

# A Genomic and Proteomic Study of Sea Anemones and Jellyfish from Portugal

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“An expert is a person who has made all the mistakes that can be made in a very narrow field”

Niels Bohr

(Nobel Prize in Physics in 1922)



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## Abstract

Sea anemones and jellyfish are marine organisms belonging to the phylum Cnidaria. Cnidarians are one of the most promising sea organisms due to their endless source of bioactive molecules with biotechnological interest. Despite the amount of scientific data published on Atlantic cnidarians, there still exists a great lack of knowledge about these organisms. This thesis compiles the recent advances in sea anemone toxins diversity and methods/techniques for studying jellyfish toxins. These subjects were a gap in the scientific literature and such wide-ranging works are of major importance for future investigations of the toxicity of cnidarians. Sea anemone toxins comprise mainly proteins and peptides that are cytolytic or neurotoxic. It includes voltage-gated Na<sup>+</sup> and K<sup>+</sup> channels toxins, acid-sensing ion channel toxins, cytolysins, toxins with Kunitz-type protease inhibitors activity and toxins with phospholipase A2 activity. Furthermore, the phylogenetic relationships of sea anemone toxins were assessed and the genes encoding toxins and the toxins three-dimensional structures were gathered. Jellyfish proteomic and transcriptomic subjects were also organized in detail. The proteomic content refers to animal collection, nematocysts extraction, rupture and methods for purifying the crude extract such as SDS-PAGE, 2DE, gel chromatography, HPLC, DEAE-Sepharose Fast Flow anion-exchange chromatography, LC-MS, MALDI and Western blot. Other assays performed in jellyfish research such as hemolytic assays, antimicrobial assays, protease activity assays, among others, were as well contemplated. Moreover, it was made reference to RNA extraction, construction of cDNA libraries, RACE and Northern blot procedures relevant for jellyfish transcriptome studies based in the RNA encoding toxins. The transcriptomic approach collects information about deep sequencing platforms used in cnidarians, and therefore in jellyfish, such as 454-pyrosequencing or Illumina. This work has the purpose to explain the methodologies employed in high throughput sequencing for the *in silico* bioprospecting. Additionally, it was investigated the all protein content of the jellyfish *Pelagia noctiluca*. Concerning to sea anemones, a phylogenetic analysis was performed with the most ubiquitous species encounter as *Actinia equina*, *Actinia fragacea*, *Anemonia viridis*, *Aulactinia verrucosa*, *Anthopleura krebsi*, *Calliactis parasitica* and *Cereus pedunculatus*. Sequences from one nuclear (18S) and four mitochondrial (16S, COI, COIII, ND6) markers were analyzed in the referred sea anemone species collected from three sampling areas separated from each other 250Km, namely North, Center and South of Portugal. Results showed more robust phylogenetic groups with better resolved tree nodes compared to previous studies. Our

results did not reveal evidence of significant genetic differentiation among organisms from the three sampling areas considered or among the haplotypes here obtained and others deposited in public databases from elsewhere. The phenotype variation is also homogeneous comparing the three sampling areas. These results suggest the absence of considerable population structure within these organisms. A method for the DNA extraction of sea anemones was improved, which facilitated the subsequent molecular assays, with the employment of a mechanic homogenizer with beads. The sea anemone tissue preserved with 99% ethanol retrieved higher quality and quantity of DNA, compared to -80°C preserved tissue. Regarding the jellyfish *P. noctiluca* study, it was performed a comprehensive proteomic evaluation of its crude extract. The body constitutive proteins were characterized using the nanoLC-MS/MS and MALDI-ToF/ToF techniques, which gave an output of 68 different proteins, notably highlighting a zinc metalloproteinase protein, a red fluorescent protein (RFP) and a peroxiredoxin, all identified for the first time in *P. noctiluca*. The zinc metalloproteinase has a ShK toxin domain and therefore could be implicated in the sting toxicity of this species. A RFP, which could be similar to other fluorescent proteins that are nowadays used in molecular assays, was also identified and can be now extracted from a new source (*P. noctiluca*). Finally, peroxiredoxin is a natural antioxidant and its identification make this species a natural resource of anti-UV radiation agents, for example with relevancy in preventing cell damage following the exposure to X-ray irradiation. This work also summarizes the biological significance of the other 65 proteins found in *P. noctiluca*, which can have major relevance to explore the clinical potential of jellyfish bioactive compounds. Altogether, the compilation of the sea anemone toxins diversity and the methods employed for toxins assessment in jellyfish will assist subsequent studies in cnidarians bioprospecting. Moreover, the phylogenetic estimation of sea anemones from the Portuguese coast and the study of all body protein content of the jellyfish *P. noctiluca*, beyond the evident input in the scientific knowledge, will boost the study of these cnidarians for possible biomedical/biotechnological applications subjects.

**Keywords:** Sea anemone, Jellyfish, Toxin, Method, Proteomic, Deep sequencing, Phylogenetic



## Resumo

As anémons e as medusas (alforrecas ou águas vivas), são organismos marinhos que pertencem ao phylum Cnidaria. Estes organismos têm sido ao longo do tempo objeto de estudo por parte dos cientistas, em grande parte devido aos compostos bioativos presentes no seu organismo. Os espécimes do Oceano Atlântico, apesar de serem estudados há várias décadas, ainda têm muito para desvendar. Esta tese reúne os mais recentes trabalhos científicos em toxinas de anémons e métodos/técnicas de estudo de toxinas de medusas. Esta compilação representa um importante recurso para aqueles que estudam a toxicologia de cnidários. As toxinas das anémons são maioritariamente proteínas ou péptidos com atividade citolítica ou neurotóxica. Incluem toxinas de canais  $\text{Na}^+$  e  $\text{K}^+$ , canais iónicos sensíveis aos ácidos, citolisinas, toxinas com atividade de proteases do tipo kunitz e de fosfolipases A2. Aqui, foram também abordadas as relações filogenéticas entre as toxinas produzidas por anémons, os genes que codificam essas mesmas toxinas e as suas estruturas tridimensionais. Nesta tese foram também organizados e sistematizados os métodos e técnicas laboratoriais, usados em medusas, para extrair e identificar toxinas. Esta abordagem proteómica reuniu os métodos e técnicas de maior sucesso para a preparação do material biológico, extração e rutura dos nematocistos e métodos de purificação do veneno como SDS-PAGE, 2DE, cromatografia em gel, HPLC, DEAE-Sepharose Fast Flow anion-exchange chromatography, LC-MS, MALDI e Western blot. Outros recursos como ensaios hemolíticos, ensaios antimicrobianos, ensaios de atividade proteásica, entre outros foram também contemplados. Para além destas técnicas, foram também considerados os trabalhos sobre a extração de ARN, construção de bibliotecas de cDNA, RACE e Northern blot, relevantes para o estudo do transcriptoma de medusas baseado no ARN que codifica toxinas. Esta abordagem transcriptómica sistematiza informação sobre a chamada sequenciação de última geração usada em cnidários com ênfase nas medusas, nomeadamente a *454-pyrosequencing* e a *Illumina*. Este trabalho tem como intuito, explicar as novas tecnologias de sequenciação para a bioprospecção *in silico*. Em relação ao trabalho efetuado com anémons, destaca-se a análise filogenética revelando como as espécies mais ubíquas, na zona intertidal, da costa Portuguesa, a *Actinia equina*, a *Actinia fragacea*, a *Anemonia viridis*, a *Aulactinia verrucosa*, a *Anthopleura krebsi*, a *Calliactis parasitica* e o *Cereus pedunculatus*. Estas espécies, recolhidas em três zonas distintas separadas entre si por 250Km, enunciadas como Norte, Centro e Sul, foram analisadas com os seguintes marcadores genéticos: um nuclear (18S) e 4

mitocondriais (16S, COI, COIII, ND6). Os resultados mostraram a presença de grupos filogenéticos robustos e mais resolvidos relativamente a trabalhos anteriormente publicados. O trabalho revelou ausência de diferenciação genética entre os organismos das três áreas, e mesmo entre os haplótipos obtidos neste estudo e outros depositados nas bases de dados provenientes de outras regiões. A variação fenotípica foi também homogênea comparando as três áreas amostradas. Os resultados sugerem a ausência de uma estrutura populacional entre os organismos estudados. Neste trabalho foi implementado um método para a extração de ADN das anémonas, que possibilitou ADN em quantidade e qualidade para as análises subsequentes, através da utilização de um homogeneizador mecânico com esferas. O tecido de anémonas preservado em etanol a 99% proporcionou melhor qualidade e quantidade de ADN extraído comparado com tecido preservado a -80°C. Relativamente ao trabalho com a medusa *P. noctiluca*, este consistiu em fazer um estudo proteómico completo ao extrato bruto do corpo do animal. Neste sentido, recorrendo à espectrometria de massa (nano-LC-MS/MS e ao MALDI-ToF/ToF) identificou-se as proteínas existentes neste organismo, tendo sido identificadas 68 proteínas diferentes. Destas, destaca-se uma metaloproteínase de zinco, uma proteína fluorescente vermelha (PFV) e uma peroxirredoxina, todas descritas pela primeira vez em *P. noctiluca*. A metaloproteínase de zinco tem um domínio tóxico idêntico à toxina ShK e como tal está implicada na toxicidade da picada desta medusa. A PFV poderá ser semelhante a outras proteínas fluorescentes atualmente bastante usadas em ensaios moleculares e pode agora ser extraída de uma nova fonte, a *P. noctiluca*. Finalmente a peroxirredoxina é uma proteína antioxidante e a sua identificação nesta espécie faz deste organismo uma fonte natural deste agente de radiação anti-UV. Esta proteína é importante por exemplo para prevenir danos celulares após exposição sob radiação-X. Adicionalmente, este trabalho cita a importância clínica das 65 proteínas encontradas e a possibilidade do uso destes organismos como modelos para o estudo destas biomoléculas. Esta tese, compilando a diversidade de toxinas de anémonas e métodos aplicados ao estudo de toxinas de medusas, poderá ser de elevada relevância para estudos futuros de bioprospeção em cnidários. O estudo filogenético das anémonas da costa portuguesa e a caracterização das proteínas existente em *P. noctiluca*, para além de um óbvio acréscimo do conhecimento científico, vai possibilitar a utilização dos cnidários como objeto de estudo de compostos com aplicações biomédicas ou biotecnológicas.

**Palavras-chave:** Anémone, Medusa, Toxina, Método, Proteómica, Filogenia, Deep-sequencing, Filogenética



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## List of abbreviations

2DE	Two dimensional electrophoresis gel
ABI	Applied Biosystems
ACN	Acetonitrile
AIDS	Acquired immune deficiency syndrome
BCA	Bicinchoninic acid
cDNA	complementary DNA
CHAPS	3- ((3-Cholamidopropyl)dimethylammonio)-1-Propanesulfonic Acid
COI	Cytochrome c oxidase subunit I
COII	Cytochrome c oxidase subunit II
COIII	Cytochrome c oxidase subunit III
DIG	Digoxigenin-11-dUTP
DNA	Deoxyribonucleic acid
DEAE	Diethyl-aminoethyl groups
DPBS	Dulbecco's Phosphate Buffered Saline
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethyleneglycoltetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FPLC	Fast Protein Liquid Chromatography
DGE	Digital Gene Expression
HPLC	High-performance liquid chromatography
HU	Hemolytic unit
IEF	Isoelectric focusing
IPG	Immobilized pH gradient
KRP	Krebs Ringer phosphate
LC	Liquid Chromatography
LTQ	Linear Trap Quadrupole mass spectrometer
MALDI	Matrix-assisted laser desorption-ionization
MDLC	Multidimensional Liquid Chromatography
MOPS	3-(n-Morpholino) Propanesulfonic Acid
MS	Mass spectrometry
MWCO	Molecular weight cut off
NBT/ BCIP	Nitro-blue tetrazolium chloride/ 5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt
NGS	Next Generation sequencing
ODS	Octadecylsilane
OGE	OFFGEL electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PE	Paired-end

PLA	Phospholipase A
PMF	Peptide Mass Fingerprinting
PMSF	Phenylmethylsulfonyl fluoride
Ppi	inorganic Pyrophosphate
PSQ-1	Protein sequencer
PTM marker	Post Translational Modification Marker
RACE	Rapid Amplification cDNA ends
RNA	Ribonucleic Acid
RP- HPLC	Reverse phase High-performance liquid chromatography
RT	Reverse transcription
SAGE	Serial Analysis of Gene Expression
SB	StableBond
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SE	Single-end
SMART	Simple modular architecture research tool
SNP	Single Nucleotide Polymorphism
SOLiD	Sequencing by Oligonucleotide Ligation and Detection
SV	Spin or Vacuum
TBS	Tris-buffered saline
TBST	Tris-Buffered Saline and Tween 20
TCA	Trichloroacetic acid
TEAB	Tetraethylammonium bromide
TFA	Trifluoroacetic acid
TMED	Tetramethylethylenediamine
UV	Ultra-violet

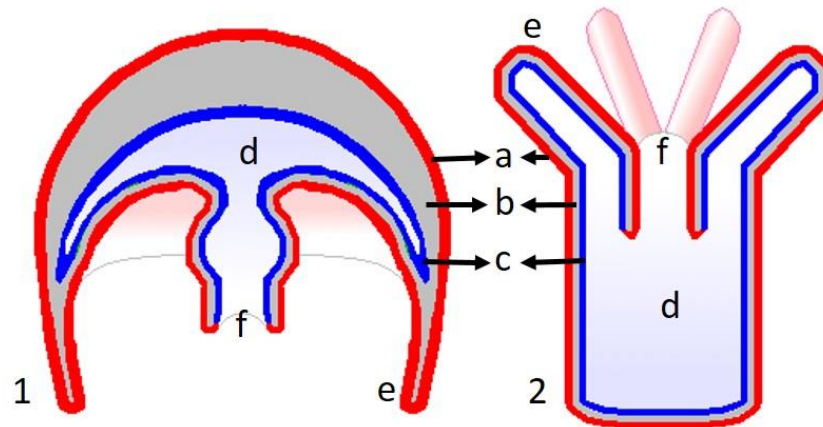




# I General introduction

## General Introduction

Cnidarians are one of the most diverse groups of animals in terms of morphology, lifecycles, ecology, and development. While they are often presented as “simple” animals, many features of supposed simplicity are actually based on misunderstandings of their biology (Zapata et al. 2015). For example, it is often claimed that cnidarians are radially symmetrical, but most have bilateral symmetry, some have directional asymmetry, and only some members have radial symmetry (Manuel 2009). Cnidaria is a phylum from the Animal Kingdom, which comprehends around 11287 species and is formed by two groups, Anthozoa and Medusozoa. Anthozoa are exclusively polypoid while Medusozoa can have polyp or medusa life stages (Daly et al. 2007). In figure I.1 it is shown a generic body plan anatomy of a polyp and a medusa. Polyps can be solitary or colonial; if colonial, they can be monomorphic or polymorphic; they may or may not have a mineralized skeleton; they can be benthic or pelagic; and tentacles, although commonly present, may be absent (Daly et al. 2007). Anthozoa is represented by sea anemones, soft corals, hard corals, gorgonians, sea pens, among others, with around 7500 species. Medusozoa is formed by five classes: Hydrozoa represented by hydras and siphonophores with around 3500 species; Scyphozoa that includes the “true” jellyfish with around 200 species; Staurozoa represented by stalked jellyfish with 50 species, Cubozoa formed by cubo-shaped medusae with 36 species and Polyplodiozoa composed by only one species, that is a parasite which attacks the eggs of fish such as sturgeon or paddlefish (Rocha et al. 2011). All the representatives are marine with the exception of the *Polypodium hydriforme* that spends most of its life cycle in the oocytes of fishes (Evans et al. 2008) and the freshwater *Hydra* or *Craspedacusta* from the Hydrozoa class (Evans et al. 2008, Zapata et al. 2015).



**Figure I.0-1. Generic diagram of the medusa (1) and polyp (2) life forms. a) epidermis, b) mesoglea, c) gastrodermis, d) gastrovascular cavity, e) tentacle and f) mouth/anus (adapted from (Wikimedia commons)).**

Due to the different subjects considered and to the different work performed, it is important to mention this thesis layout. The thesis comprehends two distinct works depending on the subject of study, sea anemones or jellyfish: which are contemplated in five main parts: Part I- General Introduction, Part II- Sea anemones, Part III- Jellyfish, Part IV- General Discussion and Part V-Final Conclusion and Future Perspectives. Parts II and III, are divided into chapters. Both parts are arranged in papers, submitted or published. Chapter II.1 is an assessment of sea anemones toxins and chapter II.2 refers to the phylogeny of sea anemones from Portugal. Chapter III.1 is an assessment of the methodology employed on jellyfish for toxins study and chapter III.2. is an indeep study of the jellyfish *Pelagia noctiluca* all body protein content. The annex is composed by a fold-out and bookmarks for biology enthusiasts, having the general purpose of field guide identification of the intertidal sea anemones. Having that in mind and to avoid redundancy, once several aspects of sea anemones and jellyfish, such as toxins produced and methods employed to its study are contemplated in the chapters II.1 and III.1, in the Part I-General Introduction, it will be made a brief reference to other members of the cnidarian phylum, as well as other aspects of sea anemones or jellyfish not contemplated in the chapters mentioned. Moreover, since each chapter has as a detailed discussion and conclusion, we have opted to finalize this thesis with a brief general discussion and final conclusion and future perspectives.

Anthozoa has morphological synapomorphies, characters that are shared by the common ancestral and are present in all members of a clade. These characters includes the actinopharynx, which is an ectodermally-lined tube that extends from the mouth into the gastric cavity; the siphonoglyph that is a ciliated groove in the actinopharynx and the mesenteries, which are formed by sheets of gastrodermal tissue

that extend from the body wall into the coelenteron and have musculature of gastrodermal origin (Won et al. 2001, Daly et al. 2007, Reft & Daly 2012). All anthozoans have bilaterally symmetric polyps (Zapata et al. 2015).

The order Scleractinia, from the Anthozoa Class, includes hard corals, which are marine corals with a hard skeleton composed by calcium carbonate. Hard corals are the base of reefs and are among the most productive and biologically diverse marine ecosystems on earth. They are considered an endanger species due to global warming and ocean acidification that by itself make their skeletons vulnerable. Furthermore, coral bleaching is also affected by these environmental changes. This type of corals lives in symbiosis with a dinoflagellate alga (zooxanthellae- namely *Symbiodinium*) that provides to coral enormous amounts of energy acquired via photosynthesis, as well as by the absorption of dissolved nutrients. This symbiotic relationship enhances the ability of corals to synthesize a calcium carbonate skeleton. Coral bleaching is the breakdown of this symbiosis between the cnidarian host and the dinoflagellate symbionts (Lehnert et al. 2012, Pernice et al. 2014). Soft corals live, unlike hard corals, in nutrient-rich waters with less intense light. It has been demonstrated that the secondary metabolites produced by these animals have HIV-inhibitory, anti-inflammatory, anticancer and antimicrobial activities, as well as cardiac and vascular responses. Due to these special features, they are mostly investigated by addressing their potential as pharmaceutical and antifouling compounds (Rocha et al. 2011). Other representatives of the Anthozoa class are the Gorgonians that have also zooxanthella symbiotic associations. They can live in shallow waters with strong currents, exhibiting in this case a more flexible and fan-shaped. By contrast, when living in deeper and quieter waters they present a taller, thinner, and stiffer structure. Gorgonians also produce compounds with anti-inflammatory, antitumor, antifouling and antimicrobial characteristics (Rocha et al. 2011). Finally, sea pens or sea feathers have a worldwide geographic and bathymetric distributions, that range from polar seas to the equatorial tropics and from intertidal flats to over 6100m in depth (Williams & One 2011). This group does not arouse scientist's curiosity as others and there are just a few related papers.

Concerning to Medusozoa members, we have Hydras, which in opposition are one of the most studied cnidarians members. This can be due to their endless regenerative ability. They are the smallest organisms from this phylum, being their study only possible with a microscope (Li et al. 2015). Siphonophores that also belong to this class, are colonial organisms in which certain species resembles jellyfish. *Physalia physalis* or Portuguese man-o-war, is one of the most studied due to its toxicity (Haddad et al. 2013).

Staurozoa organisms resembles jellyfish but with the tentacles projecting upward and fixed to a substrate. They are most studied in terms of phylogeny (Miranda et al. 2016).

Box jellyfish belonging to the Cubozoa Class are among the most venomous creatures in the world. They include species as *Chironex fleckeri*, *Carukia barnesi* and *Malo kingi* that causes the so called irukandji syndrome (Berling et al. 2015). The envenomation causes severe headache, backache, muscle pains, chest and abdominal pain, nausea and vomiting, sweating, anxiety, hypertension, tachycardia and pulmonary edema, in within 30 minutes. Two weeks are necessary to vanish completely the symptoms. Normally these attacks occur in Australia between October to May (Berling et al. 2015)

Regarding to Scyphozoa jellyfish, Pelagiidae family includes 17 recognized species and among them we can find the *Pelagia noctiluca*. This group is less venomous to humans compared to Scyphozoa species and the major concern is when they form blooms having a negative impact in fisheries or tourism. These organism outbreaks are increasingly viewed as a deterministic response to escalating levels of environmental degradation and climate extremes. In a study of Benedetti-Cecchi et al. (2015) *P. noctiluca* outbreaks in the Mediterranean Sea, occurred in the proximity of two canyons located in the northern part of the Catalan coast. This result supports the hypothesis that canyons can funnel *P. noctiluca* blooms towards shore during upwelling events. Relating to individual maturation, the canonical Pelagiidae life cycle begins when a fertilized egg develops into a ciliated non-feeding larva, termed a planula, which settles to the bottom and forms an asexually reproducing polyp. Weeks to years later, this polyp divides perpendicular to the oral-aboral axis in a process called strobilation (asexual reproduction), giving rise to several ephyrae (juvenile medusae), which grow into sexually reproductive adult medusae (Collins 2002, Bayha et al. 2010). However, the species here studied has a simpler life cycle. Over several days, the ciliated larva of *P. noctiluca* transforms into a single small ephyra, omitting a benthic stage. This species spends its entire life in the water column. This is the only species within Pelagiidae that has a simpler life cycle (Benedetti-Cecchi et al. 2015, Helm et al. 2015). Figure 1.2 shows a canonical life cycle from a Pelagiidae family member and the *P. noctiluca* life cycle.

Medusozoa is characterized by the existence of a linear mitochondrial DNA genome (Shao et al. 2006). This class display bilateral or radial symmetry, or even directional asymmetry (Dunn 2005, Manuel 2009).

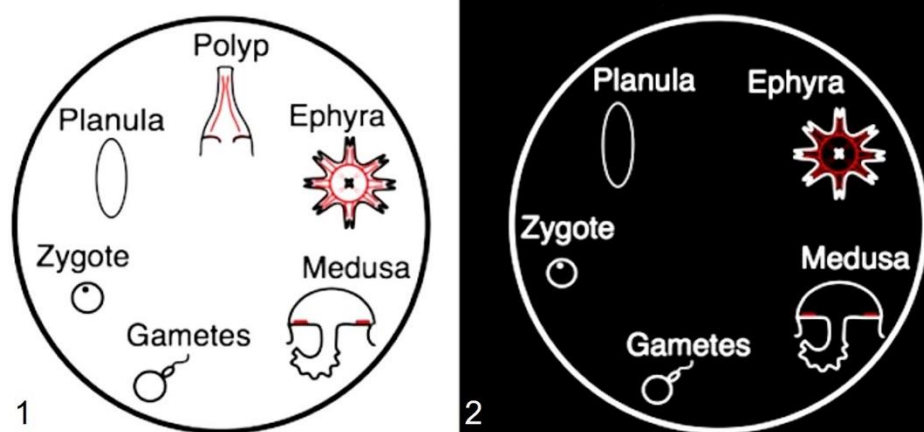


Figure I.0-2. Scyphozoan life cycles of a canonical life cycle from a Pellagiidae family member (1) and a *Pelagia noctiluca* life cycle (2) (adapted from Helm et al. (2015))

Cnidarians are one of the most promising sea organisms. They can be an endless source of bioactive molecules with biomedical, pharmaceutical and technological interest (Frazão & Antunes 2016). These compounds can be associated with their venomous cells and with other body parts, such as mucus coat or body constituent proteins (Santos et al. 2013, Stabili et al. 2015) or with microorganisms that are associated with cnidarians (Liu et al. 2016). Moreover, as simple animals, they are at the base of the tree of life, and in that sense, their study prompts investigators to the knowledge of the animal roots, and to the speciation events that conduct to us, humans. Apart from that, very little is known about the representatives of this phylum worldwide in particular those that can be encountered in Portugal. With the exception of some Atlantic and Mediterranean Sea studies on cnidarians, very few were devoted to these organisms in a biogeography perspective in Portugal. So far, there is only one work on sea anemones from Portugal (Pereira et al. 2014), which includes just the *A. equina* species.

Since the scientific knowledge related to the genome and proteome of these organisms is still very limited, we have targeted as subject of study for this PhD dissertation: “A Genomic and Proteomic Study of Sea Anemones and Jellyfish from Portugal”. The biological questions underlying this work are very pragmatic. Jellyfish found in the Portuguese coast presents new bioactive proteins? Is there a population bottleneck in the population of sea anemones from the Portuguese coast? The present study has three principal aims. First: Compile information on relevant subjects that lack systematized information, namely sea anemone toxins and wet-lab methods employed for jellyfish toxins study. Second: Infer about the phylogeny of sea anemones from the Portuguese coast. Third: Characterize all body proteins from *P. noctiluca* and identify the bioactive molecules found, based on its biological importance.

The study of toxins found in sea anemones (chapter II.1.) could be of major importance to science, once toxins influence human activities and public health negatively. On the other hand, toxins can also have several therapeutically purposes as it was demonstrated in tumor cells (Fujiki et al. 1989), microorganisms proliferation (Mariottini et al. 2013) or in anti-inflammatory activities (Wei et al. 2013). In that manner, an assessment of sea anemone toxins, apart from systematize information important to scientists, is of major relevance to further understand the data work presented on this thesis by providing the reader a broader concept on the theme under study and an in deep knowledge of the studied species.

In chapter II.2. it was evaluated in terms of phylogeny, sea anemones from our coast, using genetic and bioinformatic approaches. Portuguese sea coast has several geographical events that could influence intertidal organisms, such as upwelling phenomena (Sibaja-Cordero & Cortés 2008) or undersea canyons. Upwelling phenomena is represented by the upsurge of more cold and nutrient-rich water from the bottom of the sea, replacing the warmer, usually nutrient-depleted surface water, by the action of the wind. These upwelled waters create a bloom in the primary producers as phytoplankton. In that way, animals that feed on phytoplankton such as sea anemones, could be influenced by these phenomena (Clayton & Lasker 1984, Purcell 1991, Sabatés et al. 2010). Undersea canyons are reported as very important, influencing taxonomic composition and biodiversity patterns, as abundance and community structure (Cunha et al. 2011, Amaro et al. 2012, Ferraris et al. 2012). Portugal has three undersea canyons in Nazaré, Cascais and Setúbal, being the most promising the Nazaré canyon. Nazaré canyon is situated more or less in the middle of the Portuguese coast line and has around 5000 meters depth and 170 kilometers long (Instituto\_Hidrográfico 2016). From all the representatives of the phylum Cnidaria, we have chosen the sea anemones collected from three different areas separated by the previous mentioned canyons and distanced 250Km from each other to perform the investigation here presented. Our work is the first phylogenetic study in several species of sea anemones from Portugal. The phylogenetic assessment of cnidarians is of major importance once these organisms are in the base of metazoan common ancestry. Sea anemones arise more than 600 million years ago and are in the base of the Animal Kingdom. Immortality, for example, is a characteristic shared by sea anemones that stimulated the curiosity of many scientists. These organisms exhibit an almost unlimited regeneration capacity and therefore immortality (Li et al. 2015). In fact, *Nematostella vectensis* is a model organism to study body plan evolution (Bause et al. 2016). As cnidarians are early metazoans and humans are higher metazoans, the study of cnidarians phylogeny will certainly give an input to human genetic

differentiation. Furthermore, the input of the new sequences here obtained for the NCBI database will allow other scientists to go deeper in the sea anemone evolutionary history. Large-scale phylogeography, large-scale haplotypes or even a large-scale checklist could be performed. Altogether, this phylogenetic knowledge can guide scientists to the origin of molecules and systems in higher metazoans.

The assessment of wet-lab procedures used in jellyfish toxin study (chapter III.1), could be of major importance for those that choose to work with these organisms once there was a major gap in the literature on this subject. Beginners or experienced scientists, who needs to go deeper on the transcriptome study of these species, can now look for reliable information on the subject within this thesis.

Finally, and about chapter III.2., *P. noctiluca* was chosen as a representative of the Scyphozoa class. The choice of this species relies, apart from being easily obtained, on the fact of its important features. First it is toxic, and therefore could causes negative impacts in the bathers. This is a relevant fact for countries that has tourism as source of income as Portugal. Second, it forms dense blooms in the warm waters of the Mediterranean Sea causing negative impacts in aquaculture (Licandro et al. 2010). Since the globe temperatures are rising it is clearly a legitimate concern that this species could soon invade our waters, provoking several negative effects. In fact D'Ambra and Malej (2015) already reported an increase in scyphozoan outbreaks in the Mediterranean. Third and last, *P. noctiluca* also have benefits to the ecosystem such as being a food source for fishes with huge commercial value (Mariottini & Pane 2010) or even by providing nursery habitats to early life stages of ecologically and economically important prey fishes and other organisms which shelter underneath their bells (D'Ambra & Malej 2015). In addition, Portuguese coast has the already mentioned undersea canyons and *P. noctiluca* outbreaks are associated with the canyons proximity during upwelling events (Benedetti-Cecchi et al. 2015). In that manner, could be important to study this organism in a geographical area that has geological phenomena that influences its abundance. In this chapter, it was used a nanoLC-MS/MS and MALDI-ToF/ToF techniques to study the body constitutive proteins, which may allow to highlight proteins with biotechnological relevance.

Beyond what has been said, sea anemones and jellyfish can be exploited for other purposes besides toxin or evolutionary studies. They can be a source of food for human consumption. Sea anemones that are ubiquitous in our coast, such as *Anemonia viridis* are already feat as a food source in Spain, where it is consumed as a delicacy. Jellyfish such as *Stomolophus meleagris* or *Rhopilema esculentum*, among many others, are also consumed in Asian countries. In fact, recent scientific and media articles claim that cnidarians could be a food source in the future, due to the



exponential growth of the population compared to the scarcity of food in the ocean/world (Golden 2016). Human population has been grown exponentially since the mid-20<sup>th</sup> century. To resolve the human demands for food, scientist have been implementing improvements in genetic engineering to accelerate the agronomical traits (Shih et al. 2016). Moreover, alternative approaches to recovering depleted fisheries are being made, for improving fish abundance while increasing food security and profits (Costello et al. 2016). The fact that jellyfish have always been an important fishery product in Asian countries (Khong et al. 2016) with theirs high protein and low calorie content (Khong et al. 2016) plus the food globalization phenomena, make these organisms to be likely important as a future food source.

Ultimately this PhD thesis can be an important tool to those who want to start studying cnidarians or for those who already work in the field but want to follow different research approaches in these organisms. Finally, this is an innovating work; once sea anemones from Portugal or *P. noctiluca* all body constituents were never studied using these approaches.



## II Sea Anemones



## II.1 Chapter

Sea Anemone (Cnidaria, Anthozoa, Actiniaria)  
Toxins: An Overview

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Review

## Sea Anemone (Cnidaria, Anthozoa, Actiniaria) Toxins: An Overview

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**Abstract:** The Cnidaria phylum includes organisms that are among the most venomous animals. The Anthozoa class includes sea anemones, hard corals, soft corals and sea pens. The composition of cnidarian venoms is not known in detail, but they appear to contain a variety of compounds. Currently around 250 of those compounds have been identified (peptides, proteins, enzymes and proteinase inhibitors) and non-proteinaceous substances (purines, quaternary ammonium compounds, biogenic amines and betaines), but very few genes encoding toxins were described and only a few related protein three-dimensional structures are available. Toxins are used for prey acquisition, but also to deter potential predators (with neurotoxicity and cardiotoxicity effects) and even to fight territorial disputes. Cnidaria toxins have been identified on the nematocysts located on the tentacles, acrorhagi and acontia, and in the mucous coat that covers the animal body. Sea anemone toxins comprise mainly proteins and peptides that are cytolytic or neurotoxic with its potency varying with the structure and site of action and are efficient in targeting different animals, such as insects, crustaceans and vertebrates. Sea anemones toxins include voltage-gated Na<sup>+</sup> and K<sup>+</sup> channels toxins, acid-sensing ion channel toxins, Cytolysins, toxins with Kunitz-type protease inhibitors activity and toxins with Phospholipase A2 activity. In this review we assessed the phylogentic relationships of sea anemone toxins, characterized such toxins, the genes encoding them and the toxins three-dimensional structures, further providing a state-of-the-art description of the procedures involved in the isolation and purification of bioactive toxins.

# Sea Anemone (Cnidaria, Anthozoa, Actiniaria) Toxins: An Overview

## Abstract

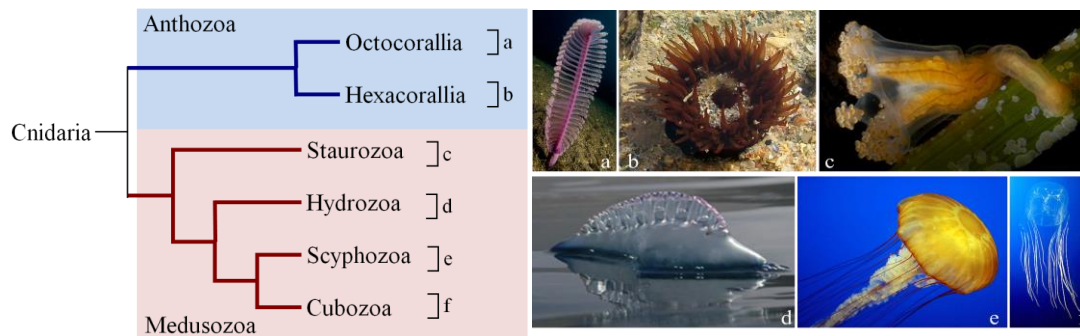
The Cnidaria phylum includes organisms that are among the most venomous animals. The Anthozoa class includes sea anemones, hard corals, soft corals and sea pens. The composition of cnidarian venoms is not known in detail, but they appear to contain a variety of compounds. Currently around 250 of those compounds have been identified (peptides, proteins, enzymes and proteinase inhibitors) and non-proteinaceous substances (purines, quaternary ammonium compounds, biogenic amines and betaines), but very few genes encoding toxins were described and only a few related protein three-dimensional structures are available. Toxins are used for prey acquisition, but also to deter potential predators (with neurotoxicity and cardiotoxicity effects) and even to fight territorial disputes. Cnidaria toxins have been identified on the nematocysts located on the tentacles, acrorhagi and acontia, and in the mucous coat that covers the animal body. Sea anemone toxins comprise mainly proteins and peptides that are cytolytic or neurotoxic with its potency varying with the structure and site of action and are efficient in targeting different animals, such as insects, crustaceans and vertebrates. Sea anemones toxins include voltage-gated Na<sup>+</sup> and K<sup>+</sup> channels toxins, acid-sensing ion channel toxins, Cytolysins, toxins with Kunitz-type protease inhibitors activity and toxins with Phospholipase A2 activity. In this review we assessed the phylogentic relationships of sea anemone toxins, characterized such toxins, the genes encoding them and the toxins three-dimensional structures, further providing a state-of-the-art description of the procedures involved in the isolation and purification of bioactive toxins.

**Keywords:** Cnidaria; sea anemone; phylogeny; toxin; toxin gene

## 1. Introduction

Cnidarians are simple animals with radial symmetry that contain two layers of cells, ectoderm and endoderm. Mesoglea, a non-cellular matrix, is present between the two layers. Cnidarians are mostly predators but certain species may also scavenge dead animals or obtain nourishment from intracellular, photosynthetic unicellular algae, named zooxanthellae.

At least four toxic living classes of cnidarians are currently recognized by most systematists: Anthozoa, Hydrozoa, Scyphozoa and Cubozoa. Molecular phylogenetic methodologies based on DNA sequencing, allowed to determine that the Anthozoa are the basal group of cnidarians (Technau & Steele 2011) (Figure II.1-1). In fact, Anthozoa has a circular mitochondrial DNA, while Hydrozoa, Scyphozoa and Cubozoa have a linear molecule. Likewise, the polyp preceded the medusoid form in the course of evolution (Turk & Kem 2009).



**Figure II.1-1. Simplified cladogram of the phylum Cnidaria (adapted from (Collins 2009)). Photos a, e and f were retrieved from Wikimedia (Wikimedia commons)**

There are two main types of life cycles in cnidarians. In Anthozoans, the polyp is the gamete-producing form and the cycle is embryo > larva > polyp. Medusozoans generally have an embryo > larva > polyp > medusa life cycle, in which the medusa is typically the sexual form. Figure II.1-2 shows a typical life cycle of Anthozoa (Technau & Steele 2011).

Cnidaria feeding success relies on the presence of specialized poisonous cells, the nematocysts. These organisms have specialized subcellular organelles called cnidae with several structures and functions. Cnidae can be classified into three types: nematocysts, spirocysts, and ptychocysts. Nematocysts deliver the venom through the skin, whereas spirocysts are adhesive and ptychocysts are involved in protection. While Anthozoans have the three types of cnidae, medusozoans (Scyphozoans and

Cubozoans) contain only nematocysts. The biological roles of toxins delivered by nematocysts include the capture and killing of prey, digestion, repelling of predators and intraspecies spatial competition (Nevalainen et al. 2004). Cnidarians are not just studied by their toxins and venoms, they are a source of marine natural compounds with therapeutically properties, namely antitumor activity (Rocha et al. 2011). Furthermore, voltage-gated ion channels toxins are studied as an inspiration for drugs design, not only therapeutic but also as insecticides (Bosmans & Tytgat 2007).

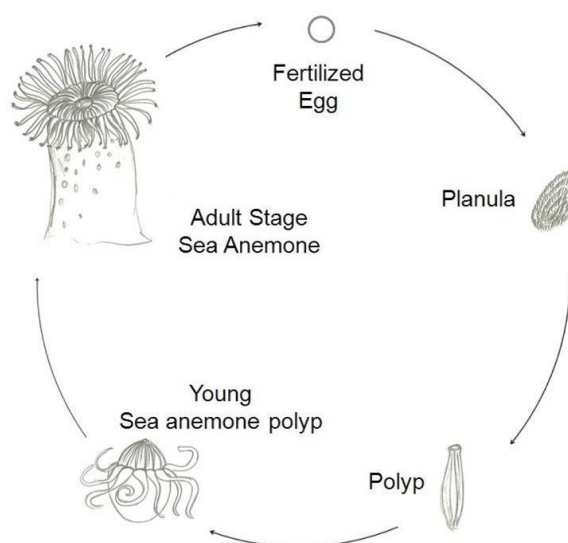


Figure II.1-2. Schematic representation of a typical life cycle of an Anthozoa.

The composition of cnidarian venoms is not known in detail, but they appear to contain a variety of proteinaceous (peptides, proteins, enzymes and proteinase inhibitors) and non-proteinaceous substances (purines, quaternary ammonium compounds, biogenic amines and betaines) (Martins et al. 2009). As an example, palytoxin is a polyether from *Palythoa*, and caissarone is an iminopurine from *Bunodosoma caissarum* (Šuput 2009).

