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Diversity of intertidal marine sponges from the western coast of Portugal (North-east Atlantic)

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Abstract

Sponges are important components of intertidal marine communities. There is a lack of information about intertidal marine sponge diversity in the western coast of Portugal (North-east Atlantic). In the present work we identified the most common intertidal sponges of the western coast of Portugal, and made a comprehensive list of the intertidal species described so far for this region. Sponges belonging to the Classes Calcarea and Demospongiae were identified, the former class for the first time at these locations. Demospongiae are the most common intertidal sponges, present in all sampling locations. We used an integrative approach for Demospongiae identification, using both morphological and molecular characters. Molecular identification, using a CO1 marker proved to be helpful in the identification to the genus level, despite some limitations, such as difficulty in amplification experienced for sponges as well as non-target organisms. A total of 170 specimens were collected. Seven specimens (five species) belonged to the Class Calcarea and 163 specimens (23 species) to the Class Demospongiae. The demosponge *Hymeniacidon perlevis* was present at all sample locations. Calcarean species were primarily found in samples taken along the south-western coast.

Introduction

Porifera is the oldest metazoan group still extant on our planet and one of the most abundant groups of animals. These organisms are key members of shallow- and deep-water benthic ecosystems, occupying all aquatic environments, from marine to freshwater, tropical, temperate and polar areas (Sarà & Vacelet, 1973; Van Soest *et al.*, 2012). There are more than 8500 species (according to World Porifera Database; Van Soest *et al.*, 2017) of Porifera accepted and an additional 2300–3000 species already identified but undescribed (Appeltans *et al.*, 2012). The Class Demospongiae comprises 83% of all living sponges (Van Soest *et al.*, 2012; Morrow & Cárdenas, 2015). Sponges play crucial steps in the cycle of dissolved nutrients and organic matter in marine environments (Bell, 2008; Maldonado *et al.*, 2012), and are a vast source of compounds with biotechnological applications (Leal *et al.*, 2012).

Economically, Porifera are also of major importance due to the extensive production of secondary metabolites, either by their own chemistry or that of their symbionts. Cyanobacteria, a common sponge symbiont, and known for their active secondary metabolism, have already been reported in intertidal sponges from this geographic location (Alex *et al.*, 2012, 2013; Alex & Antunes, 2015; Regueiras *et al.*, 2017). New secondary metabolites from Porifera, all from Demospongiae, are among the most promising to use for pharmaceutical applications (Leal *et al.*, 2012). Intertidal sponges can also be used as bioindicators for water quality monitoring (Cebrian *et al.*, 2007; Mahaut *et al.*, 2013).

Hooper & van Soest (2002) published a revised book on sponge classification, improving our knowledge of sponge biodiversity. This classification relies greatly on spicules morphology and their arrangement in sponge tissue (Morrow *et al.*, 2013). The problem with this classification is that sponges are invertebrates with a high degree of ecophenotypic plasticity, influenced by parameters such as light, sedimentation, substratum type and orientation, and water-flow regime (Bell & Barnes, 2000; Erpenbeck *et al.*, 2006, 2016; Van Soest *et al.*, 2012). Also, many of these morphological characters can be non-homologous, resulting in unresolved and ambiguous classification (Boury-Esnault, 2006). Problems related to identification resulted in disregarding sponges in large-scale surveys. In order to overcome this issue, molecular characters are being used as an aid for resolving these limitations (Wörheide *et al.*, 2005, 2007; Cárdenas *et al.*, 2009, 2012; Pöppe *et al.*, 2010; Vargas *et al.*, 2012; Boury-Esnault *et al.*, 2013). Although phylogenetic studies have shown that the four Porifera classes are monophyletic, many major clades of sponges appear to be paraphyletic, leading to a revision of traditional sponge classification (Cárdenas *et al.*, 2012; Hill *et al.*, 2013; Thacker *et al.*, 2013; Morrow & Cárdenas, 2015; Alvizu *et al.*, 2018).

In sponge phylogenetic studies, many different molecular markers have been used, both nuclear and mitochondrial. A 5′ partition of the mitochondrial cytochrome oxidase subunit 1 (CO1) (Folmer *et al.*, 1994) is among the most popular markers, being used for the 'barcoding of life' initiative. The Sponge Barcoding Project (Wörheide *et al.*, 2007) was the first one on

any non-bilateral taxon, aiming to cover all sponge taxa using primarily the 5′ partition of the CO1 marker.

The western coast of Portugal extends for more than 600 km and has some particular biogeographic circumstances (Boaventura *et al.*, 2002), being one of the warmest European countries, with climatic influences from the Atlantic Ocean and Mediterranean Sea (Kottek *et al.*, 2006). As a result, biodiversity is a mixture of the one present in the North-eastern Atlantic and the Mediterranean (Boaventura *et al.*, 2002). Although sponges can be dominant members of some communities and play important roles in a variety of ecosystem functions (Rützler, 2012; Wulff, 2012), our knowledge of the intertidal and subtidal marine sponges in western Portugal derives especially from the works of Hanitsch (1895), Lévi & Vacelet (1958), Saldanha (1974), Lopes (1989) and Pereira (2007). In recent years, and due to difficulties in sponge identification, most intertidal diversity studies performed in this area (e.g. Monteiro Marques *et al.*, 1982; Boaventura *et al.*, 2002; Pereira *et al.*, 2006) neglected the phylum Porifera, and improving our understanding of their biodiversity can be essential for habitat protection.

The aim of the present study is to characterize sponge diversity from the western coast of Portugal (NE Atlantic) using both morphological and molecular characters.

Materials and methods

Study site

Sampling locations were selected along the entire western coast of Portugal (Figure 1). All beaches had a combination of sand and rocks. Only rocky shore locations were selected as sponges are sessile animals that settle on hard surfaces. Figure 1A–C show three different sampling locations. Sampling periods were restricted to a few hours because of tidal regimes. To gain access to the largest possible intertidal area, sampling was always scheduled during spring tide (0.5 m below the mean sea level).

Sampling took place between September 2010 and August 2013 in Portugal (North-east Atlantic). Collected sponges inhabit the rocky intertidal region and were predominant in sheltered areas, protected from the strong sun and tide, often lying at the base of the rocks.

Sponge samples were collected from 12 different intertidal sites (Figure 1). Table 1 summarizes the information about the sampling locations (geographic coordinates, number of sampling trips and number of specimens collected). A total of 31 collection trips were made and 170 sponges sampled. Sponges were on rock overhangs, and were collected through wading and with the help of a knife. After collection, sponges were immediately carried to the laboratory and processing usually began within 1 h after collection (up to maximum of 28 h after collection).

To maximize the diversity of the sponges analysed we covered, as much as possible, all the rocky areas of each beach.

Samples were photographed and preserved in 96% ethanol both for molecular analysis and morphological identification.

