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**An integrative approach to investigate the biodiversity of food-web
transmitted endoparasites in European waters: the case of anisakids and
trypanorhynch**

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1. Introduction

European waters encompass a diverse and ecologically significant network of marine ecosystems, including the Mediterranean Sea, the North Sea, Baltic sea, Norwegian Sea, and the northeastern Atlantic Ocean, each of which plays a critical role in shaping the region's biodiversity and ecological dynamics. The European waters are recognized as a hotspot of marine biodiversity, currently subjected to numerous anthropogenic pressures. These include seawater warming, pollution, overexploitation of marine resources, bycatch, intensified maritime activity, and the introduction of non-native species. Among its diverse biological biodiversity, European waters ecosystems encompass a significant aspect of the marine environment: food-webs transmitted endoparasites (TTHs). These parasites form a major component of both pelagic and benthic food webs. The TTHs community includes numerous species of digeneans, trematodes, cestodes, nematodes, and acanthocephalans, previously described by various authors (Euzet, 1959; Petter and Radujkovic, 1989; Bona et al., 1995; Paggi, 2008; Radujkovic and Sundic, 2014; Gay et al., 2018; Cipriani et al., 2015). Studying TTHs biodiversity and their ecological roles offers valuable insights into the functioning and health status of European waters marine ecosystems. Among these, Anisakid (Nematodes) and Trypanorhynch (Cestodes) exhibit complex life cycles that depend on specific intermediate and definitive hosts to successfully complete their developmental stages.

The present thesis aimed to investigate by using an integrated approach, food-webs transmitted endoparasites (TTHs) to add knowledge on the state of the biodiversity within the marine ecosystems of European waters. Anisakidae (Nematoda) and Trypanorhyncha (Cestoda), endoparasites, as adults, of cetaceans and elasmobranchs respectively, with crustaceans, squids, and fish as intermediate/transport hosts, are the target parasite species. Furthermore, considering those target parasites as indirect ecological bioindicators to assess the marine food webs integrity and therefore the health status of marine ecosystems in European waters. This is based on the hypothesis that the parasitic load, genetic variability, and host effects of these endoparasites are correlated with the abundance of host populations, specifically target fish, squid, and invertebrate species, which are interconnected through trophic relationships (Mattiucci et al., 2018).

State of the Art

European waters represent a complex and interconnected network of marine ecosystems, spanning several Sea waters. The North Sea, represents a highly productive and dynamic marine region, distinguished by its shallow bathymetry and pronounced tidal currents. This ecosystem sustains extensive commercial fisheries, significant populations of seabirds, and diverse marine mammal communities. However, it is also subject to substantial anthropogenic pressures, including industrial

activities, offshore energy extraction, and agricultural nutrient runoff, which have contributed to environmental challenges such as eutrophication and habitat degradation. The northeastern Atlantic Ocean, which serves as a biogeographical connector to the broader Atlantic basin, encompasses a wide range of ecologically significant habitats, including deep-sea environments, cold-water coral reefs, and pelagic zones. This region functions as a critical migratory pathway for numerous species, such as cetaceans and seabirds, while also playing a pivotal role in global thermohaline circulation and climate regulation processes. The Mediterranean Sea, covering just 0.8% of the global ocean surface, is a region of exceptional ecological significance, hosting approximately 7.5% of the world's marine species (Coll et al., 2010) with a high degree of endemism due to its unique geological history and environmental conditions. Its warm, nutrient-poor waters support a wide range of habitats, from seagrass meadows to deep-sea corals, making it a vital area for marine life.

Despite extensive research focusing on its biodiversity and complex ecosystem dynamics, the community of food-web-transmitted endoparasites (TTHs) remains an understudied yet ecologically significant component of these marine systems. These parasites occupy multiple trophic levels, cycling through hosts that range from crustaceans and teleost fish to apex predators like cetaceans and sharks. Despite their ecological importance, much of the diversity within this group remains unexplored. Advances in taxonomy, particularly the integration of molecular and morphological methods, have led to significant progress in identifying these species in these taxonomic groups (Mattiucci et al., 2018). However, substantial gaps persist, not only in identifying their biodiversity but also in understanding their ecological roles and responses to environmental change.

European water ecosystems are subjected to an array of anthropogenic pressures, including global warming, overfishing, bycatch, intensified maritime traffic, eutrophication, and land-based pollution (Mandić and Piraino, 2023). These stressors not only disrupt the population dynamics and behavior of marine organisms but also influence critical ecological processes such as migration routes, reproductive strategies, and habitat availability for species serving as hosts in the life cycle of TTHs. As a result, such disruptions have negative effects on the stability and structure of parasite populations (Marcogliese, 2005; Lafferty et al., 2008; Timi and Poulin, 2020). Examining the intricate relationships between TTHs and their hosts within the European waters, we can gain valuable insights into the broader ecological impacts of human-induced stressors. These host-parasite dynamics offer a unique data for assessing the 'health status' of the marine ecosystem and its resilience to environmental pressures, providing a novel approach to ecosystem monitoring and management.

According to a recent study by Palomba et al. (2023), nematodes and cestodes have been identified as the dominant taxa of food-web-transmitted endoparasites (TTHs) in Mediterranean waters. Over the past decades, these helminths have gained scientific interest due to their frequent presence, particularly in their larval stage, in commercially important marine species such as fish and mollusks from the

Mediterranean Sea (Cancellieri et al., 2023). Research on certain TTHs has been driven by the zoonotic significance of some nematode species, such as *Anisakis pegreffii* (Mattiucci et al., 2018), which are widespread in Mediterranean wild fisheries. Other TTHs, such as Trypanorhynch cestodes, are not considered zoonotic but can make seafood less appealing to consumers, reducing the quality and market value of these products (Bao et al., 2020; Rudders et al., 2023). Among the larval trypanorhynchs (cestodes) frequently found with high infection rates are *Gymnorhynchus gigas*, *Molicola* spp., and *Grillotia* spp., in various intermediate and definitive hosts (Palomba et al 2021; Santoro 2018, 2021, 2022a, 2022b). These parasites represent a widely distributed group in the Mediterranean Sea, making them potential candidates for use as bioindicators or sentinel species to assess the health of marine ecosystems. Furthermore, recent advancements in genetic and molecular methodologies have enabled species-level identification of many of these parasites, enhancing their relevance (Isbert et al., 2023). The life cycle of *Anisakis* spp. inhabit a wide range of pelagic and coastal environments. Unlike trypanorhynchs that develop in several intermediate/paratenic and definitive hosts (e.g., teleost fish, small to mid-sized benthonic shark) that inhabit deep-water habitats. This dual focus on pelagic/coastal and benthic ecosystems provides a comprehensive framework for evaluating the impacts of environmental stressors across different habitats within the Mediterranean Sea.

This thesis investigates the current state of knowledge regarding the biodiversity and ecological aspects of target food-web-transmitted helminths (TTHs), with a particular focus on Anisakids and Trypanorhynchs in European waters. The taxonomic status of these parasites has been evaluated using integrated morphological and molecular genetic methodologies. The chosen TTHs are heteroxenous parasites that rely on several hosts to complete their life cycle. They can be influenced by hosts densities, ranging from different meta-populations of target invertebrates, through fish/squid species (intermediate/paratenic hosts of both the endoparasite groups), to cetaceans and sharks, when possible (their respective definitive hosts).

Anisakid nematodes are parasites of the alimentary tract of aquatic vertebrates, with indirect life cycles that involve various hosts at different levels across the food webs. In general, marine mammals (mainly cetaceans) serve as definitive hosts, fish and squid serve as intermediate or paratenic hosts, while planktonic or semiplanktonic crustaceans act as first intermediate hosts in the life-cycle of species of the genus *Anisakis* (Mattiucci & Nascetti, 2008; Mattiucci et al., 2018). Humans can be “accidental host”, acquiring infection through consumptions of raw, smoked, marinated salted, or undercooked fish and squids, infected by larval stages (Chai et al., 2005; Mattiucci et al., 2017, 2018). So far, nine species belonging to the genus *Anisakis* have been identified worldwide (Mattiucci *et al.* 2014, 2018). All species have been characterized by distinct diagnostic genetic markers, possess distinct gene pools, and they are reproductively isolated. The epidemiology and distribution of anisakid nematodes is determined

by multiple environmental factors such as the demographic density of the hosts (both intermediate and definitive) that intervene in the biological cycle of the parasites, the parasite load in their hosts, the characteristics of fish stocks and fishing areas (Angot and Brasseur 1995; Mattiucci et al., 2018; Levsen et al., 2018). However, any epidemiological survey on anisakid nematodes is incomplete if inferred only from morphology (Mattiucci & Nascetti, 2008). This highlights the importance of proper identification of biological species within anisakid nematodes inferred from (a multilocus) molecular approach, as a prerequisite for precise assessment of biodiversity.

The order Trypanorhyncha Diesing, 1863 comprises one of the most ubiquitous group of marine metazoan parasites, with more than 270 recognized species inhabiting all the world's oceans (Beveridge & Campbell, 2013; Olson et al., 2010; Palm, 1997). Their life cycle includes several hosts, linked by a prey-predator relationship and thus included in a trophic-web of the marine ecosystem. As adults, they infect the digestive tract of elasmobranchs (sharks and rays), while marine invertebrates (i. e. crustaceans) and teleost fishes act as first intermediate and second intermediate and/or paratenic hosts, respectively (Palm & Caira, 2008). The larval forms of Trypanorhyncha are generally found encysted in visceral organs and/or musculature of marine teleosts. Their occurrence in the muscles alters the fish flesh quality, reducing the marketability of commercially important fish species (Santoro et al., 2018; Oliveira et al., 2019; Palomba et al., 2021). Heavily infected fish fillets result unappealing to consumers; thus, the presence of these parasites causes economic loss to the fishing industry (Abdelsalam et al., 2016; Deardorff et al., 1984; Giarratana et al., 2014; Samn et al., 2014; Santoro et al., 2018; Palomba et al., 2021). Few cases of accidental human ingestion of Trypanorhyncha larvae were reported, showing no clear evidence of pathogenic effects in humans (Bates, 1990; Lima dos Santos & Howgate, 2011).

Aims of the thesis

The research aims to investigate by using an integrated approach, food-webs transmitted endoparasites to add knowledge on the state of the biodiversity of the marine ecosystem in the European waters. Anisakidae (Nematoda) and Trypanorhyncha (Cestoda), endoparasites are the target parasites.

Main objectives of the research work have been the following:

- 1) Infer data on biodiversity, (at species and gene level), distribution patterns, and parasitic load of some anisakids and trypanorhynchs species from sharks, teleosts fish, and squids from different seas across diverse marine environments within European waters. This step was essential for selecting the most suitable candidates for ecological bioindicators. The identification process involved a combination of morphological and molecular techniques to ensure precise taxonomic resolution.

- 2) Investigate the genetic structure of selected endoparasites in European marine waters using different genetic (mitochondrial and nuclear) markers. Following the identification of the predominant species in the region, some members of Trypanorhynchs were selected as target species to be proposed as indirect

ecological bioindicators. Their potential as bioindicators is assessed based on their role within the trophic web, providing insights into the health status of the marine ecosystem. This approach also aimed to analyze how host population structure may shape and drive parasite genetic structure.

After an Introduction on the general knowledge on the selected TTHs parasites, the main results achieved concerning the systematic, genetic structure and ecological aspects of the target parasite taxa, as published in different papers within the three years of the PhD course, are presented.

1.1. Anisakids and Trypanorhynchs as ecological indicators in the European waters

1.1.1. Chosen TTHs among nematodes: biodiversity and ecology

Adult *Anisakis* and *Skryabinisakis* nematodes reside in the stomachs of marine mammals, primarily cetaceans. Female nematodes release fertilized eggs into seawater via the feces of their definitive hosts. These eggs embryonate in the water, undergo one or two molts within the egg, and subsequently hatch as motile, sheath-covered larvae (Gomes et al., 2023). After a relatively undefined free-living phase, larvae of these genera are ingested by their first intermediate hosts, typically small crustaceans. For *Anisakis* species, these hosts often include krill (Køie et al., 1993, 2001). Specific euphausiids, such as the North Pacific krill (*Euphausia pacifica*, *E. similis*, and *Nyctiphanes couchii*) and Arctic krill (*Thysanoessa raschii*), have been reported to host *Anisakis simplex* (s.s.) (Rudolphi, 1809) and *A. pegreffii* along the Spanish Atlantic coastline (Gregori et al., 2015; Cresson et al., 2023). The third-stage larvae (L3) of *Anisakis* species develop in their first intermediate hosts, which are subsequently consumed by various paratenic hosts, including pelagic fish, demersal fish, and cephalopods (Mattiucci et al., 2018b; Tedesco et al., 2018). Once ingested by these paratenic hosts, L3 larvae may either remain encapsulated outside the visceral cavity or migrate into the muscle tissues, as observed in *A. pegreffii*, *A. simplex* (s.s.), and *Anisakis typica* (s.l.) (Diesing, 1860) (e.g., Mattiucci et al., 2018; Levsen et al., 2018; Cipriani et al., 2022).

Third-stage larvae (L3) of *Skryabinisakis physeteris* are commonly found in stranded sperm whales along the coasts of the Tyrrhenian, Ionian, and Aegean Seas, as well as the Croatian Adriatic coast (Mattiucci et al., 1986; Cipriani et al., 2022b; Blažeković et al., 2015). Significant infestations of *A. physeteris* were observed during sperm whale stranding events in 2009 and 2013 along Italy's southeastern coast, where up to 600 worms were found in individual whales (Mazzariol et al., 2018; Cipriani et al., 2022b). Adult *S. physeteris* was also identified in a stranded dwarf sperm whale along the Tyrrhenian Sea coast (Santoro et al., 2018a). This parasite appears to prefer hosts within the Physeteridae family, likely due to co-evolutionary factors and the feeding behaviors of these whales (Mattiucci and Nascetti, 2008). While *S. physeteris* has been rarely detected in fish, higher infection rates have been observed in deep-sea squids, especially those from the Histiotethiidae family, which are an important food source for sperm whales (Palomba et al., 2021a). This suggests that large, deep-dwelling cephalopods play a key role in the parasite's life cycle and distribution. Additionally, stranding records showing sperm whale stomachs filled with beaks of various squid species support the hypothesis that *A. physeteris* follows a bathypelagic life cycle (Mattiucci et al., 2018b). These findings underscore the critical role of cephalopods in the parasite's transmission and ecological dynamics.

