Jellyfish impact on aquatic ecosystems: warning for the development of mass occurrences early detection tools

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Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

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À minha avó que me ensinou que para alcançar algo é necessário muito trabalho e sacrifício.

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Abstract

Jellyfish is the free-floating stage of the Medusozoa clade (Phylum Cnidaria). They are animals with an ancient origin in the ocean, generally toxic and considered key organisms in a marine ecosystem. When favorable conditions met, these organisms can proliferate causing mass occurrence events. These phenomena are becoming more frequent and are associated with economic and ecologic losses. Formerly considered only by the negative aspects, the unique characteristics of jellyfish have begun to be explored in many areas of study such as biochemistry, medicine, aquaculture, among others. In Portugal, there are reports of medusozoans throughout the mainland coast and archipelagos. However, there are few information about mass occurrences of these organisms. Interestingly, 2019 was one of the years with the largest number of mass sightings of jellyfishes in Portugal, leading to the temporary closure of some beaches. Information about jellyfish in a national panorama is still scarce and, not only its impacts but also its valences, are poorly studied. In this thesis, we alert for the development of a consistent monitoring net, considering oceanographic parameters and contemplating an environmental deoxyribonucleic acid (eDNA) approach, using deoxyribonucleic acid (DNA) sequencing and bioinformatics methodologies to gather information on jellyfishes and deal with the possible rise of its mass occurrences. Therefore, this work serves as a guide for the study of jellyfish in Portugal and as the basis for the development of mass occurrences early detection tools; as the main goals of this thesis are to establish the Portuguese state of the art of medusozoans, with special focus in jellyfish, and to provide new sequences for the online database GenBank to assist in future studies of jellyfish's early-detection. A detailed revision of the scientific literature and magazine records, showed that jellyfishes occur systematically in Portugal, being *Catostylus tagi* one of the most common species. This fact, associated with its high potential and the genetic information available in the databases, limited to only three genes – 18S ribosomal RNA (18S rRNA), 28S ribosomal RNA (28S rRNA) and internal transcribed spacer 1 (ITS1) – , contributed to the choice of this species as a case study for the optimization of experimental molecular procedures. We selected 16S ribosomal RNA (16S rRNA), cytochrome *c* oxidase subunit I (COX1), cytochrome *c* oxidase subunit III (COX3), 18S rRNA, 28S rRNA, ITS1, and NADH dehydrogenase subunit 6 (NAD6) as target genes to be amplified as they are frequently used in environmental and evolutionary studies. The molecular optimizations performed, resulted in the first amplification of the genes 16S rRNA (MN364410, MN364412, MN364413, MN364414), COX1 (available January 2020), and COX3 (available January 2020) sequences, and new sequences of the genes

18S rRNA (MN128961, MN128962), 28S rRNA (MN128946, MN128947), and ITS1 (MN161198, MN128949) from *C. tagi*. This thesis serves as a guide for the study of medusozoans in Portugal and as a basis for the development of early detection tools of jellyfishes' mass occurrences, as well as, to raise awareness of the society and scientific community on the subject.

Keywords: Cnidaria, Medusozoa, *Catostylus tagi,* jellyfish, aquatic ecosystems, mass occurrences, molecular markers.

Resumo

A medusa é o estado de vida livre do clado Medusozoa (Filo Cnidaria). São animais com uma origem antiga no oceano, geralmente tóxicos e considerados organismos-chave num ecossistema marinho. Quando condições favoráveis se reúnem, estes organismos podem proliferar causando fenómenos de ocorrência em massa. Estes fenómenos estão a tornar-se cada vez mais frequentes sendo associados a perdas económicas e ecológicas. Consideradas antigamente apenas pelos aspetos negativos, as características únicas das medusas começaram a ser exploradas em diversas áreas de estudo como a bioquímica, medicina, aquacultura, entre outras. Em Portugal, existem relatos de medusozoários em toda a costa continental e arquipélagos. No entanto, existe pouca informação sobre ocorrências em massa destes organismos. Curiosamente, 2019 foi um dos anos com o maior número de avistamentos em massa de medusas em Portugal, levando ao encerramento temporário de algumas praias. A informação sobre medusas num panorama nacional ainda é escassa estando ambos, os seus impactos e as suas valências pouco estudadas. Nesta tese, alertamos para a necessidade de desenvolvimento de uma rede de monitorização consistente, considerando parâmetros oceanográficos e contemplando uma abordagem de ácido desoxirribonucleico (ADN) ambiental (*eDNA*), usando sequenciação de ADN e metodologias de bioinformática para recolher informações acerca das medusas e lidar com o possível aumento das suas ocorrências em massa. Portanto, este trabalho serve como um guia para o estudo das medusas em Portugal e como base para o desenvolvimento de ferramentas de deteção precoce de ocorrências em massa; uma vez que os principais objetivos desta tese são estabelecer o estado da arte dos medusozoários em Portugal, com especial enfoque nas medusas, e fornecer novas sequências para o banco de dados online GenBank visando auxiliar em estudos futuros de deteção precoce de medusas. Uma revisão detalhada da literatura científica e dos registos de revistas, mostrou que as medusas ocorrem sistematicamente em Portugal, sendo *Catostylus tagi* uma das espécies mais comuns. Este facto, associado ao seu elevado potencial e à informação genética disponível nas bases de dados estar limitada a apenas três genes – 18S ácido ribonucleico ribossomal (*18S rRNA*), 28S ácido ribonucleico ribossomal (*28S rRNA*) e espaçador interno transcrito 1 (*ITS1*) – contribuiu para a escolha desta espécie como caso de estudo para a otimização de procedimentos moleculares experimentais. Selecionámos o 16S ácido ribonucleico ribossomal (*16S rRNA*), citocromo c oxidase subunidade 1 (*COX1*), citocromo c oxidase subunidade 3 (*COX3*), *18S rRNA*, *28S rRNA*, *ITS1* e o gene que codifica a subunidade 6 da NADH desidrogenase mitocondrial

(*NAD6*) como genes alvo a serem amplificados pois são frequentemente utilizados em estudos ambientais e de evolução. As otimizações moleculares realizadas, resultaram na primeira amplificação dos genes *16S rRNA* (MN364410, MN364412, MN364413, MN364414), *COX1* (disponível em janeiro de 2020) e *COX3* (disponível em janeiro de 2020), e em novas sequências dos genes *18S rRNA* (MN128961, MN128962), *28S rRNA* (MN128946, MN128947) e *ITS1* (MN161198, MN128949) de *C. tagi*. Esta tese serve de guia para o estudo dos medusozoários em Portugal e de base para o desenvolvimento de ferramentas de deteção precoce de ocorrências em massa de medusas, serve também como forma de aumentar a consciencialização da sociedade e da comunidade científica sobre o assunto.

Palavras-chave: Cnidaria, Medusozoa, *Catostylus tagi*, medusa, ecossistemas aquáticos, ocorrências em massa, marcadores moleculares.

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List of Abbreviations

5.8S rRNA	5.8S ribosomal RNA
12S rRNA	12S ribosomal RNA
16S rRNA	16S ribosomal RNA
18S rRNA	18S ribosomal RNA
28S rRNA	28S ribosomal RNA
ATP6	ATP synthase membrane subunit 6
ATP8	ATP synthase membrane subunit 8
BLAST	Basic Local Alignment Search Tool
bp	Base pairs
BSA	Bovine Serum Albumin
CBOL	Consortium for the Barcode of Life
Cf	Final concentration
Ci	Initial concentration
COX1 to COX3	Cytochrome *c* oxidase subunit I to III
CoL	Catalogue of Life
CYTB	Cytochrome b
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphate
eDNA	Environmental DNA
ESS	Environmental Shotgun Sequencing
G	Gonads

GBIF	Global Biodiversity Information Facility
gDNA	Genomic DNA
GFP	Green fluorescence protein
IPMA	Instituto Português do Mar e da Atmosfera
ITS1	Internal transcribed spacer 1
ITS2	Internal transcribed spacer 2
$MgCl_2$	Magnesium chloride
NAD1 to NAD6	NADH dehydrogenase subunit 1 to 6
NCBI	National Center for Biotechnology Information
NEMESIS	National Exotic Marine and Estuarine Species Information System
NGS	Next Generation Sequencing
NJ	Neighbor Joining
NOAA	National Oceanic and Atmospheric Administration
OBIS	Ocean Biogeographic Information System
OMARE	Observatório Marinho de Esposende
PCR	Polymerase Chain Reaction
RNA	Ribonucleic acid
T	Tentacles
UV	Ultraviolet
Vf	Final volume
Vi	Initial volume
WoRMS	World Register of Marine Species

1. General Introduction

1.1 Background

In the eyes of the present scenario, the relationship between mankind and ecosystems is not a typical parasite/host relationship, because human actions, while always seeking their own advantage, are degrading ecosystems to the point that they also cause strong impacts to humankind. Thus, we can instead say that they live an “unbalanced ecological relationship”. The anthropogenic causes of ecosystem destruction can be reduced to just two terms: overpopulation and overexploitation. The second only exists in response to the needs of the former, which in turn is ecologically unavoidable. Analyzing from this perspective, the destiny of Humanity seems to be traced. However, fortunately, science is not ruled by fatalism, instead, it seeks the solution. It is pivotal to understand that the planet Earth has existed for about 4.54 billion years, while the modern human has only been for approximately 300 thousand years (Dalrymple, 1991; Schlebusch *et al.*, 2017). In this way, it is very presumptuous of us to think that who has to be saved is the Earth. Humanity has the duty to protect and preserve Earth’s ecosystems that make it habitable and marine ecosystems are no exception.

Marine ecosystems cover more than 70 % of the Earth's surface and constitute over 99 % of the living space on the planet (Rick and Erlandson, 2008). Moreover, the National Oceanic and Atmospheric Administration (NOAA) estimated that about 95 % of the world's oceans and 99 % of the ocean floor is still unexplored. Endowed with an unequaled biological richness, they are habitat of an immense faunal and floral diversity. Unfortunately, they are increasingly exposed to different threats. The introduction of invasive species, whether accidental or deliberate, coupled with overfishing and pollution, are, by themselves, some of the main factors that impact the well-functioning of marine ecosystems. In addition, they give rise to the most favorable conditions for the emergence of mass occurrence events.

1.2 Organisms that produce mass occurrence events

In the bibliography there is a broad nomenclature used to refer to jellyfish distributions and occurrences, namely “aggregations”, “swarms” and the commonly used “bloom” (see Lucas and Dawson, 2014). In the present thesis, “mass occurrence” will be used to encapsulate all those terms. Mass occurrence events are characterized by large aggregations of organisms that arise in a short period of time causing negative impacts (**Figure 1**). For instance, the so-called red tides are the result of the mass occurrence of protozoans and unicellular algae and its impacts are associated with the release of

toxins (Anderson, 1997). There is a panoply of organisms that can occur in mass. In fact, there are reports of mass occurrence events provoked by ciliates (Olsen *et al.*, 2019), dinoflagellates (Roselli *et al.*, 2019), diatoms (Santhanam *et al.*, 2018), cyanobacteria (Mancini *et al.*, 2010), bryozoans (Rorig *et al.*, 2017) and gelatinous zooplankton such as ctenophores (Sullivan *et al.,* 2001) and jellyfish (Dong, 2018).

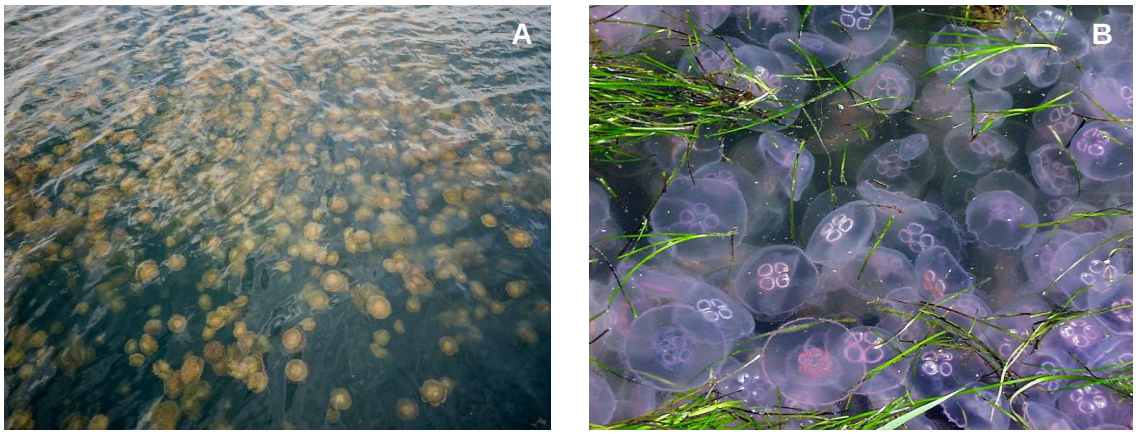

Figure 1 Representative images of jellyfish mass occurrences. **A:** *Cotylorhiza tuberculata* mass occurrence in Mar Menor, Spain. **B:** *Aurelia aurita* mass occurrence in Limfjord, Denmark. Photographs credits: **A** licensed by Stephanie Booth, 2012 under the CC BY-NC-SA 2; **B** licensed by Malene Thyssen, 2002 under the CC BY-SA 3.0, source Wikimedia Commons (https://bit.ly/2kEct0R).

1.3 Ecological role and impact of jellyfishes

According to Lucas and Dawson (2014), gelatinous zooplankton is represented by the organisms that move slowly, are transparent but big enough to be spotted at naked eye. Gelatinous zooplankton classification incorporates very different metazoan groups, between them the jellyfishes (see Lucas and Dawson, 2014).

In the present work, the term “jellyfish” refers exclusively to the organisms that present a medusa stage and belong to the clade Medusozoa. They are members of the phylum Cnidaria, an old group that includes more than 11,000 species. Cnidaria is comprised by three monophyletic clades: Anthozoa, Myxozoa and Medusozoa. Anthozoa is the representative group of corals and anemones, Myxozoa is a group of obligatory endoparasites, while Medusozoa is the one that encompasses the jellyfish (Kayal *et al.*, 2018; Naldoni *et al.*, 2019a; Zhang, 2013). The general life cycle of medusozoans is basically comprised by three stages: planula, sessile polypoid, and pelagic medusoid stage. Medusozoans life cycles either have one annual sexual reproduction event and an overwintering benthic stage (metagenetic life cycle, *i.e.,* medusoid and polypoid), or continuous reproduction and a holoplanktonic life cycle (Ortman *et al.*, 2010; Schnedler-Meyer *et al.*, 2018).

Jellyfishes represent one of the oldest lineages of animals in the ocean, being free-swimming organisms, generally carnivorous, considered key organisms in an aquatic ecosystem as they are voracious predators of planktonic organisms, crustaceans, small fishes, fish eggs and larvae (Doyle *et al.*, 2014; Berwald, 2018). In fact, their sting is the fastest known motion in the animal kingdom (Quiñones *et al.*, 2018). They also serve as food for sea turtles, fishes, sea slugs and birds. Jellyfishes can occur in many aquatic ecosystems, though most are from marine environments. Their distribution is considered seasonal and depends on several biotic and abiotic factors (Quiñones *et al.*, 2018).

Jellyfish not only play a fundamental part in the ecosystems but also represents an important asset for humans (for an extensive review see Doyle *et al.*, 2014). There are a lot of biomedical research on jellyfish that explore its toxins chemical properties and other compounds like mucins and collagen. Research on jellyfish led to two Nobel Prizes. The first was awarded to Charles Richet (Nobel Prize in Medicine, 1913) for his study of anaphylaxis using the poison of the Portuguese man o' war, *Physalia physalis*, as a natural substance to diminish the resistance of organisms to the poison when applied in nonlethal doses (Pitt and Lucas, 2014). The second was awarded to Shimomura, Chalfie and collaborators (Nobel Prize in Chemistry, 2008) for the discovery and development of green fluorescence proteins (GFPs) present in the species *Aequorea victoria* (Prasher *et al.*, 1992). In gastronomy, despite only the Rhizostomae in the medusa stage are considered edible (Armani *et al.*, 2014), jellyfishes are eaten as part of Chinese culture for centuries. This consumption has spread to other Asian countries and is starting to be found in Europe (Armani *et al.*, 2014). More recently, the jellyfish body form was an inspiration to the field of robotics. Cheng *et al.* (2019), have built a soft robotic jellyfish that can be used to surveil and help protect reefs.

Although these organisms can play an important role in aquatic ecosystems and have several benefic applications, they can also provoke many negative impacts. Those negative impacts are mostly associated with their occurrence in mass. Despite it is still not fully understood which abiotic, biotic environmental features, and functional biological characters of these organisms are interacting to cause jellyfish mass occurrences, we can associate them with some factors (Graham *et al.*, 2001; Hamner and Dawson, 2008; Madin and Deibel, 1998). The life history of jellyfish characterized by rapid rates of population increase (Madin and Deibel, 1998), aggregation of sexually mature adults, synchronized spawning, self-fertilization in hermaphrodites and high fecundity, associated with their capability to reproduce both sexually and asexually (Purcell *et al.*, 2007), grants them an extended lifetime fecundity. Moreover, their capability to grow fast (3.5x than non-gelatinous organisms of correspondent size) allows them to exploit different feeding environments in order to better find prey populations (Acuña *et al.*,

2011). Furthermore, due to their relatively large stomachs, they can accumulate food and feed at maximum clearance rate in either very high prey concentration for a brief time, or low prey concentration for an extended time. Moreover, their body composition high in water (96 %) and low in carbon (0.5 %) (Arai, 1997; Schneider, 1992), permits them to have low metabolic rates (Schneider, 1992).

Notwithstanding the increase in the number of works on jellyfish in the last decade (Condon *et al.*, 2012), ecological and evolutionary studies persist poorly combined (see *e.g.,* Dawson and Martin, 2001; Hamner and Dawson, 2009). However, it is known that global warming, overfishing, eutrophication and the widening of invasive species can affect the dynamics of these organisms (Purcell *et al.*, 2007; Stevens, 2010). Overfishing, which is responsible for the devastation of entire fish communities, coupled with the global impact caused by increased invasive species dispersal and the effects of global warming, are unbalancing factors in aquatic population dynamics. In any given ecosystem, if the population of any predator declines drastically, the repercussions on prey populations will be overwhelming (Stevens, 2010). This imbalance favors the growth and proliferation of better adapted organisms, such as jellyfish. The rapid and exacerbated increase in jellyfish populations has brutal impacts in various sectors. The fisheries sector may experience a marked decrease in productivity levels caused by the blocking of nets by large jellyfish species (Dong *et al.*, 2010; Kawahara *et al.*, 2006) and, on the other hand, the birth and survival levels of fish communities may be markedly decreased due to predation of fish eggs, larvae, and even small fishes by jellyfishes (Lynam *et al.*, 2006; Purcell, 1989). The tourism sector is affected in situations of jellyfish species proliferation in bathing areas, leading to the banning of bathing by the competent authorities and, in cases of mass strandings, access to the beach may be restricted (Fautin, 2009; Houghton *et al.*, 2007). These situations are entirely linked to the danger potential that jellyfish poses to human health, and even if only a few species are considered a threat, they all have toxicity potential due to the presence of nematocysts, the capsule that contains the stinging cells (Fautin, 2009). For instance, Dong *et al.* (2010) compiled the published hospital-based records of jellyfish stings in Chinese seas since 1983 and the obtained results showed over 2000 cases of jellyfish stings in the popular coastal areas of China with 13 fatal cases included. The industry sector may also suffer a decrease in its productivity caused by the mass occurrence of jellyfish. In fact, there are reports of industries having to stop their activity because of blockage caused by jellyfish from water pipe systems (Dong *et al.*, 2010; Purcell, 2012). The aquaculture sector in turn, can suffer huge losses from the intrusion of jellyfish into cultures. In fact, there are several reports of whole culture losses caused by the invasion of jellyfish (OBIS, 2019; Purcell *et al.*, 2013).

Therefore, though jellyfishes play a key role in aquatic ecosystems, when they occur in mass, they can cause a vast number of negative impacts. Unfortunately, due to increasing anthropogenic pressure and consequent effects, mass occurrence phenomena of these organisms are becoming increasingly frequent.

1.4 Objectives and thesis outline

Since the conditions driving mass-occurring phenomena of jellyfish tend to worsen over time, allied with the lack of effective methods to predict and address these phenomena, the general objective of this thesis will be to sensitize the scientific and social community to the problematic of jellyfish's mass occurrences.

This thesis aims to: *(i)* create a state of the art on the jellyfishes reported in Portugal and their capability to occur in mass, *(ii)* contribute with new sequences for jellyfish with few genetic markers available at online databases, namely through the optimization of molecular protocols of DNA extraction and amplification for medusozoan species, that can be used as reference for future studies of jellyfish's early-detection, and *(iii)* compare classical methods (PCR) and high-throughput methodologies (metagenomic) in order to develop in the future suitable tools to face the impacts of jellyfishes.

This thesis is structured in ten major parts:

1. **General introduction** in which the theme of the thesis, the general concepts associated with it, the problematic targeted and the objectives of this work are presented;

2. **Materials and Methods** that address the methodologies applied in the design of this thesis. The strategy applied to get to the objective traced and to respond to the problematic targeted;

3. **Results** obtained when applied the knowledge and the methods, which are subdivided into three chapters: chapter 1 – Review article entitled "Medusozoans reported in Portugal and its ecological and economical relevance"; chapter 2 – Experimental section entitled "DNA extractions, Polymerase Chain Reactions and Bioinformatics analyses"; chapter 3 – Review of molecular tools for early-detection of jellyfish entitled: "Advances in the study of ecosystems using an eDNA approach";

4. **Discussion** of the results obtained;

5. **Conclusion** of the results obtained;

6. **Future perspectives** that indicate future guidelines to pursue the work initiated in this thesis;

7. **Publications** that resulted from the work developed in the scope of the present thesis;

8. **References** used throughout the thesis;

9. **Appendices** with a list of the medusozoans reported in Portugal and the genetic information available for these species;

10. **Appendices references** used in Appendix A and B.

2. Materials and Methods

2.1 Chapter 1. Review article entitled: "Medusozoans reported in Portugal and its ecological and economical relevance"

As previously mentioned, the first aim of this thesis was to create a state of the art on the jellyfishes reported in Portugal and their capability to occur in mass. In order to reach that goal, two main tasks were defined. The first was to compile in a list the jellyfish species reported in Portugal, and the second was to compile the genetic information available at public databases for the species previously listed. The work developed, culminated in a review article entitled "An update of medusozoan reported in Portugal and its impacts based on mass occurrence events". The search criteria used are described below.

2.1.2 Updated list of the medusozoans reported in Portugal

The list of the medusozoans reported in Portugal (**Appendix A**) was constructed through an extensive search on scientific literature, databases and websites, having been consulted 170 scientific articles, 6 online databases and 4 websites. The species that met the following conditions were added to the list: *(i)* be a cnidarian belonging to the clade Medusozoa and *(ii)* be registered or reported at least once in mainland Portugal or in the archipelago of Azores or Madeira.

In order to ensure that the information obtained was as reliable as possible, data were cross-referenced from several different sources. Thus, the bibliographic search performed until 28 May 2019, used approximately 50 different articles (see references included in **Appendix A**) and the following online databases/websites: Ocean Biogeographic Information System – OBIS (WoRMS Editorial Board, 2019), World Register of Marine Species – WoRMS (Palomares and Pauly, 2019), SeaLifeBase (GBIF.org, 2019), Global Biodiversity Information Facility – GBIF (Fofonoff *et al.*, 2019), National Exotic Marine and Estuarine Species Information System – NEMESIS (Roskov *et al.*, 2019), Catalogue of Life – CoL (Azores Bioportal, 2019), Azores Bioportal (OMARE, 2019), Observatório Marinho de Esposende – OMARE (GelAvista, 2019), GelAVista (Naturdata, 2019), and Naturdata (Benson *et al.*, 2018).

2.1.3 Genetic information available for medusozoans reported in Portugal

The compilation of the genetic information was performed using the GenBank Nucleotide database (Benson *et al.*, 2018) in May 2019. The search terms consisted in the name of each of the species previously listed. The information retrieved corresponded both to mitochondrial and nuclear DNA markers, as well as to complete and incomplete sequences. The obtained results are described in **Appendix B**.

