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Evaluation of the diversity of deep-water sponges of the Selvagens Islands through DNA *barcoding*.

Avaliação da Diversidade de Esponjas de Mar Profundo das Ilhas Selvagens através de DNA *barcoding*

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Resumo

As esponjas (Filo Porifera) estão agrupadas em quatro classes, sendo a Demospongiae a maior e mais diversificada. Os habitats compostos por esponjas de profundidade são considerados hotspots de biodiversidade, sendo parte das comunidades bentónicas onde desempenham um papel muito importante no ecossistema. Estes são, no entanto, considerados ecossistemas marinhos vulneráveis (VMEs) de prioridade de conservação. O objetivo neste trabalho foi identificar, através de códigos de barras de ADN, esponjas de profundidade recolhidas numa área sub estudada do Atlântico Nordeste, as Ilhas Selvagens. Foram analisados dezoito espécimes recolhidos na campanha EMEPC/M@rBis/Selvagens2010. Destes, oito espécimes foram identificados e atribuídos a cinco géneros - *Axinella*, *Topsentia*, *Characella*, *Petrosia* e *Calyx* - representando cinco famílias (Axinellidae, Halichondriidae, Pachastrellidae, Petrosiidae, Phloeodictyidae) com base nos seus códigos de barras COI mtDNA e 28S rRNA. Discutimos ainda as afinidades filogenéticas e biogeográficas dos taxa identificados, à luz da fauna de esponjas do Atlântico Nordeste e do Mediterrâneo. Os dados gerados serão ainda complementados com a caracterização morfológica para permitir identificações precisas ao nível das espécies. Esta abordagem integrativa é fundamental para compreender a diversidade e distribuição das espécies de águas profundas e contribuir para a sua conservação.

Porifera + Demospongiae +Barcode + Filogenia + Mar Profundo

Abstract

Sponges (Phylum Porifera) are grouped into four classes with Demospongiae being the largest and most diverse. The habitats composed of deep-sea sponges are considered biodiversity hotspots, being part of the benthic communities where they play a very important role in the ecosystem. These are, however, considered vulnerable marine ecosystems (VMEs) of conservation priority. This study aimed to identify, by means of DNA barcoding, deep-sea sponges collected in an understudied area of the Northeast Atlantic, Selvagens Islands. Eighteen specimens collected in the EMEPC/M@rBis/Selvagens2010 campaign were analyzed. Of these, eight specimens were identified and assigned to five genera - *Axinella*, *Topsentia*, *Characella*, *Petrosia* and *Calyx* - representing five families (Axinellidae, Halichondriidae, Pachastrellidae, Petrosiidae, Phloeodictyidae) on the basis of their COI mtDNA and 28S rRNA barcodes. We further discuss the phylogenetic and biogeographic affinities of the identified taxa in light of the sponge fauna of the Northeast Atlantic and Mediterranean. The data generated will be further complemented with morphological characterization to allow accurate species-level identifications. This integrative approach is fundamental to understand the diversity and distribution of deep-sea species and contribute to their conservation.

Porifera + Demospongiae +Barcode + Phylogeny + Deep Sea

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1. Introduction

The Selvagens Islands are a set of oceanic islands located in the southernmost point of Portugal (Friedlander et al.,2016, 2017; Santana, 2016). Their geological history is related to the opening and expansion of the Atlantic Ocean (~200 million years) and they originated from volcanic activity (Santana, 2016). The Selvagens Nature Reserve is listed in The International Union for Conservation of Nature (IUCN) Category Ia Protected Area. The Nature Reserve is home to representative habitats and species such as deep-sea sponges, organisms that due to their ecological function are very important for the marine ecosystem, which makes their conservation imperative.

Sponges are fully aquatic invertebrates belonging to the Porifera phylum and are ecologically important animals. They constitute benthic environments and can occupy 80% of the available substrate (Webster et Thomas, 2016; González et al., 2019). This characteristic makes sponges habitat providers for a large number of species. In addition, their filter-feeding mechanism links benthic and pelagic zones. Sponges are involved in mediating biogeochemical fluxes by respiration of organic matter, facilitating the consumption and release of nutrients such as nitrate, nitrite, ammonium, and phosphate (Webster et Thomas, 2016). Despite the simplistic constitution of sponges, these organisms can harbor a high number of microorganisms in their cells. These symbionts can reach 35% of the biomass of a sponge, contributing to the physiological and ecological aspects of the sponges (Webster et Thomas, 2016).

Deep-sea sponges are the oldest metazoans that are present in the deep-sea environment, which is currently considered one of the most important biodiversity hotspots (Borchiellini et al., 2000; Van Soest et al.,2012; Cathalot et al., 2015; Kahn et al., 2015; Robertson et al., 2017). Due to their limited movement capacity, sponges are frequently endemic to regional or local areas (Van Soest et al.,2012). They occupy a wide variety of ecosystems in all latitudes and depths from the eulittoral zone to the deepest zone, the hadal layer (Araújo et al., 2003; Van Soest et al.,2012). They also play a key role in nutrient recycling, acting at the microscale level on the ecosystem and impact the remineralization and bioturbation processes (Beazley et al., 2013).

Moreover, deep-sea sponges are key elements in habitat construction. Some authors consider them as ecosystem engineers. A significant part of sponge communities is found in deep waters as sponge aggregations or sponge grounds, resulting in important 3D complexes that constitute almost 90% of the invertebrate biomass of these ecosystems (Kutti et al., 2013; Beazley et al.,2013). By generating microhabitats, these aggregations increase the local biodiversity to the point of being considered biodiversity hotspots (Kutti et al., 2013; Beazley et al.,2013). The structural benefits of these sponge grounds are not limited to providing habitats. They also serve as breeding, spawning, nursery, feeding, and refuge areas for a diverse array of fish and crustaceans, among others (Bell, 2008; Van Soest et al., 2012; Beazley et al.,2013; 2015; Howell et al.,2017).

While long-lived, deep-sea sponges have slow growth rates (Kutti et al., 2013) and are highly susceptible to disturbance. These characteristics make them highly vulnerable and fragile animals (Kutti et al., 2013; Beazley et al., 2015). It is well understood that deep-sea sponges have a sporadic recruitment time (Bell et al., 2017); however, information about reproduction, biogeographic patterns, and distribution is still sparse (Xavier et al., 2015; Bell et al.,2017). Taking into account aspects of the life history of sponges and highlighting their fragility, they are now considered as vulnerable marine ecosystems (VME) following resolution 61/105, in discussion at the United Nations General Assembly (UNGA) and The FAO International Guidelines for the Management of Deep-sea Fisheries (Beazley et al.,2013,2015).

It is important to highlight that there are many aspects that are poorly known about these organisms, biotic and abiotic factors that have become environmental stressors and generate a negative impact on these communities. Anthropogenic activity has also impacted deep-sea sponges resulting in substrate loss, habitat fragmentation and changes in geochemical, topographical, and chemical composition (Gollner et al., 2017; Clark et al., 2020), some of which may be fatal for these organisms (Bell et al., 2017; Vad et al., 2018). It is essential to obtain this information on sponge species so that policy decisions can be made with a full understanding of the likely impacts on these organisms.

For all the above-cited reasons, it is important to increase the knowledge of the species present in these environments. The overall aim of this study was to perform molecular characterization of deep-sea sponges from the Selvagens Islands, using a DNA barcoding approach. Specific aims were to generate a voucher-linked barcode library of deep-sea sponges of the Selvagens and to investigate their systematic and biogeographic affinities through phylogenetic reconstructions.

2.Materials and Methods

2.1. Study area and sponge sampling

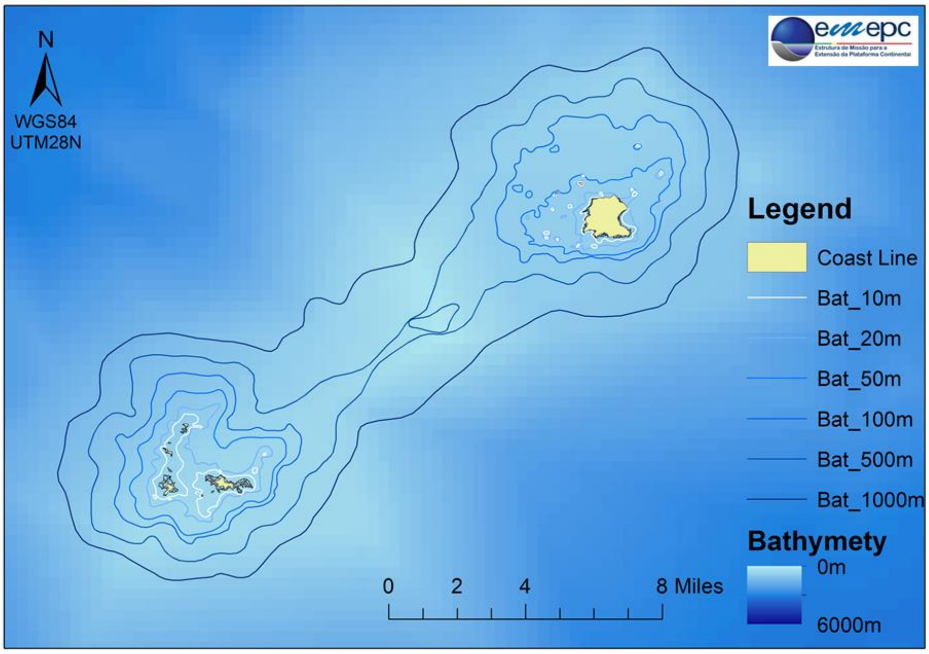

Figure 1.Selvagens Islands deep sea sponge sampling area, Lat (N) 30° 06.284' Long (W) -15° 54.982', 699-848m depth. Adapted from Estrutura de Missão para a Plataforma Continental (EMEPC).

Samples were collected in the Selvagens Islands (Selvagem Pequena and Selvagem Grande) in June 2010, during the oceanographic campaign EMEPC/M@rBis/Selvagens2010 of the Portuguese Task Group for the Extension of the Continental Shelf (EMEPC). The deep sea sampling was made at four different stations (see Table 1) on board the oceanographic vessel Navio República Portuguesa Almirante Gago Coutinho, using the Remotely Operated Vehicle (ROV) Luso.

The voucher specimens were preserved in alcohol 97%, and kept in a freezer at -80ºC. A total of 18 specimens were provided by EMEPC for the elaboration of this work.

Table 1 Samples collected in Selvagens used in the present study

Specimen code	Location	Station	Lat.	Long.	Depth (m)
E03	Selvagem Grande	L10D05	30° 06.284'	-15° 54.982'	699
E04	Selvagem Grande	L10D05	30° 06.284'	-15° 54.982'	699
E11	Selvagem Pequena	L10D04	30° 00.680’	-16° 00.164’	848
E12	Selvagem Pequena	L10D04	30° 00.680’	-16° 00.164’	848
E15	Selvagem Grande	L10D06	30° 06.284'	-15° 54.982'	699
E16	Selvagem Grande	L10D06	30° 06.284'	-15° 54.982'	699
E17	Selvagem Grande	L10D06	30° 06.284'	-15° 54.982'	699
E18	Selvagem Pequena	L10D04	30° 00.680’	-16° 00.164’	848

2.2. DNA Extraction,amplification, and sequencing

For the genomic DNA extraction, REDExtract-N-Amp™ Tissue PCR Kit by Sigma-Aldrich was used following the manufacturer’s instructions. Two independent gene fragments were selected for this work: the mitochondrial cytochrome c oxidase subunit 1 (COI) and the nuclear large ribosomal subunit 28S rRNA. These fragments are usually used in sponge systematics, due to the paucity of complex morphological characters, and in many sponges high degrees of homoplasy, which makes classification ambiguous and in some groups unresolved (Hooper & Van Soest 2002; Erpenbeck et al., 2006)). The mtDNA COI is generally used as a barcode, with adequate resolution at the family level but insufficient variability at lower levels (Erpenneck et al., 2006). The 28S has been used to resolve inter-species relationships in sponges of the order Tetractinellida (Cardenas et al., 2011).

The two markers, COI and 28S rRNA, were amplified using the primer pairs LCO1490 - HCO2198 (5’ -GGT CAA CAA ATC ATA AAG ATA TTGG-3’; 5’-TAA ACT TCA GGG

TGA CCA AAA AAT CA-3’) and C1’ASTR-D2 (‘5-ACC CGC TGA ACT TAA GCAT-3’; D2 5’- TCC GTG TTT CAA GAC GGG-3’), respectively. PCR amplification reactions were performed in a 20 µl total reaction volume with 10 µl of REDExtract-N-ampl PCR reaction mix (Sigma–Aldrich), 0.8 µl of each primer (10 µM), 4.4 µl of Sigma-water and 4 µl of template DNA. PCR profiles were: 94 °C /5 min ; 37 cycles [94°C 1min, 47 °C / 1 min ,72 °C / 1 min 30s]; 72°C / 6 min ; 16 hold for the mtDNA COI; and (95 °C /3 min ; 35 cycles [95°C 30s, 56-59 °C / 45s ,72 °C / 1 min]; 72°C / 5 min ; 16 hold) for 28S rRNA. The same primers were used for the sequencing reaction, and the PCR products were purified and sequenced in STABVIDA (http://www.stabvida.net/).

2.3.Phylogenetic Analyses

Obtained sequences were checked visually for errors and editing was performed with CodonCode Aligner version 9.0.2.. Closest matches to sequences available on GenBank were made by BLAST searches (http:// blast.ncbi.nlm.nih.gov), and all sequences within the same taxon (and taxonomic rank) available were downloaded for the phylogenetic reconstructions (https://www.ncbi.nlm.nih.gov/genbank/) (See Table S 1). For alignment, MUSCLE was used in MEGAX version 11.0.9. Sequence alignments were performed separately for each gene (COI and 28S). Outgroups for the various analyses were selected to ensure they were close to (but never part of) the ingroup taxa. So, in most cases, we selected members of another genus of the same family as the ingroup. For this selection, we followed the most recently proposed classification of the class Demospongiae (Morrow & Cárdenas, 2015) and several molecular systematics studies performed with mitochondrial and ribosomal markers within the class (e.g. Gazave et al. 2010, Cárdenas et al., 2011, Redmond et al. 2013).

MEGAX version 11.0.9 was used to find the model that best fit the data. The models suggested by the software for COI mtDNA analysis, HYC+ G+l and T92+ G and for 28S rRNA analysis were the K2+G model and T92+G. Phylogenetic reconstructions were performed for the individual markers under Bayesian Inference as implemented in MrBayes v3.2 (Ronquist et al., 2012) and Maximum Likelihood as implemented in RaxML v.8 (Stamatakis,2014) through MEGAX. Bayesian inference reconstruction was obtained with Metropolis-coupled Markov chains (MCMC) over 2000 and 14000 generations, sampled every 100 generations and with a burnin of 10%, using the models suggested by MEGAX. For the Maximum Likelihood analysis, the Kimura 2-parameter model was used, and 1000 fast bootstrapping replicates were performed. The phylogenetic trees obtained were visualized in FigTree v.1.4.4 and edited in Inkscape 0.92.4.

The haplotype networks were constructed using the method of Templeton, Crandall and Sing (1992) implemented in TCS. This algorithm calculates the characters of each sequence, recording the raw number of changes between sequences (Clement *et al.*, 2002). Finally, the haplotype network was visualized in PopArt software version 1.7 (Leigh and Bryant, 2015), for editing and visualization in Inkscape 0.92.4.

3. Results

After a few attempts, of the 18 initial specimens it was only possible to obtain, in total, 13 sequences for eight specimens analyzed. Of these, six were of the mtDNA COI gene, representing species across the families Axinellidae, Halichondriidae, Pachastrellidae, Petrosiidae, Phloeodictyidae. Seven sequences were obtained for the partial 28S rRNA gene representing species across the families Axinellidae, Pachastrellidae, Petrosiidae, Phloeodictyidae (Table 2).

