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DIVERSIDADE DAS ALGAS CALCÁRIAS CROSTOSAS  
DO BRASIL BASEADA EM MARCADORES  
MOLECULARES E MORFOLOGIA

DIVERSITY OF CRUSTOSE CORALLINE ALGAE FROM  
BRAZIL BASED ON MOLECULAR MARKERS AND  
MORPHOLOGY

São Paulo, SP

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Orientador(a)



# Dedication

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To my family;  
To honor the memory of  
Dr. Rafael Riosmena-Rodriguez



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## List of abbreviation

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ABGD	Automatic Barcode Gap Species Discovery
AIC	Akaike Information Criterion
BI	Bayesian Inference
BS	Bootstrap value
CCA	Crustose (or non-geniculate) coralline algae
COI	Mitochondria-encoded Cytochrome Oxidase Subunit I gene
ESS	Effective Sample Size
GMx	Gulf of Mexico
GMYC	General Mixed Yule Coalescence
IBC	Instituto de Biociências (field code of the collected samples)
MAAT	Molecular-assisted Alpha Taxonomy
MCMC	Markov chain Monte Carlo
ML	Maximum Likelihood
NEGMx	northeastern Gulf of Mexico
NJ	Neighbor Joining
NWGMx	northwestern Gulf of Mexico
pc	pit connection
PCR	Polymerase Chain Reaction
PP	posterior probability
<i>psbA</i>	Chloroplast-encoded Photosystem II Reaction Center Protein D1 gene
PTP	Poisson Tree Process
<i>rbcL</i>	Ribulose biphosphate carboxylase large
RuBisCO	Ribulose-1,5-biphosphate carboxylase/oxygenase
SEM	Scanning Electron Microcopy
SSU rDNA	genes coding for small-subunit rRNA
SWGMr	southeastern Gulf of Mexico
UPA	Universal Plastid Amplicon
WTA	Western Tropical Atlantic

## Resumo

As algas calcárias crostosas ou CCA (do inglês Crustose Coralline Algae) têm como principal característica a impregnação de carbonato de cálcio em suas paredes celulares.

Este grupo é formado atualmente por três ordens, Corallinales, Hapalidiales e Sporolithales, cuja taxonomia é historicamente problemática por se basear na fase tetrasporofítica, fundamental para qualquer identificação até mesmo em nível de ordem.

Em virtude disso, diversos estudos, principalmente nos últimos 10 anos, têm incluído ferramentas moleculares como auxílio à taxonomia morfoanatômica deste grupo.

O objetivo deste estudo foi investigar a diversidade e a distribuição das CCA ao longo da costa brasileira, através de dados moleculares e morfoanatômicos. Para isso, foram utilizados quatro marcadores moleculares, UPA, *rbcL-3P*, COI-5P e *psbA*, aliados à imagens de microscopia óptica e microscopia eletrônica de varredura, que resultaram na identificação de 38 espécies entre Corallinales, Hapalidiales e Sporolithales.

Os resultados obtidos a partir das análises de agrupamento dos quatro marcadores demonstraram que as ordens Corallinales e Sporolithales são monofiléticas, e Hapalidiales constitui um grupo não-monofilético (com exceção do marcador *psbA*, que resolveu a ordem como grupo monofilético). Os resultados também revelaram existência de uma grande diversidade de espécies e gêneros destas ordens no Brasil, além de espécies novas e ao menos um potencial gênero novo para ciência. O estudo também revelou relações filogeográficas entre espécies do Brasil e do Golfo do México e do Indo-Pacífico. Considerando as três ordens de CCA (Corallinales, Hapalidiales e Sporolithales), este estudo representa a primeira tentativa de desvendar de forma mais ampla a diversidade de espécies CCA encontradas ao longo da costa brasileira, utilizando dados moleculares.

## **Abstract**

The Crustose Coralline Algae (CCA) has as a main distinguishing characteristic the calcium carbonate impregnation in their cell walls. This group currently encompasses three orders, the Corallinales, Hapalidiales and Sporolithales, whose taxonomy is historically problematic because it is based on the tetrasporophytic phase, fundamental to any classification, even at the ordinal level. Therefore, many studies, especially in the last 10 years, have included molecular tools to assist the morphological taxonomy of this group. This study aims to investigate the diversity and distribution of the CCA along the Brazilian coast, through molecular and morphoanatomical data. In order to achieve this aim, four markers were used, UPA, *rbcL*-3P, COI-5P and *psbA*, allied to light and scanning electron microscopy, that resulted in the identification of 38 species between Corallinales, Hapalidiales and Sporolithales. The results of the cluster analyses of the four markers showed that Corallinales and Sporolithales are monophyletic, and Hapalidiales comprises a non-monophyletic group (with the exception of *psbA*, which resolved the order as a monophyly). Our results also revealed a great diversity of species and genera of these three orders in Brazil, as well as putative new species and at least a new genus. This study also revealed phylogeographic relationships between Brazilian species and species from Gulf of Mexico and from Indo-Pacific oceans. Considering all the three orders of CCA, this study represents the first broad attempt and effort to unveil the diversity of CCA species found on the Brazilian coast using molecular data.

## Introdução geral

As algas calcárias crostosas ou CCA (do inglês Crustose Coralline Algae) têm como principal característica a impregnação de carbonato de cálcio em suas paredes celulares (Silva & Johansen 1986, Bailey & Chapman 1998), na sua maioria na forma de calcita, diferente de outros grupos, nos quais este mineral aparece principalmente na forma de aragonita (*e. g. Halimeda* spp.) (Littler 1976).

A distribuição das algas calcárias é conhecida em todos os oceanos, dos trópicos às regiões polares (Littler *et al.* 1991, Foster 2001, Konar *et al.* 2006), como um constituinte do bentos conspicuo, ou mesmo dominante, particularmente em locais de alta herbivoria e hidrodinamismo (Steneck & Paine 1986, Woelkerling 1988). Estas algas são comumente encontradas desde a zona entre-marés até grandes profundidades (Littler *et al.* 1985, Littler *et al.* 1991, Foster 2001, Konar *et al.* 2006), sendo que sua ocorrência já foi reportada para aproximadamente 265 m de profundidade (Littler *et al.* 1985).

Em áreas tropicais, por exemplo, as CCA são fundamentais para o estabelecimento e construção dos recifes de corais, protegendo esse ecossistema contra a ação erosiva das ondas e possibilitando a manutenção e crescimento dos recifes (Steneck & Testa 1997, Piller & Rasser 1996).

Um atributo interessante destas algas é a formação de rodolitos (algas vermelhas calcificadas de vida livre que crescem independentes do substrato) formando bancos naturais, conhecidos como bancos de rodolitos (ou maërl, ou nódulos calcários) (Irvine & Chamberlain 1994, Harvey *et al.* 2005, Harvey & Woelkerling 2007). Esses bancos formam um ecossistema único que abriga grande diversidade de outras algas,

invertebrados e vertebrados associados direta ou indiretamente a eles (Steller et al. 2003, Littler & Littler 2008, Riul et al. 2009). A maior extensão de bancos de rodolitos está na costa brasileira, onde estão distribuídos em mosaicos, desde o estado do Pará até o estado de Santa Catarina, havendo uma concentração relevante na região do alargamento da plataforma que vai do sul da Bahia ao norte do Espírito Santo (Milliman & Amaral 1974, Foster 2001, Foster et al. 2013). Recentemente, o maior banco de rodolitos do mundo foi descoberto na região da Plataforma Continental de Abrolhos. Este banco ocupa uma área de mais de 20.000 km<sup>2</sup>, e tem uma importância global na produção de carbonato de cálcio comparável aos recifes coralíneos do Caribe e à Grande Barreira de Corais da Austrália (Amado Filho et al. 2012, Moura et al. 2013). Outra recente descoberta é de um extenso recife carbonático de aproximadamente 10.000 km<sup>2</sup> na região entre a Guiana Francesa e o Brasil, que compreende a região da Foz do Rio Amazonas, sendo este o primeiro recife descoberto sob a pluma de um grande rio (Moura et al. 2016).

O potencial econômico destes bancos já foi apontado por alguns autores, principalmente para aplicações como fertilizantes e corretores de solos ácidos, ou como aditivos à ração animal (Oliveira 1997, Foster 2001). Outras atividades de exploração de recursos naturais marinhos podem ser prejudiciais aos bancos de rodolitos (Riosmena *et al.* 2010), sendo exemplos a exploração de moluscos, a dragagem e a ancoragem de embarcações, além da exploração de petróleo e gás que tem sido feita quase que indiscriminadamente (à exemplo dos bancos dos Abrolhos – Moura et al. 2013). É importante que levantamentos de biodiversidade sejam realizados nestes ambientes para que medidas de mitigação de impactos possam ser embasadas e propostas, a fim de preservar estes organismos que são “bioengenheiros” (terminologia Bruno & Bertness

2001) e provedores de micro-habitats para diversos organismos marinhos de interesse farmacêutico e de bioprospecção (Amado-Filho & Pereira-Filho 2012).

Hoje em dia, são reconhecidas três ordens para as algas calcárias crostosas do filo Rhodophyta: Corallinales, Hapalidiales, Sporolithales (Nelson *et al.* 2015). Essas três ordens, juntamente com a ordem Rhodogorgonales formam um agrupamento monofilético definido como a sub-classe Corallinophycideae e se distinguem das demais Florideophyceae através evidências moleculares e celulares. Todas as ordens pertencentes à subclasse Corallinophycideae precipitam calcita na parede celular, entretanto, os membros da ordem Rhodogorgonales precipitam este mineral apenas em células vegetativas específicas, enquanto que as demais ordens o fazem por todo o talo.

A distinção das três ordens de CCA se dá através da observação de sua fase tetrasporofítica, fase na qual os representantes da ordem Corallinales apresentam tetrasporângios zonados produzidos em conceptáculos uniporados sem a presença de tampão apical, os da ordem Hapalidiales apresentam tetrasporângios zonados produzidos em conceptáculos multiporados com tampão apical, já os representantes da ordem Sporolithales, apresentam tetrasporângios cruciados produzidos em compartimentos calcificados que geralmente estão agrupados em soros e apresentam tampão apical, (Silva & Johansen, 1986, Le Gall *et al.* 2010, Nelson *et al.* 2015). Uma descrição mais detalhadas das ordens e como se chegou à classificação taxonômica atual serão abordadas no Capítulo 1.

A proposta inicial desta pesquisa era investigar a diversidade da ordem Sporolithales ao longo da costa do Brasil. No entanto, nas coletas em campo, na maioria das vezes não era possível distinguir nem mesmo a ordem à qual pertenciam as amostras. Apenas após o trabalho de triagem feita com base nos marcadores

moleculares em laboratório foi possível verificar o grupo taxonômico das amostras. A dificuldade inerente de se trabalhar com CCA, em todos os níveis, da obtenção das amostras em campo à análise molecular e morfológica, aliado ao limitado conhecimento sobre a diversidade real das espécies de CCA na costa do Brasil, fez com que este conjunto de amostras das demais ordens e os dados moleculares obtidos formassem um conjunto de informações bastante valiosas. Por outro lado, os resultados obtidos a partir de espécimes de Sporolithales coletados no entre marés e no infra litoral mostraram que a diversidade encontrada era menor que a esperada. Portanto, optamos por ampliar o escopo desse trabalho para incluir as duas outras ordens de CCA de forma a terem uma visão mais ampla sobre a diversidade de CCA ao longo da costa brasileira.

Desta forma, no Capítulo 1, tratamos de forma mais abrangente a diversidade de táxons encontrados nas coletas realizadas ao longo da costa do Brasil, mas focando nas ordens Corallinales e Hapalidiales; no Capítulo 2, encontra-se o manuscrito já submetido para publicação, onde tratamos dos resultados obtidos para a ordem Sporolithales, incluindo a descrição de duas novas espécies para a ciência; e no Capítulo 3, encontra-se outro manuscrito em vias de submissão para publicação que trata das espécies do gênero *Lithothamnion* do Golfo do México e que apresenta uma espécie em comum com o Brasil, sendo estes os primeiros resultados que serão publicados em parceria com a University of Louisiana at Lafayette.

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## First Chapter

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# DIVERSITY AND DISTRIBUTION OF CCA ALONG THE BRAZILIAN COAST WITH EMPHASIS ON CORALLINALES AND HAPALIDIALES

## Introduction

The red algae or Rhodophyta are a distinct group of eukaryotic photosynthetic organisms, containing about 5,000–7,000 species of mostly multicellular, marine algae (De Clerk *et al.* 2012; Guiry & Guiry 2016). This form a unique group is distinguished from other eukaryotic lineages by a combination of biochemical and ultrastructural features (Cavalier-Smith 2007) such as chloroplasts that lack chlorophyll b and c and instead contain phycoerythrin, phycocyanin, and allophycocyanins as accessory photosynthetic pigments, and the complete absence of flagella and centrioles in all life stages (Graham *et al.* 2009; Maggs *et al.* 2007; Saunders & Hommersand 2004; van den Hoek *et al.* 1995; Woelkerling 1990, Yoon *et al.* 2006; Yoon *et al.* 2010).

Rhodophyta are a monophyletic phylum currently divided into two subphyla, the Cyanidiophytina (previously segregated from the phylum Rhodophyta to an upper level as the Cyanidiophyta proposed by Saunders & Hommersand 2004) and the Rhodophytina; the latter divided into six classes: the Stylonematophyceae, Porphyridiophyceae; Rhodellophyceae; Compsopogonophyceae; Bangiophyceae; and Florideophyceae (Yoon *et al.* 2006; Le Gall & Saunders 2007).

The Florideophyceae is the most taxon-rich red algal class, comprising 95% of currently described species of Rhodophyta (Guiry & Guiry 2016). They are characterized primarily by 1) their triphasic life cycle, consisting of a carposporophyte, gametophyte and tetrasporophyte, 2) possessing pit-plugs between adjacent cells within filaments and 3) postfertilization cell-cell fusion mechanisms (Garbary & Gabrielson

1990). Pit connections linking neighboring cells are one of the diagnostic features characterizing red algal orders and diverse combinations of pit connection compartments (i.e., plug core with different number of cap layers and membranes) with molecular data have been used to define the ordinal boundaries of the Florideophyceae (Pueschel 1989; Pueschel 1994).

Recent phylogenetic studies based on molecular data have resulted in a revised classification system that recognizes 29 orders in five subclasses: Ahnfeltiophycidae, Corallinophycidae, Hildenbrandiophycidae, Nemaliophycidae, and Rhodymeniophycidae (Saunders & Hommersand 2004; Le Gall & Saunders 2007; Yoon *et al.* 2010). The Corallinophycidae was proposed by Le Gall & Saunders (2007) to encompass the orders Corallinales and Rhodogorgonales. Subsequently, (Le Gall *et al.* 2010) revised the order Corallinales based on a reassessment of tetrasporangial cleavage pattern and on multigene analyses (SSU, LSU, and EF2), and erected a third order in the Corallinophycidae, the Sporolithales, based on the type family Sporolithaceae (Verheij 1993). More recently, Nelson *et al.* (2015) proposed that the family Hapalidiaceae, previously belonging to the order Corallinales, should be raised to the ordinal level, and thus established the Hapalidiales. Therefore, 4 orders are currently recognized as belonging to this subclass, the Corallinales, Hapalidiales, Sporolithales, and Rhodogorgonales.

Members of the Corallinales, Hapalidiales and Sporolithales are also known as “corallines” or “calcareous algae”. The “corallines” are an artificial group composed of those multicellular red algae (Rhodophyta) that are characterized by a rigid stony thallus due to the presence of heavy calcium carbonate deposition in the form of calcite in their cell walls. Based solely in their external morphology, they can be divided in two main

groups: the crustose (or non-geniculate) and the articulated (or geniculate) corallines (Figures 1A, 1B).

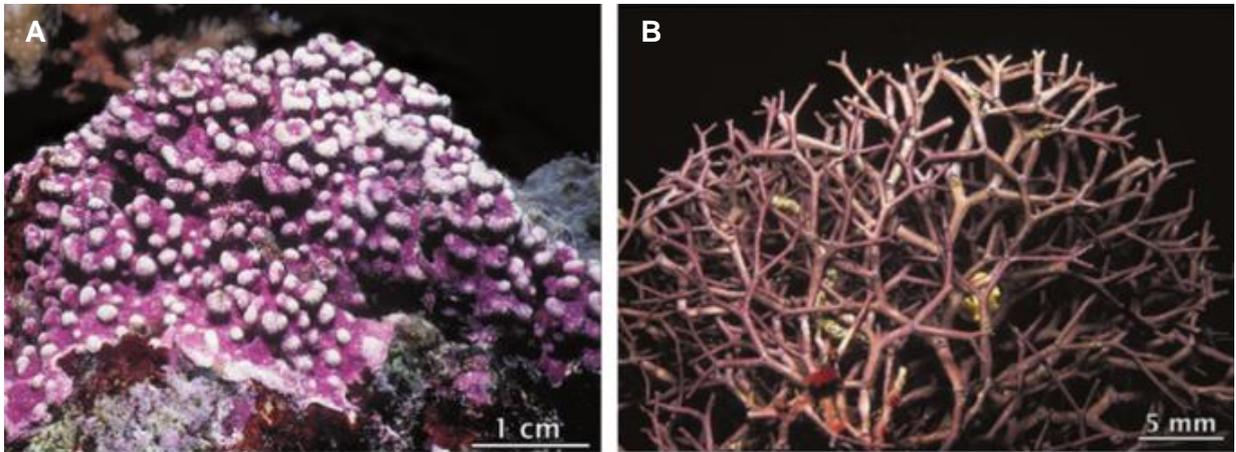


Figure 1. Main morphological habits that coralline algae can exhibit. . Photos from D. Littler (Littler & Littler 2013). A. Crustose or non-geniculate coralline algae. B. Articulated or geniculate coralline algae.

The articulated (geniculate) corallines are upright or pendulously branched, bushy plants with calcified portions of the thallus intercalated with non-calcified portions or joints called genicula (in latin, literally little knees). Crustose coralline algae (CCA) may exhibit a high degree of phenotypic plasticity, along with the occurrence of cryptic species (Steneck & Adey 1976; Woelkerling *et al.* 1993), as is the case of many other groups in Rhodophyta (Hindi *et al.* 2015). Growth forms vary greatly among the CCA, and can be smooth and encrusting, discoid, shelf-forming/layered, foliose, and fruticose (or branched with knoblike excrescences), and all growth forms may or may not possess warty or lumpy surfaces (Figure 2). They are largely epilithic (i.e. growing directly on the rocky substratum), epiphytic (i.e. growing on other algae or marine angiosperms), or even epizoic (i.e. growing on animals) (Maneveltdt *et al.* 2010). In the

absence of hard substrata, many CCA species can propagate as free-living nodules known as *rhodoliths* (literally “red stones”) (Littler & Littler 2013).

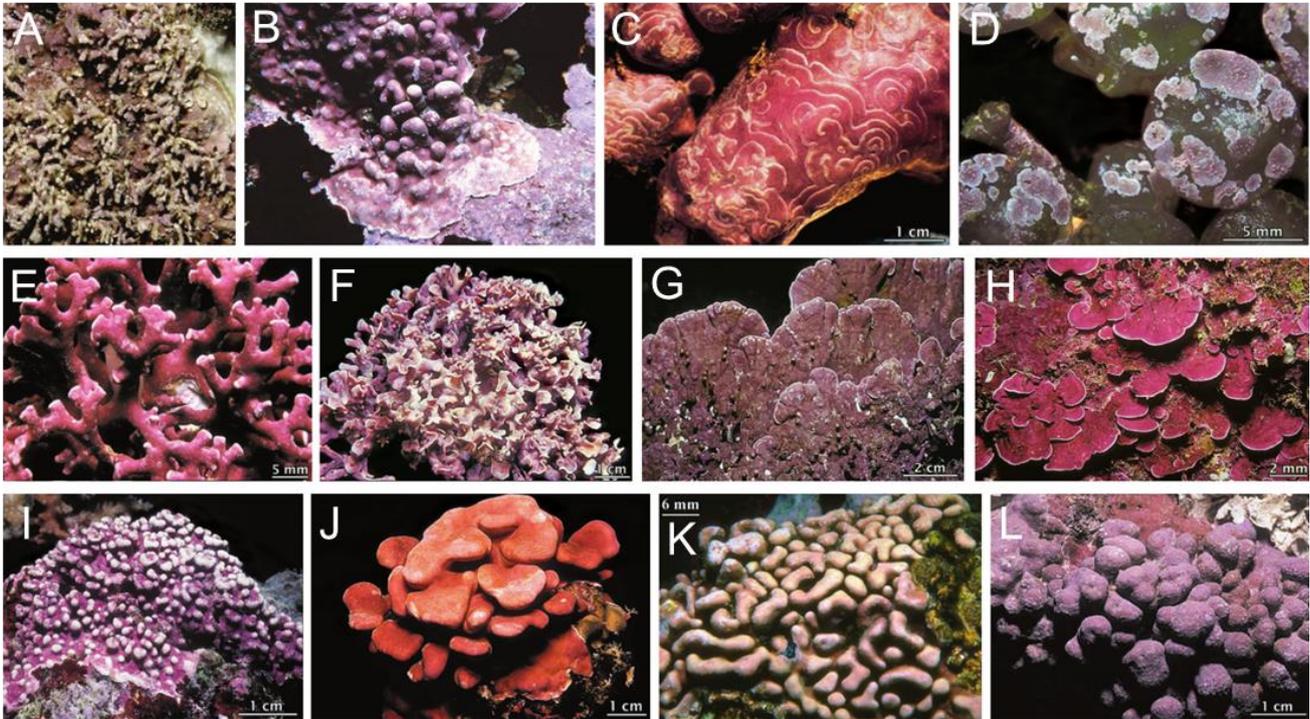


Figure 2. Spectrum of shapes and ramification exhibited by CCA. Photos from D. Littler (Littler & Littler 2013). A. Fruticose with upright spike-like protuberances. B. Fruticose with knob-like protuberances. C. Layered smooth to lumpy crusts. D. Thin smooth crusts. E. Fruticose almost dichotomous excrescences. F. Foliose G. Layered fan-like crusts. H. Layered branches I. Warty protuberances. J-K. Knoblike branched excrescences. L. Knoblike to lumpy protuberances.

### ***The CCA: Biological, ecological and geological aspects***

The CCA are distributed worldwide, ranging from polar to tropical regions and reaching their highest diversity in tropical reef environments. There they occupy the entire depth range inhabited by photosynthetic organisms, from the upper intertidal to subtidal depths as great as 295 m (Littler *et al.* 1985, Littler & Littler 2013). They show characteristic distributions (Aguirre *et al.* 2000; Bosence 1983): (1) Sporolithoideae, low latitude/mainly deep waters; (2) Melobesioideae, high latitude/shallow waters to

low latitude/deep waters; and (3) Lithophylloideae/Mastophoroideae, mid to low latitude/ shallow waters.

### *Life history*

Generally, the CCA reproduce by a triphasic life history in which the diploid tetrasporophyte (2N) produces haploid tetraspores (N) through meiosis of the sporangial mother cell, a specialized cell giving rise to the tetrasporangium. Tetrasporangia are borne in individual calcified compartments (in the Sporolithales), uniporate conceptacles (in the Corallinales) and multiporate conceptacles (in the Hapalidiales). Haploid tetraspores germinate to produce the male and female gametophytes. Both female and male gametes are borne in uniporate conceptacles (for all three orders) and only the male gametes (spermatia), which lack flagella, are released to the environment to fertilize the female carposogonium within the female conceptacle. The gametes fuse and form a parasitic diploid carposporophyte stage (2N) within the female conceptacle. Lastly, the carposporophyte produces carpospores via mitosis, and the resultant carpospores germinate into diploid tetrasporophytes (2N), completing this life cycle (Hommersand & Fredericq 1990; Irvine & Chamberlain 1994; Harvey *et al.* 2005; Littler & Littler 2013). A representative scheme is presented in the Fig. 3.

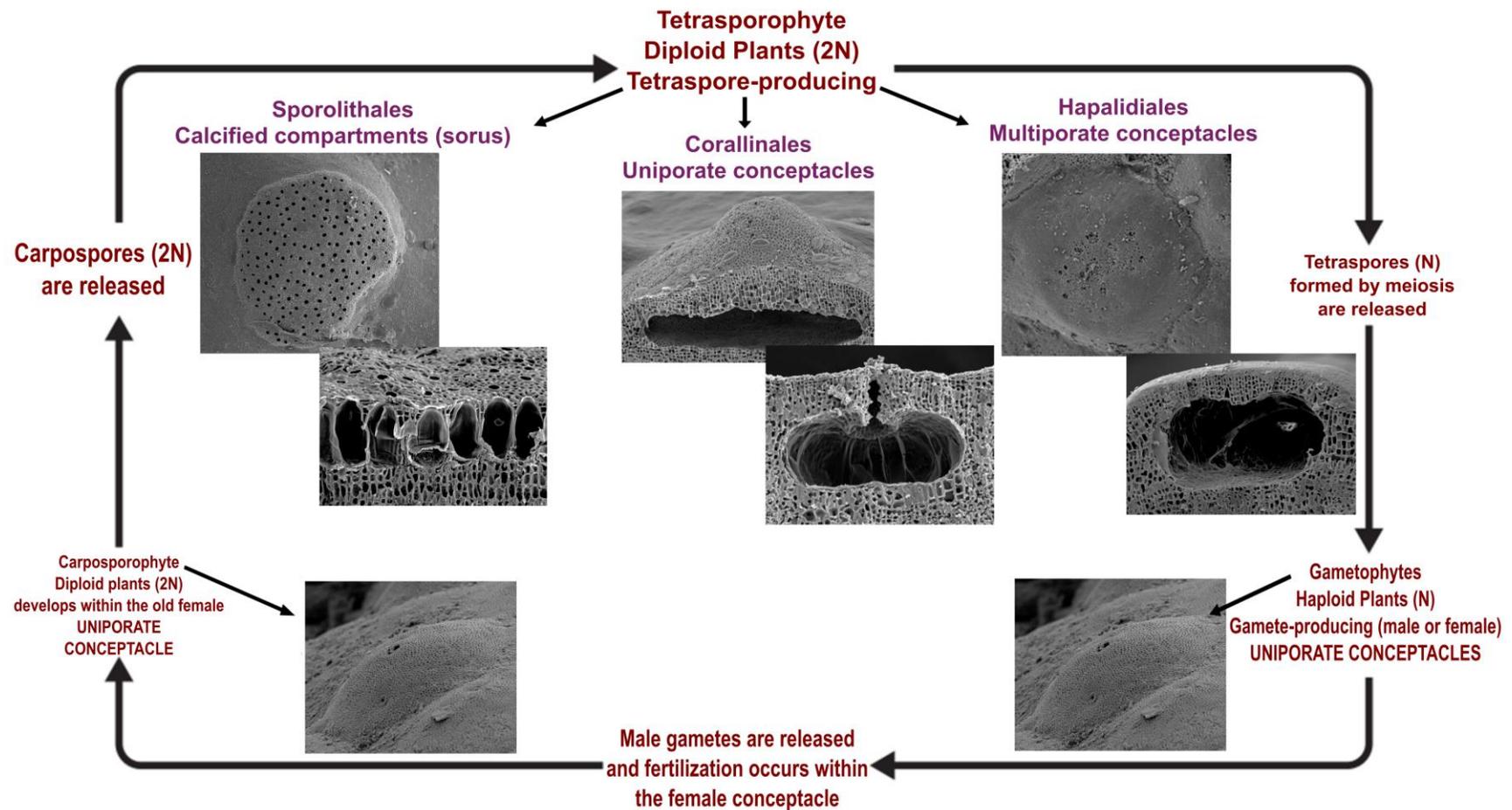


Figure 3. Simplified general sexual cycle/life history of the CCA (adapted from Harvey *et al.* 2005). The images were all newly generated in this study and are only representative, variations may occur in different genera.

### *Ecological Importance*

The CCA are a conspicuous constituent in coral reefs where they play a very important role in cementation and building reefs, offering structure and protection to this fragile ecosystem (Adey 1978b, Adey 1998, Harrington *et al.* 2004, Littler & Littler 1997). In the absence of hard substrata, many nongeniculate species can propagate as free-living rhodolith nodules colonizing sedimentary seafloors. Some of the attached crustose corallines break free from areas such as continental shelves and then continue to grow in three dimensions as they are tumbled by wave action and bioturbation and ultimately cover vast areas as free-living rhodoliths (also called nodules, rhodolites, maërl, red algal balls, algaliths). Rhodolith beds have been found throughout all of the world's oceans (Foster 2001, Amado-Filho *et al.* 2012). Globally, rhodoliths fill an important niche in marine ecosystems, serving as transitional habitats between rocky substrates and barren sedimentary areas (Littler & Littler 2008). Under favorable preservation conditions, rhodoliths can be the predominant contributors of carbonate sediments, often forming rudstone or floatstone depositional beds consisting of large fragments of rhodoliths contained in grainy matrices (Corda & Brandano 2003). Although they can be rolled occasionally by infrequent tropical storms, stationary rhodoliths nevertheless also provide a three dimensional microhabitat to a wide variety of species (Littler & Littler 2013). Sauvage *et al.* (2016) recently published a study using rhodoliths as a new resource for environmental biomonitoring, providing a framework that enables the use of high throughput sequencing to accelerate biodiversity characterization of microbial/algal assemblages from endolithic communities found in coral reef and rhodolith ecosystems, emphasizing the ecological importance of these organisms and showing the diversity of endolithic organisms (phototrophs and other

miscellaneous groups) that a rhodolith can bear inside; serving as a “seedbank” for the marine community (Fredericq *et al.* 2014).

Furthermore, the most important contribution of coralline algae worldwide may prove to be in ameliorating the greenhouse gas CO<sub>2</sub> buildup associated with global climate change (Littler & Littler 2013), especially in Brazil, where the world’s largest rhodolith beds are found, and where they are major CaCO<sub>3</sub> bio-factories (Amado-Filho *et al.* 2012). The resultant carbon stores may be among the most important in the biosphere as neutralizers of global oceanic acidification and as reservoirs of excess greenhouse CO<sub>2</sub>.

### *Geological Importance*

Crustose coralline algae have an extensive fossil record, in contrast to most algal groups. The modern corallines have appeared in the early Cretaceous (100.5–145.0 Mya) and have been important components of shallow marine communities throughout the Cenozoic (present–66.0 Mya), mostly showing long-term increases in its species richness (Aguirre *et al.* 2000). Fossil corallines are also widespread globally, and exhibited their greatest species richness during the early and middle Cenozoic (2.58–56.0 Mya), with a collapse to a late Pleistocene (11.7–126.0 Kya). In fact, corallines have shown a long term overall increase in species richness throughout most of their history. Despite this, coralline species diversification was not sustained after the Miocene (5.3–23.0 Mya) (Aguirre *et al.* 2000) and coralline algae suffered a series of extinction events, each of which eliminated at least 20% of the species. During the two largest extinction events of the late Cretaceous (66.0–100.5 Mya) and late Miocene–Pliocene (2.5–5.3 Mya), about 66% of all coralline species became extinct.

There are currently over 1,600 described species of crustose coralline algae (Woelkerling 1988) and approximately 649 fossil species (Aguirre *et al.* 2000). Paleoecological information for this group may be one of the greatest and provide a great proxy to understand the evolution in this group.

### *Biomineralization*

All crustose coralline algae belonging in the Corallinales, Hapalidiales, and Sporolithales (Rhodophyta) are characterized by the presence of calcium carbonate in their cell walls, which is often in the form of highly soluble high-magnesium-calcite (Adey 1998; Adey *et al.* 2013; Diaz-Pulido *et al.* 2014; Nelson *et al.* 2015; Krayesky-Self *et al.* 2016). Besides the Rhodogorgonales (Fredericq & Norris 1995), whose members also precipitate calcite, all other calcified red and green macroalgae deposit calcium carbonate in the form of aragonite (reviewed in Adey 1998, Nelson 2009).

The calcification process in corallines begins with the first cell divisions of the germinating spore, giving rise to a new plant (Cabioch & Giraud 1986); it is a photosynthesis-dependent process guided by a polysaccharide matrix. In this case, the calcite crystals are oriented along the direction of the polysaccharide fibrils of the developing cell walls. Numerous ultrastructural studies of coralline algae did not reveal any special features in their cell structures (in comparison with noncalcareous red algae) that may result in the calcification of their thalli (Cabioch & Giraud 1986; Bilan & Usov 2001). The reasons for the mineralization in these algae may be provided by the polysaccharide composition of their cell walls, which radically differs from that of other groups of red algae (Bilan & Usov 2001). Recently, Krayesky-Self *et al.* (2016) observed that the vegetative cell crystals are composed of high-magnesium calcite and that the crystals within the empty conceptacles are comprised of aragonite in some *Lithothamnion* species. Previous studies have also shown that the more stable

carbonates, i.e., aragonite and dolomite, may be present in the thallus of coralline algae filling in vegetative cells and pores (Alexandersson 1974; Diaz-Pulido *et al.* 2014; Nash *et al.* 2011, 2015). Walker and Moss (1984) showed that aragonite is precipitated in between the coralline crusts and their substratum, often when a space occurs between them. Understanding biomineralization and mechanisms that triggers it in the CCA is an important and timely topic as high Mg-calcite skeleton-bearing organisms may be more vulnerable to dissolution in response to increasing ocean acidification compared to other marine calcifying organisms that precipitate aragonite (Nelson 2009; Basso 2012).

### ***Taxonomy and Phylogeny***

#### *Worldwide scenario*

The taxonomy of the coralline algae has historically been very challenging due to its unique characteristics (e.g. calcified thallus) and often confusing due to morphological plasticity as well as cryptic diversity. Several classifications have been proposed based solely on comparative morphological and anatomical features (Cabioch 1972, 1988, Johansen 1976, Silva & Johansen 1986, Woelkerling 1988). Subsequently, Bailey & Chapman (1996, 1998) published the first molecular phylogenies of the Corallinales and confirmed the evolutionary scenario hypothesized by Cabioch (1988) that the geniculate forms had evolved independently in distinct lineages in the order. Since then, molecular approaches have greatly improved the resolution of red algal phylogenies as a whole (e.g. Freshwater *et al.* 1994, Le Gall & Saunders 2007, Le Gall *et al.* 2010) and as a consequence, some phylogenetic relationships between the different lineages of CCA have been clarified.

Presently, the CCA are divided into three different orders, the Corallinales, Sporolithales and Hapalidiales (Nelson *et al.* 2015). The Corallinales comprise one

family, the Corallinaceae with seven subfamilies (Corallinoideae, Hydrolithoideae, Lithophylloideae, Mastophoroideae, Metagoniolithoideae, Neogoniolithoideae and Porolithoideae) (Kato *et al.* 2011). The Hapalidiales comprise one family, the Hapalidiaceae, with three subfamilies (Austrolithoideae, Choreonematoideae and Melobesioideae) (Harvey *et al.* 2003). The Sporolithales comprise a single family, the Sporolithaceae, with two genera, *Sporolithon* and *Heydrichia* (Verheij 1993; Townsend *et al.* 1994). Table 1 summarizes most updated orders, families and subfamilies scheme for the CCA and related informative morphological characters.

Table 1. Summary of Orders, Families and Subfamilies of CCA and morphological informative characters (Adapted from Henriques 2016 based on Harvey *et al.* 2003; Harvey *et al.* 2005; Harvey & Woelkerling 2007; Le Gall *et al.* 2010; Kato *et al.* 2011).

Order/Family	Tetrasporangial structure and development	Spore cleavage arrangement	Apical plug	Subfamily	Connections between adjacent filaments	Cells at conceptacle roof	Spermatangial filaments
Corallinales Silva & Johansen, 1986/ Corrallinaceae Lamouroux, 1812	Uniporate conceptacle	Zonate	Absent	Hydrolithoideae	Only cell fusions	Pore surrounding cells perpendicular to thallus surface	Not branched; at conceptacle base
				Mastophorideae	Cell fusions; secondary pit connections rare	Pore surrounding cells parallel to thallus surface	Not branched; at conceptacle base
				Neogoniolithoideae	Only cell fusions	Pore surrounding cells parallel to thallus surface	Not branched; at conceptacle base and roof
				Porolithoideae	Only cell fusions	Pore surrounding cells perpendicular to thallus surface	Branched at the base and roof
				Lithophylloideae	Secondary pit connections	Pore surrounding cells perpendicular or parallel to thallus surface	Not branched; at conceptacle base
Hapallidiales Nelson <i>et al.</i> , 2015/ Hapalidiaceae Gray, 1864	Multiporate conceptacles	Zonate	Present	Austrolithoideae	No connection	Several layers	Not branched; at conceptacle base and roof
				Chreonematoideae	No connection	Single layer	Not branched
				Melobesioideae	Only cell fusions	Several layers	Branched or not branched; at the conceptacle base and roof
Sporolithales Le Gall <i>et al.</i> , 2010/ Sporolithaceae Verheij, 1993	Calcified compartments (sorus)	Cruciate	Present	Does not apply	Cell fusions and secondary pit connections	Does not apply	Branched or not branched; at the conceptacle base and roof

Though previously published red algal phylogenies based on *rbcL* and LSU included the geniculate coralline *Amphiroa* (Freshwater *et al.* 1994; Freshwater *et al.* 1999), crustose coralline algal phylogenies published until 2008 were inferred from a single marker, the nuclear small subunit ribosomal RNA (18S rRNA) (Bailey & Chapman 1996, 1998; Bailey 1999; Harvey *et al.* 2002). In 2008, Broom *et al.* proposed the plastidial gene *psbA* (encoding the D1 protein of photosystem II) as a novel marker to be used in combination with SSU data to improve the phylogenetic resolution within the order. Robba *et al.* (2006) and Walker *et al.* (2009) also showed the relevance of using a mitochondrial marker to obtain new insights into the genetic diversity at lower taxonomic levels using the barcode marker *cox1* (5' end of COI, the cytochrome c oxidase subunit I) in this and other groups of red algae. Chloroplast-encoded *rbcL* (large subunit of ribulose-1,5-bisphosphate carboxylase) is another informative molecular marker that can be applied to this group, and has been shown to have high phylogenetic resolution in the red algae as a whole (e.g., Freshwater *et al.* 1994). There is a section of *rbcL* that has been considered as a barcoding region for plants (at the 3' end) according to CBOL (The Consortium for the Barcode of Life). Additionally, this gene region has already been sequenced from historical type material (Herbarium specimens) of some CCA with the proposal to use it as an alternative DNA barcode for this group of algae (Gabrielson 2008; Torrano-Silva 2015).

Clearly, the use of multiple molecular markers will change many concepts in the taxonomy of the coralline algae. Despite all current efforts, a lack of information persists in relation to the systematics and phylogeny of CCA; for example, in the largest database of DNA sequences, GenBank, there are approximately 3,000 sequences available of Corallinales, Hapalidiales and Sporolithales combined, compared to more than 2,000 sequences for the single red algal genus *Gracilaria* alone.

### *Brazilian scenario*

In Brazil, from 2000 to date, there are 41 species of CCA reported to the Brazilian coast (see Table 2 for a list). The first studies of CCA started in the middle of the 19th century and *Melobesia mamillaris* Harvey (currently *Neogoniolithon mamillare* (Harvey) Setchell & L.R. Mason) and *M. scabiosa* Harvey (currently *Lithothamnion scabiosum* (Harvey) Foslie) were described by Harvey in 1849 based on specimens collected by Darwin in Bahia state, during the HMS Beagle expedition. After that, other naturalists cited CCA species in their studies (Dickie 1875; Piccone 1886; Möbius 1889, 1890). In 1900, Foslie reviewed specimens analyzed before by Dickie and in this same year he published the species *Lithothamnion erubescens* Foslie (currently *Mesophyllum erubescens* (Foslie) Me.Lemoine) from Fernando de Noronha Archipelago and posteriorly extended the occurrence for this species to São Paulo (Southeastern Brazilian coast).

In the 20th century many CCA species were reported for the Brazilian coast in floristic surveys conducted by Lemoine (1917), Taylor (1931), Setchell & Mason (1943), Joly (1965), Adey (1970), Ugadim (1970), Kempf (1970) and Câmara Neto (1971). Taylor (1960) also published a book referencing the occurrence of nine species. Yamagushi-Tomita (1976) referenced eight species of *Sporolithon* in her PhD dissertation, five of them as new combinations along the Brazilian Tropical region; although some of these species were misidentified, she provided a huge contribution to Brazilian phycology as these studies were a crucial milestone to the CCA studies in the country, being the first that described species in detail. One year later, Oliveira-Filho (1977) referenced the occurrence of 16 CCA species along the Brazilian coast, three of them reported as new occurrences.

Horta (2000) reported in his PhD dissertation seven CCA species for the Brazilian Southwestern and Southern regions (Temperate region), with three of them being reported as new occurrences. Figueiredo & Steneck (2002) reported 11 species in their floristic survey of the Abrolhos reefs, which are located along the Northeastern Brazilian coast (Tropical region). Also in 2002, Horta published a study with new perspectives to the Brazilian CCA studies.

Villas-Boas *et al.* (2005) first recorded *Porolithon pachydermun* (Foslie) Foslie from the Rocas Atoll (Northwestern Brazilian coast), emphasizing that this species is the major component of the shallow reefs in this area. In the same year, Tâmega & Figueiredo (2005) first reported the occurrence of *Hydrolithon samoëense* (Foslie) D. Keats & Y. Chamberlain and two other Corallinaceae from Buzios, in the Southeastern Brazilian coast and Nunes (2005) reported in his dissertation the occurrence of three species of CCA from Bahia state (Northeastern Brazilian coast), one of them being *Lithophyllum stictaeforme* (Areschoug) Hauck as the first report to the country. In 2006, Rocha *et al.* referenced three CCA species from Arvoredo Island on the Southern Brazilian coast, although no detailed descriptions were provided.

During the last 10 years, the efforts to identify and describe CCA species have been exponentially increasing along the Brazilian coast. Collaborations with researchers all over the world have also contributed to establish methods and approaches regarding to the studies of this group. As a result, many manuscripts have already been published and many more investigations are currently ongoing. Most studies in these past few years are filled with detailed descriptions, first reports that increased the number of species cited for the Brazilian coast, a few synonyms proposed and a significant number of species new to science.

Amado-Filho *et al.* (2007) showed the importance of the structure of the rhodolith beds in the Espírito Santo state (Southeastern Brazilian coast) and identified five genera from these rhodolith beds. Nunes *et al.* (2008) reported three species for Bahia state in a publication based on the findings from his thesis, being them *Lithophyllum stictaeforme*, *Mesophyllum erubescens* and *Sporolithon episorum* (M.Howe) E.Y.Dawson. Villas-Boas (2008) cited in his PhD dissertation seven species of CCA to Espírito Santo state and as a result for these findings in 2009, Villas-Boas *et al.* published a study referencing four species of *Lithophyllum*, one of them new to science. Farias (2009) analyzed two Hapalidiaceae species for her Master's thesis and Mariath (2009) analyzed ecological succession in a reef in Bahia state and identified some CCA as major components of that reef.

Bahia *et al.* (2010) published a study about the composition of a rhodolith bed along depth gradients on the northern coast of Bahia state. Amado-Filho *et al.* (2010) published a recent study about rhodolith beds in Espírito Santo state and identified two species in the study area. Farias *et al.* (2010) proposed to synonymize *Lithothamnion heteromorphum* (Foslie) Foslie with *Lithothamnion superpositum* Foslie based on collections from the South Atlantic coast; Henriques (2010) investigated the taxonomy and composition of the deep water rhodolith beds in the central zone of the Brazilian exclusive economic zone.

In 2011, Burgos wrote his PhD dissertation about the subtidal macrophytobenthic composition and structure from Fernando de Noronha Archipelago, Pernambuco state (Northeastern Brazilian coast); Horta *et al.* 2011 published a study regarding the morphology and reproduction of *Mesophyllum erubescens* on the Southern Brazilian coast; Bahia *et al.* (2011) first reported *Sporolithon ptychoides* Heydrich to the Atlantic;

Vieira-Pinto (2011) studied the biological, ecological and reproductive aspects of the genus *Lithophyllum* from the Southern Brazilian coast.

In 2012, Khader studied the distribution of the CCA on the Cabo Frio (Southeastern Brazilian coast) in an area with upwelling of cold water and reported nine species to this locality; Mariath *et al.* (2012) published a study first recording *Pneophyllum conicum* (E.Y. Dawson) Keats, Y.M. Chamberlain & M. Baba to Brazilian waters and proposed the new species, *Lithothamnion steneckii* Mariath & Figueiredo; Henriques *et al.* (2012) first reported rhodolith-forming species of CCA from deep waters in the Espírito Santo state.

In 2013, Sissini wrote in her Master's thesis about the diversity and biogeography of the Hapalidiaceae from the Brazilian coast; Costa (2013) wrote about the taxonomy of the CCA on the north coast of Bahia state (Northeastern Brazilian coast); Crespo (2013) studied the distribution of CCA in São Pedro and São Paulo Archipelago in a depth gradient; Pascelli *et al.* (2013) published a study about the changes in rhodolith beds in the Southeastern Brazilian.

In 2014, Borges wrote her Master's thesis about the taxonomy of epiphytic CCA; Bahia (2014), in his PhD dissertation wrote about the taxonomy and did a floristic survey on the rhodolith-forming CCA from the Brazilian coast and subsequently published the results, first reporting *Sporolithon molle* (Heydrich) Heydrich (Bahia *et al.* 2014a) and describing *Sporolithon tenue* R.G. Bahia, G.M. Amado-Filho, G.W. Maneveldt & W.H. Adey Bahia *et al.* (2014 b); Costa *et al.* (2014a) reported the occurrence of *Spongites yendoi* (Foslie) Y.M.Chamberlain from Bahia state; Woelkerling *et al.* (2014) described *Heydrichia (?) poignatii* Woelkerling, Granier &

Dias-Brito, a fossil species from the Cretaceous found on Riachuelo Formation, Sergipe Basin, Northeastern Brazil.

Also, in 2014 Riosmena-Rodrigues *et al.* organized and published a special volume in the journal *Phytotaxa*, focusing on the systematics and biogeography of the Corallinophycidae. Bahia *et al.* (2014c) cited *Porolithon improcerum* (Foslie & M. Howe) M. Howe and *Mesophyllum macroblastum* (Foslie) W.H. Adey for the first time in the Atlantic. Costa *et al.* (2014b) published a study with the CCA found on the northwestern Brazilian coast and characterized in details 5 species. Henriques *et al.* (2014a,b) published *Lithophyllum*, Mastophoroideae and *Sporolithon* species from the Brazilian continental shelf, additionally proposing *Sporolithon elevatum* M.C. Henriques & R. Riosmena-Rodriguez as a new species. Tâmega *et al.* (2014) described *Neogoniolithon atlanticum* Tâmega, Riosmena-Rodríguez, Mariath & M. Figueiredo for the northwestern Brazilian coast. Vieira-Pinto *et al.* (2014) studied the genus *Lithophyllum* in the Southern Brazilian coast and described *Lithophyllum atlanticum* T. Vieira-Pinto, M.C. Oliveira & P.A. Horta as a species new to science; Sissini *et al.* (2014) studied *Mesophyllum erubescens* from the Brazilian coast and revealed cryptic species under this epithet; Torrano-Silva *et al.* (2014) studied the genus *Pausilvella* (an intermediate between CCA and the geniculate corallines) and first reported the occurrence of this genus in Brazil. Crespo *et al.* (2014) conducted a floristic survey of the CCA from the São Pedro and São Paulo oceanic islands. In 2015, Jesionek, in his Master's thesis, studied the taxonomy of CCA in the reefs of the Abrolhos Continental Shelf. And finally, in 2016, Henriques completed her PhD dissertation about the taxonomy and phylogeny of the CCA from Campos basin and the importance of biodiversity management of rhodolith beds in Brazil and Moura *et al.* 2016 reported an

extensive coral reef in the Amazon River Mouth and in this study referred 3 species of CCA to the area.

Overall, the distribution of CCA comprises the entire Brazilian coast from the Amazon River mouth (Bahia 2014) to Torres beach further South along the Brazilian coast (Vieira-Pinto *et al.* 2014), including oceanic islands (Rocas Atoll, Fernando de Noronha Archipelago, Vitória-Trindade chain, São Pedro and São Paulo islands) (Villas-Boas *et al.* 2005; Amado-Filho *et al.* 2012; Crespo *et al.* 2014; Sissini *et al.* 2014) (see Table 2 and Fig. 4 for more details).

Table 2: List of CCA species referred to Brazilian coast after 2000 for which there are at least a taxonomic description or molecular data; with localities and references. AL= Alagoas state; AMZ = Mouth of Amazon river; AR = Rocas Atoll; BA= Bahia state; CE= Ceara state; ES= Espírito Santo state; FN = Fernando de Noronha Island; IT = Trindade Island; PE= Pernambuco state; RJ= Rio de Janeiro state; RN= Rio Grande do Norte state; SC= Santa Catarina state; SP= São Paulo state; SPSP = São Pedro and São Paulo Archipelago. \*Studies that included molecular analyses.\*\*Epiphytic species.

Taxonomic group		Distribution in Brazil	Type locality	References
Corallinaceae	<i>Lithophyllum atlanticum</i>	RS, SC, SP, RJ, AL	Brazil	Vieira-Pinto (2011)*, Vieira-Pinto <i>et al.</i> (2014)*, Torrano-Silva (2015)*, Henriques (2016)*
	<i>Lithophyllum corallinae</i>	RJ, ES, BA, AL, FN	France	Villas-Boas (2008), Villas-Boas <i>et al.</i> (2009), Khader (2012) Amado-filho <i>et al.</i> (2012b), Bahia (2014), Torrano-Silva (2015)*, Henriques <i>et al.</i> (2014b)
	<i>Lithophyllum depressum</i>	ES, BA, IT	Brazil	Villas-Boas <i>et al.</i> (2009), Torrano-Silva (2015)*
	<i>Lithophyllum johansenii</i>	ES	Australia	Villas-Boas <i>et al.</i> (2009)
	<i>Lithophyllum margaritae</i>	SC, SP, RJ, ES, AL	Mexico	Horta (2000), Vieira-Pinto (2011)*, Pascelli <i>et al.</i> (2013), Vieira-Pinto <i>et al.</i> (2014)*, Torrano-Silva (2015)*, Henriques (2016)*
	<i>Lithophyllum stictaeforme</i>	SC, RJ, ES, BA, PE	Mediterranean Sea	Nunes <i>et al.</i> (2008), Villas-Boas <i>et al.</i> (2009), Burgos (2011), Khader (2012), Amado-Filho <i>et al.</i> (2012a), Pascelli <i>et al.</i> (2013), Bahia (2014), Costa <i>et al.</i> (2014b), Henriques <i>et al.</i> (2014b)
	<i>Titanoderma prototypum</i>	IT, BA	U.S. Virgin Islands	Pereira Filho <i>et al.</i> (2012), Bahia (2014), Torrano-Silva (2015)*
	<i>Titanoderma pustulatum</i>	SP, ES, BA, SPSP, FN	France	Horta (2000), Crespo (2013), Bahia (2014), Henriques <i>et al.</i> (2014b) Torrano-Silva (2015)*
	<i>Hydrolithon rupestre</i>	ES, IT, BA, AR, FN, SPSP	Australia	Villas-Boas (2008), Amado-Filho <i>et al.</i> (2012 a,b), Crespo (2013), Bahia (2014), Villas-Boas <i>et al.</i> (2015), Moura <i>et al.</i> (2016)
	<i>Hydrolithon breviclavum</i>	IT	-	Henriques <i>et al.</i> (2014b)
	<i>Hydrolithon onkodes</i>	IT	Papua New Guinea	Henriques <i>et al.</i> (2014b)
	<i>Hydrolithon samoense</i>	RJ	Samoa	Tâmega & Figueiredo (2005)
<i>Neogoniolithon brassica-</i>	BA, ES	South Africa	Villas-Boas (2008), Amado-Filho <i>et al.</i> (2012a), Villas-Boas <i>et al.</i> (2014a)	

Cont. Tab. 2

Taxonomic group		Distribution in Brazil	Type locality	References
Corallinaceae	<i>florida</i>			
	<i>Neogoniolithon fosliei</i>	SP, BA	Egypt	Bahia (2014)
	<i>Neogoniolithon atlanticum</i>	BA	Brazil	Tâmega <i>et al.</i> (2014)
	<i>Pneophyllum conicum</i>	BA	Mexico	Mariath <i>et al.</i> (2012)
	<i>Pneophyllum fragile</i>	from NE to S Brazil	Mediterranean Sea	Bahia (2014)
	<i>Porolithon improcerum</i>	IT	Jamaica	Bahia <i>et al.</i> (2014c)
	<i>Porolithon pachydermum</i>	AR	U.S. Virgin Islands	Villas-Boas <i>et al.</i> (2005)
	<i>Spongites fruticulosa</i>	ES, IT, SPSP	Mediterranean Sea	Crespo (2013), Bahia (2014), Henriques <i>et al.</i> (2014b)
	<i>Spongites yendoi</i>	ES, BA	Japan	Villas-Boas (2008), Marins (2009), Henriques <i>et al.</i> (2012), Costa <i>et al.</i> (2014a)
Hapalidiaceae	<i>Lithothamnion brasiliense</i>	SP, BA	Brazil	Horta (2000), Bahia <i>et al.</i> (2010), Costa <i>et al.</i> (2014b)
	<i>Lithothamnion crispatum</i>	RJ, ES, IT, BA, RN, FN, AR, MA	Adriatic Sea	Bahia <i>et al.</i> (2010), Khader (2012), Amado-Filho <i>et al.</i> (2012a), Pascelli <i>et al.</i> (2013), Bahia (2014), Henriques (2016)*, Moura <i>et al.</i> (2016)
	<i>Lithothamnion glaciale</i>	ES	-	Henriques <i>et al.</i> (2012)
	<i>Lithothamnion muelleri</i>	RJ, ES, BA	Australia	Amado-Filho <i>et al.</i> (2012a), Henriques <i>et al.</i> (2012), Khader (2012), Bahia (2014)
	<i>Lithothamnion heteromorphum</i>	SP, SC, CE	Brazil	Farias <i>et al.</i> (2010, as <i>L. superpositum</i> ), Sissini (2013)*
	<i>Lithothamnion steneckii</i>	BA	Brazil	Mariath <i>et al.</i> (2012)
	<i>Mesophyllum erubescens</i>	SC, SP, ES, IT, BA, CE, FN	Brazil	Rocha <i>et al.</i> (2006), Figueiredo <i>et al.</i> (2007), Nunes <i>et al.</i> (2008), Bahia <i>et al.</i> (2010), Burgos (2011), Horta <i>et al.</i> (2011), Bahia (2014), Costa <i>et al.</i> (2014b), Sissini <i>et al.</i> (2014)*

Cont. Tab. 2

Taxonomic group		Distribution in Brazil	Type locality	References
Hapalidiaceae	<i>Mesophyllum engelhartii</i>	SC, RJ, ES, BA, FN	Australia	Farias (2008), Marins (2009), Khader (2012), Figueiredo <i>et al.</i> (2012), Amado-Filho <i>et al.</i> (2012a,b), Tâmeiga <i>et al.</i> (2013), Bahia (2014), Sissini (2013)*
	<i>Mesophyllum macroblastum</i>	RJ	Italy	Bahia <i>et al.</i> (2014c)
	<i>Phymatolithon calcareum</i>	SC, SP, BA	England	Horta (2000), Costa <i>et al.</i> (2014b)
	<i>Phymatolithon masonianum</i>	BA	Australia	Costa <i>et al.</i> (2014b)
	<i>Melobesia membranaceae</i> **	SC	France	Borges <i>et al.</i> (2014)
	<i>Melobesia rosanofii</i> **	SC	-	Borges <i>et al.</i> (2014)
Sporolithaceae	<i>Sporolithon elevatum</i>	RJ	Brazil	Henriques <i>et al.</i> (2014a)
	<i>Sporolithon episoredion</i>	IT	Hawaii	Henriques <i>et al.</i> (2014a)
	<i>Sporolithon episporum</i>	BA, CE, FN	Panama	Nunes <i>et al.</i> (2008); Bahia <i>et al.</i> (2010), Burgos (2011), Amado Filho <i>et al.</i> (2012b), Costa (2013), Costa <i>et al.</i> (2014b)
	<i>Sporolithon molle</i>	IT	Red Sea	Bahia <i>et al.</i> (2014a)
	<i>Sporolithon ptychoides</i>	ES, RJ, IT, BA, FN, AR, AMZ	Egypt	Bahia <i>et al.</i> (2011), Pereira Filho <i>et al.</i> (2012), Amado Filho <i>et al.</i> (2012b), Bahia (2014), Henriques <i>et al.</i> (2014a), Moura <i>et al.</i> (2016)
	<i>Sporolithon tenue</i>	BA, AL	Brazil	Bahia <i>et al.</i> (2014b)*
	<i>Sporolithon yoneshigueae</i>	BA	Brazil	Bahia <i>et al.</i> (2015)*

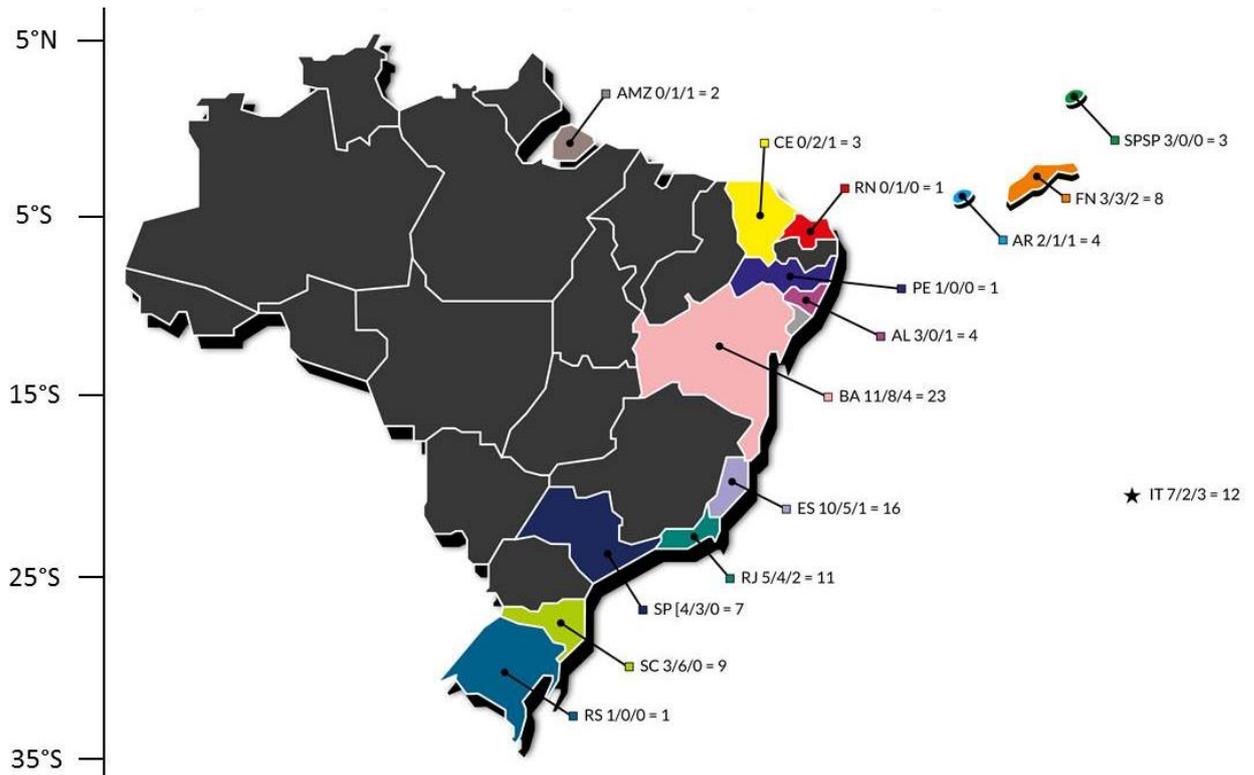


Figure 4. Distribution of CCA species referred to Brazilian coast; AMZ = Mouth of Amazon river, AL= Alagoas state, AR = Rocas Atoll; BA= Bahia state, CE= Ceara state, ES= Espírito Santo state, FN = Fernando de Noronha Island, IT = Trindade Island, PE= Pernambuco state, RJ= Rio de Janeiro state, RN= Rio Grande do Norte state, SC= Santa Catarina state, SP= São Paulo state, SPSP = São Pedro and São Paulo Archipelago. Number correspond to Corallinales/Hapalidiales/Sporolithales = Total number of species.

Despite all the efforts, very few studies analyzed the taxonomy and phylogeny based on modern techniques and fewer combined morphological and molecular analyses for this group of algae (Vieira-Pinto 2011; Sissini 2013; Bahia *et al.* 2014a; Sissini *et al.* 2014; Vieira-Pinto *et al.* 2014; Torrano-Silva 2015; Bahia *et al.* 2015, Henriques 2016); hence, the diversity of CCA is probably grossly underestimated.

Considering the importance of CCA and its difficult taxonomy, the present study aims to contribute to the knowledge of the taxonomy and phylogeny through the use of molecular markers and morphological analyses of the CCA on the Brazilian coast in order to gain a better understanding of biodiversity conservation, ecosystem services, the effects of global climate changes, and ecological modeling.

## **Aims**

The main aim of this research was to elucidate the diversity of CCA species along the Brazilian coast, using a molecular approach coupled with alpha taxonomy (molecular-assisted alpha taxonomy, known as MAAT, Hind *et al.* 2014), in order to better understand distribution patterns and phylogenetic relationships between species.

We propose to address the following specific targets in order to achieve the main aim of this project:

- 1) To identify and describe the CCA diversity from the Brazilian coast based on DNA sequences and morphological and anatomical observations;
- 2) To use molecular markers such as UPA, *psbA*, COI-5P and *rbcL*-3P to generate DNA sequences and construct matrices to compare sequences from Brazilian specimens to those available in the public databases;
- 3) To infer the informativeness and effectiveness of the molecular markers used in this study;
- 4) To generate, analyze and compare SEM images of vegetative and reproductive structures that are phylogenetically relevant to species that have already been described, especially for the Atlantic;
- 5) To contribute to the knowledge of the CCA and their distribution along the Brazilian coast.

## **Materials and Methods**

Sampling of CAA was performed along the Brazilian coast and from three Oceanic Islands (Fig. 5) in a total of 52 sample sites; more than 350 specimens were collected and will be/are being incorporated in the SPF Herbarium at the University of São Paulo (Thiers, 2016) (Appendix I – see the CD attached in the back cover).

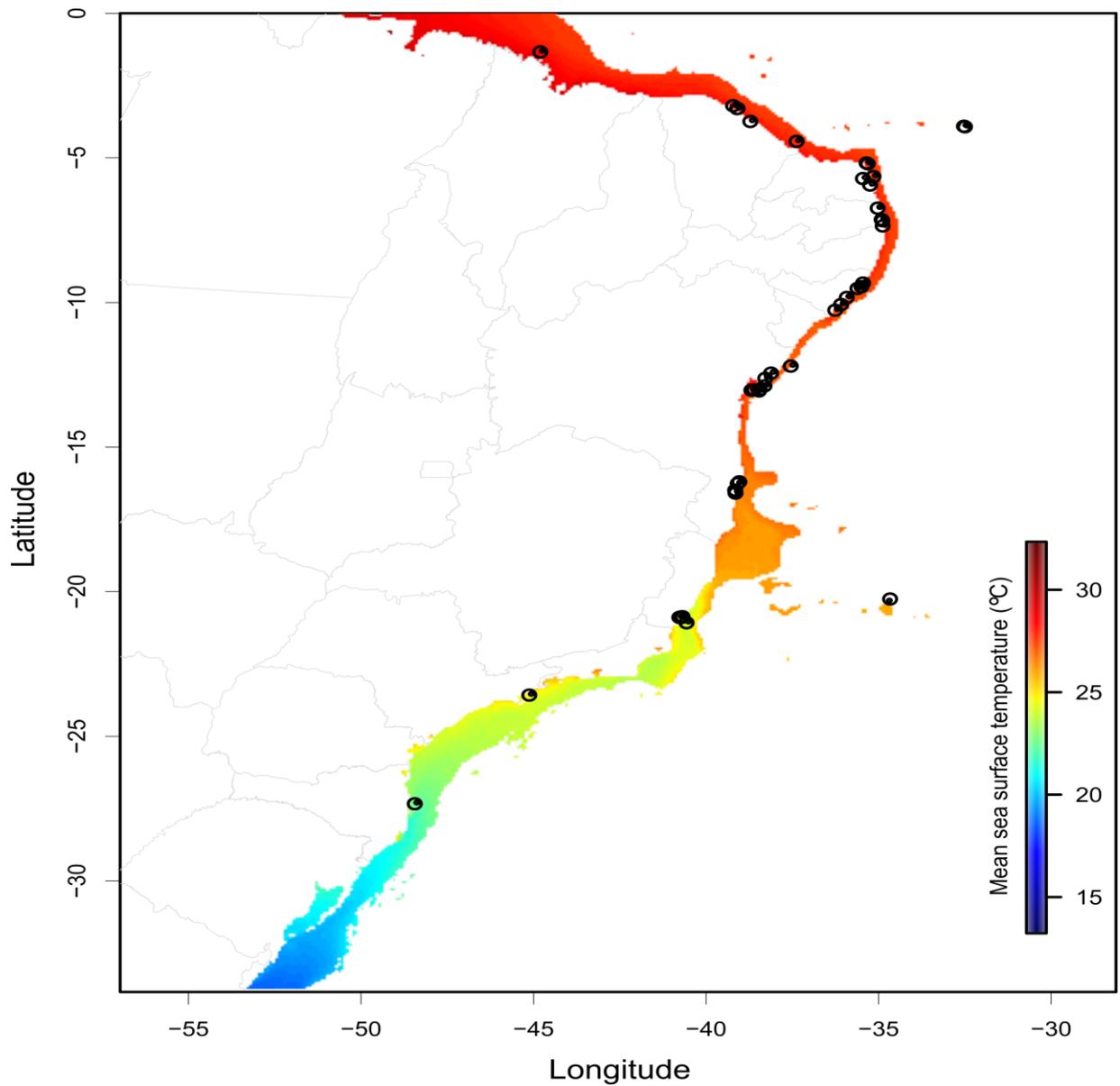


Figure 5. Sample sites along the Brazilian coast and surrounding Oceanic Islands.

Specimens were collected in the intertidal at low tide or by snorkeling and SCUBA diving. To collect epilithic specimens (attached to rocks) that were firmly attached to the substratum, a hammer and chisel were used to remove pieces of rocks or coral along with their attached CCA. For both rhodoliths and epilithic samples, prior to preservation, a careful cleaning was performed on each specimen in order to remove the associated fauna and flora from the samples.

After cleaning, each specimen was broken in half, with half the specimen stored in silica gel to preserve the DNA for molecular analyses and the other half preserved in a 4% formalin/seawater solution for morphological and anatomical studies. Before a specimen was fractured in half, we looked at general morphology under a magnifying glass. When fertile, it was sometimes possible to tentatively identify the specimens to the ordinal level. For example, specimens possessing a sorus belong to the Sporolithales, specimens with multiporate conceptacles belong to the Hapalidiales, and specimens with uniporate conceptacles may or may not belong to the Corallinales (depending on whether the conceptacles are gametangial or tetrasporangial). We also took photos of the specimens' general shape and morphological aspects.

### ***Morphological and Anatomical study***

In the Laboratory of Marine Algae “Édson José de Paula” at the University of São Paulo, the specimens were analyzed under a dissecting scope and small fragments, preferably fertile, were selected for anatomical studies.

Permanent slides were prepared using Metacrilatoglicol (Leica®) resin, in which we embedded the sample fragments (after decalcification and ethanolic dehydration following Horta (2002), and after positioning them in accordance to the orientation we wanted the view), after that, samples were sectioned in a rotary microtome with thickness varying from 5-15  $\mu\text{m}$ . The slides were stained in Toluidine blue 5% and analyzed using light microscopy, focusing mainly on the reproductive structures when present. Light microscopy images were obtained with a photomicroscope.

SEM images were obtained from portions of the thallus of dried specimens previously preserved in Si-gel or 4% formalin/seawater. Cross sections and longitudinal sections were made using a razor blade, and the resulting sections were mounted in

stubs that varied in size, using liquid graphite to glue the small fragments. Coating was made with 10-14 nm of gold. Specimens were viewed using a Hitachi S-3000N scanning electron microscope (SEM) at a voltage of 15 kV, in the Microscopy Center at UL Lafayette, following the manufacturer's instructions.

### ***Molecular studies***

#### *DNA Extraction and PCR protocols*

Total genomic DNA was extracted from cleaned fragments of silica gel-dried samples using either the Chelex resin protocol (Goff & Moon 1993) or the "NucleoSpin Plant II" kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. In all cases DNA was extracted from the same specimens used for morphological analysis. Overall, we extracted DNA from 262 samples and out of the 4 markers we used in this study, a total of 172 samples were sequenced for at least one of them (Tab. 3).

Table 3. List of samples extracted and successfully amplified in this study. For all specimens to which the DNA was extracted we tried to amplify at least UPA and COI-5P, but not always obtained good quality reads; in this table only the samples that resulted in good quality sequences (clean reads, passible of alignment) were marked with a “x”.

Institution Code	Locality (beach, city, state)	Lat/long	Collectors	Date (D/M/Y)	DNA extraction	COX	UPA	psbA	rbcL
IBC 1141	Brazil (Ilha da Rapada, Ubatuba/SP)	23°51'76.79"S; 45°04'10.72"W	P. Horta	12/01/2012	x				
IBC 1188	Brazil (Iriri, Anchieta/ES)	20°83'13.67"S; 40°69'35.10"W	C. E. Amancio, Beatriz Silva	04/05/2012	x	x	x		x
IBC 1189	Brazil (Iriri, Anchieta/ES)	20°83'13.67"S; 40°69'35.10"W	C. E. Amancio, Beatriz Silva	04/05/2012	x		x		
IBC 1191	Brazil (Iriri, Anchieta/ES)	20°83'13.67"S; 40°69'35.10"W	C. E. Amancio, Beatriz Silva	04/05/2012	x		x		
IBC 1196	Brazil (Anchieta/ES)	20°83'49.47"S; 40°62'41.48"W	C. E. Amancio, Beatriz Silva	05/05/2012	x	x	x		x
IBC 1202	Brazil (Anchieta/ES)	20°83'49.47"S; 40°62'41.48"W	C. E. Amancio, Beatriz Silva	05/05/2012	x	x	x		
IBC 1203	Brazil (Anchieta/ES)	20°83'49.47"S; 40°62'41.48"W	C. E. Amancio, Beatriz Silva	05/05/2012	x	x		x	
IBC 1207	Brazil (Anchieta/ES)	20°83'49.47"S; 40°62'41.48"W	C. E. Amancio, Beatriz Silva	05/05/2012	x		x		
IBC 1209	Brazil (Maratáízes, ES)	21°01'58.2"S; 40°48'43.9"W	C. E. Amancio, Beatriz Silva	07/05/2012	x	x	x		
IBC 1210	Brazil (Maratáízes, ES)	21°01'58.2"S; 40°48'43.9"W	C. E. Amancio, Beatriz Silva	07/05/2012	x	x	x		
IBC 1211	Brazil (Maratáízes, ES)	21°01'58.2"S; 40°48'43.9"W	C. E. Amancio, Beatriz Silva	07/05/2012	x	x	x	x	
IBC 1212	Brazil (Maratáízes, ES)	21°01'58.2"S; 40°48'43.9"W	C. E. Amancio, Beatriz Silva	07/05/2012	x		x		
IBC 1213	Brazil (Maratáízes, ES)	21°01'58.2"S; 40°48'43.9"W	C. E. Amancio, Beatriz Silva	07/05/2012	x		x		x
IBC 1216	Brazil (Maratáízes, ES)	21°01'58.2"S; 40°48'43.9"W	C. E. Amancio, Beatriz Silva	07/05/2012	x	x		x	
IBC 1222	Brazil (Maratáízes, ES)	21°01'58.2"S; 40°48'43.9"W	C. E. Amancio, Beatriz Silva	07/05/2012	x		x	x	
IBC 1228	Brazil (Maratáízes, ES)	21°01'58.2"S; 40°48'43.9"W	C. E. Amancio, Beatriz Silva	07/05/2012	x	x		x	x
IBC 1245	Brazil (Picãozinho, João Pessoa/PB)	7°11'73.61"S; 34°80'80.19"W	C. Azevedo; F. Nauer; A. Medeiros	22/07/2012	x		x	x	
IBC 1246	Brazil (Picãozinho, João Pessoa/PB)	7°11'73.61"S; 34°80'80.19"W	C. Azevedo; F. Nauer; A. Medeiros	22/07/2012	x		x	x	
IBC 1247	Brazil (Picãozinho, João Pessoa/PB)	7°11'73.61"S; 34°80'80.19"W	C. Azevedo; F. Nauer; A. Medeiros	22/07/2012	x		x	x	
IBC 1248	Brazil (Picãozinho, João Pessoa/PB)	7°11'73.61"S; 34°80'80.19"W	C. Azevedo; F. Nauer; A. Medeiros	22/07/2012	x				
IBC 1501	Brazil (Pirambúzios, Nízia Floresta/RN)	5°59'12.35"S; 35°06'49.54"W	P. Horta	12/05/2011	x				
IBC 1502	Brazil (Pirambúzios, Nízia Floresta/RN)	5°59'12.35"S; 35°06'49.54"W	P. Horta	12/05/2011	x				

Cont. Tab. 3

Institution Code	Locality (beach, city, state)	Lat/long	Collectors	Date (D/M/Y)	DNA extraction	COX	UPA	psbA	rbcl
IBC 1503	Brazil (Pirambúzios, Nízia Floresta/RN)	5°59'12.35"S; 35°06'49.54"W	P. Horta	12/05/2011	x				
IBC 1504	Brazil (Pirambúzios, Nízia Floresta/RN)	5°59'12.35"S; 35°06'49.54"W	P. Horta	12/05/2011	x		x		
IBC 1506	Brazil (Pirambúzios, Nízia Floresta/RN)	5°59'12.35"S; 35°06'49.54"W	P. Horta	12/05/2011	x				
IBC 1507	Brazil (Ponta Negra, Natal/RN)	5°88'14.03"S; 35°16'34.18"W	P. Horta	17/05/2011	x			x	
IBC 1508	Brazil (Ponta Negra, Natal/RN)	5°88'14.03"S; 35°16'34.18"W	P. Horta	17/05/2011	x	x	x	x	
IBC 1509	Brazil (Ponta Negra, Natal/RN)	5°88'14.03"S; 35°16'34.18"W	P. Horta	17/05/2011	x	x	x	x	
IBC 1510	Brazil (Ponta Negra, Natal/RN)	5°88'14.03"S; 35°16'34.18"W	P. Horta	17/05/2011	x	x	x	x	
IBC 1512	Brazil (Itapoã, Salvador/BA)	12°95'10.46"S; 38°36'39.24"W	P. Horta	29/09/2011	x				
IBC 1513	Brazil (Itapoã, Salvador/BA)	12°95'10.46"S; 38°36'39.24"W	P. Horta	29/09/2011	x				
IBC 1514	Brazil (Salvador/BA)	12°95'10.46"S; 38°36'39.24"W	P. Horta	29/09/2011	x		x		
IBC 1515	Brazil (Ilha de Santo Aleixo, Recife/PE)	8°61'27.35"S; 35°02'32.65"W	E. Bastos; D. Burgos	12/09/2010	x				
IBC 1516	Brazil (Ilha de Santo Aleixo, Recife/PE)	8°61'27.35"S; 35°02'32.65"W	E. Bastos; D. Burgos	12/09/2010	x				
IBC 1517	Brazil (Ilha de Santo Aleixo, Recife/PE)	8°61'27.35"S; 35°02'32.65"W	E. Bastos; D. Burgos	12/09/2010	x				
IBC 1518	Brazil (João Pessoa/PB)	7°07'72.29"S; 34°82'95.49"W	P. Horta	01/09/2010	x		x		
IBC 1519	Brazil (Bessa, João Pessoa/PB)	7°07'72.29"S; 34°82'95.49"W	P. Horta	01/09/2010	x		x		
IBC 1520	Brazil (Bessa, João Pessoa/PB)	7°07'72.29"S; 34°82'95.49"W	P. Horta	01/09/2010	x				
IBC 1525	Brazil (Irirí, Anchieta/ES)	20°83'13.67"S; 40°69'35.10"W	B. Silva; C. Amâncio	07/04/2012	x	x		x	x
IBC 1526	Brazil (Irirí, Anchieta/ES)	20°83'13.67"S; 40°69'35.10"W	B. Silva; C. Amâncio	07/04/2012	x	x	x	x	
IBC 1527	Brazil (Ilha da Rapada, Ubatuba/SP)	23°51'76.79"S; 45°04'10.72"W	P. Horta	12/01/2012	x		x	x	
IBC 1528	Brazil (Guaiú, Sta Cruz de Cabralia/BA)	16°14'30.83"S; 38°95'27.85"W	C. Azevedo; F. Nauer	17/09/2012	x		x		
IBC 1530	Brazil (Macugê, Arraial d'Ajuda/BA)	16°48'98.54"S; 39°06'76.71"W	C. Azevedo; F. Nauer	18/09/2012	x				
IBC 1531	Brazil (Macugê, Arraial d'Ajuda/BA)	16°48'98.54"S; 39°06'76.71"W	C. Azevedo; F. Nauer	18/09/2012	x		x		
IBC 1532	Brazil (Macugê, Arraial d'Ajuda/BA)	16°48'98.54"S; 39°06'76.71"W	C. Azevedo; F. Nauer	18/09/2012	x		x		
IBC 1533	Brazil (Macugê, Arraial d'Ajuda/BA)	16°48'98.54"S; 39°06'76.71"W	C. Azevedo; F. Nauer	18/09/2012	x	x			
IBC 1534	Brazil (Macugê, Arraial d'Ajuda/BA)	16°48'98.54"S; 39°06'76.71"W	C. Azevedo; F. Nauer	18/09/2012	x		x		

Cont. Tab. 3

Institution Code	Locality (beach, city, state)	Lat/long	Collectors	Date (D/M/Y)	DNA extraction	COX	UPA	psbA	rbcl
IBC 1536	Brazil (Ponta Grande, Porto Seguro/BA)	16°43'35.80"S; 39°07'27.80"W	C. Azevedo; F. Nauer	16/09/2012	x		x		
IBC 1537	Brazil (Ponta Grande, Porto Seguro/BA)	16°43'35.80"S; 39°07'27.80"W	C. Azevedo; F. Nauer	16/09/2012	x		x	x	
IBC 1538	Brazil (Ponta Grande, Porto Seguro/BA)	16°43'35.80"S; 39°07'27.80"W	C. Azevedo; F. Nauer	16/09/2012	x	x	x		
IBC 1539	Brazil (Ponta Grande, Porto Seguro/BA)	16°43'35.80"S; 39°07'27.80"W	C. Azevedo; F. Nauer	16/09/2012	x		x		x
IBC 1540	Brazil (Ponta Grande, Porto Seguro/BA)	16°43'35.80"S; 39°07'27.80"W	C. Azevedo; F. Nauer	16/09/2012	x		x		
IBC 1541	Brazil (Coroa Vermelha, Sta Cruz de Cabralia/BA)	16°20'27.29"S; 39°00'11.09"W	C. Azevedo; F. Nauer	15/09/2012	x		x		
IBC 1542	Brazil (Coroa Vermelha, Sta Cruz de Cabralia/BA)	16°20'27.29"S; 39°00'11.09"W	C. Azevedo; F. Nauer	15/09/2012	x		x		
IBC 1544	Brazil (Coroa Vermelha, Sta Cruz de Cabralia/BA)	16°20'27.29"S; 39°00'11.09"W	C. Azevedo; F. Nauer	15/09/2012	x		x		
IBC 1545	Brazil (Coroa Vermelha, Sta Cruz de Cabralia/BA)	16°20'27.29"S; 39°00'11.09"W	C. Azevedo; F. Nauer	15/09/2012	x				
IBC 1547	Brazil (Coroa Vermelha, Sta Cruz de Cabralia/BA)	16°20'27.29"S; 39°00'11.09"W	C. Azevedo; F. Nauer	16/09/2012	x		x	x	x
IBC 1551	Brazil (Tartarugas, Ilha de Trindade/ES)	20°51'74.80"S; 29°30'13.66"W	M. Sissini	26/06/2012	x		x		
IBC 1553	Brazil (Calheta, Ilha de Trindade/ES)	20°50'77.24"S; 29°30'97.38"W	M. Sissini	18/06/2012	x			x	
IBC 1554	Brazil (Cachoeira, Ilha de Trindade/ES)	20°49'50.91"S; 29°32'87.98"W	M. Sissini	16/06/2012	x		x		
IBC 1555	Brazil (Cabritas, Ilha de Trindade/ES)	20°52'18.13"S; 29°31'61.20"W	M. Sissini	14/06/2012	x		x		
IBC 1556	Brazil (Risca do Meio/CE)	3°70'72.92"S; 38°46'80.99"W	M. Sissini	18/04/2012	x				
IBC 1557	Brazil (Risca do Meio/CE)	3°70'72.92"S; 38°46'80.99"W	M. Sissini	18/04/2012	x		x		
IBC 1558	Brazil (Risca do Meio/CE)	3°70'72.92"S; 38°46'80.99"W	M. Sissini	18/04/2012	x		x		
IBC 1559	Brazil (Risca do Meio/CE)	3°70'72.92"S; 38°46'80.99"W	M. Sissini	18/04/2012	x		x		
IBC 1560	Brazil (Risca do Meio/CE)	3°70'72.92"S; 38°46'80.99"W	M. Sissini	18/04/2012	x		x		
IBC 1561	Brazil (Parcel Manoel Luís/MA)	1°28'16.78"S; 44°72'14.48"W	P. Horta	12/04/2012	x				
IBC 1562	Brazil (Parcel Manoel Luís/MA)	1°28'16.78"S; 44°72'14.48"W	P. Horta	12/04/2012	x	x	x	x	
IBC 1563	Brazil (Parcel Manoel Luís/MA)	1°28'16.78"S; 44°72'14.48"W	P. Horta	12/04/2012	x				
IBC 1564	Brazil (Picãozinho, João Pessoa/PB)	7°11'73.61"S; 34°80'80.19"W	C. Azevedo; F. Nauer; A. Medeiros	22/07/2012	x		x		