The venom is spread all over the body, in a mucous coat, that also protects them from predators, or it is in the nematocysts. In a recent work, Moran et al. (2012), reported that neurotoxin 1 from *Nematostella vectensis* is confined to ectodermal gland cells. Moreover, in *Anthopleura elegantissima* this toxin also appears in gland cells, whereas in *Anemonia viridis* is associated with both nematocytes and ectodermal gland cells. Previously, Honma et al. (2003) also gave a hint for the same phenomenon when describing that gigantoxins were mostly derived from unknown organelles other than nematocysts. Nematocysts are found mostly on the tentacles, but also exist in other organs such as in acrorhagi and acontia, particularly in certain species of the Actiniidae family, where they are used to fight with nonspecific non-clonemates or for purposes of



defence or predation, respectively. Acrorhagi are in a ring around the base of the tentacles (Figure II.1-3a). Acontia are thin white or color threads attached at one end to the borders of the mesenteries. They can be protruded through the mouth, and in some cases through special pores (cinclides) in the body-wall, for purposes of defence or paralyzes of prey (Figure II.1-3b).

The Anthozoa class include sea anemones, and other anemone-like groups with skeletons (such as the “stony” scleractinian corals) and without skeletons (such as tube anemones), as well as sea pens, sea fans, blue corals, and black corals. The word Anthozoa comes from greek *anthos*, flower + *zoon*, animal, as sea anemones resemble flowers (Figure II.1-3c).



**Figure II.1-3. General aspects of the sea anemone morphology. (a) Acrorhagi, the blue vesicles in *Actinia equina*, green variety (also called *Actinia prasina*), are used to fight against space towards other individuals (see arrow); (b) Acontia, the white threads secreted by *Calliactis parasitica* are used as defensive organs when disturbed (see arrow); (c) *Bunodactis verrucosa* specimens with tentacles retracted and fully expanded, illustrating the characteristic column with adhesive verrucae and short tentacles.**

Nematocysts possess a high concentration of polypeptides and proteins that act as neurotoxins, hemolysins and enzymes, which are responsible for a variety of harmful effects to humans. These toxins/venoms are only injected in the prey or predator after a mechanical or chemical stimulation (Mariottini & Pane 2010). In humans, toxins cause cardiotoxicity, dermatitis, local itching, swelling, erythema, paralysis, pain and necrosis (Martins et al. 2009). *In vivo* effects of sea anemone toxins include neurotoxicity and cardiotoxicity.

Summarily, the cnidarians venom includes 3.5–6.5 kDa voltage-gated sodium ( $Na_v$ ) channels toxins and 3–5 kDa voltage-gated potassium ( $K_v$ ) channel toxins and ~20 kDa pore-forming toxins. The first type prevents inactivation of  $Na_v$  channels by stabilizing the open state conformations. This fact is due to the binding of the toxin to neurotoxin receptor site 3 (Honma & Shiomi 2006).  $K_v$  channel toxins reversible blocks potassium current and can block acid-sensing ion channels, which are permeable to several cations. The cardiotoxic effects of toxins includes arrhythmias, triggered by

early after depolarizations resulting from incomplete  $\text{Na}_v$  channel inactivation, and systolic arrest due to myocardial cell calcium ion overloading (Šuput 2009).

Besides toxins, there are several other non-toxic proteins from sea anemones that are studied by its biological activities, such as fluorescent properties (Ip et al. 2007), but they will not be included in this review. However, we will discuss the importance of protease inhibitors as they adopt a structure that inhibits potassium channels.

In this review, we begin with a brief description of the Anthozoa phylogeny, followed by a general characterization of the sea anemone toxins and afterwards we focus on the major groups of toxins. We then refer to the state of the art techniques used for venom extraction. Afterwards we present the structure of the genes involved in toxin production and the three-dimensional (3D) structures of cnidarian toxins described to date. This review will be solely focused in the molecular diversity of sea anemone toxins. Other cnidarian toxins, as those from coral or jellyfish, will not be considered. More comprehensive information is available in a number of specific papers for jellyfish (Brinkman & Burnell 2009, Mariottini & Pane 2010), cnidarians in general (Nevalainen et al. 2004, Messerli & Greenberg 2006, Castaneda & Harvey 2009, Turk & Kem 2009) and sea anemones (Honma & Shiomi 2006, Moran et al. 2009a, Norton 2009).

## 2. Phylogenetic Relationships of Anthozoa and Sea Anemone Toxins

Cnidarians are scattered around the world and have around 10,000 estimated species. The majority of the phylogenetic studies classified cnidarians based on morphological characters (Daly et al. 2003). At the molecular level, the classification of cnidarians is not yet well established, namely for the order Actiniaria. The phylogeny of Actiniaria is at a suboptimal estimation level (Collins 2009) and has been retrieved from the sequencing analyses of *12S rRNA*, *16S rRNA*, *18S rRNA*, *28S rRNA* and *COIII* genes (Daly et al. 2010, Gusmao & Daly 2010, Rodríguez & Daly 2010, Rodríguez et al. 2012). As referred by Turk and Kem (2009), the comprehension of the phylogenetic relationships among Anthozoa members will give insights into the evolution of their toxins. Thus, a review about sea anemone toxins could not be dissociated from the Anthozoa phylogenetic characterization.

Besides the few studies on the phylogeny of Actiniaria, some other studies have also been done on the population genetics of these animals. Nonetheless, the majority of those works focus on other Orders, especially on corals. Indeed, few studies were done at the intraspecific level on Actiniaria. Population genetics of *Actinia* spp. assessed with enzyme electrophoresis showed that *Actinia nigropunctata* from Madeira Island (Portugal) is in fact a different species from all the others in the study, as well as *Actinia equina* from Africa (Schama et al. 2004). Darling et al. (2006) studied the *Nematostella vectensis* introduced along the Pacific coast of North America and the southeast coast of England, using 10 polymorphic microsatellite loci, and find high variability from Hardy-Weinberg equilibrium as a result of population genetic structure and reproductive plasticity (Reitzel et al. 2008).

Considering the molecular markers surveyed in cnidarians until now, the variation in mitochondrial Cytochrome Oxidase I (*COI*), within and between species, is much lower in Anthozoa compared to Medusozoa. Low identification success and substantial overlap between intra- and interspecific *COI* distances render the Anthozoa unsuitable for DNA barcoding (Huang et al. 2008), with *COI* p-distances among Anthozoa species being equal to 1% (Kartavtsev 2011). Shearer et al. (2002) showed that nuclear markers in Anthozoa have much higher substitution rates and therefore should be used instead of mitochondrial genes.

The reduced knowledge on sea anemones phylogeny make it difficult a direct comparison with the toxin genes phylogeny. While previous studies showed a reduced level of congruence between species phylogeny and the toxin gene phylogeny, further research is needed to better clarify this pattern. Such findings may not be unusual due to distinct patterns of toxin gene evolution (e.g., gene duplication/gene loss, horizontal gene transfer, and lineage sorting and diversification). However, future studies are needed to better elucidate the phenomena behind the acquisition and evolution of the toxin genes in Anthozoa.

Concerning the phylogeny of toxins, we assessed the phylogenetic relationships of Nav channel and Kv channel toxins. In order to systematize the information, we have assessed a phylogenetic tree of cytolysins using only Actinoporins with evidence at transcript level and with full-length sequences. A multiple sequence alignment of amino acids with 533 sites, was made with WebPrank (Loytynoja & Goldman 2010) followed by an analysis to choose the best fit model for protein evolution with ProtTest (Abascal et al. 2005), that gave WAG model. A Maximum Likelihood tree reconstruction was made in Mega 5 (Tamura et al. 2013) using 100 bootstrap inferences. A discrete Gamma distribution was used to model evolutionary rate differences among sites (4

categories), Figure II.1-4. (The alignment is available upon request to the corresponding author.)

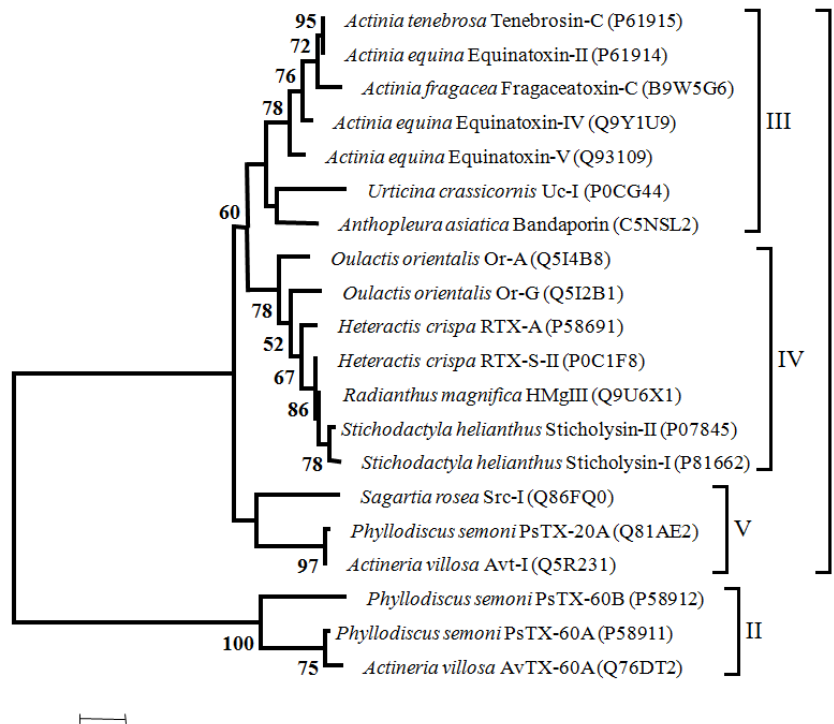


Figure II.1-4. Maximum likelihood tree of Cytolysins with 100 bootstrap replicates (only bootstrap values > 50 are shown). I—proteins without the MACPF domain, II—proteins with the MACPF domain, III—toxins from Actiniidae family members, IV—toxins from Stichodactilidae family members and *Oulactis orientalis* (Actiniidae), V—toxins from Sagartiidae and Alisiidae family members. Toxins are also referred on the Cytolysins chapter.

Considering the phylogenetic tree of cytolysis, two major groups can be defined; one including the proteins without the MACPF domain (I) and the other comprehending those with the MACPF domain (II). Within the major group “I” three clusters can be identified (III to V). Toxins from Actiniidae family members are clustered in group III. The Group IV cluster toxins from Stichodactilidae family members and toxins from *Oulactis orientalis*, (Actiniidae). In fact, toxins from *Oulactis* are more closely related to Stichodactilidae than to Actiniidae toxins. As mentioned previously, Or-A and Or-G and RTX-S-II and RTX-A from *Heteractis crispa* have in common (albeit others characteristics), the substitution of a Trp by a Leu in the position Trp<sup>112</sup> of Equinatoxin-II. Moreover, the conserved RGD sequence that occurs in Sticholysin-II, RTX-A and Equinatoxin-II, in the toxins from *Oulactis* is replaced by the GGD sequence. The cluster V includes the Src-I and the toxins from Alisiidae family. The only member of Sagartiidae family (Src-I), has the EGD sequence instead of the RGD motif. Toxins of Alisiidae family members, share a similar gene organization with three exons (two

introns). In addition, the RGD motif is replaced by the KPS tripeptides in PsTX-20A and Avt.

Regarding the sea anemones phospholipases toxins, the study of Romero et al. (2010) comparing PLA2 from *Condylactis gigantea* (Actiniidae family member), CgPLA2, with the other PLA2s from five animal phyla, suggested that sea anemones PLA2s form a monophyletic group. Within this group, CgPLA2 showed to be closer to the *Adamsia carcinoapados* (Hormathiidae family member) PLA2, AcPLA2, than to others of *Nematostella vectensis*, suggesting a significant divergence from the latter.

### 3. General Aspects of Sea Anemone Toxins

In the first decades of the 20th century, it was practically impossible to isolate and chemically characterize venom compounds, as the biochemical techniques for isolating such natural products hardly existed (Turk & Kem 2009). However, nowadays scientists developed several techniques to obtain the venom of particular structures such as acrorhagi or nematocysts, and to separate the venom into fractions. In this sense, there has been an increase in the number of publications on the subject of “cnidarian toxins”. Figure II.1-5 shows the number of publications in Pubmed, retrieved using the query “Cnidaria toxins” on 5 March 2012. In fact, it is expectable that with the deep sequence platforms, much more data will become available at genomic level allowing to better understand the evolution of cnidarians toxins and the discover of the pharmacologic and therapeutic properties of such compounds. Deep sequencing transcriptomics is the sequencing of the complete set of cellular transcripts at a specific stage or condition, and in that sense Johansen et al. (2010) and Rodríguez et al. (2012), pioneered the use of cDNA high-throughput sequencing with 454 pyrosequencing in the discovery of new toxins. The first publications on “cnidarian toxins” were about crude extracts (e.g., jellyfish crude extracts) and not about isolated toxins. In the following years, sea anemones toxins started to gain some relevance. A partial purification of a toxin from the tentacles of *Condylactis gigantea* was made by gel filtration (Shapiro 1968). Afterwards, three neurotoxic peptides were isolated from *Anemonia viridis* by cm-cellulose and sephadex chromatography (Beress et al. 1975). The ATX-II amino acid sequence published by Wunderer et al. was the first cnidarian toxin to be determined (1976b). At same time, in another laboratory, peptides from *Anthopleura* were also studied (Turk & Kem 2009).

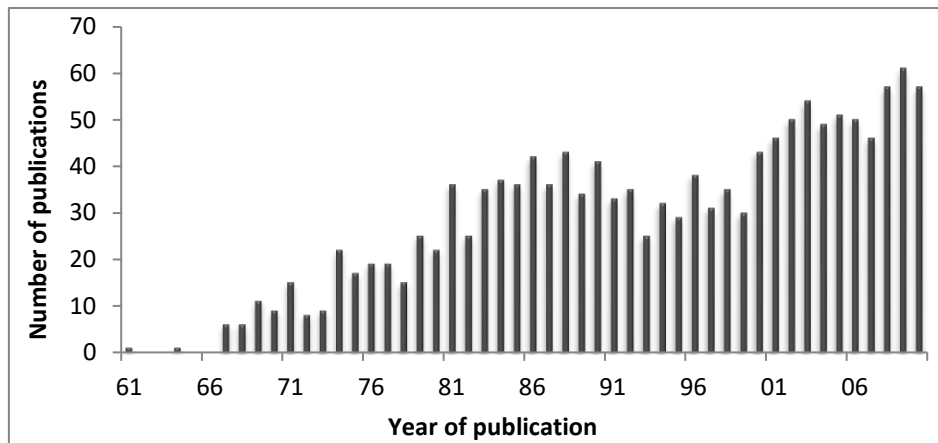


Figure II.1-5. Number of publications from 1961 to date on cnidarians toxins (retrieved from the Pubmed in May 2012).

Voltage-gated ion channels underlie electrical excitability in cells, and they also play important roles in non-excitable cells. Voltage-gated channels open in response to changes in membrane potential, allowing ions to flow down the electrochemical gradient across the cell membrane, being thus gated (by voltage) and forming an ion-selective pore (Messerli & Greenberg 2006). Voltage-gated channels are critical to normal neuromuscular transmission and disruption of their normal function can lead to rapid paralysis. Toxins that target these components (Messerli & Greenberg 2006), are a valuable tool for understanding the structure and function of ion channels (Honma & Shiomi 2006). In this review, we will only refer to  $K_v$  and  $Na_v$  channel toxins, from all the ion channel toxins known.

Besides neurotoxins, cytolytic sea anemone toxins attracted considerable interest starting in the 1970s. The first report was of a phospholipase A in *Aiptasia pallida* venom (Hessinger et al. 1973). Equinatoxin (Eq<sub>t</sub>), named following its source, the sea anemone *Actinia equina* (Ferlan & Lebez 1974), was the first actinoporin described in cnidarians.

As referred previously, not all the toxins are related to nematocyst (Honma et al. 2003, Moran et al. 2012). According to Anderluh et al. (2000), those that are, have a common signal directing them to a maturing cnidocyst (Anderluh et al. 2000). Between the signal peptide and mature region, toxins contain a propeptide of 9–17 residues long, always ending with Lys-Arg (Anderluh et al. 2000). The propeptide is composed mainly of polar and negatively charged amino acids, having the role to conduct the toxin to the nematocyst. However, in more recent works it was showed that Nv1 from *Nematostella vectensis* (Moran et al. 2008) and *Anthopleura elegantissima* toxins have propeptides that also end in a Lys-Arg tandem but are not localized in the nematocysts. This suggests that the propeptide may have another role other than conducting toxins to the

nematocysts. In another work, it was found that this cleavage sequence is not always conserved. Indeed, in the AvTX-20 (belonging to Cytolysins Type II) from *ActinERIA villosa* the propart terminate with a Lys-Lys sequence (Nagai et al. 2002a).

### 3.1. Na<sub>v</sub> Channel Toxins

The first representatives of the Na<sub>v</sub> channel binding proteins were isolated in the 1970s and from all the sea anemone toxins studied, Na<sub>v</sub> channel toxins are the most thoroughly studied, in part because they constitute a major fraction of the venom (Moran et al. 2009a). There are four types of these toxic polypeptides of 3.5–6.5 kDa and they bind to the receptor site three of Na<sub>v</sub> channel during the depolarization procedure.

Type I and II have 46–51 amino acids and anti-parallel  $\beta$ -sheet with four  $\beta$ -strands and a highly flexible loop, named “Arg-14 loop”, after its most conserved residue, lacking any  $\alpha$ -helix (Smith & Blumenthal 2007). Members of Type I and II have similar locations of the six half-Cys (which form three disulfide bonds), as well as several other residues thought to play a role in biological activity or maintenance of the tertiary structure (Norton 2009). In addition, they have basic C-terminal sequences (Messerli & Greenberg 2006).

Type III have 27–32 amino acids and rigid  $\beta$  and  $\gamma$  turns. ATX-III and PaTX are representatives of this group and are cross-linked by three and four disulfide bridges, respectively, implying that they do not share the structural scaffold (Honma & Shiomi 2006). Moreover, Moran et al. studied the bioactive surface of ATX-III and found it consisting mainly of aromatic residues and did not resemble other site-3 toxins, but it also binds the receptor of the site-3 on Na<sub>v</sub> channels (Moran et al. 2007). Type III toxins were identified only in a few species unlike Type I and Type II, which are common in the venom of various cnidarians (Messerli & Greenberg 2006).

Apart from these groups, there is another type of toxins that do not have anything in common with the classic type 3 toxins and therefore are classified as “others”. Calitoxin I and II (79 amino acid residues) resemble Type I and II in the long chain length and in the number of disulfide bridges, three, but not in the amino acid sequence. They act on voltage-gated sodium channels in a similar manner to Type I–III toxins (Honma & Shiomi 2006).

Table II.1-1 indicates all the Na<sub>v</sub> channel toxins diversity with the described amino acid sequence, their accession numbers, their classification group (toxin family), the channel targeted and the Lethal Dose (LD<sub>50</sub>).

**Table II.1-2. Sea anemone Nav channel toxins with amino acid sequence described, accession number, their classification group (toxin family), the channel targeted, the LD<sub>50</sub> and reference**

Species	Toxin	UniProt/ GenBank Accession Number	Toxin Family	Target	LD <sub>50</sub> (µg/kg)/ Tested Organism	Reference
<i>Actinia equina</i>	Ae I	Q9NJK2/ AF130344	Type I	-	-	(Lin et al. 1996)
<i>Anemonia erythraea</i>	AETX-I	P69943/ -	Type I	-	2.2/Mice	(Shiomi et al. 1997)
<i>Anemonia viridis</i>	ATX-I	P01533/ -	Type I	Nav1	-	(Wunderer & Eulitz 1978)
<i>Anemonia viridis</i>	ATX-II	P01528/ -	Type I	Binds to site 3. DmNav, SCN2A and SCN5A	-	(Wunderer et al. 1976b)
<i>Anemonia viridis</i>	ATX-III	P01535/ -	Sea anemone short toxin family	Nav1	-	(Martinez & Kopeyan 1977)
<i>Anemonia viridis</i>	ATX-V	P01529/ -	Type I	-	-	(Scheffler et al. 1982)
<i>Antheopsis maculata</i>	Am-3	P69928/ AB180687	Type I	-	70/ Crabs	(Honma et al. 2005a)
<i>Anthopleura elegantissima</i>	Anthopleurin-C	P01532/ -	Type I	-	-	(Norton 1981)
<i>Anthopleura elegantissima</i>	APE 1-1	P0C1F0/ -	Type I	-	10/ Crabs	(Bruhn et al. 2001)
<i>Anthopleura elegantissima</i>	APE 1-2	P0C1F1/ -	Type I	-	-	(Bruhn et al. 2001)
<i>Anthopleura elegantissima</i>	APE 2-1	P0C1F2/ -	Type I	-	1/ Crabs	(Bruhn et al. 2001)
<i>Anthopleura elegantissima</i>	APE 2-2	P0C1F3/ -	Type I	-	-	(Bruhn et al. 2001)
<i>Anthopleura fuscoviridis</i>	AFT-I	P10453/ -	Type I	-	-	(Sunahara et al. 1987)
<i>Anthopleura fuscoviridis</i>	AFT-II	P10454/ -	Type I	-	-	(Sunahara et al. 1987)
<i>Anthopleura</i> sp. (strain 'Zhanjiang')	Toxin Hk16	P0C5F7/ -	Type I	-	-	(Wang et al. 2004)
<i>Anthopleura</i> sp. (strain 'Zhanjiang')	Toxin Hk2	P0C5F4/ -	Type I	-	-	(Wang et al. 2004)
<i>Anthopleura</i> sp. (strain 'Zhanjiang')	Toxin Hk7	P0C5F5/ -	Type I	-	-	(Wang et al. 2004)
<i>Anthopleura</i> sp. (strain 'Zhanjiang')	Toxin Hk8	P0C5F6/ -	Type I	-	-	(Wang et al. 2004)
<i>Anthopleura xanthogrammica</i>	Anthopleurin-A	P01530/ -	Type I	Nav1	-	(Tanaka et al. 1977)
<i>Anthopleura xanthogrammica</i>	Anthopleurin-B	P01531/ -	Type I	Nav1	-	(Reimer et al. 1985)
<i>Anthopleura xanthogrammica</i>	Toxin PCR1	P0C5F8/ -	Type I	-	-	(Kelso & Blumenthal 1998)
<i>Anthopleura xanthogrammica</i>	Toxin PCR2	P0C5F9/ -	Type I	-	-	(Kelso & Blumenthal 1998)
<i>Anthopleura xanthogrammica</i>	Toxin PCR3	P0C5G0/ -	Type I	-	-	(Kelso & Blumenthal 1998)
<i>Anthopleura xanthogrammica</i>	Toxin PCR4	P0C5G1/ -	Type I	-	-	(Kelso & Blumenthal 1998)
<i>Anthopleura xanthogrammica</i>	Toxin PCR5	P0C5G2/ -	Type I	-	-	(Kelso & Blumenthal 1998)
<i>Anthopleura xanthogrammica</i>	Toxin PCR6	P0C5G3/ -	Type I	-	-	(Kelso & Blumenthal 1998)
<i>Anthopleura xanthogrammica</i>	Toxin PCR7	P0C5G4/ -	Type I	-	-	(Kelso & Blumenthal 1998)
<i>Bunodosoma caissarum</i>	Bc-III	Q7M425/ -	Type I	Nav1.5	600/ Mice	(Malpezzi et al. 1993)
<i>Bunodosoma cangicum</i>	Cangitoxin	P82803/ -	Type I	-	-	(Cunha et al. 2005)
<i>Bunodosoma cangicum</i>	Cangitoxin-2	P0C7P9/ -	Type I	Nav1.1/SCN1A, Nav1.5/SCN5A and Nav1.6/SCN8A	-	(Zaharenko et al. 2008a)
<i>Bunodosoma cangicum</i>	Cangitoxin-3	P0C7Q0/ -	Type I	SCN1A/Nav1.1	-	(Zaharenko et al. 2008a)
<i>Bunodosoma granulifera</i>	Neurotoxin Bg-2	P0C1F4/ -	Type I	Site 3. SCN2A/SCN1B, SCN4A/SCN1B, SCN5A/SCN1B and para/tipE	0.4/ Mice	(Loret et al. 1994)
<i>Bunodosoma</i>	Neurotoxin	P0C1F5/ -	Type I	Site 3.	21/ -	(Loret et al. 1994)



<i>granulifera</i>	Bg-3	-		SCN4A/SCN1B, SCN5A/SCN1B, and para/tipE	Mice	1994)
<i>Calliactis parasitica</i>	Calitoxin 1	P14531/ S69403	Others			(Cariello et al. 1989)
<i>Calliactis parasitica</i>	Calitoxin 2	P49127/ S69399	Others			(Spagnuolo et al. 1994)
<i>Condylactis gigantea</i>	CgNa	P0C20/ -	Type I	Nav1 TTX-sensitive Nav	1/ Crabs	(Standker et al. 2006)
<i>Condylactis passiflora</i> (syn- <i>Condylactis gigantea</i> )	Cp I	P0CH42/ -	Type I	Site 3	-	(Wanke et al. 2009)
<i>Cryptodendrum adhaesivum</i>	Ca I	D2KX90/ AB512761	Type II	-	20/ Crabs	(Maeda et al. 2010)
<i>Halcurias carlgreni</i>	Halcurin	P0C5G6/ -	Type II	-	5.8/ Crabs	(Ishida et al. 1997)
<i>Heteractis crispa</i>	Rm1	P30831/ -	Type II	-	-	(Zykova & Kozlovskaja 1989a)
<i>Heteractis crispa</i>	Rm2	P30783/ -	Type II	-	-	(Zykova et al. 1988a)
<i>Heteractis crispa</i>	Rm3	P30832/ -	Type II	-	-	(Zykova & Kozlovskaja 1989b)
<i>Heteractis crispa</i>	Rm4	P30784/ -	Type II	-	-	(Zykova et al. 1988b)
<i>Heteractis crispa</i>	Rm5	P30785/ -	Type II	-	-	(Zykova et al. 1988b)
<i>Heteractis crispa</i>	Toxin Rc-1	P0C5G5/ -	Type I	-	-	(Shiomi et al. 1996)
<i>Heterodactyla hemprichi</i>	Hh x	D2KX91/ AB512762	Type II	-	-	(Maeda et al. 2010)
<i>Nematostella vectensis</i>	Neurotoxin Nv1-116.25.1	B1NWS4/ EU124461	Type II	Binds to site 3 of voltage-gated sodium channels. DmNav1/TipE	(PD <sub>50</sub> ) 76 nmol/kg Blowfly larvae	(Moran et al. 2008)
<i>Nematostella vectensis</i>	Neurotoxin Nv1-116.27.1	B1NWS6/ EU124463	Type II	Binds to site 3 of voltage-gated sodium channels. DmNav1/TipE	(PD <sub>50</sub> ) 76 nmol/kg Blowfly larvae	(Moran et al. 2008)
<i>Nematostella vectensis</i>	Neurotoxin Nv1-116.28.1	B1NWS7/ EU124464	Type II	Binds to site 3 of voltage-gated sodium channels. DmNav1/TipE	(PD <sub>50</sub> ) 76 nmol/kg Blowfly larvae	(Moran et al. 2008)
<i>Nematostella vectensis</i>	Neurotoxin Nv1-116.37.1	B1NWS5/ EU124462	Type II	Binds to site 3 of voltage-gated sodium channels. DmNav1/TipE	(PD <sub>50</sub> ) 76 nmol/kg Blowfly larvae	(Moran et al. 2008)
<i>Nematostella vectensis</i>	Neurotoxin Nv1-116.39.1	P0CH46/ DS469622	Type II	Binds to site 3 of voltage-gated sodium channels. DmNav1/TipE	(PD <sub>50</sub> ) 76 nmol/kg Blowfly larvae	(Putnam et al. 2017)
<i>Nematostella vectensis</i>	Neurotoxin Nv1-116.40.1	B1NWS8/ EU124465	Type II	Binds to site 3 of voltage-gated sodium channels. DmNav1/TipE	(PD <sub>50</sub> ) 76 nmol/kg Blowfly larvae	(Moran et al. 2008)
<i>Nematostella vectensis</i>	Neurotoxin Nv1-116.41.1	A7SCE5/ DS469622	Type II	Binds to site 3 of voltage-gated sodium channels. DmNav1/TipE	(PD <sub>50</sub> ) 76 nmol/kg Blowfly larvae	(Putnam et al. 2017)
<i>Nematostella vectensis</i>	Neurotoxin Nv1-116.45.1	B1NWR7/ EU124454	Type II	Binds to site 3 of voltage-gated sodium channels. DmNav1/TipE	(PD <sub>50</sub> ) 76 nmol/kg Blowfly larvae	(Moran et al. 2008)
<i>Parasicyonis actinostoloides</i>	PA-TX	P09949/ -	Sea anemone short toxin family	-	-	(Nishida et al. 1985)
<i>Radianthus paumotensis</i>	Rp-II	P01534/ -	Type II	-	-	(Schweitz et al. 1985)
<i>Radianthus paumotensis</i>	Rp-III	P08380/ -	Type II	-	-	(Metrione et al. 1987)
<i>Stichodactyla helianthus</i>	Sh1	P19651/ -	Type II	Nav1	-	(Kem et al. 1989)
<i>Stichodactyla gigantea</i>	Gigantoxin-2	Q76CA3/ AB110012	Type I	-	70/Crabs	(Shiomi et al. 2003)
<i>Stichodactyla gigantea</i>	Gigantoxin-3	Q76CA0/ AB110015	Type II	-	120/Crabs	(Shiomi et al. 2003)
<i>Stichodactyla haddonii</i>	SHTX-4	B1B519/ AB362570	Type II	-	93/Crabs	(Honma et al. 2008)
<i>Thalassianthus aster</i>	Ta I	D2KX92/ AB512763	Type II	-	24/Crabs	(Maeda et al. 2010)

### 3.2. K<sub>V</sub> channel Toxins

K<sub>V</sub> channel toxins were discovered in the 1990's. These 3–5 kDa polypeptide toxins can be grouped into four structural classes: Type I with 35–37 amino acid residues and three disulfide bridges; Type II with 58–59 residues and three disulfide bridges; Type III with 41–42 residues and three disulfide bridges; and Type IV with 28 residues and two disulfide bridges. In table II.1-2, is indicated all K<sub>V</sub> channel toxins with the amino acid sequence described, and with the same descriptors mentioned previously for Na<sub>V</sub> channel toxins. Besides the classification in types, it was included the toxin family reference and the channel type targeted. Even within the same structural class, toxins can differ in selectivity for different subtypes of channels. Furthermore, and as it happens with Na<sub>V</sub> channel toxins, many of the sea anemone K<sub>V</sub> channel toxins have not yet their channel selectivity fully characterized (Castaneda & Harvey 2009).

Several of the sea anemone toxins were discovered for its ability to inhibit the binding of radiolabelled  $\alpha$ -dendrotoxin to synaptosomal membranes.  $\alpha$ -Dendrotoxin from the green mamba snake, binds to K<sub>V</sub>1.1, 1.2 and 1.6 subunits (Castaneda & Harvey 2009). After this approach, several other different toxins were consequently investigated. The first K<sub>V</sub> channel blockers isolated from marine sources were ShK from *Stichodactyla helianthus* and BgK from *Bunodosoma granulifera*, both from Type I. Since the detection of these two toxins, others have been discovered in *Anemonia viridis* in 1995 and 1998 (Schweitz et al. 1995, Diochot et al. 1998), *Radianthus magnifica* in 1997 (Gendeh et al. 1997b), *Actinia equina* in 1998 (Minagawa et al. 1998), *Anthopleura elegantissima* in 2003 and 2004 (Diochot et al. 2003, 2004), *Antheopsis maculata* in 2005 (Honma et al. 2005a), *Anemonia erythraea* in 2006 (Hasegawa et al. 2006), *Bunodosoma caissarum* in 2006 and 2008 (Oliveira et al. 2006, Zaharenko et al. 2008b) and *Stichodactyla haddoni* in 2008 (Honma et al. 2008).

**Table II.1-3. Sea anemone Kv channel toxins with amino acid sequence described, their accession number, their classification group (toxin family), the channel targeted, the LD<sub>50</sub> and reference**

Species	Toxin	UniProt/ GenBank Accession Number	Toxin Family	Target	LD <sub>50</sub> (µg/kg)/ Tested Organis m	Reference
<i>Actinia equina</i>	AeK	P81897/ -	Type I	Kv1	-	(Minagawa et al. 1998)
<i>Anemonia viridis</i>	SA5 II	P10280/ -	Cnidaria kunitz-type proteinase inhibitor/Type II	-	-	(Wunderer et al. 1976a)
<i>Anemonia viridis</i>	kalicludin-1	Q9TWG0/ -	Cnidaria kunitz-type proteinase inhibitor/Type II	Kv1.2	-	(Schweitz et al. 1995)
<i>Anemonia viridis</i>	kalicludin-2	Q9TWF9/ -	Cnidaria kunitz-type proteinase inhibitor/Type II	Kv1.2	-	(Schweitz et al. 1995)
<i>Anemonia viridis</i>	kalicludin-3	Q9TWF8/ -	Cnidaria kunitz-type proteinase inhibitor/Type II	Kv1.2	-	(Schweitz et al. 1995)
<i>Anemonia viridis</i>	BDS-I	P11494/ -	Cnidaria kunitz-type proteinase inhibitor/Type II	Kv3.1, 3.2, 3.4	-	(Diochot et al. 1998)
<i>Anemonia erythraea</i>	AETX-K	Q0EAE5/ AB259113	Type I	Kv1	-	(Hasegawa et al. 2006)
<i>Anemonia viridis</i>	kaliseptin	Q9TWG1/ -	Type I	Kv1.2	-	(Schweitz et al. 1995)
<i>Anemonia viridis</i>	BDS-II	P59084/ -	Type III	Kv3.1, 3.2, 3.4	-	(Diochot et al. 1998)
<i>Antheopsis maculata</i>	Am-2	P69930/ AB180686	Type III	-	(PD <sub>50</sub> ) 420/ Crabs	(Honma et al. 2005a)
<i>Anthopleura elegantissima</i>	APET x1	P61541/ -	Type III	KCNH2 (HERG1) KCNH6 (HERG2), KCNH7 (HERG3)	0/ Mice	(Diochot et al. 2003)
<i>Anthopleura elegantissima</i>	APET x2	P61542/ -	Type III	H(+)-gated Nav ASIC3	-	(Diochot et al. 2004)
<i>Anthopleura aff. xanthogrammica</i>	AXPI-I	P81547/ -	Cnidaria kunitz-type proteinase inhibitor/Type II	-	-	(Minagawa et al. 1997)
<i>Bunodosoma granulifera</i>	Bgk	P29186/ -	Type I	Kv1.1, Kv1.2 Kv1.3, Kv1.6 Kv3.2	-	(Aneiros et al. 1993)
<i>Bunodosoma caissarum</i>	BcIV	P84919/ -	Type III	-	-/ Crabs	(Oliveira et al. 2006)
<i>Bunodosoma caissarum</i>	Bc-V	P86470/ -	Type III	-	-	(Zaharenko et al. 2008b)
<i>Bunodosoma cangicum</i>	Toxin Bcg III 31.16	P86461/ -	Type III	-	-	(Zaharenko et al. 2008b)
<i>Heteractis crispa</i>	Analgesic Polypeptide HC1	B2G331/ AM933240	Cnidaria kunitz-type proteinase inhibitor/Type II	Polypeptide inhibitor of vanilloid receptor 1 (TRPV1)	-	(Andreev et al. 2008)
<i>Heteractis crispa</i>	Kunitz-type Trypsin inhibitor IV	P16344/ -	Cnidaria kunitz-type proteinase inhibitor/Type II	-	-	(Zykova et al. 1985)
<i>Metridium senile</i>	Metridin	P11495/ -	Type I	-	-	(Krebs & Habermehl 1987)
<i>Radianthus magnifica</i>	HmK	O16846/ U58107	Type I	Kv1.2	-	(Gendeh et al. 1997b)
<i>Stichodactyla helianthus</i>	SHPI-1	P31713/ -	Cnidaria kunitz-type proteinase inhibitor/Type II	-	-	(Delfin et al. 1996)
<i>Stichodactyla helianthus</i>	SHPI-2	P81129/ -	Cnidaria kunitz-type proteinase inhibitor/Type II	-	-	(Diaz et al. 1998)
<i>Stichodactyla haddoni</i>	SHTX-3	B1B5I8/ AB362569	Cnidaria kunitz-type proteinase inhibitor/Type II	-	-	(Honma et al. 2008)
<i>Stichodactyla helianthus</i>	ShK	P29187/ -	Type I	Kv1.1, Kv1.2 Kv1.3, Kv1.4 Kv1.6	-	(Castaneda et al. 1995)
<i>Stichodactyla haddoni</i>	SHTX-1/ SHTX-2	P0C7W7/ -	Type IV	-	430/ Crabs	(Honma et al. 2008)

Type I toxins interfere with binding of radiolabelled dendrotoxin to synaptosomal membranes and block currents through channels with various  $K_V1$  subunits and also intermediate conductance  $K(Ca)$  channels. The residues Ser<sup>20</sup>, Lys<sup>25</sup> and Tyr<sup>23</sup>, are responsible for the binding of ShK to the rat brain  $K_V$  channels (Honma & Shiomi 2006). Corresponding residues conserved in other toxins are also responsible for the same binding process. The dyad Lys-Tyr is thus considered to be essential for the binding of toxins to  $K_V$  channels. In fact, scorpion toxins that block  $K_V1$  channels, have the similar dyad, with the same function (Honma & Shiomi 2006).

Type II toxins, are homologous to Kunitz-type inhibitors of serine proteases. Sea anemone protease inhibitors have been considered to function by inhibiting endogenous proteases in animals themselves or to protect the toxins injected into prey animals or predators from rapid degradation. However, the finding of potassium channel toxins with protease inhibitory activity, such as kalicludines, leads to assume that sea anemone protease inhibitors serve not only as defensive substances but also as offensive substances to paralyze prey animals (Honma & Shiomi 2006). Thus, Kunitz-type protease inhibitor toxins, besides serine protease inhibition, also block various types of cation permeating channels, namely the  $K_V1.2$  channels (Gonzalez et al. 2009).

Type III toxins are not active on  $K_V1$  subunits. They block currents involving  $K_V3$  subunits or ERG (ether-a-go-go,  $K_V11.1$ ) channels. The human ERG is an essential component of cardiac cells that controls the duration of the plateau phase of the action potential (Messerli & Greenberg 2006). Type III toxins, such as BDS-I and II, showed to act by modifying channel gating rather than by directly blocking the channel pore. APETx1 blocks the ERG channels (Castaneda & Harvey 2009).

APETx2 is functionally quite unique. Although sharing 36% to 64% sequence identities with Type III  $K_V$  channel toxins, BDS-I and II and APETx1, it inhibits not potassium channels but acid-sensing ion channels (ASIC3,  $H^+$ -gated  $Na_V$  channels) in sensory neurons, which are implicated in the modulation of pain sensation. ASICs are formed by homomeric or heteromeric association of six different subunits (ASIC1a, ASIC1b, ASIC2a, ASIC2b, ASIC3, and ASIC4) but only ASIC3 channels and ASIC3-containing channels are affected by APETx2 (Honma & Shiomi 2006). This discovery by Diochot et al. (2004) was very important in the toxinology field. However, more recently Blanchard et al. (2012) it was found that this toxin also affects the voltage-gated sodium channel  $Na_V1.8$ , which raises its value as an analgesic tool, while reducing the value as a specific pharmacological tool, as  $Na_V1.8$  is also involved in pain-sensing as ASIC3.

