Identification of Ascarophis sp. *in Scorpaena porcus by morphological analysis*

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Bachelor's thesis

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Identification of *Ascarophis* sp. in *Scorpaena porcus* by morphological analysis

Abstract

Purpose The objective of this bachelor thesis is the morphological identification of *Ascarophis* sp. in *Scorpaena porcus* from a local museum sample. The identification of *Ascarophis* sp. occurred by the use of different microscopical techniques.

Methods Throughout three months, 24 specimens of *S. porcus* were used for dissection and analysis. The *S. porcus* specimens were caught in two locations, Punta Maricones and Chafarinas Islands (Northern Africa). The nematode specimens were identified with the use of both optical microscopy techniques and a scanning electron microscope (SEM).

Results The specimens of *Ascarophis* sp. were obtained of *S. porcus* from the stomach wall, stomach lumen, or intestine. The total length of the *Ascarophis* sp. males was measured: 9.73mm long and 0.08mm width. The left spicule was measured, with a length of 319.7µm and the right spicule at 105.5µm.

Conclusion The *Ascarophis* sp. founded in *S. porcus* is *A. filiformis*. By comparing the locations and hosts on different databases such as *WORMS*, and EOL, a second conclusion was made. *A. filiformis* has a new host, *S. porcus ,* and a new location, the Mediterranean Sea.

Keywords: *Scorpaena porcus* | Mediterranean Sea | Parasitic nematode | *Ascarophis filiformis* | Morphological identification

Preface

During my internship and working on my bachelor thesis I acquired knowledge about fish parasites and learnt how fish parasites are morphologically different from each other and how to recognize these parasites.

I would like to say thanks to some people. First of all, I would like to thank the University of Valencia Science Park for allowing me to do my internship and work on my bachelor thesis. Secondly, I would like to say thanks to my mentors Professor Francisco E. Montero, Professor Ana Pérez del-Olmo, and Ph.D. Marta Valmaseda Angulo for helping and reading my thesis, and for the guidance during practical experiments in the lab. Thank you to my mentor sir Jan Van Doorsselaere (Vives Roeselare, Belgium) for reading through my bachelor thesis and the guidance during my internship.

I would like to say thanks to all of the co-workers at the lab, for helping me, and answering my questions when my mentors were not around.

Finally, I want to say thank you to my family, specifically my parents. They allowed me to go to Erasmus, they gave me the chance to study Biotechnology and I appreciate everything they gave done for me over the three years.

Decaluwe Matisse
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List of abbreviations

ICBiBE	Cavanilles Institute of Biodiversity and Evolutionary Biology
O_2	Oxygen
CO_2	Carbon dioxide
SEM	Scanning electron microscope
SE	Secondary electron detector
BSE	Backscattered electron detector
EDX	Energy-dispersive X-ray spectroscopy
LUCA	Last universal common ancestor
Lt	Total length
Ls	Standard length
Worms	World Register of Marine Species
EOL	Encyclopaedia of Life
ERMS	European Register of Marine Species
HCl	Hydrogen chloride
DMP	Dimethyl phthalate
Ar	Argon
Os	Oral sucker
Vs	Ventral sucker

Technical file internship placement

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Sector:	Evolutionary Biology

Department/Group within the internship placement:	Marine Zoology

A number of employees:	13

Turnover:	Depends on year, no regular amount. Around 100.000euro per year. 180.000euro (Sub team – marine zoology department) per year. The money, can be shared with other projects.

Products:	Saline solution (9%), Ethanol (70%), Formaldehyde

Specialization:	Study of marine fish parasite and aquaculture.

Two relevant publications from the internship placement:	*Cardicola mediterraneus* n. sp. (Trematoda, Aporocotylidae): a new species infecting the gilthead seabream, Sparus aurata L., from the Western Mediterranean Sea (Palacios-Abella, J., Montero, F.E., Meralla, P., Mele, S., Raga, J.A., Repullés-Albelda., June, 2021) Considerations on the taxonomy and morphology of *Microcotyle* spp.: redescription of M. *erythrini* van Beneden & Hesse, 1863 (sensu stricto) (Monogenea: Microcotylidae) and the description of a new species from *Dentex dentex* (L.) (Teleostei: Sparidae) (Montero, M.V., Pérez-Del-Olmo, A., Georgieva, S., Raga, J.A., Montero, F.E., January, 2020)

Additional data:	

1. Introduction

The Marine Zoology unit of the Cavanilles Institute of Biodiversity and Evolutionary (ICBiBE) is dedicated to the research on parasites that use marine fish species as the hosts. Their activities consist on dissecting the specimens, collecting, and dyeing parasites, and identifying parasites using molecular techniques. Their effects on fish of humans by fish consumption are further studied among other research lines.

Everywhere on Earth, parasites occur in plants and animals. As parasitism is the most popular lifestyle on Earth. According to ScienceDirect the definition of parasitism is “a nonmutual relationship amongst two organisms where one of the organisms benefits at the expense of the other” (Wu., 2017). Because parasites adapt to the environment, the parasite and the host evolve simultaneously. The parasite uses the host for its one benefit, while the host gets damaged along the way. Due to evolution or protection, the host can get rid of the parasite before getting infected. The protection can occur by building up a symbiotic relationship with the organism to help remove the parasite (NECSI., 2022).

Parasites can be divided into two main groups ectoparasites and endoparasites. The ectoparasites are found on the surface of the host, while endoparasites are commonly found within the body of the host. If a parasite occurs in or outside the organism, there is a possibility of health damage in the host. The balance between host and parasite can be disturbed by environmental changes, often due to human activity, which leads to diseases. An example of environmental change is temperature increase, mostly by human interference, the growth of human population. The environmental change can lead to a change in dynamics between host and parasite and result in mechanical harm to the host. For example tissue replacement or physiological damage (cell proliferation, altered growth) and reproductive damage (Iwanowicz., 2011; Lazcozornago., 2018; Wu., 2017).

Besides the effect of mechanical, physiological, and reproductive injury, parasites can also affect the ecosystem, as they are present all over the food chain. In the view of ectoparasites, it can be said that they are in direct contact with the water and can reflect possible negative effects of pollutants. This can also have a reversed effect, if the ectoparasite is resistant to the pollutants, the population of ectoparasites can grow (Lazcozornago., 2018).

1.1. Description of organisms

1.1.1.Specimen *Scorpaena porcus*

The black scorpionfish, *Scorpaena porcus* (Linnaeus, 1758) (figure 1.A-1.B) is one of the most common fishes in the family of Scorpaenidae. *S. porcus* is commonly found within marine subtropical waters and is widespread in the Eastern Atlantic from the British Isles to the Azores, the Canary Islands near the coasts of Morocco, the Mediterranean Sea, and the Black Sea. (Ferri, J., *et al*, 2010; iNaturalist., 2022; Oceana., 2021).

The black scorpionfish can grow up to a length of 37cm, but the more common length is 15 to 20cm. The body of this species is covered with feathery fins or skin flaps for camouflage in the surrounding coral rocks. The color of the scorpionfish can diversify between mottled brown or yellow, other species are bright red or orange. *S. porcus* is not venomous, but is traumatogenic by punction, equipped with poisonous spines. When spines pierce through a predator, the venom gets injected immediately at the point of contact. A sting of one of those spines can be fatal for other animals and is extremely painful to humans. The scorpionfish is a sit–and–wait, predator. They stay in the shadow of rocks or reefs waiting for prey to pass by. The diet consists of small fishes, crustaceans, and snails which live within the coral reefs (Oceana., 2021; Reeve., 2007).

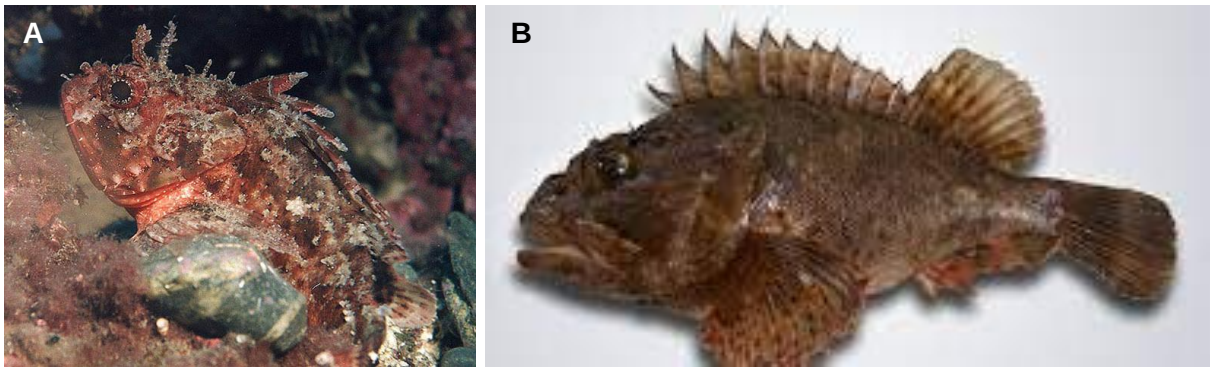

Figure 1. Species under study *Scorpaena porcus*. **A,** Specimen in natural environment (image taken by Pontes, M.); **B,** Specimen ready for dissection (Arias., 2002).

1.1.2.Nematodes

Species in the phylum Nematoda are better known as nematodes or roundworms (figure 2.A-2.D). More than 25.000 species of nematodes have been discovered by scientists. The typical morphology of a nematode is elongated, tapered at both ends, and bilaterally symmetrical. The length varies between nematodes. Most nematodes are microscopically small but some nematodes can reach up to 1meter. These organisms are one of the most common multicellular organisms on Earth. These organisms can infect different species of aquacultured and wild fish. Nematodes can be free-living organisms in freshwater, marine environments, or even in unusual places like vinegar, and beer malt. Besides, they are common fish parasites and can be found on the surface of the specimens or in the internal organs (Campbell., 2021). The morphology of nematode is smooth, cylindrical, and in some cases a long worm (Yanoung., 2003).

Figure 2. Nematode diversity. **A,** Rhabditidae (adapted from Calacademy, 2012); **B,** Hoplolaimidae (image taken by Grabau, Z.); **C,** Criconematidae (image taken by Esquivel, A.); **D,** Thelaziidae (image taken from Public Health Image Library (PHIL).

1.2. Morphology

1.2.1.Hydrostatic skeleton

Most worms have two bands of muscles: longitudinal and circular. Nematodes only have longitudinal muscles. Nematodes move by contracting the long muscles on either side of their body. The whole interior of the nematode acts like a hydrostatic skeleton and allows the nematode to move around and maintain its shape against forces, such as surface tension. When the longitudinal muscles constrict the organism's body gets shorter, just contracting one side of the muscles, nematodes bend. Nematodes belong to the pseudocoelomates with a body cavity filled with fluid, called pseudocoel. This fluid is evenly situated, and the forces of the muscle are spread throughout the organism and the organism moves around (Yekutieli, *et a*l., 2009; Alexander., 2017).

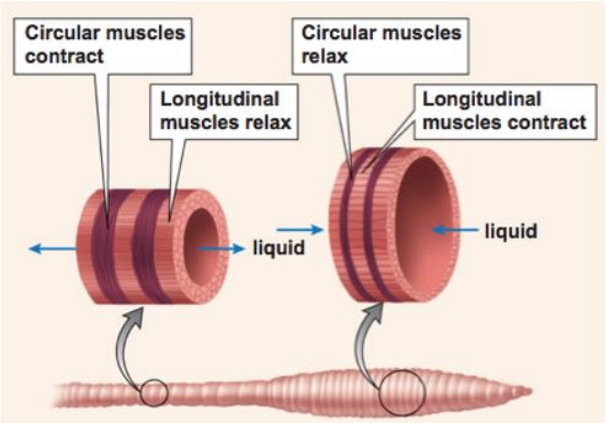

Figure 3. Hydrostatic skeleton.
When the circular muscles constrict the organism's body grows in length, if the longitudinal muscles constrict the organism's body gets shorter. Fluid in the organism is concentrated evenly and spread out through the muscles so the whole organism can move around (Hunt, R., 2015).

1.2.2.Digestive system

The digestive system of nematodes is complete. It has a mouth at the beginning and an anus at the end. Some animals for example starfish, take food in and dispose of their waste through the same opening. A complete digestive system is a system where food travels one way. This type of system, presents some advantages, such as efficiency. Each section of the digestive pathway performs a specific function (Friedl., 2022).

The digestive system of nematodes (figure 4) consists of three main parts: stomodeum, intestine, and proctodaeum. The start of the digestive system is the stomodeum. The inside of the mouth consists of the mouth opening, buccal cavity and oesophagus (often composed by pharynx and glandular oesophagus). In the stomodeum, the food is mixed with enzymes in preparation for the next compartment, the intestine, leading to the last tract, the proctodaeum. At the end of the nematode, there is the proctodaeum that serves as the anus and removes the waste. At this point, all the nutrients are absorbed from the food. The structure of the intestine is a small tube-like structure running the length of the nematode. Enzymes are produced within the intestine and help with the absorption of nutrients in the food. Unlike the intestine in humans, the intestine of nematode has no muscle to move the food which travels from start to end by movement of the nematode (Friedl., 2022).