Sponge identification

Sponges were identified based on shape, consistency, texture, colour, habitat and spicules morphology, dimensions and arrangement. All sponge species collected were identified according to Lopes (1989), Hooper & van Soest (2002) and Van Soest *et al.* (2017). Spicules temporary preparations and permanent slides of sponges cross sections were made according to the methods described by Lopes (1995). Preparations were analysed under optical microscopy (Olympus BX41 microscope; Olympus Europe). Spicules were photographed and measured using CellB (Olympus Europe) software. Permanent slides were only made for one specimen of each identified species.

All collected specimens and permanent slides are deposited at BBE (Blue Biotechnology and Ecotoxicology) laboratory, CIIMAR-UP (Interdisciplinary Centre of Marine and Environmental Research – University of Porto).

Molecular analyses

DNA extraction

Total genomic DNA was extracted from sponge tissue (choanosomal tissue) using a commercially available Purelink™ Genomic DNA mini Kit (Invitrogen, San Diego, CA) and stored at −20° C until further analysis. gDNA integrity was checked by agarose gel electrophoresis with GelRed™ (Biotium) staining.

PCR and sequencing of demosponge specimens

PCR amplification was done only for sponges belonging to the Class Demospongiae, using a fragment located at the 5′ site of the mitochondrial cytochrome oxidase subunit 1 (CO1). Primers used were designed by Meyer *et al.* (2005) (dgLCO1490: 5′-GGTCAACAAATCATAAAGAYATYGG-3′; dgHCO2198: 5′-TAAACTTCAGGGTGACCAAARAAYCA-3′) and based on the ones described by Folmer *et al.* (1994). PCR conditions employed were as follows: initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 40 s, annealing at 50°C for 40 s and extension at 72°C for 1 min and a final extension step at 72°C for 10 min. When necessary, amplification was done using primer forward from Meyer *et al.* (2005), combined with the reverse from Xavier *et al.* (2010) (PorCOI12rev: 5′-ACTGCCCCCATNGATAAAACAT-3′). This reverse primer amplifies an alternative partition of the CO1 gene that overlaps ∼60 bp with Folmer's 3′ partition and includes Erpenbeck's 'I3-M11' (Erpenbeck *et al.*, 2006), a partition known to be more informative in cases of shorter divergence times. The incorporation of the primer designed by Xavier *et al.* (2010), showed to be more sponge specific, helping overcome problems related with amplification of non-target DNA. The following protocol was used: initial denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 94°C for 40 s, annealing at 54°C for 45 s and extension at 72°C for 90 s and a final extension step at 72°C for 10 min. Between 5–10 ng of DNA were used for the PCR amplification. All PCR reactions were prepared in a 50 µl volume using Supreme NZYTaq 2x Green MasterMix (NZYTech, Lisboa, Portugal). Thermal cycling was carried out using Biometra T-Professional standard thermocycler (Biometra, Göttingen, Germany). PCR products were separated by 1.5% (w/v) agarose gel in 1× TAE buffer (Invitrogen, San Diego, CA, USA). The gels were stained with GelRed™ (Biotium, Fremont, CA, USA) and photographed under UV transillumination. For DNA sequencing each amplified product was purified using an Invitrogen PureLink™QuickGel Extraction and PCR Purification Combo Kit (Invitrogen, San Diego, CA, USA) according to the manufacturer's protocol followed by direct sequencing of the amplicons in both directions (GATC Biotech, Cologne, Germany).

Phylogenetic analysis

The sequences obtained were inspected, edited and aligned using Geneious® v9.1.5 software (Kearse *et al.*, 2012). The final sequences were used for a similarity search using BLAST and the NCBI nucleotide database (http://www.ncbi.nlm.nih.gov/BLAST) in order to complement sponge morphological identification. The nucleotide sequences were aligned with Muscle (Edgar, 2004). The unedited aligned file is provided in supplementary material (S1). Alignments were manually inspected and curated using BioEdit (Hall, 1999). Maximum-likelihood (ML)

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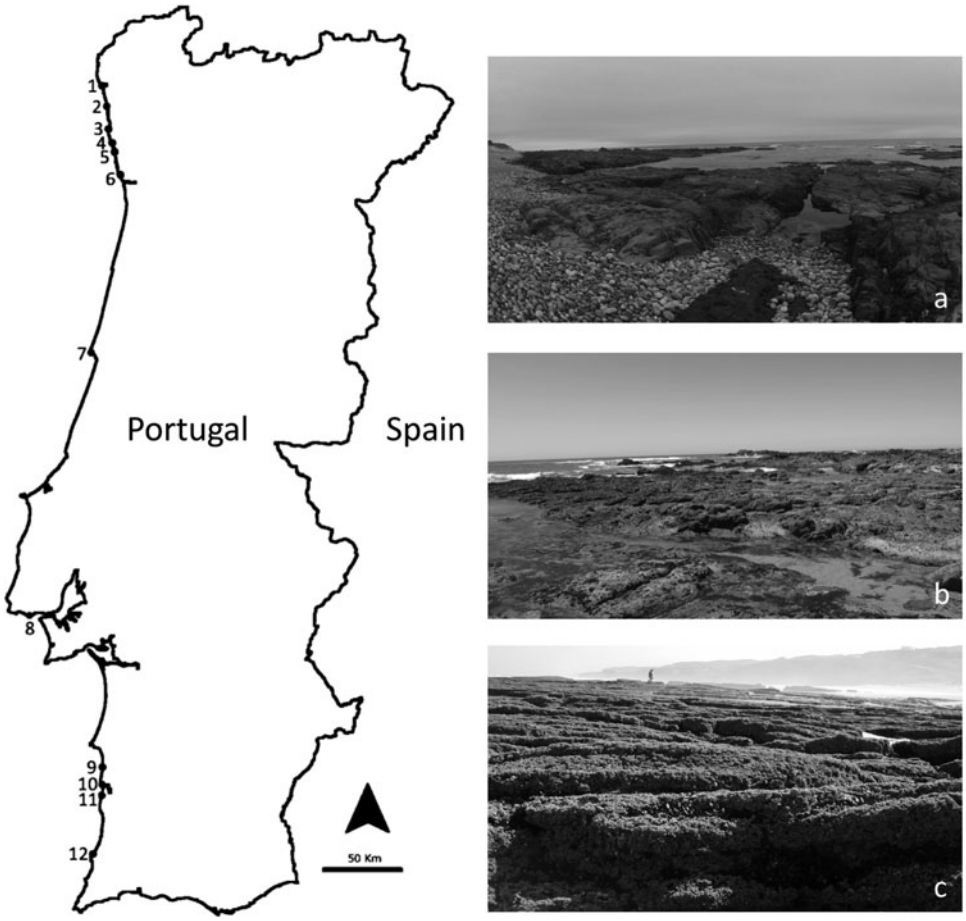

Fig. 1. Sampling locations in Portugal: (1) Viana do Castelo, (2) Esposende, (3) Apúlia, (4) Angeiras, (5) Memória, (6) Aguda, (7) Buarcos, (8) S. João do Estoril, (9) Porto Côvo, (10) Vila Nova de Milfontes, (11) Almograve, (12) Monte Clérigos. Pictures (a), (b) and (c) illustrate three of the sampling locations: Esposende (a), Memória (b) and Porto Côvo (c).

