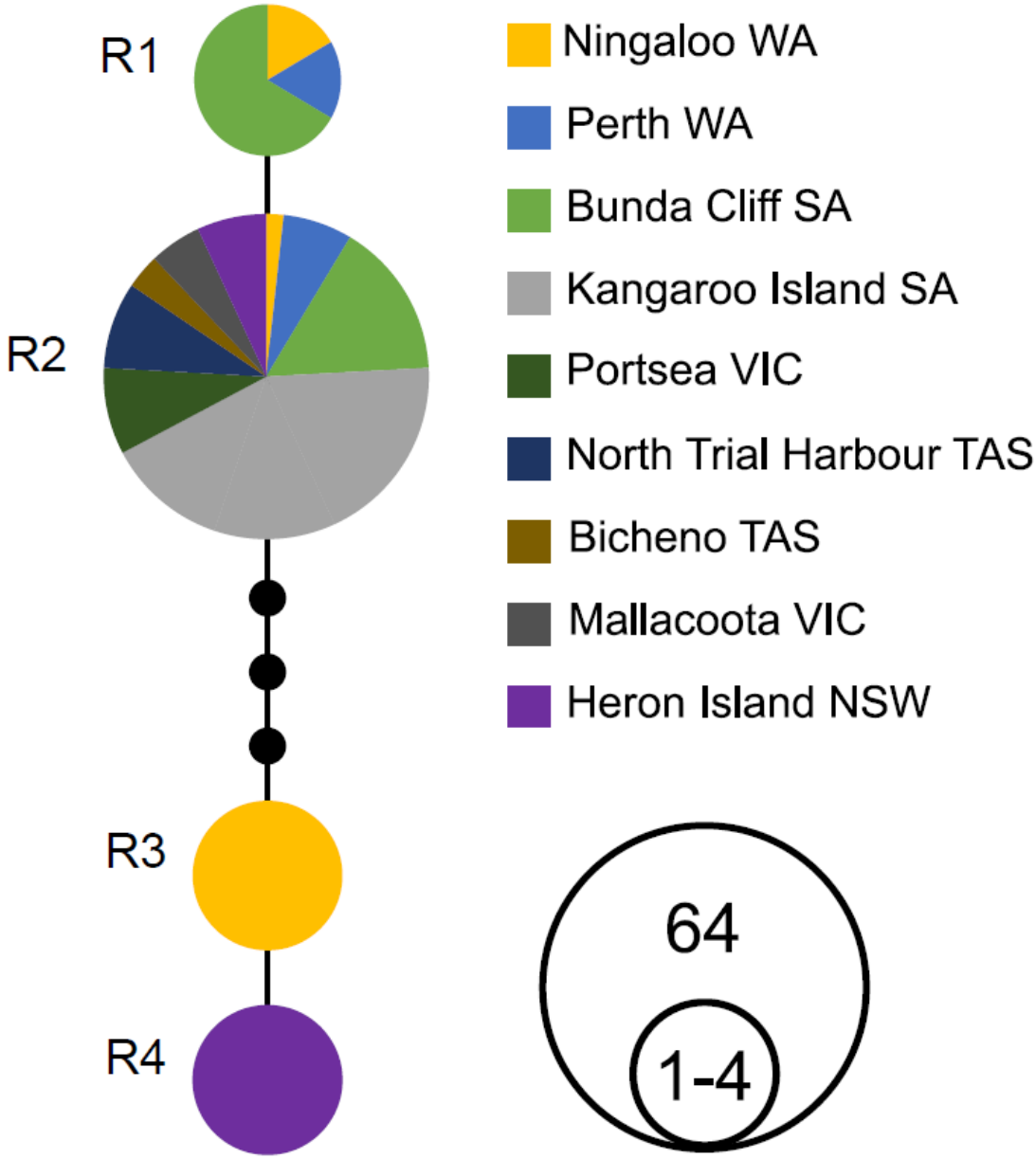

Figure 1. Statistical parsimony network (95% interval confidence) of *Colpomenia sinuosa* *rbc*L-S sequences obtained from organisms collected at 11 sites along the Australian coast. Size of the circle indicate the minimum number of sequences.