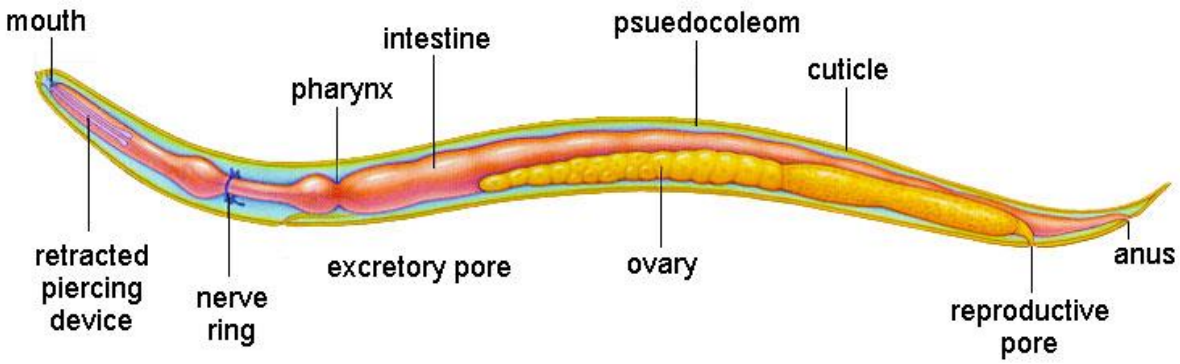

Figure 4. Digestive system nematode. The nematode's digestive system consists of three main parts: the stomodeum, intestine, and proctodaeum (Slidetodoc, 2022).

1.2.3.Respiratory system

The outer body exists of a thick, protective, non-cellular cuticle. This waxy cover is secreted by the epidermis (hypodermis) and forms the outermost cellular tissue (Basyoni and Rizk., 2016). The presence of this waxy cover prevents gas exchange. Nematodes make use of diffusion through their skin, a process where substances move from an area with a higher concentration to an area with a lower concentration. The exchange of gasses keeps the nematode alive. Like humans, nematodes use oxygen and remove carbon dioxide as a waste product. Because of the use of O_2 an imbalance occurs between the amount of O_2 inside and outside of the body, so-called a concentration gradient. The driving force behind diffusion is the concentration gradient (Corsini., 2022).

1.2.4.Nervous system

Nematodes have a central nervous system that consists of a toroidal brain, called a nerve ring (figure 5). The nerve ring consists of neuropil and encloses the pharynx. It is a collection of axonal and dendritic neurons whose cell bodies lie in bundles. These bundles are named ganglia. All nematodes have one major cord, called the ventral nerve cord. This nerve cord contains cell bodies and performs a series of actions that lead to a result (Schafer., 2016).

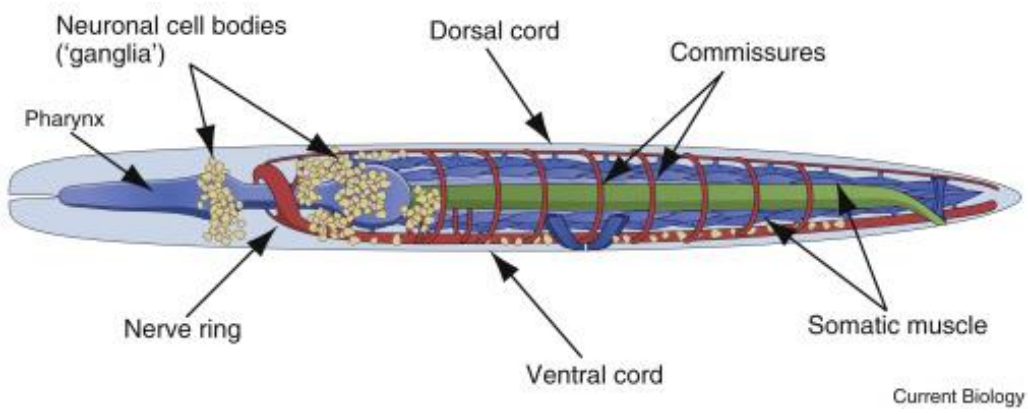

Figure 5. Nervous system. Consists of a circular brain also named a nerve ring which consists of the neuropil and encloses the pharynx (Schafer, 2016).

1.2.5.Reproductive system

The reproductive system of a male nematode (figure 6.A) includes one testis, a seminal vesicle, and a vas deferens opening into a cloaca. Most common amongst male nematodes is the existence of one testis. The production of sperm cells happens in the testes and is stored in the seminal vesicle until the nematodes reproduce. A male nematode is equipped with spicules (figure 6.C), a cuticular copulatory organ used to attach to the female nematode and deposit the sperm cells directly into the female (Basyoni., *et al*, 2016)

Female nematodes (figure 6.B) have one or two ovaries, seminal receptacles, uterus, ova-injector, and a vulva (figure 6.D). The ovary ensures the production of oogonia. The oogonia which are immature female germ cells forming the oocytes (Collins., 2022). The seminal receptacles sometimes develop into spermathecae and their purpose is to store or receive sperm from a male nematode (Martins *et al*., 2008). When an oocyte is fertilized, the development of an egg starts in the uterus. The ovijector is muscular and uses body movement combined with high internal body pressure to remove eggs out of the vagina. The most common between nematodes is syngamy, which means the formation of zygotes after the fusion of gametes. On the other hand, cross-fertilization can happen too, the fertilization of gametes that originate from different species (Biology online., 2022). Besides syngamy and cross-fertilization, hermaphroditism or parthenogenesis occur amongst nematodes also. Hermaphroditism is the production of spermatozoa and the sperm cells are getting stored until the production of eggs. Parthenogenesis is a natural form of asexual reproduction. Embryos grow and develop without the fertilization of sperm (Normark., 2013; Nematology University Nebraska., 2022).

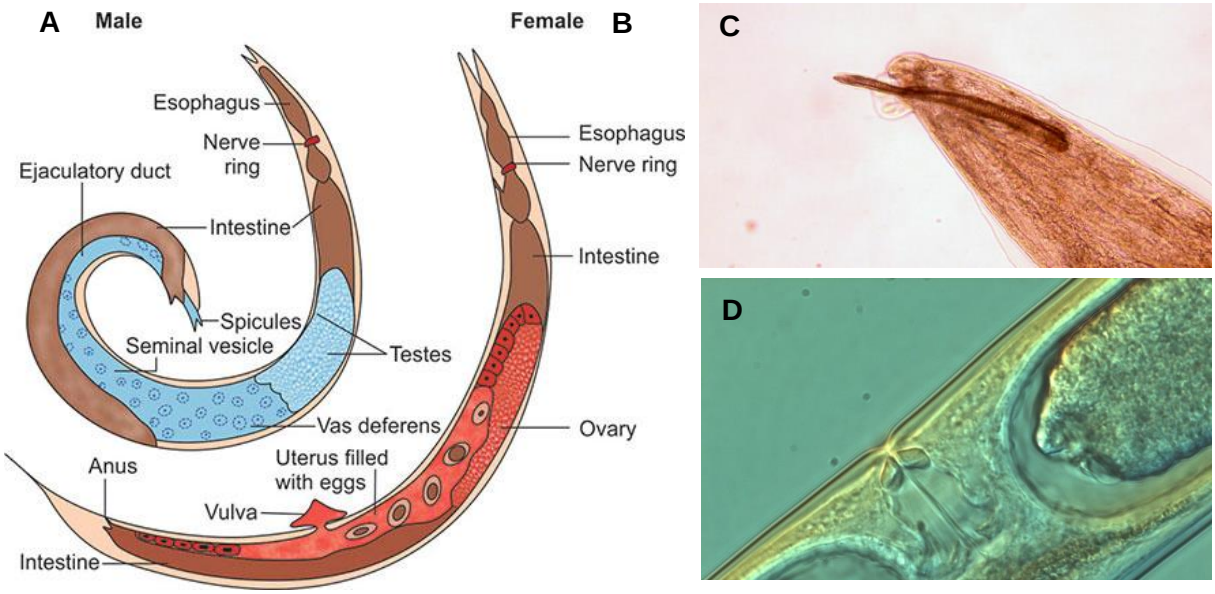

Figure 6. Nematodes r*eproductive system.* **A,** Female reproductive system, it consists of one or two ovaries, seminal receptacles, uterus, ova-injector, and a vulva (Sankar and Bhat2014); **B,** male nematode reproductive system, it consists of one testis, a seminal vesicle, and a vas deferens (Sankar and Bhat, 2014); **C,** spicules detail (adapted from Stuart *et al*., 2010); **D,** vulva detail (image taken by van Megen, H.).

1.2.6.Life cycle

The parasitic nematode can reach the adult stage in the final host, sometimes using an intermediate host to complete the life cycle. The life cycles of nematodes can be distinguished into two categories; indirect and direct. In the indirect life (figure 7.A), the nematodes lay the eggs in an intermediate host. When the intermediate host is eaten by the correct fish, the nematode can reach the adult form. In a direct life cycle (figure 7.B), the nematode does not need an intermediate host. During the infection of a fish, the infection can spread to other fishes without the need for an intermediate host (Yanoung., 2003).

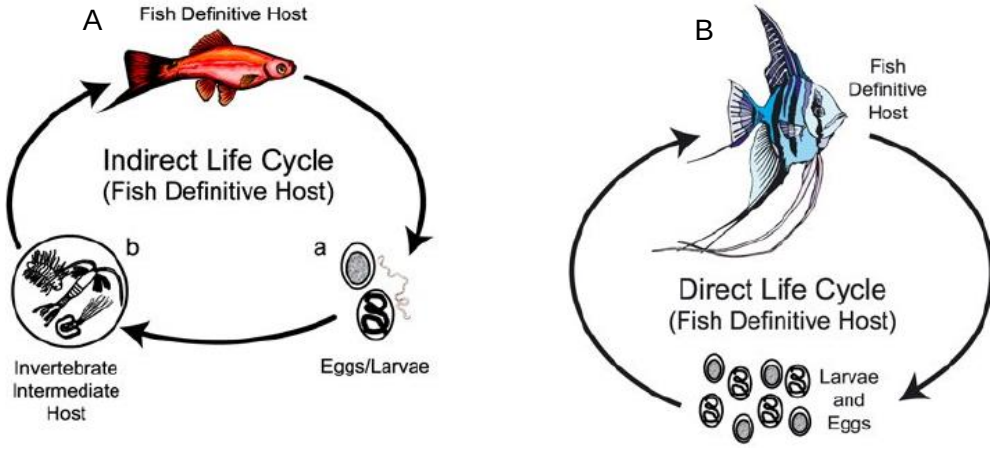

Figure 7. Nematodes infection cycles. **A,** Indirect lifecycle nematode, the nematode will put his eggs into an invertebrate where they can develop. In the final fish host, the nematode can reach sexual maturity (Yanoung, 2003); **B,** direct lifecycle nematode, the infection can spread from one fish to another without the need of an intermediate host (Yanoung, 2003).

The life cycle of the nematodes (figure 8) exists of three main stages: egg – larvae – adult. The adult female lay their eggs and undergo different metamorphoses. The first is the embryonation inside the egg. The embryonation of an egg forms the first stage, L1 larvae. who grows by following metamorphoses (L2-L4). The L1 and L2 stadia remain in the eggs. The L1 and L2 remaining in the faeces is only valid for nematodes of the digestive system, those living in internal organs. The L3 stadia do not feed but live on energy reserves. The infective stage is the L3 stadia and can be consumed by the host to continue the life cycle. During the L2-L4 stadia, there is an increase in the size of the nematode. The L4 stadia is an immature adult and later matures into an adult nematode. The adult nematode can reproduce once more with the formation of new eggs (Mkandawire., *et al*, 2022).

In the life cycle of nematodes, eggs have a plural role. At first, the egg provides an environment where embryonation and loss of the shell can occur. Secondly, eggs provide shelter as it acts as a protective shield. Eggshells consist of three components: a barrier of lipids, chitin, and a vitelline layer (glycoprotein). In some species, uterine layers can be presented. Because of the three layers, the egg is resistant to stress, but also permeable. Eggs can survive for a long period within the environment. *Trichuris suis* and *Ascaris suum* eggs can survive up to 10 years. Finally, the egg is responsible for the transport of the larvae for reaching the host and start the infection (Mkandawire., *et al*, 2022).