Cont. Tab. 3

Institution Code	Locality (beach, city, state)	Lat/long	Collectors	Date (D/M/Y)	DNA extraction	COX	UPA	psbA	rbcl
IBC 1566	Brazil (Orelhas, Ilha de Trindade/ES)	20°48'90.78"S; 29°33'94.62"W	M. Sissini	22/06/2012	x		x		
IBC 1569	Brazil (Noroeste, Ilha de Trindade/ES)	20°50'58.37"S; 29°31'59.81"W	M. Sissini	04/07/2012	x				
IBC 1574	Brazil (Ponta Verde, Maceió/AL)	9°39'54.00"S; 35°41'42.00"W	T. Vieira-Pinto, C. Azevedo, B. Silva	10/03/2013	x				
IBC 1575	Brazil (Ponta Verde, Maceió/AL)	9°39'54.00"S; 35°41'42.00"W	T. Vieira-Pinto, C. Azevedo, B. Silva	10/03/2013	x				
IBC 1577	Brazil (Francês, Marechal Deodoro/AL)	9°77'10.35"S; 35°84'06.89"W	T. Vieira-Pinto, C. Azevedo, B. Silva	10/03/2013	x				
IBC 1579	Brazil (Francês, Marechal Deodoro/AL)	9°77'10.35"S; 35°84'06.89"W	T. Vieira-Pinto, C. Azevedo, B. Silva	10/03/2013	x				
IBC 1580	Brazil (Francês, Marechal Deodoro/AL)	9°77'10.35"S; 35°84'06.89"W	T. Vieira-Pinto, C. Azevedo, B. Silva	10/03/2013	x				
IBC 1582	Brazil (Francês, Marechal Deodoro/AL)	9°77'10.35"S; 35°84'06.89"W	T. Vieira-Pinto, C. Azevedo, B. Silva	10/03/2013	x				
IBC 1583	Brazil (Francês, Marechal Deodoro/AL)	9°77'10.35"S; 35°84'06.89"W	T. Vieira-Pinto, C. Azevedo, B. Silva	10/03/2013	x				
IBC 1584	Brazil (Francês, Marechal Deodoro/AL)	9°77'10.35"S; 35°84'06.89"W	T. Vieira-Pinto, C. Azevedo, B. Silva	10/03/2013	x	x		x	
IBC 1585	Brazil (Francês, Marechal Deodoro/AL)	9°77'10.35"S; 35°84'06.89"W	T. Vieira-Pinto, C. Azevedo, B. Silva	10/03/2013	x			x	
IBC 1591	Brazil (Peba, Piaçabuçu/AL)	10°21'07.68"S; 36°17'45.24"W	T. Vieira-Pinto, C. Azevedo, B. Silva	11/03/2013	x				
IBC 1594	Brazil (Peba, Piaçabuçu/AL)	10°21'07.68"S; 36°17'45.24"W	T. Vieira-Pinto, C. Azevedo, B. Silva	11/03/2013	x	x			
IBC 1596	Brazil (Peba, Piaçabuçu/AL)	10°21'07.68"S; 36°17'45.24"W	T. Vieira-Pinto, C. Azevedo, B. Silva	11/03/2013	x				
IBC 1597	Brazil (Peba, Piaçabuçu/AL)	10°21'07.68"S; 36°17'45.24"W	T. Vieira-Pinto, C. Azevedo, B. Silva	11/03/2013	x				x
IBC 1598	Brazil (Peba, Piaçabuçu/AL)	10°21'07.68"S; 36°17'45.24"W	T. Vieira-Pinto, C. Azevedo, B. Silva	11/03/2013	x	x			x
IBC 1599	Brazil (Peba, Piaçabuçu/AL)	10°21'07.68"S; 36°17'45.24"W	T. Vieira-Pinto, C. Azevedo, B. Silva	11/03/2013	x	x		x	
IBC 1600	Brazil (Peba, Piaçabuçu/AL)	10°21'07.68"S; 36°17'45.24"W	T. Vieira-Pinto, C. Azevedo, B. Silva	11/03/2013	x				
IBC 1601	Brazil (Peba, Piaçabuçu/AL)	10°21'07.68"S; 36°17'45.24"W	T. Vieira-Pinto, C. Azevedo, B. Silva	11/03/2013	x				
IBC 1602	Brazil (Peba, Piaçabuçu/AL)	10°21'07.68"S; 36°17'45.24"W	T. Vieira-Pinto, C. Azevedo, B. Silva	11/03/2013	x				
IBC 1603	Brazil (Peba, Piaçabuçu/AL)	10°21'07.68"S; 36°17'45.24"W	T. Vieira-Pinto, C. Azevedo, B. Silva	11/03/2013	x	x			
IBC 1604	Brazil (Peba, Piaçabuçu/AL)	10°21'07.68"S; 36°17'45.24"W	T. Vieira-Pinto, C. Azevedo, B. Silva	11/03/2013	x	x			
IBC 1605	Brazil (Peba, Piaçabuçu/AL)	10°21'07.68"S; 36°17'45.24"W	T. Vieira-Pinto, C. Azevedo, B. Silva	11/03/2013	x				
IBC 1607	Brazil (Jequiá, Jequiá da Praia/AL)	10°01'18.20"S; 36°00'48.0"W	T. Vieira-Pinto, C. Azevedo, B. Silva	12/03/2013	x				

Cont. Tab. 3

Institution Code	Locality (beach, city, state)	Lat/long	Collectors	Date (D/M/Y)	DNA extraction	COX	UPA	psbA	rbcL
IBC 1608	Brazil (Jequiá, Jequiá da Praia/AL)	10°01'18.20"S; 36°00'48.0"W	T. Vieira-Pinto, C. Azevedo, B. Silva	12/03/2013	x				
IBC 1609	Brazil (Jequiá, Jequiá da Praia/AL)	10°01'18.20"S; 36°00'48.0"W	T. Vieira-Pinto, C. Azevedo, B. Silva	12/03/2013	x	x		x	x
IBC 1610	Brazil (Jequiá, Jequiá da Praia/AL)	10°01'18.20"S; 36°00'48.0"W	T. Vieira-Pinto, C. Azevedo, B. Silva	12/03/2013	x				
IBC 1611	Brazil (Jequiá, Jequiá da Praia/AL)	10°01'18.20"S; 36°00'48.0"W	T. Vieira-Pinto, C. Azevedo, B. Silva	12/03/2013	x				
IBC 1612	Brazil (Jequiá, Jequiá da Praia/AL)	10°01'18.20"S; 36°00'48.0"W	T. Vieira-Pinto, C. Azevedo, B. Silva	12/03/2013	x				
IBC 1613	Brazil (Jequiá, Jequiá da Praia/AL)	10°01'18.20"S; 36°00'48.0"W	T. Vieira-Pinto, C. Azevedo, B. Silva	12/03/2013	x	x		x	
IBC 1617	Brazil (Recife de Paripueira, Paripueira/AL)	9°45'82.90"S; 35°53'05.99"W	T. Vieira-Pinto, C. Azevedo, B. Silva	13/03/2013	x				
IBC 1618	Brazil (Recife de Paripueira, Paripueira/AL)	9°45'82.90"S; 35°53'05.99"W	T. Vieira-Pinto, C. Azevedo, B. Silva	13/03/2013	x				
IBC 1621	Brazil (Recife de Paripueira, Paripueira/AL)	9°45'82.90"S; 35°53'05.99"W	T. Vieira-Pinto, C. Azevedo, B. Silva	13/03/2013	x				
IBC 1624	Brazil (Recife de Paripueira, Paripueira/AL)	9°45'82.90"S; 35°53'05.99"W	T. Vieira-Pinto, C. Azevedo, B. Silva	13/03/2013	x				
IBC 1625	Brazil (Recife de Paripueira, Paripueira/AL)	9°45'82.90"S; 35°53'05.99"W	T. Vieira-Pinto, C. Azevedo, B. Silva	13/03/2013	x				
IBC 1626	Brazil (São Miguel dos Milagres/AL)	9°27'00.55"S; 35°36'74.40"W	T. Vieira-Pinto, C. Azevedo, B. Silva	14/03/2013	x	x			
IBC 1632	Brazil (São Miguel dos Milagres/AL)	9°27'00.55"S; 35°36'74.40"W	T. Vieira-Pinto, C. Azevedo, B. Silva	14/03/2013	x	x			
IBC 1633	Brazil (São Miguel dos Milagres/AL)	9°27'00.55"S; 35°36'74.40"W	T. Vieira-Pinto, C. Azevedo, B. Silva	14/03/2013	x	x			
IBC 1634	Brazil (São Miguel dos Milagres/AL)	9°27'00.55"S; 35°36'74.40"W	T. Vieira-Pinto, C. Azevedo, B. Silva	14/03/2013	x	x			
IBC 1635	Brazil (São Miguel dos Milagres/AL)	9°27'00.55"S; 35°36'74.40"W	T. Vieira-Pinto, C. Azevedo, B. Silva	14/03/2013	x				
IBC 1637	Brazil (São Miguel dos Milagres/AL)	9°27'00.55"S; 35°36'74.40"W	T. Vieira-Pinto, C. Azevedo, B. Silva	14/03/2013	x				
IBC 1638	Brazil (Pedra rachada, Paracuru/CE)	3°23'54.34"S; 39°00'50.43"W	T. Vieira-Pinto, C. Azevedo, B. Silva	24/04/2013	x				
IBC 1639	Brazil (Pedra rachada, Paracuru/CE)	3°23'54.34"S; 39°00'50.43"W	T. Vieira-Pinto, C. Azevedo, B. Silva	24/04/2013	x	x		x	x
IBC 1642	Brazil (Pedra rachada, Paracuru/CE)	3°23'54.34"S; 39°00'50.43"W	T. Vieira-Pinto, C. Azevedo, B. Silva	24/04/2013	x				
IBC 1643	Brazil (Pedra rachada, Paracuru/CE)	3°23'54.34"S; 39°00'50.43"W	T. Vieira-Pinto, C. Azevedo, B. Silva	24/04/2013	x	x		x	
IBC 1644	Brazil (Pedra rachada, Paracuru/CE)	3°23'54.34"S; 39°00'50.43"W	T. Vieira-Pinto, C. Azevedo, B. Silva	24/04/2013	x				
IBC 1645	Brazil (Pedra rachada, Paracuru/CE)	3°23'54.34"S; 39°00'50.43"W	T. Vieira-Pinto, C. Azevedo, B. Silva	24/04/2013	x	x			
IBC 1646	Brazil (Muguba, Paracuru/CE)	3°24'16.18"S; 39°01'46.65"W	T. Vieira-Pinto, C. Azevedo, B. Silva	24/04/2013	x				

Cont. Tab. 3

Institution Code	Locality (beach, city, state)	Lat/long	Collectors	Date (D/M/Y)	DNA extraction	COX	UPA	psbA	rbcl
IBC 1647	Brazil (Muguba, Paracuru/CE)	3°24'16.18"S; 39°01'46.65"W	T. Vieira-Pinto, C. Azevedo, B. Silva	24/04/2013	x	x		x	
IBC 1648	Brazil (Muguba, Paracuru/CE)	3°24'16.18"S; 39°01'46.65"W	T. Vieira-Pinto, C. Azevedo, B. Silva	24/04/2013	x	x			
IBC 1649	Brazil (Muguba, Paracuru/CE)	3°24'16.18"S; 39°01'46.65"W	T. Vieira-Pinto, C. Azevedo, B. Silva	24/04/2013	x	x		x	
IBC 1650	Brazil (Muguba, Paracuru/CE)	3°24'16.18"S; 39°01'46.65"W	T. Vieira-Pinto, C. Azevedo, B. Silva	24/04/2013	x				
IBC 1651	Brazil (Muguba, Paracuru/CE)	3°24'16.18"S; 39°01'46.65"W	T. Vieira-Pinto, C. Azevedo, B. Silva	24/04/2013	x	x		x	
IBC 1655	Brazil (Muguba, Paracuru/CE)	3°24'16.18"S; 39°01'46.65"W	T. Vieira-Pinto, C. Azevedo, B. Silva	24/04/2013	x	x		x	
IBC 1656	Brazil (Guajiru, Trairi/CE)	3°14'20.86"S; 39°13'46.03"W	T. Vieira-Pinto, C. Azevedo, B. Silva	25/04/2013	x				
IBC 1657	Brazil (Guajiru, Trairi/CE)	3°14'20.86"S; 39°13'46.03"W	T. Vieira-Pinto, C. Azevedo, B. Silva	25/04/2013	x	x			
IBC 1659	Brazil (Guajiru, Trairi/CE)	3°14'20.86"S; 39°13'46.03"W	T. Vieira-Pinto, C. Azevedo, B. Silva	25/04/2013	x	x			
IBC 1660	Brazil (Guajiru, Trairi/CE)	3°14'20.86"S; 39°13'46.03"W	T. Vieira-Pinto, C. Azevedo, B. Silva	25/04/2013	x				x
IBC 1661	Brazil (Guajiru, Trairi/CE)	3°14'20.86"S; 39°13'46.03"W	T. Vieira-Pinto, C. Azevedo, B. Silva	25/04/2013	x	x			
IBC 1662	Brazil (Guajiru, Trairi/CE)	3°14'20.86"S; 39°13'46.03"W	T. Vieira-Pinto, C. Azevedo, B. Silva	25/04/2013	x	x		x	
IBC 1664	Brazil (Guajiru, Trairi/CE)	3°14'20.86"S; 39°13'46.03"W	T. Vieira-Pinto, C. Azevedo, B. Silva	25/04/2013	x				
IBC 1665	Brazil (Guajiru, Trairi/CE)	3°14'20.86"S; 39°13'46.03"W	T. Vieira-Pinto, C. Azevedo, B. Silva	25/04/2013	x	x			
IBC 1666	Brazil (Guajiru, Trairi/CE)	3°14'20.86"S; 39°13'46.03"W	T. Vieira-Pinto, C. Azevedo, B. Silva	25/04/2013	x	x		x	
IBC 1667	Brazil (Guajiru, Trairi/CE)	3°14'20.86"S; 39°13'46.03"W	T. Vieira-Pinto, C. Azevedo, B. Silva	25/04/2013	x	x		x	
IBC 1669	Brazil (Guajiru, Trairi/CE)	3°14'20.86"S; 39°13'46.03"W	T. Vieira-Pinto, C. Azevedo, B. Silva	25/04/2013	x				x
IBC 1670	Brazil (Guajiru, Trairi/CE)	3°14'20.86"S; 39°13'46.03"W	T. Vieira-Pinto, C. Azevedo, B. Silva	25/04/2013	x	x		x	x
IBC 1671	Brazil (Pacheco, Caicaia/CE)	3°68'52.57"S; 38°63'51.20"W	T. Vieira-Pinto, C. Azevedo, B. Silva, P. Carneiro	26/04/2013	x	x			
IBC 1672	Brazil (Pacheco, Caicaia/CE)	3°68'52.57"S; 38°63'51.20"W	T. Vieira-Pinto, C. Azevedo, B. Silva, P. Carneiro	26/04/2013	x	x		x	
IBC 1673	Brazil (Pacheco, Caicaia/CE)	3°68'52.57"S; 38°63'51.20"W	T. Vieira-Pinto, C. Azevedo, B. Silva, P. Carneiro	26/04/2013	x				
IBC 1675	Brazil (Pacheco, Caicaia/CE)	3°68'52.57"S; 38°63'51.20"W	T. Vieira-Pinto, C. Azevedo, B. Silva, P. Carneiro	26/04/2013	x				
IBC 1676	Brazil (Pacheco, Caicaia/CE)	3°68'52.57"S; 38°63'51.20"W	T. Vieira-Pinto, C. Azevedo, B. Silva, P. Carneiro	26/04/2013	x				x
IBC 1677	Brazil (Pacheco, Caicaia/CE)	3°68'52.57"S; 38°63'51.20"W	T. Vieira-Pinto, C. Azevedo, B. Silva, P. Carneiro	26/04/2013	x				
IBC 1679	Brazil (Pacheco, Caicaia/CE)	3°68'52.57"S; 38°63'51.20"W	T. Vieira-Pinto, C. Azevedo, B. Silva, P. Carneiro	26/04/2013	x	x		x	

Cont. Tab. 3

Institution Code	Locality (beach, city, state)	Lat/long	Collectors	Date (D/M/Y)	DNA extraction	COX	UPA	psbA	rbcL
IBC 1680	Brazil (Pacheco, Caicaia/CE)	3°68'52.57"S; 38°63'51.20"W	T. Vieira-Pinto, C. Azevedo, B. Silva, P. Carneiro	26/04/2013	x	x		x	x
IBC 1682	Brazil (Pacheco, Caicaia/CE)	3°68'52.57"S; 38°63'51.20"W	T. Vieira-Pinto, C. Azevedo, B. Silva, P. Carneiro	26/04/2013	x				x
IBC 1684	Brazil (Ponta Grossa, Icapuí/CE)	4°37'35.80"S; 37°29'58.83"W	T. Vieira-Pinto, C. Azevedo	27/04/2013	x				
IBC 1685	Brazil (Ponta Grossa, Icapuí/CE)	4°37'35.80"S; 37°29'58.83"W	T. Vieira-Pinto, C. Azevedo	27/04/2013	x	x		x	x
IBC 1687	Brazil (Ponta Grossa, Icapuí/CE)	4°37'35.80"S; 37°29'58.83"W	T. Vieira-Pinto, C. Azevedo	27/04/2013	x	x			
IBC 1688	Brazil (Ponta Grossa, Icapuí/CE)	4°37'35.80"S; 37°29'58.83"W	T. Vieira-Pinto, C. Azevedo	27/04/2013	x	x		x	
IBC 1689	Brazil (Ponta Grossa, Icapuí/CE)	4°37'35.80"S; 37°29'58.83"W	T. Vieira-Pinto, C. Azevedo	27/04/2013	x	x			
IBC 1691	Brazil (Ponta Grossa, Icapuí/CE)	4°37'35.80"S; 37°29'58.83"W	T. Vieira-Pinto, C. Azevedo	27/04/2013	x				
IBC 1697	Brazil (Stella Maris, Salvador/BA)	12°56'41.50"S; 38°20'04.70"W	T. Vieira-Pinto, C. Azevedo, B. Silva, M. Jamas	22/05/2013	x				
IBC 1698	Brazil (Stella Maris, Salvador/BA)	12°56'41.50"S; 38°20'04.70"W	T. Vieira-Pinto, C. Azevedo, B. Silva, M. Jamas	22/05/2013	x				
IBC 1699	Brazil (Stella Maris, Salvador/BA)	12°56'41.50"S; 38°20'04.70"W	T. Vieira-Pinto, C. Azevedo, B. Silva, M. Jamas	22/05/2013	x				
IBC 1703	Brazil (Banco da Panela, Salvador/BA)	12°96'61.19"S; 38°52'66.75"W	T. Vieira-Pinto, C. Azevedo, B. Silva, M. Jamas	22/05/2013	x	x		x	x
IBC 1704	Brazil (Banco da Panela, Salvador/BA)	12°96'61.19"S; 38°52'66.75"W	T. Vieira-Pinto, C. Azevedo, B. Silva, M. Jamas	22/05/2013	x		x	x	
IBC 1706	Brazil (Banco da Panela, Salvador/BA)	12°96'61.19"S; 38°52'66.75"W	T. Vieira-Pinto, C. Azevedo, B. Silva, M. Jamas	22/05/2013	x				
IBC 1708	Brazil (Banco da Panela, Salvador/BA)	12°96'61.19"S; 38°52'66.75"W	T. Vieira-Pinto, C. Azevedo, B. Silva, M. Jamas	22/05/2013	x			x	
IBC 1710	Brazil (Banco da Panela, Salvador/BA)	12°96'61.19"S; 38°52'66.75"W	T. Vieira-Pinto, C. Azevedo, B. Silva, M. Jamas	22/05/2013	x			x	x
IBC 1711	Brazil (Jauá, Camaçari/BA)	12°82'75.68"S; 38°22'42.38"W	T. Vieira-Pinto, C. Azevedo, B. Silva, M. Jamas	23/05/2013	x				
IBC 1712	Brazil (Jauá, Camaçari/BA)	12°82'75.68"S; 38°22'42.38"W	T. Vieira-Pinto, C. Azevedo, B. Silva, M. Jamas	23/05/2013	x	x		x	x
IBC 1713	Brazil (Jauá, Camaçari/BA)	12°82'75.68"S; 38°22'42.38"W	T. Vieira-Pinto, C. Azevedo, B. Silva, M. Jamas	23/05/2013	x				
IBC 1714	Brazil (Jauá, Camaçari/BA)	12°82'75.68"S; 38°22'42.38"W	T. Vieira-Pinto, C. Azevedo, B. Silva, M. Jamas	23/05/2013	x				
IBC 1715	Brazil (Jauá, Camaçari/BA)	12°82'75.68"S; 38°22'42.38"W	T. Vieira-Pinto, C. Azevedo, B. Silva, M. Jamas	23/05/2013	x				x
IBC 1717	Brazil (Jauá, Camaçari/BA)	12°82'75.68"S; 38°22'42.38"W	T. Vieira-Pinto, C. Azevedo, B. Silva, M. Jamas	23/05/2013	x	x			

Cont. Tab. 3

Institution Code	Locality (beach, city, state)	Lat/long	Collectors	Date (D/M/Y)	DNA extraction	COX	UPA	psbA	rbcL
IBC 1720	Brazil (Coroa, Ilha de Itaparica/BA)	13°00'35.30"S; 38°38'27.40"W	T. Vieira-Pinto, C. Azevedo, B. Silva, M. Jamas	24/05/2013	x				
IBC 1721	Brazil (Coroa, Ilha de Itaparica/BA)	13°00'35.30"S; 38°38'27.40"W	T. Vieira-Pinto, C. Azevedo, B. Silva, M. Jamas	24/05/2013	x				
IBC 1722	Brazil (Coroa, Ilha de Itaparica/BA)	13°00'35.30"S; 38°38'27.40"W	T. Vieira-Pinto, C. Azevedo, B. Silva, M. Jamas	24/05/2013	x	x		x	
IBC 1723	Brazil (Coroa, Ilha de Itaparica/BA)	13°00'35.30"S; 38°38'27.40"W	T. Vieira-Pinto, C. Azevedo, B. Silva, M. Jamas	24/05/2013	x	x		x	
IBC 1726	Brazil (Coroa, Ilha de Itaparica/BA)	13°00'35.30"S; 38°38'27.40"W	T. Vieira-Pinto, C. Azevedo, B. Silva, M. Jamas	24/05/2013	x			x	x
IBC 1727	Brazil (Coroa, Ilha de Itaparica/BA)	13°00'35.30"S; 38°38'27.40"W	T. Vieira-Pinto, C. Azevedo, B. Silva, M. Jamas	24/05/2013	x	x		x	
IBC 1728	Brazil (Coroa, Ilha de Itaparica/BA)	13°00'35.30"S; 38°38'27.40"W	T. Vieira-Pinto, C. Azevedo, B. Silva, M. Jamas	24/05/2013	x				x
IBC 1729	Brazil (Coroa, Ilha de Itaparica/BA)	13°00'35.30"S; 38°38'27.40"W	T. Vieira-Pinto, C. Azevedo, B. Silva, M. Jamas	24/05/2013	x				
IBC 1730	Brazil (Coroa, Ilha de Itaparica/BA)	13°00'35.30"S; 38°38'27.40"W	T. Vieira-Pinto, C. Azevedo, B. Silva, M. Jamas	24/05/2013	x	x			
IBC 1731	Brazil (Recife de Caramuanas, Ilha de Itaparica/BA)	12°97'54.85"S; 38°60'34.95"W	T. Vieira-Pinto, C. Azevedo, B. Silva, M. Jamas	25/05/2013	x	x			
IBC 1732	Brazil (Recife de Caramuanas, Ilha de Itaparica/BA)	12°97'54.85"S; 38°60'34.95"W	T. Vieira-Pinto, C. Azevedo, B. Silva, M. Jamas	25/05/2013	x				
IBC 1733	Brazil (Recife de Caramuanas, Ilha de Itaparica/BA)	12°97'54.85"S; 38°60'34.95"W	T. Vieira-Pinto, C. Azevedo, B. Silva, M. Jamas	25/05/2013	x				
IBC 1734	Brazil (Recife de Caramuanas, Ilha de Itaparica/BA)	12°97'54.85"S; 38°60'34.95"W	T. Vieira-Pinto, C. Azevedo, B. Silva, M. Jamas	25/05/2013	x		x		
IBC 1735	Brazil (Recife de Caramuanas, Ilha de Itaparica/BA)	12°97'54.85"S; 38°60'34.95"W	T. Vieira-Pinto, C. Azevedo, B. Silva, M. Jamas	25/05/2013	x				
IBC 1736	Brazil (Recife de Caramuanas, Ilha de Itaparica/BA)	12°97'54.85"S; 38°60'34.95"W	T. Vieira-Pinto, C. Azevedo, B. Silva, M. Jamas	25/05/2013	x				
IBC 1737	Brazil (Recife de Caramuanas, Ilha de Itaparica/BA)	12°97'54.85"S; 38°60'34.95"W	T. Vieira-Pinto, C. Azevedo, B. Silva, M. Jamas	25/05/2013	x	x		x	x
IBC 1741	Brazil (Guarajuba, Camaçari/BA)	12°38'58"S; 38°03'37"W	T. Vieira-Pinto, C. Azevedo, B. Silva	26/05/2013	x	x			x
IBC 1742	Brazil (Guarajuba, Camaçari/BA)	12°38'58"S; 38°03'37"W	T. Vieira-Pinto, C. Azevedo, B. Silva	26/05/2013	x				
IBC 1750	Brazil (Subaúma, Entre Rios/BA)	12°14'26.40"S; 37°46'26.80"W	T. Vieira-Pinto, C. Azevedo, B. Silva	27/05/2013	x	x			
IBC 1752	Brazil (Subaúma, Entre Rios/BA)	12°14'26.40"S; 37°46'26.80"W	T. Vieira-Pinto, C. Azevedo, B. Silva	27/05/2013	x			x	
IBC 1753	Brazil (Subaúma, Entre Rios/BA)	12°14'26.40"S; 37°46'26.80"W	T. Vieira-Pinto, C. Azevedo, B. Silva	27/05/2013	x	x			
IBC 1754	Brazil (Subaúma, Entre Rios/BA)	12°14'26.40"S; 37°46'26.80"W	T. Vieira-Pinto, C. Azevedo, B. Silva	27/05/2013	x				

Cont. Tab. 3

Institution Code	Locality (beach, city, state)	Lat/long	Collectors	Date (D/M/Y)	DNA extraction	COX	UPA	psbA	rbcl
IBC 1755	Brazil (Subaúma, Entre Rios/BA)	12°14'26.40"S; 37°46'26.80"W	T. Vieira-Pinto, C. Azevedo, B. Silva	27/05/2013	x				
IBC 1756	Brazil (Subaúma, Entre Rios/BA)	12°14'26.40"S; 37°46'26.80"W	T. Vieira-Pinto, C. Azevedo, B. Silva	27/05/2013	x				
IBC 1765	Brazil (Fernando de Noronha/RN)	3°85'09.89"S; 32°44'18.85"W	T. Vieira-Pinto, C. Azevedo, B. Silva	08/11/2011	x	x		x	x
IBC 1789	Brazil (Coroa, Ilha de Itaparica/BA)	13°00'35.30"S; 38°38'27.40"W	T. Vieira-Pinto, C. Azevedo, B. Silva	24/05/2013	x				
IBC 1790	Brazil (Subaúma, Entre Rios/BA)	12°14'26.40"S; 37°46'26.80"W	T. Vieira-Pinto, C. Azevedo, B. Silva	27/05/2013	x	x	x	x	x
IBC 1792	Brazil (Guarajuba, Camaçari/BA)	12°38'58"S; 38°03'37"W	T. Vieira-Pinto, C. Azevedo, B. Silva	26/05/2013	x	x			
IBC 1793	Brazil (Ponta Verde, Maceió/AL)	9°39'54.00"S; 35°41'42.00"W	T. Vieira-Pinto, C. Azevedo, B. Silva	10/03/2013	x	x		x	x
IBC 1794	Brazil (Ponta Verde, Maceió/AL)	9°39'54.00"S; 35°41'42.00"W	T. Vieira-Pinto, C. Azevedo, B. Silva	10/03/2013	x	x			
IBC 1795	Brazil (Ponta Verde, Maceió/AL)	9°39'54.00"S; 35°41'42.00"W	T. Vieira-Pinto, C. Azevedo, B. Silva	10/03/2013	x			x	
IBC 1798	Brazil (Trindade, Vitória/ES)	20°52'33.28"S; 29°32'41.14"W	M. Sissini	07/09/2014	x			x	
IBC 1799	Brazil (Trindade, Vitória/ES)	20°52'33.28"S; 29°32'41.14"W	M. Sissini	07/09/2014	x				
IBC 1801	Brazil (Cardeiro, São Miguel do Gostoso/RN)	5°6'57.89"S; 35°37'14.16"W	T. Vieira-Pinto, M. Jamas, M. Fuji, D. Milstein, A. Leite	29/03/2014	x				
IBC 1802	Brazil (Cardeiro, São Miguel do Gostoso/RN)	5°6'57.89"S; 35°37'14.16"W	T. Vieira-Pinto, M. Jamas, M. Fuji, D. Milstein, A. Leite	29/03/2014	x	x		x	x
IBC 1803	Brazil (Cardeiro, São Miguel do Gostoso/RN)	5°6'57.89"S; 35°37'14.16"W	T. Vieira-Pinto, M. Jamas, M. Fuji, D. Milstein, A. Leite	29/03/2014	x	x			
IBC 1804	Brazil (Cardeiro, São Miguel do Gostoso/RN)	5°6'57.89"S; 35°37'14.16"W	T. Vieira-Pinto, M. Jamas, M. Fuji, D. Milstein, A. Leite	29/03/2014	x	x		x	
IBC 1805	Brazil (Cardeiro, São Miguel do Gostoso/RN)	5°6'57.89"S; 35°37'14.16"W	T. Vieira-Pinto, M. Jamas, M. Fuji, D. Milstein, A. Leite	29/03/2014	x				
IBC 1806	Brazil (Cardeiro, São Miguel do Gostoso/RN)	5°6'57.89"S; 35°37'14.16"W	T. Vieira-Pinto, M. Jamas, M. Fuji, D. Milstein, A. Leite	29/03/2014	x	x			
IBC 1807	Brazil (Cardeiro, São Miguel do Gostoso/RN)	5°6'57.89"S; 35°37'14.16"W	T. Vieira-Pinto, M. Jamas, M. Fuji, D. Milstein, A. Leite	29/03/2014	x	x			
IBC 1812	Brazil (Tourinhos/RN)	5°12'12.24"S; 35°26'56.45"W	T. Vieira-Pinto, M. Jamas, M. Fuji, D. Milstein, A. Leite	29/03/2014	x				
IBC 1813	Brazil (Tourinhos/RN)	5°12'12.24"S; 35°26'56.45"W	T. Vieira-Pinto, M. Jamas, M. Fuji, D. Milstein, A. Leite	29/03/2014	x	x			
IBC 1814	Brazil (Rio do Fogo/RN)	5°14'51.91"S; 35°23'41.15"W	T. Vieira-Pinto, M. Jamas, M. Fuji, D. Milstein, A. Leite	30/03/2014	x	x			
IBC 1815	Brazil (Rio do Fogo/RN)	5°14'51.91"S; 35°23'41.15"W	T. Vieira-Pinto, M. Jamas, M. Fuji, D. Milstein, A. Leite	30/03/2014	x	x			x
IBC 1820	Brazil (Rio do Fogo/RN)	5°14'51.91"S; 35°23'41.15"W	T. Vieira-Pinto, M. Jamas, M. Fuji, D. Milstein, A. Leite	30/03/2014	x	x		x	x

Cont. Tab. 3

Institution Code	Locality (beach, city, state)	Lat/long	Collectors	Date (D/M/Y)	DNA extraction	COX	UPA	psbA	rbcL
IBC 1821	Brazil (Rio do Fogo/RN)	5°14'51.91"S; 35°23'41.15"W	T. Vieira-Pinto, M. Jamas, M. Fuji, D. Milstein, A. Leite	30/03/2014	x	x		x	
IBC 1822	Brazil (Rio do Fogo/RN)	5°14'51.91"S; 35°23'41.15"W	T. Vieira-Pinto, M. Jamas, M. Fuji, D. Milstein, A. Leite	30/03/2014	x				
IBC 1823	Brazil (Rio do Fogo/RN)	5°14'51.91"S; 35°23'41.15"W	T. Vieira-Pinto, M. Jamas, M. Fuji, D. Milstein, A. Leite	30/03/2014	x	x			
IBC 1827	Brazil (Baía da Traição/PB)	6°68'30.63"S; 34°94'48.70"W	T. Vieira-Pinto, M. Jamas, M. Fuji, D. Milstein, A. Leite	31/03/2014	x	x		x	
IBC 1828	Brazil (Baía da Traição/PB)	6°68'30.63"S; 34°94'48.70"W	T. Vieira-Pinto, M. Jamas, M. Fuji, D. Milstein, A. Leite	31/03/2014	x				
IBC 1833	Brazil (Carapibus, Conde/PB)	7°29'84.02"S; 34°79'89.15"W	T. Vieira-Pinto, M. Jamas, M. Fuji, D. Milstein, A. Leite	01/04/2014	x	x			
IBC 1836	Brazil (Carapibus, Conde/PB)	7°29'84.02"S; 34°79'89.15"W	T. Vieira-Pinto, M. Jamas, M. Fuji, D. Milstein, A. Leite	01/04/2014	x	x		x	
IBC 1839	Brazil (Carapibus, Conde/PB)	7°29'84.02"S; 34°79'89.15"W	T. Vieira-Pinto, M. Jamas, M. Fuji, D. Milstein, A. Leite	01/04/2014	x	x		x	
IBC 1842	Brazil (Carapibus, Conde/PB)	7°29'84.02"S; 34°79'89.15"W	T. Vieira-Pinto, M. Jamas, M. Fuji, D. Milstein, A. Leite	01/04/2014	x				
IBC 1843	Brazil (Carapibus, Conde/PB)	7°29'84.02"S; 34°79'89.15"W	T. Vieira-Pinto, M. Jamas, M. Fuji, D. Milstein, A. Leite	01/04/2014	x				
IBC 1849	Brazil (Pirambúzios, Nízia Floresta/RN)	5°59'12.35"S; 35°06'49.54"W	T. Vieira-Pinto, M. Jamas, M. Fuji, D. Milstein, A. Leite	02/04/2014	x	x			
IBC 1850	Brazil (Pirambúzios, Nízia Floresta/RN)	5°59'12.35"S; 35°06'49.54"W	T. Vieira-Pinto, M. Jamas, M. Fuji, D. Milstein, A. Leite	02/04/2014	x	x			
IBC 1865	Brazil (Parati-Ilhote de Ubu, Anchieta/ES)	20°80'83.66"S; 40°59'88.26"W	T. Vieira-Pinto, M. Mungioli, M. Fuji, P. Diaz, H. Verbruggen, V. R. Marcelino	08/09/2014	x	x		x	x
IBC 1869	Brazil (Parati-Ilhote de Ubu, Anchieta/ES)	20°80'83.66"S; 40°59'88.26"W	T. Vieira-Pinto, M. Mungioli, M. Fuji, P. Diaz, H. Verbruggen, V. R. Marcelino	08/09/2014	x	x		x	x
IBC 1871	Brazil (Parati-Ilhote de Ubu, Anchieta/ES)	20°80'83.66"S; 40°59'88.26"W	T. Vieira-Pinto, M. Mungioli, M. Fuji, P. Diaz, H. Verbruggen, V. R. Marcelino	08/09/2014	x				
IBC 1872	Brazil (Parati-Ilhote de Ubu, Anchieta/ES)	20°80'83.66"S; 40°59'88.26"W	T. Vieira-Pinto, M. Mungioli, M. Fuji, P. Diaz, H. Verbruggen, V. R. Marcelino	08/09/2014	x	x		x	
IBC 1873	Brazil (Parati-Ilhote de Ubu, Anchieta/ES)	20°80'83.66"S; 40°59'88.26"W	T. Vieira-Pinto, M. Mungioli, M. Fuji, P. Diaz, H. Verbruggen, V. R. Marcelino	08/09/2014	x	x		x	
IBC 1875	Brazil (Parati-Ilhote de Ubu, Anchieta/ES)	20°80'83.66"S; 40°59'88.26"W	T. Vieira-Pinto, M. Mungioli, M. Fuji, P. Diaz, H. Verbruggen, V. R. Marcelino	08/09/2014	x				x
IBC 1876	Brazil (Parati-Ilhote de Ubu, Anchieta/ES)	20°80'83.66"S; 40°59'88.26"W	T. Vieira-Pinto, M. Mungioli, M. Fuji, P. Diaz, H. Verbruggen, V. R. Marcelino	08/09/2014	x	x			
IBC 1877	Brazil (Parati-Ilhote de Ubu, Anchieta/ES)	20°80'83.66"S; 40°59'88.26"W	T. Vieira-Pinto, M. Mungioli, M. Fuji, P. Diaz, H. Verbruggen, V. R. Marcelino	08/09/2014	x	x		x	x

Cont. Tab. 3

Institution Code	Locality (beach, city, state)	Lat/long	Collectors	Date (D/M/Y)	DNA extraction	COX	UPA	psbA	rbcl
IBC 1878	Brazil (Parati-Ilhote de Ubu, Anchieta/ES)	20°80'83.66"S; 40°59'88.26"W	T. Vieira-Pinto, M. Mungioli, M. Fuji, P. Diaz, H. Verbruggen, V. R. Marcelino	08/09/2014	x				x
IBC 1882	Brazil (Castelhanos, Anchieta/ES)	20°81'21.83"S; 40°63'87.60"W	T. Vieira-Pinto, M. Mungioli, M. Fuji, P. Diaz, H. Verbruggen, V. R. Marcelino	09/09/2014	x	x		x	
IBC 1885	Brazil (Castelhanos, Anchieta/ES)	20°81'21.83"S; 40°63'87.60"W	T. Vieira-Pinto, M. Mungioli, M. Fuji, P. Diaz, H. Verbruggen, V. R. Marcelino	09/09/2014	x				
IBC 1886	Brazil (Castelhanos, Anchieta/ES)	20°81'21.83"S; 40°63'87.60"W	T. Vieira-Pinto, M. Mungioli, M. Fuji, P. Diaz, H. Verbruggen, V. R. Marcelino	09/09/2014	x			x	x
IBC 1896	Brazil (Praia da Cruz, Marataízes/ES)	21°01'58.2"S; 40°48'43.9"W	T. Vieira-Pinto, M. Mungioli, M. Fuji, P. Diaz, H. Verbruggen, V. R. Marcelino	10/09/2014	x	x			x
IBC 1904	Brazil (Fernando de Noronha/PE)	3°85'09.89"S; 32°44'18.85"W	P. Horta	09/08/2013	x				
IBC 1906	Brazil (Laje Dois Irmãos, Fernando de Noronha/RN)	3°86'80.94"S; 32°41'38.03"W	P. Horta	09/08/2013	x				
IBC 1907	Brazil (Risca do Meio/CE)	3°85'09.89"S; 32°44'18.85"W	P. Horta	09/08/2013	x	x		x	
IBC 1908	Brazil (Parcel Manoel Luís/MA)	1°28'16.78"S; 44°72'14.48"W	P. Horta	09/08/2013	x				
IBC 1909	Brazil (Risca do Meio/CE)	3°70'72.92"S; 38°46'80.99"W	Sisbiota Mar	18/04/2012	x			x	
IBC 1910	Brazil (Picãozinho, João Pessoa/PB)	7°11'73.61"S; 34°80'80.19"W	C. Azevedo; F. Nauer; A. Medeiros	22/07/2012	x			x	
IBC 1911	Brazil (Picãozinho, João Pessoa/PB)	7°11'73.61"S; 34°80'80.19"W	C. Azevedo; F. Nauer; A. Medeiros	22/07/2012	x				
IBC 1912	Brazil (Risca do Meio/CE)	3°70'72.92"S; 38°46'80.99"W	Sisbiota Mar	18/04/2012	x				
IBC 1913	Brazil (Risca do Meio/CE)	3°70'72.92"S; 38°46'80.99"W	Sisbiota Mar	18/04/2012	x				
IBC 1914	Brazil (Risca do Meio/CE)	3°70'72.92"S; 38°46'80.99"W	Sisbiota Mar	18/04/2012	x				
IBC 1915	Brazil (Risca do Meio/CE)	3°70'72.92"S; 38°46'80.99"W	Sisbiota Mar	18/04/2012	x				
IBC 1916	Brazil (Fernando de Noronha/RPE)	3°85'09.89"S; 32°44'18.85"W	P. Horta; E. Bastos	07/01/2013	x				
IBC 1917	Brazil (Fernando de Noronha/PE)	3°85'09.89"S; 32°44'18.85"W	P. Horta; E. Bastos	07/01/2013	x			x	
IBC 1918	Brazil (Fernando de Noronha/PE)	3°85'09.89"S; 32°44'18.85"W	P. Horta; E. Bastos	07/01/2013	x	x			
IBC 1919	Brazil (Fernando de Noronha/PE)	3°85'09.89"S; 32°44'18.85"W	P. Horta; E. Bastos	07/01/2013	x			x	
IBC 1920	Brazil (Fernando de Noronha, PE)	3°85'09.89"S; 32°44'18.85"W	P. Horta; E. Bastos	07/01/2013	x				x
IBC 1921	Brazil (Arraial do Cabo/RJ)	22°96'79.78"S; 42°01'60.98"W	T. Vieira-Pinto, I.M. Martins	02/01/2013	x	x		x	x
IBC1922	Brazil (Ilha do Arvoredo/SC)	27°27'54.22"S; 48°35'91.44"W	E. Bastos; M. Peres	12/01/2013	x		x		
IBC1923	Brazil (Ilha do Arvoredo/SC)	27°27'54.22"S; 48°35'91.44"W	E. Bastos; M. Peres	12/01/2013	x		x		
IBC1924	Brazil (Ilha do Arvoredo/SC)	27°27'54.22"S; 48°35'91.44"W	E. Bastos; M. Peres	12/01/2013	x		x		
IBC1925	Brazil (Ilha do Arvoredo/SC)	27°27'54.22"S; 48°35'91.44"W	E. Bastos; M. Peres	12/01/2013	x		x		

Cont. Tab. 3

Institution Code	Locality (beach, city, state)	Lat/long	Collectors	Date (D/M/Y)	DNA extraction	COX	UPA	psbA	rbcL
IBC1926	Brazil (Ilha do Arvoredo/SC)	27°27'54.22"S; 48°35'91.44"W	E. Bastos; M. Peres	12/01/2013	x		x		
IBC1927	Brazil (Ilha do Arvoredo/SC)	27°27'54.22"S; 48°35'91.44"W	E. Bastos; M. Peres	12/01/2013	x		x		
IBC1928	Brazil (Ilha do Arvoredo/SC)	27°27'54.22"S; 48°35'91.44"W	E. Bastos; M. Peres	12/01/2013	x		x		
IBC 2508	Brazil (Espelho, Porto Seguro/BA)	16°43'35.80"S; 39°07'27.80"W	B.N.T. Silva, J. Pires, A.S.Santos	13/05/2013	x	x		x	x
IBC 2509	Brazil (Espelho, Porto Seguro/BA)	16°43'35.80"S; 39°07'27.80"W	B.N.T. Silva, J. Pires, A.S.Santos	13/05/2013	x	x		x	
IBC 2551	Brazil, Corumbau, Prado/BA)	16°53'52.00"S; 39°06'34.70"W	B.N.T. Silva, J. Pires, A.S.Santos	13/05/2013	x	x		x	x

### *PCR amplifications, cleaning/purification and cycle sequencing*

For PCR, four markers were selected based on the available comparable data and/or based on their convenience: the Universal Plastid Amplicon 23S rRNA gene (UPA), the chloroplast-encoded photosystem II reaction center protein D1 gene (*psbA*) and the chloroplast-encoded gene *rbcL*, which encodes the large subunit of the enzyme ribulose-1, 5-bisphosphate carboxylase/oxygenase (RuBisCO), and the mitochondria-encoded cytochrome oxidase subunit I gene (COI). In the Table 4 we list all the primers used during this study, although not all of them worked well in our dataset as we show in the discussion section.

PCR for UPA was performed using the primers and PCR protocol referenced in Sherwood and Presting (2007). PCR for *psbA* was performed using the primers referenced in Yoon *et al.* (2002) under the following thermal profile: an initial denaturation at 94 °C for 2 min followed by 35 cycles at 94 °C for 30 sec (denaturation), 47 °C for 1 min (primer annealing), and 72 °C for 2 min (extension) followed by a final extension at 72°C for 7 min. PCR for COI was conducted using the primers referenced in Table 4, with an initial denaturation at 94°C followed by 40 cycles at 94°C for 1 min (denaturation), 45°C for 1 min (primer annealing), and 72°C (extension) for 1 min followed by a final extension at 72°C for 5 min. PCR for *rbcL* was performed using the primers referenced in Table 4, with an initial denaturation of 94°C for 4 min followed by 2 cycles at 94°C for 1 min (denaturation), 40°C for 1 min (primer annealing), and 72°C (extension) for 2 min then 40 cycles at 94°C for 1 min (denaturation), 42°C for 1 min (primer annealing), and 72°C (extension) for 2 min followed by a final extension at 72°C for 5 min.).

PCR products were purified with ExoSAP-IT® (USB®) or with the Illustra™ GTX™ PCR DNA and Gel Band Purification Kit (GE Healthcare, Buckinghamshire, UK). Both methods worked well in our dataset, therefore no more comments about the cleaning are made in the discussion section.

Purified PCR products were subsequently cycle sequenced using the BigDye Terminator v 3.1 kit (Life Technologies) following the protocol specified by the supplier. Resulting cycle sequence reactions were purified with ETOH/EDTA precipitation and were either sequenced in-house at University of São Paulo campus on an ABI Model 3730 Genetic Analyzer or at the UL Lafayette campus on an ABI Model 3130xl Genetic Analyzer. Resulting chromatograms were assembled using Sequencher 5.1 (Gene Codes Corp., Ann Arbor, MI, USA) or Geneious R9 (<http://www.geneious.com>, Kearse *et al.*, 2012).

Table 4. List of all the primers used for PCR and cycle sequencing during this study.

<b>Marker</b>	<b>Primers</b>	<b>Sequence 5'→3'</b>	<b>Reference</b>
<b>COI-5P</b>	GazF1	TCAACAAATCATAAAGATATTGG	Saunders 2005
	GwsFn	TCAACAAAYCAYAAAGATATYGG	Le Gall & Saunders 2010
	GHalF	TCAACAAATCATAAAGATATYGG	Saunders 2008
	GazR1	ACTTCTGGATGTCCAAAAAYCA	Saunders 2005
	GwsRin	GGRTGTCCRAARAAYCARAA	McDevit & Saunders 2013
	GwsRi	GGRTGICCRAARAAYCARAA	McDevit & Saunders 2013
<b>psbA</b>	psbA-F	ATGACTGCTACTTTAGAAAGACG	Yoon <i>et al.</i> 2002
	psbA500F	CTCTGATGGWATGCCWYTAGG	Yoon <i>et al.</i> 2002
	psbA550R	CCRAAYACACCHGCWACACC	Sissini <i>et al.</i> , 2014; Torrano-Silva <i>et al.</i> 2014
	psbA600R	CCAAATACACCAGCAACACC	Yoon <i>et al.</i> 2002
	psbA-R2	GCTAAATCTARWGGGAAGTTGTG	Yoon <i>et al.</i> 2002
<b>rbcL</b>	F57	GTAATCCATATGCTAAAATGGG	Freshwater & Rueness 1994
	F577	GTATATGAAGGTCTAAAAGGTGG	Freshwater & Rueness 1994
	F753a	GCTCTTTCRTACATATCYTC	Cassano, 2009
	F993	GGTACTGTTGTAGGTAAATTWGAAGG	Freshwater <i>et al.</i> 1994
	R1150	GCATTTGTCCGCAGTGAATACC	Freshwater <i>et al.</i> 1994
	RrbcS-Start	GTTCTTGTGTTAATCTCAC	Freshwater <i>et al.</i> 1994
<b>UPA</b>	p23SrV_f1 (UPAF)	GGACAGAAAGACCCTATGAA	Sherwood & Presting 2007
	p23SrV_r1 (UPAR)	TCAGCCTGTTATCCCTAGAG	Sherwood & Presting 2007

## Alignments

Consensus DNA sequences were aligned using the CLUSTAL W (Thompson *et al.* 1994) program in MEGA 5.2.2 (Tamura *et al.* 2011). In the Table 5 is a summary of the single-genes alignment matrices used in this chapter. The final UPA alignment comprised 78 sequences (370 bp), 54 newly generated in this study, 23 sequences from GenBank, including 1 sequences of *Gracilaria cuneata* as outgroup. The final *rbcL*-3P alignment comprised 100 sequences (307 bp), 47 newly generated sequences, 53 sequences from GenBank, including 2 sequences of Rhodogorgonales as outgroups. The final COI-5P alignment comprised 229 sequences (604 bp), 69 newly generated sequences, 160 sequences from GenBank, including 3 sequences of Rhodogorgonales as outgroups. The final *psbA* alignment comprised 243 sequences (867 bp) comprised 96 newly generated sequences, 174 published sequences and a sequence of *Renouxia* sp. as outgroup. For more information about the specimens from genbank used in all matrices, see Appendix II (Appendix I – see the CD attached in the back cover).

Table 5. Summary of single-gene alignment matrices. Complete alignment matrices are available in the Appendices III-VI (see the CD attached in the back cover).

Matrix/ Marker	Number of sequences	Number of sequences THIS STUDY	Number of sequences Genbank	Number of base pairs	Outgroups (Genbank acc. number)
UPA	78	54	23	370	<i>Gracilaria cuneata</i> (KM455585)
<i>rbcL</i> -3P	100	47	53	307	<i>Renouxia antillana</i> (U04181) <i>Rhodogorgon carriebowensis</i> (U04183)
COI-5P	229	69	160	604	<i>Renouxia</i> sp. (GQ917305) <i>Rhodogorgon</i> sp. (GQ917306)
<i>psbA</i>	243	96	174	867	<i>Renouxia</i> sp. (GQ917503)

### *Phylogenetic and Barcoding Analyses*

The all four single-gene alignment matrices were analyzed with the NJ distance-based method in MEGA 5.2.2 (Tamura *et al.* 2011) with 1,000 bootstrap replicates. Analyses of the COI-5P and *psbA* were conducted using Bayesian (BI) and Maximum Likelihood (ML) methods, both with a GTR+I+G model of evolution partitioned per codon position (best-fitting model identified by PhyML-aLRT v.2.4.5 (Anisimova & Gascuel 2006) in TOPALI 2.5 (Milne *et al.* 2004). Bayesian analysis were conducted using MrBayes (Huelsenbeck & Ronquist 2001) also in TOPALI 2.5 consisting of two MCMC chains with  $1 \times 10^7$  generations. Resampling was performed every 1,000 generations. The first 10% of each run was discarded as “burn-in”, and a consensus tree was built with remaining data. ML analyses were conducted with the RAxML-HPC2 program using the online server ‘The CIPRES Science Gateway V. 3.3’ (Miller *et al.* 2010) with 1,000 topological searches from random restarts, and 1,000 bootstrap replicates.

### *Species Delimitation Analyses*

Species delimitation analyses were performed on each dataset with Automatic Barcode Gap Species Discovery (ABGD). The ABGD analysis run online at <http://www.wabi.snv.jussieu.fr/public/abgd> (Puillandre *et al.* 2012) with minimum (pmin) and maximum (pmax) intraspecific distance priors comprised between 0.001 and 1 in 0.01 steps, and with a relative gap width values of 1.

### *Evaluation of Pairwise Distance Distribution for Markers*

The numbers of pairwise differences were calculated in Geneious version R9 (<http://www.geneious.com>, Kearse *et al.* 2012). The distribution of raw pairwise distances was computed for the four markers utilized in the present study in order to evaluate their phylogenetic informativeness. Distances were calculated by dividing the number of base pair differences by the alignment length. Alignments were cropped at their 5' and 3' ends when missing data was present and short sequences were removed as to not overinflate pairwise distances.

## Results

### *Molecular results*

Based on the combined use of four molecular markers we investigated the diversity of CCA found along the Brazilian coast. The resulting tree for the markers COI-5P and *psbA* is presented split in subtrees as they are our most data-rich and taxarich sets, while UPA and *rbcL*-3P trees are presented as single figures as they are our less diversity-rich taxa datasets.

The backbones of the cluster analyses we obtained from each of the four markers were overall poorly resolved (Fig. 6-8 and 12; UPA, *rbcL*-3P, COI-5P and *psbA* respectively). Although the markers we used are not the most appropriate to address or infer phylogenetic relationships on their own (with the exception of *psbA*), we observed a pattern showing the monophyly of the orders Corallinales and Sporolithales with low bootstrap support. The Hapalidiales was not monophyletic in the analyses among markers, with the exception of the *psbA* tree that showed the Hapalidiales as a monophyletic lineage and Corallinales as being non-monophyletic. Numerous lineages in the shallow branch tips, especially in the results we obtained from COI-5P and *psbA* analyses (see next sections for the detailed lineages in zoomed-in trees) received a higher support in the analyses and revealed previously undocumented molecular diversity of CCA in Brazil.

Unidentified species annotations are the same in all trees when specimens overlap among the different markers.

## UPA

The NJ analysis of UPA (Fig. 6) showed a diversity of 22 species on the Brazilian coast, based on the data generated in this study, with nine of them belonging to Corallinales, 10 to Hapalidiales, one to the Sporolithales; the specimens IBC1551 and IBC1555 from Trindade Island were isolated in two different branches separate from Corallinales, Hapalidiales or Sporolithales. All these 22 putative species were supported by ABGD analysis with the exception of IBC1526, which appears in the tree by itself as very closely related with other Brazilian species and which in ABGD results is clustered with Hapalidiaceae sp. 9 – see details further in the text.

In the Corallinales clade, the samples IBC1527 and IBC1927 grouped with *Lithophyllum atlanticum* T.Vieira-Pinto, M.C.Oliveira & P.A.Horta from Brazil with no intraspecific variation, and IBC1928 formed a cluster with *Lithophyllum margaritae* (Hariot) Heydrich also from Brazil. *Lithophyllum* sp. 1 (IBC 1734) was sister to *Lithophyllum dentatum* (Kützing) Foslie from Spain with a divergence of 12.4% between each other; *Neogoniolithon* sp. 1 (IBC1202) grouped with *Neogoniolithon* sp. from Guadalupe and *Neogoniolithon fosliei* (Heydrich) Setchell & L. R. Mason from French Polynesia with 6.8-8.8% variation. Corallinaceae sp. 1 (IBC1247) was sister to *Spongites* sp. from Hawaii with 6.2% divergence; the sample IBC1554 was grouped with *Lithophyllum kotschyianum* Unger from Australia with 0.3% of intraspecific variation. Corallinaceae sp. 2 (IBC1209) and Corallinaceae sp. 3 (IBC1566) were clustered together with 13% divergence. Corallinaceae sp. 4 (IBC1222) comprised a clade that is sister to *Pneophyllum conicum* (E. Y. Dawson) Keats, Y. M. Chamberlain & M. Baba specimens from Hawaii (HQ421025) and Australia (KM073333). Specimens of *Pneophyllum conicum* from Genbank showed 1.9% intraspecific variation

(probably due to misapplied name), while Corallinaceae sp. 4 was 3-3.2% divergent from these specimens.

In the Hapalidiales, *Mesophyllum erubescens* (Foslie) Me. Lemoine IBC1926 and KM877298 from the database comprised a clade and showed 100% similarity between the two specimens. *Lithothamnion* sp. 1 cluster has three specimens (IBC1564, IBC1557 and IBC1704) grouped together with 0.3% intraspecific variation. Hapalidiaceae sp. 1 (IBC1790) did not form a clade with any other species, neither did Hapalidiaceae sp. 2 (IBC1196) and Hapalidiaceae sp. 3 (IBC1207), and each of the three taxa were represented by only one specimen. Hapalidiaceae sp. 4 (IBC1246 and IBC1562) grouped with Hapalidiaceae sp. from Brazil and were 0.5% divergent. Hapalidiaceae sp. 5 (IBC1559) did not group with any other species, neither did Hapalidiaceae sp. 6 (IBC1526). Hapalidiaceae sp. 7 formed a cluster with three specimens (IBC1188, IBC1245 and IBC1547) with no intraspecific variation. The Hapalidiaceae sp. 8 cluster was composed of seven specimens (IBC1191, IBC1558, IBC1560, IBC1922, IBC1923, IBC1924 and IBC1925) with intraspecific variation of 1-1.3%. However, the ABGD analysis indicated that this may represent a complex of species, as it splits this clade into four different putative species. Considering we cannot confirm this hypothesis with other markers, we decided to treat this cluster as one species.

Specimens of Sporolithales are all grouped together with an intraspecific variation ranging from 0.3-2%.



0,01

Fig. 6. Phylogram based on NJ analysis of UPA sequences. Node values indicate bootstrap values out of 1,000 replicates. Newly generated sequences shown with a bullet point in bold.

## *rbcL*-3P

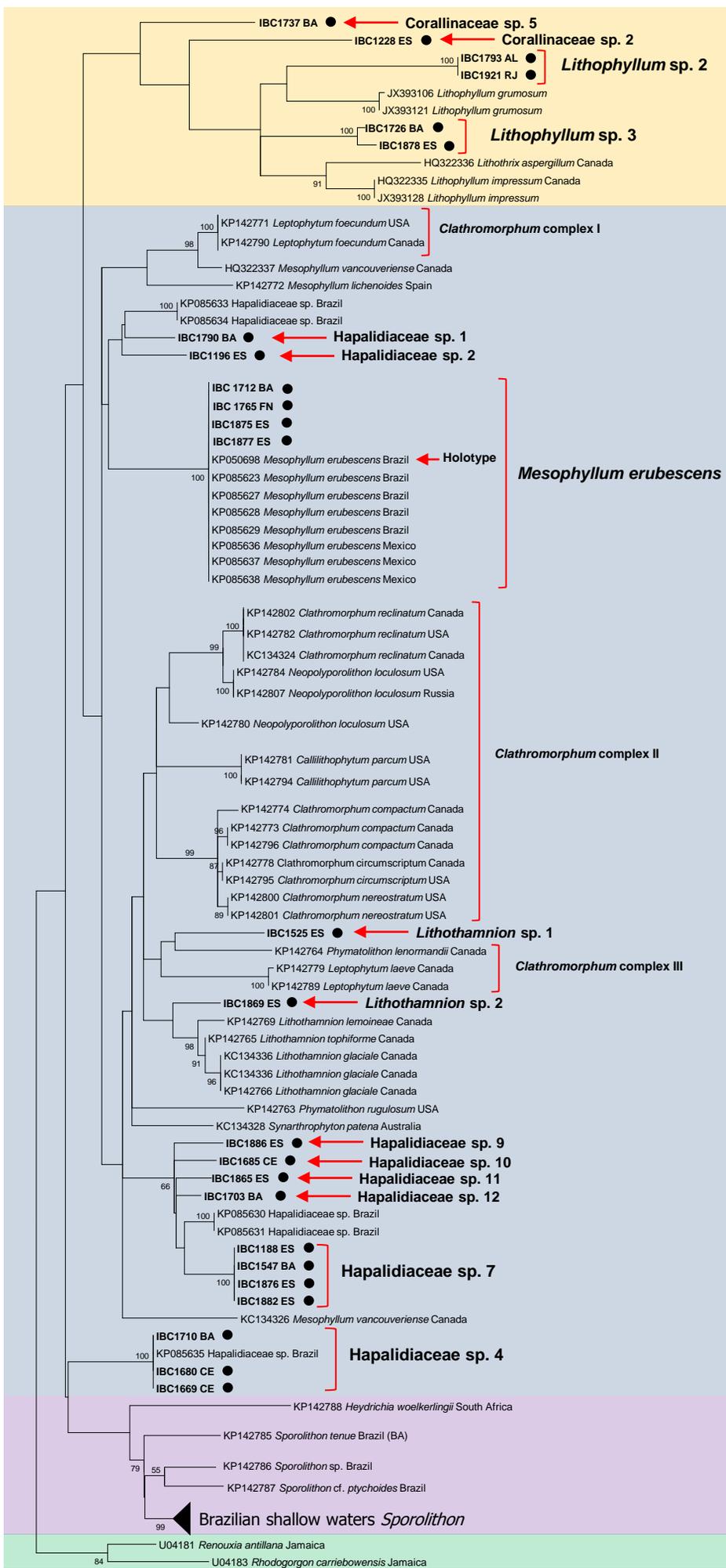
The results of the ML analysis of *rbcL*-3P (Fig. 7) revealed a diversity of 16 species on the Brazilian coast, based on the data generated in this study, with four of them belonging to Corallinales, 11 to Hapalidiales and one to the Sporolithales. All the groups presented in this tree are supported by ABGD analysis.

The clade comprised of species of Corallinales shows Corallinaceae sp. 5 (IBC1737) and Corallinaceae sp. 2 (IBC1228) were not grouped with any other specimen. *Lithophyllum* sp. 2 cluster consists of two individuals (IBC1793 and IBC1921) with 0.7% intraspecific variation, and is sister to *Lithophyllum grumosum* (Foslie) Foslie from the Pacific with interspecific variation of 5.3-6%. *Lithophyllum* sp. 3 is also comprised of two individuals (IBC1726 and IBC1878) with 3.9% intraspecific variation.

In the Hapalidiales clade, Hapalidiaceae sp. 1 (IBC1790) and Hapalidiaceae sp. 3 (IBC1196) were nested in a cluster with a Hapalidiaceae sp. from Brazil (KP085633 and KP085634) although they are 9 – 11 % divergent from each other. Specimens IBC1712, IBC1765, IBC1875 and IBC1877 were grouped with *Mesophyllum erubescens* from Brazil and Mexico with no intraspecific variation. *Lithothamnion* sp. 1 (IBC1525 – annotation based on further analyses shown further) is sister to a specimen of *Phymatholithon lenormandii* (Areschoug) W. H. Adey from Canada with 12.1% interspecific variation. *Lithothamnion* sp. 2 (IBC1869 - annotation based on further analyses shown further) is sister to a cluster including *Lithothamnion lemoineae* Adey, *L. tophiforme* (Esper) Unger and *L. glaciale* Kjellman from Canada (3.3-3.7% divergence between the three species) with 8.2-8.6% divergence between Brazilian and Canadian species. Hapalidiaceae sp. 9, 10, 11 and 12 were nested in the same cluster in

which Hapalidiaceae species from Brazil (KP085630 and KP085631) and Hapalidiaceae sp. 7 (IBC1188, IBC1547, IBC1876 and IBC1882) were grouped. Hapalidiaceae sp. from Brazil (KP085630 and KP085631) and Hapalidiaceae sp. 7 are sister to each other with 8.9% divergence. Another Hapalidiaceae sp. from Brazil (KP085635) formed a clade with Hapalidiaceae sp. 4 (IBC1669, IBC1680 and IBC1710 – annotation according to further; see next sections for details) with no intraspecific variation, that appeared to be a separate lineage in the analyses for this marker and was more closely related to Sporolithales.

Specimens of *Sporolithon* from the Brazilian shallow waters were all grouped together with 0.2% intraspecific variation and were sister to *Sporolithon* sp. (KP142785) and *Sporolithon* cf. *ptychoides* Heydrich (KP142787) both from Brazil with a divergence percentage of 18.5-18.8.



Corallinales

Hapalidiales

Sporolithales

0.05

Fig. 7. Phylogram based on NJ analysis of *rbcL*-3P sequences. Node values indicate bootstrap values out of 1,000 replicates. Newly generated sequences shown with a bullet point in bold.

## COI-5P

The general NJ tree obtained from COI-5P analysis (Fig. 8) is edited to show the backbone and the overall diversity; black dots at the terminals indicate sequences generated in this study while red dots indicate sequences downloaded from Genbank.

The Corallinales formed a monophyletic clade with no support and is shown in detail in Fig. 9. The Lithophylloideae formed a monophyletic clade also with no support. *Lithophyllum* sp. 4 cluster consists of four specimens (IBC1633, IBC1792, IBC1872 and IBC1873) with strong support (99/1/99 ML, BI and NJ respectively), and with intraspecific variation of 0.3-0.8% and in accordance with ABGD results. This species is sister to *Lithophyllum yemense* Basso, Caragnano, Le Gall & Rodondi (KP976401) from Yemen with moderate support (52/0.99/82 ML, BI and NJ respectively) and with a divergence of 9.5%. *Lithophyllum* sp. 2 consisted of three specimens from Brazil (IBC1793, IBC1794 and IBC1921) that formed a strongly supported clade (100/1/99 ML, BI and NJ respectively) with no intraspecific variation that was delimited as a single species in the ABGD analysis. This species is sister to *Lithophyllum* cf. *bamleri* from Fiji with 10.8% interspecific variation. *Lithophyllum* sp. 5 (IBC1717) was sister to *Lithophyllum insipidum* (HQ423075 and HQ422710) from Hawaii with strong support (98/1/99 ML, BI and NJ respectively) and shows 9% of interspecific variation. Corallinaceae sp. 5 (IBC1737) was isolated in a long branch, sister to the Lithophylloideae clade. Corallinaceae sp. 2 (IBC1209 and IBC1228) shows 0.3% intraspecific variation and is delimited as a single species in ABGD. This species was nested next to a specimen of unidentified crustose Corallinaceae from Vanuatu with 12-12.3% of interspecific variation. *Neogoniolithon* sp. 1 (IBC1202 and IBC1216) was nested in a clade with Neogonolithoideae species that was moderately supported in the ML and NJ analyses (70 and 65 respectively) and fully supported in the BI analysis

(PP=1). This species has no intraspecific variation and is sister to *Neogoniolithon brassica-florida* (KM392368) from France with strong support (100/1/99 ML, BI and NJ respectively) and 7.6 % interspecific divergence between them. The ABGD analysis also delimited the two individuals of *Neogoniolithon* sp. 1 as one species apart from *Neogoniolithon brassica-florida* (KM392368).

The Hapalidiales was not a monophyletic order in this analysis and was split into two clades, shown separately for a better visualization – Hapalidiales I (Fig. 10) and Hapalidiales II (Fig. 11). The Hapalidiales clade I (Fig. 10) was composed of *Mesophyllum*, *Phymatolithon* and *Lithothamnion* species with no support in the deeper node. However, a clade formed by *Phymatolithon* and *Lithothamnion* species received no support in the ML and NJ analyses but did receive strong support in the BI analysis (0.96).

*Lithothamnion* sp. 2 (IBC1869) was nested in a cluster with *Lithothamnion* species and an unidentified Hapalidiaceae from Indonesia with moderate support for NJ (67) and no support for ML and BI. *Lithothamnion* sp. 10 is sister to *Lithothamnion corallioides* from UK (KC861487) and France (KC861447) with moderate support for ML and NJ (57 and 87 respectively) and no support for BI and 10.2% of divergence. *Lithothamnion* sp. 1 is nested in a clade with *Phymatolithon* species from North Atlantic with low to moderate BI support (0.65) and no ML and NJ support. This species is comprised of two individuals (IBC1907 and IBC1525) grouped with strong support (100/1/99 ML, BI and NJ respectively) and showed 2.7% intraspecific variation. ABGD also delimited both specimens of *Lithothamnion* sp. 1 as a single species.

The Hapalidiales clade II (Fig. 11) is not supported in any of the analyses and is comprised of *Mesophyllum*, *Lithothamnion*, *Melobesia*, *Clathromorphum*,

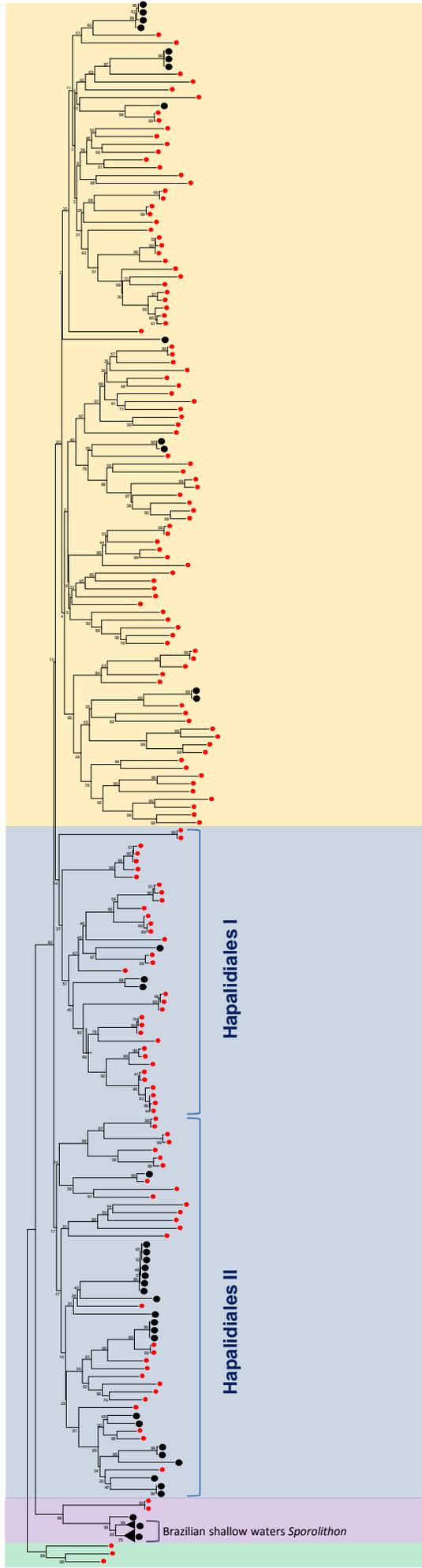
*Phymatholithon* and *Synarthrophyton* species and also a specimen reported as *Lithophyllum kotschyanum* (Corallinales) from Spain which we believe is a misidentified or mislabeled specimen. Hapalidiaceae sp. 3 (IBC1207) was grouped in a cluster with *Melobesia* sp. from USA and *Lithothamnion* sp. from New Caledonia (GQ917298) with low to moderate support (68/68/58 ML, BI and NJ respectively). *Lithothamnion* sp. (GQ917298) from Genbank grouped with the Brazilian species with strong support (100/1/99 ML, BI and NJ respectively); the ABGD analysis also supported the two specimens as a single species, with a 1.7% of divergence between them. Hapalidiaceae sp. 1, sp. 2 and sp. 4 comprised a cluster with *Lithothamnion* sp. from Vanuatu, but with no support for any analyses. Hapalidiaceae sp. 4 consisted of seven specimens (IBC1562, IBC1657, IBC1662, IBC1665, IBC1680, IBC1804 and IBC1850) in a strongly supported clade (100/1/99 ML, BI and NJ respectively) with 0.2% of divergence. Although the ABGD analysis delimited this clade as two putative species, (IBC1657, IBC1804 and IBC1850 as one species and IBC1562, IBC1662, IBC1665 and IBC1680 as a second species), we will treat them as one species in this study. Hapalidiaceae sp. 1 was sister to Hapalidiaceae sp. 4 with no support and with a divergence of 11.4-12.1%. Hapalidiaceae sp. 2 shows 14.8-15.1% interspecific variation from Hapalidiaceae sp. 1 and sp. 4.

*Mesophyllum erubescens* from Brazil (IBC1765, IBC1877 and IBC1712) grouped with several specimens that are reported under the same epithet and a specimen of *Mesophyllum sphaericum* in a clade with strong support for ML and NJ (97 and 94 respectively) but no support in the BI analysis. *Mesophyllum erubescens* from Brazil grouped in a clade with strong support (100/0.95/99 ML, BI and NJ respectively) with no intraspecific variation and was supported as a single species in the ABGD analysis; the species is sister to *Mesophyllum erubescens* from Hawaii (HQ422717 and

HQ422718) with strong support in ML and NJ analyses and moderate support in BI (99/.79/99 ML, BI and NJ respectively) and showed 2.9% intraspecific variation between species from Brazil and Hawaii, although ABGD results suggest they are separate entities.

Hapalidiaceae sp. 6, Hapalidiaceae sp. 7, Hapalidiaceae sp. 8, Hapalidiaceae sp. 10, Hapalidiaceae sp. 11 and Hapalidiaceae sp. 12 from Brazil, *Synarthrophyton patena*, *Mesophyllum* sp. and a unidentified Hapalidiaceae specimen comprised a clade with low to moderate support for ML and moderate support for BI and NJ (0.70 and 81 respectively). Hapalidiaceae sp. 6 (IBC1526) is sister to Hapalidiaceae sp. 11 (IBC1865) with no support in ML and BI and low to moderate support in NJ (65) and showed an interspecific variation of 4.8%; species were delimited as different species in ABGD analysis as well. Hapalidiaceae sp. 7 comprised two specimens (IBC1188 and IBC1876) that showed 0.4% of intraspecific variation and strong support for all analyses (100/1/99 ML, BI and NJ respectively) and was supported by results of the ABGD analysis as a single entity. Hapalidiaceae sp. 12 (IBC1703) is sister to Hapalidiaceae sp. 7 with strong support (100/1/95 ML, BI and NJ respectively) and with 11.4% of interspecific variation. Hapalidiaceae sp. 8 (IBC1882 - annotation based on *psbA* analyses shown further) is sister to Hapalidiaceae sp. 10 with no support and they are 7.8% divergent. The two clades called Hapalidiaceae sp. 7 in this analysis are 7.8-8% divergent. Hapalidiaceae sp. 10 comprised two specimens (IBC1685 and IBC1688) with no intraspecific variation.

The Sporolithales are represented in the general tree for COI-5P; however the detailed results for this order will be presented in the next chapter.



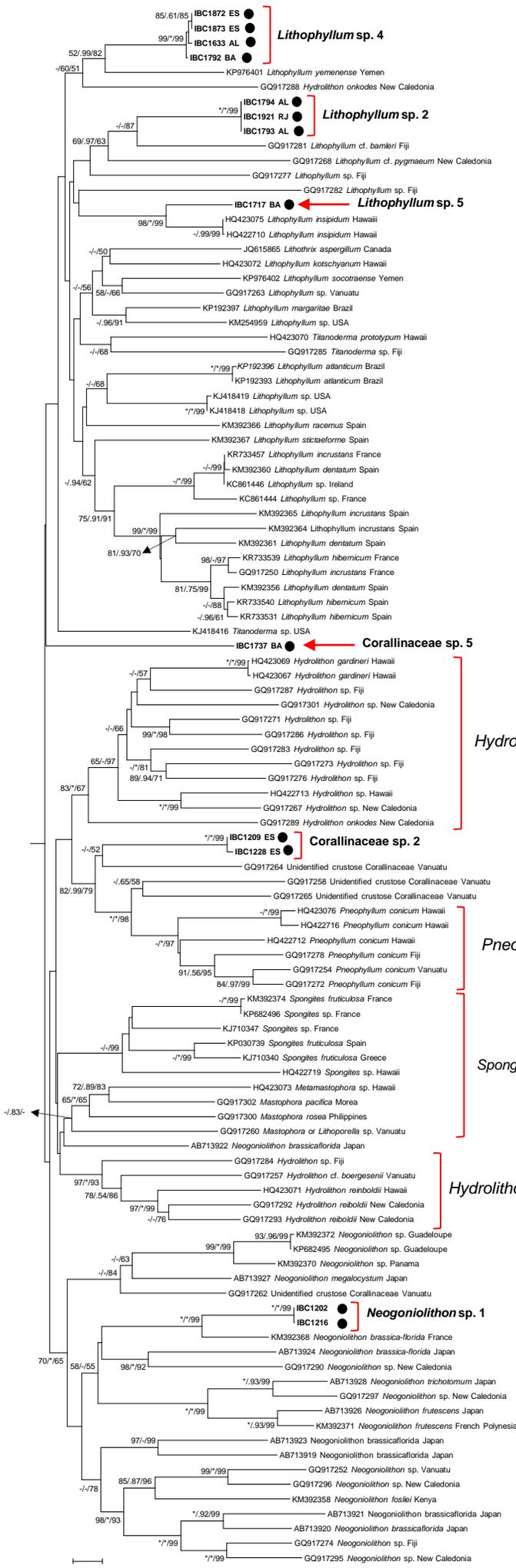
Corallinales

Hapalidiales

Sporolithales

Outgroup

Fig. 8. Backbone of the phylogram based on ML analysis of COI-5P sequences. The tree is divided into the three orders of CCA. Newly generated sequences shown with a black bullet points and sequences from Genbank are shown in red bullet points. Further trees (Figs. 9-11) will show clades in detail.



Lithophylloideae spp.

Hydrolythion I

Pneophyllum

Spongites/Mastophora

Hydrolythion II

Neogoniolithon spp.

0.01

Fig. 9. Detail of the Corallinales clade from the phylogram based on ML analysis of COI-5P sequences. Node values indicate bootstrap values out of 1,000 replicates from ML, BI and NJ analyses respectively, \* indicates full support. Newly generated sequences shown with a bullet point in bold.

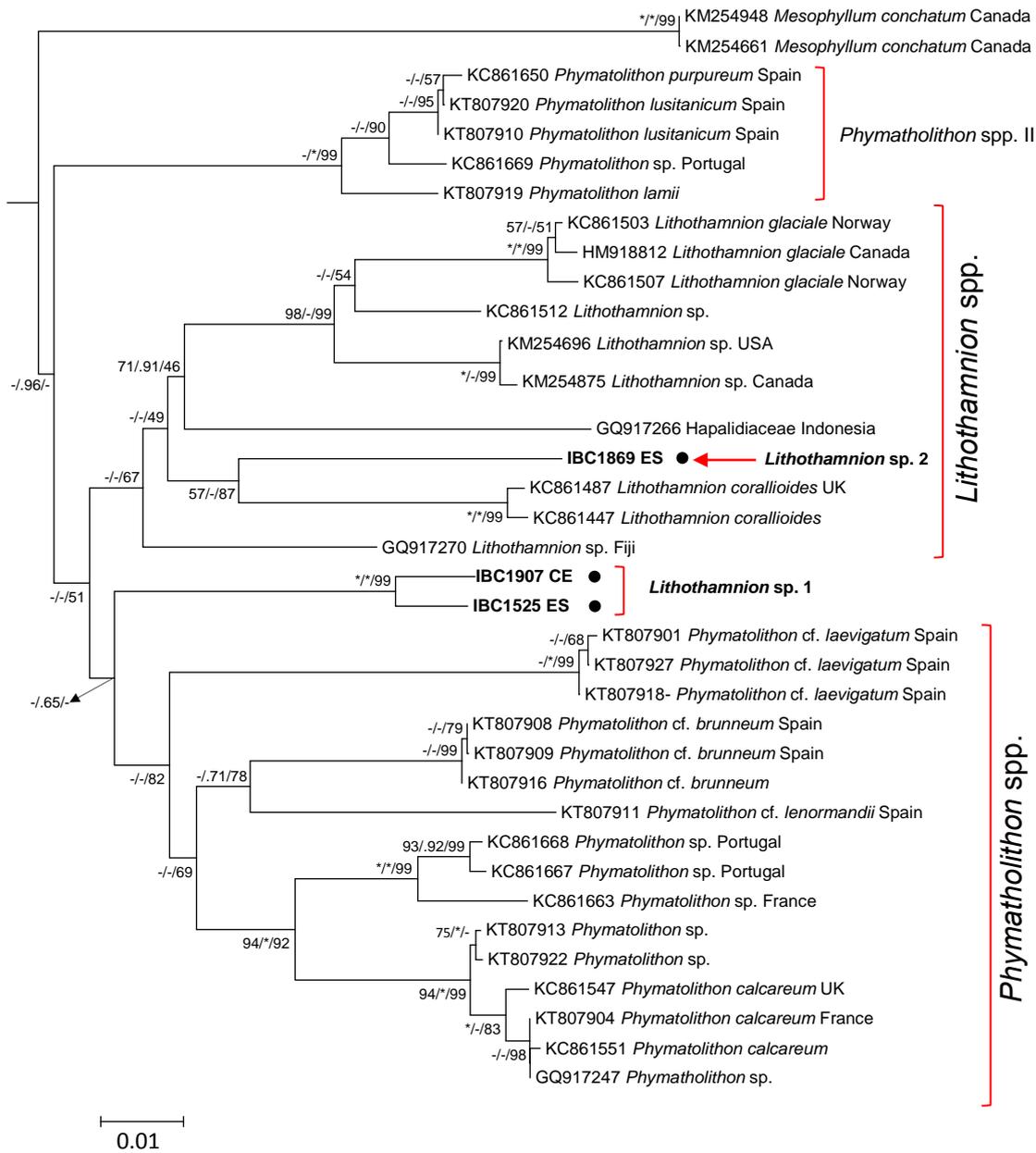


Fig. 10. Detail of the Haplidiales clade I from the phylogram based on ML analysis of COI-5P sequences. Node values indicate bootstrap values out of 1,000 replicates from ML, BI and NJ analyses respectively, \* indicates full support. Newly generated sequences shown with a bullet point in bold.

