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EVOLUTION OF NORTHEAST ATLANTIC AND MEDITERRANEAN SCORPIONFISH *SCORPAENA* AND THEIR VENOM PROTEIN PROFILES

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Resumo

Os vertebrados marinhos venenosos permanecem relativamente pouco estudados quando os comparamos com os seus homólogos terrestres. Os venenos são um recurso valioso para a pesquisa de novos compostos com potencial biotecnológico, nomeadamente na área da saúde. Contudo, para conhecer novas proteínas de interesse farmacêutico, é necessário conhecer as espécies e os seus "cocktails" venenosos. A filogenia pode ser uma ferramenta para conduzir a investigação nesta área, ao perspetivar a existência de uma molécula bioativa no veneno de espécies relacionadas que ainda não houve oportunidade de estudar.

O género *Scorpaena* inclui peixes marinhos venenosos que se distribuem por todas as bacias oceânicas. Em alguns países do Nordeste do Oceano Atlântico e do Mar Mediterrâneo, são conhecidas por fazerem parte do leque de espécies capturadas na pesca artesanal, e pelos episódios de envenenamento que causam.

Neste trabalho foi estudada a filogenia de 8 das 9 espécies de *Scorpaena* do Nordeste do Oceano Atlântico e do Mar Mediterrâneo, sendo esta a primeira filogenia a englobar um número tão abrangente de espécies deste género. Paralelamente, os perfis proteicos dos venenos de cinco destas espécies foram investigados, concluindo-se que diferem entre si inclusive quando são comparados perfis de venenos de espécies irmãs. Esta diversidade pode ser suportada por hipóteses alternativas, nomeadamente a de que os venenos estão sob ação de seleção natural ou estes venenos representam traços antigos de divergência rápida. Os resultados desvendam informação acerca da evolução destas espécies e dos seus venenos, permitindo assim reconhecer a utilidade da filogenia como um mapa a utilizar em estudos biotecnológicos no futuro.

Palavras-chave: *Scorpaena*, filogenia, venenos, biotecnologia azul, peixes venenosos

Abstract

Venomous marine vertebrates remain poorly studied compared to their terrestrial counterparts. Venoms are valuable resources for the discovery of new compounds with biotechnological potential, namely, in health sciences. However, to discover new proteins with pharmaceutical interest, it is necessary to acquire basic knowledge on venomous species and their venom "cocktails". Phylogeny can be used as a roadmap to guide research in this field, by allowing to know the relationships between species and prospect about the existence of similar proteins in closely related species not yet studied.

Scorpaena are known marine venomous fish, with species distributed throughout all the oceanic basins. In some NE Atlantic and Mediterranean Sea countries, they are known to be part of the range of species caught by artisanal fishing, and for causing episodes of envenomation.

In this work, the phylogeny of 8 of the 9 *Scorpaena* species from the NE Atlantic and Mediterranean Sea was studied. This was the first phylogeny to encompass such a wide number of species of this genus. In parallel, protein profiles from five *Scorpaena* species were investigated, concluding that their venom profiles seem to differ in protein composition, even comparing sister species. This result supports the hypothesis of fish venoms suffering natural selection and allows to recognize the utility of phylogeny in guiding biotechnological studies with venoms from marine organisms.

Key words: *Scorpaena*, phylogeny, venoms, blue biotechnology, venomous fishes

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1. Introduction

Venomous organisms are widespread among marine fauna. They include cnidaria (e.g. jellyfish), molluscs (e.g. cone snails), annelida (e.g. fire worm) and fish (Smith and Wheeler, 2006). Venoms play a vital role as key traits in fitness by acting as an adaptation to different prey species or to protect animals from predation, and consequently facilitating survival and reproductive success (Casewell *et al*., 2013; Harris and Jenner, 2019).

Venomous marine organisms are much less explored than their terrestrial counterparts and are increasingly viewed as valuable sources of bioactive molecules with economic interest, a research field recently coined as blue biotechnology. An example is the discovery of new drugs (Smith and Wheeler, 2006). From all the fish species in the world, about 7-9% are expected to be venomous (Smith et al., 2016), 2000 of which were already identified as venomous cartilaginous or ray-finned fishes (Smith and Wheeler, 2006; Smith et al., 2016; Wright, 2009).

Scorpaenidae is one of the best-known families of venomous fish (Andrich et al., 2010). Their prefix "scorpion" refers to the existence of chemical weapons and venom spines (Nelson, 2006; Poss and Eschmeyer, 2002). The genus *Scorpaena* is included in this family, encompassing 61 species (Froese and Pauly, 2019), that can be found in all oceanic basins, where they occupy variable habitats. They differ from other genera by the presence of occipital pit and palatine tooth (Hureau and Litvinenko, 1986).

In the Northeast Atlantic and Mediterranean Sea, there are nine *Scorpaena* species described in the literature - *Scorpaena notata* Rafinesque 1810, *Scorpaena elongata* Cadenat 1943, *Scorpaena porcus* Linnaeus 1758, *Scorpaena scrofa* Linnaeus 1758, *Scorpaena loppei* Cadenat 1943, *Scorpaena laevis* Troschel 1866, *Scorpaena maderensis* (Valenciennes, 1833), *Scorpaena azorica* Eschmeyer 1969 and *Scorpaena canariensis* Sauvage 1878 (Hureau and Litvinenko, 1986; Motomura, 2005) – with different distributions, from the British Islands to Senegal, including the Macaronesian region and Mediterranean Sea (Hureau and Litvinenko, 1986).

These species show a benthonic and sedentary lifestyle, sometimes solitary, and a body with several spines and variable colouration between rosy-reddish and brownish. *Scorpaena* shows opportunistic and generalist feeding habits, with crustaceans and fishes being the most common preys (Mesa *et al*., 2016). Due to their wide distribution and the fact that they are being captured by artisanal fishing or as by-catch (Fisher et al., 1987; Maricchiolo et al.,

2014; Ordines *et al*., 2009), some species such as *S. notata, S. porcus, S. scrofa* and *S. maderensis*, are easily observed along the Portuguese coast. Other species are rare (*S. loppei* and *S. laevis*), are difficult to identify (*S. canariensis*), or its existence is considered dubious, as in the case of *S. azorica*, with only one known holotype specimen described to date.

Scorpaena have a complex venom system composed by medium sized venom glands where the venom is produced (Halstead et al., 1995; Wright, 2009) and by venom spines that can be found in the dorsal fin, the anal fin and the opercula (Smith and Wheeler, 2006). Venom glands are covered by an integumentary sheath located on the anterolateral groove present on the venom spines (Smith and Wheeler, 2006; Kizer et al., 1985). When the spine is pressed against the predator's skin, the integumentary sheath is mechanically disrupted and the venom is released inside the victim's wound, through the spine's anterolateral groove (Ziegman and Alewood, 2015). In general, the fish venom is predominantly composed by protein toxins, and causes disruption at a physiological and biochemical level on the victim (Casewell et al., 2013; Fry et al., 2009). *Scorpaena*'s venom's main objective is to create discomfort and immediate intense pain on the predator, with the purpose of opening an opportunity window for the venomous fish to escape (Casewell et al., 2013; Harris and Jenner, 2019).

Venoms evolved convergently through an "arms race" evolutive system (Dawkins and Krebs, 1979; Endler 1986; 1991; Van Valen, 1973), and are used for predation, competition and defence (Casewell et al., 2013). *Scorpaena* are included in a group of more than 2000 species , that use their venom as defence against predators (Li et al., 2018; Smith et al., 2006; 2016; Wright, 2009). Venoms are thought to evolve via gene duplication, by which protein genes involved on key regulatory process were duplicated, and the copy is also expressed in the venom gland (Fry et al., 2003; Kordiš and Gubenšek, 2000).

Some researchers have already unveiled the functions of proteins from the venom of *Scorpaena*'s species. It is the case of a venomous specie from western Atlantic, *Scorpaena plumieri*, a well-studied venomous fish, whose venom has proteins such as the Sp-CTx (121 kDa), the plumieribetin (14 kDa) and C-type isolectins called SP-CL 1-5 (16,8 – 17 kDa) (Andrich et al., 2015; Evangelista et al., 2009; Gomes et al., 2016).

Phylogeny can be used to prospect which species may possess a certain compound with economic interest (Smith and Wheeler, 2006). In turn, venoms can also be used to study relations between species and the diversity between venomous organisms (Casewell et al., 2013).

In this study, the main objectives are: (1) infer the phylogeny of *Scorpaena* from the Northeast Atlantic and Mediterranean Sea; (2) describe the venom protein profiles of five species of *Scorpaena* from the Northeast Atlantic and Mediterranean Sea. As secondary objectives I will also: (3) contribute with the first DNA sequences from two of these species - *S. canariensis* and *S. azorica* -, being the results reported here their first molecular validation; (4) update the identification key from Northeast Atlantic and Mediterranean Sea *Scorpaena* with the *S. canariensis* description provided from Motomura et al., (2005); (5) evaluate the efficiency of fish venom extraction method using a sponge in a tube; (6) show the lability of fish venosm comparing protein profiles of fresh and frozen venom samples; (7) and investigate if the dorsal fin can regenerate after being cut.

2. Experimental Design

2.1. Phylogenetic analyses

The present work began with an exhaustive search for nuclear and mitochondrial sequences available in Genbank and in the literature for the genus *Scorpaena*. Based on that search four mitochondrial fragments - 16S, D-loop, *cytochrome oxidase subunit I* (COI) and *cytochrome b* (Cytb) – and one nuclear fragment - S7 ribosomal protein gene (S7) – were selected for the phylogenetic analyses. Samples from eight *Scorpaena* species were used for the laboratory work and phylogenetic analyses: *S. azorica, S. canariensis, S. elongata, S. laevis, S. maderensis, S. notata, S. porcus* and *S. scrofa*. The molecular work was divided into three steps: DNA extraction, PCR amplification/sequencing and phylogenetic analysis (see methods section below).

2.2. Venom studies

The majority of the venom samples were extracted using the sponge-in-a-tube technique ensuring that neither the specimens were sacrificed, nor the venom spiny rays were surgically removed. The exceptions to this case were two *S. notata* specimens maintained in captivity at Vasco da Gama Aquarium, in Lisbon. The first two dorsal spines from these individuals were cut to extract venom and to evaluate their regeneration capacity (see Annexs).

Venom samples were immediately frozen after collection. Posteriorly, the venom was extracted from the sponges, concentrated and submitted to an SDS-PAGE electrophoresis. Furthermore, to compare protein profiles between fresh and frozen venom samples, I collected fresh venom from *S. notata* specimens maintained at Vasco da Gama aquaria.

3. Methods

3.1. Molecular data

3.1.1. Sampling

Specimens of *S. azorica* (*N*=1), *S. canariensis* (N=3), *S. elongata* (N=2), *S. laevis* (N=1), *S. maderensis* (N=30), *S. notata* (N=39), *S. porcus* (N=29) and *S. scrofa* (N=31) were obtained at fish markets, from fishermen, scuba diving or museum samples (Funchal Municipal Museum) (see Table A1 in Annex I for detailed information). The 136 tissue samples were collected in Portugal (central and southern Portugal, Madeira Islands and Azores Islands), Spain (northern and southern Spain and Canary Islands), Cape Verde Islands and Cyprus. The species identification was made visually, according to the morphological identification described in FNAM (Hureau and Litvinenko, 1986), except for the recently described *S. canariensis*, whose morphological identification was based on the description by Motomura et al. (2005). Sampling involved collecting a small portion of caudal fin or muscle tissue (20-50 mg), preserved in 96% ethanol at room temperature.

3.1.2 DNA extraction and PCR amplification

Laboratory work was carried out at ISPA's Evolutionary Genetic laboratory. Genomic DNA extractions were performed with the REDExtract-N-Amp kit (Sigma-Aldrich) following the manufacturer's instructions. DNA quantification (ng/µl) and purity level assessment were performed with a NanoDrop spectrophotometer (Thermo Scientific™), using the absorbance ratios 260/280 and 260/230.

The different molecular fragments were amplified with polymerase chain reaction (PCR), following the conditions expressed in Table 1. DNA amplifications were obtained in a total volume of 20 µl, containing 4,4 µl of Sigma water, 10 µl of REDExtract-N-Amp PCR reaction mix, 0,8 µl of each primer (forward and reverse) and 4 µl of template DNA.

From a total of 136 samples submitted to a 16S fragment amplification, a subgroup of 3 specimens per species was chosen to perform the amplification of the remaining fragments. The choice was based on both the DNA extraction quality obtained with NanoDrop™ and 16S sequencing results.

In order to solve some constraints in the optimization of COI and Cytb amplifications, new primers were designed using the Primer-BLAST tool from NCBI (Ye, 2012). The new

primer pairs (ScoCOI-F / ScoCOI-R and ScoCytb-F / ScoCytb-R, respectively) were obtained using *Scorpaenopsis cirrosa* (NCBI Reference Sequence: NC_027735) as PCR template and Scorpaeniformes for specificity check (Table 1).

Table 1. Primers and polymerase chain reaction (PCR) conditions.

Fragment	Primer name	Sequence	Source	PCR conditions
16S	16SFor	5'-AAGCCTCGCCTGTTTACCAA-3'	Almada et al., 2005	94 °C 4’
	16SRev	5'-CTGAACTCAGATCACGTAGG-3'		30 × (94 °C 1’, 55 °C 1’, 72 °C 1’)
				72 °C 10’
COI	FishF1	5’-TCAACCAACCACAAAGACATTGGCAC-3’	Ward et al., 2005	95 ˚C 2’
	FishR2	5’-ACTTCAGGGTGACCGAAGAATCAGAA-3’		35 × (94 ˚C 30’’, 52 ˚C 30’’, 72 ˚C 1’)
				72 ˚C 10’
COI	ScoCOI-F	5’-AGCTGGAATGGTCGGAACAG-3’	Scorp COI marta	94 ˚C 2’
	ScoCOI-R	5’-TGGCCCACACTATCCCCATA-3’		35 × (94 ˚C 30’’, 52 ˚C 30’’, 72˚C 45’’)
				72 ˚C 10’
Cytb	FishcytB-F	5'-ACCACCGTTGTTATTCAACTACAAGAAC-3'	Sevillha et al., 2007	94 ˚C 3’
	TruccytB-R	5'-CCGACTTCCGGATTACAAGACCG-3'		35 × (94 ˚C 30’’, 55 ˚C 35’’, 72˚C 2’)
				72 ˚C 10’
Cytb	ScoCytb-F	5'-GCCTTCGAAAACACACCCC-3'	Scorp COI Cytb Marta	94 ˚C 2’
	ScoCytb-R	5'-GCAAGGGTTGTCAGTGCAAG-3'		35 × (94 ˚C 30’’, 52 ˚C 30’’, 72˚C 45’’)
				72 ˚C 10’
D-loop	L-PRO1	5’-ACTCTCACCCCTAGCTCCCAAAG-3’	Ostellari et al., 1996	94 ˚C 7’
	HDL1	5’-CCTGAAGTAGGAACCAGATGCCAG-3’		35 × (94 ˚C 30’’, 55 ˚C 30’’, 72˚C 1’)
				72 ˚C 7’
S7	S7RPEX1F	5’-TGGCCTCTTCCTTGGCCGTC-3’	S7 Scorpaena	94 ˚C 7’
	S7RPEX2R	5’-AACTCGTCTGGCTTTTCGCC-3’		30 × (94 ˚C 45’’, 55 ˚C 45’’, 72˚C 1’)
				72 ˚C 7’

3.1.2.1 DNA extraction with ammonium acetate

An alternative DNA extraction protocol was employed to solve the amplification difficulties of both COI and Cytb molecular markers. The chosen protocol was the ammonium acetate DNA extraction protocol, adapted from Maniatis *et al.* (1982). In the first step, 10-20 mg of fin tissue was placed inside an 1,5 ml eppendorf tube, and cellular lysis was induced using 600 µl of cellular lysis buffer (0.5M Tris, 0,1M EDTA, 2% SDS, pH 8), followed by incubation at 65˚C for 15 minutes. This was followed by protein digestion with 10 µl of Proteinase K (20mg/ml) (Promega), with a subsequent incubation at 55˚C for 3 hours. Protein precipitation was obtained with 200 µl of precipitation buffer (5M ammonium acetate, pH 8) added to the sample and centrifuged at 13000 rpm for 10 minutes. The supernatant was removed to a new tube and mixed with 750 µl of isopropyl alcohol. The tubes were maintained at -20˚C overnight and centrifuged at 13000 rpm for 15 minutes. Afterwards, the supernatant was discarded, and the DNA sample was cleaned with successive degrees of hydrated ethanol (96% and 70% ethanol), with 13000 rpm centrifugation (15 minutes) between the cleaning procedures. Following 10 minutes at room temperature to evaporate the remaining ethanol, 30 µl of Sigma water were added to the sample for DNA hydration.

3.1.2.2 Amplification and verification

Gel electrophoresis was employed for visualization of amplified PCR products (Fig. 1). Electrophoresis was performed in 1,5% agarose gels (with TBE buffer), stained with GelRed® for nucleic acids (Biotium) for 30minutes (100 volts; 400 mA). The gel was loaded with 4 µl of each amplification product.. The approximate length of the amplified fragments was determined running the samples alongside 4 µl of DNA ladder Microzone 100-1000bp ladder). Visual inspection of the gel was done with an UV transilluminator device and successfully amplified samples (i.e. with fluorescently dyed bands) were selected for sequencing.

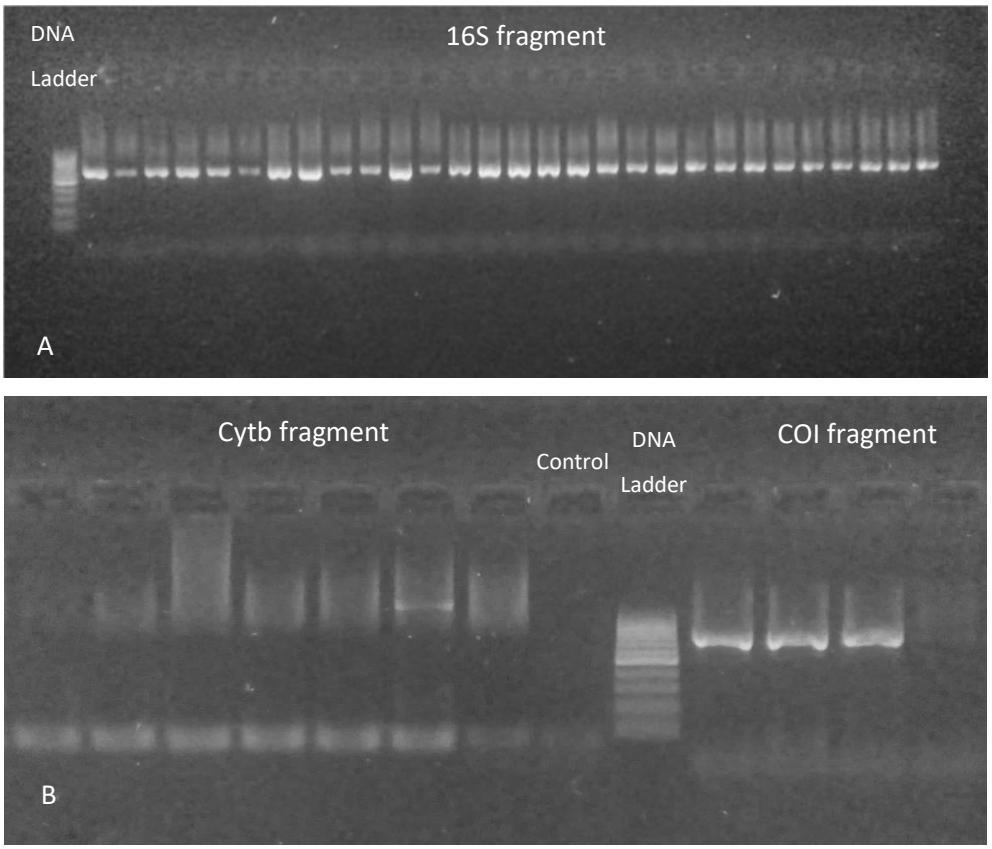

Figure 1. Electrophoresis gel showing PCR amplification results from: A) 16S DNA and B) Cytb and COI rDNA.