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Table 2. *Rbc*L-*rbc*S compilation results of *Colpomenia sinuosa* phylogeographic data along the Australian coast. Where N = sequences, h = haplotypes, S = polymorphic sites, m = number of mutations, k = average number of nucleotide differences, Hd = haplotype diversity, and Pi = nucleotide diversity. Tajima's D and Fu & Li's D and F. Asterisk (*) represents statistically significant results ($p < 0.05$). N/A means not applicable. Haplotypes represent total number of R1, R2, R3 and R4 haplotypes sequences.

Population	N	h	S	m	k	Hd	Pi	Tajima's D	Fu & Li's D	Fu & Li's F	Haplotypes			
Ningaloo	3	3	5	5	3.333	1.000	0.00613	N/A	N/A	N/A	1	1	1	0
Perth	5	2	1	1	0.400	0.400	0.00074	-0.81650	-0.8165	-0.77152	1	4	0	0
Twin Rocks	13	2	1	1	0.462	0.462	0.00085	0.95051	0.73235	0.88867	4	9	0	0
Penington Bay KI	11	1	0	0	0.000	0.000	0.00000	N/A	N/A	N/A	0	11	0	0
Vivonne Bay KI	7	1	0	0	0.000	0.000	0.00000	N/A	N/A	N/A	0	7	0	0
Stokes Bay KI	8	1	0	0	0.000	0.000	0.00000	N/A	N/A	N/A	0	7	0	0
Portsea	5	1	0	0	0.000	0.000	0.00000	N/A	N/A	N/A	0	5	0	0
North Trial Harbour	5	1	0	0	0.000	0.000	0.00000	N/A	N/A	N/A	0	5	0	0
Bicheno	2	1	0	0	0.000	0.000	0.00000	N/A	N/A	N/A	0	2	0	0
Mallacoota	3	1	0	0	0.000	0.000	0.00000	N/A	N/A	NA	0	3	0	0
Heron Island	8	2	5	5	2.857	0.571	0.00525	2,18406*	1.36768	1,71515*	0	4	0	4
Total	70	4	6	6	0.786	0.27600	0.00144	-0.86798	1.14615	0.57743	R1	R2	R3	R4

Table 3. *Cox*1 compilation results of *Colpomenia sinuosa* phylogeographic data along the Australian coast. Where N = sequences, h = haplotypes, S = polymorphic sites, m = number of mutations, k = average number of nucleotide differences, Hd = haplotype diversity, and Pi = nucleotide diversity. Tajima's D and Fu & Li's D and F. No statistically significant results ($p < 0.05$) were detected. N/A means not applicable. Haplotypes represent total number of C1, C2, C3, C4, C5, C6, C7, C8 and C9 haplotypes sequences.

Population	N	h	S	m	k	Hd	Pi	Tajima's D	Fu & Li's D	Fu & Li's F	Haplotypes								
Penington Bay KI	9	3	4	4	1.056	0.417	0.00181	0.07978	-1.03151	-1.17694	1	7	1	0	0	0	0	0	0
Vivonne Bay KI	2	1	0	0	0.000	0.000	0.00000	N/A	N/A	N/A	0	0	0	2	0	0	0	0	0
Stokes Bay KI	5	4	11	11	6.000	0.900	0.01031	0.98145	0.98145	1.03783	2	1	0	1	1	0	0	0	0
Hanson Bay KI	11	3	4	4	0.876	0.473	0.00150	-1.32167	-1.32167	-1.39473	1	8	2	0	0	1	0	0	0
Portsea	3	3	7	7	4.667	1.000	0.00802	N/A	N/A	N/A	0	0	0	0	0	0	1	1	0
North Trial Harbour	5	1	0	0	0.000	0.000	0.00000	N/A	N/A	N/A	0	0	0	0	0	0	0	0	5
Mallacoota	3	1	0	0	0.000	0.000	0.00000	N/A	N/A	N/A	0	0	0	0	0	0	0	0	3
Total	38	9	15	15	3.603	0.762	0.00619	0.04999	-0.15361	-0.10251	C1	C2	C3	C4	C5	C6	C7	C8	C9