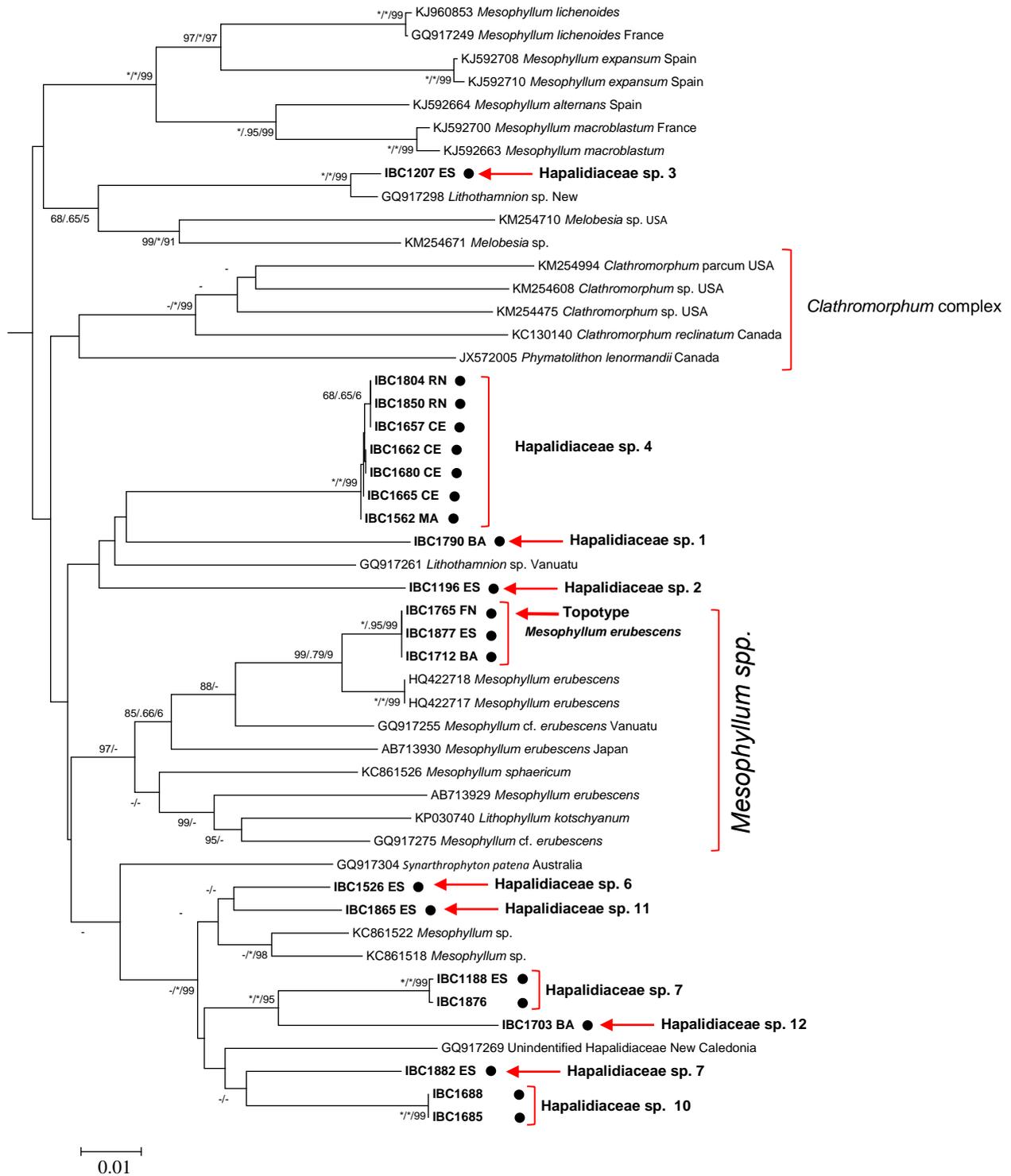


Fig. 11. Detail of the Hapalidiales clade II from the phylogram based on ML analysis of COI-5P sequences. Node values indicate bootstrap values out of 1,000 replicates from ML, BI and NJ analyses respectively, \* indicates full support. Newly generated sequences shown with a bullet point in bold.

*psbA*

The general ML tree obtained from *psbA* analysis (Fig. 12) is edited to show the backbone and the overall diversity; black dots at the terminal tips indicate sequences generated in this study while red dots indicate sequences downloaded from Genbank.

Corallinales were not monophyletic and the two clades formed were split into two subtrees (Figs. 13 and 14) for a better visualization. The *Neogoniolithon* clade is shown in Fig. 13 and was formed by two species from Brazil and 10 specimens from the database and received low to moderate support in the BI analysis (0.50), strong support in the NJ analysis (99) and was not supported in the ML analysis. *Neogoniolithon brassica-florida* from Spain grouped with IBC1216 in a clade with strong support (99/1/100 ML, BI and NJ respectively) and also supported as a single species in the ABGD analysis; they present 2.1% of variation but because this specimen (IBC1216) appeared in the COI-5P tree (Fig. 9) and was named as *Neogoniolithon* sp. 1, we have kept the annotation from the previous tree. *Neogoniolithon spectabile* from Mexico grouped with our *Neogoniolithon* sp. 2 (IBC1585) with strong support (99/1/100 ML, BI and NJ respectively), although the ABGD analysis split them into two different entities and they are 4.1% divergent.

The second clade containing species of Corallinaceae (Fig. 14) was not supported in any of the analyses. Corallinaceae sp. 6 (IBC1917) was nested in a clade with species of *Hydrolithon* from the Pacific with strong support in ML and NJ analyses (99 and 99) but no support for BI; the species from Brazil is sister to *Hydrolithon* sp. (GQ917475) from Fiji with strong support in ML and NJ analyses (98 and 96 respectively) but no support for BI (probably due to missing data from the species from Fiji – if we consider the same length for both sequences the divergence is 0.8%); ABGD

analysis supported the two specimens as a single species. Corallinaceae sp. 7 (IBC1537) formed a clade with *Hydrolithon onkodes* from New Caledonia and is close related to this *Hydrolithon* clade I with low support for ML (50) and no support for BI and NJ; the two species grouped with strong support (99/1/100 ML, BI and NJ respectively) and ABGD analysis split them as separate entities; interspecific variation when the sequences were cropped to the same length is 4.3%. Corallinaceae sp. 2 (IBC1228) and Corallinaceae sp. 4 (IBC1222) were nested in a clade with no support with *Metagoniolithon* spp. and *Pneophyllum* spp. The two species were sister to each other in a clade with strong support for ML and NJ analyses (99 and 100 respectively) and showed 9.3% interspecific variation.

Corallinaceae sp. 8 (IBC1798 and IBC1919) is nested in a second clade with *Hydrolithon* species also from the Pacific. This clade received moderate support in the ML analysis (67) and strong to moderate support in BI and NJ (.94 and 72 respectively). The specimens IBC1798 and IBC1919 (Corallinaceae sp. 8) clustered with strong support (99/1/98 ML, BI and NJ respectively) and showed only 0.3% divergence; ABGD also supported them as a single entity. Corallinaceae sp. 9 (IBC1752), Corallinaceae sp. 1 (IBC1247) and Corallinaceae sp. 5 (IBC1737) formed a clade with moderate to strong support (82/1/86 ML, BI and NJ respectively) although the three taxa represent different entities, with Corallinales sp. 1 shown as being sister to Corallinales sp. 5 with moderate support for ML (78) and no support for BI and NJ; the three species are 9.2-11.8% different from each other.

The Lithophylloideae clade is monophyletic, but with no support; *Lithophyllum* sp. 2 (IBC1793, IBC1795 and IBC1921) comprised a strongly supported clade (99/1/100 ML, BI and NJ respectively) with 0.2% of intraspecific variation and was sister to a clade of *Lithophyllum* sp. from USA with low to moderate support for ML

and no support for BI and NJ. *Lithophyllum* sp. 4 (IBC1507, IBC1872 and IBC1873) comprised a strongly supported clade (99/1/100 ML, BI and NJ respectively) with no intraspecific variation and was sister to a clade formed by *Titanoderma* sp. from USA with no support from ML, BI and NJ. *Lithophyllum* sp. 3 (IBC1726) was also nested in the larger Lithophylloideae clade and was sister to *Lithophyllum margaritae* from Mexico with strong support (99/1/100 ML, BI and NJ respectively); the two specimens are 1.9% divergent, although they are delimited as a single species by ABGD, but for now we are going to keep the annotation from *rbcL*-3P results and maintain IBC1726 as *Lithophyllum* sp. 3 in the tree. *Lithophyllum atlanticum* (IBC1527) was also nested in the larger Lithophylloideae clade and was sister to another *Lithophyllum margaritae* also from Mexico with strong support (99/1/100 ML, BI and NJ respectively) but showed interspecific variation of 5.5%.

The Hapalidiales in the analyses for *psbA* were monophyletic but there is no support for the deeper nodes (Fig. 15). Hapalidiaceae sp. 4 comprised a cluster with strong support (99/1/100 ML, BI and NJ respectively) that was composed of IBC1246, IBC1562, IBC1662, IBC1680, IBC1710, IBC1804 and IBC1909, with intraspecific variation of 0.2-0.3% and supported by ABGD; the species is sister to Hapalidiaceae sp. 1 (IBC1790) and the two species diverge 7.2-7.3%.

Hapalidiaceae sp. 9, Hapalidiaceae sp. 13, Hapalidiaceae sp. 7, Hapalidiaceae sp. 12, Hapalidiaceae sp. 10, Hapalidiaceae sp. 6 and Hapalidiaceae sp. 8 formed a strongly supported clade (97/0.95/100 ML, BI and NJ respectively) with *Lithothamnion*, *Mesophyllum* and an unidentified species from the North Atlantic and Pacific. Hapalidiaceae sp. 9 (IBC1886) grouped with *Lithothamnion* sp. (KJ710353) from France in a clade with strong support (98/1/90 ML, BI and NJ respectively) and with 16.4% divergence between each other, although the Genbank sequence is shorter due to

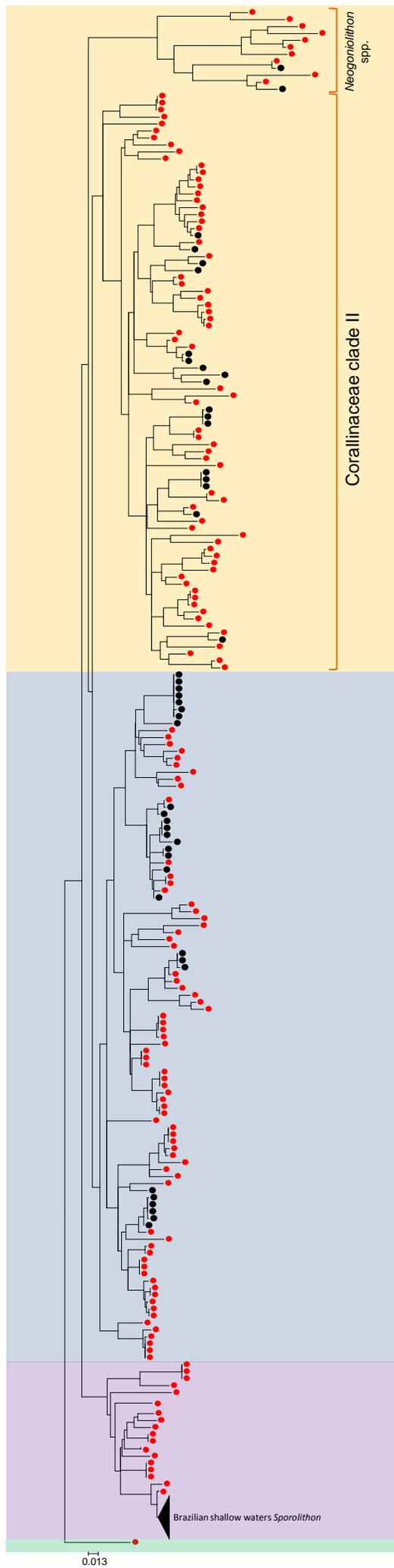
missing data and when cropped to the same length, the divergence between the two sequences is only 0.4%; Hapalidiaceae sp. 13 (IBC1553) was sister to this clade also with strong support (98/1/99 ML, BI and NJ respectively) and was 1.3% different from Hapalidiaceae sp. 9 (IBC1886) and 1.2% divergent (when excluding missing data) from Hapalidiaceae sp. from France; nevertheless, ABGD supports the three species as a single entity. Hapalidiaceae sp. 7 (IBC1245, IBC1547 and IBC1882) showed intraspecific variation of 0.1-0.2% and was sister to Hapalidiaceae sp. 12 (IBC1703) with moderate to strong support for ML and BI (86 and .96 respectively) and no support for NJ and showed 5.2% of interspecific variation. Hapalidiaceae sp. 10 (IBC1685 and IBC1688) is an independent clade with no intraspecific variation. Hapalidiaceae sp. 6 is also nested in this clade and is more closely related to Hapalidiaceae sp. 8. Hapalidiaceae sp. 8 (IBC1869) grouped with *Mesophyllum* spp. from Spain and Portugal in a clade with low to moderate support for NJ (64) and no support for ML and BI; the four specimens in this clade were 1.2-2% divergent and ABGD supports them as a single entity.

*Mesophyllum erubescens* (IBC1712, IBC1765 and IBC1877) grouped with a species from Brazil with the same epithet from Genbank (KM983046) and shows 0.2% intraspecific variation; ABGD also delimited them being the same species.

*Lithothamnion* sp. 1 and *Lithothamnion* sp. 2 were nested in a clade with *Phymatholithon* spp. and *Lithothamnion* spp. Hapalidiaceae sp. 11 (IBC1865) was sister to a species of *Lithothamnion* from Fiji (GQ917461) with no support in any analysis, and with an interspecific variation of 7.2% (when missing data were excluded). *Lithothamnion* sp.1 comprised the specimens IBC1704, IBC1708, IBC1907, IBC1910 and IBC1525 within a clade with strong support (87/1/99 ML, BI and NJ respectively) and with intraspecific variation of 2.3%; ABGD supported the four specimens as a

single species. *Lithothamnion* sp. 1 was sister to an unidentified Hapalidiaceae specimen (JQ896242) from Mexico with strong support (99/1/100 ML, BI and NJ respectively) with interspecific variation of 3.9-4.4%.

The Sporolithales were represented in the general tree for *psbA*, but the results for this order will be presented in the next chapter; as explained previously in the COI-5P section.



**Corallinales**

**Hapalidiales**

**Sporolithales**

**Outgroup**

Fig. 12. Backbone of the phylogram based on ML analysis of *psbA* sequences. The tree is divided into the three orders of CCA. Newly generated sequences shown with a black bullet points and sequences from Genbank are shown in red bullet points. Further trees (Figs. 13-15) will show clades in detail.

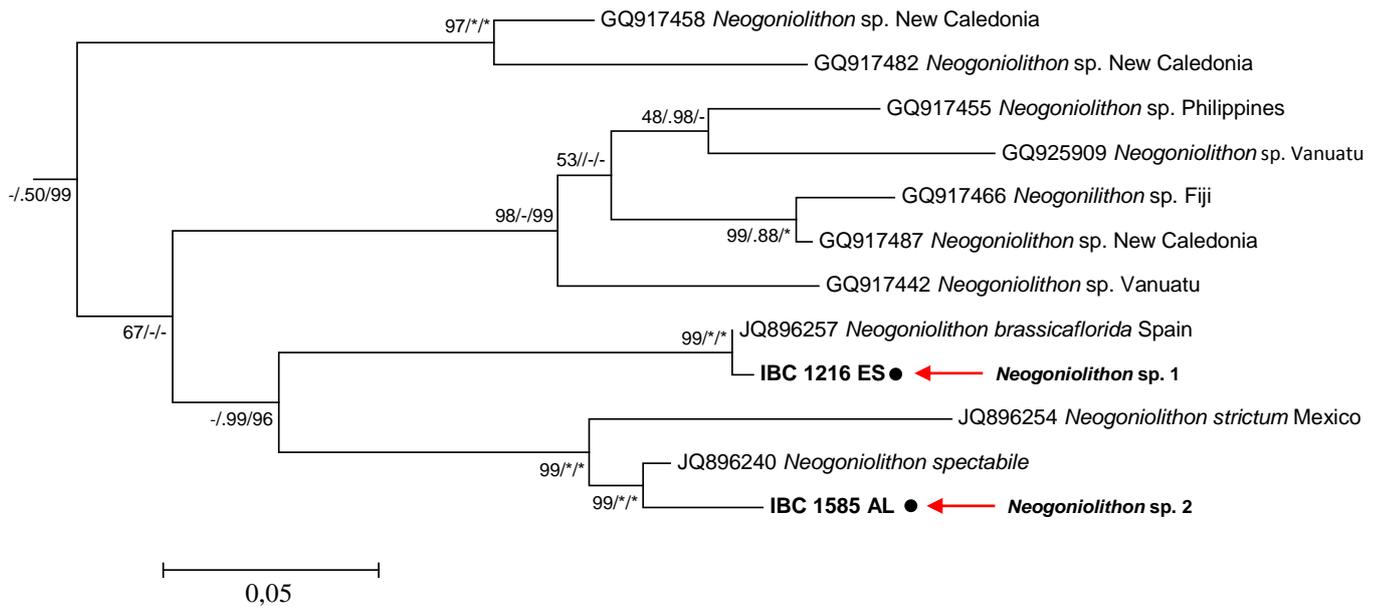


Fig. 13. Detail of the Corallinales – *Neogoniolithon* clade from the phylogram based on ML analysis of *psbA* sequences. Node values indicate bootstrap values out of 1,000 replicates from ML, BI and NJ analyses respectively, \* indicates full support. Newly generated sequences shown with a bullet point in bold.

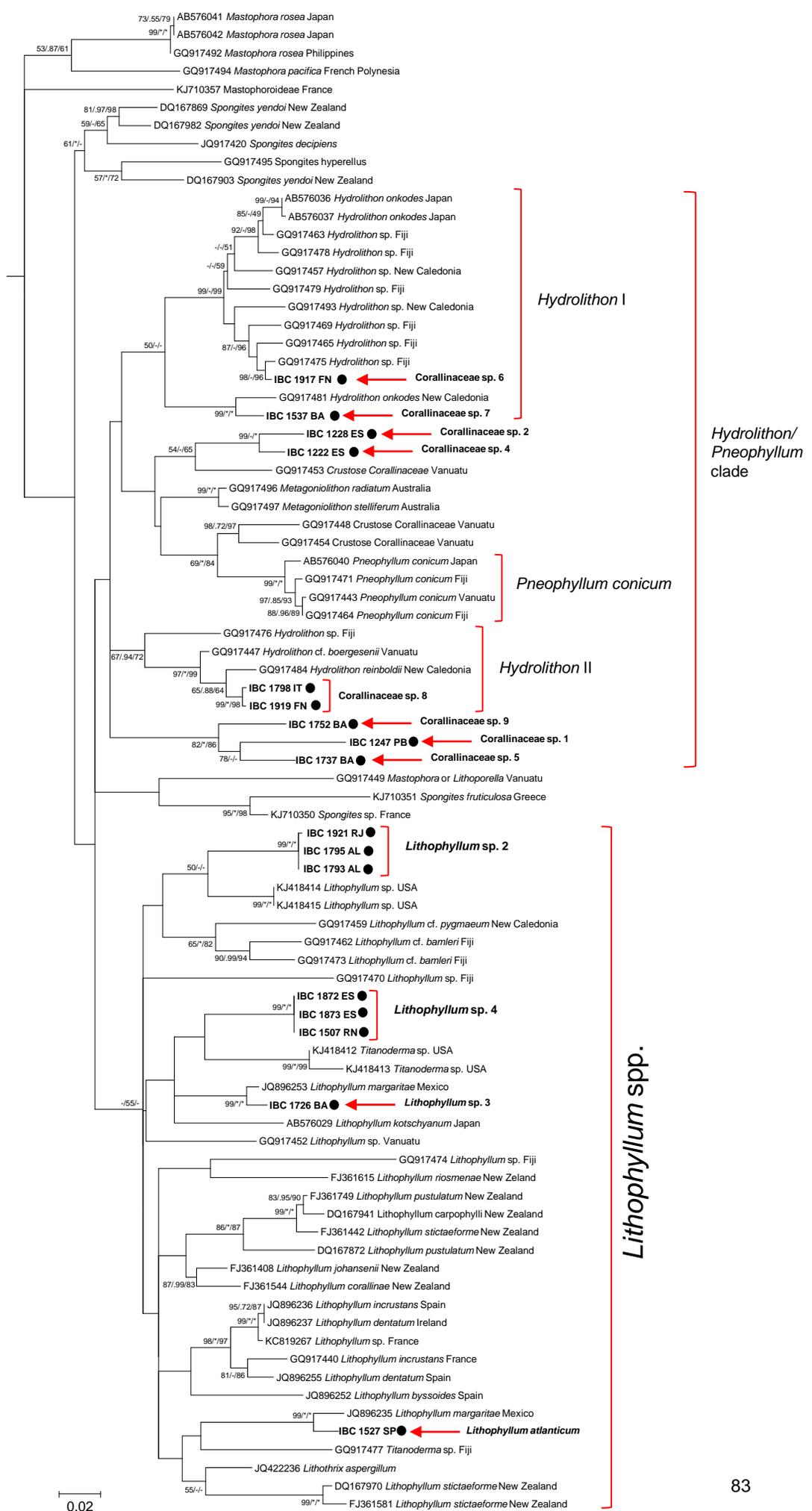


Fig. 14. Detail of the Corallinales clade II from the phylogram based on ML analysis of *psbA* sequences. Node values indicate bootstrap values out of 1,000 replicates from ML, BI and NJ analyses respectively, \* indicates full support. Newly generated sequences shown with a bullet point in bold.

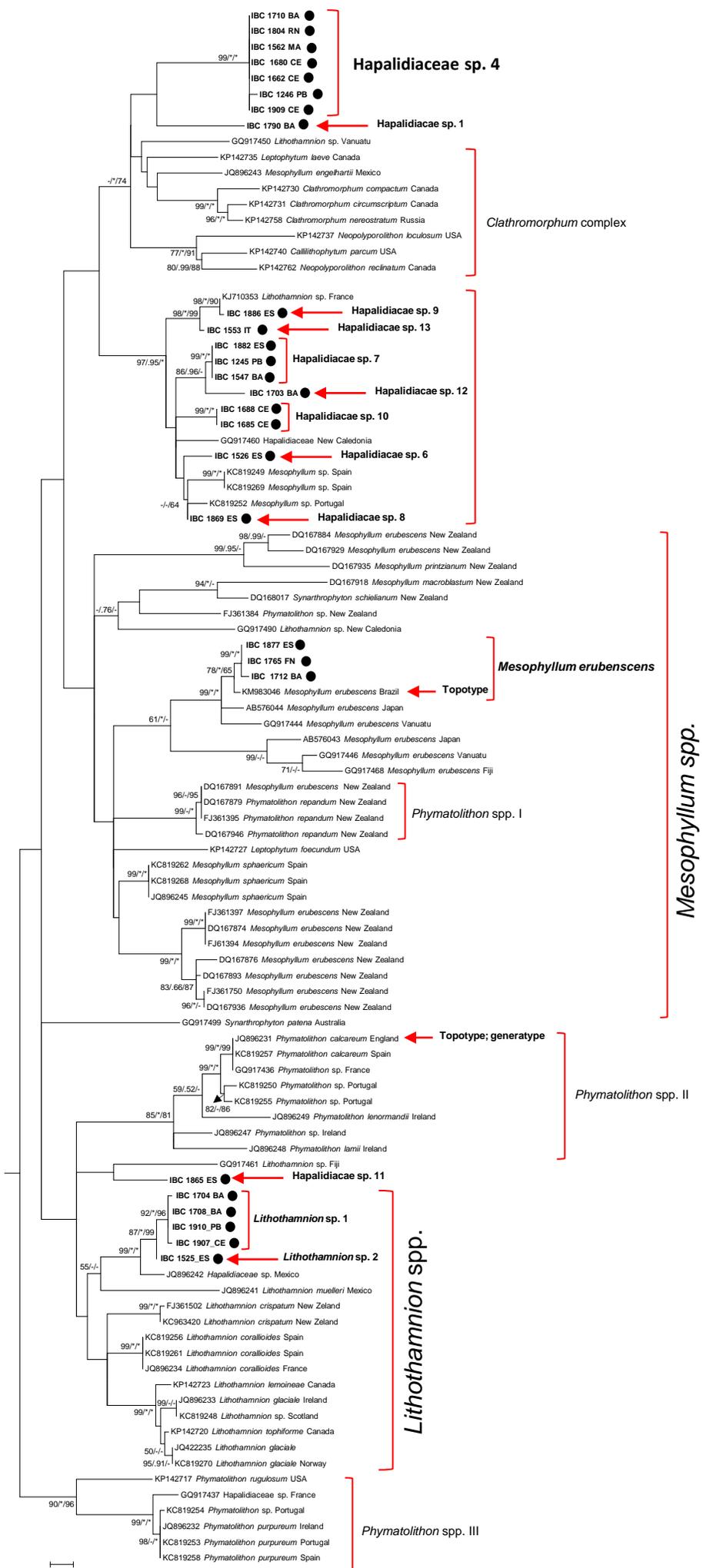


Fig. 15. Detail of the Hapalidiales from the phylogram based on ML analysis of *psbA* sequences. Node values indicate bootstrap values out of 1,000 replicates from ML, BI and NJ analyses respectively, \* indicates full support. Newly generated sequences shown with a bullet point in bold.

### ***Morphological and Anatomical results***

Here in we show the morphological and anatomical results obtained in this study. Most part of the SEM images was generated at UL Lafayette in collaboration with Dr. Suzanne Fredericq and Dr. Joseph Richards.

All specimens analyzed in this study were non-parasitic, calcified and showed non-geniculate thalli. The samples were first analyzed using molecular markers and separated in the different molecular clades (i.e. species). We tried to obtain good images to least one specimen of each different clade in order to cover the anatomical diversity of CCA along the Brazilian coast. Unfortunately, some specimens were very degraded, or had only one specimen (i. e. small pieces) or the images generated were not good or informative enough to be shown, or even all of the above; therefore for some species we could not show anatomical observations.

*Synopsis of studied taxa*

Phylum Rhodophyta

Class Florideophyceae

Subclass Corallinophycideae

Unidentified sp. 1

Unidentified sp. 2

Order Corallinales

Family Corallinaceae

Corallinaceae sp. 1

Corallinaceae sp. 2

Corallinaceae sp. 3

Corallinaceae sp. 4

Corallinaceae sp. 5

Corallinaceae sp. 6

Corallinaceae sp. 7

Corallinaceae sp. 8

Corallinaceae sp. 9

Genus *Lithophyllum* (autores)

*Lithophyllum margaritae*

*Lithophyllum kotschy anum*

*Lithophyllum* sp. 1

*Lithophyllum* sp. 2

*Lithophyllum* sp. 3

*Lithophyllum* sp. 4

*Lithophyllum* sp. 5

Genus *Neogoniolithon* (autores)

*Neogoniolithon* sp. 1

*Neogoniolithon* sp. 2

Phylum Rhodophyta

Class Florideophyceae

Subclass Corallinophycideae

Order Hapalidiales

Family Hapalidiaceae

Hapalidiaceae sp. 1

Hapalidiaceae sp. 2

Hapalidiaceae sp. 3

Hapalidiaceae sp. 4

Hapalidiaceae sp. 5

Hapalidiaceae sp. 6

Hapalidiaceae sp. 7

Hapalidiaceae sp. 8

Hapalidiaceae sp. 9

Hapalidiaceae sp. 10

Hapalidiaceae sp. 11

Hapalidiaceae sp. 12

Hapalidiaceae sp. 13

Genus *Mesophyllum*

*Mesophyllum erubescens*

Genus *Lithothamnion*

*Lithothamnion* sp.1

*Lithothamnion* sp.2

Phylum Rhodophyta

Class Florideophyceae

Subclass Corallinophycideae

Order Sporolithales

Family Sporolithales

Genus *Sporolithon*

*Sporolithon pseudoepisporum*

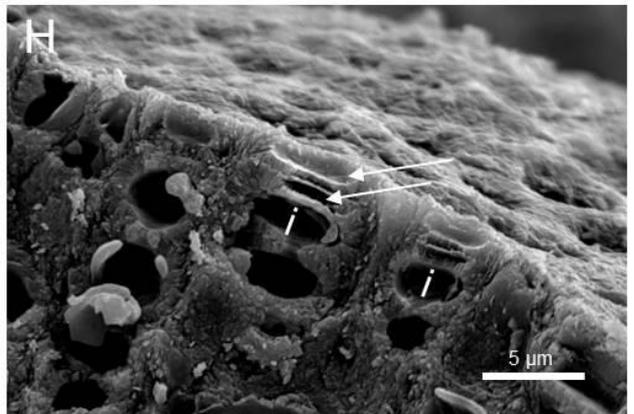
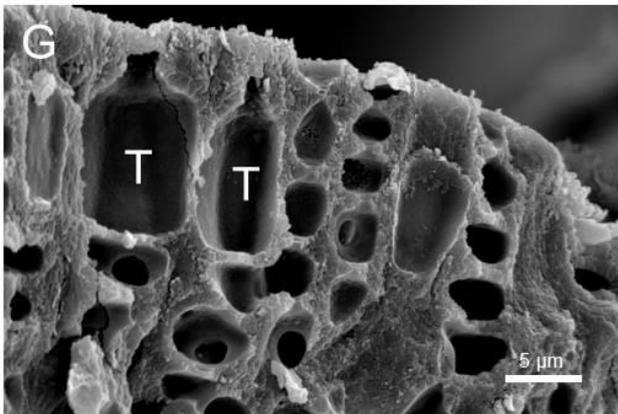
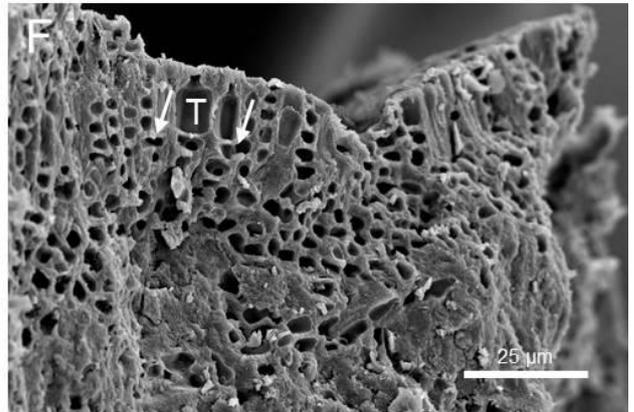
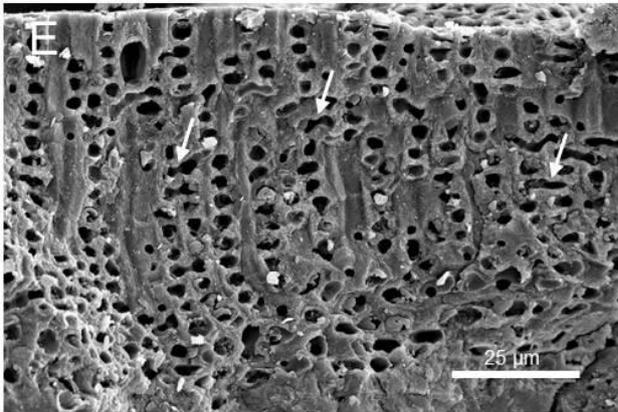
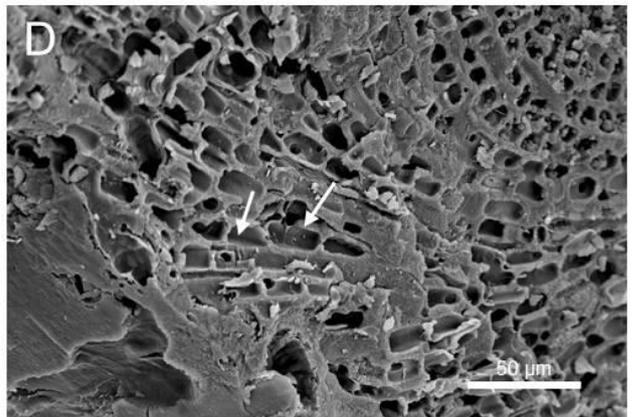
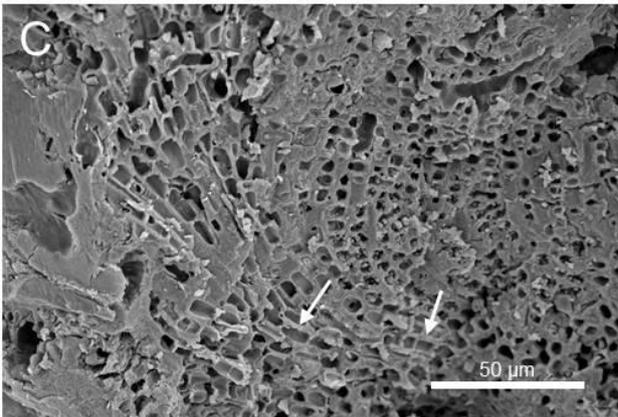
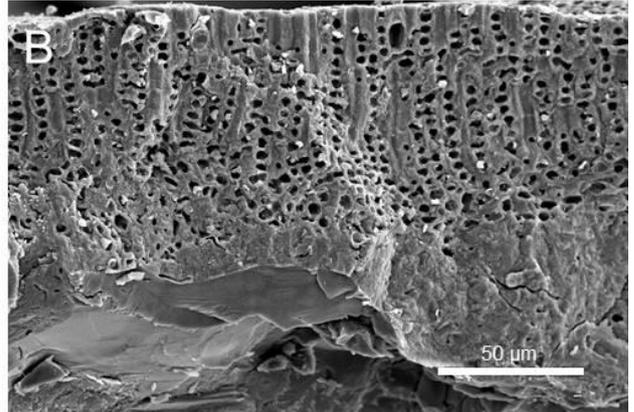
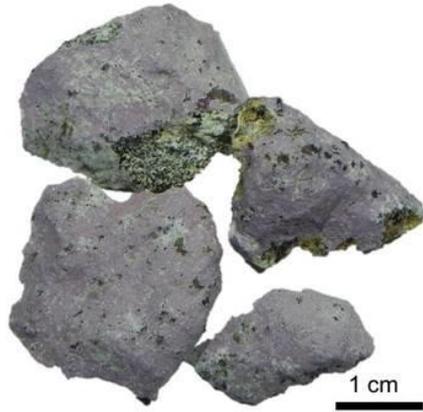
*Sporolithon tomitae*

*Corallinaceae sp. 2*

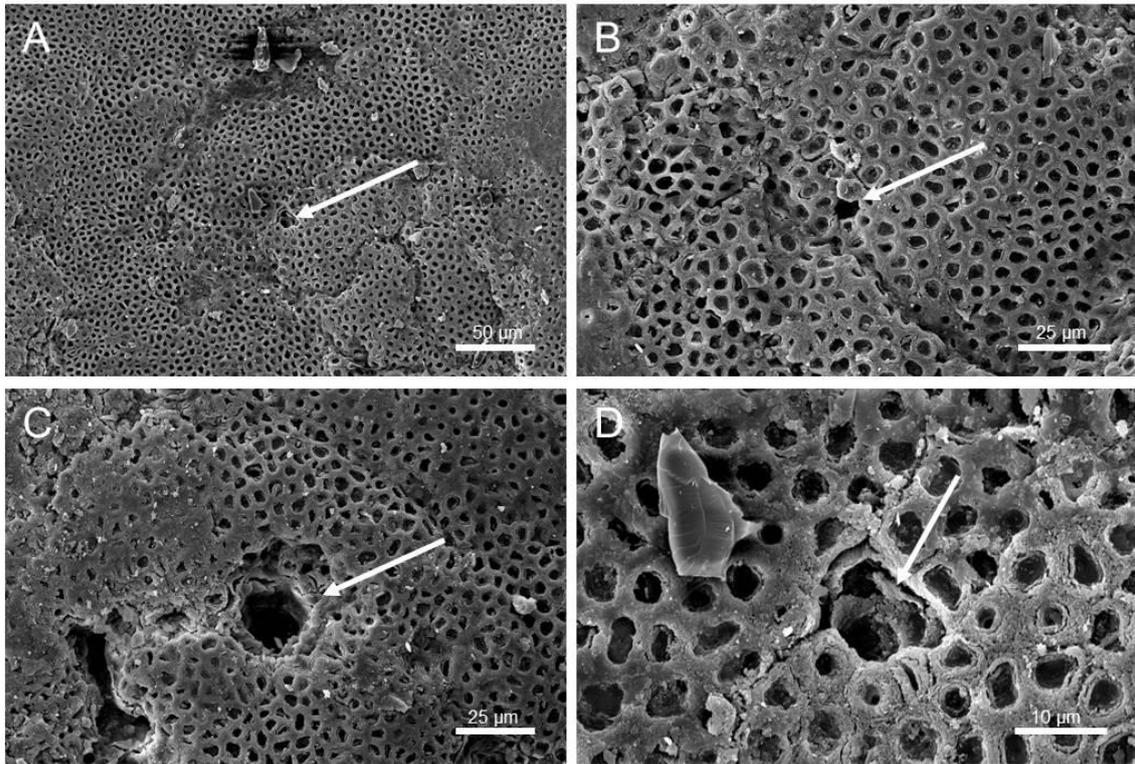
Specimen IBC1228 (Fig. 16A) was collected in Espírito Santo state, growing attached to a rock in the intertidal (for more collection information see Tab.3). Thallus encrusting, smooth with no protuberances forming a very thin crust; color is pale purple with white spots (Fig. 16A). Thallus construction consists of a multilayered hypothallus comprised of hypothallial filaments (Figs. 16B-D) with rectangular-shaped cells growing parallel to the substratum and towards thallus surface (Figs. 16C-D; arrows) and perithallial filaments (Fig. 16E) growing perpendicular to the substratum. Adjacent perithallial filaments linked by cell fusions only (Figs. 16E, 16F, arrows). Trichocytes present (Figs. 16E, 16F, "T") but not found buried. Epithallial cells (Fig. 16H) with two layers, heavily calcified cell walls but not at the roof (roof sloughed-off), with short flattened-shaped lumens (Fig. 16H, arrows); initials cells (Fig. 16H, "i" initial cells) short but at least two times the size of the epithallial cells. Uniporate conceptacles (Figs. 17A-17D; arrows) were observed from surface view and were not possible to be interpreted as being tetrasporangial or gametangial.

Distribution: This species was only found in Espírito Santo state (for more collection information see Tab.3).

A



**Figure 16. Corallinaceae sp. 2; specimen IBC1228 – External morphology and vegetative features.** **A.** Thallus habit showing a smooth thin crust. **B-D.** Fracture showing thallus construction of the hypothallus with rectangular-shaped cells (arrows). **E-F.** Adjacent perithallial filaments linked by cell fusions only (arrows); trichocytes present (T). **G.** Detail of the trichocytes (T). **H.** Epithallial cells flattened and possess two layers (arrows); initials cells short but at least two times the size of the epithallial cells (i).

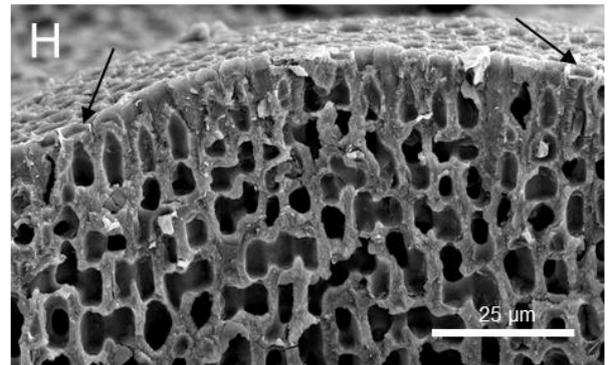
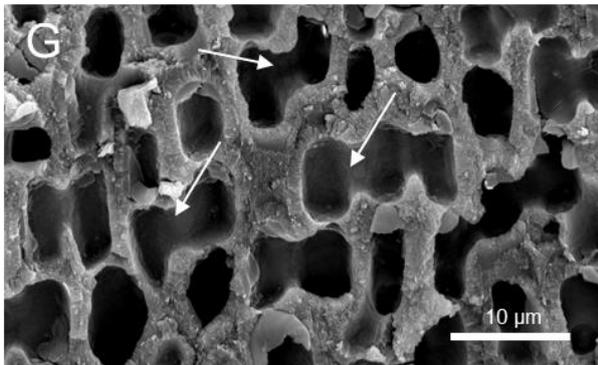
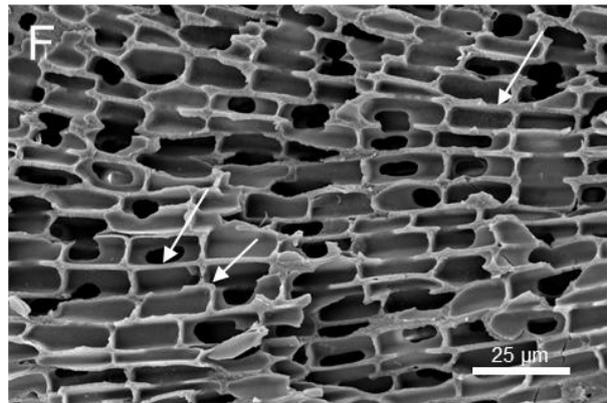
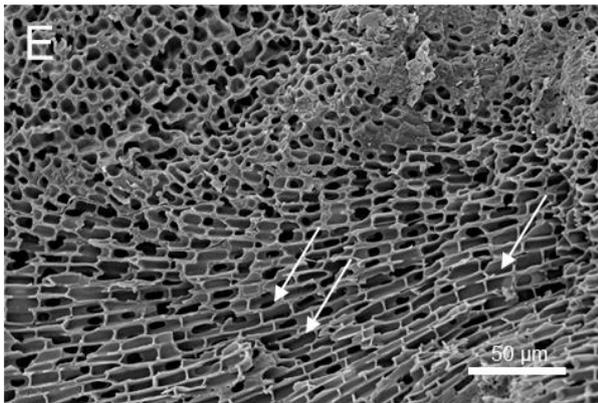
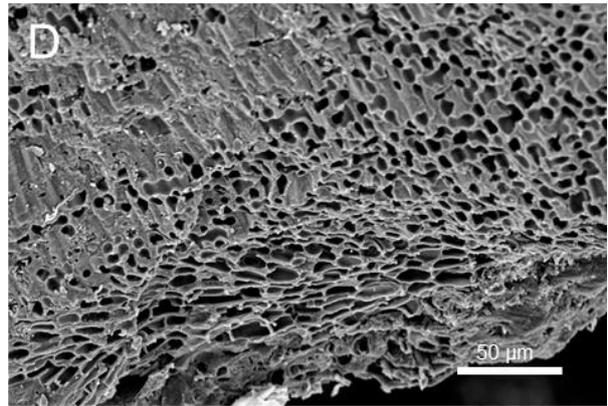
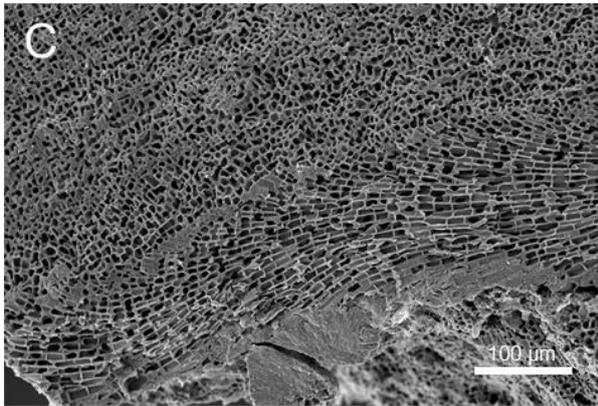
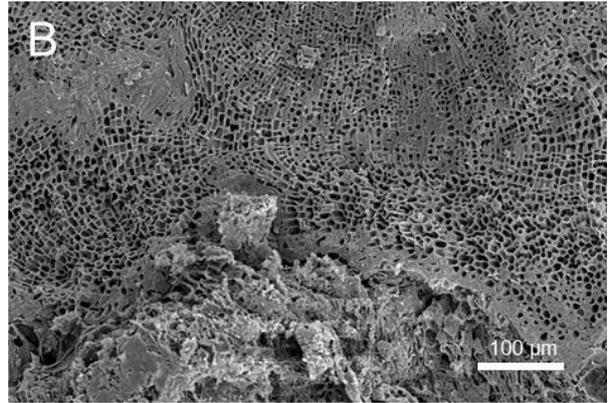


**Figure 17. Corallinaceae sp. 2; specimen IBC1228 – Reproductive features. A-D.**  
Uniporate conceptacles (arrows) observed from surface view.

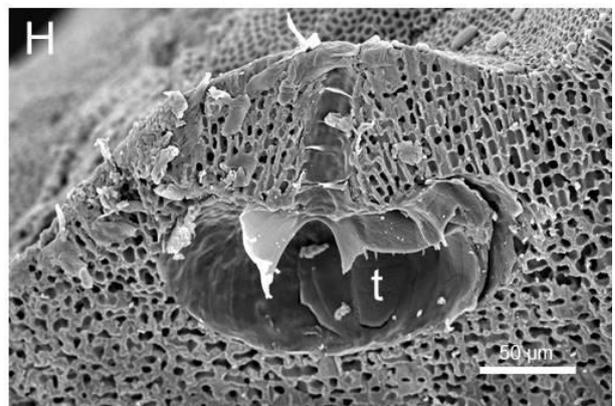
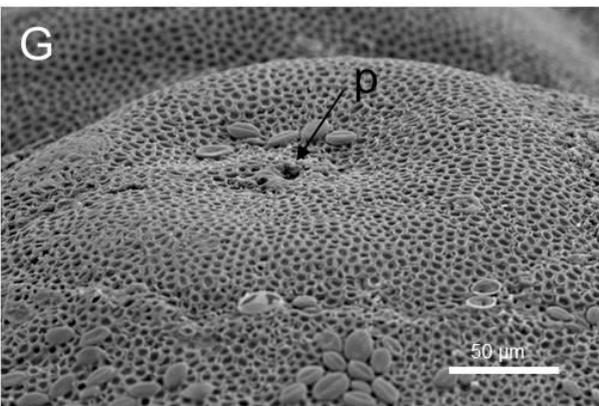
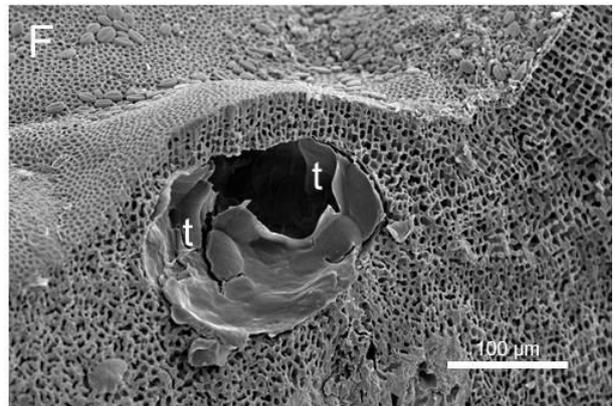
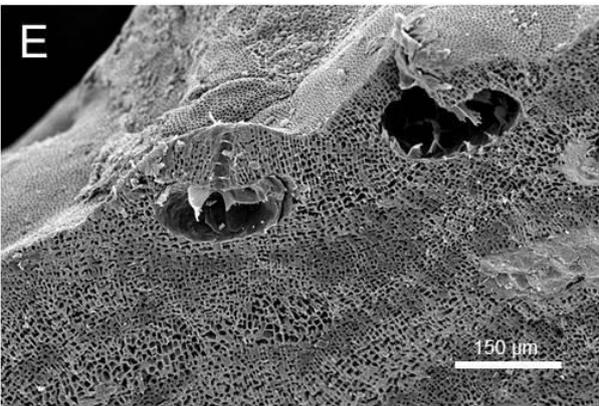
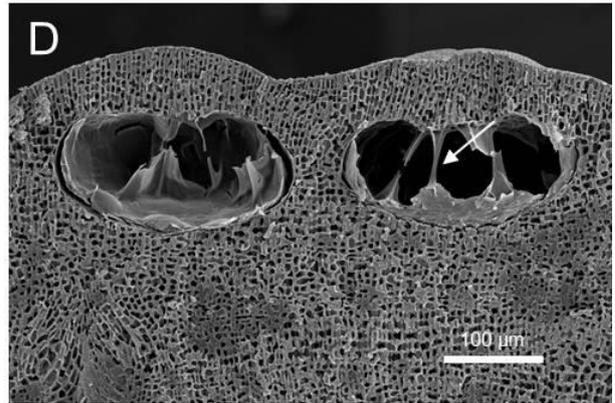
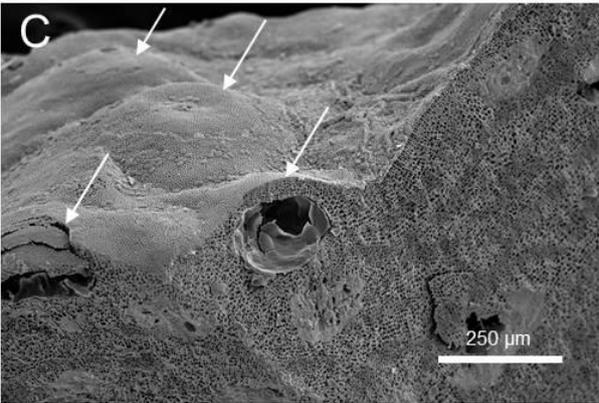
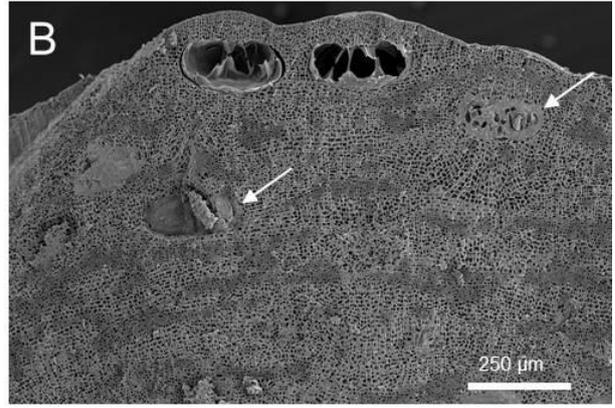
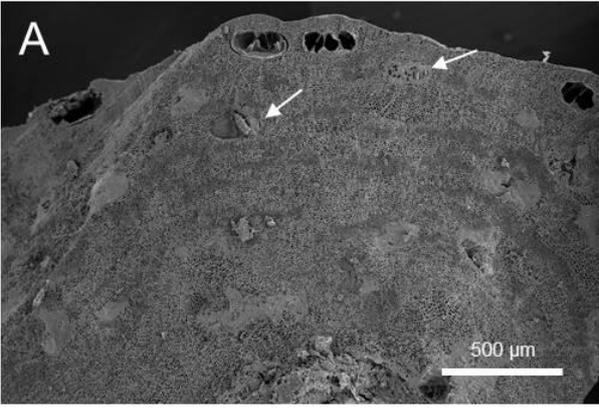
*Corallinaceae sp. 4*

Specimen IBC1222 was collected in Espírito Santo state in the intertidal, growing detached from the substratum, as a rhodolith (Fig. 18A) in the intertidal (for more collection information see Tab.3). Thallus lumpy with a few protuberances; color is pale purple to purple with white spots (Fig. 18A). Thallus construction consists of a multilayered hypothallus comprised of hypothallial filaments (Figs. 18B-18D) with rectangular-shaped cells growing parallel to the substratum and towards thallus surface (Fig. 18E, 18F, arrows) and perithallial filaments (Fig. 18E) growing perpendicular to the substratum. Adjacent perithallial filaments linked by cell fusions only (Figs. 18G, arrows). Trichocytes present (data not shown). Epithallial cells with one layer, heavily calcified cell walls but not at the roof (roof sloughed-off), short flattened-shaped lumens (Fig. 18H, arrows). Slightly raised uniporate conceptacles were found at the surface, (Figs. 19A, 19G, 19H; pore “p”), and old conceptacles were found buried throughout the thallus (Figs. 19A-B, arrows). Conceptacles were found usually in clusters of two or more (Fig. 19C, arrows). Vegetative filaments observed filling empty post-spore release conceptacles (Fig. 19D). Uniporate conceptacles were interpreted as being tetrasporangial as some of them showed zonately divided mature tetraspores (Figs. 19F and 19H, tetraspores “t”); single pore channel formed by filaments 7-8 cells long (Fig. 19H).

Distribution: This species was only found in Espírito Santo state (for more collection information see Tab.3).



**Figure 18. Corallinaceae sp. 4; specimen IBC1222 – External morphology and vegetative features.** **A.** Thallus habit showing small warty protuberances. **B-D.** Fractures showing a thick thallus and a multilayered hypothallus. **E-F.** Detail of hypothallus showing rectangular-shaped cells (arrows). **G.** Adjacent perithallial filaments linked by cell fusions (arrows). **H.** Epithallial cells with one layer (roof sloughed-off), short flattened-shaped lumens (arrows).

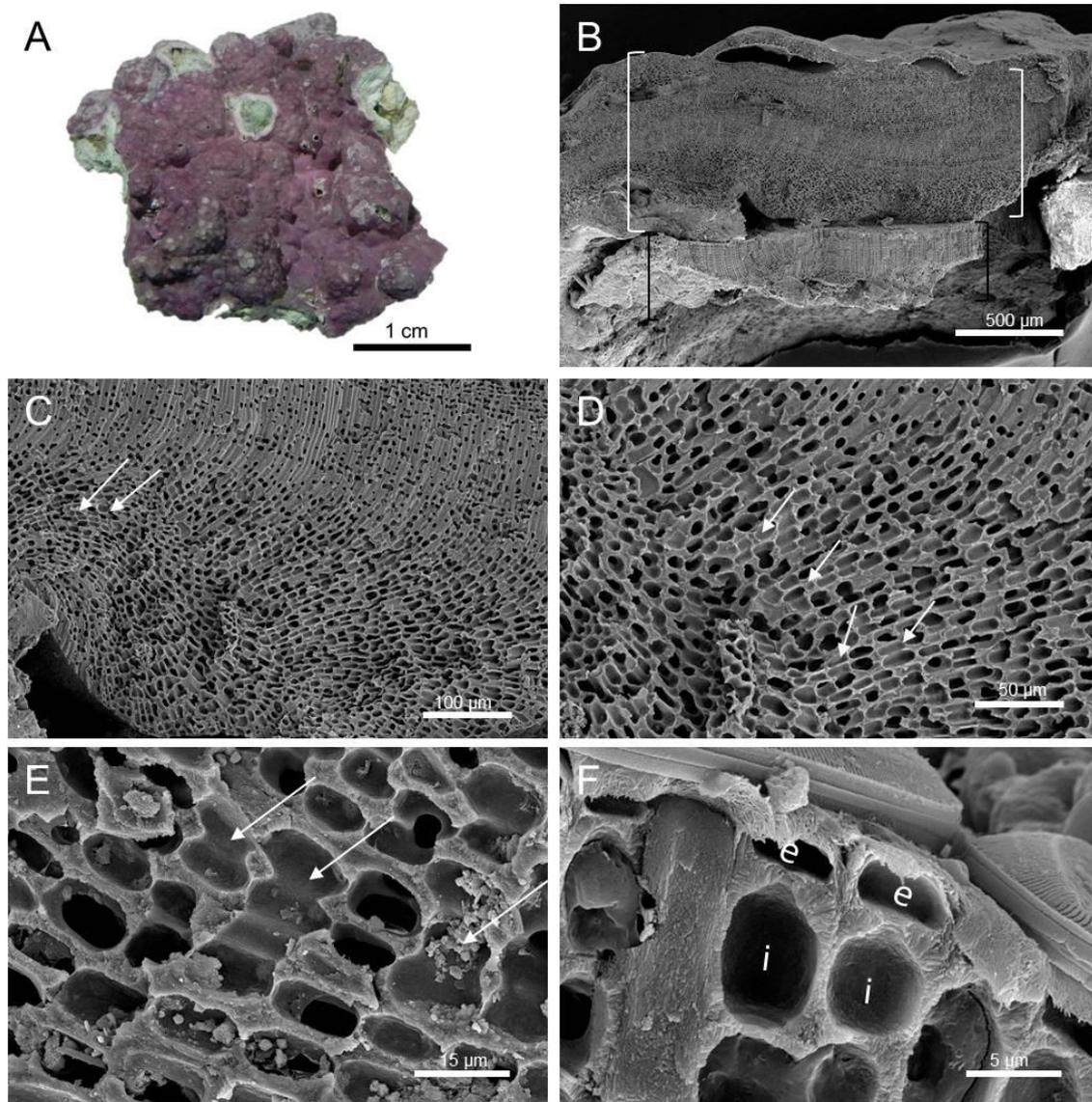


**Figure 19. Corallinaceae sp. 4; specimen IBC1222 – Reproductive features. A-B.** Fracture of the thallus showing new conceptacles at the surface and old buried conceptacles (arrows). **C.** Fracture and partial surface view showing slightly raised conceptacles (arrows). **D.** Fracture showing conceptacles with senescent vegetative cells infill (arrow). **E.** Fracture showing two conceptacles. **F.** Fracture and partial surface view showing a conceptacle with putative tetraspores (t). **G.** Surface view of a conceptacle with a single pore (p). **H.** Fracture showing a uniporate conceptacle with putative tetraspore (t).

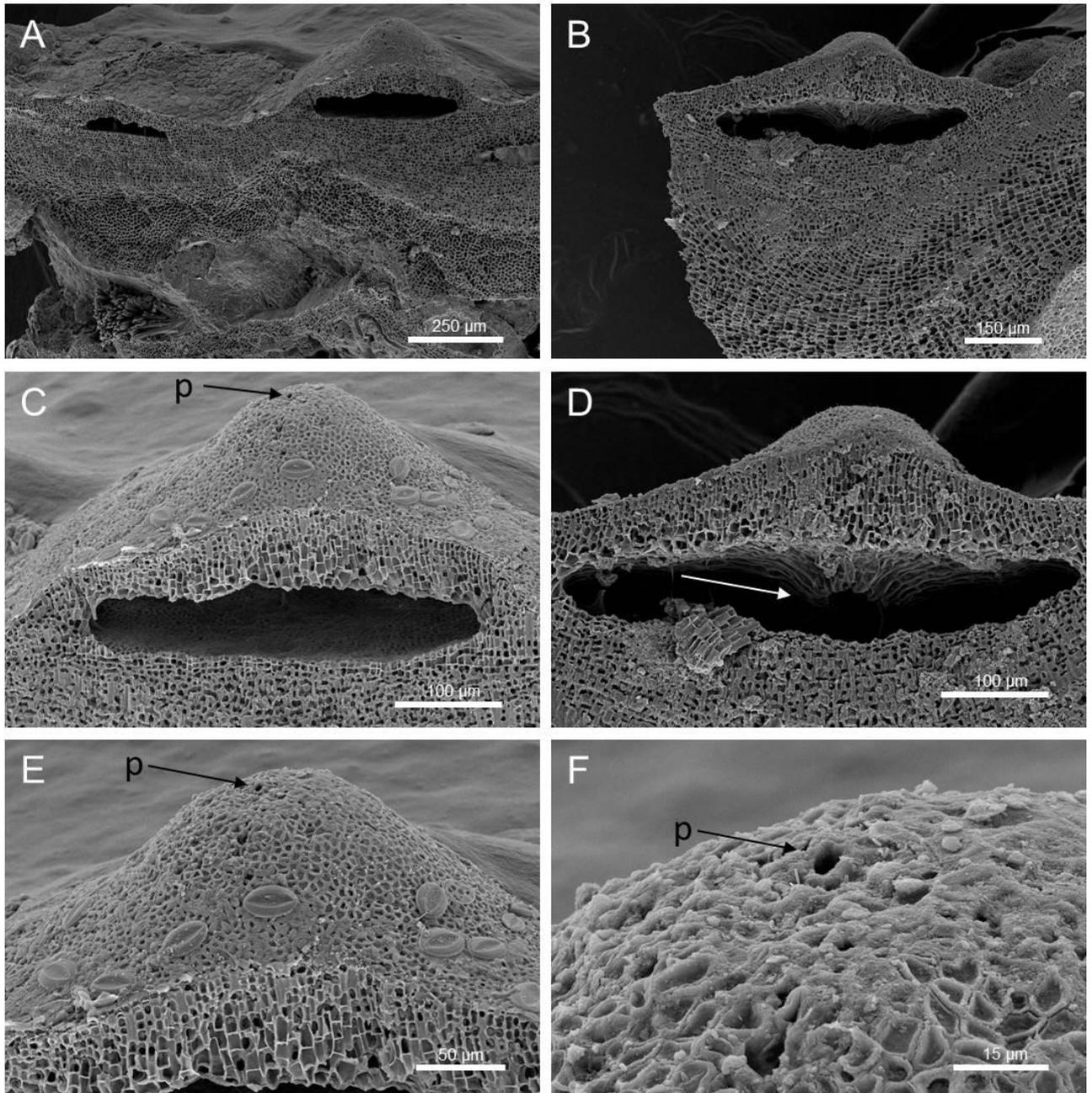
*Corallinaceae sp. 5*

Specimen IBC1737 was collected in Bahia state in the subtidal (6m depth - for more collection information see Tab. 3). Specimen was found growing detached from the substratum, as a rhodolith (Fig. 20A – shows the rhodolith already broken). Thallus external morphology is lumpy with protuberances; color is pink with white spots (Fig. 20A). The species possesses a thick thallus (Fig. 20B, upper white brackets) shown growing over another coralline alga (Fig. 20B, lower black brackets). Thallus construction consists of a multilayered hypothallus comprised of hypothallial filaments with rectangular-shaped cells growing parallel to the substratum and some filaments curving upward towards the thallus surface (Fig. 20C, arrow) and others curving downwards to form to the contours of the substratum (Fig. 20D, arrows) and perithallial filaments (Fig. 20G) growing perpendicular to the substratum. Adjacent perithallial filaments linked by cell fusions only (Figs. 20E, arrows). Trichocytes were not present. Epithallus consisted of a single layered of calcified cells with flattened-shaped lumens (Fig. 20H, “e”-epithallial cell). Initials cells long; at least three times the size of the epithallial cells (Fig. 20H, “i”-initial cells). Raised uniporate conceptacles were found at the surface, raised (Figs. 21A-21F, “p”- pore) and were empty, therefore not possible to be interpreted as being tetrasporangial or gametangial; pore channel projects into the conceptacle (Fig. 21D, arrow).

Distribution: This species was only found in Bahia state (for more collection information see Tab.3).



**Figure 20. Corallinaceae sp. 5; Specimen IBC1737 – External morphology and vegetative features.** **A.** Thallus habit showing lumpy protuberances. **B.** Fracture showing a thick thallus (upper white brackets) growing over another coralline alga (lower black brackets). **C-D.** Fractures showing a thick thallus and a multilayered hypothallus rectangular-shaped cells (arrows). **E.** Adjacent perithallial filaments linked by cell fusions only (arrows). **F.** Epithallial cells with short flattened-shaped lumens and one layer (e); initials (i) longer than epithallial cells.

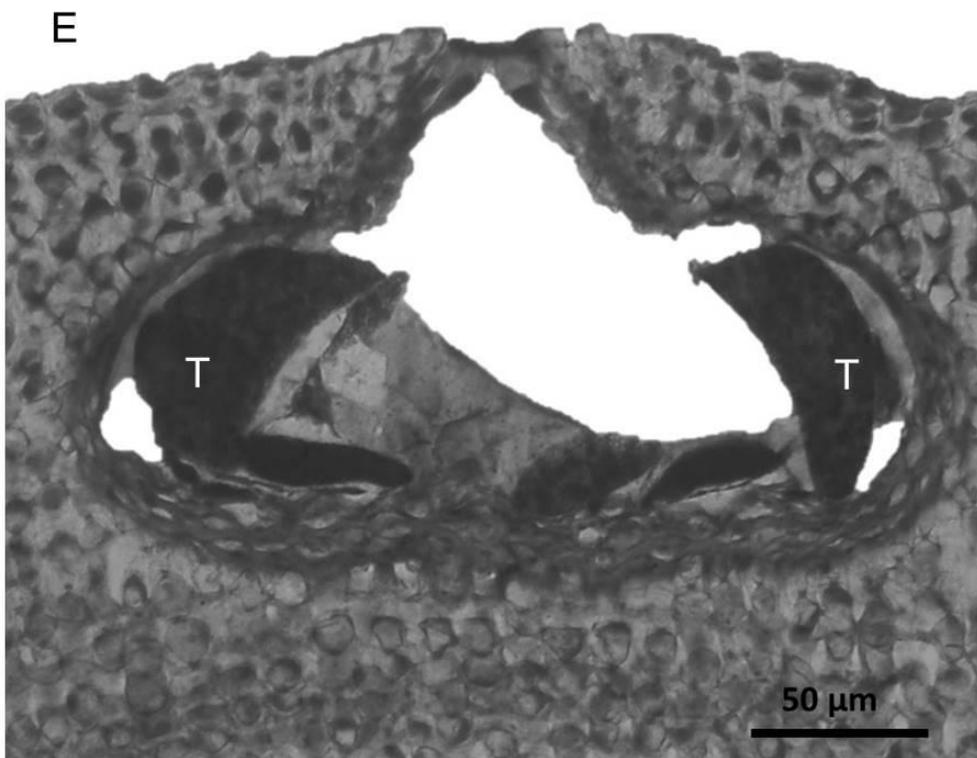
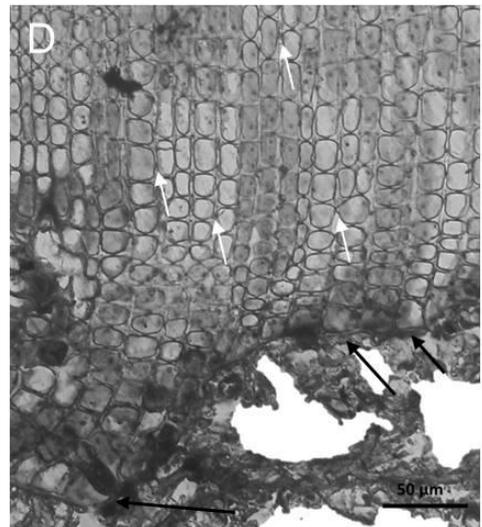
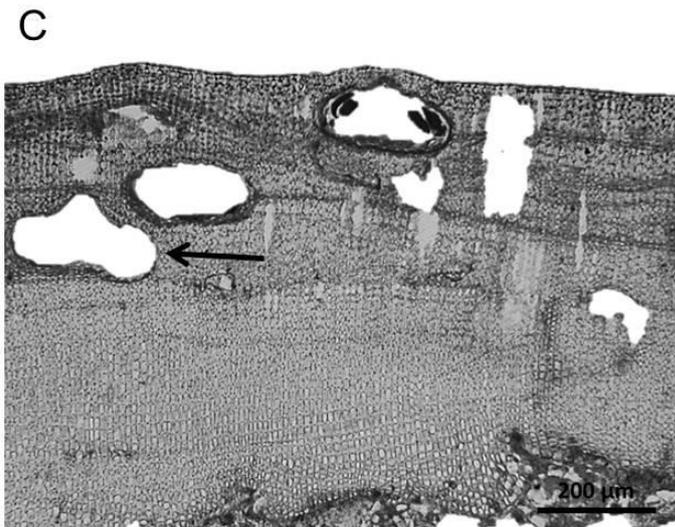
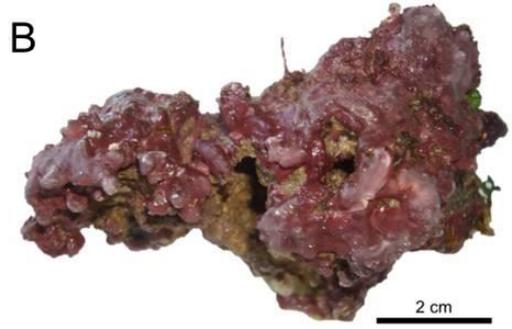
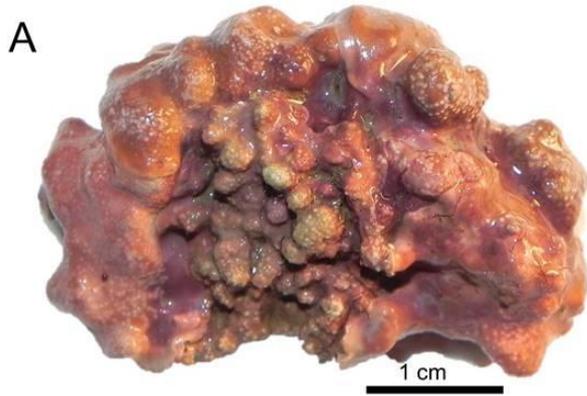


**Figure 21. Corallinaceae sp. 5; specimen IBC1737 – Reproductive features. A-B.** Fractures of the thallus showing new conceptacles at the surface. **C.** Fracture and partial surface view showing conceptacle with raised roof and a single pore (p). **D.** Fracture showing a conceptacles with pore channel that projects into the chamber (arrow). **E.** Superficial view of a conceptacle roof showing a single pore (p). **F.** Detail of the superficial view of a conceptacle roof showing a single tiny pore (p).

*Lithophyllum sp. 3*

Specimen IBC1726 (Fig. 22A) was collected in Bahia state and specimen IBC1878 (Fig. 22B) was collected in Espírito Santo state (for more collection information see Tab.3). Both specimens were collected in the intertidal, growing detached from the substratum as rhodoliths. Thalli smooth to lumpy with protuberances, dark red to purple (Fig. 22A) and smooth to warty, light pink (Fig. 22B). The species possesses a thick thallus (Fig. 22C) and its construction consists of a single layered hypothallus comprised of filaments with squared to rectangular-shaped cells (black arrows) growing parallel to the substratum in the z-axis and perithallial filaments growing towards thallus surface (Figs. 22D, black arrows). Adjacent filaments linked by secondary pit connections only (Figs. 22D, white arrows). Trichocytes were not observed. Epithallial cells with flattened or rounded lumens (Fig. 22E). Uniporate conceptacles were interpreted as being tetrasporangial (Fig. 22E, “t” - tetraspores); buried empty conceptacles present (Fig. 22A- arrow).

Distribution: This species was found in Bahia and Espírito Santo state (for more collection information see Tab.3).

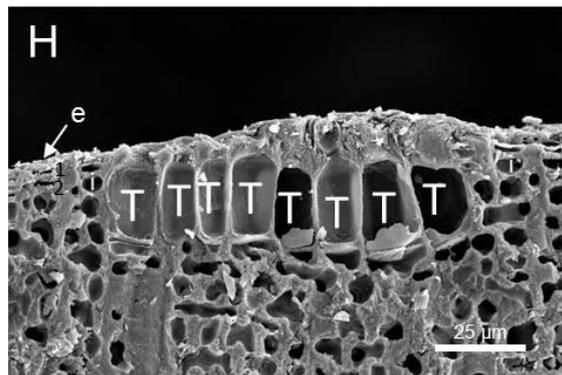
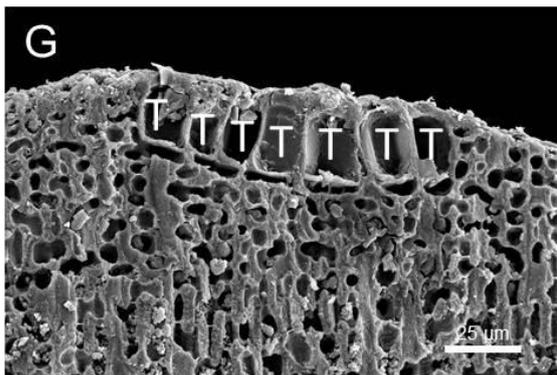
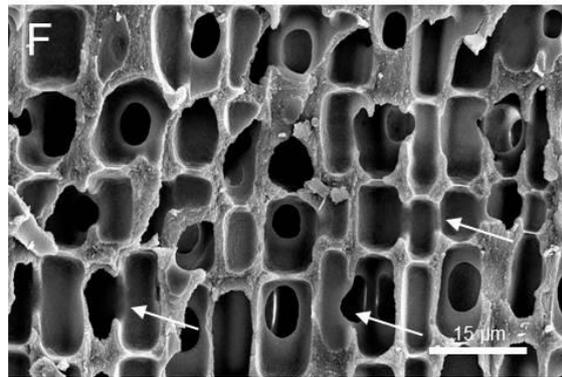
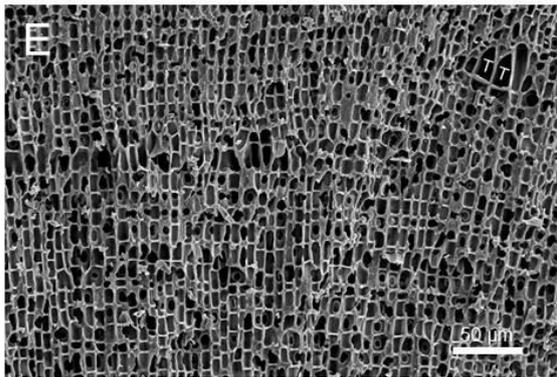
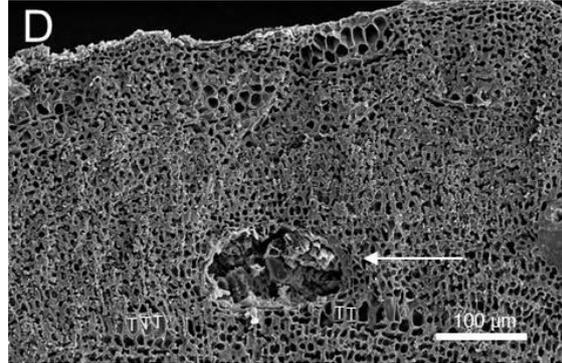
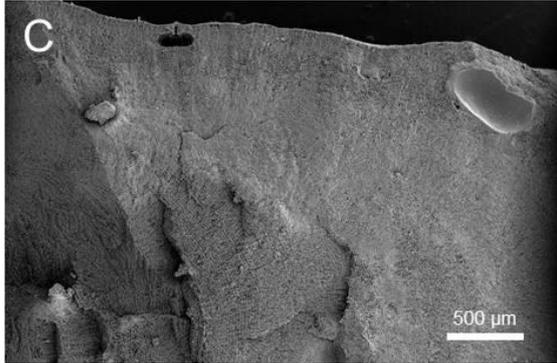
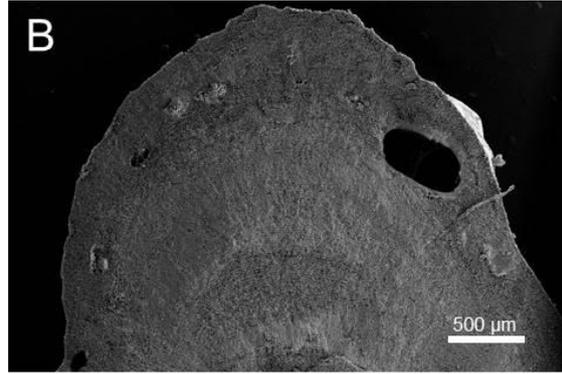
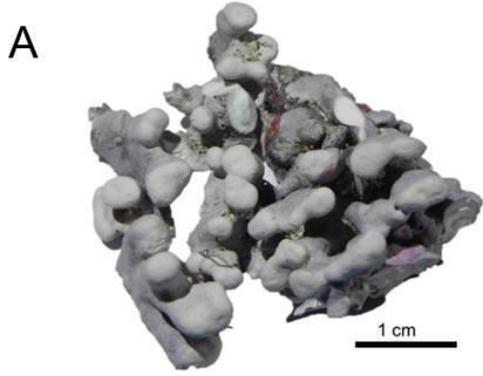


**Figure 22. *Lithophyllum* sp. 3; specimens IBC1726 and IBC1878 - External morphology, vegetative and reproductive features.** **A.** Thallus habit showing warty protuberances. **B.** Thallus habit showing smooth to warty protuberances. **C.** Section showing a thick thallus with new conceptacles and old empty buried conceptacles (arrow). **D.** Section at the hypothallus showing a single layered hypothallus comprised of filaments with squared to rectangular-shaped cells (black arrows) growing parallel to the substratum in the z-axis and perithallial filaments growing towards thallus surface; adjacent perithallial filaments mainly linked by secondary pit connections (white arrows). **E.** Section showing a tetrasporangial uniporate conceptacle; tetraspores (t).

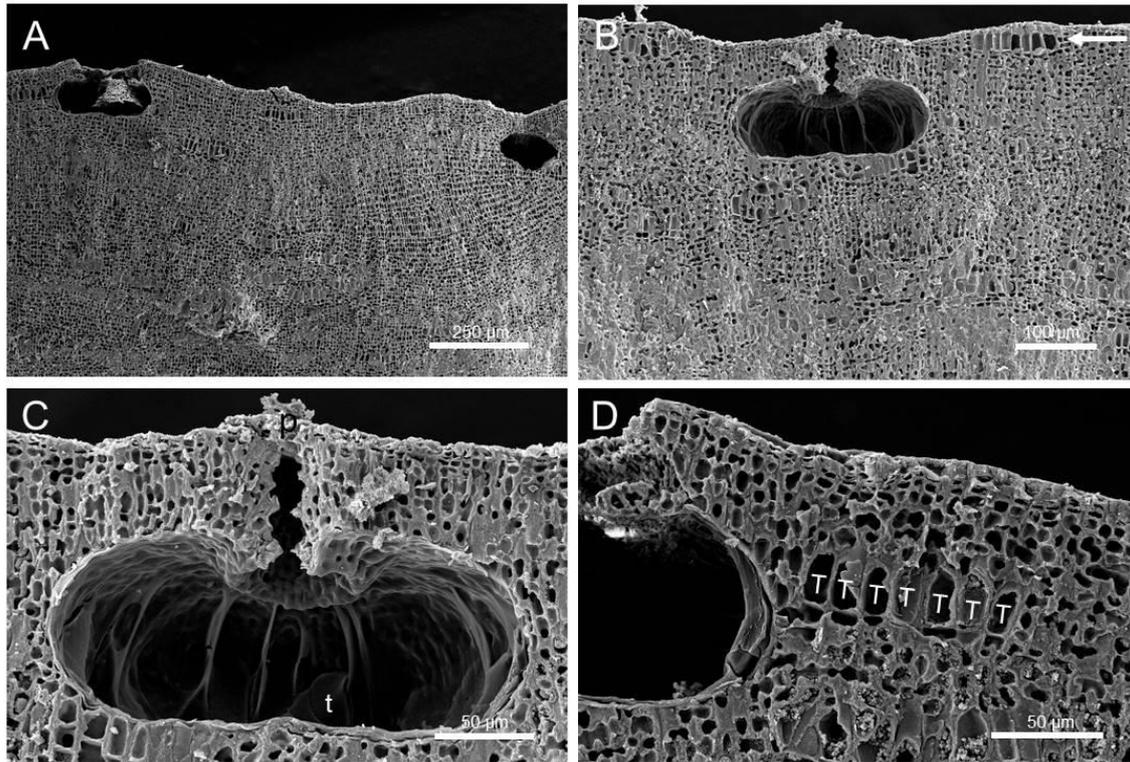
*Corallinaceae sp. 6*

Specimen IBC1917 was collected in Fernando de Noronha Island in the subtidal (10-15m depth - for more collection information see Tab.3). Specimen was found growing detached from the substratum, as a rhodolith (Fig. 23A– shows the rhodolith already broken). Thallus is fruticose; color is pale purple to gray (Fig. 23A). Thallus very thick (Figs. 23B, 23C); construction and orientation of hypothallial filaments not clearly observed. Perithallial filaments (Figs. 23D-F) grow perpendicular to the substratum. Adjacent perithallial filaments linked by cell fusions only (Fig. 23F, arrows). Trichocytes present and tightly packed horizontal fields (that lack vegetative filaments between them) (Figs. 23G-H, “T” - trichocytes) and also found buried (Fig 23D, “T” - trichocytes). Epithallial cells (Fig. 23H) with one to two layers (Fig. 23H, 1 and 2 “e”- epithallial cells), calcified cell walls with roofs sloughed-off, flattened or rounded-shaped lumens (Fig. 23H, “e” – epithallial cell); initials cells short (Fig. 23H, “i” – initial cell). Uniporate conceptacles found at the surface, flushed (Figs. 24A-C); putative old conceptacles were found buried and infilled (see Fig. 24B, arrows in previous plate). Uniporate conceptacles were interpreted as being tetrasporangial as they showed mature, zonately divided tetraspores (Figs. 24A, 24B; tetraspore “t”); single pore channel formed by filaments at least 10 cells long (Fig. 24C). Trichocytes present at the surface (Fig. 24B, arrow) and buried (Fig. D, “T”- trichocytes) next to conceptacles.

Distribution: This species was only found in Fernando de Noronha Island (for more collection information see Tab.3).