Type IV displaces dendrotoxin binding from synaptosomal membranes but their channel blocking specificity is not yet known (Castaneda et al. 1995).

### 3.3. Cytolysins

Cellular life is dependent on the integrity of cellular membranes that is responsible for controlling the proper transmembrane distribution of solutes. Thus, it is not surprising that membrane permeabilization induced by specifically designed peptides has evolved as a common strategy (Anderluh et al. 2011).

Several sea anemone species have been reported to produce cytolytic peptides. However, in this review we only refer to the 13 species that have the Cytolysins amino acid sequence described. Cytolysins are important as they serve as model proteins to study protein-lipid membrane interaction (Anderluh & Macek 2002). In addition, they are also used to study the eradication of tumour cells and parasites (Tejuca et al. 2009) and have also cardio-stimulating, dermatonecrotic properties and antihistamine activity (Klyshko et al. 2004).

Based on their primary structure and functional properties, Cytolysins have been classified in four polypeptide groups. Type I, consists of 5–8 kDa peptides that form pores in phosphatidylcholine containing membranes and have antihistamine activity.

Type II, the most numerous toxins within Cytolysins, have been extensively studied and comprise 20 kDa proteins, which are inhibited by sphingomyelin. These type II Cytolysins are also called Actinoporins due to its ability to bind the membrane phospholipids domains of the host organism, oligomerizing and forming cation selective pores (Šuput 2009). They belong to the unique family of the  $\alpha$ -pore-forming toxins (PFTs) (Monastyrnaya et al. 2010). The cations-selective hydrophilic pores of around 1nm cause haemolysis. As referred previously, in contrast with Type II, Type I are not inhibited by sphingomyelin, and are less hemolytical. In fact, Type II toxins have a preference for sphingomyelin containing membranes and are all cysteineless proteins with high isoelectric points (>9.5) (Alvarez et al. 2009).

Type III toxins have 30–40 kDa and are formed by Cytolysins with or without PLA activity, being only represented to date by the cytolytic proteins from the genus *Urticina* (Anderluh et al. 2011).

Type IV toxins are thiol-activated Cytolysins with 80 kDa. *Metridium senile* produces metridiolysin that is so far the only representative of this group of toxins (Anderluh & Macek 2002).

There is also another group of Cytolysins that have the membrane-attack complex/perforin (MACPF) domain agents. The MACPF family is best studied in the

immune system. The membrane-attack complex (MAC) of the complement system and perforin (PF) produced by T-cell and killer cells, form pores of up to 20 nm on the target membrane, which leads to cell lyses and death. PsTX-60A and PsTX-60B from *Phyllodiscus semoni* and AvTX-60A from *ActinERIA villosa*, from Japanese sea anemones, belong to this group of Cytolysins. Like perforin, these Cytolysins possess an EGF-like domain next to the MACPF domain (Anderluh et al. 2011). These sea anemone toxins were the first report of MACPF proteins in non-mammalian metazoans. Previously, this membrane-attack complex has been also described in bacteria (Oshiro et al. 2004). Furthermore, the presence of these toxins produced by nematocysts was the first reported case of MACPF proteins recruited into venoms. In this sense, the mode of action in the venom might be explained with the pore-forming action in the same way as the MACPF proteins do it in the mammal's host defence immune system (Sato et al. 2007).

Table II.1-3 summarizes all the sea anemone Cytolysins with amino acid sequence described, and with the same descriptors mentioned for Na<sub>v</sub> channel toxins.

**Table II.1-4. Sea anemone Cytolysins, their accession no., the cluster that share with them 50% similarity, their classification group (toxin family), the LD<sub>50</sub> and reference**

Species	Toxin	UniProt/GenBank Accession Number	Toxin family	LD <sub>50</sub> (µg/Kg)/ Tested Organism	Reference
<i>ActinERIA villosa</i>	Avt-I	Q5R231/ AB175824	II	-	(Uechi et al. 2005a, b)
<i>ActinERIA villosa</i>	Avt-II	D2YZQ3/ AB512460	II	-	(Uechi et al. 2010)
<i>ActinERIA villosa</i>	AvTX-60A	Q76DT2/ AB107916	MACPF	LDmin <250/Mice	(Oshiro et al. 2004)
<i>Actinia equina</i>	Equinatoxin-I	POC1H0/ -	II	23/Mice	(Macek & Lebez 1988)
<i>Actinia equina</i>	Equinatoxin-Ia	POC1H1/ -	II	23/Mice	(Macek & Lebez 1988)
<i>Actinia equina</i>	Equinatoxin-II	P61914/ U41661	II	35/Mice	(Macek & Lebez 1988, Anderluh et al. 1996)
<i>Actinia equina</i>	Equinatoxin-III	POC1H2/ -	II	83/Mice	(Macek & Lebez 1988)
<i>Actinia equina</i>	Equinatoxin-IV	Q9Y1U9/ AF057028	II	-	(Anderluh et al. 1999)
<i>Actinia equina</i>	Equinatoxin-V	Q93109/ U51900	II	-	(Pungercar et al. 1997)
<i>Actinia fragacea</i>	Fragaceatoxin C	B9W5G6/ FM958450	II	-	(Bellomio et al. 2009)
<i>Actinia tenebrosa</i>	Tenebrosin-A	P30833/ -	II	-	(Norton et al. 1990)
<i>Actinia tenebrosa</i>	Tenebrosin-B	P30834/ -	II	-	(Norton et al. 1990)
<i>Actinia tenebrosa</i>	Tenebrosin-C	P61915/ -	II	-	(Simpson et al. 1990)
<i>Anthopleura asiatica</i>	Bandaporin	C5NSL2/ AB479475	II	LD100 0.58/Crayfish	-
<i>Heteractis crispa</i>	Cytolysin RTX-A	P58691/ AY855350	I	50/Mice	(Il'ina et al. 2005)
<i>Heteractis crispa</i>	Cytolysin RTX-S-II	POC1F8/ -	I	70/Mice	(Klyshko et al. 2004)
<i>Oulactis orientalis</i>	Actinoporin Or-A	Q514B8/ AY856481	II	-	(Il'ina et al. 2005)
<i>Oulactis orientalis</i>	Actinoporin Or-G	Q512B1/ AY861662	II	-	(Il'ina et al. 2005)
<i>Phyllo-discus semoni</i>	Pstx-20A	Q81AE2/ AB063314	II	50/Shrimp	(Nagai et al. 2002a)
<i>Phyllo-discus semoni</i>	PsTX-60A	P58911/ AB063315	MACPF	-	(Nagai et al. 2002b)
<i>Phyllo-discus semoni</i>	PsTX-60B	P58912/ AB201429	MACPF	-	(Nagai et al. 2002b)
<i>Radianthus magnifica</i>	HMgI	P58689/ -	II	140/Mice	(Khoo et al. 1993)
<i>Radianthus magnifica</i>	HMgII	P58690/ -	II	320/Mice	(Khoo et al. 1993)
<i>Radianthus magnifica</i>	HMgIII	Q9U6X1/ AF170706	II	-	(Wang et al. 2000)
<i>Radianthus magnifica</i>	Hemolytic toxin	P39088/ -	II	-	(Kristan et al. 2009)
<i>Sagartia rosea</i>	Cytolysin Src-I	Q86FQ0/ AY247033	II	-	(Jiang et al. 2002)
<i>Stichodactyla helianthus</i>	Sticholysin-I	P81662/ AJ009931	II	-	(Huerta et al. 2001)
<i>Stichodactyla helianthus</i>	Sticholysin-II	P07845/ AJ005038	II	-	(Huerta et al. 2001)
<i>Urticina crassicornis</i>	Uc-I	POCG44/ -	III	-	(Razpotnik et al. 2009)
<i>Urticina crassicornis</i>	Urticinatoxin	C9EIC7/ GQ848199	III	-	(Razpotnik et al. 2010)
<i>Urticina piscivora</i>	Up-1	POC1G1/ -	III	-	(Cline et al. 1995)

Given the toxins described to date, the production of Cytolysins does not exclude the production of other toxin types, like neurotoxins. Moreover, Cytolysins may have several isoforms namely five in *Actinia fragacea* (Bellomio et al. 2009) and *Actinia equina* (Anderluh et al. 1999), three in *Actinia tenebrosa* (Norton et al. 1990), two in *Oulactis orientalis* (Il'ina et al. 2005) and at genomic level, more than 50 different gene sequences have been cloned from *Radianthus magnifica* (Wang et al. 2008). In terms of genetic sequence differences there are Actinoporins that are coded by multiple genes that lacks introns, as equinatoxins (Anderluh et al. 1999) and Or-A and Or-G, but there are also some genes, namely Avt-I and PsTX-20A, that have 2 introns with 242 bp and around 600 bp long, respectively (Uechi et al. 2010).

Regarding the Actinoporins in which the protein 3D structures have been already elucidated, it is worthwhile to mention that there is a conserved putative *N*-terminal amphiphilic  $\alpha$ -helix (essential for pore forming activity (Nagai et al. 2002a), a tryptophan-rich stretch (that binds to erythrocyte membranes (Uechi et al. 2010) and a RGD-motif Arg-Gly-Asp (that provides affinity for certain types of cells (Nagai et al. 2002a)), in the primary structure (Anderluh & Macek 2002). The conserved RGD sequence/motif, a peculiar property of some Actinoporins, is located on the surface of protein globule nearby POC (phosphocholine) binding site (Monastyrnaya et al. 2010). In this way, the binding of cytolyisin to the membranes integrin(s) is made not only by the RGD motif but also by this complementary binding site, the POC. However, not all the Actinoporins share the RGD motif, common to RTX-A, Sticholysin-II and Equinatoxin-II. There are exceptions, with motifs in equal positions but with differences in the amino acid sequence, such as Or-A and Or-G (from *Oulactis*), Src-I (from *Sagartia*), PsTX-20A (from *Phyllodiscus*) and Avt-I (from *Actinaria*). They present GGD, EGD and KPS tripeptides, respectively. In the same study, it was found the following differences in functionally regions of *Heteractis crispera* (RTX-A and RTX-S-II), *Oulactis* (Or-A and G), and some other Actinoporins: (i) Trp is substituted by Leu in the position equivalent to Trp<sup>112</sup> in the POC binding site of Equinatoxin-II; (ii) 13 and five residues are truncated in *N*-terminal regions of Or-A and Or-G, respectively (Monastyrnaya et al. 2010).

The pore formation produce by Actinoporins, is conducted by a series of steps. First the toxin attaches to the membrane by the specific recognition of sphingomyelin (but neither cholesterol nor phosphatidylcholine) using the aromatic rich region and the adjacent POC binding site. Then the *N*-terminus hydrophobic face is embedding in the lipid-water interface. This is accompanied by extending the *N*-terminus segment, which is oriented in parallel with the membrane and increases the *N*-terminus helicity. Finally, when the toxin oligomerises on the surface of the membrane, the  $\alpha$ -helices of three or



four monomers insert into the membrane, forming an ion conductive pathway. So, the walls of functional pore consist in  $\alpha$ -helices and lipid molecules (Kristan et al. 2009, Monastyrnaya et al. 2010).

### 3.4. PLA2 Toxins

Phospholipases A2 (PLA2s) catalyze the hydrolysis of 2-acyl ester bonds of 3-*sn*-phospholipids producing fatty acids and lysophospholipids. These enzymes have several important roles in the dietary lipid catabolism, in cell membrane metabolism and inflammatory diseases (Romero et al. 2010). They can be associated with the toxicity of several animal groups, such as snakes, insects, mollusks, cnidarians and sponges (Razpotnik et al. 2010). PLA2 are presynaptic neurotoxins, blocking nerve terminals by binding to the nerve membrane and hydrolyzing stable membrane lipids. The products of the hydrolysis cannot form bilayers leading to a change in membrane conformation and ultimately blocking the release of neurotransmitters. PLA2 may form dimers or oligomers.

There is a family of secreted PLA2s comprising low molecular weight (13–15 kDa) disulfide-linked proteins that depend on  $Ca^{2+}$ -ion for enzymatic activity. PLA2s secreted by the pancreas function as digestive enzymes, while others PLA2 are components of venoms. In addition to secreted PLA2s, there are cytosolic  $Ca^{2+}$ -dependent and independent PLA2-proteins. Based on the molecular structure, PLA2s are classified into various groups numbered from I to XIV and numerous subgroups (Nevalainen et al. 2004). Additional types of Phospholipases include phospholipase A1, phospholipase B, phospholipase C, and phospholipase D.

Albeit PLA2s venom properties have been reported for several cnidarians (Nevalainen et al. 2004), only in a few cases they have been deeply studied. In table II.1-4, we show all the PLA2 that have the amino acid sequence described.

**Table II.1-5. Sea anemone PLA2 toxins, accession number and reference**

Species	Toxin	UniProt/GenBank Accession Number	Reference
<i>Adamsia palliata</i>	AcPLA2	Q8WS88/ AF347072	(Talvinen & Nevalainen 2002)
<i>Bunodosoma caissarum</i>	Cationic protein C1	P0C2M4/ -	(Martins et al. 2009)
<i>Condylactis gigantea</i>	Phospholipase A2	D2X8K2/ GU046515	(Romero et al. 2010)
<i>Urticina crassicornis</i>	UcPLA2	A7LCJ2/ EU003992	(Razpotnik et al. 2010)

The first cnidarians PLA2 fully sequenced was published in 2002 for *Adamsia carcinoapados*, AcPLA2 (Talvinen & Nevalainen 2002). Although AcPLA2 share common features with others PLA2s, such as the *N*-terminal, 12 Cys for putative disulfide formation, and conserved residues found in the sites of activity and  $\text{Ca}^{2+}$ -binding in the catalytically active PLA2s, it differs in others, lacking two extra Cys (specific structural features of group I) and the *C*-terminal extension (of group II and X). Curiously, it resembles group V PLA2 in respect to the number of Cys and the absence of the *C*-terminal extension, but it does contain a *N*-terminal prepropeptide not found in group V. Additionally, a unique Phe is found in the active site instead of Tyr (Talvinen & Nevalainen 2002).

The PLA2 from *Bunodosoma caissarum* has a high amino acid sequence identity to the PLA2 group III proteins isolated from the Mexican lizard and the honey bee (Martins et al. 2009).

UcPLA2 is a PLA2 belonging to group I, isolated from *Urticina crassicornis* inhabiting the northern Pacific Ocean. It is homologous to the AcPLA2, and similar to the Elapidae snake neurotoxic PLAs, suggesting an identical functional role in snake and cnidarians venoms. However, UcPLA2 has some unusual structural features, most notably an Asn at position 27 (instead of a Cys), which is present in the majority of known group I and group II PLA2s. This replacement is rare in invertebrate PLA2s, and has not been found yet in vertebrate toxic and nontoxic PLA2s of group I and group II, with the single exception of the sea lamprey PLA2, which has an Asn at position 27. Also, in UcPLA2 there is a *C*-terminal truncation of six amino acids, including a Cys, so the usual pairing between Cys<sup>27</sup> and Cys<sup>126</sup> is not possible. Recently, several similar proteins were also detected in the *Nematostella vectensis*, implying that this type of PLA2 might be more widespread among cnidarians (Razpotnik et al. 2010).

Recently it was found a PLA2 in *Condylactis gigantea* from Cuba, which is 84% and 61% similar to the *Adamsia carcinoapados* and the *Nematostella vectensis* PLAs, respectively (Romero et al. 2010).

The toxins from *Condylactis gigantea* and *Adamsia carcinoapados* are more closely related to each other, compared to toxins from *Condylactis* and *Urticina crassicornis*, both belonging to the Actiniidae family, although *Condylactis* and *Adamsia* belong to different superfamilies, Endomyaria (namely Actiniidae family) and Acontinaria (namely Hormathiidae family), respectively. In this sense the phylogeny of the species may not be congruent with its toxins phylogeny, as previously mentioned.

### 3.5. Other Toxins

Besides the toxins described above, some others have not been yet fully characterized, and so the classification types previously referred are not yet known for such toxins.

Apart from these “other toxins” there are others that are classified site-3 sodium channel toxins or KV1 potassium channel toxins but are structurally and/or functionally distinct peptides. These include the APETx1 that inhibit an ether-a-go-go related gene potassium channel and the BDS-I and II that show selectivity for KV3.4 channels. APETx2 act on acid-sensing ion channels (Honma et al. 2008).

Until 2005, all the toxins were isolated from the whole body, tentacles or secreted mucus, but Honma & Shiomi (2006) have been able to isolate toxins from the Acrorhagi, special aggressive organs. These toxins, acrorhagins, have no sequence homologies with other toxins from sea anemones, and a low similarity with toxins from other venomous animals, such as spiders and cone snails. In fact, the low similarities and the location of Cys residues suggest a different conformation (Honma & Shiomi 2006). Such differences between acrorhagins and the others toxins suggest that they do not belong to any previously described group of toxins. Bartosz et al. (2008) implemented a study also in acrorhagi from *Actinia equina* and found that the toxins involved in this conspecific aggression induce tissues necroses by intracellular formation of reactive oxygen species (ROS), being also devoid of paralytic-neurotoxic activity.

AETX II and III toxins, do not have yet known effects produced. However, they are supposedly neurotoxins. They are composed of 59 amino acid residues and have 10 Cys residues, probably forming five disulfide bridges and are very lethal to crabs (Honma & Shiomi 2006).

Am-I with 27 amino acid residues, differ from all the other toxins by having four Cys residues. Another peculiarity of this toxin is the six copies of the toxin gene in the precursor sequence (Honma & Shiomi 2006).

Gigantoxin-1 has 35% sequence homology with epidermal growth factors (EGF), and besides EGF activities have also toxic activities. As sea anemones are in the base of the phylogenetic root of the animal kingdom, Honma & Shiomi (2006) hypothesized that Gigantoxin-1 could be the ancestor of EGFs.

Table II.1-5 shows the toxins that are not included in the previous types with the same descriptors mentioned for Nav channel toxins but without the toxin type and target. In addition, there are features that distinguish them from other toxins.

**Table II.1-6. Sea anemone toxins not yet included in any previous classification type. Accession number, their classification group (toxin family), the LD<sub>50</sub>, the features that distinguish them from the others and the reference**

Species	Toxin	UniProt/GenBank Accession Number	LD <sub>50</sub> (µg/kg)	Features	Reference
<i>Actinia equina</i>	Acrorhagin 1	Q3C258/ AB212066	520/Crabs	Produced by acrorhagi	(Honma et al. 2005b)
<i>Actinia equina</i>	Acrorhagin 1a	Q3C257/ AB212067	-	Produced by acrorhagi	(Honma et al. 2005b)
<i>Actinia equina</i>	Acrorhagin 2a	Q3C256/ AB212068	80/Crabs	Produced by acrorhagi	(Honma et al. 2005b)
<i>Actinia equina</i>	Acrorhagin 2a	Q3C255/ AB212069	-	Produced by acrorhagi	(Honma et al. 2005b)
<i>Actinaria villosa</i>	Avt120	E9RGH6/ AB576860	0.085/Mice	Possible similar function as PsTX-115/may inhibit nerve cells	(Uechi et al. 2011)
<i>Anemonia erythraea</i>	AETX-II	P69944/ -	0.53/Crabs	Toxin Type Not Known. Possible Neurotoxin	(Shiomi et al. 1997)
<i>Anemonia erythraea</i>	AETX-III	P69945/ -	0.28/Crabs	Toxin Type Not Known. Possible Neurotoxin	(Shiomi et al. 1997)
<i>Antheopsis maculata</i>	Peptide toxin Am-1	P69929/ AB180685	830/Crabs	Inhibits ion channels	(Honma et al. 2005a)
<i>Bunodosoma granulifera</i>	Granulitoxin	P58305/ -	400/Mice	Neurotoxin	(Santana et al. 1998)
<i>Phyllodiscus semoni</i>	Nephrotoxin PsTX-115	P84851/ -	-/Rats	Nephrotoxin	(Mizuno et al. 2007)
<i>Stichodactyla haddoni</i>	EGF-like peptide SHTX-5	B1B5J0/ AB362571	-	Has both toxic and EGF activity	(Honma et al. 2008)
<i>Stichodactyla gigantea</i>	Gigantoxin-1	Q76CA1/ AB110014	>1000/Crabs	Has both toxic and EGF activity	(Shiomi et al. 2003)

#### 4. Isolation and Purification of Bioactive Toxins

When scientists first extracted venoms from cnidarians, they started with species that had the more widespread toxic effects known. Thus, medusas have been the first organism studied, and all the subsequent works have used the protocol of Bloom et al. (1998) as the major technique (Bloom et al. 1998). In that work, jellyfish tentacles were removed, stored in seawater, and vigorously shaken daily and let to settle to allow the release of the nematocysts. To recover nematocysts, the solution was filtered through a fine sieve. Glass beads, sonication or even freeze-thaw cycles were used with subsequent centrifugation of the solution to remove the cell debris from the venom.

Regarding the venom extraction in medusas, many techniques are employed since then and the techniques for extracting the venoms were obviously improved. In general, several techniques allow the extraction of the sea anemones venoms, which can be removed from the entire animal body or just from parts of the body, such as tentacles, acontia or acrorhagi. The tissue can be processed immediately, frozen or freeze-dried. Moreover, the venom can be obtained without animal injury, just by electric stimulation or gently squeezing the sea anemones. The majority of protocols use water to extract the venom, nonetheless there are other solutions that can be used, such as acetone. Most of the works purify the venom after being obtained, by gel chromatography, followed by reverse phase HPLC. Some of them even go for SDS-PAGE. This chapter does not represent an exhaustive explanation of all the

techniques used and improvements since the 1970s but instead provides a brief overview of the most used techniques to make sea anemones venom extractions, so that beginners can have a compilation of them. Table II.1-6, summarizes the most used protocols. It contains the species used in the paper referenced, the tissue type and amount that scientists used, or the number of individuals collected if they have not sacrificed the animal, the technique used for tissue storage, the solvent used for venom extraction, the mechanical treatment used for venom extraction, the technique used for toxin recovery and reference.

**Table II.1-7. Most employed protocols used for venom extraction in sea anemones. Species used, tissue type, amount of tissue used or number of individuals used, technique used for tissue storage, solvent used for venom extraction, mechanical treatment used for venom extraction, technique used for toxin recovery and reference**

Species	Tissue	Amount	Storage	Solvent	Mechanical treatment	Toxin Recovery	Reference
<i>Bunodosoma caissarum</i>	Not damage	10 individuals	Kept alive	Artificial sea water	Electric stimulation	Filtration	(Malpezzi et al. 1993)
<i>Heteractis magnifica</i>	Not damage	1 kg	Kept alive	Water	Stirred (gently)	Filtration, lyophilization	(Wang et al. 2000)
<i>Adamsia carcinopados</i>	Body, Tentacles, Acontia	-	-20 °C	Sodium chloride	Sonication	Centrifugation	(Talvinen & Nevalainen 2002)
<i>Stichodactyla gigantea</i>	Body	5 g	-20 °C	Water	Motor, blender	Centrifugation	(Shiomi et al. 2003)
<i>Heteractis crispa (syn-Radianthus macrodactylus)</i>	Body	1 kg	-20 °C	Water, acetone	Bender	Centrifugation, evaporation	(Klyshko et al. 2004)
<i>Aiptasia mutabilis</i>	Acontia	-	Kept alive and fed	Sodium citrate	Sonication	Centrifugation	(Marino et al. 2004)
<i>Bunodosoma caissarum</i>	Not damage	30 individuals	Kept alive and starved	Artificial sea water	Electric stimulation	Filtration	(Oliveira et al. 2004)
<i>Actinaria villosa</i>	Globular vesicles	-	Kept alive	Phosphate buffer	Shaken (vigorously)	Centrifugation	(Uechi et al. 2005a)
<i>Actinia equina</i>	Acorrhagi	2 g	Kept alive	Water	Motor, blender	Centrifugation	(Honma et al. 2005b)
<i>Stichodactyla gigantea</i>	Tentacles	5 g	Lyophilization	Water	Bender	Centrifugation	(Hu et al. 2011)
<i>Condylactis gigantea</i>	Body	11 kg	-20 °C	Ethanol, acid acetic, acetone	Bender	Filtration, evaporation, centrifugation	(Standker et al. 2006)
<i>Anemonia erythraea</i>	Body	5 g	-80 °C, -20 °C	Water	Motor, blender	Centrifugation	(Hasegawa et al. 2006)
<i>Bunodosoma caissarum</i>	Not damage	-	Kept alive	Artificial sea water	Electric stimulation	Filtration	(Oliveira et al. 2006)
<i>Stichodactyla hadonni</i>	Body	5 g	-80 °C, -20 °C	Water	Motor, blender	Centrifugation	(Honma et al. 2008)
<i>Bunodosoma cangicum</i>	Not damage	20 individuals	Kept alive	Artificial sea water	Electric stimulation	Filtration	(Zaharenko et al. 2008b)
<i>Actinia fragacea</i>	Not damage	50 individuals	Kept alive	Put together in a beaker	Collection of exudate and gently squeezed	Centrifugation	(Bellomio et al. 2009)
<i>Bunodosoma caissarum</i>	Tentacles	-	Kept alive and starved	Trifluoroacetic acid	Freeze-thaw cycles	Centrifugation, filtration	(Martins et al. 2009)
<i>Urticina crassicornis</i>	Not damage	-	Kept alive	Put together in a beaker	Collection of exudate and gently squeezed	Filtration, centrifugation	(Razpotnik et al. 2010)
<i>Cryptodendrum adhaesivum, Heterodactyla hemprichii, Thalassianthus aster</i>	Body	5 g	-80 °C, -20 °C	Water	Motor, blender	Centrifugation	(Maeda et al. 2010)

While there have not been any paper evaluating the technique's merit, the procedures that do not injury the animal are better for the obvious reason plus the venom seem to be better in terms of purity and it can be achieved also in good amounts. Moreover, frozen the specimens at  $-20\text{ }^{\circ}\text{C}$  is also practicable, and good results have been obtained even without using  $-80\text{ }^{\circ}\text{C}$  freezers, so this conservation method would be a good starting point. Using water as a solvent and a blender, followed by centrifugation, is an easy technique not time consuming nor demanding in terms of materials or skills, thus can be also used as a starting point for venom extraction. Regarding the toxins extraction only from the nematocysts, this can be accomplished if subjecting these structures to sonication or freeze-thaw cycles to burst and release the content.

## 5. Toxin Genes

The first toxins to be studied at genomic level were Equinatoxins and they proved to be intronless. Similarly, Or-A and Or-G also do not contain introns (Il'ina et al. 2005). Afterwards it was found that some toxins, namely Cytolysins Avt-I and Pstx-20A, have three exons (two introns). Moreover, they are coded by at least two genes. Such gene arrangement is not exclusive of Cytolysins. The neurotoxic Clx-I and II, and HmK also have genes that are interrupted by two introns and their exon-intron organization is quite similar to the Avt-I genome structure. The introns-exon junctions that are typical donor and acceptor splice sites have followed the GT/AG rule, in which the introns begin with GT and end with AG (Uechi et al. 2010). In the work of Gendeh et al. (1997a) on HmK, a similar organization on introns-exon junction in scorpion toxins has been reported, suggesting that molecules with similar functions have similar organization at genomic level, therefore implying a common evolutionary path.

More than five equinatoxins genes are found and two isoforms of Equinatoxin-I (Anderluh et al. 1999). ATX-II is encoded by at least seven genes (Moran et al. 2009a). In 2008, Wang et al. (2008) showed that magnificalyxins (HMgs) are also encoded by a multigene family, with each member encoding an isoform. They cloned more than 50 genes, all intronless. From the *Nematostella vectensis* whole genome release, it was found 13 genes that encode for the Nv1 toxin. Thus, toxins that are encoded by gene families may be more common than previously believed. Indeed, alternative splicing is not commonly assumed for toxins that have not yet the gene(s) sequenced.

The eight genes that code for the same toxin, Nv1, are arranged more or less sequentially and this supports the concerted evolution theory (Moran et al. 2009b). This theory is corroborated by what it happens in yeast- unequal crossover. In this unusual

phenomenon, the sequence of genes is homogenized through unequal crossing over and gene conversion, resulting in an arrangement where two gene family members from one species are more similar to one another, than to their corresponding homologues in other species (Moran et al. 2009b). The advantage of this mechanism, having several copies of the same gene, is to produce rapidly a huge amount of venom. The nematocyst is discarded after each discharge, and the absence of a specialized venom organ/gland provide emphasis to this hypothesis. They also add that a multigene family, give organisms two more advantages: (i) the rapid transmission of advantageous mutations and (ii) the prevention of the loss of a highly effective toxin (Moran et al. 2009a). In the same work, some putative toxins in *Anemonia viridis* and *Actinia equina* were found to evolve in the opposite manner, by accelerated evolution, similar to what happens commonly in other venomous animals. Some toxins may escape from the concerted evolution process, diverging rapidly and with mutations being influenced by its selective value and neutral genetic drift (Moran et al. 2009a). Diversifying selection or Darwinian selection promotes the fixation of non-synonymous substitutions and “accelerates” the diversification of related sequences. This high substitution rate is typical to the region encoding the mature toxin. In contrast, the regions encoding the signal peptide and pro part, which are involved in secretion, are usually highly conserved. In fact, in another work, the analysis of ATX-I and ATX-III from *Anemonia viridis*, revealed that besides the differences between the two toxins and 3D structure, the signal was conserved. This has likely been generated by gene fusion and advantageous in transcript stability and intracellular trafficking and secretion (Moran et al. 2009b).

More recently, the genes encoding Kunitz-type toxins from *Heteractis crispa* have been studied (Isaeva et al. 2011). Kunitz-type proteins are encoded by four distinct gene families (GS-, RG-, GG-, and GN-gene families). In one family studied (GS), several homologues peptides were found. Moreover, the Open Reading Frame is interrupted by a single intron located at the middle of the signal peptide. The scientists suggest that the gene family in case evolved through gene tandem duplication followed by adaptive divergence of the reactive site (a particularly group of amino acids). Furthermore, this evolution seem to be lineage-specific, increasing the ability of *Heteractis crispa* to interact with multiple preys and foes (Isaeva et al. 2011).

Genes and transcripts of toxins are determined by PCR and degenerate primers by RACE, usually from the cDNA sequence and cloning.

## 6. Three-Dimensional Toxins Structures

Descriptions of sea anemone protein structures involved in venom activity have been determined by nuclear magnetic resonance (NMR) or X-ray crystallography. Apart from three Actinoporins (Equinatoxin-II, Sticholysin-II and Fragaceatoxin C), all the other toxins were determined by solution NMR. In the same manner, all the toxins have only one chain, except the Actinoporins that have two, or six in the case of fragaceatoxin. Table II.1-7 shows all the sea anemone toxins with three-dimensional structures described, the species from which it was purified, the type, the resolution method, the number of chains of the molecule and the amino acid number, and the RCSB PDB ID.

**Table II.1-8. Sea anemone toxins with 3D-structures studied. Species where the structure was purified, type, resolution method, number of chains of the molecule and amino acid number, and Pubmed accession number**

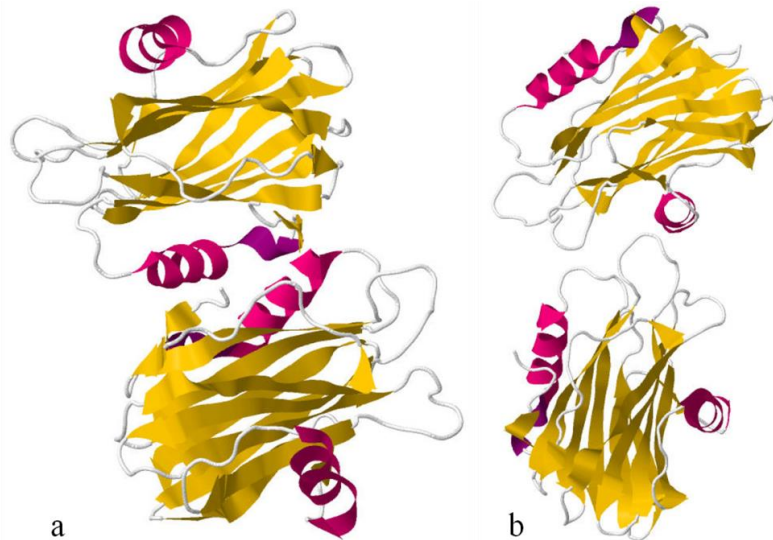
Species	Toxin	Type	Method	Number of Chains	Length (amino acid Number)	PDB ID
<i>Actinia equina</i>	Equinatoxin-II	Actinoporin	X-Ray Diffraction	2	179	1IAZ
<i>Actinia fragacea</i>	Fragaceatoxin C	Actinoporin	X-Ray Diffraction	6	178	3LIM
<i>Stichodactyla helianthus</i>	Sticholysin-II	Actinoporin	X-Ray Diffraction	2	175	1GWY
<i>Anthopleura elegantissima</i>	APETx1	K <sub>v</sub> channel	Solution NMR	1	42	1WQK
<i>Anthopleura elegantissima</i>	APETx2	K <sub>v</sub> channel	Solution NMR	1	42	1WXN
<i>Anemonia viridis</i>	BDS-1	K <sub>v</sub> channel	Solution NMR	1	43	1BDS
<i>Bunodosoma granulifera</i>	BgK	K <sub>v</sub> channel	Solution NMR	1	37	1BGK
<i>Stichodactyla helianthus</i>	ShK	K <sub>v</sub> channel	Solution NMR	1	35	1ROO
<i>Stichodactyla helianthus</i>	ShPI-1	Kunitz type proteinase inhibitor	Solution NMR	1	55	1SHP
<i>Anemonia viridis</i>	ATX-IA	Na <sub>v</sub> channel	Solution NMR	1	46	1ATX
<i>Anemonia viridis</i>	ATX-III	Na <sub>v</sub> channel	Solution NMR	1	27	1ANS
<i>Anthopleura xanthogrammica</i>	Anthopleurin-A	Na <sub>v</sub> channel	Solution NMR	1	49	1AHL
<i>Anthopleura xanthogrammica</i>	Anthopleurin-B	Na <sub>v</sub> channel	Solution NMR	1	49	1APF
<i>Condylactis gigantea</i>	CgNa	Na <sub>v</sub> channel	Solution NMR	1	47	2H9X
<i>Stichodactyla helianthus</i>	Sh1	Na <sub>v</sub> channel	Solution NMR	1	48	1SH1

The first 3D protein structures of sea anemone were obtained in the 80's by NMR analysis, for the Anthopleurin-A and ATX-IA (Gooley et al. 1984, Widmer et al. 1989). However, the first pore-forming toxin to be studied was the Equinatoxin-II in 2001.

Sea anemone Actinoporins, Equinatoxin-II and Sticholysin-II, display an extremely similar structural organisation. The molecule is composed of a tightly folded  $\beta$ -sandwich core flanked on two sides by  $\alpha$ -helices (Figure II.1-6). The first 30 amino acids encompass one of the helices. This is the only part of the molecule able to



undergo a conformational change without any structural change of the  $\beta$ -sandwich. A prominent patch of aromatic amino acids is located on the bottom of the molecule. It comprises a completely exposed Trp<sup>112</sup> (in EqtII), which was shown to participate in the initial binding of the toxin to the lipid membrane (Kristan et al. 2009). Comparing Fragaceatoxin C (FraC) with these two other actinoporins, it has a  $\beta$ -sandwich core flanked by three helices and the N-terminal domain is more “detached” from the protein core (Mechaly et al. 2009, 2011).

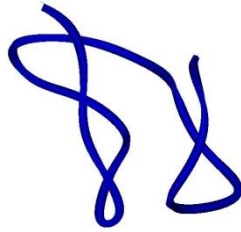


**Figure II.1-6. Ribbon view of Equinatoxin-II (a) and Sticholysin II (b), showing their secondary structure.  $\alpha$ -helices, in pink, and  $\beta$ -sandwiches, in yellow.**

As previously referred, Actinoporins form pores in membranes by rearranging themselves in a four monomers structure (Mechaly et al. 2012). For this purpose, four regions seem to be important in this step: A cluster of aromatic residues, a phosphocholine binding site, an array of basic amino acids, and the *N*-terminal  $\alpha$ -helix. Initial binding of the soluble monomers to the membrane is accomplished by the cluster of aromatic amino acids, the array of basic residues, and the phosphocholine binding site. Then, the *N*-terminal  $\alpha$ -helix detaches from the  $\beta$ -sandwich, extends, and lies parallel to the membrane. Simultaneously, oligomerization occurs. Finally, the extended *N*-terminal  $\alpha$ -helix penetrates the membrane to build a toroidal pore (Garcia-Ortega et al. 2011).

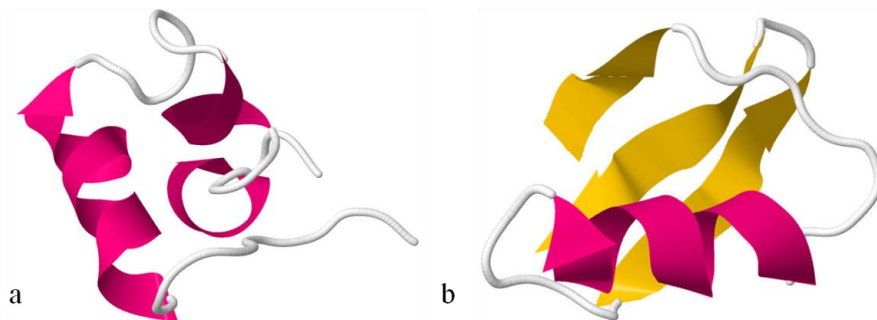
The Na<sub>v</sub> channel toxin from *Anemonia viridis* has a complete different conformation. ATX III adopts a compact structure, being the smallest of the structures, containing four reverse turns (a distorted type I  $\beta$ -turn, a type I  $\beta$ -turn, and an inverse  $\gamma$ -turn) and two other chain reversals, but no regular  $\alpha$ -helix or  $\beta$ -sheet (Figure II.1-7). In this molecule, several of the residues most affected by aggregation are located on the

surface of the molecule (Manoleras & Norton 1994), which cluster on one hemisphere and include a patch of hydrophobic residues only partially exposed (Moran et al. 2007).



**Figure II.1-7. Ribbon view of ATX-III. A peptide toxin from *Anemonia viridis* that interacts with Nav channels. It does not contain  $\alpha$ -helices or  $\beta$ -sheets.**

With the analysis of the BgK structure, Dauplais et al. (1997) verified that toxins with different structures from different organisms, like BgK from a sea anemone and ChTX, charybdotoxin (2CRD-PDB ID), from a scorpion, which bind the  $K_v$  channel, have conserved a dyad. Such dyad is composed of an essential Lys assisted by a more or less distant aromatic residue, whose precise nature (Tyr or Phe) and location may differ from one toxin to another. This fact suggests a convergent functional evolution for these small proteins (Dauplais et al. 1997). See Figure II.1-8 for structural differences between BgK and ChTx toxins and Figure II.1-9 for conserved dyad between the two toxins.