3.1.3 Sequencing, editing and aligning

The successfully amplified products were sent to STABVIDA (www.stabvida.com) for purification and sequencing analyses. Chromatograms were manually edited and initially aligned with CodonCode Aligner v.3.5.6. (CodonCode Corporation, MA, USA). For each fragment, edited sequences were aligned with additional sequences available in Genbank (NCBI).

3.1.4. Phylogenetic analyses

Bioinformatic analysis were carried out at ISPA's Evolutionary Genetic laboratory. DNA sequences were aligned with their homologous in each species and used to infer phylogenetic relationships, building Maximum Parsimony, Neighbour-Joining and Maximum Likelihood trees. In this phylogenetic study it was possible to include 8 different species of

Scorpaena: *S. notata, S. porcus, S. scrofa, S. laevis, S. azorica, S. elongata*, *S. maderensis* and *S. canariensis*.

For each molecular marker, a complete multiple alignment was obtained with Clustal X2 software (Larkin et al., 2007). MEGA X (Kumar et al., 2018) was used to compute estimates of net evolutionary divergence between species, with likelihood-distance (Kimura, 1980) and best-fit model of evolution based on the Bayesian Information Criterion (BIC) and Akaike Information Criterion (AIC) values (Kumar et al., 2018; Nei and Kumar, 2000). Phylogenetic relationships were assessed with three distinct methods: Maximum Likelihood (ML) (Kimura, 1980), Neighbor-Joining (NJ) (Saitou and Nei, 1987) and Maximum Parsimony (MP), using the best-fit model where applicable. Robustness was inferred with 500 bootstraps replicates (Felsenstein,1985), and gaps were treated as a 5^{th} state. The outgroup corresponding fragments were obtained from the mitogenomes of *Synanceia verrucosa* Bloch & Schneider 1801 (NC026989), *Sebastes inermis* Cuvier 1829 (KF725093), *Sebastes steindachneri* Hilgendorf 1880 (KJ834060), *Helicolenus hilgendorfii* Döderlein 1884 (NC003195), *Pterois miles* Bennett 1828 (NC024746) and *Scorpaenopsis cirrosa* Thunberg 1793 (NC_027735).

3.2. Venom analysis

3.2.1. Venom sampling

The venom was collected from wild specimens captured in shallow waters in Portugal (Continental west central region, Madeira and Azores Archipelagos) and Spain (Canary Islands) using 1,5 ml tubes with a synthetic sponge inside (Fig. 2A). The specimens were from five different species: *S. notata, S. porcus, S. scrofa, S. maderensis* and *S. canariensis*. Collection tubes were pressed against the dorsal and anal fin spines to maximize the amount of venom released into the sponge (Fig. 2B). Venom samples were frozen and kept at -20˚C (Fig. 1C). After venom collection, wild specimens were released in the site of capture.

To ascertained whether there was loss of protein proprieties and / or protein profile modifications, fresh samples of venom were collected from specimens of red scorpionfish (*Scorpaena notata*) maintained in captivity at Vasco da Gama Aquarium (Fig. 3).

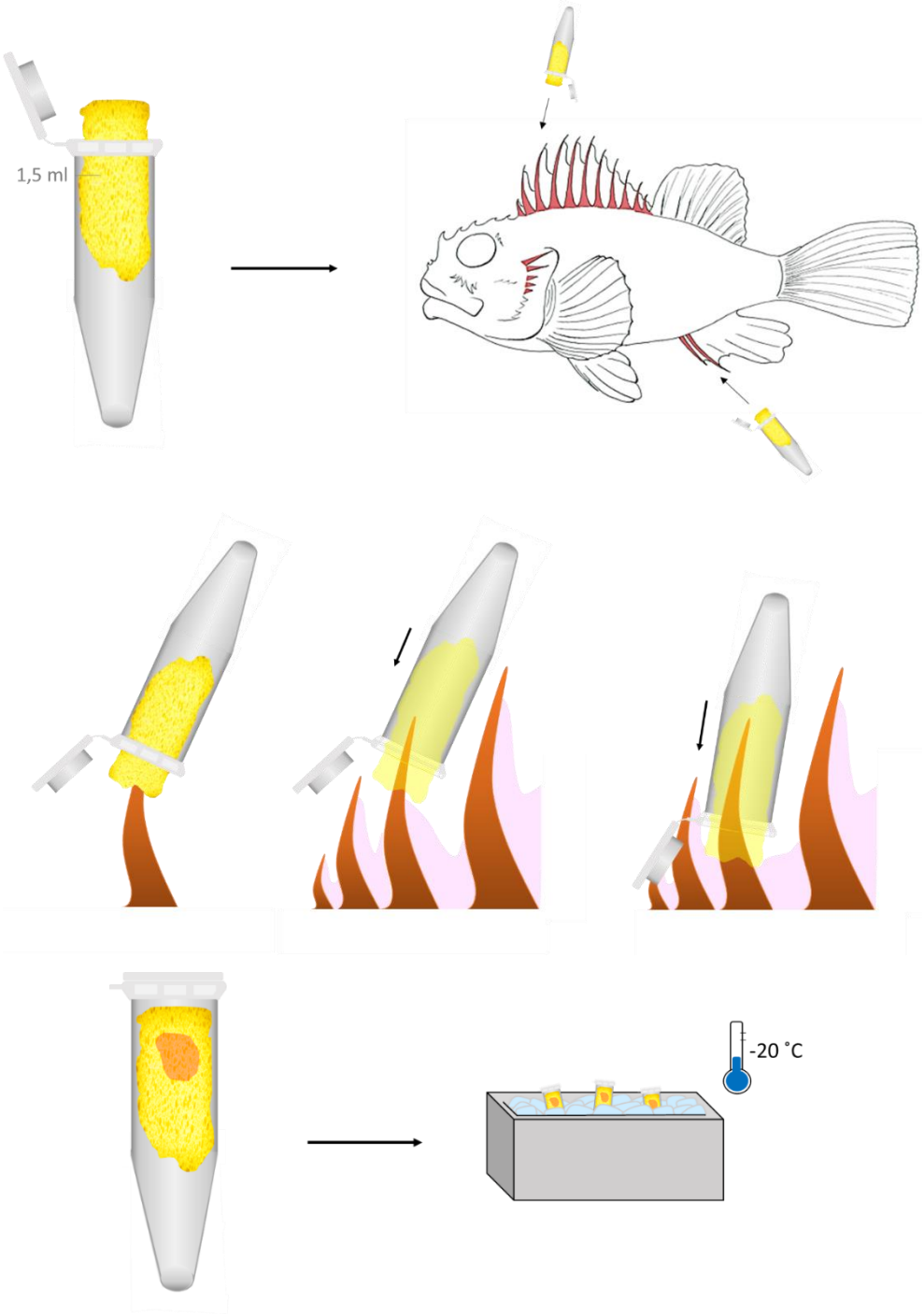

Figure 2. (A) Venom collection device (sponge-in-a-tube) and fish illustration showing the venomous spines (in red), highlighting the ones usually selected for venom collection (the first three dorsal spines and two anal spines). (B) Venom collection procedure using the sponge-in-a-tube method - the sponge is pressed against the spine, forcing the rupture of the integumentary sheath that covers it. (C) Collected venom is immediately stored at -20 ˚C. (*Scorpaena* illustration by Ruxanda Lungu).

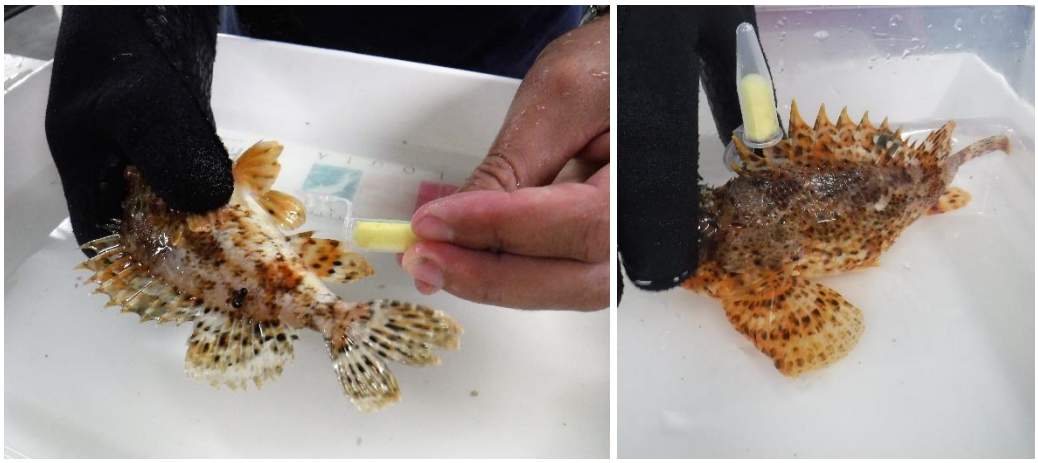

Figure 3. Venom collection procedure from a small red scorpionfish *S. notata* (above) maintained in captivity at Vasco da Gama Aquarium, in Lisbon, Portugal, and from a Madeira scorpionfish *S. maderensis* (below) from the wild at Madeira, Portugal. (A) Venom collection from the anal spine; (B) venom collection from the dorsal spine.

3.3.3. Venom Protein Extraction

Since fish venoms are described to be extremely labile (Andrich et al., 2010), to determine if the frozen samples suffered degradation during storage, two sets of crude venom were used in the analysis. One with frozen venom samples from 5 different *Scorpaena* species, and another with frozen and fresh venom samples from *S. notata*.

For the frozen samples set with the 5 *Scorpaena* species, in order to maximize the amount of crude venom for protein extraction, 2-8 individual samples per species were pooled depending on the specimen's length and venom quantity obtained in each collection tube (Table 2).

Considering *S. notata* only, fresh samples, analysed within 2h after their collection from a live fish, were replicated following two settings: 1) sample composed by the pooled venom from three specimens; 2) sample from a single specimen (Table 3). All the extractions were performed in May 2019 at ISPA's laboratory. The protein profiles from fresh and frozen samples (maintained at -20˚C for more than 6 months) were then compared..

The venom was extracted from the sponge with successive dilutions with 300 µl buffer solution (100 mM KPB pH=8), followed by centrifugations. The diluted venom was submitted to three centrifugations inside centricon tubes (Amicon® Ultra 0.5 ml centrifugal filters Ultracel® 3K), at 13.000 rpm, for 15 minutes each, and at 6˚C. The centricon tube

contains a membrane that retains proteins larger than 3 kDa. The size of the mesh was chosen based on previous studies (Andrich et al., 2015; Evangelista et al., 2009; Gomes et al., 2013, where it was demonstrated that venom proteins of interest, have a size higher than 3 kDa.

Table 2. Crude venom samples used to evaluate the venom protein profiles for each species. W – wild; C – captivity; Samples were analysed fresh or frozen at -20°C for several months; grey lines represent venom samples used to test the degradation of venom proprieties on frozen samples.

Specie	Collection site	N	Average Total Lenght (cm)	Harvest date	Provenance /Storage	Time since harvest date till extraction
Scorpaena canariensis	Canary Island (SP)	3	14,5	04/2018	W/-20°C	1 year
Scorpaena maderensis	Madeira Island (PT)	8	9,0	11/2018	W/-20°C	6 months
Scorpaena notata	Fonte da Telha (PT)	2	n/d	06/2016	W/-20°C	3 years
	Vasco da Gama Aquarium (PT)	3	16,9	05/2019	C/Fresh	2 hours
	Fonte da Telha (PT)	2	n/d	06/2016	W/-20°C	3 years
	Vasco da Gama Aquarium (PT)	3	16,9	05/2019	C/Fresh	2 hours
	Vasco da Gama Aquarium (PT)	1	17,2	05/2009	C/Fresh	2 hours
Scorpaena porcus	Peniche (PT)	2	30,25	06/2016	W/-20°C	3 years
		1	n/d	06/2016	W/-20°C	
Scorpaena scrofa	Peniche (PT)	3	47,34	05/2016	W/-20°C	3 years
		2	n/d	03/2017	W/-20°C	2 years

3.2.3. SDS-PAGE electrophoresis

Protein concentration was quantified using Nanodrop (Thermo Scientific™) at 280nm. And SDS-PAGE electrophoresis was preceded by standardization of protein concentration for each species (65 µg protein). Protein denaturation was performed adding 1µl of β-mercaptoethanol to the venom samples, followed by 10 minutes on water bath (100 ˚C). This procedure was followed to dissociate proteins with oligomeric forms and secondary and tertiary structures. Additionally, was added 5 µl of Sodium dodecyl sulfate (SDS) to the samples. The SDS is a compound responsible for transmitting electronegative charge to

proteins. As all proteins have electronegative charge, the electrophoresis run will rely on the protein molecular weight.

Venom samples were loaded into 12,5% SDS-PAGE polyacrylamide gel using Sodium SDS as buffer. Furthermore, 10 µl of standard marker with protein molecular weight between 10 kDa and 250 kDa was loaded into the gel. The electrophoresis was performed for 2 hours at 80V. After protein migration, gels were stained with blue Comassie for total protein identification.

4. Results

4.1. Phylogenetic analyses

From the total of 143 tissue samples of *S. azorica*, *S. canariensis*, *S. elongata*, *S. laevis*, *S. maderensis*, *S. notata*, S. *porcus* and *S. scrofa*, 131 sequences from the 16S fragment were obtained, with 557 bp length (420 conserved sites and 118 parsimony informative site) (see Table 4 in annex B for details).

Additional 8 sequences from COI fragment, 5 sequences from Cytochrome b fragment, 8 sequences from D-loop fragment and 8 sequences from S7 fragment were also amplified (Table 3). Three sequences of *S. canariensis* and *S. maderensis* from D-loop, and three sequences of *S. maderensis* from S7 were obtained from previous phylogeographic studies performed by Lima et al., (2018). Phylogenetic analysis did not include these additional DNA sequences because it was not possible to amplify at least one fragment for all the eight species analysed in this study.

For the 16S dataset, the best fit model was the K2 + G (gamma distribution) (Nei and Kumar, 2000) Kimura-2 parameter model (BIC= 7700,30) (AIC= 5368,41).

Table 3. Sequences obtained from 16S, COI, Cytb, D-loop and S7 for phylogenetic inference with the 8 *Scorpaena* species.

Species	Collection site	N	Mitochondrial DNA				Nuclear DNA
			16S	COI	Cytb	D-loop	S7
Scorpaena azorica	Madeira Island, Portugal	1	1				
			1				
Scorpaena canariensis	Tenerife, Spain	3	3	3		3	1
			3	3		3	1
Scorpaena elongata	Armação de Pêra, Portugal	1					
	Ilha do Sal, Cape Verde	1	1				
			1				
Scorpaena laevis	Ilha S. Nicolau, Cape Verde	1	1				
	Ilha do Sal, Cape Verde	3					
			1				
Scorpaena maderensis	Madeira Island, Portugal	20	20			1	1

	Azores Island, Portugal	5	4			1	1
	Arrábida, Portugal	1	1				
	Peniche, Portugal	1	1				
	Tenerife, Spain	3	3			1	1
	Cyprus	3	3				
			32			3	3
Scorpaena notata	Armação de Pêra, Portugal	2	2	1			
	Arrábida, Portugal	22	23	1			
	Fonte da Telha, Portugal	7	7	1	1		
	Peniche, Portugal	1	0				
	Sesimbra, Portugal	2	2	1	1		1
	Cádiz, Spain	3	1	1			
			35	5	2		1
Scorpaena porcus	Armação de Pêra, Portugal	3	1				
	Arrábida, Portugal	1	1		1	1	1
	Cascais, Portugal	2	2				
	Fonte da Telha, Portugal	1	1				
	Peniche, Portugal	14	14		1		
	Sesimbra, Portugal	3	2				
	Ferrol, Galiza	3	2				
	Galiza, Spain	3	3				
	Tenerife, Spain	2	2		1	1	1
			28		3	2	2
Scorpaena scrofa	Peniche, Portugal	27	27				1
	Setúbal, Portugal	1	1				
	Ferrol, Spain	3	2				
			30				1

4.1.1. Phylogenetic analyses

Phylogenetic analyses results were congruent between Maximum-parsimony (MP) and Neighbor-joining (NJ) methods but revealed some differences for the Maximum-likehood (ML) method (Fig. 4). However, these differences reside in nodes that are not well supported by significant bootstrap values and do not contradict the relationships of *S. porcus*, *S. laevis* and *S. notata* with the remaining species in the phylogenetic tree.

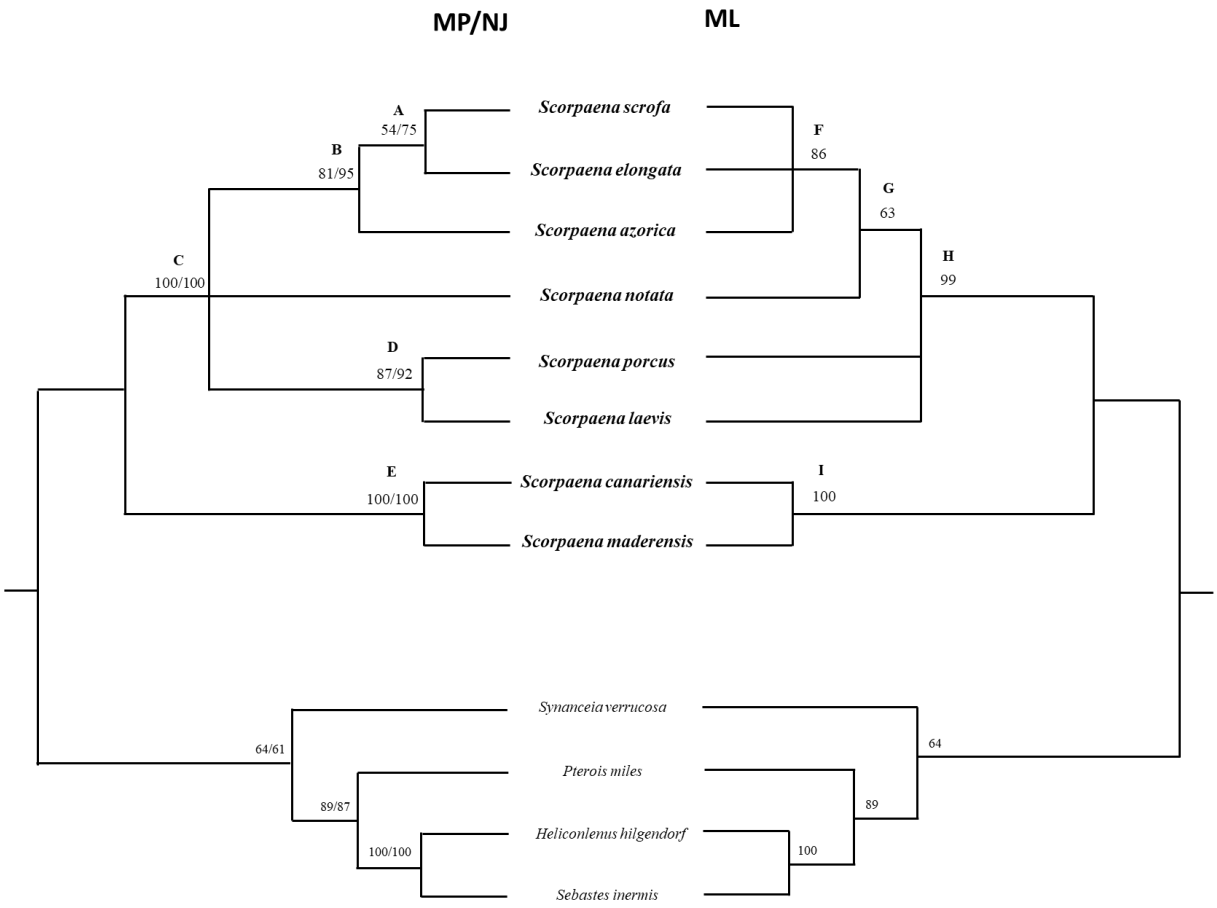

Figure 4. Phylogenetic relationships within *Scorpaena* genus. Maximum parsimony (MP) consensus tree (left). Bootstrap values for each node are shown following the order Maximum parsimony/ Neighbor-joining (MP/NJ) (Felsenstein, 1985; Kimura, 1980; Kumar et al., 2018; Nei and Kumer, 2000; Saitou and Nei, 1987); Maximum-likelihood (ML) consensus tree (right), constructed with the 16S dataset. Bootstrap values for each node (Felsenstein, 1985; Kimura, 1980; Kumar et al., 2018; Saitou and Nei, 1987).