Genetic diversity

*Rbc*L-*rbc*L

Haplotype diversity (Hd) was relatively low on *rbc*L-*rbc*S results (0.276). No genetic diversity (Hd = zero) was observed in the southern and southeastern Australian coast (Kangaroo Island, Portsea and Tasmania). West and southwestern coasts evidenced Hd values ranging from 0.4 to 1.0. On the eastern coast, the Heron Island population also evidenced high Hd (0.571). Statistical parsimony network revealed a linear relationship among haplotypes. The most common and widely distributed haplotype (R2) is centrally located, one mutational step from R1, four mutational steps apart from R3, which is one mutation step apart from R4 (Fig. 1). PERMANOVA identified the presence of three statistically different population groups: a west and southwestern group (= Ningaloo, Perth, Twin Rocks), a south-southeastern group (= Kangaroo Island, Victoria, Tasmania), and the Heron Island (Queensland) population (PERMANOVA, Pseudo-F = 13.515, $p < 0.01$, Permutations = 999, Fig. 2A). The only significant departure from demographic neutrality were positive values for Tajima's D (2.18) and Fu & Li's F (1.71) detected for Heron Island (Table 2).

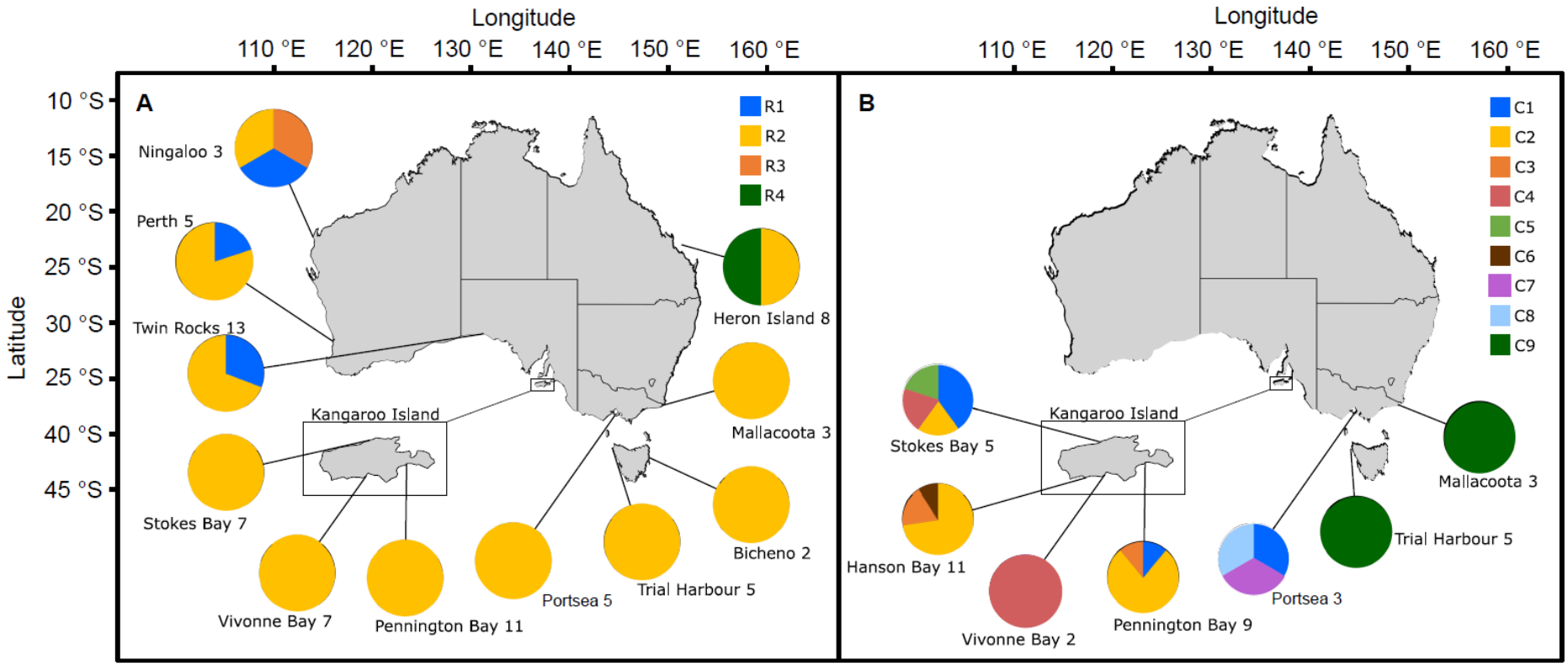

Figure 2. *Colpomenia sinuosa* (A) *rbc*L-S and (B) *cox*1 DNA sequence haplotype composition within 12 populations sampled along the Australian coast. Numbers after population names represent sample size (n). Four haplotypes were found for *rbc*L-S: R1-R4 and nine for *cox*1 C1-C9.

*Cox*1

*Cox*1 haplotype diversity was high (0.762). *Cox*1 evidenced a more complex pattern of haplotype diversity, ranging from 0.000 (at Trial Harbour, Tasmania) to 1.000 (at Portsea, Victoria). *Cox*1 statistical parsimony network also revealed a more complex scenario. The most common haplotype (C2, n = 16) is restricted to Kangaroo Island (Hanson Bay, Pennington Bay, and Stokes Bay). C4 and C5 were the most distant haplotypes with seven mutational steps apart from C3 (Fig. 3). PERMANOVA revealed the presence of significant genetic difference between the Maugean (= Kangaroo Island) and the Flindersian (= Victoria and Tasmania) marine provinces (PERMANOVA, Pseudo-F = 5,6424, p < 0,01, Permutations = 999, Fig. 2B). No significant departure from demographic neutrality was detected for *cox*1 marker (Table 3).