Table 2.Top BLAST matches of newly generated sequences with those available on GenBank

Specimen	Locality, depth (m)	Genetic marker	Sequence length (bp)	Top match	Accession no.	% identity
E03	Selvagens Islands, 699	COI	634	*Axinella verrucosa*	LN868210.1	99.37%
E11	Selvagens Islands, 848	28S	816	*Axinella damicornis*	KX688749.1	84.25%
E12	Selvagens Islands,	28S	786	*Axinella damicornis*	KX688749.1	80.69%

	848					
E04	Selvagens Islands, 699	28S	474	*Petrosia ficiformis*	KX688752.1	95.00%
E04	Selvagens Islands, 699	COI	648	*Petrosia durissima*	MT491553.1	99.69%
E15	Selvagens Islands, 699	28S	815	*Calyx* sp.	MW881149.1	98.14%
E15	Selvagens Islands,699	COI	643	*Calyx* sp.	MT491624.1	100.00%
E16	Selvagens Islands, 699	28S	797	*Characella* sp.	KC869483.1	97.61%
E16	Selvagens Islands, 699	COI	650	*Characella* sp.	MT491490.1	99.69%
E17	Selvagens Islands, 699	28S	768	*Characella pachastrelloides*	HM592778.1	100.00%
E17	Selvagens Islands, 699	COI	643	*Characella* sp.	MT491490.1	99.84%
E18	Selvagens Islands, 848	COI	635	*Topsentia ophiraphidites*	MZ487414.1	99.37%

Family Axinellidae, Genus *Axinella*

Haplotype networks were obtained separately for the mtDNA COI and the 28S rRNA regions. The 539 bp alignments of the mtDNA COI gene comprised 11 haplotypes in 22 specimens, of which one belongs to sequences obtained in this work. For specimen E03, 8 bp substitutions were necessary to connect to another *Axinella* sp. from south China (sequence retrieved from GenBank). More than 80 substitutions were necessary to connect this specimen with other *Axinella* species (Fig.2).

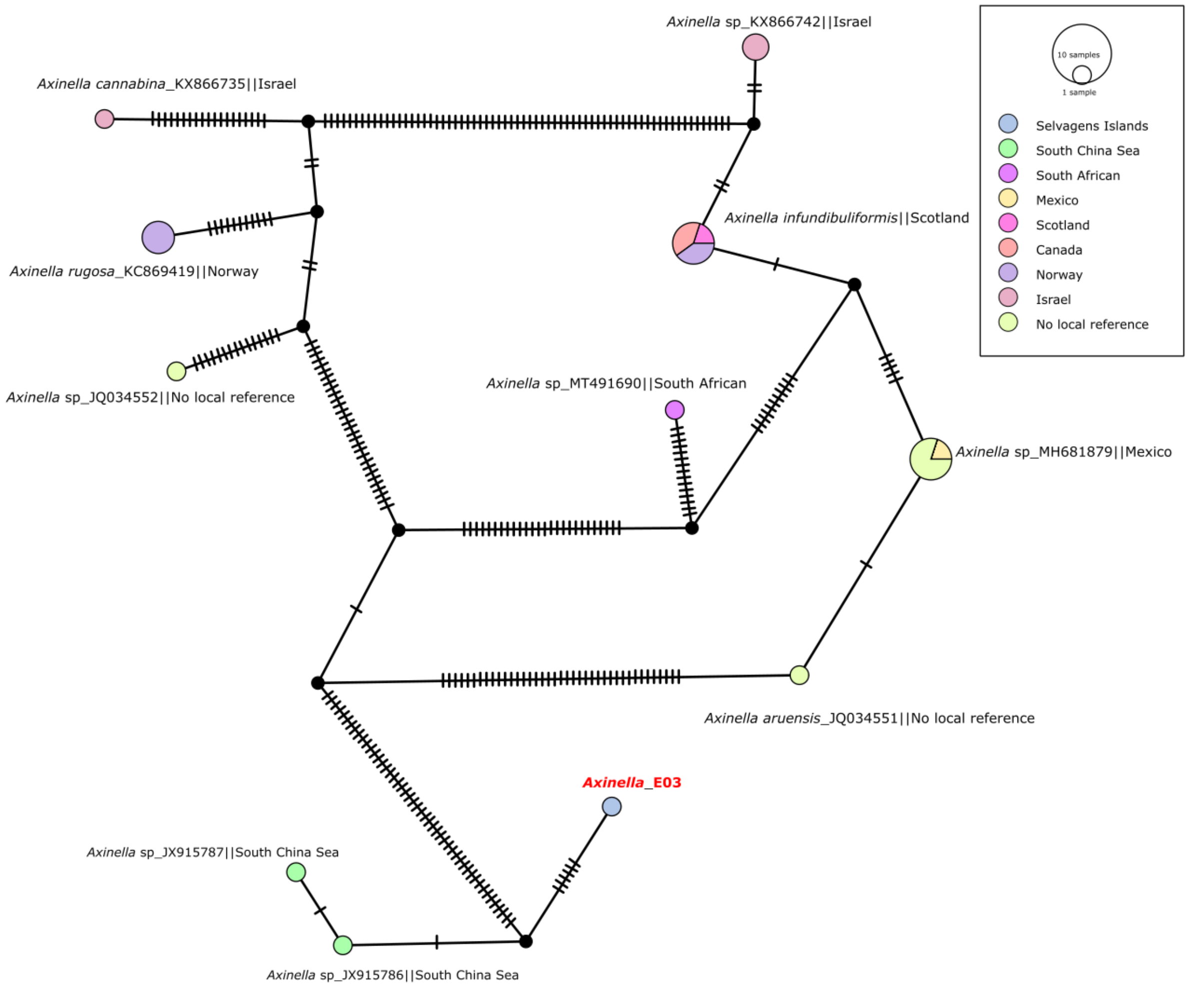

Figure 2.Haplotype network for the genus *Axinella*, constructed from COI mtDNA sequences, composed of 22 individuals from different geographic regions. Each circle represents one haplotype, and the size of the circle is proportional to the number of sequences. The lines denote 1 bp change; the black circles represent hypothetical haplotypes (not observed) that are intermediate between the observed haplotypes.

In the 28S rRNA haplotype networks, the 339 bp alignments comprised 16 haplotypes in 21 specimens, of which three belong to sequences obtained in this work. For specimen E11, a total of 63 substitutions were required to connect to *Axinella damicornis* reported for the Mediterranean. Specimen E12 required 70 substitutions to connect also with *Axinella damicornis*. Specimen E03 required a total of 40 substitutions to connect with *Axinella verrucosa* reported from the Mediterranean, with this species requiring the fewest substitutions (Fig. 3).

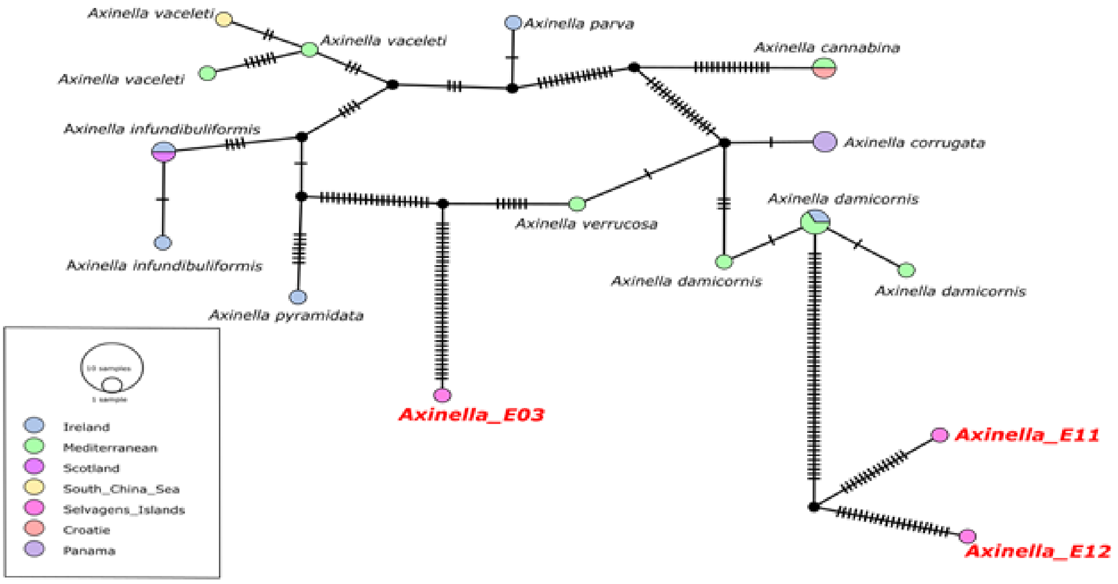

Figure 3.Haplotype network for the genus *Axinella*, constructed from sequences of the 28S rRNA region, composed of 21 individuals from different geographic regions. The circles represent the haplotypes and the size of the circles is proportional to the number of individuals. The lines denote the change of base pair, the black circles represent hypothetical haplotypes (not observed) that are intermediate between the observed haplotypes.

Phylogenetic trees were obtained separately for the mtDNA COI and the 28S rRNA. In the COI tree, the clade comprises our specimen of *Axinella* E03; other species of *Axinella* spp from South China are found. This clade is well supported (PP=1). As sister clades, the first consists of *Axinella cannabina* from the Mediterranean and *Axinella* sp (PP=0.55); the other clade was formed by 3 specimens of *Axinella rugosa* (PP=0.98) from Israel and Norway (Fig. 4).

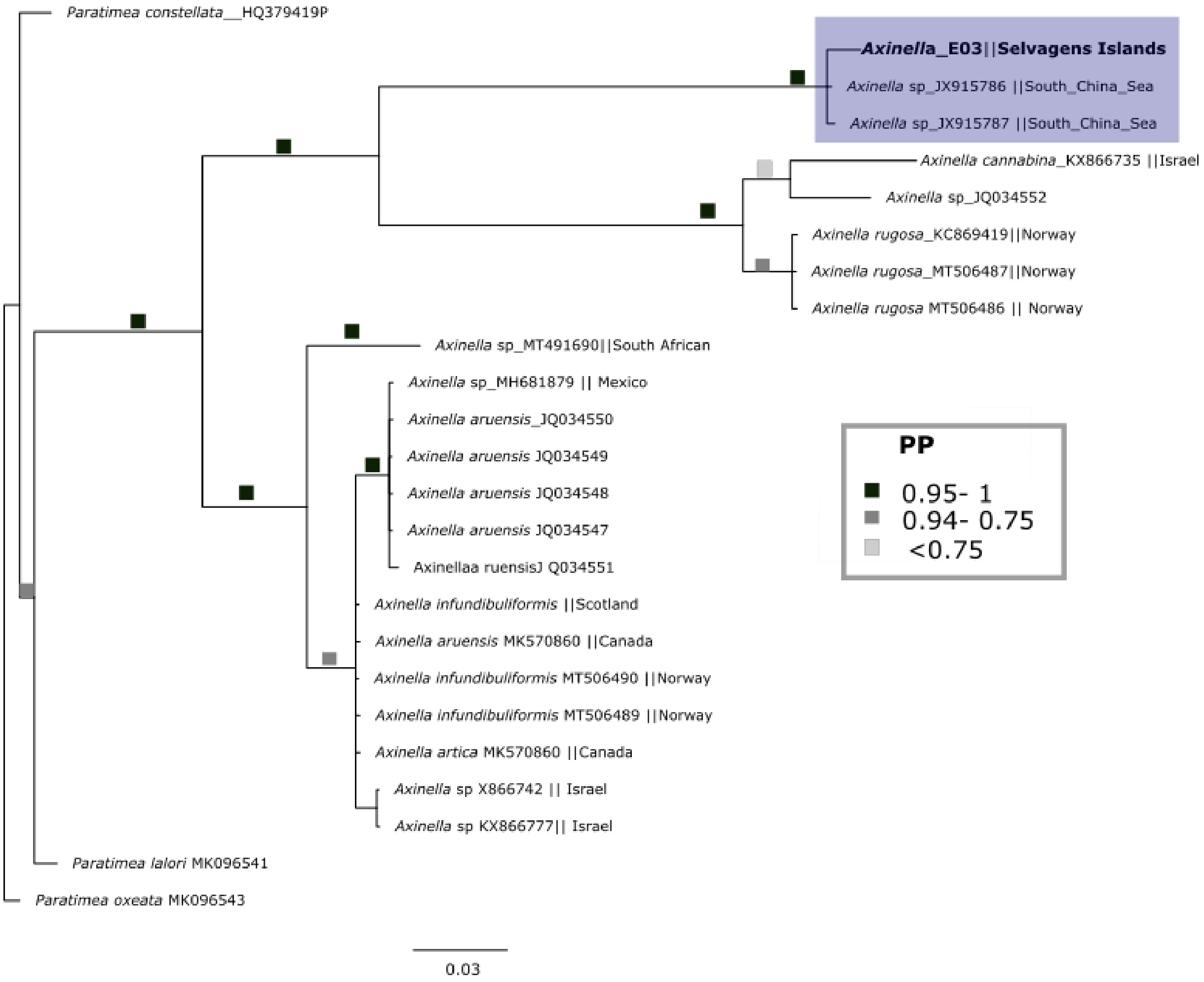

Figure 4.Tree resulting from the analyses of the mtDNA COI dataset composed of 23 individuals from different geographic regions.The topology presented corresponds to the Bayesian Inference analysis. Bayesian posterior probabilities (PP) values are provided on the branches. The tree is rooted on *Paratimea*. The clade encompassing the *Axinella* sequence generated in the present study (in bold font) are highlighted in color.

In the 28S rRNA tree, specimens E11 and E12 were placed in a highly supported clade (PP=1) with a sister clade of sequences of specimens identified as *Axinella damicornis* with low support (PP=0.56). Specimen E03 is not placed in a well-defined clade but the closest species is *Axinella verrucosa*.

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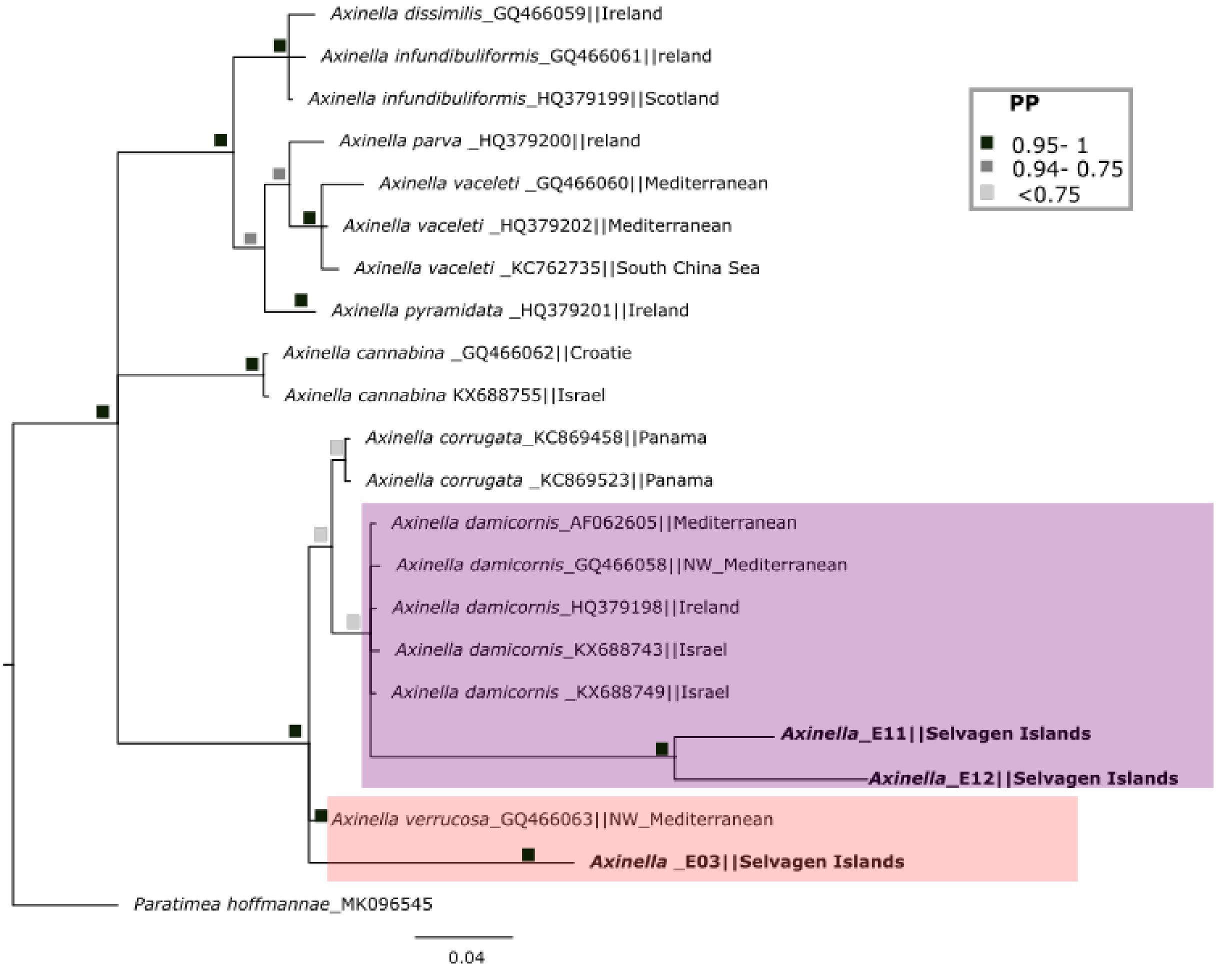

Figure 5.Tree resulting from the analyses of the 28S rRNA dataset composed of 21 individuals from different geographic regions.The topology presented corresponds to the Bayesian Inference analysis. Bayesian posterior probabilities (PP) values are provided on the branches. The tree is rooted on *Paratimea*. Genus encompassing *Axinella* species found in the present study are highlighted in color.