In figure 4 (MP/NJ methods), *S. porcus* and *S. laevis* form a well-supported clade (clade D) with bootstrap values of 87/92. These two species form another clade with *S. scrofa*, *S. elongata* and *S. azorica* (clade B) and with *S. notata* (clade C). The six species of north-eastern Atlantic compose a polyphyletic group supported by high bootstrap values of 100/100.

The ML method results are similar to the previous methods, showing only two differences: a) clade *S. porcus* and *S. laevis* (clade D in MP/NJ) is no longer supported; and b) the suggested clustering of *S. notata* with the clade *S. scrofa*, *S. elongata* and *S. azorica* (clade F), although with a weak bootstrap support (clade G; Fig. 4).

The three methods recover two basal clades with *S. scrofa*, *S. elongata*, *S. azorica*, *S. notata*, *S. porcus* and *S. laevis* (clades C and H in MP/NJ and ML methods respectively); and the clade formed by *S. canariensis* and *S. maderensis* (clades E in MP/NJ and I in ML methods). The three methods have also established that *S. scrofa* and *S. elongata* are sister-species, with *S. azorica* being the closer specie. They form the clade B (81/95) in MP/NJ methods and the clade F (86) in the ML method. *S. canariensis* and *S. maderensis* are also sister-species (clade F in MP/NJ and I in ML), with maximum bootstrap support.

As expected, these results are supported by the genetic distance (p-distance) (Table 4). The close relationship between *S. laevis* and *S. porcus* was confirmed by the genetic distance between these species (p = 0,06842). The remarkably low genetic distance between *S. elongata* and *S. scrofa* (p = 0,00873), which is even lower than the interspecific genetic distance between *S. canariensis* and *S. maderensis* (p = 0,08339), should be further evaluated in the future with additional samples of *S. elongata*.

Table 4. Genetic p-distances between species are shown below the diagonal; differences among the species are shown in the diagonal (*n/c* – represent no differences calculated due to the existence of only one specimen in the analysis) (Kimura, 1980; Kumar et al., 2018).

	Scorpaena azorica	***Scorpaena canariensis***	***Scorpaena elongata***	***Scorpaena laevis***	***Scorpaena maderensis***	***Scorpaena notata***	***Scorpaena porcus***	***Scorpaena scrofa***
Scorpaena azorica	*n/c*							
Scorpaena canariensis	0,18744	*0,00246*						
Scorpaena elongata	0,02051	0,17968	*n/c*					
Scorpaena laevis	0,08603	0,19727	0,07970	*n/c*				
Scorpaena maderensis	0,14832	0,08339	0,14450	0,16605	*0,01326*			
Scorpaena notata	0,04785	0,17361	0,05553	0,10492	0,14392	*0,00052*		
Scorpaena porcus	0,05846	0,16659	0,04781	0,06842	0,14173	0,06369	*0,01262*	
Scorpaena scrofa	0,01088	0,17508	0,00873	0,07412	0,13752	0,04401	0,05080	*0,00491*

S. maderensis showed the highest intraspecific genetic distance (p =0,01326), which was expected due to the number and broad geographic origin of the samples (from the Azores Islands to Cyprus).

S. porcus has the second higher intraspecific genetic distance (p =0,01262), with specimens from different spots in northern Spain, in Portugal and in Canary Islands.

The lowest intraspecific genetic distance was found for *S. notata* (p = 0,00052), which could be explained by the fact that a large amount of specimens are from central/southern Portugal with only one from southern Spain.

4.2. Venom analyses

Table 5. Total protein concentrations (mg/ml) quantified for the five *Scorpaena* species, in the end of the crude venom extraction protocol using Nanodrop (Thermo Scientific™) at 280 nm. Protein concentrations were obtained from the frozen and fresh crude venom extractions. W – wild; C – captivity; Storage fresh or frozen at -20°C; n/d – no data; the grey lines represent venom samples used to test the degradation of venom proprieties on frozen samples.

Specie	Collection site	N	Average Total Length (cm)	Harvest date	Provenance /Storage	Protein concentration (mg/ml) (ABS280)
Scorpaena canariensis	Canary Island	3	14,5	04/2018	W/-20°C	14,9
Scorpaena maderensis	Madeira Island	8	9,0	11/2018	W/-20°C	3,5
Scorpaena notata	Fonte da Telha	2	n/d	06/2016	W/-20°C	15,2
	Vasco da Gama Aquarium	3	16,9	05/2019	C/Fresh	
	Fonte da Telha	2	n/d	06/2016	W/-20°C	10,20
	Vasco da Gama Aquarium	3	16,9	05/2019	C/Fresh	13,90
	Vasco da Gama Aquarium	1	17,2	05/2009	C/Fresh	5,40
Scorpaena porcus	Peniche	2	30,25	06/2016	W/-20°C	12,1
		1	n/d	06/2016	W/-20°C	
Scorpaena scrofa	Peniche	3	47,34	05/2016	W/-20°C	13,8
		2	n/d	03/2017	W/-20°C	

4.2.1. Protein profiles for the five Scorpaena venoms

The analysis of the venom protein profiles revealed evident differences between the five *Scorpaena* species (Fig. 5). All the crude venom samples display a conserved set of bands at the high molecular weight region, between 75 kDa and 150 kDa. *S. notata* presents a tenuous

band at ~75 kDa with the other high molecular weight being less representative or absent. *S. canariensis* and *S. scrofa* protein profiles stand out, yielding more protein bands.

At medium molecular weight level, *S. maderensis*, *S. notata* and *S. scrofa* display a clear band between 25 kDa and 37 kDa, absent or less represented on *S. canariensis* and *S. porcus*. At low molecular weight, *S. canariensis*, *S. porcus* and *S. scrofa* show some bands at ~25 kDa. In turn, *S. notata* and *S. scrofa* show a band below 20 kDa. As the low molecular weight bands are overlapped, it became impossible to detect potential similarities and/or differences between these five species. These small molecular weight venom components must be further analysed in the future.

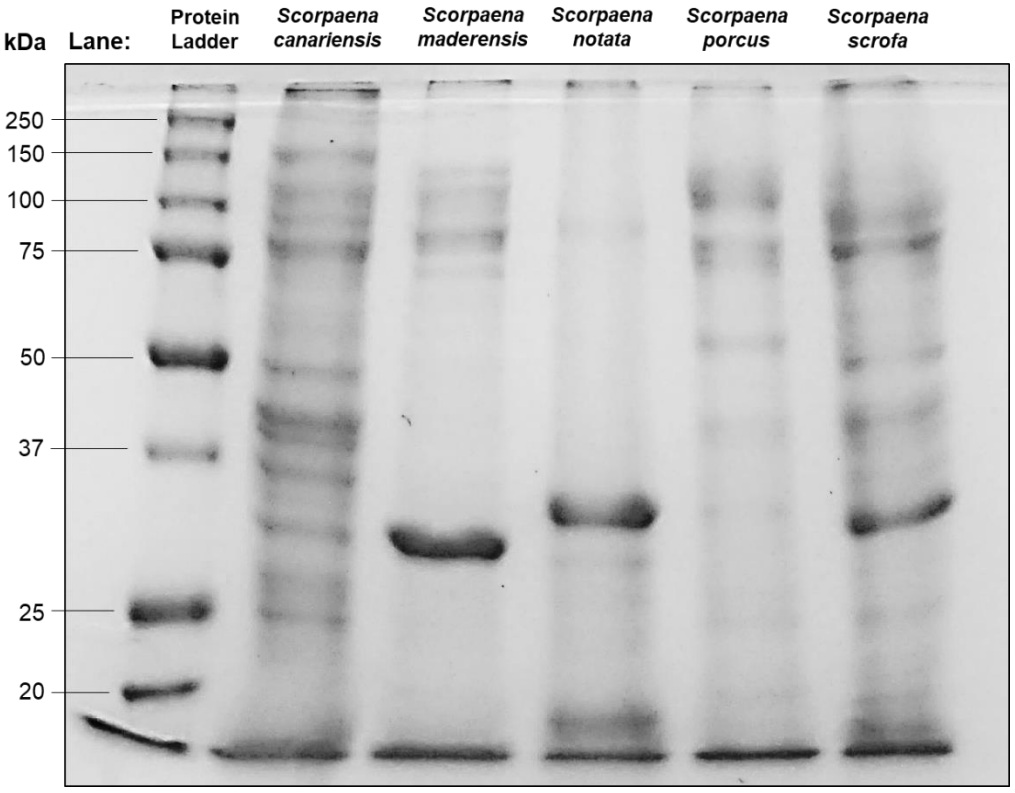

Figure 5. SDS-PAGE protein profile of the crude venom extract from *S. canariensis*, *S. maderensis*, *S. notata*, *S. porcus* and *S. scrofa* in a 12,5% polyacrylamide gel. Molecular mass marker is represented on the left.

4.2.2. Protein profile for fresh and frozen venom

Fresh venom samples show the same protein profiles as the frozen samples (Fig. 6). However, the same band profiles at the same total protein concentration show a less evident band pattern in frozen samples. This is probably related to progressive degradation of venom proteins even though they were immediately stored at -20˚ C.

Although the protein concentration estimated in the laboratory was standardized, the protein profile expressed on the intensity of the molecular mass bands obtained in the SDS-PAGE gel is clearly different between fresh and frozen samples.

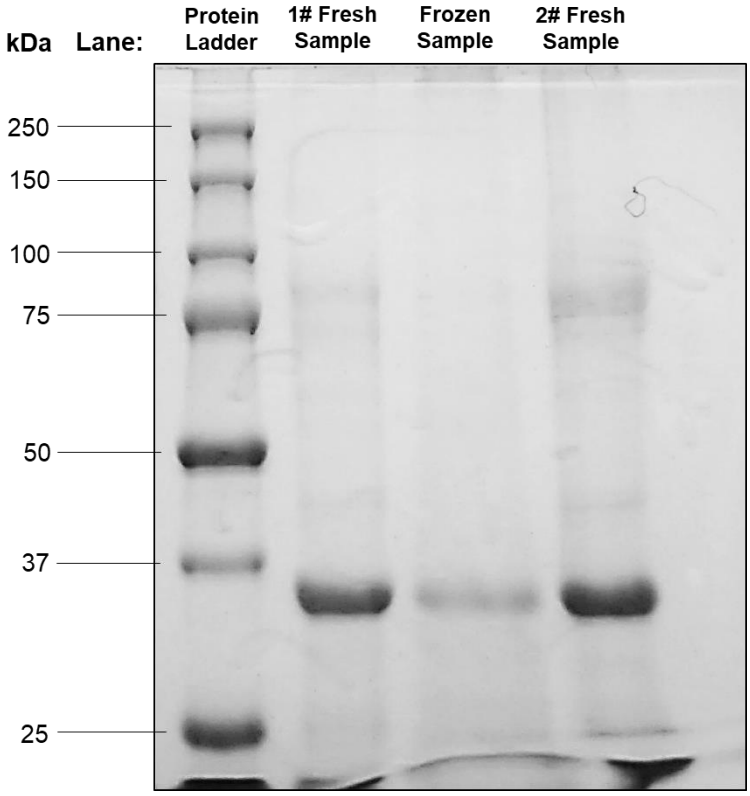

Figure 6. SDS-PAGE protein profile of the fresh and frozen venom extracts from *S. notata* in a 12,5% polyacrylamide gel. Molecular mass marker is represented on the left.

4.2.3. Spiny rays' regeneration

Spiny rays are important either as a defence mechanism, in case the individual is released in its natural environment, or if the individual is maintained in captivity for posterior milking of consecutive venom sampling. Regeneration of the spiny rays was reported to be ineffective for at least 2 years (see annex B), arguing against this classical procedure for fish venom collection. This raises concerns from different perspectives: from an animal welfare approach since proper endpoints must be defined after chirurgical removal of the venomous fin; from an ecological approach it is no longer possible to release wild animals immediately after venomous fin removal and at a biotechnological level, as it is also no longer possible to perform successive venom collections in captive individuals. On the other hand, the sponge-in-a-tube method proved to be an effective alternative for fish venom collection, limiting fish suffering and increasing the effectiveness of fish recovery.

5. Discussion

5.1. *Scorpaena* phylogeny

Scorpaena comprises 61 cryptic species recognized worldwide. The *Scorpaena's* phylogeny remains unknown and this is the first study to clarify the phylogeny of the large majority of northeastern Atlantic and Mediterranean *Scorpaena* species.

These cryptic species present variable colours patterns together with similar morphology and habitat preferences, making their identification extremely difficult. To guarantee correct identifications, practice and experienced taxonomist's opinion are needed. For smaller specimens a subsequent genetic confirmation is frequently required. Given this situation, the samples analysed in this work were identified both by their morphology and by sequencing their DNA. Additional care was taken for rarer species, such as *S. laevis, S. elongata* and *S. azorica*, with only one specimen available. Besides morphological and genetic validation, identification by an experienced taxonomist was also required (Manuel Biscoito; Funchal Municipal Museum).

Taking these precautionary methods into consideration, the results obtained in this study are discordant from the ones obtained by Turan et al., (2009), the single most comprehensive

phylogeny of this genus available in the literature. However, the results presented here are consistent with the ones described by other authors that included a small number of *Scorpaena* species in wider phylogenetic studies (Kochzius et al., 2010; Stankovic et al., 2002). According to Turan et al., (2009), *S. elongata*, *S. scrofa* and *S. maderensis* form a clade, in which the two first ones appear as sister-species. Our results also show *S. elongata* and *S. scrofa* as sister species but not forming a clade with *S. maderensis*. These authors show another clade formed by *S. porcus* and *S. notata* which was also not confirmed by our results. This is the only available reference addressing the phylogeny within the genus *Scorpaena* and is based in the same DNA fragment (16S rDNA) and similar outgroup species used in this work.

One possible explanation for the discrepancy between results, may be due to the fact that we have a more comprehensive group of species which would necessarily change the topology of the phylogenetic tree. However, a close comparison of the 16S rDNA homologous sequences suggests that some specimens could have been miss identified.

When *S. maderensis* sequences from Turan et al., (2009) (e.g. EU747077.1, EU747078.1 and EU747079.1) are aligned with *S. maderensis* sequences from this work, they differ in 80 base pairs, but display 6 differences from the *S. porcus* in this study. This means that *S. maderensis* specimens from Turan et al., (2009) could be *S. porcus*, which in that case, Turan et al., (2009) results start to be consistent with the results found in this work. It is also important to stress that we analysed and sequenced 32 specimens of S. maderensis while Turan et al., (2009) analysed only three.

Additionally, the *S. porcus* from Turan et al., (2009) (EU747075.1; EU747076.1) differ 41 base pairs from *S. porcus* from this work, but only 2 base pairs from *S. notata*. Also, *S. notata* specimens from Kochzius et al., (2010) (e.g., FN688195.1) differ 3 base pairs from *S. porcus* from Turan et al., (2009), and are similar to *S. notata* from this study. In addition, one *S. scrofa* specimen from Turan et al., (2009) (EU747071.1) is similar to our *S. elongata*, although these two species present a remarkable similarity which should be further investigated in the future. Taking this fact into consideration, there are some similarities between *S. elongata* and *S. scrofa* from Turan et al., (2009) and from this study, which was also supported by results from another study (Stankovic et al., 2002; e.g. AF518223.1).

These results emphasize the difficulty associated to identify morphological identifications of these species. They also suggest that an analysis of a representative number of species, including a large number of individuals per species, distributed by a wide geographical range is recommended when addressing the phylogeny of such a complex genus.

To confirm these results and to clarify some phylogenetic relationships that remain unresolved (the relationship between *S. notata*, *S. porcus* and *S. laevis*) additional mitochondrial and nuclear fragments, such as COI and S7 respectively, must be analysed.

Despite these difficulties it was possible to contribute with the first DNA sequences of *S. canariensis* and *S. azorica*, which were not available in GenBank database before.

5.2. Interspecific venom composition variation

Venom profiles showed that the five *Scorpaena* species conserve some proteins with similar molecular weight in their venom, which was already expected since the five species are closely related and belong to the same genus. However, the five venom profiles display bands with different molecular weights in different species, that could correspond to different proteins. This means that the crude venom extracts exhibit species-specific protein profiles both in composition and in the relative amount of venom components, or in other words, *Scorpaena* venoms exhibit a high degree of diversity.

To my knowledge, this is the first-time a comparative study of different venom protein profiles is made including several closely related fish species. It is also the most comprehensive study in *Scorpaena* showing that even sister species (such as *S. canariensis* and *S. maderensis*) can display marked differences in their venom composition and that, apparently, there is no relationship between their phylogeny and their venom similarity.

Venom proteins were already described for this genus in one single well-studied specie, mainly due to their association to public health concerns derived from dangerous or recurrent envenomation episodes. This is the case of the southwestern Atlantic *Scorpaena plumieri*. At least eight proteins were described for this species: the Plumieribetin (14 kDa; De Evangelista et al., 2009), Sp-GP gelatinase (72 – 80 kDa; Carrijo et al., 2005); SP-CL 1-5 (16,8-17 kDa; Adrich et al., 2009) and Sp-CTx (121 kDa; Adrich et al., 2010). A single reference is also available for *Scorpaena guttata* (Girard, 1854), described for the eastern Pacific, it is known that the proteins with the most potent effect have molecular mass between 50 and 800 kDa (Schaeffer et al., 1971).

In order to compare the *Scorpaena* proteins described by these authors and the ones described here for these five *Scorpaena* specie, additional information is needed. To clarify the low molecular weight proteins overlapped below 20 kDa, and to find if there are more proteins with small molecular weight in these fishes' we will need to analyse denser (possibly 15%) SDS-PAGE gel, suited to study proteins below 50 kDa (Weber & Osborn, 1975).

Fish venoms are extremely labile, which might cause loss of bioactive properties during their storage (Andrich et al., 2010). The protein concentration (mg /ml) (at 280 nm) results show the existence of differences between fresh and frozen (-20˚C) venom samples. In future research, it is recommended to use fresh venom samples or to preserve them in liquid nitrogen (−196 °C), as suggested by Menezes et al., (2012).