2.2 Chapter 2. Experimental section: "DNA extractions, Polymerase Chain Reactions and Bioinformatics analyses"

Here, are described the experimental methodologies employed in this thesis. Briefly, in order to review the molecular methodologies hitherto employed, it was performed a literature search that allowed to conclude that there was no standardization of the molecular protocols for scyphozoans and more specifically for *Catostylus tagi*. Therefore, *C. tagi* was the model species used in this study to perform the optimization of protocols for DNA extraction and amplification (molecular approach). The molecular approach was complemented with a bioinformatics approach in order to perform the primer design and further sequence editing of the sequenced targeted genes (16S rRNA, COX1, COX3, NAD6, 18S rRNA, 28S rRNA and ITS1), as well as, for their identification and validation through Basic Local Alignment Search Tool (BLAST) or phylogenetic analyses. A detailed description of this methodology can be found below.

2.2.1 Species sampled

Catostylus tagi (Haeckel, 1869) belongs to the order Rhizostomeae and is a species of jellyfish native of the Portuguese coast. This species can occur abundantly, especially in the summer, being the only Catostylidae found in the European continent. The species composition presents potential for biomedical applications and the medusa form is considered edible. Nevertheless, some of the biomarkers used for jellyfishes' identification and phylogenetic reconstruction have not been amplified for this species. In order to fill the gaps of information on this very relevant species, *C. tagi* was chosen as a model species for the work performed in the present thesis.

2.2.2 Sample collection and preparation

Three individuals of *C. tagi* (#1, #2 and #3), sampled on October 4th, 2018 in Tagus River, were preserved in ethanol 70 %(v/v) and transported in plastic flasks to the lab where they were stored at -20 ºC. Then, the specimens were dissected under aseptic

conditions: gonads and tentacles were cut in small pieces of about 25 mg or 15 mg each and then distributed into microtubes (**Figure 2**).

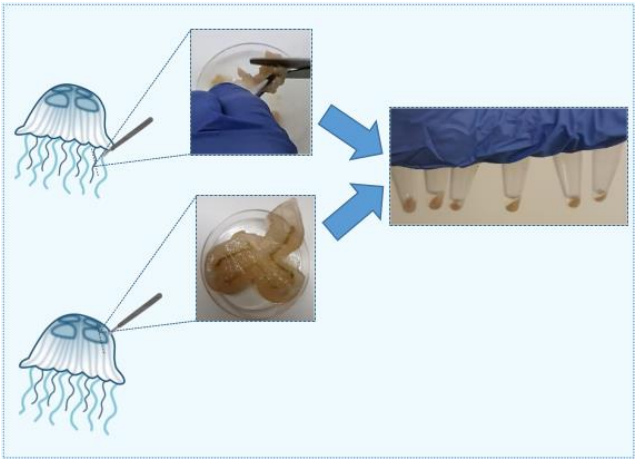

Figure 2. *C. tagi* samples preparation for DNA extraction process. Tentacles (top) and gonads (bottom). Distribution of small pieces of tentacles and gonads into several microtubes (right).

2.2.3 DNA extraction and quantification

We started the DNA extraction protocol using gonads and tentacles of just one individual of *C. tagi*. The genomic DNA (gDNA) of the previously prepared samples was extracted using the PureLink™ Genomic DNA Mini Kit (Invitrogen, Carlsbad, CA, USA), according to the manufacturers' protocol for Mammalian tissues (**Figure 3**).

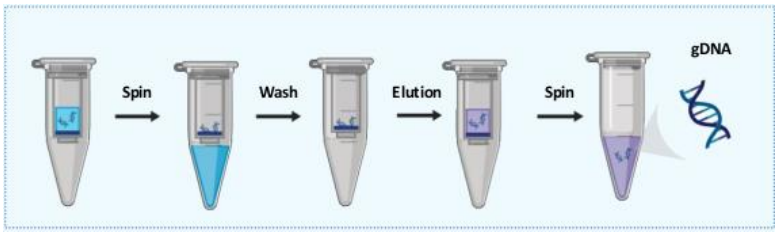

Figure 3. Genomic DNA (gDNA) extraction process.

The protocol for purifying the gDNA was the one provided in the same kit. The samples obtained using the exact approach described in the referred protocol were called original samples. Then, to recover more DNA, it was performed a second elution step that was kept in a new microtube (named replicate). Analyzing the results of the DNA quantification for the samples of this individual (please see **Table 5** of the Results 3.2 Chapter 2), allowed us to understand that it was used an excessive amount of tissue. Thus, the DNA extraction protocol for the other two specimens was slightly different: instead of using 25 mg, it was used 15mg of tissue for each sample (tentacles and gonads). Since the amount of template DNA in a reaction strongly influences PCR's performance, and according to the protocol used, a good DNA concentration value should be between 10 to 60 ng/µL, when the concentration range does not fulfill this

requirement, dilutions ($c_i \times v_i = c_f \times v_f$ with $c_f = \frac{15\ ng}{\mu L} and\ v_f = 30\ \mu L$) of the samples (named diluted samples) were performed in order to obtain optimal concentrations for further DNA amplification.

The DNA quantification was made by spectrophotometry with DeNovix DS-11 FX Spectrophotometer using of a volume of 2 µL per sample.

2.2.4 DNA amplification: Polymerase Chain Reaction optimization and primer design

For the DNA amplifications, two sets of primers were considered. Firstly, we tested a set of primers, previously used in other works with cnidarians. The complete set of these tested primers can be found in **Table 1**.

Table 1. First set of tested primers.

Target gene		Primer	Sequence (5'-3')	Product length (bp)	Reference
Mitochondrial	16S rRNA	1_F	TCGACTGTTTACCAAAAACATAGC	634	(Bridge *et al.*, 1992)
		3_R	GTCGCCCCAACTAAACTACCAAACTT		
	COX1	HCO1490	TAAACTTCAGGGTGACCAAAAAATCA	710	(Folmer *et al.*, 1994)
		LCO2198	GGTCAACAAATCATAAAGATATTGG		
	COX3	COIII F	CATTTAGTTGATCCTAGGCCTTGACC	725	(Geller and Walton, 2001)
		COIII R	CAAACCACATCTACAAAATGCCAATATC		
	NAD6	nd F	AGAGATTTAAACAGRCGTGAGC	400	(Frazão, 2016)
		nd R	GGGGCCGGTAAATCAATAAT		
Nuclear	18S rRNA	primer A_F	AACCTGGTTGATCCTGCCAGT	1750	(Medlin *et al.*, 1988)
		primer B_R	TGATCCTTCTGCAGGTTCACCTAC		
		18SFb	GCTGTATGTACTGTGAAACTGCG	1500	(Leclère *et al.*, 2009)
		18SRb	CACCTACGGAAACCTTGTTACGAC		
	28S rRNA	F	GGCGACCCGCTGAATTCAAGCATAT	300	(Chen *et al.*, 2000)
		R	GCTTTGGGCTGCAGTCCCAAGCAACCCACTC		
		LSUD1F_F	ACCCGCTGAATTTAAGCATA	1100	(Lenaers *et al.*, 1989)
		D3Ca_R	ACGAACGATTTGCACGTCAG		
	ITS1	jfITS1-5f_F	GGTTTCCGTAGGTGAACCTGCGGAAGGATC	400	(Dawson and Jacobs, 2001)
		jfITS1-3r_R	CGCACGAGCCGAGTGATCCACCTTAGAAG		

Additionally, for the genes that could not be amplified (NAD6 and 16S rRNA) using the first set of primers, a second set of primers was tested. This set consisted in specific primers designed in this study using the software Geneious Prime version 11.1.5 ("Geneious Prime," 2018). Specifically, the design methodology of NAD6 primers was developed in two different ways, giving rise to two different primer pairs. The first way started with the download of all the complete NAD6 sequences from cnidarians available in GenBank (336 nucleotide sequences). Then, it followed a translation alignment with all of those sequences. On the second method, only the complete NAD6 sequences from scyphozoans (6 nucleotide sequences) were used on the translation alignment. Similarly, to the design of NAD6 primers, two different methodologies were also adopted for the design of 16S rRNA primers. On the first method employed, it was performed a "Map to reference" using all the partial Rhizostomeae sequences available on GenBank (232 nucleotide sequences) and using the only Rhizostomeae complete sequence of the 16S rRNA available on GenBank as the reference sequence (MK157198). Then, it was performed a MUSCLE alignment with all the reads that could be mapped to the reference (225 nucleotide sequences). The other strategy was to perform a MUSCLE alignment of all the available 16S rRNA sequences from Catostylidae family species (12 nucleotide sequences). Finally, considering the above-mentioned alignments, primers were drawn using Geneious default parameters except for "size", "temperature of melting" (Tm) and "guanine-cytosine percentage" (% GC) parameters that were modified (**Figure 4**).

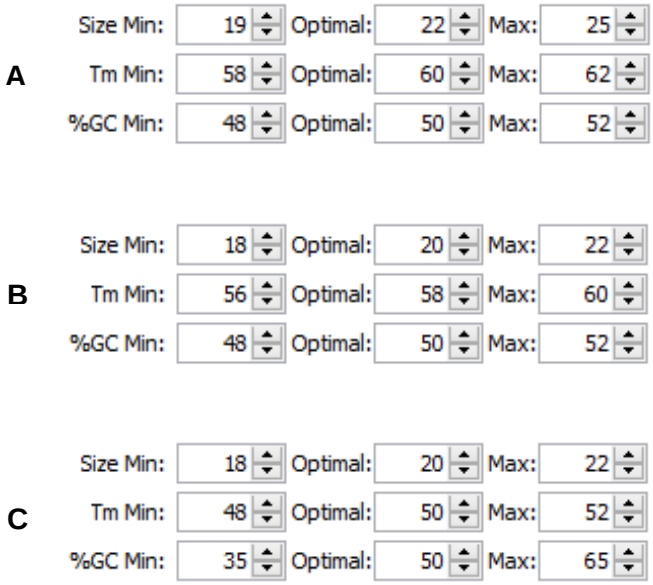

Figure 4. Primer design modified parameters. **A:** 16S rRNA primers; **B:** ND6_64 primers; **C:** ND6_14 primers. Tm – temperature of melting; % GC – guanine-cytosine percentage.

The designed primers can be found in **Table 2**.

Table 2. Second set of tested primers designed in this study.

Target gene	Primer	Sequence (5'-3')	length (bp)	% GC	Tm (ºC)	Product length (bp)
16S rRNA	16S_65F	GTTAAATAGCCGCGGTAACTCTGAC	25	48	62	504
	16S_65R	CACAATTCAACATCGAGGTGGC	22	50	60.4	
	16S_85F	AAATAGCCGCGGTAACTCTG	20	50	58.1	500
	16S_85R	AAAGCTGCTGCACCTTTAGG	20	50	58.7	
NAD6	ND6_64F	ATCTGCGCTCAATCCTGTTC	20	50	58.3	N.D.
	ND6_64R	CCTCCTATTTCATAAGGGCCAC	22	50	58.2	
	ND6_14F	TTATGTAGGAGCAATAGC	18	38.9	48.2	N.D.
	ND6_14R	AATTGCTCCTATCATAGC	18	38.9	48.6	

Polymerase Chain Reactions (PCR) that were prepared following the GoTaq protocol from Promega (USA) were performed. The methodology was executed in a UV chamber in order to avoid any contamination. The PCR mixtures had 1 x PCR GoTaq Flexi Buffer, 10 pmol of primer forward and reverse, 10 mg/mL (w/v) of Bovine Serum Albumin (BSA), 2.5 mM $MgCl_2$, 250 µM of each deoxynucleotide triphosphate (dNTP's), and 0.5 U of GoTaq® DNA polymerase (Promega, USA), performing a total volume of 20 µL. For the PCR was used the Biometra® T3000 thermal cycler and the programs were run using thermal gradients for PCR optimization purposes.

For referenced primers, standard protocols were used (see references in Table 1). In the case of the designed primers, for amplification of the 16S rRNA gene, it was used the following protocol: initial denaturation at 95 ºC for 5 min, followed by 35 cycles of 94 ºC for 30 secs, annealing temperatures were 53 ºC for the 16S 65F/R primer pair and 51 ºC for 16S 85F/R both for 40 secs, and 72 ºC for 90 secs, and a final extension at 72 ºC for 5 min. The protocol for the amplification of the NAD6 gene was the following: initial denaturation at 95 ºC for 3 min, followed by 35 cycles of 94 ºC for 30 secs, 50 ºC for 40 secs, and 72 ºC for 90 secs, and a final extension at 72 ºC for 10 min.

After performing the PCR's for all the samples, we confirmed the DNA amplification using agarose gel electrophoresis. To prepare the gel we used agarose (UltraPure™ Agarose, Invitrogen Life Technologies, UK) at 1 % (w/v) and the TAE buffer (0.4M Tris-acetate, 0.01M EDTA, pH 8.3 ± 0.1, Invitrogen™, UK) solution. We ran the electrophoresis at 100V for 30 minutes and the obtained results were then analyzed in an UV Transilluminator (GelDoc™ XR+ Imager).

Then, the DNA amplified products were sent for Sanger sequencing (Macrogen©, Madrid, Spain).

2.2.5 Sequence editing and gene annotation

After receiving the raw sequence files (.ab1) from Macrogen©, the sequence editing was performed using the software Geneious Prime version 11.1.5. For each sample there was two chromatograms of bidirectional sequences, *i.e.* forward and reverse sequences (**Figure 5**).

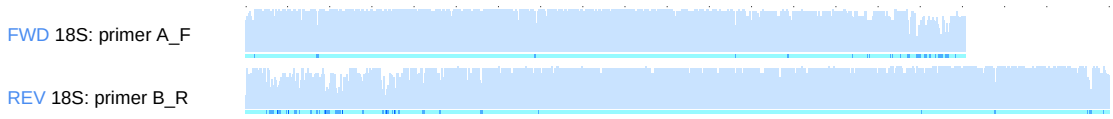

Figure 5. Example of a sequence forward and reverse for the 18S rRNA gene. These gene fragments were obtained using one of the primer pairs (forward: primer A_F and reverse: primer B_R) of the first set of primers tested, described in Table 1. FWD - means forward sequence and REV - reverse complement of the reverse sequence.

The first edition was the trimming of all the sequence ends. This procedure is made due to the low quality of the bases in the sequence ends. In the Geneious sequence view window, we inspected the chromatograms displayed with a quality measure (Phred quality scores) for each base along the sequence, representing the error probability as assessed by the base calling program used during the DNA sequencing. The quality is shown as a shaded blue bar graph overlaid on top of the chromatogram and in a horizontal bar below the same, respectively (**Figures 5** and **6**). The highest bar represents a one in a million (10^{-6}) probability of base calling error while the middle bar represents a probability of only one in a thousand (10^{-3}). Considering the blue hue present in the horizontal bar above, zones with a darker blue, represent bases with low quality (**Figures 5** and **6**) and therefore they were trimmed from the sequences.

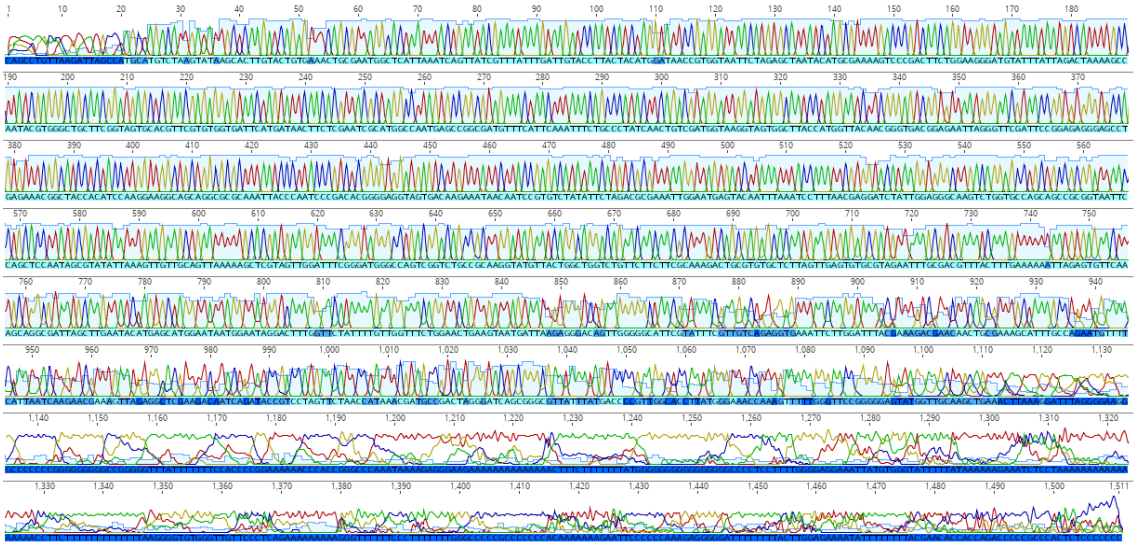

Figure 6. Chromatogram of an untrimmed sequence for the 18S rRNA gene. This sequence was obtained using the primer pairs (forward: primer A_F and reverse: primer B_R) of the first set of primers tested, described in **Table 1**.

The next step was to produce the consensus sequence from the nucleotide alignment (displayed from 5' to 3') between the forward and reverse sequences for each sample. To accomplish that, firstly we performed the reverse complement (reverse the sequence direction and replace each base by its complement) of the sequences obtained with the reverse primers. Then, these sequences were aligned (Geneious alignment) with the corresponding forward sequences (**Figure 7**).

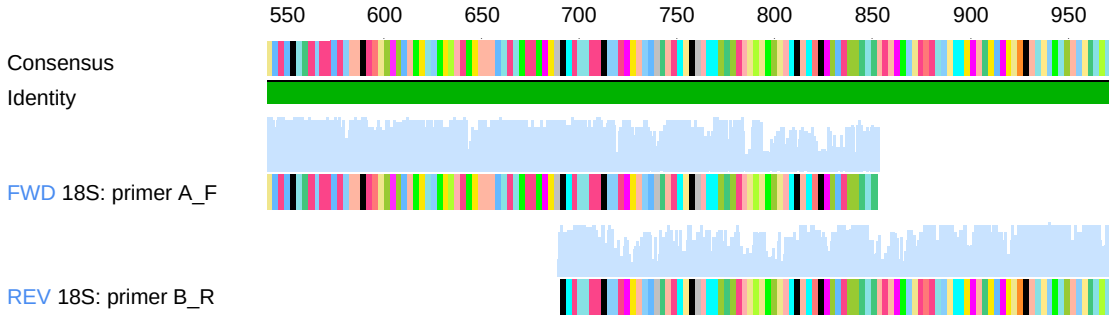

Figure 7. Example of a consensus sequence for the gene 18S rRNA gene. These gene fragments were obtained using one of the primer pairs (forward: 18Sab_1_F and reverse: 18Sab_1_R) of the first set of primers tested, described in **Table 1**. The consensus sequence ("Consensus") is at the top of the nucleotide alignment, as well as, the identity across the two sequences for every position ("Identity" - green means that the residue at the position is the same across both sequences). FWD - means forward sequence and REV - reverse complement of the reverse sequence.

The following step was to extract the consensus sequences, previously obtained, and perform a BLASTn (*i.e.,* compares nucleotide sequences used as queries with nucleotide sequences in the database) of these consensus sequences (queries), using the BLAST tool plugin of the Geneious Prime version 11.1.5, against the "Nucleotide collection" (nr) database held at NCBI (National Center for Biotechnology Information) to find sequences

that are similar to them (hits); and therefore to validate their gene annotation. To perform the BLASTn's, default parameters were considered with exception of the "e-value" that was set to $1e^{-3}$ and the "Maximum Hits" that was set to 3 (**Figure 8**).

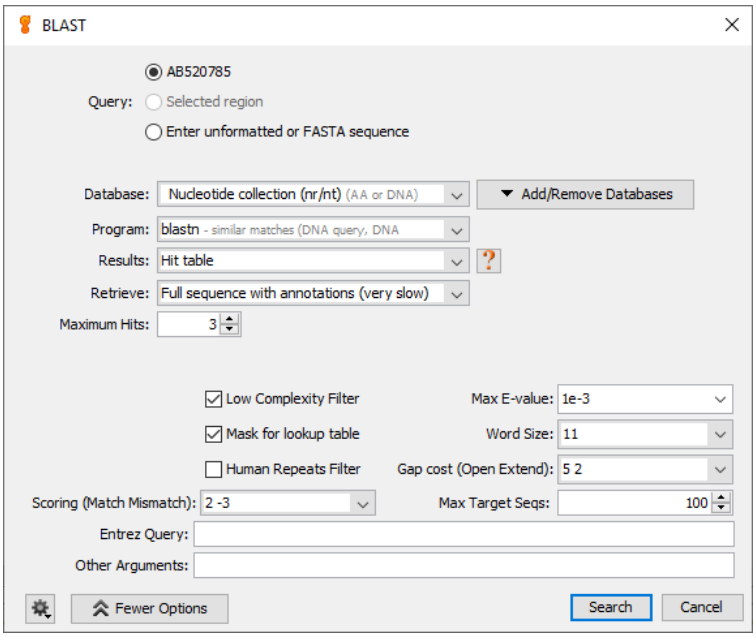

Figure 8. BLAST options used.

Finally, in order to complement the annotation of the obtained consensus sequences through BLASTn analysis, and understand their placement in an evolutionary context, phylogenetic analysis (gene trees) were performed. Phylogenetic analyses are based in the evolutionary development and diversification of a species or group of organisms, or of a particular feature of an organism (Soltis and Soltis, 2003).

In general, the phylogenetic analyses performed in this thesis, followed three main steps:
Step 1. Identification of homologous sequences and retrieval – the compilation of a dataset of homologous sequences from the groups of species studied;
Step 2. Multiple sequence alignment (MSA) and evaluation – the alignment of the sequences retrieved to allow the comparison of homologous sites between sequences;
Step 3. Phylogenetic gene tree building and editing – using a tree-inference method based on genetic distances (NJ – Neighbor joining method).
More details about this workflow can be found bellow.

Step 1 parameters:

In order to construct the datasets, we performed a search at GenBank database using the following search terms: ["*Superclass name*" + "*gene name*" + "*complete*"] and ["*Superclass name*" + "*gene name*" + "*partial genome*"]. For 18S rRNA and 28S rRNA

genes, the searches performed did not retrieved enough results. Therefore, for these genes the following search: ["*Superclass name*" + "*gene name*"] was applied. Since the last search gave us a much larger number of sequences (**Table 3**) than the previous one (because it covered partial sequences), we proceeded according to the following selection criteria to ensure the representativeness of all the taxonomic groups (*i.e.,* medusozoan superclasses) and the use of a similar number of sequences per dataset:

1. keep sequences per phylum and superclass;
2. removal of duplicate sequences;
3. removal of sequences with length <1000 base pairs (bp);
4. keep the largest sequence per species;
5. removal of unspecified species sequences;
6. keep one sequence per genera;
7. keep one sequence per family;
8. keep sequences from 1750 to 1800bp (for 18s rRNA gene) and <3250bp (for 28S rRNA gene).

Table 3. Number of obtained sequences for 18S rRNA and 28S rRNA genes using the search terms ["*Superclass name*" + "*gene name*"].

Superclass	18S rRNA gene	28S rRNA gene
Hydrozoa	1706	1364
Scyphozoa	749	697
Staurozoa	50	71
Cubozoa	79	74

Thus, the total number of sequences compiled in each dataset for further MSA, after applying the above-mentioned criteria, can be found in **Table 4**.

Table 4. Total number of sequences per dataset used in multiple sequence alignments.

Dataset	Hydrozoa	Scyphozoa	Staurozoa	Cubozoa
16S rRNA	6	6	1	0
COX1	17	15	4	3
COX3	37	17	3	3
18S rRNA	21	13	1	4
28S rRNA	24	2	2	7
ITS1	37	10	7	3

Step 2 parameters:

The sequences within each one of the above-mentioned datasets were aligned with the software Geneious Prime version 11.1.5 using the following algorithms:

- datasets containing the coding genes (COX1 and COX3) were codon-based aligned (translation alignment: transl_table=4) with MUSCLE algorithm;
- for the remaining non-coding genes (16S rRNA, ITS1, 28S rRNA and 28S rRNA) nucleotide-based alignments with MUSCLE algorithm were performed.

The alignments were visually inspected, and we have found that, apart from ITS1 gene, all the genes had a pairwise alignment percentage higher than 60 %. This value parameter ensures that the alignments have the quality required to be used for further phylogenetic analyses (Lemey *et al.*, 2009).