Family Halichondriidae, Genus: *Topsentia*

The 419 bp alignments of the mtDNA COI gene comprised five haplotypes in 43 specimens, of which one belongs to sequences obtained in this work. For specimen E18, three bp substitutions were necessary to connect to another *Topsentia ophiraphidites* from the Caribbean (sequence obtained through Genbank). More than 30 substitutions were necessary to connect this specimen with other *Topsentia* species (Fig 6).

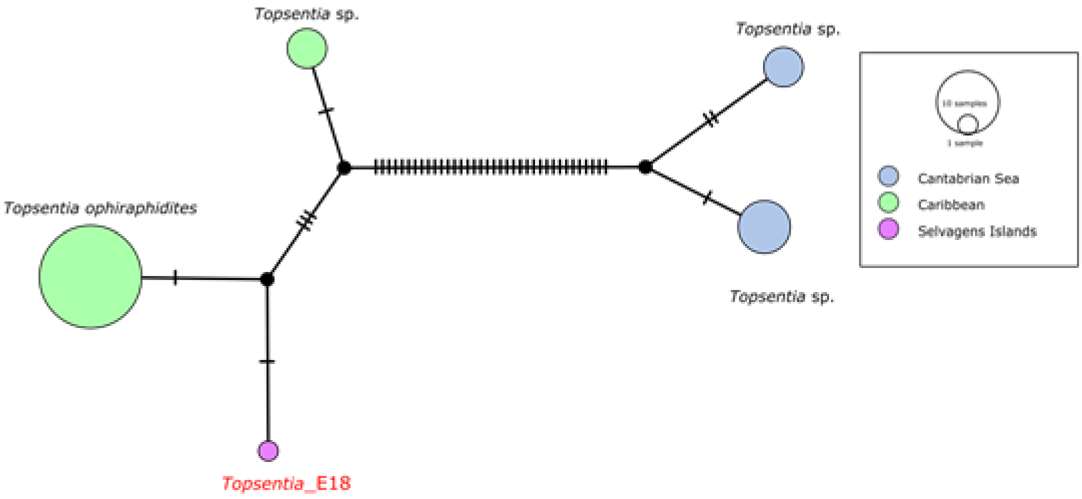

Figure 6.Haplotype network for the genus *Topsentia*, based on COI mtDNA sequences, composed of 43 individuals from different geographic regions.

In the COI tree, the clade comprising our *Topsentia* E18 specimen is a moderately supported clade (PP=0.92), which includes *Topsentia ophiraphidites* species from the Caribbean (Fig. 7). No sequences for the 28S rRNA gene were obtained.

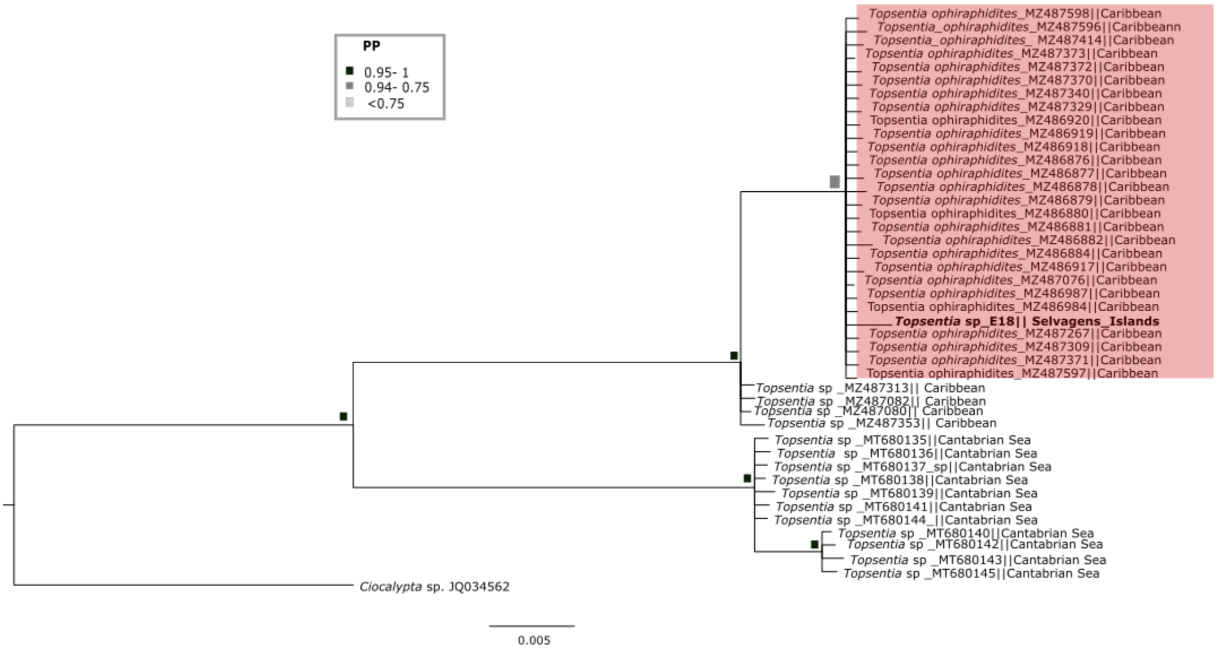

Figure 7. Tree resulting from the analyses of the mtDNA COI dataset composed of 42 individuals from different geographic regions.The topology presented corresponds to the Bayesian Inference analysis. Bayesian posterior probabilities (PP) values are provided on the

branches. The tree is rooted on *Ciocalypta*. Genus encompassing *Topsentia* species found in the present study are highlighted in color.

Family Pachastrellidae, Genus *Characella*

The 582 bp aligned mtDNA COI gene comprised five haplotypes in 17 specimens, of which two belong to sequences obtained in this work. The two specimens *Characella* E16 and E17 share the same haplotype with *Characella pachastrelloides* from different localities Ireland, Norway, and Japan (sequence obtained through Genbank) (Fig. 8).

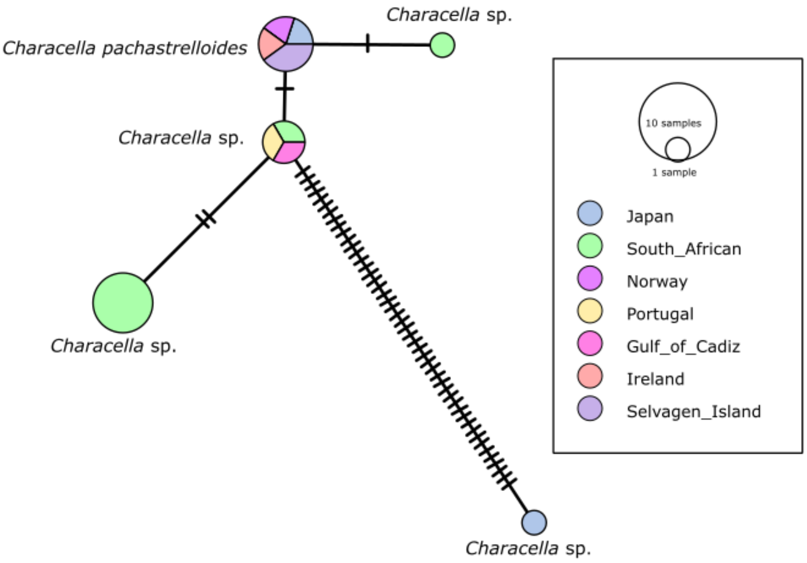

Figure 8.Haplotype network for the genus *Characella*, constructed from COI mtDNA sequences, composed of 17 individuals from different geographic regions. There is no evidence of any geographical pattern or genetic structure showing different haplotypes according to the species.

In the 28S rRNA haplotype networks, the 767 bp alignments comprised three haplotypes in seven specimens, of which two belong to sequences obtained in this work. For specimen *Characella* E16 a total of 11 substitutions were required to connect to *Characella pachastrelloides*. Specimen *Characella* E17 shares haplotypes with sequences of specimens identified as *Characella pachastrelloides* from different locations: Norway, Portugal, Gulf of Cadiz and Scotland (Fig 9).

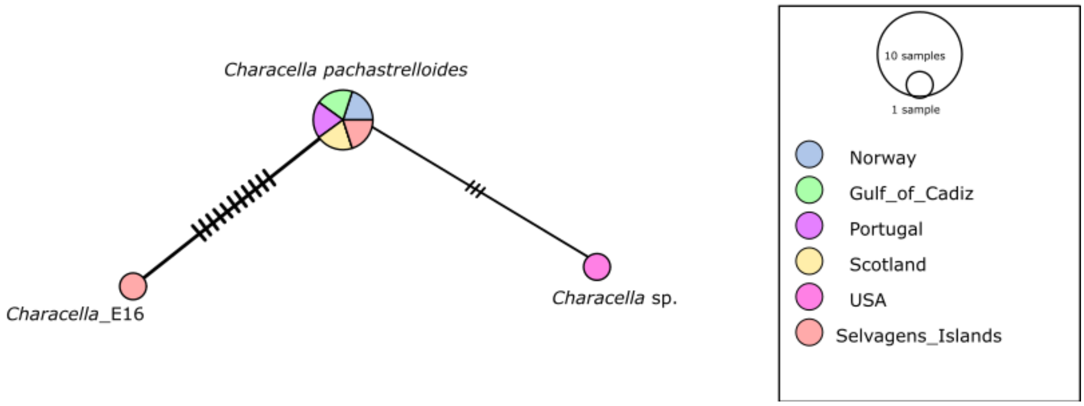

Figure 9.Haplotype network for the genus *Characella*, constructed from sequences of the 28S (rRNA) region, composed of nine individuals from different geographic regions, presenting an abundant central haplotype, showing a separation into three species.

In the COI tree, the clade comprising our *Characella* E16 and E17 specimens is a moderately supported clade (PP= 0.82), comprising sequences of specimens identified as *Characella pachastrelloides* from Norway, Japan and Ireland as well as a *Characella* spp. sequences from South Africa.

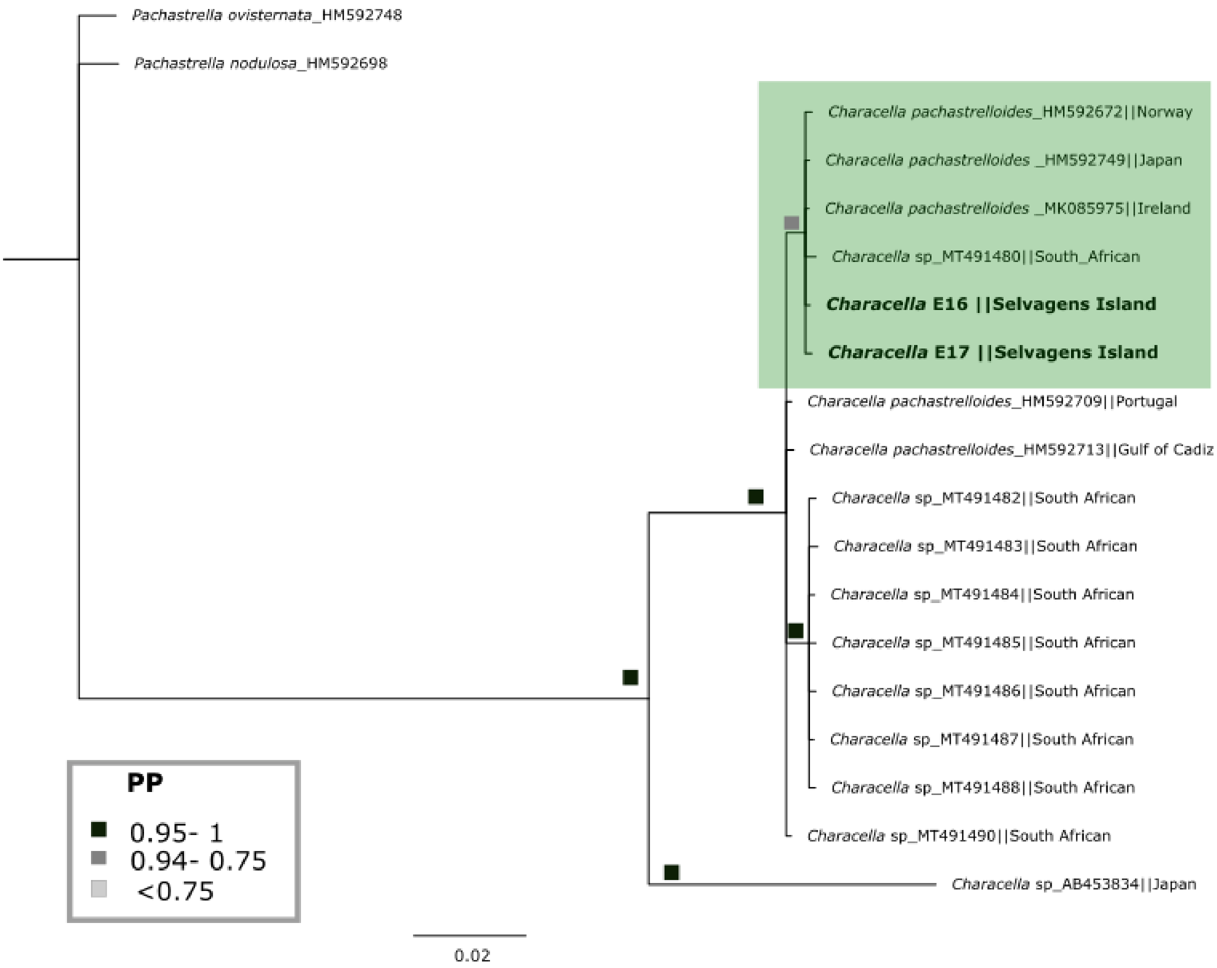

Figure 10. Tree resulting from the analyses of the mtDNA COI dataset composed of 17 individuals from different geographic regions. The topology presented corresponds to the Bayesian Inference analysis. Bayesian posterior probabilities (PP) values are provided on the branches. The tree is rooted on *Pachastrella*. Genus encompassing *Characella* species found in the present study are highlighted in color.

In the 28S rRNA tree, the clade containing our specimens as in the COI tree is moderately supported (PP= 0.93), comprising sequences of specimens identified as *Characella pachastrelloides* sequences from Norway, Gulf of Cadiz, Portugal and Scotland.