Discussion

Colpomenia sinuosa populations presented statistically significant phylogeographic structure along the Australian coast that mirrors the marine biogeographic provinces proposed by Bennett and Pope (1953). Both *rbc*L-*rbc*S and *cox*1 markers supported this pattern. *Rbc*L-*rbc*S detected an east-west population differentiation with one haplotype spread across the entire country (R2), a westerly haplotype R1 distributed along the extension of the Leeuwin Current, and two geographically restricted tropical haplotypes, one in the west (R3) and the other in the east coast (R4). The dispersal capacity of the Leeuwin Current establishing greater connectivity along the western and southwestern Australian coast, *vis a vis* the R1 haplotype distribution from Ningaloo Reef to the head of the Great Australian Bight, is anticipated due to its higher connectivity capacity, and a continuous, homogeneous, more undisturbed flow compared to the East Australian Current (Ridgway and Condie 2004, Condie et al. 2011, Suthers et al. 2011, Wernberg et al. 2013). The reduced connectivity promoted by the East Australian Current, compared to

the Leeuwin Current, is attributed to its more turbulent flow, its offshore turn towards New Zealand at latitude 34 ºS, and a greater occurrence of eddies (Ridgway and Condie 2004, Condie et al. 2011, Suthers et al. 2011, Wernberg et al. 2013). Haplotype R4 limited to the tropical east coast does not seem extend southwards along the Australian coast. As a consequence, east coast temperate populations are expected to be either more isolated from low latitude tropical populations or to present greater genetic structure along the coast (Banks et al. 2007, Wernberg et al. 2013).