**Figure 23. Corallinaceae sp. 6; specimen IBC1917 - External morphology and vegetative features.** **A.** Thallus habit showing fruticose protuberances. **B-C.** Fractures showing a thick thallus. **D.** Fracture showing buried filled conceptacles (arrow) and buried trichocytes (T). **E-F.** Fracture at the perithallus showing adjacent filaments linked by cell fusions (arrows). **G-H.** Fractures showing tightly packed trichocytes (T) and epithallial cell (e).

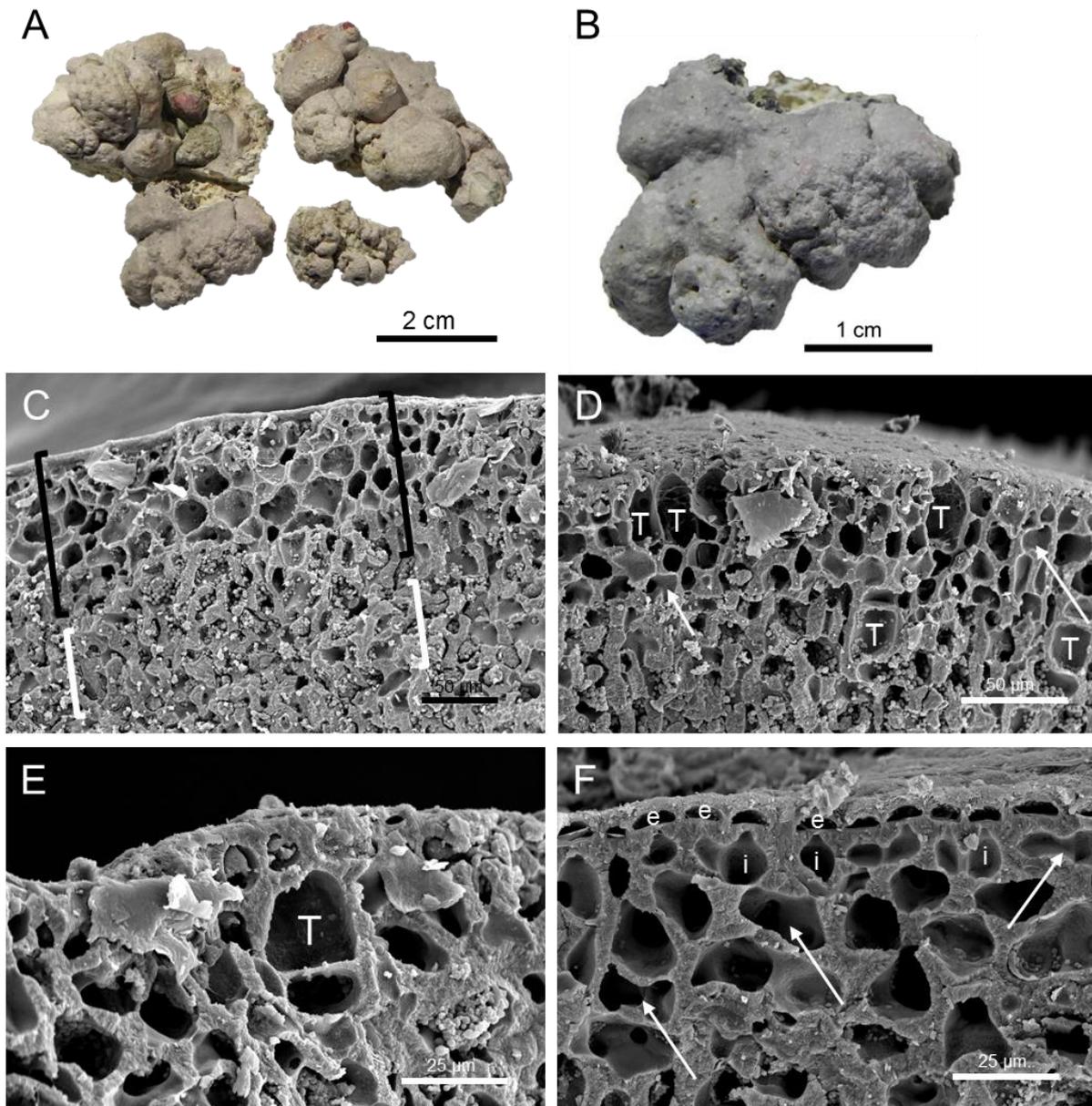


**Figure 24. Corallinaceae sp. 6; specimen IBC1917 – Reproductive features. A.** Fracture of the thallus showing new conceptacles at the surface. **B.** Fracture showing conceptacle with a single pore channel and trichocytes tightly packed. **C.** Detail of the Fracture showing a conceptacles with pore channel (p). **D.** Buried trichocytes (T) tightly packed next to a conceptacle.

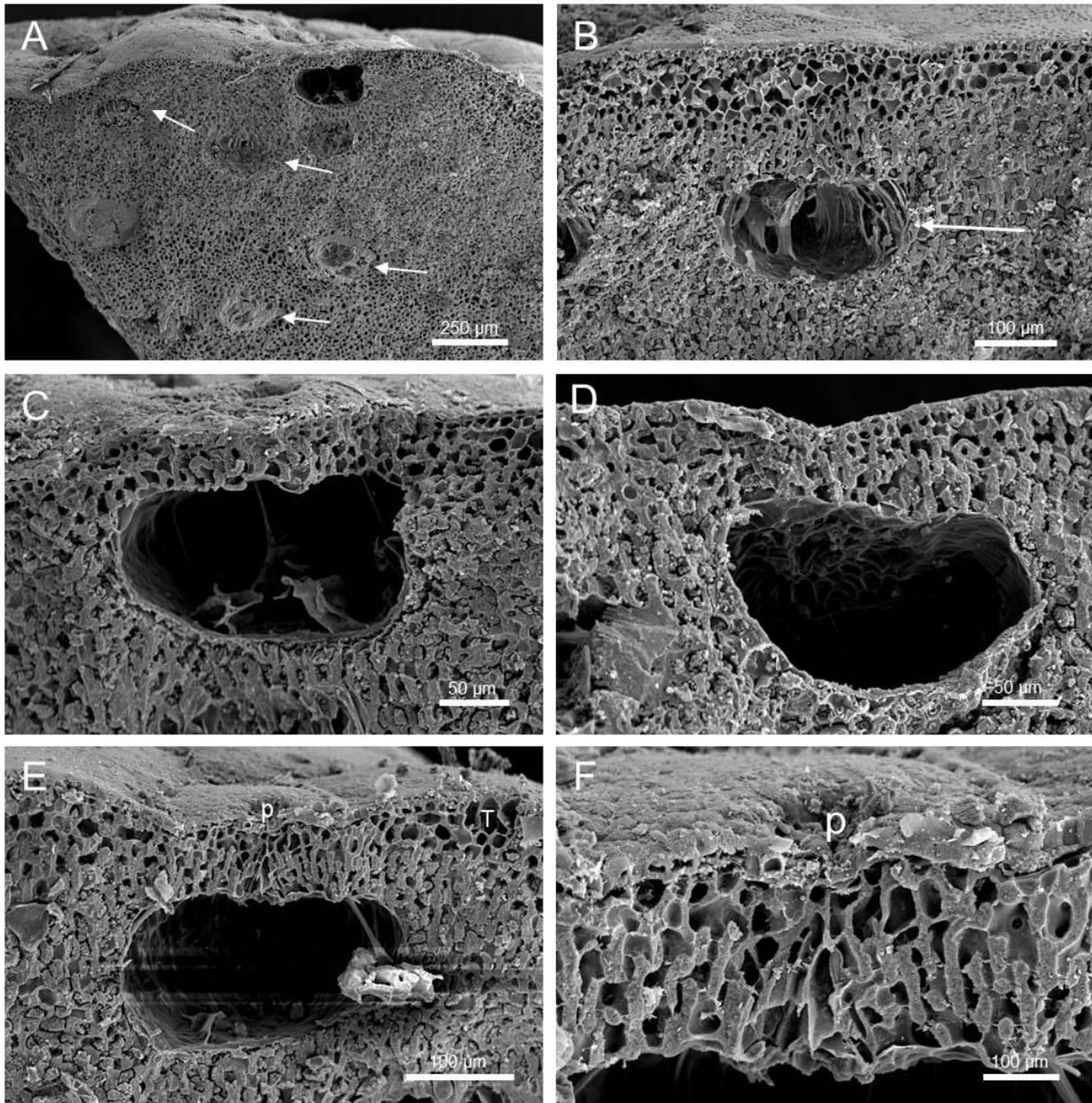
*Corallinaceae sp. 8*

Specimen IBC1919 was collected in Fernando de Noronha Island in the subtidal (10-15m depth - for more collection information see Tab.3). Specimen was found growing detached from the substratum, as a rhodolith (Figs. 25A, 25B – shows the rhodolith already broken). Thallus is lumpy to warty with large protuberances; color is pale purple with white spots (Figs. 25A, 25B). Thallus construction not clearly observed. Less calcified large cells were observed forming the epithallus (Fig. 25C, upper black brackets). Perithallial cells heavily calcified (Fig. 25C, lower white brackets). Adjacent perithallial filaments linked by cell fusions only (Figs. 25D, 25F, arrows). Trichocytes present (Figs. 25D, 25E; “T”-trichocytes) and were also found buried (Fig. 25D, “T” -trichocytes). Epithallus consists of a single layer of calcified cells with rounded-shaped lumens (Fig. 25F, “e”- epithallial cell); initials cells long; at least two times the size of the epithallial cells (Fig. 25F, “i” – initial cell). Uniporate conceptacles found at the surface, flushed (Figs. 26A, 26C) or sunken (Figs. 26D, 26E); old conceptacles were found buried throughout the thallus (Figs. 26A and 26B, arrows) completely (Fig. 26A) or partially (Fig. 26B) filled with remnant cellular material. Uniporate conceptacles at the surface were empty and therefore not possible to be interpreted as being tetrasporangial or gametangial. Trichocytes found next to uniporate conceptacles (Fig. 26E, “T”-trichocytes; “p”-pore). Pore channel formed by filaments at least 7 cells long (Fig. 26F, “p”-pore).

Distribution: This species was also found in Trindade Island (specimen IBC1798) (for more collection information see Tab.3).



**Figure 25. Corallinaceae sp. 8; specimen IBC1919 - External morphology and vegetative features. A-B.** Thallus habit showing lumpy to warty protuberances. **C.** Fracture showing less calcified large cells (upper black brackets) and perithallial cells heavily calcified (lower white brackets) **D.** Fracture showing trichocytes (T) and also buried ones; adjacent filaments linked by cell fusions (arrows). **E.** Fracture showing the detail of a trichocyte (T). **F.** Fracture showing rounded epithallial cells (e) and initial cell (i); adjacent filaments linked by cell fusions (arrows).

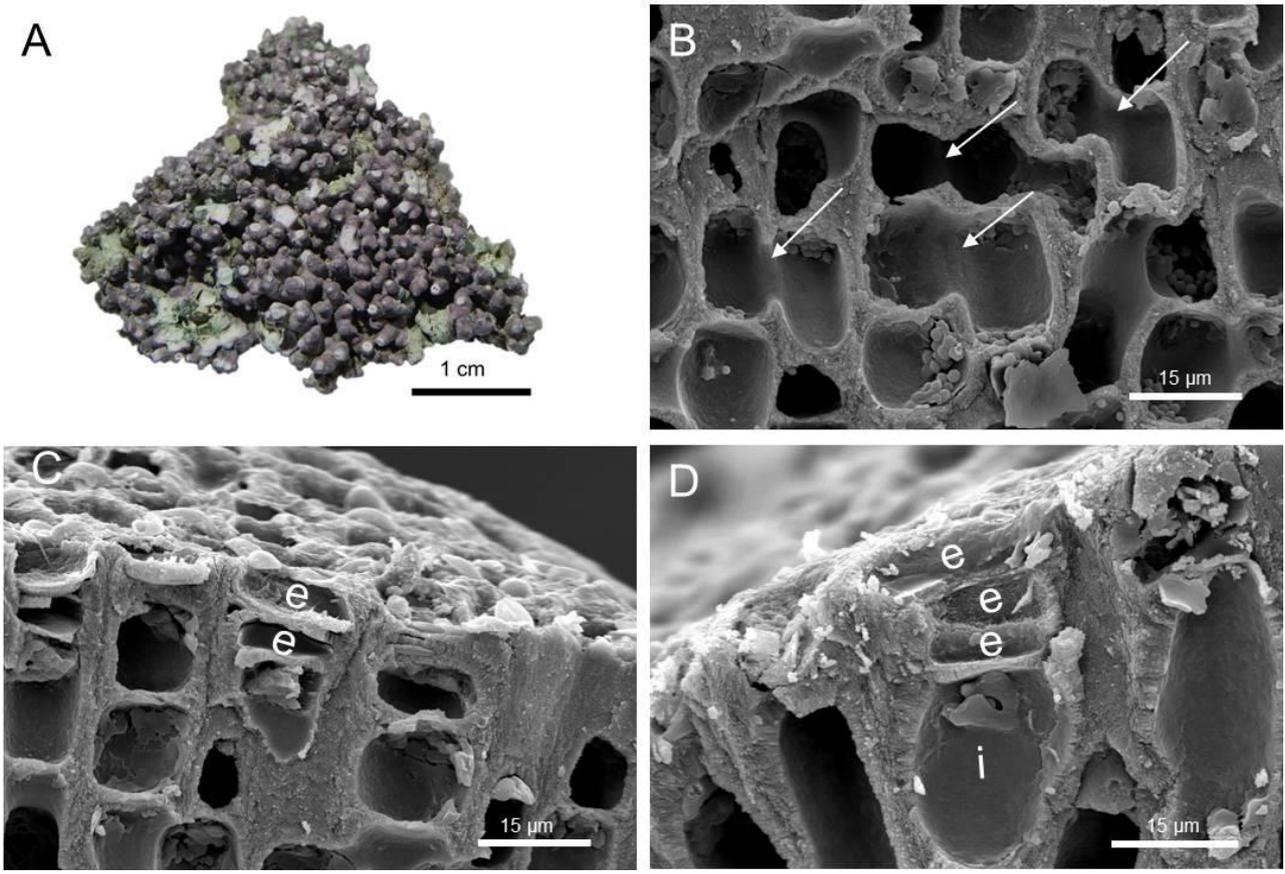


**Figure 26. Corallinaceae sp. 8; specimen IBC1919 – Reproductive features. A-B.** Fractures of the thallus showing new conceptacles at the surface and old, infilled buried conceptacles (arrows). **C-D.** Fracture showing flushed conceptacle at the surface. **E.** Fracture of a conceptacle showing a single pore channel (p). **F.** Detail of the Fracture showing the conceptacle pore channel (p).

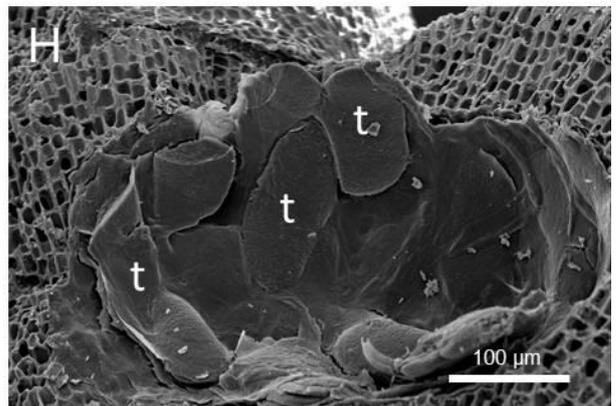
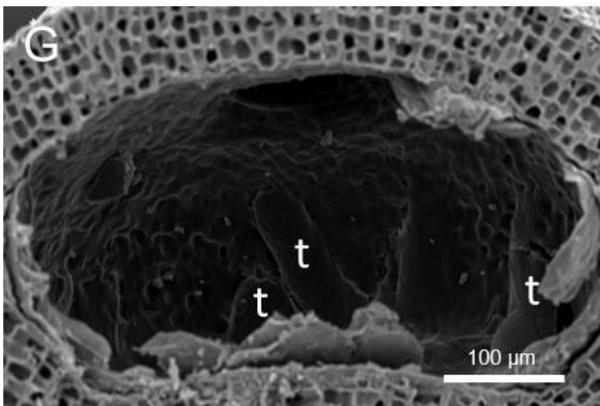
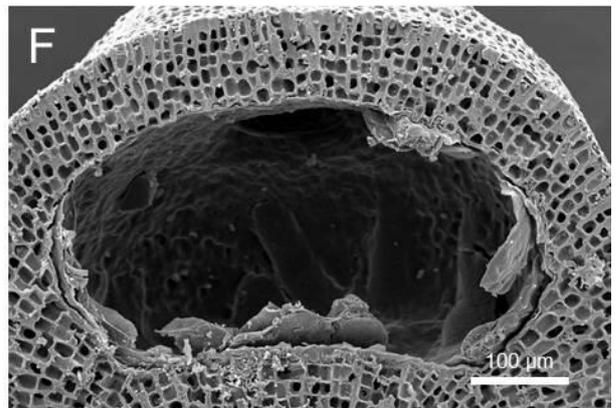
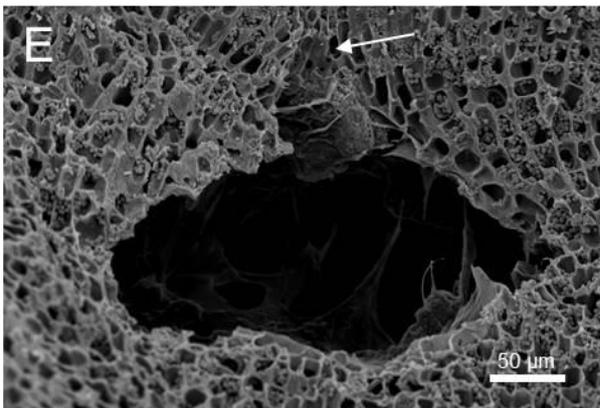
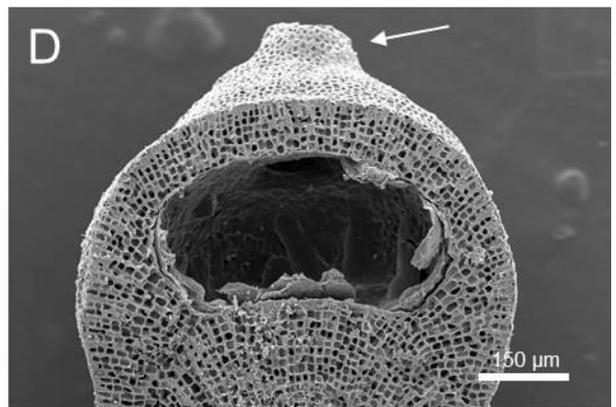
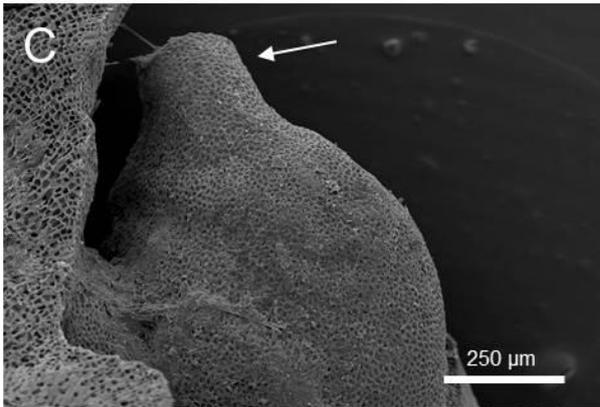
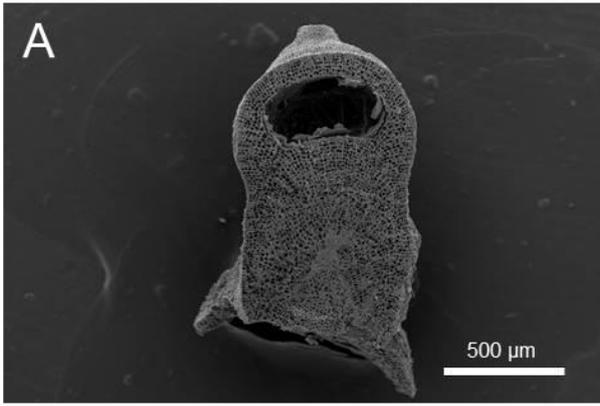
*Neogoniolithon sp. 1*

Specimen IBC1216 was collected in Espírito Santo state in the intertidal (for more collection information see Tab.3). Specimen was found growing detached from the substratum, as a rhodolith (Fig. 27A). Thallus is lumpy to warty with small spike-like protuberances; color is pale purple (Fig. 27A). Fractures throughout the thallus did not show thallus construction clearly. Adjacent perithallial filaments linked by cell fusions only (Fig. 27B, arrows). Trichocytes not observed. Epithallial cells (Figs. 27C, 27D) with up to three layers, calcified cell walls, rounded-shaped lumens (Fig. 27D, “e” – epithallial cell); initials cells long; at least two times the size of the epithallial cells (Fig. 27D, “i” – initial cell). Uniporate conceptacles found at the surface, raised and round-shaped (Figs. 28A, 28C). Fractures in a spike-like branch shows uniporate conceptacles and it is interpreted as being tetrasporangial as it showed divided mature tetraspores (Figs. 28G, 28H, tetraspore “t”); single pore (Figs. 28C-28E, arrows).

Distribution: This species was also found in a different location in the same state (specimen IBC1202 - for more collection information see Tab.3).



**Figure 27. *Neogoniolithon* sp. 1; specimen IBC1216 - External morphology and vegetative features.** **A.** Thallus habit showing lumpy to warty with small spike-like protuberances. **B.** Fracture showing perithallial adjacent filaments linked by cell fusions (arrows). **C-D.** Fracture showing flattened epithallial cells (e) with two-three layers; initial cell longer than epithallial cells (i).

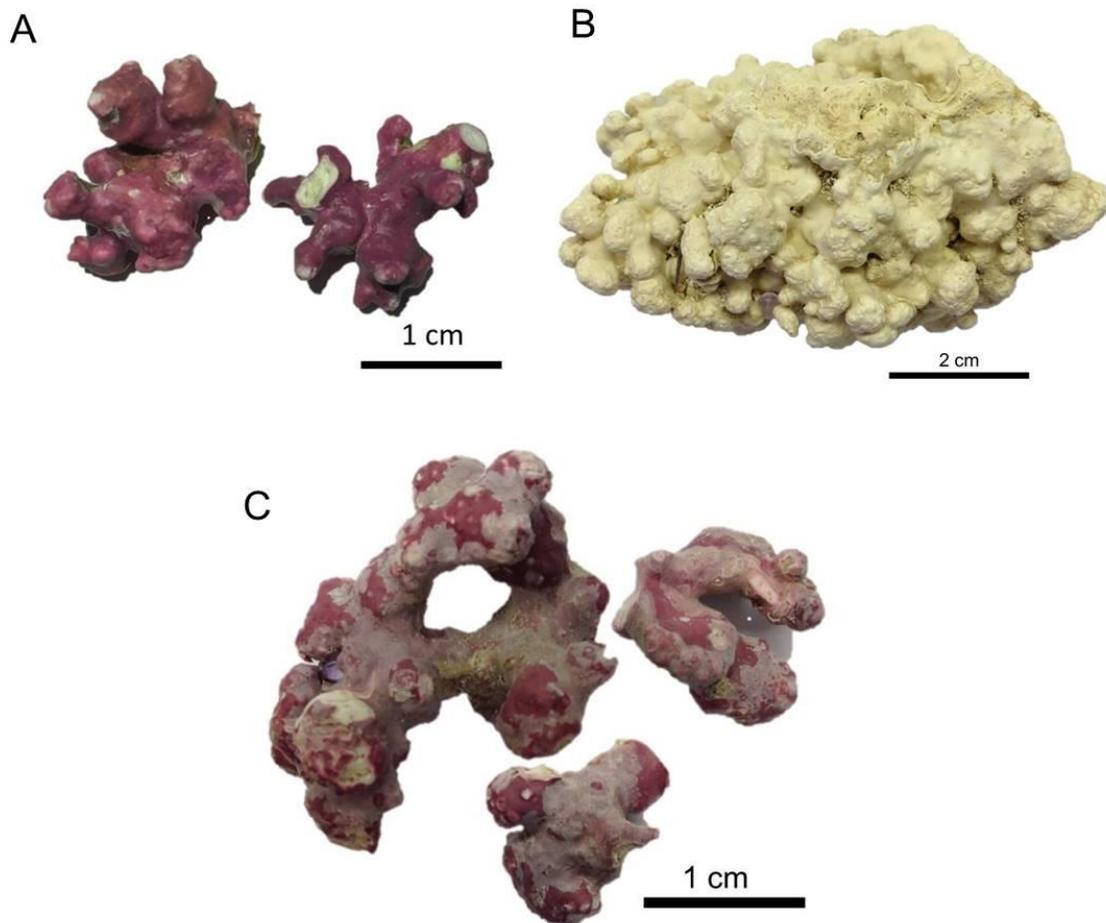


**Figure 28. *Neogoniolithon* sp. 1; specimen IBC1216 – Reproductive features. A-B.** Fractures of protuberance showing a conceptacle. **C-D.** Surface view and Fracture view showing conceptacles with a single pore channel (arrows). **E.** Fracture of an old buried conceptacle showing a single pore channel (arrow). **F.** Fracture of conceptacles showing putative tetraspores (t).

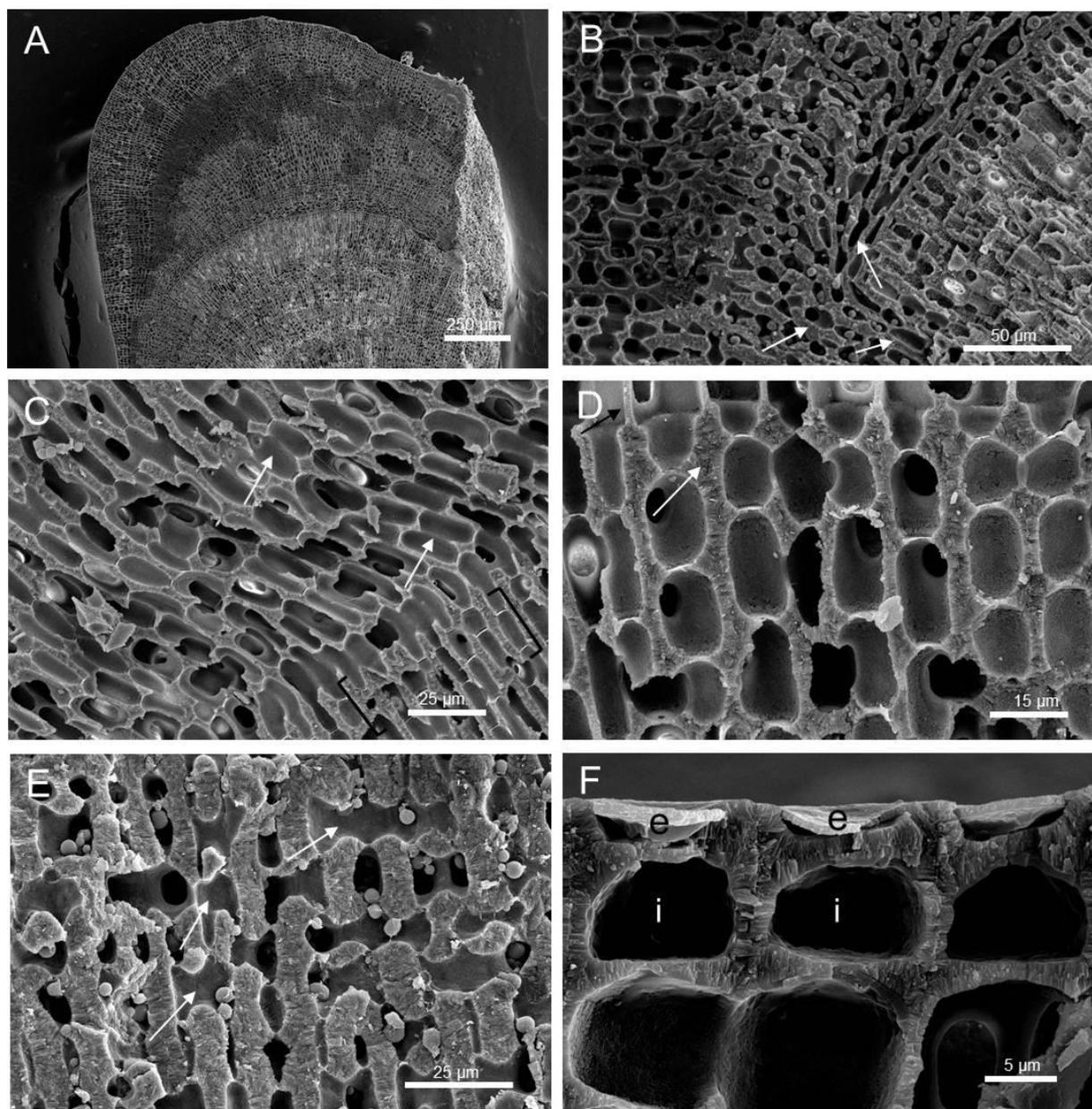
*Lithothamnion sp. 1*

Specimen IBC1704 (Fig. 29A) was collected in Bahia state whereas specimen IBC1564 (Fig. 29B) was collected in Paraíba state, and both were collected in the intertidal. Specimen IBC1557 (Fig. 29C) was collected in Ceará state in the subtidal (8m depth). All three specimens were found growing detached from the substratum, as rhodoliths (for more collection information see Tab.3). Thalli fruticose with numerous protuberances (Fig. 29A, 29B) or warty (Fig. 29C); color dark pink to purple (Fig. 29A, 29B). Fractures showing protuberances were composed largely of vegetative filaments (Figs. 30A) with radial construction. Newly formed hypothallus consisting of multiple layers of hypothallial filaments comprised of rectangular shaped cells (Fig. 30B, arrow) was observed growing over the surface of the older growth layer. Newly formed rectangular hypothallial cells apparently cut off from cells of the perithallus in the older growth layer. The hypothallial filaments grow parallel to the surface of the older growth layer and give rise to the next layer of perithallus. Adjacent perithallial filaments linked by cell fusions only (Fig. 30C, 30E, arrows). Fractures of protuberances show the perithallus consisted of filaments with alternating layers of cells with thick, heavily calcified cell walls (Fig. 30D, brackets; Fig. 30F arrows) and cells with thin, weakly calcified cell walls (Fig. 30D black arrow). Epithallus consists of short, flattened epithallial cells with heavily calcified lateral and proximal cell walls, a trapezoidal shaped cell lumen and a thinner, weakly calcified epithallial cell roof (Fig. 30F, “e”-epithallial cells); initial cells appeared to be recently divided as they are shorter than the cells right below them (Fig. 30F, “i”-initial cells). A single putative buried multiporate conceptacle was identified (Figs. 31A-B) although multipore channels were not shown clearly.

Distribution: This species was also found in Espírito Santo (IBC1525) (for more collection information see Tab.3).

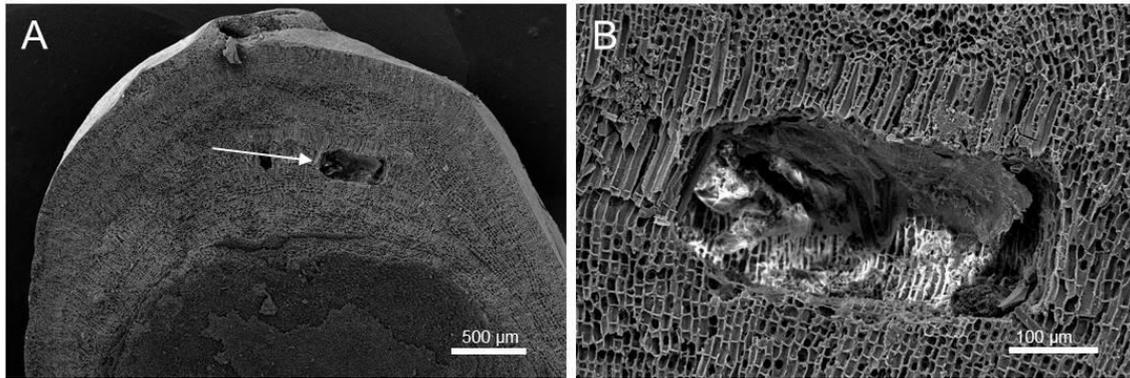


**Figure 29.** *Lithothamnion* sp. 1; specimens IBC1704, IBC1564 and IBC1557 - External morphology. **A.** Thallus habit showing fruticose protuberances (IBC1704). **B.** Thallus habit showing warty protuberances (IBC1564). **C.** Thallus habit showing fruticose protuberances (IBC1557).



**Figure 30. *Lithothamnion* sp. 1; specimens IBC1704 and IBC1907 - Vegetative features.**

**A.** Fracture showing general aspect of the thallus. **B.** Fracture showing newly generated hypothallus with rectangular-shaped hypothallial cells (arrows). **C.** Fracture showing the perithallus with a heavy calcified cell walls (lower black bracket) and a portion less calcified cell walls (upper portion in the image). **D.** Fracture showing the perithallus with a heavy calcified cell walls (white arrow) and less calcified cell walls (upper black arrow). **E.** Fracture showing the perithallus with heavy calcified cells; adjacent filaments linked by cell fusions (arrows). **F.** Fracture showing a trapezoidal shaped cell lumen and a thinner, weakly calcified epithallial cell roof (e); initial short (i).



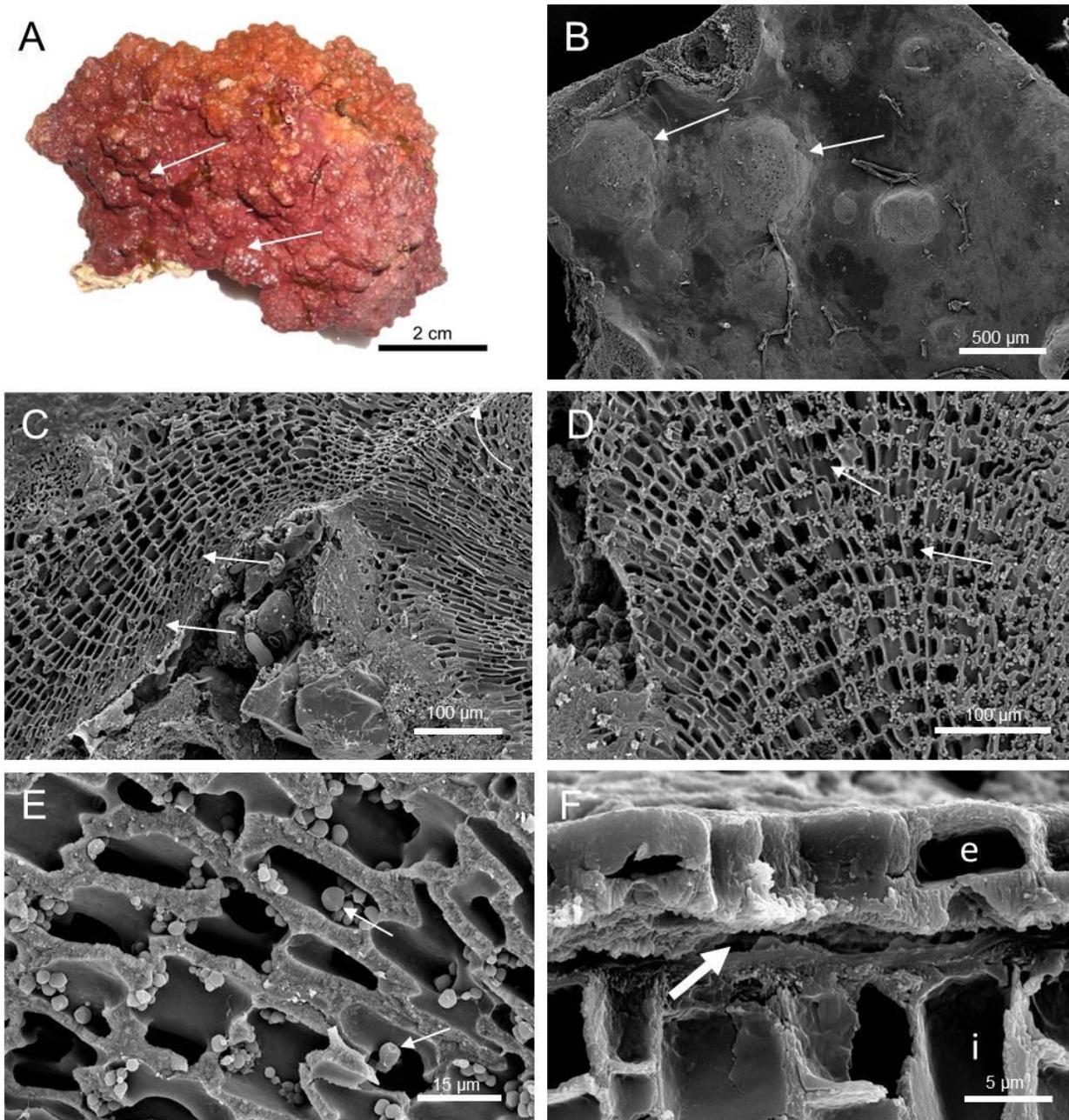
**Figure 31.** *Lithothamnion* sp. 1; specimens IBC1907 – Reproductive features. **A.** Fracture showing a putative buried multiporate conceptacle. **B.** Detail of a putative buried multiporate conceptacle.

*Hapalidiaceae sp. 1*

Specimen IBC1790 was collected in Bahia state in the intertidal (for more collection information see Tab.3). The specimen was found growing detached from the substratum, as a rhodolith (Fig. 32A). Thallus is lumpy to warty with small protuberances; color is orange to dark red white spots (Fig. 32A, arrows). Surface view shows that the white spots are multiporate conceptacle plates (Fig. 32B, arrows). Fractures show thallus construction consisted of a multilayered hypothallus comprised of hypothallial filaments with rectangular-shaped cells (Fig. 32C, straight arrows) growing parallel to the substratum and towards thallus surface (Fig. 32C, upward arrow). Adjacent perithallial filaments linked by cell fusions only (Fig. 32D, arrows). Putative dinoflagellate-like round-shaped structures were observed growing inside perithallial cells (Fig. 32E, arrows). Epithallus consists of short flattened epithallial cells with trapezoidal shaped cell lumen and a weakly calcified epithallial cell roof (Fig. 32F, “e”-epithallial cells) that are almost sloughing-off from the thallus (Fig. 32F, arrow).

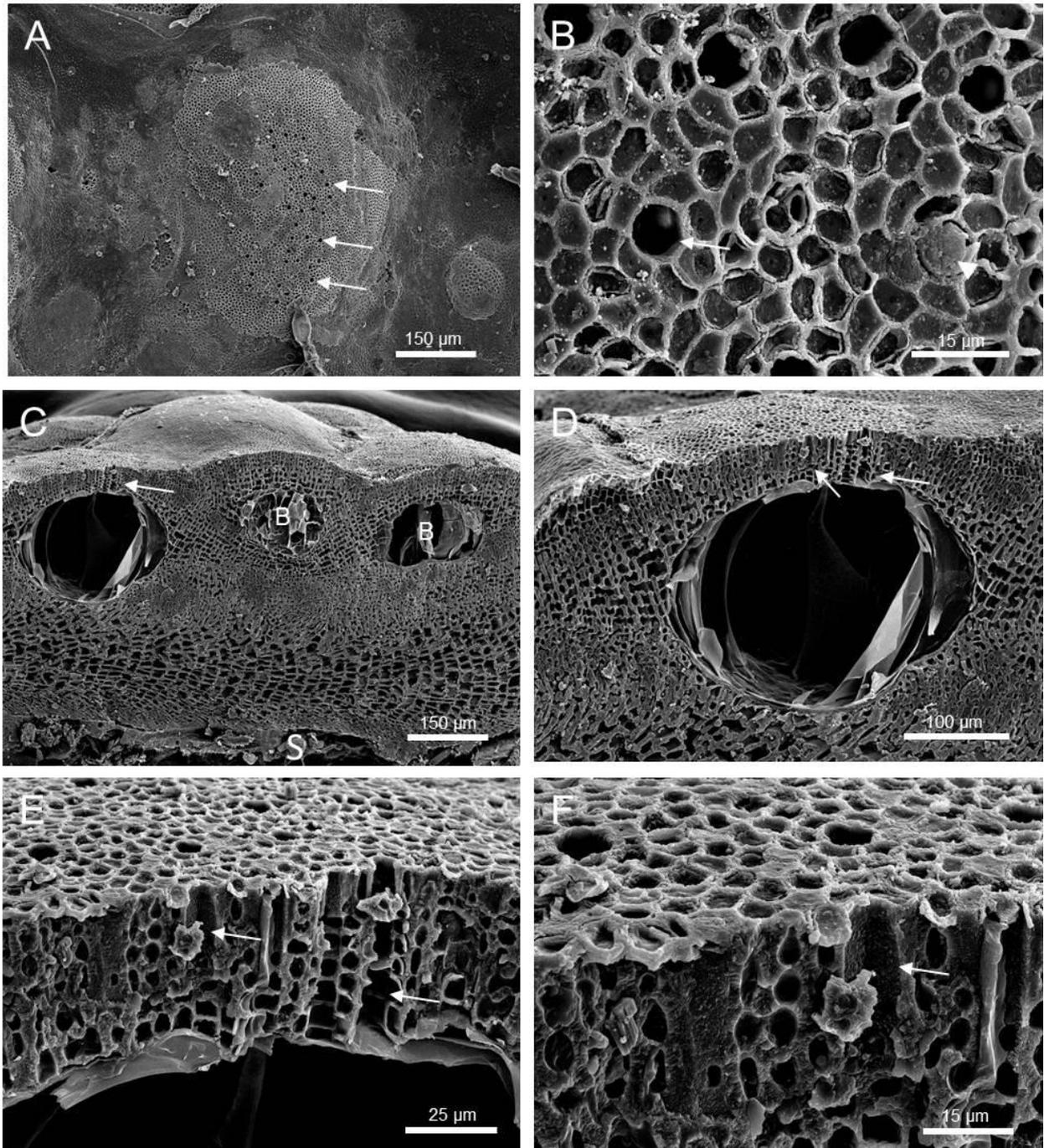
Multiporate conceptacles found at the surface, slightly raised to completely flushed (Figs. 33A, 33C, 33D). Superficial views show multiple opened pores in the conceptacle plates (Figs. 33A, 33B; arrows) and also plugged pores (Fig. 33B, arrowhead). Fractures through the protuberances show multiple pores channels in the tetrasporangial conceptacles (Figs. 33C-33F, arrows); pore channels formed by 6-8 rosettes cells. Putative recently buried conceptacles were observed (Fig. 33C, “B”-buried conceptacles) infilled by vegetative growth. Thallus is relatively thin with approximately 500µm from substratum to the epithallus (Fig. 33D, “S”-substratum).

Distribution: This species was only found in Bahia state (for more collection information see Tab.3).



**Figure 32. Hapalidiaceae sp. 1; specimen IBC1790 - External morphology and vegetative features.** **A.** Thallus habit showing lumpy to warty protuberances. **B.** Superficial view of conceptacles multiporate plates (arrows). **C.** Fracture showing multilayered hypothallus with rectangular-shaped cells (straight arrows) and perithallial filaments growing parallel to the substratum and curving towards thallus surface (upward arrow). **D.** Fracture showing adjacent filaments linked by cell fusions (arrows). **E.** Fracture showing

putative dinoflagellates inside the cells perithallus (arrows) **F**. Fracture showing flattened epithallial cells (e) and long initial cell (i); weakly calcified epithallial cell roof that are almost sloughing-off from the thallus (arrow).



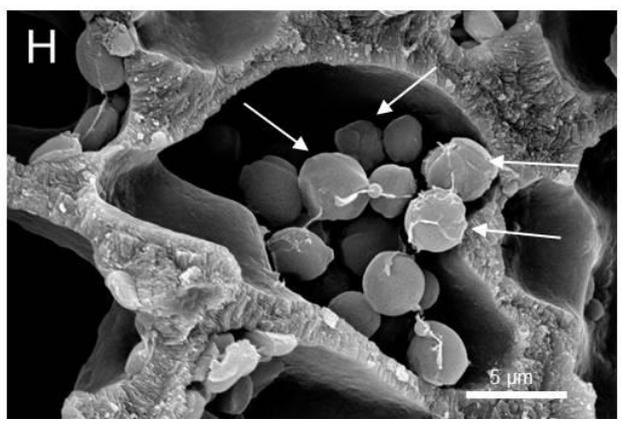
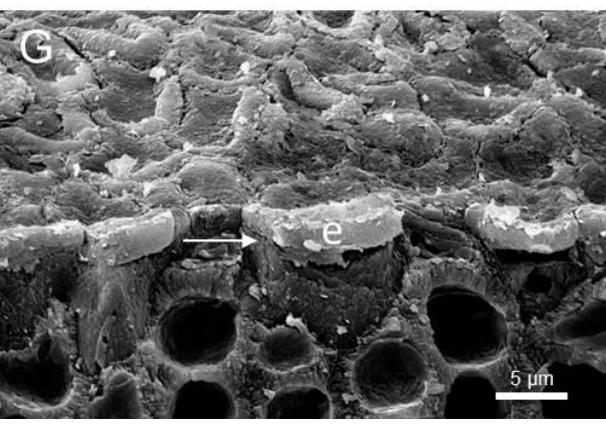
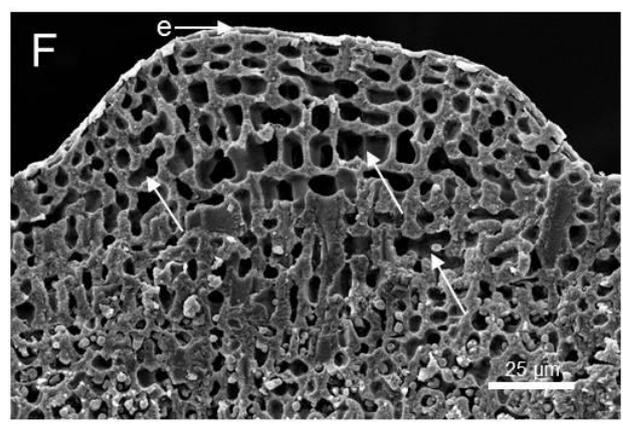
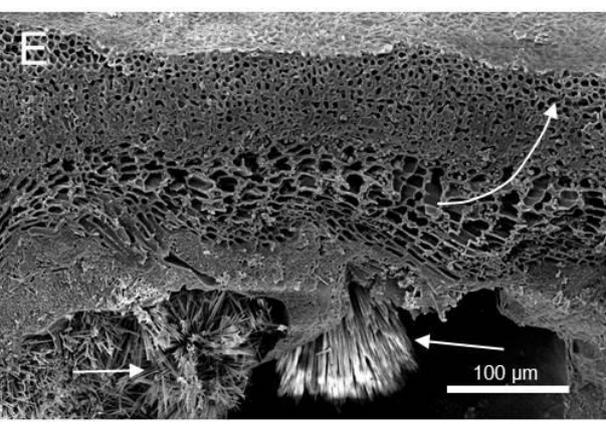
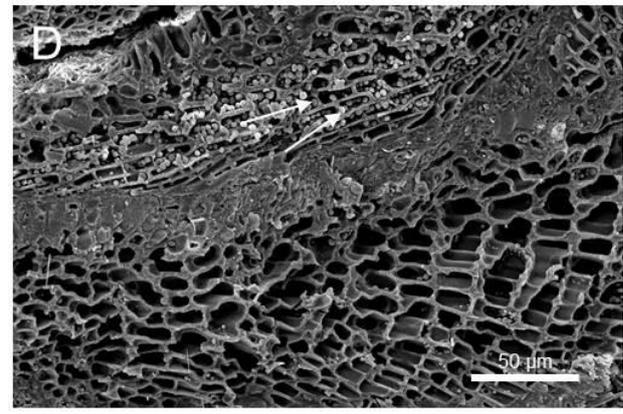
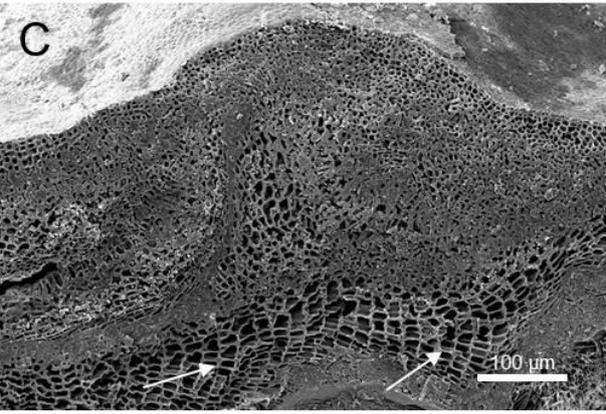
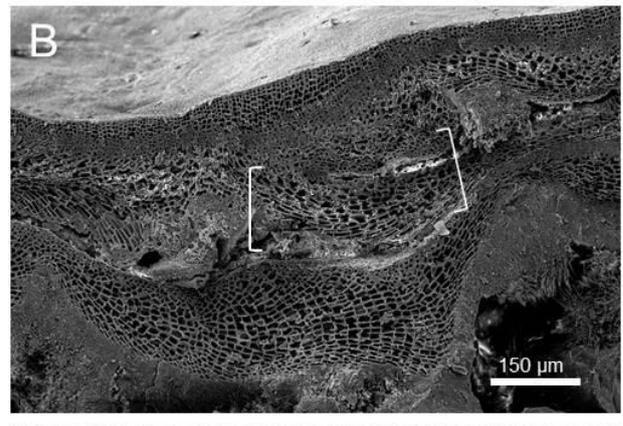
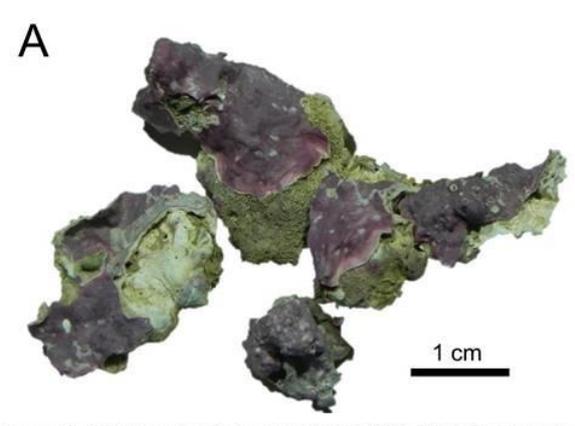
**Figure 33. Hapalidiaceae sp. 1; specimen IBC1790 - Reproductive features. A.** Surface view of conceptacles multiporate plates, showing multiple pores (arrows). **B.** Detailed surface view of the pores showing opened pores (arrow) and plugged pores (arrowhead). **C.** Fracture showing a new multiporate empty conceptacle with a

putative pore channel (arrow) and older buried, infilled conceptacles (B). **D.** Fracture showing a multiporate conceptacle with a putative pore channel (arrow). **E-F.** Fracture and partial surface view showing multiporate conceptacle and putative pore channels (arrows).

*Hapalidiaceae sp. 2*

Specimen IBC1196 was collected in Espírito Santo state in the intertidal (for more collection information see Tab.3). Specimen was found growing on rocky substratum (Fig. 34A). Thallus is encrusting, smooth with sparse protuberances and also layered; color is dark pink to purple with few white spots (Fig. 34A). Fractures show thallus is composed of a thin crust (Figs. 34B-D). Aragonite infill was observed in spaces between substrata and thallus layers (Fig. 34E, straight arrow). Thallus construction consists of a multilayered hypothallus comprised of hypothallial filaments with rectangular-shaped cells growing parallel to the substratum and curving towards thallus surface (Fig. 34B, brackets, 34C-D, arrows) and perithallial filaments growing perpendicular to the substratum (Fig. 34E, upward arrow). Adjacent perithallial filaments linked by cell fusions only (Fig. 34F, arrows). Perithallus consists of cells with thick, heavily calcified cell walls (Fig. 34F). Epithallus consists of short, flattened epithallial cells with thick weakly calcified epithallial cell roof (Fig. 34F, “e”-epithallial cells) that are almost sloughing-off from the thallus (Fig. 34F, arrow). Putative dinoflagellate-like round-shaped structures were observed growing inside perithallial cells (Fig. 34H, arrows). No reproductive structures were found for this species.

Distribution: This species was only found in Espírito Santo state (for more collection information see Tab.3).



**Figure 34. Hapalidiaceae sp. 2; specimen IBC1196 - External morphology and vegetative features. A.** Thallus habit showing layers and smooth sparse protuberances. **B.** Fracture showing multilayered hypothallus (brackets). **C-D.** Fracture showing multilayered hypothallus with rectangular-shaped cells (arrows). **E.** Perithallial filaments growing parallel to the substratum and towards thallus surface (upward arrow); spaces between substrata and thallus layers show aragonite infill (straight arrows). **F.** Fracture showing adjacent filaments linked by cell fusions (arrows) and flattened epithallial cells (e). **G.** Epithallus consisting of short, flattened epithallial cells with thick weakly calcified epithallial cell roof (e) that are almost sloughing-off from the thallus (arrow). **H.** Fracture showing putative dinoflagellates inside the cells perithallus (arrows).

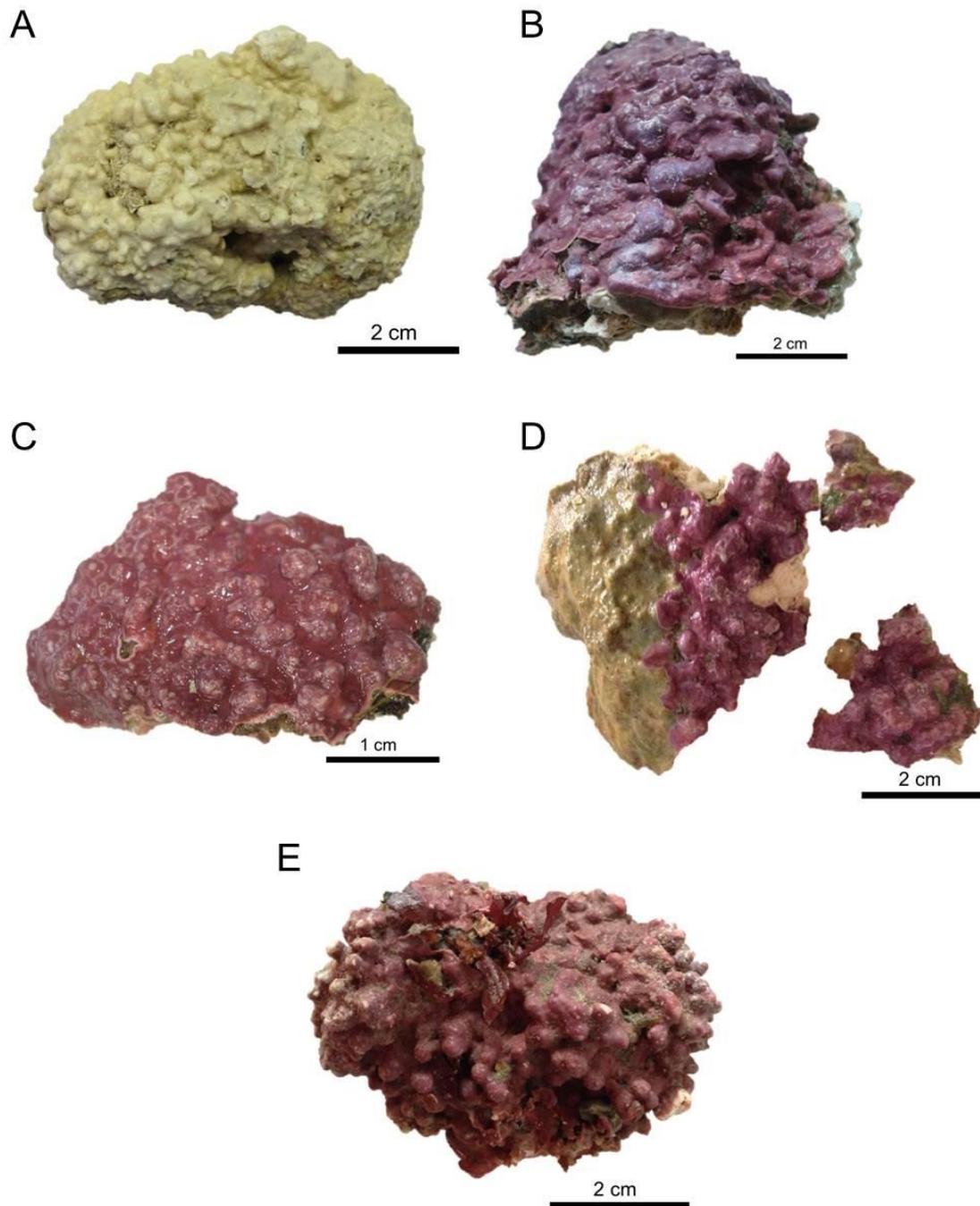
*Hapalidiaceae sp. 4*

Specimen IBC1562 was collected in Maranhão state in the subtidal (15-30m depth) and was found growing detached from the substratum, as a rhodolith (Fig. 35A), IBC1657 (Fig. 35B) and IBC1662 (Fig. 35C) were collected in Ceará state in the intertidal, both of them growing encrusting rocky substrate, IBC1804 (Fig. 35D), IBC1850 (Fig. 35E) were collected in Rio Grande do Norte in two different location, both of them were collected in the intertidal, the former was found encrusting a rocky substratum and the latter was found growing detached from the substratum, as a rhodolith (Figs. 35D, 35E, respectively) (for more collection information see Tab.3). Thalli encrusting, smooth with sparse protuberances, layered, lumpy and warty; color varied from pink, dark pink, red and purple (Fig. 35A-E). Fractures show thallus is thick (Fig. 36A). Thallus construction consists of a multilayered hypothallus comprised of hypothallial filaments with rectangular-shaped cells (Fig. 36C, arrows) growing parallel to the substratum (Fig. 36B, brackets) and towards thallus surface and perithallial filaments growing perpendicular to the substratum (Fig. 36B, upward arrow). Perithallus consists of cells with thick, heavily calcified cell walls (Fig. 36D). Adjacent perithallial filaments linked by cell fusions only (Fig. 36E, arrows). Epithallus consists of short, flattened epithallial cells (data not shown). Putative dinoflagellate-like round-shaped structures/individuals were observed growing inside perithallial cells (Fig. 36F, arrows).

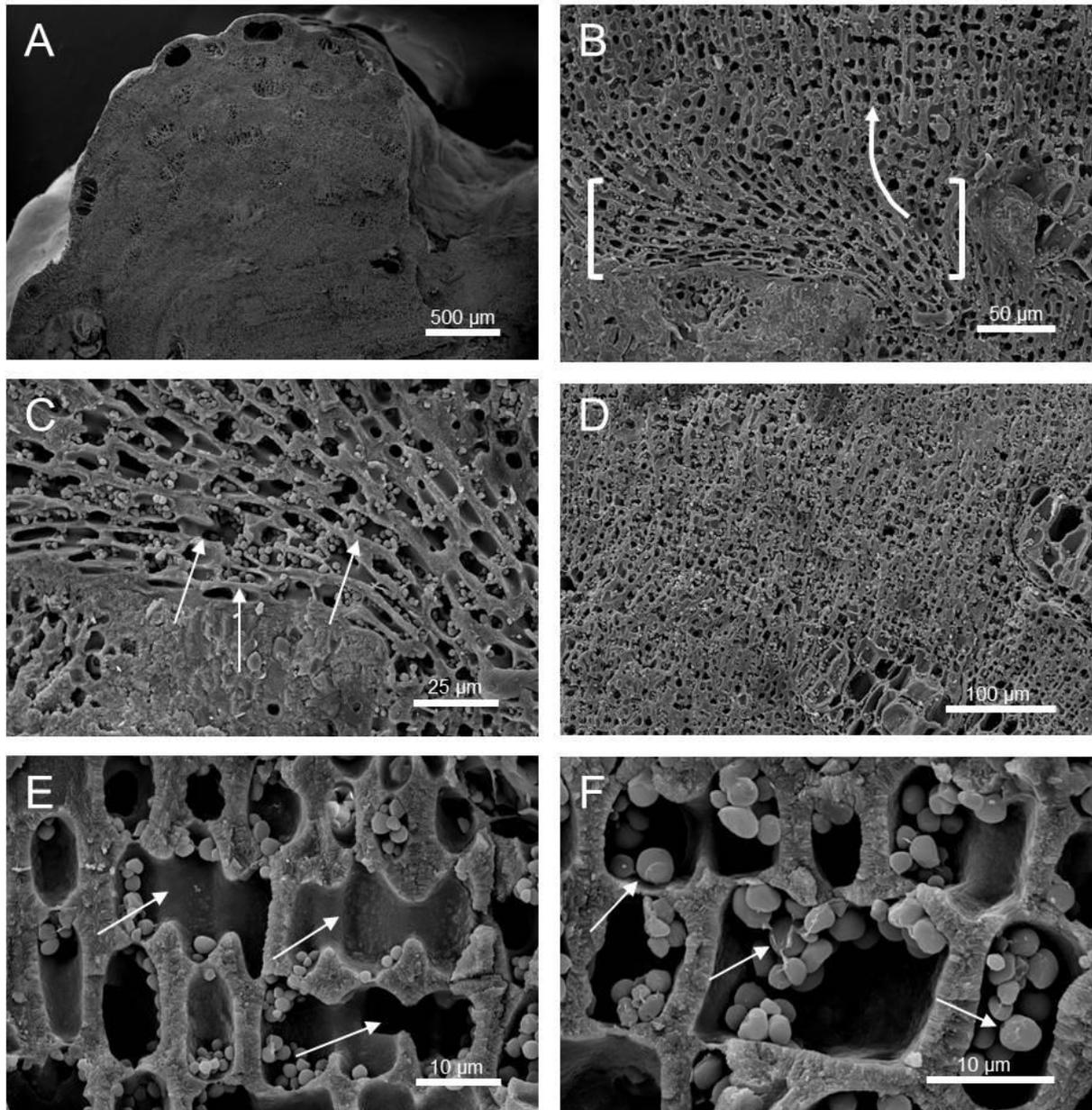
Multiporate conceptacles found at the surface, slightly raised to completely flushed (Figs. 37A, B, E). Superficial views show multiple opened pores in the conceptacle plates (Figs. 37A, C, D; arrows). Fractures through the protuberances show multiple pores channels in the tetrasporangial conceptacles (Figs. 37C-37F, arrows); pore

channels filaments were 7-8 cells long. Buried conceptacles were observed (Fig. 37C, arrows) infilled by vegetative growth.

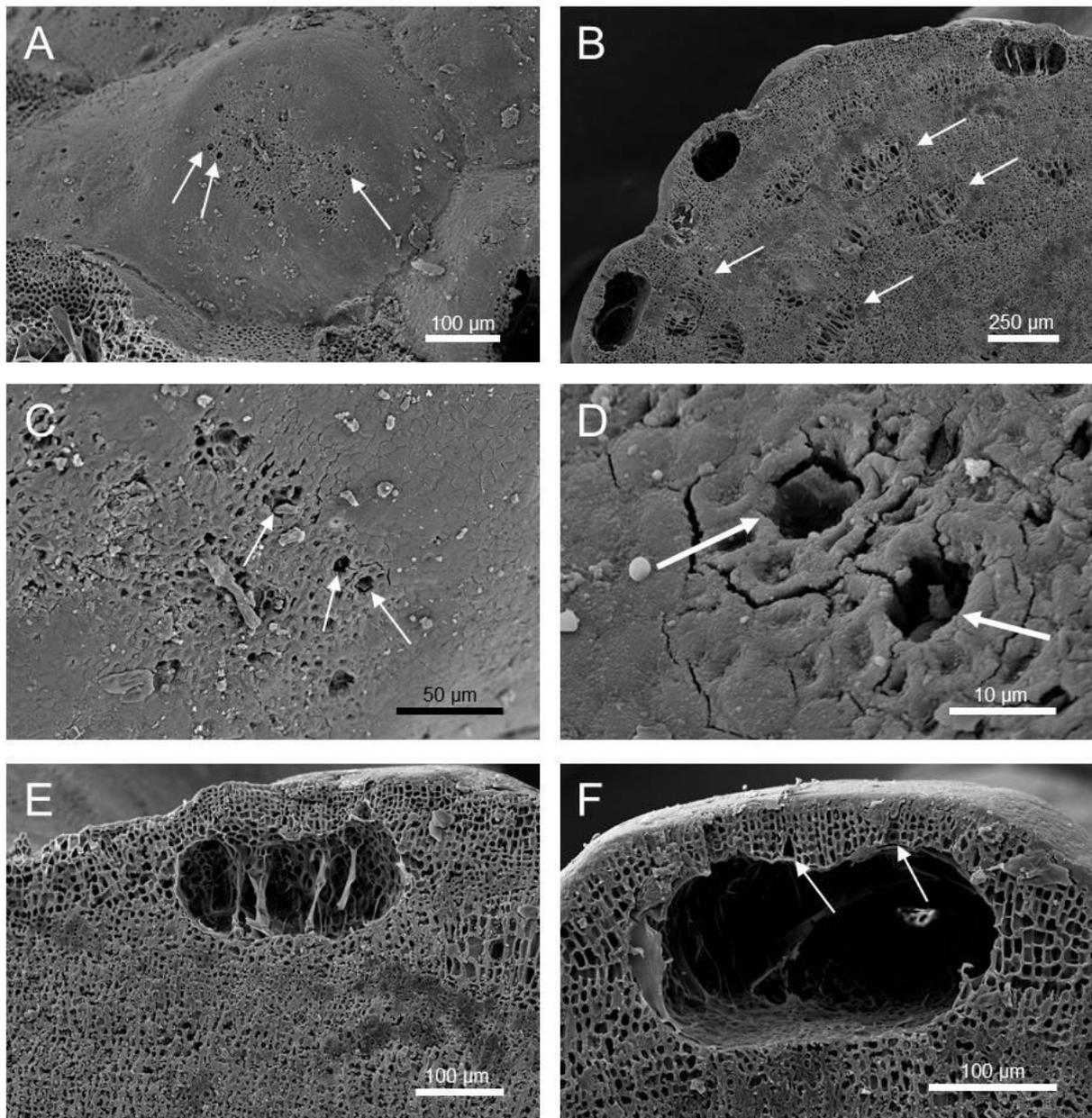
Distribution: This species was also found in Paraíba (IBC1246) and Bahia (IBC1710) states; and IBC1909 was collected in another location in Ceará state in the subtidal (8-19m depth) (for more collection information see Tab.3).



**Figure 35. Hapalidiaceae sp. 4; specimens IBC1562, IBC1657, IBC1662, IBC1804 and IBC1850 - External morphology.** **A.** Thallus habit showing lumpy to warty protuberances (IBC1562). **B.** Thallus habit showing layered smooth to warty protuberances (IBC1657). **C.** Thallus habit showing lumpy protuberances (IBC1662). **D.** Thallus habit showing layered smooth to warty protuberances (IBC1804). **E.** Thallus habit showing warty protuberances (IBC1850).



**Figure 36. Hapalidiaceae sp. 4; specimens IBC1662 - Vegetative features. A.** Fracture showing general aspect of the thallus. **B.** Fracture showing newly generated hypothallus (brackets) and perithallial filaments growing parallel to the substratum and towards thallus surface (upward arrow). **C.** Fracture showing the hypothallus with rectangular-shaped hypothallial cells (arrows). **D-E.** Fractures in the perithallus showing adjacent filaments linked by cell fusions (arrows). **F.** Fracture showing putative dinoflagellates inside the cells perithallus (arrows).

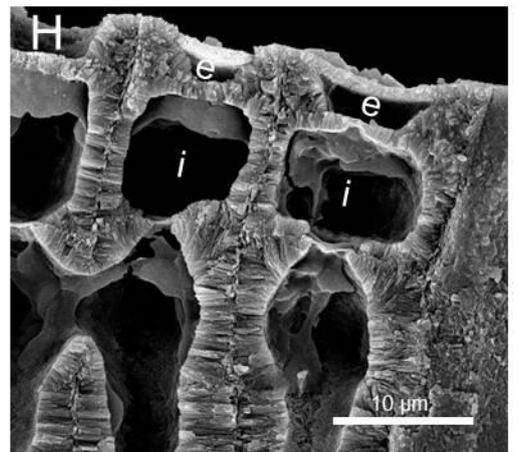
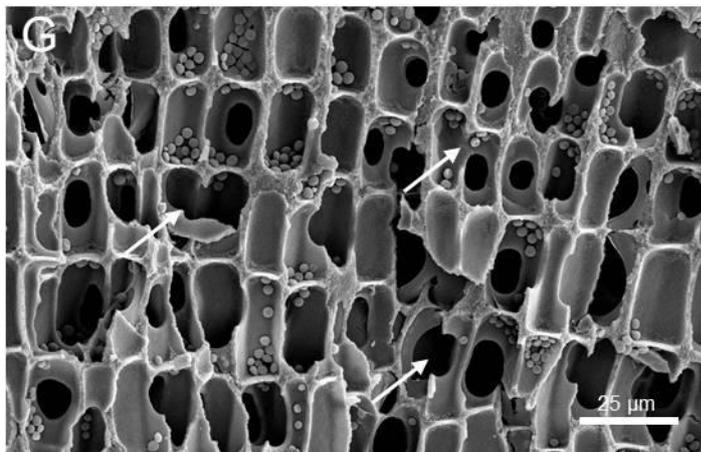
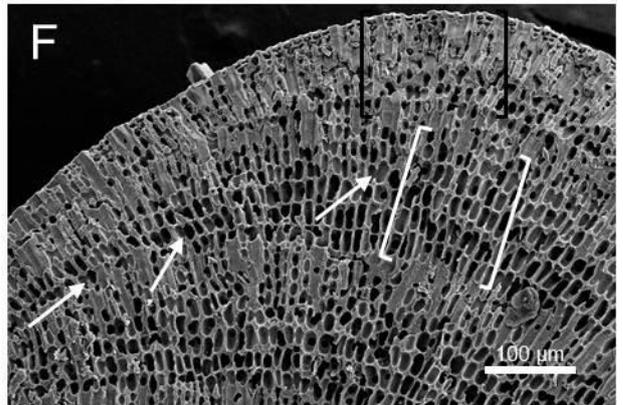
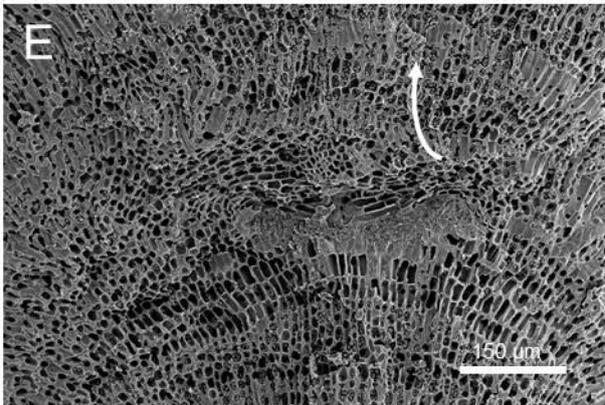
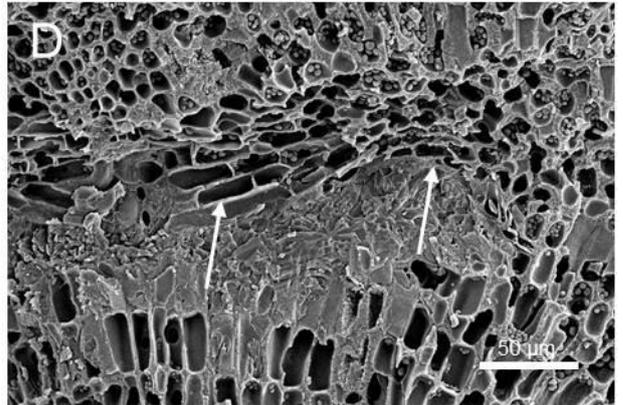
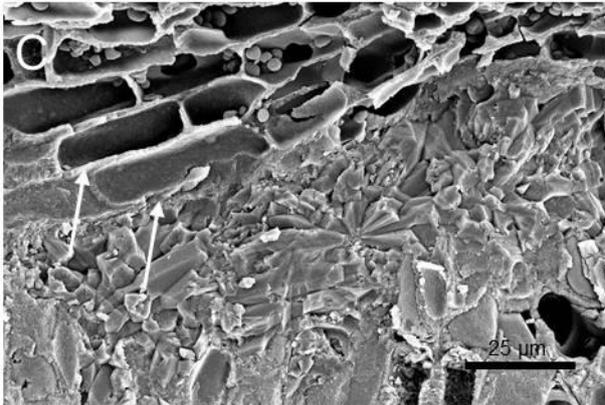
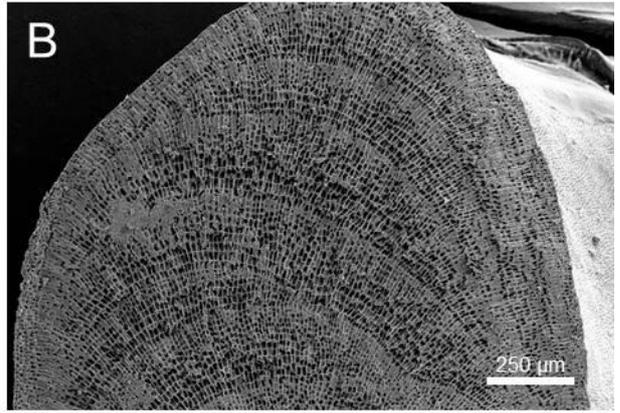
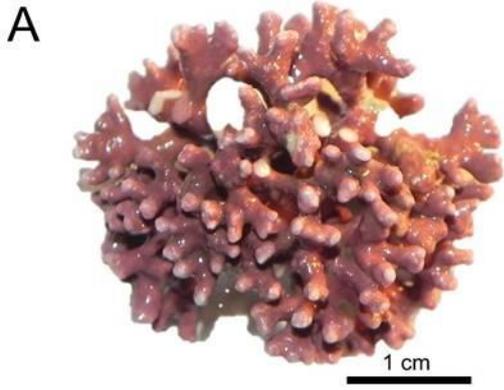


**Figure 37. Hapalidiaceae sp. 4; specimen IBC1662 - Reproductive features. A.** Superficial view of conceptacles multiporate plates, showing multiple pores (arrows). **B.** Fracture showing new conceptacles at the surface and old buried infilled conceptacles (arrows). **C-D.** Detailed superficial view of the pores showing opened pores (arrows). **E.** Fracture showing a multiporate senescent conceptacle infilled with vegetative filaments. **F.** Fracture and partial surface view showing multiporate conceptacle and putative pore channels (arrows).

*Hapalidiaceae sp. 7*

Specimen IBC1876 was collected in Espírito Santo state in the intertidal (for more collection information see Tab.3). Specimen was found growing detached from the substratum, as a rhodolith (Fig. 38A). Thallus fruticose with numerous protuberances (Fig. 38A). Fractures show protuberances composed largely of vegetative filaments (Figs. 38B, E, F) with radial construction. Newly formed hypothallus consisting of multiple layers of hypothallial filaments comprised of rectangular shaped cells (Fig. 38C, D, arrows) was observed growing over the surface of the older growth layer. Newly formed rectangular hypothallial cells are apparently cut off from cells of the perithallus in the older growth layer. The hypothallial filaments were observed growing parallel to the surface of the older growth layer and parallel to perithallus (Fig. 38E, upward arrow). Perithallus consists of filaments with alternating layers of cells with thick, heavily calcified cell walls (Fig. 38F, black brackets) and cells with thin, weakly calcified cell walls (Fig. 38F, white brackets). Adjacent perithallial filaments linked by cell fusions only (Fig. 38G, arrows). Epithallus consists of short, flattened epithallial cells with heavily calcified lateral and proximal cell walls, a trapezoidal shaped cell lumen and a thinner, calcified epithallial cell roof (Fig. 38H, “e”-epithallial cells); initial cells appeared to be recently divided as they are shorter than the cells right below them (Fig. 38H, “i”-initial cells). No reproductive structures were found for this species.

Distribution: This species was also found in Paraíba (IBC1245) and Bahia (IBC1547) states both of them we collected in the intertidal (for more collection information see Tab.3).



**Figure 38. Hapalidiaceae sp. 7; specimen IBC1876 - External morphology and vegetative features.** **A.** Thallus habit showing fruticose protuberances. **B.** Fracture general aspect of the thallus. **C-D.** Fractures showing multilayered hypothallus with rectangular-shaped cells (arrows). **E.** Perithallial filaments growing parallel to the substratum and towards thallus surface (upward arrow). **F.** Perithallus showing filaments with alternating layers of cells with thick, heavily calcified cell walls (black brackets) and cells with thin, weakly calcified cell walls white brackets). **G.** Fracture showing adjacent filaments linked by cell fusions (arrows). **H.** Epithallus consisting of short, flattened epithallial cells with thin weakly calcified epithallial cell roof (e); initials short (i).

*Hapalidiaceae sp. 10*

Specimens IBC1685 and IBC1688 were collected in Ceará state in the intertidal (for more collection information see Tab.3). Specimens were found growing detached from the substratum, as rhodoliths (Figs. 39A, 39B). Thalli smooth with sparse protuberances and lumpy to warty; color varies from pink to dark pink (Fig. 39A-39B). Fractures show thallus is not very thick (Fig. 39C-E). Thallus construction consists of a multilayered hypothallus comprised of hypothallial filaments with rectangular-shaped cells (Fig. 39F, arrows) growing parallel to the substratum and towards thallus surface and perithallial filaments growing perpendicular to the substratum (Fig. 39F, upward arrow). Perithallus consists of cells with thick, heavily calcified cell walls (Fig. 39G). Adjacent perithallial filaments linked by cell fusions only (Fig. 39G, arrows). Epithallus consists of short, flattened epithallial cells (Fig. 39H, “e”-epithallial); initial cells appeared to be recently divided as they are shorter than the cells right below them (Fig. 39H, “i”-initial cells).

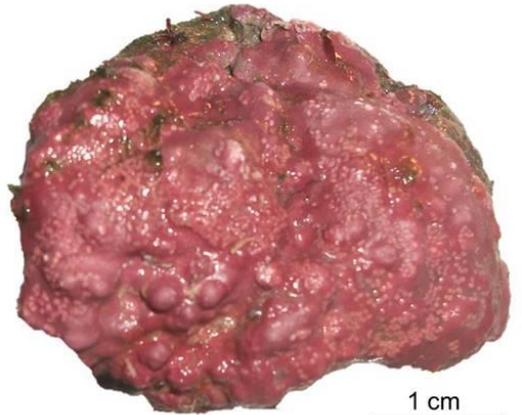
Multiporate conceptacles found at the surface, slightly raised to raised (Figs. 40A-40G). Surface views show multiple opened pores in the conceptacle plates (Figs. 40D-40F, arrows); rosettes that surround pore channels were composed of 4-6 cells (Figs. 40E-40H). Fractures through the multiporate conceptacles (Figs. 41A-41F) showed multiple pore channels in the tetrasporangial conceptacles (Figs. 41E, arrows); pore channels filaments 7-8cells long. Buried conceptacles were observed (Fig. 41C, arrows) infilled by vegetative growth. Some buried empty conceptacles (Fig. 41C, black arrow) were also observed to be infilled by putative aragonite crystals (Fig. 41C, white arrow).

Distribution: This species was only found in Ceará state (for more collection information see Tab.3).