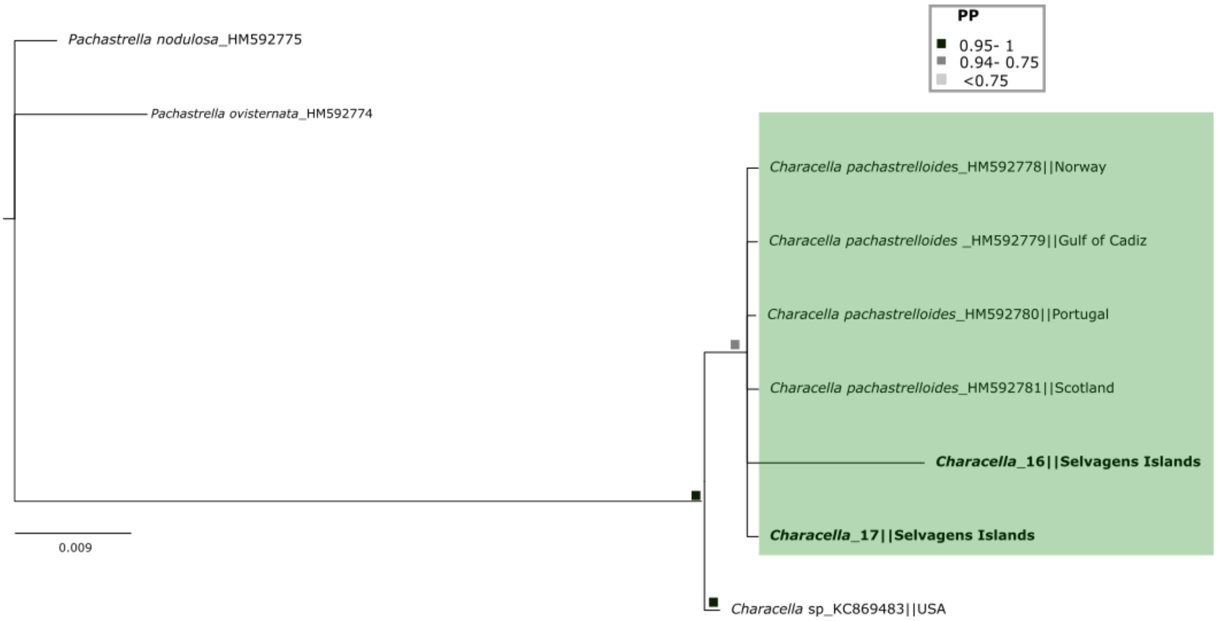

Figure 11.Tree resulting from the analyses of the 28S rRNA dataset composed of seven individuals from different geographic regions. The topology presented corresponds to the Bayesian Inference analysis. Bayesian posterior probabilities (PP) values are provided on the branches. The tree is rooted on *Pachastrella*. Genus encompassing *Characella* species found in the present study are highlighted in color.

Family Petrosiidae, Genus *Petrosia*

The 524 bp alignments of the mtDNA COI gene comprised nine haplotypes in 43 specimens, of which one belongs to sequences obtained in this work. For specimen *Petrosia* E04 a eight bp substitution was necessary to connect with *Petrosia ficiformis* from the Mediterranean, the sequence was retrieved from Genbank)(Fig. 12).

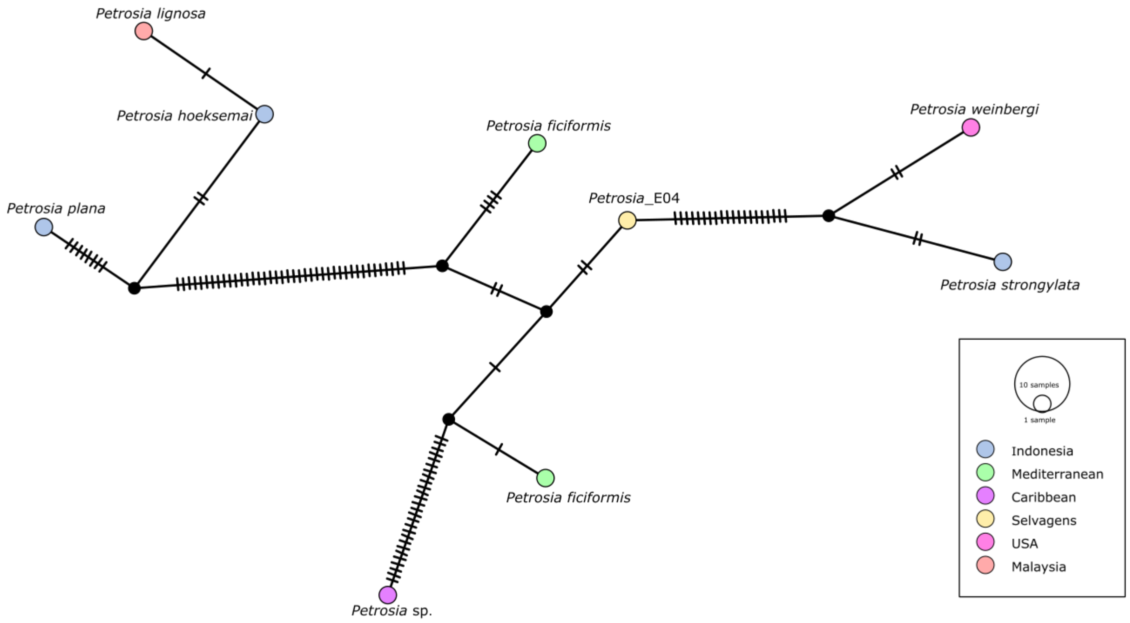

Figure 12.Haplotype network for the genus *Petrosia*, based on sequences of the 28S region (rRNA), composed of nine individuals from different geographic regions. No geographic pattern or genetic structure showing different haplotypes according to the species is evidenced.

In the 28S rRNA haplotype networks, the 232 bp alignments comprised nine haplotypes in 13 specimens, one of which belongs to sequences obtained in this work. For specimen *Petrosia* E04 a total of two substitutions were required to connect with *Petrosia ficiformis* reported for the Mediterranean (Fig. 13)**.**

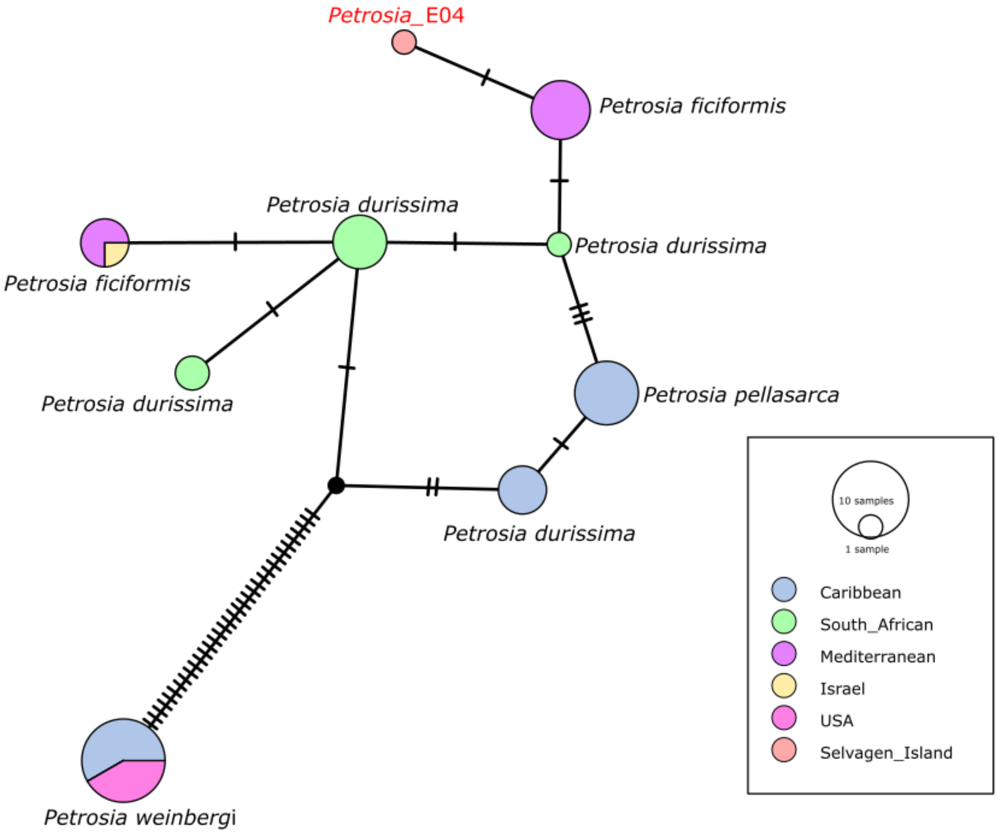

Figure 13.. Haplotype network for the genus *Petrosia*, constructed from sequences of the 28S region (rRNA), composed of nine individuals from different geographic regions. No geographic pattern or genetic structure presenting different haplotypes according to the species is evidenced.

In the COI tree, the clade containing our specimen, *Petrosia* E04, is moderately supported (PP= 0.92), comprising *Petrosia ficiformis* species from the Mediterranean.

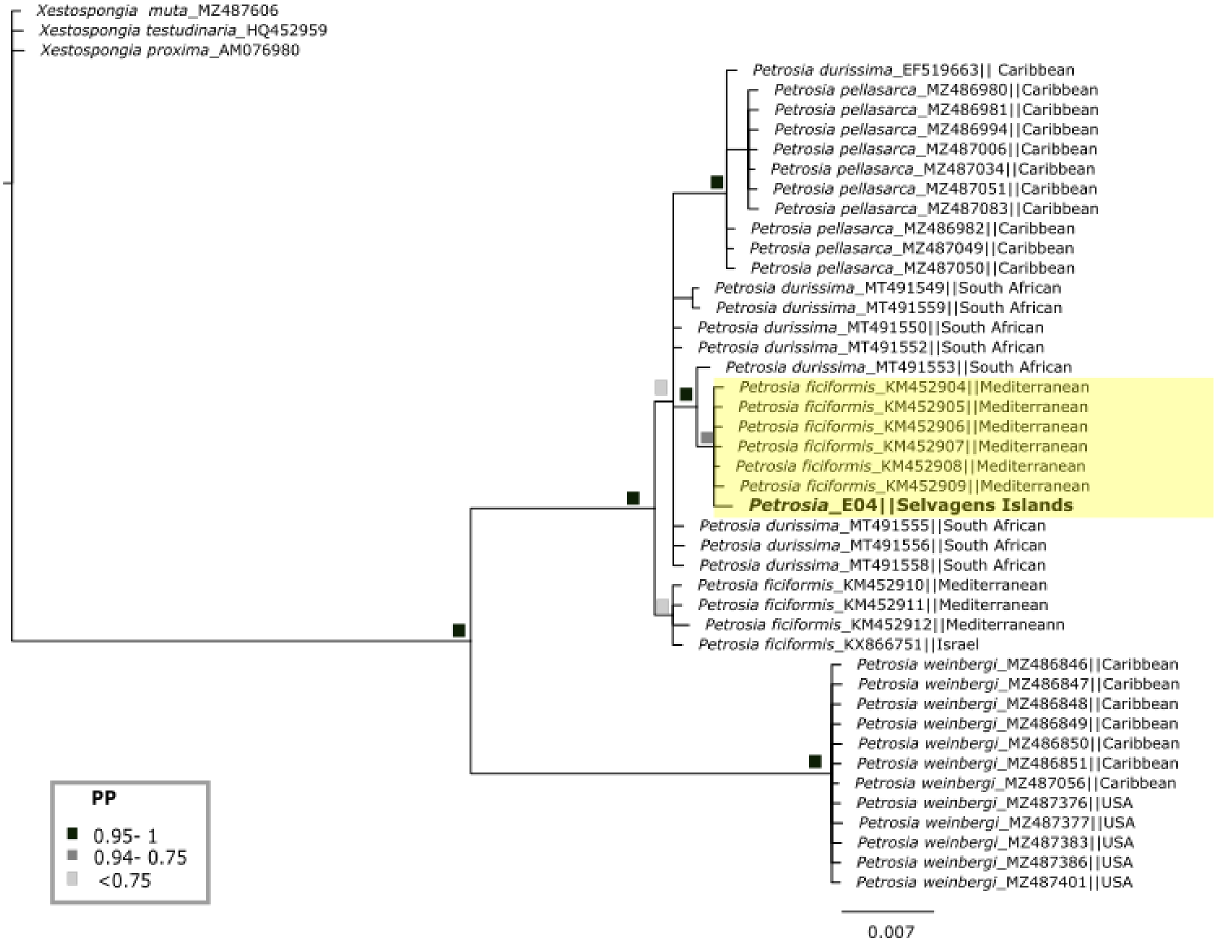

Figure 14.Tree resulting from the analyses of the mtDNA COI dataset composed of 17 individuals from different geographic regions. The topology presented corresponds to the Bayesian Inference analysis. Bayesian posterior probabilities (PP) values are provided on the branches. The tree is rooted on *Xestospongia*. Genus encompassing *Petrosia* species found in the present study are highlighted in color.

In the 28S rRNA tree, the clade containing our specimen, is highly supported (PP= 1), comprising *Petrosia ficiformis* species from the Mediterranean, Israel and *Petrosia* sp. from the Caribbean.

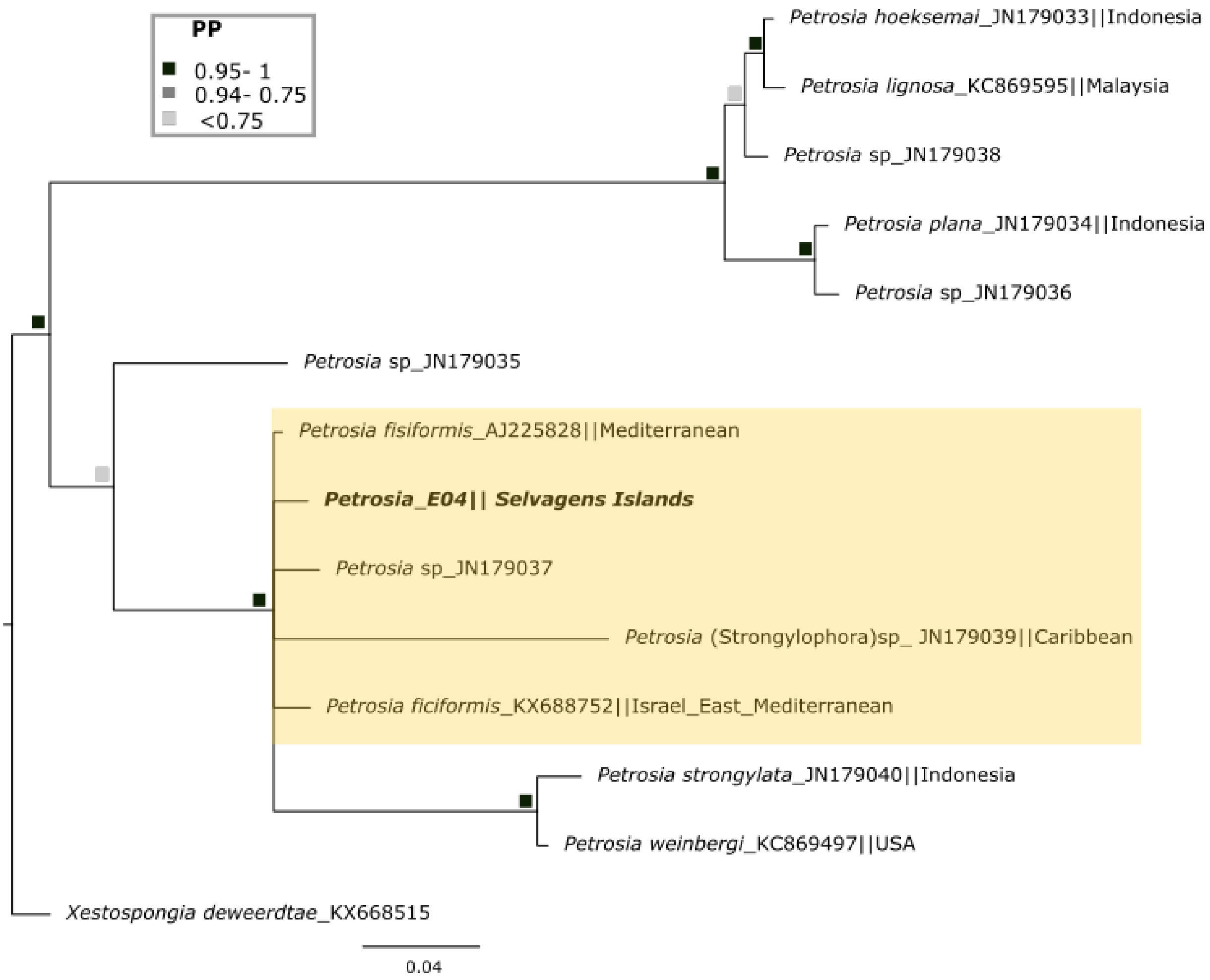

Figure 15. Tree resulting from the analyses of the 28S rRNA dataset composed of 13 individuals from different geographic regions. The topology presented corresponds to the Bayesian Inference analysis. Bayesian posterior probabilities (PP) values are provided on the branches. The tree is rooted on *Xestospongia*. Genus encompassing *Petrosia* species found in the present study are highlighted in color.