The SDS-PAGE electrophoresis is useful to separate and characterize proteins, as this technique permits proteins to run according to their molecular weight, by denaturing all the proteins. SDS-PAGE gels have allowed us to know the *Scorpaena* protein's molecular weight, and how many possible different proteins the five different species would be present in each venom crude extract. However, although the molecular weight is widely used in biotechnology to characterize proteins (Feng et al., 1991), it would not be correct to state that each band represents one different protein, since this method presents some limitations. For example, slight changes in the protein amino acid composition (e.g. the presence of isomorphs) may change their molecular weight and affect their mobility on the gel. Also, a protein not completely desaturated, as a result from insufficient disulphide bonds dissociated, would have different mobility in the gel.

To define which bands correspond to different proteins it would be necessary to select each band and perform Mass Spectrometry (MS) analysis, a more expensive technique. MS allows the isolation of each protein present in *Scorpaena* venoms profile, as well as the identification of their partial amino acid sequence and the comparison of results with the ones found in public protein databases.

Another step to be taken is to evaluate *Scorpaena* venom activity and potency. Haemolytic tests already performed by Rosa (2016). Rosa (2016) tested *S. porcus* and *S. notata* venoms in rabbit erythrocytes and concluded that *S. notata* venom presents a high cytolitic activity compared to *S. porcus* venom. This author also concluded that the venom of these two northeastern Atlantic species produce milder venoms with weaker haemolytic activity compared to the southwestern Atlantic species *S. plumieri*.

5.3. Phylogeny based on neutral markers and venom evolution

The results obtained on the venom profiles do not follow the same line as the phylogenetic results, i.e., closely related species may display very different protein profiles. As an example, *S. maderensis* and *S. canariensis* formed a highly supported clade and are in fact sister-species. However, their protein profiles are different, and their venoms present

different proteins. Other examples are *S. notata*, *S. porcus* and *S. scrofa*, which form a well-supported clade. However, *S. notata* and *S. scrofa* possess a band (between 25 kDa and 37 kDa) that is absent in *S. porcus*.

The differences in venom profiles and the fact that no clear relationship between venom profiles and the phylogeny of this group was found, could be explained by different hypothesis:

1) this defensive trait is under the effect of natural selection via "arms race" evolution (Richards et al., 2012), that lead to venom speciation. In fish, since venom is mostly a defensive adaptation against predators, it would be interesting to evaluate if these differences are related to different predominant predators along the distribution area of each species;

2) it is on a fast evolutionary track due to distinct genomic mechanisms (e.g. due to gene duplication later co-opted for defensive purposes by *sensu* (Vonk et al. 2013);

3) interspecific variations in venom composition could also be related to alimentary shifts and/or trophic ecology, although no evidence was found to support this hypothesis since no differences between wild and captive conspecifics were found in this work;

4) or represent divergence in ancient group of fish.

However, testing these hypotheses is beyond the scope of this work.

Although the protein profiles inside the *Scorpaena* genus could be very different from each other, they share some proteins. So, it is possible to use phylogenetic to design a "road map", where we can prospect which species could have certain venom's protein, by knowing the phylogenetic relationships between those species, and the species that we already know to produce that protein.

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Annexes

Annexes A

This section presents the revision of literature available for *Scorpaena* phylogeny, marine venomous species and their venoms. The revision of literature is requested by ISPA's dissertation regulation.

List of figures

A. State of the Art

1. Phylogeny

In the Classical Antiquity (Aristotle (c. 384 B.C. to 322 B.C)), taxonomy had only used common or homologous characteristics, for example morphologic, ecologic, biogeographic and behavioural features, to classify distinct groups of organisms (Kozlov et al., 2016; Ridley, 1996). Centuries later, still based on homologous characteristics, Linnaues (1707-1778) introduced hierarchical levels on species classification (e.g. kingdom, file, class, order, family) and binomial nomenclature, that is still used today (Kozlov et al., 2016). With Darwin's evolutionary theory, taxonomy adopted phylogenetic methods to support their classification (Futuyma, 1998). In most cases, phylogenetic classifications support classification based on phenetic criteria, but, in some cases, the classifications are discordant (Ridley, 1996).

Phylogeny is based on molecular biology methods (e.g. DNA and amino acid sequences), on morphological characters in living and fossil species, and on statistics methods (distance methods, parsimony and maximum likelihood), to achieve the phylogenetic relations between species (Ridley, 1996). Phylogeny allows us to understand the evolutive processes that species had suffered and comprehend the evolutive relationships between them, i.e., it permit know the evolutive history of one group of organisms (Futuyma, 1998; Ridley, 1996).

When it comes to research, phylogeny can also serve as tool for guide one line of study, by allowing to prospect the distribution of certain characteristics between related species, before they are studied (Raven *et al*., 1994), an example, it enables the prospection of existing venoms inside *Scorpaena* genus, or even, if the toxins of the venom were already known, prospect about which related species could contain the same toxin/proteins in their venom (Smith and Wheeler, 2006).

If we think ahead, we can easily comprehend that, if a particular venom protein for certain specie has pharmaceutical utility, and knowing the genera or family phylogenetic tree of that specie, it's possible to conduct a research towards exploring the existence of that same protein in another specie, possibly with other advantages, an example, being easily accessible or captured or possessing a bigger quantity of venom (Smith and Wheeler, 2006; Smith et al., 2016).

As I previously mentioned, in phylogenetic studies molecular biology methods can be applied based on DNA fragments analyses, also called molecular markers, that could be mitochondrial or nuclear DNA fragments.

The mitochondrial DNA (mtDNA), contained inside the mitochondria, holds only maternal inheritance information, rarely suffers genetic recombination, and demonstrates high prevalence in the cells (Rokas et al., 2003; Avise 2000; Gissi et al. 2008). In particular, some non-codified fragments could quickly suffer mutations (neutral selection). Furthermore, as nuclear DNA occurs in a higher quantity in the cells, their extraction and amplification are simplified (Futuyma, 1998). The nuclear DNA contained inside the cell nucleus, evolve slowly, having control regions with more conserved information (Durand *et al*. 2002; Kocher and Stepien, 1997).

The choice of which DNA fragments are more suitable to specific phylogenetic studies, is based on which type of question you want to answer, or phylogenetic relationship you wish to clarify. In other words, highly variable regions, like DNA mitochondrial regions, are useful to determine relationships between related species that have diverged recently (Durand et al., 2002; Kocher and Stepien, 1997), i.e., intraspecific studies, while conserved regions, such as nuclear DNA, are more advantageous for studies of more diverged taxa (e.g. family, orders) (Reyes et al. 2003).

1.1. *Scorpaena* taxonomy and phylogeny

Marine venomous fishes are distributed across the Chondrichthyes class, where you can find for example, stingrays and the ratfish, and across Actinopterygii or ray-finned fishes, being already identified around 2000 species (Smith and Wheeler, 2006; Smith et al., 2016; Wright, 2009).

With the exception of cartilaginous fishes, the venomous fish species already described include the Siluriformes (catfish) and five orders of Teleostei (teleost): Scorpaeniformes (e.g. Scorpionfish, Stonefish), Cypriniformes (e.g. Zebrafish), Trachiniformes (e.g. weeverfish, stargazer), Batrachoidiformes (e.g. toadfish) and Perciformes (e.g. blennies, surgeonfish, rabbitfish) (Churchand Hodgson, 2002; Haddad *et al*., 2003; Halstead, 1988; Russell, 1996; Russell, 1996; Smith-Vaniz *et al*., 2001; Smith and Wheeler., 2006; Vetrano *et al*., 2002).

Notice that, except for catfishes, all venomous bony fishes, around 1200 species belonging to “acanthomorphs” or spiny-rayed fishes (Smith and Wheeler, 2006), a large group of marine fishes whose knowledge of their evolutionary relationships remains scarce and contradictory (Johnson 1993; Miya *et al*. 2003; Nelson 1994; Stiassny *et al*. 2004).

Scorpaena (Linnaeus, 1758) is one of the genera that belongs to a venomous fish family recognized as *Scorpaenidae*, generally known as Scorpionfish, with the prefix "Scorpion" referring to the existence of chemical weapons and spines with venom (Machado et al., 2014; Nelson, 2006; Vieira and Barreiros, 2010; Poss and Eschmeyer, 2002). It is the most dangerous family of venomous fishes that we know about, and they are spread over different generas according to their venom apparatus morphology (Andrich et al., 2010).

The *Scorpaenidae* encompassed 30 genera and more than 350 species (Kizer et al., 1985), among which including species from well-known genera, for example, the *Pterois volitans* (o Indo-Pacific lionfish), a venomous predator, that after being introduced in some northwest Atlantic regions, it became the invasive specie responsible for strong impacts on marine ecosystems (Ferreira et al., 2015). Also, it includes species from *Synanceia* genera (Stonefish), one of the greatest venomous fish in the world, that is involved in several accidents with humans during recreative maritime activities (Smith and Heemstra, 1986; Tay *et al*., 2016). These fishes can be found in all oceanic basins, occupying diverse types of habitats, from sea bottoms from coastal zones sea bottoms, to coral reefs and kelp forests (Kizer et al., 1985).

In the Northeast Atlantic and Mediterranean Sea, species from *Scorpaenidae* family are generally associated to rocky marine bottoms and occur in depths up to or superior to 2 000 metres (Hureau and Litvinenko, 1986). The identification of *Scorpaena* genera species that belong to this study, except for *S. canariensis*, was based on the taxonomy present in *Fishes of the North-eastern Atlantic and the Mediterranean* (FNAM) (Whitehead et al. 1989).

According to Hureau and Litvinenko (1986), *Scorpaena* from Northeast Atlantic and Mediterranean Sea can display a body covered by ctenoid and cycloid scales, but ctenoid scales are predominant. The head is characterized by being highly spiny, with spines on their orbital, nasal, ocular, opercular and superior region of the head. The preopercular spine is the longest. Generally, they exhibit occipital pit and palatine teeth. Their dorsal fins have 12 spines and 10 rays, the anal fin has 3 spines and 5 rays, and the pectoral fins have 16 to 21 rays.

For the Northeast atlantic and Mediterranean,there are 8 species described: *S. notata* Rafinesque 1810, *S. porcus* Linnaeus 1758, *S. scrofa*, Linnaeus 1758, *S. azorica* Eschmeyer 1969, *S. maderensis* Valenciennes 1833, *S. elongata* Cadenat 1843, *S. laevis*, Troschel 1866, and *S. loppei* Cadenat 1943 (Hureau and Litvinenko, 1986).

More recently, on a workshop in Tenerife, Canary Islands, in 2005, Motomura (2005) described taxonomically the *S. canariensis*, Sauvage, 1878, making it 9 the number of

Scorpaena species described for the Northeast atlantic and Mediterranean region. Until now, *S. canariensis* was found only in three northeast atlantic archipelagos – Azores, Madeira and Canarys – between 100 and 120 metres of depth, on rocky reef bottoms (Motomura, 2005).

When we look at the existent literature, phylogenetic studies about venomous fishes that belong to Acanthomorphs, where are included all the spiny-rayed fishes, we found two studies by Smith and Wheeler (2006) and Smith et al., (2016). In the first mentioned study, the researchers tried to clarify the evolutionary relationships that exist between 233 species on the total of ~18000 Acanthomorphs species described in the world (Smith and Wheeler, 2006). In that study, they used species that could represent all the venomous fishes (Meiacanthus blennies, thalassophrynine toadfishes, scatophagids, scomberoidine carangids, uranoscopids, siganids, acanthurids, trachinids and scorpaenoids), and applied 12S, 16S, 28S and histone H3 genetic molecular markers (Smith and Wheeler, 2006). Additionally, they also analysed the type of venom apparatus that which specie possess. Inside the *Scorpaenidae* group, the individuals from *Scorpaena, Scorpaenodes, Scorpaenopsis, Sebastapistes, Pterois, Taenionotus, Iracundus* and *Parascorpaena* genera displayed venomous glands and anterolateral groove on the venomous spines from the dorsal and anal fins. *Neomerinthe, S*ebastolobus and *Pontinus* didn't display glandular tissue associated with the anterolateral groove (Smith and Wheeler, 2006). As a result from the phylogenetic relationships, they showed that *Scorpaeniformes* (where S. madarensis is found) and Perciformes are polyphyletic group (Smith and Wheeler, 2006), as previous studies suggest (Chen *et al.*, 2003; Johnson 1993; Miya *et al.*, 2003; Smith and Wheeler, 2004; Tang *et al.*, 1999).

In order to clarify the phylogenetic relationships of diverse Gasterosteiformes, Perciformes and Scorpaeniformes families, Lautredou et al., (2013) used seven molecular markers: the IRBP (*interphotoreceptor retinoid-binding protein*) (Chen and Mayden, 2009; Dettai and Lecointre, 2008), the MLL2 (*Mixed-lineage leukemia-like protein* 2) (Ansari *et al*., 2008; Hess, 2004; Yu *et al.*, 1998), the MLL4 (*Mixed-lineage leukemia-like protein* 4) (Dettai and Lecointre, 2005), the MC1R (*melanocortin type 1 receptor*) (Mundy, 2005; Healy *et al*., 2001; Rosenblum *et al*., 2004), the Pkd1 (*polycystic kidney disease*) (Lautredou *et al*., 2010, 2012), the *Rhodopsin retrogene* (Chen *et al*., 2003) and the RNF213 (*ring finger protein* 213) (Li *et al*., 2009). In this article, the *Scorpaena, Scorpaenopsis, Sebastapistes* and *Parascorpaena* formed one clade, and the *Scorpaenodes* and *Pteroini formed another one, being these two clades very close* (Lautredou *et al*., 2013) (Fig. A1).

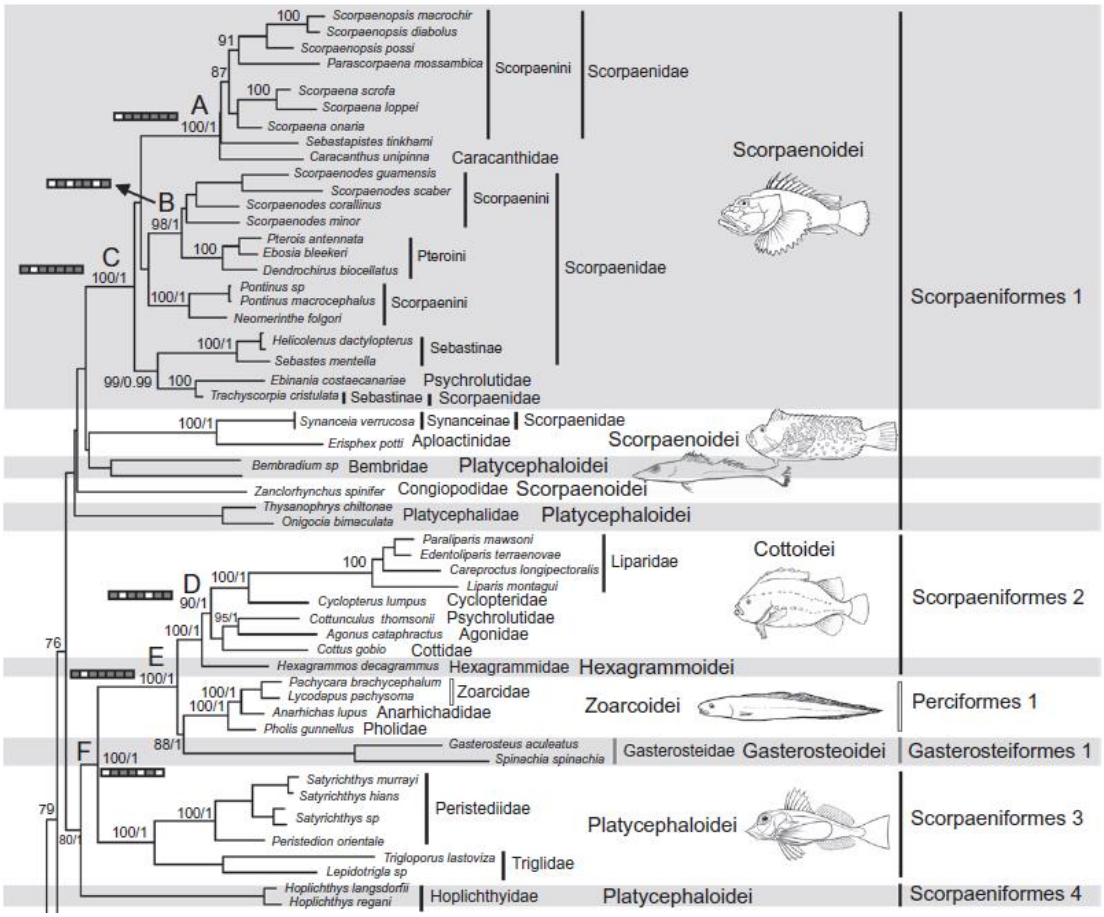

Figure A1. Bayesian and Maximum likelihood trees of Scorpaeniformes, where are inserted *Scorpaena* specimens (adapted from Latredou et al., 2013).

For the Northeast Atlantic and Mediterranean *Scorpaena*, exists one study where only 16S mitochondrial fragment was used. In this work, Turan et al., (2009) used five species of *Scorpaena* genera - *S. elongata, S. scrofa, S. porcus, S. notata* e *S. maderensis* – and one specie, the *Helicolenus dactylopterus* as outgroup, applying neighbour joining (NJ) and maximum parsimony (MP) statistic methods. The researchers obtained two different lineages, where one contained the *S. porcus* and *S. notata* species, and another confined two clades, one with *S. elongate* and *S. scrofa* as sister group, and another one with *S. maderensis*.

2. Venom

2.1.Venomous organisms

Venoms are aqueous secretions primarily composed by peptide and protein toxins, produced inside a specialized structure (e.g. gland), which possesses the capacity to disturb physiological and biochemical processes in another animal when they are released in their body (Casewell et al., 2013; Fry et al., 2009 Okulalakshmi et al., 2018).

A venomous organism has specialized tissues or organs for the production of toxic substances – the venoms – and use those substances in interactions with another organisms, using specialized structures (e.g. spines, stings, nematocysts, barbs, beaks, fangs, pinchers, proboscises) (Fry et al., 2009) for the delivery and application of the venom inside the target specie‘s tissue (Mebs, 2002). Those substances injected on the victim provoke clinical conditions known as envenomation (White and Meier, 2008) (e.g. species from *Conus* genus (Haddad et al., 2006)). This concept is important in order differentiate venomous organisms from poisonous ones, i.e., organisms that don’t possess specialized tissue or organs for the venom (in this case, poison) production and delivery, but cause intoxications, called poisoning, when they are ingested by their predator or when the predator is in contact with the poisonous organism body (White and Meier, 2008; Halstead, 1956; Daly et al., 2005). (An example of a poisonous organism could be the poison-dart frog *Phyllobates terribilis* (Dly et al., 2005).

In the animal kingdom, we find venomous organisms on (in) marine and terrestrial vertebrate and invertebrate groups. Within the marine environment, venomous organisms are distributed by cnidaria, molluscs, annelidea and fishes’ group, the latter representing more than half of all venomous vertebrate animals (Smith and Wheeler, 2006). When we compared the number of terrestrial venomous species with the number of marine ones, we noticed that most venomous species belong to the marine environment (Tse, 2016).