Step 3 parameters:

The phylogenetic gene trees were reconstructed using the software Geneious Prime version 11.1.5. The phylogenetic-inference method applied was the NJ method (Saitou and Nei, 1987; Studier and Keppler, 1988), with default parameters and 1000 bootstrap replicates, since it is one of the most commonly used methods to construct distance trees.

The obtained gene trees were then edited using the software MEGA version 5.2 (Tamura *et al.*, 2011).

2.3 Chapter 3. Review of molecular tools for early-detection of jellyfish: “Advances in the study of ecosystems using an eDNA approach”

As previously stated, the third aim of this thesis is to compare classical and high-throughput methodologies for the future development of a suitable early-predictive tool of jellyfish mass occurrence events. Thus, for this chapter a total of 17 articles were consulted in order to review the most recent and relevant methods used in ecosystem biomonitoring based on environmental DNA (eDNA). Among such methods, three different techniques for assessing eukaryotic diversity were reviewed: environmental shotgun sequencing; eDNA barcoding and metabarcoding; and capture enrichment.

3. Results

3.1 Chapter 1. Review article entitled: “Medusozoans reported in Portugal and its ecological and economical relevance”.

3.1.1 Abstract

Cnidaria is a phylum of predominantly marine organisms encompassing over 13,300 species. During around 600 million years, cnidarians evolved into two basic body forms: “polyp” characterized by a tubular body and “medusa”, the free-floating stage characterized by a bell-shaped body. The diagnostic feature of cnidarians is the presence of a capsule called nematocyst, that contains a venomous thread used for prey capture and defense. Thus, a fast increase in the abundance of these venomous species, usually known as “blooms”, can produce great impacts on fisheries, public health, tourism, the normal functioning of factories and aquaculture. Those impacts are produced by the free-living stage of cnidarians known as “jellyfish” (Medusozoa subphylum), influenced by some natural factors such as water temperature, wind and water tides, but also induced by global warming, overfishing, eutrophication and widening of invasive species habitats. These “bloom” events have also been reported in the Portuguese coast, but the information is still scarce and therefore their impacts are underestimated. Hence, we compiled the medusozoans reported in mainland Portugal and archipelagos. The data gathered showed a total of 273 species reported, being 255 hydrozoans, 15 scyphozoans, 2 staurozoans, and 1 cubozoan. We also compiled the genetic information available online of the reported species to access further ecological, diversity or genetic regional studies with this group of organisms. The data compiled revealed that, 26 % of the reported species, did not presented any genetic information in the GenBank Nucleotide database. We found that 16S rRNA and COX1 were the mitochondrial markers with more sequences available, and that the genes 18S rRNA and 28S rRNA were the most common nuclear markers for this group of species.

3.1.2 Introduction

With increasing anthropogenic pressure and the resulting consequences, marine ecosystems have been undergoing enormous changes. Cnidaria, an ancient group of mainly marine species, which is divided into three clades Anthozoa, Myxozoa and Medusozoa, comprises organisms with a diversity of life history strategies (Collins, 2009; Okamura *et al.*, 2015). For its life cycles’ features, medusozoans represented by

jellyfishes stand out. Being key organisms in the dynamic balance of ecosystems, jellyfishes are often seen pejoratively, as they cause negative impacts mostly associated with their mass occurrences and, on the other hand, due to their toxic potential that represents a threat to human health. In order to study the insertion of this group of organisms in the Portuguese regional ecological panorama and serving not only as a warning for the problems associated with the mass occurrences of these organisms, but also for their enormous valences, this review article arises. Herein, we made an in-depth review of the jellyfishes reported in mainland Portugal and archipelagos. In this review, we tried to demonstrate the state of the art of the studies performed in Portugal with this group of organisms, focusing mainly on the medusozoans reported in Portugal known to form blooms or other mass occurrence events, as well as, the species more relevant either economically and ecologically. In this work we also compile the genetic information available for molecular markers of the medusozoans reported, stablishing bases to facilitate further studies.

3.1.3 Cnidarians features

Cnidarians have evolved more than 600 million years ago and during this evolutionary process, they have created a selection of biological features, leading into an enormous variety of forms and a great diversity of life history strategies. In fact, it is one of the most diverse groups of predominantly marine organisms, encompassing around 13,300 species (Collins, 2009; Kayal *et al.*, 2018). Their diagnostic feature is the presence of nematocysts, the one of the three categories of cnidae (nematocysts, spirocysts and ptychocysts) exclusive of all cnidarians (Fautin, 2009). Nematocyst is the capsule that contains the stinging cells responsible for the production, inoculation and discharge of toxins by which cnidarians capture their preys and defend themselves (Fautin, 2009). The cells that make the capsules are cnidoblasts (nematoblasts, ptychoblasts, and spiroblasts), with the corresponding mature cells cnidocytes (and nematocytes, ptychocytes, and spirocytes) (Watson and Wood, 1988). This feature makes this phylum one of the largest of generally toxic animals.

3.1.4 Body Forms

Cnidarians exhibit two epithelial layers: an external layer (epidermis) and an internal layer (gastrodermis) separated by an extracellular mesoglea (Zapata *et al.*, 2015). They present an incomplete digestive system with only one opening that leads to a gastrovascular cavity, where the digestion and distribution of nutrients occur. There are two basic body forms among cnidarians: “polyp” characterized by a tubular body, and

"medusa" the free-floating stage characterized by a bell-shaped body (Daly *et al.*, 2007). These organisms have complex life cycles that involve one or both of the body forms above mentioned. Generally, they can reproduce both, asexually and sexually (**Figure 9**). Polyps usually reproduce asexually, giving rise to another polyp or medusa. A medusa, in turn, can originate other medusa asexually or, by means of sexual reproduction forms a zygote that will develop into a planula (larval stage) that can generate another polyp (Boero, *et al.*, 1992; Boero and Bouillon, 1993; Collins, 2002).

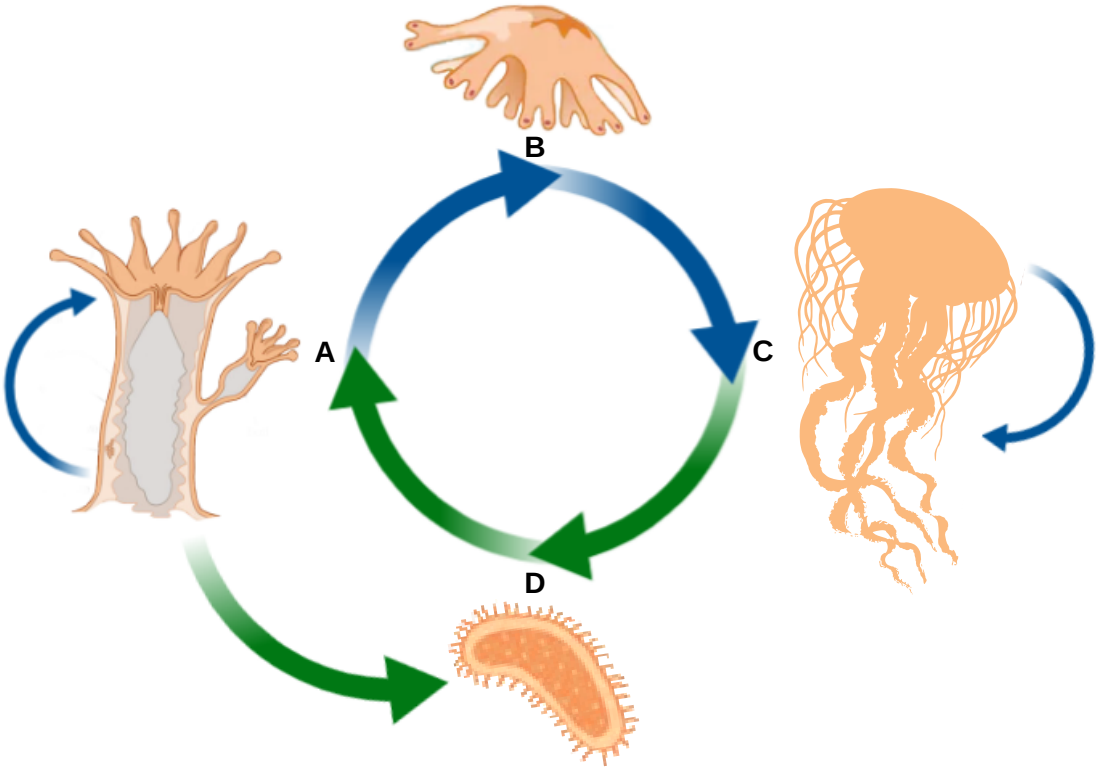

Figure 9. Medusozoan life cycle. **A:** Polyp, **B:** Ephyra, **C:** Medusa, **D:** Planula. Blue arrows: asexual reproduction. Green arrows: sexual reproduction. Image credits: **A** adapted from CK-12 Foundation under the CC-BY-NC-SA 3.0, source ck12.org (https://bit.ly/2mckZ7m); **B** adapted from Dorling Kindersley, source thoughtco.com (https://bit.ly/2m60Cca); **C** adapted from svgsilh.com under public domain (https://bit.ly/2mzbXBN).

3.1.5 Taxonomy: cnidarians clades

Cnidarians can be divided into three distinct clades: Anthozoa, Myxozoa and Medusozoa (Kayal *et al.*, 2018; Naldoni *et al.*, 2019a; Zhang, 2013). The Anthozoa clade encompasses organisms exclusively polypoid and sessile, the Myxozoa clade is a group of obligatory endoparasites while the Medusozoa clade is comprised by organisms covering a diversity of forms, including benthic and pelagic life history stages with many species having both phases within their life-cycle (Daly *et al*., 2007; Schnedler-Meyer *et al.*, 2018).

Anthozoans are distributed in three sub-classes (Kayal *et al.*, 2018): Octocorallia (Haeckel, 1866) – sea pens, sea fans and soft corals; Hexacorallia (Haeckel, 1896) – stony corals, black corals, sea anemones, zoantharians and corallimorpharians; and Ceriantharia (Perrier, 1893) – tube anemones.

Myxozoans are separated into two classes (Naldoni *et al.*, 2019b): Malacosporea and Myxosporea. In the life cycles of both classes, the intermediate host is fish. In Malacosporea, the definitive hosts are freshwater bryozoans, while in Myxosporea they are annelids.

Medusozoans are divided into four classes (Collins, 2009; Straehler-Pohl, 2017): Hydrozoa (Owen, 1843) – hydras, hydroids, hydromedusae, and siphonophores; Scyphozoa (Goette, 1887) – true jellyfishes; Cubozoa (Werner, 1973) – box jellyfishes or sea wasps; and Staurozoa (Collins and Marques, 2004) – stalked jellyfishes.

In this article, we do not intend to exhaustively review the Cnidaria phylum neither the Medusozoa clade (for an extensive review see Daly *et al*., 2007). Instead, the aims of this article are: *(i)* to briefly define the Medusozoa clade in terms of general aspects, taxonomy and phylogenetics; *(ii)* to review the most relevant jellyfish species existing in Portugal; *(iii)* to assist further studies with the compilation of an updated list of the Medusozoa reported in Portugal (mainland, Azores and Madeira), as well as, a list of the genetic information available at the NCBI database for the species reported.

3.1.6 Medusozoa clade

The Medusozoa clade, being the cnidarian clade represented with free-swimming organisms, the jellyfish, is the one responsible for jellyfish blooms as we will discuss further.

Generally, Medusozoan life cycles are three-phased: planula, sessile polypoid, and pelagic medusoid stages. Thus, those can be either a life cycle with one annual sexual reproduction event and an overwintering benthic stage (metagenetic life cycle, *i.e.,* medusoid and polypoid), or continuous reproduction and a holoplanktonic life cycle (Ortman *et al.*, 2010; Schnedler-Meyer *et al.*, 2018). We can distinguish three main life cycle patterns: strobilation (scyphozoans), metamorphosis (cubozoans) and lateral budding (hydrozoans). In strobilation, each segment formed in the parental organisms will give rise to an ephyra thus becoming a new organism (Spangenberg, 1965); in metamorphosis, the whole polyp transforms into a single medusa (Straehler-Pohl and Jarms, 2005); and in lateral budding, the formation of a new organism involves the growth of a third layer of tissue in a polyp between endoderm and ectoderm, the entocodon, that buds giving rise to a new being (Kraus *et al.*, 2015).

3.1.7 Hydrozoa superclass

The Hydrozoa is the largest and most diverse superclass within Medusozoa. It is a vast heterogeneous group comprising approximately 3,718 living species (WoRMS Editorial Board, 2019) that share rare derived features: the velum (absent only in Obelia genus) and the ectodermal “gonads”. Hydrozoans are among the most important planktonic and benthic predators (Bouillon *et al*., 2006).

The Hydrozoa superclass is distributed by two reciprocally monophyletic clades (Collins, 2002; Marques and Collins, 2004): Hydroidolina (3,563 spp.) and Trachylinae (155 spp.) subclasses.

Hydroidolina subclass

Originally named by Collins (2000), Hydroidolina (**Figure 10**) is the largest and most diverse group within Medusozoa and is the hydrozoan group characterized by highly polymorphic polyp colonies (Collins *et al*., 2006; Goffredo and Dubinsky, 2016). In fact, all the polymorphic species within Medusozoa are from this group with the exception of the limnomedusan genus Monobrachium (Bouillon and Boero, 2000; Collins, 2002). The ecto-endodermal statocysts, characteristic of cnidarians, are not present, instead they display ectodermal statocysts (Daly *et al.*, 2007). Actually, there are uncertainties regarding the Hydroidolina phylogeny. However, in terms of taxonomy the actual scenario divides the group into three orders: Anthoathecata (athecate hydroids and antho-medusae), Leptothecata (thecate hydroids and leptomedusae) and Siphonophorae (colonial siphonophores), totalizing 98 families (Daly *et al*., 2007; Cartwright *et al*., 2008; Collins, 2009).

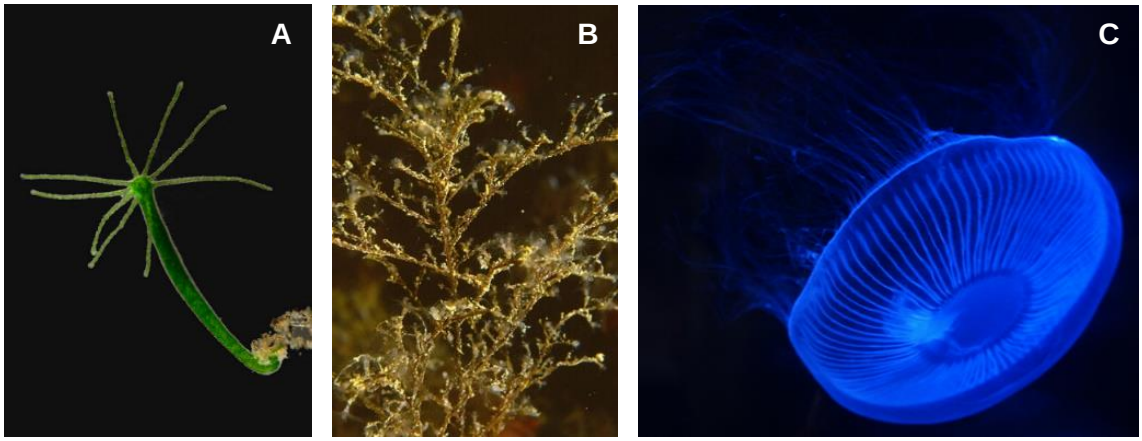

Figure 10. Representative images of the Hydroidolina subclass. **A:** *Hydra viridissima* (Order Anthoathecata, Family Hydridae). **B:** *Eudendrium ramosum* (Order Leptothecata, Family Eirenidae). **C:** *Aequorea victoria* (Order Leptothecata, Family Aequoreidae). Photographs credits: **A** licensed by Frank Fox, 2012 under the CC BY-SA 3.0 DE, source Wikimedia Commons (https://bit.ly/2Ggnb4Z); **B** licensed by Parent Géry, 2010 under public domain, source Wikimedia Commons (https://bit.ly/2SjrW2J); **C** licensed under the CC BY-SA 2.5, source OpenCage Systems (https://bit.ly/2Gif7kh).

Trachylinae subclass

Trachylinae (**Figure 11**) is a small clade divided in four orders (Actinulida, Limnomedusae, Narcomedusae and Trachymedusae) with 15 families (Goffredo and Dubinsky, 2016). This clade has a considerable loss of a polyp stage in its life history (Kayal *et al.*, 2018). In fact, most marine Trachylinae lack a benthic polyp stage and some medusae are strictly benthic and/or inhabit the interstitial environment (Gibbons *et al*., 2010).

Currently, due to limitations in the study of these organisms and the techniques used, it is very difficult to know the exact number of species of hydrozoans that present a jellyfish stage (Ames, 2018). However, it is important to notice that with the development of new tools and techniques (*e.g.,* Next Generation Sequencing), we are pathing through the right way to expand our knowledge about this group of medusozoans.

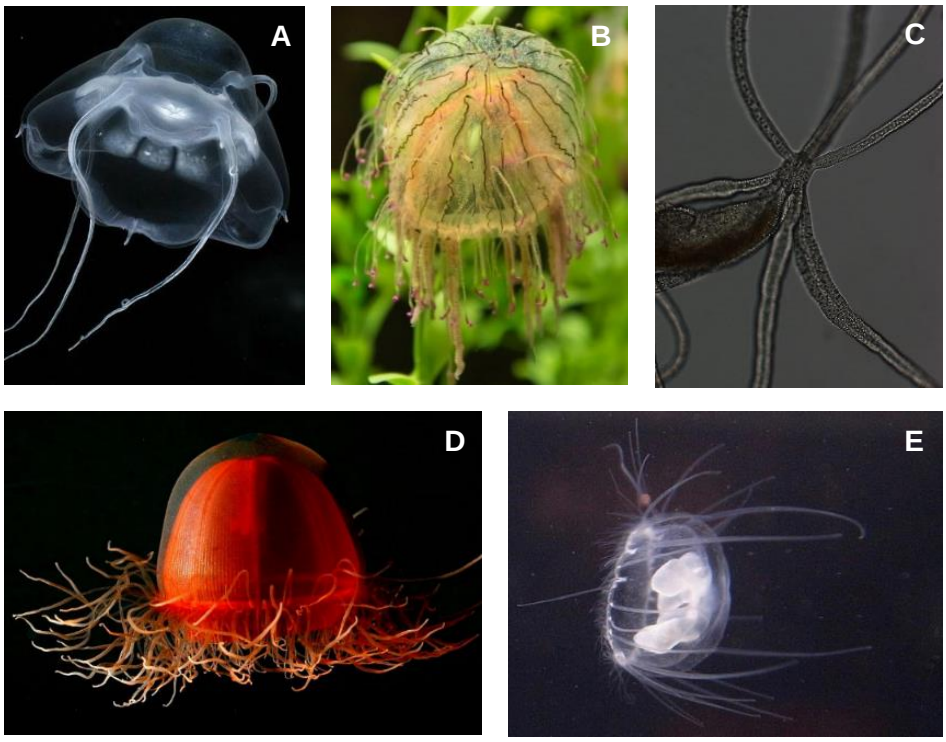

Figure 11. Representative images of Trachylinae subclass. **A:** *Bathykorus bouilloni* (Order Narcomedusae, Family Aeginidae). **B:** *Olindias formosus* (Order Limnomedusae, Family Olindiidae). **C:** *Halammohydra octopodides* (Order Actinulida, Family Halammohydridae). **D:** Crossota sp. (Order Trachymedusae, Family Rhopalonematidae) **E:** Craspedacusta sowerbyi (Order Limnomedusae, Family Olindiidae). Photographs credits: **A** licensed by Kevin Raskoff, 2005 under public domain, source Wikimedia Commons (https://bit.ly/2OpH7cn); **B** licensed by Mark Mauno, 2013 under the CC BY-SA 2.0, source Flickr (https://bit.ly/2K0WlAk); **C** licensed by LasseØ, 2013 under the CC BY-SA 3.0, source Wikimedia Commons (https://bit.ly/2Kh6jwu); **D** licensed by Kevin Raskoff, 2010 under the CC BY-SA 2.0 (https://bit.ly/2K1IyJB); **E** licensed under the CC BY-SA 2.5, source OpenCage Systems (https://bit.ly/32R1Usm).

3.1.8 Scyphozoa superclass

Scyphozoans form a very significant group of organisms that play an important role in the ecology of the many oceanic habitats. Their life cycles are mainly composed by three stages. The matured organisms produce either eggs or sperm, and in some rare cases

both (Morandini and Marques, 2010), which fuse and develop into a ciliated larva: the 'planula'. The planula settles to the benthos and originates the sessile life-cycle stage: the 'polyp'. In its turn, the polyp suffers a metamorphic process called strobilation giving rise to the juvenile medusa, the 'ephyra'. Scyphozoans are considered key organisms in many marine ecosystems as they are both predators and preys (Doyle *et al*., 2014). The group gained considerable attention in recent years due to their impact on people and ecosystems as we will see later (Kawahara *et al*., 2006; Lynam *et al*., 2006; Mills, 2001; Purcell *et al*., 2007). There are *circa* 194 living scyphozoan species that are currently divided into two subclasses (WoRMS Editorial Board, 2019): Coronamedusae (53 spp.) and Discomedusae (141 spp.).

Coronamedusae subclass

Coronamedusae (**Figure 12**) are commonly referred as deep-sea species though they exist in a wide range of depths. Most members of the Coronamedusae subclass (commonly known as crown jellyfish, thanks to the shape of their tentacles), present small polyps that live fixated in chitinous tubes to the substrate and can be distinguished by a distinct pattern on the external surface of the tube (Jarms *et al*., 2002). Coronate medusae present coronal groove, *i.e.,* a deep furrow around the umbrella, scalloped margin and the pedalia to perform their locomotion. They display simple mouth supported by a stalk 'manubrium', and, in various species, by forward-facing tentacles and also show non-pigmented oocytes (Ames, 2018; Daly *et al*., 2007; Marques and Collins, 2004; Russell, 1953). Phylogenetically, all the Coronamedusae belong to the Coronatae order.

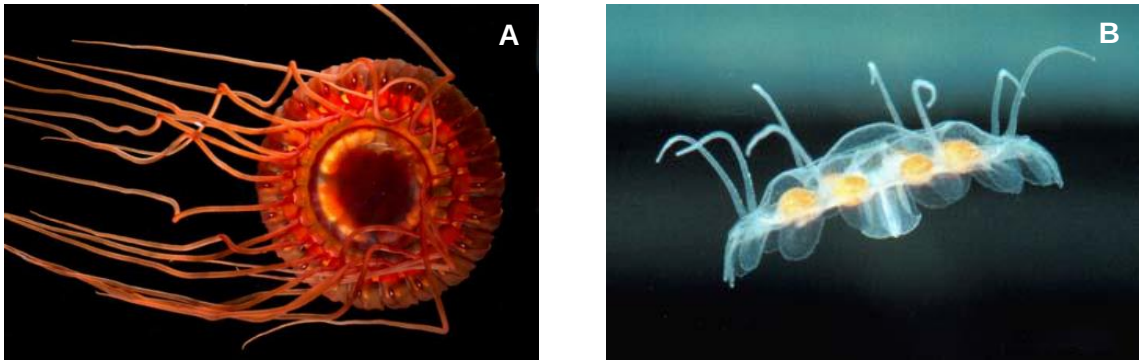

Figure 12. Representative images of the Coronamedusae subclass. **A**: *Atolla wyvillei* (Order Coronatae, Family Atollidae). **B**: *Nausithoe aurea* (Order Coronatae, Family Nausithoidae). Photographs credits: **A** licensed by NOAA Ocean Explorer, 2008 under the CC BY-SA 2.0, source Wikimedia Commons (https://bit.ly/2YsLR0H); **B** source Wikimedia Commons (https://bit.ly/2LOzE4A).

Discomedusae subclass

Discomedusae (**Figure 13**) polyps either lack a chitinous tube or possess a partial chitinous covering on the aboral stalk. Their medusae forms present elaborate oral arms,

a gastric system with canals and bells without grooves and pedalia (Marques and Collins, 2004). They are more likely to swarm or bloom than Coronamedusae (Hamner and Dawson, 2009). The reason for that may lie on the formation of 'podocysts' by some Discomedusae polyps. 'Podocysts' are cysts with stored reserves of organic compounds that contribute to the increase of polyps and their survival through periods of scarce food supply or predation high levels (Arai, 2008). Regarding their phylogeny, the group is ascribed to two orders: Semaeostomeae and Rhizostomeae (WoRMS Editorial Board, 2019).