The *rbc*L-*rbc*S marker showed differences among: *i.* the Flindersian province (characterized by haplotypes R1, R2, R3), *ii.* an area formed by both the Maugean and the Peronian provinces (characterized by the fixation of haplotype R2), and *iii.* the Solanderian province (characterized by haplotypes R2 + R4). Our *cox*1 results although more limited in space compared to our *rbc*L-*rbc*S results, corroborated the results observed in the latter maker and identified the presence of phylogeographic structure between the Flindersian and the Maugean provinces. Kangaroo Island populations (= western Flindersian province) present unique haplotypes (C2-C6) that differentiate it from the Victoria-Tasmania populations (= Maugean province) with other three unique haplotypes (C7-C9). Since the first conception of Bennett and Pope (1953)'s Australian marine biogeography, the exact border between provinces is not precise and some degree of overlap occurs, depending on the studied taxa. The border between the Flindersian and the Maugean provinces often overlaps and it is not marked by major boundary currents. Differences between the Maugean and the Flindersian provinces (including the Peronian province) are explained by historical and climatic rather than oceanographic processes (Bennett and Pope 1953).

The *cox*1 genetic structure observed today might have been produced by extinct barriers to gene flow because contemporary oceanographic processes do not explain the presence of observed *cox*1 phylogeographic breaks, but may be responsible for maintaining breaks that evolved earlier (Teske et al. 2017). Our *cox*1 results agree with

other the marine phylogeographic studies which suggested that contemporary oceanic currents do not seem to be the main factor playing a critical role on promoting genetic variation in southeastern Australia (Waters and Roy 2003). Genetic differences within the Maugean province are often regarded as signature from Pliocene and Pleistocene, due to extant factors, such as glaciations events (Waters and Roy 2003).

The search for genetic (= among markers), taxonomic (= among species) and geographic (= shared barriers to gene flow among multiple taxa) concordance is one of the major phylogeography goals (Avise 1998). Longstanding historical barriers to gene flow usually result in spatially concordant phylogeographic pattern across independent neutral loci (Kuo and Avise 2005). In this study, we found difference aspects of all three facets of phylogeographic concordance among Australia's *C. sinuosa* populations. Genetic concordance: both markers showed significant genetic differences between well-known marine biogeographic provinces, *rbc*L-*rbc*S, (the less divergent marker) in a broad spatial scale (= thousands of Km) and *cox*1 (the more divergent marker) in a smaller spatial scale (= hundreds of Km). Several studies show that marine and coastal terrestrial biotas display the imprint of the Australian marine biogeographic provinces on their biogeography, at multiple organizational scales, from genes to communities (e.g., genes: this study, Waters and Roy (2003), Waters et al. (2005) and Teske et al. (2017); communities: Waters et al. (2010); terrestrial floras: Saintilan (2009) and Gurgel et al. (2014)).