Family Phloeodictyidae, Genus *Calyx*

The 558bp alignments of the mtDNA COI gene comprised six haplotypes in 10 specimens, of which one belongs to sequences obtained in this work. For the specimen *Calyx* E15 8 bp substitutions needed to connect with *Calyx nicaeensis* from the East Mediterranean, the sequence obtained through GenBank)(Fig.16), the haplotype where our specimen was located is also shared by *Calyx* sp. from South Africa and Western Mediterranean.

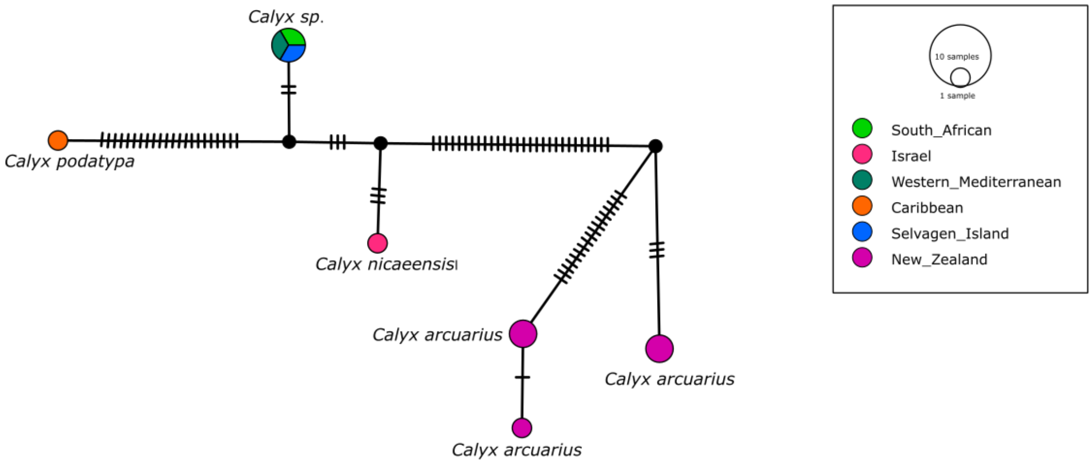

Figure 16.Haplotype network for the genus *Calyx*, based on COI mtDNA sequences, composed of 10 individuals from different geographic regions.

In the 28S rRNA haplotype networks, the 309 bp alignments comprised four haplotypes in four specimens, one of which belongs to sequences obtained in this work. For specimen *Calyx* E15 a total of five substitutions were required to connect to *Calyx nicaeensis* reported for the East Mediterranean.

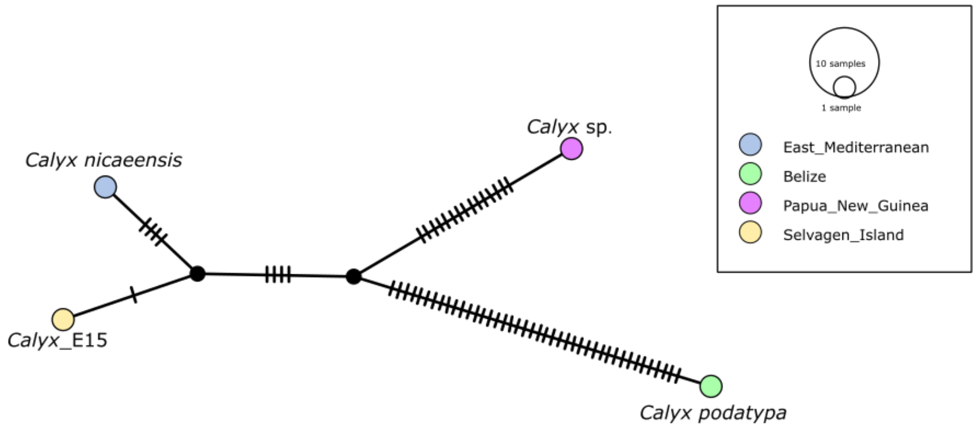

Figure 17.Haplotype network for the genus *Calyx*, constructed from sequences of the 28S (rRNA) region, composed of four individuals from four geographic regions.

In the COI tree, the clade comprising our *Calyx* E15 specimen is low-supported (PP= 0.71), comprising *Calyx nicaeensis* species from Israel, along with other *Calyx* sp. from South Africa and the Western Mediterranean.

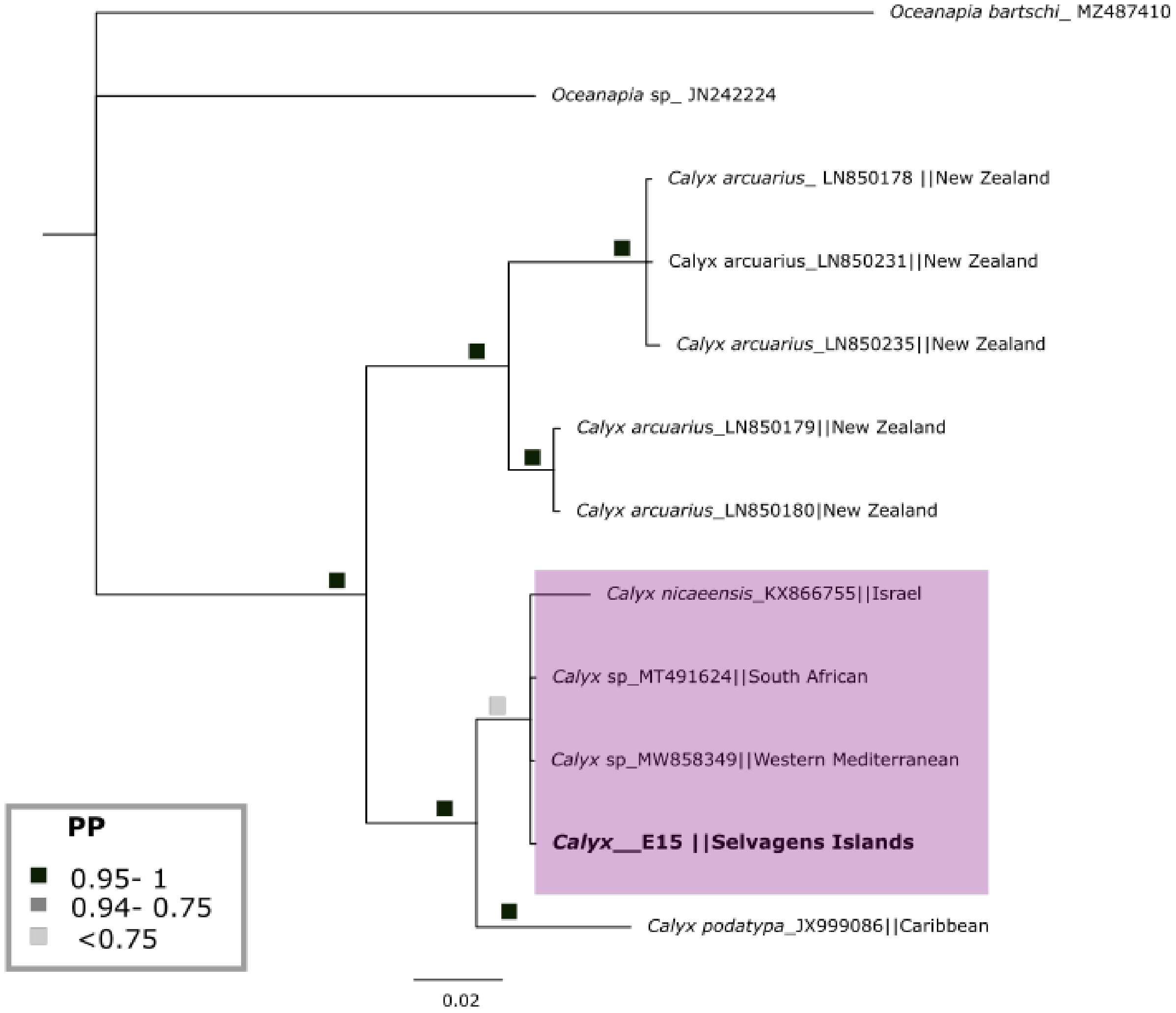

Figure 18. Tree resulting from the analyses of the mtDNA COI dataset composed of 10 individuals from different geographic regions. The topology presented corresponds to the Bayesian Inference analysis. Bayesian posterior probabilities (PP) values are provided on the branches. The tree is rooted on *Oceanapia* sp.. Genus encompassing *Calyx* species found in the present study are highlighted in color.

In the 28S rRNA tree, the clade containing our specimen is low-supported (PP= 0.71), comprising a *Calyx nicaeensis* species from the East Mediterranean.

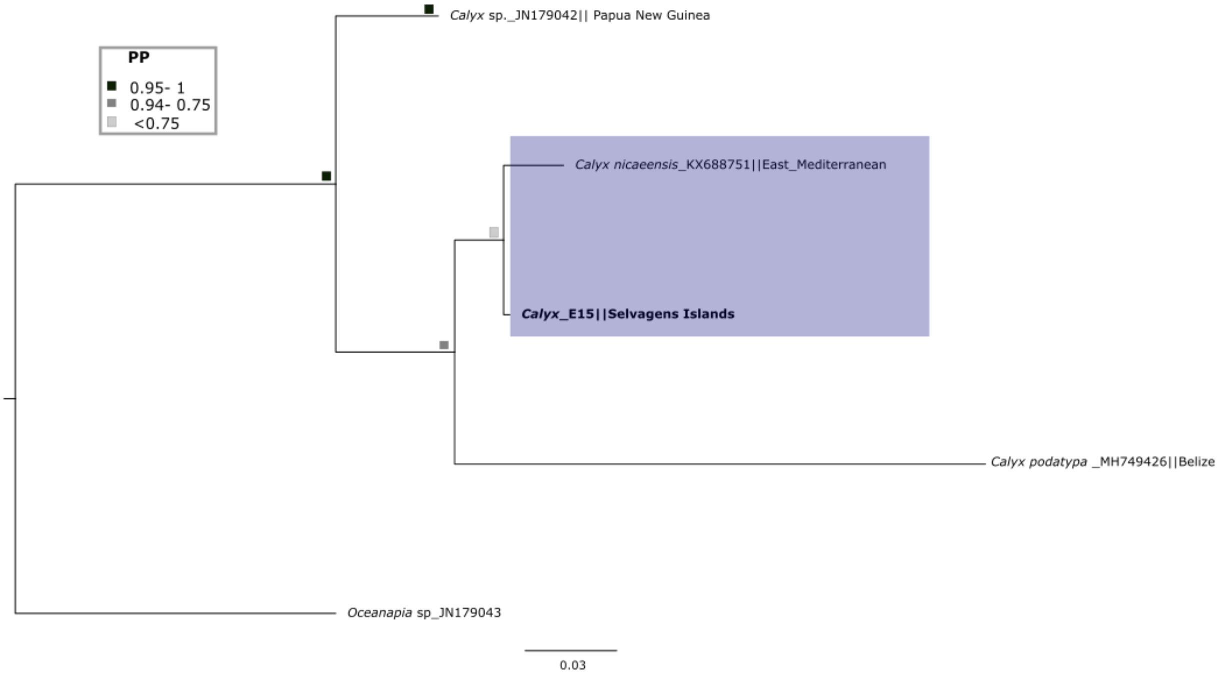

Figure 19.Tree resulting from the analyses of the 28S rRNA dataset composed of 4 individuals from different geographic regions.The topology presented corresponds to the Bayesian Inference analysis. Bayesian posterior probabilities (PP) values are provided on the branches. The tree is rooted on *Oceanapia* sp.. Genus encompassing *Calyx* species found in the present study are highlighted in color.

4. Discussion

Prior to the development of this work the knowledge on Porifera diversity for the deep-sea area of the Selvagens Islands was limited to the work by Carvalho et al. 2015 on lithistid tetractinellids, also known as rock sponges. The results therein reported resulted from the morphological analyses of specimens collected between 200–800 m depth in the scope of a campaign of the Harbor Branch Oceanographic Institute (HBOI). In that work, Carvalho et al. identified eight species, of which two - *Neophrissospongia nolitangere* (Schmidt, 1870) and *Macandrewia azorica* (Gray, 1859), representing the families Corallistidae and Macandrewiidae, were found to occur at approximately 360 m depth in Selvagens. In the present work, with the use of molecular methods, this knowledge base is considerably expanded with species across five families of Demospongiae: Axinellidae, Halichondriidae, Pachastrellidae, Petrosiidae, Phloeodictyidae, being recorded for the first time to these islands. Considerations on the taxonomic assignments of the taxa found and their biogeographic affinities are presented below.

Genus *Axinella* Schmidt, 1862 (Order Axinellida, Family Axinellidae)

Axinella E03, as observed in both the mtDNA COI and 28S haplotype networks (Fig.2 and Fig.3), does not show a strong phylogenetic affinity with other *Axinella* species obtained from the GenBank. In the mtDNA COI tree *Axinella* E03 is found in a clade that brings together other *Axinella* sp., reported elsewhere in the world, whereas for the 28S tree, specimen E03 is closer to *Axinella verrucosa*. This species is a species that although described for the Mediterranean and the region comprising the Selvagens islands, but it is also important to clarify that only between ten and fifteen species of *Axinella* are found in the GenBank, and not all are reported in the Mediterranean Sea (MED)/ Northeast Atlantic (NEA) area, and in some cases it is not possible to find the sequences of the same fragment, so the molecular identification may be a little short. For this reason, for specimen E03 it is possible to suggest that it belongs to this genus *Axinella*.

For specimens E11 and E12 it was only possible to obtain sequences for 28S. In the haplotype network the *Axinella* species that is relatively closer to our specimen is*Axinella damicornis* (Esper, 1794). On the other hand, in the phylogenetic tree for the same gene, our specimens are grouped with *Axinella damicornis*, although for this case the support values are low. Indeed, *Axinella damicornis* is a species that is reported in this region at depths of 200 m (de Voogd et al., 2021), although it is the species with which we found greater phylogenetic affinity for the two specimens, we could only suggest its identification at the genus level as is the case with specimen E03, these specimens belonging to the genus *Axinella*.

Taking into account that of the 27 species of this genus recorded in the MED/NEA region, nine have been recorded in Azores, Madeira, Selvagens, Canaries, further taxonomic studies are recommended, in order to try to reach lower taxonomic levels.

As described by Gazave et al. (2010) the family Axinellidae, and particularly the genus *Axinella*, is difficult to classify based on morphological characters since polyphyly is suspected. The use of molecular tools can provide insight into the phylogenetic relationships in these groups (Gazave et al., 2010), although the low number of sequences available may compromise the molecular approach..

Sitja & Maldonado 2014, also suggest that *Axinella* is a polyphyletic group and is constituted by two clades, a first clade that revolves around the type species, *Axinella polypoides* Schmidt, 1862, while the other, which includes species such as *Axinella damicornis* (Esper, 1794), *Axinella verrucosa* (Esper, 1794) and *Axinella corrugata* (George & Wilson, 1919), which would likely be the clade in which our specimens would be grouped (considering COI mtDNA).

Genus *Topsentia* Berg, 1899 (Order Suberitida, Family Halichondriidae)

For this specimen it was only possible to obtain sequences for the COI fragment as shown in figure.6. In the haplotype network, this specimen is three substitutions away from *Topsentia ophiraphidites*, the same species with which is grouped in the phylogenetic , although with a medium level of support (Fig.7). For the genus *Topsentia*, as described above, there are 35 known species of which eight have been recorded in MED/NEA and four have been reported for Azores, Madeira, Selvagens, Canaries, Cape Verde. In the GenBank there are some available sequences of *Topsentia* sp. and *Topsentia ophiraphidites*, but the latter species is distributed across Central and South America, with the type locality being from the Greater Antilles, between 36 -76 m depth. Therefore, it is not possible to affirm that our specimen is *Topsentia ophiraphidites*, since the number of species in the GenBank is small and it is not possible to compare with other species. It is possible to suggest that our specimen E18 belongs to the genus *Topsentia*.