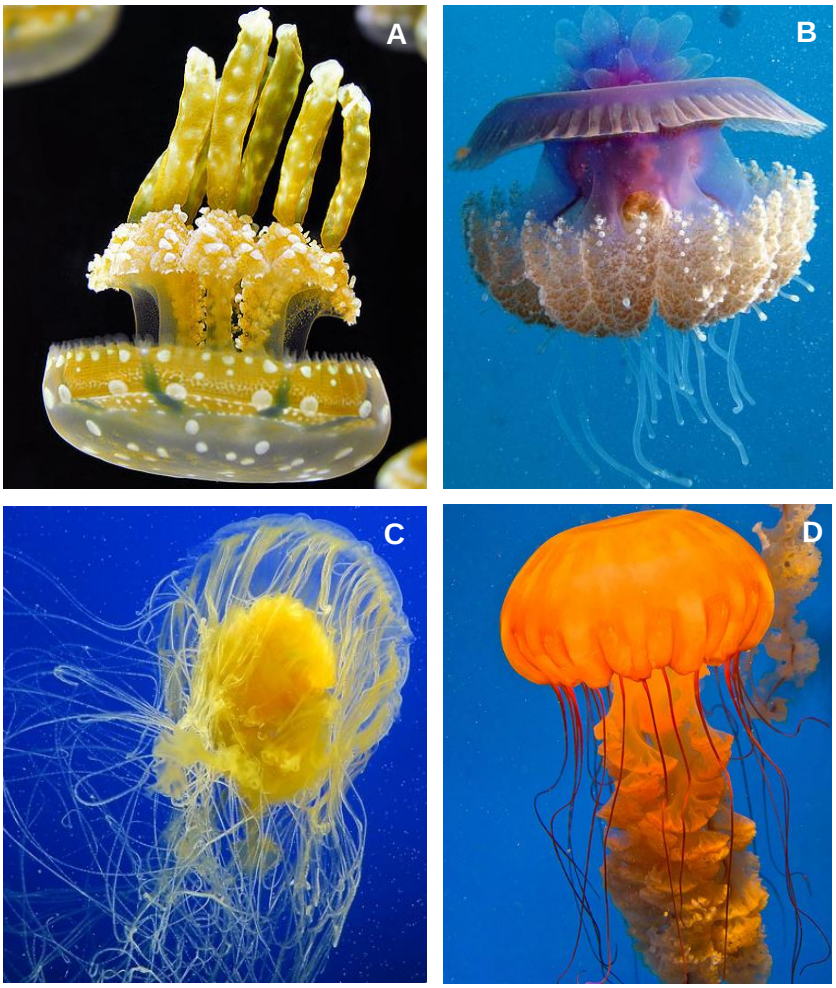

Figure 13. Representative images of the Discomedusae subclass. **A:** *Mastigias papua* (Order Rhizostomeae, Family Mastigiidae). **B:** *Cephea cephea* (Order Rhizostomeae, Family Cepheidae). **C:** *Phacellophora camtschatica* (Order Semaeostomeae, Family Phacellophoridae). **D:** *Chrysaora quinquecirrha* (Order Semaeostomeae, Family Pelagiidae). Photographs credits: **A** licensed by Adrian, 2008 under the CC BY-SA 3.0, source Wikimedia Commons (https://bit.ly/2Zj1Uzm); **B** licensed by Derek Keats, 2011 under the CC BY-SA 2.0, source Wikimedia Commons (https://bit.ly/2ysYoXj); **C** licensed by John Rusk, 2016 under the CC BY-SA 2.0, source Flickr (https://bit.ly/2yqgx8c); **D** licensed by Antoine Taveneaux, 2011 under the CC BY-SA 3.0, source Wikimedia Commons (https://bit.ly/2yoeGR5).

Semaeostomeae order

The Semaeostomeae order is composed of 4 families, 5 subfamilies, 19 genera and 65 species (Worms Editorial Board, 2019). Characterized by four oral arms around the

mouth, Semaeostomeae jellyfish have their tentacles at the umbrella margin (Nair, 2018).

Rhizostomeae order

The Rhizostomeae order is the most diverse group inside Scyphozoa and is comprised of 2 suborders, 10 families, 27 genera and 76 species (Daly *et al*., 2007; Worms Editorial Board, 2019). Rhizostomeae jellyfish are characterized by having bell margin cleft into lappet, with no tentacles on the bell margin, without a central mouth, with eight oral arms extended from the subumbrella, where each oral arm bears numerous secondary mouths. Network of canals are found beyond the stomach (Arai, 1997; Kramp, 1961).

3.1.9 Cubozoa superclass

Frequently called 'box jellyfish', cubozoans are widely distributed with reports from tropical, sub-tropical, and temperate localities in the Indian, Pacific and Atlantic Ocean (Bentlage *et al*., 2009; Lawley *et al*., 2016). Cubozoan jellyfishes (**Figure 14**), with peculiar features such as four perradial sensory rhopalia containing strikingly complex eyes with ocelli, vitreous bodies, lenses, and retinas (Coates, 2003; Pearse and Pearse, 1978), encapsulate some of the most life-threatening organisms in the world due to their powerful toxin production that caused several fatalities (Bordehore *et al*., 2011, 2014; Carrette *et al*., 2002, 2012; Carrette and Seymour, 2004, 2013; Williamson *et al*., 1996; Yanagihara *et al*., 2002).

Even so, the population dynamics of these organisms remain shortly studied (Bentlage *et al*., 2009; Gershwin *et al*., 2009; Yoshimoto and Yanagihara, 2002) compared to scyphozoans, possibly due to the large spatial and temporal variability in their abundances (Kingsford and Mooney, 2014; Lawley and Faria Júnior, 2018). The life cycle of this organisms is similar to the scyphozoans encompassing the same three main stages: polyp, planulae and medusa.

The most characterizing features of the cubozoan life cycle are the banded spotted pattern planula and the complete metamorphosis, where polyps produce secondary polyps asexually that fully transform into a free-swimming medusa without any polypoid residues left (Carrette *et al*., 2018). Recently, it was found that there are some exceptions to these characteristics within Carybdeida order. Thus, some species perform incomplete metamorphosis by leaving a regenerative polypoid residue to form new polyps (Straehler-Pohl and Jarms, 2005), others display inner structures in the polyps that split the gastric cavity into sections (Straehler-Pohl and Jarms, 2011) and even some species of the Carukiidae family reproduce by a modified strobilation, generally typical of scyphozoans (Carrette *et al*., 2018; Courtney *et al*., 2016; Toshino *et al*., 2013, 2015).

The superclass is phylogenetically divided into two orders: Carybdeida and Chirodropida.

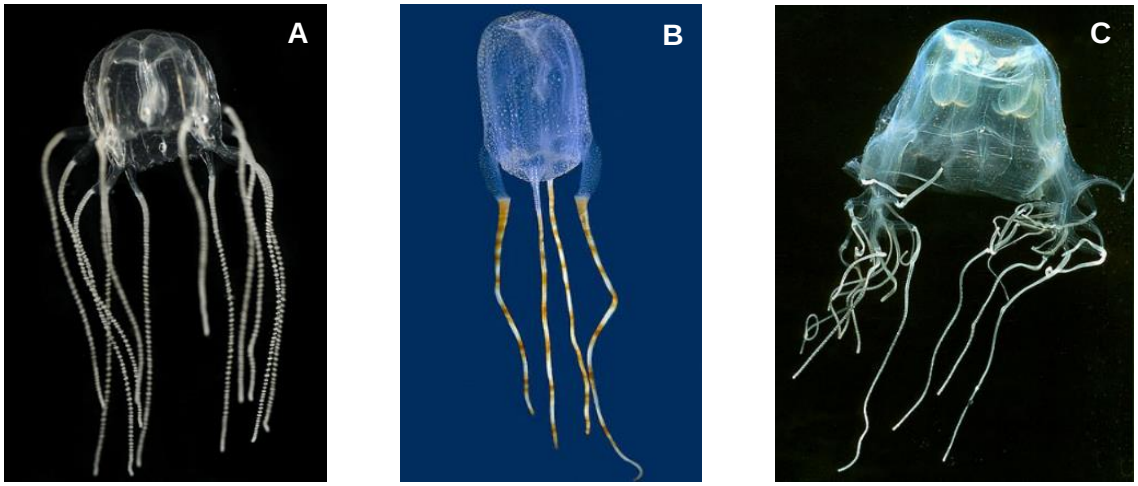

Figure 14. Representative images of the Cubozoa superclass. **A:** *Tripedalia cystophora* (Order Carybdeida, Family Tripedaliidae). **B:** *Tamoya ohboya* (Order Carybdeida, Family Tamoyidae). **C:** *Chiropsalmus quadrumanus* (Order Chirodropida, Family Chiropsalmidae). Photographs credits: **A** licensed by Jan Bielecki, 2012 under public domain, source Wikimedia Commons (https://bit.ly/2OqlOXV); **B** licensed by Ned DeLoach, 2008 under public domain, source Wikimedia Commons (https://bit.ly/2LQPuvm); **C** licensed by Alvaro E. Migotto, 2001 under the CC BY-SA 3.0, source Wikimedia Commons (https://bit.ly/2ZpVmyQ).

Carybdeida order

The Carybdeida order is composed of 5 families, 11 genera and 33 species (Worms Editorial Board, 2019). The most recognizable feature is the unbranched pedalia located at the four interradial corners of the bell margin, also, species of the Carybdeida order lack gastric saccules and most of them present four pedalia and four tentacles (Daly *et al.*, 2007).

Chirodropida order

The Chirodropida order is composed of 3 families, 7 genera and 14 species (Worms Editorial Board, 2019). Members of this order are distinguished by their branched pedalia with numerous tentacles and almost all of them possess gastric saccules.

3.1.10 Staurozoa superclass

Encompassed by 49 living species, all from the Stauromedusae order, divided into two sub-orders Amyostaurida and Myostaurida, Staurozoa (**Figure 15**) is one of the most poorly understood cnidarian clades (WoRMS Editorial Board, 2019). It is composed by benthic stalked jellyfishes with tentacles that live attached to the substrate (Frazão, 2016; Kayal *et al*., 2018), from the intertidal zones to deep hydrothermal vents. Although Stauromedusae may be locally abundant (they have a cosmopolitan distribution, mainly

in temperate and polar waters), they are rarely observed because of their relatively small size and cryptic coloration (Miranda *et al*., 2017a).

Their life cycle starts with a creeping non-ciliated larval planula stage that develops into a stauropolyp (Miranda, 2019). This polyp suffers both, apical and basal metamorphosis (without strobilation or budding) finally becoming an adult Stauromedusae (Miranda *et al*., 2016a). Regarding their distinct characters/structures, stalked jellyfishes have long been confusing to systematists. For instance, in a study by Miranda *et al*. (2017b), it was concluded that the structure called claustrum was a character exclusive to some species of Staurozoa and that the structure also called claustrum in Cubozoa corresponds to a completely different structure. The adult staurozoan body plan includes features common to both the polyp and medusa stages of other cnidarians, thus adding significance to their phylogeny (Collins, 2002; 2006; Miranda *et al*., 2016b).

Some benthic polyp forms of staurozoans exhibit characters (gastric filaments, coronal muscle, rhopalioids and gonads) also known in the medusa stages of cubozoans and scyphozoans. In other hand, the basal region (peduncle), retains polypoid characters such as gastric septa associated with four interradial longitudinal muscles (Collins *et al*., 2006; Zapata *et al*., 2015). Consequently, understanding the body plan of a stauromedusa is more complex than for other medusozoans because of its dual nature (Miranda, *et al*., 2016a). The phylogenetic position of staurozoans within Cnidaria, remains controversial although a recent study by Zapata *et al.* (2015) placed Staurozoa in a clade with Cubozoa and Scyphozoa. Moreover, both morphological and molecular studies have revealed relatively little evidence of the Staurozoa phylogenetic position (Kayal *et al*., 2018; Miranda *et al*., 2016b; 2017a; Simion *et al*., 2017).

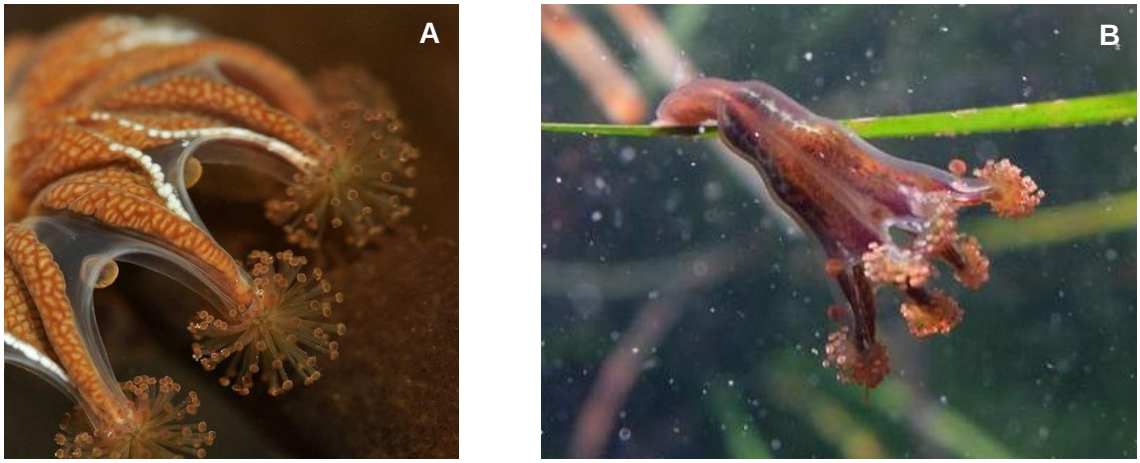

Figure 15. Representative images of the Staurozoa superclass. **A:** *Haliclystus stejnegeri* (Order Stauromedusae, Family Haliclystidae). **B:** *Haliclystus tenuis* (Order Stauromedusae, Family Haliclystidae). Photographs credits: **A** licensed by Minette Layne, 2009 under the CC BY-SA 2.0, source Wikimedia Commons (https://bit.ly/2Zm0FiQ); **B** licensed by Sarah E. Millerse under the CC BY-SA 3.0, source Wikimedia Commons (https://bit.ly/2yCwwQJ).

Amyostaurida and Myostaurida suborders

The difference between the suborders Amyostaurida and Myostaurida lies on the interradial longitudinal muscles. The last common ancestor of Staurozoa is thought to have had peduncular muscles; a loss of the character could explain what happened on the lineage leading to the Amyostaurida suborder. Therefore, the Myostaurida suborder presents interradial longitudinal peduncular muscles while Amyostaurida presents them at the base of the calyx (Miranda *et al*., 2016a).

3.1.11 Medusozoa in Portugal

Considering the ecological relevance, the impacts of jellyfishes, and the lack of information of such species in the Portuguese coast, we performed a review of the Medusozoa records in Portugal (mainland and archipelagos of Azores and Madeira), resorting to many different databases and works (consulted data can be found in **Appendix A**), resulting in a list with a total of 272 reported species (**Appendix A**). Globally, these reported species are distributed by medusozoan superclasses as follows (**Figure 16**): approximately 93 % of the species are hydrozoans, 6 % are scyphozoans, 1 % are staurozoans, and less than 1 % are cubozoans.

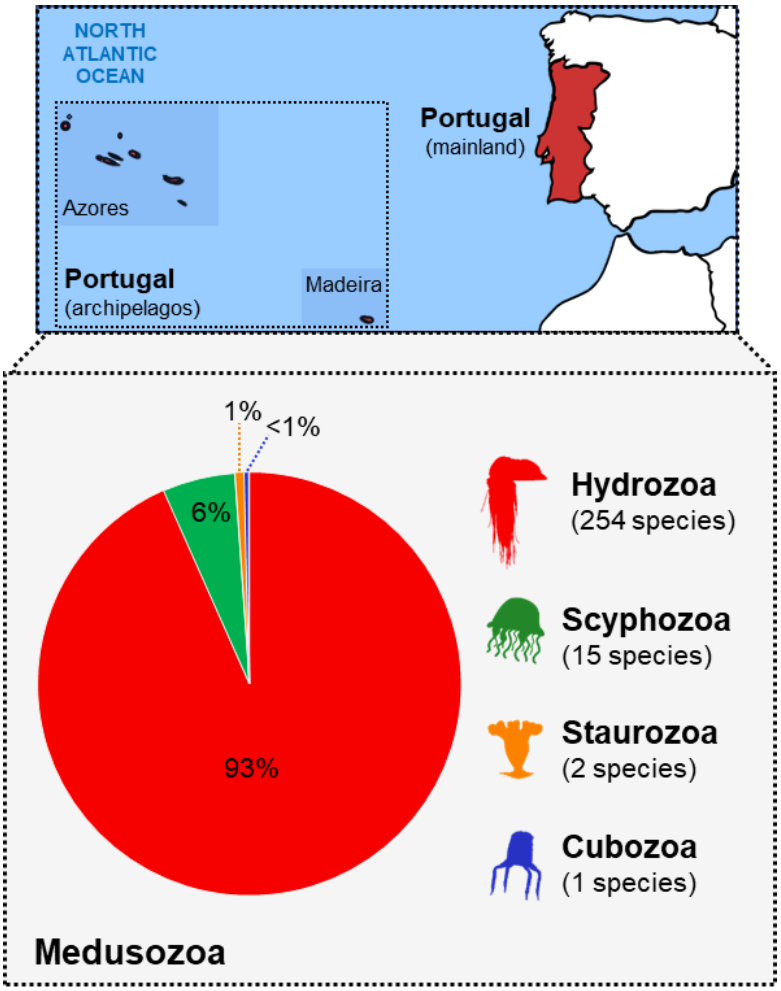

Figure 16. Number of medusozoans reported in Portugal distributed by 'Superclass'.

The 254 different hydrozoan species are divided into six orders: Leptothecata, Anthoathecata, Siphonophorae, Trachymedusae, Narcomedusae, and Limnomedusae (**Figure 17**). The 15 different scyphozoan species are divided into three orders: Rhizostomeae, Coronatae, and Semaeostomeae (**Figure 17**). The two staurozoan species reported were *Haliclystus auricula* and *Calvadosia campanulata* (Kramp, 1961*;* OMARE, 2019) (**Figure 17**). Finally, the single cubozoan reported in Portugal was *Carybdea marsupialis* (Kramp, 1961) (**Figure 17**).

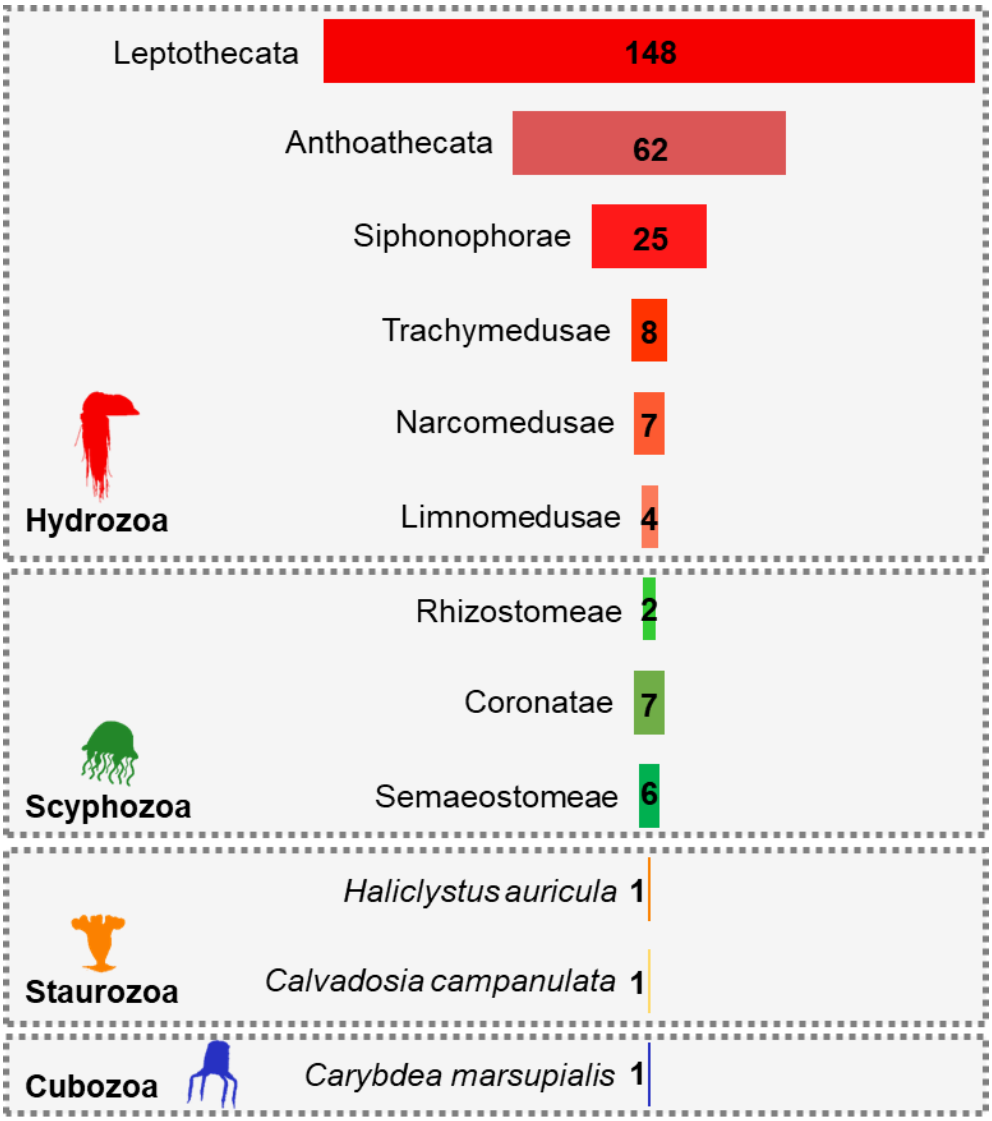

Figure 17. Graphical representation for the distribution of the medusozoan number reported in Portugal by its taxonomic rank 'Order' or 'Species' within their respective 'Superclass'.

3.1.12 Genetic information available for jellyfishes occurring in Portugal

With the species list completed (**Appendix A**), it was compiled the genetic information regarding available nucleotide sequences for all the species previously listed (**Appendix B**). The molecular markers were selected according to their use in prior studies (Günther *et al.*, 2018; Machida and Knowlton, 2012; Zheng *et al.*, 2014). Thus, resorting to the GenBank Nucleotide database, a search was performed using the terms "*species name*" (May, 2019). The data compiled for the species listed include 15 mitochondrial DNA markers, 16S rRNA, COX1, COX3, 12S ribosomal RNA (12S rRNA), ATP synthase membrane subunit 6 (ATP6), ATP synthase membrane subunit 8 (ATP8), NADH dehydrogenase subunit 1 to 6 (NAD1 to NAD6), and Cytochrome b (CYTB), and five nuclear DNA markers, 18S rRNA, 28S rRNA, ITS1, Internal transcribed spacer 2 (ITS2),

and 5.8S ribosomal RNA (5.8S rRNA). The information gathered consisted in all the sequences available, *i.e.,* complete and incomplete sequences (**Figure 18**).

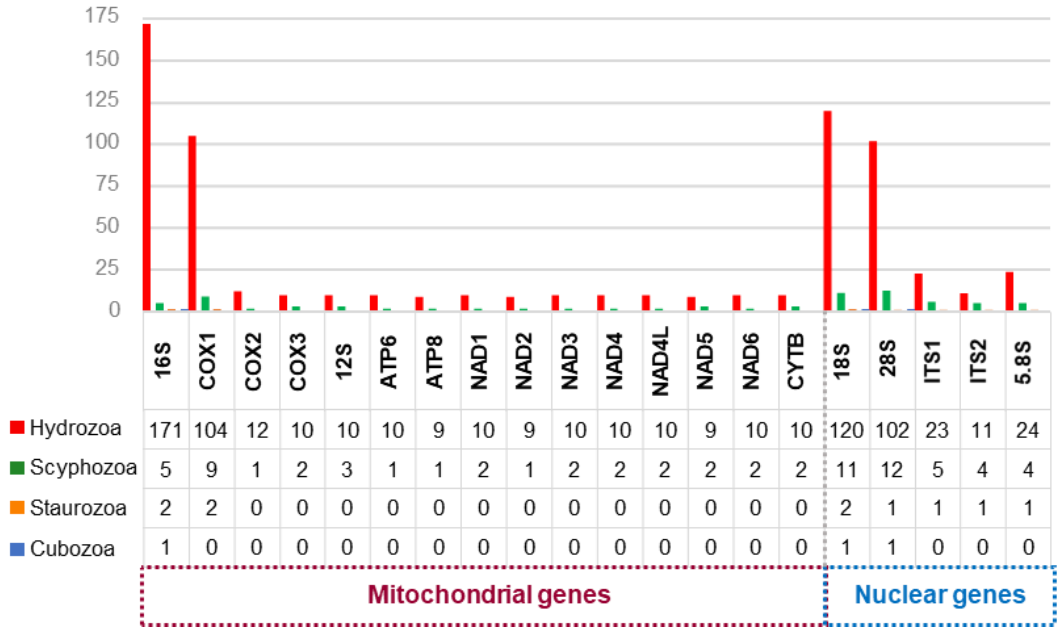

Figure 18. Graphical representation of the genetic information available of the medusozoans reported in Portugal.