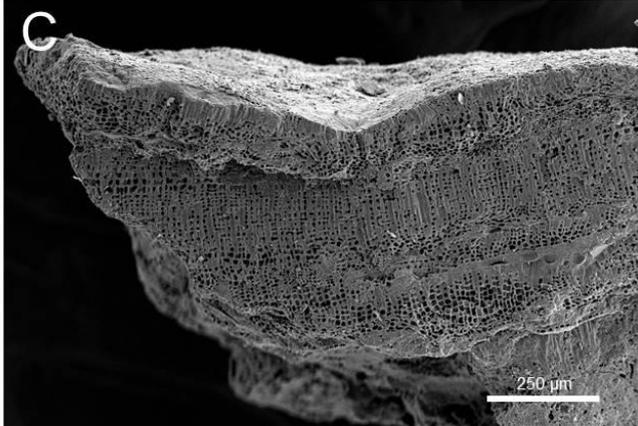
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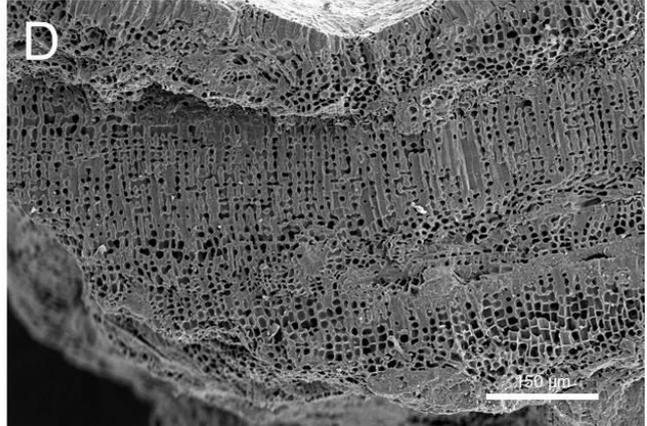
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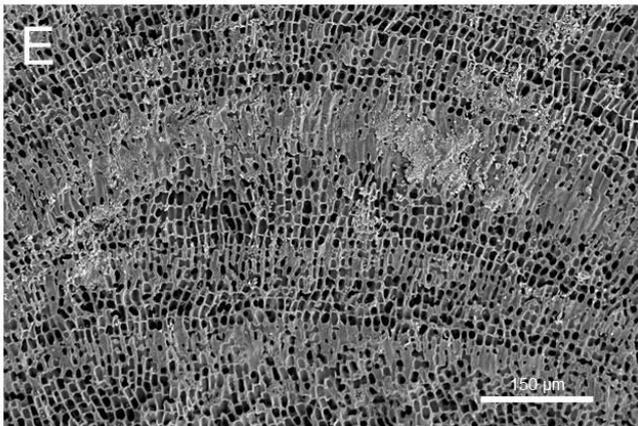
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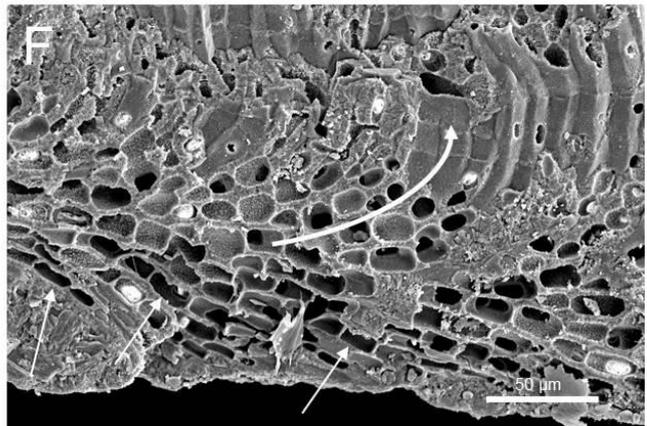
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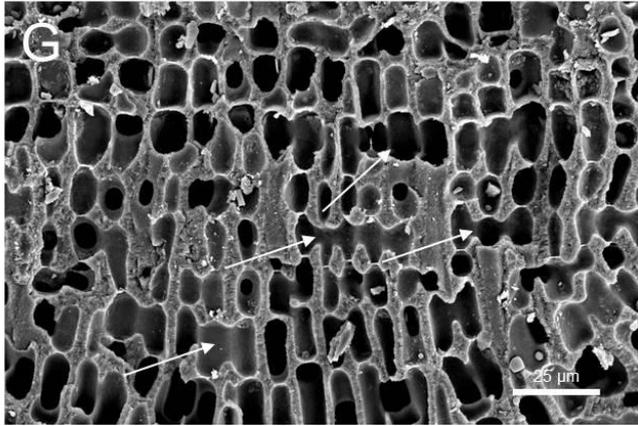
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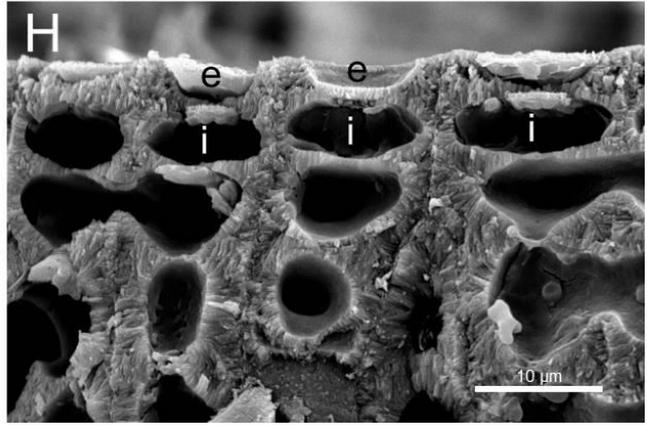
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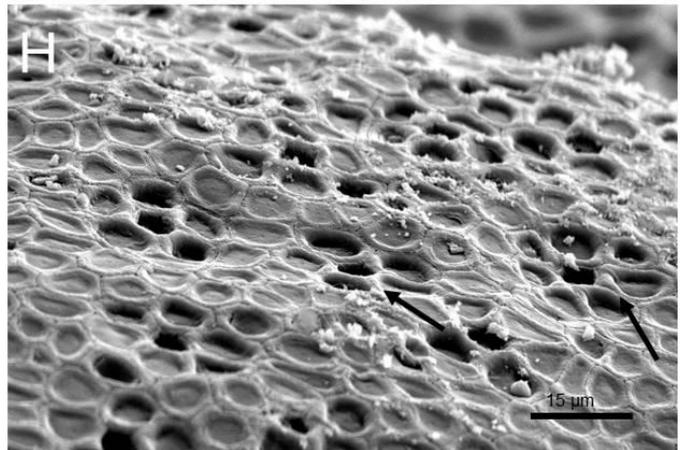
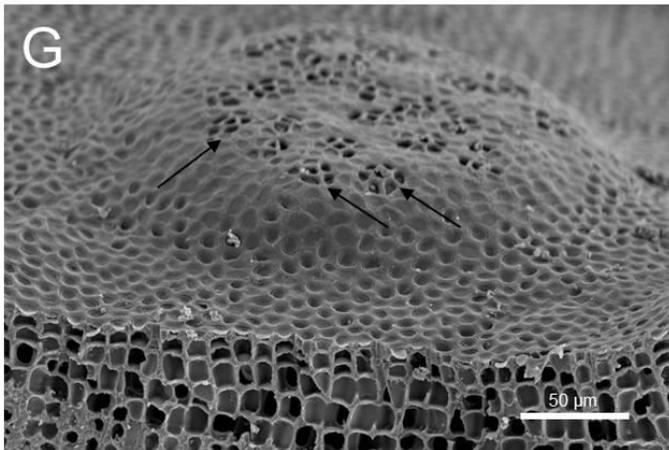
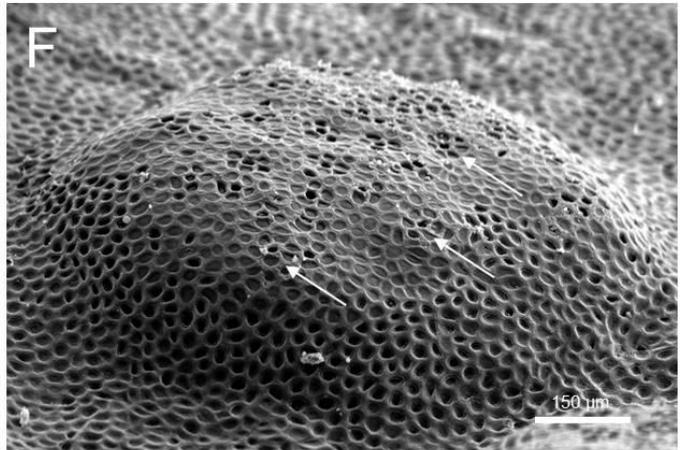
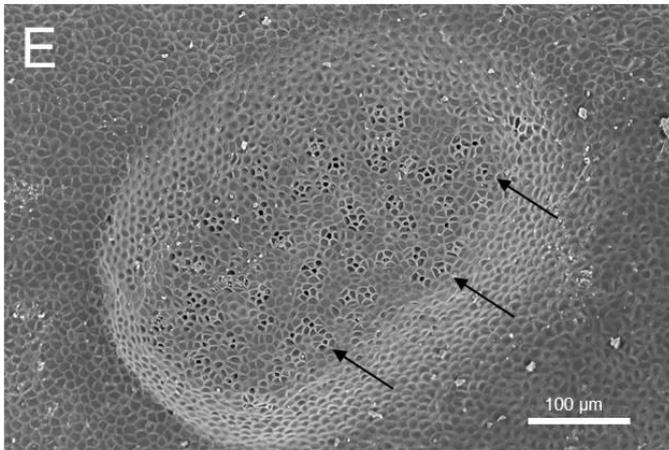
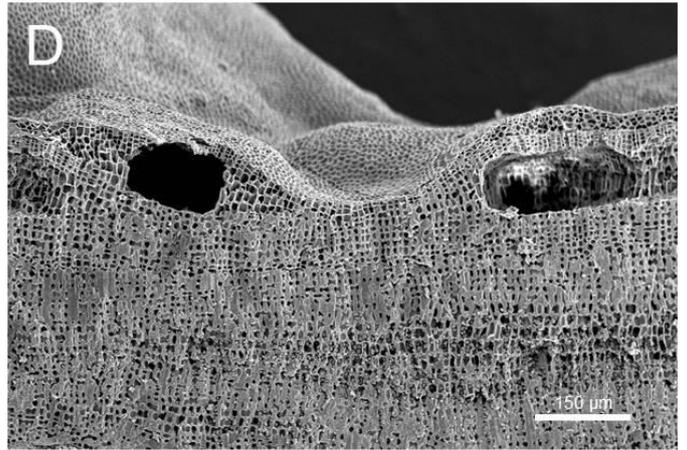
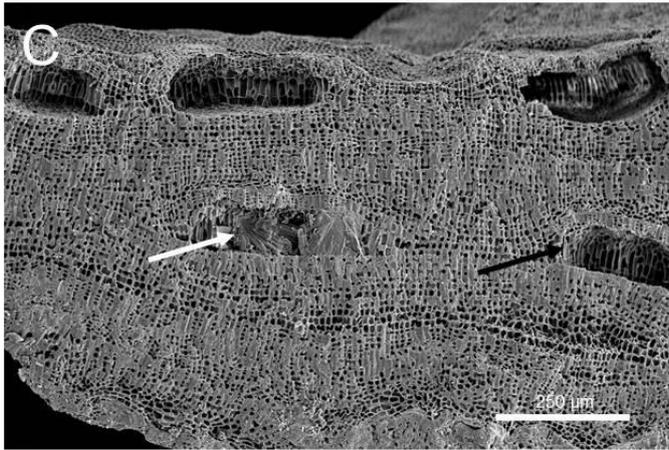
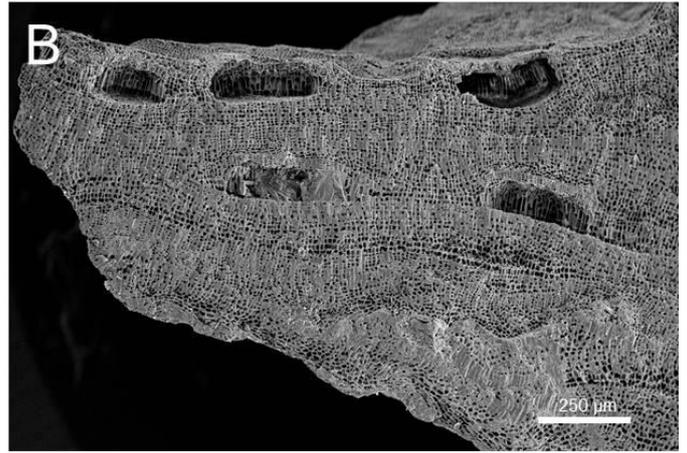
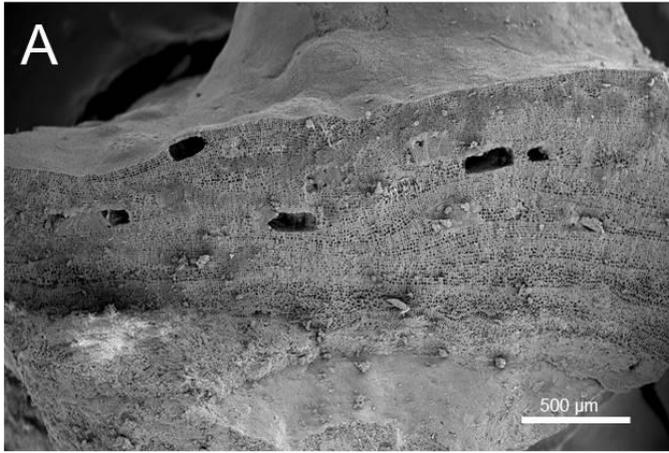
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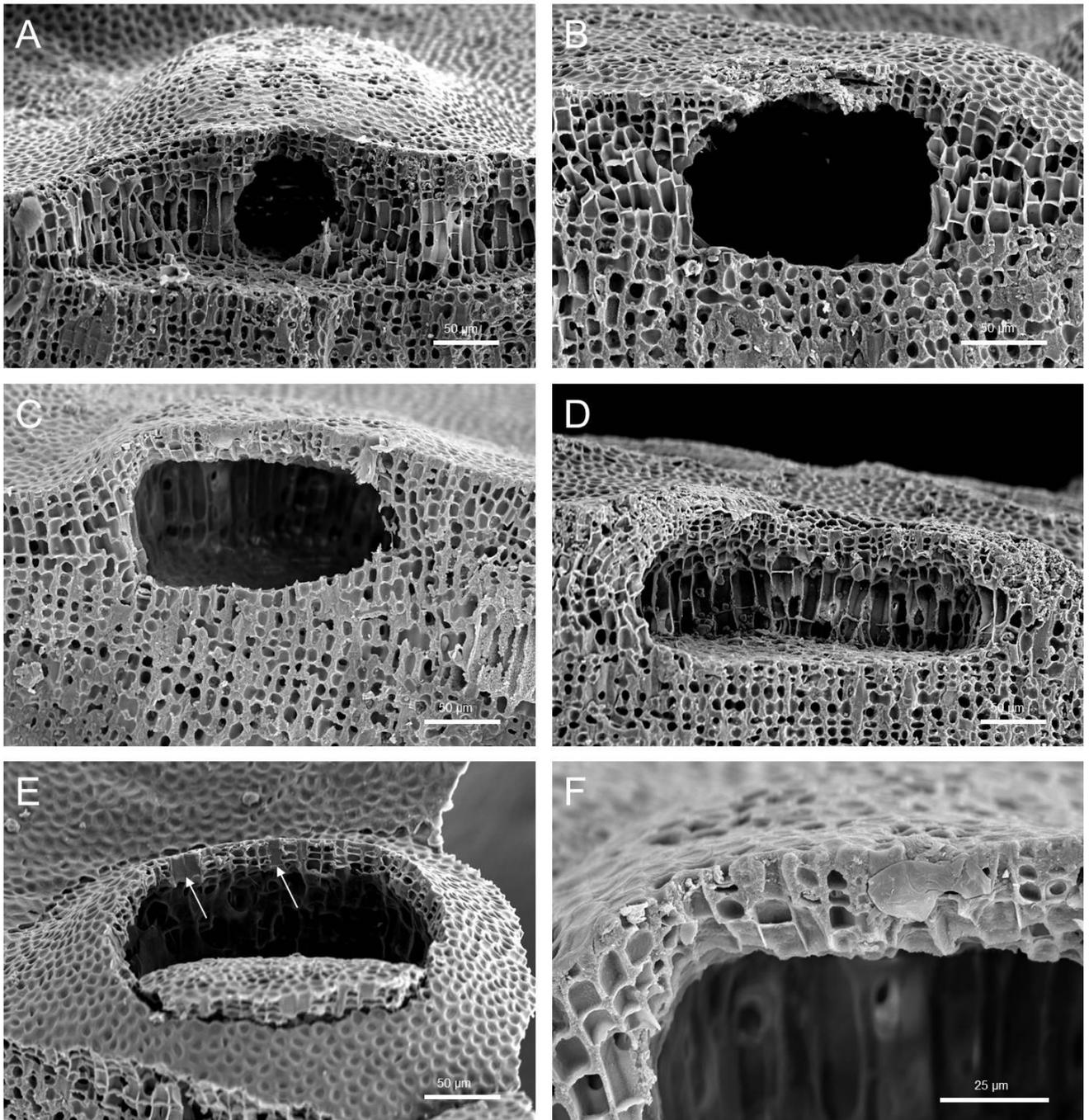
H



**Figure 39. Hapalidiaceae sp. 10; specimens IBC1685 and IBC1688 - External morphology and vegetative features.** **A.** Thallus habit showing lumpy to warty protuberances. **B.** Thallus habit showing smooth sparse to warty protuberances. **C-D.** Fractures showing general aspect of the thin thallus **E.** Fracture showing perithallial filaments. **F.** Fracture showing multilayered hypothallus with rectangular-shaped cells (arrows) and growing parallel to the substratum and towards thallus surface (upward arrow). **G.** Fracture showing adjacent filaments linked by cell fusions (arrows). **H.** Epithallus consisting of short, flattened epithallial cells with thin weakly calcified epithallial cell roof (e); initials very short (i).



**Figure 40. Hapalidiaceae sp. 10; specimen IBC1685 - Reproductive features. A-D.** Fracture showing new conceptacles at the surface; and old buried conceptacles infilled (white arrow) and empty (black arrow). **E-F.** Surface view of conceptacles multiporate plates, showing multiple pores (arrows). **G-H.** Detail of the rosettes cells (arrows).



**Figure 41. Hapalidiaceae sp. 10; specimen IBC1685 - Reproductive features. A-D.** Fracture showing new conceptacles at the surface. **C-D.** Detailed surface view of the pores showing opened pores (arrows). **E-F.** Fracture and partial surface view showing multiporate conceptacle and putative pore channels (arrows).

## Discussion

### *Success of amplification and sequencing among molecular markers*

Out of 232 samples for which DNA was extracted, a total of 172 were successfully amplified for at least one of the four markers used in this study, therefore our success rate was 75% overall. We expected this number to be higher considering the markers we used targeted the genomes of different organelles and the primers used were universal, designed for red algae specifically (or for red, green, and brown algae). However, considering the endophytic microalgal (see Figs. 34H, 36F component of coralline algae and the difficulty in obtaining clean amplicons, extraction and amplification in this group was relatively more problematic compared to other groups (personal communication from many researchers who study corallines).

For UPA we obtained 54 sequences out of nearly 180 samples we attempted to amplify (30% success rate). UPA has been shown to be a marker that is relatively easy to amplify and sequence (Sherwood *et al.* 2010a), and was used here for an initial screening of samples despite its lower rates of variation compared to other molecular markers such as COI-5P (Sherwood *et al.* 2010b) and *psbA* (Vidal *et al.*, 2003, Costa *et al.* 2012, Sissini *et al.* 2014, Iha *et al.* 2015, Torrano-Silva, 2015). We began this study focusing on the Sporolithales and as we advanced, UPA, which is a more conserved marker (Sherwood *et al.*, 2010a, b), did not show enough signal to delimit species boundaries for this group. The PCR success rate was also lower than expected, which further limited the utility of this marker. Even after modifications of PCR conditions and the application of newly synthesized primers, little change was observed regarding the rates of success for PCR, so we decided not to continue using UPA as our primary

marker for DNA barcoding. Furthermore, we observed that after 2010, the amount of sequences available for comparison in Genbank did not increase much, and relatively few studies utilized this marker from 2011 through 2015 (Sissini *et al.* 2014).

The *rbcL*-3P is a very important marker as some types have been successfully sequenced for this region of *rbcL* (Gabrielson *et al.* 2011, Hind *et al.* 2014). We attempted to amplify this region for almost 180 samples, but were successful for only 47 sequences (25% success rate). This low success rate may be due to the high level of diversity of samples from different orders we were working with, making it very difficult to keep track of what combination of primers and PCR conditions could work better for which order/family/genera. In fact we tried a large amount of primer combinations (see Tab. 4 for more details), but overall we only obtained good quality sequences with the following pairs of primers: F-753/R-rbcSstart and F-993/R-rbcSstart (Freshwater & Rueness, 1994).

For the COI-5P we obtained 101 sequences out of 232 samples (43% success rate). This region is considered the DNA barcode of most organisms that possess mitochondria (Hebert *et al.* 2003) and has been recommended to be used for the Rhodophyta (Saunders 2005) and Corallinophycidae (Walker *et al.* 2009). Although the success rate for this marker is less than 50%, the effort to obtain good quality sequences was justified as it has proven to be the more variable marker when compared to *psbA*, *rbcL*-3P and UPA (see Table 6 for more details). The most successful combination of primers among the ones we tested was GwsFn x GwsRi (Le Gall & Saunders, 2010) (see Tab. 4 to see the list of primers we tested). This specific marker is known to be difficult to amplify even for other groups of Rhodophyta as many authors have reported (for Gracilariales - Costa 2013, Medeiros 2013, for *Hypnea* - Nauer 2013, for Gelidiales - Iha 2014). The discrepancy in the number of good quality sequences

obtained to this marker versus the number of sequences in the matrix constructed to this chapter analyses is due to *Sporolithon* specimens, for which we generated and used more sequences to construct a matrix with only Sporolithales taxa (results are shown in the next chapter).

For the *psbA* we obtained 96 sequences out of approximately 180 samples (53% success rate), the same set that we tried for the *rbcL*-3P. This marker was successfully applied to other groups of Rhodophyta (Seo *et al.*, 2003; Yang & Boo, 2004) and to the CCA (Broom *et al.* 2008, Bittner *et al.* 2011, Pardo *et al.* 2014, Richards *et al.* 2014, Sissini *et al.* 2014), and although none of these studies reported the successful rates of amplification, most of them reported that it is an easily amplifiable marker.

Considering that we worked with a very wide set of samples from different genera and orders, these resulting success rates are satisfactory. Also the use of a combination of different markers enabled us to obtain molecular data for a larger array of samples (65%) than each individual marker.

### ***Intraspecific and Interspecific divergence among molecular markers x orders***

We compared intraspecific variation among molecular markers and orders, with the exception of Sporolithales species (discussed in detail in the next chapter). Intraspecific variation presented in Table 6, represents the divergences found in our own dataset; the interspecific variation was calculated based on the species we found in Brazil compared to species from the same order downloaded from Genbank. In general, all of the interspecific divergence values we observed are in accordance to those found in previous studies for the Rhodophyta, including other CCA (Saunders 2005, Cassano 2009, Broom *et al.* 2008, Clarkston & Saunders 2010, Freshwater *et al.* 2010, Milstein

*et al.* 2012, Hind & Saunders 2013, Iha 2014, Nauer 2013, Sissini *et al.* 2014, Vieira-Pinto *et al.* 2014).

In order to not overestimate the diversity of species found, we used a conservative approach. Therefore, some intraspecific variation values need to be taken in consideration carefully as the values may in fact represent the divergence between very closely related species, especially with UPA and *psbA* values, as in the case of Hapalidiaceae sp. 8 that presented 1.3% intraspecific variation for UPA, whereas referenced studies presents 0-0.8% intraspecific variation and 0.6-8.4% of interspecific variation, and *Lithothamnion* sp. 1 that presented 2.3% intraspecific variation for *psbA*, whereas referenced studies presents 0-0.3% intraspecific variation and 2.5-13.1% of interspecific variation (see Table 6 for details).

Intraspecific divergence observed among the Corallinales in this study for UPA and COI-5P was in accordance to what was found in previous studies (Table 6). *RbcL*-3P and *psbA* showed a relatively high-end intraspecific divergence (0.7% and 0.9% respectively) nearly overlapping with the low-end of interspecific divergences previously found for these markers (Table 6). Values for *rbcL*-3P are not well established. For example, a recent study that used this region in a single locus-analysis did not report any intraspecific divergence (Sissini *et al.*, 2014), therefore it was not possible to evaluate if the divergence values we found for *rbcL*-3P among the Corallinales represent in fact two separate species or if it correspond to a real intraspecific variation.

The intraspecific divergence observed among the Hapalidiales for *rbcL*-3P and for the COI-5P was in accordance to what was found in previous studies (Table 6). UPA and *psbA* showed the relatively high-end of divergence (1.3 and 2.3%, respectively)

overlapping or almost overlapping with the values for the low-end of interspecific divergences previously found for these markers.

Unfortunately, as we did not obtain all four markers for the exact same samples, it is impossible to directly compare the diversity found for each marker.

Considering the rates of success and the divergence values for each marker, the most applicable markers to use in a more comprehensive approach are COI-5P and *psbA* followed by *rbcL*-3P. These results corroborate similar previous studies that used COI-5P and *psbA* to reveal diversity among the CCA in other regions of the world (Peña *et al.* 2014 for the Caribbean, Pardo *et al.* 2014, for Europe). Overall, there were no notable differences between the orders considering the markers tested in this diversity survey.

Table 6. Intraspecific and interspecific variation found in this study versus divergence rates found in the following references for the Rhodophyta: Saunders 2005, Cassano 2009, Clarkston & Saunders 2010, Freshwater *et al.*, 2010, Hind & Saunders 2013, Hind *et al.* 2014, Iha 2014, Nauer 2013, Sissini *et al.* 2014, Vieira-Pinto *et al.* 2014, Peña *et al.* 2015 – Adapted from Torrano-Silva (2015).

Markers	Intraspecific divergence (this study)	Intraspecific divergence (this study)	Intraspecific divergence (this study)	Intraspecific divergence (References)	Interspecific divergence (this study)	Interspecific divergence (this study)	Interspecific divergence (this study)	Interspecific divergence (References)
	CORALLINALES	HAPALIDIALES	SPOROLITHALES		CORALLINALES	HAPALIDIALES	SPOROLITHALES	
<b>UPA</b>	0.3%	0.3 - 1.3%	0.2%	0 - 0.8%	6.8 - 13%	3.4-4%	4.7-5.4%	0.6 - 8.4%
<b><i>rbcL- 3P</i></b>	0.7%	0%	0.2-0.4%	0%	5.3 - 6%	8.2 - 11%	0.8%-12%	0.9 - 12%
<b>COI-5P</b>	0.3 - 0.8%	0.2 - 0.4%	0.2-0.7%	0 - 2.6%	7.6 - 12.3%	7.8 - 15.1 %	3-15%	3.3 - 18.2%
<b><i>psbA</i></b>	0.1 – 0.9%	0.1 - 2.3%	0	0 - 0.3%	4.1 - 11.8%	3.9 - 7.3%	0.1-11.4%	2.5 - 13.1%

### *Diversity of CCA along the Brazilian coast*

This study provides an assessment of the taxonomic diversity of the encrusting and rhodolith-forming species collected on the coast of Brazil using molecular tools and SEM. Our molecular analyses based on four molecular markers (UPA, *rbcL*-3P, COI-5P and *psbA*) have revealed the occurrence of 37 different species belonging to Corallinales and Hapalidiales on the Brazilian coast (Sporolithales are shown in the chapter 2). Most importantly, very few of the sequences generated in this study matched publicly available sequences. This observation suggests that some species studied are potentially new to science, or belong to described species for which no sequence data is yet available. In either case, our results show that CCA is a diverse group in Brazil and in the Tropical Western Atlantic and will be a significant contribution to the sequence databases (e.g. GenBank) for this group of organisms.

Applying specific epithets and even genus names was very challenging and most of the times not yet possible, mainly because for most species found in this study, we only collected one or few individuals. In the field, most of the time it was not possible to distinguish the CCA by their external morphology, and while we tried to collect an abundance of samples, we could not be sure of their identity even at the ordinal level (due to the very similar external morphology they present between orders; compare the similarity between Figs. 22A – Corallinales and 39A, B – Hapalidiales). Besides that, many of the samples were not fertile, therefore applying names correctly becomes even more difficult. Also, recent DNA-based studies have revealed multiple, cryptic species passing under a single name in the same geographic area (e.g., southern Japan, Kato *et al.*, 2013; central New Zealand, Broom *et al.*, 2008, Brazilian coast, Sissini *et al.*, 2014), in part because of misapplied names. Therefore, only by sequencing type material, or

less desirably, samples from a species' type locality, can names be applied with certainty (Hughey *et al.*, 2001, Hughey & Gabrielson, 2012).

In the following section we discuss some of the applied binomial and generic names and the putative species for each family.

The results of the UPA analysis (Fig. 6) show three applicable names for the clade including Corallinales species and one for the clade containing the Hapalidiales. Based on this marker, we could identify *Lithophyllum atlanticum* and *L. margaritae*. Both species were already previously sequenced and well described for Santa Catarina state (Vieira-Pinto *et al.* 2014), therefore no morpho-anatomical investigations were made. The third species is *Lithophyllum kotschyanum* (sequence available from Australia, Rösler *et al.* 2016) that grouped with a specimen from the oceanic island Trindade (located 1.167 kilometers from the Brazilian coast) with a strong support in the NJ analysis, supported by ABGD analysis and with a low intraspecific variation (0.3%). Unfortunately, the Brazilian specimen was much damaged and not reproductive, and it was not possible to obtain good images from it. Based on the molecular evidence, we are going to tentatively apply this epithet and therefore this is the first record of *L. kotschyanum* for the Atlantic coast. However UPA is a less variable marker (Sherwood *et al.*, 2010a, 2010b, Milstein *et al.* 2012) and *L. kotschyanum* was originally described from the Persian Gulf (Woelkerling *et al.* 2005). Furthermore, Rösler *et al.* (2016) in their multigene analysis, show several paraphyletic branches of specimens assigned to *L. kotschyanum* and morphologically related species (Pacific and Indian ocean), suggesting that the type needs to be sequenced to better address the relationship between specimens from outside the Indian ocean.

In the Hapalidiales, we could also apply a name to *Mesophyllum erubescens*, as this species grouped with the holotype in the *rbcL*-3P analysis (see Fig. 7 in the results section) and is very well described from the Atlantic (Horta *et al.*, 2011, Costa *et al.*, 2014b, Sissini *et al.* 2014), therefore no morpho-anatomical investigations were made for this species.

For three other specimens, using the UPA, we could apply a name at the generic rank: *Lithophyllum* sp. 1, *Neogoniolithon* sp. 1 and and *Lithothamnion* sp. 1. *Lithophyllum* sp. 1 grouped with other known species of this genus, but unfortunately the sample was non-reproductive. Based on the cell shapes and the predominant occurrence of secondary pit connections and absence of cell fusions (data not shown), we concluded that this is the appropriate genus to apply to this specimen. The specimen *Neogoniolithon* sp. 1 was fertile (tetrasporophyte - Fig. 28) and all the characters exhibited matched the genus description (e.g. presence of cells fusions and absence of secondary pit connection) and therefore we also concluded that this is the appropriate genus to apply to this specimen. *Lithothamnion* sp. 1 was identified based on further analyses of *psbA* and also due characteristic generic features of this genus (e. g. flared epithallial cells [i.e. with a trapezoidal lumen] – Fig. 30-F).

Based on the data we have so far, we were uncertain of genus and species names, therefore all remaining species shown in this tree were named only at the family rank. We chose to take a more conservative approach as to not misapply any names that might lead readers to confusion when using these data for comparison.

The results of the *rbcL*-3P analysis (Fig. 7) show four specimens to which we could apply a genus name. *Lithophyllum* sp. 2 and *Lithophyllum* sp. 3 grouped with other *Lithophyllum* species and we further show these specimens are nested in a large

clade of Lithophylloideae species. Also, *Lithophyllum* sp. 3 grouped with other *Lithophyllum* species in the *psbA* analysis, and its anatomy matches that of this genus. In the Hapalidiales clade, *Mesophyllum erubescens* was the only species identified as the specimens of this clade grouped with a holotype (voucher TRH C15-3212; Genbank acc. number KP050698; type locality Fernando de Noronha, Brazil – Sissini *et al.* 2014) from Genbank. *Lithothamnion* sp. 1 and *Lithothamnion* sp. 2 were identified based on further analyses of *psbA* and also due characteristic generic features of this genus (e. g. flared epithallial cells [ie. with a trapezoidal lumen] – Fig. 30-F for *Lithothamnion* sp. 1 and data not shown for *Lithothamnion* sp. 2).

All the remaining species shown in this tree were named only to family rank due to insufficient or lack of comparable data obtained so far.

The results of the COI-5P analysis (Figs. 8-11) show seven specimens to which available names can be applied. One specimen can be identified to the genus and species level and six others can be identified to the genus level. In the Hapalidiales I (Fig. 10), *Mesophyllum erubescens* consisted of a clade, previously shown in *rbcL*-3P, which grouped with a holotype from Genbank (KP050698 – Sissini *et al.* 2014), therefore we were confident to apply this name to those specimens. In the Corallinales (Fig. 8), *Lithophyllum* sp. 2, *Lithophyllum* sp. 4 and *Lithophyllum* sp. 5 are nested in a larger clade with other Lithophylloideae species. A “*Hydrolithon onkodes*” (GQ917288 – Bittner *et al.* 2011) is also nested in this clade, but this could be a case of a misapplied name as there are two other clades containing *Hydrolithon* species in this tree and there is no morphological description for this specimen (Bittner *et al.* 2011). *Neogoniolithon* sp. 1 is nested in a larger clade with other *Neogoniolithon* species with moderate support (70/1/65 for ML, BI and NJ respectively), and for this species we also generated high quality images (Figs. 27-28). The morpho-anatomical features we observed are

also in accordance with the circumscription of this genus and therefore we were confident to apply this genus name. *Lithothamnion* sp. 1 and *Lithothamnion* sp. 2 were identified based on further analyses of *psbA* and also due to characteristic generic features of this genus (e. g. flared epithallial cells [ie. with a trapezoidal-shaped lumen] – Fig. 30-F for *Lithothamnion* sp. 1 and data not shown for *Lithothamnion* sp. 2).

All the remaining species shown in this tree were named only to family rank due to insufficient or lack of comparable data obtained so far.

The results of the *psbA* analysis (Figs. 12-15) show two applicable specific epithets and four genus names that can be applied to Brazilian specimens. Among the Corallinales, the *Neogoniolithon* clade (Fig. 13) received moderate support in BI and NJ (0.50 and 99 respectively) whereas species of *Neogoniolithon* from different oceans, e.g. Pacific and North Atlantic, grouped together. Therefore, we could name *Neogoniolithon* sp. 1 and *Neogoniolithon* sp. 2 with confidence. *Neogoniolithon* sp. 1 is sister to *N. brassica-florida* from Spain, but showed 2.1% of divergence, moreover this species is often confused with *Neogoniolithon fosliei*, a species that has been referred to Brazil (Bahia 2014) and as our species did not present all reported features to confirm the identity, we applied the genus name only. *Neogoniolithon* sp. 2 grouped with *Neogoniolithon spectabile* from Mexico, but showed 4.1% of divergence, indicating that it might be a different species, and therefore we applied only the genus name for this specimen. *Lithophyllum atlanticum* was identified based on the UPA tree; sample IBC1527 grouped with *Lithophyllum atlanticum* (KP192377) from Genbank. *Lithophyllum* sp. 2, *Lithophyllum* sp. 3 and *Lithophyllum* sp. 4 are nested in the same clade as *Lithophyllum atlanticum*, and were also shown to belong to *Lithophyllum* in the other gene trees in this study, therefore we were confident to name these species at genus level. In the Hapalidiales clade (Fig. 15), *Mesophyllum erubescens* consisted of a

clade previously shown in both *rbcL*-3P and COI-5P, therefore we were confident to apply this name to those specimens.

*Lithothamnion* sp. 1 and *Lithothamnion* sp. 2 grouped with a specimen identified as *L. muelleri* (JQ896242) in this analysis. However, the type locality of *L. muelleri*, which is the generitype of *Lithothamnion*, is in Australia and the specimen available for comparison from Genbank was collected in Mexico. Therefore, the sequence available on GenBank is not ideal, considering it would be better to have the type itself or at least a topotype to compare. However, we have decided to tentatively apply this genus name in all of our previous trees, based also on the characteristic generic features that the species showed (e. g. flared trapezoidal epithallial cells – Fig. 30-F for *Lithothamnion* sp. 1 and data not shown for *Lithothamnion* sp. 2). Furthermore, we discuss more about *Lithothamnion* sp. 1 in chapter 3 (as *Lithothamnion* sp. I).

All of the remaining species shown in this tree were named only to the rank of family, due to insufficient or lack of comparable data obtained so far.

### *Specimens named to rank of family*

Corallinaceae sp. 1, Corallinaceae sp. 5 and Corallinaceae sp. 9 grouped together in the *psbA* analysis (Fig. 14). Though anatomical data were generated only for Corallinaceae sp. 5, the results of the *psbA* analysis indicate the three species likely belong to the same genus, considering the clade they formed has a relative high support in all three analyses and the interspecific divergence among those three species is 7.8% to 10.3%. Anatomical features of Corallinaceae sp. 5 correspond with descriptions of Mastophoroideae genera, although is not possible to determine to which one it belongs as most genera differ based on spermatangial development and on carposporophyte features (Penrose, 1991, Penrose & Woelkerling, 1992, Kato *et al.*, 2011) and no gametophytes were found.

In the UPA analysis, Corallinaceae sp. 1 grouped with *Spongites* and in *psbA* analysis Corallinaceae sp. 1 and Corallinaceae sp. 5 are nested in a clade with other Mastophoroideae (*Hydrolithon* spp. and *Pneophyllum* spp.), but apart from *Spongites*. Therefore, until more sequences are generated from additional specimens and anatomical features from gametophytes of these taxa are documented, it is not possible to resolve their identity.

Corallinaceae sp. 2 and Corallinaceae sp. 4 likely belong to the same genus, and based on the *psbA* analysis they are nested in a larger clade with other Mastophoroideae spp. Both species possess cell fusions and lack secondary pit connections between adjacent filaments. Specimens also present trichocytes and flattened epithallial cells, but due to the lack of gametophytes we were not able to identify both specimens to genus rank.

Also based on the *psbA* results, Corallinaceae sp. 6 and Corallinaceae sp. 7 are nested in a clade with *Hydrolithon* species (Fig. 14 - *Hydrolithon* I) and Corallinaceae

sp. 8 is nested in another clade containing species of *Hydrolithon* (Fig. 14 – *Hydrolithon* II). None of them were named as *Hydrolithon* because we could not be sure which clade in fact corresponds to the real *Hydrolithon*. Corallinaceae sp. 6 shows some features that matched with the Mastophoroideae circumscription (e. g. presence of exclusively cell fusions and lack of secondary pit connection with the adjacent filaments, presence of trichocytes, uniporate conceptacles), but it is most likely that it belongs to the *Porolithon* genus as it presents tightly packed trichocytes (Fig. 23 – G-H) which is diagnostic for this genus (Kato *et al.* 2011). However, we decided to not identify Corallinaceae sp. 6 as *Porolithon* sp. due to the fact that the circumscription and relationship between *Porolithon* and *Hydrolithon* are not well resolved and need further research (Kato *et al.* 2011, Bittner *et al.* 2011, Woelkerling *et al.* 2012). Regarding Corallinaceae sp. 7, it was not possible to confirm whether this species belongs to *Hydrolithon* because no images of this taxon were generated. In regards to Corallinaceae sp. 8, this species is nested in the clade that we called *Hydrolithon* II, including a specimen identified as *H. reinboldii* (GQ917484, Bittner *et al.* 2011), which is the species that typifies the genus. Although this specimen was collected in New Caledonia and relatively close to the type locality (Indonesia), the anatomical features shown by Corallinaceae sp. 8 (Figs. 26-27) are not in accordance to what is described as diagnostic for *Hydrolithon*. Harvey *et al.* (2006) state that species belonging to *Hydrolithon* show tetra/bisporangial conceptacle pore canals lined by cells that are orientated perpendicularly to the thallus surface, whereas our specimen (Corallinaceae sp. 8 – Figs 25 and 26) that grouped in the clade with *H. reinboldii* shows tetrasporangial conceptacle pore canals lined by cells that are orientated parallel to the thallus surface (Fig. 26). Therefore, we maintained our specimen as Corallinaceae sp. 8 instead of naming it a *Hydrolithon* species.

As shown previously in the results section, the Hapalidiales clade was not monophyletic with the exception of the *psbA* analysis, and a lot of inconsistencies and polyphyly were found among genera (e. g. *Phymatholithon* clades – Fig. 15), which made it more difficult to match specimens to a genus in this order. Based on *psbA* analysis, Hapalidiaceae sp. 1 and Hapalidiaceae sp. 4 are likely to belong to the same genus and both species nested in a clade with the *Clathromorphum* complex. But genera among the *Clathromorphum* complex are restrictively found in cold waters while Hapalidiaceae sp. 1 and Hapalidiaceae sp. 4 occur in the Tropical Western Atlantic which may indicate that these taxa are different genera.

Hapalidiaceae sp. 2, Hapalidiaceae sp. 3 and Hapalidiaceae sp. 5 were not successfully amplified for *psbA*, but they nested within the Hapalidiales in the UPA and/or *rbcL* and COI-5P analyses. For Hapalidiaceae sp. 3 and Hapalidiaceae sp. 5, no informative images were generated and unfortunately no more specimens of these species were identified in our collections. For Hapalidiaceae sp. 2, vegetative features were observed, but no informative characters were found. This specimen exhibited some structures that might be benthic dinoflagellate stages inside its cells walls (Fig. 34H) and similar round/disk-shaped structures were observed in Hapalidiaceae sp. 1 (Fig. 32E). Kraysky-Self (2015) documented similar structures in rhodoliths and reported that studying endolithic relationships between coralline red algae and dinoflagellates (or Haptophytes, as she found in her study) could lead to deeper understanding of benthic marine ecological patterns in general. Though this was not our focus when performing SEM on our samples, we believe it could shed light on studies in the future.

In the *psbA* analysis (Fig. 15), Hapalidiaceae sp. 6, Hapalidiaceae sp. 7, Hapalidiaceae sp. 8, Hapalidiaceae sp. 9, Hapalidiaceae sp. 10, Hapalidiaceae sp. 12 and

Hapalidiaceae sp. 13 are all nested in one clade with strong support in all analyses; therefore we believe that they might represent a single genus that is possibly new to science. Between the species in this clade, the interspecific variation ranged from 3.3% to 13% in the *psbA* matrix. As we observed in the *psbA* tree (Fig. 15), all sequences from Genbank that grouped within this clade are not identified and appear as *Mesophyllum* spp. (KC819249, KC819252 and KC819269, Pardo *et al.*, 2014), *Lithothamnion* sp. (KJ710353 – in the publication as *Lithothamnion* cf. *ruptile*, Peña *et al.*, 2014) or Hapalidiaceae (GQ917460, Bittner *et al.* 2011). Pardo *et al.* 2014 documented that these *Mesophyllum* species possessed anatomical characters congruent with *Lithothamnion*, though the authors concluded the specimens belonged to *Mesophyllum* because they grouped with *Mesophyllum sphaericum*. Peña *et al.* (2014) showed in the results of their *psbA* analysis that the species of *Lithothamnion* mentioned above comprised a clade separate from other *Lithothamnion* species. We obtained anatomical images only for Hapalidiaceae sp. 7 (Fig. 38) and Hapalidiaceae sp. 10 (Figs 39-40), but unfortunately no obvious distinguishing characters were observed. Therefore, further DNA analyses and thorough anatomical investigations of additional specimens need to be done to characterize these species. DNA sequencing of the generitype specimen of *Lithothamnion muellerii* needs to be performed before we can confirm if this is in fact a new genus. More discussion on this clade is shown in Chapter 3.

Hapalidiaceae sp. 11 grouped with a sequence from Genbank of *Lithothamnion* sp. collected in Fiji, but considering this clade did not group with the other *Lithothamnion* spp., we kept this specimen as Hapalidiaceae sp. 11 instead of treating it as *Lithothamnion*.

### *Distribution of CCA along the Brazilian coast*

The distribution of coralline algae found in our study, despite the low number of specimens found for some species, is consistent with a general pattern observed in many taxonomic groups where a majority of species have small geographic ranges, whereas a few species have larger ranges (Gaston, 1996). This distribution pattern is also similar to what was found for rhodolith forming species in Europe (Pardo *et al.* 2014) and the Caribbean (Peña *et al.* 2014).

The Corallinales and Hapalidiales species diversity found in this study is represented in maps where we show the species' distributions along the Brazilian coast (Fig. 42 – Corallinales; Fig. 43 - Hapalidiales). In this study we could not address diversity or species richness in the ecological definition of these terms; by diversity we are referring to the number of species collected during a limited single expeditions to each sample site, and this should be taken in consideration when we discuss diversity herein.

Considering our sampling effort and results obtained in this study, we conclude that members of the Corallinales are distributed from Rio Grande do Norte state (RN, 5°S) to Santa Catarina state (SC, 27°S) and also in the vicinity of Fernando de Noronha Island (3°S) (Fig. 42). Therefore, according to Spalding *et al.* (2007), we collected in the Tropical Atlantic with a southern limit in the Temperate South American realm. When we compiled the information available in the literature for the occurrence of Corallinales along the Brazilian coast (Table 2, Introduction section), a similar distribution was observed for this order; therefore we corroborate with molecular data that in fact the order is widespread in Brazil. We did not obtain any sample from Rio Grande do Sul state in this study, which is the southern limit of the

Brazilian coast (approximately 30°S). However, a species of *Lithophyllum*, *L. atlanticum*, which is a member of the Corallinales, has been reported for this state based on morphological and molecular data (Vieira-Pinto *et al.* 2014). Regarding the northern limit, a recently published study reported a species of CCA belonging to the Corallinales (*Hydrolithon ruprestre*) from the region of the Amazon River mouth (between French Guiana border and Maranhão state in Brazil) (Moura *et al.* 2016). Although we obtained a few samples from a nearby area in the region, we were unable to successfully amplify some of the specimens and the other samples sequenced did not belong to Corallinales.

The genus *Lithophyllum* seems to be the most widespread based on our dataset, with *Lithophyllum* sp. 2 and *Lithophyllum* sp. 4 being the most common ones within the genus. Corallinaceae sp. 8 seems to be restricted to Oceanic Islands (Fernando de Noronha and Trindade Islands).

The Hapalidiales samples collected and investigated in this work are distributed from Maranhão state (MA, 1°S) to Santa Catarina state (SC, 27°S) (Fig. 43). Therefore, according to Spalding *et al.* (2007), they range from their northern limit in the Tropical Atlantic to the southern limit in the Temperate South American realm. Likewise, similar to what was observed for the Corallinales in the literature, the Hapalidiales has a wide distribution along the Brazilian coastline; thus we corroborate with molecular data that in fact the order is widespread in Brazil, including Maranhão state, which is near the region of the Amazon river mouth. Hapalidiaceae sp. 8 and *Mesophyllum erubescens* seem to be the most widespread species occurring from the Northeastern to the Southern Brazilian coastline (from 5°S to 27°S), corroborating the previously published data shown by Sissini *et al.* (2014), followed by Hapalidiaceae sp. 4, occurring from the Northern to the Southeastern coastline (nearly 0° to 12°S).

Overall, the greatest number of Corallinales species we found in this study was in Bahia state (BA – 7 species) followed by Espírito Santo state (ES – 5 species). However, for Hapalidiales, the most species were observed in Espírito Santo state (ES – 10 species) followed by Bahia state (BA – 6 species). This higher number of species, in comparison to the other Brazilian states, was not unexpected considering a similar pattern was shown before by Horta *et al.* (2001) who proposed that Espírito Santo state is the transition zone between the two macroalgal phytogeographic zones in Brazil (the tropical and the warm temperate subtropical). This transition zone encompasses Espírito Santo and the south of Bahia state and includes a variety of different habitats (e.g. coral reefs, rocky shores, rhodolith beds) (Oliveira Filho 1969, Oliveira Filho 1977; Horta *et al.* 2001) that are unique to this part of the Brazilian coast, (Nunes, personal communication) and this may be the explanation for the higher level of diversity found in this particular area.

Comparing the number of species found for Corallinales and Hapalidiales with the list of previously reported species (see Tab. 2 in the introduction section), a total of 32 species (after excluding exclusively epiphytic species, which is beyond of the scope of this study) of these two orders were reported for Brazil. Of these species, only nine were reported based on molecular studies (Vieira-Pinto 2011; Sissini 2013, Sissini *et al.* 2014; Vieira-Pinto *et al.* 2014; Torrano-Silva 2015; Henriques 2016), and of those nine species only three of the sequences were deposited in public databanks (Sissini *et al.* 2014; Vieira-Pinto *et al.* 2014). In this study, we found at least 37 putative different species (see Tab. 7 in this section). For the Corallinales, prior to this study, 21 species were reported, only six of these reports were corroborated with molecular data. In this study, we generated molecular data for 19 different species in this order. For the Hapalidiales, 11 species were previously reported, only four of which were based on

molecular data. In this study, we found 16 putative different molecular species for this order.

The results found in this study corroborate our hypothesis that the diversity for CCA is underestimated. However, it is not clear if the species names reported in previous studies are applicable to specimens investigated in this study. Therefore, future investigations need to be performed to link the molecular data to taxonomic names. This study forms an important foundation for future work and is a significant contribution for enriching the sequence databanks for this group of algae, especially for the South Atlantic, from where few sequences are available. Further research will be needed, particularly obtaining sequences from types or topotypes, in order to fully understand the species and generic diversity of CCA occurring along the Western South Atlantic.

Also, some of our specimens appear to be closely related to species from the Indo Pacific (i.e. Corallinaceae sp. 7, *Mesophyllum erubescens*, Hapalidiaceae sp. 11). Considering it has been previously hypothesized that a portion of the Brazilian marine macroflora has an Indo-Pacific origin (Horta *et al.* 2001), applying different markers in future studies could help to clarify its origin and the biogeographical pathways that led to the present distribution of CCA in Brazil.



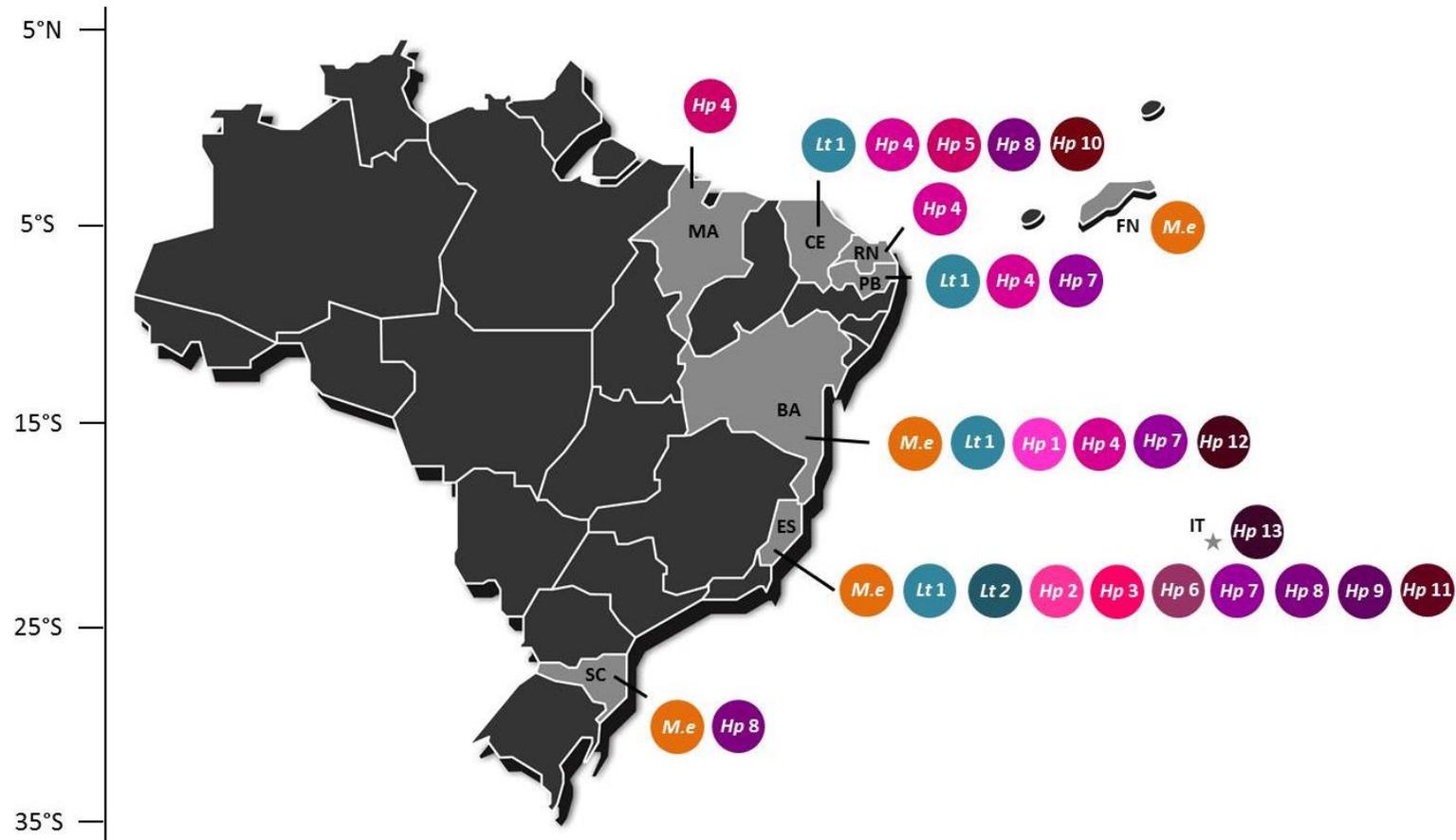


Figure 43. Map of distribution of Hapalidiales spp. found in this study. Highlighted in light gray are the states where samples were collected. States and oceanic Islands abbreviations are as follows: MA=Maranhão, CE=Ceará, FN=Fernando de Noronha Island, RN=Rio Grande do Norte, PB= Paraíba, BA=Bahia, ES=Espírito Santo, IT= Trindade Island, SC=Santa Catarina. Species abbreviations are as follows: M.e=*Mesophyllum erubescens*, Lt1=*Lithothamnion* sp.1, Lt2=*Lithothamnion* sp.2, Hp1=Hapalidiaceae sp. 1, Hp2=Hapalidiaceae sp. 2, Hp3=Hapalidiaceae sp. 3, Hp4=Hapalidiaceae sp. 4, Hp5=Hapalidiaceae sp. 5, Hp6=Hapalidiaceae sp. 6, Hp7=Hapalidiaceae sp. 7, Hp8=Hapalidiaceae sp. 8, Hp9=Hapalidiaceae sp. 9, Hp10=Hapalidiaceae sp. 10, Hp11=Hapalidiaceae sp. 11, Hp12=Hapalidiaceae sp. 12, Hp13=Hapalidiaceae sp. 13.

Table 7. Species and distribution of samples analyzed in this study.

Species	Samples	Distribution
<i>Lithophyllum atlanticum</i>	IBC1527; IBC1927	SC, SP
<i>Lithophyllum margaritae</i>	IBC1928	SC
<i>Lithophyllum kotschyianum</i>	IBC1554	IT
<i>Lithophyllum</i> sp. 1	IBC1734; IBC1795	BA, AL
<i>Lithophyllum</i> sp. 2	IBC1793; IBC1794; IBC1921	RJ, AL
<i>Lithophyllum</i> sp. 3	IBC1726; IBC1878	ES, BA
<i>Lithophyllum</i> sp. 4	IBC1507; IBC1633; IBC1792; IBC1872; IBC1873	ES, BA, AL, RN
<i>Lithophyllum</i> sp. 5	IBC1717	BA
<i>Neogoniolithon</i> sp. 1	IBC1202; IBC1216	ES
<i>Neogoniolithon</i> sp. 2	IBC1585	AL
<i>Mesophyllum erubescens</i>	IBC1712; IBC1765; IBC1875; IBC1877; IBC1926	SC, ES, BA, FN
<i>Lithothamnion</i> sp.1	IBC1525; IBC1557; IBC1564; IBC1704; IBC1708; IBC1907; IBC1910	ES, BA, PB, CE
<i>Lithothamnion</i> sp.2	IBC1869	ES
<i>Corallinaceae</i> sp. 1	IBC1247	PB
<i>Corallinaceae</i> sp. 2	IBC1209; IBC1228	ES
<i>Corallinaceae</i> sp. 3	IBC1566	IT
<i>Corallinaceae</i> sp. 4	IBC1222	ES
<i>Corallinaceae</i> sp. 5	IBC1737	BA
<i>Corallinaceae</i> sp. 6	IBC1917	FN
<i>Corallinaceae</i> sp. 7	IBC1537	BA
<i>Corallinaceae</i> sp. 8	IBC1798; IBC1919	IT, FN
<i>Corallinaceae</i> sp. 9	IBC1752	BA
<i>Hapalidiaceae</i> sp. 1	IBC1790	BA
<i>Hapalidiaceae</i> sp. 2	IBC1196	ES
<i>Hapalidiaceae</i> sp. 3	IBC1207	ES
<i>Hapalidiaceae</i> sp. 4	IBC1246, IBC1562, IBC1657, IBC1662, IBC1665, IBC1669, IBC1680, IBC1710, IBC1804, IBC1850, IBC1909	BA, PB, RN, CE, MA
<i>Hapalidiaceae</i> sp. 5	IBC1559	CE
<i>Hapalidiaceae</i> sp. 6	IBC1526	ES
<i>Hapalidiaceae</i> sp. 7	IBC1188; IBC1245; IBC1547; IBC1876; IBC1882	ES, BA, PB
<i>Hapalidiaceae</i> sp. 8	IBC1191, IBC1558, IBC1560, IBC1922, IBC1923, IBC1924, IBC1925	SC, ES, CE
<i>Hapalidiaceae</i> sp. 9	IBC1886	ES
<i>Hapalidiaceae</i> sp. 10	IBC1685, IBC1688	CE
<i>Hapalidiaceae</i> sp. 11	IBC1865	ES
<i>Hapalidiaceae</i> sp. 12	IBC1703	BA
<i>Hapalidiaceae</i> sp. 13	IBC1553	IT
Unidentified sp. 1	IBC1551	IT
Unidentified sp. 2	IBC1555	IT

## Final remarks

- Considering the informativeness and cost-benefit based on our data, the most suitable markers to use in comprehensive and broad studies of CCA are *psbA* and COI-5P, followed by *rbcL*-3P.
- Very few of the sequences generated in this study matched publicly available sequences, suggesting that some species studied are potentially new to science, or belong to described species for which no sequence data are yet available. Therefore this study provides a significant contribution for enriching the sequence databanks for these algae, particularly for the South Atlantic, from where few sequences are available.
- Our results demonstrated that the diversity might be underestimated, especially within the Hapalidiales. Before this study there were 32 CCA species between Corallinales and Hapalidiales referred to the Brazilian coast, with only nine based on molecular data. After this study we have generated molecular data for at least 35 different species of Corallinales and Hapalidiales. Before this study, there were 21 species of Corallinales referred to Brazil, with only six for which molecular data were generated; in this study we generated molecular data for 19 different species of this order. For the Hapalidiales 11 species were previously reported for the Brazilian coast, with molecular data generated for only four species; in this study we generated molecular data for 16 different species of this order.
- Applying specific epithets and even genus names was not practical for our entire dataset, mainly because for most species found in this study, we only collected one or a few individuals of which many of them were not fertile (it was especially difficult to find gametophytes). Therefore future investigations will be

needed to link the sequences generated in this study to the applicable names and/or to propose new species and genera.

- Applying molecular tools, such as DNA barcoding, to studies of the diversity of CCA is fundamental and opens a new perspective in this particular group which has been historically difficult to study considering the challenging techniques and time-consuming preparations necessary to properly study their anatomy.
- Some of our specimens seem to be closely related to species from the Indo-Pacific. Applying different markers could help to understand this relationship between the flora of the Atlantic and Indo Pacific and clarify the biogeographical pathways that led to their present distribution.
- Considering all the three orders of CCA, this study represents the first broad attempt and effort to unveil the diversity of CCA species found on the Brazilian coast using molecular data.

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## Second Chapter

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# DIVERSITY OF THE SHALLOW WATER *SPOROLITHON* (SPOROLITHALES, RHODOPHYTA) FROM THE SOUTH ATLANTIC COAST

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## Abstract

*Sporolithon* (Sporolithales, Rhodophyta) is a commonly found crustose coralline algal (CCA) genus. It is one of the main genera found in tropical waters forming either rhodoliths or crusts. The genus presents as distinguishing characteristic the development of spores in delicate calcified compartments. Despite efforts in the past decade to investigate and document the diversity of CCA as a whole along the Western Atlantic coast, especially in Brazil, few studies have combined molecular-based analyses with morphological/anatomical approaches; hence the diversity of the group is probably underestimated. In the present study, we combined DNA sequences (COI-5P and *psbA*) and Scanning Electron Microscopy (SEM) anatomical characterization to identify *Sporolithon* species that occur in the shallow waters along the Brazilian coast across a range extension of approximately 2,000 km (from Ceará to Espírito Santo states). Based on the results of GMYC and ML analyses for COI-5P sequences we were able to distinguish two different species, both new to science that are described herein as *Sporolithon pseudoepisporum* sp. nov. and *Sporolithon tomitae* sp. nov., and help clarify the species boundaries in this genus. These results reinforce the need of using molecular approaches in CCA studies to reveal the true level of diversity, especially to closely related and cryptic species.

**Key words:** Brazil, COI-5P, coralline algae, diversity, *Sporolithon*, *psbA*, SEM

**Running title:** *Sporolithon* from Western Atlantic shallow waters

## Introduction

The taxonomy of the crustose coralline algae (CCA) has historically been very challenging and several classifications have been proposed based solely on comparative morphological and anatomical features (Cabioch 1972, 1988, Johansen 1976, Silva & Johansen 1986, Woelkerling 1988, Harvey & Woelkerling 1995). Bailey and Chapman (1996, 1998) published the first molecular phylogenies of the Corallinales and confirmed the evolutionary scenario hypothesized by Cabioch (1988) that the geniculate taxa had evolved independently in distinct lineages in the order. Since then, molecular approaches have greatly improved the resolution of red algal phylogenies as a whole (e.g. Freshwater *et al.* 1999, Le Gall & Saunders 2007, Broom *et al.* 2008, Le Gall *et al.* 2010, Bittner *et al.* 2011, Kato *et al.* 2011) and as a consequence, some phylogenetic relationships between the CCA are being clarified. Presently, the CCA are divided into three orders, the Corallinales, the Hapalidiales and the Sporolithales (Nelson *et al.* 2015). The Sporolithales comprise a single family, the Sporolithaceae, with two genera, *Sporolithon* and *Heydrichia* (Verheij 1993, Townsend *et al.* 1994) both presenting cruciately divided tetrasporangia occurring individually in calcified compartments rather than in conceptacles (Verheij 1993). The family was previously included in the Corallinales (Verheij 1993) but was elevated to ordinal rank as the Sporolithales by Le Gall *et al.* (2010) because of its unique tetrasporangial development with a strong molecular argument based on molecular phylogenies that showed it is more closely related to the Rhodogorgonales than to the Corallinales (Harvey *et al.* 2002, Le Gall *et al.* 2010).

The genus *Sporolithon* is characterized by possessing thick-walled epithallial cells that are flared at their upper margins; cells of contiguous vegetative filaments joined by both secondary pit connections and cell fusions; tetrasporangia produced within

calcified sporangial compartments; tetrasporocytes that undergo cruciate cleavage; tetrasporangial compartments that bear apical pore plugs; and the absence of an involucre surrounding the calcified sporangial compartments (Verheij 1993; Townsend et al. 1995; Harvey et al. 2002; Le Gall et al. 2010).

Although the genus *Sporolithon* is generally considered a deep water (>20 m) genus of CCA (Verheij 1993, Lund et al. 2000, Braga & Bassi 2007, Bahia et al. 2011, Bahia et al. 2014a,b), it is commonly found growing epilithically or forming rhodoliths in shallow tropical and sub-tropical waters (Verheij 1993, Basso et al. 2009, Kaewsuralikhit et al. 2012) and in Brazil it seems to be one of the major constituents in coral reefs along the coastline (as shown in the present study). In Brazil, the genus is represented by the following species: *S. africanum* (Foslie) J. Afonso-Carillo, *S. australasicum* (Foslie) N. Yamaguishi-Tomita ex M.J. Wynne, *S. durum* (Foslie) Townsend & Woelkerling, *S. erythraeum* (Rothpletz) Kylin, *S. howei* (Lemoine) N. Yamaguishi-Tomita ex M.J. Wynne, *S. pacificum* E.Y. Dawson, *S. ptychoides* Heydrich (Bahia et al. 2011; Wynne 2011; Guiry & Guiry 2013, Bahia et al. 2014a, Henriques et al. 2014), *S. episorum* (M.A.Howe) E.Y. Dawson (Costa et al. 2014), *S. tenue* R.G. Bahia, G.M. Amado-Filho, G.W. Maneveldt & W.H. Adey (Bahia et al. 2014a), *S. elevatum* M.C. Henriques & R. Riosmena-Rodriguez (Henriques et al. 2014), *S. episoredion* (W.H.Adey, R.A.Townsend & Boykins) Verheij (as *S. episoredium* in Henriques et al. 2014) and *S. yoneshigueae* R.G. Bahia, G.M. Amado-Filho, G.W. Maneveldt & W.H. Adey (Bahia et al. 2015). The latter six species have been consistently identified based on the modern understanding of the genus and *Sporolithon durum* was reported as a new occurrence for the Atlantic Ocean (Wynne 2011); however, a detailed description of this record was not provided and is still needed as pointed out by Bahia et al. (2014a). The remaining five *Sporolithon* species were

reported in the doctoral dissertation of Dr. Tomita (1976), but have never been published. Most species were cited for depths greater than 20 meters up to 133 meters (Bahia *et al.* 2011, 2013, 2014a, Henriques *et al.* 2014), and only *Sporolithon episporum* was cited to shallow waters (Costa *et al.* 2014). From all the citations and descriptions for Brazilian waters, only three species were described based on molecular data, i.e. *Sporolithon ptychoides* and *S. tenue* (Bahia *et al.* 2013), and *S. yoneshigueae* (Bahia *et al.* 2015)

Even though the knowledge of the diversity of CCA as a whole in Brazil has improved recently, few studies have combined molecular analyses with morphological/anatomical approaches (Bahia *et al.*, 2013, 2015; Sissini *et al.*, 2014, Vieira-Pinto *et al.*, 2014, Bahia *et al.*, 2015); hence the diversity of the group is probably grossly underestimated.

Considering the importance of revealing the diversity of CCA, our aim in this study was to investigate the genus *Sporolithon* along the Brazilian coast based on morphological/anatomical and molecular data. Whereas our morphological data shows a low diversity for this genus in the Brazilian shallow waters, and only minor anatomical features appear to differ from the currently known species, our molecular data revealed two new species proposed in this study as *Sporolithon pseudoepisporum* T. Vieira-Pinto, P.A. Horta & M.C. Oliveira and *S. tomitae* T. Vieira-Pinto, P.A. Horta & M.C. Oliveira.

## **Material and methods**

Specimens were collected in 24 locations along the Brazilian coast. The majority of specimens were collected in the intertidal as rhodoliths (as loose nodules on the bottom) but when attached to the substrata, a hammer and chisel was used to remove samples.

For the molecular analyses, material was dried in the shade and stored in silica gel. Whenever possible, the samples were fractured in half and fixed partly in 4% formaldehyde-seawater solution to aid in preservation of the soft structures for morphological analysis and partly in silica gel. All specimens' data including vouchers, sampling localities, collectors, dates and sequenced markers are summarized in Table 1.

### *Molecular studies*

#### *DNA Extraction and PCR protocols:*

Total DNA was extracted from silica gel-dried samples using the Chelex resin protocol (adapted from Goff & Moon 1993). The mitochondrial COI-5P fragment was PCR-amplified using primer pairs GazF1/GazR1 or GWSFn/GWSRi (Saunders, 2005; Saunders & McDevit, 2012). The *psbA* locus was amplified using primer pairs *psbA-F1/psbA-R2* or *psbA-F1/psbA600R* (Yoon et al., 2002) or *psbA550R* (Sissini et al. 2014) whenever there were ambiguous bases from resulting *psbA600R* sequences. Thermal cycle profiles for PCR amplification of COI-5P and *psbA* fragments followed those in Saunders & McDevit (2012) and Bittner (2010), respectively. Resulting PCR products were cleaned with the column PCR<sup>TM</sup>GTX DNA and Gel Band Purification Kit (GE Healthcare, Pittsburgh, USA), according to manufacturer's protocol of the and sequenced in-house at the University of São Paulo on an ABI 3130xl or 3730 and/or at UL Lafayette campus on an ABI Model 3130xl Genetic Analyzer.

#### *Assembling, Alignments and Analyzes:*

Forward and reverse sequences were assembled either in Sequencher 5.0 software (Gene Codes Corporation, Ann Arbor, MI) or BioEdit 7.0.9.0 (Hall, 1999). For each

marker, a multiple alignment excluding PCR primers was generated with Clustal W (Thompson *et al.* 1994) available in BioEdit 7.0.9.0 (Hall, 1999).

The *psbA* matrix was constructed with 60 sequences, 33 newly generated in this study and 27 downloaded from GenBank, including *Renouxia* sp. (GQ917503) and *Rhodogorgon* sp. (GQ917504) that were used as outgroups. The matrix was cropped at the 5' and 3' ends to minimize missing data in sequences from GenBank and the final alignment was 832 base pairs (bp) in length. The COI-5P matrix was constructed with 77 sequences, 65 newly generated in this study and 12 publicly available from GenBank, including one sequence of *Rhodogorgon* sp. and one of *Renouxia* sp. each used as outgroups. This alignment, which was cropped at the 5' and 3' ends, was 577 bp in length and included no missing data in any of the sequences. It included a COI-5P sequence from a specimen of *Sporolithon episporum* (M.Howe) E.Y.Dawson which was collected at the type locality (Bocas del Toro, Panama). Comparative analyses of the *rbcL* sequence generated from this specimen revealed it is identical to an *rbcL* sequence (data not shown) generated from the type material of *Sporolithon episporum* (M.Howe) E.Y.Dawson (personal communication with P.W. Gabrielson, manuscript in prep.) (Table 1). Phylogenetic relationships were inferred using Maximum likelihood (ML) conducted in MEGA6 (Tamura *et al.* 2013) as well as Neighbor-Joining cluster analysis; Bayesian inference (BI) was generated in MrBayes (Huelsenbeck & Ronquist 2001) using TOPALI v.2.5 software (Milne *et al.* 2008). Models of sequence evolution were estimated using the Akaike Information Criterion (AIC) obtained in MEGA 5.2.2. Maximum likelihood and Bayesian analyses for the COI-5P and *psbA* alignments were performed under a generalized time-reversible with gamma+invariant sites heterogeneity model (GTR + G + I). The Bayesian analyses were performed under the same model with four Markov Chain Monte Carlo for 10 million generations, and tree

sampling every 1000 generations. Bayesian analysis was performed using Mr. Bayes 3.2.6 (Ronquist *et al.* 2012). Two parallel analyses were conducted, each consisting of four MCMC chains (3 heated and 1 cool) with  $1 \times 10^7$  generations. Resampling was performed every 1,000 generations. The first 10% of each run was discarded as “burn-in”, and a consensus tree was built with remaining data.

The general mixed Yule-coalescent (GMYC) model was applied for the delimitation of species (Pons *et al.* 2006, Fujisawa & Barraclough 2013). Species boundaries are defined based on the ultrametric tree obtained from a Bayesian phylogenetic under the same model we used for the phylogenies.

#### *Morphological studies*

SEM images were obtained from portions of the thallus of dried specimens previously preserved in Si-gel and/or 4% formalin/seawater. Cross sections and longitudinal sections were made using a razor blade, and the resulting sections were mounted using liquid graphite and coated with 10-14 nm of gold. Specimens were viewed using a Hitachi S-3000N scanning electron microscope (SEM) at a voltage of 15 kV, housed in the Microscopy Center at UL Lafayette, following the manufacturer’s instructions. Identified samples, including permanent slides, were deposited on the Herbarium of University of São Paulo (SPF). Herbarium abbreviation follows Thiers (2016, continuously updated).

#### **Results**

The *psbA* consensus tree was generated by ML analyses (Fig. 1). The clade of Sporolithales comprised collections of *Sporolithon* and *Heydrichia* from Brazil, Costa Rica, South Africa, Australia, New Zealand, Vanuatu and New Caledonia. Using the

outgroups *Renouxia* sp. and *Rhodogorgon* sp., the Sporolithales formed a monophyletic group with full support (BI, ML and NJ). Members of *Sporolithon* also formed a monophyletic clade but with no support in all analyzes, while members of *Heydrichia* were basal and paraphyletic in relation to *Sporolithon*. *Sporolithon durum* from New Zealand formed two distinct clades that were not closely related. In one of these clades a specimen from Australia (DQ168023) presented a divergence of 29 bp for the *psbA* from the other specimens from New Zealand. These results show the occurrence of cryptic species under the name *S. durum* was also separated in a different clade. *S. ptychoides* from Brazil and New Caledonia also are divided in two different clades and presenting a difference of 36 bp, also indicating the occurrence of cryptic species. The clade corresponding to *S. yoneshigueae* from Brazil was comprised of 4 specimens with full support in all analyzes; *S. tenue*, also from Brazil, formed a clade with strong support (.84/100/100 for BI, ML and NJ, respectively). Representatives of *Sporolithon* from Brazilian shallow waters formed a monophyletic clade (.94/92/99 for BI, ML and NJ, respectively). All analyzes resolved *Sporolithon episporum* from Costa Rica as a sister taxon to Brazilian shallow waters *Sporolithon* with full support (1/99/100 for BI, ML and NJ, respectively) and genetic diversity among them are shown in Table 2.

The phylogenetic tree obtained from the ML analysis of the COI-5P alignment (Fig. 2) resolved *Sporolithon* in a clade with strong support (1/99/97 for BI, ML and NJ, respectively). Species were distinguished into 7 lineages; *S. ptychoides* from Hawaii was resolved separated from *S. ptychoides* from New Caledonia; an unidentified species from Fiji grouped in a clade with species from Panama and Brazil with strong support (.89/95/96 for BI, ML and NJ, respectively); and finally, Brazilian shallow water *Sporolithon* was resolved into a strongly supported clade with *S. episporum* from Panama (.82/94/95 for BI, ML and NJ, respectively). The Brazilian shallow water

*Sporolithon* was resolved as a monophyletic clade with moderate to strong support (.90/84/85 for BI, ML and NJ, respectively), that was further split into two lineages with moderate to high support. The GMYC model fit was significantly better than a null model driven by coalescence only and reported 2 ML entities (represented by thicker branches in Fig. 2). All analyzes of COI-5P dataset were congruent in delimiting the Brazilian specimens as an independent lineage separated from crustose specimen of *S. episporum* from Panama, with a genetic divergence ranging from 3.2-3.9% (Table 2); although as shown in *psbA* results, the three species are very closely related. Based on our collections, the diversity of *Sporolithon* taxa in the Brazilian shallow waters is low (2 species), but shows us two previously unknown species.

Given the molecular evidence shown above, we propose to describe two new species of CCA from the Brazilian coast shallow waters as follows.

***Sporolithon pseudoepisporum* Vieira-Pinto, Oliveira et Horta sp. nov.**

FIGURES 3-16

Holotype: Tetrasporic plant (IBC 1613). Brazil, Jequiá da Praia, AL, 10°01'18.20"S; 36°00'48.0"W, intertidal (T. Vieira-Pinto, C. Azevedo, B. Torrano, 12 March , 2013).

Isotype: Tetrasporic plants (IBC 1667, IBC 1727, IBC 1836)

Etymology: “*pseudoepisporum*” was named as a reference to the resemblance and close relation to the species *S. episporum*.

Diagnosis: The new species proposed segregates from others based on its COI-5P DNA sequences.

Distribution: This species is widespread along the Brazilian coastline from Ceará (Tropical Atlantic realm) to Espírito Santo state (Temperate South American realm), more than 2,000 km of range extension.

### **Description**

**Vegetative features:** Calcified nongeniculate thalli, exhibiting growth forms such as rhodoliths found loose on the sandy bottom, or crusts fully attached to the substrata (Fig. 3). Surfaces are warty to lumpy, protuberances 5-20 mm in diameter and 3-10 mm high (Fig. 3 and 4). Thallus pseudoparenchymatous with monomerous organization in older portions (Fig. 5 and 6). Epithallium is composed of a single layer of cells that are 2.5-3.5  $\mu\text{m}$  high and 3.5-6  $\mu\text{m}$  in diameter with flared walls (Figs. 7 and 8). Subepithallial cells are 6.5-10  $\mu\text{m}$  high and 3.5-8  $\mu\text{m}$  in diameter. Perithallial cells 8-20  $\mu\text{m}$  high and 3.5-8  $\mu\text{m}$  in diameter. Cells of contiguous (adjacent) filaments are joined predominantly by secondary pit connections (Fig. 7).

**Reproductive features:** Tetrasporangial sori (Figs. 9-12) are raised and present 4-5 cell layers above the surrounding vegetative thallus (Fig. 11). Individual tetrasporangial calcified chambers are uniporate (Figs. 9-12), occur mostly in clusters (Figs. 9-11). Tetrasporangial chambers are 80-110  $\mu\text{m}$  in height and 35-45  $\mu\text{m}$  in diameter, and are separated from one another by none to 2 paraphyses composed of 5-6 elongate cells (Fig 12). Tetrasporangial pores measure 10-15  $\mu\text{m}$  in diameter and are surrounded by 10-14 rosette cells (Fig. 12) that are often slightly sunken towards the pore opening (Fig. 12 – arrow). Within sori, each chamber bears only one tetrasporangium that is cruciately divided or undivided when not mature (Fig. 12 - te) and measures 50–75  $\mu\text{m}$  in height and 30–40  $\mu\text{m}$  in diameter. Senescent tetrasporangial sori are sloughed off from the outer thallus and no scars are visible. Gametangial conceptacles are uniporate and

raised in relation to the thallus surface (Fig. 13-16), carpospores are arranged in the chamber floor (Fig. 15 - c); conceptacles are 120-130  $\mu\text{m}$  in diameter and 150-185 in height. Pore channel is 30  $\mu\text{m}$  in diameter and 50  $\mu\text{m}$  (or 6 cells) in height (Fig. 16).

**Ecological observations:** Species were found as rhodoliths at 2-12 m depth and as epilithic crusts in the intertidal growing on rocky shores and coral reefs.

***Sporolithon tomitae* Vieira-Pinto, Oliveira et Horta sp. nov.**

FIGURES 17-28

Holotype: Tetrasporic plant (IBC 1508). Brazil, Pirambúzios beach, Nízia Floresta, RN, 5°59'12.35"S; 35°06'49.54"W, intertidal (P. Horta, May 17th, 2011).

Isotype: Tetrasporic plants (IBC 1509, IBC 1639, IBC 1820).

Etymology: “*tomitae*” was named after a Brazilian phycologist Dr. Noemy Yamagushi-Tomita in honor of her memory and legacy of her algal contributions and pioneering studies of the genus *Sporolithon* and the CCA in Brazil and South America.

Diagnosis: The new species proposed segregates from others based on its COI-5P DNA sequences.

Distribution: This species was found at a restricted area from CE to RN states in the Brazilian coastline (Tropical Atlantic realm).

Description

**Vegetative features:** Calcified nongeniculate thalli, growth forms mostly as rhodoliths found loose on the sandy bottom, and rarely as crusts fully attached to the substrata (Fig. 17). Surface smooth to lumpy, protuberances 0,5-1 cm in diameter and 0.3-0.5 cm high (Fig. 17). Thallus pseudoparenchymatous with monomerous organization in older and younger portions (growth margins) (Figs. 18-20). Perithallial cells 5-18  $\mu\text{m}$  high

and 3.5-7.5µm in diameter (Fig. 21- p). Cells of contiguous (adjacent) filaments are joined by mainly secondary pit connections (Fig. 21- arrows) in a ratio of 2:1. Epithallium is composed of a single layer of cells that are 3.5-4 µm high and 3.5-4 µm in diameter with flared walls (Fig. 22 - e). Subepithallial initial cells are 5.5-8 µm high and 3.5-6 µm in diameter (Fig. 22 - i).

**Reproductive features:** Tetrasporangial sori (Fig. 23) are flush to slightly raised in relation to the thallus surface and present 5–7 cell layers above the surrounding vegetative thallus (Fig. 26). Individual tetrasporangial calcified chambers are uniporate and occur mostly in clusters (Figs. 23-26), they present a single differentiated stalk cells (data not shown). Tetrasporangial chambers are 80-110 µm in height and 35-45 µm in diameter (Fig. 27 - t), and are separated from one another by none to 2 paraphyses comprised of 5-6 elongate cells (Fig. 27 - arrows) Tetrasporangial pores measure 10-15 µm in diameter and are surrounded by 10-14 rosette cells (Fig. 28) that are often slightly sunken towards the pore opening, which is raised as a halo (Fig. 28 - arrow). Senescent tetrasporangial sori are sloughed off from the outer thallus and no scars were observed.

**Ecological observations:** Species were found as rhodoliths and as epilithic crusts in the intertidal growing on rocky shores and coral reefs.

## Discussion

Presently, there are eight extant species of *Sporolithon* for which detailed taxonomic information exists (Bahia *et al.* 2015); among them seven species have been recorded for the southwestern Atlantic, Brazil. It can be very difficult to distinguish species within the genus *Sporolithon* as most characters used to delimit species in this genus can show a high degree of plasticity (Verheij, 1993, this study) and often overlap (see Table 3 for details). Despite these limitations, *Sporolithon pseudoepisporum* and *S.*

*tomitae* can be distinguished from *S. yoneshigueae*, *S. molle*, *S. episoredion* and *S. ptychoides* by not presenting old buried tetrasporangial compartments, but that instead these compartments are flaked off, although *S. pseudoepisporum* presents buried empty old gamentangial conceptacles. Both species can also be distinguished from *S. episporum* by not presenting scars of old tetrasporangial compartments throughout the deeper parts of the thalli like it has been extensively reported for *S. episporum* (Verheij, 1993, Keats & Chamberlain, 1993; Bahia et al. 2015b); they can also be differentiated from *S. elevatum* by presenting a monomerous thallus organization whereas the latter presents a dimerous organization.