**Figure II.1-8. Ribbon structures of BgK (a) and ChTx toxins (b). BgK lacks the  $\beta$ -sheet secondary structure, while ChTx and most of the scorpion toxins have  $\beta$ -sheet at both ends of the molecule. Also, the molecular scaffolds for the  $K_v$  channel-binding surfaces of each toxin are of different type: helix (in pink) for BgK and  $\beta$ -sheet (in yellow) for ChTx (Kem et al. 1999).**

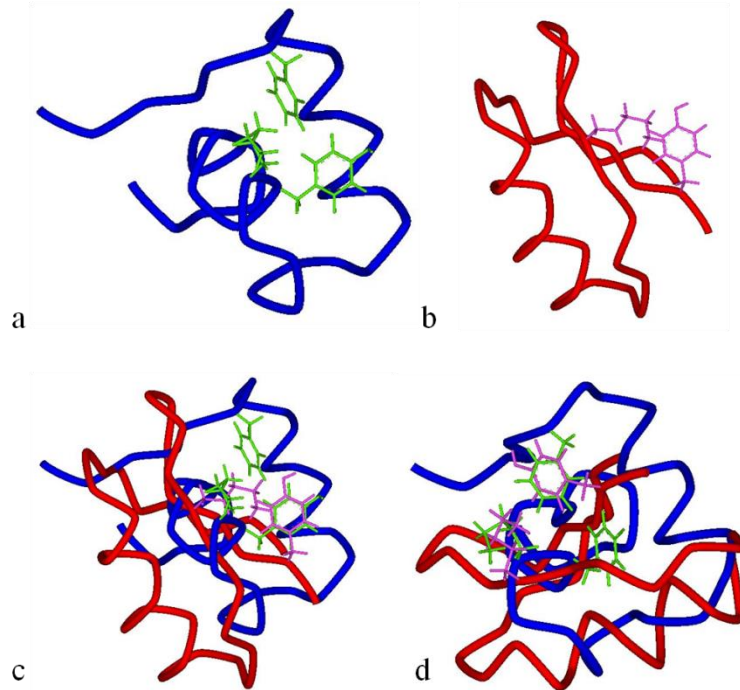
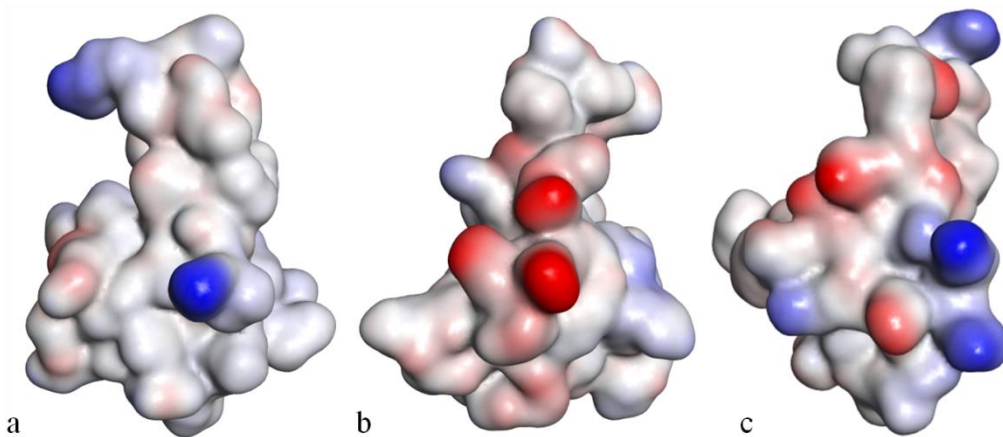


Figure II.1-9. View in tube style of BgK in blue (a) and ChTx in red (b) molecules, with the residues Lys<sup>25</sup>/Tyr<sup>26</sup>/Phe<sup>6</sup> in green and Lys<sup>27</sup>/Tyr<sup>36</sup> in pink, respectively. Superimposition of the functional dyad Lys<sup>27</sup>/Tyr<sup>36</sup> (in pink) from ChTx with the functional dyad of BgK Lys<sup>25</sup>/Tyr<sup>26</sup> in (c) and with the functional dyad Lys<sup>25</sup>/Phe<sup>6</sup> in (d) (adapted from (Dauplais et al. 1997)).

The structure of CgNa, which was solved by NMR spectroscopy, is somewhat atypical and display significant homology with both type I and type II anemone toxins in amino acid sequence. CgNa also displays a considerable number of exceptions to the canonical structural elements that are thought to be essential for the activity of this group of toxins. Furthermore, unique residues, as Asp<sup>36</sup>, Glu<sup>37</sup> and Glu<sup>43</sup> in CgNa (instead of Trp<sup>45</sup> in Anthopleurin-B), define a characteristic structure with strong negatively charged surface patches (Figure II.1-10). These patches disrupt a surface-exposed cluster of hydrophobic residues present in all anemone-derived toxins described to date. CgNa preferentially binds to TTX-S (tetrodotoxin-sensitive) Nav channels in the resting state. The specific structural features of CgNa may explain its weaker inhibitory capacity when compared with the other type I and II anemone toxins (Salceda et al. 2007).



**Figure II.1-10.** Solvent-accessibility surface representations of Anthopleurin-B (a), CgNa (b) and Sh1 (c), evidencing the electrostatic potential at the surface of the molecule. The color blue represents highly positive and red, highly negative, in grade (adapted from Salceda et al. (2007).

ATX-IA, Anthopleurin-A and B and Sh1 are constituted by four-stranded  $\beta$ -sheets. In the ATX-IA they are connected by two loops and there is an additional flexible loop consisting of 11 residues (Widmer et al. 1989). In Anthopleurin-A and Sh1 they are connected by three loops (Fogh et al. 1990, Pallaghy et al. 1995) and in Anthopleurin-B by several  $\beta$ -turns (Monks et al. 1995). Anthopleurin-A, B and Sh1 have antiparallel  $\beta$ -sheets. APETx1 and BDS-I have three-stranded anti-parallel  $\beta$ -sheets. In addition, BDS-I has one more mini antiparallel  $\beta$ -sheet at the *N*-terminus. The  $\beta$ -sheet is connected by a long exposed loop (Driscoll et al. 1989).

The calculated structure of APETx1 belongs to the disulfide-rich all- $\beta$  structural family, in which a three-stranded anti-parallel  $\beta$ -sheet is the only secondary structure. APETx1 is the first Ether-a-go-go effector discovered to fold in this way (Chagot et al. 2005). The hERG (the **h**uman **E**ther-**à**-go-go-**R**elated **G**ene) is a gene (KCNH2) that codes for a protein known as  $K_{v11.1}$  potassium ion channel. This ion channel (sometimes simply denoted as “hERG”) is best known for its contribution to the electrical activity of the heart that coordinates the heart’s beating.

The structures of the  $K_v$  channel toxins, as BDS-I, APETx1 and APETx2 are similar to those of the  $Na_v$  channel toxins such as Anthopleurin-A, but quite different from the ShK/BgK family of  $K_v$  channel toxins. This evidence clearly shows that sea anemones are capable of using a common structural scaffold to create blockers of distinct targets, e.g. Anthopleurin-A, APETx1 and APETx2 act on  $Na_v$  channel, hERG and ASIC channels, respectively, while also using different scaffolds (all- $\beta$  in APETx1 vs. all- $\alpha$  in ShK) to block similar channels (hERG and  $K_{v1}$ , respectively) (Norton 2009).

## 7. Conclusions

Despite the recent increasing effort to study cnidarians venoms, much more is yet to be done to characterize these compounds in this diverse group of animals. The venom from each species of cnidarians is supposed to contain around 100 compounds, but not more than 1% is currently known even in the better studied species. Indeed, a recent work described 156 peptide venom compounds in a single species (Rodriguez et al. 2012). In this sense, toxins such as Actinoporins or PLA2s could be particularly interesting. First, they are less studied than other toxins in cnidarians and second, concerning PLA2s, they are a wide group of toxins also encountered in other animals, as in the better studied snakes. However, much more effort is also needed to pursue the study of ion channel toxins, which will allow a better understanding, not only of the diversity of those toxins, but also of the function of ion channels.

Another field starting to gain relevance is toxin gene detection. The increase in availability of genome sequences for venomous animals or cnidarians in general, added to the development of deep sequencing technology, will enable in depth study of genes encoding toxins. Such genomic studies will not only shed light on the evolutionary mechanisms influencing venom evolution but also, more broadly, on the genetic processes that underline the evolution of novel functionalities.

Cnidarians have impressive strategies for locomotion, feeding and reproduction. Its detailed study may allow unraveling the key for new medical drugs, as well as better understand the diversification of the molecular novelties of these unique metazoan species. For all such reasons, the study of cnidarians in whatever field is of great importance. From genetic and ecological studies to more applied pharmacological and toxicological assessments, these soft body animals should be a target of future scientific research.

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## II.2 Chapter

Phylogenetic study of the intertidal sea anemones from Portugal

# Phylogenetic study of the intertidal sea anemones from Portugal

## Abstract

Sea anemones are one of the most ubiquitous organisms in the rocky shores of the Atlantic Ocean. Here, we studied the intertidal sea anemones from several beaches of the Portuguese coast (sampled in three different areas separated by 250Km from each other). The inventory of the most common species revealed the presence of three families, namely (i) Actiniidae, which includes *Actinia equina*, *Actinia fragacea*, *Anemonia viridis*, *Aulactinia verrucosa* (*Bunodactis verrucosa*), and *Anthopleura krebsi* species, (ii) Hormathiidae, which includes *Calliactis parasitica*, and (iii) Sagartiidae, including *Cereus pedunculatus*. We analyzed sequences from one nuclear (18S) and four mitochondrial (16S, COI, COIII and ND6) markers to perform the first phylogenetic assessment of the Portuguese intertidal sea anemones. Our study revealed 58 new haplotypes, which have allowed us to generate a more robust and better-resolved phylogenetic tree relatively to previous works. The intertidal sea anemones here studied did not reveal phenotypic or genotypic heterogeneity within species among the different locations sampled. The sea anemones tissue preserved with ethanol and in particular, anemones column tissue, yielded higher amounts of DNA compared to -80°C preserved tissue and to other parts of the sea anemone body such as tentacles, gastrodermis or ectodermis.

**Keywords:** Anthozoa, Phylogeny; nuclear markers, mitochondrial markers, DNA extraction

## 1. Introduction

The phylum Cnidaria is a diverse group of relatively simple animals characterized by a specialized cell called the cnidocyte. This cell can have three types of cnidae (nematocysts, ptychocysts, and spirocysts). Actiniidae is the family from the class Anthozoa that harbors sea anemones. Anthozoa includes also corals, hydroids, jellyfishes and sea fans, which are abundant and common in marine environments and well known by the scientific community and public in general.

Sea anemones name comes from their resemblance with terrestrial flowers. They are predatory animals that live majority in seawater, having huge ecological success, including an enhanced ability to form symbiotic relationships. Such associations can be seen with hermit crabs (e.g. the sea anemone *Calliactis parasitica* (COUCH) gains food while the hermit crab obtains protection from predators (Pretterebner et al. 2012)), with fish (e.g. the sea anemone *Heteractis* or *Stichodactyla* gains protection from predators and parasites while the fish gets refuge plus food (Litsios et al. 2014)), and even with the zooxanthellae algae (e.g. the *Anemonia viridis* (FORSSKÅL) sea anemone benefits by receiving oxygen and food in the form of glycerol, glucose and alanine from photosynthesis, while the algae receives carbon dioxide from the host respiration, and nutrients such as nitrogen and phosphorus from the hosts metabolism, plus a shelter to live in (Revel et al. 2016)).

Cnidarians are a source of marine natural compounds with potential biomedical application (Mariottini & Grice 2016). An important feature of these animals, studied by several scientists, is their high regenerative ability. An individual polyp may exceed 50 years in nature and 150 years in captivity (Daly et al. 2008).

Cnidarians are structurally simple at level of tissue organization, being composed of two epithelia, the ectoderm and endoderm. The ectoderm is also referred as epidermis and the endoderm as the gastrodermis (Fautin 2009). Due to their simple organization, these organisms held polysaccharides in their body constitution (Dellacorte 1994, Stabili et al. 2015), as well as pigments (Sahu et al. 2012) or polyphenols (Leone et al. 2015) along with other molecules that interfere negatively with genomic DNA extraction and downstream DNA applications (Pinto et al. 2000), which makes difficult the genetic study of these animals.

Morphological characters used in traditional taxonomy such as the nematocysts of the acontia, the marginal sphincter muscle, and the mesenteries, are highly homoplasious, meaning that are shared by several anemone taxa, but not evolved from a common ancestor, and therefore unreliable for an accurate classification (Rodríguez et al. 2012). Moreover, due to high phenotypic variability within species, morphological

approaches can failed to recognize natural taxa (Huang et al. 2009). Therefore, researchers have increasingly used molecular techniques to study cnidarian groups. However, as referred previously, DNA extraction from invertebrates and marine organisms can be challenging, with only a few reports focusing to date on this matter (Schizas et al. 1997, Pinto et al. 2000). In addition, the mitochondrial genome in cnidarians is more slowly evolving than that of bilaterians (Daly et al. 2008), which complicates the study of different species but morphological similar (cryptic species) with common markers. This may explain why just one work has been published to date on intertidal Portuguese sea anemones (Pereira et al. 2014).

Portuguese sea coast has several geographical events that could influence marine organisms, such as upwelling phenomena (Sibaja-Cordero & Cortés 2008) or undersea canyons. Undersea canyons can considerable influence taxonomic composition and patterns in biodiversity, abundance and community structure (Cunha et al. 2011, Amaro et al. 2012). In Portugal there are three major undersea canyons, in Nazaré, Cascais and Setúbal, being the most promising, the Nazaré canyon. Nazaré canyon is situated more or less in the middle of the Portuguese coast line and has around 5000 meters depth and 170 kilometers long (Instituto\_Hidrográfico 2016). In that sense, we collected specimens from three different areas separated by 250Km from each other. The previous mentioned canyons also separate these areas.

In this work we have try to address the following questions: Which are the most common sea anemone species encounters in the intertidal Portuguese coast? Are there phenotypic or genotypic differences in the species from the three separate locations across the Portuguese coast? Relatively to the genotypes, are sea anemones from the Portuguese coast different from others found worldwide? Which is the best approach to store sea anemone tissue for DNA extraction and what is the most appropriate DNA extraction method?

Aiming to answer such questions we assessed the phylogeny of the sea anemones species commonly found in the beach shores of Portugal, by amplifying one nuclear (18S rRNA gene) and four mitochondrial markers (16S rRNA gene, the cytochrome oxidase I gene (COI), the cytochrome oxidase III gene (COIII) and the NADH-ubiquinone oxidoreductase chain 6 gene (ND6)) in three families of sea anemones encompassing seven species. Furthermore, we also improved the method for DNA extraction in sea anemones, as well as determined the best long storage method and the best body part (tentacles, gastrodermis, column or ectodermis) to obtain a higher concentration and good DNA quality.

Briefly, we conclude that Actiniidae, Hormathiidae and Sagartiidae were the more common families of sea anemones. The species are homogeneous regarding to their



genetic composition independent of the geographic location. The Portuguese specimens are phenotypically homogeneous and the best approach for conducting genomic assays on these organisms is to store their tissues in ethanol.

## 2. Material and Methods

### 2.1. Taxon sampling

Collection of sea anemone specimens was performed during 2010 and 2011 in the intertidal shore of 12 beaches from the Portuguese coast (see Figure II.2-1). A total of 42 samples corresponding to seven species and three families were used in this study. Based on the geographical location, samples were divided in three groups: North, Center and South. Overall we considered six specimens of *Actinia equina* (LINNAEUS), 12 of *Actinia fragacea* (TUGWELL), four of *Anemonia viridis* (FORSKAL) 10 of *Aulactinia verrucosa* (PENNANT), four of *Anthopleura krebsi* (DUCHASSAING & MICHELOTTI), four of *Calliactis parasitica* (COUCH) and two of *Cereus pedunculatus* (PENNANT). Our specimens were identified based on its morphological diagnostic characters according to the literature (Campbell et al. 1994, Saldanha 2003, Wirtz et al. 2003, Boyra et al. 2008, Prados 2008, Monteiro et al. 2010) and by its genetic identification with the primers used.

The samples were stored as soon as possible at -80°C or in 99% ethanol until use.

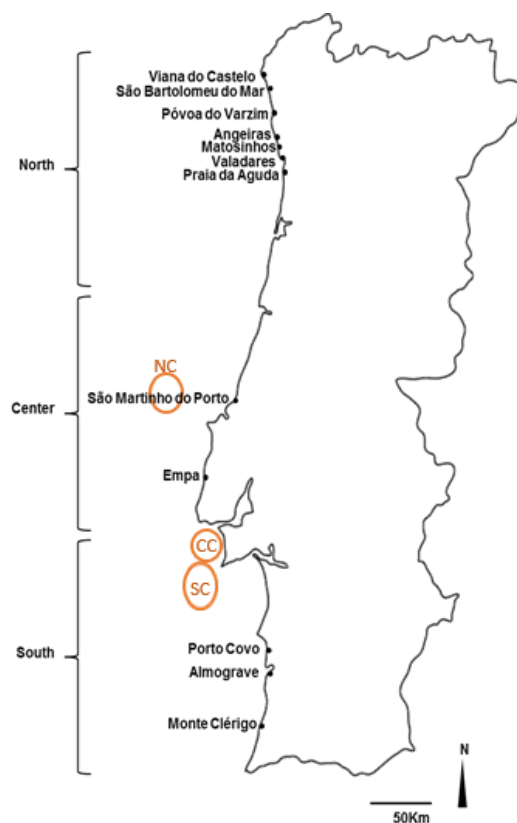


Figure II.2-1. Map showing the 12 sampling site locations in the Portuguese coast. Based on the geographical location samples were divided in three groups: North, Center and South. Orange circles represent canyons: NC-Nazaré Canyon, CC- Cascais Canyon and SC- Setúbal Canyon

## 2.2. Molecular data collection

Due to the difficulty in extracting large amounts of good quality DNA from sea anemones, we conducted an experiment with the purpose of inferring the best method for DNA storage, as well as the best source of tissue to obtain the higher amount and good quality DNA. In that sense, we conducted our analysis in 17 individuals preserved in either 99% ethanol or at  $-80^{\circ}\text{C}$  and collected tissue samples from four body parts: tentacles, gastrodermis, column and ectodermis. DNA extraction was performed according to the method described below and its quantification ( $\text{ng}/\mu\text{l}$ ) and contamination inference (Abs260/280 ratio) was measured in NanoDrop™.

Genomic DNA was isolated from the organism by the phenol-chloroform method with the use of the Precellys® homogenizer. This mechanical digestion is critical to the success of the subsequent steps. Cnidarians tissue, revealed to be very difficult to digest only by chemical digestion or even using a mortar and pestle with liquid nitrogen, likely due to the considerable amount of polysaccharides present in the samples (Dellacorte 1994, Stabili et al. 2015). Mechanical digestion was processed at 6800 rpm, for 2 times at 30s or until complete digestion. The tissue was dissolved in digestion

buffer composed of Tris 10 mM, pH 8.0, ethylenediaminetetraacetic acid 100 mM, sodium dodecylsulfate 0.5% and 1 mg/ml proteinase K (Applichem Lifescience). Afterwards there was an addition of 10% Cetrimonium (hexadecyltrimethylammonium) bromide (Sigma) (CTAB) in 0.7 M NaCl to a final concentration of 0.3% of the sample volume (Moran et al. 2008). The CTAB remove polysaccharides and polyphenols that co-precipitate and interfere negatively with the DNA extraction (Leone et al. 2015). The samples were then incubated 20 minutes at 65°C and then an equal volume of chloroform/isoamyl alcohol (24:1) was added, mixed, and centrifuged at 16000 g for 20 min. This emulsion forms an interphase of macromolecules and polysaccharides that cannot be disturbed when pipetting the supernatant. This CTAB addition and centrifugation steps were repeated until the interphase became invisible. Finally, an addition of phenol: chloroform: isoamyl alcohol (25:24:1) in equal volume was preformed, mix and centrifuged as previously described. To the supernatant was added 1/10 NaCl 6M, and 2.5 volumes of absolute ethanol. The DNA was left to precipitate at -80°C for 1h. Afterwards a centrifugation was made at 16000g, 20min at 4°C. The pellet was washed with 70% ethanol and centrifuged as described previously. The supernatant was drained off at maximum volume possible. The pellet was left to air dry for 10 min and resuspended with TE buffer.

PCR products for one nuclear (18S) and four mitochondrial markers (16S, COI, COIII and ND6) were amplified on a thermocycler using the primers referred in table II.2-1. Markers were chosen based on previous works. COI gene in anthozoans has a characteristic that is worth to mention, has a group I intron. Previous works (Fukami et al. 2007) on corals and in the sea anemone *Metridium senile* showed the presence of this group I intron. In our work, we have used primers spanning the region of the first exon of the COI gene.

**Table II.2-1. List of primers used in this study**

Primer	Sequence	Reference
<b>anem16sa</b>	CACTGACCGTGATAATGTAGCGT	(Geller & Walton 2001)
<b>anem16sb</b>	CCCCATGGTAGCTTTTATTTCG	(Geller & Walton 2001)
<b>16PF</b>	TCGACTGTTTACCAAAAACATAGC	(Bridge et al. 1992)
<b>16PR</b>	ACGGAATGAACTCAAATCATGTAAG	(Bridge et al. 1992)
<b>dgLCO1490:</b>	GGTCAACAAATCATAAAGAYATYGG	(Meyer et al. 2005)
<b>dgHCO2198:</b>	TAAACTTCAGGGTGACCAARAAYCA	(Meyer et al. 2005)
<b>COIIIF</b>	CATTTAGTTGATCCTAGGCCTTGACC	(Geller & Walton 2001)
<b>COIIIR</b>	CAAACCACATCTACAAAATGCCAATATC	(Geller & Walton 2001)
<b>ND6F</b>	CATTTGGGRCYRTTGCTCT	Design for NADH chain 6 from sea anemone
<b>ND6R</b>	CTGWYCKATYTCTTGRG	Design for NADH chain 6 from sea anemone
<b>18S_A</b>	AACCTGGTTGATCCTGCCAGT	(Schmitt et al. 2005)
<b>18S_B</b>	TGATCCTTCTGCAGGTTACCTAC	(Schmitt et al. 2005)
<b>18S_2F</b>	CTGGTGCCAGCAGCCGCGG	(Schmitt et al. 2005)
<b>18S_1R</b>	TGGTGCCCTCCGTCATTCTCT	(Schmitt et al. 2005)

For designing the primers for ND6 gene, the two phylogenetic nearest of the available sequences from Actiniaria species were downloaded from the NCBI and the primers were designed with Primaclade (Gadberry et al. 2005). The primers were further analyzed, concerning secondary structures, by the online program OligoAnalyzer® - Integrated DNA Technologies.

For the published primers, standard protocols were used. For ND6 gene amplification it was used the following protocol: initial denaturation at 95 °C for 3 min, followed by 35 cycles of 94 °C for 30 secs, 50 °C for 40 secs, and 72 °C for 90 secs, and a final extension at 72°C for 10 min. Annealing temperatures were 46°C for COIII gene, 55°C for 18S\_A/1R primer pair and COI gene and 60°C for 18S\_2F/B primer pair. All PCRs were amplified using the BIOTAQ™ DNA Polymerase from Bioline. PCR products were cleaned with Diffinity RapidTip® from GRISP. PCR products were sequenced by Macrogen company. Sequence data were submitted to the GenBank under accession numbers according to table II.2-2.

**Table II.2-2. List of samples included in this study, with sample location and subset and GenBank accession numbers (AN). Samples are organized alphabetically within each family**

Family	Sample ID	Species	Group	Sample location	Marker				
					16S	18S	COI	COIII	ND6
<b>Actiniidae</b>	Aequina1C	<i>Actinia equina</i>	Center	Empa	AN	AN	AN	AN	AN
	Aequina2C	<i>Actinia equina</i>	Center	Empa	AN	AN	AN	AN	AN
	Aequina3C	<i>Actinia equina</i>	Center	Empa	-	AN	-	-	-
	Aequina1S	<i>Actinia equina</i>	South	Porto Covo	AN	AN	AN	AN	AN
	Aequina2S	<i>Actinia equina</i>	South	Porto Covo	AN	AN	AN	AN	AN
	Aequina3S	<i>Actinia equina</i>	South	Porto Covo	AN	AN	AN	AN	AN
	Afragacea1C	<i>Actinia fragacea</i>	Center	São Martinho do Porto	AN	-	AN	-	AN
	Afragacea2C	<i>Actinia fragacea</i>	Center	Empa	AN	AN	AN	AN	AN
	Afragacea3C	<i>Actinia fragacea</i>	Center	Empa	AN	AN	AN	AN	AN
	Afragacea4C	<i>Actinia fragacea</i>	Center	Empa	AN	AN	AN	AN	AN
	Afragacea1N	<i>Actinia fragacea</i>	North	Praia da Aguda	AN	AN	AN	AN	AN
	Afragacea2N	<i>Actinia fragacea</i>	North	Praia da Aguda	AN	AN	AN	AN	AN
	Afragacea3N	<i>Actinia fragacea</i>	North	Póvoa de Varzim	AN	AN	AN	AN	AN
	Afragacea4N	<i>Actinia fragacea</i>	North	Valadares	AN	AN	AN	AN	AN
	Afragacea5N	<i>Actinia fragacea</i>	North	Valadares	AN	AN	AN	AN	AN
	Afragacea6N	<i>Actinia fragacea</i>	North	Valadares	AN	AN	AN	AN	AN
	Afragacea1S	<i>Actinia fragacea</i>	South	Almogrove	AN	AN	AN	AN	AN
	Afragacea2S	<i>Actinia fragacea</i>	South	Almogrove	AN	AN	AN	AN	AN
	Aviridis1S	<i>Anemonia viridis</i>	South	Monte Clérigo-Aljezur	AN	AN	AN	AN	AN
	Aviridis2S	<i>Anemonia viridis</i>	South	Monte Clérigo	AN	AN	AN	AN	AN
Aviridis3S	<i>Anemonia viridis</i>	South	Monte Clérigo	AN	-	-	AN	AN	
Aviridis4S	<i>Anemonia viridis</i>	South	Monte Clérigo	-	-	-	-	AN	
Averrucosa1C	<i>Aulactinia verrucosa</i>	Center	Empa	AN	AN	AN	AN	AN	
Averrucosa2C	<i>Aulactinia verrucosa</i>	Center	Empa	AN	AN	AN	AN	AN	
Averrucosa3C	<i>Aulactinia verrucosa</i>	Center	Empa	-	AN	-	-	-	
Averrucosa1N	<i>Aulactinia verrucosa</i>	North	São Bartolomeu do Mar	AN	AN	AN	AN	AN	
Averrucosa2N	<i>Aulactinia verrucosa</i>	North	Valadares	AN	AN	AN	AN	AN	
Averrucosa3N	<i>Aulactinia verrucosa</i>	North	Valadares	AN	AN	AN	AN	AN	
Averrucosa4N	<i>Aulactinia verrucosa</i>	North	Viana do Castelo	-	-	-	-	AN	
Averrucosa1S	<i>Aulactinia verrucosa</i>	South	Porto Covo	AN	AN	AN	AN	AN	
Averrucosa4S	<i>Aulactinia verrucosa</i>	South	Monte Clérigo	AN	AN	AN	AN	AN	
Averrucosa5S	<i>Aulactinia verrucosa</i>	South	Porto-Covo	AN	-	-	AN	AN	
Akrebsi1C	<i>Anthopleura krebsi</i>	Center	Empa	-	-	AN	AN	AN	
Akrebsi2C	<i>Anthopleura krebsi</i>	Center	Empa	-	-	AN	AN	AN	
Akrebsi1S	<i>Anthopleura krebsi</i>	South	Porto Covo	-	-	-	-	AN	
Akrebsi2S	<i>Anthopleura krebsi</i>	South	Porto Covo	-	-	-	-	AN	
<b>Hormathiidae</b>	Cparasitica1N	<i>Calliactis parasitica</i>	North	Praia da Aguda	AN	AN	AN	AN	AN
	Cparasitica2N	<i>Calliactis parasitica</i>	North	Matosinhos-Open sea	AN	AN	AN	AN	AN
	Cparasitica3N	<i>Calliactis parasitica</i>	North	Matosinhos-Open sea	AN	AN	AN	AN	AN
	Cparasitica4N	<i>Calliactis parasitica</i>	North	Matosinhos-Open sea	-	-	AN	AN	AN
<b>Sagartiidae</b>	Cpedunculatus1S	<i>Cereus pedunculatus</i>	South	Porto Covo	AN	AN	AN	AN	AN
	Cpedunculatus1N	<i>Cereus pedunculatus</i>	North	Praia da Memória	AN	-	AN	-	AN

### 2.3. Statistical analysis

Data from the DNA extraction assay were analyzed by fitting a general linear model, with DNA concentration as dependent variable, and preservation method and body tissue used as fixed factors. The DNA concentration data were logarithmically transformed. The interaction between the fixed factors was tested in a first version of the model, and because its effect was non-significant, it was removed from the final model. A Shapiro – Wilks test applied to the model residuals confirmed the normal distribution ( $W = 0.98$ ,  $p = 0.29$ ). An analysis of variance was used for testing the significance of model factors. All these analyses were performed with R software. Table II.2-3 show the results of variance analysis.

### 2.4. Molecular data analysis

All sequences obtained ( $n = 172$ ) (see table II.2-4) were assembled and edited using Geneious Pro 5.6.6® software (Biomatters) and were blasted against GenBank to check for successful amplification and species identification (cnidarian or associated organisms). For each specimen was given a code. The first letter is the first letter of the genus, followed by the specific epithet and the number distinguishing specimens from the same sample location; finally, a letter stands for the geographical group (North, Center or South). Aiming to increase the phylogenetic tree resolution, we concatenated the nuclear marker 18S with the maternally-inherited mitochondrial markers (COI, COIII, 16S and ND6) (Huang et al. 2009). In that sense, we construct our phylogenetic trees with specimens that have the 5 genes sequenced, with the exception of *Anthopleura* specimens that have three genes sequenced, which makes a total of 151 sequences. GenBank sequences from *Nematostella vectensis* (STEPHENSON 1935) (Order Actiniaria, Edwardsiidae family) were included to root our analysis. In addition, we have used three species with NCBI sequences of the genes included in this study. Regardless of none of the NCBI conspecifics have the COI or ND6 gene, *A. fragacea*, *A. viridis* and *C. parasitica* have the remaining three genes (16S, 18S and COIII) sequenced. *A. krebsi* was not included once it has only two genes sequenced and different from those used in the concatenated dataset (see table II.2-2). Although *C. pedunculatus* and *A. verrucosa* have three genes deposited in the NCBI, our Blast search retrieved a different species that was meant to be, even being amplified from the same DNA that gave positive results for the other genes. *C. pedunculatus* and *A. verrucosa*, retrieved an erroneous result of the 18S and COIII genes respectively. In this sense, these two conspecific sequences were not included in the concatenated

dataset. Finally, *Actinia equina* have sequences from 16S and 18S genes, but from different individuals, in opposition as it is for the others conspecifics and therefore, was not included in this study. Sequences were aligned using webPRANK (Loytynoja & Goldman 2010). To identify the best-fit model of nucleotide sequence evolution for the data set we used Mega 6.06 (Tamura et al. 2013).

Phylogenies were estimated using Maximum Likelihood (ML) as implemented in Mega 6.06 (Tamura et al. 2013), with 1000 bootstraps samples and with Kimura 2 parameter (K2P) plus gamma, nucleotide model substitution. This metric model is most effective for sequences having low divergence rates (Tamura et al. 2013). Bayesian inference was calculated in MrBayes (Ronquist & Huelsenbeck 2003) under the HKY model with fix stationary state frequencies to get K2P model and set the genetic code to mycoplasma. The analysis was performed with the following settings: four Markov chains were run for  $1 \times 10^6$  generations with a sampling frequency of 500 generations. The log-likelihood score of each saved tree was plotted against the number of generations to establish the point at which the log-likelihood scores of the analysis reached their asymptote and the posterior probabilities for clades established by constructing a majority rule consensus tree for all trees generated after the completion of the burn-in phase. The trees were visualized in Seaview4 (Guindon & Gascuel 2010) and annotated using GIMP (GNU Image Manipulation Program) and Mega 6.06 (Tamura et al. 2013). A study on the characteristics of the datasets was performed in Mega 6.06. We calculated the proportion of invariant sites, the number of parsimony informative characters and singletons, the average distance and finally the translation/transversion bias (see table II.2-4).

### 3. Results

#### 3.1. Species identification

12 rocky shores along the Portuguese coast were sampled for the most ubiquitous sea anemones species. Three families were found in total (representing seven species): Actiniidae with *Actinia equina*, *Actinia fragacea*, *Anemonia viridis*, *Aulactinia verrucosa* (*Bunodactis verrucosa*) and *Anthopleura krebsi* species, Hormathiidae with *Calliactis parasitica* species and Sagartiidae with *Cereus pedunculatus* species (see Figure II.2-2).

The collected organisms did not reveal any phenotypic differences among specimens from the same species. This analysis was based on morphological



distinguishing characters, according to the literature and genetic identification with used primers.

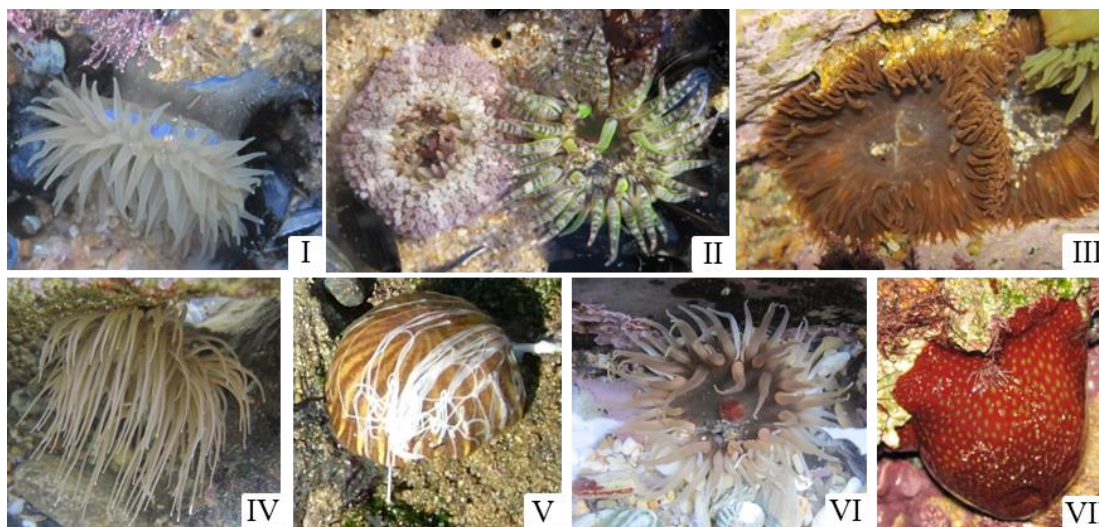


Figure II.2-2. Species collected in this study. I) *Actinia equina*, II) *Aulactinia verrucosa*, III) *Cereus pedunculatus*, IV) *Anemonia viridis*, V) *Calliactis parasitica*, VI) *Anthopleura krebsi*, VII) *Actinia fragacea*

### 3.2. DNA extraction and statistical analysis

The DNA extraction results obtained for each of the 17 samples considering the amount (ng/ $\mu$ L) and purity (Abs 260/280 ratio) were subjected to an analysis of variance. Data from the two methods of storage (99% ethanol and  $-80^{\circ}\text{C}$ ) and the four different body parts (tentacles, gastrodermis, column and ectodermis) are shown in the table II.2-1 and Figure II.2-3. Values of Absorbance 260/280 ratio below 1.8 were considered to have phenol or protein contamination; values higher than 2.0 to have RNA contamination and values between 1.8 and 2.0 indicate good DNA pureness with absence of contaminants that could influence negatively downstream applications.

Concerning the purity of DNA, DNA extracted from 99% ethanol preserved tissues showed higher frequency of absorbance values indicating high DNA pureness (Figure II.2-3a, upper panel). Differences between tissues, however, were not significant ( $F_{3,60} = 0.2$ ,  $p = 0.9$ ), probably due to low number of replicates and high dispersion. Differences between tissues did not show to be so remarkable, although tentacles showed most of its values concentrated inside the interval indicating high quality DNA. On the other hand, Epidermis showed the highest dispersion (Figure II.2-3a, lower panel). Tentacle tissue displayed larger absorbance values (average = 222.62, inter-quartile range = 202.36) compared to the other tissues: column (average

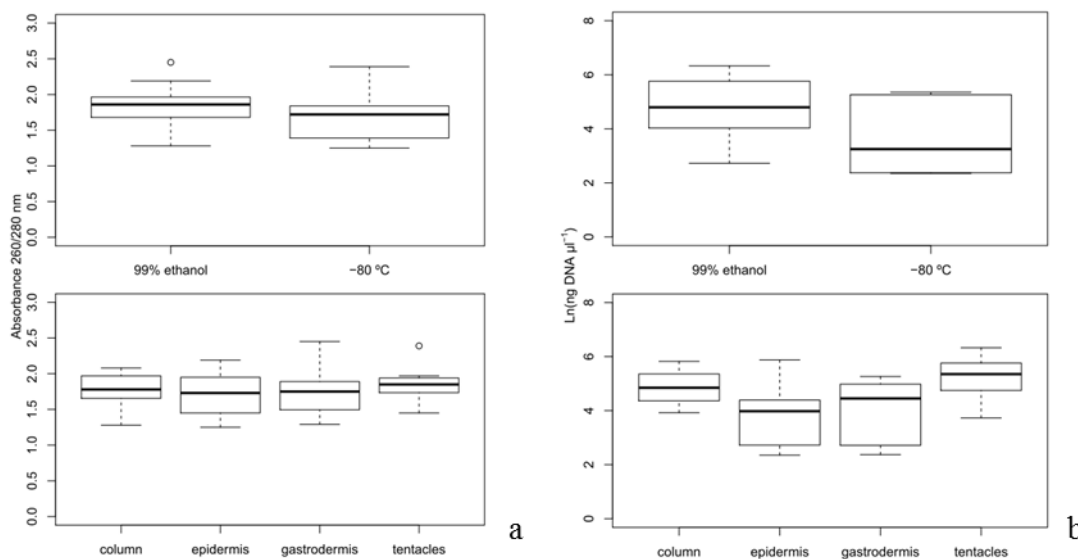


= 159.94, iqr = 80.28), epidermis (average = 93.96, iqr = 50.49), gastrodermis (average = 88.94, iqr = 130.3), indicating higher quality of DNA extracted from tentacles.

Concerning to the amount of DNA, the extraction method using ethanol preserved tissues shows larger average values and less dispersion than the tissues preserved at -80°C (Figure II.2-3b, upper panel). The extraction procedure comparing different body parts showed slightly different average values, and overlapping dispersion (Figure II.2-3b, lower panel). An analysis of variance, using only the data from high quality DNA, showed significant effect of the factor “tissue type preservation”, but not for the factor “body part” (table II.2-3). This is probably due to this overlapped dispersion.

**Table II.2-3. Results of a two-way analysis of variance applied to absorbance data, as a proxy for DNA extraction quality, using preservation method and tissue of origin as factors. F: Fischer - Snedecor contrast statistic, df: degrees of freedom, p: associated probability value**

Factor	F	df	p
Tissue type preservation	18.3	1	< 0.001
Body part	0.2	3	0.9
Residuals		60	



**Figure II.2-3. Box whiskers plot of the DNA extraction results by tissue type preservation (upper panel) and body part (lower panel). a) Absorbance values 260/280 indicate the purity of the extracted DNA and b) Ln of DNA concentration (ng/μl)**

### 3.3. Phylogenetic study

For the phylogenetic analysis, we have applied all the five markers in one concatenated data (CD) alignment dataset, with the exception of *A. krebsi* specimens that has only three markers, and constructed a Maximum-Likelihood with 1000 bootstrap replicates and a Bayesian Inference tree with  $1 \times 10^6$  generations (see Figure II.2-4). In the phylogenetic tree, it was also considered the NCBI sequences of the species collected (labeled by its NCBI Accession Nos), with the exception of *A. equina*, *A. krebi*, *A. verrucosa* and *C. pedunculatus* by the reasons mentioned previously. *Nematostella vectensis* is a sea anemone from the Edwardsiidae family and was used as an outgroup. Table II.2-4 shows the characteristics of each marker dataset. We analyzed a total of 172 sequences from 42 samples, and all of them (with 58 new haplotypes) were submitted to the NCBI (accession numbers in table II.2-2). Our phylogenetic trees were constructed with 152 sequences once only 31 specimens have the five genes sequenced, apart from *A. krebsi* specimens.