Another species of *Anisakis* that meets the criteria of an indicator taxon is *Anisakis typica* (s.l.). Originally described in the Central Atlantic (off the coast of Brazil) and the Indian Ocean (Mattiucci et al., 2002), it has recently been identified as a complex of sibling species based on genetic and molecular

studies (Cipriani et al., 2022a). These include two distinct species, *A. typica* sp. A and *A. typica* sp. B, both of which occur in the Indian Ocean at the larval stage in various fish species. This species complex is found in warmer temperate and tropical waters, ranging from 35–40°N to 36°S. Currently, only *A. typica* sp. A has been recorded in the Mediterranean, where it has been detected in the warmer waters off Cyprus, Crete, the Aegean Sea, and Tunisia (Mattiucci et al., 2004, 2018, 2018; Farjallah et al., 2008). This species has been observed as an adult in striped dolphins and at the larval stage in fish from both pelagic and mesopelagic habitats (Mattiucci et al., 2004, 2018; Cipriani et al., 2018a).

In comparison, *Anisakis ziphidarum* is less frequently found in the Mediterranean, mainly associated with its definitive hosts, the beaked whales (Mattiucci et al., 2018b). The first Mediterranean record of this species was in a Cuvier's beaked whale (Paggi et al., 1998). It is rarely found in fish, but some deep-sea fish, such as the myctophid *Diaphus metopoclampus* in the Ionian Sea, have been infected (Gaglio et al., 2018). This species is known to prefer deeper waters (>200 m), which aligns with habitat modeling for Cuvier's beaked whale populations in the Mediterranean (Podestà et al., 2016), indicating that deep-sea cephalopods likely play a role in the parasite's life cycle. The low frequency of *A. ziphidarum* records in the Mediterranean is probably due to the limited presence of beaked whale populations in the region, as well as the limited focus on parasitological studies of deep-sea species.

Anisakis simplex (s.s.) is typically found in Arctic and Boreal regions and is seldom reported in Mediterranean waters. It has occasionally been detected in its larval form in fish from the Alboran Sea (Mattiucci et al., 2018b; Molina-Fernández et al., 2018; Roca-Geronès et al., 2021), which is more connected to Atlantic waters than the Mediterranean. This species is also present in the eastern Mediterranean, likely linked to the migratory pathways of intermediate and definitive hosts, such as bluefin tuna and striped dolphins (Mladineo and Poljak, 2014; Blažeković et al., 2015). Adult *A. simplex* (s.s.) is extremely rare in the Mediterranean, likely due to temperature constraints that limit the hatching of eggs and the survival of larvae (Gomes et al., 2023). An adult specimen was recorded in a striped dolphin from the Sicilian coast, where it was co-infected with *A. pegreffii* (Cavallero et al., 2014).

1.1.2. Chosen TTHs among cestodes: biodiversity and ecology

The diversity of cestodes in the European waters is mainly studied in relation to those found in elasmobranchs, though much of the available data is based on older records. Most of the documented cases involve these parasites in intermediate or paratenic fish species. Recent research utilizing a more comprehensive taxonomy approach has shown that the distribution and variety of cestodes in the Mediterranean are more extensive than previously thought. Among these, trypanorhynch tapeworms are the most commonly reported. Other groups such as Tetraphyllidea, Oncoproteocephalidea, Rhinebothriidea, and Phyllobothriidea have historical significance but have not been the focus of recent investigations. Historical data suggest that the Mediterranean is a key area for trypanorhynch diversity,

containing about 14% of the known species globally. The most abundant families in the region are Lacistorhynchidae (14 species), Eutetrarhynchidae (12 species), and Tentaculariidae (5 species), collectively accounting for around 85% of all trypanorhynchs found in the Mediterranean.

These cestodes typically have complex life cycles, involving various marine organisms at different trophic levels. Different species have adapted unique life-cycle strategies, allowing them to occupy distinct ecological niches, including in terms of their vertical distribution (Palm, 2004). Many species show a clear preference for certain definitive hosts, which aligns with the feeding habits and depth range of those hosts (Palm and Caira, 2008). Their life cycle generally consists of several stages, such as proceroid, plerocercus, and adult forms, with some species also including a merocercoid stage.

Two potential life cycle models for cestodes have been suggested (Palm, 2004). In the first model, the cycle involves three hosts: copepods as the initial host, followed by invertebrates as the second intermediate host, as seen in Eutetrarhynchidae. Alternatively, copepods can be the first host, with pelagic euphausiids as the second intermediate host, as observed in Gymnorhynchidae. In the second model, copepods are the first host, and fish serve as the second intermediate host, a pattern commonly found in Lacistorhynchidae. A well-documented example of this is the life cycle of *Grillotia erinaceus* (Lacistorhynchidae), where operculate eggs released into seawater hatch into coracidia, which are consumed by copepods, the first intermediate hosts. The plerocercus then develops in fish, particularly Gadiformes, and the definitive hosts are Raja species (Palm, 2004).

The most frequently reported cestodes in the Mediterranean Sea include *Molicola uncinatus* (Gymnorhynchidae) and *Grillotia gigas* (Lacistorhynchidae). *M. uncinatus* has been recorded in several global regions, such as New Zealand, South Africa, and the Mediterranean. In the latter, it has been found in species like the silver scabbardfish (*Lepidopus caudatus*) and the Atlantic pomfret (*Brama brama*), with high parasitic burden indicating that the species' life cycle is functioning well in the Mediterranean (Palm, 2004; Palomba et al., 2021b; Santoro et al., 2022b). The common thresher shark (*Alopias vulpinus*) is considered its definitive host, though no infection data from stranded individuals in the Mediterranean are available. This shark species is listed as critically endangered in the region.

G. gigas has been observed in Mediterranean sharks, including the great white shark (*Carcharodon carcharias*) and the porbeagle shark (*Lamna nasus*). Larvae of *G. gigas* have been found in the Atlantic pomfret and silver scabbardfish in both the Tyrrhenian and Ionian Seas (Manfredi et al., 1993; Giarratana et al., 2014; Palomba et al., 2021b; Santoro et al., 2022b). Larvae of *M. uncinatus* and *G. gigas* can coexist in the same environments, but identifying them through morphology alone can be challenging, especially when their tentacles are invaginated. In such cases, molecular techniques are essential for accurate species identification. Furthermore, *Gymnorhynchus isuri* has been recently reported in *Mola mola* in the Mediterranean (Santoro et al., 2020b). This species, typically found in the Atlantic and Pacific, likely reached the Mediterranean through the migration of sunfish.

The genus *Grillotia* (family Lacistorhynchidae, Guiart, 1927), comprises several species that infect the digestive tract of elasmobranchs (Beveridge & Campbell, 2013; Palm, 2004). The tentacular apparatus serves as a dichotomous key, allowing the genus to be divided into two well-defined groups (the true species of *Grillotia* and those in the subgenus *Christianella* Guiart, 1931), along with a third, more heterogeneous group, *Grillotia* sensu lato (Beveridge & Campbell, 2007, 2010, 2013). Morphologically, the larvae and adults of *Trypanorhyncha* are characterized by the presence of two or four bothria and a tentacular apparatus comprising four retractile tentacles equipped with hooks (Palm et al., 2007; Rohde, 2005; Palm et al., 2009). According to the most recent taxonomic revision, 18 species are currently recognized within the genus *Grillotia* (Beveridge and Campbell, 2007; 2013).

Nine species of *Grillotia* (Lacistorhynchidae) have been recorded in the Mediterranean, with *G. heptanchi*, *G. adenoplusia*, and *G. erinaceus* being the most notable. *G. heptanchi* and *G. adenoplusia* have been found in sharks, while *G. erinaceus* occurs in skates and rays (Beveridge and Campbell, 2013). Conversely, *Grillotia scolecina* (Rudolphi, 1819), previously described in this region, has been designated as a species inquirenda (Beveridge and Campbell, 2013). Furthermore, two additional undescribed representatives of *Grillotia* have been recently recorded from the Mediterranean hosts *Lophius piscatorius* and *Galeus melastomus* (Santoro et al., 2018; 2021). Molecular data are available for these two unidentified taxa from the Mediterranean, as well as for three other species: *G. pristiophori* Beveridge and Campbell, 2007, *G. erinaceus*, and *G. (Christianella) yuniariae* Palm, 2004, from the North Atlantic and West Pacific (Palm et al., 2007; 2009).

Until recently, molecular identification of *Grillotia* in European waters was limited, with *G. erinaceus* being the only species previously identified. However, advancements in molecular techniques have expanded our understanding. Plerocerci of *G. adenoplusia* have been detected in species like the anglerfish (*Lophius piscatorius*) and deep-water sharks (Dallarés, 2016; Santoro et al., 2021). These plerocerci were confirmed to be *G. adenoplusia* through both morphological and molecular methods (Isbert et al., 2023). *G. adenoplusia* plerocerci were found in the blackmouth catshark (*Galeus melastomus*), Portuguese dogfish (*Centroscymnus coelolepis*), and velvet belly lanternshark (*Etmopterus spinax*) off Spain's western Mediterranean coast (Dallarés, 2016; Isbert et al., 2023). Infection rates ranged from 17% to 100%, with the blackmouth catshark showing the highest abundance. In the Tyrrhenian Sea, similar infection rates were reported (Santoro et al., 2021a). The larvae are linked to the consumption of myctophid fish, which could act as intermediate hosts (Dallarés, 2016; Santoro et al., 2022). The definitive host for *G. adenoplusia* is the bluntnose sixgill shark (*Hexanchus griseus*) (Beveridge and Campbell, 2013).

1.1.3. Life cycle and host- preference of Trypanorhyncha

The life cycle of is heteroxenous, involving a primary host (typically an invertebrate, such as a crustacean or mollusk), a secondary intermediate or paratenic host (often a teleost or elasmobranch), and a definitive host, usually an elasmobranch (Palm, 2004). As noted by Rohde (2005), some Trypanorhyncha exhibit a ciliated membrane around the first larval stage. This larval stage, known as the coracidium, emerges from the egg and remains free-living for a short period, awaiting predation by the appropriate intermediate host. Upon ingestion, it develops into a proceroid, a larval stage primarily found in invertebrates. These initial intermediate hosts are then preyed upon by vertebrates acting as secondary intermediate/paratenic hosts, where development continues into the plerocercoid stage. Plerocercoids can survive through one or more paratenic hosts, eventually maturing into adults when the host is consumed by a suitable definitive host, where reproduction occurs. Eggs are then released in the definitive host's feces. The Trypanorhyncha's life cycle typically includes a proceroid stage, followed by either a plerocercoid stage in some species or a plerocercus stage in others, and finally the adult stage. Thus, a single Trypanorhyncha traverses at least three different hosts over the course of its life (Dollfus, 1942; Mattis, 1986). Generally, Trypanorhyncha exhibit relatively low host specificity across each life stage (Palm et al., 1997, 2007; Beveridge et al., 2000; Palm, 2004; Palm and Klimpel, 2007). However, detailed studies on certain species have suggested significant variations (Beveridge and Jones, 2000; Beveridge and Duffy, 2005). Relative host specificity of the larval and adult stages was determined based on the host specificity index (HSs), where for each species the HSs value of the adult was subtracted from the HSs value of the final larval stage (Palm and Caira, 2008). Therefore, if the difference value is between -1 and 1, larvae and adults have similar specificity; if it is negative and greater than 1, adults are less specific than larvae; if it is positive and greater than 1, adults are more specific than larvae. Palm and Caira (2008), tell us that, in general, the plerocercoid and plerocercus stages of Trypanorhyncha are significantly less host-specific than the adult stage (Figure 1).

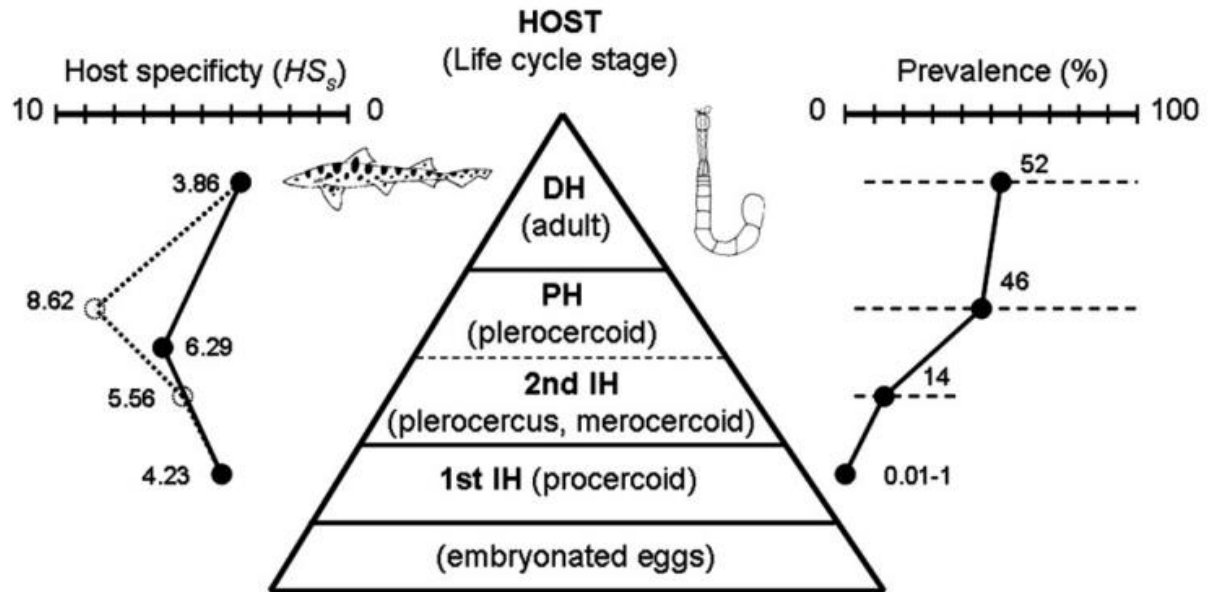


Figure 1: Schematic representation of the relationship between host specificity (HS_x) and prevalence of Trypanorhyncha life cycle stages in the respective hosts. Specificity is highest for adults in the definitive host, lowest for plerocercoid, plerocercus, in the second intermediate host or paratenic host, and intermediate for proceroid in the first intermediate host. The solid line on the left represents the average HS_x value when the second intermediate and paratenic hosts are combined; the dashed line shows the separate HS_x values. The horizontal dashed lines on the right indicate the prevalence ranges reported in the literature. DH: definitive host; PH: paratenic host; 1st IH: first intermediate host; 2nd IH: second intermediate host (Palm and Caira, 2008).