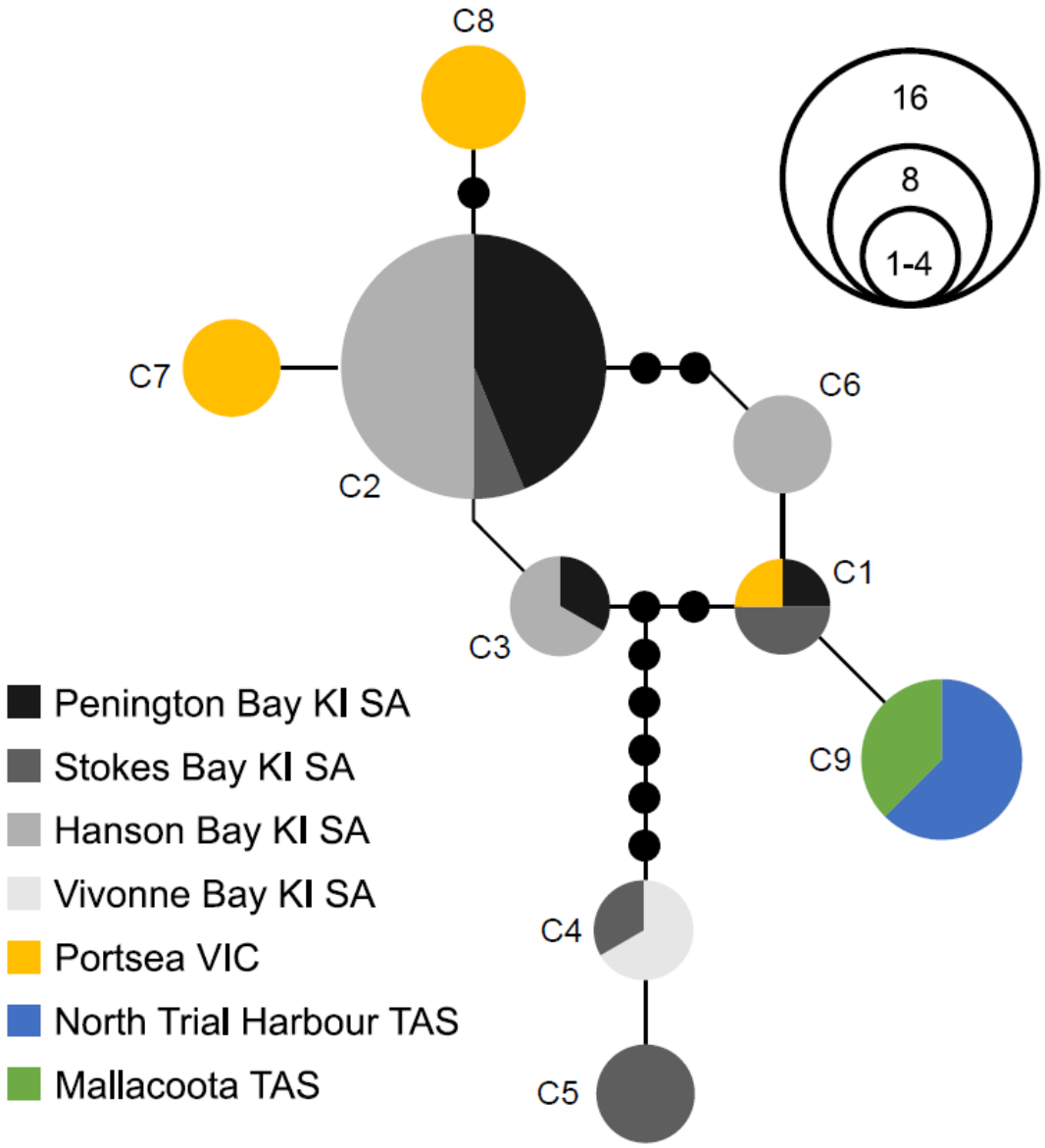

Figure 3. Statistical parsimony network (95% interval confidence) of *Colpomenia sinuosa* *cox*1 sequences obtained from organisms collected at 7 sites along the Australian coast. Size of the circle indicate the minimum number of sequences.

The observed difference in spatial resolution between *rbc*L-*rbc*S and *cox*1 is attributable to the difference in genetic divergence between these two markers. *Cox*1 divergence is usually greater than *rbc*L or *rbc*L-*rbc*S divergence. This difference is found not only in *C. sinuosa* as reported in this study, but also in other brown macroalgae (Hoshino et al. 2021), diatoms (Evans et al. 2007) and red algae (Gurgel et al. 2004). In this study, *cox*1 was 2.8 and 4.3 times more divergent than *rbc*L-*rbc*S spacer, in terms of haplotype and nucleotide diversity, respectively. High levels of *cox*1 divergence have been reported in other brown algae as well, such as *Alaria* (Lane et al. 2007), *Fucus* (Kucera and Saunders 2008), *Laminaria* and *Saccharina* (McDevit and Saunders 2009).