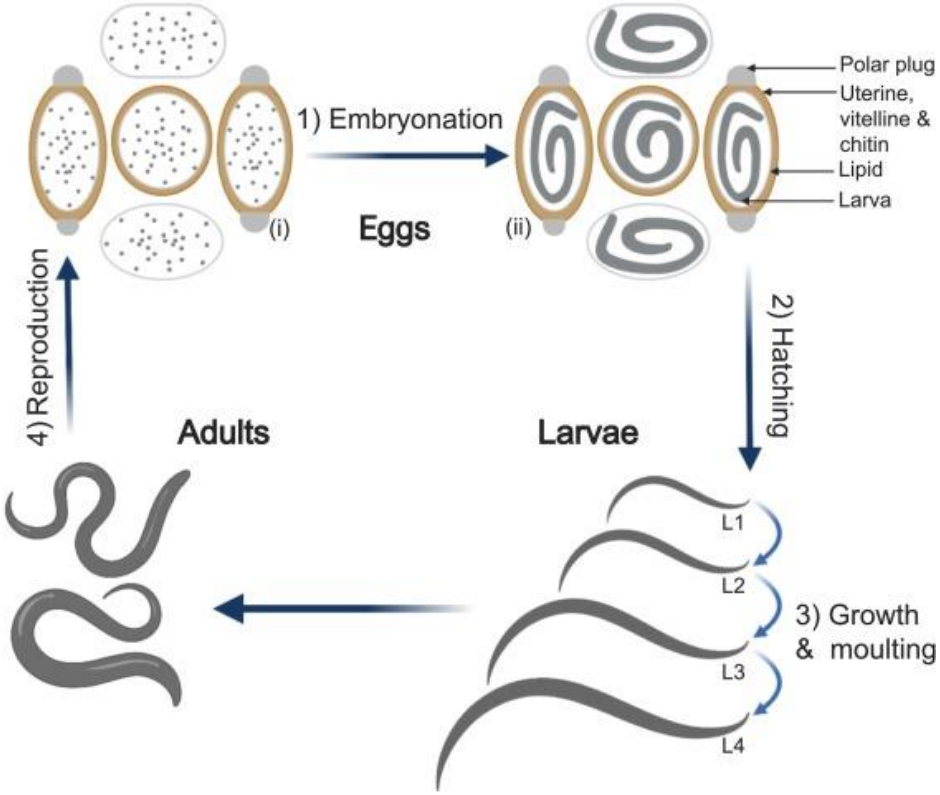

Figure 8. Life cycle of a nematode. The life cycle of the nematode exists of three main stages: egg – larvae – adult. L2 – L4 stadia will increase in size. L4 is the immature adult form, which will develop into a mature nematode that can reproduce sexually to lay eggs and continue the life cycle (BioRender, 2022).

1.3. Materials of determination

1.3.1.Stereomicroscope

The stereomicroscope (figure 9) is an optical microscope that is used to give a three–dimensional view of the specimen. The stereo head is the moveable part of the microscope that holds the 2 eyepieces. A diopter is used to compensate for the focus between the left and right eyes. The focus knob is used to move the head of the microscope up or down and give a sharper image of the sample. Stage clips helps to keep the sample in his place. The stage plate is located directly under the objective lens and is the place where the specimen is viewed. The eyepieces and the objective lens determine the magnification of the microscope (10x to 50x). Many microscopes are using bottom and top lighting. The top lighting shines down on the specimens and reflects lights, the bottom lighting transmits light up to show translucent specimens (New York Microscope Company., 2022).

The principle of the stereomicroscope is based on the two different patterns of lights originating from the objective lens and the eyepiece. Using two eyepieces, a view of the sample is created from different angles simultaneously. The stereomicroscope includes a camera that is used for viewing the specimen on the computer and for closer observation (Yard., 2021).

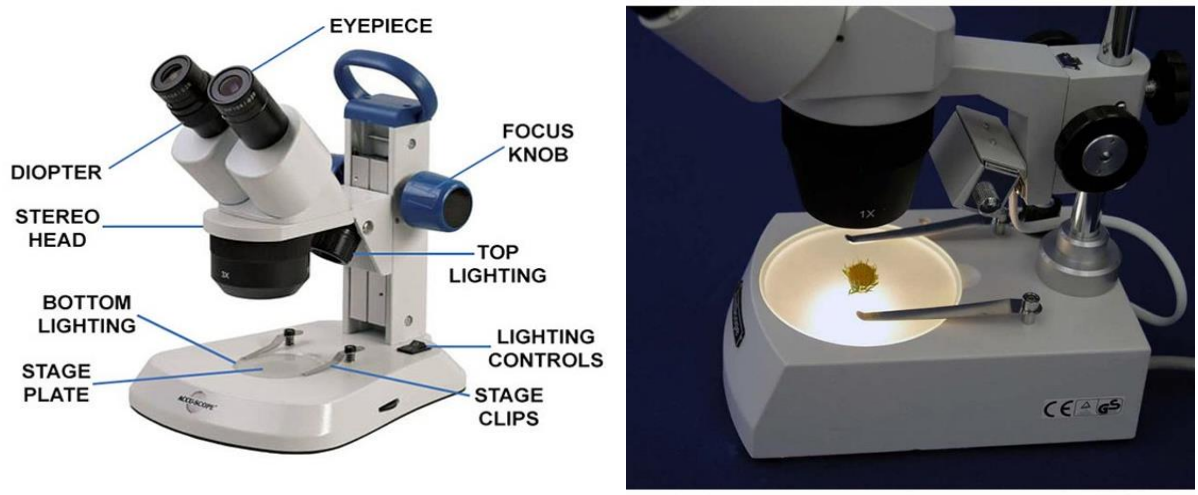

Figure 9. Stereomicroscope, its parts and an example of samples observation (Kroening, 2018).

1.3.2.Scanning electron microscope (SEM)

Scanning electron microscope (SEM) has become a powerful tool, that can be used in the morphological analysis for the identification of parasites. Electron microscopes emit a beam of electrons that scans the sample in a raster pattern. Electrons are generated at the top of the column by an electron source. The electron source is under vacuum, like all of the components of the electron microscope. The electron source is placed in a special chamber to preserve the vacuum and protect against contamination, vibrations, and noise. The absence of a vacuum can result in the presence of other atoms, and molecules (Nankoudis., 2019). The interaction between the electron beam and the sample surface takes place as a “pear shape”. The interactions lead to the production of different electrons (figure 10.A): secondary, backscattered electrons, and characteristic X-rays (Nanoscience., 2022). An anode plate attracts the electrons originating from the beam. After the attraction of electrons, the size of the beam is controlled by a condenser lens and determines the number of electrons in the beam. The size of the beam will determine the image resolution. Scan coils are used to deflect the beam along the x and y axes. The beam will scan in a raster formation over the sample surface. The last lens, the objective lens focuses the beam on a specific spot on the sample (SciMed., 2020).

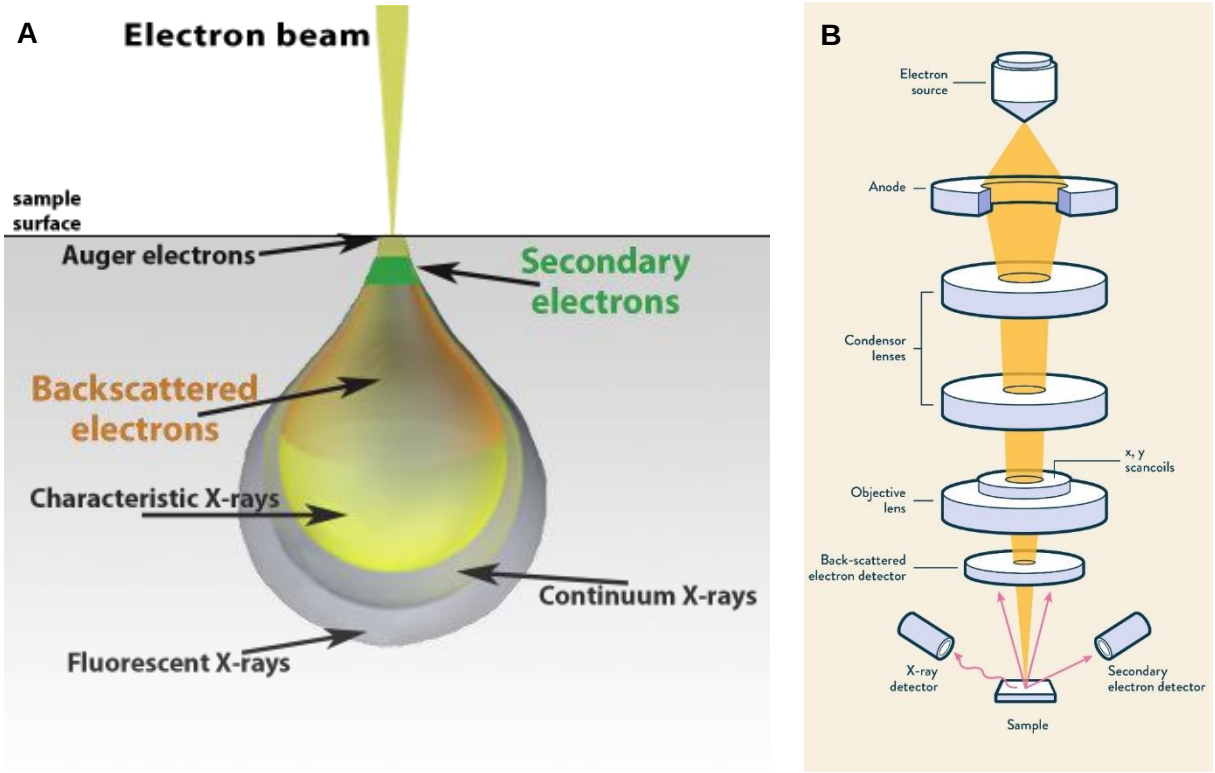

Figure 10. Scanning electron microscope (SEM) operation. **A,** The transmit of electrons originating of an electron beam (Nanoscience, 2022); **B,** construction of SEM, the electron sources sends out electrons what are getting attracted to the anode plate. From there the electrons move through different lenses and interacts with the sample. Afterwards the electrons are getting detects by different detectors (Technology network, 2022).

Electrons are detected by different detectors (figure 10.B). The secondary electrons are detected by a secondary electron detector (SE). The SE captures the energy from the secondary electrons generated in the sample by interaction with the electron beam. Secondary electrons give information about the texture or topography of the outermost layer. Backscattered electrons are detected with a backscattered electron detector (BSE). A BSE – detector captures the outcoming energy originating from the backscattered electrons (second layer of the "pear"). The surface resolution is less, but the sensitivity to variations in atomic numbers is higher. Detection of backscattered electrons will result in a gray shade according to the atomic weight. When the emitted energy of backscattered electrons is higher, the gray shade is lighter. At last the X-ray detector. This type of detector captures the emitted energy of X – rays generated from the surface originating from the third layer. Capturing the energy of X-ray will give information about the elemental composition, and unlikely to the BSE – detector, the X-ray detector provides more information about the sample. An example of an X-ray detector is the Energy-dispersive X-ray spectroscopy (EDX). EDX – detector can be applied to a specific point on the sample surface. By focussing the electron beam on an area, a map can be created of the different elements of that selected area. Each element is represented by a different color (Atria Innovation., 2020).

1.4. Preserving marine species

According to the article: *Molecular resolution of marine nematodes for improved assessment of biodiversity* (Punyasloke., 2013) formalin is a fixative and is the preferred choice for preserving marine species, including free-living nematodes for long-term studies. The reason for the preferred choice of formalin is inexpensive, effective, and low maintenance. Formalin maintains the morphological structure of the specimens. The chemical composition of formalin is a 37% aqueous solution of formaldehyde (CH_2O) and is the most reactive of all the aldehydes. Formalin is the preferred choice, because of the advantages, but has also a couple of disadvantages. Specimens fixed in formalin for a long period are considered unsuitable for DNA work because of the effect the fixative has on the components including nucleic acids. The way formalin works during the fixation process are by combining functional groups of amino acids, thereby denaturing proteins. During the reactions, oxygen atoms undergo hydrogen bonding with primary amines to crosslink the proteins. The reaction is dependent on physical factors such as pH, buffer, concentration, temperature, fixation time, etc. Formaldehyde has been shown to cause the formation of DNA – DNA, DNA – protein, and protein-protein hybrids through crosslinking. Some of these crosslinks can be partially broken which causes an effect on the success during polymerase chain reaction (PCR), and electrophoresis experiments. At a neutral pH (pH 7), formaldehyde can react with three DNA bases, cytosine, guanine, and adenine, which leads to the formation of a reactive compound. The reactive compound group can cause problems with primer annealing, inhibit the renaturation, and suppresses the replication procedure during PCR (Technology, F. O.S.A., 2013).

1.5. Cladogram

To determine how species are related to each other, a phylogenetic tree (figure 11) is needed. The phylogenetic tree or evolutionary tree is a schematic representation to think clearly about the differences between species and examine these differences (Felsenstein., 2004). Study, phylogenetics can be defined as, “the systematic study of relationships between organisms which lead to a taxonomical classification based on how close organisms are related (Wyant., 2011).

The interpretation of a phylogenetic tree is as follows, a phylogenetic tree can be rooted or unrooted. A rooted tree has a root, that indicates the last universal common ancestor (LUCA). From the LUCA other taxonomic groups can descend (Choudhouri., 2014). Starting at the root, the phylogenetic tree is developing into branches. Branches represent the transmission of genetic information through time from one generation to the next (EMBL – EBI., 2022). At the end of the branches, there are nodes present, points that represent the last common ancestor of organisms at the end of the branch. Within phylogenetic threes, a cluster can appear. One node can have a cluster of different species that are related. These clusters are called clades, a cluster of organisms that includes a node of all the lineage that descended from that one node (Scott., *et al*, 2016).