Table 1. Summary of sampling locations: latitude, longitude, number of sampling trips and number of specimens collected

Study site	Latitude	Longitude	No. of sampling trips	No. of specimens collected
(1) Viana do Castelo	N 41° 41′ 48,79″	W 8° 51′ 4,03″	2	8
(2) Esposende	N 41° 34′ 25,59″	W 8° 47′ 54,81″	3	5
(3) Apúlia	N 41° 29′ 17.34″	W 8° 46′ 59.38″	1	1
(4) Angeiras	N 41° 16′ 6.08″	W 8° 43′ 33.39″	2	1
(5) Memória	N 41° 13′ 52.27″	W 8° 43′ 18.34″	12	109
(6) Aguda	N 41° 2′ 58.35″	W 8° 39′ 19.22″	2	14
(7) Buarcos	N 40° 9′ 22.36″	W 8° 52′ 18.49″	2	11
(8) S. João do Estoril	N 38° 41′ 31.68″	W 9° 21′ 57.74″	1	3
(9) Porto Côvo	N 37° 52′ 3.04″	W 8° 47′ 37.19″	1	1
(10) Vila Nova de Milfontes	N 37° 42′ 58.61″	W 8° 47′ 4.79″	1	6
(11) Almograve	N 37° 39′ 2.7″	W 8° 48′ 10.8″	1	2
(12) Monte Clérigos	N 37° 20′ 29.35″	W 8° 51′ 10.05″	1	9

phylogenetic trees (Felsenstein, 1981) were constructed in PhyML (Guindon & Gascuel, 2003); with 100 bootstrap replicates using nearest-neighbour interchanges (NNIs) tree search criteria. The best fit evolutionary model TrN + I + G under Akaike Information Criterion with correction (AICc) implemented in MrAIC v1.4.6 (Nylander, 2004) was selected for ML analysis. As the point of the phylogenetic analysis was not to make any evolutionary inference, focusing on sponge diversity rather than evolutionary relationships, an unrooted tree was used.

All sequences were submitted to the GenBank database (accession numbers KY492518–KY492600).

Results

A total of seven specimens (five species) were identified as belonging to the class Calcarea and 163 specimens (26 species) to the class Demospongiae. Although sampling locations were distributed along all the western coast of Portugal, due to

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proximity to our laboratory, most samplings were made on the north-western coast, mainly at Memória beach. Among Demospongiae, all species identified belonged to the subclasses Heteroscleromorpha, Keratosa and Verongimorpha. Table 2 shows the distribution of the identified species across the study area. All calcarean sponges were only present in one or two different locations, especially on the south-western coast. For Demosponges, only six different species were present in at least three different locations. *Hymeniacidon perlevis*, *Ophlitaspongia papilla*, *Clathria* sp. and *Ircinia variabilis* were the only sponges belonging to the class Demospongiae identified in the southern locations. *Hymeniacidon perlevis* was the most prevalent sponge along the sampled area, identified in 11 of the 12 sampling locations. Memória beach had the highest diversity of sponges (25 species) with 15 species only here identified.

The last column of Table 2 shows the known distribution of the identified sponges, made in accordance to the information available at World Porifera Database (Van Soest *et al.*, 2017) and based on the Marine Ecoregions of the World (Spalding *et al.*, 2007). From this information, it is possible to see that all species here identified have already been described in the North-east Atlantic or the Atlanto-Mediterranean region.

Figure 2 shows photos of the 31 identified sponge species. This identification is based on the morphological characters (Table S2 in supplementary material shows information on morphological identification of the demosponges, as spicules diversity and their measurements) and, when sequences obtained, further confirmed by molecular analyses (Table S3 in supplementary material shows data from the sequenced demosponges specimens and similarities with other CO1 sequences available at the nucleotide database at NCBI).

From the 163 demosponges collected, we were only able to retrieve high quality sponge DNA for 83 of them. Sequences ranged from 278 bp to 1084 bp, representing 18 species across 14 genera and 10 families. From the remaining specimens, obtained sequences had poor quality or amplified DNA from other small invertebrates or marine algae, and were discarded. Molecular analysis was carried out to apply an integrative taxonomic approach, complementing the morphological identification (see Table S2 in supplementary material). Molecular analysis allowed comparison of obtained sequences with those available at the GenBank nucleotide database (NCBI) (Table S3).

The phylogenetic tree (Figure 3) revealed a well-supported topology, by Maximum likelihood tree-reconstruction approach, clearly separating different sponge genera. All sequences obtained belong to the subclass Heteroscleromorpha and there is a clear distinction between the different orders. At the orders level, phylogenetic analysis (Figure 3) showed to be monophyletic, with high bootstrap support values for orders Suberitida and Poecilosclerida and moderate support values for other orders. Specimens from the genera *Hymeniacidon*, *Halichondria* and *Aaptos*, belonging to the order Suberitida, formed a distinctive clade. In this clade it is also possible to distinguish between different families (*Hymeniacidon* and *Halichondria* belong to the family Halichondriidae and *Aaptos* belongs to the family Suberitidae) and different genera. Also, the genera *Tedania*, *Hymedesmia*, *Myxilla*, *Phorbas*, *Antho*, *Clathria*, *Ophlitaspongia* and *Amphilectus* all belong to the order Poecilosclerida and form a distinctive clade. At the family level, cladistic separations are also in most cases possible to differentiate.

Here, we present a list of all intertidal demosponges reported to date in the western coast of Portugal. This checklist comprises information from the works of Hanitsch (1895), Lévi & Vacelet (1958), Saldanha (1974), Lopes (1989), Pereira (2007) and Costa *et al.* (2012). Lopes (1989) already made a compilation of the intertidal sponge diversity, which was used as a basis for our list, with all data checked and complemented with more recent published information.

List of intertidal sponges from the western coast of Portugal

Species with an asterisk (*) correspond to the ones found in the present work. After the name of the species, the reference for the first record for the western coast of Portugal is given. For demosponges, the classification system followed was according to Morrow & Cárdenas (2015).