Although we could detect differences between the Maugean and the Flindersian provinces for the *cox*1 marker, the sampling design was scarce and results should be considered with care. The lack of samples on east side of Wilson's Promomtory, also known as the Bassian landbridge, did not allow us to test the effect of this extant Pleistocene glaciation-related barrier to gene flow on *C. sinuosa*. However, the existence of a single *cox*1 haplotype east of this geographic region (C9, n = 3, Mallacoota), indicates a possible genetic discontinuity. C9 also occurs in western Tasmania (n = 5, Trial Harbour) but it could have reached such western distribution after secondary contact during interglacial periods as observed for *Durvillea* species (Fraser et al. 2012).

Despite the differences in oceanography, eastern and western Australian coasts are roughly 7,000 Km apart, which alone is enough to promote isolation by distance in most marine macroalgal populations. R3 and R4 seem to be exclusively tropical haplotypes. Larger sampling in tropical latitudes will reveal more information about the extent of the spatial distribution of these two haplotypes along the tropics, and other uniquely tropical genetic diversity. Tropical sampling will also reveal how vast really is the distribution of the R2 haplotype into tropical latitudes.

Widespread marine taxa in Australia usually display phylogeographic breaks coincident with provincial boundaries. Thus, it looks like the physical differences between Dampierian and Flindersian and between Solarian and Flindersian at the east coast, results in genetic differences observed by the presence of exclusive tropical haplotypes. However, these regions are not separated by a rigid barrier (Bennett and Pope 1953). Indeed, *C. sinuosa* is a saccate macroalgae which may be filled with air during low tide that could confer flotation, which facilitate dispersal in detached adult macroalge (Blackler 1967, Mathieson et al. 2016).

We have detected genetic structure for *Colpomenia sinuosa* along the Australian coast. Probably due to saccate habit and possibility of dispersion via drift *C. sinuosa* genetic structure are often related to the main Australian oceanic currents. Even though several patterns could be addressed, further studies will elucidate *C. sinuosa* diversity in tropical Australia, in addition to the southeastern marine hotspot and will help to elucidate the main geographic events/accidents playing role on genetic divergence.

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Concluding remarks

Considerações finais

We have extensively investigated *Colpomenia sinuosa* diversity in Brazil and Australia. In both countries, we detected different lineages with relatively high genetic divergence, suggesting the presence of cryptic species.

In Brazil, by applying *cox*3 DNA sequences and species delimitation methods (SDM), we identified five overall *Colpomenia* lineages: the true *C. sinuosa* and four putative new species passing under the name of *C. sinuosa.* All five linages lack morphological differences among them. We concluded that *C. sinuosa* is a complex of cryptic species. Similar pattern was described by Lee et al. (2013) on a worldwide *C. sinuosa* study. Morphologically, the specimens analyzed in this study correspond to descriptions historically made for *C. sinuosa* from the Brazilian coast (Semir 1977; Széchy 1986; Gomes et al. 1989; Széchy and Cordeiro-Marino 1991; Crispino 2000; Nunes and Paula 2004; Ouriques and Cordeiro-Marino 2004).

In Australia, however, by applying SDM in COI-5P DNA sequences, there was no evidence of cryptic speciation in *C. sinuosa*, even though this is the most widely distributed species. Indeed, we detected three different *Colpomenia* species in Australia: *C. sinuosa*, *C. claytoniae* and *C. peregrina*. The latter two evidenced also intraspecific genetic variation that might correspond to cryptic species. *C. peregrina* and *C. claytoniae* require further studies that consider the inclusion of other molecular markers, more widespread sampling, and comparative morphological data. Fortunately, for the three Australian species, morphological characters are proving to be reliable.