1.1.4. Biology and Ecology of *Grillotia adenoplusia* (Family Lacistorhynchidae)

Grillotia species are organisms are widely distributed, from brackish waters to great ocean depths, with the highest species diversity found in the tropical coastal waters of the Indo-Australian region (Palm, 2004). Among these, *G. heptanchi* and *G. adenoplusia* have been found as adults in the digestive tracts of pelagic sharks, while *G. erinaceus* has been observed as an adult in the digestive tract of rays. *G. scolecina*, on the other hand, has been described based on plerocercoids collected from *Scyliorhinus stellaris* (Linnaeus, 1758) in the Gulf of Naples; however, available data on this species remain limited (Beveridge and Campbell, 2007, 2010, 2013). All these species, except *G. erinaceus*, are included in the *G. sensu lato* group. These species are characterized by a heteroxenous lifecycle, with unique morphological features at each life stage (Campbell and Beveridge, 1994; Palm, 2004). Adults are typically found in the stomach and intestines of sharks and rays, while larval forms infect a wide range of marine invertebrates and teleosts, with infection sites that can differ from the digestive tract. Recent morphological and molecular evidence indicates that larval stages exhibit low host specificity (Palm & Caira, 2008) and have a broad, sometimes cosmopolitan, geographic distribution (Palm, 2004; Palm et al., 2007). Therefore, these cestodes can serve as model organisms for better understanding ecological and coevolutionary histories with their definitive hosts, given the high host specificity shown by adults. The morphological characteristics distinguishing the order include a scolex (head) with 2 or 4 bothria

(Jones et al., 2004), from which a tentacular apparatus extends, consisting of four retractable, hook-adorned tentacles that likely aid in attachment to host tissues (Dollfus, 1942). Retractor muscles allow for the eversion and retraction of these structures used for movement. This complex attachment apparatus, unique among the cestodes, represents a clear example of synapomorphy, supporting the monophyly of the order. Regarding the life cycle of *G. adenoplusia*, adults inhabit the digestive tract of the host, regularly producing proglottids that mature over time. An adult exhibits a progression of proglottids, starting from the neck (production zone) to the terminal end of the body, where gravid proglottids are located. Gravid proglottids and/or eggs are released into the marine environment through the host's feces. Contact with seawater causes the proglottid body wall to rupture in one or more areas, allowing eggs to escape. These organisms are generally hermaphroditic, although cross-fertilization events -reciprocal fertilization between two hermaphroditic individuals- also appear possible (Olson et al., 2021). Such events promote genetic diversity; however, this theory is still under investigation due to limited data and challenges in studying these organisms and parasites in general.

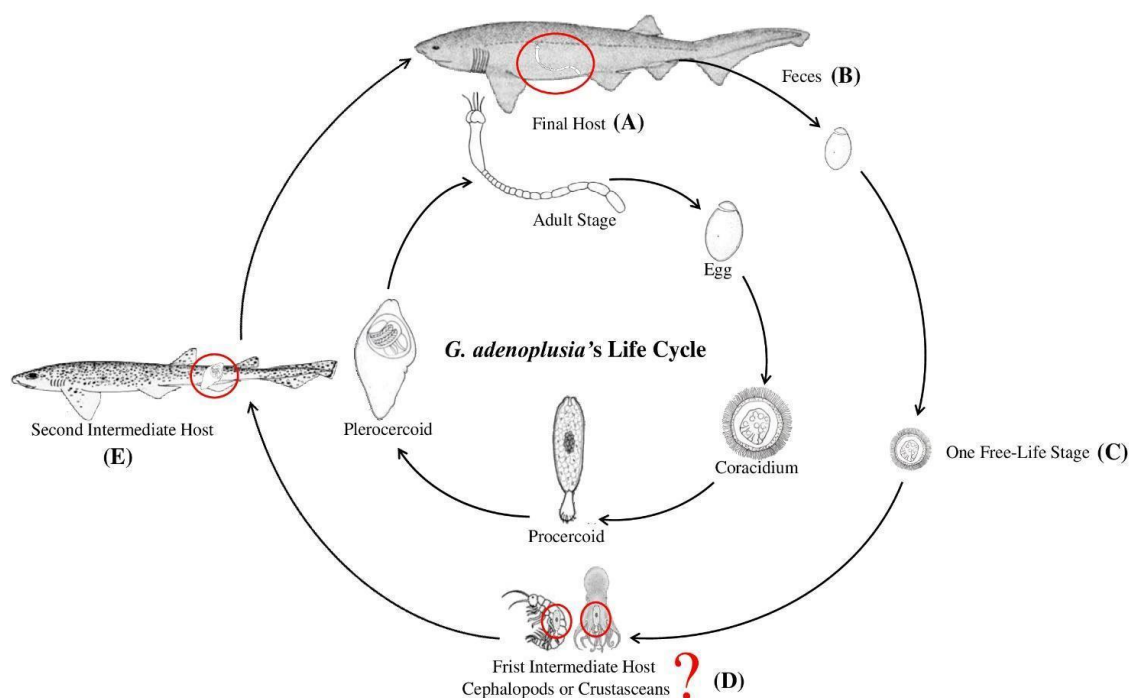


Figure 2: Life Cycle of *G. adenoplusia* . (A) Adult hermaphroditic tapeworms in the gastrointestinal tract of *H. griseus* produce eggs, released via apolysis or anapolysis.(B) Eggs enter seawater through feces, releasing the free-living ciliated coracidium. (C) The coracidium adheres to substrates, increasing chances of ingestion by the unknown first intermediate host (likely a copepods). (D) In the first host, the larva develops into a proceroid stage. (E) When the first host is preyed upon, the plerocercoid develops in the second intermediate or paratenic host, migrating to the caudal tissues. Upon predation by the definitive host, the parasite matures into an adult, completing the cycle.

Considering the factors influencing host infection patterns, parasites can serve as valuable bioindicators. As natural biological tags, parasites provide critical insights into various aspects of host life history (Caira, 1990; Williams et al., 1992) and are particularly recommended for studies focusing on deep-sea and rare marine species (MacKenzie and Abaunza, 1998). Parasites can offer evidence on ecological and biological aspects such as population separation or connectivity, migration patterns, and localized host movements (e.g., Grutter, 1998; MacKenzie and Abaunza, 1998; Mattiucci et al., 2015). These studies hold substantial importance for fisheries management and conservation by aiding the identification of demographically independent fish populations and facilitating an understanding of population differentiation (Gubili et al., 2016). Mattiucci et al. (2015) highlighted the complementary nature of fish population genetics and parasitic bioindicators, where genetic studies reveal changes over evolutionary timescales, and parasites provide insights into host movements on finer temporal and spatial scales.

Phylogeographic approaches based on homologous genes of both hosts and their parasitic biomarkers have been particularly emphasized for investigating fish population structure. In eukaryotic species, nuclear ribosomal genes, which consist of numerous tandemly repeated copies, are frequently employed in phylogenetic studies due to their conserved nature and utility across diverse taxa. The large ribosomal subunit gene (28S rDNA) demonstrates higher variability and divergence among phyla compared to the small subunit gene (18S rDNA) (Hillis and Dixon, 1991). However, the concerted evolution of these ribosomal units results in high intragenomic similarity (Arnheim, 1983), where individual copies within a species are nearly identical, even as interspecies differences accumulate rapidly (Hillis and Dixon, 1991).

Despite its relatively low level of variation compared to other molecular markers, the variability observed in the 28S rDNA region is particularly useful for reconstructing recent evolutionary events. Palm et al. (2007) investigated the genetic diversity of the cestode *Tentacularia coryphaenae* by analyzing the 28S rRNA gene. Their results revealed a high degree of genetic homogeneity across populations, suggesting that the species exhibits a wide distribution and low host specificity. However, the limited intraspecific variation in the 28S rRNA gene restricted the ability to distinguish finer genetic structures within the species. To address this limitation and obtain more detailed insights into the genetic diversity and evolutionary history of parasitic cestodes, the authors emphasized the importance of incorporating additional molecular markers, such as the mitochondrial cytochrome c oxidase subunit I (*cox1*) gene. The mtDNA *cox1* gene, with its higher resolution, offers a more effective tool for examining genetic differentiation and understanding the population structure of these parasites.

Isbert et al. (2023) investigated the genetic structure of *Grillotia* larvae found in the velvet belly lantern shark (*Etmopterus spinax*) in the Mediterranean Sea and Northeast Atlantic Ocean. To understand the genetic diversity and relationships of these parasites, the researchers used the 28S ribosomal DNA

(rDNA) marker. Their analysis revealed that the 28S rDNA sequences from *Grillotia* larvae were almost identical to a species previously identified in the Mediterranean Sea. Two distinct genetic genotypes were found: one associated with the deep-sea shark *Dalatias licha*, and another shared by multiple host species, including *Etmopterus spinax*, *Galeus melastomus*, and *Scyliorhinus canicula*. This genetic variation suggests that these two genotypes may occupy different ecological niches or interact with distinct host species. The study highlights the effectiveness of using the 28S rDNA marker to explore the genetic structure and diversity of parasites, providing valuable insights into their ecology and the broader marine ecosystem.

The main results achieved in target TTHs taxa investigated during the PhD thesis are presented, as following:

2. I - Distribution and genetic diversity of *Anisakis* spp. in cetaceans from the Northeast Atlantic Ocean and the Mediterranean Sea. Published in: *Scientific Reports* 12, 13664 (2022)

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Abstract - Parasite biodiversity in cetaceans represents a neglected component of the marine ecosystem. This study aimed to investigate the distribution and genetic diversity of anisakid nematodes of the genus *Anisakis* sampled in cetaceans from the Northeast Atlantic Ocean and the Mediterranean Sea. A total of 478 adults and pre-adults of *Anisakis* spp. was identified by a multilocus genetic approach (mtDNA *cox2*, EF1 α -1 nDNA and *nas* 10 nDNA gene loci) from 11 cetacean species. A clear pattern of host preference was observed for *Anisakis* spp. at cetacean family level: *A. simplex* (s.s.) and *A. pegrefii* infected mainly delphinids; *A. physeteris* and *A. brevispiculata* were present only in physeterids, and *A. ziphidarum* occurred in ziphiids. The role of cetacean host populations from different waters in shaping the population genetic structure of *A. simplex* (s.s.), *A. pegrefii* and *A. physeteris* was investigated for the first time. Significant genetic sub-structuring was found in *A. simplex* (s.s.) populations of the Norwegian Sea and the North Sea compared to those of the Iberian Atlantic, as well as in *A. pegrefii* populations of the Adriatic and the Tyrrhenian Seas compared to those of the Iberian Atlantic waters. Substantial genetic homogeneity was detected in the Mediterranean Sea population of *A. physeteris*. This study highlights a strong preference by some *Anisakis* spp. for certain cetacean species or families. Information about anisakid biodiversity in their cetacean definitive hosts, which are apex predators of marine ecosystems, acquires particular importance for conservation measures in the context of global climate change phenomena.

2.1. Introduction

Anisakid nematodes of the genus *Anisakis* Dujardin, 1845 are marine parasites with a worldwide distribution. Their life cycle is indirect, involving several hosts at different trophic levels of marine food webs. The parasite's adult stage lives in marine mammals, mainly cetaceans, while planktonic or semi-planktonic crustaceans act as first intermediate hosts of the parasite, and fish and squid represent intermediate/paratenic hosts (Aguirre & Lutz, 2004; Fayer & Eberhard, 1997; Lopez & Becerra, 2002). Specifically, *Anisakis* spp. parasitize the digestive tracts of cetaceans, being commonly found in the stomachs of both toothed and baleen whales (Aguirre & Lutz, 2004; Lopez & Becerra, 2002; Smith & Bell, 2013; Willis & Calder, 2015; Turner & Silva, 2009; Thompson & Green, 2001). The definitive hosts acquire *Anisakis* through ingestion of infected crustaceans, fish, or squid. Once in the stomach of cetacean hosts, the third larval stages (L3) of *Anisakis* spp. moult into the fourth stage larvae (L4), that further develop into sexually mature adults, a process taking from 40 to 60 days (Olsen & Jensen, 2006; Bellamy & Foster, 2011; Marshall & Hanson, 2010). The adult stages of *Anisakis* “swim” in the ingesta, probably feeding on them (Bellamy & Foster, 2011). In many cases, they attach themselves to the stomach mucosa, forming clusters and sometimes generating gastric granulomatous ulcers (Sharp & Porter, 2004; Williams & Williams, 2012; Montgomery & Herrero, 2015).

The application of genetic/molecular methodologies to *Anisakis* morphospecies has advanced our understanding of their systematics, taxonomy, and phylogeny, allowing the detection and description of sibling species and the discovery of new species (Fayer & Eberhard, 1997; Lopez & Becerra, 2002; Williams & Williams, 2012), and generally furthering our knowledge of their ecology, geographical distribution, and host preferences (Aguirre & Lutz, 2004; Fayer & Eberhard, 1997; Lopez & Becerra, 2002; Smith & Bell, 2013). The existence of nine nominal species of *Anisakis* as distinct phylogenetic units has been demonstrated by various phylogenetic analyses, using both nuclear and mitochondrial genes (Fayer & Eberhard, 1997; Lopez & Becerra, 2002; Williams & Williams, 2012). PCR–RFLP of the ITS rDNA region (Williams & Williams, 2012) and the sequencing of the mitochondrial mtDNA *cox2* gene (Bellamy & Foster, 2011) are used to identify all the species of *Anisakis*, at both larval and adult stages. Several nuclear gene loci have been recently validated on specimens of the *A. simplex* s.l. complex (i.e. *A. pegreffii*, *A. simplex* (s.s.) and *A. berlandi*): sequence analysis of the EF1 α – 1 nDNA region revealed two diagnostic nuclear sites (SNPs) at which the nucleotides differed between *A. simplex* (s.s.) and *A. pegreffii* (Bellamy & Foster, 2011); the metalloproteinase 10 (*nas10* nDNA) locus exhibits diagnostic SNPs, at which the nucleotides differ between the three species, and for which ARMS-PCR assays were developed (Marshall & Hanson, 2010). Panels of DNA microsatellite (SSR) loci have been developed in recent years (Williams & Williams, 2012; Bellamy & Foster, 2011), which include some 100% diagnostic loci with fixed alternative alleles which differ between the three species (Williams & Williams, 2012; Lopez & Becerra, 2002).

Studies on the epidemiology of *Anisakis* spp. in their intermediate/paratenic hosts (i.e. fish and squid) from Northeast (NE) Atlantic Ocean and Mediterranean Sea are abundant, and this field is regularly updated (Fayer & Eberhard, 1997; Lopez & Becerra, 2002; Williams & Williams, 2012; Thompson & Green, 2001). However, there is scant epidemiological data on *Anisakis* spp. in their definitive hosts (i.e., cetaceans) and consequently, knowledge is lacking on their biodiversity, distribution, and ecology at the pre-adult and adult stages. To date, investigations on parasites of cetaceans in the NE Atlantic and Mediterranean Sea have relied on samples from stranded individuals (Sharp & Porter, 2004; Williams & Williams, 2012; Montgomery & Herrero, 2015) or, more rarely, on samples obtained during whaling activities (Olsen & Jensen, 2006).