Venomous fishes possess a venom apparatus with variable degree of complexity among species, inclusive (including) between related species (Halsead et al., 1955; Wright, 2009). They are composed by a venom tissue or gland, excretory ducts and venom spines (Church and Hodgson, 2002). Venom spines can be located on dorsal, pectoral pelvic and anal spines, as on the opercule and tails (e.g. stingrays) (Ziegman and Alewood, 2015). Some exceptions to this venom apparatus profile exist, for example on *sabre-toothed blenny*, *Meiacanthus nigrolineatus*, where we can find canine teeth in the place of spines (Fishelson, 1974).

In *Scorpaena* genera, the venom apparatus is formed by medium sized venom glands, constituted by glandular cells surrounded by support cells (Halsead et al., 1955; Wright, 2009), and by spines located on the opercule and on the dorsal and anal fins. The spines hold an anterolateral groove, where the venom glands are associated (Church and Hodgson, 2002; Smith and Wheeler, 2006; Ziegman and Alewood, 2015). It is important to note that the anterolateral groove doesn't exist in all venomous fish species, neither in all *Scorpaenidae* (Smith and Wheeler, 2006). Finally, covering the spine and the venom gland, there is an integumentary sheath (Kizer et al., 1985).

During an envenomation, the predator/prey's tissue puts pressure on the spines, which causes the integumentary sheath and tissue glands to break, and release the venom inside the victim's body, guided by the anterolateral groove (Halstead et al., 1956;1955; Smith and Wheeler, 2006; Smith et al., 2016; Wright, 2009). It is important to emphasize the lack of muscular tissue associated to the venom apparatus in *Scorpaena* genera, which implicates an involuntary release of the venom, and without any control of the quantity liberated (Ziegman and Alewood, 2015).

The symptom dimension and severity of envenomation varies according to the individual's susceptibility, it differs among individuals, among species and relative to the number of injuries and their seriousness (Ziegman and Alewood, 2015). Usually, the symptoms described in the case of envenomation are intense pain disproportional to the wound size, erythema and severe edema (Church and Hodgson, 2002; Haddad and Martins, 2006; Ziegman and Alewood, 2015). Frequently, vesicles around the wound will occur (Patel and Wells, 1993; Auerbach *et al.*, 1987), and in extreme cases, the victim dies in the first hours following envenomation, normally due to severe hypotension and cardiac or respiratory failure (Church and Hodgson, 2002).

Common treatments for envenomation caused by fish venoms are immersion of the zone of injury in hot water (45 -50 °C), in order to relieve the pain (Haddad and Martins, 2006), and when possible, use antivenoms (Ziegmand and Alewood, 2015). It is thought that the extreme fish venom's lability to temperature, which makes hot water the most efficient on pain relief (Schaeffer *et al*., 1971). Interestingly, morphine, one of the most used analgesic against intense pain, is inefficient when it comes to the pain caused by envenomation (Patkin and Freeman, 1969; Smith, 1951). All scorpaenoids venoms provoke vasodilatation, hypotension, muscular weakness and neuromuscular paralysis (Cohen and Olek, 1989; Khoo, 2002), and maintain their total potency for 48 hours after the venomous animal dies (Kizer et al., 1985; Lyon, 2004). The envenomation caused by *Scorpaenidae* species, occurs due to

negligence on manipulating those fishes, mainly by fishermen and aquariologists (Kizer et al., 1985), and in occasional meetings with bathers and divers (Moser and Sturchler, 1979; Prentice et al., 2008).

2.2.Venom evolution

Venoms are important for the venomous fishes' fitness and reproductive success (Smith et al., 2006; 2016; Ziegman and Alewood, 2015). These bioactive compounds suffered a convergent evolution 19 times to be used on defence, predation and competition (Casewell et al., 2013), being the competition, the rarest role played by these venoms (Buchheim and Hixon, 1992; Koppel, 1988; Stephens et al., 1970). A species could produce a single venom that has more than one purpose (Barlow et al., 2009; Boyer et al., 2015; Dutertre et al., 2014; Inceoglu et al., 2003; Whittington *et al*., 2008), or even produce more than one venom. This convergent evolution was particularly important in Scorpaeniformes and Siluriformes, that represent the majority of venomous fish species (Smith et al., 2016).

Almost 3000 species of venom fishes use venom as defence weapon (Smith and Wheeler, 2006; Smith et al., 2016; Li et al., 2018; Wright, 2009).

The defensive venoms's evolution occurs from antagonistic coevolution (Endler 1986; 1991), where the predator-prey interactions provoked selective pressures through the evolutive system of "arms race" (Dawkins and Krebs, 1979; Endler 1986; 1991; Van Valen, 1973), with the predation being the motor for venoms evolution (Casewell et al., 2013). In this way, when the target-predator develops resistance to the venom's toxins, the selective pressure selects venoms with higher toxicity, which in turn will select target-species with higher resistance (Richards et al., 2012).

According to Endler (1986), predation is divided into five stages: detection, identification, approximation, subjugation and consumption. The strategies or adaptations of chemical weapons that evolved in these fishes, occurred in order to allow the venom specie's escape from these phases, in particular, to avoid the last two stages (the subjugation and consumption) (Harris and Jenner, 2019; Nelsen *et al*., 2014). The venom's defensive function has the purpose of causing discomfort or intense and immediate pain on the target, without immobilizing it, giving the fish the opportunity to escape (Casewell et al., 2013, Harris and Jenner, 2019). This is proven when we analyse the chemical proprieties of the venom, and the disturbance types that the venom causes on the target. The venom used for defence is effective to a large range of predators, having each one different susceptibility to it, which

suggests that it wasn't a single predator specie to lead to the evolution of defensive venoms (Mumby et al., 2011; Paxton et al., 1998; Smith et al., 2006; 2016; Ziegman and Alewood, 2015).

To avoid the first stages of predation (detection, identification and approximation), the venomous organisms evolved another type of adaptation, more visual, discouraging the predator to attack. The aposematism (shiny and contrasting colour patterns) and the crypsis are the most common, but some organisms also use camouflage and mimicry (Cott, 1940; Harris and Jenner, 2019; Hoese et al., 2006; Ruxton et al., 2004). This type of visual adaptations is well-succeeded, and some venom species serve as mimicry model to non-venom species (Alexandrou et al., 2011; Casewell et al., 2017; Greene, 1981; Wüster et al., 2004).

A big disadvantage in the evolution of the defensive venoms, comes with its the energetic waste. The chemical defences are more expensive at an energetic level, namely on the venom production phase, thus venomous species only use them as last resource (Endler, 1986; Mappens et al., 2005; Speed and Ruxton, 2005; Wüster et al., 2004; Enzor et al., 2011). After provoking an envenomation, the animal remains chemically defenceless, which reduces their fitness and affect their survival (Boyer *et al*., 2015; Currier *et al*., 2012; Nisani et al., 2007; 2012). The absence of control/dosage mechanisms for the quantity of venom is also an energetic disadvantage factor on these organisms (Morgenstern and King, 2013). Therefore, to economize energy for growth and reproduction, many species adopt sedentary lifestyles, in where they camouflage among substrate detritus and keep their spines erect, which can be observed on scorpionfish, *stonefish, waspfish* e *weeverfish* (Cameron and Endean, 1973; Ziegmand and Alewood, 2015). These species end up benefiting from their chemical to take advantage of their sedentary life (Ziegman and Alewood, 2015). Additionally, the fact that venoms are efficient on a large number of predators, is also a way to economize metabolic costs (Richards et al., 2012).

The predation pressure leads to the evolution of glands that produce venom, from epidemic cells that produce toxins with antiparasitic proprieties (e.g. icthiocrinotoxins), that suffered thickening and aggregation (Mangoni et al., 2000; Rinaldi, 2002). On the other hand, icthiocrinotoxins have originated toxin development in venoms, although there are studies that prove their distinct composition (Cameron and Endean, 1973; Ramos et al., 2012). The continuing predatory pressure caused the association of venom glands with spines, structures already used on defence, and that began to serve as venom delivery structure (Sismour et al., 2013; Smith et al., 2006; 2016; Wright, 2012).

Fishes segregate a mucus through their skin, composed by bioactive and toxic substances – the icthiocrinotoxins – different from those that exist on venoms (Ramos et al., 2012; Klaassen and Watkins, 1999; Thulesius et al., 1983). It is believed that ichtiocrinotoxins possess antibiotic and antiparasitic proprieties (Halsted and Courville, 1970), that protect fishes from microorganisms (Church and Hodgson, 2002) and reduce the peeling (Ziegman and Alewood, 2015). Borges et al., (2018) concluded that the toxins existent in the skin mucus and the venom of *Scorpaena plumieri* are similar. In spite of the fact that the mucus and the venom have similarities, the mucus isn't considered a venom, due to the lack of a specialized deliver system (Church and Hodgson, 2002; Klaassen and Watkins, 1999). Frequently, on envenomation, the skin mucus goes into the wound along with venom (Klaassen and Watkins, 1999; Thulesius et al., 1983; Wright, 2009;). If the predator-prey interactions lead to the evolution of venoms on fishes, the hostage-parasite interactions lead to the evolution of mucus with antimicrobial proprieties (Harris and Jenner, 2019).

Scorpaena have a sedentary lifestyle and their hunt mode isn't dependent of venom use. Moreover, their mechanisms of expulsion of venom are totally involuntary, together with ecological characteristics previous mentioned, supports the hypothesis that this genus possess the venom for defence purposes (Church and Hodgson, 2002; Ziegman and Alewood, 2015).

The evolution of venom's toxins present in Scorpaeniformes occurs through genes duplication (Chuang and Shiao, 2014; Ellisdon et al., 2015). In this theory, normal physiological proteins genes suffer duplication, usually those involved with key regulatory processes or bioactivity, and the duplicated copy is selectively expressed on venom glands (Fry et al., 2003; Kordiš and Gubenšek, 2000). These physiological proteins could be previously expressed in a variety of types of tissue (e.g. brain, liver, muscle) and show a diversity of activities (Fry et al., 2012). When a gene is recruited to a venom gland, an additional duplication occurs, with a neofunctionalization of the protein, resulting on a multi locus genes family, that codifies proteins with functional activities and variable potency (Casewell et al., 2011; Chang and Duda, 2012; Fry et al., 2003; Kordiš and Gubenšek, 2000; Weinberger et al., 2010;). Segundo Casewell et al., (2012). This protein recruitment from the tissues to a venom gland also occur on a reserved way, with the proteins being able to suffer "reverse recruitment" from the glands to the tissues where they play physiological roles. Furthermore, the proteins can be expressed on venom glands and in other tissues (Casewell et al., 2012)

This theory is also known as via “birth and death”, is followed by an accelerated evolution and positive selection (Kordiš and Gubenšek, 2000). The positive selection seems to be common on several venomous taxa, including cone snails (Chang and Duda, 2012; Duda and Palumbi, 1999). The gene duplication, the positive selection and the protein neofunctionalization, seem to work together to evolve the venom in order to adapt to different targets (Duda and Palumbi, 1999).

2.3.Venom studies

In the middle of the 20th century, it has witnessed an increase of scientific studies with biomolecules, including proteins. In the 40’s and 50’s, studies with venom proteins began to appear, and late proteomic and transcriptomic studies (Fox, 2013).

As mentioned in previous chapters, it’s the marine environment that has a higher number of venomous species (Tse, 2016). However, the majority of the studies performed about venomous animals were performed with terrestrial animals (sp*iders, snakes, scorpions*) (Church and Hodgson, 2002). This disproportion in the number of studies, with the lack of studies with marine venomous animals, is due to various factors. One of them is the fact that terrestrial animals are closer to humans, which facilitates the tissue and venom samples collecting (Church and Hodgson, 2002). Although, proximity also makes possible a bigger number of unpleasant meetings, where envenomation can occur (Church and Hodgson, 2002). Terrestrial animals represent an imminent threat to public health, and consequently it will be necessary to know their venoms and prospect the treatments that can be applied to victims. Additionally, the most studied marine animals are the jellyfish, sea anemones and cone snails (Smith and Wheeler, 2006), It can be verified a scarce number of studies with venomous fishes, which represent the majority of venomous vertebrates (Smith and Wheeler, 2006; Smith et al., 2016), although it is estimated that many envenomation cases occur.

Venoms can respond to a great diversity of targets and contain toxins with high specificity to cellular receptors (Valentin and Lambeau, 2000), membranes (Hains et al., 1999) and ionic canals (Kordis and Gubensek, 2000). Those characteristics make venoms useful in the study of physiological functions to learn more about the location of ionic canals and cellular receptors (Baron et al., 2013; Dutertre and Lewis, 2010; Terlau and Olivera, 2004), to develop vaccines (Zenouaki et al., 1997) and antitoxins (Devaux et al., 1997; Guatimosim *et al*., 2000), terapeutical agents and drugs (Bowersox and Luther, 1998; Braud

et al., 2000; Harvey, 2000; Harvey et al., 1998; Menez, 1998; Rogers, 1996; Wang et al., 2000; Zieler *et al*., 2001), for allergic treatments (Hunt et al., 1978) for investigating blood coagulation mechanisms (Hoffman et al., 1992), for creating insecticide's formulas (Froy et al., 2000; Zlotkin et al., 2000), and for carrying out evolutive studies (Possani *et al*., 2000). In particular, venom can be useful to study the inter-relations between natural selection and genetic and molecular process responsible for creating diversity.

Nowadays, according to PubMed's data base, more than 1000 articles on venomous organisms are published annually (Utkin, 2015), centred mainly on biochemical, genetic, evolution and pharmacology areas (Clark, 1996; Fernandez et al., 2004; Fry et al., 2005; Smith and Wheeler, 2006; Smith et al., 2016; Triplitt et al., 2006; Ziegman and Alewood, 2015).

2.4. Studies on marine venomous species

The ocean and the marine species are considered important resources for economic growth. Thereby, governmental organisms have been supporting biotechnology studies related with marine animal's venoms, principally on the health area, that could lead to the emergence of new drugs (Smith and Wheeler, 2006), useful in the treatment of "modern diseases". This is proved with the growing number of toxin sequences identified for marine species, which are around 4455 (Xie et al., 2017). About 87% of these sequences belong to the species from the Conus genus (Xie et al., 2017), one of the most studied groups and whose venom is used to create medicines. For example, the medicine ziconotide (Prialt®) ($C_{102}H_{172}N_{36}O_{32}S$), used on severe chronic pains, is developed from one ω-conotoxin M-VII-A, from *Conus magus* venom (Mtewa et al., 2019). Compounds from sea anemones venom are already synthesized, namely Stichodactylatoxin shK (dalazatide, shK-186), from *Stichodactyla helianthus*, and the soricidin, from *Blarina brevicauda* (Mtewa et al., 2019).

According to Ziegman and Alewood (2015) diverse toxins were already isolated on venomous fishes: Dracotoxin (Trachinus draco; Chhatwal and Dreyer (1992); Trachinine (Trachinus vipera; Perriere et al., (1988); Nocitoxin (Notesthes robusta; Hahn and O'Connor (2000); Toxin-PC (Plotosus canius; Auddy et al., (1995); Verrucotoxin (VTx) (Synanceia verrucosa; Garnier et al. (1995); Stonustoxin (SNTX) (Synanceia horrida; Colasante et al., 1996); Trachunilysin (TLY) (Synanceia trachynis; Poh et al. (1991); Neoverrucotoxin (neoVTX) (Synanceia verrucosa (Ueda et al., 2006); Cardioleputin (Notesthes robusta; Abe et

al., 1996); Karatoxin (Hypodytes rubripinis; Nagasaka et al., 2009); SA-HT (Scatophagus argus; Karmakar et al., 2004), TmC4-47.2 (Thalassophryne maculosa; Sosa-Rosales et al., 2005), Nattectin (Thalassophryne nattereri; Lopes-Ferreira et al., 2014); Wap65 (Cathrops spixii; Ramos et al., 2012).

Currently, the specie for which exists some data within the *Scorpaena* genus is the *Scorpaena plumieri* from SW Atlantic. The isolated toxins were the Sp-CTx (Andrich et al., 2010), the Plumieribetin (Evangelista et al., 2009) and the SP-CL 1-5 (Andrich et al., 2015). The Sp-CTx (Scorpaena plumieri Cytolytic Toxin) is a glycoprotein with a molecular mass of 121 kDa and dimeric constitution, in which each subunit weighs approximately 65 kDa (Aldrich et al., 2010). In a study with rats, Gomes et al., (2016) proved that Sp-CTx is a cytolytic vasoactive toxin, that provokes disruption on the cardiovascular system with an increase of calcium influx, provoked by the release of endogenous noradrenalin. Andrich et al., (2015) found five amino acid sequences with molecular mass between 16,800 – 17,000 Da (16,8 – 17 kDa) and terminal sequences from the type N in S. plumieri‘s venom. The characteristics found on these sequences were consistent with lectines type C isomorphs or isolectins called SP-CL 1-5 (Scorpaena plumieri C-type Lectin 1, 2, 3, 4 and 5) (Andrich et al., 2015).

Evangelista et al., (2009) discovered a homotetramer protein, the Plumieribetin with a molecular mass of 14 kDa, constituted by large quantities of anti-parallel β-strands, similar to mannose-binding monocot B-lectins found on monocotyledonous plants, that binds to α1β1 integrins through protein-protein binding, independently integrin’s divalent cations. Another curious fact is that this protein was isolated both from the mucus and venom of *S. plumieri* (Evangelista et al., 2009). Integrins are receptor proteins from the cell membrane, composed by two subunits, α e β, and are abundant in hepatic and vascular cells of smooth tissue. They are essential to ensure tissue integrity and are involved in physiological and pathological situations, which makes them one common target to various toxins. One type of integrins is the α1β1 integrin, that possess receptors for collagen molecules (Evangelista et al., 2009). There was the idea that venom toxins mimic collagen structures, so they could connect with those receptors, but these authors showed that was unverified in the case of plumieribetin. On *S. plumieri*‘s envenomation, it is recorded the formation of vesicles and cell detachment from the endothelium of the subendothelial membrane, with subsequent increase of blood vessels(‘s) permeability and erythema formation, and haemorrhages (Theakston and Kamiguti, 2002), symptoms consistent with complications at the level of cell membrane

integrins (Carrijo et al., 2005; Boletini-Santos et al., 2008; Haddad et al., 2003; Loyo et al., 2008).

In the venom from venomous fishes, it is also present another type of compounds apart from toxins, such as salts, organic molecules (e.g. amino acids), polyamines, neurotransmitter and enzymes (e.g. oxidases and hydrolases) (Foz and Serrano, 2008; Fry, 2005; *Inceoglu et al.*, 2003; Kalia et al., 2015; Sollod et al., 2005). Some molecules are histamines, catecholamines, cholinesterase, 5-hydroxytryptamine, 5-nucleotidase, fosfodiesterase, acetylcholine and cholinomimetic (Church and Hodgson, 2000; Garnier et al., 1996; Haavaldsen and Fonnum, 1963; Khoo et al., 1992; Rodriguez, 1972; Russel and Van Harreveld, 1954; Ziegman and Alewood, 2015).

The fish‘venom has lesser toxins and higher lability to temperature and pH compared with terrestrial venomous animals and differ in the type of active compounds and chemical and pharmacological proprieties (Church and Hodgson, 2002; Russel, 1996; Schaeffer et al., 1971). For example, phospholipase A2 PLA2 contributes to cytolytic activity in several venoms but is absent in fishes' venoms (Grotendorst and Hessinger, 1999; Gull and Smith, 1974; Valdez-Cruz et al., 2004). Generally, the effect provoked by venoms are caused by synergetic actions (White and Meier, 2008).