The data compiled revealed that 72 of the reported species, corresponding to approximately 26 % of the total species, do not present genetic information in the GenBank Nucleotide database (May, 2019). Furthermore, regarding the mitochondrial markers, it was verified that the most representative for these species are the genes 16S rRNA and COX1, with 66 % (180) and 42 % (116) of the species having at least a partial sequence of these markers available from the GenBank, respectively. As far as nuclear markers are concerned, the most common for these species are the genes 18S rRNA and 28S rRNA, with 49 % (134) and 42 % (116) of the species having at least a partial sequence of these markers available from the GenBank, respectively. From the total of 255 species of hydrozoans and 15 species of scyphozoans reported, only four (*Clava multicornis, Craspedacusta sowerbyi, Hydra oligactis, Laomedea flexuosa*) and two (*Aurelia aurita, Chrysaora quinquecirrha*), respectively, present the complete mitochondrial DNA sequence in GenBank. These results reveal that there is a lack of genetic information on a substantial part of the species reported in Portugal and that the available data is majorly focused in just two mitochondrial and two nuclear markers.

3.1.13 Bloom-producing medusozoans and other mass occurrences reported in Portugal: a warning perspective for future events

Firstly, we highlight the importance of properly define the concept of *"bloom"* as the term is increasingly used in the bibliography. We can expect that with such common use of the term its definition is plain and simple, however it is not the case. In the biological sciences, the term began to be applied to the appearing of flowers in a plant or the opening of the flowers. Later, the term gained a new meaning: *"bloom"* as a rapid and excessive growth of an algal or phytoplankton population (Smayda, 1997). Posteriorly, the term was employed to define a large aggregation of gelatinous free-swimming organisms (Lucas and Dawson, 2014). In the present work, we will refer to "*bloom"* as the third definition with focus on *jellyfish blooms*.

The general definition of jellyfish includes organisms that are gelatinous and constituted mostly by water. Gelatinous zooplankton comprise a vast panoply of animal groups such as: ctenophores, cnidarians, pelagic tunicates (salps, larvaceans), mollusks, and worms (Prieto, 2018). In this review article, we use the term "*jellyfish"* to describe just cnidarian medusae. These organisms have an osmotic and biomechanical versatility, allowing the adaptation of jellyfish to many different marine ecological environments such as brackish water and freshwater habitats (Ames, 2018). Their amazing adaptability to different environments, with different conditions, is the cause for the large space-time fluctuations in their abundance. The body composition, high in water and low in carbon, and the abundance of medusae liberated from polyps (*i.e.*, strobilation) are connected to the potential for a jellyfish species to cause a *bloom*. The so-called *jellyfish blooms* are a natural phenomenon of seasonal abundance featured by a rapid population increase in a certain area. Many are the factors that contribute to the occurring of this phenomenon: environmental changes caused by human exploitation plays a very important role on the occurrence of this phenomenon. Jellyfishes' researches are increasing not only due to the growth of jellyfishes' mass occurrences that resulted in several economic losses, but also due to the growing awareness of jellyfishes' pivotal roles in the marine ecosystems (Brotz, 2001; Condon *et al*., 2012,2013 ; Dawson *et al*., 2014; Lucas and Dawson, 2014; Lucas *et al*., 2014a; Palmieri *et al.*, 2014; Pitt and Lucas, 2014; Schnedler-Meyer *et al*., 2018).

With the species listed (**Appendix A**), we resorted to the historical records in scientific literature, magazines and information from websites, to select the species reported in Portugal more relevant both ecologically and economically (**Figure 19**). Thus, since our focus were the Scyphomedusae capable to occur in mass, we selected the *Aurelia, Chrysaora, Pelagia*, and *Rhizostoma* genus. We also chose *Catostylus tagi* species for

being one of the most common jellyfishes in Portugal and for its valences. The hydrozoans were selected for different reasons. *Blackfordia virginica* for being an invasive species, *Physalia physalis* for the danger that represents to the human health, and *Velella velella* for its unpredictable behavior and peculiar features that sometimes translate into *blooms* and massive strandings worldwide.

Figure 19. Compilation of the most relevant medusozoan species occurring in Portugal representing the taxonomic groups Hydrozoa (**A**-**C**) and Scyphozoa (**D**-**H**). **A:** *Blackfordia virginica* (Blackfordiidae). **B:** *Physalia physalis* (Physaliidae). **C:** *Velella velella* (Porpitidae). **D:** *Aurelia aurita* (Ulmaridae). **E:** *Chrysaora hysoscella* (Pelagiidae). **F:** *Rhizostoma luteum* (Rhizostomatidae). **G:** *Catostylus tagi* (Catostylidae). **G:** *Pelagia noctiluca* (Pelagiidae). Photographs credits: **A** by image courtesy of Mariah Meek, source NEMESIS Databases (https://s.si.edu/2X7aZy7); **B** licensed by Biusch under the CC BY-SA 3.0 Unported license, source Wikimedia Commons (https://bit.ly/1VUgOTc); **C** licensed by Jonathan Lidbeck under the CC BY 4.0, source Flickr (https://bit.ly/2FqL5dR); **D** licensed by Alexander Vasenin, 2010 under the CC BY-SA 3.0 Unported license, source Wikimedia Commons (https://bit.ly/2IKa39Z); **E** licensed by Francesco Crippa, 2007 under the CC BY 2.0 Generic license, source Wikimedia Commons (https://bit.ly/2YbReln); **F** licensed by Roberto Pillon, under the CC BY-NC-SA 4.0, source OMARE (https://bit.ly/2sLFIYA); **G** by image courtesy of Mauro Hilário, source Flickr (https://bit.ly/2XvnQts); **H** licensed by Hans Hillewaert, 2008 under the CC BY-SA 4.0, source Wikimedia Commons (https://bit.ly/2YfWeWm).

Aurelia genus

Studies on the phylogeny of *Aurelia* revealed that the genus has at least 16 phylogenetic branches with 13 cryptic species (Dawson *et al.*, 2005; Ki *et al.*, 2008). *Aurelia* belongs

to the Semaeostomeae order and encompasses the common scyphozoan jellyfish *Aurelia aurita* (Lucas, 2001). *A. aurita*, the most notorious species of the genus, is known to establish their populations in several different environmental conditions, to have big inter-population differences, and to have inter-annual variability inside a population. All these features between others make *A. aurita* a potential *bloom* forming species. In fact, there are several reports of *A. aurita blooms* throughout the world (Dong *et al*., 2010, 2012; Dong, 2018; Lucas, 2001; Mills, 2001; Purcell *et al*., 2007). According to Dong (2018), the majority of the Aurelia species or subspecies appears to occur regionally. Actually, *A. aurita* is mostly distributed in the North Atlantic Ocean and in the Black Sea (Dong, 2018; Lucas, 2001). In a Portuguese perspective, this is very important, as Portugal is geographically located in the North East part of the Atlantic Ocean. There are few studies on this species in Portugal (Araújo *et al*., 2014; Pereira *et al*., 2014) and there is a study that reports *blooms* of the species on the Guadiana Estuary, South East Portugal (Muha *et al.*, 2012). The potential of the moon jellyfish is starting to be explored in Portugal (Chambel *et al.*, 2016) and the awareness on this fascinating organism is increasing.

Chrysaora genus

According to Collins *et al.* (2019), the *Chrysaora* genus, commonly known as sea nettle, is comprised of 15 accepted species. The systematics and taxonomy of some species of the genus are poorly described, while others have not been reported regularly. The global studies with this genus are not much and the Portuguese studies are practically inexistent with one exception (Cruz, 2015). There are only reports of two species of the *Chrysaora* genus to occur in Portuguese waters: *Chrysaora hysoscella* and *Chrysaora quinquecirrha*. Even those, lack information and the data available is dubious. In fact, about *Chrysaora hysoscella* Junior and Barreiros (2007) mentioned that “This species does not occur in the Azores or Madeira and is considered rare in the Portuguese continent”; the species was reported in Lisbon by Morandini and Marques (2010) and in Esposende, in “OMARE” (2019). About *Chrysaora quinquecirrha*, there is no reports in mainland Portugal, but Habermehl (1981) writes that the species is distributed in Azores, though we failed to find evidence of it. However, Cruz (2015) developed a thesis about the growth and development of this species in aquaculture, as they have value for aquarium and potential value for pharmacological and medical applications.

Pelagia genus

The *Pelagia* genus lies exclusively on the *Pelagia noctiluca* species, commonly referred as mauve stinger. This species has been increasing in frequency and abundance in the

Mediterranean, being the most common oceanic jellyfish in the Mediterranean Sea (Canepa *et al.*, 2014; Lucas *et al.*, 2014b; Mariottini *et al.*, 2008). The species is responsible for many *blooms* along warm and temperate seas; those outbreaks are responsible for substantial negative impacts on aquaculture, fisheries and tourism. Since a long time ago, it is known that the occurrence of this species is quite unpredictable; the biology population of *P. noctiluca* is known to suffer drastic fluctuations throughout time (Stopar *et al.*, 2010). Despite of the species occurrence unpredictability , it is known that climatic factors such as water temperature, rainfall and atmospheric pressure are intrinsically related to it (Goy *et al.*, 1989) since those factors may enhance the survival and growth of the ephyrae (Morand *et al.*,1992; Sabatés *et al.*, 2018). In fact, Morand *et al.*(1992), developed a model proving that the annual occurrence of *P. noctiluca* in the Mediterranean Sea, was controlled by environmental changes. In Portugal mainland, the species is considered rare, although there are some reports in the press and in the national institution “IPMA - Instituto Português do Mar e Atmosfera”. Despite their annual occurrence in the Azores, and even with an investigation center that monitors the waters of Azores “OKEANOS”, there are few studies in Portugal. With climate change and the natural unpredictability of the species, the scenario currently found in Portuguese waters could change in the upcoming future.

Rhizostoma genus

The *Rhizostoma* genus belongs to the Rhizostomeae order and comprises three different species: *Rhizostoma luteum*, *Rhizostoma octopus* and *Rhizostoma pulmo.* Both species are known to occur in mass (Prieto *et al.*, 2013; Ramšak *et al.*, 2012) but curiously, *R. luteum* was thought to be a very rare species until, recently, Kienberger and Prieto (2018) detected that, in the past, this species was frequently misidentified as *R. pulmo* and *Catostylus tagi*. They even state that “Rhizostomeae is very abundant during summer on the shores of Portugal”. There is a lack of works made in Portugal with these species, in order to understand its ecological behavior and to explore the resources that these species may provide us, we need to follow the example of the work that has been done with *C. tagi* in Portugal.

Catostylus tagi species

Also belonging to the Rhizostomeae order, the *Catostylus* genus is comprised of 10 different species though only *Catostylus tagi* is present in Portugal. *C. tagi* is native of our coast and considered very common in Portugal, especially in the Tagus and Sado estuaries where they can occur abundantly in the summer (Morais *et al.*, 2009; Saldanha, 1997). Although the studies on this species are very recent (of the last 10 years), they

are made mainly in Portugal, making it the currently most studied scyphozoan species in our country. The first studies done with *C. tagi*, began to explore the benefits that could be extracted from this animal (Morais *et al.*, 2009). Parracho and Morais (2015) optimized the sample preparation for DNA extraction for further works with this species and Calejo *et al.* (2009; 2012), explored and characterized a new collagen from *C. tagi* with the potential to be used for biomedical applications. The *in vitro* results obtained by Morais *et al.*, 2009 showed that *C. tagi* had potential for the manufacturing of aquaculture feeds and also for human consumption, in fact, of all the scyphozoans, only Rhizostomae are considered edible (Kimura *et al.*, 1983). This was followed by the development of new studies to evaluate the viability of *C. tagi* as food (Amaral *et al.*, 2018; Raposo *et al.*, 2018); the primary results were promising showing that the jellyfish intake is safe and its organoleptic properties were accepted by the study population (Raposo *et al.*, 2018).

***Blackfordia virginica* species**

The non-indigenous hydroid *Blackfordia virginica* has a controversial origin, some authors suggest that the species is native of the North West Atlantic coast (Zaitsev and Ozurk, 2001), where the medusa was first described in 1910 (Mayer, 1910), while others state that it is endemic of the Black Sea (Graham and Bayha, 2007; Mills and Sommer, 1995). Its life cycle includes a benthic polyp stage and a planktonic medusa stage (Kimber, 2014; Mills and Sommer, 1995). The seasonal production of medusae can reach high peaks of abundance during the warmer periods of the year (Bardi and Marques, 2009; Marques *et al*., 2015; Wintzer *et al*., 2013). The species is considered a zooplankton predator that feeds primarily on copepods (Mills and Sommer, 1995; Wintzer *et al*., 2013), and has the potential to alter planktonic food webs. *B. virginica* is considered an invasive species that has been introduced worldwide due to its high potential for dispersal; some authors suggest ballast water exchanges and hull fouling as the main vectors (Golemansky, 2007; Zaitsev and Ozurk, 2001), and its capacity to live in waters with a wide range of temperatures and salinities (Bardi and Marques, 2009; Moore, 1987). In Portugal, the species is well documented and it was firstly observed in May 1984 in the Mira estuary where since then it is thriving (Marques *et al*., 2015; 2017; Moore, 1987), and later in the Guadiana estuary (Chícharo *et al.*, 2009). Due to its invasive nature, it is important to monitor the places where the species occur, especially during the warmer periods, in order to control the species dispersal and to avoid possible negative shifts in the ecosystems provoked by the species proliferation.

Physalia physalis species

Physalia physalis Linnaeus, 1758, commonly known as the Portuguese man o' war or blue bottle, is a colonial pleustonic siphonophore that is easily recognizable by its huge asymmetric purplish-blue pneumatophore, a gas-filled float developed from one of the polyps used for navigation and floating (Bouillon *et al*., 2006). The other polyps that constitute the colony, differentiate into digesting polyps (gastrozooids), reproductive polyps (gonozooids) and long hunting tentacles (dactylozooids) (Iosilevskii and Weihs, 2009). The Portuguese man o' war was the first siphonophore ever to be formally described; is a voracious carnivore very important to the pleustonic community, and it is a ubiquitous species common in the tropical and subtropical waters (Lopes *et al.*, 2016). The species was profoundly studied by Totton (1960), Shannon and Chapman (1983) and Pagès and Gili (1992). The venom of this species is what turns it so "relevant" for us. The main toxin of its venom is called physaliatoxin, a powerful cytotoxin and hemotoxin. There are many reports of Portuguese man o' war envenomation's worldwide causing different symptoms from intense pain and skin inflammation to cardiac and neurological manifestations, and even fatal occurrences (Stein *et al*., 1989). Due to the characteristics of the pneumatophore, specimens are sometimes found far away from their usual warm water habitats (see Iosilevskii and Weihs, 2009). In fact, there have been reports of the species in the North Atlantic including in European coastal waters. For instance, there are reports of the species in France (Labadie *et al.*, 2012) and in Spain (Prieto *et al*., 2015). However, few or no studies were developed specifically for the species occurrence in the Portuguese coast, although there are many media reports of sightings of the species in the Portuguese coast.

Velella velella species

Velella velella Linnaeus, 1758, also known as wind sailor is a carnivorous colonial pleustonic siphonophore that occurs in temperate and tropical waters (Bouillon *et al*., 2006; Lopes *et al*., 2016; Purcell *et al*., 2012). The wind sailor presents a characteristic blue-hued float and a chitinous triangular sail that extends above the surface (Kirkpatrick and Pugh, 1984). They have an important ecological role on marine food webs since they prey on fish eggs, euphausiid eggs, crustaceans and copepods (Evans, 1986; Purcell *et al*., 2015; Zeman *et al*., 2018) and they are preyed by fish, sea turtles, and birds (Arai, 2005; Phillips *et al*., 2017). In addition to that, algal symbionts associate with *V. velella* and may provide supplementary nutrition to the colonies (Banaszak *et al*., 1993; Lopes *et al*., 2016). *V. velella* distribution is quite unpredictable; it has a seasonal distribution, being the warmer periods when they occur, grow and reproduce more (Bieri, 1977, Purcell *et al*., 2012) possibly due to food and light availability and wind conditions

(Bigelow, 1911, Bieri, 1977, Purcell *et al*., 2015); the ocean circulation and the wind regimes also contribute to their erratic dispersal (Bieri, 1977). The species is responsible for huge *blooms* and mass strandings throughout the world's oceans (Evans, 1986; Flux, 2009; Purcell *et al*., 2015; Pires *et al*., 2018). Those large *blooms* can reach the shore and impact the coastal systems as they are responsible for the deposit of big amounts of nitrogen and carbon (Bieri, 1977; Purcell *et al*., 2015; Savilov, 1968). In Portugal, the studies on the species are scarce though there is a recent study by Pires *et al*., 2018 where the authors evaluate the distribution of *V. velella* in the Portuguese shore through a citizen science and oceanographic approach.

3.1.14 Conclusions

In the elaboration of this review there were some difficulties in obtaining information about studies made in Portugal with medusozoans. In fact, most of the Portuguese studies on Cnidaria are way more focused in the clade Anthozoa. We consider that the reason for this may lie in the difficult accessibility and underestimation of the value, diversity and abundance of the medusozoans present in Portugal. With the compiled data, we gained a wider view of the Portuguese panorama on the species that represent this group of cnidarians. Moreover, this work also serves to display the valences of an integral part of this group: jellyfishes. On the other hand, with the problematics associated with the occurrence of these organisms increasing, this review article also serves as reference to assist further investigations on the dynamics of this group of organisms in Portugal, and to alert for putative mass occurrence events as well.

Therefore, to the best of our knowledge, we presented for the first time a list of the Medusozoa reported in Portugal (mainland and archipelagos), as well as a list of the genetic information (nucleotide sequences) available in the GenBank (May 2019) for those species.

We think that the number of species on the list may be undervalued due to the limited number of studies with these organisms. More diversity of studies and monitoring programs throughout the entire country should be implemented for a better diversity evaluation. Regarding the genetic information available in GenBank for this group of organisms, we conclude that the available information is still very scarce. We verified that the most represented markers are 16S rRNA and COX1 (mitochondrial), and 18S rRNA and 28S rRNA (nuclear) genes.

In this study, we also reviewed the works made in Portugal with the jellyfish species with more economic and ecological relevance. The hydrozoans *Physalia physalis* and *Velella velella* and the scyphozoan *Aurelia*, *Chrysaora*, *Pelagia* and *Rhopilema* genus, as well as, the most common jellyfish species in Portugal, *Catostylus tagi*. The information here

provided about the jellyfish species capable of producing mass occurrence events in Portugal serves as a “wake-up” call to the authorities about the importance of monitoring these species, as well as to create strategies to face putative ecological and economic losses caused by them. On the other hand, this work also attends to change the way people think about jellyfish; often seen as worthless venomous creatures, jellyfishes must be studied and monitored, since they play a very important role in the ecosystem food webs, and therefore, in our fisheries, and can also be used to test compounds with a therapeutic purpose. The two lists displayed in this review article, compile information that may assist further works, and may serve as the basis for a future Portuguese database of Medusozoa, or even to an Iberian database of these organisms. The future of our changing oceans may lie in jellyfishes.

3.2 Chapter 2. Experimental section: "DNA extractions, Polymerase Chain Reactions and Bioinformatics analyses"

3.2.1 DNA quantification and amplification results

The quantification of the extracted DNA showed that the amount of DNA yielded by tentacles was higher than that from the gonads, ranging from 1 to over 600 ng/µL. Moreover, six of the samples did not had enough DNA to be quantified and five of those samples were from the gonad tissue (**Table 5**). The quality of the extracted DNA was relatively good, showing a ratio 260/280nm higher than 1.8 in most replicates.

Table 5. DNA quantification results from original and replicate samples.

Organism identifier	Sample*	DNA concentration [ng/µL]	Ratio 260/280 nm
#1	T1	48.449[a]	1.680
	G1	3.992	1.180
#2	T2	2.028	0.400
	G2	1.302	2.500
#3	T3.1	622.868[b]	1.804
	G3.1	3.656	1.000
	T3.2	333.387	1.840
	G3.2	0.000	0.000
	T3.3	539.858	1.843
	G3.3	77.509[b]	1.782
#1	T1R	1.326	0.640
	G1R	0.000	0.000
#2	T2R	0.000	0.000
	G2R	0.000	0.000
#3	T3.1R	93.076[b]	1.815
	G3.1R	0.000	0.000
	T3.2R	60.477[b]	1.813
	G3.2R	0.000	0.000
	T3.3R	92.637[b]	1.837
	G3.3R	24.250[a]	1.640

#C. tagi individual number. * tissue sample: T (tentacles), G (gonads), R (replicates - corresponding to the 2nd elution). [a] good concentration values. [b] high values selected for dilution.

From the quantification results obtained (see **Table 5**), only the original sample T1 and the replicate sample G3.3R are within the appropriate DNA concentration range for further DNA amplification. The dilution of the samples DT3.1, DG3.3, DT3.1R, DT3.2R and DT3.3R allowed to obtain the recommended DNA concentration values (**Table 6**).

Table 6. DNA quantification results of diluted samples.

Sample*	DNA concentration [ng/µL]	Ratio 260/280 nm
DT3.1	17.017	1.650
DG3.3	24.250	1.640
DT3.1R	22.814	1.520
DT3.2R	15.599	1.670
DT3.3R	28.589	1.590

***tissue sample: T (Tentacles), G (Gonads), D (Dilutions).

The results displayed on **Table 7** correspond to only the purified bands posteriorly sent for sequencing. These results show the difference between using gonads or tentacle tissue, tentacle is the tissue with more DNA (strong bands).

Table 7. Schematic representation signaling the intensity of the purified bands from the electrophoresis gel sent for sequencing.

Ladder (bp)	16S rRNA		COX1		COX3		NAD6		18S rRNA		28S rRNA		ITS1	
	G	T	G	T	G	T	G	T	G	T	G	T	G	T
2000														
1500									-	-				
1000											-	+		
850														
650			-	+	+	+								
500	+	+												
400													-	+
300							+	-						
200														
100														

[-] thin band, [+] strong band, [G] gonad, [T] tentacle.

3.2.2 Bioinformatics analyses

From the BLASTn analyses, it was obtained a sorted table of the sequences more similar (hit sequences) to the queried sequence obtained in this study (**Table 8**). This table shows the name of the sequences obtained (Query), the hit sequence code at GenBank database (Accession number), the average percent identity over the alignment (% Pairwise Identity), an indication of how good the alignment is, calculated from a formula that takes into account the alignment of similar or identical residues, as well as any gaps introduced to align the sequences (Bit-Score), the number of hits with at least this score that you would expect purely by chance, given the size of the database and query sequence (E-Value), the organism of the hit sequence (Organism) and the percent of the query that is covered by the hit (% Query coverage).

Table 8. BLASTn results.