Knowledge of the biodiversity (at both species and genus level) of *Anisakis* in different cetacean hosts has important implications for the understanding of the parasite/host ecology. The aim of the present work was to apply a multilocus genetic approach to the study of anisakid endoparasites of cetaceans from European seas, to: (i) assess the biodiversity of *Anisakis* species at their adult stage in various cetacean hosts, from different areas of the NE Atlantic Ocean and the Mediterranean Sea; (ii) provide insights into the distribution of *Anisakis* species in cetacean populations; (iii) analyse the host-parasite association between cetacean hosts and their *Anisakis* parasites; (iv) investigate the population genetic structure of *Anisakis* spp. from different hosts and geographical ranges.

2.2. Methods

2.2.1. Cetacean parasites sampling data

Samples of *Anisakis* spp. were collected from 34 individuals belonging to 11 cetacean species stranded in locations along the Mediterranean Sea and NE Atlantic Ocean coast, between 2004 and 2019 (see Table 4 and Fig. 7). All cetacean individuals were necropsied following a standardised method (Mattiucci et al., 2013). All parasites were recovered from the stomach chambers of their hosts, usually during necropsy, and generally it was possible to obtain only a random subsample of the *Anisakis* spp. specimens infecting the host. On a few occasions it was possible to count or estimate the total burden of parasites infecting the host.

In total, N=478 anisakid nematodes were identified to genus level based on morphological characters reviewed by Mattiucci et al. (2013). Mature adults were separated from pre-adults, based on the visibility in the former of caudal papillae and spicules in males and eggs in females. The sampled nematodes were stored frozen at -20°C , or in 70% ethanol, for further molecular analyses.

2.2.2. Genetic identification of anisakid parasites

The nematode specimens were identified by sequence analysis of the mitochondrial cytochrome c oxidase II (mtDNA *cox2*) gene locus (Mattiucci et al., 2013; Palomba et al., 2017). Specimens of *A. pegreffii* and *A. simplex* (s.s.) (N=134), as inferred from the mitochondrial marker, collected from cetaceans stranded in sympatric areas for these two sibling species, were also identified using nuclear markers. In particular, sequence analysis of the diagnostic gene locus elongation factor EF1 α – 1 of nDNA (Mattiucci et al., 2015) and direct genotyping determination of the diagnostic nuclear metalloproteinase *nas10* locus by ARMS-PCR (Palomba et al., 2017) were performed on these 134 specimens. In addition, sequence analysis of the same gene locus (*nas 10* nDNA) was carried out on those individuals which showed a heterozygote pattern.

Total DNA was extracted from ~2 mg of tissue from each specimen by using the Quick-gDNA Miniprep Kit (ZYMO RESEARCH), following the manufacturer's instructions. For sequencing of the *cox2* gene, polymerase chain reaction (PCR) amplification was performed using the primers 211F (5'-TTTCTAGTTATATAGATTGRTTTYAT-3') and 210R (5'-CACCAACTCTTAAATTA TC-3') (Mattiucci et al., 2013). PCR was carried out according to the procedures described by Mattiucci et al. (2013).

The EF1 α – 1 nDNA gene was amplified using the primers EF-F (5'-TCCTCAAGCGTTGTTATCTGTT-3') and EF-R (5'-AGTTTTGCCACTAGCGGTTCC-3') (Mattiucci et al., 2015). PCR conditions and procedures followed those reported by Mattiucci et al. (2015). The EF1 α – 1 nDNA sequences were compared with those previously deposited in GenBank, at the diagnostic positions (i.e., 186 and 286), as previously detailed by Mattiucci et al. (2015).

For sequencing the *nas10* gene, PCR amplification was performed using the primers *nas10*F (5'-GATGTCCTGCAAGTGATTG-3') and *nas10*R (5'-CGCTATTAAGAGAGGGATCG-3') (Palomba et al., 2017). PCR was carried out according to the procedures provided by Palomba et al. (2017). A direct genotyping determination of the nuclear *nas10* locus was performed by ARMS-PCR at the gene locus *nas10* by the combined use of OUT-F1 (5'-TATGGCAAATATTATTATCGTA-3'), OUT-R1 (5'-TATTTCCGACAGCAAACAA-3'), INN-F1 (5'-GCATTGTACACTTCGTATATT-3'), INN-R1 (5'-ATTTCTYCAGCAATCGTAAG-3'), following the procedures reported in Palomba et al. (2017). The PCR products were separated by electrophoresis using agarose gel (1.5%) stained with GelRed; 3 μ L of the amplification products were visualized. The distinct banding patterns were detected using ultraviolet transillumination (Palomba et al., 2017).

The sequences obtained at the mtDNA *cox2* locus were aligned with other *Anisakis* spp. sequences obtained in previous studies and deposited in GenBank, using ClustalX v2.0 (Larkin et al., 2007). Alignments were manually edited and trimmed with BioEdit v7.0.5.3 (Hall, 1999), resulting in 580 characters and 9 taxa.

2.2.3. Phylogenetic analysis inferred from results for the mtDNA *cox2* gene locus

A phylogenetic tree of the *cox2* mtDNA gene locus was constructed using Bayesian Markov Chain Monte Carlo (MCMC) analysis in BEAST v1.10.4 (Suchard et al., 2015). The best-fit substitution model was selected using the Akaike information criterion (AIC) as implemented in jModeltest v2.1.7 (Darriba et al., 2012). GTR + I + G substitution model, strict molecular clock (Drummond et al., 2006) and a Yule speciation process were used as the tree priors (Yule, 1925), with default parameters. A run of 10 million iterations was completed, logging parameter values after every 10,000 iterations, and checking for stationarity and effective sample sizes (ESS) from all the available parameters (cut-off > 200) with Tracer v1.7 (Rambaut et al., 2018). The first 10% of trees were discarded as burn in, and the remaining 9000 were analysed and visualized using TreeAnnotator v1.10.4 (Suchard et al., 2015), and FigTree v1.4.2 (<http://tree.bio.ed.ac.uk/software/figtree/>), respectively. *Toxocara canis* (AP017701) and *Ascaris suum* (X54253) were used as outgroups (Mattiucci et al., 2013).

2.2.4. Intraspecific genetic diversity of *Anisakis* spp. inferred from results at the mtDNA *cox2* gene locus

The mean pairwise differences, among and within populations of *A. simplex* (s.s.), *A. pegreffii* and *A. physeteris* from the different cetacean samples were calculated from mtDNA *cox2* sequences. Spatial analysis of molecular variance (AMOVA), conducted with ARLEQUIN version 3.5 (Excoffier & Lischer, 2010), with 1000 permutations, was applied to the genetic data sets obtained for the populations of *A. simplex*(s.s.), *A. pegreffii* and *A. physeteris*. The *F_{st}* values were estimated by ARLEQUIN V3.5 (Excoffier & Lischer, 2010) at the intraspecific level, among and within samples of *A. simplex* (s.s.), *A. pegreffii* and *A. physeteris* from different sampling areas. The *F_{st}* value ranges from a maximum of 1, indicating complete differentiation among sequences of pre-defined populations of the same *Anisakis* species collected from different regions to a value equal to a minimum of 0, which indicates no differentiation among the populations. Pairwise comparisons of *F_{st}* (assuming that $p < 0.05$ indicates a significant difference) were based on 1000 permutations of the data matrix. Intraspecific population genetic diversity of *A. simplex* (s.s.), *A. pegreffii* and *A. physeteris* among sampling areas was estimated based on the following standard statistical parameters: number of haplotypes (Nh), number of unique haplotypes (Nuh), nucleotide diversity (π), haplotype diversity (Hd), average number of differences (K), and number of polymorphic sites (S), using DnaSP V5.10.01 (Librado & Rozas, 2009). Haplotype network construction was carried out using PopART V1.7 (Leigh & Bryant, 2015). The analysis was performed using the statistical parsimony procedure (95% parsimony connection limit), implemented in TCS V3.5.1 (Clement et al., 2000).

2.3. Results

2.3.1. Parasite samples

A total of N=478 anisakid nematodes was obtained from stranded cetaceans and, according to their morphological characteristics, they were all assigned to the *Anisakis* genus. Only adult (showing developed reproductive structures, such as caudal papillae and spicules in male worms and ovaries in females) and pre-adult (presence of labia, absence of boring tooth) specimens were found. It was not possible to count or estimate the total burden of worms during most of the necropsies of cetaceans, only to obtain a subsample of parasites. Hence, we could not perform a comprehensive epidemiological analysis of the parasites for each host species.

2.3.2. Genetic identification of *Anisakis*

According to the BLAST analysis performed on the 478 mtDNA cox2 sequences (580 bp) obtained, five species were detected: *A. simplex* (s.s.), *A. pegreffii*, *A. physeteris*, *A. brevispiculata* and *A. ziphidarum*. The numbers of *Anisakis* specimens genetically identified in the definitive hosts from different sampling areas are reported in Table 1. The relative proportions of *Anisakis* spp. in cetaceans in different geographical areas of the NE Atlantic Ocean and the Mediterranean Sea, and in their different definitive hosts families, are reported in Figs. 1 and 2, respectively.

The sympatric (in some cases even syntopic, such as in two specimens of common dolphins *Delphinus delphis*, one striped dolphin *Stenella coeruleoalba*, one bottlenose dolphin *Tursiops truncatus* and three harbour porpoises *Phocoena phocoena*) occurrence of 134 adult specimens of *A. pegreffii* and *A. simplex* (s.s.) was recorded from dolphin species stranded along the Iberian Atlantic coast (Table 1). These specimens were also identified using nuclear diagnostic loci, i.e. the EF1 α - 1 nDNA and nas10 nDNA loci. Among them, 134 specimens showed 100% concordance with the mtDNA cox2 data set for the identification of the two parental taxa, i.e. *A. simplex* (s.s.) (N = 107) and *A. pegreffii* (N = 27). Indeed, at the EF α - 1 nDNA locus, 107 individuals were found to be homozygous C/C and T/T, respectively, at the fixed alternative nucleotide positions 186 and 286, i.e. diagnostic for the parental species *A. simplex* (s.s.). A further 25 showed the homozygote genotypes T/T and C/C at the same SNPs, corresponding to the parental species *A. pegreffii* (Suppl. Fig. 1). Additionally, the ARMS-PCR analysis enabled us to genotype 107 individuals as belonging to *A. simplex* (s.s.), and 25 to the species *A. pegreffii*. The combined use of the nas 10 primers generated a specific PCR product of 296 bp in *A. simplex* (s.s.), amplifying the T-allele, and a specific PCR fragment of 117 bp in *A. pegreffii*, amplifying the C-allele. In contrast, two specimens obtained respectively from a harbour porpoise and a short-beaked common dolphin, which at the mtDNA cox2 locus corresponded to the species *A. pegreffii*, showed a heterozygote pattern at those diagnostic positions (SNPs)—thus falling between *A. pegreffii* and *A. simplex* (s.s.), in both the two nuclear loci EF1 α - 1 nDNA and nas10 nDNA (Suppl. Fig. 1). In particular, the sequence

analysis of these two specimens revealed the occurrence of a double peak at these diagnostic positions (i.e., 186 and 286) at the EF1 α – 1. For the ARMS analysis, two specific PCR fragments of both 296 and 117 bp were generated in the species with a heterozygote pattern, amplifying both the C and T allele. This latter finding showed 100% concordance with the direct sequence analysis of the gene locus nas 10 nDNA. These two specimens, showing a heterozygous pattern at all the diagnostic SNPs of the EF1 α – 1 and nas10 nDNA loci, seem to be the result of hybridization events between *A. pegreffii* and *A. simplex* (s.s.).

Table 1. Number (N_i) of *Anisakis* specimens genetically identified in the definitive hosts from different sampling area.

Host family	Cetacean species	Samplin garea	N ₁	A. simplex(s.s)	A.pegreff ii	A.simpl/A.peg Heterozygote	A.physeteri s	A.brevispicula ta	A.ziphidarum
Mysticeti									
Balenopteridae	Balenoptera acutorostrata	Norwegian Sea	25	25	–	–	–	–	–
Physeteroidea									
Physeteridae	Physeter macrocephalus	Scotland coast	7	2	–	–	5	–	–
		Adriatic Sea	60	–	–	–	60	–	–
		Aegean Sea	1	–	–	–	1	–	–
Kogiidae	Kogia breviceps	Iberian Atlantic coast	3	1	–	–	–	2	–
	Kogia sima	Tyrrhenian Sea	107	–	19	–	88	–	–
Ziphiidae									
Ziphiidae	Ziphius cavirostris	Aegean Sea	4	–	1	–	3	–	–
		Ionian Sea	2	–	–	–	–	–	2
Delphinoidea									
Delphinidae	Delphinus delphis	Iberian Atlantic coast	34	23	10	1	–	–	–
	Lagenorhynchus albirostris	Scotland coast	22	22	–	–	–	–	–
	Stenella coeruleoalba	Scotland coast	21	21	–	–	–	–	–
		Iberian Atlanticcoast	22	18	4	–	–	–	–
		Adriatic Sea	44	–	44	–	–	–	–
	Tursiops truncatus	Iberian Atlanticcoast	3	1	2	–	–	–	–
		Tyrrhenian Sea	6	–	6	–	–	–	–
	Globicephala melas	Faroe Islands	34	34	–	–	–	–	–
		Iberian Atlantic coast	27	27	–	–	–	–	–
Phocoenidae	Phocoena phocoena	Scotland coast	9	9	–	–	–	–	–
		Iberian Atlantic coast	47	37	9	1	–	–	–
		Tot.	478	220	96	2	157	2	2

The DNA sequences of the presently identified *Anisakis* species were deposited in GenBank under the following accession numbers: mtDNA *cox2*: OM142467–OM142471 (*A. pegreffii*); OM142472–OM142476 (*A. simplex* (s.s.)); OM14247–OM142481 (*A. physeteris*). EF1 α – 1 nDNA: OM210033–OM210037 (*A. pegreffii*); OM210038 (*A. simplex* (s.s.)).