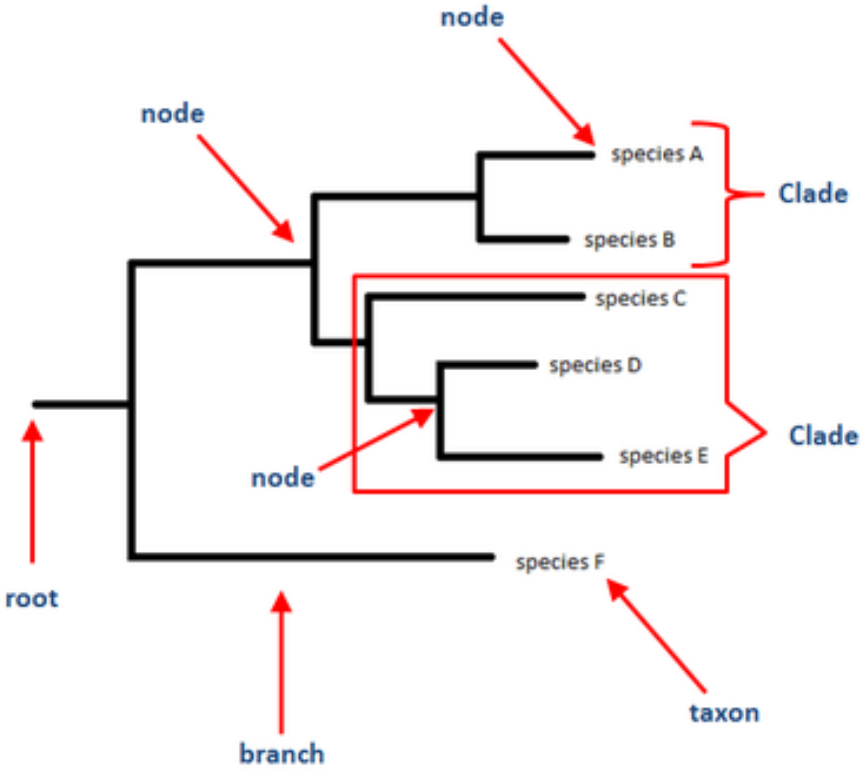

Figure 11. Example of a phylogenetic three. A phylogenetic tree starts at a root, which will stretch into different branches. At the end of a branch, a node is present. Within one node different clusters are present, called clades (Crother, 2020).

1.5.1.Phylogenetic tree

As an example, the article *A New Species Of Ascarophis (Nematoda: Cystidicolidae) Parasitizing Clinocottus analis (Pisces: Cottidae) From Baja California, Mexico (R. Aguilar-Aguilar., et al, 2019)* makes a phylogenetic tree to determine if they discovered a new *Ascarophis* species (figure 12).

To make the phylogenetic tree of the new *Ascarophis* sp., a variability of *Ascarophis* nematodes was collected from different hosts caught in the same location. DNA extraction was performed using releasing reagent GeneReleasert DNA Full Size (BioVentures, Murfreesboro, Tennessee). The small subunit rRNA (SSU rRNA) was amplified by PCR using a forward primer, 103F, and a reverse primer 18SR. Sequences of *Ascarophis* nematodes were obtained using Geneious Pro v.5.1.7. The examined nematodes have shown major characteristics shared with members of the genus *Ascarophis* and conclude that it represents a new species (*A. morronei*). This conclusion was made based on morphological features: morphology of the eggs, combined with other features such as the spicule length of males, and the distances of nerve rings *(Aguilar-Aguilar., et al, 2019).*

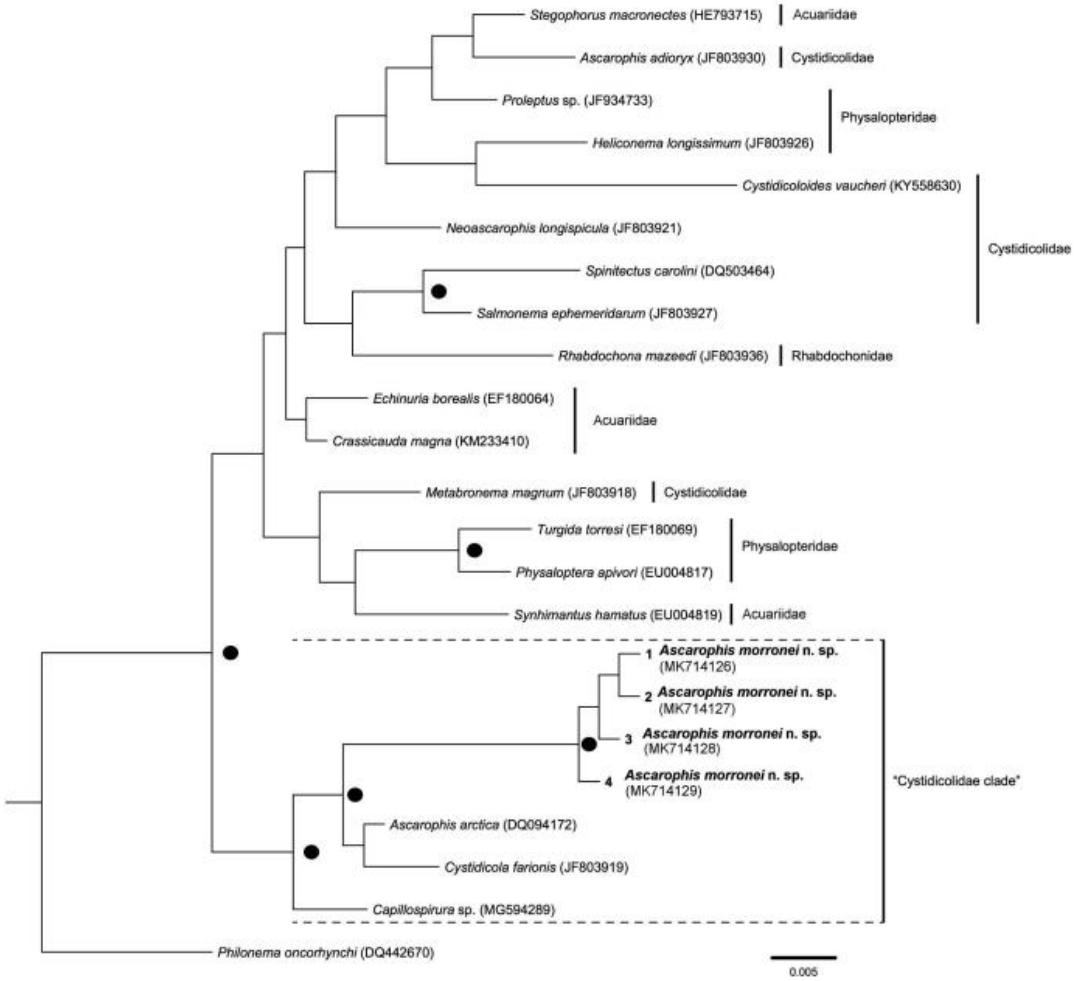

Figure 12. Phylogenetic tree of *Ascarophis* spp. and its relative position (Aguilar-Aguilar., *et al*, 2019).

2. Objective

The morphological analysis of parasites in *S. porcus* allows us to understand which kind of parasites harbour this host. Using a Leica DMR optical microscope and a scanning electron microscope, the morphological identity of the parasite can be tracked down.

The main objective of this bachelor thesis is to track down the morphological identification of collected parasites found in the specimen *S. porcus* which can be split into two specific parts:

Primarily, the identification of fish parasites especially nematodes (*Ascarophis* spp.) in *S. porcus* employing a Leica DMR optical microscope and scanning electron microscope (SEM) by performing a morphological analysis of a collection of parasites found this fish.

Secondly, learning different staining methods such as iron acetocarmine and hematoxylin-eosin staining.

Material and methods

3.1. Geographical localization

The Islas Chafarinas (35°10'55.0"N, 2°25'48.0"W) is formed by three other small islands (figure 13), Isla del Congreso, Isla de Isabel II, and Isla del Rey. The islands are located in the Mediterranean Sea, at 48km east of Melilla and 3.5km from the city of Morocco, Ra'su I-Ma. The specimens of *S. porcus* used for examination was caught on the 27th, 31th of July, 5th of August 1991. Specimen caught in an unknown location of this islands on the 23rd of February 1994. The making of the report is shown in table 1.

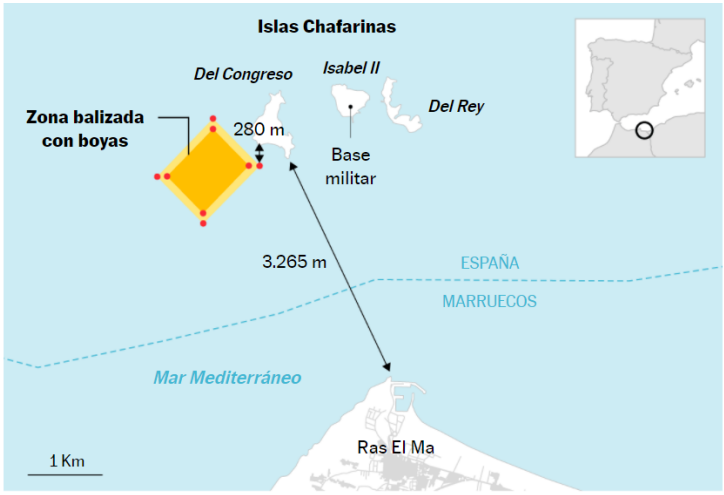

Figure 13. Geographical location of the samples.

Table 1. Origin and dissection data *Scorpaena porcus*
The dissection and examination date is the day when the specimen got dissected and examed.
The last column is where the fish was obtained.

Specimen code	Date of Fishing	Date of Dissection	Date of Examination	Locality
Sp20220223	27th July '91	23rd February '22	23rd February '22	Pta. Maricones
Sp20220224	27th July '91	24th February '22	24th February '22	Pta. Maricones
Sp20220225	27th July '91	25th February '22	25th February '22	Pta. Maricones
Sp20220228	27th July '91	28th February '22	28th February '22	Pta. Maricones
Sp20220301	27th July '91	1st March '22	1st March '22	Pta. Maricones
Sp20220302	27th July '91	2nd March '22	1st March '22	Pta. Maricones
Sp20220303	27th July '91	3rd March '22	3rd March '22	Pta. Maricones
Sp20220304	27th July '91	4th March '22	4th March '22	Pta. Maricones
Sp20220307	27th July '91	7th March '22	7th March '22	Pta. Maricones
Sp20220308.1	27th July '91	8th March '22	8th March '22	Pta. Maricones
Sp20220308.2	27th July '91	8th March '22	8th March '22	Pta. Maricones
Sp20220309	5th August '91	9th March '22	9th March '22	Pta. Maricones
Sp20220310	5th August '91	10th March '22	10th March '22	Pta. Maricones
Sp20220311	5th August '91	11th March '22	11th March '22	Pta. Maricones
Sp20220321	5th August '91	21th March '22	21th March '22	Pta. Maricones
Sp20220322	5th August '91	22th March '22	22th March '22	Pta. Maricones
Sp20220323	5th August '91	23rd March '22	23rd March '22	Pta. Maricones
Sp20220324	23rd February '94	24th March '22	24th March '22	Unknown
Sp20220325	23rd February '94	25th March '22	25th March '22	Unknown
Sp20220331	23rd February '94	31st March '22	31st March '22	Unknown
Sp20220401	23rd February '94	1st April '22	1st April '22	Unknown
Sp20220404	23rd February '94	4th April '22	4th April '22	Unknown
Sp20220405	23rd February '94	5th April '22	5th April '22	Unknown
Sp20220413	31st July '91	13th April '22	13th April '22	Unknown

3.2. Morphological analysis

3.2.1. Determination of parameters

For the morphological analysis, 24 specimens of *S. porcus* were used. These specimens were caught in different locations and on different dates. The measurements consist of weighing the specimen and taking the total and standard length of the specimen. The total length (Lt) is referred to the length of the fish from the head to the tail fin. The standard length (Ls) can be defined as the length from the head until the start of the tail fin (figure 14).

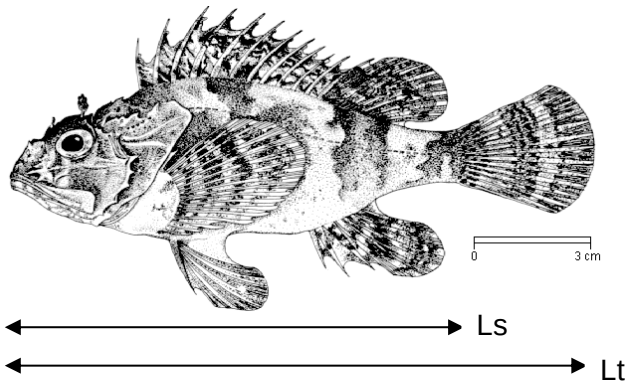

Figure 14. Total and standard length measurements (Bauchot., 1987).

3.2.2.Dissection *Scorpaena porcus*

After taking measurements, an external examination was performed to look for ectoparasites, such as leeches, looking between the fins, scales, into the mouth and nostrils. Once the external examination has been performed, the organs were extracted and separate in petri dishes with 9% saline solution. The organs used for examination were eyes, brain, gills, heart, liver, stomach, intestine, and gonads (figure 15A-H). The eyes were carefully studied for parasitic nematodes and preserved in 70% ethanol for later histological analysis.

Organs were examinated using a Leica M6-stereoscope looking for parasites. All of the parasites were collected in an eppendorf filled with 70% ethanol for morphological analysis.