Although *Sporolithon pseudoepisporum* and *S. tomitae* cannot be easily morphologically distinguished from all other species of this genus, as shown above, we relied on DNA barcoding technique to corroborate our findings. It is now widely recognized that COI-5P and *psbA* are useful and informative markers to resolve species boundaries (Yang & Boo, 2004; Broom et al., 2008; Le Gall et al., 2010; Bittner et al., 2011; Kato et al., 2011; 2013; Bahia et al., 2014a; Pardo et al., 2014; Peña et al., 2014, 2015; Richards et al., 2014, Sissini et al., 2014; Vieira-Pinto et al., 2014); the former is considered the DNA barcode for Rhodophyta (Saunders, 2005). Using these markers, we have shown that *S. pseudoepisporum* and *S. tomitae* represent distinct taxonomic entities separate from all other *Sporolithon* species currently recognized for which DNA sequences are available, such as *S. tenue* and *S. yoneshigueae*, species recently recognized for the Brazilian coast; and *S. durum* from Australia. Regarding genetic divergences, the newly proposed species, which are very closely related to *S. episporum*, show interspecific divergences of 0.6% and 0.8% for *psbA* from *S. episporum*, which is higher than the minimum value of interspecific divergence between other coralline species reported in previous studies (Broom et al. 2008, Hindi &

Saunders 2013); between the Brazilian species the divergence is 0.1%, a very low divergence that could be interpreted as a single species. Divergence values of COI-5P for *S. pseudoepisporum* and *S. tomitae* compared to *S. episporum* is 3.2% and 3.9% respectively (or 19 and 23 bp), and therefore congruent with interspecific divergence values reported in previous studies using this marker (Saunders, 2005, Robba *et al.* 2006, Hind & Saunders 2013, Hind *et al.* 2014, Peña *et al.* 2015), and between the two Brazilian species divergence is 3%. *Phymatholithon lamii* and *P. lusitanicum* (Peña *et al.*, 2015) showed exactly the same divergence range, which can be considered as a gray zone to delimit species boundaries for this marker. *Phymatholithon lamii* and *P. lusitanicum* (Peña *et al.*, 2015), were segregated based on the same genetic divergence for COI-5P that we found in this study along with ecological observations regarding these species habit, such as *P. lamii* was only found as crusts species and *P. lusitanicum* as rhodolith-forming species. Although both Brazilian species were found as crusts and rhodoliths and therefore no distinction in growth form was observed, we did find a separate distributional pattern, in that *S. tomitae* has a more restricted distribution in the Tropical Atlantic realm and *S. pseudoepisporum* is widely distributed along the Brazilian coast from the Tropical Atlantic with a southern limit in the Temperate South American realm (following the Marine Ecoregions of the World - Spalding *et al.* 2007) (Fig. 29).

## **Conclusions**

Our COI-5P analyses revealed that specimens of *Sporolithon* from Brazilian shallow waters represent two new CCA species. These specimens were resolved as taxa sister to *S. episporum* with full support, indicating that the three species share a very recent common ancestor. DNA barcodes proved to be essential for delimiting species within

this challenging group of nongeniculate corallines where phenotypic plasticity and overlapping character measurements complicate the use of conventional, morphology-based approaches. As pointed out by Bahia *et al.* (2015a), recognizing, describing and documenting the CCA species is the first step to understanding the complexity of tropical habitats dominated by coralline algae. More importantly, our understanding of the diversity of nongeniculate coralline algae in Brazil has been improving in the past few years by studies that focus on combining different approaches and has great potential to help and provide scientific-based information to national legislation in order to ensure appropriate protection for marine habitats.

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Table 1. Sample information for specimens included in molecular analyses. Sequences generated in this study are in bold, other available sequences were downloaded from GenBank. (\*Genbank accession number for the data generated in this study will be provided soon)

Taxa	Voucher	Locality	Lat/long	Collector	Date (D/M/Y)	Genbank Accession No.	
						COI-5P	psbA
<i>Sporolithon pseudoepisporum</i>	IBC 1203	Brazil (Anchieta, ES)	20°83'49.47"S; 40°62'41.48"W	C. E. Amancio, Beatriz Torrano	05/05/2012	<b>x</b>	<b>x</b>
	IBC 1210	Brazil (Marataízes, ES)	21°01'58.2"S; 40°48'43.9"W	C. E. Amancio, Beatriz Torrano	07/05/2012	<b>x</b>	-
	IBC 1211	Brazil (Marataízes, ES)	21°01'58.2"S; 40°48'43.9"W	C. E. Amancio, Beatriz Torrano	07/05/2012	<b>x</b>	<b>x</b>
	IBC 1533	Brazil (Arraial d'Ajuda, BA)	16°48'98.54"S; 39°06'76.71"W	C. Azevedo; F. Nauer	18/09/2012	<b>x</b>	-
	IBC 1538	Brazil (Porto Seguro, BA)	16°43'35.80"S; 39°07'27.80"W	C. Azevedo; F. Nauer	16/09/2012	<b>x</b>	-
	IBC 1584	Brazil (Marechal Deodoro, AL)	9°77'10.35"S; 35°84'06.89"W	T. Vieira-Pinto, C. Azevedo, B. Torrano	10/03/2013	<b>x</b>	<b>x</b>
	IBC 1594	Brazil (Piaçabuçu, AL)	10°21'07.68"S; 36°17'45.24"W	T. Vieira-Pinto, C. Azevedo, B. Torrano	11/03/2013	<b>x</b>	-
	IBC 1598	Brazil (Piaçabuçu, AL)	10°21'07.68"S; 36°17'45.24"W	T. Vieira-Pinto, C. Azevedo, B. Torrano	11/03/2013	<b>x</b>	-
	IBC 1599	Brazil (Piaçabuçu, AL)	10°21'07.68"S; 36°17'45.24"W	T. Vieira-Pinto, C. Azevedo, B. Torrano	11/03/2013	<b>x</b>	<b>x</b>
	IBC 1603	Brazil (Piaçabuçu, AL)	10°21'07.68"S; 36°17'45.24"W	T. Vieira-Pinto, C. Azevedo, B. Torrano	11/03/2013	<b>x</b>	
	IBC 1604	Brazil (Piaçabuçu, AL)	10°21'07.68"S; 36°17'45.24"W	T. Vieira-Pinto, C. Azevedo, B. Torrano	11/03/2013	<b>x</b>	-
	IBC 1609	Brazil (Jequiá da Praia, AL)	10°01'18.20"S; 36°00'48.0"W	T. Vieira-Pinto, C. Azevedo, B. Torrano	12/03/2013	<b>x</b>	<b>x</b>
	<b>IBC 1613 TYPE</b>	Brazil (Jequiá da Praia, AL)	10°01'18.20"S; 36°00'48.0"W	T. Vieira-Pinto, C. Azevedo, B. Torrano	12/03/2013	<b>x</b>	<b>x</b>

Cont. Tab. 1

<i>Sporolithon pseudoepisorum</i>	IBC 1626	Brazil (São Miguel dos Milagres, AL)	9°27'00.55"S; 35°36'74.40"W	T. Vieira-Pinto, C. Azevedo, B. Torrano	14/03/2013	x	-
	IBC 1632	Brazil (São Miguel dos Milagres, AL)	9°27'00.55"S; 35°36'74.40"W	T. Vieira-Pinto, C. Azevedo, B. Torrano	14/03/2013	x	-
	IBC 1634	Brazil (São Miguel dos Milagres, AL)	9°27'00.55"S; 35°36'74.40"W	T. Vieira-Pinto, C. Azevedo, B. Torrano	14/03/2013	x	-
	IBC 1645	Brazil (Paracuru, CE)	3°24'16.18"S; 39°01'46.65"W	T. Vieira-Pinto, C. Azevedo, B. Torrano	24/04/2013	x	-
	IBC 1647	Brazil (Paracuru, CE)	3°24'16.18"S; 39°01'46.65"W	T. Vieira-Pinto, C. Azevedo, B. Torrano	24/04/2013	x	x
	IBC 1648	Brazil (Paracuru, CE)	3°24'16.18"S; 39°01'46.65"W	T. Vieira-Pinto, C. Azevedo, B. Torrano	24/04/2013	x	-
	IBC 1649	Brazil (Paracuru, CE)	3°24'16.18"S; 39°01'46.65"W	T. Vieira-Pinto, C. Azevedo, B. Torrano	24/04/2013	x	x
	IBC 1651	Brazil (Paracuru, CE)	3°24'16.18"S; 39°01'46.65"W	T. Vieira-Pinto, C. Azevedo, B. Torrano	24/04/2013	x	x
	IBC 1655	Brazil (Paracuru, CE)	3°24'16.18"S; 39°01'46.65"W	T. Vieira-Pinto, C. Azevedo, B. Torrano	24/04/2013	x	x
	IBC 1659	Brazil (Trairi, CE)	3°14'20.86"S; 39°13'46.03"W	T. Vieira-Pinto, C. Azevedo, B. Torrano	25/04/2013	x	-
	IBC 1661	Brazil (Trairi, CE)	3°14'20.86"S; 39°13'46.03"W	T. Vieira-Pinto, C. Azevedo, B. Torrano	25/04/2013	x	-
	IBC 1666	Brazil (Trairi, CE)	3°14'20.86"S; 39°13'46.03"W	T. Vieira-Pinto, C. Azevedo, B. Torrano	25/04/2013	x	x
	IBC 1667	Brazil (Trairi, CE)	3°14'20.86"S; 39°13'46.03"W	T. Vieira-Pinto, C. Azevedo, B. Torrano	25/04/2013	x	x
	IBC 1670	Brazil (Trairi, CE)	3°14'20.86"S; 39°13'46.03"W	T. Vieira-Pinto, C. Azevedo, B. Torrano	25/04/2013	x	x
	IBC 1671	Brazil (Caucaia, CE)	3°41'11.10"S; 38°37'57.47"W	T. Vieira-Pinto, C. Azevedo, B. Torrano, P. Carneiro	26/04/2013	x	-
	IBC 1672	Brazil (Caucaia, CE)	3°41'11.10"S; 38°37'57.47"W	T. Vieira-Pinto, C. Azevedo, B. Torrano, P. Carneiro	26/04/2013	x	x

Cont. Tab. 1

<i>Sporolithon pseudoepisorum</i>	IBC 1679	Brazil (Caucaia, CE)	3°41'11.10"S; 38°37'57.47"W	T. Vieira-Pinto, C. Azevedo, B. Torrano, P. Carneiro	26/04/2013	x	x
	IBC 1699	Brazil (Salvador, BA)	12°56'41.50"S; 38°20'04.70"W	T. Vieira-Pinto, C. Azevedo, B. Torrano- Silva, M. Jamas	22/05/2013	x	x
	IBC 1722	Brazil (Ilha de Itaparica, BA)	13°00'35.30"S; 38°38'27.40"W	T. Vieira-Pinto, C. Azevedo, B. Torrano- Silva, M. Jamas	24/05/2013	x	x
	IBC 1723	Brazil (Ilha de Itaparica, BA)	13°00'35.30"S; 38°38'27.40"W	T. Vieira-Pinto, C. Azevedo, B. Torrano- Silva, M. Jamas	24/05/2013	x	x
	IBC 1727	Brazil (Ilha de Itaparica, BA)	13°00'35.30"S; 38°38'27.40"W	T. Vieira-Pinto, C. Azevedo, B. Torrano- Silva, M. Jamas	24/05/2013	x	x
	IBC 1730	Brazil (Ilha de Itaparica, BA)	13°00'35.30"S; 38°38'27.40"W	T. Vieira-Pinto, C. Azevedo, B. Torrano- Silva, M. Jamas	24/05/2013	x	-
	IBC 1731	Brazil (Ilha de Itaparica, BA)	13°00'35.30"S; 38°38'27.40"W	T. Vieira-Pinto, C. Azevedo, B. Torrano- Silva, M. Jamas	25/05/2013	x	-
	IBC 1741	Brazil (Camaçari, BA)	12°38'58"S; 38°03'37"W	T. Vieira-Pinto, C. Azevedo, B. Torrano-Silva	26/05/2013	x	-
	IBC 1750	Brazil (Entre Rios, BA)	12°14'26.40"S; 37°46'26.80"W	T. Vieira-Pinto, C. Azevedo, B. Torrano-Silva	27/05/2013	x	-
	IBC 1753	Brazil (Entre Rios, BA)	12°14'26.40"S; 37°46'26.80"W	T. Vieira-Pinto, C. Azevedo, B. Torrano-Silva	27/05/2013	x	-
	IBC 1802	Brazil (São Miguel do Gostoso, RN)	5°6'57.89"S; 35°37'14.16"W	T. Vieira-Pinto, M. Jamas, M. Fuji, D. Milstein, A. Leite	29/03/2014	x	x
	IBC 1803	Brazil (São Miguel do Gostoso, RN)	5°6'57.89"S; 35°37'14.16"W	T. Vieira-Pinto, M. Jamas, M. Fuji, D. Milstein, A. Leite	29/03/2014	x	-

Cont. Tab. 1

<i>Sporolithon pseudoepisorum</i>	IBC 1806	Brazil (São Miguel do Gostoso, RN)	5°6'57.89"S; 35°37'14.16"W	T. Vieira-Pinto, M. Jamas, M. Fuji, D. Milstein, A. Leite	29/03/2014	x	-
	IBC 1807	Brazil (São Miguel do Gostoso, RN)	5°6'57.89"S; 35°37'14.16"W	T. Vieira-Pinto, M. Jamas, M. Fuji, D. Milstein, A. Leite	29/03/2014	x	-
	IBC 1813	Brazil (Tourinhos, RN)	5°12'12.24"S; 35°26'56.45"W	T. Vieira-Pinto, M. Jamas, M. Fuji, D. Milstein, A. Leite	29/03/2014	x	-
	IBC 1827	Brazil (Baía da Traição, PB)	6°68'30.63"S; 34°94'48.70"W	T. Vieira-Pinto, M. Jamas, M. Fuji, D. Milstein, A. Leite	31/03/2014	x	x
	IBC 1833	Brazil (Conde, PB)	7°29'84.02"S; 34°79'89.15"W	T. Vieira-Pinto, M. Jamas, M. Fuji, D. Milstein, A. Leite	01/04/2014	x	
	IBC 1836	Brazil (Conde, PB)	7°29'84.02"S; 34°79'89.15"W	T. Vieira-Pinto, M. Jamas, M. Fuji, D. Milstein, A. Leite	01/04/2014	x	x
	IBC 1839	Brazil (Conde, PB)	7°29'84.02"S; 34°79'89.15"W	T. Vieira-Pinto, M. Jamas, M. Fuji, D. Milstein, A. Leite	01/04/2014	x	x
	IBC 1849	Brazil (Nízia Floresta, RN)	5°59'12.35"S; 35°06'49.54"W	T. Vieira-Pinto, M. Jamas, M. Fuji, D. Milstein, A. Leite	02/04/2014	x	-
	IBC 1896	Brazil (Marataízes, ES)	21°01'58.2"S; 40°48'43.9"W	T. Vieira-Pinto, M. Mungioli, M. Fuji, P. Diaz, H. Verbruggen, V. R. Marcelino	10/09/2014	x	-
	IBC 1918	Brazil (Fernando de Noronha, PE)	3°85'09.89"S; 32°44'18.85"W	P. Horta; E. Bastos	07/01/2013	x	-
	IBC 2508	Brazil (Porto Seguro, BA)	16°43'35.80"S; 39°07'27.80"W	B.N. Torrano-Silva, J. Pires, A.S.Santos	13/05/2013	x	x

Cont. Tab. 1

<i>Sporolithon pseudoepisorum</i>	IBC 2509	Brazil (Porto Seguro, BA)	16°43'35.80"S; 39°07'27.80"W	B.N. Torrano-Silva, J. Pires, A.S.Santos	13/05/2013	x	x
	IBC 2551	Brazil (Prado, BA)	16°53'52.00"S; 39°06'34.70"W	B.N. Torrano-Silva, J. Pires, A.S.Santos	13/05/2013	x	x
<i>Sporolithon tomitae</i>	<b>IBC 1508 TYPE</b>	Brazil (Nízia Floresta, RN)	5°59'12.35"S; 35°06'49.54"W	P. Horta	17/05/2011	x	x
	IBC 1509	Brazil (Nízia Floresta, RN)	5°59'12.35"S; 35°06'49.54"W	P. Horta	17/05/2011	x	x
	IBC 1510	Brazil (Nízia Floresta, RN)	5°59'12.35"S; 35°06'49.54"W	P. Horta	17/05/2011	x	x
	IBC 1639	Brazil (Paracuru, CE)	3°23'54.34"S; 39°00'50.43"W	T. Vieira-Pinto, C. Azevedo, B. Torrano	24/04/2013	x	x
	IBC 1643	Brazil (Paracuru, CE)	3°23'54.34"S; 39°00'50.43"W	T. Vieira-Pinto, C. Azevedo, B. Torrano	24/04/2013	x	x
	IBC 1687	Brazil (Icapuí, CE)	4°37'35.80"S; 37°29'58.83"W	T. Vieira-Pinto, C. Azevedo	27/04/2013	x	-
	IBC 1689	Brazil (Icapuí, CE)	4°37'35.80"S; 37°29'58.83"W	T. Vieira-Pinto, C. Azevedo	27/04/2013	x	-
	IBC 1814	Brazil (Rio do Fogo, RN)	5°14'51.91"S; 35°23'41.15"W	T. Vieira-Pinto, M. Jamas, M. Fuji, D. Milstein, A. Leite	30/03/2014	x	-
	IBC 1815	Brazil (Rio do Fogo, RN)	5°14'51.91"S; 35°23'41.15"W	T. Vieira-Pinto, M. Jamas, M. Fuji, D. Milstein, A. Leite	30/03/2014	x	-
	IBC 1820	Brazil (Rio do Fogo, RN)	5°14'51.91"S; 35°23'41.15"W	T. Vieira-Pinto, M. Jamas, M. Fuji, D. Milstein, A. Leite	30/03/2014	x	x
	IBC 1821	Brazil (Rio do Fogo, RN)	5°14'51.91"S; 35°23'41.15"W	T. Vieira-Pinto, M. Jamas, M. Fuji, D. Milstein, A. Leite	30/03/2014	x	x
IBC 1823	Brazil (Rio do Fogo, RN)	5°14'51.91"S; 35°23'41.15"W	T. Vieira-Pinto, M. Jamas, M. Fuji, D. Milstein, A. Leite	30/03/2014	x	-	

Cont. Tab. 1

<i>Sporolithon eposporum</i> Topotype	Spo Epi	Bocas del Toro, Panama				x	
<i>Sporolithon durum</i>	NZC2396	New Zealand (North Island, Northland, Haraweka Island)	-	-	-	-	FJ361599
<i>Heydrichia homalopasta</i>	NZC2015	New Zealand (North Island, Auckland, west coast, Te Henga)	-	-	-	-	FJ361383
<i>Heydrichia homalopasta</i>	NZC0757	New Zealand: (Chatham Islands, Port Hutt)	-	-	-	-	DQ167937
<i>Heydrichia woelkerlingii</i>	NZC2014	New Zealand (North Island, Auckland, west coast, Te Henga)	-	-	-	-	FJ361382
<i>H. woelkerlingii</i>	-	South Africa (Western Cape)	-	-	-	-	JQ917415
<i>S. durum</i>	NZC0837	New Zealand (North Island, Wellington, Kapiti Island)	-	-	-	-	DQ167995
<i>S. durum</i>	NZC0805	New Zealand (North Island, Wellington, Kapiti Island)	-	-	-	-	DQ167961
<i>S. durum</i>	ND393	New Zealand (North Island, Northland, Haraweka Island)	-	-	-	-	FJ361599
<i>S. durum</i>	NZC0310	New Zealand (South Island, Nelson, Cable Bay)	-	-	-	-	DQ167887
<i>S. durum</i>	NZC0228	New Zealand (South Island, Nelson, Cable Bay)	-	-	-	-	DQ167955
<i>S. durum</i>	RHO2203	New Zealand (North Island, Bay of Islands)	-	-	-	-	KC963421
<i>S. episporum</i>	Steneck USAJ-A-73233	Atlantico Costa Rica (Punta Cocies, Prov. Limon).	-	-	-	-	KC870925
<i>Sporolithon ptychoides</i>	US Amado-FilhoBrazil 8	Brazil (Fernando de Noronha archipelago)	-	-	-	-	KC870926

Cont. Tab. 1

<i>S. ptychoides</i>	US Amado-FilhoBrazil 7	Brazil (Fernando de Noronha archipelago)	-	-	-	-	KC870927
<i>S. cf. ptychoides</i>	GM AF6	Brazil (Fernando de Noronha archipelago)	-	-	-	-	KP142753
<i>Sorolithon</i> sp.	NZC2175	New Zealand (North Island, Raglan, Whale Bay)	-	-	-	-	FJ361509
<i>Sporolithon tenue</i> (type)	US BahiaBrazil 12512-4	Brazil (Bahia, Salvador)	-	-	-	-	KC870923
<i>Sporolithon tenue</i>	US 170943	Brazil (Bahia, Salvador)	-	-	-	-	KP142751
<i>Sporolithon yoneshigueae</i> (type)	RB600359	Brazil (Bahia, Abrolhos)	-	-	-	-	KM20384 0
<i>S. yoneshigueae</i>	RB600360	Brazil (Bahia, Abrolhos)	-	-	-	-	KM20384 1
<i>S. yoneshigueae</i>	RB600362	Brazil (Bahia, Abrolhos)	-	-	-	-	KM20384 2
<i>S. yoneshigueae</i>	RB570782	Brazil (Bahia, Abrolhos)	-	-	-	-	KC953094
<i>Sporolithon</i> sp.	LBC0695	Fiji	-	-	-	GQ917279	-
<i>Sporolithon</i> sp.	LBC0567	Vanuatu	-	-	-	GQ917259	-
<i>S. ptychoides</i>	ARS02819	Hawaii	-	-	-	HQ422711	-
<i>S. ptychoides</i>	LLG0745	New Caledonia	-	-	-	GQ917307	-
<i>Rhodogorgonales</i> sp.	LLG0730	New Caledonia	-	-	-	GQ917305	-
<i>Rhodogorgonales</i> sp.	LLG0743	New Caledonia	-	-	-	GQ917306	-

Table 2. Genetic divergence for *psbA* and COI-5P; percentage and base pairs (bp) for *Sporolithon pseudoepisporum*, *Sporolithon tomitae* and *S. episporum* species; “n” is the number of specimens. In black are values for *psbA* and in gray the values for COI-5P.

	<i>S. pseudoepisporum</i> <i>psbA</i> n=26/COI-5P n=52	<i>S. tomitae</i> <i>psbA</i> n=7/ COI-5P n=12	<i>S. episporum</i> type ID n=1
<i>S. pseudoepisporum</i>	- (0bp)/ 0.7% (4pb)	-	-
<i>S. tomitae</i>	.1% (1bp)/ 3% (17pb)	-.% (0bp)/ 0.2% (1pb)	-
<i>S. episporum</i> type ID	.8% (6bp)/ 3.2% (19pb)	.6% (5bp)/ 3.9% (23pb)	0

Table 3. Comparative taxonomic features of *Sporolithon* species for which detailed descriptions are available. Adapted from Bahia *et al.* 2015 - *S.* <sup>a</sup>Verheij (1993); <sup>b</sup>Keats & Chamberlain (1993); <sup>c</sup>Type of *S. episorum* in Keats & Chamberlain (1993); <sup>d</sup>Townsend *et al.* (1995); <sup>e</sup>Bahia *et al.* (2014a); <sup>f</sup>Henriques *et al.* (2014); <sup>g</sup>Alongi *et al.* (1996); <sup>h</sup>Bahia *et al.* (2011); <sup>i</sup>as *Archaeolithothamnion episorion* in Adey *et al.* (1982); <sup>j</sup>Costa *et al.* 2014; <sup>k</sup> Bahia *et al.* (2015a).

Character	<i>S. pseudoepisorum</i> (present study)	<i>S. tomitae</i> (present study)	<i>S. episorum</i> a,c,j,k	<i>S. yoneshigueae</i> <sup>k</sup>	<i>S. durum</i> <sup>d</sup>	<i>S. molle</i> <sup>a</sup>	<i>S. episorion</i> a,i, f	<i>S. ptychoides</i> a,b,g,h,f	<i>S. elevatum</i> <sup>f</sup>	<i>S. tenue</i> <sup>e</sup>
Samples localities	Brazil	Brazil	Panama, Indian Ocean, Brazil	Brazil	Australia	Red Sea and Indian ocean	Hawaii, Indonesia, Brazil	Red sea, Indian Ocean, Hawaii, Mediterranean Sea, Brazil	Brazil	Brazil
Number of rosette cells surrounding tetrasporangial chamber pore	10-15	8-12	8-12	19-24	14-15	10-12	11-13	8-11	ND	8-12
Thallus organization	Monomeric	Monomeric	Monomeric	Monomeric	Monomeric	Monomeric	Monomeric	Dimerous	Dimerous	Monomeric
Ratio of secondary pit connections/cell fusions	Secondary pit connections predominant	Secondary pit connections predominant	4:1	1:3	Secondary pit connection rare or absent	2-3:1	1-4:1	2-3:1/2:1 Mainly pit connections/Mainly cell fusions	2:3	Secondary pit connections predominant
Tetrasporangium compartment height × diameter (µm)	80-110 x 35-50	60-80 x 25-40	(50) 70-100 x (25) 30-55	90-140 x 80-100	92-105 x 38-54	63-85 x 25-48	180-220 x 100-135	55-130 x 25-60	60-76 x 34-45	50-75 x 30-40
Tetrasporangial compartment pore diameter (µm)	10-15	12-20	(9) 16-30	35-43	13-21	10-17	10-20 <sup>a</sup>	9-15	ND	9-14
Old tetrasporangial compartment buried x sloughed off	sloughed off	sloughed off	sloughed off	Become buried	sloughed off	Become buried	Become buried	Become buried	sloughed off	sloughed off
Number of cells in the paraphyses	5-6	4-5	3-5 <sup>a</sup> 4-8 <sup>b,c</sup>	4-6	6-7	3-4	6-9	3-5 <sup>a</sup> /7-9 <sup>b</sup> / 4-6 <sup>b,f</sup> /3-5 <sup>h</sup>	2-3	4-6
Position of sori relative to surrounding vegetative surface	Raised	Flush to slightly raised	Raised	Raised	Raised	Flush to slightly raised	Raised	Raised	Raised	Raised

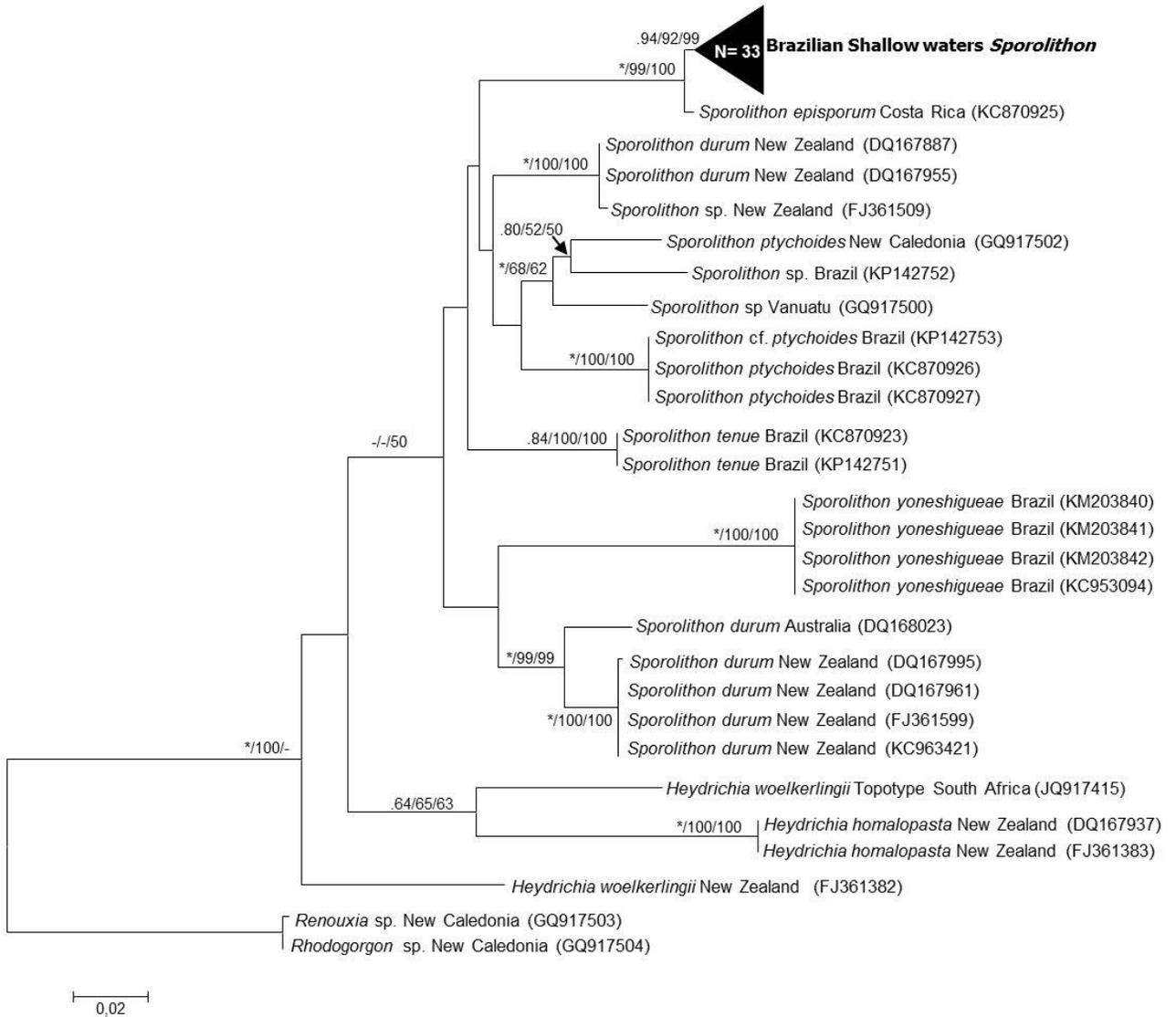


Fig. 1. Phylogram inferred from ML analyses of *psbA* sequences. Numbers at branches indicate posterior probabilities for Bayesian analysis (BI) and Bootstrap values for Maximum Likelihood (ML) and Neighbor-Joining (NJ) respectively. Scale bar indicates number of substitutions per site. The clade in bold represent sequences generated in this study; “N” represents number of sequences in the compressed clade.

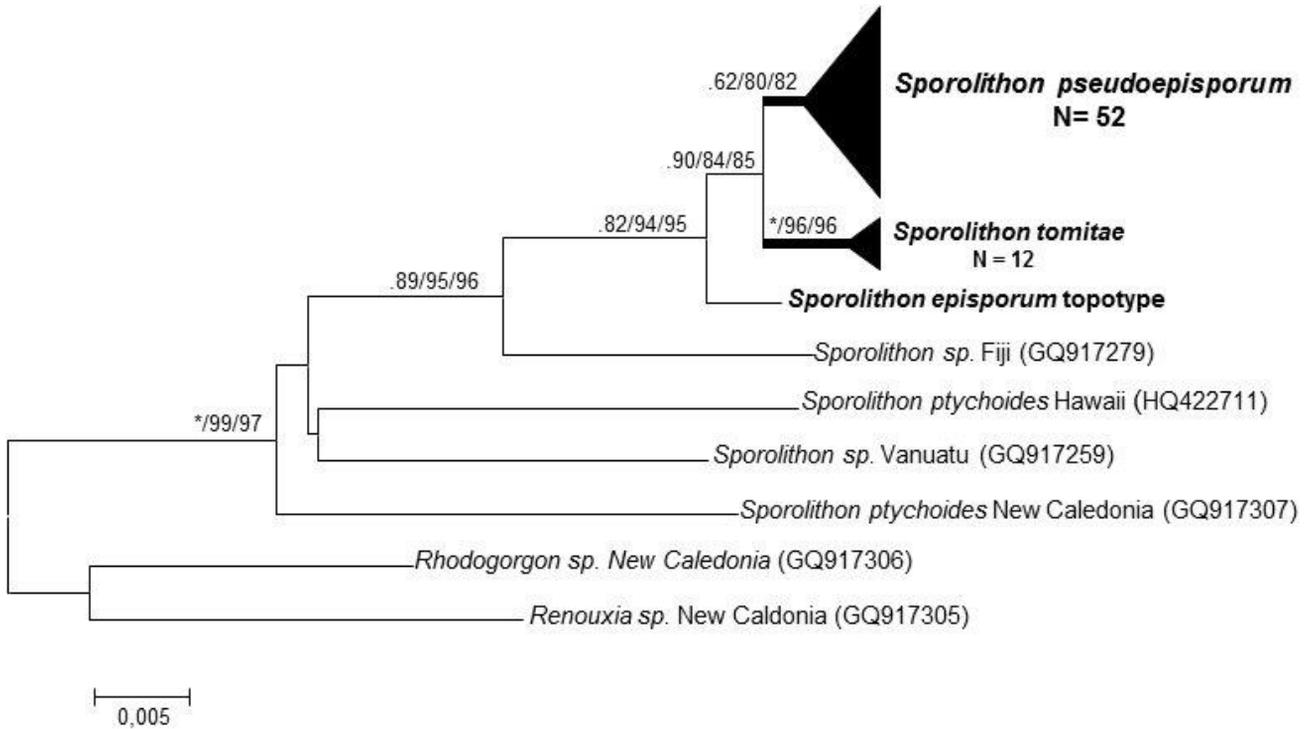
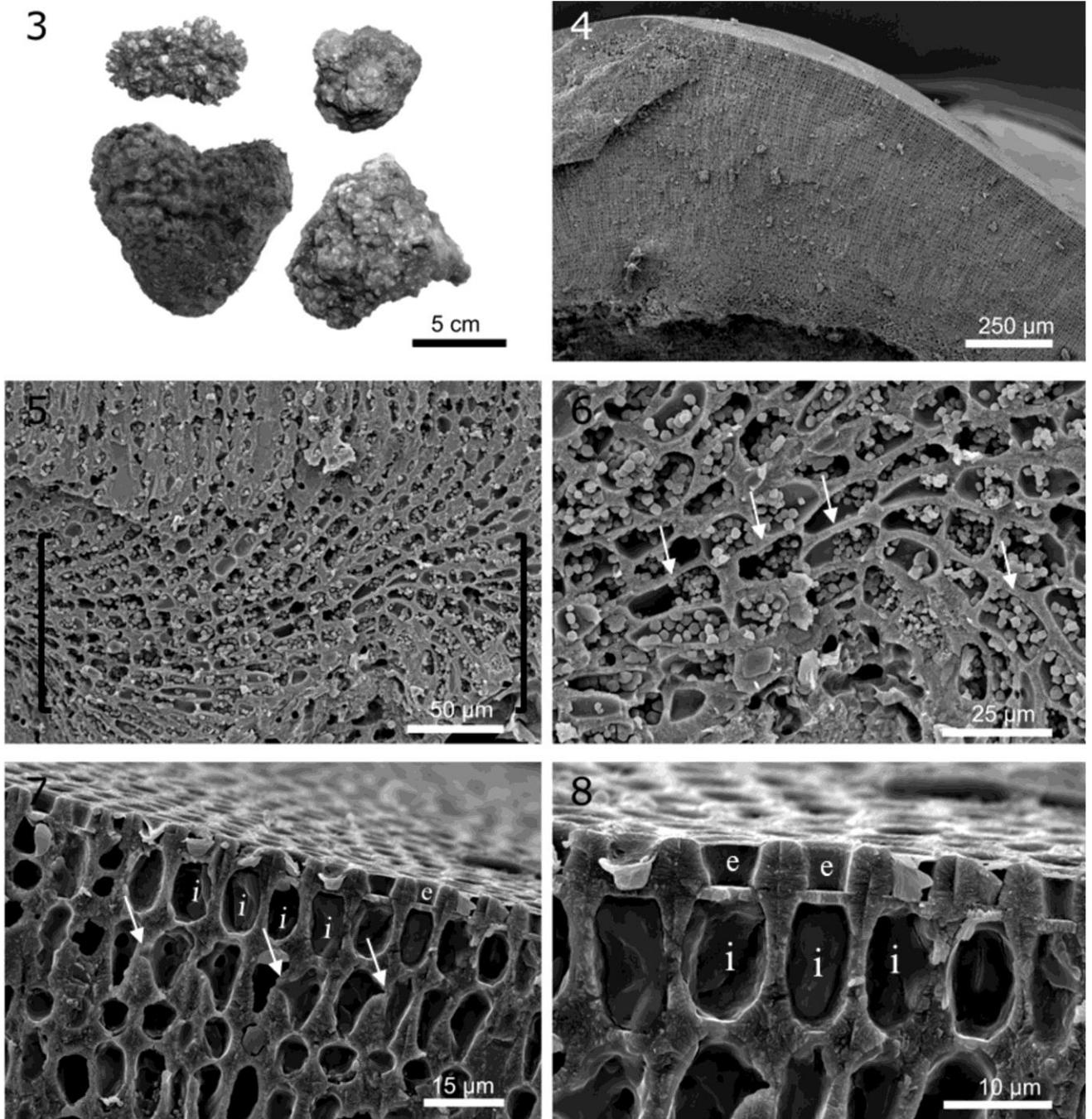
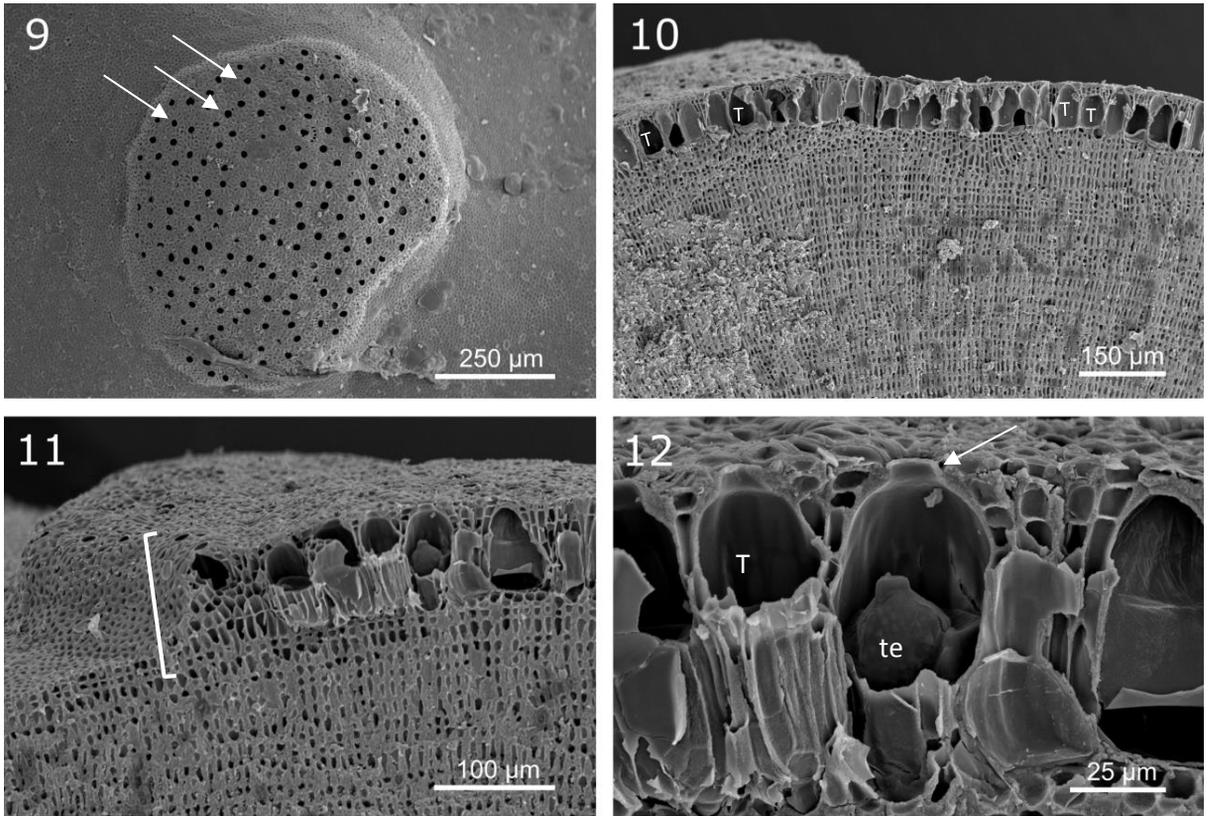


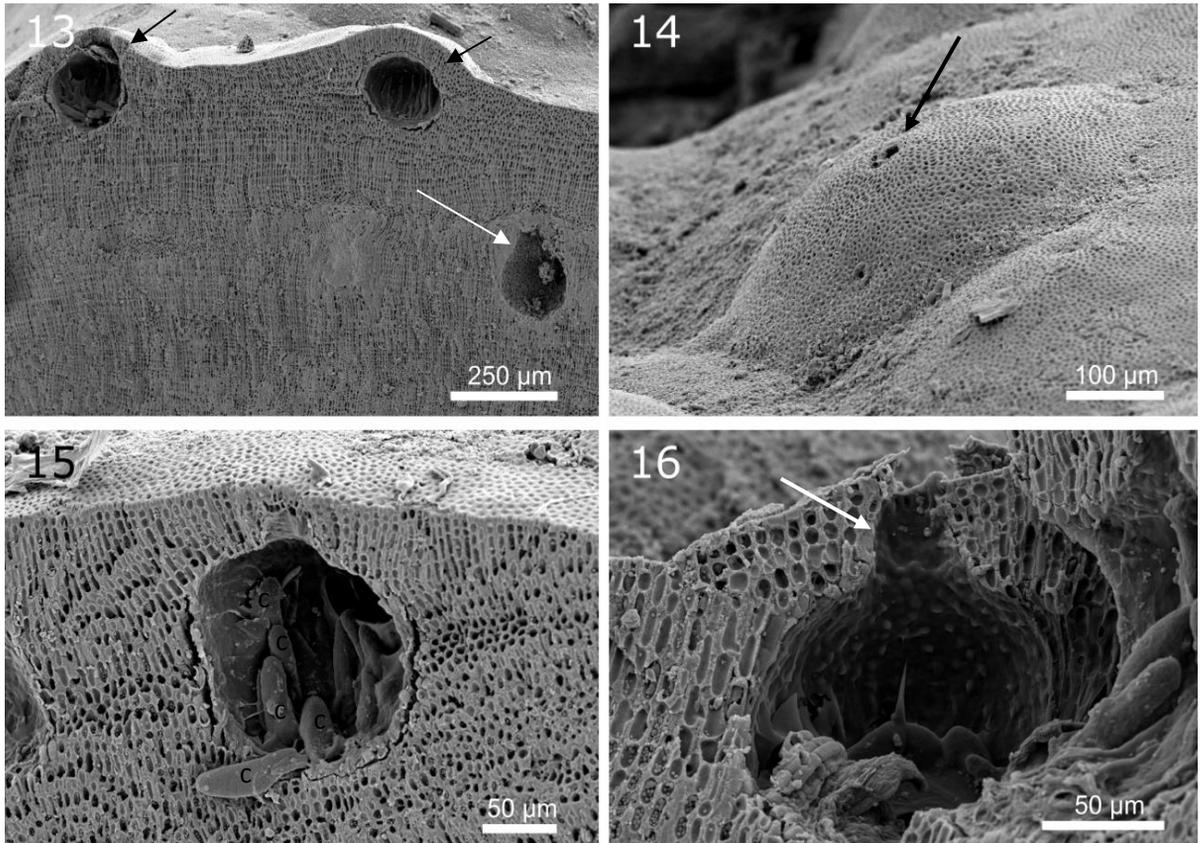
Fig. 2. Phylogram inferred from ML analyses of COI-5P sequences. Numbers at branches indicate posterior probabilities for Bayesian analysis (BI) and Bootstrap values for Maximum Likelihood (ML) and Neighbor-Joining (NJ) respectively. Thicker branches represent support from the GMYC analysis. Scale bar indicates number of substitutions per site. The clades in bold represent sequences generated in this study; “N” represents number of sequences in the compressed clades.



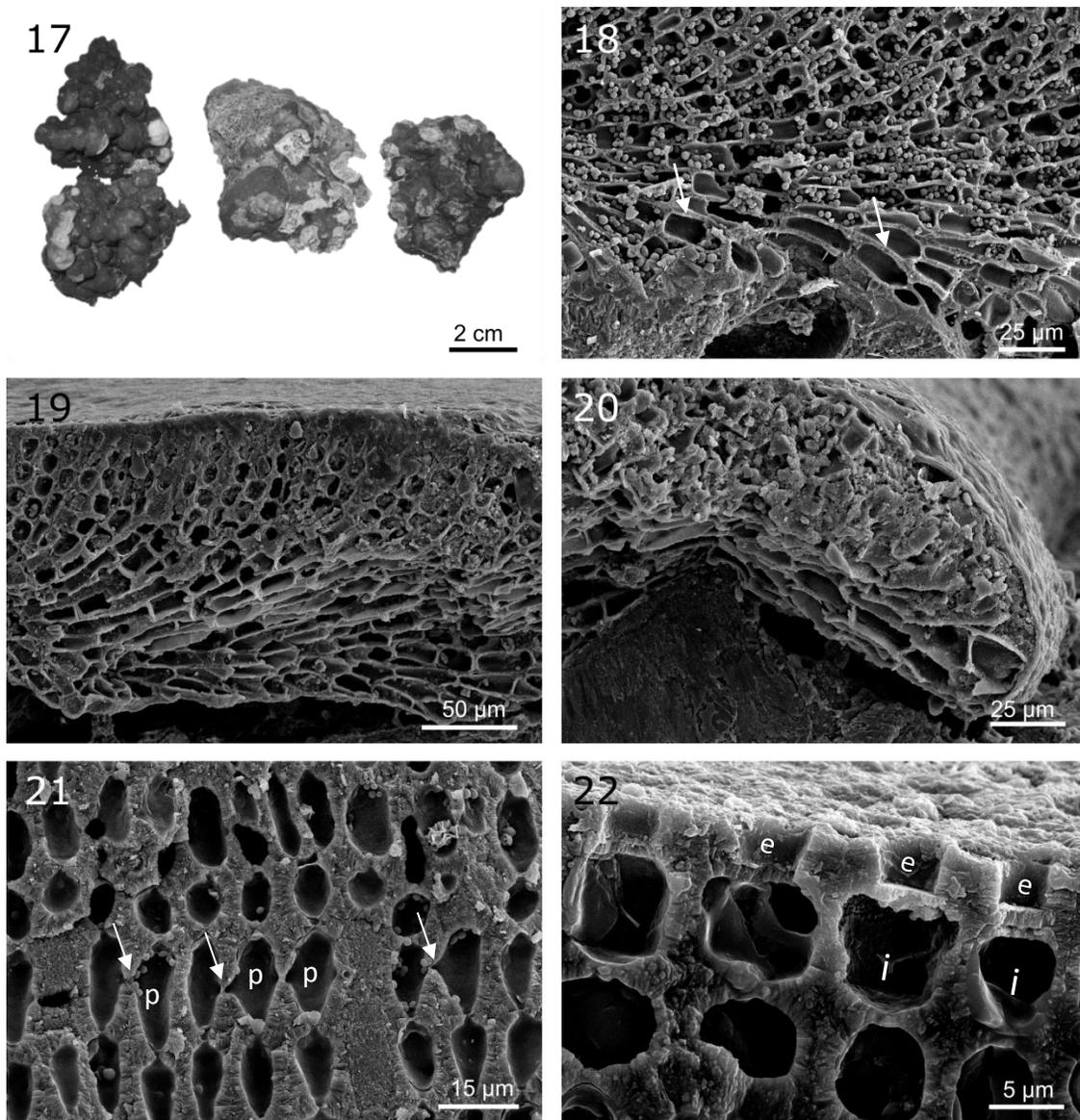
Figs. 3-8. Specimen IBC1609. Vegetative features of *Sporolithon pseudoepisorum*. Fig. 3. Habit of specimens showing different growth forms. Fig. 4. Section of the thallus showing thallus thickness. Fig. 5. Section of thallus showing location of hypothallial filaments (brackets). Fig. 6. Magnified view of hypothallus showing enlarged cells (arrows). Fig. 7. Section of thallus showing epithallial cells (e) and initials of subepithallial cells (i) and secondary pit connections (arrows). Fig. 8. Magnified view of the epithallial cells (e) and initials of subepithallial cells (i).



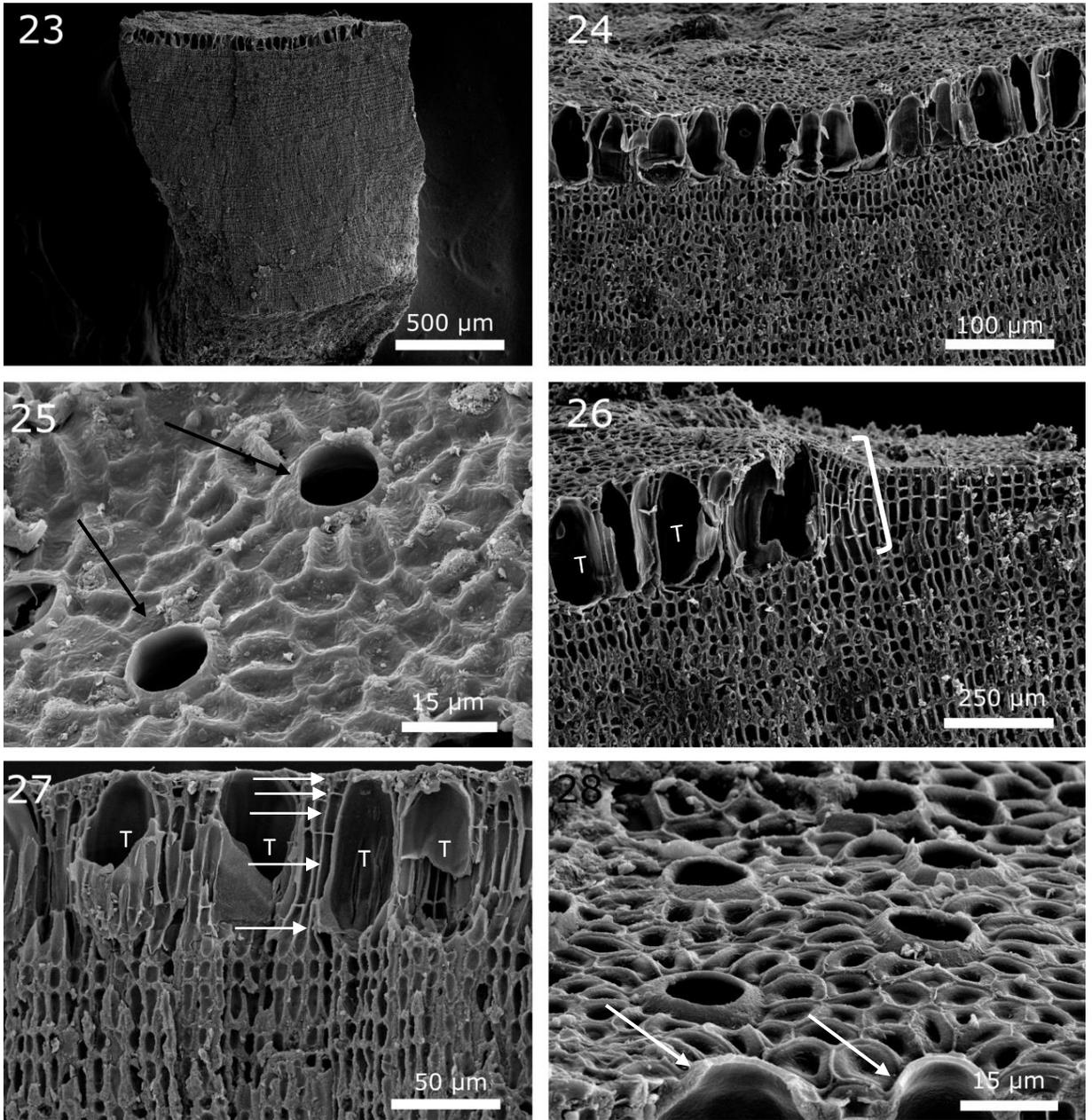
Figs. 9-12. Specimen IBC1613. Reproductive features of *Sporolithon pseudoepisporum* – Tetrasporophyte. Fig. 9. Surface view of the sori (clustered; pores - arrows). Fig. 10. Section through a sorus cluster showing empty tetrasporangial compartment (T). Fig. 11. Section through a sorus cluster showing the raised sori in relation to the thallus surface (bracket) and empty tetrasporangial compartment (T). Fig. 12. Section through a sori cluster showing empty tetrasporangial compartment (T); filled tetrasporangial compartment with tetrasporangia (te) and a sunken halo forming the pore (arrow).



Figs. 13-16. Specimen IBC1801. Reproductive features of *Sporolithon pseudoepisporum*-Gametophyte. Fig. 13. Section of the thallus showing carposporangia (black arrows) and an old buried empty carposporangium (white arrow). Fig. 14. Surface view of an raised uniporate conceptacle; Detail of the pore (arrow). Fig. 15. Section of a carposporangia filled with multiple carpospores in the arranged on the conceptacle floor (c). Fig. 16. Section of a carposporangia showing the inside aspect of the conceptacle walls and a pore channel (arrow).



Figs. 17-22. Specimen IBC1820. Vegetative features of *Sporolithon tomitae*. Fig. 17. Habit of specimens showing different growth forms. Fig. 18. Section of thallus showing hypothallial filaments with enlarged cells (arrows). Fig. 19. Magnified detail of hypothallial filaments showing upward growth. Fig. 20. Magnified detail of hypothallus showing the growth margin. Fig. 21. Section of thallus showing perithallial cells (p) and secondary pit connections (arrows). Fig. 22. Magnified view of the epithallial cells (e) and initials of subepithallial cells (i).



Figs. 23-28. Specimen IBC1643. Reproductive features of *Sporolithon tomitae* – Tetrasporophyte. Fig. 23. General aspect of a cross section through the thallus. Fig. 24. Magnified view of the cross section showing sori. Fig. 25. Magnified surface view of the sori showing pores (arrows) and rosettes. Fig. 26. Section through a sorus cluster showing the sunken/slightly raised sori in relation to thallus surface (bracket). Fig. 27. Section through a sorus cluster showing empty tetrasporangial compartment (sorus) - (T); paraphyses filaments comprised of five cells (arrows). Fig. 28. Superficial view of pore channel showing raised halos forming the pore.

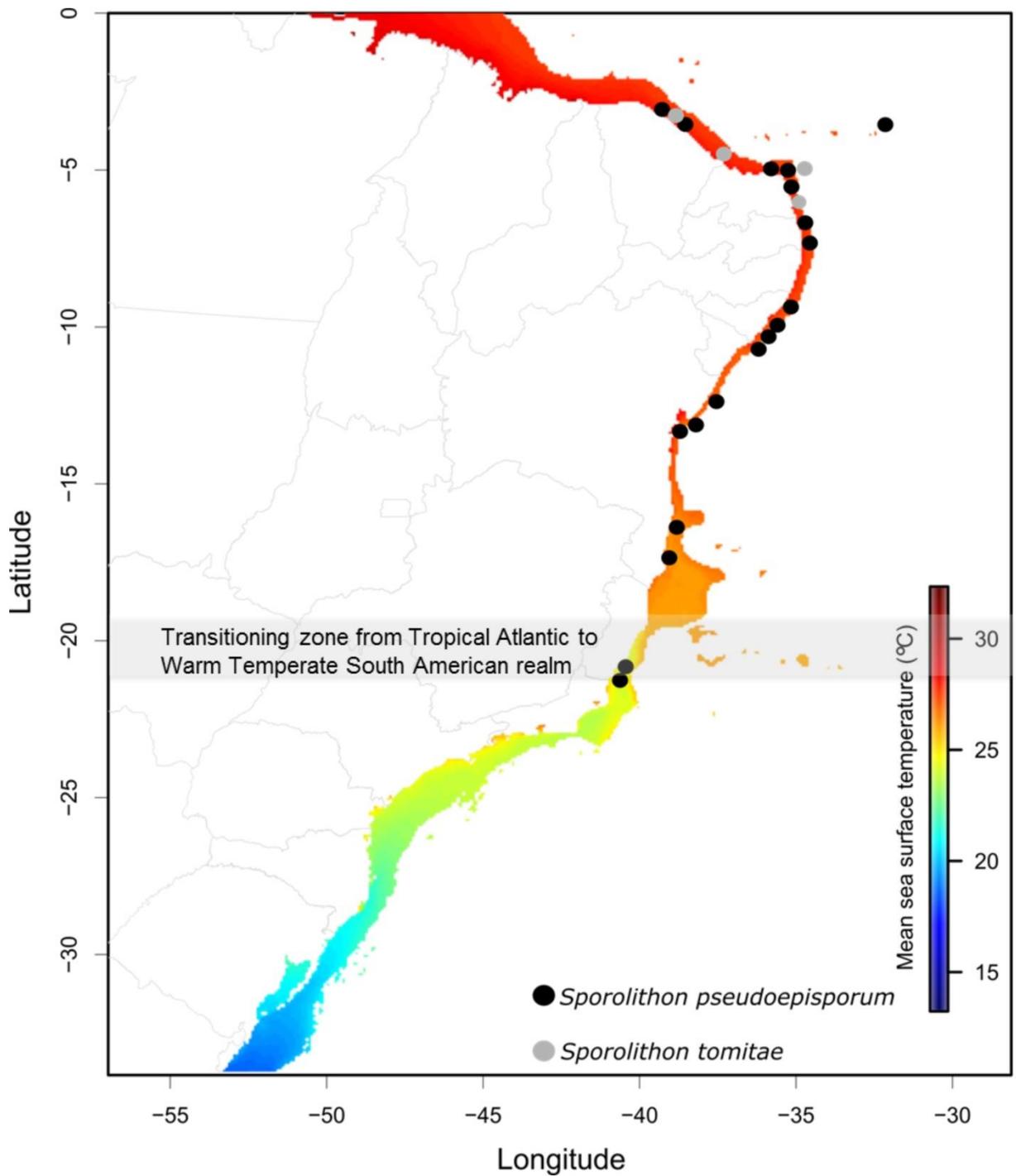


Fig. 29. Map of species distribution along the Brazilian coast. Mean SST are provided to show the specific distribution of *Sporolithon tomitae* (gray dots) in the Tropical Atlantic realm compared to the wide distribution of *S. pseudoepisporum* (black dots).

## Third Chapter

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[Prepared as a coauthored manuscript (J.L. Richards, T. Vieira-Pinto, W.E. Schmidt, T. Sauvage, P.W. Gabrielson, M.C. Oliveira & S. Fredericq) to be submitted to *Phytotaxa*]

MOLECULAR AND MORPHOLOGICAL DIVERSITY OF *LITHOTHAMNION* SPP.  
RHODOLITHS (HAPALIDIACEAE, HAPALIDIALES) FROM DEEPWATER  
RHODOLITH BEDS IN THE NORTHWESTERN GULF OF MEXICO

[Prepared as a coauthored manuscript (J.L. Richards, T. Vieira-Pinto, W.E. Schmidt, T. Sauvage, P.W. Gabrielson, M.C. Oliveira & S. Fredericq) to be submitted to *Phytotaxa*]

**Abstract**

In the Northwestern Gulf of Mexico (NWGMx), subtidal rhodolith beds offshore Louisiana at 45-80 m depth harbor a diverse community of non-geniculate coralline algae including both biogenic and autogenic rhodoliths and other encrusting taxa. The coralline algal flora of this region has yet to be fully characterized, and identifying specimens to their correct genus and species is an ongoing process because many available names remain to be validated by comparison to historical type specimens. Here, comparative DNA sequencing (*psbA*, UPA, and COI) and scanning electron microscopy (SEM) were used to assess the molecular and morphological diversity of the rhodolith-forming specimens belonging to the generic concept of *Lithothamnion* (Melobesioideae, Hapalidiaceae, Hapalidiales) in the NWGMx. Phylogenetic and barcoding analyses of the newly generated sequences from recently dredged specimens at Ewing and Sackett Banks offshore Louisiana revealed the presence of at least six species of *Lithothamnion*, whose SEM imaging confirmed the presence of characters described for the genus. More broadly, our analyses indicate the presence of at least eight species found in the Gulf of Mexico and at least ten species in the Western Atlantic Ocean for this form genus. Further comparisons with specimens from the coast of Brazil indicate the occurrence of a widespread *Lithothamnion* sp. I in the Western Tropical Atlantic.

*Key words:* COI, coralline algae, diversity, Gulf of Mexico, Hapalidiaceae, Hapalidiales, *psbA*, rhodolith, Rhodophyta, SEM, UPA

*Running title:* *Lithothamnion* in the Gulf of Mexico

*Abbreviations:* BS= bootstrap value; GMx = Gulf of Mexico; NEGMx = northeastern Gulf of Mexico; NWGMx = northwestern Gulf of Mexico; PP= posterior probability; SEM = Scanning Electron Microscope; SEGMx = southeastern Gulf of Mexico; SWGMx = southwestern Gulf of Mexico; UPA =Universal Plastid Amplicon; WTA = Western Tropical Atlantic

## Introduction

The Northwestern Gulf of Mexico (NWGMx) offshore Louisiana, harbors subtidal rhodolith beds at depths of 45–80m comprising a diverse assemblage of non-geniculate coralline algae spanning multiple lineages (Richards *et al.* 2014, Fredericq *et al.* 2014, Krayesky-Self *et al.* 2016). These rhodolith communities consist of free-living marine nodules primarily accreted by coralline algae precipitating calcium carbonate, or biogenic rhodoliths, and secondarily colonized nodules of geobiological origin, or autogenic rhodoliths (Foster 2001, Fredericq *et al.* 2014). The latter begin as calcium carbonate nodules formed by differential erosion of cap rock that originated from anaerobic bacteria acting on the minerals within the underlying salt domes, which subsequently become overgrown by a suite of encrusting macroalgae (e.g., members of Corallinales, Peyssonneliales, or Dictyotales) (Gore 1992, Fredericq *et al.* 2014). These rhodolith beds harbor a diverse assemblage of *Bivalvia* (Turgeon *et al.* 2009), whose shells provides substrata for encrusting coralline algae (Richards *et al.* 2014, present study).

*Lithothamnion* Heydrich (1897) is conserved against *Lithothamnium* Philippi (1837). Philippi (1837) based his genus *Lithothamnium* on five specimens of “rigid calcareous plants” from the Sicilian coast, Mediterranean Sea (Woelkerling 1983, p. 165). Four of these were newly described species (*L. crassum* Philippi, *L. gracilis* Philippi, *L. ramulosum* Philippi, and *L. rubrum* Philippi). The fifth species was *L. byssoides* (Lamarck) Philippi (Woelkerling 1983). Philippi merged the taxa *Nullipora byssoides* Lamarck and *Millepora polymorpha* var. *globosa* Esper (previously considered to be animals by Lamarck and Esper) into *Lithothamnium*. Philippi also synonymized both taxa, assigned the specific epithet *byssoides* based on priority, and assigned this specific epithet to one of the five specimens in his Sicilian collection (Woelkerling 1983, p. 165). Philippi’s original

description was later augmented by subsequent authors (Heydrich 1897b, Mason 1953, Adey 1966) who began considering three characters as diagnostic for *Lithothamnium*: 1) multiporate tetrasporangial conceptacles, 2) a multilayered noncoaxial hypothallus (= hypothallium *sensu* Woelkerling) and 3) a single layer of “angular” epithallial cells (reviewed in Woelkerling 1983) which are sometimes referred to as having “flattened and flared outermost cell walls” (Woelkerling, 1988 p. 171) or being armored (Adey *et al.* 2015). This concept of *Lithothamnium*, however, was not based on any material from Philippi’s original collection, which was found by Woelkerling in 1980 in Kützing’s herbarium (Woelkerling 1983). Woelkerling showed that none of the specimens in Philippi’s original collection possessed multiporate tetrasporangial conceptacles, and thus that all of Philippi’s species belonged to other generic concepts. However, because the modern concept of *Lithothamnium* had been adopted by many authors (albeit with some disagreement between authors in regards to how the genus is circumscribed) and many described species names had already relied on it, Woelkerling (1983, pp. 193-194) proposed to conserve *Lithothamnion* Heydrich 1897b, noting he was the first to establish the presence of multiporate tetrasporangial conceptacles as a defining character. Thus, Woelkerling established a lectotype specimen for the genus, *Lithothamnion muelleri* Lenormand ex Rosanoff (1866), which has a type locality near Victoria, Australia (Woelkerling 1983).

Few studies on *Lithothamnion* Heydrich have been conducted in the Gulf of Mexico (Dawes 1974, Minnery 1990, Mateo-Cid *et al.* 2014) and until recently relied solely on thallus morpho-anatomy rather than being integrated with additional characters such as molecular data (i.e. DNA sequences). Excluding taxa since transferred to other genera, Dawes (1974) reported a single species of *Lithothamnion*, *L. sejunctum* Foslie, from offshore

the west coast of Florida. *Lithothamnion* was reported in a preliminary investigation into the diversity and zonation of non-geniculate coralline algae in the NWGMx for the Flower Garden Banks National Marine Sanctuary offshore Texas by Minnery (1990); however, no specific epithets were assigned with confidence to the specimens which were identified as *Lithothamnium* sp. A, B and ‘*Lithothamnium sejunctum?*’ (Minnery, 1990 p. 996). Mateo-Cid *et al.* (2014a) reported three species, *L. occidentale* (Foslie) Foslie, *L. sejunctum*, and *L. crispatum* Hauck (Hauck), from offshore the southwestern Gulf of Mexico near Campeche Banks and from the intertidal zone along the Atlantic coast of Mexico. Recently, Kraysky-Self *et al.* (2016) reported two unnamed species of *Lithothamnion* from the NWGMx and provided the first published *psbA* sequence of *Lithothamnion* from this location.

Recent DNA-based studies have shown a wealth of previously undocumented diversity of non-geniculate coralline algae in the Northeast Pacific (Adey *et al.* 2015), North Atlantic Ocean (Peña *et al.* 2014a, Pardo *et al.* 2014), the Mediterranean Sea (Peña *et al.* 2015), the Caribbean (Peña *et al.* 2014b), the South Atlantic (Bahia *et al.* 2014, Sissini *et al.* 2014, Vieira-Pinto *et al.* 2014) as well as in the Gulf of Mexico (Mateo-Cid *et al.* 2014b, Richards *et al.* 2014, Kraysky-Self *et al.* 2016) and the Gulf of California (Hernández-Kantún *et al.* 2015). These studies demonstrated the importance of integrating DNA sequence analyses with morpho-anatomical studies when assessing biodiversity, and provided a preliminary reference framework for comparison with newly generated data.

Following the April 2010 Deepwater Horizon oil spill, seven box-dredging expeditions conducted in NWGMx rhodolith beds for the purpose of biomonitoring led to comparative DNA sequencing and morphological analyses of non-geniculate Corallinales and Hapalidiales specimens (Fredericq *et al.* 2014, Richards *et al.* 2014, Kraysky-Self *et al.*

2016). Often non-geniculate corallines were the only visible algal species present in the dredges post-spill (see Fredericq *et al.* 2014) and a substantial portion of these specimens identified as members of the subfamily Melobesiodeae (Hapalidiaceae, Hapalidiales) awaited further investigations.

Herein we focus on the molecular and morphological characterization of six unnamed *Lithothamnion* species from these NWGMx collections based on phylogenetic analyses of DNA sequence data and high magnification imaging of thallus anatomy using scanning electron microscopy (SEM). The diversity of specimens fitting the generic concept of *Lithothamnion* in the NWGMx is addressed in the context of newly generated and publicly available data.

## **Material and methods**

Specimens (Table 1) were collected with an Hourglass-design box dredge using minimum tow periods (usually 10 minutes or less) (Joyce & Williams 1969) deployed by the *R/V Pelican*, the UNOLS (University-National Oceanographic Laboratory System) research vessel stationed at LUMCON (Louisiana Universities Marine Consortium), during seven post-Deepwater Horizon oil spill (DWH) expeditions in the vicinity of Ewing Bank (~28° 05.737'N; 91° 01.608'W) and Sackett Bank (~28° 38.019'N; 89° 33.262'W). Expedition collection dates were December 2-6, 2010; April 19-24, 2011; August 26-30, 2011; August 24-26, 2012; November 15-17, 2012; October 17-22, 2013; and September 7-14, 2014. During the course of the September 2014 cruise, samples were also collected offshore Florida in the southeastern Gulf of Mexico in the vicinity of the Dry Tortugas. Collected specimens were desiccated in silica gel aboard the *R/V Pelican* and/or preserved in 5%

formalin/seawater, and deposited in the University of Louisiana at Lafayette Herbarium (LAF). Another portion of the samples was transported to the laboratory as “live rocks” (Delbeek & Sprung 2005) and grown in 75-liter microcosm tanks prior to harvesting and preservation as described in Fredericq *et al.* (2014), Richards *et al.* (2014) and Felder *et al.* (2014). Exploratory DNA sequencing and SEM imaging led to the identification of eight specimens from the NWGMx belonging to the genus *Lithothamnion*. For phylogeographic context, additional sequences generated from three rhodolith-forming samples of *Lithothamnion* from the surrounding areas of the Gulf of Mexico (vicinity of the Florida Middle Grounds in the NEGMx, Campeche Banks in the SWGMx, and near the Dry Tortugas areas in the SEGMx) were also included as well as two samples from Pacific Panama and three samples from the Brazilian states of Ceará and Bahia (both in the Tropical region of the Brazilian coast).

**DNA Extraction and PCR protocols.** Total genomic DNA was extracted from samples from the Gulf of Mexico and Panama according to the protocol of Richards *et al.* (2014), while Brazilian samples were extracted using the protocol of Vieira-Pinto *et al.* (2014). In all cases DNA was extracted from the same specimens used for morphological analysis. Overall, 17 specimens of *Lithothamnion* spp. were sequenced (Table 1).

Three markers were selected for PCR: the chloroplast-encoded photosystem II reaction center protein D1 gene (*psbA*) and Universal Plastid Amplicon 23S rRNA gene (UPA), and the mitochondria-encoded cytochrome oxidase subunit I gene (CO1). PCR for *psbA* was performed using the primers referenced in Yoon *et al.* (2002) under the following thermal profile: an initial denaturation at 94°C for 3 min followed by 39 cycles at 94°C for 30 sec (denaturation), 52°C for 50 sec (primer annealing), and 72°C (extension) for 1 min

followed by a final extension at 72°C for 5 min. PCR for COI was conducted using the primers referenced in Saunders (2005) with an initial denaturation at 94°C followed by 40 cycles at 94°C for 1 min (denaturation), 45°C for 1 min (primer annealing), and 72°C (extension) for 1 min followed by a final extension at 72°C for 5 min. PCR for UPA was performed using the primers and PCR protocol referenced in Sherwood & Presting (2007). PCR products were purified with ExoSAP-IT® (USB®) or by excision on a Agarose II (Amersco) TAE (Tris-acetate-EDTA) gel followed by digestion with GELase™ (Epicentre®). Purified PCR products were subsequently cycle sequenced using the BigDye Terminator v 3.1 kit (Life Technologies). Resulting cycle sequence reactions were purified with ETOH/EDTA precipitation and were either sequenced in-house at the UL Lafayette campus on an ABI Model 3130xl Genetic Analyzer, at the University of São Paulo campus on an ABI Model 3730 Genetic Analyzer, or outsourced (Beckman Coulter Genomics Danvers, MA). Resulting chromatograms were assembled using Sequencher 5.1 (Gene Codes Corp., Ann Arbor, MI, USA).

**Alignment.** Newly generated and previously available DNA sequences from GenBank (Table 1) were aligned using the CLUSTAL W (Thompson *et al.* 1994) program in MEGA 5.2.2 (Tamura *et al.* 2011). For UPA, a small ambiguous region was cropped to the nearest conserved region, and the alignment was truncated to minimize missing data at the 3' end. The final UPA alignment was 352 base pairs (bp) in length and included 14 newly generated sequences, 12 available Hapalidiaceae sequences, and two sequences of *Sporolithon* spp. as the outgroup. *PsbA* and COI aligned unambiguously. The final *psbA* alignment (863 bp) comprised 13 newly generated sequences, 62 available sequences and 3 sequences of the Sporolithales. The final COI alignment (652 bp) included eight newly generated sequences,

33 available sequences, and three sequences of *Sporolithon* as the outgroup. Alignments were not concatenated due to a lack of corresponding markers available on GenBank.

**Multi-gene Alignment.** CO1, UPA, and *psbA* alignments were imported to MacClade 4.08 (Maddison & Maddison 2000), blank cells were added in place of missing data where needed, and exported as NEXUS files for assembly using the application Sequence Matrix 1.7.8 (Vaidya *et al.* 2011). The alignment included concatenated sequences from three members of the Sporolithales and 60 concatenated sequences of the Hapalidiales. The final concatenated alignment was exported as a PHYLIP file for analysis with RAxML and was 1,867 base-pairs in length including a 652 base-pair portion of COI, a 352 base-pair portion of UPA, and a 863 base-pair portion of *psbA*.

**Phylogenetic and Barcoding Analyses.** The UPA alignment was analyzed with the NJ distance-based method in MEGA 5.2.2 (Tamura *et al.* 2011) with 1,000 bootstrap replicates to assess branch support. Phylogenetic analyses of the *psbA* and COI alignments were conducted using Bayesian (BI) and Maximum Likelihood (ML) methods, both with a GTR+I+G model of evolution partitioned per codon position. Phylogenetic analysis of the multi-gene alignment was conducted using ML method, with a GTR+I+G model of evolution partitioned per codon position. Bayesian analysis was performed using Mr. Bayes 3.2.6 (Ronquist *et al.* 2012). Two parallel analyses were conducted, each consisting of four MCMC chains (3 heated and 1 cool) with  $1 \times 10^7$  generations. Resampling was performed every 1,000 generations resulting in a total of 2,002 trees for both runs. The first 10% of each run was discarded as “burn-in”, and a consensus tree was built with remaining data. Convergence was determined with Tracer v1.6 (Rambaut *et al.* 2014). ML analyses were conducted with the RAxML-HPC2 program using the online server ‘The CIPRES Science

Gateway V. 3.3' (Miller *et al.* 2010) with 1,000 topological searches from random restarts, and 1,000 bootstrap replicates to assess branch support.

**Species Delimitation Analyses.** Species delimitation analyses were performed on each of the *psbA* and COI datasets with both Automatic Barcode Gap Species Discovery (ABGD) and General Mixed Yule Coalescence (GMYC). For ABGD, branch lengths were extracted from the *psbA* and COI RAxML trees with the function *cophenetic.phylo* of the package APE in R (Paradis *et al.* 2004, R Core Team, 2015) to produce a distance matrix as input. The latter was run with minimum (pmin) and maximum (pmax) intraspecific distance priors comprised between 0.001 and 1 in 100 steps, and with a relative gap width values of 0.003. Alternative species boundaries hypotheses were produced with the (GMYC) model with the package SPLITS in R (Fujisawa & Barraclough 2013), with the single threshold method based on an ultrametric tree generated in BEAST v2.0 (Bouckaert *et al.* 2014) using a relaxed log-normal clock with a constant population coalescent as prior, and a GTR+I+G model of evolution partitioned per codon position. MCMC chains were run for 30 million generations (sampled every 1000th generations) and the quality of the run assessed in Tracer v1.6 (Rambaut & Drummond 2007) to ensure that ESS values were > 200 with the default burnin (3000 trees).

**Evaluation of Pairwise Distance Distribution for Three Markers.** The distribution of raw pairwise distances was computed for the three markers utilized in the present study in order to evaluate their phylogenetic informativeness. Distances were calculated by dividing the number of base pair differences by the alignment length. Alignments were cropped at their 5' and 3' ends when missing data was present and short sequences were removed as to not overinflate pairwise distances. Distance matrices were constructed for each of the UPA,

*psbA*, and COI alignments using Geneious version R9 (<http://www.geneious.com>, Kearse *et al.*, 2012).

**Preparation of Material for Scanning Electron Microscopy.** Portions of the thallus from silica gel-dried specimens were removed using a razor blade and forceps. Sections were made manually using a razor blade and mounted using liquid graphite and coated with 15 nm of gold. To ensure even distribution of the gold over the three dimensional features in the sections, coating was performed in two applications. First, 8 nm of gold was applied with the stub lying flat on the stage of the coating chamber. After the first application, the specimen was tilted using a coin placed underneath the stub and a second application of 7 nm of gold was performed. Specimens were viewed using a Hitachi S-3000N scanning electron microscope (SEM) at a voltage of 15 kV, housed in the Microscopy Center at UL Lafayette, following the manufacturer's instructions.

## Results

**DNA sequence Analyses.** The backbones of the three phylogenies are overall poorly resolved (Fig. 1-3). Numerous lineages harboring specimens fitting the morphological concept of *Lithothamnion* spp. (see next sections) receive high support with both analyses and overall, UPA, *psbA*, and COI trees revealed previously undocumented molecular diversity found in the Gulf of Mexico, WTA, and Pacific Panama. While UPA is less sampled and less resolved than *psbA* and COI, all trees, including the multi-gene tree (Fig. 4), demonstrate the polyphyly of specimens identified as *Lithothamnion* species interspersed among (or sister to) other genera of Hapalidiaceae. In the best sampled trees (taxa-wise, i.e. *psbA* and COI), *Lithothamnion* spp. are found in early branching paraphyletic lineages and a

monophyletic lineage sister to several lineages including *Mesophyllum*, “*Leptophyllum*” *foecundum*, *Synartrophyton*, and members of the *Clathromorphum* complex of genera (*sensu* Adey *et al.* 2015). *Phymatholithon* spp. are nested among early branching lineages of *Lithothamnion* spp. Several unresolved sequences labeled as Hapalidiaceae sp. branch within *Lithothamnion* clades, in *psbA* and COI (LBC0640, Bittner *et al.* 2011; E58, Hernández-Kantún *et al.* 2015), and in the UPA tree especially (FLOR14925-30, Sissini *et al.* 2014). The results of the ABGD and GMYC analyses showed some discrepancies in the number of delimited molecular species for the entire data set, especially for *psbA*, 41 vs. 52 species (respectively, Fig. S1), whereas results for COI were more congruent, 27 vs. 28 species (respectively, Fig. S2). However, delimitation for the newly investigated specimens (GMx, WTA, Pacific Panama), reported in the *psbA* tree and the COI tree as Hapalidiaceae sp. A to J were all congruent in revealing nine of these ten separate entities found in GMx (spp. A, B, C, D, E, F, G, H, I) and one in Brazil (sp. I). Nonetheless, whether sister sequences to the above taxa that are found in other locations are conspecifics is unclear based on the present data set. For instance in the COI tree, *Lithothamnion* sp. I from the Gulf of Mexico and specimens from Bahia and Ceará in Brazil split in two entities with GMYC analyses, where ABGD delimit a single species. Likewise, in the *psbA* tree, LBC0640 and Hapalidiaceae sp. D were split in the GMYC analyses but delimited as a single species in the ABGD analyses. Species annotation for the newly generated specimens were transferred to the UPA, *psbA* and COI trees (Figs. 1-3) based on the above *psbA* and COI delimitation results and herein are referred to as *Lithothamnion* spp. A-J. The multi-gene tree was also congruent in revealing Hapalidiaceae sp. A to J as ten separate entities (Fig. 4). Finally, evaluation of pairwise distance distribution for the three markers as a proxy for these markers’ speed of evolution

and phylogenetic informativeness indicated that UPA is the most conserved, followed by *psbA* and the most variable, COI (Fig. 5).

The NJ analysis of the UPA alignment shows multiple sequences labeled as Hapalidiaceae sp. among which our *Lithothamnion* spp. are interspersed. Three clades receive moderate to strong support (BS= 77-99%, Fig. 1, *Lithothamnion* spp. A-J). Six of these clades include specimens from the NWGMx, with one of the six (*Lithothamnion* sp. I) also represented by a specimen from the NEGMx and a specimen from Brazil. UPA sequences of specimens of *Lithothamnion* sp. I from the GMx and Brazil were 100% identical (Table S1). Two clades (*Lithothamnion* spp. D and J) each include a specimen from Pacific Panama and two clades each include a single specimen from the Campeche Banks and vicinity of the Dry Tortugas, respectively.

The results of the *psbA* analysis revealed 9 clades (*psbA* for *Lithothamnion* sp. H was not successfully amplified). Though several lineages within the Hapalidiales received high support (Fig. 2), branching order in the deeper nodes was unresolved. The *psbA* sequence of *Lithothamnion* sp. G is sister to *L. coralloides* (P. Crouan & H. Crouan) P. Crouan & H. Crouan (97.9% similarity, Table S2) with moderately good support in the Bayesian analysis (PP=0.82) but low support in the ML analysis (BS= 51%). *Lithothamnion* sp. G and *L. coralloides* are sister to *L. crispatum*, which are nested with a clade of other available sequences of *Lithothamnion* spp. including a specimen identified as *L. muellerii* from Isla San Jose, Mexico and specimens of *L. glaciale* from the North Atlantic. Sequences of *Lithothamnion* spp. I and J were sister to each other and comprised a lineage with full support in both Bayesian and ML analyses. The *psbA* sequence of Hapalidiaceae sp. E58, from La Paz, Mexico, is sister to *Lithothamnion* sp. J from Pacific Panama with strong

support (PP= 0.98, BS=94%) and these sequences show high similarity (98.8%). *Phymatolithon calcareum* is sister to the lineage comprised of *Lithothamnion* sp. I, J, and E58. This node is strongly supported in the Bayesian analysis (PP=0.99) but not in the ML analysis. The sequence of *P. calcareum* differed from sequences of *Lithothamnion* spp. I, J by 7.5-8.3%. Sequences of Brazilian specimens of *Lithothamnion* sp. I differed from sequences of the GMx specimens by 3-5 base pairs (0.35-0.58% difference). *Lithothamnion* sp. E and F comprise a clade at the base of the Hapalidiales, with *Lithothamnion* sp. (LBC0642) nested in between *Lithothamnion* sp. E and F.