2.3.3. Phylogenetic analysis inferred from mtDNA *cox2* gene locus

The Bayesian inference (BI) phylogenetic tree based on mtDNA *cox2* sequences (Fig. 3) indicated that a total of 220 nematodes, including preadult and adult specimens recovered in minke whale (*Balaenoptera acutorostrata*) and six delphinid species (striped dolphin *Stenella coeruleoalba*, bottlenose dolphin *Tursiops truncatus*, harbour porpoises *Phocoena phocoena*, long-finned pilot whale *Globicephala melas*, white-beaked dolphin *Lagenorhynchus albirostris*, and common dolphin *Delphinus delphis*) and a few preadults found in physeterids (sperm whale *Physeter macrocephalus* and pygmy sperm whale *Kogia breviceps*), clustered together within a well-supported clade (100% probability value), together with a reference sequence of *A. simplex* (s.s.) (DQ116426) (Fig. 3). Similarly, a total of 96 *Anisakis* preadult and adult specimens grouped in a highly (100%) supported phylogenetic lineage represented by *A. pegreffii*, which also included a reference sequence (DQ116428) of the parasite species (Fig. 3). Additionally, 157 specimens collected in physeterids from the Mediterranean Sea and the Atlantic Ocean clustered in a well-supported distinct phylogenetic clade formed by the species *A. physeteris* (Fig. 3). Two adult *Anisakis* specimens collected from pygmy sperm whale (*Kogia breviceps*) in Atlantic waters clustered in a clade represented by *A. brevispiculata* (Fig. 3, Table 1). Two adult worms collected from Cuvier's beaked whale (*Ziphius cavirostris*) from the Aegean Sea were placed in a highly supported clade comprising sequences of *A. ziphidarum* previously deposited in GenBank (Fig. 3, Table 1).

2.3.4. Genetic diversity inferred from mtDNA *cox2* gene locus

The genetic diversity analysis was performed at intraspecific level in *A. simplex* (s.s.), *A. pegreffii* and *A. physeteris*. It was not possible to give estimates of genetic diversity of *A. ziphidarum* and *A. brevispiculata* because of the low number of worms identified for those species. The hierarchical gene diversity analysis by AMOVA (Suppl. Table 1) indicated that in *A. simplex* (s.s.) 92.77% of the genetic variation was expressed within populations and only 2.52% was variation among populations of this parasite species collected from cetacean hosts of different geographic areas of the NE Atlantic Ocean (Suppl. Table 1). Similar results were obtained for *A. pegreffii*, with 97.48% of variation occurring within populations of the parasite species collected in cetaceans from different areas (Suppl. Table 1). In the case of *A. physeteris* 94.62% of variation occurred within populations (Suppl. Table 1).

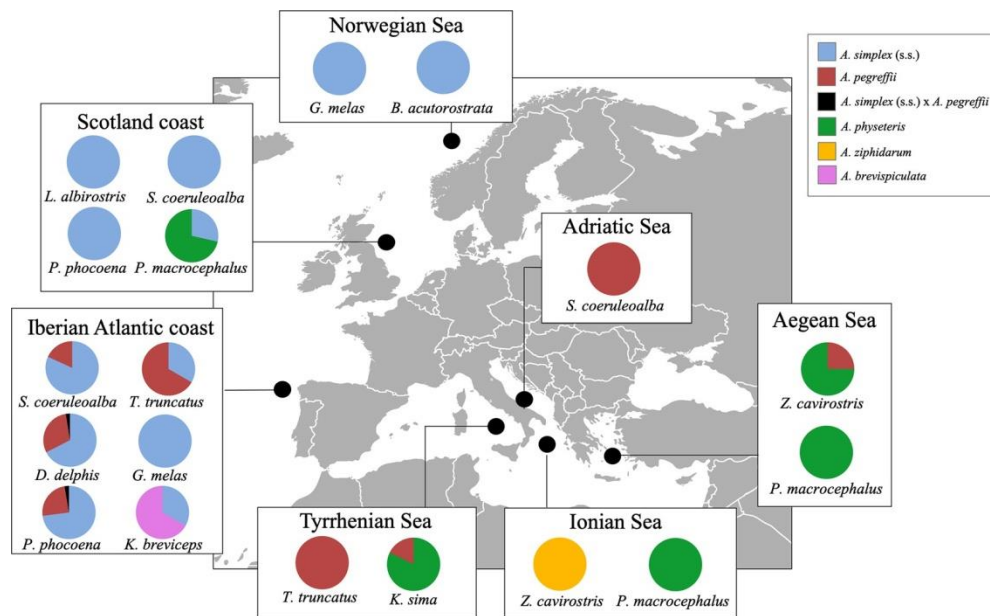


Figure1. Relative proportions of *Anisakis* spp. in cetaceans according to different geographical areas of the NE Atlantic Ocean and the Mediterranean Sea. The map was obtained from Wikimedia Commons, licensed-free (<https://commons.wikimedia.org/wiki/File:BlankMap-Europe-v4.png>), Roke was the unofficial uploader of the derivative map CC BY-SA 3.0.

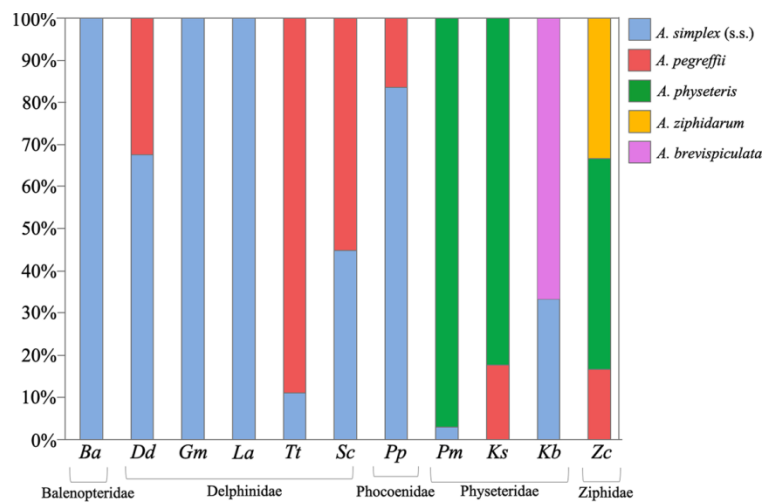


Figure 2. Relative proportions of *Anisakis* spp. according to definitive hosts belonging to species of families Balenopteridae, Delphinidae, Phocoenidae, Physeteridae and Ziphiidae. Host codes are reported by alphabetical order: Ba: *Balenoptera acutorostrata*, Dd: *Delphinus delphis*, Gm: *Globicephala melas*, La: *Lagenorhynchus albirostris*, Tt: *Tursiops truncatus*, Sc: *Stenella coeruleoalba*, Pp: *Phocoena phocoena*, Pm: *Physeter macrocephalus*, Ks: *Kogia sima*, Kb: *Kogia breviceps*, Zc: *Ziphius cavirostris*.

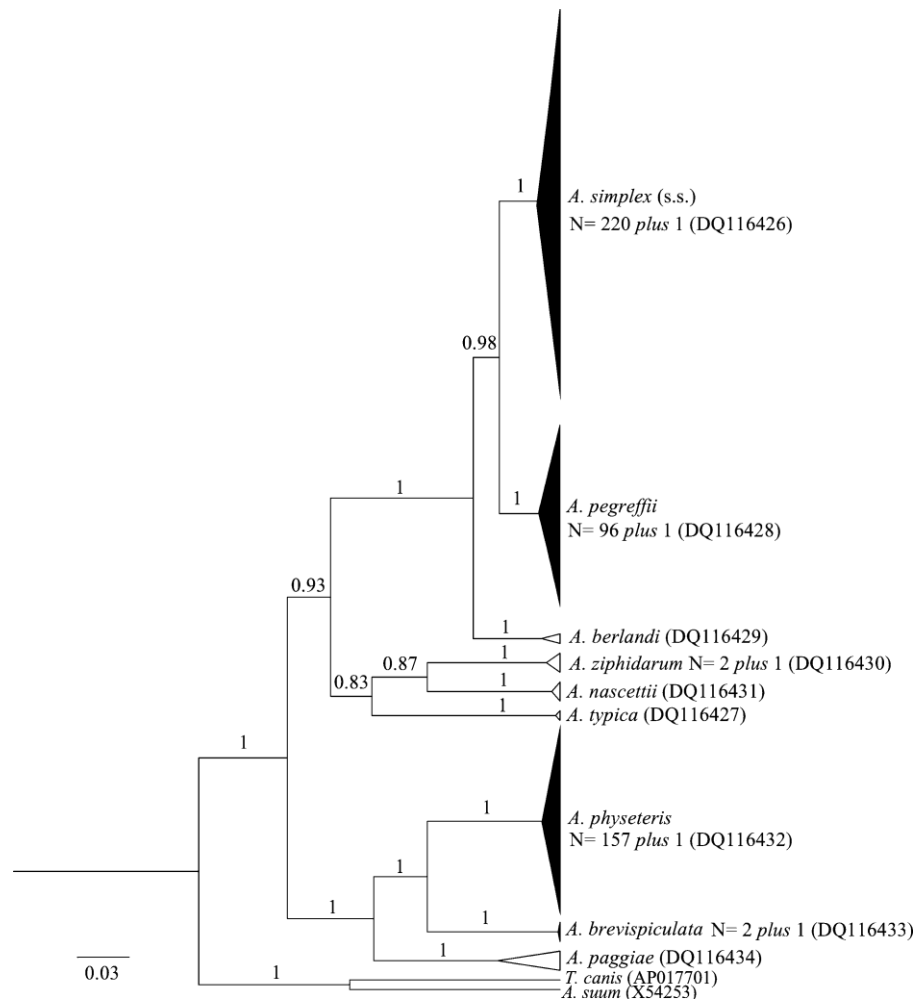


Figure 3. Phylogenetic tree of cox2 mtDNA gene locus constructed using Bayesian Markov Chain Monte Carlo (MCMC) analysis in BEAST v1.10.4¹⁰⁹. GTR + I + G substitution model, strict molecular clock¹⁰³ and Yule speciation process were used as the tree priors, Bayesian inference (BI) on mtDNA cox2 gene sequences of *Anisakis* spp. obtained from cetaceans. *Toxocara canis* and *Ascaris suum* were used as an outgroup.

Estimates of the genetic differentiation (Fst) between populations of the presently identified *Anisakis* species, as inferred from the fixation index, are shown in Table 2. At the intraspecific level, a significant genetic differentiation (Fst) was found between *A. simplex* (s.s.) metapopulations from the Norwegian Sea compared to those from Iberian Atlantic coast (Fst $\approx$ 0.12, $p < 0.00001$), as well as between populations of the same species from the Scottish and Iberian Atlantic coast (Fst $\approx$ 0.07, $p < 0.00001$). However, *A. simplex* (s.s.) individuals collected from the Norwegian Sea had a lower pairwise Fst when compared to its conspecifics in Scottish waters (Fst $\approx$ 0.02, $p = 0.03$) (Table 2). Comparing host species, significant Fst values were found between *A. simplex* (s.s.) metapopulations from long-finned pilot whales (Faroes Islands and Iberian Atlantic) and those collected from the minke whale from Norwegian Sea (Fst $\approx$ 0.10, $p < 0.00001$) and white-beaked dolphin from Scottish waters (Fst $\approx$ 0.07, $p < 0.00001$).

(data not shown). However, a higher F_{st} pairwise value ($F_{st} \approx 0.15$, $p < 0.001$) was recorded when comparing parasite metapopulations from the same cetacean species, i.e. pilot whale subpopulation from Faroes Island and Iberian Atlantic coast. Similarly, a significant genetic differentiation was found when comparing the metapopulations of *A. simplex* (s.s.) from striped dolphins of Iberian Atlantic coast and Scottish waters ($F_{st} \approx 0.09$, $p < 0.00001$).

Table 2. Population pairwise F_{ST} estimates inferred from mtDNA *cox2* sequences analysis among *A. simplex* (s.s.) (a) and *A. pegreffii* (b) and *A. physeteris* (c) collected from cetacean of different localities. Below the diagonal: conventional F_{ST} from haplotype frequencies; above the diagonal: p values of F_{ST} .

	IberianAtlanticcoast	AdriaticSea	TyrrhenianSea
a. <i>A. pegreffii</i>			
IberianAtlanticcoast	–	0.00000±0.0000	0.01465±0.0039
AdriaticSea	0.07006	–	0.10449±0.0081
TyrrhenianSea	0.04070	0.01569	–
	IberianAtlanticcoast	Scottishcoast	NorwegianSea
b. <i>A. simplex</i> (s.s.)			
IberianAtlanticcoast	–	0.00000±0.0000	0.00000±0.0000
Scottishcoast	0.07611	–	0.02637±0.0061
NorwegianSea	0.12307	0.02101	–
	IonianSea	TyrrhenianSea	AegeanSea
c. <i>A. physeteris</i>			
IonianSea	–	0.03418±0.0056	0.03809±0.0044
TyrrhenianSea	0.01228	–	0.13574±0.0094
AegeanSea	0.12020	0.05631	–

Intraspecific genetic variation between metapopulations of *A. pegreffii* from the Adriatic and the Tyrrhenian Seas was not significant ($F_{st} \approx 0.015$, $p = 0.10$) (Table 2). In contrast, significant genetic substructuring was observed when comparing the Mediterranean (Adriatic and Tyrrhenian) populations of *A. pegreffii* with those from the Iberian Atlantic coast ($F_{st} \approx 0.07$ ($p = 0.0001$) and $F_{st} \approx 0.04$ ($p = 0.001$), respectively (Table 2)). Due to the low number of *A. pegreffii* found in the different definitive hosts examined, except for striped dolphins, it was not possible to compare the F_{st} value at intraspecific level by host species.

Due to the low number of specimens of *A. physeteris* obtained from the NE Atlantic Ocean, F_{st} at the intraspecific level was calculated only for the comparison of specimens collected from stranded sperm whales along the coast of the Adriatic Sea (of Ionian Sea origin, Klamper et al., 2015) and those carried by the dwarf sperm whale (*Kogia sima*) from the Tyrrhenian Sea, with the finding of a generally low and slightly significant level of differentiation ($F_{st} \approx 0.012$, $p = 0.03$) (Table 2).

At the interspecific level, significant values of genetic differentiation were observed between *A. simplex* (s.s.) and *A. pegreffii* ($F_{st} \approx 0.836$), with the highest values being those found between *A. physeteris* and *A. simplex* (s.s.) ($F_{st} \approx 0.945$) and between *A. physeteris* and *A. pegreffii* ($F_{st} \approx 0.940$).

The haplotype parsimony network (TCS) inferred from mtDNA *cox2* haplotypes of *A. simplex* (s.s.) (Fig. 4) shows that the presence of two major haplotypes, i.e. Hap25 and Hap48. Hap25 was shared by several *A. simplex* (s.s.) individuals collected in the cetacean hosts from the Norwegian Sea and Scottish coast of the NE Atlantic Ocean, whereas Hap48 was shared mostly by the *A. simplex* (s.s.) metapopulations collected in the Iberian and Scottish Atlantic (Fig. 4). The TCS analysis revealed the existence of several unique haplotypes in the Norwegian metapopulation of *A. simplex* (s.s.) and in Iberian Atlantic waters. Moreover, TCS also shows concordance between the haplotype clustering and geographic origin of the endoparasite samples (Fig. 4).