Query	Accession*	% Pairwise Identity	Bit-Score	E Value	Organism	% Query coverage
16S_65_T5	KY610595.1	87	526	4e-145	*Lychnorhiza* sp. 3	100
16S_65_T6	KY610584.1	87	546	5e-151	Catostylidae sp. 2	100
16S_85_T7	KY610595.1	87	567	1e-157	*Lychnorhiza* sp. 3	100
16S_85_T8	KY610584.1	87	566	5e-157	Catostylidae sp. 2	99
COI_G3R	KY611026.1	84	588	9.10e-164	Catostylidae sp. 1 LGD-2017	100
CO3_DT2R	KY454767.1	80	329	1.88e-85	*Nemopilema nomurai*	97
CO3_G3R	KY454767.1	82	575	8.12e-160	*Nemopilema nomurai*	100
18Sab_DT1R	KY610760.1	98	2923	0	Lobonematidae sp. 3 LGD-2017	100
18Sab_DT2R	HM194795.1	86	823	0	*Rhizostoma pulmo*	78
18Sab_DT3R	HM194795.1	85	931	0	*Rhizostoma pulmo*	96
18Sab_G3R	KY610760.1	98	2942	0	Lobonematidae sp. 3 LGD-2017	100
18SFR_DT1R	HM194795.1	92	956	0	*Rhizostoma pulmo*	80
18SFR_DT2R	KY610755.1	85	636	3.92e-178	*Catostylus townsendi*	99
18SFR_DT3R	MH059775.2	87	298	2.31e-76	*Aurelia aurita*	35
18SFR_G3R	KY610785.1	85	690	0	*Lychnorhiza lucerna*	89
28SFR_DT1R	AY935211.1	93	402	1.07e-107	*Aurelia* sp. 10 sensu Dawson *et al.* (2005)	52
28SFR_DT2R		93	387	1.42e-103		99
28SFR_DT3R		93	402	1.09e-107		51
28SFR_G3R		93	387	2.83e-103		53
28SL_DT1R	KY610905.1	94	1373	0	*Catostylidae* sp. 2 LGD-2017	97
28SL_DT2R			1395			95
28SL_DT3R			1390			93
28SL_G3R			1392			94
ITS1_DT1R	KM519755.1	100	594	1.20e-165	*Catostylus tagi*	82
ITS1_DT2R		99	575	4.52e-160		80
ITS1_DT3R		100	594	1.17e-165		84
ITS1_G3R		100	594	1.26e-165		79

* All the obtained accession numbers corresponded to the expected gene.

All the obtained accession numbers (**Table 8**) corresponded to the expected genes, allowing us to annotate each one of the sequences with the corresponding gene name. Furthermore, the phylogenetic analyses of the aligned datasets (containing the sequenced genes obtained in this study and the sequences available in GenBank database) through the NJ method resulted in one gene tree per dataset except for ITS1 gene (**Figures 20** to **24**).

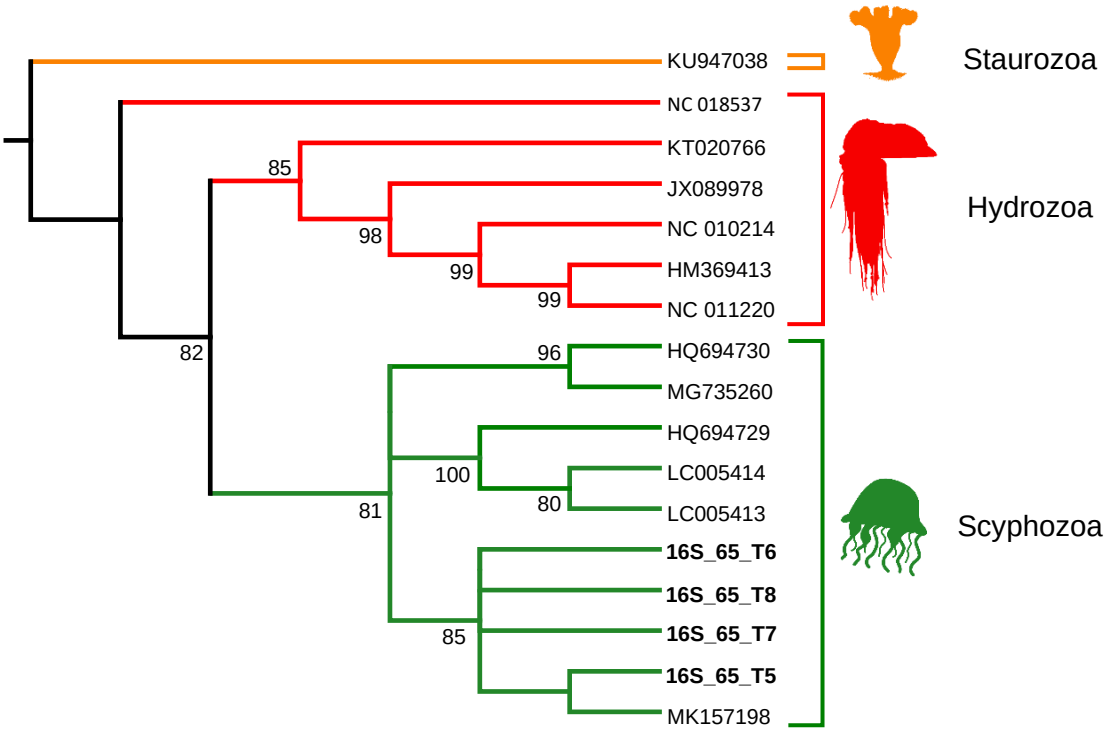

Figure 20. Evolutionary relationships of the 16S rRNA gene among the four medusozoan superclasses. The phylogenetic tree was built in the software Geneious Prime version 11.1.5 using the Neighbor joining method, with 1000 bootstrap replicates and default parameters. The percentage of replicate trees in which the associated taxa cluster together in the bootstrap test is shown next to the branches. Values less than 70 % support are not shown. The codes on the right correspond to the GenBank accession numbers. The codes in bold correspond to the sequences obtained in this study.

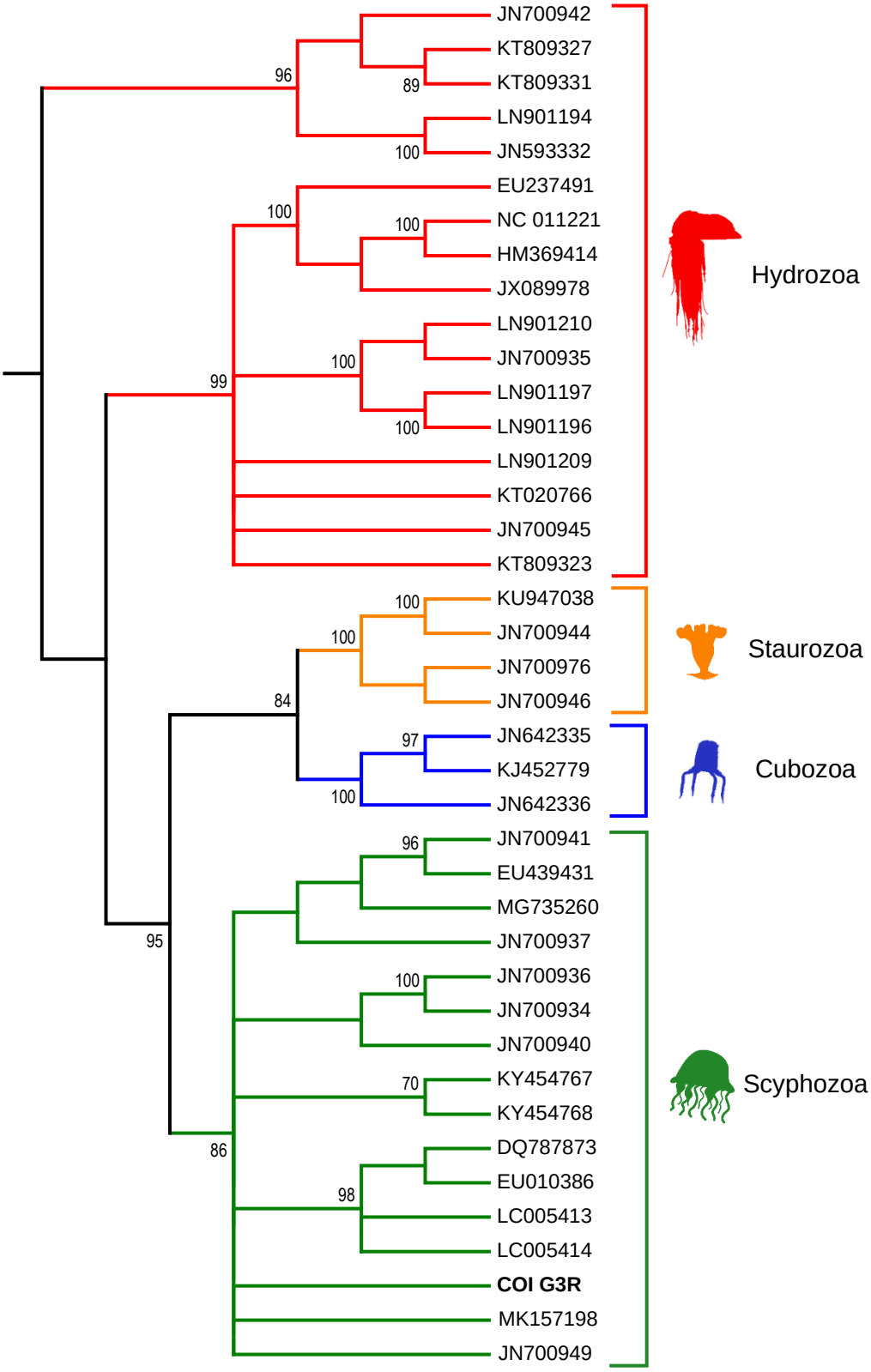

Figure 21. Evolutionary relationships of the COX1 gene among the four medusozoan superclasses. The phylogenetic tree was built in the software Geneious Prime version 11.1.5 using the Neighbor joining method, with 1000 bootstrap replicates and default parameters. The percentage of replicate trees in which the associated taxa cluster together in the bootstrap test is shown next to the branches. Values less than 70 % support are not shown. The codes on the right correspond to the GenBank accession numbers. The codes in bold correspond to the sequences obtained in this study.

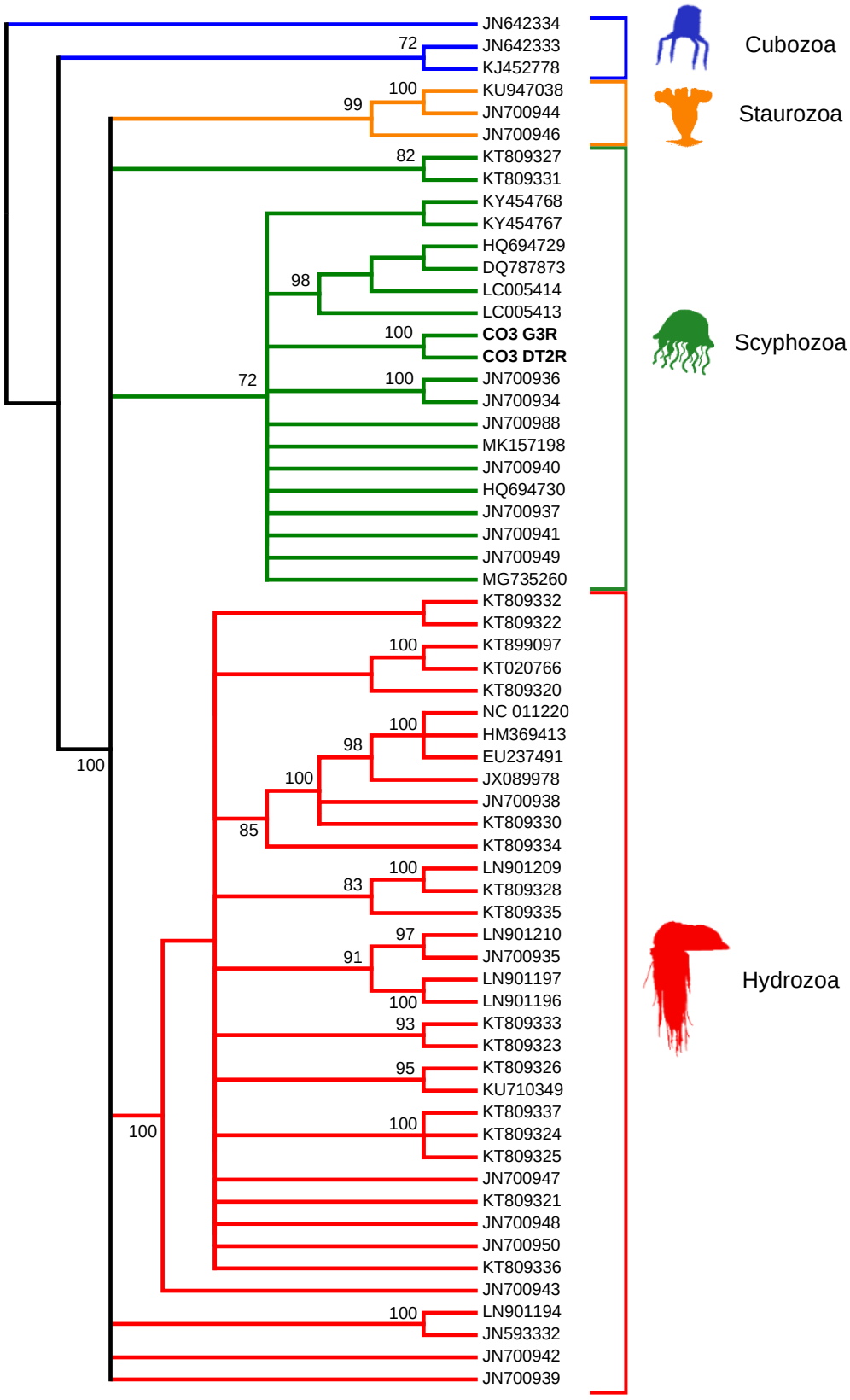

Figure 22. Evolutionary relationships of the COX3 gene among the four medusozoan superclasses. The phylogenetic tree was built in the software Geneious Prime version 11.1.5 using the Neighbor joining method, with 1000 bootstrap replicates and default parameters. The percentage of replicate trees in which the associated taxa cluster together in the bootstrap test is shown next to the branches. Values less than 70 % support are not shown. The codes on the right correspond to the GenBank accession numbers. The codes in bold correspond to the sequences obtained in this study.

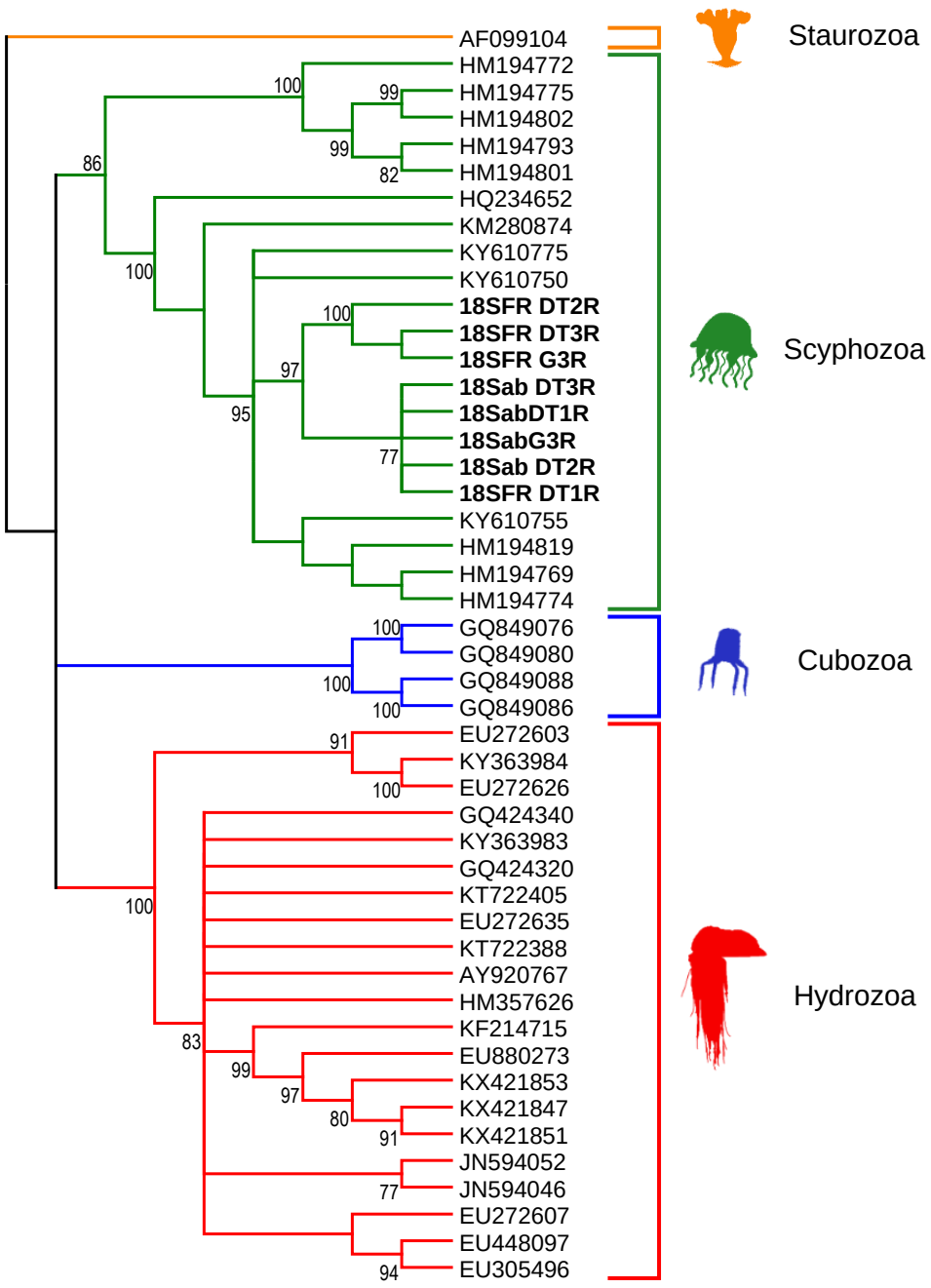

Figure 23. Evolutionary relationships of the 18S rRNA gene among the four medusozoan superclasses. The phylogenetic tree was built in the software Geneious Prime version 11.1.5 using the Neighbor joining method, with 1000 bootstrap replicates and default parameters. The percentage of replicate trees in which the associated taxa cluster together in the bootstrap test is shown next to the branches. Values less than 70 % support are not shown. The codes on the right correspond to the GenBank accession numbers. The codes in bold correspond to the sequences obtained in this study.

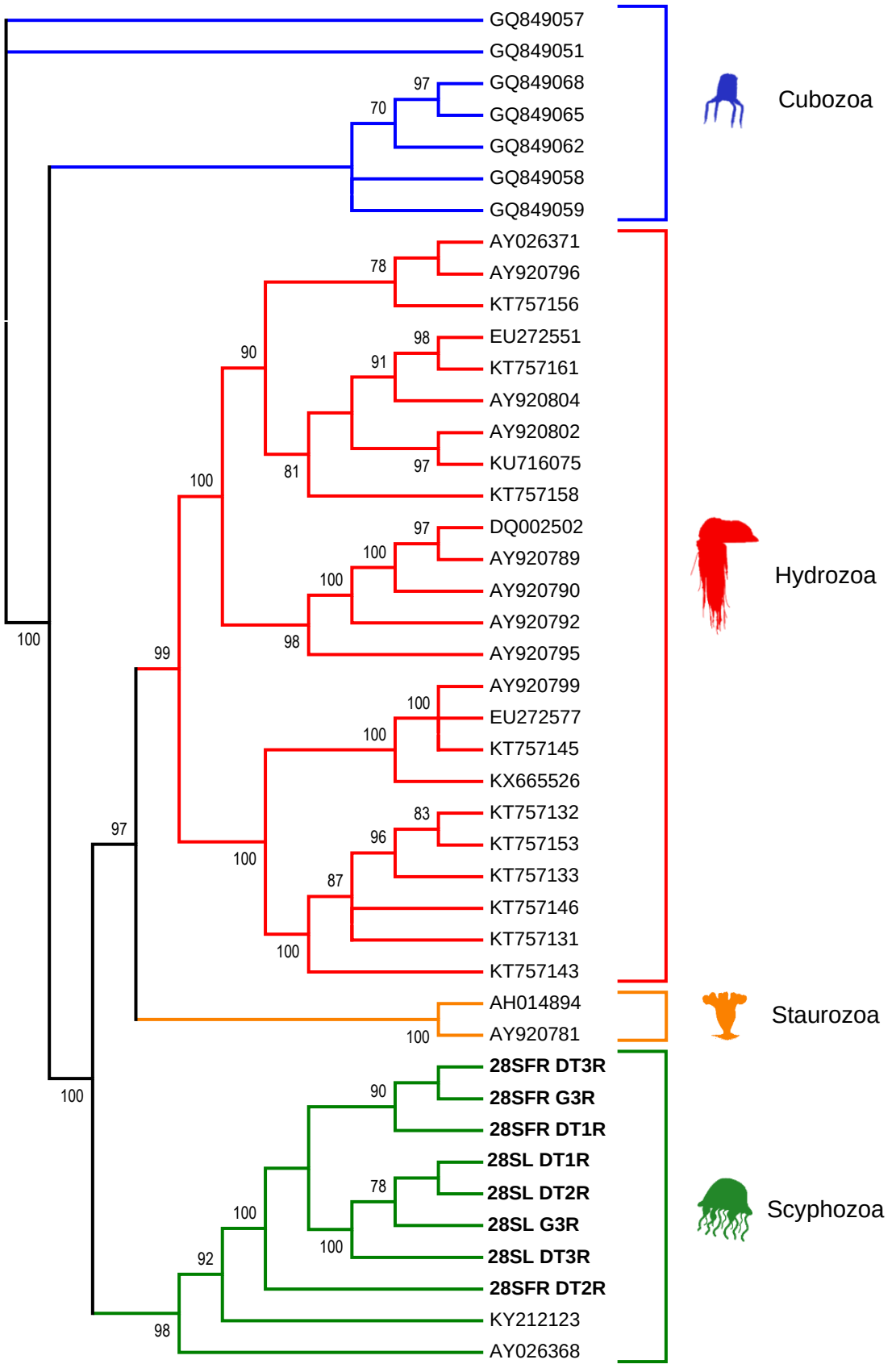

Figure 24. Evolutionary relationships of the 28S rRNA gene among the four medusozoan superclasses. The phylogenetic tree was built in the software Geneious Prime version 11.1.5 using the Neighbor joining method, with 1000 bootstrap replicates and default parameters. The percentage of replicate trees in which the associated taxa cluster together in the bootstrap test is shown next to the branches. Values less than 70 % support are not shown. The codes on the right correspond to the GenBank accession numbers. The codes in bold correspond to the sequences obtained in this study.

The resulting gene trees confirmed that our sequences belong to the superclass Scyphozoa and corroborate the previous gene annotation via BLASTn.

From the 27 sequences obtained in this study, the 10 with the better quality were selected to be submitted to GenBank. This way, we made available for the first time *C. tagi* sequences of the COX1, COX3 and 16S rRNA genes, as well as, new sequences of the 18S rRNA, 28S rRNA and ITS1 genes (**Table 9**). COX1 and COX3 sequences will only be available in January 2020 due to the waiting time of the GenBank submission portal nonetheless the sequences were already accepted and validated.

Table 9. Genetic information obtained in the present study and made available in GenBank.

Target gene	Sample name	Accession Number	Primers reference
16S rRNA	16S_65_T5	MN364410	Designed in the present study
	16S_65_T6	MN364412	
	16S_65_T7	MN364413	
	16S_65_T8	MN364414	
COX1	COI G3R	Available January 2020	(Folmer *et al.*, 1994)
COX3	CO3 G3R		(Geller and Walton 2001)
18S rRNA	18SabDT1R	MN128961	(Medlin *et al.*,1988)
	18SabG3R	MN128962	(Leclère *et al.*, 2009)
28S rRNA	28SL DT1R	MN128946	(Chen *et al.*, 2000)
	28SL DT2R	MN128947	
	28SL DT3R	MN128948	(Lenaers *et al.,* 1989)
	28SL G3R	MN128949	
ITS1	ITS1_G3R	MN161198	(Dawson and Jacobs 2001)

3.3 Chapter 3. Review of molecular tools for early detection of jellyfish: "Advances in the study of ecosystems using an eDNA approach"

Monitoring the biodiversity of a certain river, sea or soil using the traditional methods is a very time consuming and laborious task and requires a lot of experience and knowledge on the morphology/taxonomy of the organisms sampled (Baird and Hajibabaei, 2012; Wood *et al.*, 2013). Additionally, operational costs, difficulties associated with hard to reach environments, and the possibility of non-observation of certain organisms using traditional sampling, are all very valid reasons for the emergence of new methodologies (Rees *et al.*, 2014; Valentini *et al.*, 2016). Thanks to the recent advances in genomic technologies, bio-monitorization became "easier". Those advances allowed the definition of environmental DNA (eDNA), *i.e.*, the joint genetic material (whole microorganisms or cellular material) of all the organisms present in an environmental sample (water, soil or air) (Deagle *et al.*, 2014; Thomsen and Willerslev, 2015). However, Ogram *et al.* (1987) were the first to speak of eDNA referring to a method for extracting microbial DNA from sediments.