*Lithothamnion* spp. A-D includes specimens from the GMx and a specimen from Pacific Panama. These specimens, together with specimens identified as *Lithothamnion* (“Species1-3”) from Guadeloupe, French West Indies (FWI), an unidentified Hapalidiaceae specimen from New Caledonia, specimens identified as *Mesophyllum* sp. 1 and 2 from Macaronesia, and a specimen identified as *Lithothamnion* cf. *ruptile*, comprised a monophyletic lineage with strong support (PP= 1, BS= 99%). Within this lineage, *Lithothamnion* sp. D is sister to the unidentified Hapalidiaceae sp. (LBC0640) with strong support (PP=1, BS= 95%), and these sequences show high similarity (98.7%). This lineage is sister to the *Clathromorphum*-complex with moderate support in the Bayesian analysis (PP=0.71), but with low support in the ML analysis (BS <50%). The *Clathromorphum*-complex formed a monophyletic lineage with strong support (PP=1, BS=97%). The lineage including *Lithothamnion* spp. A-D and the *Clathromorphum*-complex lineage are both sister in a larger clade including specimens identified as *Synarthrophyton patena*, sequences of specimens belonging to *Mesophyllum*, including *M. lichenoides* (J. Ellis) Lemoine, *M. erubescens* (Foslie) Lemoine, and *M. sphaericum* Peña, Bárbara, Adey, Riosmena-Rodríguez

& H.G. Choi and "*Leptophytum*" *foecundum* (Kjellman) Adey. This clade is well supported (PP=1, BS= 92%) but has unresolved branching order (ie. a polytomy) at the base.

The results of the COI analysis revealed 6 clades (COI for *Lithothamnion* sp. B, C, D, and G were not successfully amplified). The sequence of specimen LAF6970C, *Lithothamnion* sp. H (KU514416), from offshore Florida in the SEGMx, is identical to the sequence of the specimen identified as *Lithothamnion*, "Species 5 PC0144250" (KJ710337) published by Peña *et al.* (2014) from Guadeloupe, FWI. The COI tree (Fig. 3) shows the Hapalidiaceae lineage received full support in both the Bayesian and ML analyses, and is comprised of two sister lineages that are each well supported in the Bayesian analysis (PP=1, .99) but lack support in the ML analysis (BS<50%). The clade comprised of *Lithothamnion* spp. E, F, and *Lithothamnion* sp. LBC0642 shows a different topology than in the *psbA* tree. This clade is sister to *L. coralloides* and nested within a larger clade including *L. glaciale*, *L. sp.*, and *L. sp.32* with *Lithothamnion* sp. H/"Species 5 PC0144250" comprising a clade at the base of this lineage. COI sequences of the Brazilian and GMx specimen of *Lithothamnion* sp. I differed by 2.6% (Table S3). *Lithothamnion* sp. I and J comprise a clade sister to *Phymatolithon calcareum*, however this lineage received only moderate support in the Bayesian analysis (PP=0.86) and was not supported in the ML analysis (BS<50%). The COI sequence of *P. calcareum* differed from the COI sequences of *Lithothamnion* spp. I and J by 12.2-12.7%. *Lithothamnion* sp. A, which is sister to the unidentified Hapalidiaceae sp. LBC0640 from New Caledonia, comprises a clade that is well supported in the Bayesian analysis (PP=.99) but not supported in the ML analysis (BS= <50%). Although sample size in this clade is smaller than in the *psbA* tree, the specimens for which both COI and *psbA* data are available, including the unnamed *Lithothamnion* spp. ("Species1-3") from

Guadeloupe, FWI and ‘*Mesophyllum*’ sp. 1 and 2 from Macaronesia, show similar groupings in the COI tree as in the *psbA* tree. This clade is nested within a larger clade including three other branches, one comprised of a specimen identified as *Synarthrophyton patena*, a second comprised of *Mesophyllum* spp., and a third comprised of two specimens identified as *Lithothamnion* sp. (LBC0845 and “Species 4-PC0144248”) that were not present in the *psbA* analysis. No *psbA* data are available for “Species 4-PC0144248” and the corresponding *psbA* sequence for LBC0845 comprised a long branch with low support in preliminary analyses (data not shown) and was excluded from the final analysis.

The ML analysis of the concatenated multi-gene alignment resulted in a tree with a backbone that is more resolved overall than the individual gene trees (Fig. 4) and revealed 10 clades of Hapalidiales within the newly generated data for this study (*Lithothamnion* spp. A-J). In this analysis, Hapalidiales comprised a monophyletic clade with full bootstrap support (100%) and several lineages within the Hapalidiales received high support. The clades are overall congruent with the *psbA* and COI single-gene analyses (Figs. 2, 3), although in this analysis, the clade comprised of *Lithothamnion* spp., including specimens of *L. muelleri*, *L. crispatum*, *L. corallioides*, *L. glaciale*, *L. lemoineae*, *L. tophiforme*, and *Lithothamnion* spp. E-H, is more resolved (BS=97%) and is sister to the clade including *Lithothamnion* spp. I-J and *Phymatholithon calcareum* with moderate support (BS=73%). The clade including *Lithothamnion* spp. A-D, *Lithothamnion* and *Mesophyllum* species from the North Atlantic and Caribbean, and unidentified Hapalidiaceae species from Brazil and the Indo-Pacific, comprised a monophyletic lineage with strong support (BS= 99%).

**Species delimitation:** The ABGD analyses of both *psbA* and COI delimited the specimens LAF6521, LAF1437, SPF57882, SPF57883 and SPF57884 (*Lithothamnion* sp. I) as being a single species, whereas the GMYC analyses of *psbA* and COI delimited LAF6521/LAF1437 and SPF57882/SPF57883/SPF57884 as separate species. Both ABGD and GMYC analyses delimited PHYKOS7249 (*Lithothamnion* sp. J) and Hapalidiaceae sp. E58 as being separate species. The ABGD analyses of *psbA* delimited the sequence of LBC0640 as being the same species as specimen LAF6631 (*Lithothamnion* sp. D), whereas the GMYC analyses of *psbA* indicated LBC0640 and LAF6631 are different species. Both GMYC and ABGD analyses delimited specimen LAF6970C (*Lithothamnion* sp. H) and “Species 5-PC0144250” as being a single species.

**Morphological analysis:** Scanning electron microscopy identified 16 specimens with characters corresponding to the genus *Lithothamnion*. All specimens analyzed were non-parasitic, calcified, non-geniculate thalli forming biogenic rhodoliths or encrusting autogenic rhodoliths or other substrata. A non-coaxial, monomerous thallus construction was observed in all specimens, that consistently exhibited cell fusions linking cells of adjacent filaments and lacked secondary pit connections, palisade cells and trichocytes (see Table 2 for complete list of vegetative characters). All crustose specimens possessed dorsiventral orientation with a multilayered hypothallus consisting of hypothallial filaments with rectangular-shaped hypothallial cells that are approximately isodiametric in cross section. Protuberant rhodolith specimens showed radial construction in vegetative protuberances (sometimes interspersed by new vegetative growth layers), whereas reproductive protuberances possessed radial construction interspersed by conceptacles and new vegetative growth layers. Crustose portions of protuberant rhodoliths were not sectioned to preserve the

intact rhodolith as a morphological voucher specimen. All specimens displayed a single layer of epithallial cells with thick, heavily calcified cell walls that varied in size, shape, and degree of thickness of the cell walls. Large secondary interfilament crystals characteristic of *Clathromorphum* were not observed in any of the specimens examined. Considering the molecular and morphological data, existing names could not be assigned with confidence to any of the species described below.

***Lithothamnion* sp. A** Specimens LAF6547 and LAF6549 are separate thalli with no protuberances encrusting the same autogenic rhodolith (Fig. 6A). Sections show a multilayered hypothallus comprised of hypothallial filaments (Fig. 6B, lower bracket) with rectangular-shaped cells growing parallel to the substratum (Fig. 6B, larger arrows) and arching tiers of perithallial filaments (Fig. 6B, upper bracket) growing perpendicular to the substratum. Adjacent perithallial filaments linked by cell fusions (Fig. 6B smaller arrows, Fig. 6C, F). Epithallial cells (Fig. 6C) with thick, heavily calcified cell walls and cylinder-shaped lumens. Surface views show multiporate conceptacles (Fig. 6D, E) that are interpreted as being tetrasporangial. Spores were not shown in conceptacle sections (data not shown), and the conceptacles appeared post-spore release. Pores surrounded by 5-7 rosette cells (Fig. 5F).

***Lithothamnion* sp. B** Specimen LAF6957B possesses a thallus with some protuberances (Fig. 7A, black arrows) encrusting an eroded bivalve shell (Fig. 7A, brackets). Crustose portion loosely adhered to substratum with lobed margins (Fig. 7A white arrow). Sections show multilayered hypothallus comprised of filaments growing parallel to the substratum (7B, lower bracket), that gives rise to arching tiers of perithallial filaments growing perpendicular to the substratum (7B, upper bracket). Perithallus with numerous cell fusions

often linking only two adjacent filaments (Fig. 7C arrows). Sections show conceptacles which are raised with respect to the surrounding thallus surface (Fig. 7D), some becoming filled in with crystals from secondary mineralization (Fig. 7C black arrow). It was not evident if conceptacles were uniporate or multiporate. Epithallial cells with a polygonal outline, trapezoidal shaped lumens (Figs. 7E, F) and epithallial cell roofs that may remain intact (Fig. 7C circle arrow) or become collapsed (Fig. 7D circle arrow).

***Lithothamnion* sp. C** Specimen 6820 is a rhodolith that possesses numerous unbranched protuberances (Fig. 8A). Median periclinal sections show protuberances composed of abundant overgrown conceptacles (Fig. 8B), many with needle-like crystals of putative aragonite (Fig. 8C, arrow). Conceptacles are uniporate (Fig. 8C, circle arrow) and are interpreted to be either male or female gametangial conceptacles. Sections show conceptacles become overgrown by continuation of growth of the perithallial cells, aided by elongation of perithallial cells at the periphery of conceptacles (Fig. 8D, arrows). Cells bordering the conceptacles show infill with small, centripetally formed calcite crystals (Fig. 8E, F arrows). Small, unidentified spherical inclusions observed in cells near the empty conceptacles may be life history stages of unicellular organisms (Fig. 8F, circle arrows). Sections infrequently show newly formed hypothallial filaments consisting of rectangular-shaped cells (Fig. 8G, H, circle arrow, upper bracket) growing over older parts of the thallus (Fig. 8G, H, lower bracket). Perithallus with numerous cell fusions often fusing several adjacent filaments (Fig. 8A, "F"). In some locations sections show intercalary meristematic cells (Fig. 8B, "M") between the epithallus (Fig. 8B, upper bracket) and perithallus (Fig. 8B, lower bracket) while other locations show cells below the epithallus that appear to be recently divided meristematic cells (Fig. 8B, \*). Epithallial cells with heavily calcified cell walls, which are

thicker at the proximal end of the cell and thinner at the distal end, a trapezoidal-shaped cell lumen (Fig. 8B arrows) and a thinner epithallial cell roof which may or may not remain intact (Fig. 8B circle arrows).

***Lithothamnion* sp. C** Specimen 6956B is a rhodolith that possesses numerous, sometimes branched, protuberances (Fig. 9C, arrow). Median periclinal sections show thallus construction similar to LAF6820, excepting that putative aragonite crystals which infill the conceptacles are in the form of spherical masses (Fig. 9D, arrows). Some portions of the thallus are very heavily calcified; sections show the polygonal outline of filaments where cells were cleaved apart from their adjacent filaments in the z-axis (Fig. 9E, black arrows) and reveal the cell fusions in the z-axis (Fig. 9E, “F”). Some locations in the heavily calcified areas show the sections of individual cells, revealing epithallial cells (Fig. 9E, F, brackets) with a trapezoidal shaped cell lumen (Fig. 9E, F, white arrows) and meristematic cells (Fig. 9F, “M”) similar in size and shape to those of LAF6820.

***Lithothamnion* sp. D** Specimen LAF6631 possesses a thallus with no protuberances encrusting a small putative limestone nodule (Fig. 10A). Thallus sections show multiple layers of hypothallial filaments (Fig. 10B, lower bracket) that give rise to arching tiers of perithallial filaments (Fig. 10B, upper bracket). Perithallus with abundant cell fusions (Fig. 10C, “F”) sometimes occurring between more than two adjacent filaments. Surface views of epithallus (Fig. 10D, right bracket, Fig. 10E, left bracket, arrows, Fig. 10F, arrows) show cells possess a polygonal outline. Partial section views show flattened epithallial cells each with a small lumen (Fig. 10D, arrow). Epithallial cell roofs (Fig. 10F, arrows) appear thin and collapsed into the cell lumen. Cells subtending the epithallial cells are not clearly identified as meristematic cells (Fig. 10D, E, F, \*) and in some location the cells subtending

the epithallial cells appear as possible pairs of recently divided meristematic cells (Fig. 10E, F, \*).

***Lithothamnion* sp. E** Specimen LAF5421 possesses a thallus with no protuberances, encrusting an autogenic rhodolith fragment (Fig. 11A, bracket). Sections show the thallus (Fig. 11B, upper bracket) and substratum (Fig. 11B, lower bracket). Hypothallus with multiple layers of hypothallial filaments (Fig. 11C arrow, lower bracket) with rectangular shaped cells that grow parallel to the substratum, and give rise to arching tiers of perithallial filaments (Fig. 11C, upper bracket) that grow perpendicular to the substratum. Sections and surface views show remnant, heavily calcified proximal cell walls with primary pit connections in the center of the walls appearing as small round structures (Fig. 11D, arrows) and do not show intact cells of the epithallus or clearly show meristematic cells (Fig. 11D).

***Lithothamnion* sp. F** Specimen LAF6882 is a rhodolith that possesses numerous protuberances (Fig. 12A). Median periclinal sections of protuberances show overgrown conceptacles (Fig. 12B, arrow, circle arrow) that in some locations become filled in with putative aragonite crystals (Fig. 12B, circle arrow) from secondary mineralization. Sections show conceptacles and older parts of the thallus (Fig. 12C, lower left bracket) become overgrown by new vegetative growth through the formation of a new multilayered hypothallus oriented parallel to the older parts of the thallus (Fig. 12C, lower right bracket). Hypothallial filaments give rise to the arching tiers of perithallial filaments (Fig. 12C, upper right bracket) that form newly developed protuberances (Fig. 12C, circle arrow) or add volume to existing protuberances. Perithallus with abundant cell fusions (Fig. 12C, inset, arrows). Intercalary meristematic cells (Fig. 12D, “M”) shown between the epithallus (Fig. 12D, upper bracket) and perithallus (Fig. 12D, lower bracket). Epithallial cells with thick,

heavily calcified cell walls and cylinder-shaped lumens. (Fig. 12E, arrows) that have a polygonal outline in surface view and an epithallial cell roof (Fig. 12E, circle arrow) that is missing from cells shown in cross section. In some locations, sections show cells exhibiting pseudodichotomous branching at the distal end of filaments of the perithallus, near the epithallus, with primary pit connections linked to a distal cell and a sub-lateral cell (Fig. 12F, arrows). Lumens of some perithallial cells with secondary mineralization (Fig. 12F, circle arrow).

***Lithothamnion* sp. G** Specimen LAF6548 is a biogenic rhodolith with numerous protuberances (Fig. 13A inset). Median periclinal sections of protuberances show overgrown conceptacles (Fig. 13A, arrows; \* denotes area magnified in Fig. 13C.) with remnant organic material (Fig. 13B, arrow). Sections show conceptacle overgrowth resulted from newly formed hypothallial filaments (Fig. 13C, arrow) that developed over the conceptacle roof and give rise to new perithallial filaments. Cross sections of protuberances show radial construction (Fig. 13D), with overgrown conceptacles showing some secondary mineral infill (Fig. 13D, arrow). Perithallus with abundant cell fusions (F) that link more than two adjacent filaments (Fig. 13E). Epithallial cells (Fig. 13F) with thick, heavily calcified cell walls and slightly trapezoidal shaped lumens (arrows) with a polygonal cell outline, varying between pentagonal and hexagonal. Section and surface views show intercalary meristematic cells subtend the epithallial cells (Fig. 13F, “M”).

***Lithothamnion* sp. H** Specimen LAF6970C is a small rhodolith with numerous protuberances (Fig. 14A). Median periclinal sections show protuberances composed of vegetative filaments interspersed by numerous overgrown conceptacles (Fig. 14B, black arrows). Sections show overgrowth is achieved by small rectangular cells that are cut off

from perithallial cells bordering the conceptacles and develop over the conceptacle roof (Fig. 14B, C, white arrow). Sections did not show conspicuous elongated perithallial cells bordering the conceptacles' periphery. Cross sections of protuberances show radial construction interspersed by overgrown conceptacles, with one conceptacle including an unidentified organism (Fig. 14D, Fig. 14D inset, white arrow) and others that become filled in with putative aragonite crystals (Fig. 14D, E, black arrows). Perithallus with numerous cell fusions (Fig. 14E, "F") in some locations linking more than two filaments. Sections show an intercalary meristem (Fig. 14G, arrow) between the epithallus (Fig. 14G, upper brackets) and perithallus (Fig. 14G, lower bracket) with meristematic cells (Fig. 14G, H, "M") and pairs of recently divided meristematic cells (Fig. 14G, H, \*). Section views (Fig. 14H, black bracket) and surface views (Fig. 14H, white brackets) show epithallial cells possess heavily calcified lateral and proximal cell walls, a very small round cell lumen, and a thin, weakly calcified epithallial cell roof. Section views reveal the round shaped lumen in part (Fig. 14H, arrows) and surface views of cells lacking the epithallial cell roof reveal the round shaped lumen (Fig. 14H, arrow, "L"). Surface views of cells with an intact epithallial cell roof show primary pit connections in the center of the roof (Fig. 14H, circle arrow, "P") appearing as a very small circular dark spot.

***Lithothamnion* sp. I** Specimen LAF6521 is a rhodolith with protuberances (Fig. 15A). Median periclinal sections show protuberances composed largely of vegetative filaments (Fig. 15B) with a single putative overgrown conceptacle (Fig. 15B, circle arrow). Sections show a new growth layer (Fig. 15B, arrow) over the older thallus surface. Newly formed multilayered hypothallus comprised of hypothallial filaments with rectangular shaped cells (Fig. 15C middle right bracket) that grow over the older layer of thallus (Fig. 15C lower right

bracket) and give rise to arching tiers of perithallial filaments (Fig. 15C upper right bracket). Surface views (Fig. 15C left bracket) and section views show epithallial cells (Fig. 15D) with heavily calcified lateral and proximal cell walls, a trapezoidal shaped cell lumen (Fig. 15D arrows), and a thinner, weakly calcified epithallial cell roof (Fig. 15D, white arrows). Sections show intercalary meristematic cells (Fig. 15D, “M”) between the perithallus and epithallus. Perithallus with abundant cell fusions (Fig. 15D) linking cells of adjacent filaments on all lateral sides, shown in the x-axis (black “F”) and z-axis (white “F”) of sections. Cross sections of protuberances (Fig. 15E, F) show radial construction and revealed the new growth layer (Fig. 15F, upper bracket, also shown in Fig. 15B, arrow) formed from filaments that “erupt” from the perithallus of the older growth layer (Fig. 15E, F arrow) and develop into a newly formed hypothallus. The newly formed hypothallus grows up and over the older part of the thallus and gives rise to newly formed arching tiers of perithallial filaments.

***Lithothamnion* sp. I** Specimen LAF1437 is a rhodolith with numerous protuberances (Fig. 16A). Median periclinal sections of protuberances show numerous overgrown conceptacles (Fig. 16A, inset, white arrows) with aragonite infill in the form of needle-like crystals (Figs. 16B, C, black arrows) and spherical masses (Fig. 16B, C, black circle arrows). Sections show conceptacles possess a single pore in the conceptacle roof (Fig. 16C, white circle arrow) indicating they are either male or female gametangial conceptacles. Newly formed hypothallus (Figs. 16C, D, white arrow, Fig. 16D, upper right bracket) develops from perithallial cells surrounding the conceptacle, and grows over the roof of old conceptacles (Fig. 16D, lower right bracket) giving rise to newly formed arching tiers of perithallial filaments (Fig. 16D, left bracket). Perithallus with numerous cell fusions (Figs. 16E, F, G,

“F”) and in some locations with abundant unidentified spherical inclusions (Fig. 16E). Sections show intercalary meristematic cells (Figs. 16F, G, “M”) between the perithallus (Fig. 16F, lower bracket, Fig. 16G, arc) and epithallus (Fig. 16F, upper bracket). Epithallial cells (Figs. 16F, G, H) with a trapezoidal shaped cell lumen (Figs. 16F, G white arrow, Fig. 7H, \*) and thick, heavily calcified cell walls, which are thickest at the proximal end of the lateral walls (Fig. 16H, horizontal arrows) and become thinner near the distal end (Fig. 16H, vertical arrows), and an epithallial cell roof (Figs. 16F, G, H, circle arrow) which is sometimes missing in sections (Figs. 16F, G, white arrows).

***Lithothamnion* sp. I** Specimen SPF57882 is a rhodolith with numerous protuberances (Fig. 17A). Median periclinal sections show protuberances composed largely of vegetative filaments (Fig. 17B) with radial construction. Sections show a new growth layer (Figs. 17B, C, D, white brackets) over the surface of the older portion of the protuberance (Figs. 17B, C, D, black brackets). Newly formed hypothallus with multiple layers of hypothallial filaments comprised of rectangular shaped cells (Figs. 17C, D, arrow), apparently cut off from cells of the perithallus in the older growth layer, develops over the surface of the older growth layer and gives rise to newly formed arching tiers of perithallial filaments. Perithallus with abundant cell fusions (Fig. 17E, “F”) fusing cells of adjacent filaments from all lateral sides, shown in the x-axis (black “F”) and z-axis (white “F”) of sections. Sections of protuberances show the perithallus consists of filaments with alternating layers of cells with thick, heavily calcified cell walls (Fig. 17F, lower bracket) and cells with thin, weakly calcified cell walls (Fig. 17F, upper bracket). Epithallial cells (Fig. 17G, bracket) with heavily calcified lateral and proximal cell walls, a trapezoidal shaped cell lumen (Fig. 17G arrows), and a thinner, weakly calcified epithallial cell roof (Fig. 17G, circle arrows). Sections show recently

divided meristematic cells subtending the epithallial cells in some locations (Fig. 17G, \*). Epithallial cells remain intact (Fig. 17H, arrow) upon being buried by newly formed hypothallial filaments, which appear round or square in cross section (Fig. 17H, bracket). Sections showed several putative buried conceptacles lacking aragonite infill (data not shown) but no diagnostic characters were evident to indicate if conceptacles were gametangial or tetrasporangial.

***Lithothamnion* sp. J** Specimen PHYKOS7249 is a rhodolith with numerous protuberances (Fig. 18A). Median periclinal sections of protuberances show overgrown conceptacles (Fig. 18B arrows), which frequently become filled in with aragonite crystals (Fig. 18B perforated arrows). Newly formed hypothallus (Fig. 18C right lower bracket) develops over old conceptacle roofs (Fig. 18C left bracket) and gives rise to the next layer of perithallus (Fig. 18C upper right bracket). Sections show conceptacles are multiporate, indicated by the presence of pores within conceptacle roofs (Fig. 18C arrows) and are interpreted as tetrasporangial conceptacles. Intercalary meristematic cells were identified (Fig. 18D “M”), as well as recently divided meristematic cells (Fig. 18D, black outline). Perithallus with abundant cell fusions, in some locations linking cells of two or more adjacent filaments (Fig. 18D, “F”, circle arrow). Epithallial cells (Fig. 18D,E) with a trapezoidal shaped lumen (Fig. 18D, white arrows). In some locations sections show recently divided epithallial cells (Fig. 18E) with very little space between the epithallial cell roof (Fig. 18E, circle arrows) and the proximal epithallial cell wall (arrows). Sections show aragonite crystals (Fig. 18F, G) infill empty conceptacles and grow from the roof and floor (Fig. 18F, arrows). Aragonite crystals have two forms; spherical masses, appearing fan-shaped in cross section (Fig. 18F arrows) and needle-like crystals with a hexagonal outline (Fig. 18G, arrow).

## Discussion

We show the likely polyphyly of the genus *Lithothamnion* as recently demonstrated by Peña *et al.* (2014), and identify additional species that were previously unreported for the northern GMx, and more broadly the WTA. Our extensive SEM investigation of specimens spanning the two clades of *Lithothamnion* spp. (i.e. sp. A, B, C, D vs. sp. E, F, G, H, I, J) showed that both demonstrated characteristic generic features of the genus, but also lacked obvious distinguishing morpho-anatomical characters that would support their segregation into two genera. In light of these results, it is currently impossible to provide a taxonomic solution by assigning *Lithothamnion sensu stricto*, nor a new genus name, to any of the lineages depicted on the tree until DNA sequencing of the generitype specimen of *L. muelleri* is performed, or less preferably, conducted on samples collected at or near the type locality in South Australia.

Currently, only two DNA sequences (one *psbA* and one SSU) are available from a single specimen conforming to the morphological concept of *L. muelleri* (collected from Isla San José, Baja California). DNA sequences generated from the lectotype specimen of *L. muelleri*, housed at CN (Woelkerling 1983), may help clarify the relationship between this southern hemisphere specimen and the northern hemisphere specimen identified as *L. muelleri*, and may also shed light on the relationships between other southern and northern hemisphere species of *Lithothamnion*.

The results of the UPA analysis (Fig. 1) show that none of the newly generated sequences (*Lithothamnion* spp. A-J) in this study are closely related to the rhodolith-forming species *Mesophyllum erubescens* (Foslie) Me. Lemoine (type locality: Fernando de Noronha

Archipelago, Brazil). Interestingly, the recently published sequences of two unidentified Hapalidiaceae specimens, FLOR14925 and FLOR14926 from Brazil (Sissini *et al.* 2014) are nested within the clade comprised of *Lithothamnion* sp. A, B, C, D in both the UPA tree and multi-gene tree (Fig. 4), though no further comparisons to these specimens could be made due to a lack of other corresponding molecular markers and morphological descriptions.

The results of the *psbA* analysis (Fig. 2) indicate that the lineage harboring *Lithothamnion* spp. A, B, C, and D represents a well-supported lineage separate from other *Lithothamnion* spp. Included in this lineage are the specimens collected from subtidal depths (5-50m) offshore Guadeloupe, F.W.I investigated in Peña *et al.* (2014) and identified based on morphology as *Lithothamnion* spp. (tentatively assigned as Species 1, 2, and 3), *L. cf. ruptile*, as well as *L. cf. crispatum* collected from the Mediterranean Sea. Peña *et al.* (2014) showed in their *psbA* analysis that these specimens comprised a strongly supported lineage separate from the lineage comprised of *L. muellerii*, *L. glaciale*, and *L. coralloides*, and noted obtaining DNA sequences from the type specimens of *L. ruptile*, *L. crispatum*, and *L. occidentale* may aid in clarifying relationships between this lineage and other *Lithothamnion* spp. Interestingly, sequences of specimens identified as *Mesophyllum* sp. 1 and 2, from subtidal depths in the vicinity of Macaronesia (20 and 15m, respectively) investigated by Pardo *et al.* (2014) are also nested within this lineage and comprise a clade with *Lithothamnion* spp. (Species 1, 2, and 3) from Guadeloupe. Pardo *et al.* (2014) reported that these specimens shared morphological characters with *Lithothamnion* spp. but concluded that the specimens belonged in *Mesophyllum* based on the results of the molecular analyses, which indicated that these specimens were sister to *Mesophyllum sphaericum* Peña, Bárbara, Adey, Riosmena-Rodríguez et Choi with strong support. An unidentified Hapalidiaceae

specimen from New Caledonia is also nested within this lineage, which is sister to *Lithothamnion* sp. D, specimen LAF6631, from Pacific Panama, indicating that this lineage has a wide distribution. The *psbA* sequences of this specimen LBC0640 shows a high similarity with the sequence of *Lithothamnion* sp. D (98.7%). ABGD analyses indicated these specimens represent separate species while GMYC analyses delimited the specimens as a single species. Future collections in the tropical Pacific may aid in clarifying the relationship between these two specimens. The results of the concatenated ML analysis (Fig. 4) support that the lineage harboring *Lithothamnion* spp. A, B, C, and D represents a lineage separate from other *Lithothamnion* spp. (E-I).

With a larger sample size, including the recently published sequences shown by Sissini *et al.* (2014) to correspond to the holotype of *M. erubescens* (Foslie) Me. Lemoine 1928, we show here that *M. sphaericum* is sister to *M. erubescens* with strong support in the *psbA* tree (PP=0.99, BS=92%) (Fig. 3) and in the multi-gene tree (BS=98%) (Fig. 4). The *Clathromorphum*-complex comprised a lineage with full support. The branching order of *Mesophyllum sphaericum*, *M. erubescens*, and *M. lichenoides*, as well as specimens identified as *Synarthrophyton patena*, was unresolved (i.e. a polytomy) in the *psbA* tree (Fig. 2). In the COI tree (Fig. 3) *M. lichenoides* was sister to *M. sphaericum* and *M. erubescens* with strong support in the Bayesian analysis (PP=0.97), though this node was not supported in the ML analysis (BS <50%).

*Lithothamnion* spp. E and F show a close relationship with *Lithothamnion* spp. LBC0642 in both the *psbA* and COI trees (Figs. 2 and 3) and comprise a lineage that is well supported in both Bayesian (*psbA* PP=.98, COI PP= .94) and ML analyses (*psbA*, BS=80%, COI, BS=94%). In the *psbA* tree this lineage comprises a clade at the base of the

Hapalidiales. However, the branching pattern is quite different in the COI tree, with this clade comprising a lineage sister to *L. corallioides*. Taking into account the results of the pairwise distance distribution analysis (Fig. 5), this may be a result of *psbA* being more conserved than COI, considering studies in other taxonomic groups have shown conserved genes lead to paraphyletic relationships and unresolved phylogenies (Maia *et al.* 2012, Givnish *et al.* 2011). The close relationship between *Lithothamnion* sp. G and *L. corallioides* is intriguing and *psbA* sequences of *L. corallioides* varied from the *psbA* sequence of *Lithothamnion* sp. G by only 2.1%. *Lithothamnion* sp. G and *L. corallioides* are nested within a larger group of *Lithothamnion* spp., including *L. glaciale* Kjellman, the *psbA* sequences of which have been corroborated by comparisons to the holotype specimen (Adey *et al.* 2015).

The COI tree revealed that the COI sequence of *Lithothamnion* sp. H (LAF6970C), collected from 69m depth in the SEGMx, is 100% identical to the COI sequence of a specimen (“Species 5 PC0144250”) collected from a deep depth (110m) offshore Guadeloupe, F.W.I investigated in Peña *et al.* (2014). In the COI tree (Fig. 3) and concatenated gene tree (Fig. 4) these two specimens are sister to other *Lithothamnion* spp., including *L. glaciale* and *L. corallioides*.

The close relationship between specimens of *Lithothamnion* sp. I and J and specimen E58 is noteworthy, as this lineage received full support in both Bayesian and ML analyses. Though UPA sequences of specimens of *Lithothamnion* sp. I from the GMx and Brazil were identical (Fig. 1, Table S1), considerable distance was observed in both the *psbA* and COI datasets (Figs. 2, 3, Tables S2, 3). *PsbA* and COI sequences of specimens from the GMx varied from the specimens from Brazil by 0.35-0.58% and 2.6% respectively. ABGD analyses for both *psbA* and COI delimited the specimens from the GMx and Brazil as being

conspecific whereas GMYC analyses for both markers delimited the specimens from the GMx as being a species separate from the specimens from Brazil. These results indicate that the specimens comprising *Lithothamnion* sp. I may represent a single species, separate species or separate entities below the rank of species, though additional samples from other localities are needed to examine the range of DNA sequence variation in this clade and neighboring clades. The close relationship between *Lithothamnion* sp. J and the unidentified Hapalidiaceae sp. E58 (Hernández-Kantún *et al.* 2015) is intriguing considering Hernández-Kantún *et al.* (2015) showed that the specimen E58 possessed “flared” epithallial cells. *Phymatolithon calcareum* (Pallas) Adey & McKibbin recently has been clarified by DNA comparisons with its neotype specimen (Peña *et al.* 2014a, Hernández-Kantún *et al.* 2015, Woelkerling & Irvine, 1986). The close relationship between the sequences of specimens comprising *Lithothamnion* spp. I, J, Hapalidiaceae sp. E58 and *Phymatolithon calcareum* - the generitype species of *Phymatolithon* Foslie (Woelkerling & Irvine, 1986) - indicates further studies of these taxa are necessary to resolve the evolutionary relationships between these two genera. Results of the *psbA* analysis (Fig. 2) are similar to the results of the *psbA* analysis presented by Hernández-Kantún *et al.* (2015) (see fig. 2. p. 53) with regards to the relationship between *Lithothamnion* spp., *Phymatolithon calcareum*, and Hapalidiaceae sp. E58.

Results of the phylogenetic and barcoding analyses, as well as the ABGD and GMYC species delimitation analyses, indicate that the newly generated sequences in this study represent at least ten species, with eight being present in the GMx. These results show that there are more species corresponding to the genus *Lithothamnion* than there are available names for this genus in the GMx. *Lithothamnion* sp. C, G, F, and I, which are all collected

from deepwater, unlikely conform to the species concept of *L. occidentale* (Foslie) Foslie, described from Cruz Bay, St. John Island, US Virgin Islands, and reported for the Gulf of Mexico (Dawes 1974, Fredericq *et al.* 2009, Mateo Cid *et al.* 2014). Images of the holotype specimen fragment of *L. occidentale* (sweetgum.nybg.org) show that the specimen possess a different habit, which does not have a spherical/round shape with many protuberances growing from all sides of this specimen, and instead appears to consist of two branched branches which appear to be growing erect and attached to a central branch. DNA sequencing needs to be performed on the type specimen of *L. occidentale* and the morphology of the specimen needs to be examined carefully before this species epithet can be assigned with confidence to any of the specimens examined.

*L. crispatum*, reported by Mateo-Cid *et al.* (2014) from the Atlantic Coast of Mexico in the SWGMx, may correspond to *Lithothamnion* spp. F, I, and J in this study. The habit of *Lithothamnion* sp. F (LAF6882) from Campeche Banks, Mexico, is similar in habit to specimens identified as *L. crispatum* studied by Mateo-Cid *et al.* (2014), including specimens collected on the same expedition to Campeche Banks. However, the *psbA* sequence of a specimen collected in the Mediterranean Sea, Spain, identified as *L. cf. crispatum* (Peña *et al.* 2014) and the *psbA* sequences of the taxon identified as *L. crispatum* (Nelson *et al.* 2014, Smith *et al.* 2012) appear distantly related and are not conspecific with any of the newly sequenced taxa presented in this study. DNA sequencing of the lectotype specimen of *L. crispatum*, originally collected from the Adriatic Sea and housed in the Rijksherbarium (L) in Leiden, Netherlands (Basso *et al.* 2011), needs to be performed before this name can be applied with confidence to specimens from the GMx.

Minnery (1990) reported three species of *Lithothamnion* for the Flower Garden Banks, including two unnamed species and one reported as '*L. sejunctum?*'. *Lithothamnion sejunctum* (type locality: U.S. Virgin Islands), reported by Taylor (1960) to be epilithic and encrusting, may correspond to *Lithothamnion* spp. A, B, or E. *Lithothamnion* sp. B, specimen LAF6957B from Sackett Bank, possesses lobed, white margins, a character reported for *L. sejunctum* (Taylor, 1960), but it also has a loosely adherent crust, which is in contrast to Taylor's description of *L. sejunctum* as "strongly adherent" (Taylor 1960, p. 381). *Lithothamnion* spp. A, E possess strongly adherent crusts but do not possess lobed margins. DNA sequencing of the type specimen of *L. sejunctum* collected by Børgesen from St. Croix, U.S. Virgin Islands, and housed at TRH (Woelkerling *et al.* 2005), will help clarify if this species corresponds to specimens in the current study.

*Lithothamnion ruptile* (Foslie) Foslie, whose type locality is the Dominican Republic (Woelkerling *et al.* 2005) and which is also reported for the Virgin Islands is described as having an encrusting morphology (Taylor 1960). This species may correspond to the encrusting specimens *Lithothamnion* spp. A, B, or E. Interestingly, the specimen identified as *L. cf. ruptile* is closely related to *Lithothamnion* sp. A, and included in this lineage is *Lithothamnion* sp. B. However, both ABGD and GMYC analyses indicate that the *psbA* sequence of *L. cf. ruptile* is not conspecific with *Lithothamnion* sp. A or B, nor is it conspecific with any other taxon in the *psbA* tree. DNA sequencing of the holotype specimen of *L. ruptile* (basionym: *Lithothamnion syntrophicum* f. *ruptile*), collected by Bock and housed at TRH (Woelkerling *et al.* 2005), may help clarify if any of the specimens in the current study correspond to *L. ruptile*.

More recently, Athanasiadis & Ballantine (2011) described a new shallow water species of *Lithothamnion* from the Caribbean, *L. carpoklonium*. This species, which possesses a crustose morphology and also bears protuberances, is similar in habit to *Lithothamnion* sp. B. However, *L. carpoklonium* was discovered growing on mangrove prop roots, whereas *Lithothamnion* sp. B was found encrusting a bivalve shell from deepwater. Peña *et al.* (2014) noted that DNA sequencing of the holotype specimen of *L. carpoklonium* may help clarify the application of species names in the Caribbean. The holotype collection of *L. carpoklonium* is a contemporary collection housed at MSM and includes air-dried tetrasporangial and gametangial specimens (Athanasiadis & Ballantine 2011). Sequencing, and sequences generated from these specimens may help clarify if this specific epithet can be applied to specimens from the GMx.

The close relationship between specimens from the GMx and the specimens from Brazil (*Lithothamnion* sp. I, *Lithothamnion* sp. A and FLOR14925/FLOR14926) indicates that names applied to taxa from areas in or near Brazilian waters should also be considered for the specimens collected throughout the GMx. Surface views of the epithallial cells and of multiporate conceptacles (which show depressions around each pore) of *Lithothamnion* sp. A (LAF6549) are similar to the SEM images of a specimen identified as *L. crispatum* from the northeastern coast of Brazil (Costa *et al.* 2014, see p. 146, figs. 9D, G, H), and surface views of the epithallial cells are also similar to SEM images of a specimen identified as *L. brasiliense* Foslie (Costa *et al.* 2014, p. 148 fig. 10C). DNA sequencing of *L. brasiliense*, collected from São Sebastiao, Brazil and housed at TRH (Woelkerling *et al.* 2005) may help clarify if this species is related to taxa from the GMx. Mariath *et al.* (2012) recently described a protuberant crustose species from Bahia, Brazil which grows in shallow water on

corals, *Lithothamnion steneckii* Mariath and Figueiredo. This specimen also shows the habit of *Lithothamnion* sp. B. DNA sequencing of the holotype specimen of *L. steneckii* is not ideal due formalin preservation (Mariath *et al.* 2012); however, DNA sequencing of topotype collections may be an informative alternative.

Terminology referring to the type of epithallial cells considered diagnostic of the genus *Lithothamnion* has been used inconsistently in coralline literature. These cells have been referred to as being “angular” (Woelkerling 1983, Johansen 1976), “flattened and flared” (Woelkerling, 1988), “eared” (Johansen, 1976, Johansen 1981) and “armored” (Adey *et al.* 2015). Adey (1966, p. 364) presented excellent illustrations of *Lithothamnion* (as *Lithothamnium*) epithallial cells and described that the uppermost portion of the lateral cell walls “tend to flare-out” (Adey, 1966 p. 329). The term “flared” appears to be used most widely in modern coralline literature (Peña *et al.* 2014, Oliveira-Costa *et al.* 2014, Mariath *et al.* 2012, Robinson *et al.* 2013) though not exclusively (Adey *et al.* 2015). Using transmission electron microscopy, Wegeberg & Pueschel (2002) corroborated the work of Adey (1966) showing that the “flared” appearance of epithallial cells in *Lithothamnion* spp. is the result of the trapezoidal shape of the epithallial cell lumen (p. 230-36, figs.1-13), formed in part by the very thick lateral and proximal cell walls, the lateral walls becoming thinner near the distal end of the cell. Wegeberg & Pueschel (2002) also showed that the epithallial cell roof possesses a primary pit connection and consists of a cross wall with two layers, one being the layer of the distal end of the intact epithallial cell and the other layer being a remnant of the proximal end of the previously sloughed epithallial cell.

Using SEM and illustrations based on light microscopy observations, Adey *et al.* (2005) documented epithallial cell morphology in three species of *Lithothamnion*, showing

high magnification images and providing excellent illustrations of *L. tophiforme*, *L. lemoineae*, and *L. glaciale*. The epithallial cell roof is sometimes absent in published SEM images of *Lithothamnion* spp. (Adey *et al.* 2005, p.1016, fig. 7C, Robinson *et al.* 2013 p.66 fig. 3B, left arrowhead) but may remain intact (Robinson *et al.* 2013 p.66 fig. 3B, right, arrowhead) or remain intact but collapses into the cell lumen (Basso *et al.* 2011, p. 148, fig. 12). Adey recently avoided using the term flared in morphological descriptions, and described the epithallial cells as “armored” (with heavy calcification and thick walls). Adey *et al.* (2005) also showed that epithallial cells of *Lithothamnion glaciale* do not show a pronounced trapezoidal shape in sections (p. 1014, fig. 4).

Surface and section views of epithallial cells of *Lithothamnion* sp. A (LAF6549), *Lithothamnion* sp. F (LAF6882), and *Lithothamnion* sp. H (LAF6548) resemble SEM images and illustrations of *L. glaciale* (Adey *et al.* 2005) and surface and section views *Lithothamnion* sp. G (LAF6548) resembles images and illustrations of *L. lemoineae* (Adey *et al.* 2005). Images of epithallial cells of *Lithothamnion* sp. C, I, and J correspond to decalcified sections of the lectotype of *Lithothamnion crispatum* (Basso *et al.* 2011) viewed with light microscopy and resemble SEM images of epithallial cells of specimens identified as *L. crispatum* by Basso *et al.* (2011). *Lithothamnion* sp. B, C, I, and J are also similar to images and illustrations of *L. tophiforme* (Adey *et al.* 2005). Images of *Lithothamnion* sp. D (Fig. 10) indicate epithallial cells are very short with a roof that collapses into the lumen of the cell. Desiccation during preservation in silica gel may play a role in the collapsing of the epithallial cell roof. Epithallial cells in *Lithothamnion* sp. D also appear to be covered in mucilage or a cuticle. Other groups of coralline algae have been reported to have epithallial cells that “secrete cuticular material” (Johansen 1981, p. 31). Completely intact epithallial

cells in *Lithothamnion* sp. E appear to be absent, with images showing only the calcified proximal cell walls with some portions of the lateral walls remaining and with primary pit connections in the center. Images of epithallial cells of *Lithothamnion* sp. H (LAF6970C) are quite unique (Fig. 14), showing epithallial cells possess proximal and lateral walls which are very heavily calcified, accounting for more than half of the volume of the cell. Cell lumens are small and round and epithallial cell roofs are thin and weakly calcified with a primary pit connection in the center. Images of this species are shown in Peña *et al.* (2014, p. 206 fig. 9D) and the epithallial cells are reported as being “flared” in the figure legend (p. 205) but further described as having “somewhat flared outermost cell walls” (p. 205). The images lack proper contrast to show the primary pit connections in the epithallial cell roofs, however, the small circular epithallial cell roof can be seen in surface view and a single cell (second from the right, left of the cell indicated by the arrow) shows the small round lumen possessed by this species. The newly generated images of this species presented in the study indicate that referring to these unique epithallial cells as “flared” does not adequately describe their structure.

Krayesky-Self *et al.* (2016) analyzed the elemental composition of three specimens investigated in this study. Images of specimens LAF1437, *Lithothamnion* sp. I (Fig. 16) and PHYKOS7249, *Lithothamnion* sp. J (Fig. 18) illustrate the infill of needle-like crystals in empty, overgrown conceptacles, which was confirmed with analyses of SEM-EDS (energy dispersive spectrometry) and x-ray diffraction data by Krayesky-Self *et al.* (2016) to be aragonite. Alexandersson (1974) documented two forms of “aragonite cement” (i.e. aragonite infill) in empty conceptacles of specimens identified as *Lithothamnion glaciale* from the North Sea and referred to these forms as “aragonite needles” (see Alexandersson 1974, fig.

7A, 8D-F) and “aragonite spherulitic cement” (see Alexandersson 1974, fig. 7C). Specimen PHYKOS 7249 possesses both of these forms of aragonite (Figs. 6F-H), including a spectrum of sizes and morphology of aragonite needles which correspond to the sizes and descriptions documented by Alexandersson. Alexandersson also documented the presence of small, centripetally formed calcium carbonate crystals in individual cells of *Lithothamnion*. Likewise, such small, centripetally formed calcium carbonate crystals of uncertain elemental composition were also observed within individual cells (Figs. 8, 17). Secondary mineral infill appears to be a common occurrence in specimens from the GMx, and putative aragonite infill is shown in several other specimens in the present study (Figs. 7, 8, 9, 12, 14). This phenomenon has been speculated to be associated with the development of newly formed hypothallial filaments which grow over empty conceptacles (Krayesky-Self *et al.* 2016), which is a common development pattern in specimens investigated in the present study. Alexanderson (1974) speculated that formation of mineral infill in *Lithothamnion* specimens from the North Sea is associated with the metabolic activity of the living coralline alga and noted that dissolution of minerals within the thallus occurs if the living outer layer of the rhodolith dies.

*L. carpoklonium* also has protuberances comprised of overlapping conceptacles. However, the structure of protuberances in *L. carpoklonium*, which are comprised of newly formed conceptacles directly overlapping the conceptacles below with little vegetative growth in between (see p. 408, figs. 20-21, Athanasiadis & Ballantine, 2011), appears to differ from specimens from the GMx.

Krayesky-Self *et al.* (2016) also determined that no significantly detectable amount of aragonite was present in the thallus of LAF6521. Here, we show that the lack of aragonite

corresponds with a lack of empty, overgrown conceptacles embedded within the protuberances, and that protuberances consisted primarily of vegetative filaments. Specimen LAF6521, which was collected on 16.xi.2012, may have been among those rhodoliths living in the Gulf during and in the aftermath of the Deepwater Horizon oil spill event. Considering the fact that many rhodolith specimens observed in the field following the Deepwater Horizon oil spill were bleached and denuded of associated macroalgae (Fredericq *et al.* 2014, Felder *et al.* 2014), it can be speculated that this specimen may have been bleached after the spill as well. The development of the new growth layer “erupting” out of the perithallus of the older growth layer, which apparently began below the intercalary meristem, may represent a strategy that allows for vegetative regeneration following a natural disaster and bleaching of the intercalary meristem. However, as this phenomenon was observed only in this specimen, this is only speculative at this point.

The morphology of the intercalary meristematic cells has been reported to be one of the defining characters of the genus *Lithothamnion*, and is reported to be “as long or longer than their immediate inward derivatives” (Woelkerling 1988, p. 171). In this study, cells confidently identified as meristematic cells were indeed usually as long or longer than the inward perithallial cells (Figs. 9, 12, 13, 14, 15, 16, 18). In some locations of certain specimens, cells that were proximal to surface cells appeared to be pairs of recently divided meristematic cells (Figs. 9, 10, 14, 17, 18), prior to elongation of either resulting cell. In other specimens meristematic cells could not be identified with confidence (Figs. 6, 7, 11).

Peña *et al.* (2014) reported that specimens investigated in their study of Caribbean specimens identified as *Lithothamnion* spp. (Fig. 2, 3) did not have meristematic cells that fit the description of being “as long or longer than their inward derivatives” and that the putative

meristematic cells were often shorter than the inward derivatives. Peña *et al.* (2014) attributed this to a possible “active cell division process in the subepithallial initials” (i.e. meristematic cells). Meristematic cells which are approximately the same size as the inward derivatives were also reported by Athanasiadis & Ballantine (2011) in *L. carpoklonium*. Intercalary meristematic cells are thought to divide in one of two ways; the meristematic cell can divide to form a proximal cell of the perithallus, with the resulting distal cell remaining meristematic, or the meristematic cell can divide to form a small epithallial cell distally, with the proximal cell remaining meristematic (Johansen 1981). However, due to the inherent difficulties of conducting microscopy on calcified specimens and the differences in the resulting images of calcified and decalcified specimens, and also considering the current lack of method for viewing cell division in real time, it could be speculated that different meristematic developmental patterns may occur and have gone unreported. Perhaps meristematic cells may divide, with the proximal cell remaining meristematic, while the distal cell also remains meristematic, dividing again without elongation, forming very small, flattened epithallial cells such as the cells illustrated in the current study (Figs. 7, 9, 10) and in Peña *et al.* (2014). Apart from members of the Corallinophycidae, the only other red algal group which possesses an intercalary meristem is the family Delesseriaceae (Ceramiales). Members of the Delesseriaceae possess sophisticated development at sites of intercalary meristematic activity, sometimes involving complex branching patterns which vary between groups (Jeong *et al.* 2016, Lin *et al.* 2015, Wynne 2014). Complex intercalary meristematic activity may also be possible in the Corallinophycidae and intercalary branching could be involved. Integrated studies involving multiple types of microscopy conducted on both

calcified and uncalcified specimens may elucidate the developmental pattern of meristematic activity in the Corallinophycidae.

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**Table 1. Collection data of voucher specimens and GenBank accession numbers for newly generated sequences and sequences of referenced studies included in the analyses of this study. Newly generated sequences shown in boldface. \*Not analyzed in present study.**

Id. No.	Taxa	Locality	Collector/ Reference	GenBank Accession No.		
				UPA	psbA	CO1
LAF6547 (11-16-12)	<i>Lithothamnion</i> sp. A (Hapalid. sp. A)	Ewing Bank, NWGMx 28° 5.936'N; 91° 2.112'W 55-58 meters	J. Richards 16.xi.2012, collected from site 18.ii.2013, collected from microcosm	<b>KU514425</b>	<b>KU557496</b>	-
LAF6549 (11-16-12)	<i>Lithothamnion</i> sp. A (Hapalid. sp. A)	Ewing Bank, NWGMx 28° 5.936'N; 91° 2.112'W 55-58 meters	J. Richards 16.xi.2012, collected from site 18.ii.2013, collected from microcosm	<b>KU514426</b>	<b>KU557497</b>	<b>KU514420</b>
LAF6957B (9-7-14-1-3)	<i>Lithothamnion</i> sp. B (Hapalid. sp. B)	Sackett Bank, NWGMx 28° 38.0'N; 89° 33.028'W 65-68m	J. Richards S. Fredericq 7.ix.2014	<b>KU514429</b>	<b>KU557501</b>	-
LAF6820	<i>Lithothamnion</i> sp. C (Hapalid. sp. C)	Ewing Bank, NWGMx 28° 05.041'N; 91° 01.648'W 70-75m	J. Richards 19.x.2013, collected from site 10.xii.2013, collected from microcosm	<b>KU514427</b>	<b>KU557498</b>	-

Cont. Tab. 1

LAF6956B (9-7-14-1-2)	<i>Lithothamnion</i> sp. C (Hapalid. sp. C)	Sackett Bank, NWGMx 28° 38.0'N; 89° 33.028'W 65-68m	J. Richards S. Fredericq 7.ix.2014	<b>KU514428</b>	<b>KU557499</b>	-
LAF6631	<i>Lithothamnion</i> sp. D (Hapalid. sp. D)	Pacific Panama Gulf of Chiriquí 7° 28' N; 81° 14' W, 16m	W.E. Schmidt 11.v.2013	<b>KU519740</b>	<b>KU557500</b>	-
LAF5421 (8-29-11-4-1)	<i>Lithothamnion</i> sp. E (Hapalid. sp. E)	Ewing Bank, NWGMx 28° 06.066'N; 91° 02.146'W 58-91m	J. Richards 29.viii.2011, collected from site 23.vii.2012, collected from microcosm	-	<b>KU557493</b>	<b>KU514417</b>
LAF6494	<i>Lithothamnion</i> sp. E (Hapalid. sp. E)	Sackett Bank, NWGMx 28 38.012'N 89 33.587'W 60-90m	J. Richards 24.viii.2012, collected from site viii.2012, collected from microcosm	<b>KU514423</b>	<b>KU557494</b>	<b>KU514418</b>
LAF6882 (NSFII-26-3)	<i>Lithothamnion</i> sp. F (Hapalid. sp. F)	Campeche Banks, SWGMr 21° 04.41'N; 92° 08.05'W 50m	S. Fredericq 9.vi.2005	<b>KU514424</b>	<b>KU557495</b>	<b>KU514419</b>
LAF6548 (8-26-12-3)	<i>Lithothamnion</i> sp. G (Hapalid. sp. G)	Ewing Bank, NWGMx 28° 06.00'N;	J. Richards 26.viii.2012	<b>KU514421</b>	<b>KU557492</b>	-

Cont. Tab. 1

		91°02.21'W 53-54m				
LAF6970C (9-10-14-22-2)	<i>Lithothamnion</i> sp. H (Hapalid. sp. H) ("Species 5" <i>sensu</i> Pena <i>et al.</i> 2014)	Dry Tortugas Vicinity, SWGMx 24°31.494'N; 83°19.793'W 69m	J. Richards S. Fredericq 10.ix.2014	<b>KU514422</b>	-	<b>KU514416</b>
LAF6521 (11-16-12)	<i>Lithothamnion</i> sp. I (Hapalid. sp. I)	Ewing Bank, NWGMx 28°05.845'N; 91°01.817'W, 54-58m	J. L. Richards 16.xi.2012 (Krayesky-Self <i>et al.</i> 2015)	<b>KU504274</b>	KP844864	<b>KU504276</b>
LAF1437A (7-5-06-2-2)	<i>Lithothamnion</i> sp. I (Hapalid. sp. I)	Florida Middle Grounds, NEGMx 28°10.27'N; 84°02.07'W, 42-43m	S. Fredericq 5.vii.2006 (Krayesky-Self <i>et al.</i> 2015)	<b>KU504273</b>	KP844863	-
SPF57882 (IBC1704)	<i>Lithothamnion</i> sp. I (Hapalid. sp. I)	Banco da Panela Salvador, Bahia, Brazil 10-15m	T. Vieira-Pinto, C. Azevedo, B. Torrano-Silva, M. Jamas 22.v.2013	<b>KU519741</b>	<b>KU529477</b>	-
SPF57883 (IBC1708)	<i>Lithothamnion</i> sp. I (Hapalid. sp. I)	Banco da Panela Salvador, Bahia, Brazil 10-15m	T. Vieira-Pinto, C. Azevedo, B. Torrano-Silva, M. Jamas 22.v.2013	-	<b>KU529478</b>	-
SPF57884 (IBC1907)	<i>Lithothamnion</i> sp. I (Hapalid. sp. I)	Cabeço do arrastado Fortaleza, Ceará, Brazil	R. M. Araújo & G. O. Longo 19.iv.2012	-	<b>KU529479</b>	<b>KU529476</b>

Cont. Tab. 1

		Subtidal, >10m				
PHYKOS7249	<i>Lithothamnion</i> sp. J (Hapalid. sp. J)	Pacific Panama 7° 28' N; 81° 14' W, 16m	W. E. Schmidt 11.v.2013 (Krayesky-Self <i>et al.</i> 2015)	<b>KU504275</b>	KP844865	<b>KU504277</b>
NCU 588631	<i>Callolithophytum parcum</i>	Washington, U.S.A.	Adey <i>et al.</i> 2015	-	KP142742	-
US 169083	<i>Clathromorphum circumscriptum</i>	Labrador, Canada	Adey <i>et al.</i> 2015	-	KP142731	-
US 170929	<i>Clathromorphum compactum</i>	Labrador, Canada	Adey <i>et al.</i> 2015	-	KP142730	-
US 170930	<i>Clathromorphum compactum</i>	Newfoundland Canada	Adey <i>et al.</i> 2015	-	KP142729	-
NCU 601308	<i>Clathromorphum compactum</i>	Maine, U.S.A.	Adey <i>et al.</i> 2015	-	KP142757	-
NCU 627718	<i>Clathromorphum nereostratum</i>	Commander Islands, Russia	Adey <i>et al.</i> 2015	-	KP142758	-
NCU 597128	<i>Clathromorphum nereostratum</i>	Alaska, U.S.A.	Adey <i>et al.</i> 2015	-	KP142759	-
US 170931	<i>Clathromorphum nereostratum</i>	Alaska, U.S.A.	Adey <i>et al.</i> 2015	-	KP142733	-
NCU 627106	<i>Clathromorphum nereostratum</i>	Alaska, U.S.A.	Adey <i>et al.</i> 2015	-	KP142760	-
NCU 627110	<i>Clathromorphum nereostratum</i>	Alaska, U.S.A.	Adey <i>et al.</i> 2015	-	KP142761	-

Cont. Tab. 1

NCU 597127	<i>Heydrichia woelkerlingii</i>	Cape Province, South Africa	Mateo-Cid <i>et al.</i> 2014b	-	JQ917415	-
US 169189	" <i>Leptophytum fecundum</i> "	Labrador, Canada	Adey <i>et al.</i> 2015	-	KP142726	-
US 169242	<i>Leptophytum laeve</i>	Labrador, Canada	Adey <i>et al.</i> 2015	-	KP142735	-
FRA1993	<i>Lithothamnion ruptile</i> cf.	Guadeloupe, F.W.I.	Peña <i>et al.</i> 2014	-	KJ710353	-
GALW15750	<i>Lithothamnion corallioides</i>	Bay of Brest, France 6.2 m	Hernández-Kantún <i>et al.</i> 2014	-	JQ896234	-
CPVP-563	<i>Lithothamnion corallioides</i>	Galicia, Spain	Pardo <i>et al.</i> 2014	-	KC819256	KC861460
CPVP-691	<i>Lithothamnion corallioides</i>	Galicia, Spain	Pardo <i>et al.</i> 2014	-	KC819261	*KC861467
CPVP-802	<i>Lithothamnion corallioides</i>	Galicia, Spain	Pardo <i>et al.</i> 2014	-	-	KC861447
CPVP-808	<i>Lithothamnion corallioides</i>	Brittany, France	Pardo <i>et al.</i> 2014	-	KC819264	*KC861452
CPVP-817	<i>Lithothamnion corallioides</i>	Brittany, France	Pardo <i>et al.</i> 2014	-	KC819265	KC861448
CPVP-1167	<i>Lithothamnion corallioides</i>	Wales, United Kingdom	Pardo <i>et al.</i> 2014	-	-	KC861487
NZC2315	<i>Lithothamnion crispatum</i>	North Island, New Zealand	Nelson <i>et al.</i> 2015	-	FJ361502	-
RHO2099	<i>Lithothamnion crispatum</i>	North Island New Zealand		-	KC963420	-

Cont. Tab. 1

VPF00148	<i>Lithothamnion</i> cf. <i>crispatum</i>	Mediterranean Sea, Spain	Peña <i>et al.</i> 2014	-	KJ710356	-
US 170935	<i>Lithothamnion glaciale</i>	Quebec, Canada	Adey <i>et al.</i> 2015	-	KP142721	-
US 170936	<i>Lithothamnion glaciale</i>	Newfoundland, Canada	Adey <i>et al.</i> 2015	-	KP142722	-
GALW15742	<i>Lithothamnion glaciale</i>	Kingstown Bay, Ireland	Hernández-Kantún <i>et al.</i> 2014	-	JQ896233	-
CPVP-91	<i>Lithothamnion glaciale</i>	Skarsundet, Norway	Pardo <i>et al.</i> 2014	-	KC819244	*KC861508
CPVP-93	<i>Lithothamnion glaciale</i>	Skarsundet, Norway	Pardo <i>et al.</i> 2014	-	-	KC861503
CPVP-1401	<i>Lithothamnion glaciale</i>	Norway	Pardo <i>et al.</i> 2014	-	-	KC861504
CPVP-1443	<i>Lithothamnion glaciale</i>	Norway	Pardo <i>et al.</i> 2014	-	KC819270	KC861507
CPVP-1444	<i>Lithothamnion glaciale</i>	Norway	Pardo <i>et al.</i> 2014	-	KC819271	* KC861509
GWS007542	<i>Lithothamnion glaciale</i>	Newfoundland and Labrador, Canada	Unpublished	-	-	HM918812
US 170936	<i>Lithothamnion</i> <i>lemoineae</i>	Newfoundland, Canada	Adey <i>et al.</i> 2015	-	KP142722	-
US 169116	<i>Lithothamnion</i> <i>lemoineae</i>	Labrador, Canada	Adey <i>et al.</i> 2015	-	KP142723	-
US 170937	<i>Lithothamnion</i> <i>lemoineae</i>	Labrador, Canada	Adey <i>et al.</i> 2015	-	KP142724	-

Cont. Tab. 1

GALW15734	<i>Lithothamnion muelleri</i>	Baja California Sur, Mexico	Hernández-Kantún <i>et al.</i> 2014	-	JQ896241	-
LBC0642	<i>Lithothamnion</i> sp.	Fiji	Bittner <i>et al.</i> 2011	-	GQ917461	GQ917270
LBC0845	<i>Lithothamnion</i> sp.	New Caledonia	Bittner <i>et al.</i> 2011	-	GQ917490	GQ917298
CPVP30	<i>Lithothamnion</i> sp.	Hvalfjoerdur, Iceland	Pardo <i>et al.</i> 2014	-	-	KC861510
CPVP92	<i>Lithothamnion</i> sp.	Skarsundet, Norway	Pardo <i>et al.</i> 2014	-	KC819245	KC861512
CPVP305	<i>Lithothamnion</i> sp.	Scotland, UK	Pardo <i>et al.</i> 2014	-	KC819248	*KC861517
FRA1148 PC0144033	<i>Lithothamnion</i> sp. 1 "Species 1"	Guadeloupe, F.W.I.	Peña <i>et al.</i> 2014	-	KJ710352	KJ710343
FRA1152 PC0144055	<i>Lithothamnion</i> sp. 1 "Species 1"	Guadeloupe, F.W.I.	Peña <i>et al.</i> 2014	-	-	KJ710342
FRA1059 PC0142655	<i>Lithothamnion</i> sp. 1 "Species 1"	Guadeloupe, F.W.I.	Peña <i>et al.</i> 2014	-	-	KJ710345
FRA2172 PC0144249	<i>Lithothamnion</i> sp. 2 "Species 2"	Guadeloupe, F.W.I.	Peña <i>et al.</i> 2014	-	KJ710354	KJ710344
FRA1211 PC0144042	<i>Lithothamnion</i> sp. 3 "Species 3"	Guadeloupe, F.W.I.	Peña <i>et al.</i> 2014	-	KJ710355	KJ710346
FRA2164 PC0144248	<i>Lithothamnion</i> sp. 4 "Species 4"	Guadeloupe, F.W.I.	Peña <i>et al.</i> 2014	-	-	KJ710341
FRA2177 PC0144250	<i>Lithothamnion</i> sp. 5 "Species 5"	Guadeloupe, F.W.I.	Peña <i>et al.</i> 2014	-	KJ710349	KJ710337

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GWS020939	<i>Lithothamnion</i> sp. 32BC	British Columbia, Canada	Saunders 2014	-	-	KM254875
US 170938	<i>Lithothamnion</i> <i>tophiforme</i>	Labrador, Canada	Adey <i>et al.</i> 2015	-	KP142720	-
ARS02826	<i>Mesophyllum</i> <i>erubescens</i>	Hawaii	Sherwood <i>et al.</i> 2010	HQ420974	-	HQ422718
ARS02835	<i>Mesophyllum</i> <i>erubescens</i>	Hawaii	Sherwood <i>et al.</i> 2010	HQ420979	-	HQ422717
FLOR 14900	<i>Mesophyllum</i> <i>erubescens</i>	ES, Brazil	Sissini <i>et al.</i> 2014	KM877275	KM983038	-
FLOR 14901	<i>Mesophyllum</i> <i>erubescens</i>	ES, Brazil	Sissini <i>et al.</i> 2014	KM877276	KM983039	-
US 170940	<i>Mesophyllum</i> <i>lichenoides</i>	Spain	Adey <i>et al.</i> 2015	-	KP142728	-
LBC0031	<i>Mesophyllum</i> <i>lichenoides</i>	France	Bittner <i>et al.</i> 2011	-	GQ917439	GQ917249
GALW15775	<i>Mesophyllum</i> <i>lichenoides</i>	Kingstown Bay, Ireland	Hernández-Kantún <i>et al.</i> 2014	-	JQ896244	-
CPVP-464	<i>Mesophyllum</i> sp. 1	Algarve, Portugal	Pardo <i>et al.</i> 2014	-	KC819252	KC861519
CPVP-467	<i>Mesophyllum</i> sp. 1	Algarve, Portugal	Pardo <i>et al.</i> 2014	-	-	KC861521
CPVP-514	<i>Mesophyllum</i> sp. 1	Algarve, Portugal	Pardo <i>et al.</i> 2014	-	-	KC861518
CPVP-1157	<i>Mesophyllum</i> sp. 2	Canary Islands, Spain	Pardo <i>et al.</i> 2014	-	KC819269	KC861522

Cont. Tab. 1

CPVP-307	<i>Mesophyllum</i> sp. 2	Canary Islands, Spain	Pardo <i>et al.</i> 2014	-	KC819249	KC861523
CPVP-776	<i>Mesophyllum sphaericum</i>	Galacia, Spain	Pardo <i>et al.</i> 2014	-	KC819262	KC861526
AM-C-20	<i>Neopolyporolithon loculosum</i>	Alaska, USA	Adey <i>et al.</i> 2015	-	KP142737	-
AM-IP-I	<i>Neopolyporolithon loculosum</i>	Alaska, USA	Adey <i>et al.</i> 2015	-	KP142738	-
AM-SM-I	<i>Neopolyporolithon loculosum</i>	Alaska, USA	Adey <i>et al.</i> 2015	-	KP142750	-
NCU 588641	<i>Neopolyporolithon reclinatum</i>	Washington, USA	Adey <i>et al.</i> 2015	-	KP142743	-
BM000712373	<i>Phymatolithon calcareum</i>	Cornwall, UK	Pardo <i>et al.</i> 2014, Peña <i>et al.</i> 2014a	-	JQ896231	KF808323
US 170885	<i>Phymatolithon lenormandii</i>	Nova Scotia	Adey <i>et al.</i> 2015	-	KP142718	-
ARS02350	<i>Phymatolithon</i> sp.	Hawaii, USA	Sherwood <i>et al.</i> 2010	HQ421548	-	-
USAJ-A-73233	<i>Sporolithon episporum</i>	Atlantic Costa Rica	Bahia <i>et al.</i> 2014	-	KC870925	-
ARS02819	<i>Sporolithon ptychoides</i>	Hawaii, USA	Sherwood <i>et al.</i> 2010	*HQ420971	-	HQ422711
GM AF5	<i>Sporolithon</i> sp.	Bahia, Brazil	Adey <i>et al.</i> 2015	-	KP142752	-
LBC0567	<i>Sporolithon</i> sp.	Vanuatu	Bittner <i>et al.</i> 2011	-	GQ917500	GQ917259

Cont. Tab. 1

LBC0695	<i>Sporolithon</i> sp.	Fiji	Bittner <i>et al.</i> 2011	-	GQ917501	GQ917279
IBC 1519	<i>Sporolithon</i> sp. 1	Paraíba, Brazil	Vieira-Pinto <i>et al.</i> 2014	KP192382	-	-
LLG0081	<i>Synarthrophyton patena</i>	Australia	Bittner <i>et al.</i> 2011	-	GQ917499	GQ917304
DH20	<i>Synarthrophyton patena</i>	Australia	Nelson <i>et al.</i> 2015	-	KM369060	-
NZC0899	<i>Synarthrophyton patena</i>	North Island, New Zealand	Nelson <i>et al.</i> 2015	-	DQ168000	-
LBC0640	Unidentified Hapalidiaceae	New Caledonia	Bittner <i>et al.</i> 2011	-	GQ917460	GQ917269
GALW15736	Unidentified Hapalidiaceae	Baja California Sur, Mexico	Hernández-Kantún <i>et al.</i> 2014	-	JQ896242	-
FLOR 14925	Unidentified Hapalidiaceae	Ceará, Brazil	Sissini <i>et al.</i> 2014	KM877299	-	-
FLOR 14926	Unidentified Hapalidiaceae	Santa Catarina, Brazil	Sissini <i>et al.</i> 2014	KM877300	-	-
FLOR 14927	Unidentified Hapalidiaceae	Rio de Janeiro, Brazil	Sissini <i>et al.</i> 2014	KM877301	-	-
FLOR 14928	Unidentified Hapalidiaceae	Santa Catarina, Brazil	Sissini <i>et al.</i> 2014	KM877302	-	-
FLOR 14929	Unidentified Hapalidiaceae	Santa Catarina, Brazil	Sissini <i>et al.</i> 2014	KM877303	-	-
FLOR 14930	Unidentified Hapalidiaceae	Paraíba, Brazil	Sissini <i>et al.</i> 2014	KM877304	-	-

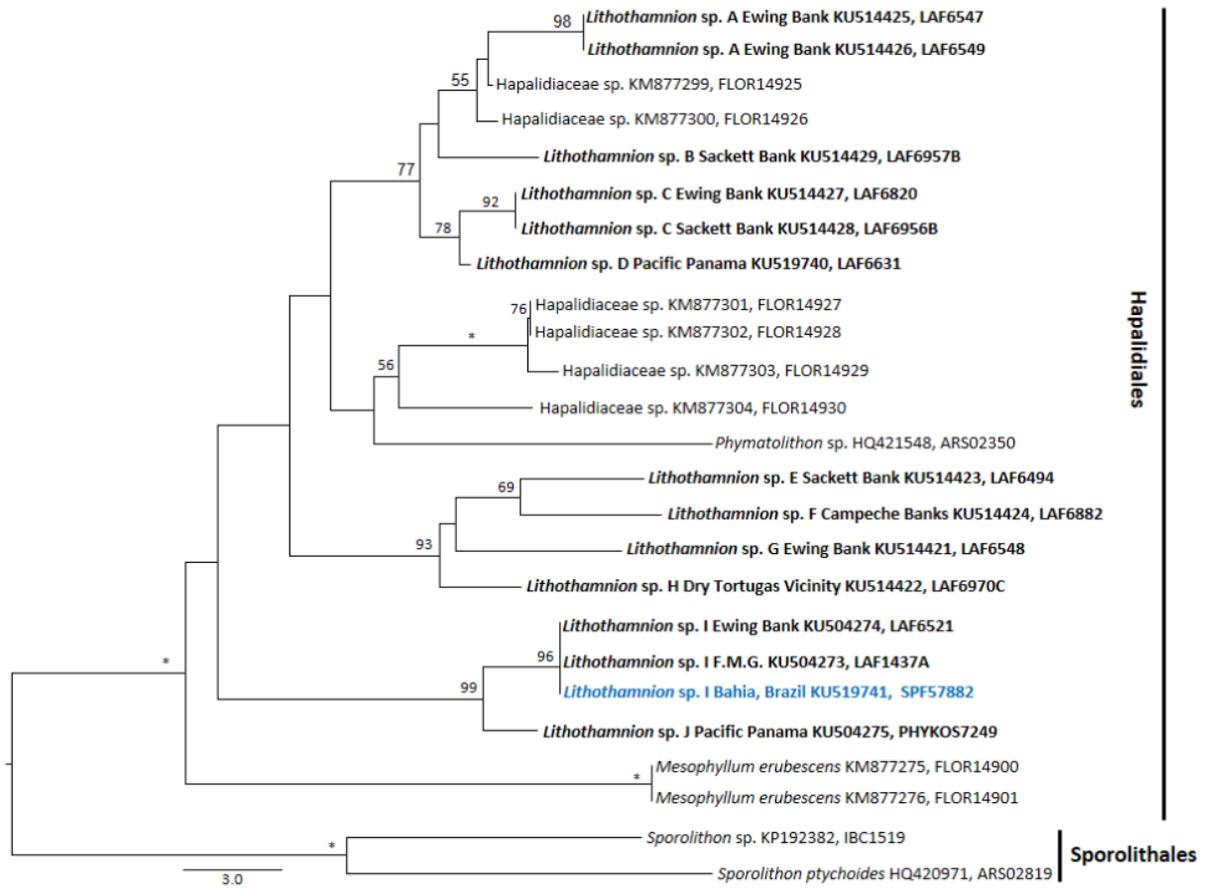
**Table 2.** Morphological characters of the Melobesiodeae specimens observed in this study. \*Data not available.

Abbreviation pc =pit connection

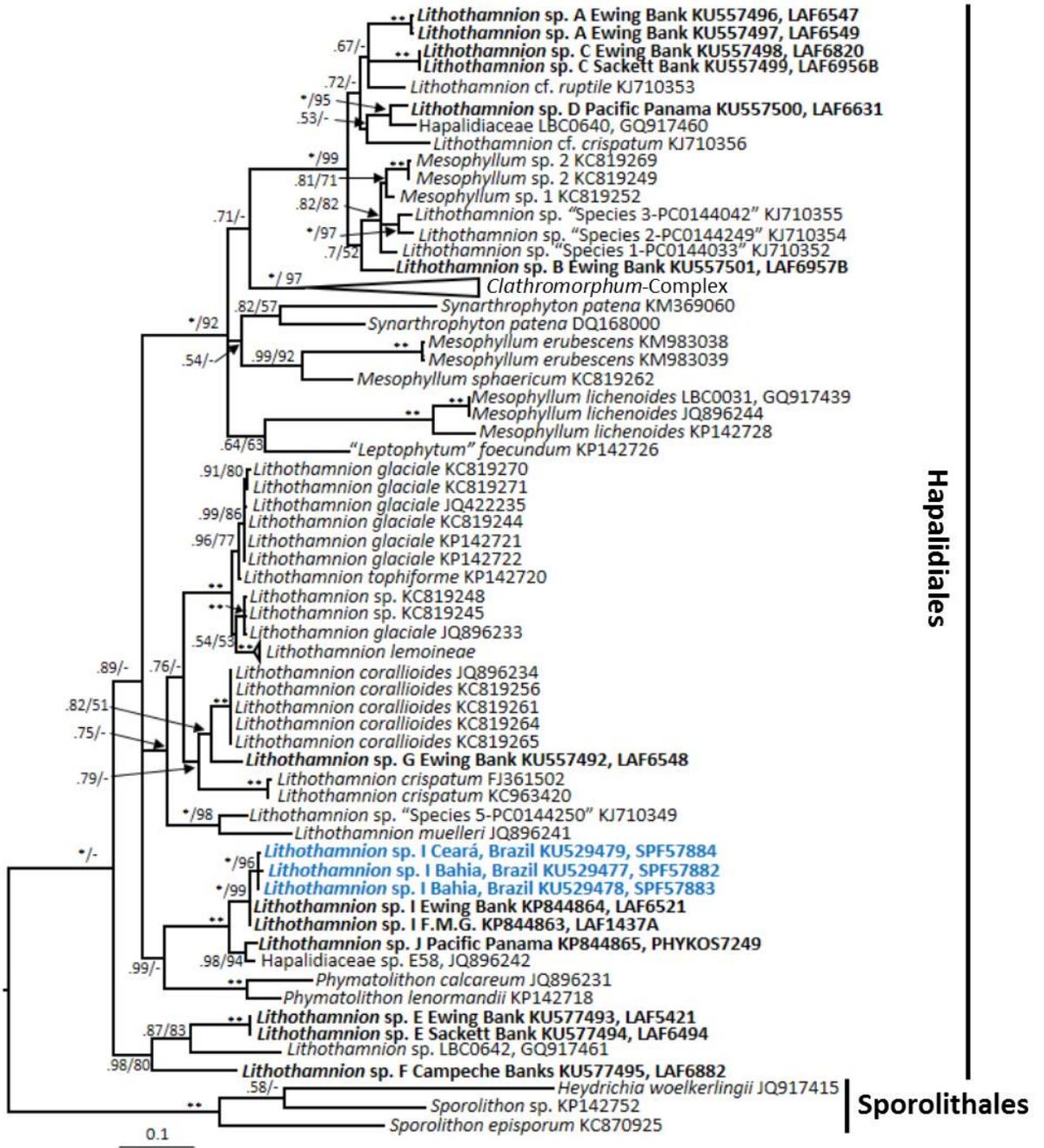
Herbarium Accession No.	Taxa	Rhodolith type/ Substratum	No. of epithallial cell layers/ epithallial lumen shape	Thallus construction	2 <sup>nd</sup> pc	Cell fusions	Palisade cells	Tricho- cytes
LAF6547	<i>Lithothamnion</i> sp. Hapalidiaceae sp. A	Autogenic rhodolith	*	*	*	*	*	*
LAF6549	<i>Lithothamnion</i> sp. Hapalidiaceae sp. A	Autogenic rhodolith	1/cylinder	Monomeric	-	+	-	-
LAF6957B	<i>Lithothamnion</i> sp. Hapalidiaceae sp. B	Encrusting bivalve shell	1/trapezoid	Monomeric	-	+	-	-
LAF6820	<i>Lithothamnion</i> sp. Hapalidiaceae sp. C	Biogenic rhodolith	1/trapezoid	Monomeric	-	+	-	-
LAF6956B	<i>Lithothamnion</i> sp. Hapalidiaceae sp. C	Biogenic rhodolith	1/trapezoid	Monomeric	-	+	-	-
LAF6631	<i>Lithothamnion</i> sp. Hapalidiaceae sp. D	Putative limestone fragment	1/*	Monomeric	-	+	-	-
LAF5421	<i>Lithothamnion</i> sp. Hapalidiaceae sp. E	Autogenic rhodolith	1/*	Monomeric	-	+	-	-
LAF6494	<i>Lithothamnion</i> sp. Hapalidiaceae sp. E	Autogenic rhodolith	*	*	*	*	*	*
LAF6882	<i>Lithothamnion</i> sp. Hapalidiaceae sp. F	Biogenic rhodolith	1/cylinder	Monomeric	-	+	-	-
LAF6548	<i>Lithothamnion</i> sp. Hapalidiaceae sp. G	Biogenic rhodolith	1/trapezoid	Monomeric	-	+	-	-
LAF6970C	<i>Lithothamnion</i> sp. Hapalidiaceae sp. H ("Species 5" <i>sensu</i> Peña <i>et al.</i> 2014)	Biogenic rhodolith	1/cylinder	Monomeric	-	+	-	-

Cont. Tab. 2

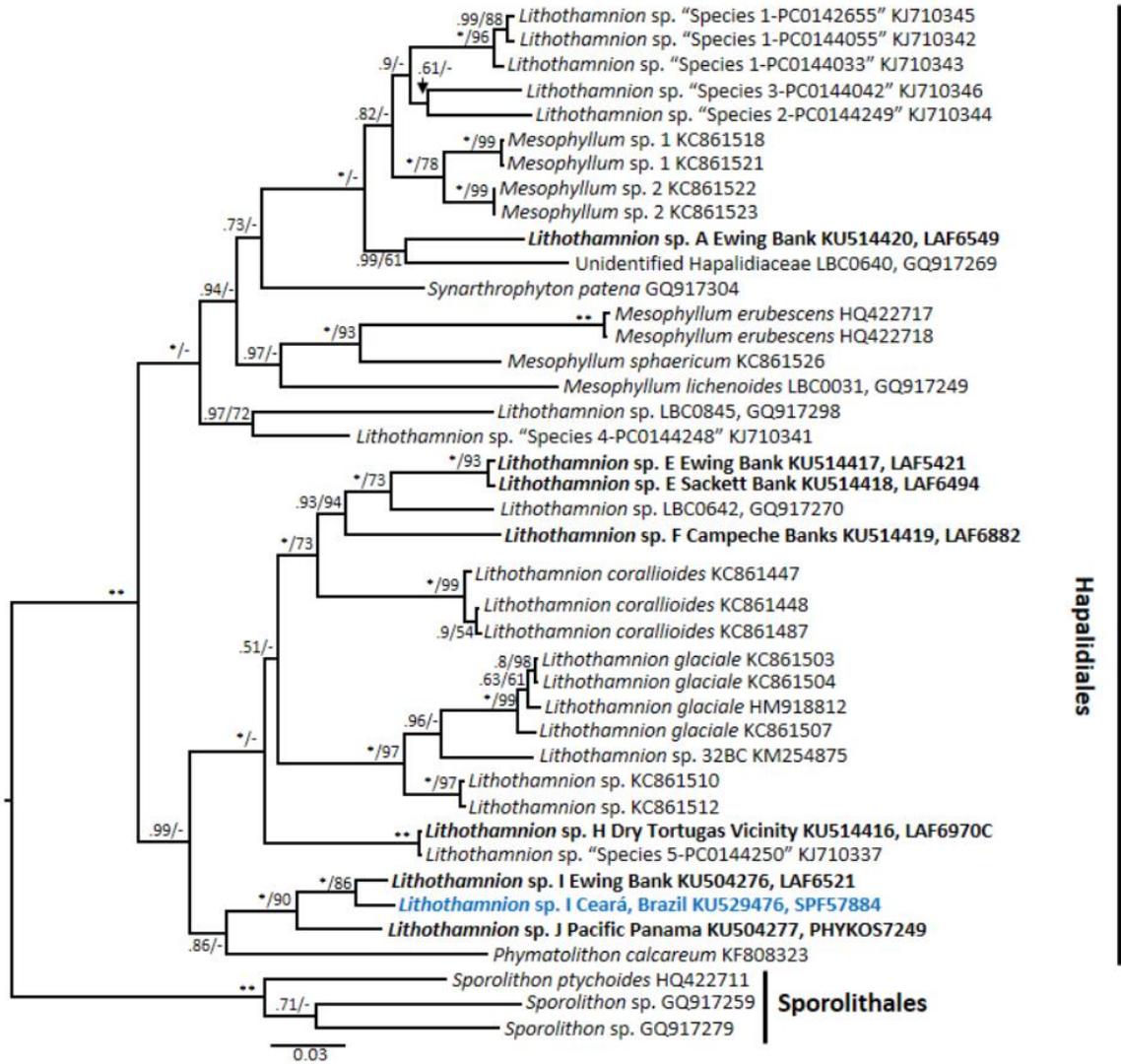
LAF6521	<i>Lithothamnion</i> Hapalidiaceae sp. I	sp.	Biogenic rhodolith	1/trapezoid	Monomeric	-	+	-	-
LAF1437A	<i>Lithothamnion</i> Hapalidiaceae sp. I	sp.	Biogenic rhodolith	1/trapezoid	Monomeric	-	+	-	-
SPF57882	<i>Lithothamnion</i> Hapalidiaceae sp. I	sp.	Biogenic rhodolith	1/trapezoid	Monomeric	-	+	-	-
SPF57883	<i>Lithothamnion</i> Hapalidiaceae sp. I	sp.	Biogenic rhodolith	*	*	*	*	*	*
SPF57884	<i>Lithothamnion</i> Hapalidiaceae sp. I	sp.	Biogenic rhodolith	1/*	Monomeric	-	+	-	-
PHYKOS 7249	<i>Lithothamnion</i> Hapalidiaceae sp. J	sp.	Biogenic rhodolith	1/trapezoid	Monomeric	-	+	-	-



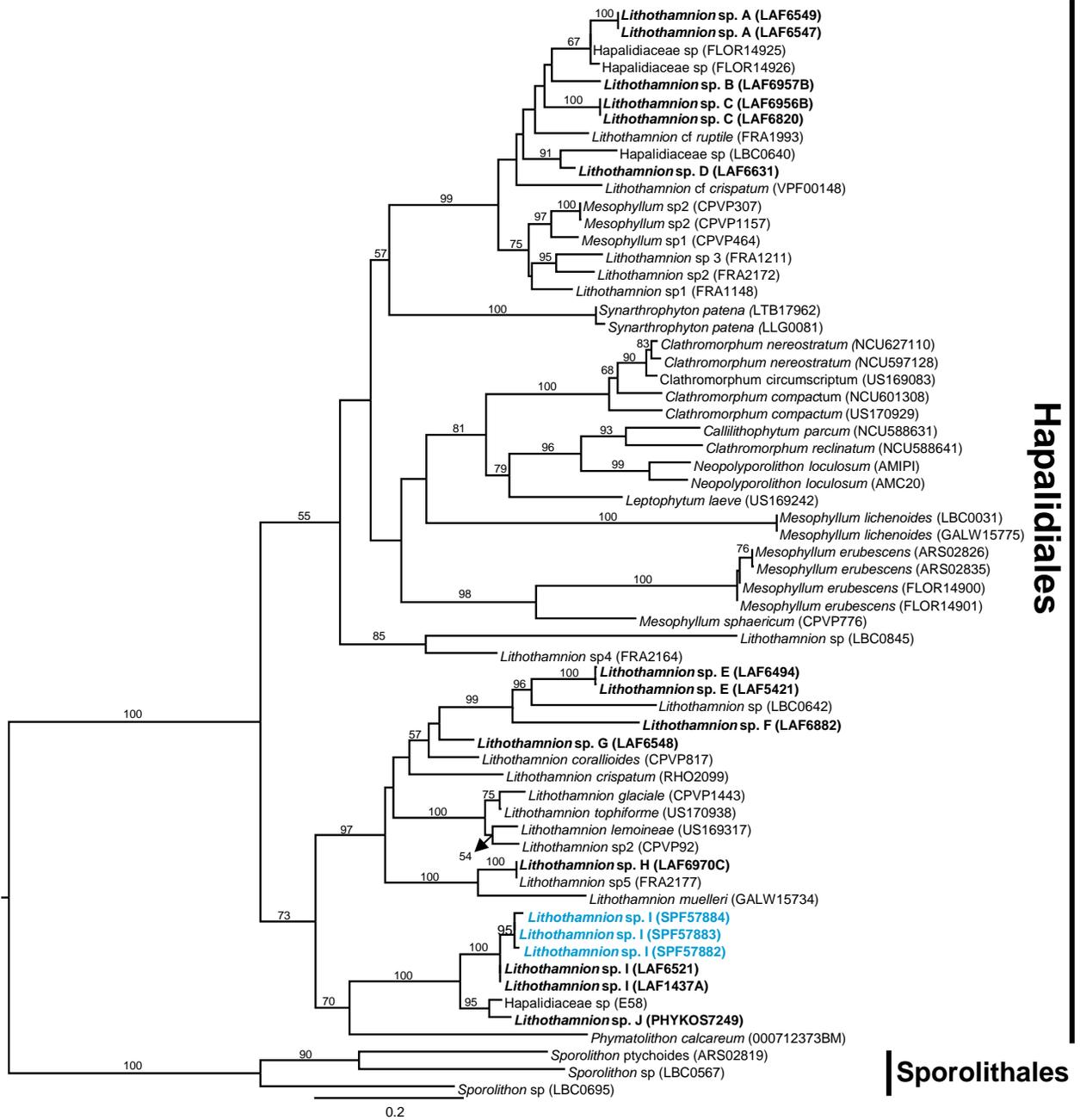
**Figure 1.** Phylogram based on NJ analysis of UPA sequences. Node values indicate bootstrap values out of 1,000 replicates, \* indicates full support. Newly generated sequences shown in bold.