The phylogenetic tree retrieved the same clades constructed under the Maximum Likelihood criterion or the Bayesian Inference (Figure II.2-4), with just one exception. The analysis of the phylogenetic tree shows that *Cereus pedunculatus* groups with *Calliactis parasitica* and form a distinct clade from the Actiniidae family members. *Cereus* and *Calliactis* are from different families Sagartiidae and Hormathiidae, respectively, but from the same superfamily Acontiaria. The others species of the analysis belong to the same family, Actiniidae and Endomyaria superfamily. Among these, there are two clades. One composed by *A. krebsi* and *A. verrucosa* and the other by *A. viridis* and *Actinia equina* and *Actinia fragacea*. This last clade is not well supported. It has a bootstrap value below 50 and in the Bayesian inference *Anemonia viridis* forms a distinct clade from *Actinia*. The support values found in this study were: 97, 90, 89 and 83 for the pair of groups Acontiaria/Endomyaria, *C. pedunculatus*/*C. parasitica*, *A. krebsi*/*A. verrucosa* and *Actinia fragacea*/*Actinia equina*. Phylogenetic tree constructed under the Bayesian Inference, shows the same clades with higher values of posterior probabilities.

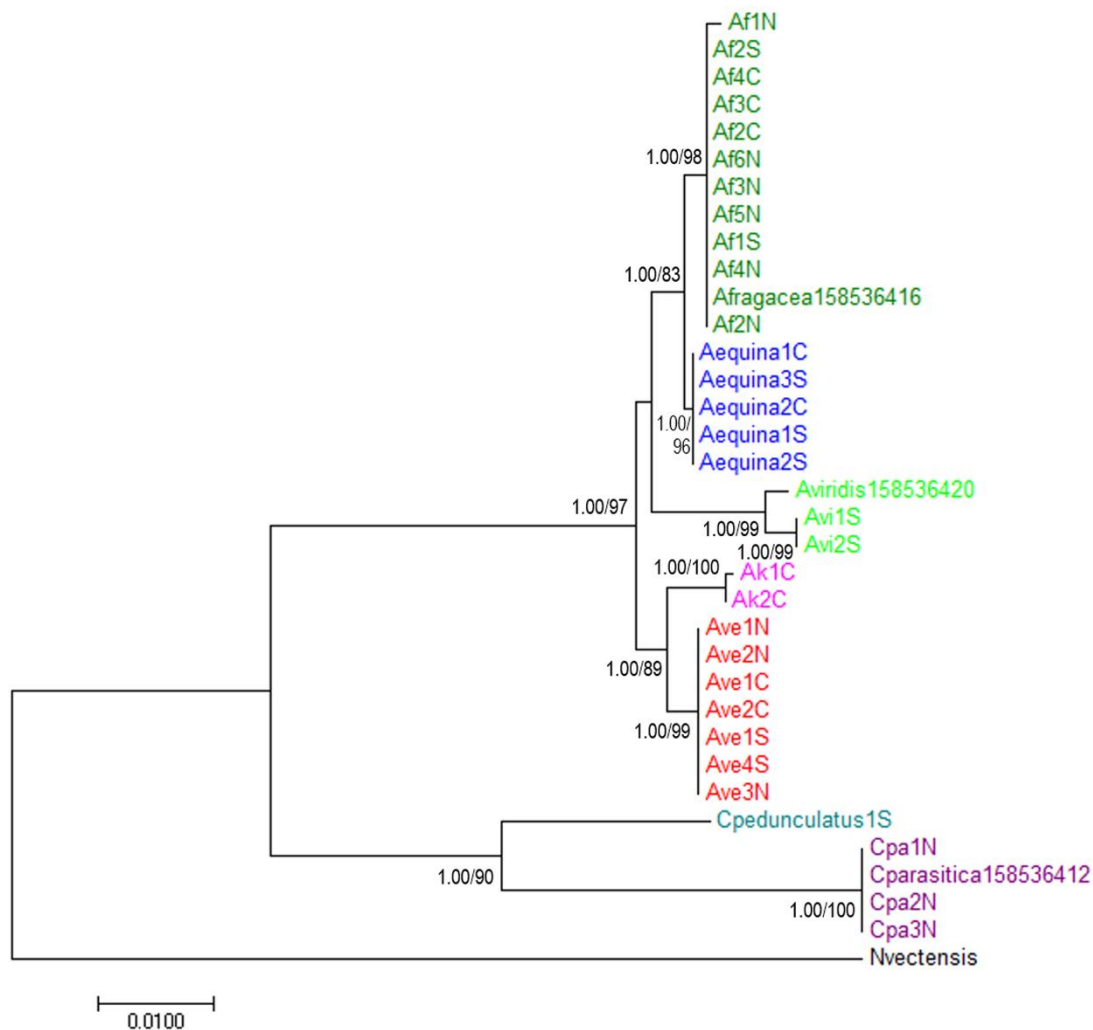


Figure II.2-4. Maximum-Likelihood tree with 1000 bootstrap values and Bayesian posterior probability values (shown first), of the concatenated dataset with five markers (18S, 16S, COI, COIII and ND6). The taxa are highlighted with different colors representing the different species studied. The taxa are represented by codes that are elucidated in table II.2-2. NCBI sequences are represented by its accession numbers

By the analysis of table II.2-4, considering the CD dataset, cytosine is in low abundance compared to thymine, nonetheless transition/ transversion bias is 0.5, meaning there is no bias towards either transitional or transversional substitution. This happens when the two kinds of substitutions are equally probable, and therefore there are twice as many possible transversions as transitions (Tamura et al. 2013). It also can be seen that this dataset has a higher value of parsimony informative characters, therefore providing a more robust analysis. Another parameter analyzed was the proportion of invariant sites. In this last dataset (CD), there is the smallest value, meaning that if there are less invariant sites compared to the other datasets (18S, 16S, COI, COIII, ND6), this CD dataset have most sites evolving at a same single rate.

Summarizing, CD dataset with 4116bp in length shows more phylogenetic informative sites, relatively to fewer sites when considering each marker alone. Comparing markers among each other, the COIII was the one showing greater variability with a value of 0.1592. Comparing the coding (COI, COIII and ND6) with the non-coding (16S and 18S) markers, it can be seen that the coding genes showed more variability. The values ranged from 0.14 to 0.16. The longest marker (18S) was one of the least variables (0.0831). All of these results followed the previous trend in marker variability reported for actinarians (Rodríguez & Daly 2010). Finally comparing the nuclear (18S) with the mitochondrial markers, the first is one of the less variable with a value of 0.0831. This fact is in accordance to Sinnigar *et al.* (2008) that state that COI and 16S were more promising, distinguishing Zoanthinaria species groups. In contrast, two works stated that the mitochondrial genome of some scleractinians evolve five times more slowly than the nuclear genome (Chen *et al.* 2009), and 50–100 times slower than the mitochondrial genomes of most animals (Hellberg 2006).

**Table II.2-4. Length of PCR products aligned and characteristics of each marker dataset**

Dataset	Length (bp)	% T	%C	%A	%G	ts/tv bias	Proportion of invariant sites	Proportion of parsimony informative characters
<b>18S</b>	1611	27.29	19.87	26.18	26.66	2.21	0.8504	0.0831
<b>16S</b>	421	34.71	19.18	24.33	21.77	2.56	0.9299	0.0453
<b>COI</b>	662	36.37	19.03	24.04	20.55	2.30	0.8338	0.1435
<b>COIII</b>	672	34.83	18.67	25.10	21.41	4.05	0.7247	0.1592
<b>ND6</b>	669	27.45	17.96	29.64	24.95	2.85	0.7743	0.1510
<b>Concatenated data (CD)</b>	4116	31.04	19.26	25.76	23.94	0.51	0.0477	0.9039

## 4. Discussion

A phylogenetic study of intertidal sea anemones from Portugal was conducted with five markers (18S, 16S, COI, COIII, ND6). The selection of these markers was based on previous work (Daly *et al.* 2008, 2010, Rodríguez & Daly 2010). In sea anemones phylogenetic studies, it is commonly used the 18S to examine subclass-level and ordinal-level (Berntson *et al.* 1999). The 18S marker is not used for population studies. Nonetheless, due to its robustness and easiness amplification we have choose to incorporate this marker in the set of the used markers. In the same sense, the NADH subunit ND6 is known to be suitable to resolve phylogenetic relationships among families and some genera of Octocorallia, but insufficient for most species or population-level studies (McFadden *et al.* 2004). The COI marker, also showed to be inappropriate for species-specific barcode in anthozoans (Hebert *et al.*

2003). Mcfadden et al. (2011) based on previous works (Shearer et al. 2002, Fukami & Knowlton 2005, Concepcion et al. 2006) suggested that “No regions of the anthozoan mitochondrial genome have sufficient variation to distinguish populations of conspecifics, and few gene regions exhibit enough variability to separate congeneric species”. Moreover, other reports on corals demonstrated that different genera share identical COI sequences (Huang et al. 2008, Shearer et al. 2008). However, Sinniger *et al.* (2008) used COI and 16S markers and state that these sequences have been promising in distinguishing both species groups and most morphospecies of Zoanthinaria (colonial anemones). This justifies the usefulness of an integrated study considering all these five markers, which has not been considered previously in other studies.

Integrate data from different sources is known to be more likely to accurately reconstruct evolutionary history (Rodríguez et al. 2012). Moreover, nuclear and mitochondria data usually give concordant trees in actinarians. For that reason, it was construct a phylogenetic tree under the Maximum Likelihood analysis and under the Bayesian Inference with the concatenated dataset. In this work is presented the ML tree with posterior probability values (shown first) and bootstrap values higher than 50. The results are concordant with previous works (Daly et al. 2008, 2010, Rodríguez & Daly 2010, Rodríguez et al. 2012), but our results retrieved a more resolved phylogeny. From the four well resolved nodes for the pair of groups described previously, Rodríguez *et al.* (2012)) recovered lower bootstrap values for all the nodes, compared to ours 97, 90, 89 and 83 bootstrap values. In Rodríguez & Daly (2010) the less resolved node from our work, namely the position of *A. viridis* clade, also retrieve a low bootstrap value of 54. In the work of Daly et al. (2008) two nodes retrieved values higher than ours, namely 100 and 98 for the pair of clades Acontiarina/ Endomyaria and *A. krebsi*/ *A. verrucosa*. Summarizing, from 15 nodes comparisons, only three nodes from other works gave better results.

To our knowledge this is one of the first works on Portuguese sea anemones. The previous work (Pereira et al. 2014) referred only to one anemone species - *A. equina*. In this case, a haplotype network was constructed using two genetic markers (16SrRNA and 28S). Our study is the first were all the species enunciated previously, collected in the Portuguese coast, are studied phylogenetically.

One of the questions raised before this work was: Which are the most common sea anemone species encounters in intertidal Portuguese coast? Our study provided the first checklist of the most common sea anemones found in the intertidal zone of Portugal mainland. There are seven anemones species with highest incidence in intertidal Portuguese coast: some species are buried in sand, such as *Aulactinia*

*verrucosa*, others are well exposed to the sun and waves, such as *Actinia equina*, *Actinia fragacea* or *Anemonia viridis*, found in cracks such as *Cereus pedunculatus*, or even sheltered from the sun light as the *Anthopleura krebsi*. All the *Calliactis parasitica* specimens collected were not associated with hermit crabs.

Apart from the phylogenetic relationships between the most ubiquitous sea anemones, another question raises: Sea anemones that are separated by 250 Km have genetic differences? From our results, the species found in at least two collection groups (*Actinia fragacea*, *Actinia equina* and *Aulactinia verrucosa*), all the five markers did not reveal evidence of any genetic structure across North, Center and South groups in Portugal (also supported by the haplotypes networks resolved in TCS (Clement et al. 2000); data not shown). This fact can also be due, not only by the choice of the markers, but to the phylogenetic proximity of the sampled groups. Indeed, corals that dist 3000 km apart, also did not showed any population difference with the COI gene marker, despite high levels of variation and population subdivision from allozymes (Hellberg 2006). As mentioned previously, mitochondrial molecular evolution in basal lineages, such as cnidarians, is slower than in bilaterians. Furthermore, within cnidarians, anthozoans are the most slow evolving (France & Hoover 2002, Goddard et al. 2006, Daly et al. 2008), even in geographically distant or potentially isolated populations (Snell et al. 1998, Medina et al. 1999, Hellberg 2006). This would also explain why some of the haplotypes found in the sea anemones from Portugal are similar to others found abroad (*Actinia fragacea* and *Anemonia viridis* from California and *Aulactinia verrucosa*, *Anthopleura krebsi*, *Cereus pedunculatus* and *Calliactis parasitica* from Kansas).

Although there has been an increase number in molecular works on sea anemones, there are still a limited number of haplotypes in databases (e.g., NCBI, <http://www.ncbi.nlm.nih.gov/>). This can be due to insufficient taxon sampling, as well as difficulties in handling the DNA from these organisms. This raises one last question that was: Which is the best approach to store sea anemone tissue for further DNA extraction and what is the best method to perform this DNA extraction? The difficulty in extracting large amounts of good quality DNA from sea anemones was improved by using an optimized phenol extracting method with the support of a mechanic homogenizer with beads, the Precellys® tissue homogenizer. Our study reveals that the best protocol is to store sea anemone tissue in 99% ethanol. Nonetheless, the different body parts did not reveal correlation between DNA amount and tissue type.

Summarizing, our exploratory work found the most common species to be the *Actinia equina*, *Actinia fragacea*, *Anemonia viridis*, *Aulactinia verrucosa* *Anthopleura krebsi*, *Calliactis parasitica* and *Cereus pedunculatus*. The integrated study of five

genetic markers (18S, 16S, COI, COIII and ND6) did not showed evidence of genetic structure among intertidal sea anemones from North, Center and South groups, or genetic divergence from other NCBI haplotypes collected elsewhere (California and Kansas from the United States of America). The ML tree gave similar results, but with improved resolution and more robustly supported nodes, compared to previous studies on these species. Concatenated data gave better resolution of the tree. Altogether, this study increased the number of haplotypes in NCBI (58 haplotypes), enlarging the expertise and information available for future genetic studies on this group of organisms. The best way for obtaining DNA from sea anemones in quantity and quality is by using the Precellys® homogenizer and following the phenol: chloroform DNA extraction method described earlier. Moreover, the use of 99% ethanol preserved tissue is the best option to have good DNA yields, independently of the tissue type.

Given the low levels of genetic variation using mitochondrial markers in sea anemones, population genetic studies should consider more variable markers, such as microsatellites (Thornhill et al. 2013) or consider more rapidly evolving nuclear DNA regions, such as introns sequences (McFadden et al. 2004). Alternatively, amplified fragment length polymorphism (AFLP) markers could also be used for phylogenetic reconstruction, which has been successfully used for intrageneric relationships, to study species boundaries and for intraspecific genetic variation in anthozoans (Douek et al. 2002, McFadden et al. 2004, Chomsky et al. 2009). Finally, next-generation sequencing is also an alternative technique. Despite the difficulty of the data analysis, NGS has gaining strength among scientists. In fact, some researchers already propose this methodology for population genetic differentiation (Fumagalli et al. 2013), which can provide massive data on fine-scale genetic structure of the sampled individuals.

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### III Jellyfish



### III.1 Chapter

Jellyfish Bioactive Compounds: Methods for Wet-Lab Work





Review

## Jellyfish Bioactive Compounds: Methods for Wet-Lab Work

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**Abstract:** The study of bioactive compounds from marine animals has provided, over time, an endless source of interesting molecules. Jellyfish are commonly targets of study due to their toxic proteins. However, there is a gap in reviewing successful wet-lab methods employed in these animals, which compromises the fast progress in the detection of related biomolecules. Here, we provide a compilation of the most effective wet-lab methodologies for jellyfish venom extraction prior to proteomic analysis—separation, identification and toxicity assays. This includes SDS-PAGE, 2DE, gel chromatography, HPLC, DEAE, LC-MS, MALDI, Western blot, hemolytic assay, antimicrobial assay and protease activity assay. For a more comprehensive approach, jellyfish toxicity studies should further consider transcriptome sequencing. We reviewed such methodologies and other genomic techniques used prior to the deep sequencing of transcripts, including RNA extraction, construction of cDNA libraries and RACE. Overall, we provide an overview of the most promising methods and their successful implementation for optimizing time and effort when studying jellyfish.

**Keywords:** jellyfish; toxin; methods; proteomic; transcriptomic; deep sequencing

### 1. Introduction

Many investigations in marine animals target the study of bioactive compounds for various purposes including the discovery of novel drugs, nutritional supplements and applications for industrial biotechnology [1]. This can be accomplished *in vivo* and *in vitro*, for example by the direct study of produced venoms or, more indirectly, by deep sequencing and *in silico* analyses of the genome of these animals. Marine bioprospecting in tropical and sub-tropical species has resulted in the discovery of several bioactive compounds which are of great benefit to human medicine, including cancer therapy, vascular diseases and infectious viral diseases such as AIDS. For example, some soft corals have been exploited for the isolation of products with anti-inflammatory activities [2]. Bioprospecting in species found in Arctic and sub-Arctic waters has allowed us to discover products adapted to extreme environmental conditions such as low temperatures or seasonal lighting [3–5].

Jellyfish (or medusae) comprise a group of free-swimming animals that belong to the phylum Cnidaria and have representatives in the Schyphozoa, Cubozoa and Hydrozoa classes. Cnidarians have special cells, cnidocytes, which gave the name to the phylum and contain a special organelle called the cnidocyst. Research on cnidocysts can have several purposes, but often they are studied for their toxic potential. Nonetheless, toxicity is not exclusively associated to this kind of cell [4]. In fact neurotoxins are also associated with ectodermal gland cells [6]. Therefore, exhaustive studies often consider not only the nematocyst venom but also the toxicity of the animal's body parts [6].

# Jellyfish Bioactive Compounds: Methods for Wet-Lab Work

## Abstract

The study of bioactive compounds from marine animals has provided overtime an endless source of interesting molecules. Jellyfish are commonly targets of study due to its toxic proteins. However, there is a gap in reviewing successful wet-lab methods employed in these animals, which compromises the fast progress on the detection of related biomolecules. Here, we provide a compilation of the most effective wet-lab methodologies for jellyfish venom extraction, prior to proteomic analysis – separation, identification and toxicity assays. This includes SDS-PAGE, 2DE, gel chromatography, HPLC, DEAE, LC-MS, MALDI, Western blot, hemolytic assay, antimicrobial assay and protease activity assay. For a more comprehensive approach, jellyfish toxicity studies should further consider transcriptome sequencing. We reviewed such methodology and other genomic techniques used prior to the deep sequencing of transcripts, including RNA extraction, construction of cDNA libraries and RACE. Overall, we provide an overview of the most promising methods and their successful implementation for optimizing time and effort when studying jellyfish.

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## 1. Introduction

Many investigations in marine animals target the study of bioactive compounds for various purposes including the discovery of novel drugs, nutritional supplements and applications for industrial biotechnology (Urbarova et al. 2012). This can be accomplished *in vivo* and *in vitro* for example by the direct study of produced venoms or, more indirectly, by deep-sequencing and *in silico* analysis of the genome of those animals. Marine bioprospecting in tropical and sub-tropical species has resulted in the discovery of several bioactive compounds of great benefit to human medicine, including cancer therapy, vascular diseases and infectious viral diseases such as AIDS. For example, some soft corals have been exploited for the isolation of products with anti-inflammatory activities (Wei et al. 2013). Bioprospecting in species found in Arctic and sub-Arctic waters have allowed discovering products adapted to extreme environmental conditions such as low temperature or seasonal lighting (Johansen et al. 2010, Leal et al. 2012, Moran et al. 2012).

Jellyfish (or medusae) comprise a group of free-swimming animals that belong to the phylum Cnidaria and have representatives in the Scyphozoa, Cubozoa and Hydrozoa classes. Cnidarians have special cells, cnidocytes, which gave the name to the phylum and contain a special organelle called cnidocyst. Research on cnidocysts can have several purposes, but often they are studied by its toxic potential. Nonetheless, toxicity is not exclusively associated to this kind of cells (Moran et al. 2012). In fact neurotoxins are also associated to ectodermal gland cells (Frazão et al. 2012). Therefore, exhaustive studies often consider not only the nematocyst venom but also the toxicity of the animal's body parts (Frazão et al. 2012).

The direct study of the jellyfish venom requires several common steps. Firstly, the protein sample should be obtained by isolating the nematocysts and releasing its venom from the interior of the organelle (or from the tissue samples). Then, the purification of the crude extract may rely on several methods including, gel electrophoresis, gel chromatography, HPLC, among others. For better quality results, the liquid chromatography LC-MS or HPLC may be used. Western blot is a technique allowing the detection of specific proteins in a sample, being used when the toxin is more or less well characterized. Hemolytic, antimicrobial and protease activity assays, among others, are also a common approach for these studies. When the interest relies on the amino acid sequence identification there are direct methods, such mass spectrometry, or indirect methods, such as RNA extraction, construction of cDNA libraries, Northern blot analysis and RACE amplifications, which will lead to the nucleotide sequence of the gene.

Besides this direct study of the venom, the protein content of a species can also be inferred by sequencing its transcriptome or genome. The transcriptome study can be simple since it allows more directly to characterize protein-coding genes. This review also systematizes the deep sequencing platforms used in cnidarians for optimizing their appropriate use and choice.

Given the broadness of this review, some contents while relevant will not be discussed in detail (e.g. toxicity assays in animals, data handling and bioinformatics analyses - *in silico* lab work). Overall, this review will enable the reader to choose the most appropriate methods for the evaluation of bioactive compounds in jellyfish by using either genomic, proteomic or deep sequencing platform tools.

## 2. Proteomics in jellyfish

Here we describe how to prepare the biological material for proteomic analysis, including the appropriate kits, type of columns and technologies needed for obtaining and processing crude extracts. Bioassays requiring highly specialized facilities or authorizations will not be contemplated. Sea anemones and jellyfish procedures were reviewed previously in 2012 (Frazão et al. 2012) and 2004 (Carrette & Seymour 2004), respectively.

Here we reviewed relevant information on several jellyfish species including *Aurelia aurita* (Ovchinnikova et al. 2006, Lee et al. 2011, Rastogi et al. 2012, Ponce et al. 2013), *Carybdea alata* (Nagai et al. 2000b), *Carybdea rastoni* (Nagai et al. 2000a), *Chironex fleckeri* (Kintner et al. 2005, Brinkman & Burnell 2007, Winter et al. 2007, 2010, Brinkman et al. 2012, Chaousis et al. 2014), *Chiropsalmus quadrigatus* (Nagai et al. 2002c), *Chiropsalmus* sp. (Kintner et al. 2005), *Chiropsella bronzie* (Winter et al. 2007), *Cyanea capillata* (Xiao et al. 2009, 2011), *Cyanea nozakii* (Feng et al. 2010, Lee et al. 2011, Li et al. 2011), *Nemopilema nomurai* (Kim et al. 2006, Lee et al. 2011, Kang et al. 2014), *Pelagia noctiluca* (Marino et al. 2008, Sánchez-Rodríguez & Lucio-Martínez 2011, Ayed et al. 2012, Morabito et al. 2012, 2015, Maisano et al. 2013, Bruschetta et al. 2014), *Rhopilema esculentum*, (Bakhtiar et al. 2003, Yu et al. 2007, Lee et al. 2011), *Rhopilema nomadica* (Gusmani et al. 1997), *Stomolophus meleagris* (Li et al. 2005, 2012a, b, 2013, 2014) and deep-sea jellyfish (Kawabata et al. 2013). Apart from the true jellyfish, there are also Hydrozoa members that have different evolutionary histories (Collins et al. 2006) are colonial free-swimming organisms resembling jellyfishes. *Olindias sambaquiensis* (Weston et al. 2013, Junior et al. 2014) and *Physalia physalis* (Diaz-Garcia et al. 2012) are two of such examples. When considering jellyfish sample collection, the most important step is to quickly preserve

proteins from any kind of degradation. For that purpose, all the procedures should be kept in ice to avoid loss of efficacy (Kintner et al. 2005), or proceed as explained by the authors. Jellyfish can be transported live to the laboratory in plastic buckets with seawater (Weston et al. 2013) or processed in the field. Tentacles can be immediately processed and stored in seawater at 4°C (Winter et al. 2007) prior to nematocyst extraction. Alternatively, the tissues can be immediately stored at -20°C (Xiao et al. 2009). The transport of jellyfish to the laboratory facility should consider the use of ice (Lee et al. 2011) or dry ice (Nagai et al. 2000a, b, 2002c). Dry ice (Xiao et al. 2011) or lyophilized material (Radwan et al. 2001) is also suitable for shipping material. Another option is to snap freeze tentacles in liquid nitrogen and store them at -80°C (Nagai et al. 2000a, b, Li et al. 2012b, 2014) until use.

## 2.1. Nematocysts extraction

### 2.1.1. Autolysis

The nematocysts extraction can be accomplished using several protocols. One of the most commonly used methods is that proposed by Bloom et al. (1998), which refrigerate the tentacles in two volumes of seawater from one to four days. Once a day the containers should be vigorously shaken and an aliquot filtered through a fine kitchen sieve. The decision on whether or not to continue digestion of individual samples is based on microscopic examination of the tentacles and the filtered sediment. If digestion is to be continued the original fluid is decanted, replaced with fresh seawater, and the filtered material washed with seawater allowing three hour settling periods under refrigeration. The final sediments are then lyophilized and stored at -80°C until use.

Several works (Radwan et al. 2001, Kim et al. 2006, Winter et al. 2007, Chaousis et al. 2014) used this well establish method (or with slight adaptations). Feng et al. (2010) has frozen the tentacles placed in 5 x vol. of fresh seawater at 4°C and set the autolysis for four days. The resulting suspension was filtered through a fine sieve (54 µm). The sediments (where most of the nematocysts are contained) were then collected and washed several times with 0.9% NaCl solution. Xiao et al. (2011) set the mass: volume ratio to 1:1. The mixture was stirred for 30 min twice daily and the autolyzed mixture was centrifuged thrice. Nagai et al. (2000b) isolated the nematocysts using a modification of the Burnett method (1992). After 4 days, the sample was filtered through a nylon stocking and the nematocysts in the filtrate allowed settling in a 50-ml conical centrifuge tube. More recently, the method has been simplified and performed

faster (Li et al. 2011, 2014) with an overnight autolysis at 4°C, and afterwards the tentacles are stirred for 10 min and filtered through a plankton net (approximately 280 µm) to remove most of the tentacle debris. Brinkman et al. (2007) purified the nematocysts from tentacle debris by centrifugation in a discontinuous Percoll gradient diluted with 35 g/L NaCl.

In other works (Marino et al. 2008, Morabito et al. 2012, 2015, Maisano et al. 2013, Bruschetta et al. 2014), the nematocysts were isolated as described by Salleo et al. (1983). Briefly, the oral arms were excised and submerged in distilled water for 2h at 4°C. After a complete detachment of the epidermis the tissue was removed from the suspension, containing both epidermis and undischarged nematocysts (organoids) deriving from the osmotic rupture/ lysis of nematocytes. The nematocysts that were still attached to the epidermal tissue were separated by stirring. The nematocysts suspension was repeatedly washed in distilled water and filtered through plankton nets to remove most of the tissue debris, and then centrifuged. In a similar way, Lee et al. (2011) and Kang et al. (2013, 2014) gently swirled the tentacles with distilled water, and then stood still for 1–2 h to remove debris and seawater. After decanting the supernatant, tentacle pellets settled down at the bottom were resuspended in about a 2 x vol. of distilled water and shaken vigorously for 3 min. However, in this procedure the detached nematocysts were separated by filtering the tentacle preparation through four layers of medical gauze.

In the study of the tentacle venom fraction from the jellyfish *Aurelia aurita* (Ponce et al. 2013), cold deionized water and a protease inhibitor cocktail has been used for nematocysts extraction. Previously, Gusmani et al. (1997) used the scapular and mouth filaments suspended in cold sterilized distilled water containing 1 M glycerol for 2 hr at 5°C, and then stirred it. The resulting suspension was filtered through a plankton net and the mesogleal component discarded. After centrifugation the supernatant was discarded and the pellet was resuspended in the same medium. The procedure was repeated twice and the final suspension containing undischarged nematocysts was deep frozen for further use. In brief, autolysis can be performed in fresh seawater, artificial seawater, filtered seawater or reverse-osmosis purified water to different concentration of saline solutions (Kintner et al. 2005, Xiao et al. 2009, Rastogi et al. 2012).

### 2.1.2. *Alternative to Autolysis*

In alternative to autolysis with different types of water, Weston et al. (2013) isolated the nematocysts using a modification of the Weber et al. (1987) method. The tentacles were gently homogenized in a pestle and mortar in cold SuFi (sucrose + Ficoll-Paque Plus) solution. The material was kept at 4°C for 30 min and then passed through a 2 mm diameter sieve. The sample was centrifuged and the supernatant containing debris and cell fragments was removed. The pellet containing intact nematocysts was carefully suspended and washed three times in cold SuFi solution. The final material was submitted, after microscopic inspection, for lyophilisation. In a different approach, Ovchinnikova et al. (2006) excised pieces of *Aurelia aurita* and placed them for extraction into 5% of acetic acid. The obtained extract was consecutively passed under pressure, three times, through a stirred ultrafiltration cell and dried in vacuum. On the other hand, Radwan et al. (2001) attempt to dissociate the nematocysts from both, tentacle tissue and dinoflagellates, by renograffin density gradient centrifugation according to Calton and Burnett (1973).

The simpler the method the better, as long as it performs well. Indeed, simpler methods are faster and less susceptible to errors. Autolysis overnight at 4°C, tentacles stirred for 10 min and filtered through a plankton net are suitable to obtain a nematocyst solution.

## 2.2. Venom extraction

### 2.2.1 *Sonication*

For venom extraction, several protocols can be used. Venom can be obtained by sonication of the nematocysts suspension in cold extraction buffer (Marino et al. 2008, Li et al. 2011, 2012a, b, 2014, Morabito et al. 2012, 2015, Maisano et al. 2013) followed by centrifugation. Bloom et al. (1998) proposed a widely used protocol. Freeze dried samples are resuspended in 1:6 with cold deionized water and sonicated for three 20s periods. Sonications are intercalated with cooling periods of at least 1min on ice. The degree of nematocyst rupture is examined microscopically. The suspension is clarified by centrifugation at 20 000xg for 1h at 4°C, being the supernatant composed of the nematocyst proteins.

Nematocysts sonication can be performed in the presence of different buffers. Weston et al. (2013), disrupted in a sonic bath, freeze dried nematocysts in TEAB. Whereas Gusmani et al. (1997) sonicated the nematocysts in acetate buffer containing

NaCl and the protease inhibitors benzamidine and iodoacetic acid. Nagai et al. (2000a, b) sonicated nematocysts and frozen tentacles in a phosphate buffer solution.

### 2.2.2. *Nematocysts mechanical disruption*

#### *Glass beads*

Extraction of venom according to Carrette and Seymour (2004) using glass beads in an ice-cold (4°C) solution, has been adopted by several authors (Kintner et al. 2005, Kim et al. 2006, Lee et al. 2011, Kang et al. 2014). The samples incubated with PBS solution were shaken in a mini bead mill with intervals for five times with intermittent cooling on ice. The venom extract was then transferred to a new Eppendorf® tube and centrifuged. Both the supernatant and the venom were used. Winter et al. (2007, 2010) applied this method, but replaced the PBS solution by distilled water. In a different approach, Brinkman et al. (Brinkman & Burnell 2007, Brinkman et al. 2012) resuspended the nematocysts in ice-cold buffer (MOPS, NaCl and protease inhibitors) and ruptured using 0.5 mm glass beads. Nematocyst disruption was monitored microscopically and >90% rupture of nematocysts was achieved with 4–6 cycles of homogenization. Crude nematocyst extracts were clarified by centrifugation. In the case of Feng et al. (2010) lyophilized nematocysts were placed into screw-top vials with Tris–HCl buffer and glass beads. Samples were shaken four times in a mini-bead beater with intervals on ice. The venom samples were then separated from the glass beads with a pipette and centrifuged.

#### *Other processes*

Pestle and mortar can be used to homogenize tentacles for venom studies (Ponce et al. 2013). Kawabata et al. (2013) crushed with PBS. The samples were then centrifuged and the supernatant was filtered through a cellulose acetate membrane filter to obtain the crude extract. A blender can also be used with ice-cold water (Diaz-Garcia et al. 2012). In this procedure the crude lysate was clarified through a layer of spun glass and subsequently centrifuged. Using 0.1 % trifluoroacetic acid as incubation solution, Junior et al. (2014) was able to obtain venom from *Olindias sambaquiensis* tentacles. After freeze-thaw cycles and centrifugation, the supernatant was recovered and filtered through a 0.45 µm filter, followed by a second ultrafiltration using a 0.22 µm filter.



When the whole body of the jellyfish is used to obtain the venom content, mincing can be used to obtaining the venom (Carrette & Seymour 2004). Another approach was used by Brinkman et al. (2012). For venom collection, Percoll-cleaned nematocysts were washed with Tris-HCl and resuspended in SDS-sample buffer containing DTT until nematocyst discharge.

The accumulated knowledge revealed the use of glass beads as the most effective approach. Compared to sonication, both techniques require special equipment, but when using glass beads the risk of sample contamination is minor and the time to rupture the cells is less, therefore causing reduced protein degradation when rupturing the nematocysts. The use of a blender or a mortar and pestle, also increases the risk of protein degradation and contamination, compared to glass beads.

### 2.2.3. Concentration of venom proteins

The concentration of total protein in venom extracts can be determined with the Bradford method (Bradford 1976, Chaousis et al. 2014), or measuring absorbance at 280 nm and using bovine serum albumin as a reference standard (Brinkman & Burnell 2007) or by a Pierce BCA assay kit (Nagai et al. 2000a, b, 2002c, Kim et al. 2006, Diaz-Garcia et al. 2012, Ponce et al. 2013, Badré 2014). Alternatively, Nanodrop™ also estimates total protein from extracts (Weston et al. 2013). Winter et al. (2010) have quantified the amount of protein obtained after filtered the supernatant through a micro 0.2 µm syringe filter and read at 562 nm in a Fusion α microplate reader. Other authors quantified the proteins based on the techniques of Waddell (1956) or Lowry et al. (1951). For the identification and quantification of the different venom components, immunodetection (ELISA and Western Blot) or proteomics methodologies combining sample fractionation (liquid chromatography and/or gel electrophoresis) and mass spectrometry could be used. These methodologies are explained in more detail below.

## 2.3. Toxin purification, detection and identification

For toxin purification and identification several methods can be applied. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), two-dimensional gel electrophoresis (2DE), gel filtration, liquid chromatography–mass spectrometry (LC-MS), reverse-phase high performance liquid chromatography (RP-HPLC), DEAE-Sepharose Fast Flow anion-exchange chromatography, matrix-assisted laser desorption-ionization mass spectrometry (MALDI-MS), are commonly used.

### 2.3.1. Electrophoresis

Gel electrophoresis allows discriminating proteins according to size or to purify a protein extract, by picking proteins out of the gel. Briefly, samples are mixed with 4x loading buffer (Tris–HCl, SDS, glycerol, bromophenol blue,  $\beta$ -mercaptoethanol). The samples are reduced at 100°C and then cooled. The gels of polyacrylamide contained acrylamide/bis solution, Tris–HCl, SDS, TMED and ammonium persulphate. Broad range molecular weight markers are used for quantification. The electrophoretic buffer contains Tris base, glycine and SDS. The protein bands are visualized with Coomassie R-250 staining (Brinkman & Burnell 2007, Xiao et al. 2009), silver nitrate (Brinkman & Burnell 2007, Li et al. 2012a, b) or both to elucidate any bands that could be missed with just one stain (Kintner et al. 2005). The gels are then destained in KCl. This SDS-PAGE procedure description follows Laemmli (Laemmli 1970, Xiao et al. 2009). Other authors (Feng et al. 2010, Li et al. 2011) make a SDS-PAGE with slightly modifications, namely a sample dilution (v/v = 1:1) with 2x loading buffer. Prior to 2DE, the venom could be treated with cold acetone for 2h. The samples are centrifuged and the pellets dried in the lyophilizer. The dried pellets are dissolved in the sample buffer (containing urea, thiourea, CHAPS, DTT and IPG buffer) and afterwards in rehydration buffer (containing the previous described reagents plus bromophenol blue) and applied to an IPG strip. After isoelectric focusing (IEF), the IPG strips are first equilibrated with DTT and then with iodoacetamide. Thereafter, the IPG strips are placed onto 12% SDS-PAGE gel and sealed with agarose, before begin run (Brinkman & Burnell 2007).

According to several authors, jellyfish venoms assessed by SDS-PAGE (Nagai et al. 2000b, 2002c, 2000a, Radwan et al. 2001, Kintner et al. 2005, Ovchinnikova et al. 2006, Brinkman & Burnell 2007, Winter et al. 2010, Sánchez–Rodríguez & Lucio–Martínez 2011, Lee et al. 2011, Li et al. 2011, 2012a, Rastogi et al. 2012, Diaz-Garcia et al. 2012, Ponce et al. 2013, Maisano et al. 2013, Badré 2014, Kang et al. 2014) can be analyzed using diverse percentages of polyacrylamide. In a stacking gel, it can range from 4% to 5% (Feng et al. 2010, Li et al. 2011, Rastogi et al. 2012), and in a separating gel from 10% to 20% (Brinkman & Burnell 2007, Feng et al. 2010, Li et al. 2011, Brinkman et al. 2012, Rastogi et al. 2012, Weston et al. 2013). Gusmani et al. (1997) made a SDS-PAGE on a continuous gel gradient from 6% to 15% polyacrylamide and Ovchinnikova et al. (2006) applied a preparative continuous acid-urea–PAGE. Tricine gels are also used to detect smaller sized proteins, compared to glycine gels (Kintner et al. 2005). A Native PAGE analysis (Switzer 3rd et al. 1979, Li et al. 2013) can also be made. In this case, the SDS is abolished from the resolving

gel and electrophoretic buffer, while the loading buffer lack  $\beta$ -mercaptoethanol and SDS.

### 2.3.2. Gel extraction

Proteins can be obtained through in-gel digestion. Briefly, after electrophoresis each band (SDS-PAGE) or dot (2DE) of interest is excised. In-gel reduction, alkylation, and proteolytic digestion with trypsin is performed for each band/dot gel piece (Weston et al. 2013). After digestion, the supernatants are recovered with formic acid and acetonitrile (ACN). The extracts are dried in a vacuum centrifuge (Brinkman et al. 2012, Kang et al. 2014). Other authors (Li et al. 2012b, 2014) digested the venom prior to any separation method. The lyophilized venom is dissolved in guanidine hydrochloride with DTT in Tris-HCl and then reacted at 37°C. Subsequently, iodoacetic acid is added and reacted at room temperature in the dark.  $\text{NH}_4\text{HCO}_3$  is added and then centrifuged with an ultrafiltration device (Molecular Weight Cut Off - 3 kDa). The sample is then digested with trypsin for 20h at 37°C. Finally, the digested proteins are lyophilized and stored at  $-80^\circ\text{C}$  until use. Brinkman et al. (2012) used OFFGEL electrophoresis (OGE). Tryptic digested fragments were diluted in peptide-focusing buffer, without the addition of ampholytes. The samples were focused and peptide fractions were harvest and lyophilized.