There is a vast number of methodologies that can be used in the analysis of eDNA such as environmental shotgun sequencing (ESS), DNA metabarcoding and Capture enrichment. In this chapter it will be explored the applicability and the pros and cons of each one of these methods.

3.3.1 Environmental Shotgun Sequencing

The application of environmental shotgun sequencing in seawater samples was firstly applied to prokaryotes not so long ago (Venter *et al.*, 2004). The use of this methodology has been increasing though their use in the study of eukaryotes is still very fresh (Cowart *et al.*, 2017; Stat *et al.*, 2017). ESS arbitrarily sequences fragmented DNA directly from an environmental sample without enriching the target DNA (Eisen, 2007). Thus, this technique avoids problems related with the use of primers, however, its use is unaffordable due to the high costs associated with sequencing the entire DNA complement present in a sample. The applicability of this technique will depend on the technological advances associated with sequencing, for now, its routine use is unsustainable.

3.3.2 eDNA barcoding and metabarcoding

The main practical difference between using eDNA barcoding and eDNA metabarcoding, is that in metabarcoding, the resulting information, will reveal the species present in the

sample without the need of having a prior knowledge of the species most likely to be found (Taberlet *et al.*, 2012). This is because, unlike DNA barcoding that uses short DNA fragments as “fingerprints” for each species (Hebert *et al.*, 2003), DNA metabarcoding amplifies and sequences marker genes, using primers specific for a certain taxonomic group, resulting in the amplification of DNA fragments from very distinct species.

Opposite to ESS, this approach works with sequencing of the enriched DNA using next-generation sequencing (NGS), surpassing the costs and DNA quantity issues that ESS has (Taberlet *et al.*, 2012). According to Stat *et al.* (2017), metabarcoding is better than ESS on representing ocean biodiversity and specifically to access eukaryotic organisms in an environmental sample. Metabarcoding have long been used for single-celled organisms in water samples to access the composition and diversity of microbial and phytoplankton communities. However, the use of DNA metabarcoding applied to multicellular organisms is recent. In fact, Ficetola *et al.* (2008) were the first to use DNA barcoding to trace a eukaryotic being (frog) from an aquatic environment.

The Consortium for the Barcode of Life (CBOL) has adopted the mitochondrial COX1 gene has standard for DNA barcoding of single animal specimens (Deagle *et al.*, 2014). The use of eDNA metabarcoding in the future, will depend on the standardization of eDNA collection and isolation protocols, the multiple PCR assays and the bioinformatics works. Moreover, as the application of these methodologies depends on the databases available, the increase of the information provided by them is crucial.

3.3.3 Capture enrichment

Instead of amplifying specific regions of interest using PCR, this technique, commonly used as an alternative to ESS and metabarcoding, (Dowle *et al.*, 2016), hybridizes the targeted region, using synthetic DNA or RNA probes bound to a magnetic bead. After that, the rest of the sequences are washed way, leaving the targeted regions isolated (Wilcox *et al.*, 2018). The DNA captured can then be sequenced using High Throughput Sequencing without the need for using primers (Maricic *et al.*, 2010; Mertes *et al.*, 2011).

4. Discussion

This thesis culminated in three chapters of results, being the first the making of a review article about the medusozoans reported in Portugal and its ecological and economical relevance, also covering its corresponding genetical information publicly available. The extensive bibliographic review revealed gaps in the genetic information from molecular markers, for most of the reported species, and a lack of studies on jellyfishes in Portugal. However, in the year 2019 there was in Portugal a large number of occurrences of these organisms, and some of the events deserved prominence on national television. The lack of data does not allow to conclude whether this increase is due to a real rise in the number of occurrences of these organisms or due to increased monitoring and awareness. Following the world trend (Mills, 2001; Purcell *et al.*, 2007), the possible increase of jellyfish occurrences in Portugal, may be related to climate change. In 2019 some beaches were closed due to jellyfish occurrences on the coast. As far as we know, with the exception of the citizen science program GelAVista (GelAvista, 2019), Portugal does not present a consistent medusozoan monitoring program. The list of medusozoans reported in Portugal, has allowed to verify that this group of organisms is well represented in Portuguese waters. We found that 93 % of the reported species were hydrozoans, data that meet the enormous diversity and variety of this superclass (Bouillon *et al.*, 2006). We also confirmed records of staurozoans and cubozoans; and even reports of scyphozoans known to be responsible for mass occurrence events around the world, such as Aurelia aurita (Dong *et al.*, 2012), Chrysaora hysoscella (Lynam *et al.*, 2006), Rhizostoma luteum (Prieto *et al.*, 2013) and Pelagia noctiluca (Milisenda *et al.*, 2018). Thus, it is important to emphasize that these phenomena can also occur in Portugal. The genetic information compiled, allowed to conclude that the information available on the mitochondrial and nuclear markers of the reported species is scarce. The review of Portuguese studies with medusozoans confirmed that there are few national studies on these species. Fortunately, the most part of the existing studies, have been carried out over the last 10 years, thus showing that these organisms are starting to be acknowledged by the Portuguese scientific community.

With the research performed, we verified that there was no standard molecular protocol for the study of medusozoans. Thus, the second chapter of the thesis was focused into the development of a molecular protocol for the identification of jellyfishes. *C. tagi* was chosen as a case study species for being a native, very common species in Portugal, and also for having little information available in the databases. The DNA extraction

method allowed us to conclude that, for the *C. tagi* species, the DNA extraction from tentacles yielded better quantity and quality than that from the gonads. These results can be related with the populations of cells (cells agglomerates, batteries of cnidocytes) found in this organ, which contain more nucleus, hence more DNA. On the other hand, the low concentration of DNA yielded by the gonads could be explained considering the incipient stage of development observed in this organ. Indeed, other authors have previously extracted enough amount of DNA from jellyfishes' gonads (Cho and Kim, 2007; Parracho and Morais, 2015; Stopar *et al.*, 2010). From our research, to the present work, there was seven studies with *C. tagi*, all Portuguese studies since the species is endemic of this coast (Amaral *et al.*, 2018; Calejo *et al.*, 2009; Morais *et al.*, 2009; Muha *et al.*, 2012; Parracho, 2013; Parracho and Morais, 2015; Pintão *et al.*, 2005; Raposo *et al.*, 2018). Even so, the molecular information available in the GenBank database for *C. tagi*, was limited to only three partial sequences of the 18S rRNA, 28S rRNA and ITS1 markers. In order to increase the genetic information available for this species the COX1, COX3, 18S rRNA, 28S rRNA and ITS1 genes were amplified using existing universal primers (Dawson and Jacobs, 2001; Folmer *et al.*, 1994; Geller and Walton, 2001; Leclère *et al.*, 2009; Medlin *et al.*, 1988). Since the amplification of the 16S rRNA and NAD6 genes was unsuccessful using existing primers, specific primers for them were designed. For the 16S rRNA gene, the primer set designed provided a successful gene amplification. To note that for cnidarian works the commonly used universal primers for 16S rRNA gene are the ones of Bridge *et al.* (1992), primers designed more than 27 years ago not suitable for most of medusozoans species. In turn, the NAD6 amplification was unsuccessful, even with new designed primers. This can be related with the fact that this gene is one of the least conserved genes in Cnidaria, presenting a high nucleotide substitution rate (Zou *et al.*, 2012). We can conclude that NAD6 is not a good molecular marker to be used in future molecular works. The molecular protocol optimization and the bioinformatics tools employed validated this approach as a good method for identifying jellyfish.

In the third chapter of results, recent studies using molecular tools that implement eDNA to monitor marine environments and thus, medusozoans among other taxonomic groups, were reviewed (Cowart *et al.*, 2017; Günther *et al.*, 2018; Stat *et al.*, 2017). From the three approaches found, used for ecosystem monitorization, i.e., "ESS", "eDNA barcoding and metabarcoding", and "targeted gene enrichment", the "eDNA barcoding and metabarcoding" revealed to be a robust tool. However, the suitability of such approach depends on consistent databases. "ESS" has a principal limitation its high costs and "targeted gene enrichment" is very promising since it does not require PCR

amplification. Both eDNA approaches and traditional methods have limitations, thus, the best monitorization approach must be a combination of the traditional sampling and laboratory molecular assays with a highly advanced metagenomic approach as a useful monitoring tool.

5. Conclusion

Globally, this work gives a remarkable input to the knowledge of medusozoans species in Portugal through an updated list of recorded species and a compilation of their respective genetic information. In this respect, the review chapter constitutes a great contribution releasing a state-of-the-art reference for local and worldwide researchers, interested in jellyfish's ecological aspects, taxonomy, phylogeny and population genetics. Moreover, this work also listed those species with any kind of mass occurrence behavior from a warning perspective. Among them, should be considered *Physalia physalis* as the most dangerous for tourism and human health in the current context of the climate changes. Besides, the standard protocols tested, using universal and new primers designed for molecular markers, provided 13 new sequences submitted in GenBank (16S rRNA: MN364410, MN364412, MN364413, MN364414; COX1: 1 sequence available online January 2020; COX3: 1 sequence available online January 2020; 18S rRNA: MN128961, MN128962; 28S rRNA: MN128946, MN128947, MN128948; ITS1: MN161198, MN128949) for the little explored and endemic specie *C. tagi*, also capable of producing mass occurrence events. Even though representing a small contribution, it is a start point to produce or filling genetic information gaps for non-model species of jellyfishes such as *C. tagi*. This work demonstrates how difficult is to obtain some genetic information in short term, taking into account sampling, specific primers design and successful DNA sequencing. The lack of information is one of the most important barriers for future development of early detection tools from a molecular biological point of view. In this sense, our outcomes should not be underestimated, on the contrary should be considered as a first step to design those molecular tools to predict and face jellyfishes' blooms. In fact, we can conclude that the lack of monitorization and genetic information are the main obstacles to overcome, and that the combination of classical and NGS approaches would be needed. Finally, this work calls the attention to authorities of the vulnerabilities against incipient jellyfishes' blooms.

6. Future perspectives

In order to increase the genetic information needed for an early detection tool development, firstly, an extension of the sample collection will be performed and the existing gaps for molecular markers used to identify medusozoans will be filled. In this sense, different conditions like PCR programs and specific primer design will be tested to all individuals comprising the sample collection. This will contribute to the discovering of genes suitable for metabarcoding as a fast species identification approach.

To increase the knowledge of cnidarian populations and the dynamics of early living stage (jellyfishes' larvae, planula), metagenomics and NGS using water samples (eDNA) from different sampling sites will be seasonally carried out.

The results from PCR and NGS will contribute to the creation of a genetic database for the reported jellyfishes in Portugal, that will be used finally to establish a monitoring program based on molecular early detection tools.

Develop an Iberian partnership to extend jellyfish's monitoring programs across the Iberian Peninsula aiming the creation of an Iberian database.

7. Publications

The results obtained in the scope of this Master thesis originated the following publications:

- **Rodrigues T**, Almeida D, Domínguez-Pérez D, Matos A, and Antunes A (2019). *Through the records of jellyfish blooms in Portugal*. IJUP2019 - 13th to 15th of February 2019, Porto, Portugal (poster);

- **Rodrigues T**, Matos A, Domínguez-Pérez D, Falcão J, Marques SC, Leandro S, Almeida D and Antunes A. *Medusozoa in Portugal: impact on the ecosystems and development of DNA-based tools for the early forecasting of mass occurrences*. Front. Mar. Sci. Conference Abstract: XX Iberian Symposium on Marine Biology Studies (SIEBM XX) (poster).

- Rodrigues T, Domínguez-Pérez D, Almeida D, Matos A, and Antunes A (2019). *Medusozoans reported in Portugal and its ecological and economical relevance* (Review article – submitted before the thesis defense).

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Appendices

Appendix A – List of the medusozoans reported in Portugal (mainland and archipelagos of Madeira and Azores). Here are displayed their taxonomic information (class, subclass, order, suborder, family, and species) and the reference of their mention.

Appendix B– Genetic information available at GenBank database considering mitochondrial and nuclear markers from the medusozoans recorded in Portugal (May, 2019).

Appendix A. List of the medusozoans reported in Portugal (mainland and archipelagos of Madeira and Azores). Here are displayed their taxonomic information (class, subclass, order, suborder, family, and species) and the reference of their mention.

Species	Reference
Class Hydrozoa	
Subclass Hydroidolina	
Order Anthoathecata	
Suborder Aplanulata	
Family Candelabridae	
Candelabrum phrygium	(Santos, 2018)
Family Corymorphidae	
Corymorpha sp.	(Santos, 2018)
Family Hydridae	
Hydra circumcincta	(Ramos, 2010)
Hydra oligactis	(D'Ambrosio *et al.*, 2016)
Hydra viridissima	(Santos, 2018)
Hydra vulgaris	(Ramos, 2010)
Family Margelopsidae	
Margelopsis haeckelii	(D'Ambrosio *et al.*, 2016)
Family Tubulariidae	
Ectopleura crocea	(Cornelius, 1992; Wirtz, 1995; 2007)
Ectopleura dumortierii	(Da Cunha, 1944; Cardigos *et al.*, 2006)
Ectopleura larynx	(Da Cunha, 1944; OMARE, 2019)
Tubularia crocea	(Cardigos *et al.*, 2006)
Tubularia indivisa	(Cornelius, 1992; OMARE, 2019)
Suborder Capitata	
Family Cladocorynidae	
Cladocoryne floccosa	(Da Cunha, 1944; Wirtz, 2007)
Family Corynidae	
Codonium proliferum	(D'Ambrosio *et al.*, 2016)
Coryne eximia	(Moura, 2015)
Coryne muscoides	(Da Cunha, 1944; OMARE, 2019)
Coryne pusilla	(Da Cunha, 1944)
Sarsia tubulosa	(Rees and White, 1966; Borges *et al.*, 2010)
Stauridiosarsia gemmifera	(D'Ambrosio *et al.*, 2016)
Stauridiosarsia ophiogaster	(D'Ambrosio *et al.*, 2016)
Family Moerisiidae	
Odessia maeotica	(D'Ambrosio *et al.*, 2016)
Family Pennariidae	
Pennaria disticha	(Rees and White, 1966; OMARE, 2019)
Family Porpitidae	
Velella velella	(OMARE, 2019)
Family Rosalindidae	
Rosalinda incrustans	(Kramp, 1961)
Family Zancleidae	
Zanclea alba	(Schuchert, 2010)
Zanclea costata	(Borges *et al.*, 2010; D'Ambrosio *et al.*, 2016)
Zanclea sessilis	(Wirtz, 2007)
Suborder Filifera	
Family Bougainvilliidae	
Bougainvillia muscus	(Schuchert, 2007; Muha *et al.*, 2012)
Bougainvillia pyramidata	(D'Ambrosio *et al.*, 2016)
Koellikerina fasciculata	(Kramp, 1961)
Pachycordyle michaeli as P. navis	(Borges *et al.*, 2010)
Silhouetta uvacarpa	(Schuchert, 2007; Borges *et al.*, 2010)
Family Bythotiaridae	
Calycopsis typa	(Schuchert, 2009)
Sibogita geometrica	(Schuchert, 2009)
Family Cordylophoridae	
Cordylophora caspia	(Cancela da Fonseca, 1989; Fuller and Cannister, 2013)

Family Filifera incertae sedis	
Kinetocodium danae	(Schuchert, 2007)
Family Hydractiniidae	
Clava multicornis	(Da Cunha, 1944; Schuchert, 2008)
Hydractinia echinata	(Nobre, 1937; Schuchert, 2008)
Podocoryna carnea	(D'Ambrosio *et al.*, 2016; OMARE, 2019)
Family Hydrichthyidae	
Hydrichthys cyclothonis	(Schuchert, 2007; Borges *et al.*, 2010)
Family Oceaniidae	
Oceania armata	(Kramp, 1961; Schuchert, 2004)
Family Pandeidae	
Amphinema dinema	(Moura, 2015; D'Ambrosio *et al.*, 2016)
Amphinema rugosum	(Schuchert, 2007)
Leuckartiara grimaldii	(Schuchert, 2007)
Leuckartiara octona	(Machado and Fonseca, 1997; D'Ambrosio *et al.*, 2016)
Neoturris pileata	(Schuchert, 2007)
Pandea conica	(Orrell, 2019)
Pandea rubra	(Schuchert, 2007)
Family Rathkeidae	
Lizzia blondina	(Kramp, 1961; D'Ambrosio *et al.*, 2016)
Podocorynoides minima	(D'Ambrosio *et al.*, 2016)
Rathkea octopunctata	(D'Ambrosio *et al.*, 2016)
Family Stylasteridae	
Crypthelia affinis	(Schuchert, 2008; Institute of Marine Research, 2019)
Crypthelia medioatlantica	(Schuchert, 2008; Eibye-Jacobsen *et al.*, 2019)
Crypthelia tenuiseptata	(Schuchert, 2008; Institute of Marine Research, 2019)
Crypthelia vascomarquesi	(Schuchert, 2008; Museum national d'Histoire naturelle, 2019)
Errina atlantica	(Schuchert, 2008; Institute of Marine Research, 2019)
Errina dabneyi	(Schuchert, 2008; Institute of Marine Research, 2019)
Lepidopora eburnea	(Schuchert, 2008; Museum national d'Histoire naturelle, 2019)
Pliobothrus symmetricus	(Schuchert, 2008; Institute of Marine Research, 2019)
Stenohelia maderensis	(Museum national d'Histoire naturelle, 2019)
Stylaster erubescens	(Rogers and Hall-Spencer, 2005)
Stylaster sp.	(Orrell, 2019)
Order Leptothecata	
Family Aequoreidae	
Aequorea victoria	(Casassovici and Brosens, 2019)
Zygocanna vagans	(Orrell, 2019)
Family Blackfordiidae	
Blackfordia virginica	(Moura, 2015; Ranson, 1936)
Family Campanulariidae	
Campanularia hincksii	(Rees and White, 1966; OMARE, 2019)
Campanularia volubilis	(Da Cunha, 1944; Rees and White, 1966)
Clytia brunescens	(Borges *et al.*, 2010)
Clytia gracilis	(Da Cunha, 1944; OMARE, 2019)
Clytia hemisphaerica	(Rees and White, 1966; OMARE, 2019)
Clytia linearis	(Da Cunha, 1944; Wirtz, 2007)
Clytia noliformis	(Rees and White, 1966; Wirtz, 2007)
Clytia paulensis	(Wirtz, 2007)
Clytia striata	(Rees and White, 1966)
Gonothyraea loveni	(Rees and White, 1966)
Hartlaubella gelatinosa as Laomedea gelatinosa	(Da Cunha, 1944)
Laomedea angulata	(OMARE, 2019)
Laomedea calceolifera	(Da Cunha, 1944; Borges *et al.*, 2010)
Laomedea flexuosa	(Rees and White, 1966; OMARE, 2019)
Laomedea pseudodichotoma	(Borges *et al.*, 2010)
Obelia bidentata	(Rees and White, 1966; Borges *et al.*, 2010)
Obelia dichotoma	(Rees and White, 1966; Borges *et al.*, 2010)
Obelia geniculata	(Da Cunha, 1950; OMARE, 2019)

Obelia longissima	(Da Cunha, 1944; Rees and White, 1966)
Orthopyxis crenata	(Wirtz, 2007)
Orthopyxis integra	(OMARE, 2019)
Family Campanulinidae	
Calycella syringa	(Da Cunha, 1944)
Family Cirrholoveniidae	
Cirrholovenia tetranema	(Wirtz, 2007)
Family Eirenidae	
Eutima gegenbauri	(Kramp, 1961)
Eutima gracilis	(Kramp, 1961; D'Ambrosio *et al.*, 2016)
Eudendrium armatum	(Moura, 2015)
Eudendrium merulum	(Moura, 2015)
Eudendrium rameum	(Da Cunha, 1944; OMARE, 2019)
Eudendrium ramosum	(Da Cunha, 1944; OMARE, 2019)
Family Haleciidae	
Halecium beanii	(Rees and White, 1966; OMARE, 2019)
Halecium delicatulum	(Rees and White, 1966; Borges *et al.*, 2018)
Halecium halecium	(Da Cunha, 1944; OMARE, 2019)
Halecium labrosum	(Rees and White, 1966; Borges *et al.*, 2010)
Halecium mediterraneum	(Borges *et al.*, 2010)
Halecium nanum	(Wirtz, 2007)
Halecium profundum	(Ramirez-Llodra and Blanco, 2005)
Halecium pusillum	(Wirtz, 2007)
Halecium sessile	(Rees and White, 1966; Institute of Marine Research, 2019)
Halecium tenellum	(Rees and White, 1966; OMARE, 2019)
Family Hebellidae	
Anthohebella parasitica as *Hebella parasitica*	(Da Cunha, 1944)
Bedotella armata	(Royal Belgian Institute of Natural Sciences, 2017; Borges *et al.*, 2018)
Hebella scandens	(Rees and White, 1966; Wirtz, 2007)
Scandia gigas	(OMARE, 2019)
Scandia mutabilis	(Wirtz, 2007)
Family Lafoeidae	
Acryptolaria conferta	(Rees and White, 1966; Institute of Marine Research, 2019)
Acryptolaria crassicaulis	(Rees and White, 1966; Institute of Marine Research, 2019)
Acryptolaria longitheca	(Borges *et al.*, 2018)
Cryptolarella abyssicola	(Rees and White, 1966; IFREMER BIOCEAN database)
Cryptolaria exserta	(Rees and White, 1966)
Cryptolaria pectinata	(Borges *et al.*, 2018; Institute of Marine Research, 2019)
Filellum serpens	(Wirtz, 2007; Royal Belgian Institute of Natural Sciences, 2017)
Filellum serratum	(Wirtz, 2007; Institute of Marine Research, 2019)
Grammaria abietina	(IFREMER BIOCEAN database)
Lafoea dumosa	(OMARE, 2019; Institute of Marine Research, 2019)
Lafoeina tenuis	(Moura, 2015)
Zygophylax biarmata	(Rees and White, 1966; Institute of Marine Research, 2019)
Zygophylax echinata	(Ramirez-Llodra and Blanco, 2005)
Zygophylax elegantula	(Rees and White, 1966)
Zygophylax geniculata	(Rees and White, 1966; Royal Belgian Institute of Natural Sciences, 2017)
Family Laodiceidae	
Laodicea undulata	(Ranson, 1936)
Family Lovenellidae	
Eucheilota maculata	(Wirtz, 2007; Mgnify, 2018)
Hydranthea margarica	(Wirtz, 2007)
Lovenella clausa	(Medel and Lopez-Gonzalez, 1996; IFREMER BIOCEAN database, 2019)
Family Mitrocomidae	
Cosmetira pilosella	(Kramp, 1961)
Cyclocanna producta	(Rees and White, 1966)
Family Sertulariidae	