2.5. Analyses methods

The first step on venom studies is their sampling. There are various ways to extract venom from venomous fishes, being some methods more invasive than others. Some researchers maintain an alive stock and keep extracting the venom whenever they need (Ziegman and Alewood, 2015). In these cases, the venom spine is completely cut, as the venom gland, followed by homogenization and centrifugation (Nair et al., 1985), resulting in a "clean" venom extraction. Up to now, the is thought that fish spines are modified scales, and if the spine breaks during the envenomation, the fish can regenerate the spine associated with the venom gland (Church and Hodgson (2000). However, this method has the disadvantage of causing degradation of some labile compounds from the venom, and a long period for the regeneration of the fish's spines. Other methods to extract the venom directly from the venom gland, involve the use of syringes (Church and Hodgson, 2000; Poh et al., 1991). Schaeffer et al., (1971) used micropipettes connected to a vacuum source to collect venom from the

groove, what he called “aspiration method”, and decanted the venom from the spines, called “bath method”. Both methods needed a lyophilization in the end.

Or putting pressure on the venom spine against a membrane inside an Eppendorf tube, that will activate the release of the venom to the membrane, where it will be retained, with the disadvantage of a possible contamination with skin mucus (Ziegman and Alewood, 2015). In these cases, many researchers opt to perform a posterior precipitation of the venom, to remove the maximum quantity of skin mucus (Baumann et al., 2014).

After the extraction and some treatments, the samples can be analysed with proteomic and/or transcriptomic methods, depending if we want to obtain amino acid sequences from certain protein or mRNA sequences that will later code a protein (Xie et al., 2017). Some of the most applied proteomic methods on venom from marine venomous animals are the electrophoresis, the Edman’s degradation and mass spectrophotometry (MS) (Carrijo et al., 2005; Dutertre et al., 2014). 2-D eletrophoresis is the most used proteomic methods the most used to quantify proteins from venom samples. It is composed by two phases, where in the first phase the proteins are separated according to their isoelectric point (pI), with isoelectric focusing (IEF), and in the second phase, proteins are separated according to their molecular mass weight, with an SDS-PAGE gel (*sodium dodecyl sulfate-polyacrylamide gel electrophoresis*) (Santos *et al.*, 2018). The final result is a gel with bands (spots) corresponding to different proteins. In this technique, it’s possible to identify post-translation modifications that occur in proteins, such as acetylation, glycosylation and hydrolyse, because they modify the molecular weight and pI of the protein, and carbonylation caused by oxidations (Santos et al., 2018). The bands or spots can be recollected from the gel and be subsequently analysed using mass spectrometry techniques. Edman’s degradation is a traditional technique that allows us to obtain amino acid sequences from certain peptide (Xie et al., 2017). This method was substituted by mass spectrometry, but it is used more frequently to complement the information provided by MS (Xie et al., 2017).

Traditionally, to analyse biological activity from the venom compounds, the venom was submitted to a *bioassay-guided fractionation*, in which every compound mixed in the venom was extracted one by one, based on physic-chemical proprieties between them (Malviya and Malviya, 2017; Xie et al., 2017), followed by isolation and characterization of the bioactive substances (Xie et al., 2017). As this is a slow process and needs a large quantity of venom sample (crude venom) (Bringans et al., 2008), the researchers started using the *Liquid Chromatography* (LC) technique, combined with mass spectrometry (MS), that allows the gathering of great amounts of data, in a short period of time and with small amounts of

sample (Biass et al., 2009; Favreau *et al*., 2006; Tan et al., 2003). This method has the advantage (of allowing sequencing and characterization of venom peptides, by comparing their amino acid sequences with known peptides on public data base. The combination LC-MS and the use of *Next generation sequencing* (NGS), i.e., new technologies for DNA sequencing (Behjati and Tarpey, 2013), open the possibility for venom studies to become more efficient and sensitive (Fry et al., 2009; Tan et al., 2003).

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Annexes B

This section presents a more detailed approach to the spiny rays regeneration tests, a actualization of the FNAM's dichotomic key with *Scorpaena canariensis* described by Motomura et al., (2005), figures with the 9 *Scorpaena* species description, and tables with detailed information about the samples used in the phylogenetic and venoms analysis.

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List of figures

1. Spiny Rays Regeneration

According to Church and Hodgson (2000), the spine rays that possess venom can be cut during venom extraction procedure, because a posterior recovery of the spines is assured, i.e., the regeneration of the spines. In order to confirm this affirmation, we kept *Scorpaena notata* specimens in captivity at Vasco da Gama Aquarium, in Lisbon, which were marked, and their dorsal spines were cut. Photos with three years apart were taken and the dorsal fin rays did not regenerate. This result is important for fish venom studies, as the venom spine removal is the most usual technique to collect venom in fish (REFS). Removal of the venom spines results in the limitation of the defence mechanism of these fish and is therefore to avoid when the fish are released in the site of capture. On the other hand, if the fish are to be kept in captivity, successive venom milking is no longer possible if the spines were removed. Our method of venom collection with a tube and a sponge was developed to avoid these constraints allowing for an effective extraction of fish venom.

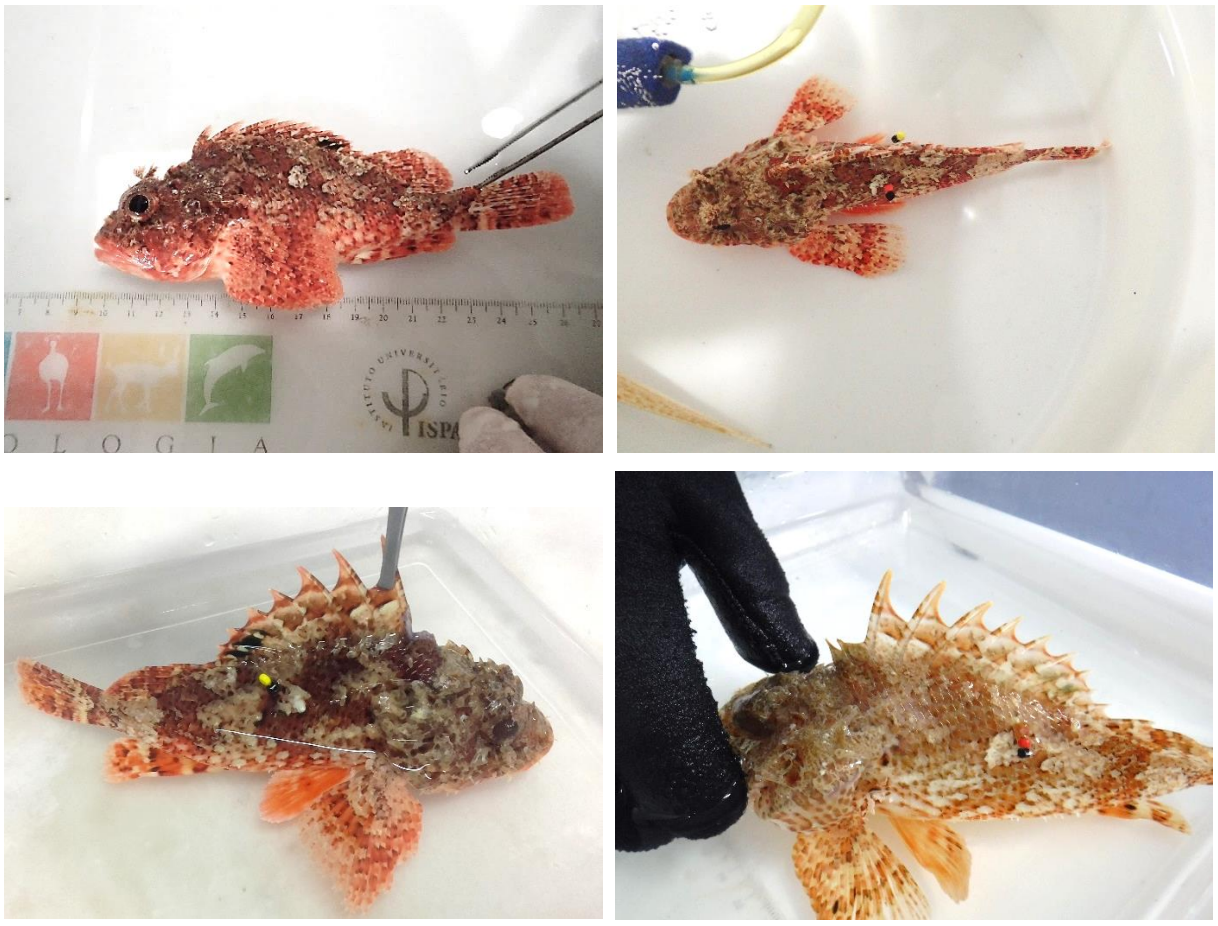


Figure 1. *Scorpaena notata* specimen maintained during three years at Vasco da Gama Aquarium. (A) Specimen before being marked chirurgical; (B) Specimen marked after being marked the colour pattern Black-Yellow/Black-Red; (C) Specimen in 2016 after the two first dorsal venomous rays being cut; (D) Specimen in 2019 showing that the rays did not recovery after three years at the same conditions.

2. *Scorpaena* dichotomous key actualization

The only dichotomous key for *Scorpaena* from the Northeast Atlantic and the Mediterranean Sea was made by Hureau & Litvinenko (1986). Many years later, Motomura et al., (2005) described a new specie, the *Scorpaena canariensis*, which still has not been included in the dichotomous key. Here I present a suggestion for the key actualization.

KEY TO SPECIES (adapted from FNAM and Motomura et al., (2005))

1a Two Mandibular white skin flaps present ……..…..…...……..… 2

1b Mandibular white skin flaps absent …….……...… 3

2a Occipital pit absent; Posterior lacrimal spine turned backward …… ***S. maderensis***

2b Occipital pit presente, with a slighty convex; Posterior lacrimal spine directed ventroanteriorly……..…….…… ***S. canariensis***

3a Vertical scale rows more than 60; scales on body emarginate ……..…..…. ***S. porcus***

3b Vertical scale rows fewer than 50 ……..…… 4

4a Scales cycloid; pectoral fin base and chest scaled ……..…..…. ***S. laevis***

4b Scales ctenoid; pectoral fin base and chest naked …….……..... 5

5a Anterior mandibular pores united into a single opening (Fig. 3) …….……..… 6

5b Anterior mandibular pores separate (Fig. 4) …….…….. 7

6a Maxilla with a longitudinal crest (Fig. 5) …….…… ***S. loppei***

6b Maxilla lacking a crest ……. ***S. azorica***

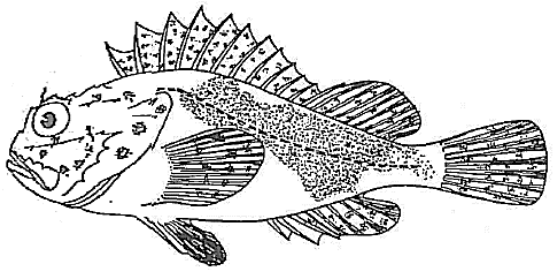
7a Cutaneous tabs present on underside of head ……..…….. ***S. scrofa***

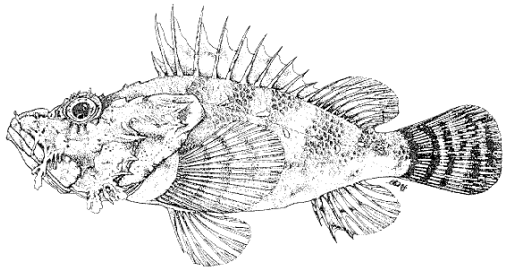
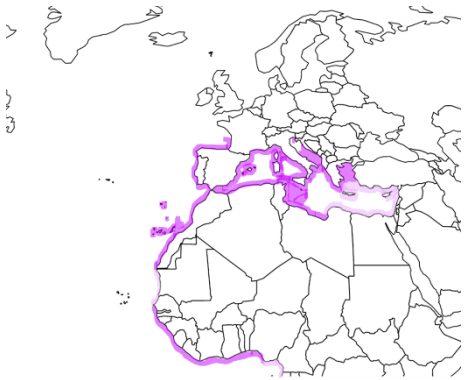
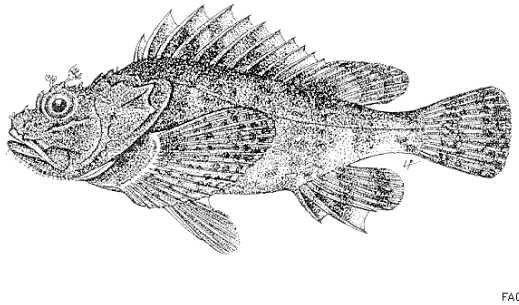
7b Lower jaw without cutaneous tabs …… 8

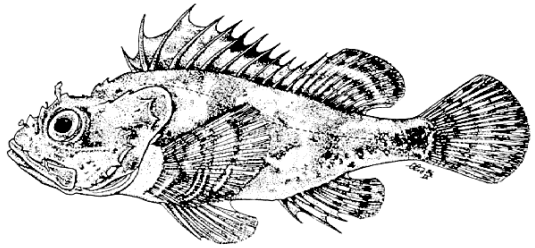
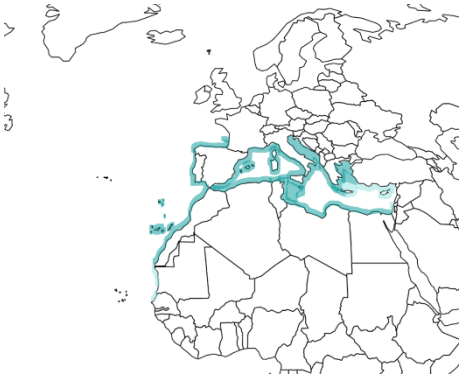
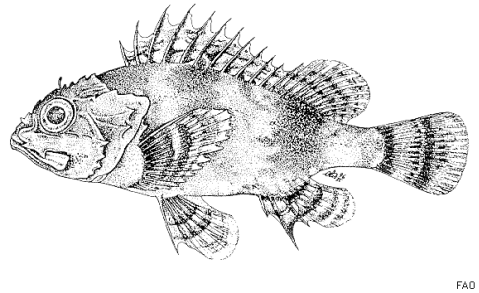
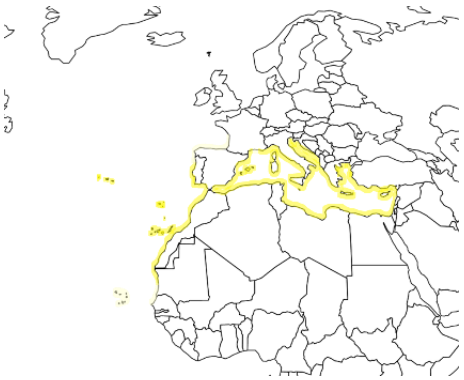
8a Spinous dorsal fin without a black spot; pectoral finrays usually 19, sometimes 18 or 20 …….…….……. ***S. elongata***

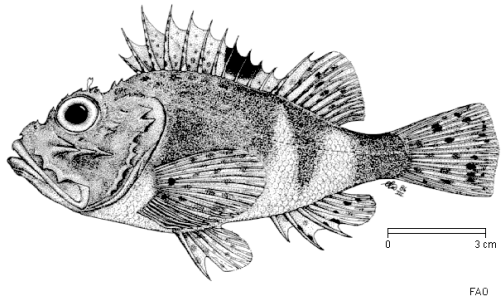
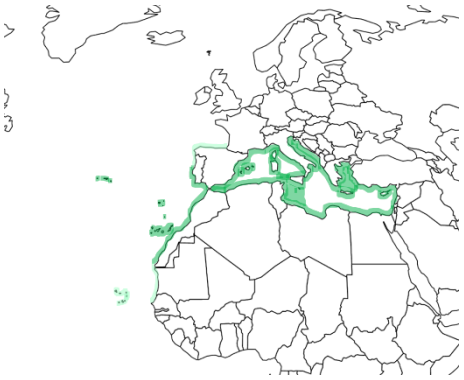
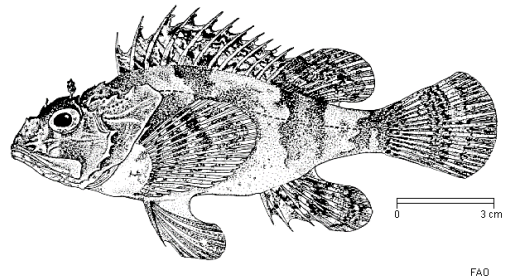
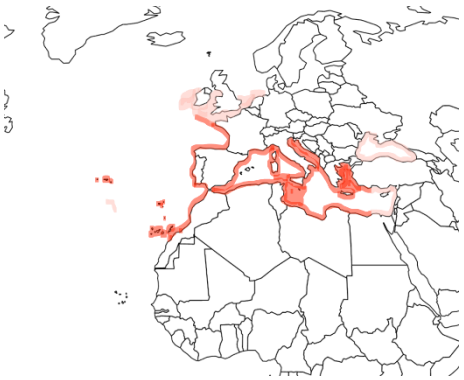
8b Spinous dorsal fin with a black spot; pectoral finrays usually 17-18, rarely 19 ……..……. ***S. notata***

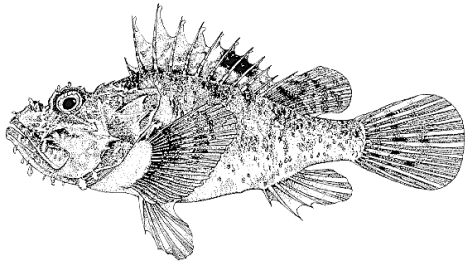
Table 1. Description and distribution of the nine *Scorpaena's* species described for the Northeast Atlantic Ocean and Mediterranean Sea, based on Motomura et al., (2005), Froese and Pauly (2019) and Hureau and Litvinenko (1986).