Class CALCAREA Bowerbank, 1862
Subclass CALCARONEA Bidder, 1898
Order LEUCOSOLENIDA Hartman, 1958
Family GRANTIIDAE Dendy, 1893
Genus *Grantia* Fleming, 1828
**Grantia compressa* (Fabricius, 1780) (Pereira, 2007)
Genus *Leucandra* Haeckel, 1872
**Leucandra gossei* (Bowerbank, 1862) (Saldanha, 1974)
Family SYCETTIDAE Dendy, 1893
Genus *Sycon* Risso, 1827
**Sycon ciliatum* (Fabricius, 1780) (Saldanha, 1974)
Subclass CALCINEA Bidder, 1898
Order CLATHRINIDA Hartman, 1958
Family CLATHRINIDAE Minchin, 1900
Genus *Clathrina* Gray, 1867
**Clathrina coriacea* (Montagu, 1814) (Hanitsch, 1895)
**Clathrina blanca* (Miklucho-Maclay, 1868) (Pereira, 2007)
Class DEMOSPONGIAE Sollas, 1885
Subclass HETEROSCLEROMORPHA Cárdenas, Pérez & Boury-Esnault, 2012
Order AXINELLIDA Lévi, 1953
Family RASPAILIIDAE Nardo, 1833
Genus *Eurypon* Gray, 1867
Eurypon clavatum (Bowerbank, 1866) (Lopes, 1989)
Eurypon coronula (Bowerbank, 1874) (Lopes, 1989)
Family STELLIGERIDAE Lendenfeld, 1898
Genus *Stelligera* Gray, 1867
**Stelligera rigida* (Montagu, 1814) (Lopes, 1989)
Order BUBARIDA Morrow & Cárdenas, 2015
Family DICTYONELLIDAE van Soest, Diaz & Pomponi, 1990
Genus *Tethyspira* Topsent, 1890
Tethyspira spinosa (Bowerbank, 1874) (Lopes, 1989)
Order CLIONAIDA Morrow & Cárdenas, 2015
Family CLIONAIDAE d'Orbigny, 1851
Genus *Cliona* Grant, 1826
**Cliona celata* Grant, 1826 (Saldanha, 1974)
Cliona viridis (Schmidt, 1862) (Saldanha, 1974)
Genus *Pione* Gray, 1867
Pione vastifica (Hancock, 1849) (Saldanha, 1974)
Order HAPLOSCLERIDA Topsent, 1928
Family CHALINIDAE Gray, 1867
Genus *Haliclona* Grant, 1841
**Haliclona* sp.1
**Haliclona* sp.2
**Haliclona* (*Rhizoniera*) *rosea* (Bowerbank, 1866)
**Haliclona* (*Haliclona*) *simulans* (Johnston, 1842)
Order POECILOSCLERIDA Topsent, 1928
Family COELOSPHAERIDAE Dendy, 1922
Genus *Lissodendoryx* Topsent, 1892
Lissodendoryx (*Lissodendoryx*) *isodictyalis* (Carter, 1882) (Saldanha, 1974)
Family CRELLIDAE Dendy, 1922
Genus *Crella* Gray, 1867
**Crella* (*Yvesia*) *rosea* (Topsent, 1892)

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Family ESPERIOPSIDAE Hentschel, 1923
Genus *Amphilectus* Vosmaer, 1880
**Amphilectus fucorum* (Esper, 1794) (Lopes, 1989)
Family HYMEDESMIIDAE Topsent, 1928
Genus *Hymedesmia* Bowerbank, 1864
Hymedesmia (*Hymedesmia*) *jecusculum* (Bowerbank, 1866)
Hymedesmia (*Hymedesmia*) *pansa* Bowerbank, 1882 (Lopes, 1989)
Hymedesmia (*Stylopus*) *coriacea* (Fristedt, 1885) (Lopes, 1989)
Genus *Phorbas* Duchassaing & Michelotti, 1864
Phorbas dives (Topsent, 1891) (Lopes, 1989)
Phorbas fictitious (Bowerbank, 1866) (Saldanha, 1974)
**Phorbas plumosus* (Montagu, 1814) (Lopes, 1989)
Family MICROCIONIDAE Carter, 1875
Genus *Antho* Gray, 1867
**Antho* (*Antho*) *granditoxa* Picton & Goodwin, 2007
Antho (*Antho*) *involvens* (Schmidt, 1864) (Lopes, 1989)
Genus *Clathria* Schmidt, 1862
Clathria (*Clathria*) *coralloides* (Scopoli, 1772) (Lopes, 1989)
Clathria (*Clathria*) *toxistricta* Topsent, 1925 (Pereira, 2007)
Clathria (*Microciona*) *atrasanguinea* (Bowerbank, 1862) (Lopes, 1989)
Clathria (*Microciona*) *strepsitoxa* (Hope, 1889) (Lopes, 1989)
**Clathria* sp.
Genus *Ophlitaspongia* Bowerbank, 1866
**Ophlitaspongia papilla* Bowerbank, 1866 (Costa, 2012)
Family MYCALIDAE Lundbeck, 1905
Genus *Mycale* Gray, 1867
Mycale (*Aegogropila*) *contarenii* (Lieberkühn, 1859) (Lopes, 1989)
Mycale (*Carmia*) *macilenta* (Bowerbank, 1866) (Lopes, 1989)
Mycale (*Carmia*) *minima* (Waller, 1880) (Lopes, 1989)
Family MYXILLIDAE Dendy, 1922
Genus *Myxilla* Schmidt, 1862
**Myxilla* (*Myxilla*) *rosacea* (Lieberkühn, 1859) (Hanitsch, 1895)
Family TEDANIIDAE Ridley & Dendy, 1886
Genus *Tedania* Gray, 1867
Tedania (*Tedania*) *anhelans* (Vio in Olivi, 1792) (Saldanha, 1974)
**Tedania* (*Tedania*) *pilarriosae* Cristobo, 2002
Order POLYMASTIIDA Morrow & Cárdenas, 2015
Family POLYMASTIIDAE Gray, 1867
Genus *Polymastia* Bowerbank, 1862
**Polymastia* sp.1
**Polymastia* sp.2
**Polymastia agglutinans* Ridley & Dendy, 1886
**Polymastia penicillus* (Montagu, 1814) (Saldanha, 1974)
Order SUBERITIDA Chombard & Boury-Esnault, 1999
Family HALICHONDRIIDAE Gray, 1867
Genus Halichondria Fleming, 1828
**Halichondria* sp.
**Halichondria* (*Halichondria*) *panicea* (Pallas, 1766) (Carter, 1876)
Genus *Hymeniacidon* Bowerbank, 1858
**Hymeniacidon perlevis* (Montagu, 1814) (Hanitsch, 1895)
Family SUBERITIDAE Schmidt, 1870
Genus *Aaptos* Gray, 1867
**Aaptos aaptos* (Schmidt, 1864)
**Aaptos papillata* (Keller, 1880) (Lopes, 1989)
Genus *Protosuberites* Swartschewsky, 1905
Protosuberites epithyum (Lamark, 1815) (Lopes, 1989)
Genus *Pseudosuberites* Topsent, 1896
Pseudosuberites mollis Topsent, 1925 (Lopes, 1989)
Genus *Suberites* Nardo, 1833
Suberites carnosus (Johnston, 1842) (Lopes, 1989)
Genus *Terpios* Duchassaing & Michelotti, 1864
Terpios fugax Duchassaing & Michelotti, 1864 (Lopes, 1989)
Order TETHYIDA Morrow & Cárdenas, 2015
Family HEMIASTERELLIDAE Lendenfeld, 1889
Genus *Adreus* Gray, 1867
Adreus fascicularis (Bowerbank, 1866) (Lopes, 1989)
Family TETHYIDAE Gray, 1848
Genus *Tethya* Lamark, 1815
Tethya aurantium (Pallas, 1766) (Hanitsch, 1895)
Family TIMEIDAE Topsent, 1928
Genus *Timea* Gray, 1867
Timea mixta (Topsent, 1896) (Lopes, 1989)
Order TETRACTINELLIDA Marshall, 1876
Family ANCORINIDAE Schmidt, 1870
Genus *Stelleta* Schmidt, 1862
Stelletta anancora (Sollas, 1886) (Lopes, 1989)
Stelletta hispida (Buccich, 1886) (Saldanha, 1974)
Family GEODIIDAE Gray, 1867
Genus *Erylus* Gray, 1867
Erylus discophorus (Schmidt, 1862) (Saldanha, 1974)
Genus *Geodia* Lamark, 1817
Geodia cydonium (Linnaeus, 1767) (Saldanha, 1974)
Order TRACHYCLADIDA Morrow & Cárdenas, 2015
Family TRACHYCLADIDAE Hallmann, 1917
Genus *Trachycladus* Carter, 1879
Trachycladus minax Topsent, 1888 (Lopes, 1989)
Subclass KERATOSA Grant, 1861
Order DICTYOCERATIDA Minchin, 1900
Family DYSIDEIDAE Gray, 1867
Genus *Dysidea* Johnston, 1842
**Dysidea fragilis* (Montagu, 1814) (Pérès, 1959)
Family IRCINIIDAE Gray, 1867
Genus *Ircinia* Nardo, 1833
**Ircinia variabilis* (Schmidt, 1862) (Hanitsch, 1895)
Genus *Sarcotragus* Schmidt, 1862
Sarcotragus spinosulus Schmidt, 1862 (Lopes & Boury-Esnault, 1981)
Sarcotragus fasciculatus (Pallas, 1766) (Saldanha, 1974)
Family SPONGIIDAE Gray, 1867
Genus *Spongia* Linnaeus, 1759
Spongia (*Spongia*) *officinalis* Linnaeus, 1759 (Lopes & Boury-Esnault, 1981)
Family THORECTIDAE Bergquist, 1978
Genus *Scalarispongia* Cook & Bergquist, 2000
Scalarispongia scalaris (Schmidt, 1862) (Lopes & Boury-Esnault, 1981)
Order DENDROCERATIDA Minchin, 1900
Family DARWINELLIDAE Merejkowsky, 1879
Genus *Aplysilla* Schulze, 1878
**Aplysilla rosea* (Barrois, 1876) (Lopes, 1989)
Subclass VERONGIMORPHA Erpenbeck, Sutcliffe, De Cook, Dietzel, Maldonado, van Soest, Hooper & Wörheide, 2012
Order CHONDRILLIDA Redmond, Morrow, Thacker, Diaz, Boury-Esnault, Cárdenas, Hajdu, Lobo-Hajdu, Picton, Pomponi, Kayal & Colins, 2013
Family CHONDRILLIDAE Gray, 1872
Genus *Thymosia* Topsent, 1895
Thymosia guernei Topsent, 1895 (Lopes, 1989)
Order VERONGIIDA Bergquist, 1978
Family APLYSINIDAE Carter, 1875
Genus *Aplysina* Nardo, 1834
Aplysina aerophoba (Nardo, 1833) (Lopes, 1989)