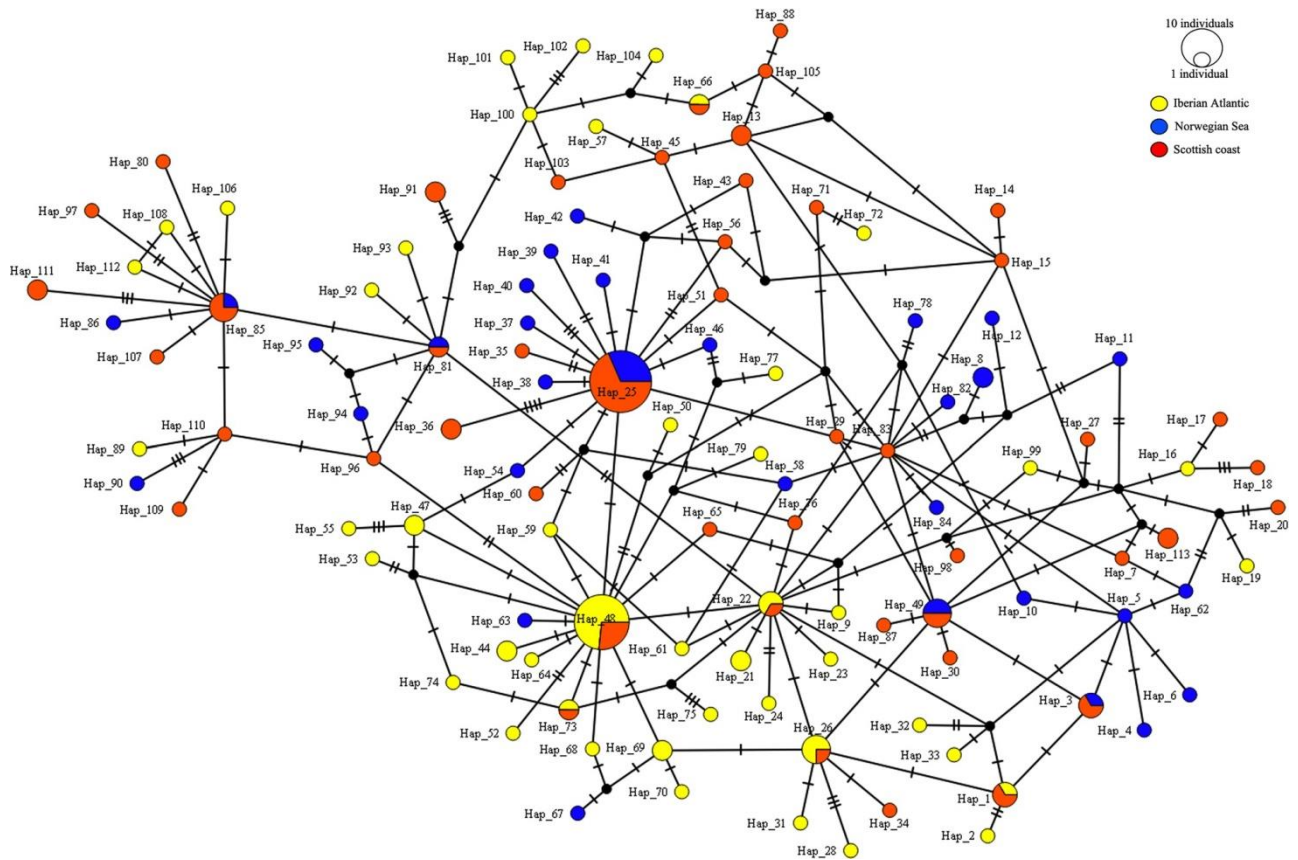


Figure 4. TCS network of *A. simplex* (s.s.) from cetaceans' definitive hosts obtained using the *cox2* sequences data set. Haplotypes (Hap1, Hap2, Hap3, etc.) are reported. Hatch marks indicate mutations. Circle sizes are proportional to the number of shared individuals per haplotypes.

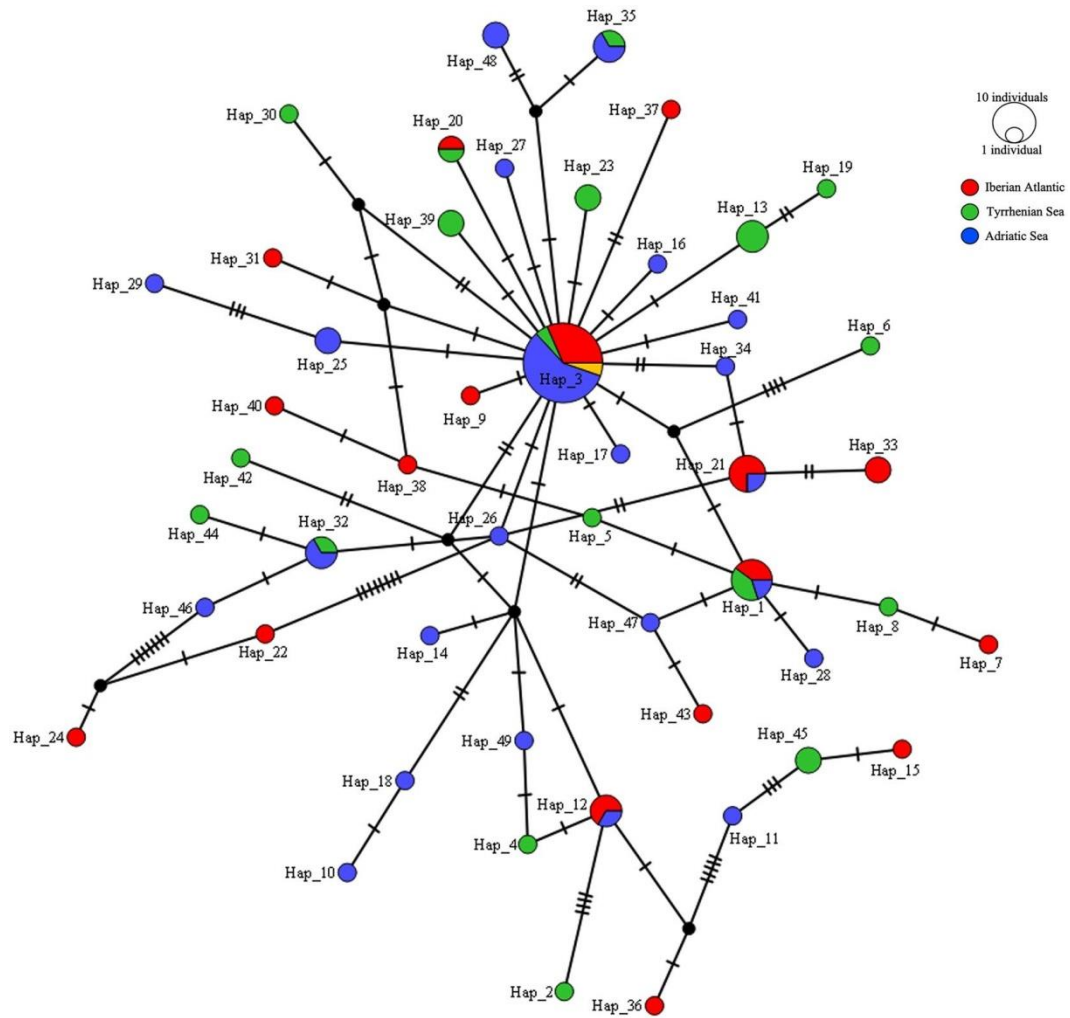


Figure 5. TCS network of *A.pegreffii* from cetaceans' definitive hosts obtained using the *cox2* sequences data set. Haplotypes (Hap1, Hap2, Hap3, etc.) are reported. Hatch marks indicate mutations. Circle sizes are proportional to the number of shared individuals per haplotypes.

TCS analysis of mtDNA *cox2* haplotypes of *A. pegreffii* resulted in a star-like tree (Fig. 5), with Hap3 being the most frequent haplotype, shared by all the metapopulations of the parasite (Fig. 5). A high frequency of certain unique haplotypes in the parasite specimens from the definitive hosts stranded on the Spanish Atlantic coast and from the Adriatic Sea was observed in *A. pegreffii* (Fig. 5).

2.4. Discussion

2.4.1. Identification of *Anisakis* spp. in cetaceans

Knowledge of the occurrence and distribution of adult stage worms of the genus *Anisakis* in their definitive hosts is mostly based on opportunistic samples, obtained during necropsy investigations of cetaceans which were stranded, bycaught in fishing gear or taken by whaling (Smith, 1983; Mattiucci et al., 1997; Klimpel et al., 2008). Use of stranding records is a valid and sustainable approach to study the diversity of local marine mammal species (Evans & Hammond, 2004; Pierce et al., 2008; Norman et al., 2010; Evans et al., 2011; Peltier et al., 2012), and to investigate the parasite fauna hosted by these animals. Stranding events can occur for several reasons, including natural causes such as illness, environmental factors, and anthropogenic causes including bycatch, vessel strikes, plastic ingestion, or acoustic trauma (Jepson et al., 2005; Wright et al., 2007; Simmonds et al., 2009; Fernández et al., 2011; Peltier et al., 2013; Dolman et al., 2014; Rogan et al., 2017; Tenan et al., 2020; Pierce et al., 2021). Despite these advantages, parasitological studies on stranded cetaceans are affected by the decomposition state of the animals. It is also possible that animals in poor nutritional condition or moribund due to disease or trauma are unlikely to have been successfully foraging before death, which may affect the parasite burden demonstrated at necropsy.

In this study, five *Anisakis* species, namely *A. simplex* (s.s.), *A. pegreffii*, *A. physeteris*, *A. brevispiculata* and *A. ziphidarum*, were detected from 11 species of cetacean stranded at various locations along the coasts of the NE Atlantic Ocean and Mediterranean Sea (Fig. 7, Table 4). In addition, a multilocus genotyping approach based on nuclear markers, applied to specimens of the sibling species *A. simplex* (s.s.) and *A. pegreffii* obtained from hosts occurring in a sympatric area (Iberian Atlantic waters), allowed the identification of two heterozygote genotypes, i.e. two F1 hybrids, showing a heterozygous pattern at all of the diagnostic nucleotide positions observed at the EF1 α – 1 nDNA and nas 10 nDNA loci (Palomba et al., 2017; Mattiucci et al., 2015) (Suppl. Fig. 1). This finding suggests that these heterozygote genotypes most likely originated from a recent hybridization event between sympatric specimens of *A. simplex* (s.s.) and *A. pegreffii*.

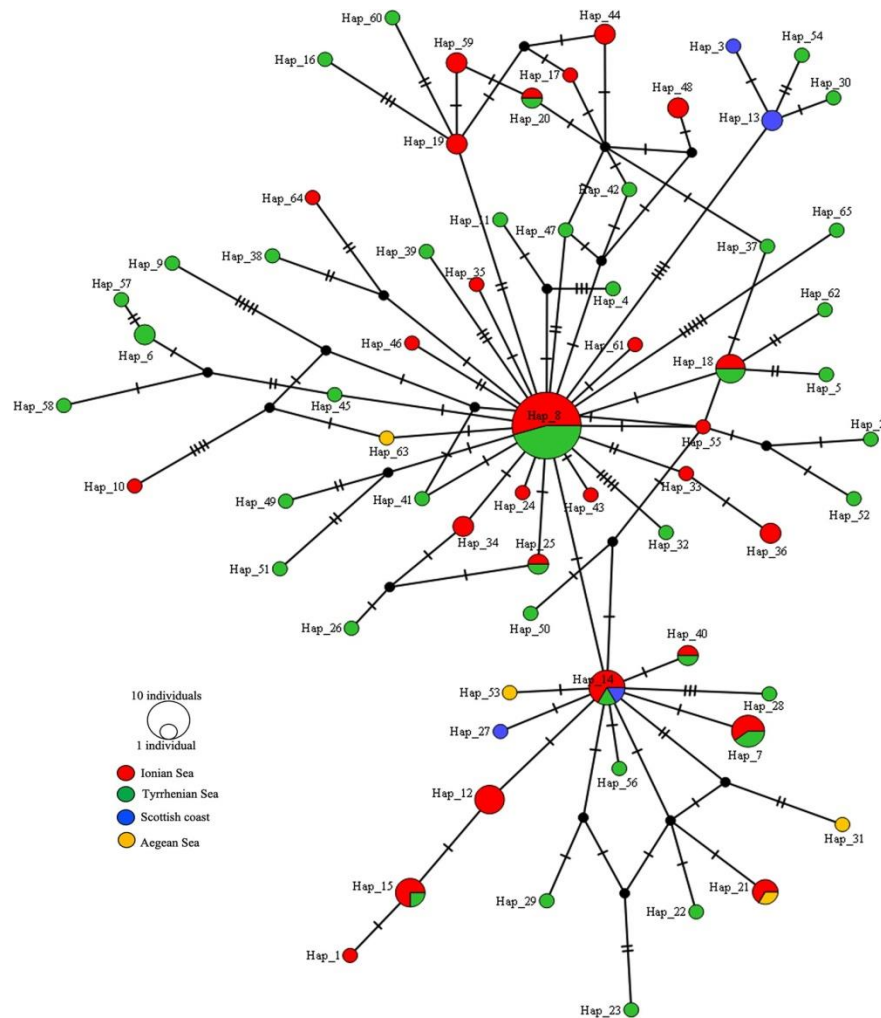


Figure 6. TCS network of *A. physeteris* from cetaceans' definitive hosts obtained using the *cox2* sequences data set. Haplotypes (Hap1, Hap2, Hap3, etc.) are reported. Hatch marks indicate mutations. Circle sizes are proportional to the number of shared individuals per haplotypes.

There have been reports of *A. simplex* (s.s.) and *A. pegreffii* L3 heterozygote genotypes in the same sympatric area from several fish species, including species which are likely to be eaten by the studied cetaceans (Mattiucci et al., 2004; Pontes et al., 2005; Abollo et al., 2007; Silva et al., 2008; Herreras et al., 2009; Borrell et al., 2010; Cavallero et al., 2014; Palomba et al., 2017; Llarena-Reino et al., 2021). Thus, hybridization events between species of the *A. simplex* (s. l.) complex are probably a common phenomenon in sympatric areas, as also recently reported from southern oceans based on a multinuclear genotyping approach (Silva et al., 2008).