**Figure. 2.** Phylogram based on Bayesian analysis of *psbA*. Node values indicate posterior probability (left) and bootstrap values for ML analyses out of 1,000 replicates (right), \* indicates full support. Newly generated sequences shown in bold.

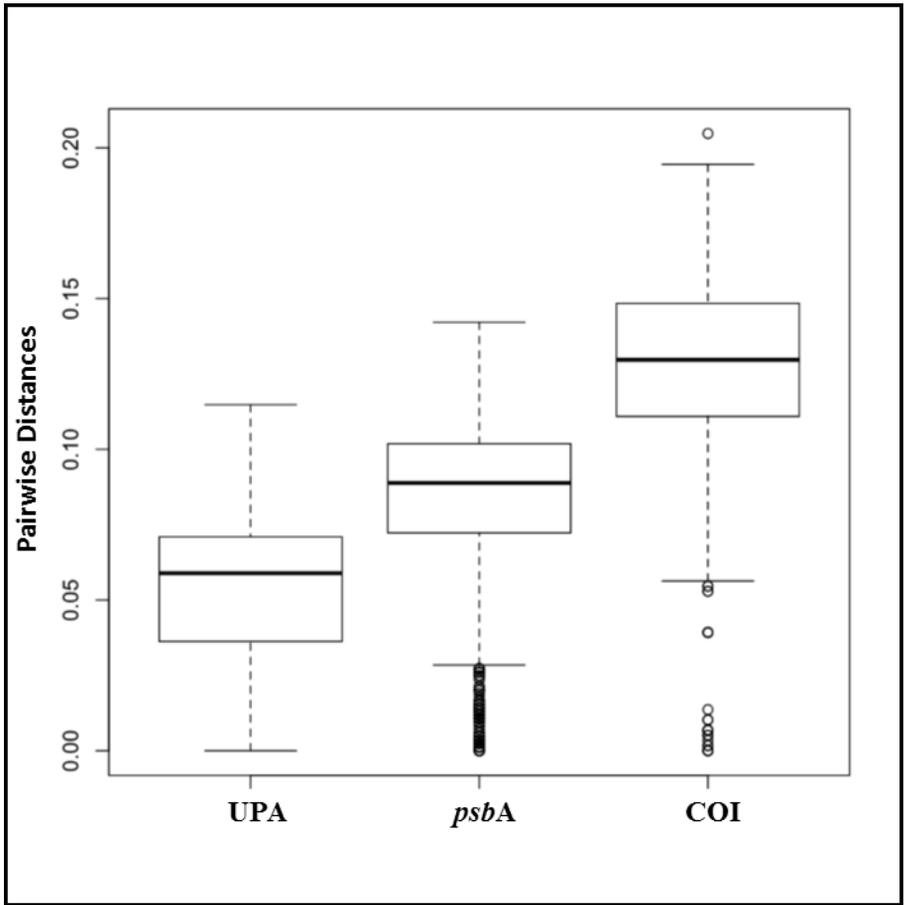


**Figure 3.** Phylogram based on Bayesian analysis of COI. Node values indicate posterior probability (left) and bootstrap values for ML analyses out of 1,000 replicates (right), \* indicates full support. Newly generated sequences shown in bold.

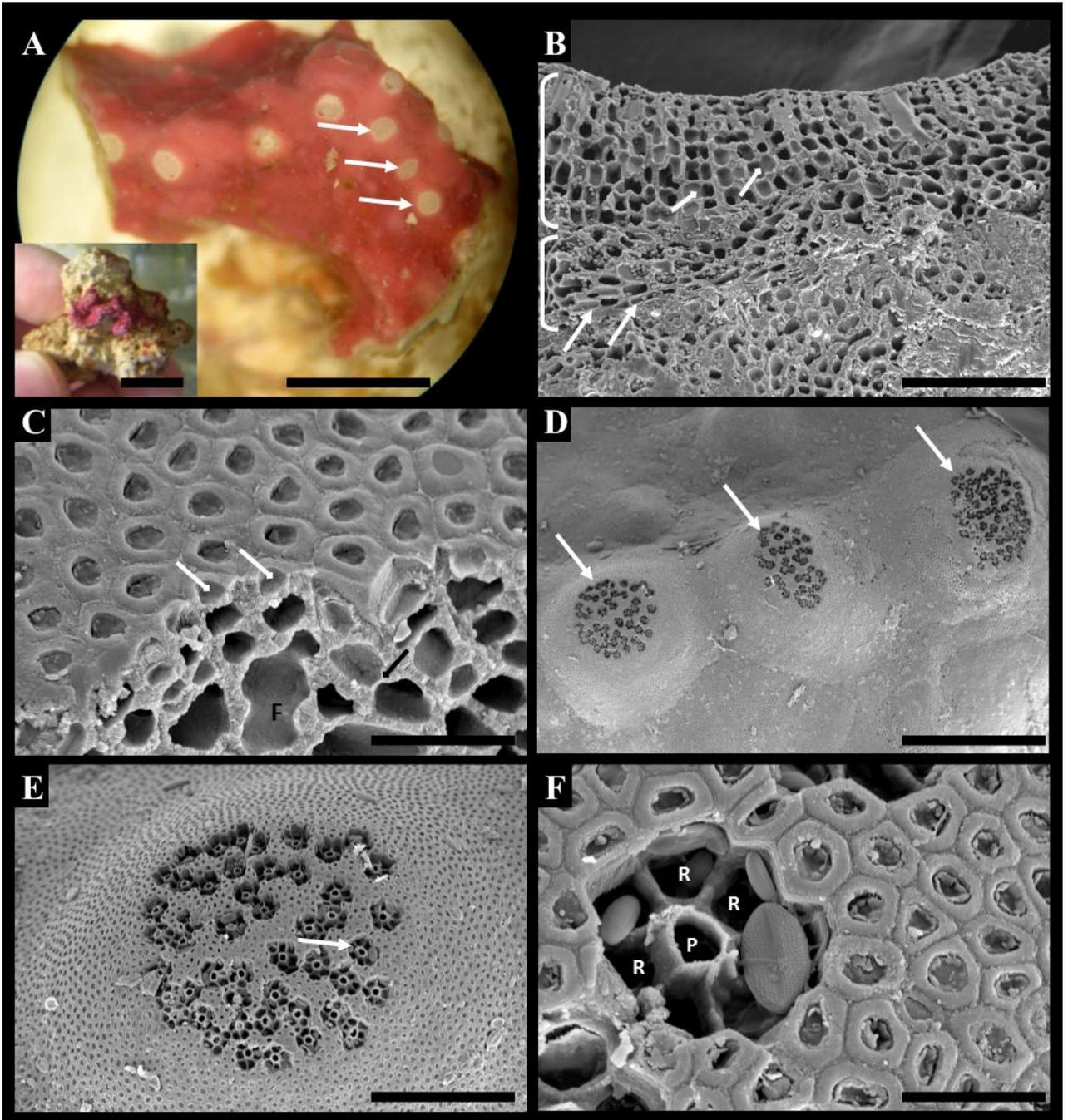


**Figure 4.** Phylogram based on ML analysis of concatenated UPA, *psbA*, and COI sequences.

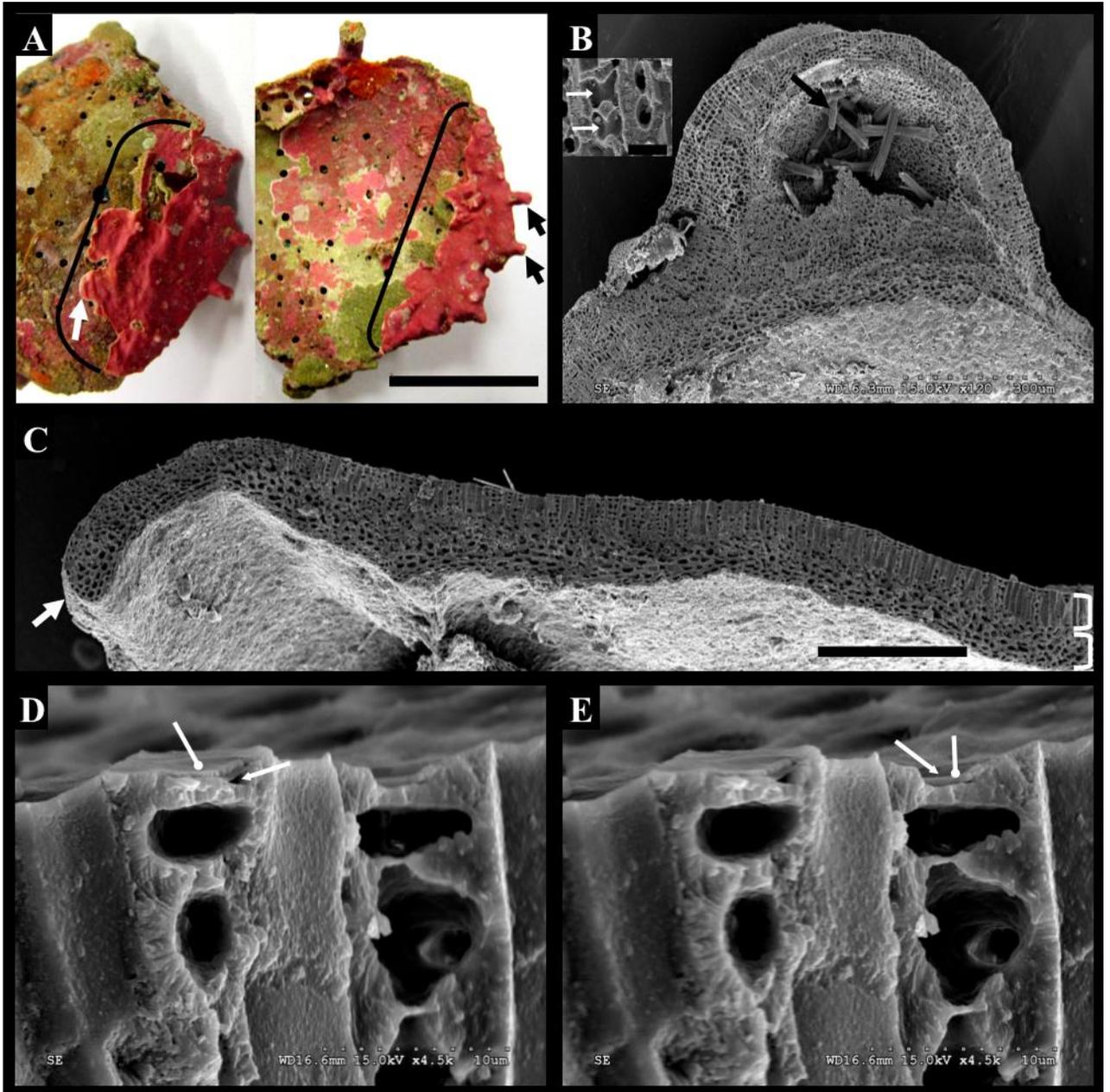
Node values indicate bootstrap values out of 1,000 replicates.



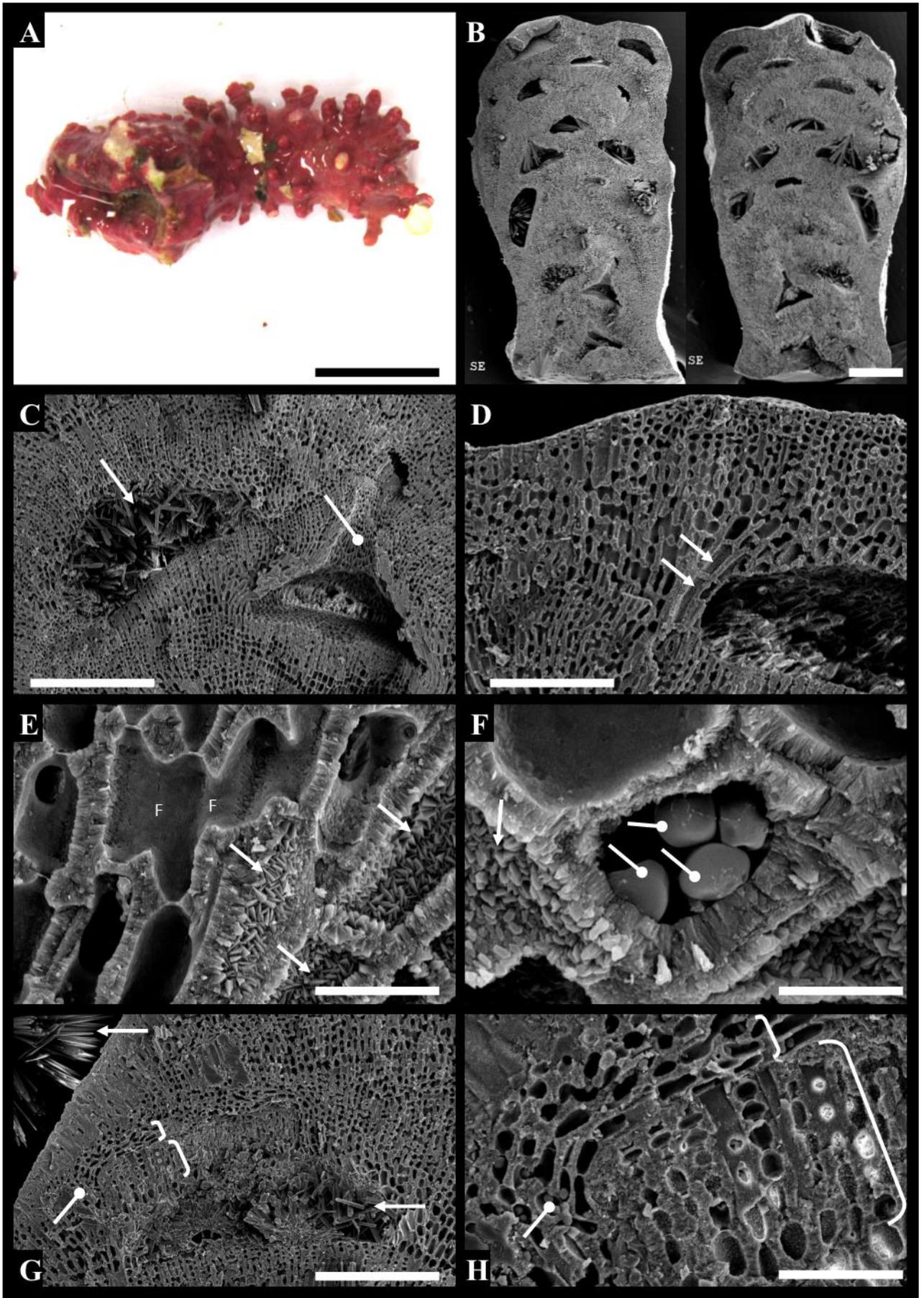
**Figure 5.** Distribution of raw pairwise distances (no. of base pair differences/alignment length) for each of the three markers analyzed in this study.



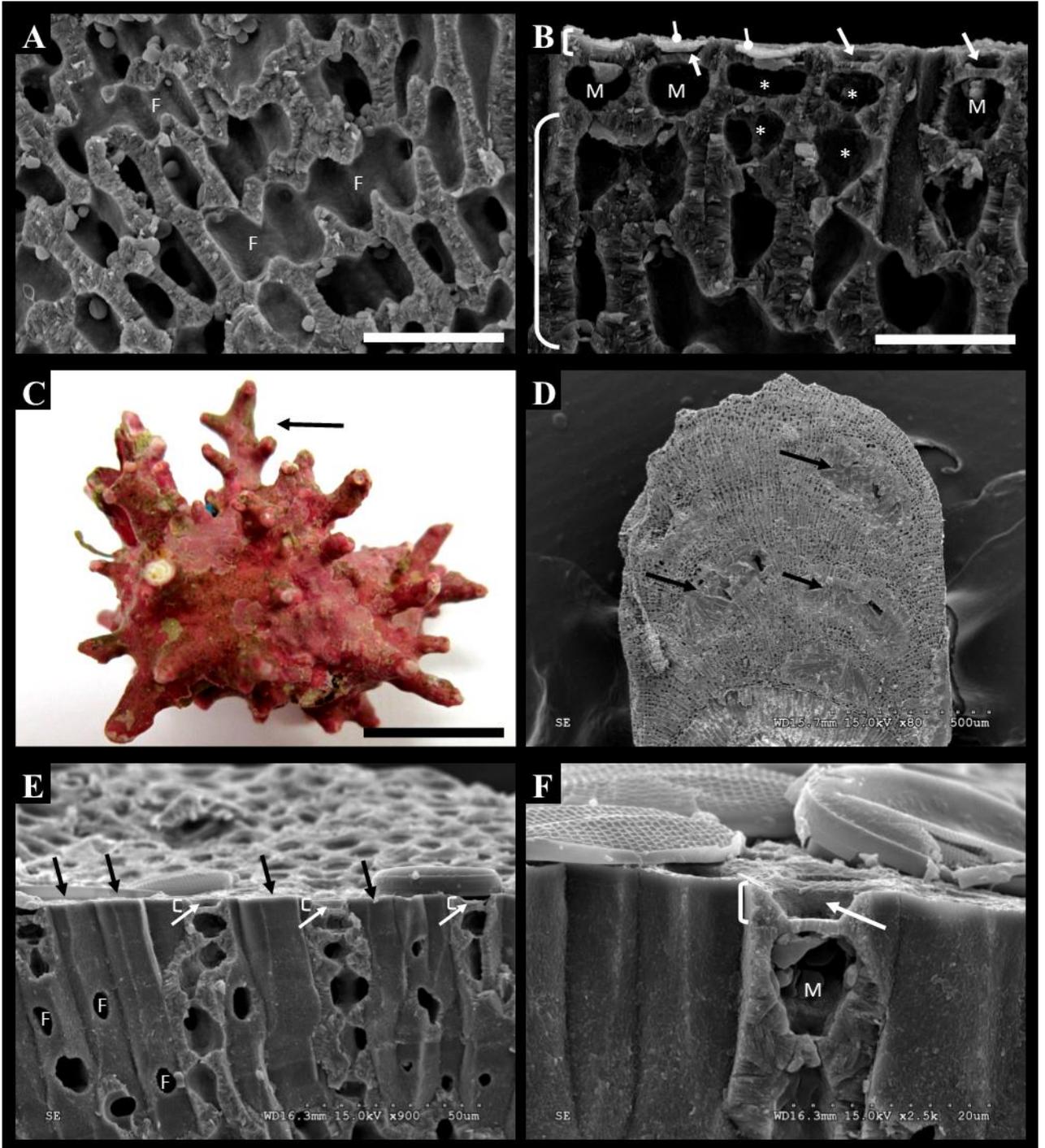
**Figure 6. Specimen LAF6549.** A. View through dissecting microscope showing multiporate conceptacles (arrows) on thallus surface. Scale bar 1 mm. Inset: Thallus habit growing on autogenic rhodolith. Scale bar 10 mm. B. Section through thallus showing hypothallus (lower bracket) with rectangular shaped cells (large arrows) and perithallus (upper bracket) with cell fusions (small arrows). Scale bar 95  $\mu\text{m}$ . C. Surface view and section of thallus showing epithallial cells (white arrows) and cell fusion (F). Scale bar 25  $\mu\text{m}$ . D. Surface view of thallus showing multiporate conceptacles (arrows). Scale bar 375  $\mu\text{m}$ . E. Surface view of conceptacle showing individual pores, each surrounded by rosette cells (arrow). Scale bar 140  $\mu\text{m}$ . F. Magnified view of pore (P) with rosette cells (R) indicated by arrow in Fig. 1E. Also shown are pennate diatoms. Scale bar 20  $\mu\text{m}$ .



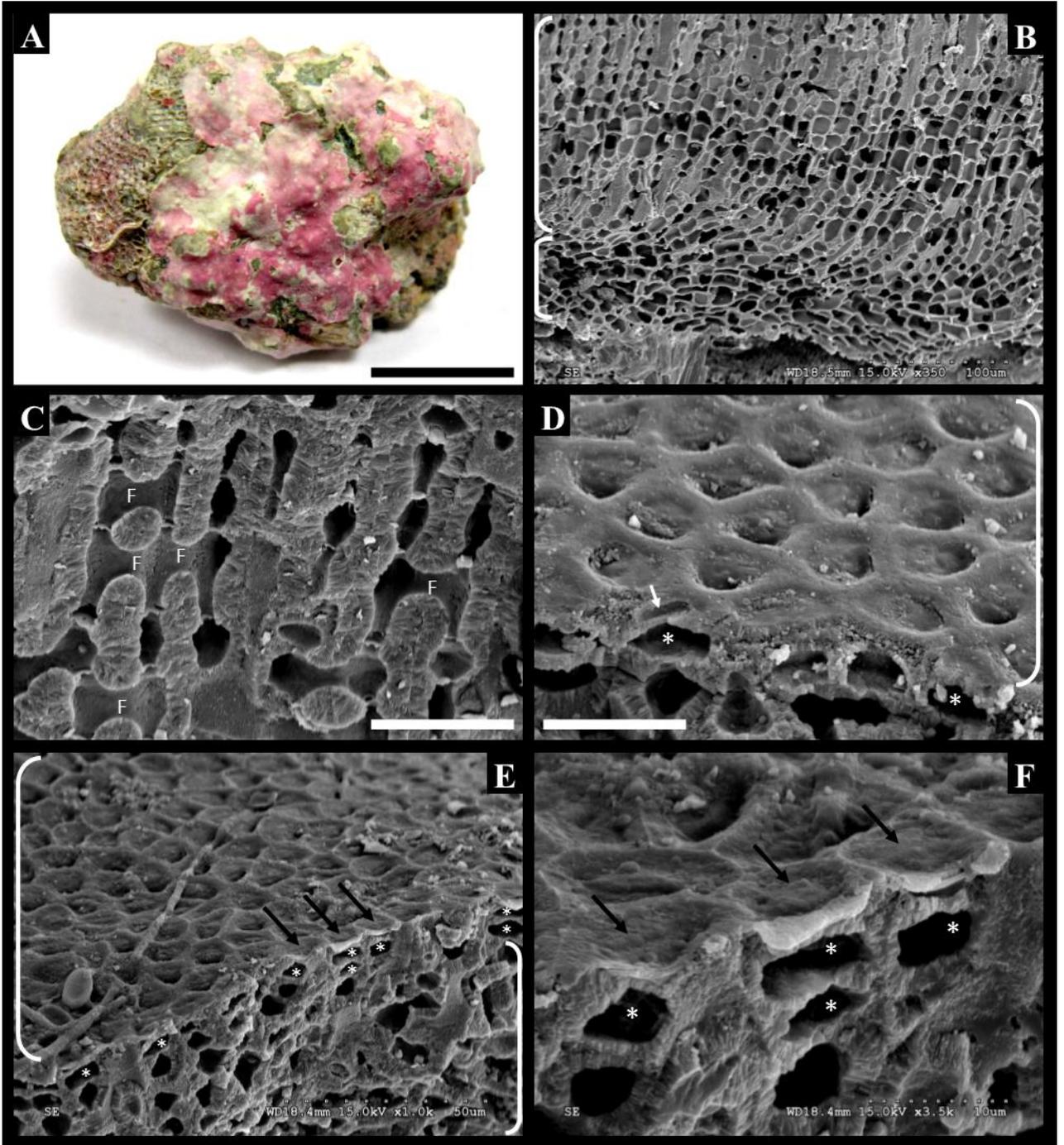
**Figure 7. Specimen LAF6957B.** A. Thallus habit growing on the top (left) and bottom (right) of an eroded bivalve shell. Scale bar 18 mm. B. Section of thallus showing hypothallus (lower bracket) and perithallus (upper bracket). Each unit of the embedded scale is 5  $\mu\text{m}$ . C. Section of perithallus showing cell fusions in the x-axis (arrows) and z-axis (circle arrows). Scale bar 24  $\mu\text{m}$ . D. Section of thallus showing a conceptacle post-spore release including crystals (black arrow) formed from secondary mineralization. Scale bar 300  $\mu\text{m}$ . E. Section of thallus showing left epithallial cell in focus with lumen (arrow) and intact epithallial cell roof (circle pointer). Each unit of embedded scale is 1  $\mu\text{m}$ . E. Same field of view as F. showing the right epithallial cell in focus with trapezoidal shaped lumen (arrow) and collapsed epithallial cell roof (circle arrow). Each unit of embedded scale bar is 1  $\mu\text{m}$ .



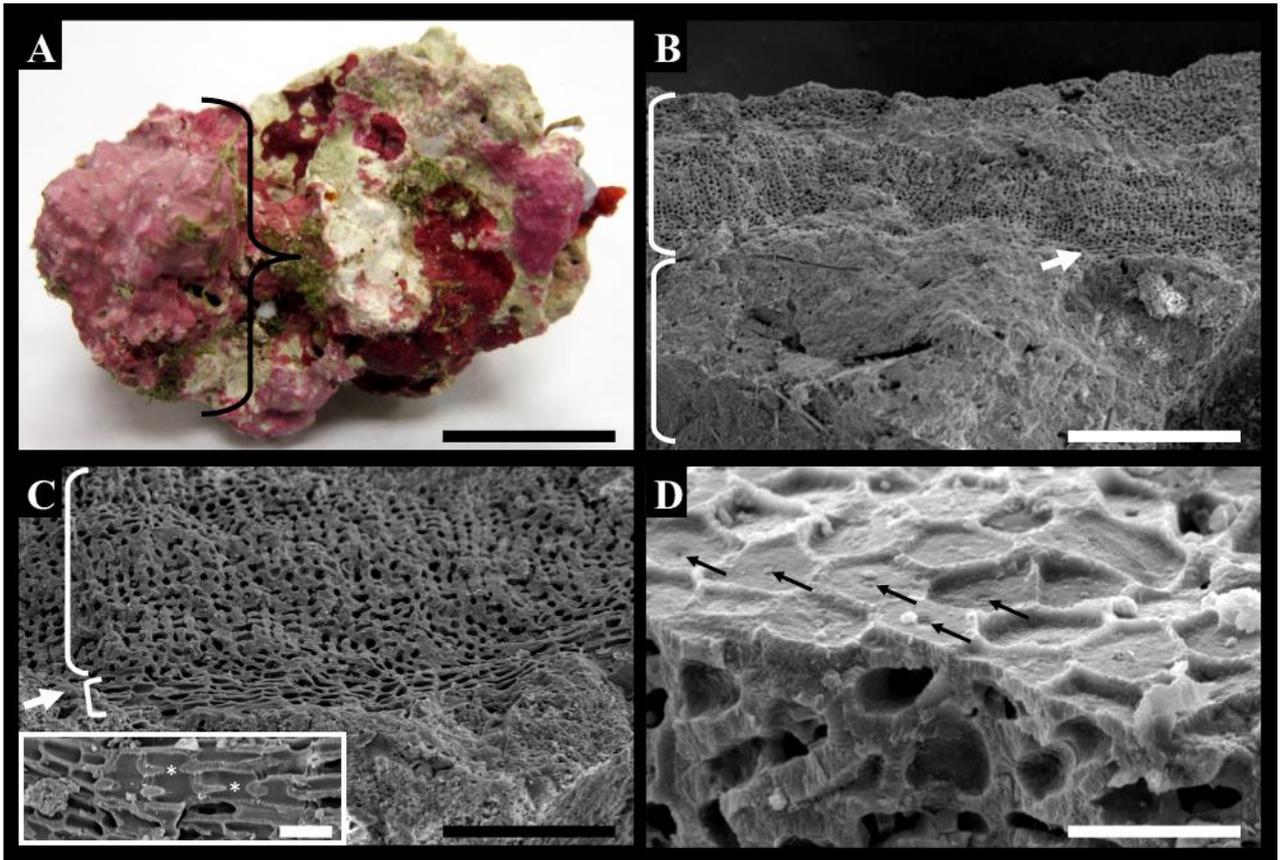
**Figure 8. Specimen LAF6820.** A. Thallus habit showing numerous protuberances. Scale bar 12.5 mm. B. Longitudinal sections of protuberance showing numerous overgrown conceptacles, many with putative aragonite infill. The sections are two halves of the same protuberance and show mirroring images of the same structures. Scale bar .5 mm. C. Magnified view of two overgrown conceptacles. Arrow indicates conceptacle with putative aragonite crystals. Circle arrow indicates section of conceptacle pore. Scale bar 240  $\mu\text{m}$ . D. Section of thallus showing elongated perithallial cells (arrows) located at the periphery of the conceptacle. Scale bar 105  $\mu\text{m}$ . E. Magnified view of area indicated by arrows in D. showing cell fusions (“F”) and centripetal infill of small, calcium carbonate crystals in cells bordering the conceptacles (arrows). Scale bar 17.5  $\mu\text{m}$ . F. Unidentified spherical inclusions (circle arrows) near an empty conceptacle and cells with centripetal infill of calcium carbonate crystals (arrow). Scale bar 7  $\mu\text{m}$ . G. Partial and complete views of overgrown conceptacles showing putative needle-shaped crystals of aragonite (arrows). Circle arrow and upper bracket indicate location of newly formed hypothallial filaments growing over an older thallus portion (lower bracket). Scale bar 175  $\mu\text{m}$ . H. Magnified view of newly formed hypothallial filaments (circle arrow, upper bracket) consisting of rectangular-shaped cells growing parallel to the older growth layer (lower bracket). Scale bar 44  $\mu\text{m}$ .



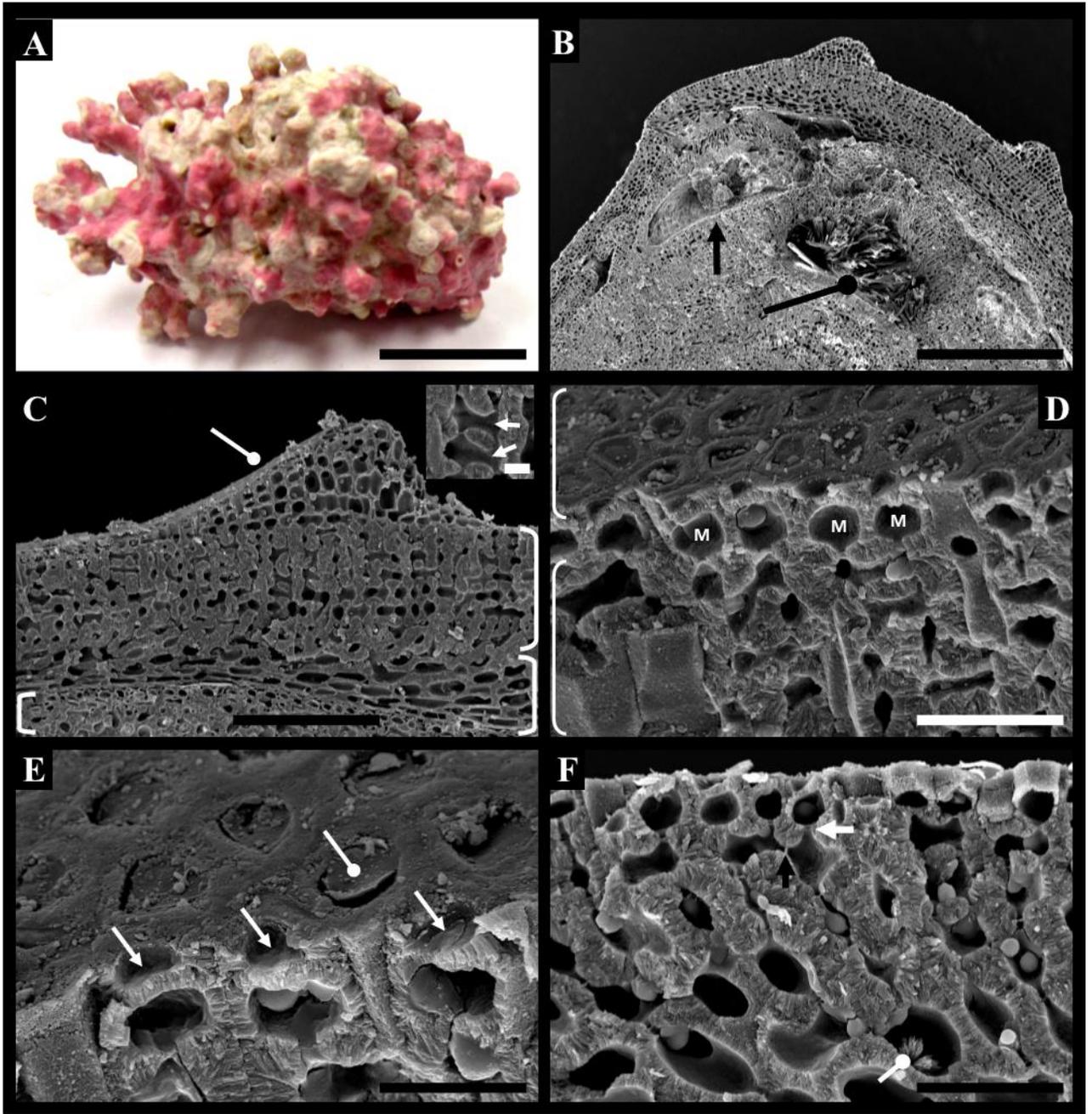
**Figure 9. Specimen LAF6820 A-B, Specimen LAF6956B C-F.** A. Section of perithallus showing cell fusions between several adjacent filaments (“F”). Scale bar 28.5  $\mu\text{m}$ . B. Section of thallus showing epithallus (upper bracket), perithallus (lower bracket), meristematic cells (“M”) and putative recently divided meristematic cells (\*). Arrows indicate epithallial cell lumens and circle arrows indicate epithallial cell roofs. Scale bar 17.5  $\mu\text{m}$ . C. Thallus habit showing numerous, sometimes branched (arrow), protuberances. Scale bar 11 mm. D. Section of thallus showing overgrown conceptacles with spherical masses of putative aragonite (arrows). Each unit of the embedded scale is 50  $\mu\text{m}$ . E. Section and partial surface view of thallus showing heavily calcified filaments which were cleaved from their neighboring filaments in the z-axis (arrows) showing polygonal outline and cell fusions in the z-axis (“F”). Brackets indicate epithallial cells and arrows indicate epithallial cell lumens. Each unit of the embedded scale is 5  $\mu\text{m}$ . F. Magnified view of epithallial cell (bracket) lacking a roof with trapezoidal-shaped cell lumen (arrow) and a meristematic cell below (“M”). Each unit of the embedded scale bar is 2  $\mu\text{m}$ . Note centric diatoms at the surface in E, F.



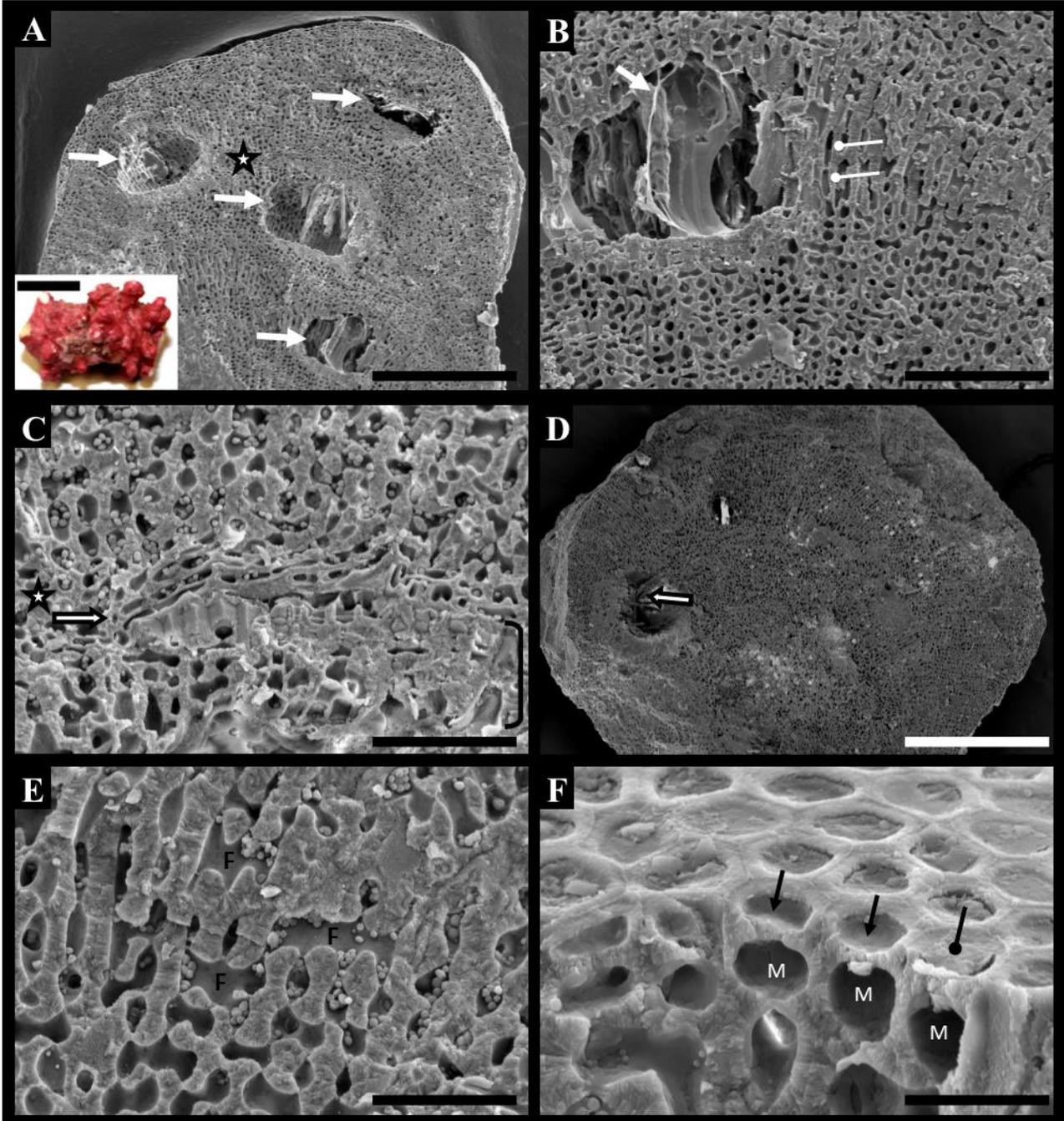
**Figure 10. Specimen LAF6631.** A. Thallus habit showing thin crust growing over putative limestone fragment. Scale bar 8 mm. B. Section of thallus showing multilayered hypothallus (lower bracket) and perithallus (upper bracket). Each unit of the embedded scale bar is 10  $\mu\text{m}$ . C. Section of thallus showing cell fusions (“F”). Scale bar 24  $\mu\text{m}$ . D. Surface view of epithallus (bracket) showing epithallial cells with polygonal outline and partial section view showing partial lumen (arrow) of epithallial cell and cells subtending the epithallial cells (\*). Scale bar 19.5  $\mu\text{m}$ . E. Section and surface view of thallus showing epithallus (left bracket) and perithallus (right bracket) and cells subtending the epithallial cells (\*). Each unit of the embedded scale bar is 5  $\mu\text{m}$ . Note algal filament and pennate diatom at thallus surface. F. Surface and section view showing magnified view of epithallial cells (arrows) indicated by arrows in E and cells subtending the epithallial cells (\*) including two cells (\*) which may represent recently divided meristematic cells. Each unit of the embedded scale bar is 1  $\mu\text{m}$ .



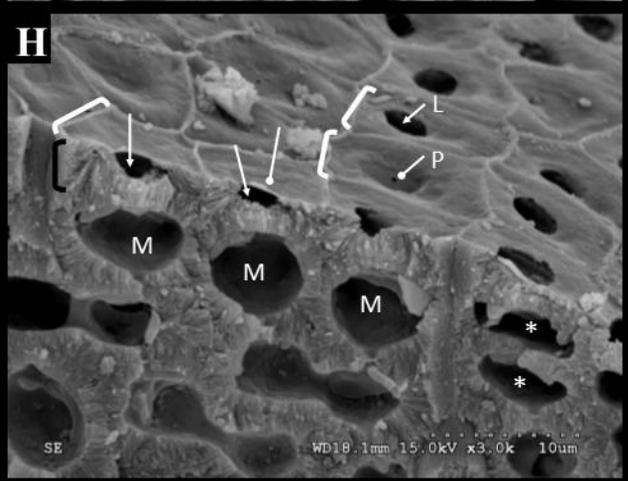
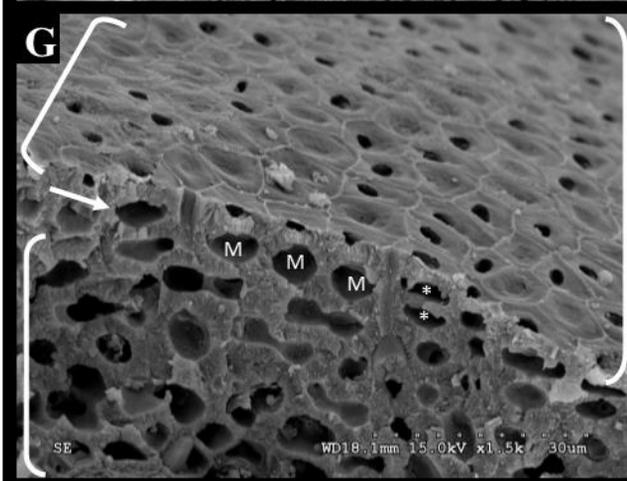
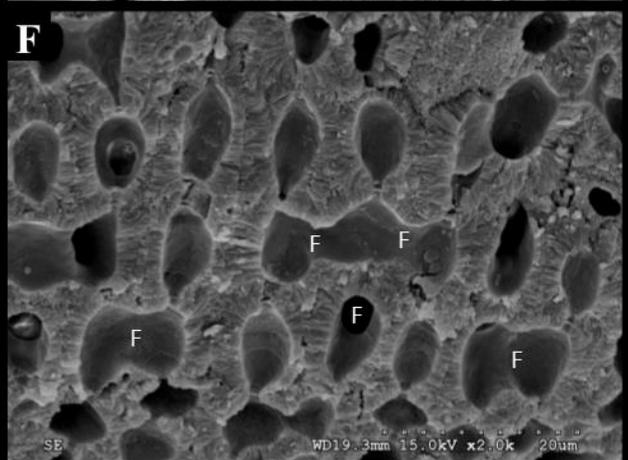
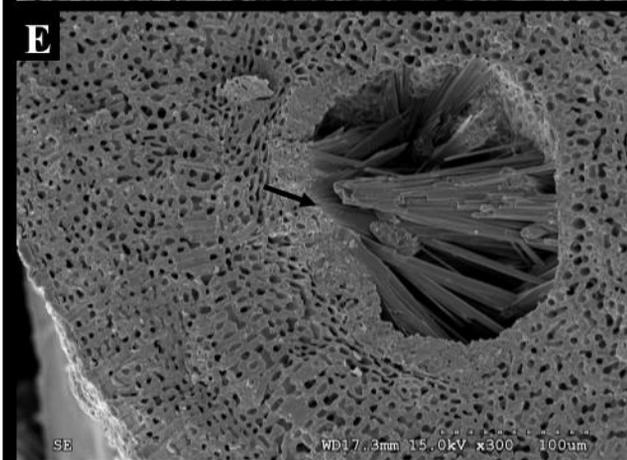
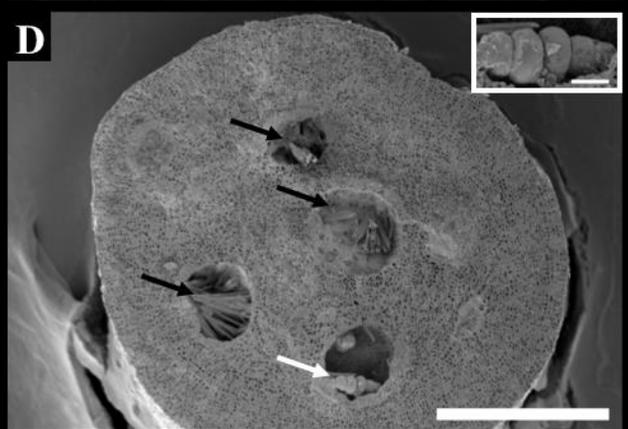
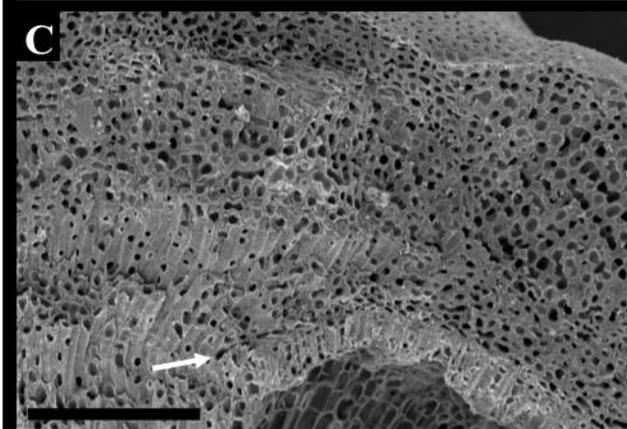
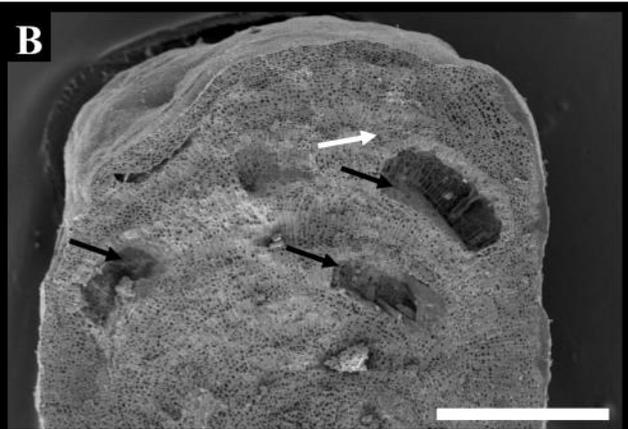
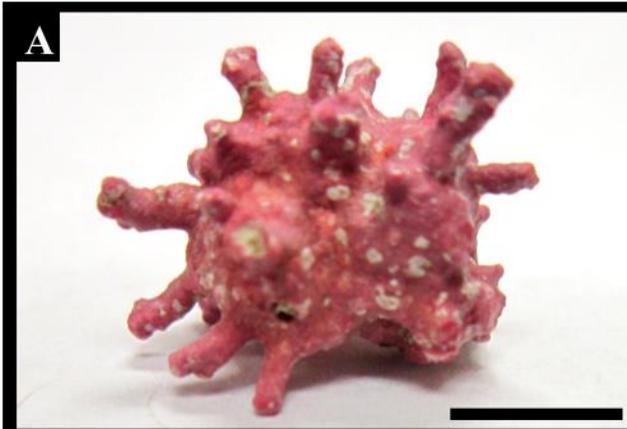
**Figure 11. Specimen LAF5421.** A. Specimen habit showing thallus at left (bracket) encrusting autogenic rhodolith fragment. Scale bar 8 mm. B. Section of thallus (upper bracket) growing over substratum (lower bracket). Scale bar 260  $\mu\text{m}$ . Arrow indicates location of hypothallus. C. Section of thallus showing magnified view of hypothallus (lower bracket) and perithallus (upper bracket). Arrow indicates same location indicated by arrow in Fig. B. Scale bar 100  $\mu\text{m}$ . Inset shows cell fusions (\*) between adjacent hypothallial filaments. Scale bar 20  $\mu\text{m}$ . D. Surface view and partial section of thallus showing remnant proximal cell walls with primary pit connections (arrows) of putative epithallial cells. Scale bar 14  $\mu\text{m}$ .



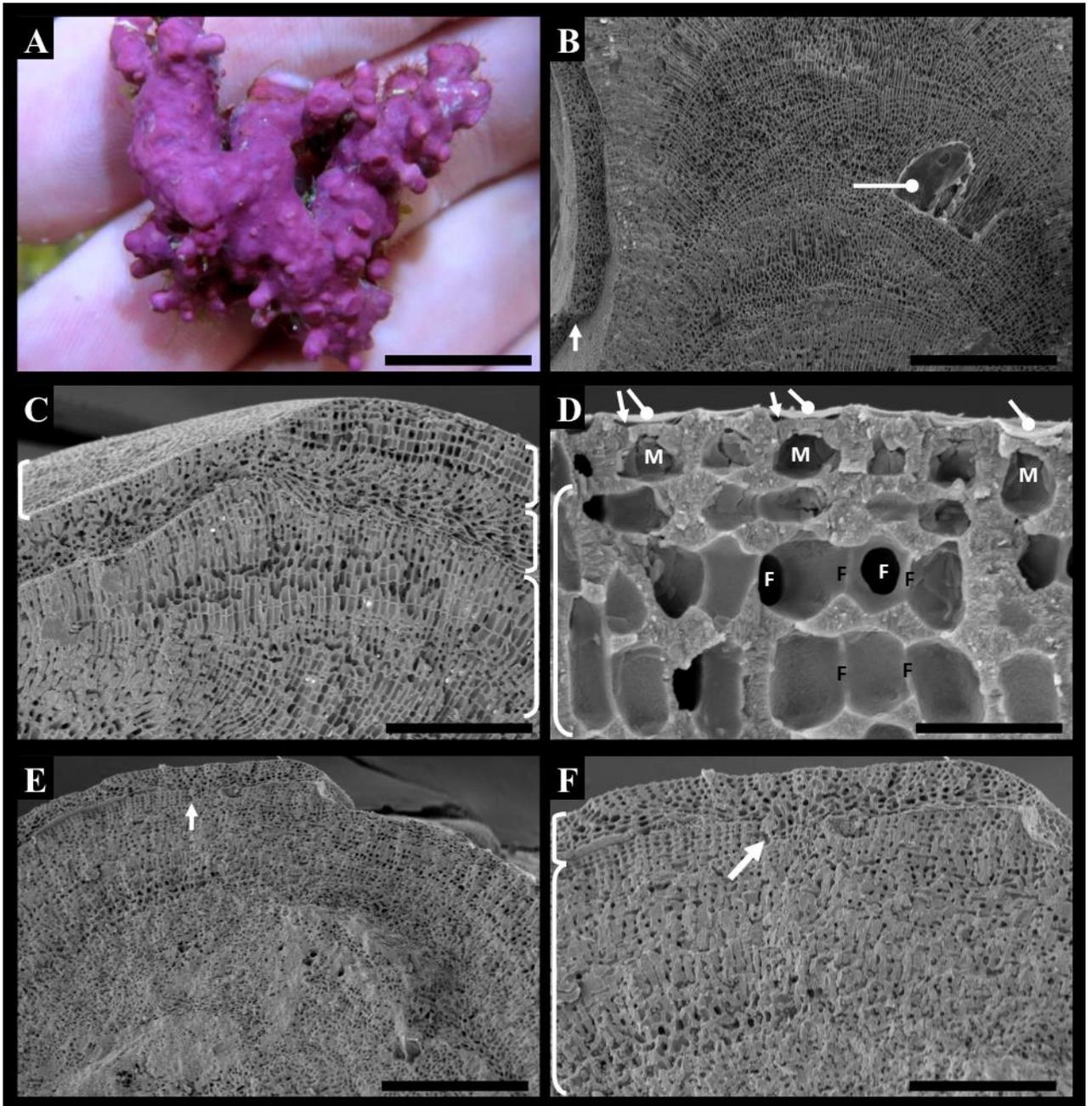
**Figure 12. Specimen LAF6882.** A. Thallus habit showing numerous protuberances. Scale bar 7 mm. B. Section of protuberance showing two overgrown conceptacles (arrow and circle arrow), one including numerous putative aragonite crystals (circle arrow). Scale bar 290  $\mu\text{m}$ . C. Newly formed growth layer growing over an older part (left bracket) of the thallus showing hypothallial filaments (lower right bracket) growing parallel to the older part of the thallus, and arching tiers of perithallial filaments growing perpendicular to the thallus (upper right bracket). Circle arrow indicates location of developing protuberance. Scale bar 90  $\mu\text{m}$ . Inset shows cell fusions (arrows). Scale bar 6  $\mu\text{m}$ . D. Section and surface view showing perithallus (lower bracket), epithallus (upper bracket) and meristematic cells (M). Scale bar 28  $\mu\text{m}$ . E. Magnified view of epithallial cells lacking epithallial cell roofs (arrows) and others with roof intact (circle arrow). Scale bar 10.5  $\mu\text{m}$ . F. Section of thallus showing primary pit connection (black arrow) at the site of lateral branching giving the false appearance of a secondary pit connection and first formed primary pit connection (white arrow). Note site of crystals formed from secondary mineralization (circle arrow). Scale bar 25  $\mu\text{m}$ .



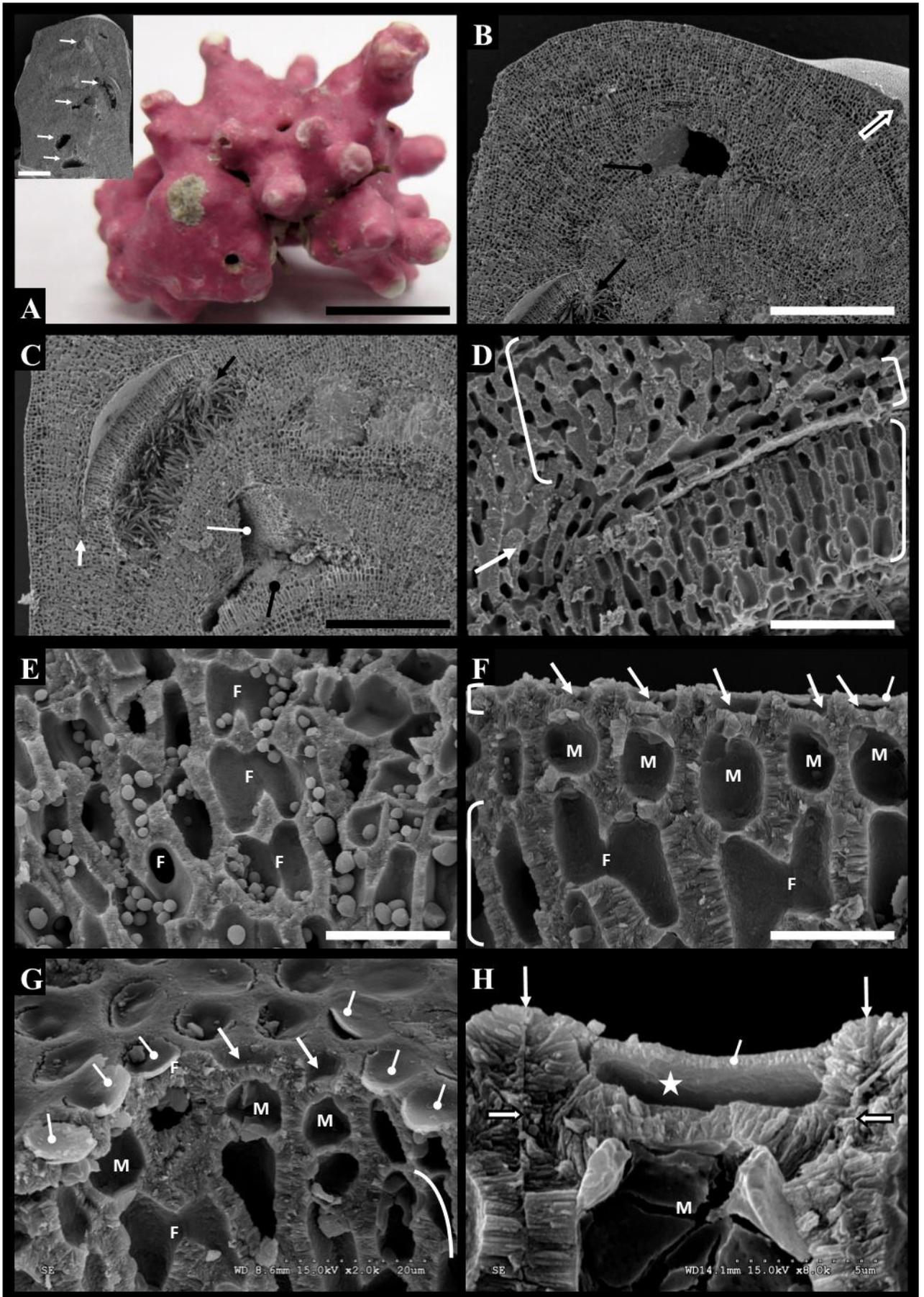
**Figure 13. Specimen LAF6548.** A. Section through protuberance showing multiple overgrown conceptacles (arrows) and location of new hypothallial growth over the conceptacle roof (star). Scale bar 250  $\mu\text{m}$ . Inset: Thallus habit; biogenic rhodolith with numerous protuberances. Scale bar 5 mm. B. Lowermost conceptacle shown in Fig. 2A (arrow) and surrounding perithallus. Scale bar 80  $\mu\text{m}$ . C. Magnified view of location indicated by the star in Fig. 2A showing newly formed hypothallus (arrow) growing over the conceptacle roof. Scale bar 45  $\mu\text{m}$ . D. Cross section through protuberance showing radial construction and overgrown conceptacle with crystals (arrow) from secondary mineral infill. Scale bar 350  $\mu\text{m}$ . E. Perithallus showing abundant cell fusions (F). Scale bar 30  $\mu\text{m}$ . F. Section and surface view showing epithallial cells (arrows) with polygonal outline. Circle arrow indicates epithallial cell with intact distal cell wall. Scale bar 13  $\mu\text{m}$ .



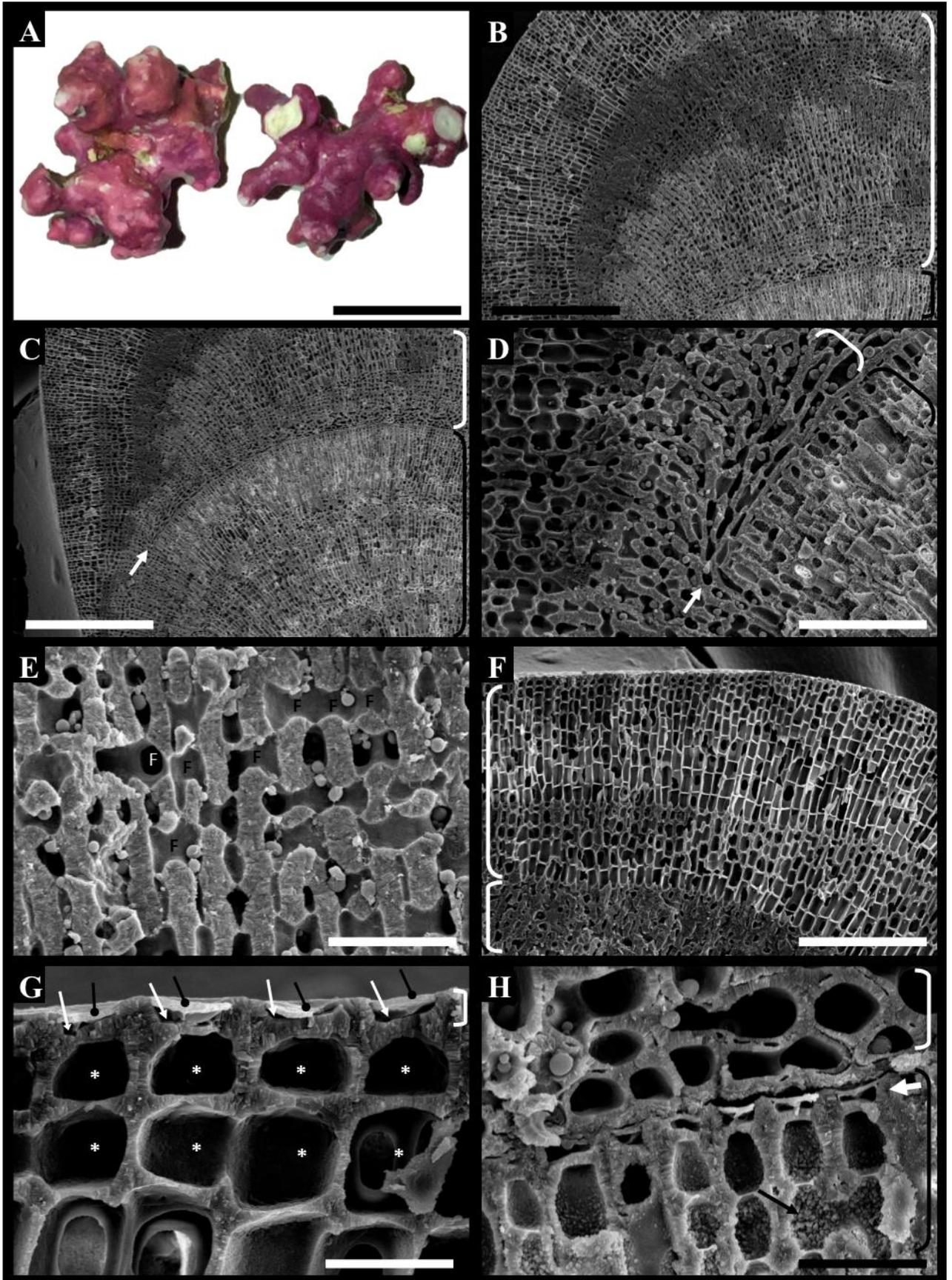
**Figure 14. Specimen LAF6970C.** A. Thallus habit showing numerous protuberances. Scale bar 5 mm. B. Median periclinal section and partial surface view of protuberance showing overgrown conceptacles (arrows). Scale bar 400  $\mu\text{m}$ . C. Magnified view of area indicated by white arrow in B. showing newly formed hypothallial filaments growing over an empty conceptacle (white arrow). Scale bar 110  $\mu\text{m}$ . D. Cross section of protuberance showing radial construction and overgrown conceptacles with putative aragonite infill (arrows) and conceptacle with an unidentified invertebrate organism (white arrow). Scale bar 450  $\mu\text{m}$ . Inset shows magnified view of unidentified invertebrate. Scale bar 32  $\mu\text{m}$ . E. Magnified view of lower left conceptacle shown in D. with needle-like crystals of putative aragonite. Each unit of the embedded scale is 10  $\mu\text{m}$ . F. Section of thallus showing perithallus with cell fusions (“F”). Each unit of the embedded scale is 2  $\mu\text{m}$ . G. Section and surface view of thallus showing epithallus (upper brackets), perithallus (lower left bracket) and intercalary meristem (arrow, “M”, \*). Each unit of the embedded scale is 3  $\mu\text{m}$ . H. Magnified view of same location shown in G. showing meristematic cells (“M”), recently divided meristematic cells (\*), and section and surface views of epithallial cells (brackets) showing a polygonal outline in surface view, thick cell walls, small round lumens (arrows, “L”), epithallial cell roofs (circle arrows) with one showing primary pit connection (“P”). Each unit of the embedded scale is 1  $\mu\text{m}$ .



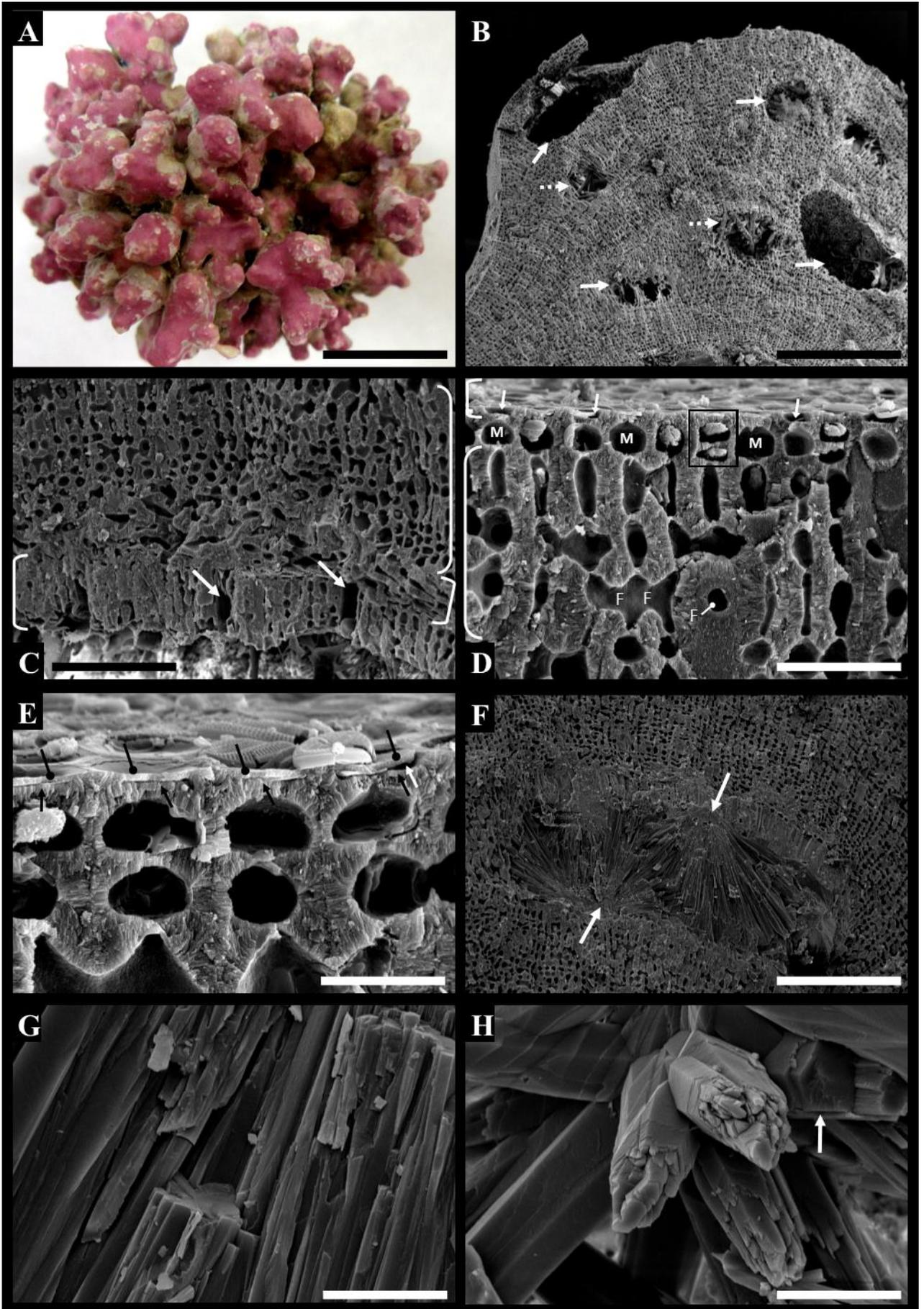
**Figure 15. Specimen LAF6521.** A. Thallus habit showing irregularly arranged protuberances. Scale bar 150 mm. B. Section and partial surface view of protuberance showing putative undeveloped conceptacle (circle arrow) and new vegetative growth layer (arrow). Scale bar 450  $\mu\text{m}$ . C. Section and partial surface view of protuberance showing magnified view of new growth layer with newly formed hypothallus (middle right bracket), perithallus (upper right bracket), and epithallus (left bracket) growing above older growth layer (lower right bracket). Scale bar 240  $\mu\text{m}$ . D. Section of thallus showing perithallus (bracket) with abundant cell fusions in the x-axis (“black F”) and z-axis (white “F”), meristematic cells (“M”), and epithallial cells with trapezoidal-shaped cell lumens (arrows) and intact epithallial cell roofs (circle arrows). Scale bar 24  $\mu\text{m}$ . E. Cross section of protuberance showing radial construction and location of new growth layer growing over older part of protuberance (arrow). Scale bar 400  $\mu\text{m}$ . F. Magnified view of new growth layer (upper bracket) growing out of perithallus (arrow) of older growth layer (lower bracket). Scale bar 195  $\mu\text{m}$ .



**Figure 16. Specimen LAF1437.** A. Thallus habit showing numerous protuberances. Scale bar 5 mm. Inset shows section of protuberance with multiple overgrown conceptacles (white arrows). Scale bar .7 mm. B. Section and partial surface view of same protuberance shown in Fig. 7A., inset, showing overgrown conceptacles with spherical mass of aragonite (circle arrow) and aragonite crystals in the form of needles (arrow). White and black arrow indicates location shown in G. Scale bar 500  $\mu\text{m}$ . C. Section of same protuberance shown in A (inset), B, showing conceptacle with aragonite crystals and conceptacle with conceptacle pore (circle arrow) and some aragonite infill (black arrow). Scale bar 400  $\mu\text{m}$ . D. Magnified view of area indicated by white arrow in Fig. C showing newly formed hypothallial filaments growing over the conceptacle roof. Scale bar 70  $\mu\text{m}$ . E. Section of thallus showing perithallus with cell fusions (“F”) and abundant unidentified spherical inclusions. Scale bar 35  $\mu\text{m}$ . F. Section of thallus showing perithallus (bracket) with cell fusions (“F”), meristematic cells (“M”), and epithallial cells with trapezoidal shaped lumens, one showing an intact epithallial cell roof (circle arrow). Scale bar 17.5  $\mu\text{m}$ . G. Section and surface view of portion of thallus indicated by white and black arrow shown in B. showing perithallus (arc) with cell fusion (“F”), meristematic cells (“M”) and epithallial cells lacking intact roofs showing trapezoidal shaped cell lumens and other epithallial cells showing intact roofs (circle arrows). Each unit of the embedded scale is 2  $\mu\text{m}$ . H. Magnified view of epithallial cell (upper cell) with intact roof (circle arrow) and partial view of meristematic cell (“M”) showing ultrastructure of calcified cell walls. Arrows indicate the four corners of the epithallial cell; white arrows indicate boundaries of adjacent filament cell walls, black arrows with white fill indicate boundary between epithallial cell wall and meristematic cell wall. Each unit of the embedded scale is 0.5  $\mu\text{m}$ .



**Figure 17. Specimen SPF57882.** A. Habit of specimen showing numerous protuberances (two fragments represent a single specimen which was fractured during preparation of material for DNA extraction and SEM). Scale bar 1 cm. B. Section of protuberance showing location of new (white bracket) and older (black bracket) growth layers. Scale bar 300  $\mu\text{m}$ . C. Section of same protuberance shown in B showing location of newly forming hypothallial filaments (white arrow) growing over surface of older growth layer (black bracket). Scale bar 350  $\mu\text{m}$ . D. Magnified view of newly formed hypothallus (arrow, white bracket) showing rectangular shaped cells. Scale bar 70  $\mu\text{m}$ . E. Section of thallus showing perithallus with cell fusions (“F”) and spherical inclusions. Scale bar 35  $\mu\text{m}$ . F. Section of thallus showing layer of perithallus with thick, heavily calcified cell walls (lower bracket) and a layer with thin, weakly calcified cell walls (upper bracket). Scale bar 140  $\mu\text{m}$ . G. Section of thallus showing epithallial cells with trapezoidal shaped cell lumens (arrows) and intact epithallial cell roofs (circle arrows). Note putative recently divided meristematic cells (\*). Scale bar 14  $\mu\text{m}$ . H. Magnified view of newly formed hypothallus shown in cross section (white bracket) growing over epithallus (arrow) of older growth layer (black bracket). Scale bar 17.5  $\mu\text{m}$ .



**Figure 18. Specimen PKYKOS7249.** A. Thallus habit showing numerous protuberances. Scale bar 20 mm. B. Section of protuberance showing overgrown conceptacles (arrows), some filled with aragonite crystals (perforated arrows). Scale bar 400  $\mu\text{m}$ . C. Section of protuberance showing newly forming hypothallus (lower right bracket) and perithallus (upper right bracket) growing over an old conceptacle roof (left bracket) including pores (arrows). Scale bar 70  $\mu\text{m}$ . D. Section of thallus showing perithallus (lower bracket) with cell fusions (“F”, circle arrow), meristematic cells (“M”) and recently divided meristematic cells (black outline), and section and partial surface view of epithallus (upper bracket). Epithallial cells lacking intact roofs (arrows) show trapezoidal shaped lumens. Scale bar 30  $\mu\text{m}$ . E. Recently divided epithallial cells with intact roof (circle arrows) and proximal cell wall (arrows). Scale bar 12 $\mu\text{m}$ . F. Overgrown conceptacle showing aragonite infill in the form of spherical masses with aragonite crystals forming from the roof (upper arrow) and floor of the conceptacle (lower arrow). Scale bar 180  $\mu\text{m}$ . G. Magnified view of aragonite crystals shown in F. Scale bar 10  $\mu\text{m}$ . H. Aragonite crystals in the form of needles showing hexagonal outline (arrows). Scale bar 14  $\mu\text{m}$ .

## Conclusões gerais

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- Baseados nos dados obtidos neste estudo, considerando a informatividade e a relação custo-benefício, os marcadores mais adequados para serem utilizados em estudos mais abrangentes das CCA são o *psbA*, o COI-5P, seguido do *rbcL*-3P.
- Poucas sequências geradas neste estudo corresponderam a sequências disponíveis nos bancos de dados públicos, o que sugere que possivelmente algumas das espécies estudadas são potencialmente novas para a ciência, ou pertencem a espécies descritas para os quais não existem sequências disponíveis. Assim, este estudo contribuirá significativamente para o enriquecimento dos bancos de dados para estas algas, especialmente considerando o Atlântico Sul, para onde poucas sequências estão disponíveis.
- Nossos resultados demonstraram que a diversidade de CCA no Brasil estava subestimada, principalmente para as Hapalidiales. Antes deste estudo, haviam 32 espécies CCA entre Corallinales e Hapalidiales referidas para a costa brasileira; apenas 10 baseadas em dados moleculares. Após este estudo, foram gerados dados moleculares para pelo menos 34 diferentes espécies pertencentes às ordens Corallinales e Hapalidiales.
- Antes deste estudo, haviam 21 espécies referidas para Brasil pertencentes à ordem Corallinales, apenas seis para as quais dados moleculares foram gerados; neste estudo foram gerados dados moleculares para 19 espécies diferentes desta ordem.
- Para as Hapalidiales, haviam sido referidas 11 espécies para a costa brasileira, apenas quatro para as quais dados moleculares foram gerados; neste estudo, foram gerados dados moleculares para ao menos 16 espécies diferentes desta ordem.

- Os caracteres morfológicos e anatômicos observados serão valiosos para estudos posteriores, servindo de base para comparação.
- Não foi possível elencar caracteres morfológicos e anatômicos de maior ou menor relevância na maioria dos espécimes analisados, principalmente por se tratar de um conjunto de amostras de diversos gêneros diferentes.
- Para a ordem Sporolithales, abordada no capítulo 2, sete espécies haviam sido citadas para a costa brasileira, três delas foram propostas nos últimos dois anos, sendo duas delas baseadas em dados moleculares. Após este estudo, foram propostas duas novas espécies para a ciência e, portanto, nove espécies desta ordem passarão a ser reconhecidas para o Brasil, após a publicação do trabalho submetido (cap. 2). Todas as espécies antes citadas foram referidas para o infralitoral em profundidades de 25 até 85 m, as duas espécies propostas no capítulo 2 são espécies encontradas no mesolitoral ou em profundidades até 15 metros.
- O uso de ferramentas moleculares, como a técnica de DNA barcoding, é fundamental e abre uma nova perspectiva para os estudos da diversidade das CCA, especialmente considerando as técnicas e preparações complexas e demoradas necessárias para estudar adequadamente a anatomia deste grupo.
- Alguns dos nossos espécimes parecem estar estreitamente relacionados com espécies do Indo-Pacífico. Serão necessários mais estudos, que utilizem diferentes marcadores, para entender esta relação entre a flora do Atlântico e do Indo-Pacífico.
- Alguns dos nossos espécimes estão estreitamente relacionados com espécies do infralitoral do Golfo do México, o que nos indica que os nomes aplicados à

táxons da costa brasileira devem ser considerados como possíveis epítetos para os espécimes desta localidade; e vice-versa.

- No capítulo 3 tratamos da diversidade molecular, morfológica e anatômica das espécies de *Lithothamnion* encontradas no Golfo do México e no Brasil e mostramos que de fato o gênero é polifilético e nessas regiões está dividido em dois grandes clados. Ainda neste capítulo discutimos a hipótese de que as Corallinophycidae podem apresentar crescimento através da divisão de células de um meristema intercalar, porém estudos envolvendo outras técnicas de microscopia serão necessários para elucidar o padrão de desenvolvimento da atividade meristemática na nesta sub-classe.
- Considerando as três ordens de CCA (Corallinales, Hapalidiales e Sporolithales), este estudo representa a primeira tentativa de desvendar de forma mais ampla a diversidade de espécies CCA encontradas ao longo da costa brasileira, utilizando dados moleculares.