### 2.3.3. Gel Filtration and columns

Gel filtration is a size exclusion technique. It includes media such as Sephacryl and Superose (e.g., 12 HR (Gusmani et al. 1997)) for wide ranges of fractionation, Superdex for high resolution, and Sephadex (e.g., G-75 (Ayed et al. 2012); G-50M (Sánchez-Rodríguez & Lucio-Martínez 2011)) for small molecules in organic solvents.

Sephadex G-50 M equilibrated with acetic acid was used by Sánchez-Rodríguez and Lucio-Martínez (2011). Thereafter, the active fraction was passed through a QAE Sephadex A-25 column and subsequently through a Fractogel EMD  $\text{SO}^{3-}$  column equilibrated with ammonium acetate. The sample was afterwards concentrated under vacuum and desalted in a Sephadex G-25 column.

Gel filtration could be a following procedure after an ion-exchange chromatography, which allows the separation of proteins based on their affinity to the ion exchanger. Indeed, Feng et al. (2010) used a Sephadex G-100 column pre-equilibrated with Tris-HCl buffer, Li et al. (2011) used a Superdex 75 column pre-equilibrated with a size-exclusion standard mixture (SERVA) or a Superdex 200

column (Li et al. 2013) and Chaoussis et al. (2014) a Superdex 200 10/300 GL Column in a Fast Protein Liquid Chromatography (FPLC). This last work (Chaoussis et al. 2014) wanted to select proteins in a range of 600 kDa-10kDa, and therefore prior to sample running, a standard curve was generated with manufacturer proteins to allow determining the proteins molecular weights. Subsequently, crude venom was dissolved in Dulbecco's Phosphate Buffered Saline (DPBS) and run through the column for eluting fractions in a 96 well plate. Winter et al. (2010) used a Superdex S-200 column with PBS solution as the mobile phase. The eluant was monitored at 280 nm. Using similar columns, Radwan et al. (2001) venom elution was performed in a Sephadex G-200 and afterwards in Sephacryl S-200 HP prepacked column. Proteins were eluted in phosphate buffer. In a different approach, Li et al. (2012a) filtered the solution of interest with a 0.45 $\mu$ m filter membrane and load it onto a Superdex 75 column preequilibrated with NaCl in PBS. The purified protein was subjected to a TSK gel G3000PWxL column. Diaz-Garcia et al. (2012) purified proteins by resuspending the lyophilized crude extract in ammonium acetate following filtration through a 0.22  $\mu$ m membrane. Aliquots were subjected to gel filtration on a Superdex 75 column. The column was previously equilibrated and eluted with the sample buffer. The low molecular weight fractions of interest were pooled and loaded onto a Phenomenex ODS-3 reverse phase column. Components were eluted using a linear gradient of ACN: TFA. All columns mentioned were connected to a HPLC system.

#### 2.3.4. Fast Flow anion-exchange chromatography

A DEAE-Sepharose Fast Flow anion-exchange chromatography is a technique frequently employed for toxin purification. Feng et al. (2010) pre-equilibrated the column with Tris-HCl buffer, and the nematocyst extract was ultrafiltered using a 10 kDa membrane. The column was stepwise eluted using a NaCl gradient ranging from 0.1 to 0.6 M in the equilibrating buffer. Fractions showing lethal activities in bioassay were collected, pooled, concentrated by ultrafiltration and proceed to gel filtration. For isolation of the hemolytic proteins from the nematocyst venom of the jellyfish *Stomolophus meleagris* (Li et al. 2013), the hemolytic fractions of the dialyzed venom sample were loaded onto a 60 mL DEAE Sepharose Fast Flow column. Afterwards the eluates were pooled and concentrated by a 3 kDa ultrafiltration device and went for a gel filtration. In another study, Li et al. (2011) applied the crude venom to a 16/20 ion-exchange chromatography column packed with DEAE-Sepharose Fast Flow, which was pre-equilibrated and connected to a UV-ribonucleoprotein detector. After removing unbound proteins with EBS, the column was then eluted using stepwise increases in NaCl in Tris-HCl. The eluted fractions were monitored by UV detection and

were then collected. Fractions of the same peak were pooled and concentrated using a 3 kDa Ultra centrifugal filter device and subjected to a hemolytic activity test. Thereafter, the toxic fractions were purified by gel filtration.

### 2.3.5. HPLC

HPLC is a technique that purifies more or less complex protein samples. It can be used as one of the first techniques for purifying proteins or be used after coarser methods, as gel filtration and toxicity assays, as explained in the following examples. Ponce et al. (2013) performed a chromatography fractionation of the venom in a Vydac C18 analytical RP-HPLC column. Maisano et al. (2013) inserted its extracts in a BioSuite 250, 10 $\mu$ mSEC, 7.5 $\times$ 300mm column. Nagai et al. (Nagai et al. 2000a, b, 2002c, Badré 2014) applied venom extracts first to an ion-exchange HPLC, TSK-GEL CM-650S column and then to a TSK-GEL CM-5PW column. Throughout the purification process, each fraction was checked for its hemolytic activity and pooled. The concentrated sample was applied to a gel-permeation HPLC, Superdex75 column. Sánchez-Rodríguez and Lucio-Martínez (2011) determined the purity of the protein fraction by HPLC on a Varian ProStar 410 Autosampler using a Nucleosil C18 reversed phase column. Ovchinnikova et al. (2006) purified by RP-HPLC, on a Macrosphere 300 C-18 column, active fractions in microbes. Nagai et al. (2000a) incubate the toxins first with lysylendopeptidase and then applied the samples onto a reversed-phase HPLC column: Bakerbond wide-pore ODS 5  $\mu$ m with MeCN: TFA as solvent system.

### 2.3.6. Mass spectrometry

Besides HPLC, nano liquid chromatography (nanoLC-MS/MS) has been also successfully applied in other studies. LC-MS/MS is one of the most powerful techniques in the field of proteomics allowing high throughput identification of proteins out of complex protein mixtures. Li et al. (2012b, 2014) for the identification of venom extracted from the jellyfish *Stomolophus meleagris*, has desalted the digested proteins on reversed phase columns (Zorbax 300 SB C18), which were then separated with an analytical RP column using Ettan MDLC system. The process of separation used a Finnigan LTQ linear ion trap MS equipped with an electrospray interface connected to the LC setup for eluted peptide detection. Brinkman et al. (2012) for protein identification, used a Dionex Ultimate 3000 HPLC with an Agilent Zorbax 300SB-C18 column. Eluates from the RP-HPLC column were directly introduced into the NanoSpray II ionisation source of a QSTAR Elite Hybrid MS/MS System operated in positive ion electrospray mode. Weston et al. (2013) reconstituted the protein extract in

ammonium bicarbonate prior to LC-MS/MS analysis and samples were analyzed on a Thermo Scientific Orbitrap Velos Pro mass spectrometer coupled to an EASY-nLC II (Proxeon) nano LC system. After mass spectrometric analysis, liquid chromatographic separation was performed. Samples were trapped on an Easy-column packed with ReproSil-Pur C18 (3  $\mu\text{m}$ ). In these procedures the separations were performed with a gradient of formic acid: ACN.

MALDI-ToF allows the identification of peptides and is commonly applied to jellyfish venom analysis (Kang et al. 2014). Li et al. (2012a) made a Peptide Mass Fingerprinting (PMF) analysis using a MALDI-ToF-MS, where the N-terminal amino acid sequence was determined by the method of Edman degradation. Diaz-Garcia et al. (2012) used this same method and an automatic gas-phase protein sequencer. Another example of the application of this technique is the peptide molecular mass determination described by Ovchinnikova et al. (2006), where peptide microsequencing was made in the Procise cLC 491 Protein Sequencing System. Likewise, Brinkman and Burnell (2007) determined the internal amino acid sequences for two jellyfish toxins. Nagai et al. (2000a) used this technique for peptide mapping of toxins, where separated peptide fragments were fractionated and their amino acid sequences analyzed by a PSQ-1 protein sequencer.

For protein purification, detection and identification, gel electrophoresis is a technique transversal to all types of works. It does not require expensive equipments, being fast and most informative. For the separation of toxin fragments, gel filtration is one of the most employed and easy to use techniques. Other methodologies here referred require more specialized skills and their selection should be based on the study purpose and the easy access to those equipments.

### 2.3.7. *Glycoproteins, phosphoproteins and antioxidant protein detection*

Brinkman et al. (2012) was able to detect glycoproteins and phosphoproteins using a GlycoProfile III fluorescent glycoprotein detection kit and a Pro-Q Diamond phosphoprotein gel stain, respectively. For glycoprotein analysis, a duplicate gel was processed omitting the oxidation step to detect any non-specific fluorescent staining. For phosphoprotein analysis, the ProteoProfile PTM marker containing phosphorylated ovalbumin and  $\beta$ -casein was included as a positive control. Fluorescently stained glycoproteins and phosphoproteins were visualized using a ChemiSmart 3000 image acquisition system.

For the purification of an antioxidant protein, Li et al. (2012a) used an ammonium sulfate precipitation procedure. For that purpose, the nematocysts were sonicated in cold extraction buffer with sodium EDTA, PMSF, pepstatin A, leupeptin and aprotinin in

PBS. Ammonium sulfate was added into aliquots of seven groups representing saturation from 20% to 80%. The precipitation was dissolved and dialyzed for further analysis (Li et al. 2012a).

#### 2.3.8. Western Blot analysis

In a *Chironex fleckeri* study (Brinkman & Burnell 2007) to detect the proteins of interest (CfTX-1 and CfTX-2), as well as in other works (Brinkman et al. 2012, Kang et al. 2014), a Western Blot analysis was performed using polyclonal antibodies purchased or raised in rabbits. The nematocyst extract proteins separated by SDS-PAGE were transferred to Immobilon-P<sup>®</sup> membranes. Membranes were blocked in non-fat milk powder in TBST and incubated overnight with either purchased antibodies or rabbit antiserum diluted in blocking solution. Membranes were washed in TBST then incubated with secondary antibodies, conjugated to alkaline phosphatase and diluted in TBST. Following membrane washing, antigenic proteins were visualized using NBT/BCIP (Brinkman & Burnell 2007). The antibody-containing serum was analyzed by ELISA assay (Kang et al. 2014). Nagai et al. (2000a) also complete their study with a Western immunoblotting assay. The blots were saturated with a 5% skim milk solution in phosphate buffer containing Tween 20, and reacted with the polyclonal antiserum against the toxin fragmented peptide. The bands were revealed using a peroxidase-conjugated anti-rabbit serum and the Western-blotting detection reagent ECL Plus.

## 2.4 Toxicity assays and others

### 2.4.1 Venom proteolytic activity

Gusmani et al. (1997) and Lee et al. (2011) assessed the proteolytic activity of jellyfish venom with gelatin, casein, and fibrin as substrates that were dissolved in sodium phosphate buffer and used in 15% polyacrylamide zymography gels. Venom extracts were prepared in non-reducing sample buffer, and then run on gels at 4°C.

After electrophoresis, SDS was removed by washing the gel twice in Triton X-100. The gel was incubated in Tris and calcium chloride and stained with Coomassie blue. Clear zones in the gel indicate regions of proteolytic activity. When required, the protease inhibitor, 1,10-phenanthroline was added to the wash and incubation buffers, and to the gel stained. For hyaluronidase activity, the hyaluronic acid was used as a substrate in SDS-PAGE following Miura et al. (1995). Residual proteins (which may interfere with gel staining) were removed by adding *S. griseus* protease and incubating.

The gel was stained with Alcian blue and the caseinolytic activity was also measured. An aliquot of casein (1%, in potassium phosphate buffer) substrate was incubated for 3h in the absence (as blank) or the presence of jellyfish venom. The reaction was stopped by the addition of trichloroacetic acid and the casein hydrolysis was measured by modifying the Folin-Ciocalteu method. For blank, the same amount of venom was added before the measurement. A standard graph was generated using standard tyrosine solution. The developed colors of reaction mixtures and standard mixtures were read at 660 nm. For the inhibition study of caseinolytic activity, metalloproteinase inhibitors (EDTA, EGTA, 1,10-phenanthroline) and a serine proteinase inhibitor (PMSF) were used.

The venoms were preincubated with an inhibitor, and then its caseinolytic activity was measured as previously described. One unit of the caseinolytic activity was defined as the amount of enzyme, which hydrolyzed casein to liberate one mg of tyrosine per minute. Li et al. (2005) study the protease activity of venom from *Rhopilema esculentum* using the Folin-phenol of Bakhtiar method (Bakhtiar et al. 2003). Briefly, casein in sodium phosphate buffer (PBS) and crude protein were preincubated. Then unhydrolyzed protein was precipitated with trichloroacetic acid and softly shaken. After centrifugation,  $\text{Na}_2\text{CO}_3$  was added to the supernatant followed by the Folin-phenol reagent and then immediately shaken up. Absorbance at 640 nm was measured for the reaction mixture. One unit of protease activity was defined as 1 ng tyrosine released from casein hydrolyzed by protease of 1 mL of crude protein at 37°C, pH 8.0, for 1 min. The effects of temperature, pH and additives on protease activity were also assessed.

#### 2.4.2. Hemolytic assay

The hemolytic assay is commonly used to determine if the protein solution has toxic properties as the hemolysis of cells (Gusmani et al. 1997, Nagai et al. 2000a, b, 2002c, Li et al. 2005, 2011, 2013, Badré 2014). Yu et al. (2007) studied the hemolytic activity of the *Rhopilema esculentum* venom accordingly to the method previously described for *C. marsupialis* (Rottini et al. 1995). Briefly, 0.5 ml of a 0.05% suspension of erythrocytes in Krebs Ringer phosphate buffer (KRP), pH 7.4, was incubated at 37°C for 30 min with different amounts of venom. After centrifugation, the hemolytic activity was evaluated spectrophotometrically at 415 nm by assaying the hemoglobin released in the supernatant. Reference samples were employed using hypotonic lysis with water as a 100% lysis reference and the supernatant of 0.05% erythrocyte suspension (0.5 ml) incubated with 4.5 ml KRP at 37°C for 30 min as the 0% reference. The  $\text{HU}_{50}$  was defined as the amount of venom required to cause 50% lysis. Junior et al. (2014)



also made a hemolytic assay slightly modified from the previous: human blood, freshly collected with heparin, was centrifuged to remove the buffy coat, and the erythrocytes obtained were washed three times in 0.85% saline and stored at 4°C. Toxins at desired concentrations were added in the first well to erythrocyte buffer, and then were serially diluted in a two-fold ratio. Red blood cells in erythrocyte buffer were added to the toxins, and hemolysis was monitored by measuring attenuation at 630 nm for 20 min at room temperature. The final volume was 200 µL per well. The hemolysis percentage was determined at the end of the assay.

#### 2.4.3. Antimicrobial assay

Ovchinnikova et al. (2006) developed an antimicrobial assay to assess the venom toxicity activity. Antimicrobial activities of peptides were measured in radial diffusion assays by the agarose gel overlay technique according to Lehrer et al. (1991). Samples were tested against *E. coli* and *Listeria monocytogenes*. The microorganisms were precultured in tryptic soy broth at 37°C for 16 h. Aliquots of the bacteria-containing medium were transferred into a freshly prepared medium and incubated at 37°C for 2.5 h to obtain mid-logarithmic phase microorganisms. Cell counts for each microorganism were determined using a spectrophotometer by the turbidity of cell suspensions at 620 nm. Suspension aliquots were mixed with sterile agarose solution in sodium phosphate buffer and poured into sterile plastic 90 mm Petri dishes. Wells made by a 3 mm applicator were filled with 5 µl test samples. The diameter of the inhibited growth zone (the microbe-free zone around a well) was measured assuming 0.1 mm as one unit of antimicrobial activity and subtracting 30 such units (the well diameter) from each result.

### 3. Genomics/ Transcriptomics

The genetic code of organisms can be used for unraveling a variety of biological processes. Deep sequencing technologies generate genomic and transcriptomic sequences (Wenger & Galliot 2013). Several subjects benefit from deep sequencing such as evolutionary (Stefanik et al. 2014), bioprospecting (Rodriguez et al. 2012, Li et al. 2014) or metagenomic (environmental genomics describing high diverse microbial communities) studies (Luo et al. 2012). In brief, Next Generation Sequencing (NGS) technology has facilitated genome re-sequencing, *de novo* genome assembly, transcriptome and non-coding RNA sequencing, transcriptome assembly, as well as sequencing of genome wide protein binding or methylation sites (ChIP-seq and Methyl-seq) (Rallapalli et al. 2014). Sequencing can include a whole individual (Rodriguez et














al. 2012), a pool of individuals (Helm et al. 2013), a specific tissue (Li et al. 2014) or even a microbial community associated with an organism, such as the microbial communities investigated by 454 pyrosequencing in cnidarians and sponges (Sun et al. 2014, Alex & Antunes 2015). Deep sequencing or NGS or High-throughput sequencing are all synonyms. Deep sequencing for transcriptome analysis, also referred as RNAseq (Oshlack et al. 2009), will be considered in further detail in this review. The transcriptome represents the full complement of RNA transcripts expressed in a cell for a specific developmental stage or physiological condition, and consists of protein-coding RNA transcripts (mRNAs) and non-protein-coding RNA transcripts. Only a fraction of the total cellular RNA is referred as mRNA, which is often at very low abundance and requires extremely sensitive analytical tools to be identified and characterized. RNAseq allow scientists to look at different populations of RNA as total RNA, miRNA, tRNA (Ingolia et al. 2012), alternative gene spliced transcripts, post-transcriptional modifications, gene fusion, mutations/SNPs (Maher et al. 2009) or even determine exon/intron boundaries and verify or amend previously annotated 5' and 3' gene boundaries. Overall, the key aims of transcriptomics are to obtain an exhaustive catalogue of transcripts, allowing determining the transcriptional structure of genes and quantifying changes in expression levels among transcriptome samples (Johansen et al. 2010).



### 3.1. Wet-lab genomics for toxin coding gene discovery

After the separation of the toxic fraction from the crude extract, it is commonly aimed to characterize the amino acid sequence of the relevant proteins and the DNA sequencing of related protein-coding genes. Relatively to the numbers of protein identified on sea anemones venoms, and therefore sequenced (Frazão et al. 2012), jellyfish have much reduced figures even though such proteins are supposedly more toxic. Table III.1-1 summarizes the toxins identified in jellyfish and the available amino acid (aa) and nucleotide (nt) sequences, or if having only information described on protein mass or bioactivity (-).

Lassen et al. (2011) work is an example of the effort placed to obtain the amino acid sequence of CcTX-I, a toxin from *Cyanea capillata*, but not the protein-coding gene sequence. This was determined by MALDI-ToF/ToF MS/MS *de novo* sequencing after a size-exclusion, cation-exchange and a reversed-phase chromatography.

**Table III.1-1. Jellyfish toxins described until date. Toxins that have amino acid (aa) or nucleotide (nt) sequences described are labeled in the table. Toxins with only its molecular mass or bioactivity action described are labeled with a dash**

Image	Species	Toxin	Sequence	Reference
 (Hillewaert)	<i>Aurelia aurita</i>	Aurelin Metalloprotease	nt -	(Ovchinnikova et al. 2006) (Rastogi et al. 2012)
 (Seymour)	<i>Carukia barnesi</i>	CbTX-I CbTX-II	nt nt	(Ávila-Soria 2009)
 (Kuwabara)	<i>Carybdea alata</i>	CaTX-A CaTX-B	nt nt	(Nagai et al. 2000b)
 (Norman)	<i>Carybdea rastoni</i>	CrTX-A CrTX-B	nt nt	(Nagai et al. 2000a)
 (Gautsch)	<i>Chironex fleckeri</i>	CfTX-1 CfTX-2 CfTX-A CfTX-B	nt nt nt nt	(Brinkman & Burnell 2007, Brinkman et al. 2008) (Brinkman et al. 2014)
 (OpenCage)	<i>Chiropsalmus quadrigatus</i>	CqTX-A	nt	(Nagai et al. 2002c)
 (Hershman)	<i>Cyanea capillata</i> ( <i>Cyanea nozakii</i> )	CcTX-1 CcNT Metalloprotease	aa -	(Lassen et al. 2011) (Lassen et al. 2012) (Lee et al. 2011)
 (Key)	<i>Cyanea lamarckii</i>	CIGP-1	-	(Helmholz et al. 2008)
 (Gershwin Lisa ann)	<i>Malo kingi</i>	MkTX-A MkTX-B	nt nt	(Ávila-Soria 2009)
 (Endlessblue Blog)	<i>Nemopilema nomurai</i>	Metalloprotease	-	(Lee et al. 2011) (Kang et al. 2014).
 (Migotto)	<i>Olindia sambaquiensis</i>	Metalloprotease	-	(Weston et al. 2013)
 (Hobgood)	<i>Phyllorhiza punctata</i>	Saxitoxin Gonyautoxin-4 Tetrodotoxin Brevetoxin-2	- - - -	(Carneiro et al. 2011)
 (Dunn)	<i>Physalia physalis</i>	Physalitoxin P1 P3 PpV9.4	- - - -	(Tamkun et al. 1981) (Menendez et al. 1990) (Mas et al. 1989) (Diaz-Garcia et al. 2012)

 (OpenCage)	<i>Rhopilema esculenta</i>	Metalloprotease	-	(Lee et al. 2011)
 (McFall)	<i>Stomolophus meleagris</i>	SmP90 C-type lectin, PLA <sub>2</sub> , K <sub>v</sub> <sup>+</sup> toxin, Hemolysin Metalloprotease	aa - - -	(Li et al. 2012a) (Li et al. 2014)

Nagai et al. (2000a, b, 2002) were among of the first works that discovered the gene encoding for jellyfish toxins. First they isolate the toxin by HPLC, complemented with SDS-PAGE and Western Blotting. Then they cloned it and performed a Northern analysis. For that purpose, total RNA was isolated from the intact tentacles using TRIzol<sup>®</sup> in a bead mill or a mortar-and-pestle. First-strand cDNA was synthesized from total RNA using SuperScript<sup>®</sup> II reverse transcriptase and an oligo(dT)12-18 primer. A degenerative reverse transcriptase- polymerase chain reaction (RT-PCR) using mixed oligonucleotide primers was performed to obtain a partial cDNA fragment. Mixed oligonucleotide primers were designed based on the amino acid sequences of the peptides. Amplification was carried out using Ex Taq polymerase and all possible combinations of the primers were used. The obtained products were cloned into a vector and nucleotide sequences were determined using the BigDye<sup>®</sup> Terminator Cycle Sequencing Kit. The cDNA was then subjected to a 5'/3'-rapid amplification of cDNA ends (RACE), using a 5'/3'-RACE kit. The secondary PCR products were subcloned into a vector and sequenced. Full-length cDNA was recloned directly from the total RNA by RT-PCR. PCR products were subcloned and nucleotide sequencing was carried out as described above. Northern analysis was processed as follows: total RNA was separated on a denaturing 1% agarose-formaldehyde gel. Separated RNAs were transferred and crosslinked onto a nylon membrane GeneScreen Plus<sup>®</sup>. The full-length cDNA of the toxin was labeled with DIG DNA Labeling Kit as a probe. Hybridization and detection were carried out according to the DIG system. The antibodies were raised in rabbits.

Similarly, to the previous works, Ovchinnikova et al. (2006) determined the nucleotide sequence of aurelin, a protein from *Aurelia aurita*. They used a SV Total RNA Isolation System and a SMART<sup>™</sup> RACE cDNA Amplification Kit. Likewise, Brinkman and Burnell (2007) screened the gene encoding for a *Chironex fleckeri* toxin, using a bead mill, TRIzol<sup>®</sup>, MicroPoly(A)Pure<sup>™</sup> Kit, ZAP-cDNA Gigapack II Gold Cloning Kit and a Ready-to-Go RT-PCR Beads.

### 3.2. Deep sequencing platforms

Deep sequencing is an outstanding improvement in the discovery of new bioactive molecules, apart from all the other advantages previously mentioned. The sequencing of these millions databases generates thousands of data that are stored in large electronic archives and processed into gene product profiles (mainly proteins, peptides and RNAs) (Johansen et al. 2010, Urbarova et al. 2012). The *in silico* analysis is another approach in bioprospecting, contrasting with the proteomic version that we explained previously. By sequencing genes, genomes and transcriptomes, the search for gene homologs, motifs or transcripts with a certain expression profile, can be identified (Urbarova et al. 2012). Deep sequencing, has one major advantage (beside others), as its relative low cost allows the sequencing of whole transcriptomes of non-model organisms in a relative short time period (Urbarova et al. 2012). Fischer et al. (2014) constructed a database only for transcriptomes of marine organisms with the purpose of sharing and searching transcriptomic data, a major tool for identifying mechanisms of development and evolution, regeneration, resistance to cancer, longevity and symbiosis, among others. This database can be accessed online at: <http://seabase.core.cli.mbl.edu/>. However, for now only the transcriptome of *Nematostella vectensis* is available (Fischer et al. 2014). Further information on this species genome can be retrieved from the works of Moran et al. (Moran & Gurevitz 2006, Moran et al. 2008, 2009b).

Several deep sequencing technologies are available such as Capillary Sequencing ABI, Ion Torrent, 454 Pyrosequencing from Roche, the Genome Analyzer platform from illumina Sequencing technologies and the SOLiD (Sequencing by Oligonucleotide Ligation and Detection) from Life Technologies, among others. All of these are multi-step processes that differ in sample preparation, sequencing methods (Velculescu et al. 1995, Hu et al. 2006, Rothberg & Leamon 2008, Lipson et al. 2009, Oshlack et al. 2009, Zhou et al. 2010, Johansen et al. 2010, Siebert et al. 2011, Luo et al. 2012, Bombarely 2013), mapping tools (Siebert et al. 2011) and also by the type of errors they generate (Luo et al. 2012, Wenger & Galliot 2013). The last three deep sequencing platforms have dominated whole transcriptome analysis (Urbarova et al. 2012).

In practical terms, SOLiD sequencing is highly preferred in small RNA and gene expression profiling, as well as in whole transcriptome re-sequencing (Zhou et al. 2010). For example, it is suitable when aiming to identify a particular mRNA that translate into a toxin. By contrast, 454 give longer reads (700bp) (Siebert et al. 2011, Bombarely 2013). In the absence of an annotated sequenced genome, Siebert et al.

(2011) showed that a hybrid long-read/short-read sequencing strategy is an effective way to collect gene expression data. As mentioned above, this is the case for the large majority of jellyfish species. Moreover, assembling raw sequence reads into a reference of gene sequences is best served by long reads, but quantifying gene abundance is easily accomplished by having many reads. However, it is less expensive to collect short reads than long reads. Thus, collecting long reads across all the samples to be analyzed (including multiple treatments and biological replicates) would therefore greatly increase the project cost or greatly reduce the number of reads that could be sequenced for quantification (Siebert et al. 2011, Bombarely 2013). Illumina and short-read sequencing, in general, may be a more appropriate method for metagenomic studies. Roche 454 may be advantageous for resolving sequences with repetitive structures or palindromes or for metagenomic analyses based on unassembled reads, given the substantially longer read length. Nonetheless both platforms (454 and illumina) provide comparable results (Luo et al. 2012).

After obtaining the digital data, there are two main different assembly methods for producing a transcriptome from raw sequence reads: *de novo* and genome-guided. *De novo* can be done with special software such as Trans-ABYSS, SOAPdenovo, Velvet/Oases, or Trinity (Tulin et al. 2013). The other approach, "easier" and relatively computationally cheaper is to align the millions of reads to a "reference genome" using for example Geneious or CodonCode Aligner software. This, however, as a major problem by depending on the quality of the reference genome (Tulin et al. 2013). Moreover, there is the problem of reads that align equally well to multiple regions of the genome. The program must then choose if these reads are excluded, which can result in gaps, or decide which alignments should be retained, which could lead to wrong assignments or incorrect predictions of transcripts (Tulin et al. 2013).

Another feature that researchers must choose, beyond the platform choice, it is the kind of reads that they want to analyze, pair-ends or single-ends. Paired-end (PE) sequencing allows users to sequence both ends of a fragment and generate high-quality, alignable sequence data. This facilitates detection of genomic rearrangements and repetitive sequence elements, as well as gene fusions and novel transcripts. Single-ends (SE), involves sequencing DNA from only one end. This solution is more rapid and affordable. Moreover, has the advantage of avoiding homopolymer sequencing errors and the G-C bias (illumina®, Tulin et al. 2013). Figure III.1-1 schematically condenses the information on deep sequencing technologies, facilitating the selection of the best approach as a subject of study. Figure III.1-2 represents a diagram to visualize the deep sequencing workflow.

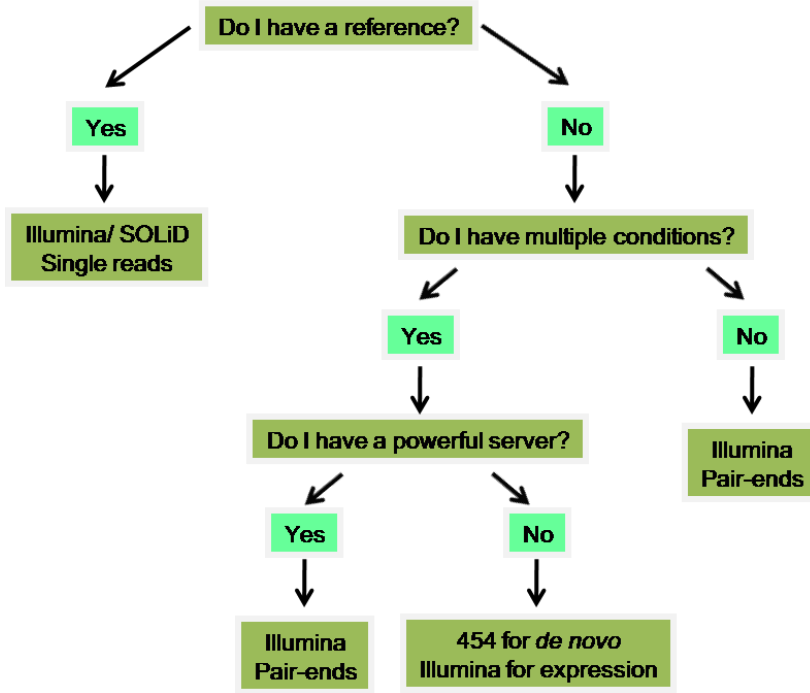


Figure III.1-1. Selecting the right sequencing technology (adapted from Tulin et al. (2013)).

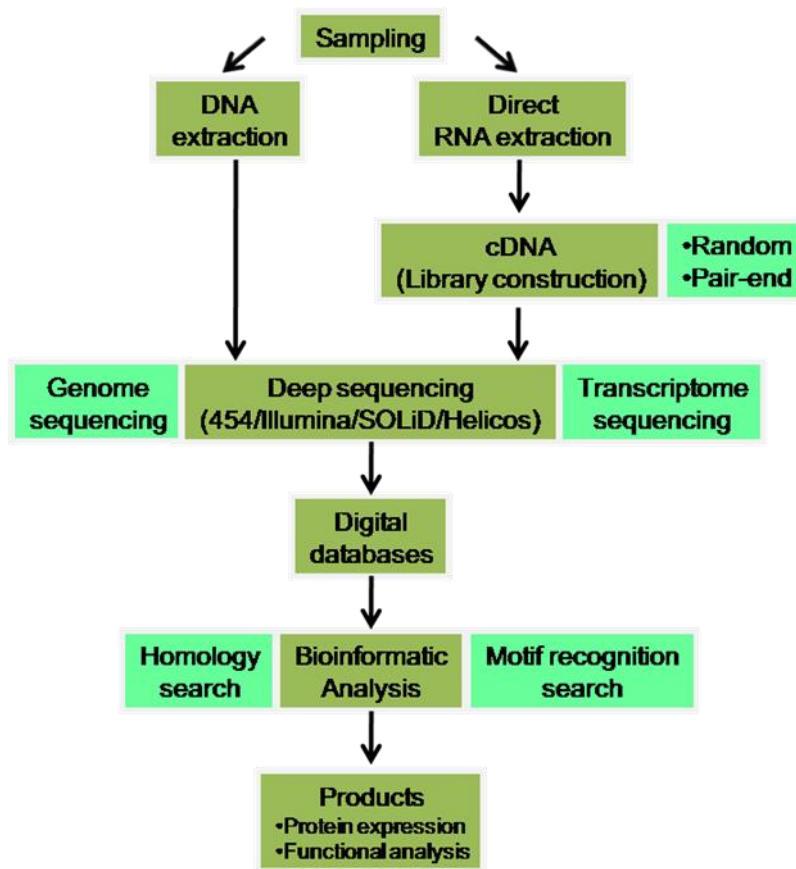






Figure III.1-2. Deep sequencing workflow (adapted from Urbarova et al. (2012)).

As an emergent technology, deep sequencing is not supported by the same volume of published work as compared to Sanger sequencing technology. Among cnidarians there is already several works on sea anemones, corals, Hydrozoa and just one in jellyfish, including the transcriptome of *Stomolophus meleagris* (obtained with illumina HiSeq™ 2000) (see table III.1-2).

Concerning jellyfish genome deep sequencing, there are more works, but all refer to mitochondrial genomes. *Alatina moseri* (*Carybdea alata*) was sequenced by Roche 454 (GS FLX Titanium) and ABI SOLiD (Smith et al. 2012). *Lophelia pertusa* by SOLiD (Emblem et al. 2012) and *Cassiopea andromeda*, *Carybdea xaymacana*, *Cassiopea frondosa*, *Chrysaora* sp. *Carukia barnesi*, *Chironex fleckeri*, *Alatina moseri*, *Chiropsalmus quadrumanus*, *Cyanea capillata*, *Nemopsis bachei*, *Catostylus mosaicus*, *Linuche unguiculata*, *Rhizostoma pulmoa* and *Pelagia noctiluca*, with nearly complete mitochondrial genomes sequenced (Kayal et al. 2012) by the Sanger method and long PCR by 454 high-throughput or illumina Sequencing platforms.



**Table III.1-2. Deep sequencing platforms used in Cnidaria. For each species is identified its Order, the tissue used, the number of Raw Reads obtained and its length and reference. The information on Paired-end (PE) or Single-end (SE) is placed in the Raw Reads column when mentioned in the reference paper**

Order	Species	Tissue	Sequencing platform	Raw Reads (milions)	Read Length (bp)	Reference
<b>Actiniaria</b>  *(Kempf)	<i>Aiptasia palliada</i> *	Adults growing under different conditions	illumina	208	-	(Lehnert et al. 2012)
	<i>Bunodosoma granulifera</i>	Adult	454	-	-	(Rodriguez et al. 2012)
	<i>Edwardsiella lineata</i>	5 developmental stages	illumina	376.2 PE	40	(Stefanik et al. 2014)
	<i>Nematostella vectensis</i>	6 developmental stages Adult stress w/ 4 heavy metals	illumina	165 SE 200 PE 15.22 SE	50 100	(Helm et al. 2013) (Tulin et al. 2013) (Elran et al. 2014)
	<i>Acropora palmata</i> *	Larvae	454 GS-FLX	0.960	398	(Polato et al. 2011) (Moya et al. 2012)
<b>Scleractinia</b>  *(Porter)	<i>Acropora millipora</i>	Larvae w/ CO <sub>2</sub> stress Larvae	illumina 454 GS-FLX	28 628 PE	38 232	(Meyer et al. 2009)
	<i>Favia corals</i>	Adult	illumina	80 PE	75	(Pooyaei Mehr et al. 2013)
	<i>Stylophora pistillata</i>	Adults growing under different conditions	454 GS-FLX	521	-	(Karako-Lampert et al. 2014)
	<i>Pocillopora damicornis</i>	Adult colonies subject to a battery of stressors	454	0.955	379	(Traylor-Knowles et al. 2011)
	<i>Platygyra carnosus</i>	Adult colonies	illumina	83 PE	90	(Sun et al. 2013)
<b>Hydrozoa</b>  *(Bryant & Campbell)	<i>Hydractinia symbiolongicarpus</i>	Adult feeding, reproductive, and defensive polyps	illumina	0.066	200	(Sanders et al. 2014)
	<i>Hydra vulgaris</i> *	Regenerating polyps	illumina 454 Titanium 454,	53.6 1.2	-	(Wenger & Galliot 2013)
	<i>Nanomia bijuca</i>	Nectophores, gastrozooids	illumina, SOLiD SAGE, Helicos DGE	943	-	(Siebert et al. 2011)
<b>Scyphozoa</b>  (McFall)	<i>Stomolophus meleagris</i>	Tentacles	illumina	108	90	(Li et al. 2014)

### 3.3. RNA procedures

Before performing deep sequencing, wet-lab procedures must be undertaken for extracting and prepare RNA. In this review, we do not approach the techniques employed for Sanger sequencing. Beginners should have a good background working with DNA before start working with RNA, which is very sensitive to heat degradation and contamination. Therefore, having an isolated physical space in the lab is advisable for RNA manipulation. Moreover, special materials such as gloves, tips and reagents, among many others, should be RNase free and be consider for RNA restrict use.

The RNA extraction is performed with two main objectives: for Rapid Amplification of cDNA Ends (RACE technology) aiming the discovery of a toxin coding gene sequence or for large-scale sequencing to unravel new bioactive compounds. This review describes both technologies. The first was already described in the 3.1 content.

As an example of large-scale sequencing, Li et al. (2014) sequenced on illumina HiSeq™ 2000 the venom gland of the jellyfish *Stomolophus meleagris*. Several procedures were conducted on short mRNA fragments that were used as templates. Random hexamer-primer was used to synthesize the first-strand of cDNA. The second-strand of cDNA was synthesized using buffer, dNTPs, RNaseH and DNA polymerase I. The short fragments were purified with a QIAQuick® PCR extraction kit and resolved with EB buffer to end reparation and added a polyA tail. The suitable fragments with sequencing adaptors were selected as templates for the PCR amplification based on the agarose gel electrophoresis results. The samples were clustered in flow cells to construct the cDNA library and loaded onto the platform for sequencing.

As only one jellyfish transcriptome is currently available (Li et al. 2014), we refer to similar works on sea anemones, which would allow establishing a parallel. *Bunodosoma granulifera* (Rodriguez et al. 2012) and *Aiptasia sp.* (Lehnert et al. 2012) were sequenced by 454 and illumina, respectively. RNA was extracted using TRIzol® and purified using RNeasy Mini Spin Column. A high-salt method of RNA precipitation can also be used to reduce proteoglycan and polysaccharide contamination. DNA digestion is performed using a DNase. The quality and quantity of the total RNA was detected using RNA 6000 pico LabChip® kit. The cDNA library was prepared with kits compatible with the platform in use. *Nematostella vectensis*, a model system for studying animal body plans evolution, has several works preformed all with illumina HiSeq (Helm et al. 2013, Tulin et al. 2013, Elran et al. 2014). Total RNA was extracted with Tri-reagent kit and purified with RNA Clean & Concentrator™ kit, or with mRNA DIRECT™ kit. Dynabeads and low adhesion microcentrifuge tubes were used. Genomic DNA residues were removed by DNase. RNA concentrations were further determined in a NanoDrop™ or in a Qubit®. Samples were prepared for multiplex sequencing using illumina TruSeq kits (Helm et al. 2013, Tulin et al. 2013, Elran et al. 2014).