Bertolino et al. 2015 made a morphological description of the genus *Topsentia* Berg, 1899 and documents the presence of four species of *Topsentia* for the Mediterranean: *T. garciae* Bibiloni, 1993, *T. lacazei* (Schmidt, 1868), *T. pachastrelloides* (Topsent, 1892) and *T. vaceleti* Kefalas & Castritsi-Catharios, 2012.

Genus *Characella* Sollas, 1886 (Order Tetractinellida, Family Pachastrellidae)

A total of 16 species are currently accepted as valid within this genus, from which three species are known to occur inNEA/MED:
Characella connectens (Schmidt, 1870) is distributed in the south of the Atlantic and in the Canaries (1300 m depth), the type locality being in Florida at 14 m depth; ***Characella pachastrelloides* (Carter, 1876)** is distributed along the Atlantic Ocean (Azores, Madeira, Selvagens, Canaries, Cabo Verde at 736 m depth), with the type locality in South European Atlantic Shelf at 684 m depth; *Characella tripodaria* (Schmidt, 1868) occurs in the Mediterranean, and the type locality is in Western Mediterranean between 700-912 m depth.

Considering the haplotype network, the two specimens E16 and 17 are grouped within *Characella pachastrelloides*. In the 28S network (Fig. 9) the specimen E17 continues in the group with *Characella pachastrelloides*, while the specimen E16 is separated. In the trees both specimens group with *Characella pachastrelloides* but with medium support. For this genus we only found sequences in GenBank of *Characella pachastrelloides* and some of *Characella* sp., which means that we will only have an unrepresentative group which probably preventing the interpretation of phylogenetic relationships. As described above, *Characella pachastrelloides* is indeed distributed in the Azores, Madeira, Selvagens, Canary Islands and Cape Verde, and the depth of collection of our specimens coincides with that indicated for this species.

Cárdenas et Rapp (2012) describe two species of *Characella* present in MED/NEA: *C. tripodaria* and *C. pachastrelloides*, claiming that the records of *C. tripodaria* are scarce and that its distribution overlaps with that of *C. pachastrelloides.* In relation to the bathymetry of these species Cárdenas and Rapp (2012) observed that the distribution of *C. pachastrelloides* and *C. tripodaria* perfectly follows the shelf break of the European and African continental margins.

It could be suggested, based on the percentages of identity and the results presented above, that specimen E17 is *Characella pachastrelloides*, and that specimen E16 belongs to the genus *Characella*, and could potentially be *C. tripodaria*, but this would need to be confirmed by observing morphological characters.

Genus *Petrosia* Vosmaer, 1885 (Order Haplosclerida, Family Petrosiidae)

The order Haplosclerida, which includes the family Petrosiidae, is an order that is widely distributed throughout the world and it is an important element in deep-sea sessile marine communities (de Weerdt, 1985). From the 58 *Petrosia* species currently accepted as valid, seven are known from the NEA/MED.

Petrosia ficiformis (Poiret, 1789) is very widely distributed across the temperate NEA (including Azores, Madeira, Selvagens, Canaries, Cabo Verde) and throughout the Mediterranean, but this is typically a shallow-water species (deep-water records are not certain); *Petrosia canariensis* de Weerdt & van Soest, 1986 is known from the Canary islands between 200-1000 m depth; *Petrosia clavata* (Esper, 1794) occurs in the Mediterranean and the Azores; *Petrosia crassa* (Carter, 1876) occurs in the NEA (including Azores and Josephine bank), the type-locality is on the Faroe Plateau at 305 m depth; *Petrosia raphida* Boury-Esnault, Pansini & Uriz, 1994 - known from the Western Mediterranean and Gulf of Cadiz, the type-locality is at the Alboran Sea at 580 m depth but can occur shallower (100-150 m depth on the Menorca Channel); *Petrosia pulitzeri* Pansini, 1996 occurs in the Eastern Mediterranean but typically in sublitoral caves; *Petrosia vansoesti* Boury-Esnault, Pansini & Uriz, 1994 occurs in the Western

Mediterranean and Gulf of Cadiz and has its type-locality in the gulf of Cadiz at 360 m depth. As could be seen in Figures 12 and 13 in the haplotype networks, our specimen E4 was placed in a position closer to *Petrosia ficiformis* than to other *Petrosia* species. For the COI and 28S trees the same occurred, clustered in clades with *Petrosia ficiformis,* however the support values were moderate and low. This suggests a phylogenetic affinity of our specimen E04 with *Petrosia ficiformis,* and given the distribution of this species as mentioned above, it could be suggested that our specimen belongs to the genus *Petrosia* and the species *P. ficiformis*. However, *P. ficiformis* is a shallow water species and for now no sequences of the deep water Petrosiids are available in GenBank. Morphological analyses will be necessary to try to reach lower taxonomic levels, and analysis of other NEA/MED petrosiids is suggested to try to determine the identity of the specimen examined here.

Genus *Calyx* Vosmaer, 1885 (Order Haplosclerida, Family Phloeodictyidae)

Sixteen *Calyx* species are known, of which only two occur in the NEA/MED: *Calyx nicaeensis* (Risso, 1827) is widely distributed across the Mediterranean and its type-locality is on the Northern Adriatic Sea; *Calyx tufa* (Ridley & Dendy, 1886) occurs in the NEA and Western mediterranean and its type-locality is in Cabo Verde (approx. 220 m depth).

As shown in Figure16 and 17, in the haplotype networks for the COI and 28S fragment, our specimen E15 is closer to the species *Calyx nicaeensis*. For the COI tree we observe that it is grouped in a clade with *Calyx* sp. and *Calyx nicaeensis*. For the 28S tree, although with a low support, our specimen shows a phylogenetic affinity with the species *C. nicaeensis*. As described above for the MED/NEA, *Calyx nicaeensis* is registered for this region, which could point to the fact that our specimen is possibly *Calyx nicaeensis*. Nevertheless it is not possible to confirm, since there is another species of *Calyx* reported for this area. Unfortunately there are no sequences of *Calyx tufa* available in GenBank, so it is not possible to be sure to which of these species our specimen belongs to. It is therefore suggested that our specimen belongs to the genus *Calyx.*

In biogeographical terms and given the location of our sampling area there are some key aspects to understand the distribution of deep-sea sponges. The presence of hard substrates, creates the ideal conditions for the deep-sea sessile fauna colonization (sponges, corals, etc.) (Sitja et al 2020). This pioneering colonization by these organisms facilitates the arrival of other organisms, triggering an increase in benthic biodiversity (Levin, 2005; Rueda et al., 2012; Gonzalez-García et al., 2020; Sitja et al 2020).

In addition to the above, this region contains an area with diverse climatic conditions similar to those of subtropical, temperate and subarctic climates. The geological history of the northeastern Atlantic and Mediterranean has gone through several relevant events, such as the Messinian Salinity Crisis (MSC). All of the above generated changes in the distribution of the species, in some cases possible extinctions and in others migrations or displacements to other areas, which has allowed a genetic record to remain in their populations (Xavier & Van Soest, 2012). Sponges are organisms that during their larval phase show movement, due to the production of lecithotrophic larvae, with a short life span and philopatric behavior (Mariani et al., 2005, 2006; Maldonado, 2006; Uriz et al., 2008; Xavier & Van Soest, 2012; Cárdenas & Rapp, 2015). Much of the current Mediterranean sponge fauna is a post-MSC event. Such fauna is composed of Atlantic migrants, species that over time have evolved in the Mediterranean basin (Xavier & Van Soest, 2012). Xavier & Van Soest (2012) confirmed that there is a strong affinity of the Mauritanian (Canary Islands) and Senegalese (Cape Verde) sponge fauna with those of the westernmost Mediterranean (Alboran, Catalonia, Algeria) (Xavier & Van Soest, 2012). The authors suggest that both geographical distance and currents are important factors determining the zoogeographic affinities of the sponge fauna, since, as mentioned above, sponges depend on currents for larval transport (Xavier & Van Soest, 2012).

Potential and limitations of the barcoding approach

With the development of this work, we have been able to recognize some of the potential of molecular techniques, although we only worked on a small part of them. However, Gavazavena et al 2010, describe how molecular techniques have helped to elucidate some enigmas in groups such as Porifera, helping in the identification of homoplasies, homology of the characters of a species, as well as the ancestral status of that character and cryptic species (Gazave et al., 2010). Since the deep-sea is characterized by harboring a large number of species that present morphological plasticity as is the case of sponges (Xavier et al., 2010), the use of molecular tools and the generation of barcodes helps to clarify the identifications and to solve problems, which are not possible to answer with classical taxonomy. An example of this is what happens with the genus *Axinella*, where morphological characters are not enough to confidently distinguish species, so it is necessary to go to deeper levels (Gazave et al., 2010).

It was also possible to identify some limitations such as the low representativeness of Porifera on GenBank compared to other taxonomic groups, which makes comparisons with this reference barcode library challenging. Furthermore, in some cases, it was not possible to obtain sequences for both markers of the same specimen(Xavier et al. 2021).

At this point, there is no method more efficient than another. What we must do is judicious and meticulous work, as proposed by integrative taxonomy, which allows us to collect as much information as possible from a specimen. This work generated new voucher-linked barcode sequences, which could be used and compared in other studies, contributing to the knowledge of deep-sea sponge species.

With most of our specimens provisionally identified to the genus level, the next step is to undertake morphological examination of each of the specimens, such as skeletal features, shape and size, as well as constituent elements such as spicules and spongin fibers (Gazave et al., 2010). In this way, we intend to reach the species level by comparing it with the descriptions found in the literature.

Final remarks

This study allowed the identification at the genus level of several deep-sea sponges collected in Selvagens Islands, by means of DNA barcoding.

The geographical location of the Selvagens, as well as the geological history of the Atlantic-Mediterranean zone, makes this area a biodiverse region, harboring groups of sponges that play a determining role in the functioning of the ecosystem, and that are also highly vulnerable organisms. Providing information on the species that inhabit this area improves the efforts in monitoring programs and conservation strategies.

As evidenced by the results here reported, the phylum Porifera is still largely underrepresented in existing reference barcoding databases such as GenBank, and therefore additional sequencing efforts are needed. Therefore, some phylogenetic relationships and biogeographic affinities in this work remained unresolved at lower taxonomic levels. A combination of molecular and morphological methods are thus the best approach to advance the knowledge on diversity and distribution patterns of this taxonomic group, particularly in the deeper areas of the oceans.

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Appendices

Appendix I -State of the art

1.Selvagens islands

The Selvagens islands are a group of small islands composed of Selvagem Grande, Selvagem Pequena and Ilhéu de Fora (Friedlander et al.,2016, 2017; Santana, 2016). The Islands are located southeast of Madeira and north of Tenerife and the Canary Islands being the southernmost point of Portugal (Friedlander et al.,2016, 2017; Santana, 2016). The Selvagens Islands are administered by the municipality of Funchal which is part of Madeira (Friedlander et al.,2016, 2017; Santana, 2016).

These oceanic islands have a volcanic origin, being their geological history related to the opening and expansion of the Atlantic Ocean (~200 million years) (Santana, 2016). The Selvagens islands were the first natural reserve in Portugal, established in 1971, and were included in the European Ecological Network "Natura 2000" in 2007, having been classified as a "site of Community importance located on national territory, belonging to the Macaronesian biogeographic region", with the code PTSEL0001 (MAOTDR, 2007; Friedlander et al.,2016, 2017; Santana, 2016). The Selvagens Nature Reserve is listed as an IUCN Category Ia Protected Area meaning that human activity in the reserve is strictly controlled. In addition, Selvagens are protected under European Commission legislation, included in the Natura 2000 Network as a Special Area of Conservation (SAC) and designated a Special Protection Area (SPA) under the European Union Birds Directive (Friedlander et al.,2016, 2017; Santana, 2016).

2.Porifera

Sponges are fully aquatic invertebrates and are part of the Porifera phylum. These organisms are the oldest metazoans and are one of the groups present in the deep-sea, being considered one of the most important biodiversity hotspots. (Borchiellini et al., 2000; Van Soest et al.,2012; Cathalot et al., 2015; Kahn et al., 2015; Robertson et al., 2017). Sponges are simple organisms without real tissues or organs, however, they have specialized cell aggregations (Huber and Castro, 2007; Van Soest et al.,2012). The structure of their skeleton can be siliceous or calcareous and, according to this, the sponges can be soft, rock hard, fragile, and with different shapes and forms like elaborate branching or cup-shaped forms, encrusting, giant or small (as small as some millimeters) being the shape different among genera and species (Van Soest et al.,2012). Additionally, the environment plays an important role with hydrodynamics, light, and turbidity (Van Soest et al.,2012). They are benthonic sessile organisms, and are designated as filter feeders which means that they feed through filtering microscopic-size food particles in the water column (Van Soest et al.,2012). Due to their limited movement capacity, sponges can occur in regional or local areas of endemism (Van Soest et al.,2012). They occupy a wide variety of ecosystems in all latitudes and depths from the eulittoral zone to hadal areas (Araújo et al., 2003; Van Soest et al.,2012).

Sponges are distributed into 4 Classes: Demospongiae, Hexactinellida, Calcarea and Homoscleromorpha (Huber and Castro, 2007): the biggest and more diverse class of Porifera being Demospongiae This kind of sponges have siliceous spicules and/or a fibrillar collagen skeleton (Van Soest et al.,2012). Hexactinellidae or glass sponges, as they are commonly known, also have siliceous spicules, but these ones are called hexatines. This kind of sponges are typical from deep-sea at bathyal and abyssal depths, mainly restricted to hard and soft substrates (Huber et Castro, 2007; Van Soest et al.,2012; Xavier et al., 2015). Sponges from: Calcarea class have calcareous spicules made of calcium carbonate that can be distributed in marine ecosystems until 800m deep (Huber and Castro, 2007). Finally, Homoscleromorpha have small encrusting or lumpy spicules that can branch (El-Bawab, 2020).

2.1Ecological functional role of Porifera Phylum

Sponges play a relevant role in the ecosystem that can be explained at different levels. First, as already mentioned, sponges feed by means of a filtering mechanism, using their pores, allowing water and particles to pass through. Since the water column is rich in utraplankton and picoplankton, feeding with these particles is an important link between carbon and benthos. Generating a carbon flux that can be directed to different trophic levels (Bell, 2008; Howell et al., 2016). For this reason sponges are considered efficient filter feeders. These mechanisms are not only favorable for sponges, but also for nearby benthic communities, as sponges favor benthic-pelagic coupling that contributes to nutrient recycling, carbon, silicon, nitrogen and other cycles (Pile and Young, 2006; Beazley et al.,2013,2015; Van Soest et al.,2012).

Sponges, and in particular deep-sea sponges, play an important role since they are capable of filtering large quantities of water efficiently, re-integrating nutrients. This process has been called "sponge loop", recycling nutrients in oligotrophic waters (Vad et al., 2018). Water movement also favors hydrodynamic changes, generating an increase in habitat heterogeneity and increasing biodiversity (Beazley et al., 2013; Maldonado et al., 2017).

Along with filtration, nutrient recycling is one of the important ecological functions of sponges, as it has an impact at the microscale level of the ecosystem with processes like remineralization and bioturbation (Beazley et al., 2013). Sponges are considered to be the best regulators of dissolved organic matter (DOM) in oligotrophic environments (Robertson et al., 2017). They have the capacity to catch and absorb DOM, and making it available to other organisms by creating a flow of particulate organic matter

(POM), which in turn will be used by other invertebrates in lower trophic levels (Maldonado et al., 2017). This nutrient recycling capacity is also due to symbioses with other organisms (bacteria, archaea, eukaryotes and prokaryotes), living in their mesohyl (Vad et al., 2018). An example of this is the symbiosis between sponges and some species of nitrifying bacteria that allow nitrogen fixation (via aerobic or anammox fixation) (Maldonado et al., 2017; Vad et al., 2018). An additional aspect to note about symbiosis is that it also occurs within or on the sponge body, with some of the associated macrofauna ranging from fish, shrimp, polychaetes, among others (Van Soest et al., 2012).