Discussion

Most sponge diversity studies focus on subtidal sponges (Carter, 1876; Topsent, 1928; Lévi & Vacelet, 1958; Saldanha, 1974;

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Table 2. Sponges collected from the western coast of Portugal. Sponges are divided in accordance to Class (Calcarea and Demospongiae) and their geographic locations are identified

		North						Centre		South				Species distribution		
Class	Species	Viana do Castelo	Esposende	Apúlia	Angeiras	Memória	Aguda	Buarcos	S. João do Estoril	Porto Côvo	V N Mil Fontes	Almograve	Monte Clérigos	NEA	MED	ATL-MED
Calcarea	*Grantia compressa* (Fabricius, 1780)										X		X	X		
	Leucandra gossei (Bowerbank, 1862)												X			X
	Sycon ciliatum (Fabricius, 1780)										X					X
	Clathrina coriacea (Montagu, 1814)	X				X					X					X
	Clathrina blanca (Miklucho-Maclay, 1868)					X										X
Demospongiae	*Stelligera rigida* (Montagu, 1814)					X										X
	Cliona celata Grant, 1826					X		X								X
	Haliclona sp.1					X										
	Haliclona sp.2					X										
	Haliclona (Rhizoniera) rosea (Bowerbank, 1866)							X								X
	Haliclona (*Haliclona*) *simulans* (Johnston, 1842)	X				X	X	X								X
	Crella (*Yvesia*) *rosea* (Topsent, 1892)					X										X
	Amphilectus fucorum (Esper, 1794)		X			X	X	X								X
	Phorbas plumosus (Montagu, 1814)					X	X	X								X
	Antho (*Antho*) *granditoxa* Picton & Goodwin, 2007					X								X		
	Clathria sp.					X							X			
	Ophlitaspongia papilla Bowerbank, 1866	X				X							X	X		
	Myxilla (*Myxilla*) *rosacea* (Lieberkühn, 1859)					X										X

(*Continued*)

Table 2. (*Continued.*)

Class	Species	North						Centre		South				Species distribution		
		Viana do Castelo	Esposende	Apúlia	Angeiras	Memória	Aguda	Buarcos	S. João do Estoril	Porto Côvo	V N Mil Fontes	Almograve	Monte Clérigos	NEA	MED	ATL-MED
	Tedania (*Tedania*) *pilarriosae* Cristobo, 2002					X								X		
	Polymastia sp.1					X										
	Polymastia sp.2					X										
	Polymastia agglutinans Ridley & Dendy, 1886					X								X		
	Polymastia penicillus (Montagu, 1814)					X										X
	Halichondria (*Halichondria*) *panicea* (Pallas, 1766)		X													X
	Halichondria sp.	X	X			X	X	X	X							X
	Hymeniacidon perlevis (Montagu, 1814)	X	X	X	X	X	X	X	X	X	X	X				X
	Aaptos aaptos (Schmidt, 1864)					X										X
	Aaptos papillata (Keller, 1880)					X										X
	Dysidea fragilis (Montagu, 1814)					X										X
	Ircinia variabilis (Schmidt, 1864)					X					X					X
	Aplysilla rosea (Barrois, 1876)						X									X
Total species per location		5	4	1	1	25	6	7	2	1	5	1	4			

Last column shows species distribution in accordance with the information provided by the World Porifera Database (Van Soest *et al.*, 2017) for the North-Eastern Atlantic (NEA), Mediterranean (MED) or Atlanto-Mediterranean (ATL-MED) distribution.