Table 3. Genetic diversity of *A. simplex* (s.s.), *A. pegreffii* and *A. physeteris* from the cetaceans' definitive host, by collection localities. Number of sequences analyzed (N), number of haplotypes (Nh), nucleotide diversity (per site) (π) and haplotype diversity (Hd) with their relative standard deviation (SD), average number of nucleotide differences (K) and number of polymorphic sites (S), are reported.

	IberianAtlanticcoast	AdriaticSea	TyrrhenianSea
a. <i>A. pegreffii</i>			
IberianAtlanticcoast	–	0.00000±0.0000	0.01465±0.0039
AdriaticSea	0.07006	–	0.10449±0.0081
TyrrhenianSea	0.04070	0.01569	–
	IberianAtlanticcoast	Scottishcoast	NorwegianSea
b. <i>A. simplex</i> (s.s.)			
IberianAtlanticcoast	–	0.00000±0.0000	0.00000±0.0000
Scottishcoast	0.07611	–	0.02637±0.0061
NorwegianSea	0.12307	0.02101	–
	IonianSea	TyrrhenianSea	AegeanSea
c. <i>A. physeteris</i>			
IonianSea	–	0.03418±0.0056	0.03809±0.0044
TyrrhenianSea	0.01228	–	0.13574±0.0094
AegeanSea	0.12020	0.05631	–

The phylogenetic tree topology, based on the mtDNA *cox2* sequences obtained (Fig. 3), differentiated *Anisakis* spp. into four well-defined clades, in accordance with previous studies (Mattiucci et al., 2013; Mattiucci & Nascetti, 2006). The first clade comprised species of the *A. simplex* complex, i.e. *A. simplex* (s.s.), *A. pegreffii* (and *A. berlandi*, not found in the current study). The second clade included *A. ziphidarum* and *A. nascettii* (the latter not found in the current study), while the third clade comprised *A. physeteris*, *A. brevispiculata*, and *A. paggiae* (the latter not found in the current study).

2.4.2. Host preference by *Anisakis* spp.

The well-known existence of a host specificity pattern in *A. simplex* (s.s.) and *A. pegreffii* for dolphins and baleen whales has been further supported by the present findings. Indeed, the three sibling species of the *A. simplex* (s.l.) complex are primarily parasites of the cetacean families Delphinidae, Monodontidae, Phocoenidae and Balaenopteridae (Abollo et al., 2001; Mattiucci et al., 2013; Smith & Wootten, 1978; Umehara et al., 2010). They parasitize these hosts in different geographical areas, depending on the range of distribution of both host and parasite species (Mattiucci & Nascetti, 2006; Valero et al., 2006).

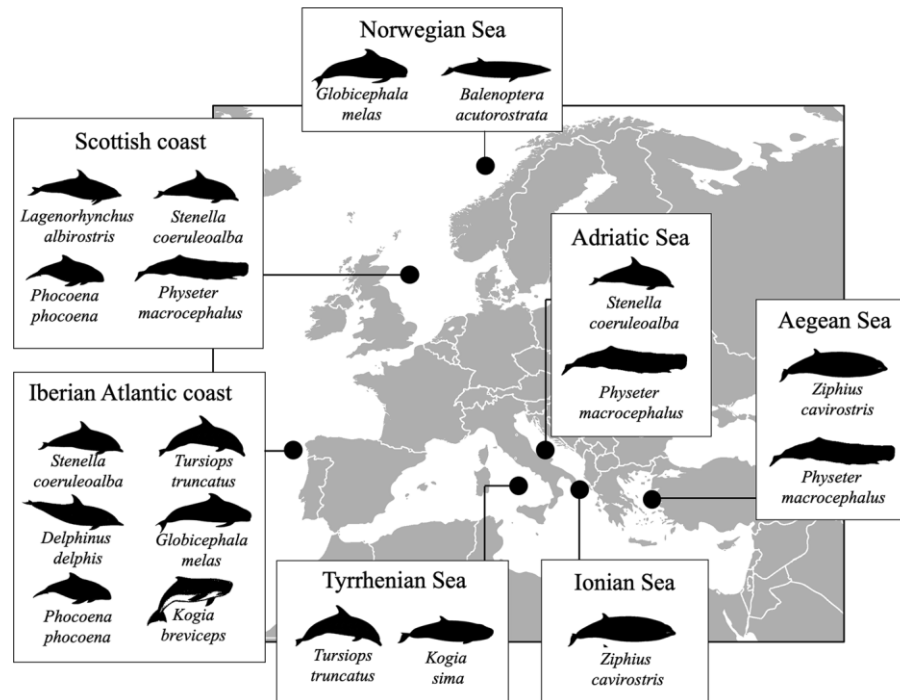


Figure7. Sampling localities of *Anisakis* spp. in the Mediterranean Sea and NE Atlantic Ocean of the occasional cetacean strandings, which resulted in 34 specimens belonging to 11 species. The map was obtained from Wikimedia Commons, licensed-free (<https://commons.wikimedia.org/wiki/File:BlankMap-Europe-v4.png>), Roke was the unofficial uploader of the derivative map CCBY-SA3.0.

Table 4. Number (N_p) of specimens of *Anisakis* spp. collected in the definitive host individuals (N_h) from different sampling area, studied through the sequence analysis of the mtDNA *cox2* and *EF1 α -1* nDNA and the direct genotyping (ARMS) of *nas* 10 nDNA.

Host family	Cetacean species	N_h	Sampling area	Coordinate	N_p	N_p mtDNA <i>cox2</i>	N_p <i>EF1α-1</i> nDNA	N_p ARMS <i>nas</i> 10nDNA
Balenopteridae	<i>Balenoptera acutorostrata</i>	1	Norwegian Sea	63°27'N–6°24'E	25	25	–	–
Physeteridae	<i>Physeter macrocephalus</i>	1	Scotland coast	58°19'N–8°17'W	7	7	–	–
		5	Adriatic Sea	42°04'N–14°46'E	60	60	–	–
		1	Aegean Sea	38°32'N–25°04'E	1	1	–	–
Kogiidae	<i>Kogia breviceps</i>	1	Iberian Atlantic coast	42°21'N–9°12'W	3	3	3	3
	<i>Kogia sima</i>	1	Tyrrhenian Sea	40°20'N–14°47'E	107	107	–	–
Ziphiidae	<i>Ziphius cavi-rostris</i>	1	Aegean Sea	38°32'N–25°04'E	4	4	–	–
		1	Ionian Sea	37°36'N–18°14'E	2	2	–	–
Delphinidae	<i>Delphinus delphis</i>	3	Iberian Atlantic coast	42°21'N–9°12'W	34	34	34	34
	<i>Lagenorhynchus alborostris</i>	1	Scotland coast	58°19'N–8°17'W	22	22	–	–
	<i>Stenella coeruleoalba</i>	1	Scotland coast	58°19'N–8°17'W	21	21	–	–
		3	Iberian Atlantic coast	42°21'N–9°12'W	22	22	22	22
		3	Adriatic Sea	42°18'N–15°00'E	44	44	–	–
	<i>Tursiops truncatus</i>	1	Iberian Atlantic coast	42°21'N–9°12'W	3	3	3	3
		2	Tyrrhenian Sea	40°20'N–14°47'E	6	6	–	–
	<i>Globicephala melas</i>	1	Faroe Islands	62°47'N–6°54'W	34	34	–	–
		2	Iberian Atlantic coast	42°21'N–9°12'W	27	27	27	27
Phocoenidae	<i>Phocoena phocoena</i>	1	Scotland coast	58°19'N–8°17'W	9	9	–	–
		3	Iberian Atlantic coast	42°21'N–9°12'W	47	47	47	47

In the current study, adult nematodes of *A. simplex* (s.s.) and *A. pegreffii* were found in all the species of delphinids that were examined and in a minke whale specimen (Table 1, Figs. 1 and 2). Adult parasites of these two species can often be found in syntopy in the same definitive hosts in areas where the geographical range of the two parasite species overlaps (Navarro et al., 2011; Zhu et al., 2002), as observed in bottlenose dolphins, striped dolphins, common dolphins, and harbour porpoises inhabiting the waters off the Iberian coast (Table 1, Figs. 1 and 2). A sympatric and syntopic distribution of these two species was previously observed in other delphinids and whales sampled along the Japanese coasts, such as bottlenose dolphin, minke whale, and sei whale (*Balaenoptera borealis*) (Abollo et al., 2001). Similarly, a syntopic occurrence of *A. pegreffii* and *A. berlandi* was reported in long-finned pilot whales from New Zealand (Valero et al., 2006), as well as in a killer whale (*Orcinus orca*) from Argentinian waters (Mattiucci & Nascetti, 2006). Another relevant observation concerning the preference of these parasite species for certain definitive hosts is the different size reached by mature specimens of *A. simplex* (s.l.) in mysticetes, killer whales, and smaller dolphins (Smith & Wootten, 1978; Umehara et al., 2010). Gomes et al. (2011) and Ugland et al. (2011) reported that *A. simplex* (s.s.) and *A. pegreffii* specimens seem to reach bigger sizes in mysticetes (sei whales and minke whales) than in Delphinidae (bottlenose dolphins, long-finned pilot whales, and striped dolphins). Bigger whales probably offer a more beneficial microhabitat to these parasite species compared to smaller delphinids, enhancing their fitness potential. However, the coevolutionary implications related to these observations have not yet been clarified.

This investigation further confirms that cetaceans of the families Physeteridae (i.e. sperm whale) and Kogiidae (i.e. dwarf sperm whale, *K. sima*, and pygmy sperm whale, *K. breviceps*) are the main definitive hosts for the species included in clade 3 (Figs. 1, 2, 3), comprising *A. physeteris*, *A. brevispiculata*, and *A. paggiae*. However, a very rare infection of (preadult) *A. simplex* (s.s.) and *A. pegreffii* was recorded in physeterids in the present study. Santoro et al. (2013) reported the presence of a few immature specimens of *A. pegreffii* in syntopy with fully developed adult specimens of *A. physeteris* located in the stomach of the same specimen of dwarf sperm whale examined in the present study. A rare case of infection with *A. simplex* (s.s.) in a pygmy sperm whale from the Caribbean Sea was also previously observed (Smith & Wootten, 1978), but the developmental stage of the worms was not reported. The rare cases of co-infection with both *A. simplex* (s.s.) and *A. pegreffii* in physeterid species, mostly at the preadult stage, strengthen the hypothesis that infections with these two parasite species in physeterids are quite uncommon and probably accidental. In this study, *A. physeteris* was found to be the most common anisakid in the sperm whale, representing 97% of the identified specimens, and 82% of the anisakids recovered from dwarf sperm whale. These proportions are similar to those previously reported in other studies (Mattiucci et al., 2013; Smith & Wootten, 1978).

2.5. Conclusions

Studies on the adult stage of nematode parasites of the genus *Anisakis* mostly rely on occasional stranding events of their definitive hosts, making sampling unpredictable and opportunistic. At the same time, the scientific data obtained are crucial to understand the biology, host specificity, and life cycle of the *Anisakis* species. In fact, the ecology, evolutionary history, geographical distribution, and migration of cetaceans are among the drivers defining their parasites' geographic range and population genetic structure. Drivers of marine speciation are rarely obvious, because the distributions of marine organisms (and changes in these distributions) tend to be poorly documented, implying a high level of uncertainty, and also because the oceans are continuous environment with few geographical barriers. Therefore, we have little understanding of the nature and mechanisms of reproductive isolation in the marine environment that determine the host-parasite association between *Anisakis* spp. and their cetacean hosts. This study indicates a congruence between the geographic distribution of the adult stages of the *Anisakis* species in their final hosts and the larval stages in their fish hosts. While *Anisakis* spp. are generalists for paratenic/transport hosts, the findings on definitive host-parasite association at the adult stage suggests instead a strong preference by some *Anisakis* spp. for certain cetacean species or families. This host preference is likely to be driven by coevolutionary aspects and mediated by definitive host feeding ecology and geographic distribution. Novel data on genetic structuring, geographical distribution, and relative proportions of different parasites species in their hosts can help in unravelling the complex life cycles of these parasites. Alongside the influence of abiotic and biotic limiting factors, the intermediate, paratenic and definitive hosts, according to their ecological characteristics, all contribute to shape the current distributions of *Anisakis* spp. in the world's oceans.

Acknowledgements

We affectionately dedicate this paper to the memory of our colleague and friend Professor Dario Angeletti from the Department of Ecological and Biological Sciences (Tuscia University) who tragically left us on December, the 7th, 2021, during the final stages of writing this paper. He was the director of the Tuscia University unit of Centro Interuniversitario di Ricerca sui Cetacei (CIRCE), and a researcher with a great fondness for cetaceans of the Mediterranean Sea. The authors are very grateful to all the researchers and collaborators who worked on the stranded cetaceans, providing the precious parasite specimens that allowed this research.

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3. II - Anisakid parasites diversity in pygmy sperm whale, *Kogia breviceps* (Cetacea: Kogiidae) stranded at the edge of its distribution range in the Northeast Atlantic. Published in: *Parasite* 31, 43 (2024)

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Abstract -Anisakid nematodes are a globally distributed group of marine mammals' parasites. They are characterized by complex life cycles, with adult stage developing in marine mammals, while planktonic or semi-planktonic crustaceans act as first intermediate hosts, and fish and molluscs representing intermediate/paratenic hosts. Kogiid whales, including pygmy sperm whale *Kogia breviceps*, host a peculiar assemblage of specific anisakid species. Currently, three species within the *Anisakidae* family are known to be specific to kogiid hosts, i.e., *Skrjabinisakis paggiae*, *S. brevispiculata*, and the less studied *Pseudoterranova ceticola*. The aim of this study was to investigate the species diversity of anisakid nematodes sampled from a pygmy sperm whale stranded in 2013 at the edge of its distribution range in the Northeast Atlantic, specifically in the North of Scotland. Nematodes were assigned to genus level based on morphology and identified by sequence analysis of the mtDNA *cox2* gene and the rDNA ITS region. The present finding represents the first observation of syntopic occurrence of adult stages of *Skrjabinisakis brevispiculata*, *S. paggiae*, and *Pseudoterranova ceticola* in a dwarf sperm whale in the Northeast Atlantic Ocean, and represent the northernmost record of these species in this area. *S. brevispiculata* was the most prevalent species, accounting for 55% of the identified nematodes, predominantly at adult stage. *Anisakis simplex* (s.s.) was also abundant, with most specimens in a preadult stage, followed by *S. paggiae* and *P. ceticola*. The pygmy sperm whale is rarely documented in Scottish waters, and its occurrence in the area could suggest a potential expansion of its geographic range. The presence of *S. brevispiculata*, *S. paggiae*, and *P. ceticola* in this whale species in this region may indicate a shift in the whole host community involved in the life cycle of these parasites in northern waters. However, it is also plausible that these parasites were acquired while the whale was feeding in more southern regions, before migrating northbound.