Also, sponges play an important role in habitat construction. Some authors consider them as key ecosystem engineers, as a significant part of these communities are found in deep waters, forming sponge aggregations or sponge grounds, making them important 3D complexes. In these areas they constitute almost 90% of the biomass, generating support and stability to the ecosystem, creating habitats for other organisms, from small invertebrates to some vertebrates. The generation of microhabitats leads to an increase in local biodiversity, turning these aggregations into important biodiversity hotspots (Kutti et al., 2013; Beazley et al.,2013). The structural benefits of these sponge grounds are not limited to providing habitat, but are also important as breeding grounds, spawning grounds, nursery grounds, feeding grounds, and refuge areas for many fish and crustaceans, among others (Bell, 2008; Van Soest et al., 2012; Beazley et al.,2013; 2015; Howell et al.,2017). Some authors have reported that sponge grounds are also breeding grounds for fish species of commercial interest such as rockfish, hake, and blue ling (Vad et al.,2018).

With so many interactions occurring, it is key to note that sponges are also part of the trophic chain, so they are predated by organisms such as fish, sea stars, among others. Being sessile organisms, their defense mechanisms rely on chemical products, morphological features (such as the spicules), or the ability to quickly regenerate affected tissues (Robertson et al.,2017). Over the years the production of such chemicals has been of great interest to science, particularly to biotechnology. Secondary metabolites are found in some marine invertebrates, but in sponges, the abundance and diversity of these compounds are remarkable (Sokolover and Ilan, 2007). These secondary metabolites in sponges may act as antifouling agents, defense against pathogens such as some bacteria, fungi, and viruses, protection against solar UV rays, larval signaling, among others (Sokolover and Ilan, 2007). In recent years, sponges have been assuming an important role in human health, as many of these secondary metabolites have been found to have anti-tumor, anti-infectious, and anti-inflammatory properties (Van Soest et al.,2012).

On the other hand and although not less important, sponges play a relevant role as bioindicators of the environment. It is believed that, due to their life history, and as great filterers, sponges may have the ability to store pollutants and contaminants from the surrounding environment, which would allow them to be used as bioindicators, monitoring the health of the ecosystem, particularly metals and pesticides (Araujo et al.2003).

2.2 Deep-sea sponges

Sponges inhabiting the deep sea have slow growth rates (Kutti et al., 2013), are long-lived, and highly susceptible to disturbance, these facts make them vulnerable and fragile animals (Kutti et al., 2013; Beazley et al., 2015). These organisms have a sporadic recruitment time (Bell et al.,2017) but are not well known in what concerns reproduction, biogeographic patterns and distribution (Xavier et al., 20151; Bell et al.,2017). As mentioned above, deep-sea sponges are found in aggregations or sponges grounds also known as ostur communities. The ostur communities can be: boreal ostur and cold water ostur; the first commonly occurs close to the Faroe Islands Norway, Sweden, parts of the western Barents Sea, and south of Iceland; the second ones occur in the north of Iceland, the Denmark Strait, off East Greenland and north of Spitsbergen (Howell et al., 2016).

Deep-sea sponges can be divided in mainly two classes of sponges: Demospongiae and Hexactinellida, being these classes the most abundant in these deep-sea ecosystems (OSPAR, 2010). In general, these organisms can be found at depths from 150 to 1,700 m (Maldonado et al., 2017), an environment where the temperature can range from 0°C to 10 °C (OSPAR, 2010). Sponge aggregations are reported in biogeographic zones I, II, III, IV, V, which means Arctic waters (Deep-sea), North Atlantic Abyssal Province (Deepsea), Norwegian Coast (Skagerrak), South Iceland – Faroe shelf; Barents Sea (OSPAR, 2010). The class Hexactinellidae is the most predominant deep-sea group, specifically glass sponges, *Pheronema carpenteri*, form the most extensive sponge aggregation from south of Iceland and west of Scotland, across the Porcupine Seabight, Azores, northern Spain, Portugal, Canary Islands, and off Morocco (OSPAR, 2010; Maldonado et al., 2017). Other hexactinellid-dominated sponge grounds occur from several areas of the North Atlantic, off the Canary Islands, off Portugal, and at the Alboran Sea (Mediterranean Sea) in seamounts at bathyal depths (Maldonado et al., 2017).

2.3 Anthropogenic impacts deep-sea sponges

Taking into account the aspects of the life history of sponges and highlighting their fragility, sponge grounds have been considered vulnerable marine ecosystems (VME) following resolution 61/105, this in discussions at the United Nations General Assembly (UNGA) and The FAO International Guidelines for the Management of Deep-sea Fisheries (Beazley et al.,2013,2015). It is important to highlight that there are aspects that are little known related to these organisms, such as biotic or abiotic factors that could have become environmental stressors and that could generate a negative impact on these communities. Abiotic factors like temperature, water flow, light intensity, sedimentation and, salinity can generate perturbation in sponges (Bell et al.,2017). Besides, when such stress factors occur, modifications in the bacterial assemblage may follow, since organisms such as cyanobacteria provide sponges with carbon through photosynthesis (Bell et al., 2017).

Anthropogenic impacts also play a determinant role. With the search for new resources, the deep-sea has become the target of many stakeholders. Mining, gas production, oil, and intensive fishing and especially trawling, are activities that generate a large negative impact on ecosystems (Bell et al., 2017). The impacts of these activities range from substrate loss and habitat fragmentation to changes in geochemical, topographical, and chemical composition (Gollner et al., 2017; Clark et al., 2020), generating disturbances in the environment. Some of these changes can be fatal for organisms as fragile as sponges (Bell et al., 2017; Vad et al., 2018). According to Gollner et al., 2017 and Miller et al., 2018, the impacts of mining are much worse than those of trawling, since there is total removal of the substrate, which generates great concern when it is known that many organisms such as sponges present slow growth taxa, thus generating irreversible changes in benthic ecosystems (Kutti et al., 2013; Bell et al.,2017).

As mentioned above, the exploration of these resources generates large disturbances, some of which can directly affect deep-sea sponges, such as temperature increase and disturbances due to the increase of sediment plumes. As mentioned, temperature in deep areas is relatively low (2 ºC, according to MIDAS, 2016). But with the start of mining activities, this can change, since there is a release of heat by the removal of sediments, which can generate up to 11ºC more in the surrounding environment (Miller et al., 2018). The deep-sea being a relatively stable environment, when this type of alterations occur, changes can be generated at many levels. In this case, temperature increases that alters the bacterial assemblage of sponges. Some studies correlate changes in bacterial communities with changes in the environment (Bell et al., 2017). Other studies also indicate that a change in the genetic functionality of sponge-associated bacterial communities may take place (Bell et al., 2017).

In addition, sediment plumes, which are clouds of detritus resulting from mining activities, can cause saturation in the feeding organs, especially in filter-feeding organisms such as sponges, as fine sediments and heavy metals are suspended in the water column and captured by these organisms (Miller et al., 2018; Clark et al., 2020). Another problem is toxicity, as much of these particles are complex mixtures of materials that can be potentially hazardous. One of the issues that arise with deep-sea mining is the number of compounds that can derive from the different steps of extraction, and how these metal ions, in a different state of oxidation, generate a cloud of toxics that can travel hundreds of kilometers and affect the ecosystem (MIDAS, 2016).

Regarding anthropogenic impacts, the direct impacts of underwater well blowouts construction and pipeline leaks at depth, have shown that in the long term, the sponge groups, after a disturbance of this magnitude, have not recovered after 10 years, due to their longevity, slow growing taxa and probably their low reproductive taxa, which generates problems in their conservation (Vad et al., 2018). Some studies have shown that power transmission cable installations generate a mortality of 100% of the glass sponges remaining under the wiring and 15% of the sponges found within 1.5 m of the cables (Dunham et al., 2015; Vad et al.,2018).

Last but not least, the impacts of climate change on sponges are mainly related by two factors: temperature and pH. As indicated by the Intergovernmental Panel on Climate Change (IPCC), the exposure of sponges to high temperatures can generate a loss of symbionts (as occurs with *Rhopaloeides odorabile*), followed by cell necrosis. Other symptoms that have been described are alterations in the host/symbiont relationship, attack by opportunistic organisms amd, holobiont stress among others (Bell et al., 2017).

2.4 Molecular ecology tool in conservation

2.4.1 Taxonomy and molecular in sponges

One of the problems of marine species, and particularly of deep-sea species, has been their identification, the existence of Cryptic species. These species prevail in these environments and are characterized by huge morphological plasticity (Xavier et al., 2010), influenced by factors such as light, sedimentation, water chemistry, ecological interactions, among others (Xavier et al., 2010; Miglietta et al., 2011). Specifically in sponges, this type of problem is well illustrated (Loukaci et al.,2004), since descriptions are based on morphological features almost 99% of the time (Castello and Chaudhary, 2017). These problems are, therefore, more evident in sponge species without a skeleton, since morphology is fundamental for taxonomic description (Loukaci et al.,2004).

With the implementation of molecular tools, many sister species have been discovered in the Phylum Porifera (Loukaci et al., 2004). Molecular studies in this phylum began in 1980, with the use of tools such as allozyme polymorphism to detect sister species (Borchiellini & Chombard, 2000). The comparison of molecular sequences to observe phylogenetic relationships among sponges occurred in the mid-1990s (Borchiellini & Chombard, 2000). In fact, since the implementation of molecular techniques in systematics, the number of morphologically but genetically divergent cryptic species has increased (Xavier et al., 2010).

In an era as accelerated as ours, in which there is an enormous loss of biodiversity, many species have disappeared before being identified (Uriz & Turon, 2012). This raises a lot of concerns in terms of conservation, aswithout knowledge of the species, few strategies in terms of management plans can be implemented (Xavier et al., 2010).

For this reason, the use of molecular tools is fundamental to try to know and recognize species, and to assess aspects such as genetic diversity, gene flow, inbreeding, among others (Uriz, et Turon, 2012). With these studies, our understanding of the ocean has changed, expanding the idea that marine organisms are as genetically distinct populations despite geographical proximity (Miglietta et al., 2011). Data from mitochondrial and nuclear DNA sequences have been relevant for phylogeny and biogeography studies, providing new knowledge (Uriz, et Turon, 2012). It is important to highlight that exploration efforts have also increased in recent times, the implementation of ROVs, diving stations, video, submarines, among others. Nevertheless, there is still much work to be done, and the deep-sea remains a mystery. With the increase in extractive activities, the characterization of these species is of paramount importance (Castello and Chaudhary, 2017).

2.4.2 **DNA Barcoding**

DNA barcoding has become one of the most important and widely used tools in the scientific arena in the last decade. The concept of DNA barcoding was introduced in 2003 by Professor Paul Hebert and collaborators, gaining popularity as a tool that allows species identification using mitochondrial DNA, Mitochondrial cytochrome c oxidase subunit 1 (COI) gene (Hebert et al., 2003; Trivedi et al. 2016). This mitochondrial gene is present in all metazoans and is mostly a highly conserved gene, making the identification of orthologous sequences a great alternative (Neigel and Stake, 2007). Being a highly conserved gene, fragments of the same gene are conserved at the amino acid level allowing the design of PCR primers of approximately 700 base pair (bp) amplifying a wide range of metazoan phylum (Folmer et al. 1994; Neigel and Stake, 2007).

Barcoding is presented as an alternative to traditional taxonomic classification methods based on morphological characters of species often facing limitations related to the morphology and distribution of organisms (Neigel and Stake, 2007). Barcoding was born with the goal of discovering new species, attributing them to already known species, identifying morphologically similar species (e.g., cryptic species), helping to understand phylogenetic relationships and genetic divergence, and in the last decade has focused on species conservation and management (Neigel and Stake, 2007). Barcoding is for example used for the monitoring of invasive species, as well as the protection of endangered species.. All this by analyzing small fragments of tissues, fins, feces, etc, collected, for example, in illegal fisheries (Trivedi et al. 2016; Galal-khallaf et al. 2019).

Particularly for the sponge group the reconstruction of phylogenetic relationships is complex due to their relative simplicity and morphological plasticity (Morrow et al. 2013). One of the difficulties is the poor understanding of whether some of the morphological characters indicate a common evolutionary origin (homology) or whether they are the consequence of convergent evolution, parallel evolution, or evolutionary reversal (homoplasy) (Morrow et al. 2013). In the case of sponges, the scarcity of morphological characters may allow the detection of such homoplasies in a meaningful way allowing their classification, compared to other organisms. These tools have been gradually developed but there are still a large number of phylogenetic relationships in the Porifera group to be solved, which sometimes hinders the attempts to achieve a stable classification for this group of organisms (Morrow et al. 2013).

3.**The oceanographic campaign EMEPC/M@rBis/Selvagens2010**

The records of the first campaigns on the Selvagens Islands date back to the 1970s with the study of birds. The campaign of Professor Luis Saldanha took place in 1980 and those of the Task Group for the Extension of the Continental Shelf (EMEPC) in 2010. The goal was the inventory of the marine biodiversity of the Selvagens Islands. 37 institutions, 77 researchers, and 220 crew members participated in the campaign EMEPC/M@rBis/Selvagens2010.

The campaign collected data from the distinct bathymetric layers in Selvagem Pequena and Selvagem Grande subtidal samples with the help of the vessel Navio de Treino de Mar Creoula, and samples collected at depth by Navio República Portuguesa Almirante Gago Coutinho, using the ROV Luso. Sampling was carried out in three lines; Intertidal: sand and platform sampling, carrying out transects and snorkelling around some points; Subtidal: NTM Creoula base, diving with transects focused on: benthic, cryptic and pelagic ichthyofauna; Deep subtidal: NRP Almirante Gago Coutinho base. ROV with marked transects. Dredging was also carried out.

The objective of this base, N.R.P. Almirante Gago Coutinho, was the characterization of marine biodiversity and the mapping of habitats in the deepest zones of Selvagens Islands. For this purpose, transects were carried out within quadrants that subdivided the islands. The ROV dives collected constant information.

3.1 ***Remotely Operated Vehicle*** **(ROV)**

The LUSO ROV is a vehicle adapted for science, which allows the collection of various types of samples (geological, biological, sediment, and water). The ROV can dive up to 6,000 m deep. The ROV has different sensors: a methane sensor and such as the CDT (Conductivity-Temperature-Depth), which allows to obtain physical-chemical parameters of the water such as conductivity, temperature, depth (Estrutura de Missão para a Plataforma Continental [EMEPC], n.d.)(Fig.20). It also makes measurements of a point, or a vertical profile. This way, the physical characterization of the water column is possible. It's equipped with Niskin bottles used to collect water samples (EMEPC, n.d.). Once the ROV has finished the dive, it returns to the ship, where a group of scientists separates the samples, which are photographed, preserved in ethanol, formaldehyde, or refrigerated at -80 ° C.

The ROV also has video cameras that permanently transmit images during each dive, allowing the crew to control and operate the ROV. It also allows you to make notes of the dive. All this thanks to the fiber optic cables that are connected to the ROV and the boat, allowing the sending of images. High-resolution video recording is of vital importance, as it will be necessary for the characterization of the collected individuals and their future identification (Calado et al., 2012). Specifically for Portugal, the LUSO ROV was acquired by the Mission for the Extension of the Continental Shelf (EMEPC) in 2008 within the scope of the Extension of the Continental Shelf project, and since then it has participated in different campaigns, carrying out approximately 17 oceanographic campaigns focused on the deep-sea (EMEPC), n.d.).

Figure 20.ROV LUSO EMEPC

4. DNA extraction and PCR

4.1 Markers

In barcoding studies, some mitochondrial fragments stand out as it is the case for Cytochrome c oxidase I (COI), It was described by Folmar 1994 for the amplification of various invertebrates highlighting for the time the description of new deep-sea invertebrates. It is a fragment highly used in genetic studies. being one of the most conservative protein-coding genes of the mitochondrial genome of animals (Brown,1985; Folmer et al.1994). In addition, it is a good quality molecular marker for barcoding studies, with good taxonomic resolution. One of the great advantages of using this mitochondrial fragment is that it has a large number of data/sequences in reference databases, which generates more robustness and confidence in subsequent analyses (Elbrecht et al. 2016).Nuclear fragments are also used in barcoding studies. In the particular case of this work were used 28S, as described by Borchiellini et al (2000), Morrow et al(2013), Elbrecht et al (2016). These markers have great potential to solve problems at the species level, and at the level of taxonomic divergence, are more conserved regions which is useful in studies of sponge phylogeny.

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Appendix II Supplementary Material

Table S 1Molecular ID and Photos of the Specimens analysed.

Specimen code	Photo	Molecular ID
E03		*Axinella* sp.
E04		*Petrosia* sp.
E11		*Axinella* sp.
E12	No picture available	*Axinella* sp.