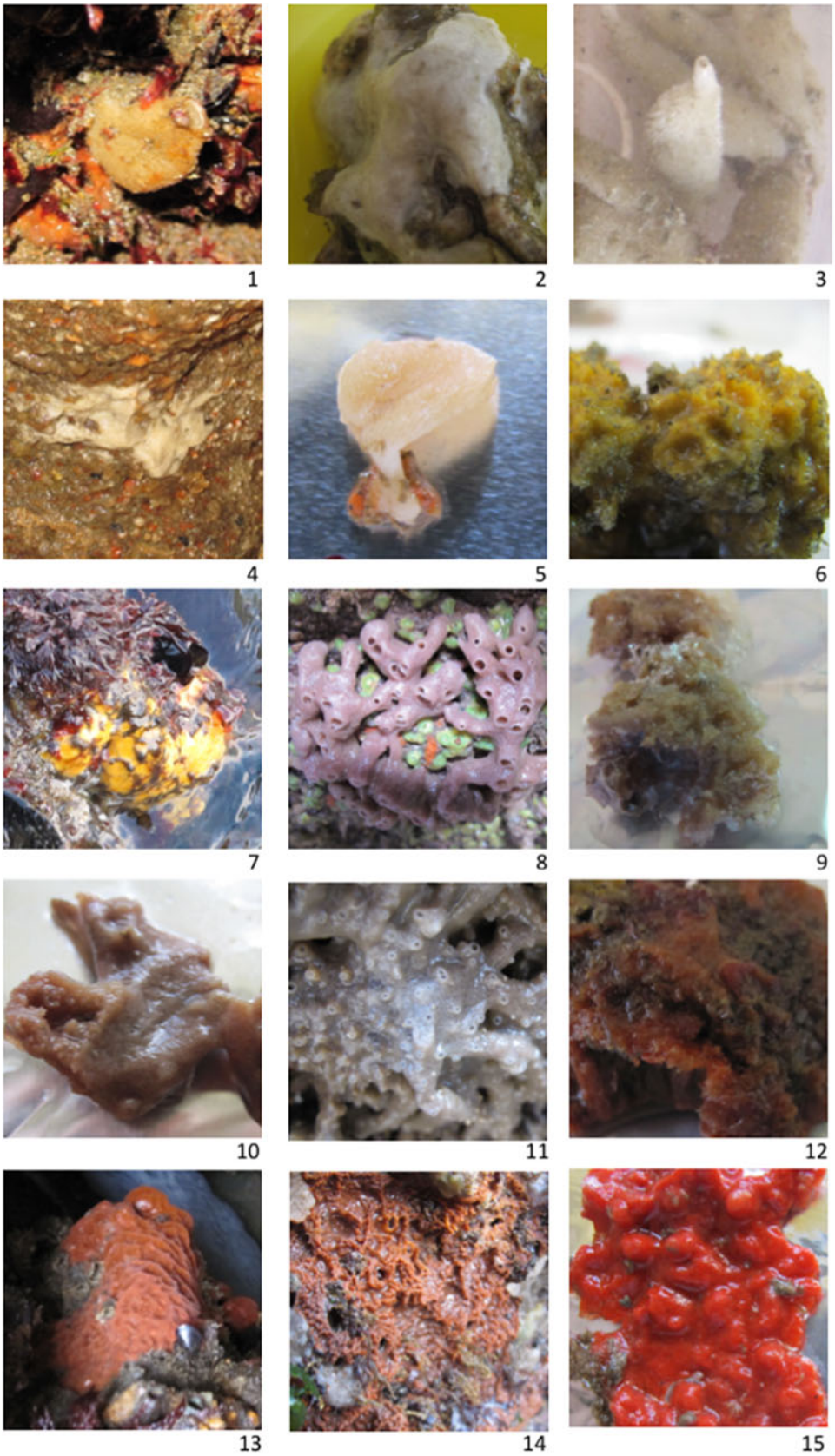

Fig. 2. Photographs of identified sponges: 1. *Grantia compressa*, 2. *Leucandra gossei*, 3. *Sycon ciliatum*, 4. *Clathrina coriacea*, 5. *Clathrina blanca*, 6. *Stelligera rigida*, 7. *Cliona celata*, 8. *Haliclona* sp.1, 9. *Haliclona* sp.2, 10. *Haliclona* (*Rhizoniera*) *rosea*, 11. *Haliclona* (*Haliclona*) *simulans,* 12. *Crella* (*Yvesia*) *rosea*, 13. *Amphilectus fucorum*, 14. *Phorbas plumosus*, 15. *Antho* (*Antho*) *granditoxa*, 16. *Clathria* sp., 17. *Ophlitaspongia papilla*, 18. *Myxilla* (*Myxilla*) *rosacea,* 19. *Tedania* (*Tedania*) *pilarriosae*, 20. *Polymastia* sp.1, 21. *Polymastia* sp.2, 22. *Polymastia agglutinans*, 23. *Polymastia penicillus*, *24. Halichondria* (*Halichondria*) *panicea*, 25. *Halichondria* sp., 26. *Hymeniacidon perlevis*, 27. *Aaptos aaptos*, 28. *Aaptos papillata*, 29. *Dysidea fragilis*, 30. *Ircinia variabilis*, 31. *Aplysilla rosea*.

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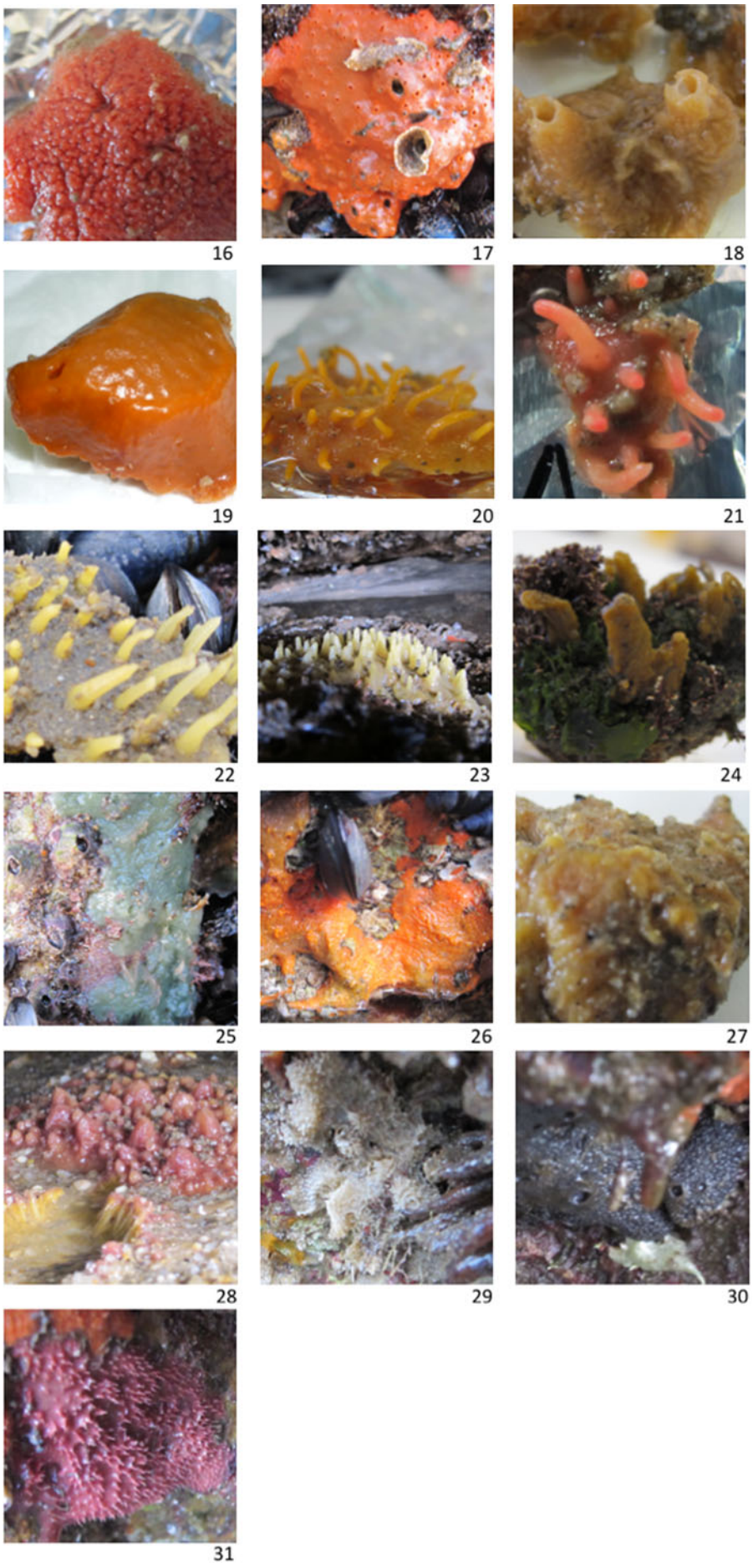