The various deep sequencing technologies share the massive sequencing of DNA in a flow cell. The flow cell is a glass slide with 1, 2, or 8 physically separated lanes, where it is load the sample. Helm et al. (2013) sequenced in a single lane. Elran et al. (2014) sequenced triplicates of the samples in two lanes. In the work of Tulin et

al. (2013) the RNAseq Library was prepared with ScriptSeq™ v2 kit using Phusion High Fidelity polymerase with barcoded illumina-compatible primers. The libraries were size selected for 450 bp. The samples run on a single lane.

#### 4. Conclusions

Bioactive components from jellyfish can be characterized by various techniques. The traditional approach used the venom extract followed by purification procedures until obtaining more or less pure toxins. Examples of such trials include the SDS-PAGE, 2DE, gel filtration, LC-MS, RP-HPLC, DEAE-Sepharose Fast Flow anion-exchange chromatography, MALDI-ToF MS, etc. Prior to purification procedures, most techniques for the venom extraction employed nematocysts that are removed from the tentacles by over-night agitation in seawater. Nonetheless, freshwater can also be used. The nematocysts rupture can be accomplished with a mortar and pestle, bead mills grinding in an electrical pulverizer or even point tip sonication. Beside these techniques, venom can be obtained by a chemical discharge of the nematocysts using buffers as glycerin or sodium citrate. All the procedures should be made on ice and the samples stored preferential at -80°C, as toxins are heat sensitive. Protein quantification can be made by various methods, but the Bradford method is well established among scientists. After isolating the toxic fraction/protein, several assays can be considered to decipher the toxin modes of action. There are proteolytic, hyaluronidase and caseinolytic activity assays, hemolytic and antimicrobial assays.

Amino acid sequencing determination is made in MALDI-ToF by the Edman degradation method. The finding and study of the mRNA translating the toxin is performed after the identification and characterization of the toxins by Western Blotting, RACE, RT-PCR, cloning and Northern analysis.

Currently, deep sequencing of a species transcriptome is emerging as a valuable approach for bioprospecting molecules *in silico*. For jellyfish, only one work used previously the illumina platform. However, other platforms have been successfully used in other cnidarians such as 454, illumina, SOLiD and Helicos.

Even with so many techniques available, major challenges still remain such as the complexity of the sample, the scarcity of the biological material, and the absence of databases for the determination of peptide and protein sequences in jellyfish (Escoubas et al. 2008).

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## III.2 Chapter

Analysis of *Pelagia noctiluca* proteome reveals a Red Fluorescent Protein, a Zinc Metalloproteinase and a Peroxiredoxin

# Analysis of *Pelagia noctiluca* proteome reveals a Red Fluorescent Protein, a Zinc Metalloproteinase and a Peroxiredoxin

## Abstract

*Pelagia noctiluca* is the most venomous jellyfish in the Mediterranean Sea where it forms dense blooms. Although there is several published research on this species, until now none of the works has been focused on a complete protein profile of the all body constituents of this organism. Here, we have performed a detailed proteomics characterization of the major protein components expressed by *P. noctiluca*. With that aim, we have considered the study of jellyfish proteins involved in defense, body constituents and metabolism, and furthered explore the significance and potential application of such bioactive molecules. *P. noctiluca* body proteins were separated by 1D SDS-PAGE and 2DE followed by characterization by nanoLC-MS/MS and MALD-ToF/ToF techniques. Altogether, both methods revealed 68 different proteins, including a Zinc Metalloproteinase, a Red Fluorescent Protein (RFP) and a Peroxiredoxin. These three proteins were identified for the first time in *P. noctiluca*. Zinc Metalloproteinase was previously reported in the venom of other jellyfish species. Besides the proteins described above, the other 65 proteins found in *P. noctiluca* body content were identified and associated with its clinical significance. Among all the proteins identified in this work we highlight: Zinc metalloproteinase, which has a ShK toxin domain and therefore should be implicated in the sting toxicity of *P. noctiluca*.; the RFP which are a very important family of proteins due to its possible application as molecular markers; and last but not least the discovery of a Peroxiredoxin in this organism makes it a new natural resource of antioxidant and anti-UV radiation agents.

**Keywords:** *Pelagia noctiluca*, jellyfish, proteomic, toxin

## 1. Introduction

*Pelagia noctiluca* is a pelagic (open water) jellyfish in which its bell (measuring 3 to 12 cm in diameter) is phosphorescent in the night darkness, whence its scientific name. During the day, *P. noctiluca* is colored pink, mauve or light brown. The tentacles, eight, appear from the bell edge (Tibballs 2006). *P. noctiluca* is ubiquitous in the Mediterranean Sea and is known to be the most venomous jellyfish in this area (Marino et al. 2004, Mariottini et al. 2008). Although, the sting is venomous, in humans usually only causes a whip-like scar across the body (Del Pozo et al. 2016). Nonetheless, allergic reactions and anaphylactic shocks, these last of major importance are also documented (Burnett 2009). On the Calabrian coast, for example, a middle-aged woman stung by this species had a loss of consciousness and required prolonged cardiopulmonary resuscitation (Andras et al. 2011).

*P. noctiluca* is eligible as a model for ecotoxicological investigations and as bio-indicator for the quality of the marine environment. For instance, the toxic potential of the *P. noctiluca* nematocysts can be affected by factors such as heavy metals contamination and water acidification (Morabito et al. 2013, 2014).

Cnidaria luminescence is currently a hot topic of research interest. Fluorescent proteins from Cnidaria, including green fluorescent protein (GFP) from *Aequorea victoria* (Zimmer & Chem Soc 2009), red FP (RFP) from *Discosoma sp.* (Fradkov et al. 2000), yellow FP (YFP) from *Zoanthus sp.* (Hoi et al. 2013), among many others, have been well characterized aiming its application as molecular markers to identify genes and protein expression in cellular and molecular biology research (Chudakov et al. 2010). *P. noctiluca* might as well be a source of novel biomolecules with technological application.

This species is documented in the Pacific Ocean, Mediterranean Sea and more recently in the Northeast Atlantic Ocean (Mariottini et al. 2008). The invasion of colder waters by this venomous species can be detrimental to aquaculture and recreational activities, thus having negative socio-economic impacts (Licandro et al. 2010, Bosch-Belmar et al. 2016). On the other hand, *P. noctiluca* is an important food resource for fishes such as *Boops boops* that have high economic value (Milisenda et al. 2014).

As already reported for other Cnidaria, the toxicity of *P. noctiluca* is not exclusively determined by the components produced by nematocysts, but is also influenced by other tissue components (Mariottini et al. 2008). Its venom is known to be cytotoxic (Mariottini et al. 2002, Morabito et al. 2015) causing osmotic lysis, and/or oxidative damage (Ayed et al. 2011, 2013, Morabito et al. 2015) and the activation of

nematocysts is calcium dependent (Salleo et al. 1994). For other jellyfish, some venom components have already been characterized. PLA2 (Phospholipase A2) is common in *Carukia barnesi*, *Carybdea rastonii*, *Chironex fleckeri*, *Cyanea capillata*, *Phyllorhiza punctata* and *Aurelia aurita*. Aurelin is found in *Aurelia aurita* (Ovchinnikova et al. 2006), CbTX-I and II in *Carukia barnesi* (Ávila-Soria 2009), CaTX-A and B are found in *Carybdea alata* (Nagai et al. 2000b), CrTX-A and B in *Carybdea rastonii* (Nagai et al. 2000a), CfTX-1 and 2 (Brinkman & Burnell 2007, Brinkman et al. 2008) and CfTX-A and B in *Chironex fleckeri* (Brinkman et al. 2014), CqTX-A in *Chiropsalmus quadrigatus* (Nagai et al. 2002c), CcTX-1 (Lassen et al. 2011) and CcNT (Lassen et al. 2012) in *Cyanea capillata*, CIGP-1 in *Cyanea lamarckii*, MkTX-A and B in *Malo kingi* (Ávila-Soria 2009), saxitoxin, gonyautoxin-4, tetrodotoxin and brevetoxin-2 in *Phyllorhiza punctata* (Carneiro et al. 2011), physalitoxin (Tamkun et al. 1981), P1 (Menendez et al. 1990), P3 (Mas et al. 1989) and PpV9.4 (Diaz-Garcia et al. 2012) in *Physalia physalis* and SmP90 in *Stomolophus meleagris* (Li et al. 2012b).

Concerning to the genetic structure of *P. noctiluca*, Stopar et al. (2010) reported that specimens of this species have greater haplotype diversity among them compared to the diversity of specimens among geographical regions. The authors assume that all the specimens studied belong to a unique large population.

While venom characterization may provide clear understanding of human envenoming (Morabito et al. 2015), and knowledge of bioactive molecules, the all body protein content (Rocha et al. 2011, Ruan et al. 2014) on the other hand, may reveal an additional repertoire of molecules with biological and biotechnological interest. Here, we have performed a detailed proteomics characterization of the major protein components expressed by *P. noctiluca*. With that aim, we have considered the study of jellyfish proteins involved in defense, body constituents and metabolism, and furthered explore the significance and potential application of such bioactive molecules. In this study, it was also performed a genotyping identification with two mitochondrial markers (16s rRNA and the cytochrome oxidase subunit I genes) to support our phenotypic identification.

## 2. Methods

### 2.1. Sample collection and crude extract preparation

*P. noctiluca* specimens were collected according to specific collection procedures (Pierce 2009). *P. noctiluca* specimen's collection respected the national guide for the care and use of laboratory animals. The samples were collected in Peniche beach (ten specimens) in October of 2010 (Figure III.2-1).

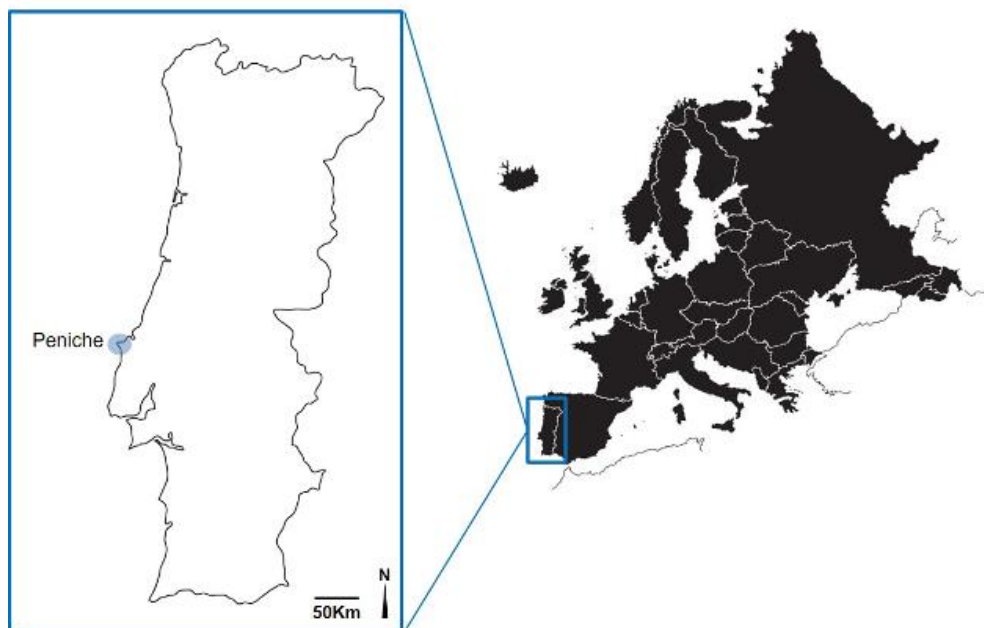


Figure III.2-1. Geographic location of the Peniche beach in the west coast of Portugal, Europe.

The specimens were left on starvation for three days in an aquarium and were then stored at  $-80^{\circ}\text{C}$  until use. The choice of keeping the animals on starvation was to ensure total digestion and waste disposal, for strict analysis to jellyfish body proteins, guarantying the exclusion from fish or other jellyfish prey proteins. Moreover, three days was the time that it was expected to not induce to much ecological stress to the individuals, meaning the least protein damage or protein expression variation. A tissue sample from two specimens was collected for genotyping identification. For proteomic analysis, the material was treated as described. All individuals were combined in a beaker and thawed. This material was centrifuged to ensure the sedimentation of cell debris, at  $4^{\circ}\text{C}$  for 10 min at 4600 g. The presence of nematocyst was inspected under a microscope. Since no intact nematocysts were found, no further treatments were done to release the venom from these cells (Maisano et al. 2013). The supernatant was freeze-dried and resuspended in a minimum volume of ultra-pure water. The

sample was desalted with PD-10 Desalting Columns (GE Healthcare). These columns are packed with Sephadex™ G-25 Medium, which allow to remove from the sample salts and low molecules with molecular mass < 5KDa. Samples were filtered through a 0.22 µm sterile filter (Millipore). The protein quantification was assessed by the Bradford Method (Bradford Reagent from Sigma-Aldrich®) following the manufacturer's instructions.

## 2.2. Protein separation by 1D SDS-PAGE

Protein separation by 1D SDS-PAGE electrophoresis was made according to Santos et al. (2009). 53 µL of sample (39 µg protein) was diluted in 72 µL of Loading Buffer (0.01% bromophenol blue, 2% SDS (Sodium-Dodecyl-Sulfate), 20% glycerol, 5% β-mercaptoethanol (w/v/v) in 62.5 mM Tris – HCl, pH 6.8). The resulting solution was heated for 3 min at 99°C. Proteins were separated by SDS-PAGE with 12% (w/v) polyacrylamide gels. Electrophoresis was carried out using the mini Protean Cell (Bio-Rad) at a constant voltage of 150 V. The separated proteins were visualized by staining with Colloidal Coomassie Brilliant Blue (CCB) (Neuhoff et al. 1988).

## 2.3. Mass spectrometry and protein identification

The identification of the proteins obtained from the 1D gel was made according to Raposo et al. (2011). Gel lanes were excised, cut into 20 equal portions and in-gel digested with trypsin. Briefly, gel bands were excised and destained in 50mM ammonium bicarbonate (Sigma) in 50% acetonitrile (HPLC grade, Fisher) and reduced with TCEP-HCl and alkylated with chloracetamide (Sigma). Gel bands were digested using 200 ng of trypsin (Promega) for 16 hours at 37°C. Digests were desalted prior to mass spectrometry using an in-house manufactured C18 reverse-phase tip.

Samples were analyzed by LC-MS/MS using a QExactive mass spectrometer (Thermo, Hemel Hempstead) coupled to a Dionex Ultimate 3000 RSLCnano system. Samples were resolved on a 60 min gradient on a home-packed 25 cm long, by 75 µm inner-diameter column, at a flow-rate of 300 nL min<sup>-1</sup> run in direct injection. The QExactive was operated in a "Top20" Data Dependent Acquisition mode using HCD fragmentation.

## 2.4. Two-dimensional gel electrophoresis (2DE) and Protein digestion

Protein sample (278 µg of protein) was diluted in 200 µL of urea (7 M), thiourea (2 M), 3-((3-cholamidopropyl) dimethylammonio)-1-propane sulfonate (CHAPS) (4%, w/v), dithiothreitol (65 mM) and ampholytes, pH 3–10 (0.8%, v/v) (Bio-Rad, Hercules, CA, USA). Then proteins were separated by 2DE as described by Puerto et al. (2011). Briefly, the sample was loaded on to 17 cm, pH 3–10 non-linear dry-strips (Bio-Rad, Hercules, CA, USA) and proteins separated by isoelectric focusing (IEF) in a Protean IEF Cell (Bio-Rad, Hercules, CA, USA) with the following program: 16 h at 50 V (strip rehydration); step 1, 15 min at 250 V; step 2, 3 h voltage gradient to 10,000 V (linear ramp); step 3, 10,000 V until achieving 60,000 V/h (linear ramp). After the first dimension IEF gel strips were equilibrated using 10 mg/mL dithiothreitol and 25 mg/mL iodoacetamide in urea (6 M), glycerol (30%, v/v), SDS (2%, w/v) (Puerto et al. 2011). Subsequently IEF gel strips were assembled in 12% (w/v) polyacrylamide SDS-PAGE gels and proteins separated in a Protean XI Cell (Bio-Rad, Hercules, CA, USA) at 24 mA. Gel was stained with CCB (Neuhoff et al. 1988).

Protein digestion followed the Santos et al. (2009) protocol. Protein spots were manually excised from the gels, washed in Milli-Q H<sub>2</sub>O, and destained in 50% acetonitrile (ACN) and subsequently in 100% ACN. The dried gel pieces were hydrated in 50mM NH<sub>4</sub>HCO<sub>3</sub> digestion buffer containing 6.7 ng/ µL of trypsin (modified porcine trypsin, sequencing grade; Promega, USA) on ice. After 15 min, the supernatant was removed and discarded, 25 µL of 50 mM NH<sub>4</sub>HCO<sub>3</sub> was added to the gel pieces and digestion was allowed to proceed at 37°C overnight. After digestion, the remaining supernatant was removed and stored at – 20°C until use.

## 2.5. MALD-ToF/ToF and Protein Identification

MALD-ToF/ToF analysis and proteins concentration was performed as Neves et al. (2013). Peptide samples were concentrated in a Centrivap (Labconco) and resuspended in trifluoroacetic acid 0,1% (v/v). Samples were concentrated and cleaned according to the manufacturer's instructions on a micro C18 column (ZipTip, Millipore, Bedford, MA, USA). The proteins were eluted in 1,7 µL directly onto the MALDI plate using the matrix α-cyano-4-hydroxycinnamic acid (α-CHCA) at 7 mg/mL prepared in ACN (50%), trifluoroacetic acid (0.1%) and ammonium phosphate (6 mM). Peptide mass spectrometry analyses were performed by MALD-ToF/ToF (4800 Plus MALDI-ToF/ToF Analyzer, AB SCIEX, Framingham, MA, USA) in reflector positive mode (700–4000 Da). The experimental mass spectra were searched against the UniprotKB

protein sequence database with the MASCOT (Matrix-Science, London, UK) algorithm, integrated in the GPS Explorer software (AB SCIEX, Framingham, MA, USA). Moreover, the peak list was also searched against three databases retrieved from NCBI. Metazoa, Cnidaria and Nucleotide. The search parameters were up to two maximum trypsin missed cleavages, mass tolerance of 50 ppm, cysteine carbamidomethylation (fixed modification), methionine oxidation (variable modification) and a charge state of +1.

For the sequences that gave uncharacterized, predicted or putative proteins, a blast search was performed in the NCBI to obtain more information by homology. For one entry it was made a PFam (Finn et al.) search. For the rest of the proteins we have obtained the molecular function, biological process and cellular component from the Gene Ontology (GO) in UniprotKb (Consortium 2014).

## 2.6. Molecular data collection

Genomic DNA was isolated by the phenol-chloroform method. The tissue was dissolved in digestion buffer (Tris 10 mM, pH 8.0, EDTA 100 mM, SDS 0.5%) with 1 mg/ml of proteinase K (Applichem Lifescience). Afterwards there was an addition of 10% CTAB (Sigma) in 0.7 M NaCl to a final concentration of 0.3 % of the sample volume (Geller & Walton 2001). The sample was then incubated 20 min at 65°C and then an equal volume of chloroform/isoamyl alcohol (24:1) was added, mixed, and centrifuged at 16000 g for 20 min. This emulsion forms an interphase of macromolecules and polysaccharides that cannot be disturbed when pipetting the supernatant. The CTAB addition and centrifugation step was repeated until the interphase became invisible. Thereafter, it was added Phenol: chloroform: isoamyl alcohol (25:24:1) in equal volume, mix and centrifuged as previously described. To the supernatant it was added 1/10 NaCl 6M, and 2.5 volumes of cold absolute ethanol for DNA precipitation. The DNA was left to precipitate at -80°C for 1h. After centrifugation at 16000 g, 20 min at 4°C, the pellet was washed with 70% ethanol and centrifuged as described previously. The supernatant was drained off at maximum volume possible. Finally, the pellet was left to air dry for 10 min and resuspended in TE buffer.

Species identification was confirmed based on the PCR products of two mitochondrial DNA markers (16SrRNA, and COI genes) that were amplified on a thermocycler using previously published primers (table III.2-1). Standard PCR protocols were used. COI (cytochrome oxidase subunit I) gene was amplified with 55°C of annealing temperature. All PCRs were performed using the BIOTAQ™ DNA Polymerase from Bioline. PCR products were cleaned with Diffinity RapidTip® from



GRISP and then sequenced by Macrogen company. Sequence data were submitted to GenBank. The corresponding accession numbers are mentioned in the results section.

**Table III.2-1. List of primers used in this study**

Primer	Sequence	Reference
16PF	TCGACTGTTTACCAAAAACATAGC	(Bridge et al. 1992)
16PR	ACGGAATGAACTCAAATCATGTAAG	(Bridge et al. 1992)
dgLCO1490	GGTCAACAAATCATAAAGAYATYGG	(Meyer et al. 2005)
dgHCO2198	TAAACTTCAGGGTGACCAARAAYCA	(Meyer et al. 2005)

## 2.7. Molecular data analysis

The sequences were assembled and edited using Geneious Pro 5.6.6® software (<http://www.geneious.com>, (Kearse et al. 2012)) and were blasted against GenBank to check for successful amplification.

## 3. Results

### 3.1. Protein profiles of *P. noctiluca*

1D SDS-PAGE analysis of *P. noctiluca* crude protein extracts revealed at least 22 protein bands of molecular weights ranging from 15 to 177 KDa (Figure III.2-2A). The complexity of *P. noctiluca* crude protein extracts was also revealed by 2D electrophoresis (Figure III.2-2B), which is a more powerful technique than 1D electrophoresis for the separation of individual proteins and protein isoforms. Two 2DE gels were run, and as they show equal resolution and protein separation, only one image is presented. At least 151 protein spots were clearly resolved with this technique. This group of proteins was analyzed by MALDI-ToF/ToF to access the molecular function. On the other hand, 1D protein bands were analyzed by nanoLC-MS/MS, a complementary MS technique with higher sensitivity and more effective than MALDI-ToF/ToF regarding the identification of individual proteins in complex mixtures.

The proteomic study revealed a total of 79 proteins of *P. noctiluca* identified with high probability (>95%) (see table III.2-2), 31 being identified from MALDI-ToF/ToF and 48 from nanoLC-MS/MS in a total of 68 different proteins. Results with redundant information were excluded relatively to those with the highest protein score (therefore we mentioned only the 68 different proteins instead of the 79 proteins identified). The functions of 36 proteins, seven from MALDI-ToF/ToF and 29 from nanoLC-MS/MS,

could be retrieved from homology search using the mass spectral data. The remaining 43 proteins showed high homology to unknown, uncharacterized or predicted proteins in databases. A Blast search in different databases was performed for this group of proteins to find homologies with other proteins with known functions. The difference between the number of analyzed proteins (22 bands plus 151 spots) and the 79 identified is due to the lack of information on *P. noctiluca* proteome or even on other jellyfish proteomes. Moreover, the lack of information related to the known protein sequences of this taxonomic group, has created an additional difficulty in the assessment of the functions of the proteins here identified.

From all the identified proteins, and given their major biological importance, we highlight: (i) the zinc metalloproteinase nas-15-like, (ii) Heat shock protein, (iii) Red fluorescent proteins, and (iv) Peroxiredoxin-4.

Concerning the molecular function of the proteins found, the vast majority has a binding function followed by a catalytic activity, regulatory activity and antioxidant activity. The metabolic processes were over-represented in this characterization of *P. noctiluca* proteome in comparison to other cellular processes and cellular/extracellular organization, response to stimulus, etc. Relatively to metabolic processes we can highlight by its high expression, processes encoded by aminopeptidases or glutamate decarboxylases, ATP or RNA binding activity processes or n-dimethylguanosine tRNA methyltransferase activity processes. Concerning to the cellular distribution of the proteins here identified, our study revealed that the cytoplasmic and nucleus proteins are the most represented (Figure III.2-3).

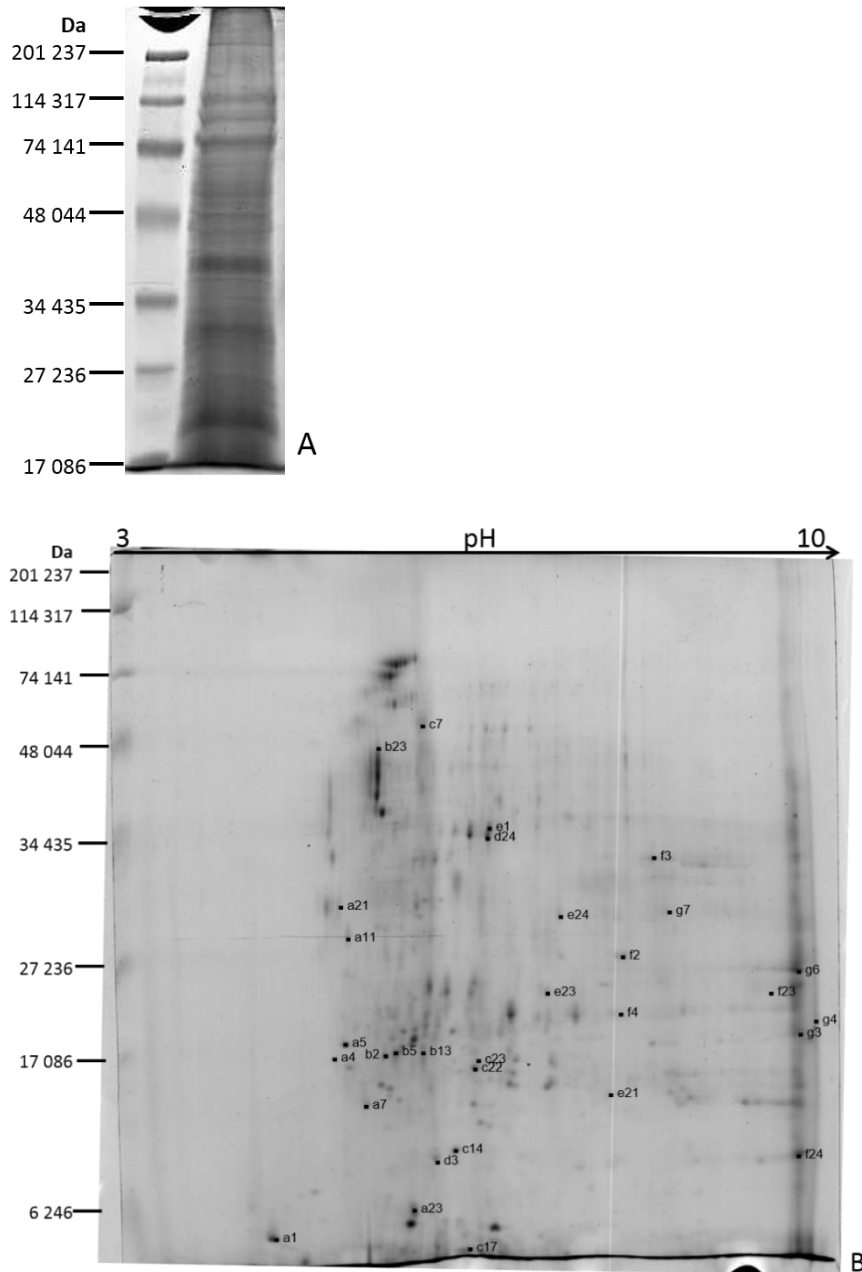


Figure III.2-2. Representative 1D and 2D gel electrophoresis of *P. noctiluca* crude protein extracts. *P. noctiluca* proteins separated by 1D (A) and 2DE (B). Gels were stained with CCB. 2DE gel dots identified by a code refer to the proteins identified by MALDI-ToF/ToF (see table III.2-2). The code refers to the MALDI plate coordinate.

**Table III.2-2. List of all proteins from *P. noctiluca* identified by nanoLC-MS/MS from the 1D SDS-PAGE gel and from MALDI-ToF/ToF from 2DE. Proteins are organized according to the platform LC-MS/MS or MALDI-ToF/ToF. Database search (Db): Nr- NCBI\_nr, C- NCBI\_Cnidaria P- UniProtKB\_Cnidaria, M- UniProtKB\_Metazoa, followed by lane No/gel dot code. Lane No refers to one of the 20 equal portions of the 1D gel cuts. Gel dot code is identified in the Figure 1B. Redundant identifications are not showed. All proteins have the percentage of identity in Blast search, Mass (kDa), protein score and the amino acid sequence with the corresponding ion score.**

Db	Lane/ Gel dot	Protein	NCBI/ UniProtKb Accession NO	% Identity (Blast)	Mass (kDa)	Protein score	Sequence	Ion score
LC-MS/MS								
Nr	7	Betaine-homocysteine S-methyltransferase 1	gi 47227619	95	54	93	FIGGCCGFEPY HIR	93
C	8	Aminopeptidase	gi 449662315	100	60	77	VGNTDAEGR DIGGSDPER IQDKEGIPDQQR	55 51 68
C	8	Ubiquitin	gi 455714954	100	93	74	TITLEVEPSDSIE NVK	34
C	8	Ubiquitin C	gi 449692233	100	61	67	TITLEVELADTIE NVK	67
C	8	Zinc finger protein 862	gi 443585256	99	1770	81	SVDETLR QITVNDLPVGR	43 73
Nr	8	Aminopeptidase	gi 156372710	62	56	128	NSVGADCYVAD EIITSAAGVR VGNTDAEGR	119 55
Nr	8	Aminopeptidase	gi 115495185	82	56	77	GITYDTGGADIK VGNTDAEGR	74 55
Nr	8	Chitinase precursor 1	gi 46016169	48	52	106	IALGMGTYGR IALGMGTYGR	68 89
Nr	8	Chitinase precursor 1	gi 156382335	48	50	68	LVLGIGTYGR	68
Nr	8	Disulfide isomerase	gi 156371686	64	54	92	ANQLPLVTEFSD ETAPK	92
Nr	8	Fructose-bisphosphate aldolase	gi 29336561	100	40	70	ATVEAIQR	70
Nr	8	Isochorismatase domain	gi 156386742	65	22	70	TAAPDSGLLSKV	70
Nr	8	Peroxidoxin-1	gi 2352262	100	22	73	QITVNDLPVGR	73
Nr	8	Polysaccharide deacetylase	gi 497542149	100	28	65	RMGFDVMSLES AR	62
Nr	8	Polyubiquitin-B	gi 511876691	100	26	75	IQDKEGIPDQQR TITLEVEPSDTIE NVK	68 67
Nr	8	Simple sugar transport system ATP-binding protein	gi 288960650	100	56	71	AALDLIAR	71
Nr	9	Transport-associated lipoprotein	gi 491621847	100	38	69	MSTSAAPGFDR	69
Nr	10	Phosphate binding protein	gi 77454721	100	39	152	GVDDLVLDGPT AAK IVTSAGPEPVEIT TETVGK	66 61
C	10	Zinc metalloproteinase nas-15-like	gi 449678102	100	34	61	IATVTVPR	53
Nr	11	Filamin-C	gi 156400860	40	367	66	FADEHVPGSPF K	66
Nr	12	Alpha-1,6-mannosylglycoprotein 6-beta-N-acetylglucosaminyltransferase B isoformX2	gi 118099612	100	92	63	NSLMNAK	63
C	13	Actin	gi 455714727	100	99	65	SYELPDGQVITI GNER	65
C	13	RNA-directed DNA polymerase from mobile element jockey	gi 443585134	94	1723	74	LILITNMLK	57
Nr	13	Actin	gi 61676569	100	26	65	SYELPDGQVITI GNER	65
Nr	13	Phosphomethylpyrimidine kinase	gi 517524103	51	55	73	LDKAIQLAK	73

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Nr	15	Glutamate decarboxylase	gi 325285142	100	51	65	GLELAQIAGK	65
Nr	15	Haloacid dehalogenase superfamily subfamily IA variant 1 with third motif having Dx(3-4)D or Dx(3-4)E	gi 524050579	100	23	62	AANIQTIAK	64
Nr	15	Hypothetical protein ARB_04071	gi 302510485	100	23	66	GDPATASRR	66
Nr	15	TPA: putative EDM2-like family protein	gi 414588820	100	87	62	GANLLPKK	62
Nr	15	Transferrin	gi 150036372	100	76	70	CLKDGAGDVAFVK	74
Nr	16	Heme exporter protein A	gi 497843781	100	21	64	SQDILCTFLSDQLIAK	64
Nr	16	Lysozyme	gi 220950	100	15	91	ATNYNAGDR STDYGIFQINSR	61 86
Nr	16	n-dimethylguanosine tRNA methyltransferase	gi 397572273	37	70	66	QAGAAGDAGR	66
Nr	16	Potassium-transporting ATPase subunit B	gi 427717747	100	75	67	LIDIVNIGK	67
C	17	Disulfide-isomerase A3	gi 163685802	73	30	76	VDCTEAGKESCSK	76
C	17	Disulfide isomerase-like 2-3	gi 219223716	84	10	111	LAPEYEEAATK RLAPEYEEAATK	80 66
Nr	17	Aminopeptidase NPEPL1	gi 260781477	71	54	87	LVLGDGVAYAAK	89
Nr	17	Aminopeptidase NPEPL1	gi 157823323	100	44	78	DCGGAAAILGAFR	81
Nr	17	C4-dicarboxylate ABC transporter substrate-binding protein	gi 517538613	36	39	63	VGSENYKMALEILK	63
Nr	17	Disulfide-isomerase A3	gi 156356029	58	55	61	VDCTEAGKDTC SK	61
Nr	17	T-complex protein 1 subunit gamma	gi 66824625	26	70	62	QMNIIEPLILK	62
Nr	17	Thymidine phosphorylase	gi 14520781	100	55	62	LDKAILLAR	62
C	18	Actin	gi 167801642	90	30	134	AGFAGDDAPR SYELPDGQVITIGNER	77 98
C	18	RNA-binding protein 39	gi 74124731	100	26	65	VLVLLDAK	54
Nr	18	Membrane protein	gi 518978269	100	82	65	ASAATLAVQR	65
Nr	18	Outer membrane protein assembly complex, YaeT protein	gi 495614929	100	84	64	SSIVISSK	64
Nr	18	Ubiquitin/actin fusion protein	gi 33346945	100	50	117	AGFAGDDAPR SYELPDGQVITIGNER	77 98
Nr	19	DNA ligase	gi 497462837	100	34	63	VGAGLAGTAGAR	63
MALDI-ToF/ToF								
P	a1	Calmodulin	tr B1PM92	100	17	213	DTDSEEEIREAF R	3
							VFDKDGNGFISAAELR	75
							EAFLSLFDKDG GTITTK	38
							EADIDGGQVNYEEFVK	55
M	a4	Proteoglycan	tr H2XZV7	42	7	109	VSTPSLPR	41
							VTTPSLPR	50
P	a5	Calbindin	tr T2MHT3	100	31	60	LWYHYDTR	53
Nr	a7	GPI inositol-deacylase-like protein	gi 379111263	31	108	82	QKAGKPNK	43
Nr	a11	E3 ubiquitin-protein ligase	gi 196136937	100	1816	86	LAPLNSNK	18
							LAPLNSNK	9
							IIFKVNR	30
							IIFKVNR	9
							QEDAEKTTITC	21



							RRTTTECQTYF	2
							HAR	
							VSKNMQKNQNL	6
							ECHGR	
<b>P</b>	e1	Intraflagellar transport protein 81 homolog	tr T2M8U1	100	78	62	IHEQEVLGK	1
							KTVSDQNVFR	33
							TVSDQNVFRDK	8
<b>Nr</b>	e21	RNA-directed DNA polymerase from mobile element jockey	gi 196141658	94	1826	81	RQNIKTQIR	9
							LILIITNMLK	72
<b>Nr</b>	e23	histone-lysine N-methyltransferase SETMAR	gi 196133572	84	1694	96	SIYAILLR	29
							EYIKSNKR	7
							NTLCEYFIR	25
							VKYSGIWLIR	11
							KNTLCEYFIR	21
							NEMGWMKSDK	17
							TNI	
							QVQLTRKHHNP	8
							LR	
							SYECCMTCRKF	1
							K	
<b>Nr</b>	e24	jerky homolog protein	gi 196138580	83	1838	110	YIDYEK	19
							IIECMPIK	9
							MLLSTILLK	35
							CVLMLKSKSK	20
							VCFVFCNYVR	1
							NTHLGQMLTAS	5
							S	
							ELSENILEFK	17
							IFLNCSRSLR	13
							YYLDTR	54
							NCGMOTLR	1
							NTKMRTPR	14
							NSISVSKFR	20
							IGFELLTVR	20
							INKNANCNI	3
<b>P</b>	f2	Class A rhodopsin-like G-protein coupled receptor	tr A7RW16	33	87	65	SPGLPTAEQLS	12
							DR	
							KSPGLPTAEQL	27
							SDR	
							KKSPGLPTAEQ	22
							LSDR	
<b>Nr</b>	f3	NACHT, LRR and PYD domains-containing protein 6	gi 262248455	85	994	82	IIRVNNK	10
							VGYEIQDER	17
							LIINTLFIL	34
							NMATVFHTRSD	5
							LR	
							NMATVFHTRSD	2
							LR	
							NIKPKISLDYLFR	9
							KITINCINQKM	10
							EI	
<b>Nr</b>	f4	RNA-directed DNA polymerase from mobile element jockey-like	gi 196152139	87	1842	84	SSGAEFEKK	9
							SSICIGLR	20
							AASSDFPGSAV	2
							T	
							SSLTEMKEINR	62
							IREFFFLDSHG	5
							KVR	
<b>Nr</b>	f23	zinc finger BED domain-containing protein 4	gi 262251512	81	1862	102	NLEVDQK	2
							CNKIYQK	7
							LKDLTNNR	23
							EQSYRARK	8
							HNFKKIQR	10
							VTPVHLMELF	0
							TTPSSILLQFK	22
							AIGMMQLFVHR	13

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							AYNGCIYIVR	1
							ISPMNERWNVN	13
							GSSDKLFTPQNI	16
							K	
							CNFQIIISKHHQ	2
							NK	
Nr	f24	Microsatellite	gi 386989807	74	164	77	LEGVGLLR	28
							IQSFVWNGR	7
P	g3	Protein domain with unknown function of	tr A7RSY1	Joubert (PF15392)	398	63	HTDDPRGVSK	8
							EQHDLFLDNS	0
							R	
							FQDIQSLLSDDS	16
							ELK	
							DPQGSRHPDDT	13
							VISSR	
							VMVQSPTTEEE	15
							DGSKR	
							MSGQNVPLGNT	4
							QQDVSGQDVH	
							GQGSK	
Nr	g4	RNA-directed DNA polymerase from mobile element jockey	gi 262250531	97	1526	82	NVLELIR	15
							VLILADFQK	7
							NVRISHCFNKN	3
							VCTSSIQDEKKL	26
							CPK	
P	g6	Spectrin chain alpha	tr T2MDE9	100	254	67	QDFNDEL	12
							NTSNIQAKR	24
							EVDEAFRAITDG	16
							GK	
							LQETVKDREVE	5
							LEK	
Nr	g7	Structural maintenance of chromosomes protein 3	gi 196149695	82	1167	80	MKGMNVK	1
							AIAEYLLR	53
							CIFGIIHYR	2
							KLFTHSDYTKM	12
							K	
							ESALKRLLDELV	24
							IK	

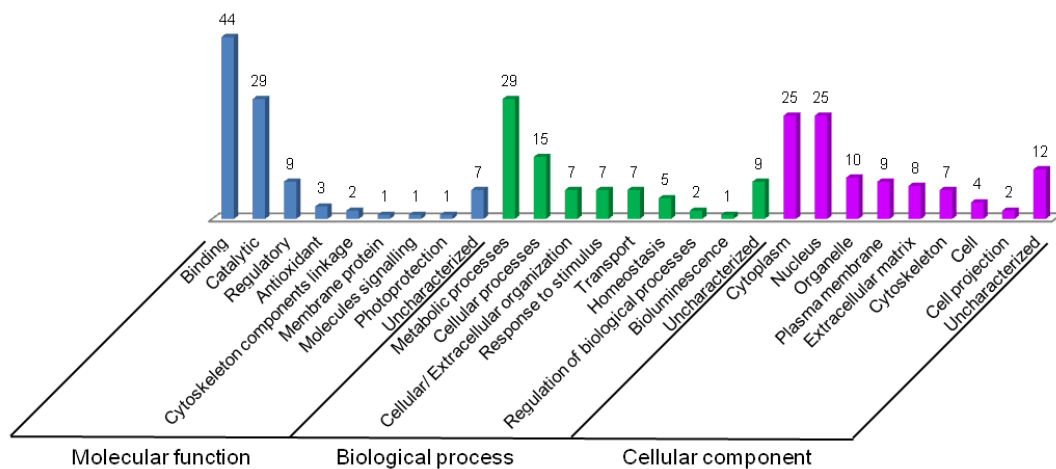


Figure III.2-3. *P. noctiluca* protein characterization relative to its molecular function, biological process and cellular distribution based on its GO annotation. Bars numbers correspond to the amount of proteins identified in this study related with the described (under the bars) characteristic (Molecular function, Biological process or Cellular component).