E15		*Calyx* sp.
E16		*Characella* sp.
E17		*Characella* sp.
E18		*Topsentia* sp.

Table S 2.List of mtDNA (COI) and rRNA (28S) sequences used in the phylogenetic reconstructions. Newly generated sequences are highlighted in bold.

Species	Voucher	Locality, depth (m)	mtDNA COI	rRNA 28S	Source
Axinella infundibuliformis	not provided	Ireland		GQ466061.1	Gazave et al. 2010
Axinella vaceleti	not provided	Mediterranean		GQ466060.1	Gazave et al. 2010
Axinella verrucosa	not provided	3PPs cave NW Mediterranean		GQ466063.1	Gazave et al. 2010
Axinella infundibuliformis	BELUM:Mc4438	Scotland		HQ379199.1	Morrow et al. 2012
Axinella parva	BELUM:Mc4606	Ireland		HQ379200.1	Morrow et al. 2012
Axinella pyramidata	BELUM:Mc3385	Ireland		HQ379201.1	Morrow et al. 2012
Axinella vaceleti	BELUM:Mc4200	Mediterranean		HQ379202.1	Morrow et al. 2012
Axinella vaceleti	not provided	South China Sea		KC762735.1	Unpublished
Axinella dissimilis	not provided	Ireland		GQ466059.1	Gazave et al. 2010

Axinella_E11		Selvagens Islands		**xxxx**	**this study**
Axinella_E12		Selvagens Islands		xxxx	this study
Axinella_E03		Selvagens Islands		xxxx	this study
Axinella cannabina	not provided	Croatie		GQ466062.1	Gazave et al. 2010
Axinella cannabina	Po.25604	Israel		KX688755.1	Unpublished
Axinella corrugata	P134	Panama		KC869458.1	Thacker et al. 2013
Axinella corrugata	P61	Panama		KC869523.1	Thacker et al. 2013
Axinella damicornis	not provided	Mediterranean		AF062605.1	Chombard et al.1997
Axinella damicornis	not provided	3PPs cave NW Mediterranean		GQ466058.1	Gazave et al. 2010
Axinella damicornis	BELUM:Mc4987	Ireland		HQ379198.1	Morrow et al 2012
Axinella damicornis	Po.25764	Israel		KX688743.1	Idan et al.2016
Axinella damicornis	Po.25924	Israel		KX688749.1	Idan et al.2016

Axinella_E03		Selvagens Islands	xxxx		this study
Axinella sp.	not provided	South China Sea	JX915786.1		Qu et al.2012
Axinella sp.	not provided	South China Sea	JX915787.1		Qu et al.2012
Axinella sp.	TS4249	South African	MT491690.1		Unpublished
Axinella sp.	EBG-2018	Mexico	MH681879.1		Carballo et al. 2018
Axinella aruensis	G301092	not provided	JQ034550.1		Erpenbeck et al. 2012
Axinella aruensis	G300635	not provided	JQ034549.1		Erpenbeck et al. 2012
Axinella aruensis	G300595	not provided	JQ034548.1		Erpenbeck et al. 2012
Axinella aruensis	G300581	not provided	JQ034547.1		Erpenbeck et al. 2012
Axinella aruensis	G312935	not provided	JQ034551.1		Erpenbeck et al. 2012

Axinella infundibuliformis	BELUM<GBR>:Mc4438	Scotland	HQ379410.1		Morrow et al 2012
Axinella aruensis	CMNI 2018-0099	Canada	MK570860.1		Dinn,2019
Axinella infundibuliformis	SPX29	Norway	MT506490.1		Unpublished
Axinella infundibuliformis	SPX28	Norway	MT506489.1		Unpublished
Axinella artica	CMNI 2018-0099	Canada	MK570860.1		Unpublished
Axinella sp.	Po.25779	Israel	KX866742.1		Idan et al. 2016
Axinella sp.	Po.26035	Israel	KX866777.1		Idan et al. 2016
Axinella cannabina	Po.25604	Israel	KX866735.1		Idan et al. 2016
Axinella rugosa	not provided	Norway	KC869419.1		Thacker et al. 2013
Axinella rugosa	SPX137	Norway	MT506487.1		Unpublished
Axinella rugosa	SPX43	Norway	MT506487.1		Unpublished

Axinella rugosa	SPX40	Norway	MT506486.1		Unpublished
Axinella sp.	not provided	not provided	JQ034552.1		Erpenbeck et al. 2012
Characella pachastrelloides	ZMBN:80248	Norway		HM592778.1	Cardenas et al. 2011
Characella pachastrelloides	ZMA:POR 18041	Gulf of Cadiz		HM592779.1	Cardenas et al. 2011
Characella pachastrelloides	ZMBN:85225	Portugal		HM592780.1	Cardenas et al. 2011
Characella pachastrelloides	ZMA:POR 20375	Scotland		HM592781.1	Cardenas et al. 2011
Characella sp.	HBOI 14-VIII-09-2-004	USA		KC869483.1	Thacker et al. 2013
Characella_E17		Selvagens Islands		xxxx	this study
Characella_E16		Selvagens Islands		xxxx	this study
Characella sp.	not provided	Japan	AB453834.1		Nishijima et al. 2010
Characella sp.	TS3999	South African	MT491480.1		Unpublished

Characella sp.	TS4025	South African	MT491482.1		Unpublished
Characella sp.	TS4026	South African	MT491483.1		Unpublished
Characella sp.	TS4377	South African	MT491490.1		Unpublished
Characella pachastrelloides	ZMBN:80248	Norway	HM592672.1		Cardenas et al. 2011
Characella pachastrelloides	ZMBN:85225	Portugal	HM592709.1		Cardenas et al. 2011
Characella pachastrelloides	ZMA:POR 18041	Gulf of Cadiz	HM592713.1		Cardenas et al. 2011
Characella pachastrelloides	ZMA:POR 20375	Japan	HM592749.1		Cardenas et al. 2011
Characella pachastrelloides	BDV1826	Ireland	MK085975.1		Unpublished
Characella_E16		Selvagens Islands	xxxxxx		this study
Characella_E17		Selvagens Islands	xxxxxx		this study
Characella sp.	TS4029	South African	MT491484.1		Unpublished
Characella sp.	TS4030	South African	MT491485.1		Unpublished

Characella sp.	TS4035	South African	MT491486.1		Unpublished
Characella sp.	TS4036	South African	MT491487.1		Unpublished
Characella sp.	TS4047	South African	MT491488.1		Unpublished
Calyx sp.	MKB1132	Papua New Guinea		JN179042.1	Redmond et al. 2011
Calyx nicaeensis	Po.25570	East_Mediterranean		KX688751.1	Unpublished
Calyx podatypa	CBC2012-051-002	Belize		MH749426.1	Unpublished
Calyx_E15		Selvagens Islands		xxxx	this study
Calyx sp.	TS4212	South African	MT491624.1		Unpublished
Calyx nicaeensis	Po.25570	Israel	KX866755.1		Unpublished
Calyx sp.	i525/CFM-IEOMA7403	Balearic-Islands_Western_Mediterranean	MW858349.1		Diaz et al. 2011
Calyx podatypa	MCZ:DNA106224	Caribbean	JX999086.1		Riesgo et al. 2014
Calyx_E15		Selvagens Islands	xxxxxx		this study

Calyx arcuarius	NIWA29140	New_Zealand	LN850178.1		Vargas et al. 2015
Calyx arcuarius	NIWA37656	New_Zealand	LN850179.1		Vargas et al. 2015
Calyx arcuarius	NIWA38732	New_Zealand	LN850180.1		Vargas et al. 2015
Calyx arcuarius	NIWA29165	New_Zealand	LN850231.1		Vargas et al. 2015
Calyx arcuarius	NIWA28878	New_Zealand	LN850235.1		Vargas et al. 2015
Petrosia hoeksemai	POR14474	Indonesia		JN179033.1	Redmond et al. 2011
Petrosia plana	POR14516	Indonesia		JN179034.1	Redmond et al. 2011
Petrosia ficiformis	not provided	Mediterranean		AJ225828.1	Lafay et al. 1992
Petrosia ficiformis	Po.25572	Israel_East_Mediterranean		KX688752.1	Unpublished
Petrosia (Strongylophora) sp.	MKB587	Caribbean		JN179039.1	Redmond et al. 2011
Petrosia strongylata	POR16747	Indonesia		JN179040.1	Redmond et al. 2011

Petrosia weinbergi	NCI209	USA		KC869497.1	Thacker et al. 2013
Petrosia_E04		Selvagens Islands		xxxx	this study
Petrosia lignosa	NCI279	Malaysia		KC869595.1	Thacker et al. 2013
Petrosia sp	MKB560			JN179035.1	Redmond et al. 2011
Petrosia sp	MKB1068			JN179038.1	Redmond et al. 2011
Petrosia sp	MKB1020			JN179036.1	Redmond et al. 2011
Petrosia sp	MKB1028			JN179037.1	Redmond et al. 2011
Petrosia durissima	B205	Caribbean	EF519663.1		Erpenbeck et al. 2008
Petrosia durissima	TS4240	South African	MT491549.1		Unpublished
Petrosia durissima	TS4828	South African	MT491550.1		Unpublished

Petrosia durissima	TS3821	South African	MT491552.1		Unpublished
Petrosia durissima	TS3640	South African	MT491553.1		Unpublished
Petrosia durissima	TS4444	South African	MT491555.1		Unpublished
Petrosia durissima	TS4602	South African	MT491556.1		Unpublished
Petrosia durissima	TS3411	South African	MT491558.1		Unpublished
Petrosia durissima	TS4228	South African	MT491559.1		Unpublished
Petrosia ficiformis	not provided	Mediterranean	KM452904.1		Burgsdorf et al. 2014
Petrosia ficiformis	not provided	Mediterranean	KM452905.1		Burgsdorf et al. 2014
Petrosia ficiformis	not provided	Mediterranean	KM452906.1		Burgsdorf et al. 2014
Petrosia ficiformis	not provided	Mediterranean	KM452907.1		Burgsdorf et al. 2014
Petrosia ficiformis	not provided	Mediterranean	KM452908.1		Burgsdorf et al. 2014

Petrosia ficiformis	not provided	Mediterranean	KM452909.1		Burgsdorf et al. 2014
Petrosia ficiformis	not provided	Mediterranean	KM452910.1		Burgsdorf et al. 2014
Petrosia ficiformis	not provided	Mediterranean	KM452911.1		Burgsdorf et al. 2014
Petrosia ficiformis	not provided	Mediterranean	KM452912.1		Burgsdorf et al. 2014
Petrosia ficiformis	Po.25572	Israel	KX866751.1		Unpublished
Petrosia pellasarca	KY082	Caribbean	MZ486980.1		Unpublished
Petrosia pellasarca	KY083	Caribbean	MZ486981.1		Unpublished
Petrosia pellasarca	KY085	Caribbean	MZ486982.1		Unpublished
Petrosia pellasarca	KY107	Caribbean	MZ486994.1		Unpublished
Petrosia pellasarca	KY129	Caribbean	MZ487006.1		Unpublished
Petrosia pellasarca	KY172	Caribbean	MZ487034.1		Unpublished
Petrosia pellasarca	KY215	Caribbean	MZ487049.1		Unpublished

Petrosia pellasarca	KY216	Caribbean	MZ487050.1		Unpublished
Petrosia pellasarca	KY217	Caribbean	MZ487051.1		Unpublished
Petrosia pellasarca	KY326	Caribbean	MZ487083.1		Unpublished
Petrosia weinbergi	CU195	Caribbean	MZ486846.1		Unpublished
Petrosia weinbergi	CU196	Caribbean	MZ486847.1		Unpublished
Petrosia weinbergi	CU197	Caribbean	MZ486848.1		Unpublished
Petrosia weinbergi	CU199	Caribbean	MZ486849.1		Unpublished
Petrosia weinbergi	CU200	Caribbean	MZ486850.1		Unpublished
Petrosia weinbergi	CU201	Caribbean	MZ486851.1		Unpublished
Petrosia weinbergi	KY233	Caribbean	MZ487056.1		Unpublished
Petrosia weinbergi	SX213	USA	MZ487376.1		Unpublished
Petrosia weinbergi	SX214	USA	MZ487377.1		Unpublished
Petrosia weinbergi	SX234	USA	MZ487383.1		Unpublished

Petrosia weinbergi	SX245	USA	MZ487386.1		Unpublished
Petrosia weinbergi	SX269	USA	MZ487401.1		Unpublished
Petrosia_E04		Selvagens Islands	xxxxxx		this study
Topsentia_E18		Selvagens Islands	xxxxxx		this study
Topsentia ophiraphidites	NIWA28878	Caribbean	MZ487267.1		Vargas et al. 2015
Topsentia ophiraphidites	SX001	Caribbean	MZ487309.1		Unpublished
Topsentia ophiraphidites	SX203	Caribbean	MZ487371.1		Unpublished
Topsentia ophiraphidites	KY206	Caribbean	MZ487597.1		Unpublished
Topsentia sp.	SX178	Caribbean	MZ487353.1		Unpublished
Topsentia sp	not provided	Cantabrian Sea	MT680135.1		Unpublished
Topsentia sp	not provided	Cantabrian Sea	MT680136.1		Unpublished
Topsentia sp	not provided	Cantabrian Sea	MT680137.1		Unpublished

Topsentia sp	not provided	Cantabrian Sea	MT680138.1		Unpublished
Topsentia sp	not provided	Cantabrian Sea	MT680139.1		Unpublished
Topsentia sp	not provided	Cantabrian Sea	MT680141.1		Unpublished
Topsentia sp	not provided	Cantabrian Sea	MT680144.1		Unpublished
Topsentia sp	not provided	Cantabrian Sea	MT680140.1		Unpublished
Topsentia sp	not provided	Cantabrian Sea	MT680142.1		Unpublished
Topsentia sp	not provided	Cantabrian Sea	MT680143.1		Unpublished
Topsentia sp	not provided	Cantabrian Sea	MT680145.1		Unpublished
Topsentia ophiraphidites	KY218	Caribbean	MZ487598.1		Unpublished
Topsentia ophiraphidites	KY146	Caribbean	MZ487596.1		Unpublished
Topsentia ophiraphidites	SX297	Caribbean	MZ487414.1		Unpublished
Topsentia ophiraphidites	SX205	Caribbean	MZ487373.1		Unpublished
Topsentia ophiraphidites	SX204	Caribbean	MZ487372.1		Unpublished

Topsentia ophiraphidites	SX202	Caribbean	MZ487370.1		Unpublished
Topsentia ophiraphidites	SX154	Caribbean	MZ487340.1		Unpublished
Topsentia ophiraphidites	SX132	Caribbean	MZ487329.1		Unpublished
Topsentia ophiraphidites	CUXm04	Caribbean	MZ486920.1		Unpublished
Topsentia ophiraphidites	CUXm03	Caribbean	MZ486919.1		Unpublished
Topsentia ophiraphidites	CU239	Caribbean	MZ486918.1		Unpublished
Topsentia ophiraphidites	CU241	Caribbean	MZ486876.1		Unpublished
Topsentia ophiraphidites	CU242	Caribbean	MZ486877.1		Unpublished
Topsentia ophiraphidites	CU243	Caribbean	MZ486878.1		Unpublished
Topsentia ophiraphidites	CU244	Caribbean	MZ486879.1		Unpublished
Topsentia ophiraphidites	CU245	Caribbean	MZ486880.1		Unpublished
Topsentia ophiraphidites	CU246	Caribbean	MZ486881.1		Unpublished
Topsentia ophiraphidites	CU248	Caribbean	MZ486882.1		Unpublished

Topsentia ophiraphidites	CUXm01	Caribbean	MZ486884.1		Unpublished
Topsentia ophiraphidites	KY299	Caribbean	MZ486917.1		Unpublished
Topsentia ophiraphidites	BZ140	Caribbean	MZ487076.1		Unpublished
Topsentia ophiraphidites	BZ140	Caribbean	MZ486587.1		Unpublished
Topsentia ophiraphidites	KY088	Caribbean	MZ486984.1		Unpublished
Topsentia sp	not provided	Caribbean	MZ487313.1		Unpublished
Topsentia sp	not provided	Caribbean	MZ487082.1		Unpublished
Topsentia sp	not provided	Caribbean	MZ487080.1		Unpublished