Fig. 2. (*Continued*)

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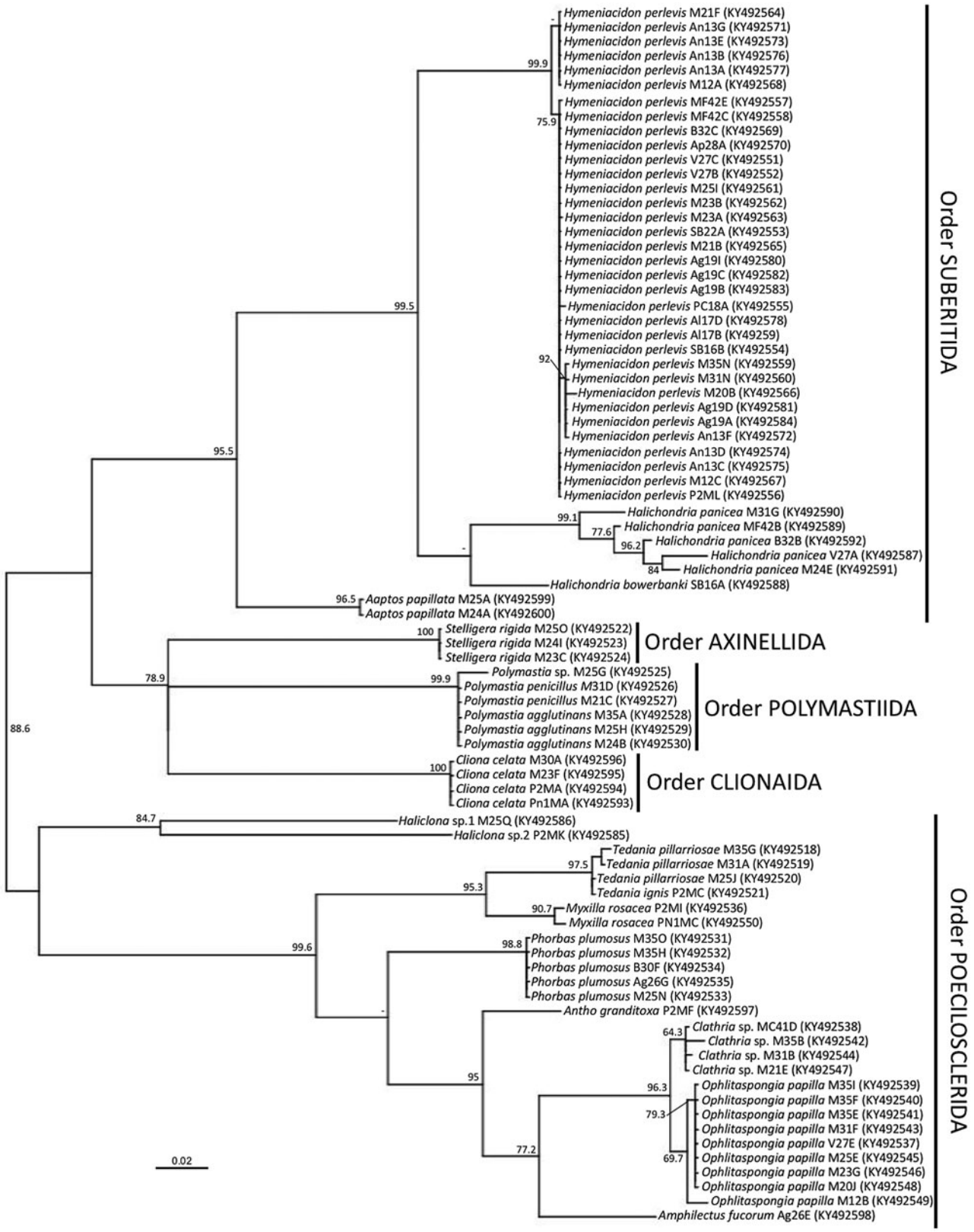

Fig. 3. Maximum likelihood (ML) phylogenetic tree based on the CO1 fragment (concatenation of both the Folmer's and the I3-M11 fragments of the gene CO1) of the sequences from Demospongiae. GenBank accession numbers are given in parentheses. The tree is unrooted. ML bootstrap support values are represented at the nodes. Only bootstrap values greater than 50% are given. The scale bar at the bottom represents 2% sequence divergence. On the right end of the tree information about sponge orders are given.

Lopes & Boury-Esnault, 1981; Naveiro, 2002; Pereira, 2007; Pires, 2007) and most intertidal diversity studies from this geographic area completely neglect the existence of sponges (e.g. Monteiro Marques *et al.*, 1982; Boaventura *et al.*, 2002; Pereira *et al.*, 2006). In Atlantic shores, sponges have been recognized as important members of the ecosystem, both in terms of biomass and species richness, playing significant roles in ecosystem functioning (Xavier & van Soest, 2012) due to being filter feeders.

The present study shows for the first time an updated list of intertidal sponges from the western coast of Portugal. Identified