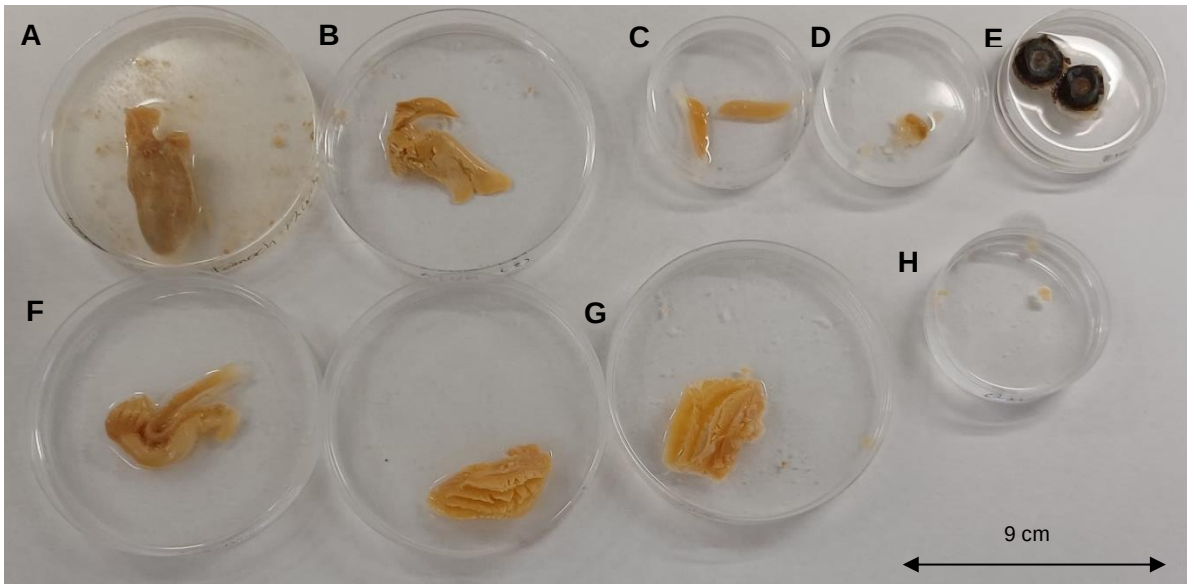

Figure 15. Organs in 9% saline solution after dissection. **A,** stomach; **B,** liver; **C,** gonads; **D,** heart; **E,** eyes; **F,** intestine; **G,** gills; **H,** brain.

3.2.3.Parasitological analysis

According to Bush *et al*. (1997) prevalence, mean intensity and mean abundance were calculated. Prevalence, expressed as a percentage, consists of the number of hosts that are infected with one or more individuals of a particular parasite species.

$$Prevalence = \frac{Total\ number\ of\ infected\ hosts}{Total\ population}\ x\ 100$$

Mean intensity is the average number of parasites per infected host.

$$Mean\ intensity = \frac{Total\ number\ of\ parasites}{Number\ of\ infected\ hosts}$$

The mean abundance is the average number of parasites per host in a sample or a population.

$$Mean\ abundance = \frac{Total\ number\ of\ individuals\ of\ a\ species\ of\ parasites}{Total\ number\ of\ hosts\ (Infected\ and\ uninfected)}$$

3.3. Iron acetocarmine staining

Monogeneans and digeneans collected from *S. porcus* and the historical collection of the Marine Zoology Department of the University of Valencia, were used for the iron acetocarmine staining. The parasites were transferred to different containers with iron acetocarmine solution and were stored one and a half to two hours in the fridge until a deep-red color is achieved. Subsequently, an ethanol increasing gradient was created (70-80-90-96-100-100) for dehydration. After the ethanol gradient, the parasites were moved to dimethyl phthalate (DMP). DMP works as a clearing substance, but also as a fixative to keep the structure of the parasite intact.

The mounting medium used was Canada balsam. Once parasites were stained and mounted, they were observed by optical microscopy (Nikon Opiphot-2 microscope; 40-400X) and subsequently identified. For the correct identification of the parasitic species, several measurements have been made using the Leica LAS software (Leica Application Suite).

3.4. Histological analysis

A histological analysis (figure 16) was performed on four *S. porcus* eyes in order to find possible pathological effects in this organ. Both eyes were preserved in 70% ethanol. Because of fixing of the specimen *S. porcus* in formalin, the procedure started with the preparation of cassettes, which were placed overnight in 70% ethanol. Place a sponge on the cassette and add the eyes on top of the sponge. Add another sponge to cover the eyes and close the cassette. After closing, the cassette is labelled with the specimen code and the name of the organ. The cassette is placed overnight in 70% ethanol (figure 16).
When the paraffin blocks were made, the Leica RM 2125 RTS microtome were used to make slices of the sample. After labeling the slides, the container was filled up with tap water and heated up to 52°C. The starting thickness of the slides were 10µm. The resultant tissue was collected and placed into slides for a final staining step.

The following methodology was performed for the hematoxylin and eosin samples staining process, xylol I for 15 min (repeat twice), absolute ethanol for 10 min, 96% ethanol for 5 min, 70% ethanol for 5 min, 50% ethanol for 5 min, hematoxyline for 5 min, water for 5 min (first wash step), eosin for 4 min, water for 5 min (second wash step), 50% ethanol for 5 min, 70% ethanol for 5 min, 96% ethanol for 5 min, absolute ethanol for 10' (repeat twice) and finally xylol for 10min. Last of all, samples were mounted. The mounting medium used is Entellan. Samples were covered with Entellan, taking care to not form bubbles. Samples were collected in a flat rack to view under the microscope for further analysis.

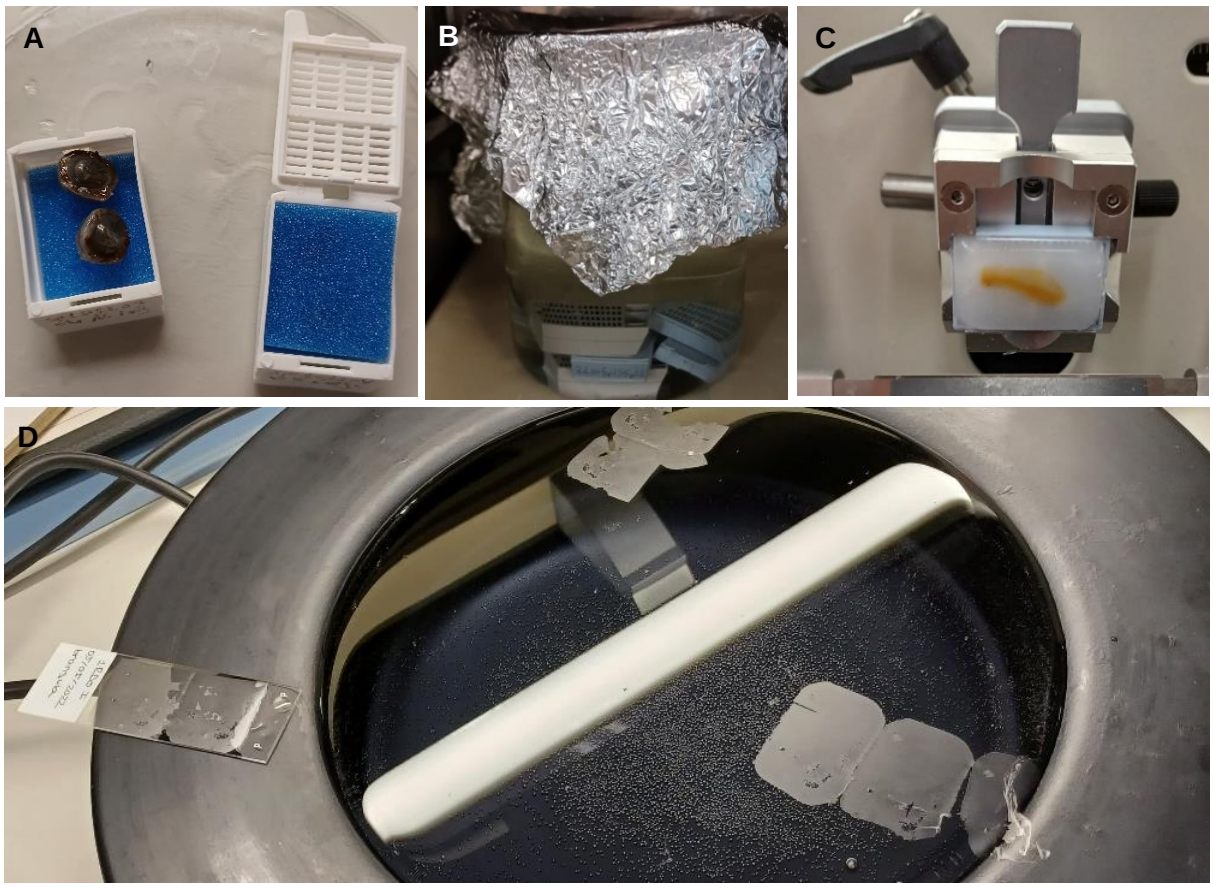

Figure 16. Histological procedure. **A,** Preparation of the cassette; **B,** paraffin inclusion; **C**-**D,** tissue samples from the microtome.

3.5. Scanning electron microscope (SEM)

3.5.1.Preparation

For the scanning electron microscope (SEM) (figure 17.A) four male and female *Ascarophis* specimens were collected. Parasites were prepared making an increasing ethanol gradient (70 – 80 – 90 – 96 – 100 – 100% ethanol) and performing the critical point for a total dehydration. Nematodes were placed in ventral view on a metal plate with a carbon strip for better observations of the reproductive system, mouth and papillae. Then, nematodes were coated with gold-palladium particles for being visualized under the SEM (figure 17.B-C).

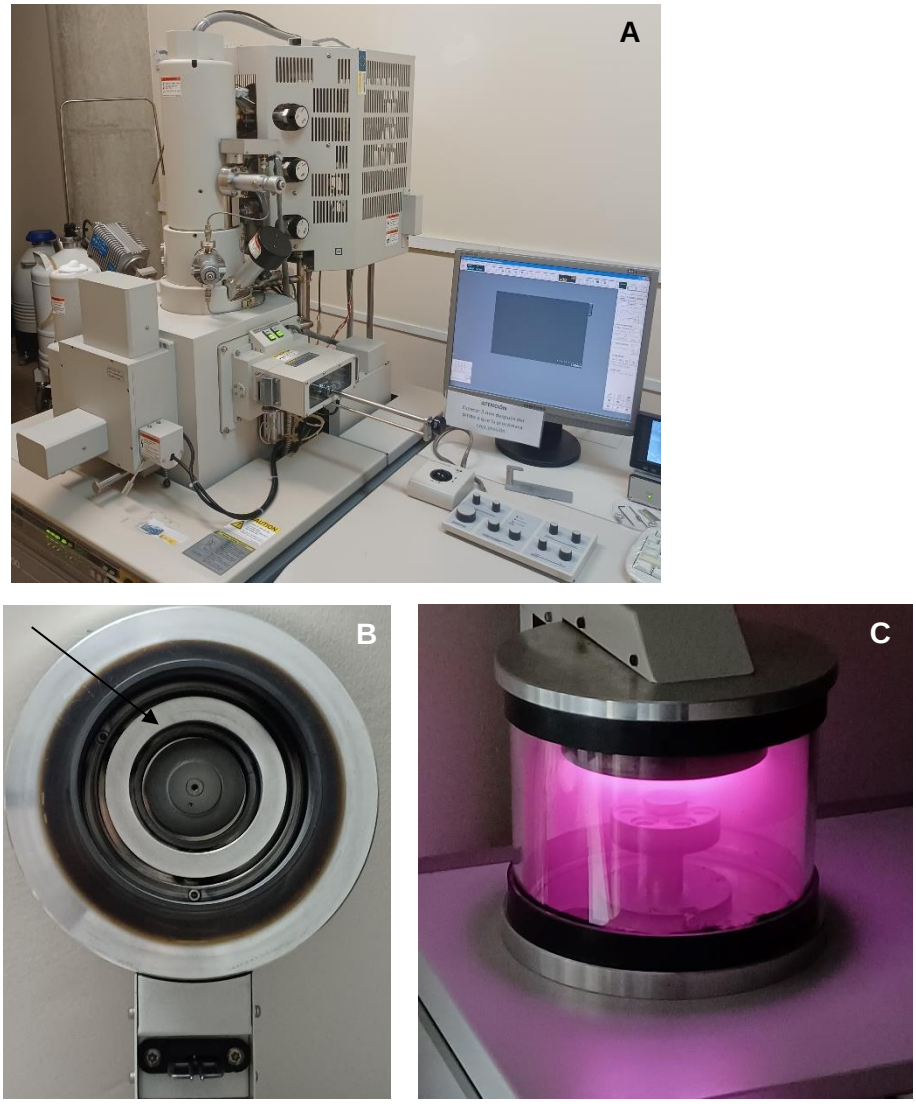

Figure 17. Sample preparation for scanning electron microscope. **A,** Scanning electron microscope; **B-C,** Coating with gold-palladium particles.