Abietinaria abietina	(Nobre, 1937; Telenius and Shah, 2016)
Amphisbetia distans	(OMARE, 2019)
Amphisbetia fasciculata	(Royal Belgian Institute of Natural Sciences, 2017)
Amphisbetia operculata	(Primo *et al.*, 2012; Borges *et al.*, 2018)
Diphasia alata	(Rees and White, 1966; Royal Belgian Institute of Natural Sciences, 2017)
Diphasia attenuata	(Cornelius, 1992; Borges *et al.*, 2010)
Diphasia delagei	(Moura, 2011)
Diphasia margareta	(Institute of Marine Research, 2019; OMARE, 2019)
Diphasia pinastrum	(Moura, 2011; Institute of Marine Research, 2019)
Diphasia rosacea	(Da Cunha, 1950; Bouillon and Boero, 2000)
Dynamena crisioides	(Royal Belgian Institute of Natural Sciences, 2017)
Dynamena disticha	(Royal Belgian Institute of Natural Sciences, 2017; OMARE, 2019)
Dynamena pumila	(Da Cunha, 1944; 1950)
Dynamena quadridentata	(Wirtz, 2007)
Hydrallmania falcata	(Ramirez-Llodra and Blanco, 2005)
Salacia desmoides	(Wirtz, 2007)
Sertularella ellisii	(OMARE, 2019)
Sertularella fusiformis	(Da Cunha, 1950; Moura, 2011)
Sertularella gayi	(Rees and White, 1966; OMARE, 2019)
Sertularia marginata	(Rees and White, 1966; Wirtz, 2007)
Sertularella mediterranea	(Da Cunha, 1950; Borges *et al.*, 2018)
Sertularella ornata	(Wirtz, 2007)
Sertularella polyzonias	(Borges *et al.*, 2018; OMARE, 2019)
Sertularella tenella	(Ramirez-Llodra and Blanco, 2005)
Sertularella unituba	(Moura, 2011)
Sertularia cupressina	(Royal Belgian Institute of Natural Sciences, 2017)
Sertularia distans	(Moura, 2011; Borges *et al.*, 2018)
Sertularia gracilis	(OMARE, 2019)
Sertularia tenera	(Telenius and Shah, 2016)
Symplectoscyphus bathyalis	(Ramirez-Llodra and Blanco, 2005)
Tamarisca tamarisca	(Institute of Marine Research, 2019)
Thuiaria articulata	(Moura, 2011)
Family Thyroscyphidae	
Sertularelloides cylindritheca	(Moura, 2011)
Family Tiarannidae	
Krampella dubia	(Cornelius, 1992; Borges *et al.*, 2018)
Stegopoma giganteum	(IFREMER BIOCEAN database, 2019)
Stegolaria geniculata	(Borges *et al.*, 2018)
Superfamily Plumularioidea	
Family Aglaopheniidae	
Aglaophenia acacia	(Rees and White, 1966; Borges *et al.*, 2018)
Aglaophenia elongata	(Rees and White, 1966; Royal Belgian Institute of Natural Sciences, 2017)
Aglaophenia kirchenpaueri	(OMARE, 2019)
Aglaophenia lophocarpa	(Rees and White, 1966; Wirtz, 2007)
Aglaophenia octodonta	(Moura, 2007; Borges *et al.*, 2018)
Aglaophenia parvula	(Moura, 2012)
Aglaophenia picardi	(Wirtz, 2007)
Aglaophenia pluma	(Da Cunha, 1950; OMARE, 2019)
Aglaophenia tubiformis	(Da Cunha, 1950; OMARE, 2019)
Aglaophenia tubulifera	(Rees and White, 1966; OMARE, 2019)
Aglaophenopsis cartieri	(Rees and White, 1966; Moura, 2012)
Cladocarpus boucheti	(IFREMER BIOCEAN database, 2019)
Cladocarpus formosus	(Ramirez-Llodra and Blanco, 2005)
Cladocarpus paraventricosus	(IFREMER BIOCEAN database, 2019)
Cladocarpus sigma	(Da Cunha, 1950; Telenius and Shah, 2016)
Gymnangium montagui	(Da Cunha, 1950; OMARE, 2019)
Lytocarpia myriophyllum	(Rees and White, 1966; OMARE, 2019)
Macrorhynchia philippina	(Wirtz, 2007)

Streptocaulus corneliusi	(Natural History Museum, 2019)
Streptocaulus pectiniferus	(Rees and White, 1966; Borges *et al.*, 2018)
Streptocaulus pulcherrimus	(Rees and White, 1966)
Family Halopteridae	
Antennella ansini	(European Nucleotide Archive, 2019)
Antennella secundaria	(Rees and White, 1966; Borges *et al.*, 2018)
Halopteris catharina	(OMARE, 2019)
Halopteris diaphana	(Wirtz, 2007)
Monostaechas quadridens	(Wirtz, 2007)
Polyplumaria flabellata	(Rees and White, 1966; OMARE, 2019)
Family Kirchenpaueriidae	
Kirchenpaueria curvata	(Royal Belgian Institute of Natural Sciences, 2017)
Kirchenpaueria pinnata	(Borges *et al.*, 2018; OMARE, 2019)
Kirchenpaueria halecioides	(Cornelius, 1992; Chainho *et al.*, 2015)
Family Plumulariidae	
Nemertesia antennina	(OMARE, 2019; Gomes-Pereira, 2019)
Nemertesia belini	(Rees and White, 1966; Borges *et al.*, 2018)
Nemertesia intermedia	(Royal Belgian Institute of Natural Sciences, 2017)
Nemertesia norvegica	(Borges *et al.*, 2018)
Nemertesia paradoxa	(Royal Belgian Institute of Natural Sciences, 2017)
Nemertesia ramosa	(Gomes-Pereira, 2019; OMARE, 2019)
Plumularia filicula	(IFREMER BIOCEAN database)
Plumularia obliqua	(Da Cunha, 1950; OMARE, 2019)
Plumularia pulchella	(Wirtz, 2007)
Plumularia setacea	(European Nucleotide Archive, 2019; OMARE, 2019)
Plumularia strictocarpa	(Mgnify, 2018)
Pseudoplumaria marocana	(IFREMER BIOCEAN database, 2019)
Pseudoplumaria sabinae	(Stocks, 2005)
Order Siphonophorae	
Suborder Calycophorae	
Family Abylidae	
Abyla trigona	(Natural History Museum, 2019)
Abylopsis tetragona	(Pugh, 2000)
Abylopsis eschscholtzii	(Natural History Museum, 2019)
Bassia bassensis	(Natural History Museum, 2019)
Family Clausophyidae	
Chuniphyes multidentata	(Natural History Museum, 2019)
Family Diphyidae	
Chelophyes appendiculata	(Pugh, 2000)
Diphyes bojani	(Pugh, 2000)
Diphyes dispar	(Natural History Museum, 2019; Telenius and Ekström, 2019)
Eudoxoides spiralis	(Natural History Museum, 2019)
Lensia conoidea	(Pugh, 2000)
Lensia multicristata	(Pugh, 2000)
Muggiaea atlantica	(Primo *et al.*, 2012)
Muggiaea kochi	(D'Ambrosio *et al.*, 2016)
Sulculeolaria chuni	(Natural History Museum, 2019)
Family Hippopodiidae	
Hippopodius hippopus	(Natural History Museum, 2019)
Family Prayidae	
Nectopyramis thetis	(Borges *et al.*, 2010)
Rosacea cymbiformis	(Bouillon and Boero, 2000)
Suborder Cystonectae	
Family Physaliidae	
Physalia physalis	(OMARE, 2019)
Suborder Physonectae	
Family Agalmatidae	
Agalma okenii	(Pugh, 2000)
Athorybia rosacea	(Mackay, 2019)
Halistemma rubrum	(Natural History Museum, 2019)

Nanomia bijuga	(Pugh, 2000)
Family Apolemiidae	
Apolemia uvaria	(OMARE, 2019)
Family Cordagalmatidae	
Cordagalma bimaculatum	(IFREMER BIOCEAN database, 2019)
Family Physophoridae	
Physophora hydrostatica	(Borges *et al.*, 2010)
Subclass Trachylinae	
Order Limnomedusae	
Family Olindiidae	
Craspedacusta sowerbyi	(Muha *et al.*, 2012; Mgnify, 2018)
Gonionemus vertens	(Kienberger and Prieto, 2018; Goulletquer *et al.*, 2002)
Maeotias marginata	(Habermehl, 1981)
Olindias sp.	(Mgnify, 2018)
Order Narcomedusae	
Family Aeginidae	
Aegina citrea	(Mgnify, 2018; Natural History Museum, 2019)
Aeginura grimaldii	(Kramp, 1961)
Family Cuninidae	
Cunina frugifera	(Mgnify, 2018)
Cunina octonaria	(Royal Belgian Institute of Natural Sciences, 2017)
Family Solmarisidae	
Pegantha clara	(Orrell, 2019)
Pegantha rubiginosa	(Kramp, 1961)
Solmaris corona	(Borges *et al.*, 2010; D'Ambrosio *et al.*,2016)
Order Trachymedusae	
Family Geryoniidae	
Liriope tetraphylla	(Borges *et al.*, 2010; D'Ambrosio *et al.*,2016)
Family Halicreatidae	
Halicreas minimum	(Natural History Museum, 2019)
Haliscera bigelowi	(Natural History Museum, 2019)
Family Rhopalonematidae	
Aglaura hemistoma	(Borges *et al.*, 2010)
Colobonema sericeum	(Natural History Museum, 2019)
Crossota rufobrunnea	(Natural History Museum, 2019)
Pantachogon haeckeli	(Mgnify, 2018; Natural History Museum, 2019)
Rhopalonema velatum	(Borges *et al.*, 2010; Royal Belgian Institute of Natural Sciences, 2017)
Class Scyphozoa	
Subclass Coronamedusae	
Order Coronatae	
Family Atollidae	
Atolla parva	(Natural History Museum, 2019)
Atolla vanhoeffeni	(Natural History Museum, 2019)
Atolla wyvillei	(Natural History Museum, 2019; Orrel, 2019)
Family Nausithoidae	
Nausithoe atlantica	(Kramp, 1961)
Nausithoe globifera	(Kramp, 1961)
Nausithoe punctata	(Borges *et al.*, 2010)
Family Periphyllidae	
Periphylla periphylla	(Kramp, 1961)
Subclass Discomedusae	
Order Rhizostomeae	
Suborder Daktyliophorae	
Family Catostylidae	
Catostylus tagi	(OMARE, 2019)
Family Rhizostomatidae	
Rhizostoma luteum	(OMARE, 2019)
Order Semaeostomeae	
Family Pelagiidae	

Chrysaora hysoscella	(Morandini and Marques, 2010; OMARE, 2019)
Chrysaora quinquecirrha	(Habermehl, 1981)
Pelagia noctiluca	(Borges *et al.*, 2010; OMARE, 2019)
Family Phacellophoridae	
Phacellophora camtschatica	(GelAvista, 2019)
Family Ulmaridae	
Aurelia aurita	(Muha *et al.*, 2012; Pereira *et al.*, 2014)
Aurelia solida	(Kramp, 1961)
Class Staurozoa	
Order Stauromedusae	
Suborder Amyostaurida	
Family Kishinouyeidae	
Calvadosia campanulata	(Kramp, 1961)
Suborder Myostaurida	
Family Haliclystidae	
Haliclystus auricula	(Kramp, 1961; OMARE, 2019)
Class Cubozoa	
Order Carybdeida	
Family Carybdeidae	
Carybdea marsupialis	(Kramp, 1961)

Appendix B. Genetic information available at GenBank database considering mitochondrial and nuclear markers from the medusozoans recorded in Portugal (May, 2019).

Species	Genetic information available in Genbank																						
	Mitochondrial DNA																	Nuclear DNA					
	Complete genome	16S	COX1	COX2	COX3	12S	ATP6	ATP8	NAD1	NAD2	NAD3	NAD4	NAD4L	NAD5	NAD6	CYTB		Complete genome	18S	28S	ITS1	ITS2	5.8S
Class Hydrozoa																							
Subclass Hydroidolina																							
Order Anthoathecata																							
Suborder Aplanulata																							
Family Candelabridae																							
Candelabrum phrygium		X																					
Family Corymorphidae																							
Corymorpha sp.																							
Family Hydridae																							
Hydra circumcincta		X	X	X															X	X	X	X	X
Hydra oligactis	EU237491.1																		X	X	X	X	X
Hydra viridissima		X	X	X															X	X	X	X	X
Hydra vulgaris	GCF_000004095.1																						
Family Margelopsidae																							
Margelopsis haeckelii																							
Family Tubulariidae																							
Ectopleura crocea		X	X																X	X	X		X
Ectopleura dumortierii		X	X																X	X			
Ectopleura larynx		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X			X	X			

Tubularia crocea																						
Tubularia indivisa		X	X															X	X			X
Suborder Capitata																						
Family Cladocorynidae																						
Cladocoryne floccosa		X																X	X			
Family Corynidae																						
Codonium proliferum		X	X																			
Coryne eximia		X	X															X	X			
Coryne muscoides		X	X															X	X			
Coryne pusilla		X	X															X	X			
Sarsia tubulosa		X	X	X	X	X	X	X	X	X	X	X	X		X	X		X	X			
Stauridiosarsia gemmifera			X																			
Stauridiosarsia ophiogaster		X	X																			
Family Moerisiidae																						
Odessia maeotica		X																X	X			
Family Pennariidae																						
Pennaria disticha		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		X	X			
Family Porpitidae																						
Velella velella		X	X															X	X	X		X
Family Rosalindidae																						
Rosalinda incrustans																						
Family Zancleidae																						
Zanclea alba																						
Zanclea costata		X																X	X			
Zanclea sessilis		X	X																			

Suborder Filifera																						
Family Bougainvilliidae																						
Bougainvillia muscus		X	X														X	X				
Bougainvillia pyramidata		X																				
Koellikerina fasciculata		X															X	X				
Pachycordyle michaeli as P. navis																						
Silhouetta uvacarpa																						
Family Bythotiaridae																						
Calycopsis typa																						
Sibogita geometrica																						
Family Cordylophoridae																						
Cordylophora caspia		X	X														X	X	X			
Family Filifera incertae sedis																						
Kinetocodium danae																						
Family Hydractiniidae																						
Clava multicornis	NC_016465.1																X	X				
Hydractinia echinata																						
Podocoryna carnea		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X						
Family Hydrichthyidae																						
Hydrichthys cyclothonis																						
Family Oceaniidae																						
Oceania armata		X	X																			
Family Pandeidae																						
Amphinema dinema		X	X																			
Amphinema rugosum																						

Leuckartiara grimaldii																						
Leuckartiara octona		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		X	X			
Neoturris pileata		X	X															X	X	X	X	X
Pandea conica		X	X															X		X		X
Pandea rubra																						
Family Rathkeidae																						
Lizzia blondina		X	X															X	X			
Podocorynoides minima		X	X															X	X			
Rathkea octopunctata		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		X	X			
Family Stylasteridae																						
Crypthelia affinis																						
Crypthelia medioatlantica																						
Crypthelia tenuiseptata																						
Crypthelia vascomarquesi																						
Errina atlantica																						
Errina dabneyi																						
Lepidopora eburnea																						
Pliobothrus symmetricus		X																				
Stenohelia maderensis																						
Stylaster erubescens		X																				
Stylaster sp.																						
Order Leptothecata																						
Family Aequoreidae																						
Aequorea victoria		X																X	X			
Zygocanna vagans																						

Family Blackfordiidae																						
Blackfordia virginica		X	X															X	X	X		
Family Campanulariidae																						
Campanularia hincksii		X	X															X	X			
Campanularia volubilis		X	X															X				
Clytia brunescens																						
Clytia gracilis		X	X															X	X			
Clytia hemisphaerica		X	X															X	X			
Clytia linearis		X	X															X	X			
Clytia noliformis		X	X															X	X			
Clytia paulensis		X	X															X				
Clytia striata																						
Gonothyraea loveni		X	X															X	X			
Hartlaubella gelatinosa as Laomedea gelatinosa		X	X															X				
Laomedea angulata		X	X															X	X			
Laomedea calceolifera		X	X															X	X			
Laomedea flexuosa	JN700945.1																					
Laomedea pseudodichotoma																						
Obelia bidentata		X	X															X	X			
Obelia dichotoma		X	X															X	X	X	X	X
Obelia geniculata		X	X															X	X			
Obelia longissima		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		X	X			
Orthopyxis crenata		X	X															X	X	X	X	X
Orthopyxis integra		X	X															X				
Family Campanulinidae																						

Calycella syringa		X	X															X	X			
Family Cirrholoveniidae																						
Cirrholovenia tetranema																		X		X		X
Family Eirenidae																						
Eutima gegenbauri		X	X															X	X			
Eutima gracilis		X	X															X				
Eudendrium armatum		X																				
Eudendrium merulum		X																				
Eudendrium rameum		X	X																			
Eudendrium ramosum		X																				
Family Haleciidae																						
Halecium beanii		X	X															X	X			
Halecium delicatulum		X																				
Halecium halecinum		X	X															X	X			
Halecium labrosum		X	X															X	X			
Halecium mediterraneum		X																X	X			
Halecium nanum		X																				
Halecium profundum																						
Halecium pusillum		X																X	X			
Halecium sessile																						
Halecium tenellum		X																				
Family Hebellidae																						
Anthohebella parasitica as *Hebella parasitica*		X																X	X			
Bedotella armata																						
Hebella scandens		X																				

Scandia gigas		X																				
Scandia mutabilis																						
Family Lafoeidae																						
Acryptolaria conferta		X																				
Acryptolaria crassicaulis		X																				
Acryptolaria longitheca																						
Cryptolarella abyssicola																						
Cryptolaria exserta																						
Cryptolaria pectinata		X																				
Filellum serpens		X																				
Filellum serratum		X																				
Grammaria abietina																						
Lafoea dumosa		X	X															X	X			
Lafoeina tenuis		X																				
Zygophylax biarmata		X																				
Zygophylax echinata																						
Zygophylax elegantula																						
Zygophylax geniculata																						
Family Laodiceidae																						
Laodicea undulata		X	X															X	X			
Family Lovenellidae																						
Eucheilota maculata		X	X															X	X			
Hydranthea margarica		X																X	X			
Lovenella clausa																						
Family Mitrocomidae																						

Cosmetira pilosella		X																				
Cyclocanna producta																						
Family Sertulariidae																						
Abietinaria abietina		X	X															X	X			
Amphisbetia distans																						
Amphisbetia fasciculata																						
Amphisbetia operculata		X																X	X			
Diphasia alata																						
Diphasia attenuata																						
Diphasia delagei		X																				
Diphasia margareta		X																				
Diphasia pinastrum		X																				
Diphasia rosacea		X																				
Dynamena crisioides		X																X	X			
Dynamena disticha		X																				
Dynamena pumila		X	X															X	X			
Dynamena quadridentata		X																				
Hydrallmania falcata		X																X	X			
Salacia desmoides		X																X	X			
Sertularella ellisii		X																X	X			
Sertularella fusiformis		X																				
Sertularella gayi		X																X	X			
Sertularia marginata		X																X	X			
Sertularella mediterranea		X																X	X			
Sertularella ornata		X																				

Sertularella polyzonias		X																				
Sertularella tenella																		X	X			
Sertularella unituba		X																				
Sertularia cupressina		X																X	X			X
Sertularia distans		X																X	X			
Sertularia gracilis																						
Sertularia tenera																						
Symplectoscyphus bathyalis																						
Tamarisca tamarisca																						
Thuiaria articulata		X																				
Family Thyroscyphidae																						
Sertularelloides cylindritheca		X																X				
Family Tiarannidae																						
Krampella dubia																						
Stegopoma giganteum																						
Stegolaria geniculata																						
Superfamily Plumularioidea																						
Family Aglaopheniidae																						
Aglaophenia acacia		X																				
Aglaophenia elongata		X																X	X			
Aglaophenia kirchenpaueri		X	X															X				
Aglaophenia lophocarpa		X	X																			
Aglaophenia octodonta		X	X															X	X			
Aglaophenia parvula		X	X																			
Aglaophenia picardi		X																				

Aglaophenia pluma		X	X															X	X			
Aglaophenia tubiformis		X	X															X	X			
Aglaophenia tubulifera		X	X																			
Aglaophenopsis cartieri		X																				
Cladocarpus boucheti																						
Cladocarpus formosus																						
Cladocarpus paraventricosus																						
Cladocarpus sigma		X	X																			
Gymnangium montagui		X	X																			
Lytocarpia myriophyllum		X	X																			
Macrorhynchia philippina		X	X															X	X	X	X	X
Streptocaulus corneliusi																						
Streptocaulus pectiniferus		X																				
Streptocaulus pulcherrimus																						
Family Halopteridídae																						
Antennella ansini		X	X															X	X			
Antennella secundaria		X	X															X	X			
Halopteris catharina		X																X	X			
Halopteris diaphana		X	X															X	X			
Monostaechas quadridens		X	X															X	X			
Polyplumaria flabellata		X	X																			
Family Kirchenpaueriidae																						
Kirchenpaueria curvata																						
Kirchenpaueria pinnata		X	X															X	X			
Kirchenpaueria halecioides		X	X															X	X			

Family Plumulariidae																						
Nemertesia antennina		X	X															X	X			
Nemertesia belini		X																				
Nemertesia intermedia																						
Nemertesia norvegica		X																				
Nemertesia paradoxa																						
Nemertesia ramosa		X	X															X				X
Plumularia filicula																						
Plumularia obliqua		X																				
Plumularia pulchella		X																				
Plumularia setacea		X	X															X	X	X	X	X
Plumularia strictocarpa		X	X															X	X	X	X	X
Pseudoplumaria marocana		X																				
Pseudoplumaria sabinae																						
Order Siphonophorae																						
Suborder Calycophorae																						
Family Abylidae																						
Abyla trigona																						
Abylopsis tetragona		X	X															X				
Abylopsis eschscholtzii			X															X				
Bassia bassensis			X																			
Family Clausophyidae																						
Chuniphyes multidentata		X	X															X				
Family Diphyidae																						
Chelophyes appendiculata		X																X				

Diphyes bojani		X	X															X		X		X
Diphyes dispar		X	X															X		X		X
Eudoxoides spiralis			X																			
Lensia conoidea		X	X															X	X			
Lensia multicristata		X																				
Muggiaea atlantica		X	X															X	X	X	X	X
Muggiaea kochi		X																				
Sulculeolaria chuni																						
Family Hippopodiidae																						
Hippopodius hippopus		X	X															X	X			
Family Prayidae																						
Nectopyramis thetis																						
Rosacea cymbiformis			X																			
Suborder Cystonectae																						
Family Physaliidae																						
Physalia physalis		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		X	X	X		X
Suborder Physonectae																						
Family Agalmatidae																						
Agalma okenii																						
Athorybia rosacea		X	X															X				
Halistemma rubrum		X	X															X	X			
Nanomia bijuga		X	X	X	X	X	X		X		X	X	X	X	X	X		X				
Family Apolemiidae																						
Apolemia uvaria		X																				
Family Cordagalmatidae																						

Cordagalma bimaculatum																						
Family Physophoridae																						
Physophora hydrostatica		X	X														X	X				
Subclass Trachylinae																						
Order Limnomedusae																						
Family Olindiidae																						
Craspedacusta sowerbyi	NC_018537.1																	X	X	X	X	X
Gonionemus vertens		X	X															X	X			
Maeotias marginata		X	X															X	X			
Olindias sp.																						
Order Narcomedusae																						
Family Aeginidae																						
Aegina citrea		X	X															X	X			
Aeginura grimaldii		X	X															X	X			
Family Cuninidae																						
Cunina frugifera		X																X	X			
Cunina octonaria		X																X	X			
Family Solmarisidae																						
Pegantha clara																						
Pegantha rubiginosa		X																X	X			
Solmaris corona																						
Order Trachymedusae																						
Family Geryoniidae																						
Liriope tetraphylla		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		X	X			
Family Halicreatidae																						

Halicreas minimum		X																X	X			
Haliscera bigelowi		X																X	X			
Family Rhopalonematidae																						
Aglaura hemistoma		X	X															X	X			
Colobonema sericeum		X	X															X	X			
Crossota rufobrunnea		X																X	X	X		X
Pantachogon haeckeli		X	X															X	X	X		X
Rhopalonema velatum		X	X															X	X	X		X
Class Scyphozoa																						
Subclass Coronamedusae																						
Order Coronatae																						
Family Atollidae																						
Atolla parva																						
Atolla vanhoeffeni		X	X															X	X			
Atolla wyvillei		X	X															X	X			
Family Nausithoidae																						
Nausithoe atlantica			X															X	X			
Nausithoe globifera																						
Nausithoe punctata																		X	X	X	X	X
Family Periphyllidae																						
Periphylla periphylla			X		X	X			X		X	X	X	X	X	X		X	X			
Subclass Discomedusae																						
Order Rhizostomeae																						
Suborder Daktyliophorae																						

Family Catostylidae																						
Catostylus tagi																		X	X	X		
Family Rhizostomatidae																						
Rhizostoma luteum			X																			
Order Semaeostomeae																						
Family Pelagiidae																						
Chrysaora hysoscella		X	X			X												X	X	X	X	X
Chrysaora quinquecirrha	NC_020459.1																	X	X			
Pelagia noctiluca		X	X	X	X	X	X	X	X	X	X	X	X	X	X			X	X	X	X	X
Family Phacellophoridae																						
Phacellophora camtschatica		X	X															X	X			
Family Ulmaridae																						
Aurelia aurita	HQ694729.1																	X	X	X	X	X
Aurelia solida			X																X			
Class Staurozoa																						
Order Stauromedusae																						
Suborder Amyostaurida																						
Family Kishinouyeidae																						
Calvadosia campanulata		X	X															X	X	X	X	X
Suborder Myostaurida																						
Family Haliclystidae																						
Haliclystus auricula		X	X															X				
Class Cubozoa																						
Order Carybdeida																						

Family Carybdeidae																						
Carybdea marsupialis		X																X	X			

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