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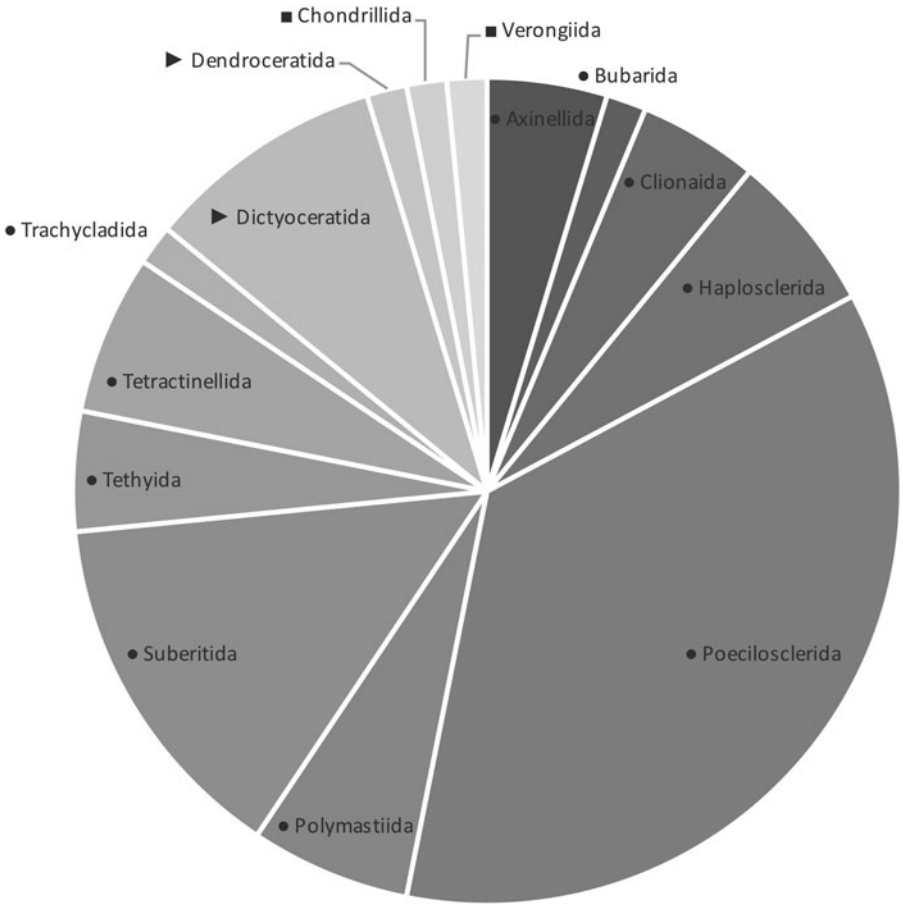

Fig. 4. Graphical distribution of number of species present in each order of the Class Demospongiae described in the intertidal area of the western coast of Portugal. In each order, information about its subclass is provided: ● Heteroscleromorpha; ► Keratosa; ■ Verongimorpha.

sponges from the present work belong to the classes Calcarea (five species) and Demospongiae (26 species). For the first time, calcarean sponges from the intertidal areas in this geographic location are described. So far, to our knowledge, there was no information on intertidal diversity of calcarean sponges (Hanitsch, 1895; Saldanha, 1974; Lopes, 1989; Pereira, 2007). Combining all information available for the class Demospongiae, the intertidal area of the western coast of Portugal has 64 different species described. Figure 4 summarizes the information present in the list presented above.

Most identified species belong to the class Heteroscleromorpha, within 10 orders, 22 families and 31 genera.

Praia da Memória, in the northern part of Portugal, seems to harbour the highest diversity of demosponges. Although the level of diversity of this place was clear when compared with other locations, Memória comprised more than 50% of all sampling trips, which can explain the discrepancies in diversity. Sponges belonging to the class Calcarea showed to be more dominant on the southern intertidal area of Portugal.

From the 26 species of Demospongiae here identified, 12 are described for the first time in the intertidal area and 11 for the first time on the western coast of Portugal. As shown in Table 2, all described sponges have already been reported in the North-east Atlantic and/or Mediterranean Sea. This information is available online at the World Porifera Database (Van Soest *et al.*, 2017). Xavier & Van Soest (2012) analysed diversity patterns of the North-east Atlantic and Mediterranean shallow water sponges, being able to identify 135 species just on the western Iberian Peninsula. These finding emphasize the need for a much deeper study of sponge diversity along the Portuguese coast, as the diversity may still be underestimated.

Hymeniacidon perlevis seems to be almost ubiquitous to all sample locations. Alex *et al.* (2012) have already reported, for the same studied area, genetic richness between different *H. perlevis* specimens in such a small distance (~500 km). Mahaut *et al.* (2013) used *H. perlevis* as a bioindicator and reported it to have a higher accumulation capacity of contaminants than the mussel *Mytilus edulis* Linnaeus. As this sponge inhabits almost all the western coast of Portugal, it can be used for water pollution studies in the future. These findings show the importance of the study of sponges, and knowing their diversity is the first step for every other study.

Plasticity in sponge morphology is very common, which makes sponge identification a challenge. Barnes & Bell (2002) found differences in sponge morphology within the same species with varying depth.

To overcome this issue, many studies have been focusing on molecular data. CO1 has been the most popular marker, as it can help in taxonomy (Pöppe *et al.*, 2010). As it has been the marker chosen for the barcoding of life and the sponge barcoding project, there is more information on public databases for this marker than for any other.

In our study, the use of CO1 helped to distinguish most of our sponges at the genus level. Although there are some limitations in the use of the gene CO1 for Porifera phylogeny resolution, as pointed out by Cárdenas *et al.* (2012), this marker has been successfully used for the Porifera Barcoding project (Wörheide *et al.*, 2007; Vargas *et al.*, 2012), allowing in the majority of cases differentiation between different species. As demonstrated here, CO1 was previously shown to have a good resolution at the family level (Erpenbeck *et al.*, 2002, 2016) and in some cases to the genus level (Erpenbeck *et al.*, 2006).

We were not able to retrieve DNA for all Demospongiae. Extracting DNA from sponge tissue can have its challenges, as it is known that some taxa require specialized protocols (Erpenbeck *et al.*, 2016) and some compounds can be present that can inhibit PCR reaction (Vargas *et al.*, 2012). Also, the

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use of CO1 can result in co-amplification and/or specific amplification of non-target organisms (Vargas *et al.*, 2012). According to Vargas *et al.* (2012) it is easier to amplify DNA in some Porifera families than others. Fifty-five per cent of our samples showed poor DNA quality and/or amplification of DNA from non-target organisms. Vargas *et al.* (2012) found amplification of non-target organisms occurred in 40% of samples.

The incorporation in the molecular analysis of the primer designed by Xavier *et al.* (2010) that includes Erpenbeck's 'I3-M11' partition (Erpenbeck *et al.*, 2006) allowed us to obtain more sequences but not for all Demospongiae. We only amplified this second region when we were not able to obtain target DNA, as this primer showed to be more sponge specific than the Folmer's one. In the future, it would be interesting to amplify all collected sponges using this partition, to help in distinguishing phylogenetically between species and to see if its resolution can separate different populations of the same species in accordance with geographic distribution.

For sponges belonging to the class Calcarea, in the present work was not performed any molecular analysis. In the future, it would be interesting to include this information using a fragment of the 28SrRNA (C-region) proposed as a Calcarea barcode by Voigt & Wörheide (2016).

In this study, we presented for the first time an annotated checklist of intertidal sponges from the western coast of Portugal, based on collection and identification and bibliography data. We presented also the first intertidal data for Calcarea intertidal sponges for the western coast of Portugal. We also showed advantages and limitations of using the CO1 DNA data to help in the identification of Demospongiae. Amplification of a bigger fragment of the CO1 gene, complemented with the use of a more specific protocol for DNA extraction for Porifera should be used in the future in order to perform amplification for all collected demosponges specimens, as well as to allow a phylogenetic study of the sponges of the western coast of Portugal.

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Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0025315419000420

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