Specie	Medium Size (max size)	Distinctive characteristic	Distribution	Profundity	
Scorpaena azorica Eschmeyer, 1969  Illustration adapted from Hureau and Litvinenko (1986)	9,8 cm	A single large pore at the symphysis of the lower jaw; Supra-ocular tentacle short, less than half the orbit diameter; no skin flaps on lower jaw. Preorbital bone with 4 spines over maxilla.	 Terceira Island, Azores (Portugal)	No data	Benthic; hard bottoms
Scorpaena canariensis (Sauvage, 1878)  Illustration adapted from Motomura (2005)	14 cm	Two small and white skin flap below the mandibula; Posterior lacrimal spine directed ventro-anteriorly.	 Madeira and Canary Islands.	to 120 m	No data

Scorpaena elongata Cadenat, 1943 Slender rockfish  Illustration adapted from Schneider (1990)	25 cm (50 cm)	Supra-ocular tentacle small; Large flap associated with posterior pre-orbital spine. Pores at symphysis of lower jaw small and separate. Occipital pit shallow.	 From Gulf of Cadiz to Namibia and Mediterranean	Demersal; Range: 75 - 800 m; Usually: 100 to 600 m	Rocky areas; Offshore continental species on soft bottoms
Scorpaena laevis Troschel, 1866 Senegalese rockfish  Illustration adapted from Schneider (1990)	20 cm (35 cm)	Supra-ocular tentacle variable, sometimes longer than orbital diameter; Tentacle well developed under the head and the lower jaw. Pores at symphysis of lower jaw separate. Occipital pit deep.	 Azores island, Cape Verde, Mauritania to Pointe de Noire, Congo, except Mediterranean Sea	Demersal; to 100 m	Benthic; Shallow Rocky areas

Scorpaena loppei Cadenat, 1943 Cadenat's rockfish  Illustration adapted from Bauchot (1987)	10 cm (15 cm)	Supra-ocular tentacle shorter than orbital diameter. Tentacles on head and body moderate or poorly developed. Pores at symphysis of lower jaw united. Maxilla with a longitudinal crest.	 Bay of Biscay to Morocco and Mauritania, Atlantic coast of Spain; Mediterranean, including Cyprus	Demersal; Range 50 – 200 m	Benthic; deep-self (Ordines et al., 2012) muddy sand or gravel
Scorpaena maderensis Valenciennes, 1833 Madeira rockfish  Illustration adapted from Schneider (1990)	10 cm (15 cm)	Two small and white skin flap below the mandibula; Posterior lacrimal spine turned backward.	 Azores, Madeira; Portugal and Morocco to Canary Islands, Cape Verde and Senegal. Mediterranean, except Black Sea. FNAM só dá ideia de alguns lugares	20 to 40 m	Benthic inshore specie; rocky areas; depressions and ledges or boulders, with algae growth

Scorpaena notata Rafinesque, 1810 Small red scorpionfish  Illustration adapted from Bauchot (1987)	15 cm (20 cm)	Preobrital bone with 3 spines over maxilla; Upper post-temporal spine present; Supra-ocular tentacle short; Variable small skin flaps on head; Pores at symphysis of lower jaw small and separate but close together;	 Bay of Biscay to Senegal, Madeira, Azores and the Canary Islands; Mediterranean, rare in northern Adriatic and present in Black Sea*	Demersal; 10 to 700 m	Rocky littoral
Scorpaena porcus Linnaeus, 1758 Black scorpionfish  Illustration adapted from Bauchot (1987)	15 cm (25 cm)	Supra-ocular tentacle, normally equal to orbital diameter; No flaps on the lower jaw; Pores at symphysis of lower jaw small and separate.	 British Isles to Morocco, including the Azores, and the Canary Islands; Mediterranean and Black Sea. (rare in Senegal FNAM)	800 m	Benthic littoral specie; among rocks and algaes

Scorpaena scrofa Linnaeus, 1758 Red scorpionfish  Illustration adapted from Schneider (1990)	30 cm (50 cm)	Pores at symphysis of lower jaw small and separate; Supra-ocular tentacle usually small or absent; Numerous tentacles on lower jaw; Large skin flap associated with posterior preorbital spine and flaps on fourth and fifth preopercle spines.	 British Isles (rare) to Senegal including Madeira, the Canary Islands, and Cape Verde. Mediterranean Sea, except Black Sea.	Demersal; 20 to 500 m	Benthic; Rocky, sandy and muddy bottoms

**Scorpaena notata afimbria* on Black Sea

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Table 2. Detailed information about the specimens include in phylogenetic analysis. The grey shaded are the samples discarded from the analysis.

Species	Collection site	Geographic	Label	N	Capture date	Origin
Scorpaena azorica	Funchal, Madeira Island, Portugal	32° 64' N, 16° 91' W	MMF047815	1	2017	Funchal Municipal Museum
Scorpaena canariensis	Tenerife, Canary Islands, Spain	28° 35' N, 16° 36' W	SCCACAN01	3	2018	Collected for this work
			SCCACAN03		2018	Collected for this work
			SCCACAN05		2018	Collected for this work
Scorpaena elongata	Armação de Pêra, Portugal	37° 10' N, 08° 35' W	SCELSP01	1	2009	ISPA's tissue collection
	Ilha do Sal, Cape Verde	16° 43' N, 23° 02' W	MMF043115	1	unknown	Funchal Municipal Museum
Scorpaena laevis	Ilha de São Nicolau, Cape Verde	16° 33' N, 24° 16' W	MMF043116	1	unknown	Funchal Municipal Museum
	Ilha do Sal, Cape Verde	16° 43' N, 23° 02' W	1	3	2019	Collected for this work by Peter Wirtz
			4		2019	Collected for this work by Peter Wirtz
			13		2019	Collected for this work by Peter Wirtz
Scorpaena maderensis	Arrábida, Portugal	38˚29' N, 08˚ 59' W	SCMACPS01	1	2017	ISPA's tissue collection
	Peniche, Portugal	39˚35' N, 09˚ 37' W	SCNOCPN03	1	2016	ISPA's tissue collection
	Funchal, Madeira Island, Portugal	32˚63' N, 16˚ 93' W	SCMAMAD01	3	2018	Collect by Patricia Carvalho
			SCMAMAD02		2018	Collect by Patricia Carvalho
			SCMAMAD06		2018	Collect by Patricia Carvalho
	Caniçal, Madeira Island, Portugal	32˚74' N, 16˚ 70' W	SCMAMAD07	11	2018	ISPA's tissue collection

		SCMAMAD08		2018	ISPA’s tissue collection
		SCMAMAD09		2018	ISPA’s tissue collection
		SCMAMAD10		2018	ISPA’s tissue collection
		SCMAMAD11		2018	ISPA’s tissue collection
		SCMAMAD12		2018	ISPA’s tissue collection
		SCMAMAD13		2018	ISPA’s tissue collection
		SCMAMAD14		2018	ISPA’s tissue collection
		SCMAMAD15		2018	ISPA’s tissue collection
		SCMAMAD16		2018	ISPA’s tissue collection
		SCMAMAD17		2018	ISPA’s tissue collection
Selvagens, Madeira Island, Portugal	30° 13' N, 15° 86' W	SCMASEL01	3	2009	ISPA’s tissue collection
		SCMASEL02		2009	ISPA’s tissue collection
		SCMASEL03		2009	ISPA’s tissue collection
Faial, Azores Island, Portugal	36°56’N, 25°08’W	SCMAAZO24	2	2016	ISPA’s tissue collection
		SCMAAZO25		2016	ISPA’s tissue collection
Faial, Azores Island, Portugal	38° 51' N, 28° 71' W	SCMAAZO01	3	2018	ISPA’s tissue collection
		SCMAAZO03		2018	ISPA’s tissue collection
		SCMAAZO04		2018	ISPA’s tissue collection
Tenerife, Canary Island, Spain	28° 35' N, 16° 36' W	SCMACAN20	3	2018	Collected by Sérgio Moreno
		SCMACAN24		2018	Collected by Sérgio Moreno
		SCMACAN29		2018	Collected by Sérgio Moreno

	Cyprus	34° 67' N, 33° 31' W	J1938	3	unknown	Extraction sample donated by Eva Velasco
			J2029		Unknown	Extraction sample donated by Eva Velasco
			J2047		unkown	Extraction sample donated by Eva Velasco
Scorpaena notata	Armação de Pêra, Portugal	37° 10' N, 08° 35' W	SCNOSP36	2	2009	ISPA's tissue collection
			SCNOSP37		2009	ISPA's tissue collection
	Arrábida, Portugal	38˚44' N, 09˚ 07' W	SCNOCPS02	24	2015	ISPA's tissue collection
			SCNOCPS03		2015	ISPA's tissue collection
			SCNOCPS04		2015	ISPA's tissue collection
			SCNOCPS05		2015	ISPA's tissue collection
			SCNOCPS06		2015	ISPA's tissue collection
			SCNOCPS07		2015	ISPA's tissue collection
			SCNOCPS09		2015	ISPA's tissue collection
			SCNOCPS10		2015	ISPA's tissue collection
			SCNOCPS11		2015	ISPA's tissue collection
			SCNOCPS12		2015	ISPA's tissue collection
			SCNOCPS13		2015	ISPA's tissue collection
			SCNOCPS14		2015	ISPA's tissue collection
			SCNOCPS15		2015	ISPA's tissue collection
			SCNOCPS16		2015	ISPA's tissue collection
			SCNOCPS17		2015	ISPA's tissue collection

		SCNOCPS18		2015	ISPA’s tissue collection
		SCNOCPS19		2015	ISPA’s tissue collection
		SCNOCPS20		2015	ISPA’s tissue collection
		SCNOCPS21		2015	ISPA’s tissue collection
		SCNOCPS22		2015	ISPA’s tissue collection
		SCNOCPS23		2015	ISPA’s tissue collection
		SCNOCPS24		2015	ISPA’s tissue collection
		SCNOCPS25		2015	ISPA’s tissue collection
Fonte da Telha, Portugal	38˚56’ N, 09˚ 19’ W	SCNOCPS27	7	unknown	ISPA’s tissue collection
		SCNOCPS29		2016	ISPA’s tissue collection
		SCNOCPS30		2016	ISPA’s tissue collection
		SCNOCPS31		2016	ISPA’s tissue collection
		SCNOCPS32		2016	ISPA’s tissue collection
		SCNOCPS33		2016	ISPA’s tissue collection
		SCNOCPS34		2016	ISPA’s tissue collection
Peniche, Portugal		SCNOCPN02	1	2016	ISPA’s tissue collection
Sesimbra, Portugal	38˚44’ N, 09˚ 05’ W	SCSCCPS01	2	2015	ISPA’s tissue collection
		SCNOCPS26		2015	ISPA’s tissue collection
Cádiz, Spain		SCNOCAD1	3	2009	Collected by Eva Velasco
		SCNOCAD2		2009	Collected by Eva Velasco

			SCNOCAD3		2009	Collected by Eva Velasco
Scorpaena porcus	Armação de Pêra, Portugal	37° 10' N, 08° 35' W	SCNOSP35	3	2009	ISPA's tissue collection
			SCPOSP01		2009	ISPA's tissue collection
			SCPOSP02		2009	ISPA's tissue collection
	Arrábida, Portugal	38˚29' N, 08˚ 59' W	SCPOCPS01	1	2015	ISPA's tissue collection
	Cascais, Portugal	38˚69' N, 09˚ 41' W	SCPOCPN10	2	2015	Vasco da Gama Aquarium
			SCPOCPN11		2015	ISPA's tissue collection
	Fonte da Telha, Portugal	38˚56' N, 09˚ 19' W	SCNOFT02	1	2016	ISPA's tissue collection
	Peniche, Portugal	39˚35' N, 09˚ 37' W	SCNOCPN04	11	2016	ISPA's tissue collection
			SCNOCPN05		2016	ISPA's tissue collection
			SCNOCPN06		2016	ISPA's tissue collection
			SCNOCPN07		2016	ISPA's tissue collection
			SCPOCPN01		2016	ISPA's tissue collection
			SCPOCPN02		2016	ISPA's tissue collection
			SCPOCPN04		2016	ISPA's tissue collection
			SCPOCPN05		2016	ISPA's tissue collection
			SCPOCPN06		2016	ISPA's tissue collection
			SCPOCPN07		2016	ISPA's tissue collection
			SCPOCPN08		2016	ISPA's tissue collection
	Sesimbra, Portugal	38˚44' N, 09˚ 05' W	SCNOCPS01	3	2015	ISPA's tissue collection
			SCPOCPS02		2015	ISPA's tissue collection

			SCPOCPS03		2015	ISPA's tissue collection
	Ferrol, Galiza, Spain	43˚48' N, 08˚ 23' W	SCPOGAL1	3	2009	ISPA's tissue collection
			SCPOGAL2		2009	ISPA's tissue collection
			SCPOGAL3		2009	ISPA's tissue collection
	Coruña, Galiza, Spain	43˚36' N, 08˚ 40' W	SCPOGAL9	3	2009	ISPA's tissue collection
			SCPOGAL10		2009	ISPA's tissue collection
			SCPOGAL11		2009	ISPA's tissue collection
	Tenerife, Canary Island, Spain	28° 35' N, 16° 36' W	SCPOCAN01	2	2018	Collected by Sérgio Moreno
			SCPOCAN02		2018	Collected by Sérgio Moreno
Scorpaena scrofa	Peniche, Portugal	39˚35' N, 09˚ 37' W	SCPOCPN03	27	2016	ISPA's tissue collection
			SCPOCPN09		2016	ISPA's tissue collection
			SCSCCPN01		2016	ISPA's tissue collection
			SCSCCPN02		2016	ISPA's tissue collection
			SCSCCPN03		2016	ISPA's tissue collection
			SCSCCPN04		2016	ISPA's tissue collection
			SCSCCPN05		2016	ISPA's tissue collection
			SCSCCPN06		2016	ISPA's tissue collection
			SCSCCPN07		2016	ISPA's tissue collection
			SCSCCPN08		2016	ISPA's tissue collection
			SCSCCPN09		2016	ISPA's tissue collection
			SCSCCPN10		2016	ISPA's tissue collection
			SCSCCPN11		2016	ISPA's tissue collection

		SCSCCPN12		2016	ISPA's tissue collection
		SCSCCPN13		2016	ISPA's tissue collection
		SCSCCPN14		2016	ISPA's tissue collection
		SCSCCPN15		2016	ISPA's tissue collection
		SCSCCPN16		2016	ISPA's tissue collection
		SCSCCPN17		2016	ISPA's tissue collection
		SCSCCPN18		2016	ISPA's tissue collection
		SCSCCPN19		2016	ISPA's tissue collection
		SCSCCPN20		2016	ISPA's tissue collection
		SCSCCPN21		2016	ISPA's tissue collection
		SCSCCPN22		2016	ISPA's tissue collection
		SCSCCPN23		2016	ISPA's tissue collection
		SCSCCPN24		2017	ISPA's tissue collection
		SCSCCPN25		2017	ISPA's tissue collection
Setúbal, Portugal	38˚51' N, 08˚ 89' W	SCSCCPS02	1	2016	ISPA's tissue collection
Ferrol, Galiza, Spain	43˚48' N, 08˚ 23' W	SCSCRGAL01	3	2009	ISPA's tissue collection
		SCSCRGAL02		2009	ISPA's tissue collection
		SCSCRGAL03		2009	ISPA's tissue collection

Table 3. Specimens from 5 *Scorpaena* species involved in venoms analysis, and respective venoms description.

Species	Collection site	Samples	Total Lenght (cm)	Exctration date	Provenance/Storage
Scorpaena canariensis	Canary Island, Spain	SCCACAN01	15,9	04/2018	W/-20°C
		SCCACAN03	14,3	04/2018	W/-20°C
		SCCACAN05	13,2	04/2018	W/-20°C
Scorpaena maderensis	Madeira Island, Portugal	SCMAMAD07	7,4	11/2018	W/-20°C
		SCMAMAD08	13,9	11/2018	W/-20°C
		SCMAMAD09	8,4	11/2018	W/-20°C
		SCMAMAD10	8,2	11/2018	W/-20°C
		SCMAMAD11	8,7	11/2018	W/-20°C
		SCMAMAD12	8,2	11/2018	W/-20°C
		SCMAMAD13	9,0	11/2018	W/-20°C
		SCMAMAD17	8,4	11/2018	W/-20°C
Scorpaena notata	Fonte da Telha, Portugal	SCNOCPS33	n/d	06/2016	W/-20°C
		SCNOCPS34	n/d	06/2016	W/-20°C
	Vasco da Gama Aquarium	SCNOAVG01	15,1	05/2019	C/Fresh
			17,4	05/2019	C/Fresh

			18,3	05/2019	C/Fresh
	Vasco da Gama Aquarium, Portugal	SCNOAVG02	17,2	05/2019	C/Fresh
Scorpaena notata	Peniche, Portugal	SCPOCPN01	31,3	06/2016	W/-20°C
		SCPOCPN02	29,2	06/2016	W/-20°C
		SCPOCPN06	n/d	06/2016	W/-20°C
Scorpaena scrofa	Peniche, Portugal	SCSCCPN11	46,6	05/2016	W/-20°C
		SCSCCPN12	44,7	05/2016	W/-20°C
		SCSCCPN17	50,8	05/2016	W/-20°C
		SCSCCPN24	n/d	03/2017	W/-20°C
		SCSCCPN25	n/d	03/2017	W/-20°C

Table 4. DNA sequences. 16S amplifications. *Extractions obtained by Cristina Lima for a previous work (Lima et al., 2018).

Specie	Collection site	Geographic	Capture date	Label	Extraction date	16S Amplification date	Origin
Scorpaena azorica	Madeira Island, Porutugal	32° 64' N, 16° 91' W	2017	MMF047815	29/May	26/June	Funchal Municipal Museum
Scorpaena canariensis	Tenerife, Spain	28° 35' N, 16° 36' W	2018	SCCACAN01	7/2018*	12/fev	Collected for this work
	Tenerife, Spain	28° 35' N, 16° 36' W	2018	SCCACAN03	7/2018*	12/fev	Collected for this work
	Tenerife, Spain	28° 35' N, 16° 36' W	2018	SCCACAN05	7/2018*	8/fev	Collected for this work
Scorpaena elongata	Armação de Pêra, Portugal	37° 10' N, 08° 35' W	2009	SCELSP01	24/Jan	7/fev	ISPA's tissue collection
	Ilha do Sal, Cape Verde	16° 43' N, 23° 02' W	unknown	MMF043115	29/May	26/June	Funchal Municipal Museum
Scorpaena laevis	Ilha São Nicolau, Cape Verde	16° 33' N, 24° 16' W	unknown	MMF043116	29/May	26/June	Funchal Municipal Museum
	Ilha do Sal, Cape Verde	16° 43' N, 23° 02' W	07/2019	1	23/July	-	Collected by Peter Wirtz
	Ilha do Sal, Cape Verde	16° 43' N, 23° 02' W	07/2019	4	23/July	-	Collected by Peter Wirtz
	Ilha do Sal, Cape Verde	16° 43' N, 23° 02' W	07/2019	13	23/July	-	Collected by Peter Wirtz
Scorpaena maderensis	Arrábida, Portugal	38˚29' N, 08˚ 59' W	2017	SCMACPS01	24/january	8/feveruary	ISPA's tissue collection
	Peniche, Portugal	39˚35' N, 09˚ 37' W	2016	SCNOCPN03	15/fev	12/fev	ISPA's tissue collection
	Madeira Island, Portugal	32˚63' N, 16˚ 93' W	2018	SCMAMAD01	*	12/fev	Collect by Patricia Carvalho
	Madeira Island, Portugal	32˚63' N, 16˚ 93' W	2018	SCMAMAD02	*	8/fev	Collect by Patricia Carvalho
	Madeira Island, Portugal	32˚63' N, 16˚ 93' W	2018	SCMAMAD06	*	12/fev	Collect by Patricia Carvalho
	Madeira Island, Portugal	32˚74' N, 16˚ 70' W	2018	SCMAMAD07	*	7/march	ISPA's tissue collection
	Madeira Island, Portugal	32˚74' N, 16˚ 70' W	2018	SCMAMAD08	*	7/march	ISPA's tissue collection
	Madeira Island, Portugal	32˚74' N, 16˚ 70' W	2018	SCMAMAD09	*	7/march	ISPA's tissue collection
	Madeira Island, Portugal	32˚74' N, 16˚ 70' W	2018	SCMAMAD10	*	7/march	ISPA's tissue collection
	Madeira Island, Portugal	32˚74' N, 16˚ 70' W	2018	SCMAMAD11	*	7/march	ISPA's tissue collection
	Madeira Island, Portugal	32˚74' N, 16˚ 70' W	2018	SCMAMAD12	*	7/march	ISPA's tissue collection
	Madeira Island, Portugal	32˚74' N, 16˚ 70' W	2018	SCMAMAD13	*	7/march	ISPA's tissue collection
	Madeira Island, Portugal	32˚74' N, 16˚ 70' W	2018	SCMAMAD14	*	7/march	ISPA's tissue collection
	Madeira Island, Portugal	32˚74' N, 16˚ 70' W	2018	SCMAMAD15	*	7/march	ISPA's tissue collection
	Madeira Island, Portugal	32˚74' N, 16˚ 70' W	2018	SCMAMAD16	*	7/march	ISPA's tissue collection
	Madeira Island, Portugal	32˚74' N, 16˚ 70' W	2018	SCMAMAD17	*	7/march	ISPA's tissue collection
	Selvagens Island, Portugal	30° 13' N, 15° 86' W	2009	SCMASEL01	24/Janeiiro	7/fev	ISPA's tissue collection
	Selvagens Island, Portugal	30° 13' N, 15° 86' W	2009	SCMASEL02	24/Jan	12/fev	ISPA's tissue collection