Results and discussion

4.1. Morphometric parameters of *Scorpaena porcus*

The specimens of *S. porcus* were caught on different locations and dates. From these 24 specimens, different measurements were taken (table 2). Over some time and caught in different locations, a difference in length and weight can be distinguished amongst the *S. porcus* specimens.

Table 2. Morphometric parameters of Scorpaena porcus
Measuring weight (g), total length (Lt), and standard length (Ls) (cm) caught at different locations and dates.

Locality	***Weight (g)***	***Total length (cm)***	***Standard length (cm)***
Islas Chafarinas (27/07/91)	5,59 – 78,9 (33,11)	7,5 – 17 (12,1)	5,5 – 13 (9,6)
Islas Chafarinas (05/08/91)	34,36 – 147,36 (76,91)	12,5 – 20 (16,1)	10 – 16,5 (13,1)
Unknown (23/02/94)	19,35 – 55,17 (29,55)	10 – 14,5 (11,6)	8,5 – 11,5 (9,3)
Unknown (31/07/91)	93,26	19,5	16,5

4.2. Occurrence of parasites in *Scorpaena porcus*

From 24 specimens of *Scorpaena porcus*, a total of 153 parasites were collected. Based on the prevalence of the parasites, can be concluded that the most dominant group is nematodes. Nematodes come with a prevalence of 87.5%. The mean intensity of nematodes is 5.95. This means that in one infected host, five nematodes are present. The prevalence of the other parasites that also have been collected are leeches 37.5%, Platylhelminthes 12,5%, and Arthropoda 4.17% (table 3). The found Arthropoda in *S. porcus* was *Gnathia*. a micropredator for fishes and works similarly to mosquitos. They attach to the surface of the host and feeds on blood. After the feeding, the micropredator will release from the host to reproduce. Only female *Gnathia* will feed in blood.

Table 3. Prevalence of different parasites
A total of 153 parasites were collected from 24 specimens of Scorpaena porcus. From these parasites, the prevalence, mean intensity, mean abundance, and standard deviation were calculated.

Variable (Parasite)	***Total***	***Prevalence (%)***	***Mean intensity***	***Mean abundance***	***Standard deviation***	***Relationship parasite – host***
Nematode	125	87,5	5,95	5,21	3,37	Parasite
Hirundinea	22	37,5	2,44	0,92	2,17	Micropredator
Platyhelminthes	5	12,5	1,67	0,21	0,66	Parasite
Arthropoda	1	4,17	1	0,04	0	Micropredator

4.3. Morhophical characterization

4.3.1.*Ascarophis filiformis* eggs

After the examination of different organs under a Leica DMR microscope, several female *Ascarophis filiformis* specimens were used for the examination of the eggs. Pictures of the *A. filiformis* eggs were taken with a Leica DMC 5400 camera. The eggs were pushed out of the uterus for examination. The eggs were oval-shaped with a small size. Figure 18.A shows immature eggs without any filaments, which are fragile and can break easily. The poles in figure 18.A are smaller than in figure 18.C. A smaller pole could mean that the pole is developing. Figures 18.B, and 18.C show a mature egg with two filaments in the same pole. The number of filaments an its position is important for the identification of the *Ascarophis* species. In figure 18.D, the eggs are organized in four rows within the uterus.

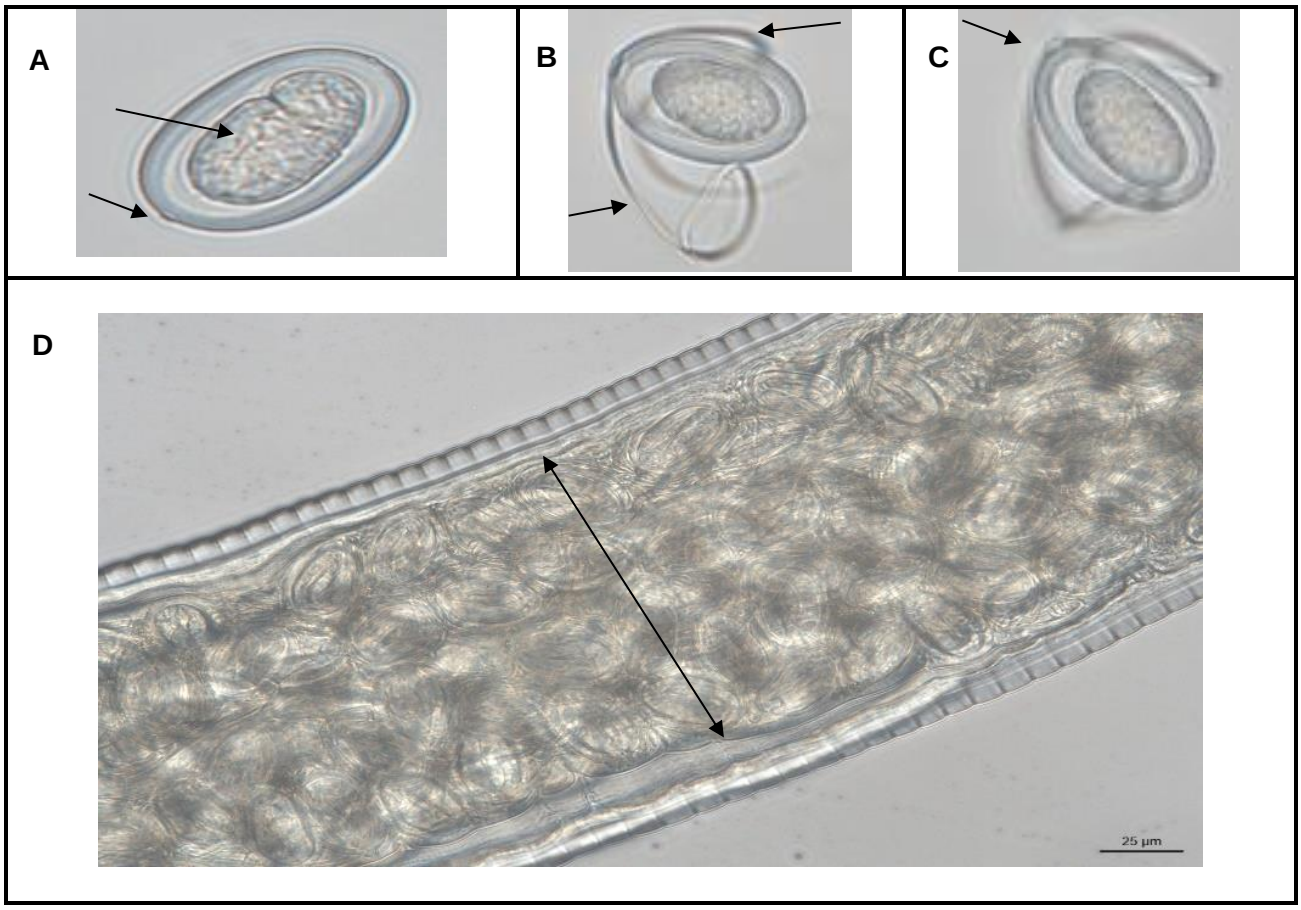

Figure 18. *Ascarophis filiformis* eggs. **A,** Non-filamented egg; **B,** two filaments at one pole; **C,** Mature egg with two filaments at one pole; **D,** a group of eggs organized in four rows within the uterus.

4.4. Morhophical characterization

4.4.1.Male *Ascarophis filiformis*

Male worms of *A. filiformis* were measured at 9.73mm with a width of 0.08mm of total length (figure 19.A). Mouth and lips were present, connected to the vestibulum followed by the oesophagus (figure 19.B). The pharynx is enclosed by the nerve ring (figure 19.B4) The most important taxonomic information occurs at the tail. The tail of the male is curved at has two spicules, the left and right ones. The right and smaller spicule has a length of 319.70 µm, the left spicule has a length of 105.46 µm (figure 19.C). In males the cloaca was located at the same position as the right spicule. A closer look at the tail of a male *A. filiformis* gives the image of two different papillae at the tail, post- and preanal papillae (figure 19.D). A detailed image of the preanal papillae is shown in figure 19.E. An image of the postanal papillae is shown in figure 19.F. The post- and preanal papillae have a sensory function. The *A. filiformis* worms are blind and makes use of the post- and preanal papillae to find the attachment site of females. According to a redescription "*Guide to the parasites of fishes of Canada part V Nematoda"* (Arai and Smith., 2016), male *A. filiformis* can grow up to a length between 9.0 – 14.7 mm long with a width of 0.077 – 0.13 mm.

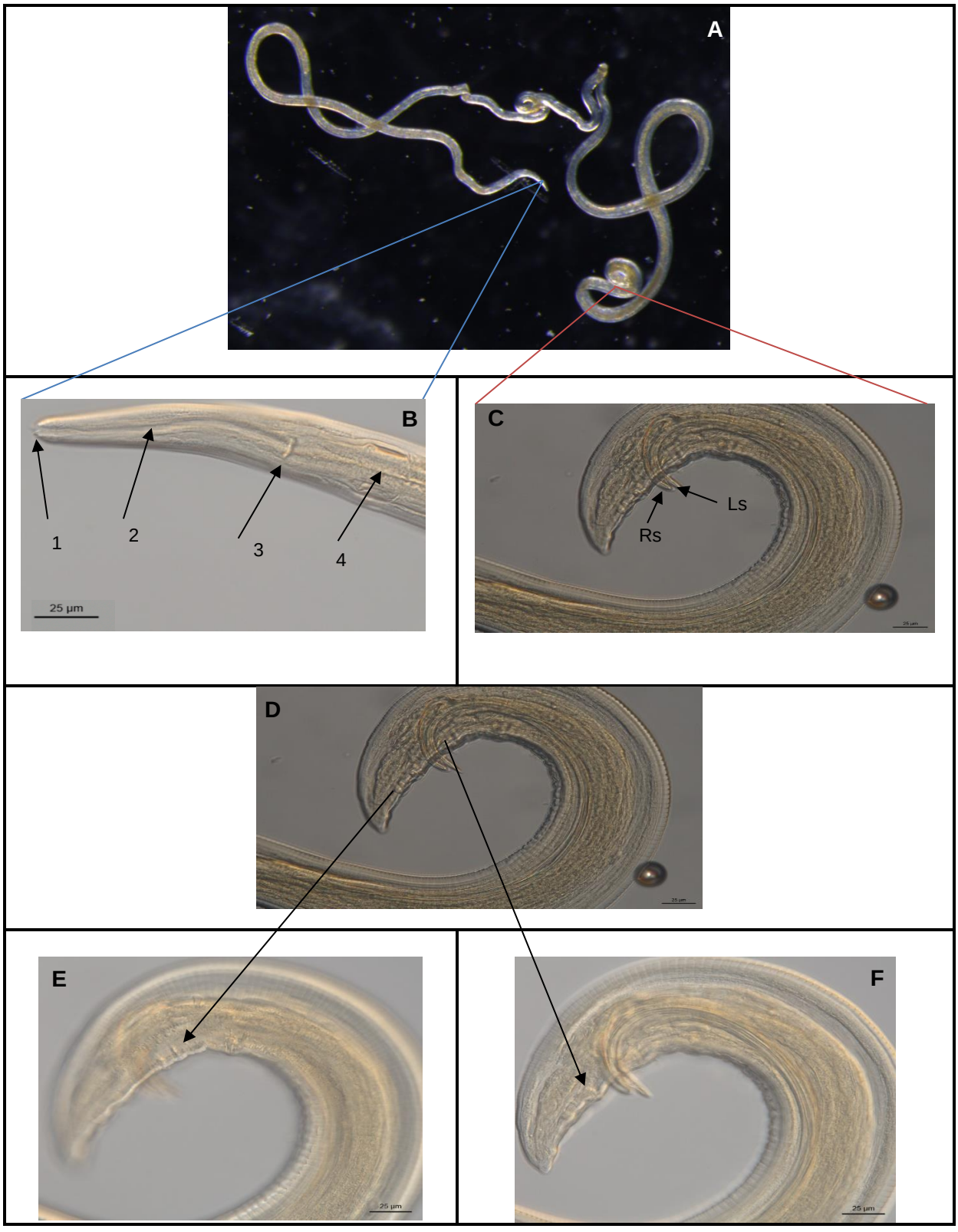

Figure 19. Morhophical identification of male *Ascarophis filiformis* in lateral view. **A,** Full body of a male; **B,** head of a male (1. Mouth with two lips, 2. Vestibulum, 3. Oesophagus, 4. Nerve ring); **C,** male tail with the right (Rs) and left spicule (Ls); **D,** post- and preanal papillae; **E,** preanal papillae; **F,** postanal papillae.

4.4.2.Female *Ascarophis filiformis*

The total length of the female was 10.97 mm with a width of 0.10 mm. Figure 20.A1 shows the mouth with lips, vestibulum, oesophagus and the nerve ring embracing the pharynx is shown in figure. The eggs in *A. filiformis* were organized in multiple rows(figure 20.B). The vulva is located near the anal region and is used to push out the eggs. Figure 20.C gives a detailed view of the female tail. The end of the uterus is a closed segment. At last, the anus is present for the removal of waste (figure 20.C). According to the keys used for the identification, *A.filiformis* females can reach a length up to 35.9 mm long with a width of 0.2 mm.