### 3.2. Genotyping Identification

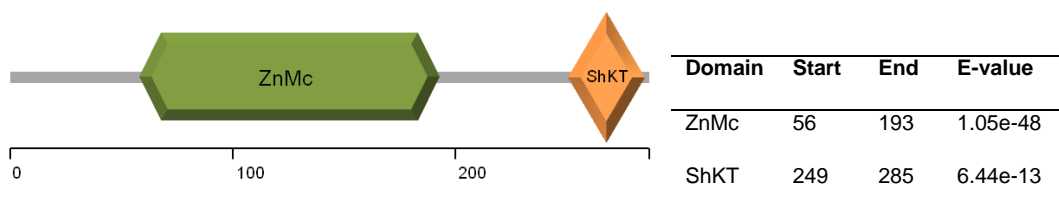
*P. noctiluca* DNA sequences have showed a good amplification of the fragment of interest, as well as a positive identification for the species. GenBank accession numbers for the 16S and COI gene are the, PN1 (accession no1, accession no2) and PN2 (accession no3, accession no4), respectively.

## 4. Discussion

To our knowledge, this work is the first study focused on the proteome of the entire jellyfish body. Kawabata et al. (2013) studied the all body protein content of 12 jellyfish species, but only in terms of bioactivity, and only a few works (Brinkman et al. 2012, Li et al. 2012b, 2014, Weston et al. 2013, Kang et al. 2014) studied jellyfish venomics on a proteomic approach. In contrast, our study also emphasizes, beside venom proteins, other proteins that constitute the jellyfish body, which can be more or as important as toxins. Since there is a lack of work on *P. noctiluca* all body proteome, this study provides a step forward to the knowledge of its body constituents not only uncovering bioactive compounds with possible biomedical application, but also improving this species mastery. Moreover, the characterization of the body constituent proteins can provide insight into the biological mechanisms allowing environmental adaptation and predatory diversification (e.g. new processes of toxin synthesis and maturation). Our work allowed the identification of 68 different proteins related with binding processes, catalytic and regulatory activity among many others. A Blast search for protein identification revealed several representatives previously described in jellyfish, including calmodulin, ubiquitin and potassium-transporting ATPase (Li et al. 2012b). From all the identified proteins, we have selected by its relevance: (i) the zinc metalloproteinase nas-15-like, (ii) Heat shock protein, (iii) Red Fluorescent Protein and (iv) Peroxiredoxin-4.

Zinc metalloproteinase or metalloproteases, are enzymes whose catalytic mechanism involves a metal, in this case zinc. Zinc metalloproteinase nas-15-like has a size of 34 KDa and show a ShK toxin domain in the end of the protein sequence (inferred in the SMART webpage (Letunic et al. 2015)) (Figure III.2-4). This domain is found in sea anemones toxins, namely metridin from *Metridium senile* and in ShK from *Stoichactis helianthus*. The toxicity associated with this domain was previously described by Lee et al. (2011). It is known that the neurotoxin ShK, which

inhibits potassium channels, block a particular white blood cell, namely the T-lymphocyte in the voltage-gate Kv1.3 channel. It has been proposed that structural analogues may be used as immunosuppressant for the prevention of transplant rejection and for autoimmune diseases, such as diabetes, multiple sclerosis or rheumatoid arthritis treatment (Letunic et al. 2015). Importantly, we have identified this protein for the first time in this species. A zinc metalloproteinase was previously found in a hydrozoan jellyfish *Podocoryne carnea* and described as having an important role in prey paralysis until ingestion (Pan et al. 1998). However, it is not the first time that zinc metalloproteases are reported in jellyfish venom. Previously, Lee et al. (2011) found metalloproteases in *Aurelia aurita*, *Cyanea nozakii*, *Nemopilema nomurai* and *Rhopilema esculenta*, and their presence was correlated with venom toxicity. In a hydrozoan jellyfish *Olindia sambaquiensis*, 29 putative toxins (mostly being metalloproteinases) were characterized by LC-MS/MS, including cytolytins, neurotoxins, phospholipases and toxic peptidases, causing the disruption of capillary vessels and tissues leading to fatal hemorrhagic lesions. Interestingly, metalloproteases from our study are described as catalysts of coagulation factor II, and some have the ability to activate only the coagulation factor X (Weston et al. 2013). Similar studies on the jellyfish *Stomolophus meleagris*, using both proteomics and transcriptomics approaches, revealed major components related to the sting, in a total of 218 toxins, including the zinc metalloprotease, C-type lectin, PLA2, potassium channel inhibitor, serine protease inhibitor, and hemolysins, among others (Li et al. 2014). MALDI-ToF analysis on *Nemopilema nomurai* revealed 18 protein families, such a matrix metalloproteinase-14 and an astacin-like metalloprotease toxin 3 precursor (Kang et al. 2014).



**Figure III.2-4. Identification of ShK domain.** Diagram representing the ShK domain (Yellow diamond) on the *Hydra magnipapillata* zinc metalloproteinase nas-15-like (LOC100213185), mRNA protein, based on SMART program webpage.

*P. noctiluca* has been studied for years due to its ecological relevance and toxicity. Since 2002, its venom has been recognized to have neurotoxic, hemolytic and cytotoxic properties. The neurotoxic activity was observed in a shore crab (Sánchez–

Rodríguez & Lucio–Martínez 2011) and hemolysis was observed in fish, chicken, rabbit and human red blood cells (Marino et al. 2007, 2008, Sánchez–Rodríguez & Lucio–Martínez 2011, Maisano et al. 2013). Results showed that hemolysis is due to a pore formation on the erythrocytes. Cytotoxicity was observed in hamster lung fibroblasts (Mariottini et al. 2002), monkey kidney cells (Ayed et al. 2013, 2014), human neuroblastoma (Morabito et al. 2012), glioblastoma (Ayed et al. 2012) and colon cancer cells (Ayed et al. 2011). Oxidative stress and apoptosis induction was revealed in these studies. Moreover, a different work referred that two venom fractions display potent anti-tumoral properties (Ayed et al. 2012), reinforcing the importance of studying this species.

Heat shock proteins are proteins that play a critical role in protein folding, protein intracellular traffic, and recovery of proteins denatured by heat and other stresses (Santoro & Biochem 2000). In that sense, they are house-keeping proteins. Nonetheless, these proteins are expressed when the organisms are under stress. The jellyfish collected for this study, were catch in the shore break, and afterwards taken to an aquarium where they starved for three days, consequently subjected to various kinds of stress (e.g. manipulation, starvation, change in the light intensity or water physiochemical parameters). In the literature it is referred that when cells are exposed to elevated temperatures or other stress such as starvation, heat shock proteins increased its concentration (Santoro & Biochem 2000). In that sense, the observation of heat shock proteins could be explained by either their housekeeping features or the stress induced in this study. In addition, previous studies (Brinkman et al. 2012, Li et al. 2012b) also documented the expression of these proteins in jellyfish.

The Red Fluorescent Protein (RFP) is another protein with high biotechnological importance. This protein was previously found in species of anthozoans and hydrozoans, in a total of 52. Anthozoans species include stony corals as *Acropora* sp. or *Montipora* sp., coral anemones as *Discosoma* sp., *Corynactis californica* or *Ricordea florida*, sea anemones as *Anemonia sulcata*, *Actinia equina*, *Condylactis gigantea*, *Condylactis passiflora*, *Entacmaea quadricolor*, *Nematostella vectensis*, *Heteractis crispa* and mat anemones as *Zoanthus* sp. Importantly, our study identified for the first time this RFP in a jellyfish. Until now it was not identified either in a scyphozoan or in a cubozoan. It is thought that this protein plays a role in the photoprotection of the animal. In the depths of the open sea, the fluorescence may convert blue light into longer wavelengths more appropriate for the photosynthesis of microalgal symbionts (Matz et al. 1999). For scientists, fluorescent proteins have become a useful and ubiquitous tool for making chimeric proteins, where they function as a fluorescent

protein tag. In general, fluorescent proteins can accommodate a ligation to other proteins in the beginning of the amino acid chain (N-terminus) or in the end (C-terminus). They have been found in various cell types and even in living cells or organisms with minor interference. They are used in a wide range of applications such as cell lineage tracer, reporter of gene expression, or as a measure of protein-protein interactions (Matz et al. 1999, Shrestha et al. 2006). Thus, the finding of a RFP in the *P. noctiluca* jellyfish is of major relevance for future works on molecular biology and biotechnology.

Jellyfishes are exposed to continuous environmental changes, such as salinity, temperature, pollution, solar radiation, microorganisms and pathogens. This can cause the activation of inner defense responses, namely the production of reactive oxygen species (ROS). Although ROS are needed in the organism, an excess of these chemical species could cause a metabolic balance disorder, damaging cellular lipids, proteins and DNA. Catalase, glutathione peroxidase, superoxide dismutase, thioredoxin and peroxiredoxins are proteins with powerful antioxidant properties. *P. noctiluca* showed to possess peroxiredoxin and superoxide dismutase. To our knowledge this is the second time that peroxiredoxin is found in a jellyfish (Ruan et al. 2014). Previous works have already demonstrated the presence of superoxide dismutase in medusa (Zhuang et al. 2009). These antioxidant proteins are likely just a few of many others possible molecules of *P. noctiluca* with powerful antioxidant properties and therefore constitute a natural resource of antioxidant and anti-UV radiation agents (Ruan et al. 2014).

Most studies focus on the characterization of the venom constituents of jellyfish. Here, we aimed a more comprehensive analysis of *P. noctiluca* proteome. This enabled us to identify several proteins, also represented in humans and known to play a major role in several diseases. The jellyfish homologous proteins could be important to provide further insights on the structure and function, and molecular evolution of Cnidaria proteins (see table III.2-3). The majority of the proteins identified in *P. noctiluca* are implicated in cancer, followed by cardiovascular diseases, diabetes, HIV, Immune system disorders, musculoskeletal disorders, neurologic system disorders, and red blood cell disorders, among others. In fact, a recent study on Nme gene family has shown that these genes are evolutionarily conserved in Metazoans. Nme gene family is implicated in cellular differentiation and in metastatic dissemination, and is used as clinical markers of tumor aggressiveness. This observation supports the importance of the genomic and proteomic studies in these animals, which can be used as models to investigate Nme functions (Desvignes et al. 2010) From these studies can

arise new biological compounds with biological significance, which could shed new light on a wide variety of key developmental and cellular processes in humans.

**Table III.2-3. Biological significance of the proteins found in *P. noctiluca* detected by nanoLC-MS/MS and MALDI-ToF/ToF**

Biological significance	Reference	Biological significance	Reference
<b>Cancer</b>		<b>Immune system disorders</b>	
Alpha-1,6-mannosylglycoprotein 6-beta-N-acetylglucosaminyltransferase B isoformX2	(Bubka et al. 2014)	Chitinase 1 precursor	(Bierbaum et al. 2005)
Aminopeptidase	(Ikeda et al. 2003)	Lysozyme	(Tomita et al. 1999)
DNA ligase	(Lamballe et al. 1988)	NACHT, LRR and PYD domains-containing protein 6	(Martinon et al. 2007)
Fructose-bisphosphate aldolase	(Khan et al. 1998)	Phosphate binding protein	(Gonzalez et al. 2014)
Histone-lysine N-methyltransferase SETMAR	(Tian et al. 2013)	Superoxide dismutase	(Muscoli et al. 2003)
Microsatellite sequence	(Sinicrope et al. 2012)	<b>Musculoskeletal disorders</b>	
n -dimethylguanosine trna methyltransferase	(Goffin et al. 2002)	Actin	(Satish et al. 2011)
Protocadherin Fat 1	(Nishikawa et al. 2011)	Haloacid dehalogenase superfamily subfamily IA variant 1 with third motif having Dx(3-4)D or Dx(3-4)E	(Roberts et al. 2008)
Red fluorescent protein gene	(Hoffman & Nat Rev 2005)	Proteoglycan	(Knudson et al. 2001)
RNA-binding protein 39	(Nash et al. 2003)	TPA: putative EDM2-like family protein	(Fiedler et al. 2002)
Serine/arginine-rich splicing factor	(Kim et al. 2014)	<b>Neurologic system disorders</b>	
Structural maintenance of chromosomes protein 3	(Zhou et al. 2012, Yadav et al. 2013)	Calbindin	(Stabler et al. 1999)
Thymidine phosphorylase	(Ogawa et al. 2014)	Potassium-transporting ATPase subunit B	(Adav et al. 2014)
Zinc metalloproteinase nas-15-like	(Krizkova et al. 2011)	<b>Red blood cell disorders</b>	
<b>Cardiovascular disorders</b>		Heme exporter protein A	(Chiabrando et al. 2012)
Disulfide isomerase	(Flaumenhaft et al. 2015)	Spectrin alpha chain	(Wolny et al. 2012)
Filamin-C	(Valdes-Mas et al. 2014)	Transferrin	(Chua et al. 1999)
Peroxiredoxin	(Maksimenco et al. 2012)	<b>Implication in various previous disorders / Other disorders not mentioned</b>	
Zinc finger BED domain-containing protein 4	(Klug & Lett 2005)	Betaine-homocysteine S-methyltransferase 1	(Pajares et al. 2006)
<b>Diabetes</b>		Calmodulin	(Neri et al. 1995)
Glutamate decarboxylase	(Ellis et al. 1996)	Class A rhodopsin-like G-protein coupled receptor	(Muller et al. 2012)
Simple sugar transport system ATP-binding protein	(Matsuo & Biosci Biotechnol 2010)	Disulfide-isomerase A3	(Rosewell et al. 2011)(Flaumenhaft et al. 2015)
<b>HIV</b>		GPI inositol-deacylase-like protein	(Ferguson et al. 1999)
Plectin	(Ding et al. 2008)	Heat shock protein	(Soti et al. 2005)
RNA-directed DNA polymerase from mobile element jockey	(Yokoyama et al. 2010)	Intraflagellar transport protein 81 homolog	(Yang & Wang 2012)
		Phospholipase C	(Li et al. 2010, Ramadan et al. 2012)
		T-complex protein 1 subunit gamma	(Bhaskar et al. 2012)
		Transposable element-derived protein	(Ivics et al. 2006)
		Ubiquitin	(Xu & Jaffrey 2013)

## 5. Conclusion

*P. noctiluca* is a jellyfish species of concern since it forms frequent blooms in the Mediterranean Sea, negatively affecting tourism and fishery. As a toxic species, the study of its venom is important for prospecting bioactive compounds. Here, we used a comprehensive proteomic analysis of the all body constituents of this medusa, which revealed an additional repertoire of molecules with biological and biotechnological interest. We used specimens from the Atlantic Ocean and found various proteins with potential biological significance (e.g. for cancer treatment). Moreover, we found a zinc metalloprotease that was described for the first time in this jellyfish. This protein has a ShK toxic domain and therefore could be implicated in the sting toxicity. Interestingly, a Red Fluorescent Protein was also identified for the first time in a jellyfish. The RFP here identified could be important as a new potential molecular marker for gene or protein expression assays. Finally, a potent antioxidant protein, peroxiredoxin, was also found for the second time in a jellyfish and for the first time in *P. noctiluca*. The identification of this protein can make of this organism a new natural resource of antioxidant and anti-UV radiation agents. Based on these results we proposed *P. noctiluca* as a promising species for further detailed studies to characterize its all body and venom proteome.

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## IV General Discussion

## General discussion

Portuguese sea coast has favorable environmental conditions for the proliferation of different marine organisms. Cnidarians have found here, an advantageous ecosystem to establish their community. The principal reasons pointed for this establishment could be that Portuguese sea coast has several geographical events that could influence positively intertidal organisms, such as the upwelling phenomena (Sibaja-Cordero & Cortés 2008) and the presence of rocky shores. As it is well known, cnidarians produce toxins, and in that manner they can affect country economy by causing negative impacts in tourisms and in aquaculture. Despite all the scientific literature on these organisms there are considerable information about their toxins that are still unrevealed. In that sense and to organize the knowledge in this topic we have created two compilations works that were published as review articles, one encompassing the diversity of sea anemones toxins and the other gathering the methods for analysis of jellyfish bioactive compounds. In fact, these works that assemble various kind of information revealed that there were for example a gap on toxin coding genes identification, as well as in the use of novel technologies in the study of cnidarians such as deep sequencing. High-throughput sequencing is an emerging technology and apart from two genomes (*Nematostella vectensis* and *Hydra vulgaris*) that have been already sequenced; only a few mitochondrial genomes and a few other transcriptomes have recently become available, which make cnidarians far over of being extensively studied.

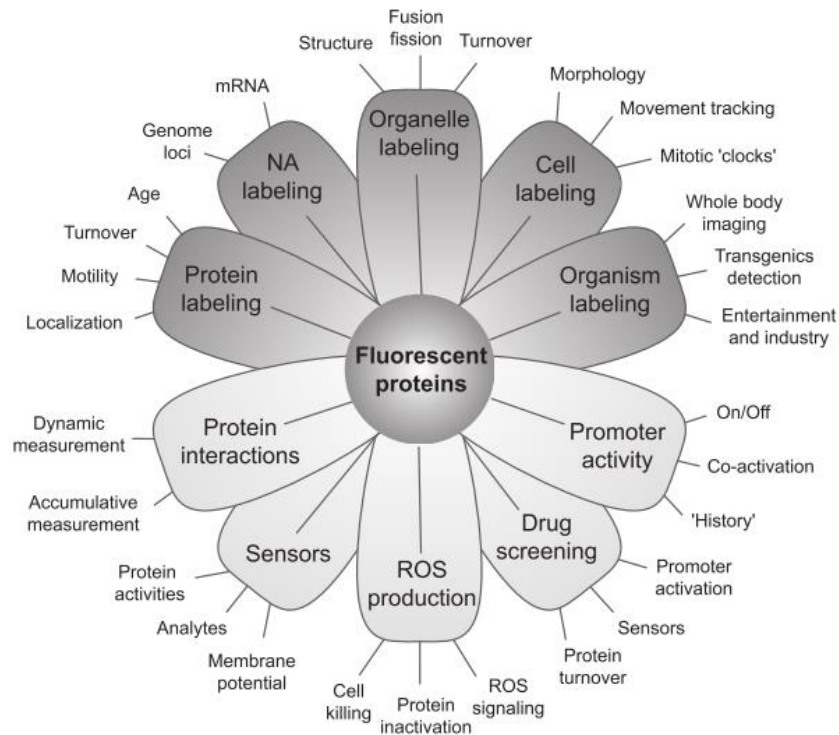
However, the so far genome assembly and annotation of non- bilaterian animals such as cnidarians, poriferans, placozoans, and ctenophores has already greatly contributed to unravel the origin and early evolution of animals (Ryan et al. 2010, 2013, Srivastava et al. 2010, Smith et al. 2012, Pan et al. 2013, Putnam et al. 2017) and the evolutionary origins of neural systems (Moroz et al. 2014). Reference transcriptomes have been produced for several species (Pooyaei Mehr et al. 2013, Wenger & Galliot 2013, Stefanik et al. 2014, Fernandez-Valverde et al. 2015) and RNAseq (RNA deep sequencing) has been used to uncover details regarding the evolution of special developmental features such as head regeneration in *Hydra magnipapillata* (Krishna et al. 2013) or the life cycle of the scyphozoan jellyfish *Aurelia aurita* (Brekhman et al. 2015). Transcriptomic data has also been used to study the response to environmental stress such as bleaching in corals (Anderson et al. 2016) and exposure to heavy metals in cnidarians (Elran et al. 2014). Other techniques, such as restriction site associated DNA (RAD markers) has been successfully used in these animals as exemplified by Reitzel et al. (2013) to uncover the phylogeographic and demographic

history of sea anemone (*Nematostella vectensis*), as well as to identifying genomic regions showing signatures of natural selection. Another interesting application of genomic approaches includes the search for bioactive compounds with potential to become novel drugs, nutritional supplements or applications for industrial biotechnology (Urbarova et al. 2012, Frazão & Antunes 2016). Deep-sequencing of the genome or transcriptome of these animals is an alternative to traditional *in vivo* and *in vitro* analysis that targeted the venoms or secreted proteins (Johansen et al. 2010, Frazão & Antunes 2016). Bioprospecting of neurotoxic drugs has been performed on sea anemones (Kozlov & Grishin 2011, Urbarova et al. 2012) and some other putative toxins were discovered using the transcriptome obtained from nematocysts localized in the tentacles of the jellyfish *Chrysaora fuscescens* (Ponce et al. 2016) or from the coral *Acropora digitifera* (Gacesa et al. 2015), as well as from the specialized tentacles called acrorhagi in aggressive and non-aggressive *Anthopleura elegantissima* polyps (Macrander et al. 2015).

As it was referred previously, cnidarian toxins are somewhat documented. Nonetheless, toxins evolution studies have still a long way to go. Previous works on coral toxins, state that toxins do not evolve rapidly by prey-driven positive natural selection. Rather, Gacesa et al. (2015) stated that the venom has a defensive role deterring predation or harm from interspecific competition and overgrowth by fouling organisms. Moran et al. (2008) also share this statement with respect to *N. vectensis* toxins. However, in the same work, it is elucidated that are other toxins evolving rapidly by diversifying selection such as snake, scorpions or cone snails venoms and is referred that both concerted evolution and accelerated evolution may occur simultaneously (Moran et al. 2008). With an increase in the study of cnidarian phylogeny and toxin characterization, as this work, other phylogenetic studies will emerge to better resolve toxins phylogeny and evolution. Another gap, is the data missing on toxin gene coding sequence. The coding gene finding is somewhat more difficult if the toxin is coded by more than one gene. For example, Nv1 from *N. vectensis* is coded by 13 genes. Gene families' sequences are more difficult to resolve compared with single gene sequences since more procedures have to be applied such as cloning, and more techniques imply time consuming and more efforts. On the other hand, toxin three-dimensional structures are gaining relevance, with 15 currently available. The importance of these studies relies on deep knowledge of its mode of action. Another interesting topic is PLA2 characterization. Nevalainen et al. (2004) determined the PLA2 activity in 22 species of cnidarians and only four have its sequenced described. These neurotoxins appear in a wide variety of venomous animals and so far, very few were characterized to date in cnidarians. The phylogeny of

these kind of toxins is at its initial stage and for now the characterized toxins are not congruent with the species phylogeny.

The investigation on marine organisms has revealed to be important for the discovery of biomolecules with pharmaceutical or industrial interest (Rocha et al. 2011), and to understand metabolism processes important to medicine, as for example the endless capability of regeneration and consequent immortality of some of these organisms (Krishna et al. 2013) as previously referred. In that sense, our proteomic analysis on *P. noctiluca* collected in the Portuguese coast, revealed three bioactive proteins that were for the first time identified in this organism. It was found a potent antioxidant molecule, a peroxiredoxin (Ruan et al. 2014), which could be used for pharmaceutical purposes. Peroxiredoxins regulates cell growth, metabolism and immune regulation and therefore are involved in pathologic regulation or in cancers suppressors mechanisms, neurodegenerative diseases and inflammatory diseases. (Park et al. 2016). The identification of this protein can make this organism a new natural resource of antioxidant and anti-UV radiation agents. In a recent study, mice exposed to peroxiredoxin 6 survived after lethal X-ray irradiation, compared to mice not injected with this compound, suggesting that peroxiredoxin 6 can be regarded as a prophylactic radioprotective agent (Sharapov et al. 2016). Besides this compound, it was also found a fluorescent protein, a Red Fluorescent Protein (RFP), which has biotechnology potential. These kind of molecules are used to monitor physiological processes, visualizing protein cellular localization, and to detect transgenic expression in vivo when tagged to other molecules or cell components (Piatkevich & Verkhusha 2011). In figure IV.1 it is shown the main areas of applications of fluorescent proteins. Since the discover and isolation of the RFP from the sea anemone *Discosoma sp.* in 2000 (Fradkov et al. 2000, Chudakov et al. 2010) this protein have been used as fluorophore (a fluorescent protein) and nowadays, laboratories have focused their efforts on identification and development of fluorescent proteins with novel characteristics and enhanced properties (Chudakov et al. 2010). With this study, it was highlighted that *P. noctiluca* could be another source of biomolecules with fluoresce characteristics.



**Figure IV. 1. Main areas of application of fluorescent proteins (adapted from (Chudakov et al. 2010))**

Lastly, it was found a protein with a toxic domain, a zinc metalloproteinase with a ShK domain, which can be implicated on the sting toxicity. This domain in sea anemone toxins is a potassium channel inhibitor of T- lymphocytes (Letunic et al. 2015). This white blood cells are responsible for the immune response that leads to the rejection of a transplanted organ, autoimmune diseases such as diabetes, multiple sclerosis, rheumatoid arthritis among others, and even allergic reactions such as gluten intolerance (Nouri-Aria & Durham 2008). In this sense, this molecule or its analogue could be important to suppress this immune response. The zinc metalloproteinase, found in *P. noctiluca* together with other known toxins can be used for the discovery of new drugs with medical application or even for anti-venom events.

This thesis revealed in the intertidal zone of the Portuguese rocky beaches, seven most common sea anemone species. They belong to the Actiniidae, Hormathiidae and Sagartiidae family. Namely, *Aulactinia verrucosa*, *Actinia equina*, *Actinia fragacea*, *Anemonia viridis*, *Cereus pedunculatus*, *Anthopleura krebsi* and *Calliactis parasitica*. These species are known to be ubiquitous (Campbell et al. 1994), but for the first time five genes were sequenced and a ML tree was constructed. This phylogenetic work did not exhibit genetic differences among populations from the three sampling sites (North, Center, and South groups). This fact could be possible due to the proximity of the sampling locations or the inefficiency of the markers chosen. In fact, previous

population studies on *Actinia equina* (Solé-Cava & Thorpe 1992, Vianna 2003, Schama et al. 2004, Chomsky et al. 2009) revealed different populations among the collected specimens. Nonetheless 58 new haplotypes were found. In favor to our work, this thesis provided a phylogenetic tree with more robust nodes compared to previous studies. Further in this work, it was accessed the best method for preserving sea anemones tissue with an optimized method for extracting DNA for subsequent molecular analysis. The result showed that 99% ethanol was the best choice compared to  $-80^{\circ}\text{C}$  preservation. As previously said, DNA extraction from tissues with high content of polysaccharides, pigments, among others (Dellacorte 1994, Pinto et al. 2000, Sahu et al. 2012, Stabili et al. 2015, Leone et al. 2015) is hard to obtain. A few works have been done to assess the best method for DNA extraction (Pinto et al. 2000). Some claim that DMSO provide good results (Dawson et al. 1998, Kilpatrick 2002) for those who do not extract with ethanol based reagents, others prefer ethanol (Bressan et al. 2014) and the majority prefers cryopreservation (Rocha et al. 2014). In this work, tissues preserved at  $-80^{\circ}\text{C}$ , did not revealed the best results. This fact can possible be due to the time of transportation of the biological material from the sample location to the lab, the absence of cryoprotectants (Fliegerova et al. 2014, Tsai et al. 2015) or even the freeze-thawed cycles that samples were subjected over the course of this thesis. Cryopreservation possible works better if live specimens were freeze over liquid nitrogen ( $-196^{\circ}\text{C}$ ) and then stored at  $-80^{\circ}\text{C}$  until use with cryoprotectants as ethanol, PBS-glycerol, Tris-EDTA, among others (Nagy 2010, Fliegerova et al. 2014, Tsai et al. 2015). In addition to the procedure referring to the best preservation method for sea anemone tissue, it was also improved the classical method of Phenol: Chloroform DNA extraction with the use of a mechanic homogenizer with glass beads.



## V Final Conclusion and Future Perspectives

## Final Conclusion and Future Perspectives

From this work, and answering to the biological questions proposed on this thesis, it can be stated that the most common cnidarian species found in the intertidal zone of the Portuguese rocky beaches, belong to the Actiniidae, Hormathiidae and Sagartiidae family. Namely, *Aulactinia verrucosa*, *Actinia equina*, *Actinia fragacea*, *Anemonia viridis*, *Cereus pedunculatus*, *Anthopleura krebsi* and *Calliactis parasitica*, in a total of seven species. There is no population bottleneck in the population of sea anemones from the Portuguese coast. *Pelagia noctiluca*, a jellyfish found in our shores, presented three bioactive proteins, found for the first time in this species. A zinc metalloproteinase that could be important to suppress immune responses, a peroxiredoxin that can be regarded as a prophylactic radioprotective agent and a fluorescent protein that can have novel characteristics and enhanced properties for biotechnological applications.

During this research several difficulties were encountered related to samples collection. For organism harvesting, special licenses are needed and facilities such as boats or specialized skills for SCUBA diving, which are expensive and time consuming. In addition, it is difficult to work with the collected biological material. Cnidarians nucleic acids are very hard to obtain, in high concentration amounts and in good quality for downstream applications. As stated on chapter II.2, possibly this is due to the presence of high amounts of polysaccharides and pigments present in the samples, which influence subsequent works. In conclusion, the major limitations that were found in this research were to obtain the most diversity of organisms in terms of species and geographic location, followed by the acquisition of genetic and proteomic material in good quality to proceed with our analysis. In opposition, these organisms are very interesting when related to toxicity, regeneration ability and phylogenetic position in the tree of life, which have retained our curiosity for working with them.

Despite our contribution with the work here presented on the cnidarians field, the sea anemones and other cnidarians members from Portugal, in intertidal or subtidal areas, should be continually investigated as few knowledge still exist for these animals in this specific location. More genetic studies should be performed and new sequences should be submitted to the NCBI database to increment and facilitate further phylogenetic analyses. Although corals are well studied; they are an endless source of biomolecules with pharmaceutical application (Rocha et al. 2011). Moreover, toxins evolution had been an interesting theme of investigation but is far from been solved. Furthermore, in addition to transcriptome studies on developmental stages or stress subjected organisms, also a gap in the scientific knowledge, nematocysts venom

transcriptome (Ponce et al. 2016) will be an important approach for future research. Finally, an emergent topic as epigenetics could be accomplished. It is known that epigenetic modifications on DNA and on chromatin are shared among eumetazoans. This suggests that most conserved genes present in humans genomes, as well as the mechanisms guiding their expression, evolved before the divergence of cnidarians and bilaterians (Technau & Schwaiger 2015, Dixon et al. 2016) and thus the study of epigenetic modifications in cnidarians (Marsh et al. 2016) can lead to a better understand of the epigenetic events in humans.

Altogether, this dissertation compile information on cnidarians and bring new insights which will assist in future cnidarians studies on population genetic structure, evolution and on the discovery of biomolecules with biological and clinical significance.



## VI References

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## VII Annex

VIII **Corynactis viridis**



Não pertence ao grupo das anêmonas verdadeiras. Até 1 cm de diâmetro. Ocorre a partir do intertidal inferior, em locais escondidos da luz como grutas. É encontrada em densos agregados. Possui uma cor brilhante como verde, rosa ou amarelo.

*Does not belong to true sea anemones group. Can reach 1 cm in diameter. Occurs from the lower shore on rocks, in caves and beneath overhangs where it is shaded from light. Found in dense aggregations. Presents bright colours, mainly green, pink or yellow.*

IX **Actinothoe sphyrodeta**

Até 3 cm de diâmetro. Espécie comum no subtidal, mas pode ser encontrada em zonas protegidas nas rochas, em maré baixa. Forma extensas colónias. Geralmente branca, com a zona oral branca ou alaranjada. Projecta longos fios brancos (aconita) quando perturbada.



*Can reach 3 cm in diameter. Common in subtidal areas, but in the low tide can be found in areas protected from light. Forms big colonies. Usually white, with the oral area white or orange. When disturbed projects long thin white threads (aconita).*



# ANÉMONAS DO INTERTIDAL DA COSTA PORTUGUESA

## INTERTIDAL SEA ANEMONES FROM THE PORTUGUESE COAST

CREDITS  
Barbara Frazão  
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DIVULGAÇÃO NO ÂMBITO DO PROJETO MARBIOTECH  
OUTREACH ACTIVITY FROM PROJECT MARBIOTECH

design/development: Feroz Leonardo Silva



**I Actinia equina**



Até 7 cm de diâmetro. Espécie ubíqua. Apresenta um anel azul na base e pode apresentar vesículas azuis (acrorhagi) junto aos tentáculos, para lutar por espaço com indivíduos da mesma espécie. Variedades: vermelha, acastanhada, verde ou esbranquiçada.

Can reach 7 cm in diameter. Common species. Presents a blue ring around the base and can show blue vesicles (acrorhagi) around the tentacles to fight for space against other individuals. Wide colour variation, from green to red.



**III Actinia cari**



Até 7 cm de diâmetro. Coluna com cores variadas, normalmente verde com linhas circundantes. Pode apresentar acrorhagi.

Can reach 7 cm in diameter. Wide colour variation, but usually green with surrounding lines. May present acrorhagi.

**IV Aulactinia verrucosa**



Até 3 cm de diâmetro. Coluna verrucosa cinzenta ou rosa, normalmente com 6 fíadas brancas. Tentáculos translúcidos geralmente com manchas verdes, cinzentas, rosa ou brancas. Encontra-se parcialmente enterrada.

Can reach 3 cm in diameter. Column covered with small grey or pink wart-like protuberances (verrucae), usually with 6 main white rows. Transparent tentacles mottled grey, pink, white and green in colour. Partially buried.

**VI Anemonia viridis**



Até 14 cm de diâmetro. Espécie ubíqua. Tentáculos não completamente retráteis. Tentáculos de cores variáveis; normalmente acinzentados ou verdes com pontas rosa.

Can reach 14 cm in diameter. Common species. Tentacles not fully retractable. Tentacles can vary in colour; usually grey or green with pink tips.

**II Anthopleura sp.**



Até 12 cm de diâmetro. Apresenta cores muito variáveis. A coluna verrucosa adere detritos como pedras e pedaços de conchas. Encontra-se parcialmente enterrada.

Can reach 12 cm in diameter. Variable in colour. The column possesses adherent vesicles which attach debris, smithereens of rocks and shells. Partially buried.

**VII Cereus pendunculatus**



Disco oral até 7 cm de diâmetro, mas pode ser maior. Base firmemente adesiva, coluna atumilada. Tentáculos curtos, por vezes com padrões variados e visíveis. Encontra-se em fendas.

The oral disc can reach 7 cm in diameter, but can be wider. Base firmly adhesive. Tapered column. Short tentacles, with attractive patterns. Found in cracks.

**V Actinia fragacea**



Até 14 cm de diâmetro. Espécie ubíqua. Coluna com cores variadas, normalmente vermelha com pontos verdes. Pode apresentar acrorhagi.

Can reach 14 cm in diameter. Common species. Wide colour variation, usually red with green spots. May present acrorhagi.



Até 7 cm de diâmetro.  
Especie dibruca. Apresenta um anel azul na base e pode apresentar vesículas azuis (acromagi) junto aos tentáculos, para lutar por espaço com indivíduos da mesma espécie. Variedades: vermilha, acastanhada, verde ou estriada/ruçada.

Can reach 7 cm in diameter. Common species. Presents a blue ring around the base and can show blue vesicles (acromagi) around the tentacles to fight for space against other individuals. Wide colour variation, from green to red.

## Actinia equina

I



Até 12 cm de diâmetro. Apresenta cores muito variáveis. A coluna verrucosa adere detritus, como pedras e pedaços de conchas. Encontra-se parcialmente enterrada.

Can reach 12 cm in diameter. Variable in colour. The column possesses adherent vesicles which attach detritus, shells/breaks of rocks and shells. Partially buried.

## Anthopleura sp.

II



Até 7 cm de diâmetro. Coluna com cones verrucos, normalmente verde com listras circulares. Pode apresentar acromagi.

Can reach 7 cm in diameter. Wide colour variation, but usually green with surrounding fringes. May present acromagi

## Actinia cari

III



Até 3 cm de diâmetro. Coluna verrucosa cinzenta ou rosa, normalmente com 6 listras brancas. Tentáculos translúcidos geralmente com manchas verdes, cinzentas, rosa ou brancas. Encontra-se parcialmente enterrada.

Can reach 3 cm in diameter. Column covered with small grey or pink wart-like protuberances (verrucae), usually with 6 main white rows. Transparent tentacles mottled grey, pink, white and green in colour. Partially buried.

## Aulactinia verrucosa

IV





**Actinia fragacea**

Até 14 cm de diâmetro. Espécie ubíqua. Coluna com cores variadas, normalmente vermelha com pontos verdes. Pode apresentar acrothagi.

Can reach 14 cm in diameter. Common species. Wide colour variation, usually red with green spots. May present acrothagi.



**Anemonia viridis**

Até 14 cm de diâmetro. Espécie ubíqua. Tentáculos não completamente retráteis, variáveis: normalmente acinzentados ou verdes com pontas rosa.

Can reach 14 cm in diameter. Common species. Tentacles not fully retractable. Tentacles can vary in colour: usually grey or green with pink tips.



**Cerereus pendunculatus**

Disco oral até 7 cm de diâmetro, mas pode ser maior. Base firmemente adesiva, coluna alfinhada. Tentáculos curtos, por vezes com padrões variadíssimos e vistosos. Encontra-se em fendas.

The oral disc can reach 7 cm in diameter, but can be wider. Base firmly adhesive. Tapered column. Short tentacles, with attractive patterns. Found in cracks.



**Corynactis viridis**

Não pertence ao grupo das anemónias verdadeiras. Até 1 cm de diâmetro. Ocorre a partir do intertidal inferior, em locais escudidos da luz como grutas. É encontrada em densos agregados. Possui uma cor brilhante como verde, rosa ou anarelô.

Does not belong to true sea anemones group. Can reach 1 cm in diameter. Occurs from the lower shore, on rocks, in caves and beneath overhangs where it is shaded from light. Found in dense aggregations. Presents bright colours, mainly green, pink or yellow.



**Actinotheres sphyrodeta**

Até 3 cm de diâmetro. Espécie comum no subtidal, mas pode ser encontrada em zonas protegidas nas rochas, em maré baixa. Forma extensas colónias. Geralmente branca, com a zona oral branca ou alaranjada. Projecta longos fios brancos (acontia) quando perturbada.

Can reach 3 cm in diameter. Common in subtidal areas, but in the low tide can be found in areas protected from light. Forms big colonies. Usually white, with the oral area white or orange. When disturbed projects long thin white threads (acontia).

**ANÉMONAS  
DO INTERTIDAL  
DA COSTA  
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*INTERTIDAL  
SEA ANEMONES  
FROM THE  
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