	Selvagens Island, Portugal	30° 13' N, 15° 86' W	2009	SCMASEL03	24/Jan	12/fev	ISPA's tissue collection
	Azores Island, Portugal	36°56'N, 25°08'W	2016	SCMAAZO24	15/fev	21/fev	ISPA's tissue collection
	Azores Island, Portugal	38° 51' N, 28° 71' W	2016	SCMAAZO25	24/Jan	8/fev	ISPA's tissue collection
	Azores Island, Portugal	38° 51' N, 28° 71' W	2018	SCMAAZO01	*	12/fev	ISPA's tissue collection
	Azores Island, Portugal	38° 51' N, 28° 71' W	2018	SCMAAZO03	*	8/fev	ISPA's tissue collection
	Azores Island, Portugal	38° 51' N, 28° 71' W	2018	SCMAAZO04	*	7/fev	ISPA's tissue collection
	Tenerife, Spain	28° 35' N, 16° 36' W	2018	SCMACAN20	*	7/fev	Collected by Sérgio Moreno
	Tenerife, Spain	28° 35' N, 16° 36' W	2018	SCMACAN24	*	8/fev	Collected by Sérgio Moreno
	Tenerife, Spain	28° 35' N, 16° 36' W	2018	SCMACAN29	*	12/fev	Collected by Sérgio Moreno
	Cyprus	34° 67' N, 33° 31' W	unknown	J1938	Provided	7/fev	Extraction sample donated by Eva Velasco
	Cyprus	34° 67' N, 33° 31' W	unknown	J2029	Provided	8/fev	Extraction sample donated by Eva Velasco
	Cyprus	34° 67' N, 33° 31' W	unkown	J2047	Provided	12/fev	Extraction sample donated by Eva Velasco
Scorpaena notata	Armação de Pêra, Portugal	37° 10' N, 08° 35' W	2009	SCNOSP36	24/Jan	12/fev	ISPA's tissue collection
	Armação de Pêra, Portugal	37° 10' N, 08° 35' W	2009	SCNOSP37	24/Jan	12/fev	ISPA's tissue collection
	Arrábida, Portugal	38˚44' N, 09˚ 07' W	2015	SCNOCPS02	24/Jan	7/fev	ISPA's tissue collection
	Arrábida, Portugal	38˚44' N, 09˚ 07' W	2015	SCNOCPS03	24/Jan	12/fev	ISPA's tissue collection
	Arrábida, Portugal	38˚44' N, 09˚ 07' W	2015	SCNOCPS04	24/Jan	12/fev	ISPA's tissue collection
	Arrábida, Portugal	38˚44' N, 09˚ 07' W	2015	SCNOCPS05	24/Jan	12/fev	ISPA's tissue collection
	Arrábida, Portugal	38˚44' N, 09˚ 07' W	2015	SCNOCPS06	24/Jan	12/fev	ISPA's tissue collection
	Arrábida, Portugal	38˚44' N, 09˚ 07' W	2015	SCNOCPS07	24/Jan	12/fev	ISPA's tissue collection
	Arrábida, Portugal	38˚44' N, 09˚ 07' W	2015	SCNOCPS08	24/Jan	12/fev	ISPA's tissue collection
	Arrábida, Portugal	38˚44' N, 09˚ 07' W	2015	SCNOCPS09	24/Jan	12/fev	ISPA's tissue collection
	Arrábida, Portugal	38˚44' N, 09˚ 07' W	2015	SCNOCPS10	24/Jan	12/fev	ISPA's tissue collection
	Arrábida, Portugal	38˚44' N, 09˚ 07' W	2015	SCNOCPS11	24/Jan	12/fev	ISPA's tissue collection
	Arrábida, Portugal	38˚44' N, 09˚ 07' W	2015	SCNOCPS12	24/Jan	12/fev	ISPA's tissue collection
	Arrábida, Portugal	38˚44' N, 09˚ 07' W	2015	SCNOCPS13	24/Jan	12/fev	ISPA's tissue collection
	Arrábida, Portugal	38˚44' N, 09˚ 07' W	2015	SCNOCPS14	24/Jan	12/fev	ISPA's tissue collection
	Arrábida, Portugal	38˚44' N, 09˚ 07' W	2015	SCNOCPS15	24/Jan	12/fev	ISPA's tissue collection
	Arrábida, Portugal	38˚44' N, 09˚ 07' W	2015	SCNOCPS16	24/Jan	12/fev	ISPA's tissue collection
	Arrábida, Portugal	38˚44' N, 09˚ 07' W	2015	SCNOCPS17	24/Jan	12/fev	ISPA's tissue collection
	Arrábida, Portugal	38˚44' N, 09˚ 07' W	2015	SCNOCPS18	24/Jan	12/fev	ISPA's tissue collection
	Arrábida, Portugal	38˚44' N, 09˚ 07' W	2015	SCNOCPS19	24/Jan	12/fev	ISPA's tissue collection
	Arrábida, Portugal	38˚44' N, 09˚ 07' W	2015	SCNOCPS20	24/Jan	12/fev	ISPA's tissue collection
	Arrábida, Portugal	38˚44' N, 09˚ 07' W	2015	SCNOCPS21	24/Jan	12/fev	ISPA's tissue collection
	Arrábida, Portugal	38˚44' N, 09˚ 07' W	2015	SCNOCPS22	24/Jan	12/fev	ISPA's tissue collection
	Arrábida, Portugal	38˚44' N, 09˚ 07' W	2015	SCNOCPS23	24/Jan	12/fev	ISPA's tissue collection
	Arrábida, Portugal	38˚44' N, 09˚ 07' W	2015	SCNOCPS24	24/Jan	12/fev	ISPA's tissue collection

	Arrábida, Portugal	38˚44’ N, 09˚ 07’ W	2015	SCNOCPS25	24/Jan	12/fev	ISPA’s tissue collection
	Fonte da Telha, Portugal	38˚56’ N, 09˚ 19’ W	unknown	SCNOCPS27	24/Jan	7/fev	ISPA’s tissue collection
	Fonte da Telha, Portugal	38˚56’ N, 09˚ 19’ W	2016	SCNOCPS29	24/Jan	12/fev	ISPA’s tissue collection
	Fonte da Telha, Portugal	38˚56’ N, 09˚ 19’ W	2016	SCNOCPS30	24/Jan	12/fev	ISPA’s tissue collection
	Fonte da Telha, Portugal	38˚56’ N, 09˚ 19’ W	2016	SCNOCPS31	24/Jan	12/fev	ISPA’s tissue collection
	Fonte da Telha, Portugal	38˚56’ N, 09˚ 19’ W	2016	SCNOCPS32	24/Jan	12/fev	ISPA’s tissue collection
	Fonte da Telha, Portugal	38˚56’ N, 09˚ 19’ W	2016	SCNOCPS33	24/Jan	12/fev	ISPA’s tissue collection
	Fonte da Telha, Portugal	38˚56’ N, 09˚ 19’ W	2016	SCNOCPS34	24/Jan	12/fev	ISPA’s tissue collection
	Peniche, Portutugal	39˚35’ N, 09˚ 37’ W	2016	SCNOCPN02	24/Jan	12/fev	ISPA’s tissue collection
	Sesimbra, Portugal	38˚44’ N, 09˚ 05’ W	2015	SCSCCPS01	24/Jan	12/fev	ISPA’s tissue collection
	Sesimbra, Portugal	38˚44’ N, 09˚ 05’ W	2015	SCNOCPS26	24/Jan	7/fev	ISPA’s tissue collection
	Cádiz, Spain		2009	SCNOCAD1	24/Jan	7/fev	Collected by Eva Velasco
	Cádiz, Spain		2009	SCNOCAD2	24/Jan	12/fev	Collected by Eva Velasco
	Cádiz, Spain		2009	SCNOCAD3	24/Jan	12/fev	Collected by Eva Velasco
Scorpaena porcus	Armação de Pêra, Portugal	37° 10' N, 08° 35' W	2009	SCNOSP35	24/Jan	21/fev	ISPA’s tissue collection
	Armação de Pêra, Portugal	37° 10' N, 08° 35' W	2009	SCPOSP01	24/Jan	21/fev	ISPA’s tissue collection
	Armação de Pêra, Portugal	37° 10' N, 08° 35' W	2009	SCPOSP02	24/Jan	21/fev	ISPA’s tissue collection
	Arrábida, Portugal	38˚29’ N, 08˚ 59’ W	2015	SCPOCPS01	24/Jan	12/fev	ISPA’s tissue collection
	Cascais, Portugal	38˚69’ N, 09˚ 41’ W	2015	SCPOCPN10	24/jan	12/fev	Vasco da Gama Aquarium
	Cascais, Portugal	38˚69’ N, 09˚ 41’ W	2015	SCPOCPN11	24/jan	12/fev	ISPA’s tissue collection
	Fonte da Telha, Portugal	38˚56’ N, 09˚ 19’ W	2016	SCNCPS28	24jan	12/fev	ISPA’s tissue collection
	Peniche, Portugal	39˚35’ N, 09˚ 37’ W	2016	SCNOCPN04	24/jan	12/fev	ISPA’s tissue collection
	Peniche, Portugal	39˚35’ N, 09˚ 37’ W	2016	SCNOCPN05	24/jan	12/fev	ISPA’s tissue collection
	Peniche, Portugal	39˚35’ N, 09˚ 37’ W	2016	SCNOCPN06	24/jan	12/fev	ISPA’s tissue collection
	Peniche, Portugal	39˚35’ N, 09˚ 37’ W	2016	SCNOCPN07	24/jan	12/fev	ISPA’s tissue collection
	Peniche, Portugal	39˚35’ N, 09˚ 37’ W	2016	SCPOCPN01	24/jan	12/fev	ISPA’s tissue collection
	Peniche, Portugal	39˚35’ N, 09˚ 37’ W	2016	SCPOCPN02	24/jan	12/fev	ISPA’s tissue collection
	Peniche, Portugal	39˚35’ N, 09˚ 37’ W	2016	SCPOCPN04	24/jan	12/fev	ISPA’s tissue collection
	Peniche, Portugal	39˚35’ N, 09˚ 37’ W	2016	SCPOCPN05	24/jan	12/fev	ISPA’s tissue collection
	Peniche, Portugal	39˚35’ N, 09˚ 37’ W	2016	SCPOCPN06	25/jan	12/fev	ISPA’s tissue collection
	Peniche, Portugal	39˚35’ N, 09˚ 37’ W	2016	SCPOCPN07	25/jan	12/fev	ISPA’s tissue collection
	Peniche, Portugal	39˚35’ N, 09˚ 37’ W	2016	SCPOCPN08	25/jan	12/fev	ISPA’s tissue collection
	Sesimbra, Portugal	38˚44’ N, 09˚ 05’ W	2015	SCNOCPS01	24/jan	12/fev	ISPA’s tissue collection
	Sesimbra, Portugal	38˚44’ N, 09˚ 05’ W	2015	SCPOCPS02	24/jan	12/fev	ISPA’s tissue collection
	Sesimbra, Portugal	38˚44’ N, 09˚ 05’ W	2015	SCPOCPS03	24/jan	12/fev	ISPA’s tissue collection
	Ferrol, Galiza, Spain	43˚48’ N, 08˚ 23’ W	2009	SCPOGAL1	24/jan	7/fev	ISPA’s tissue collection
	Ferrol, Galiza, Spain	43˚48’ N, 08˚ 23’ W	2009	SCPOGAL2	24/Jan	8/fev	ISPA’s tissue collection
	Ferrol, Galiza, Spain	43˚48’ N, 08˚ 23’ W	2009	SCPOGAL3	24/Jan	12/fev	ISPA’s tissue collection
	Coruña, Galiza, Spain	43˚36’ N, 08˚ 40’ W	2009	SCPOGAL9	24/Jan	7/fev	ISPA’s tissue collection
	Coruña, Galiza, Spain	43˚36’ N, 08˚ 40’ W	2009	SCPOGAL10	24/Jan	12/fev	ISPA’s tissue collection

	Coruña, Galiza, Spain	43˚36’ N, 08˚ 40’ W	2009	SCPOGAL11	24/Jan	12/fev	ISPA’s tissue collection
	Tenerife, Spain	28° 35' N, 16° 36' W	2018	SCPOCAN01	24/Jan	8/fev	Collected by Sérgio Moreno
	Tenerife, Spain	28° 35' N, 16° 36' W	2018	SCPOCAN02	24/jan	7/fev	Collected by Sérgio Moreno
Scorpaena scrofa	Peniche, Portugal	39˚35’ N, 09˚ 37’ W	2016	SCPOCPN03	25/jan	7/fev	ISPA’s tissue collection
	Peniche, Portugal	39˚35’ N, 09˚ 37’ W	2016	SCPOCPN09	25/jan	12/fev	ISPA’s tissue collection
	Peniche, Portugal	39˚35’ N, 09˚ 37’ W	2016	SCSCCPN01	25/jan	12/fev	ISPA’s tissue collection
	Peniche, Portugal	39˚35’ N, 09˚ 37’ W	2016	SCSCCPN02	25/jan	12/fev	ISPA’s tissue collection
	Peniche, Portugal	39˚35’ N, 09˚ 37’ W	2016	SCSCCPN03	25/jan	12/fev	ISPA’s tissue collection
	Peniche, Portugal	39˚35’ N, 09˚ 37’ W	2016	SCSCCPN04	25/jan	12/fev	ISPA’s tissue collection
	Peniche, Portugal	39˚35’ N, 09˚ 37’ W	2016	SCSCCPN05	25/jan	12/fev	ISPA’s tissue collection
	Peniche, Portugal	39˚35’ N, 09˚ 37’ W	2016	SCSCCPN06	25/jan	12/fev	ISPA’s tissue collection
	Peniche, Portugal	39˚35’ N, 09˚ 37’ W	2016	SCSCCPN07	25/jan	12/fev	ISPA’s tissue collection
	Peniche, Portugal	39˚35’ N, 09˚ 37’ W	2016	SCSCCPN08	25/jan	12/fev	ISPA’s tissue collection
	Peniche, Portugal	39˚35’ N, 09˚ 37’ W	2016	SCSCCPN09	25/jan	12/fev	ISPA’s tissue collection
	Peniche, Portugal	39˚35’ N, 09˚ 37’ W	2016	SCSCCPN10	25/jan	12/fev	ISPA’s tissue collection
	Peniche, Portugal	39˚35’ N, 09˚ 37’ W	2016	SCSCCPN11	25/jan	12/fev	ISPA’s tissue collection
	Peniche, Portugal	39˚35’ N, 09˚ 37’ W	2016	SCSCCPN12	25/jan	12/fev	ISPA’s tissue collection
	Peniche, Portugal	39˚35’ N, 09˚ 37’ W	2016	SCSCCPN13	25/jan	12/fev	ISPA’s tissue collection
	Peniche, Portugal	39˚35’ N, 09˚ 37’ W	2016	SCSCCPN14	25/jan	12/fev	ISPA’s tissue collection
	Peniche, Portugal	39˚35’ N, 09˚ 37’ W	2016	SCSCCPN15	25/jan	12/fev	ISPA’s tissue collection
	Peniche, Portugal	39˚35’ N, 09˚ 37’ W	2016	SCSCCPN16	25/jan	12/fev	ISPA’s tissue collection
	Peniche, Portugal	39˚35’ N, 09˚ 37’ W	2016	SCSCCPN17	25/jan	12/fev	ISPA’s tissue collection
	Peniche, Portugal	39˚35’ N, 09˚ 37’ W	2016	SCSCCPN18	25/jan	12/fev	ISPA’s tissue collection
	Peniche, Portugal	39˚35’ N, 09˚ 37’ W	2016	SCSCCPN19	25/jan	12/fev	ISPA’s tissue collection
	Peniche, Portugal	39˚35’ N, 09˚ 37’ W	2016	SCSCCPN20	25/jan	12/fev	ISPA’s tissue collection
	Peniche, Portugal	39˚35’ N, 09˚ 37’ W	2016	SCSCCPN21	25/jan	12/fev	ISPA’s tissue collection
	Peniche, Portugal	39˚35’ N, 09˚ 37’ W	2016	SCSCCPN22	25/jan	12/fev	ISPA’s tissue collection
	Peniche, Portugal	39˚35’ N, 09˚ 37’ W	2016	SCSCCPN23	25/jan	12/fev	ISPA’s tissue collection
	Peniche, Portugal	39˚35’ N, 09˚ 37’ W	2017	SCSCCPN24	25/jan	12/fev	ISPA’s tissue collection
	Peniche, Portugal	39˚35’ N, 09˚ 37’ W	2017	SCSCCPN25	25/jan	12/fev	ISPA’s tissue collection
	Setúbal, Portugal	38˚51’ N, 08˚ 89’ W	2016	SCSCCPS02	25/jan	12/fev	ISPA’s tissue collection
	Ferrol, Galiza, Spain	43˚48’ N, 08˚ 23’ W	2009	SCSCRGAL01	25/jan	12/fev	ISPA’s tissue collection
	Ferrol, Galiza, Spain	43˚48’ N, 08˚ 23’ W	2009	SCSCRGAL02	25/jan	12/fev	ISPA’s tissue collection
	Ferrol, Galiza, Spain	43˚48’ N, 08˚ 23’ W	2009	SCSCRGAL03	25/jan	12/fev	ISPA’s tissue collection

Table 5. Specimens and amplification dates of the sequences obtained from COI, Cytb, D-loop and S7 for phylogenetic inference with the 8 *Scorpaena* species. The 16S fragment was previously obtained for all the samples. (*amplifcations attained in previous studies by Cristina Lima).

Species	Collection site	Label	16S	COI	Cytb	D-loop	S7
Scorpaena azorica	Madeira Island, Portugal	MMF047815	26/jun	-	-	-	-
Scorpaena canariensis	Tenerife, Spain	SCCACAN01	12/fev	26/fev	-	Cris*	27/jun
	Tenerife, Spain	SCCACAN03	12/fev	26/fev	-	Cris*	-
	Tenerife, Spain	SCCACAN05		26/fev	-	Cris*	-
Scorpaena elongata	Ilha do Sal, Cape Verde	MMF043115	26/jun	-	-	-	-
Scorpaena laevis	Ilha S. Nicolau, Cape Verde	MMF043116	26/jun	-	-	-	-
Scorpaena maderensis	Madeira Island, Portugal	SCMAMAD01	12/fev	-	-	Cris*	*
	Azores Island, Portugal	SCMAAZO01	12/fev	-	-	Cris*	*
	Tenerife, Spain	SCMACAN20	7/fev	-	-	Cris*	*
Scorpaena notata	Armação de Pêra, Portugal	SCNOSP37	12/fev	26/fev	-	-	-
	Arrábida, Portugal	SCNOCPS02	12/fev	26/fev	-	-	-
	Fonte da Telha, Portugal	SCNOCPS27		26/fev	11/mar	-	-
	Sesimbra, Portugal	SCNOCPS26		26/fev	21/jun	-	27/jun
	Cádiz, Spain	SCNOCAD1		26/fev	-	-	-
Scorpaena porcus	Arrábida, Portugal	SCPOCPS01	12/fev	-	17/jul	09/jul	09/jul
	Peniche, Portugal	SCPOCPN01	12/fev	-	17/jul	-	-
	Tenerife, Spain	SCPOCAN01		-	-	09/jul	09/jul
	Tenerife, Spain	SCPOCAN02		-	17/jul	-	-
Scorpaena scrofa	Peniche, Portugal	SCSCPN21		-	-	-	27/jun