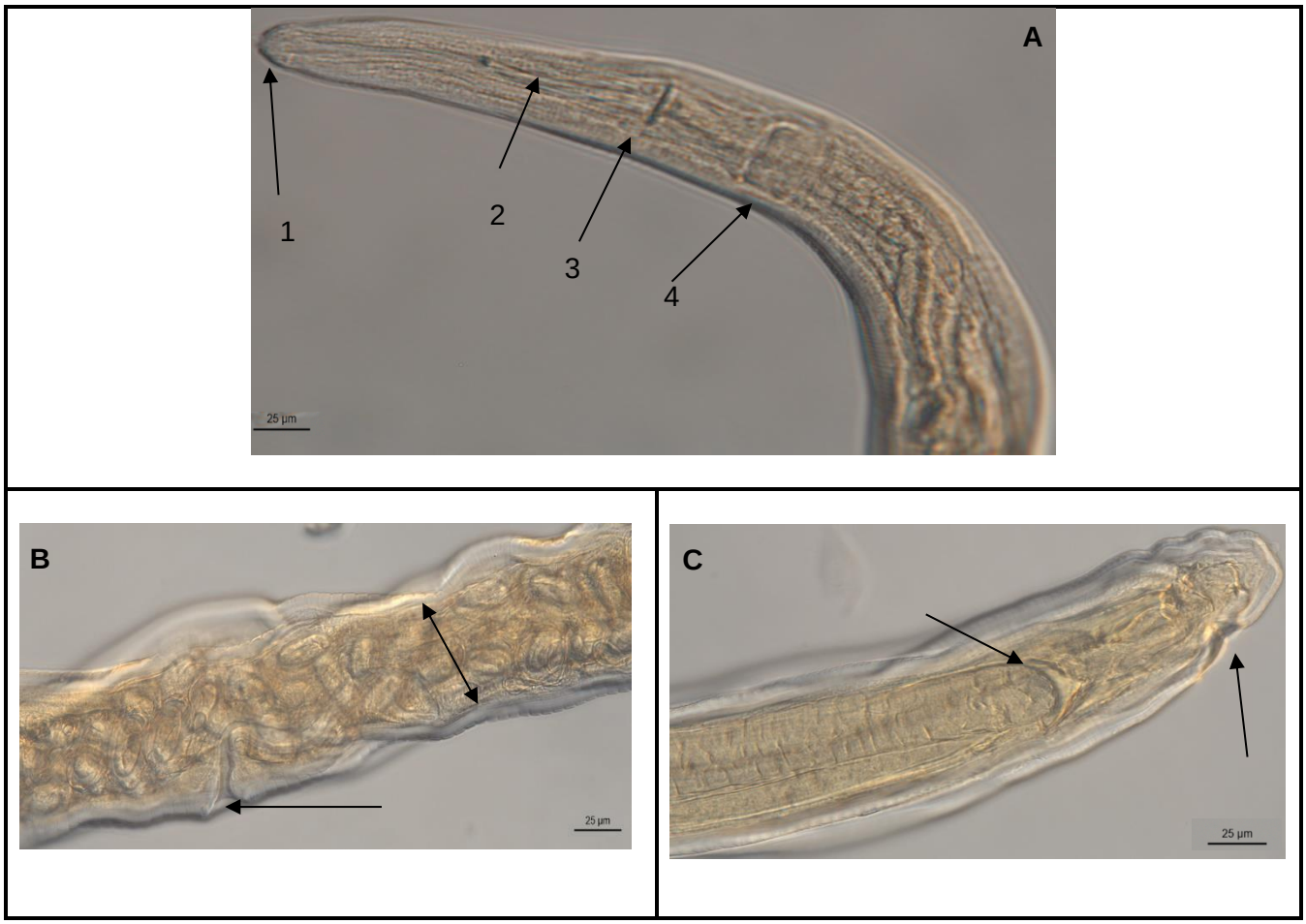

Figure 20. Morhophical identification of female *Ascarophis filiformis* in lateral view. **A,** Head of a female (1. Mouth with two lips, 2. Vestibulum, 3. Oesophagus, 4. Nerve ring); **B,** vulva with three rows of eggs in the uterus; **C,** end of the uterus with the anus.

4.5. SEM image of *Ascarophis filiformis*

To complete the morphological identification, a total of eight specimens (four males and four females) of *A. filiformis* were used for SEM analysis. The specimens were selected based on their condition and stored in 100% ethanol. The morphological identification of a female worm of *A. filiformis* consists of a mouth with two lips. Around the head, two amphids and papillae are present (figure 21.A). At the end of a females tail, there are the presents of an anus (figure 21.B).
The morphological identification of a male *A. filiformis* consisted of a mouth with two lips and two amphids and papillae around the head (figure 21.C). Male tails were curved and had two spicules that, together with some kind of suckers were used to attach to the female (figure 21.D). Underneath the male tail there were post- and preanal papillae (figure 21.E).

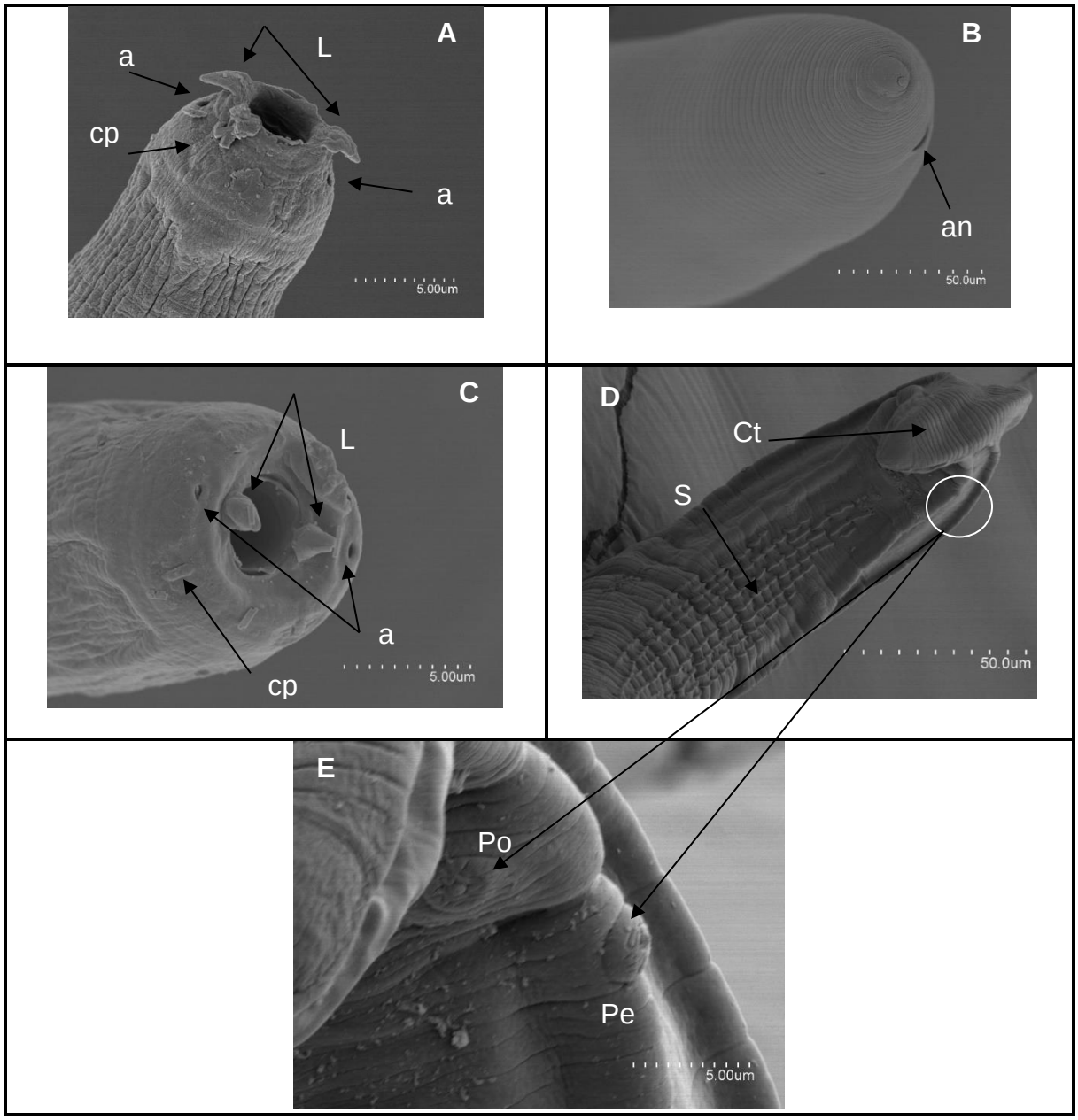

Figure 21. SEM image of a female and male *Ascarophis filiformis*. **A,** Head of a female (L, lips; a, amphid; cp, cephalic papillae); **B,** End of the female tail with the anus (an); **C,** Head of a male (L, lips; a, amphid; cp, cephalic papillae), **D,** Curved tail (Ct) with suckers (S) for attachment; **E,** Detailed image of pre (pe) – and postanal papillae (po).

4.6. Staining of *Derogenes* (*Digenean*)

Five digenean were found in *S. porcus*. The parasite *Derogenes* spp. belongs to the platyhelminth subclass of Digenean. The full body of *Derogenes* spp. (figure 22.A) consisted of a mouth with the oral sucker (figure 22.B), located at the tip of the anterior body and surrounding the mouth. The ventral sucker (figure 22.C) was located halfway through the body of *Derogenes* spp. on the ventral side. Both suckers, oral and ventral are used for attachment to the intestinal wall and blood vessels. The *Derogenes* spp. eggs (figure 22.D) were small and oval in size.

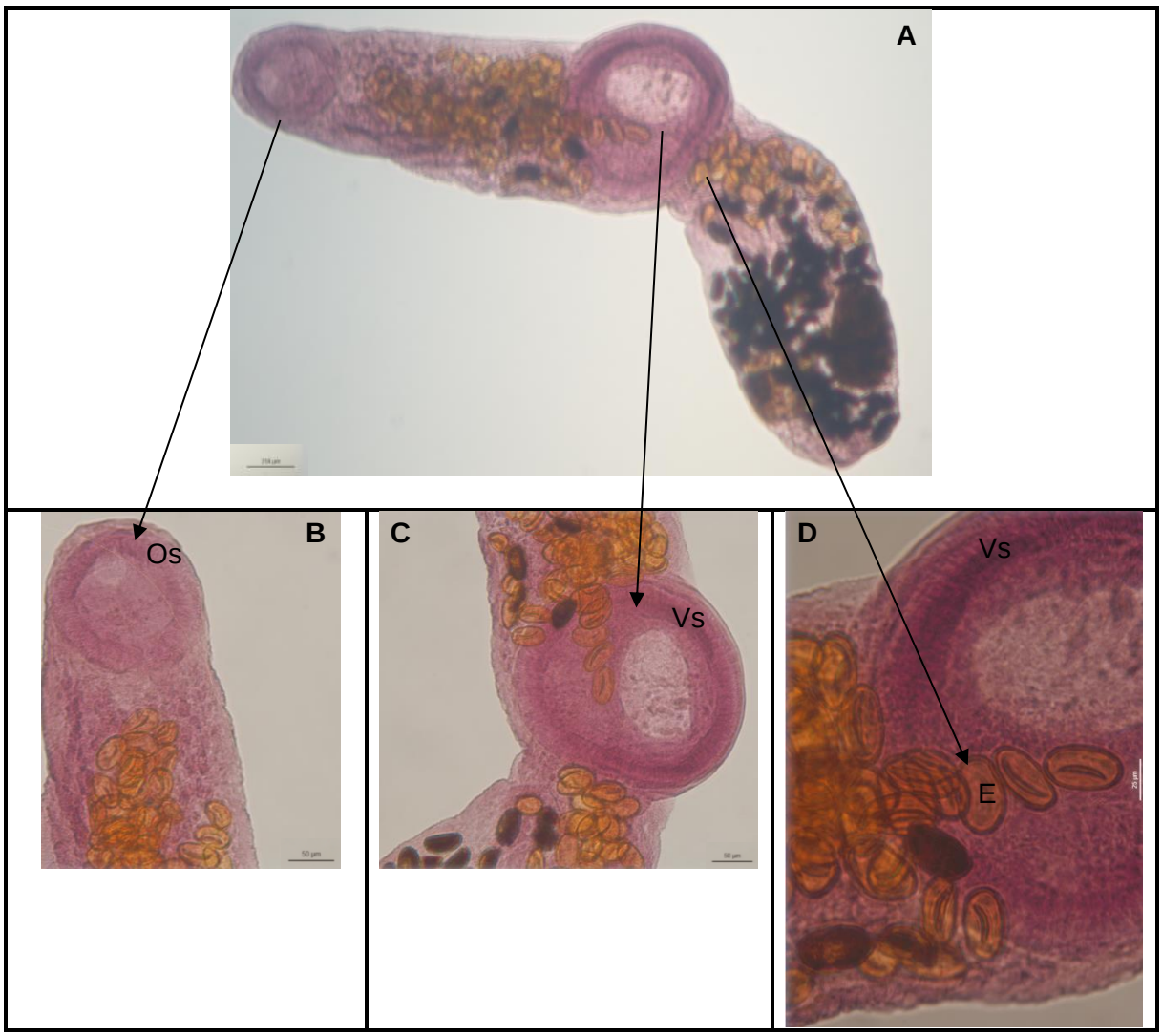

Figure 22. Iron acetocarmine stain of *Derogenes* spp. **A,** Full body; **B,** The oral sucker (Os) surrounding the mouth; **C,** The ventral sucker (Vs) is located halfway through the body; **D,** Eggs (E) are small in size and are oval-shaped.