During the molecular genetics investigation of *Colpomenia* spp. in temperate Australia, we identified the presence of *Mikrosyphar zosterae* as endophyte in this genus and in *Leathesia marina* using *rbc*L-*rbc*S and COI-5P DNA sequences. This is the first record of *M. zosterae* in the southern hemisphere and as an endophyte in the brown algal genera *Leathesia* and *Colpomenia*. There is very few information regarding *Mikrosyphar*. However, this seems to be a common species in *Leathesia marina* from southern

Australia, and to a lesser extent, in *Colpomenia* spp. thalli. Further investigations will help to clarify whether this endophyte also occurs on other Australian marine species and in other regions of the world, especially where *Leathesia* is already reported. In addition, laboratory experiments could elucidate if this relationship is harmful or not to the host. We did not detect *M. zosterae* in Brazilian samples, neither morphologically nor molecularly, even though we did not sequence the same genetic markers as we did for the Australian samples (*cox*3 vs. *rbc*L-S and COI-5P, respectively).

By performing a literature review and metadata analysis, we identified Cape São Roque, the region where the South Equatorial Current splits into two as the location that presents the largest values of overall marine taxa genetic discontinuities. Vitória-Trindade seamount chain represents the second most important region of genetic discontinuities along the Brazilian coast, especially to fishes. The Cabo Frio seasonal upwelling system, although frequently pointed in literature as potential strong barrier, presented lower levels of phylogeographic concordance. On the other hand, the meeting of the Brazil-Malvinas Current confluence, Cape Santa Marta, and the string of estuaries in southern Brazil turned out to be a region of higher levels of genetic breaks as well, representing other major drivers of genetic breaks. Due to the lack of sampling for phylogeographic studies at both extremes of the Brazilian coast, we could not test the effects of the Amazon river outflow, and the Rio Grande do Sul long extensions of unconsolidated benthic subtract correspond to major barriers to gene flow for multiple taxa.

After understanding the geographical processes promoting biodiversity in Brazilian marine taxa as a whole and studying *Colpomenia* spp. diversity in Brazil, we could properly perform the phylogeographic analyses on *Colpomenia sinuosa* populations along the Brazilian coast. Even though the Cape São Roque seems to be the main barrier to gene flow for the majority of the studied organisms along the Brazilian coast, this did not come up to be a barrier for *C. sinuosa*. Instead, our study demonstrated the existence of

one major genetic break along the Brazilian coastline, represented by the Vitória Trindade seamount chain (20.5º S), resulting in two divergent *C. sinuosa* phylogroups (north vs. south). This is also evident the impact of a complex region characterized by the interaction of changes in climate over geological time (glaciations), the Brazil Current, the Abrolhos basin, the Doce river and the Victoria-Trindade seamount chain driving genetic structuring, and potentially recent speciation, along the Brazilian coast.

The phylogeographic study of *C. sinuosa* from Australia evidenced a different pattern than those observed in Brazil. The Australian phylogeography evidenced that *C. sinuosa* is highly affected by oceanic currents. The dispersal capacity of the Leeuwin Current establishes greater connectivity along the western and southwestern Australian coast. This evidences long-distance dispersion capability probably via drift due to saccate habit of *Colpomenia* species. However, the eastern Australian Current seems to promote a reduced connectivity, attributed to its more turbulent flow, its offshore turn towards New Zealand, and a greater occurrence of eddies (Ridgway and Condie 2004; Suthers et al. 2011; Wernberg et al. 2013). Consequently, south-eastern coast temperate populations are isolated from tropical populations and present greater genetic structure.

Acknowledging the existence of multiple phylogeographical lineages is important not only for understand recent historical processes shaping genetic diversity in tropical regions, but also as subsidies for conservation and the management of natural marine resources. Therefore, we recommend further studies to test different approaches, such as mating compatibility and Next-Generation Sequencing (e.g. phylogenomics).

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