4.8. Histological analysis Scorpaena porcus eyes

Two Infected and two non-infected *S. porcus* eyes were collected out of the specimens and preserved in 70% ethanol for further examination. structures and cells were visible making a sagittal cut of the retina (figure 23.A) and the crystalline lens. The transverse cut of the sclera (figure 23.B) reveals after staining the outer layer, suprachoroid lamina. Inside the sclera, there is the presence of chondrocytes . The last part of the sclera is a dense connective tissue that lies under the chondrocytes. A transverse cut of the lateral retina (figure 23.C) reveals after staining the outer layer, the pigment epithelium. Under the pigment epithelium, is the layer of cones and rods, the photoreceptor layer . The next two layers of the retina are the inner nuclear and the inner plexiform layer. Underneath the inner nuclear and inner plexiform layer, is the layer of ganglion cells. The last layer of the retina is the layer of the optic nerve fiber.

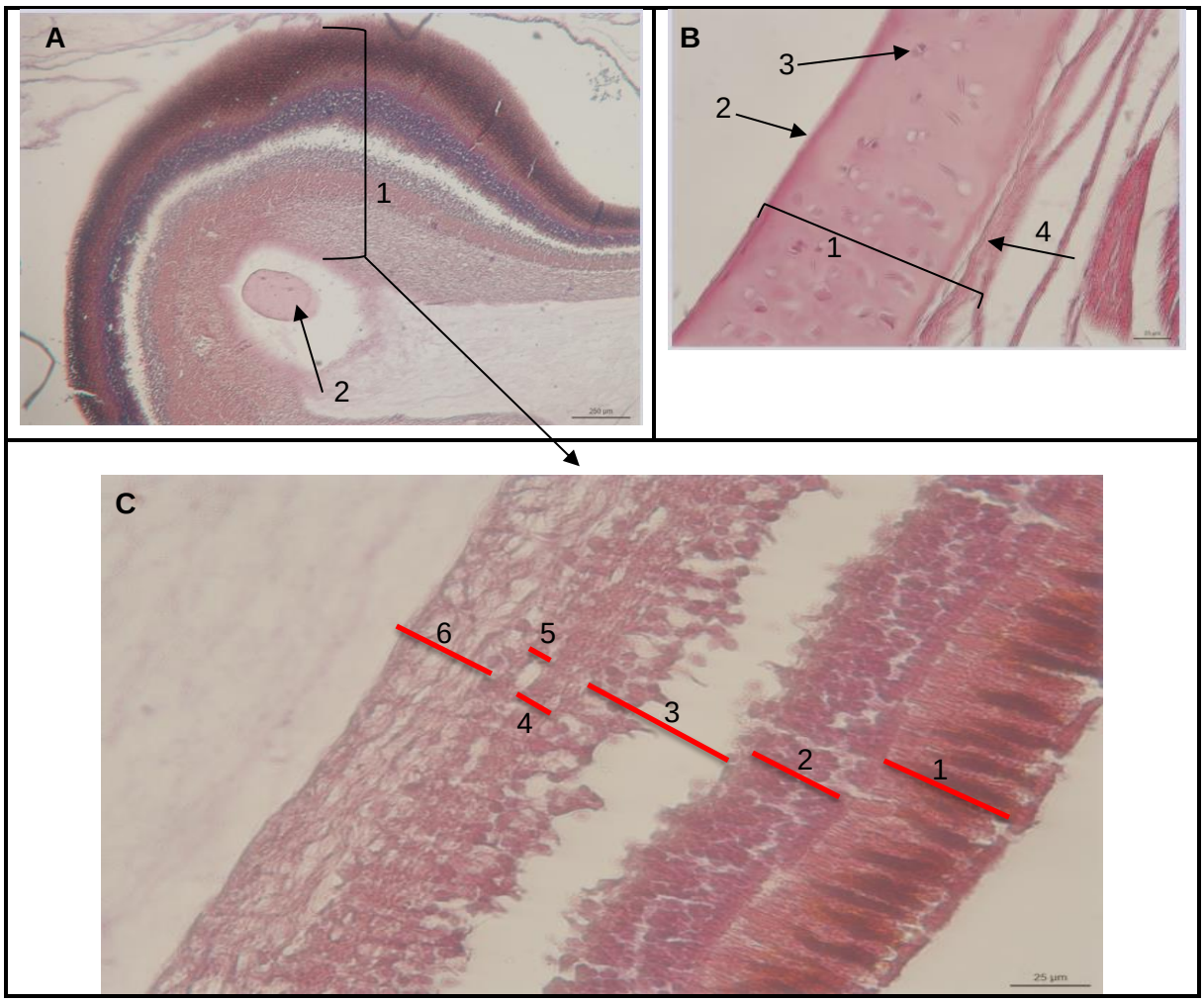

Figure 23. Hematoxylin-eosin stain of *Scorpaena porcus* eyes. **A,** Sagittal section of the retina (1) and posterior crystalline lens (2); **B,** transverse section of the sclera (3). (1. Chondrocyte, 2. Suprachoroid lamina, 4. Dense connective tissue), **C,** transverse section of the lateral retina. (1. Pigment epithelium, 2. Photoreceptor layer (cones and rods), 3. Inner nuclear layer, 4) Inner plexiform layer, 5. Ganglion cell layer, 6. Nerve fiber layer).

5. Conclusion

The main objective is the identification of *Ascarophis* spp. in *S. porcus* by morphological analysis. The specimens of *S. porcus* were caught in different locations on the Mediterranean Sea, and different dates. After the dissection of the 24 specimens, several parasites were collected. The collected parasites were nematodes, hirudineans, platyhelminthes, and arthropods. The focus of the thesis lies on the nematodes (*Ascarophis* spp.) in *Scorpaena porcus*. A total of 125 nematodes were found in the stomach wall, stomach lumen, or the intestine of *S. porcus.* To make a morphological identification, the *Ascarophis* sp. were separated based on size and gender. The identification took place using different microscopical techniques, as optical microscope and Scanning electron microscope. *Ascarophis filiformis* was identified with a Leica DMR optical microscope equipped with a Leica DMC 5400 camera. The eggs within the uterus are organized in a row of four. The *A. filiformis* eggs were removed out of the uterus by a scalpel and were examined under the optical microscope. The eggs have a small, oval-shaped size with two filaments at one pole. Both, male and female were found in the stomach. The male specimen was measured at a length of 9.73 mm with a width of 0.08 mm. The tail of the male is curved and uses two spicules for attachment to the female. The left spicule was measured at a length of 319.70 µm and the right spicule has a length of 105.46 µm. The female specimen was measured at a total length of 10.97 mm with a width of 0.10 mm. By performing a histological analysis, an attempt was made to identify the entrance of the nematode into the eyeball of *S. porcus*. The histological analysis only led to the concealment of the different layers of the retina.

The identification of *A. filiformis* in *S. porcus* is based on different microscopical techniques and the use of keys for identification. After the identification, a second conclusion was made. *Ascarophis filiformis* has a new host and a new location. This conclusion is based on comparing the locations described in *WORMS*, EOL*,* and other databases. *A. filiformis* has been sighted in the Atlantic and the Pacific Ocean, the Barents Sea, and the Sea of Japan and uses the following hosts: *Boreogadus saida, Gadus morhua, Hippoglossus stenolepis, Liparis dennyi, and Reinhardtius hippoglossoides*.

For further research of fish parasites, the aim can be DNA extraction of formalin-fixed tissue. To identify parasites in marine species, DNA extraction can be necessary in some cases. Marine species are preserved for short-term or long-term research in formalin or another fixative. By preserving marine species in formalin, the DNA can be broken off and results in extracted DNA of less quality. Many studies have shown that DNA extraction of fixed tissue is a possibility, but this occurs at a low chance. The morphological identification combined with the DNA extraction of formalin-fixed tissue can result in a more resulting identification.

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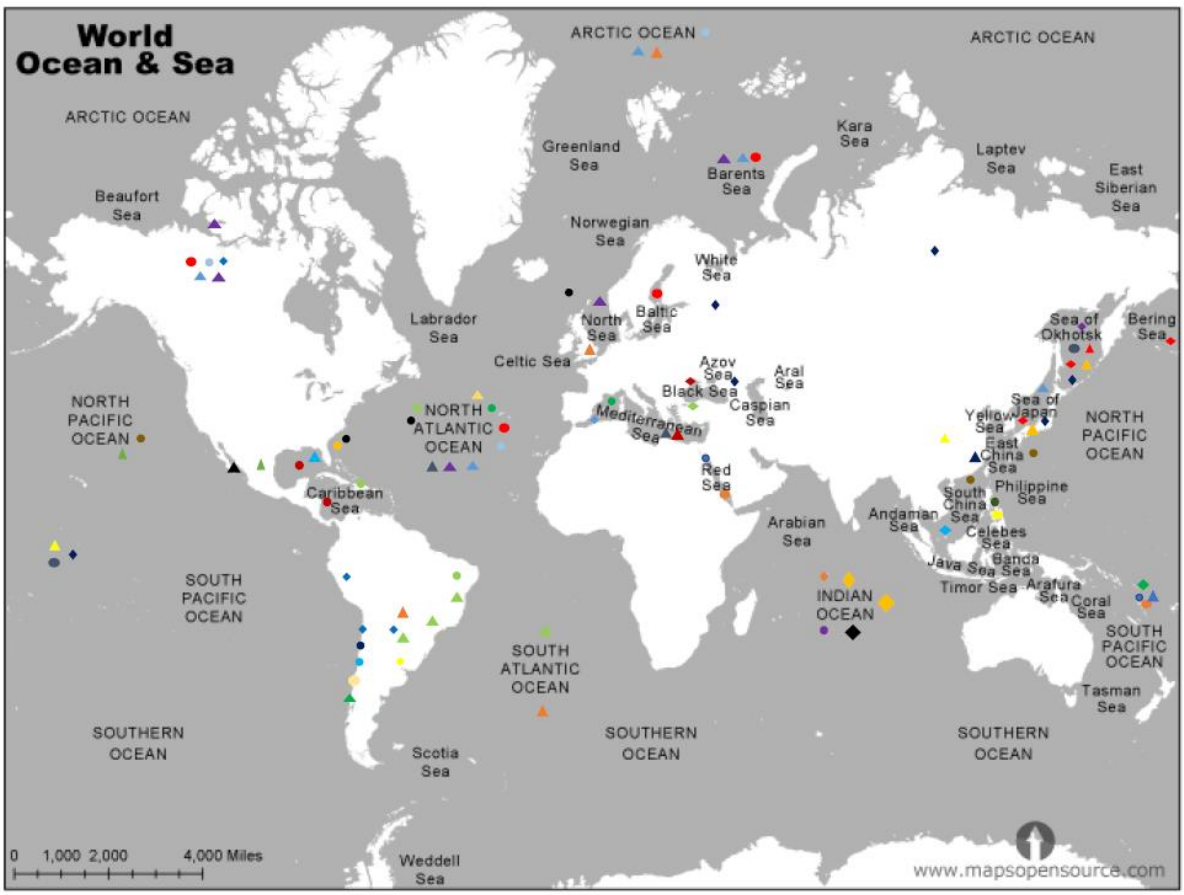
Addendum

Geographical location of all *Ascarophis* species



Legend

Ascarophis adioryx	*Ascarophis filiformis*	*Ascarophis pacifica*
Ascarophis arctica	*Ascarophis girellae*	*Ascarophis parupenei*
Ascarophis ayalai	*Ascarophis gymncranii*	*Ascarophis patagonica*
Ascarophis beaveri	*Ascarophis japonica*	*Ascarophis pontica*
Ascarophis beryx	*Ascarophis litoralica*	*Ascarophis prosper*
Ascarophis brasiliensis	*Ascarophis longiovata*	*Ascarophis richeri*
Ascarophis capelanus	*Ascarophis longispicula*	*Ascarophis scatophagi*
Ascarophis carvajali	*Ascarophis malmae*	*Ascarophis sebastodis*
Ascarophis cestus	*Ascarophis marina*	*Ascarophis skrjabini*
Ascarophis chilensis	*Ascarophis maulensis*	*Ascarophis slankisi*
Ascarophis cooperi	*Ascarophis mexicana*	*Ascarophis sobolevi*
Ascarophis crassicollis	*Ascarophis minuta*	*Ascarophis tropica*
Ascarophis curvicauda	*Ascarophis morrhuae*	*Ascarophis upenei*
Ascarophis distorta	*Ascarophis morronei*	*Ascarophis upeneichthys*
Ascarophis draconi	*Ascarophis mullus*	*Ascarophis valentina*
Ascarophis epinepheli	*Ascarophis nasonis*	
Ascarophis erythrichthys	*Ascarophis nototheniae*	
Ascarophis extalicola	*Ascarophis orientali*	