3.1. Introduction

Anisakid nematodes are a group of marine mammal parasites with global distribution. They have complex life cycles, with adult stage developing in marine mammals, predominantly cetaceans for species of the genera *Anisakis*, *Skrjabinisakis* and *Pseudoterranova*. Planktonic or semi-planktonic crustaceans serve as first intermediate hosts, while fish and molluscs act as intermediate/paratenic hosts. Among the various cetacean species hosting adult anisakid nematodes, kogiid whales harbour a distinctive assemblage of specific species. Currently, four Anisakidae species are known to be specific to kogiid hosts: *Skrjabinisakis brevispiculata* (Dollfus, 1966) Safonova, Voronova & Vainutis, 2021, *S. paggiae* (Mattiucci, Nascetti, Dailey, Webb, Barros, Cianchi & Bullini, 2005) Safonova, Voronova & Vainutis, 2021, and two less studied *Pseudoterranova* species, i.e *P. ceticola* (Deardorff & Overstreet, 1981) Gibson & Colin, 1982 and *P. kogiae* (Johnston & Mawson, 1939) Mozgovoi, 1951. Records of these species from kogiid whales have been reported in several tropical and temperate ocean regions, spanning the Atlantic and Indo-Pacific areas (Mattiucci et al., 2018; Kuhn et al., 2016; Klimpel and Palm, 2011; Cavallero et al., 2011; Quiazon et al., 2013; Di Azevedo et al., 2017; Santos and Lodi, 1998; Abollo and Santiago, 2002; Shamsi, et al., 2019).

The pygmy sperm whale, *Kogia breviceps* (de Blainville 1838), and the dwarf sperm whale, *Kogia sima* (Owen 1866), are the only two species in the Kogiidae family, within the superfamily Physeteroidea (Heyning, 1997; Rice, 1998; Plön et al., 2023). These relatively small elusive, and poorly studied whales, have a cosmopolitan distribution in tropical and warm-temperate waters. The pygmy sperm whale primarily inhabits offshore waters, feeding mainly on cephalopods while occasionally consuming fish and crustaceans (Ross, 1979a; 1984; Klages et al., 1989; Plön, 2022; Taylor et al. 2012; Santos et al., 2006). They are found continuously on the eastern side of the Atlantic Ocean, from Argentina to eastern Canada, including the Caribbean Sea, the eastern United States waters, and the Gulf of Mexico. Sightings and strandings have been recorded widely also on the western side of Atlantic, from South Africa to Scottish waters (Bloodworth & Odell, 2008). The aim of this study was to investigate the anisakid species diversity in an individual pygmy sperm whale stranded at the northern boundary of its distribution range in the Northeast Atlantic, specifically in the North of Scotland.

3.2. Methods

3.2.1. Sample collection and morphological examination

A sub-adult female pygmy sperm whale *Kogia breviceps* was found live stranded on September 6th, 2013, at Banff Aberdeenshire, in the North of Scotland (57°40'13.9"N 2°32'20.8"W). The animal died on the beach. At necropsy, the animal weighed 138 kg and measured 207 cm in length. Nematodes were collected from the cardiac section of the whale's stomach and stored in 70% ethanol. Once at the laboratory of Sapienza University of Rome, Italy, anisakid nematodes were initially subjected to a morphological examination using light microscopy (Olympus BX51

microscope). All anisakids were measured (length, mm), mature adults were separated from pre-adults based on the presence of caudal papillae and spicules in males, or the presence of eggs in females, respectively. Sex was determined whenever possible. The heads and tails were then cut and mounted on glass slides with lactophenol for morphological studies, while body fragments were stored in 70% ethanol for further molecular analyses. The nematodes were assigned to genus level according to diagnostic morphological characters (reviewed by Mattiucci et al. 2018). Studied characters included the cephalic end, ventriculus length and shape, presence or absence of the intestinal caecum, spicule length and shape, ratio between the right and left spicule lengths (R/L), male caudal end, and arrangement of caudal papillae (reviewed by Mattiucci et al. 2018).

3.2.2. Genetic identification of anisakid parasites

A subsample including all the morphospecies of nematodes were genetically identified by sequence analysis of the mitochondrial cytochrome *c* oxidase II (mtDNA *cox2*) gene and the entire internal transcribed spacer (ITS) region of the nuclear ribosomal DNA, including the first internal transcribed spacer (ITS-1), the 5.8S, and the second transcribed spacer (ITS-2).

Total DNA was extracted from ~2 mg of tissue from each specimen using the Quick-gDNA Miniprep Kit (ZYMO RESEARCH), following the manufacturer's instructions. DNA extraction was repeated in samples showing poor quality and/or low DNA sample concentration.

For *cox2* gene sequencing, polymerase chain reaction (PCR) amplification was performed using the primers 211F (5'-TTTTCTAGTTATATAGATTGRTTTYAT-3') and 210R (5'-CACCAACTCTTAAAATTA TC-3') (Nadler & Hudspeth, 2000). PCR was carried out according to the procedures described by Mattiucci et al., 2014. The ITS region rDNA (ITS-1, 5.8S rDNA, and ITS-2) was sequenced using the primers NC5 (forward; 5'-GTA GGT GAA CCT GCG GAA GGA TCA TT-3') and NC2 (reverse; 5'-TTA GTT TCT TTT CCT CCG CT-3') (Zhu et al., 2000). PCR was carried out according to the procedures reported in Zhu et al. (2000).

Purification and sequencing of PCR products were carried out by Biofab (Rome, Italy). Sequences were assembled using ChromasPro 2.1.5 (Technelysium Pty Ltd., Tewantin, Australia) and aligned using ClustalX 2.0 (Larkin et al., 2007). Identity was checked using the Nucleotide Basic Local Alignment Search Tool (BLAST, www.ncbi.nlm.nih.gov/BLAST).

3.3. Results

3.3.1. Whale condition

A full necropsy was performed to assess the condition of the whale, including a thorough examination of the stomach lining and contents. Gross examination revealed the animal to be in good nutritional condition. All sections of the stomach were unremarkable with no ulcers noted. Nematodes were present solely in the cardiac section of the stomach, accompanied by a few squid beaks; no marine debris was detected in any stomach sections.

3.3.2. Anisakid infection

Out of the 95 anisakid nematodes examined, 4 specimens showed morphology consistent with *Pseudoterranova*, with the typical intestinal cecum running along the ventriculus, 35 specimens were assigned to *A. simplex* (s.l.) species complex, showing a long and curved ventriculus, and 56 had morphology consistent with *Skrjabinisakis* spp., showing a short ventricle. Within the 56 specimens with *Skrjabinisakis* spp. morphology, 4 showed a ventriculus with a prominent median narrowing, resembling the violin-shape ventriculus characterising *S. paggiae*. Pre-adult (presence of labia, absence of boring tooth) and adult (showing developed reproductive structures, e.g., caudal papillae and spicules in male worms and ovaries in females) specimens were found in each morphospecies.

3.3.3. Genetic identification of anisakid nematodes

Readable *cox2* mtDNA and ITS rDNA sequences were obtained for only 16 specimens, likely due to suboptimal preservation of the nematode samples. Partial *cox2* mtDNA sequence (580 bp) obtained from a specimen with *A. simplex* (s.l.) morphotype showed 99% identity with *Anisakis simplex* (s.s.) deposited in GenBank (accession number: KC810004). One sequence, obtained from a specimen displaying *Skrjabinisakis* morphotype, matched 99% with the species *S. brevispiculata* (DQ116433). A nematode with *Pseudoterranova* morphotype showed >99% similarity with *P. ceticola* (OP380503). The ITS rDNA sequences obtained from nematodes with *A. simplex* (s.l.) morphotype were identical (100%) and matched 99.88% with *A. simplex* (s.s.) (AB277822). Among the seven ITS rDNA sequences obtained from individuals showing *Skrjabinisakis* morphotype, 6 were identical (100%) to a sequence of *S. brevispiculata* (MK325199), while one, displaying a violin-shaped ventriculus, matched 99.75% with *S. paggiae* (GU295973). *Skrjabinisakis brevispiculata* was the most abundant species, accounting for 55% of the identified nematodes. The specimens were found in different developmental stages, with the majority being female (65%), ranging from fully mature adults with developed eggs (49 mm) to immature pre-adults (25 mm). Two male specimens of *S. brevispiculata* measuring up to 39 mm displayed well-developed spicules. All four *Pseudoterranova* specimens (representing 4% of the identified nematodes) were female, with a mean length of 10.3 mm. Although they exhibited adult features, ovaries appeared not fully developed. *Skrjabinisakis paggiae* represented the 4% of identified nematodes. All specimens were female; one measured 37 mm in length and showed ovaries with eggs, while the immature specimens had a mean length of 20.6 mm. The relative frequency of *A. simplex* (s.s.) was 37%. All worms assigned to the *A. simplex* (s.l.) morphotype were in a pre-adult developmental stage, with a mean length below 25 mm, except for one male specimen measuring 30 mm, which possessed developed male spicules. The DNA sequences of the presently identified *Anisakis* species were deposited in GenBank under the following accession numbers: mtDNA *cox2*: PP888191 (*A. simplex* (s.s.)); PP888189 (*S. brevispiculata*); PP888190 (*P. ceticola*). ITS rDNA: PP884102 (*A. simplex* (s.s.)); PP884101 (*S. brevispiculata*); PP884100 (*S. paggiae*).

3.4. Discussion

Kogiid whales are found worldwide in warm-temperate waters and are known hosts for specific *Anisakis* parasites. This study provides data on the anisakid species diversity from a pygmy sperm whale stranded at the northern distribution limit of this species in the NE Atlantic waters in the North of Scotland, harbouring syntopically adult specimens of four nematode species, i.e. *A. simplex* (s.s.), *Skrjabinisakis brevispiculata*, *S. paggiae*, and *Pseudoterranova ceticola*. *Skrjabinisakis brevispiculata*, *S. paggiae*, and *P. ceticola* have been originally described at adult stage in kogiid whales (Mattiucci et al., 2005; Deardorff & Overstreet, 1981; Johnston & Mawson, 1939). Since then, adult specimens of these nematode species have solely been found in these definitive hosts (Santos and Lodi, 1998; Abollo and Santiago, 2002; Mattiucci and Nascetti, 2008; Cavallero et al., 2011; Klimpel and Palm, 2011; Quiazon et al., 2013; Kuhn et al., 2016; Di Azevedo et al., 2017; Shamsi, et al., 2019), suggesting a host-parasite adaptation of these anisakid species to this cetacean group.

Nematode species were recorded with different relative abundances. *S. brevispiculata* was the most common anisakid nematode identified in the pygmy sperm whale in this study, with an assemblage of few pre-adult stages and mature individuals. Mature males and females showed sizes comparable with those provided with the species description (Mattiucci et al., 2005), thus indicating a full development of the parasite in its host in the area. The current finding represents the northernmost record (57°40'13.9"N) of *S. brevispiculata* in the NE Atlantic. So far, this species was found in kogiids from warm and temperate waters of the Atlantic and Pacific Ocean (Mattiucci et al., 2001). *Skrjabinisakis brevispiculata* larvae have been rarely found, at low intensity, in fish from the North Atlantic Ocean (reviewed in Mattiucci et al., 2018), and never in northern seas. The species was recently detected in mesopelagic fish species in tropical areas of the Indian Ocean, suggesting that myctophids play a role in the transmission of this species as direct prey for kogiids or as prey for cephalopods, which themselves represent kogiids prey (Cabrera-Gil et al., 2018).

The finding of *S. paggiae* nematodes in this study represents the first report of the adult stage in the NE Atlantic. Adults *S. paggiae* have been reported in pygmy sperm whales stranded in warm tropical waters of central Eastern Atlantic Ocean (Caribbean Sea and Florida coast) (Mattiucci et al., 2005; Cavallero et al., 2011). At larval stage, *S. paggiae* has been reported from the common fangtooth (*Anoplogaster cornuta*) in the Irminger Sea (NE Atlantic Ocean) (Klimpel et al., 2011), from the European hake *Merluccius merluccius* in Morocco and Mauritania Atlantic waters (Mattiucci et al., 2004), and in the swordfish *Xiphias gladius* in tropical equatorial waters of the Atlantic Ocean, in syntopic infection with *S. physeteris* and *S. brevispiculata* (Garcia et al., 2008).

The occurrence of *P. ceticola* in pygmy sperm whale at such northern latitude (57° N) represent a rare finding. The specimens, all females, were not fully mature worms, with a size way smaller than reported for adult mature females. Adult specimens of this species have been reported in a pygmy sperm whale stranded on the Iberian coast in the NE Atlantic (Abollo and Santiago, 2002), in

tropical waters of central Eastern Atlantic Ocean (Caribbean Sea and Florida coast) (Valentini et al., 2006; Cavallero et al., 2011), and in warm Australian waters (Shamsi, et al., 2019). At larval stages, this species has only been identified in mesopelagic and bathypelagic fish species from tropical warm/temperate waters (revised in Bao et al, 2022).

The occurrence of larval stages of *S. paggiae* in meso pelagic fish (Klimpel et al., 2011), *S. brevispiculata* in myctophids (Cabrera-Gil et al., 2018), *P. ceticola* in mesopelagic and bathypelagic fish species (Bao et al, 2022) suggests that these species interact with hosts that develop in the deep-water or meso-/bathypelagic realms of marine ecosystems. Further studies on mesopelagic and bathypelagic fauna in the NE Atlantic could shed light on the intermediate/transport hosts for these species, thus elucidating the life cycles of these parasites in these northern waters.

Anisakis simplex (s.s.) is a common *Anisakis* species in the NE Atlantic area. Adult individuals of this species are found in cetacean species, mainly Mysticetes and Delphinidae (Lakemeyer, et al., 2020; Højgaard, 1999; Mattiucci, S. et al. 2014; Ryeng et al., 2021; Ugland et al., 2004; Pons-Bordas et al., 2020; Cipriani et al., 2022), whereas L3 stage larva commonly infect many fish and cephalopod species worldwide, commonly at high latitudes (reviewed in Mattiucci et al., 2018; Cipriani et al., 2021; Sayyaf Dezfuli et al., 2021). The predominance of relatively small immature specimens of *A. simplex* (s.s.) recorded in this pygmy sperm whale may suggest that this parasite has a low fitness in this host. A correlation between the size of this parasite and its fitness has indeed been documented (Ugland et al., 2004, Gomes et al., 2021), indicating the ability of *A. simplex* (s.s.) to attain bigger sizes, and eggs production, in killer whales and mysticetes, rather than in dolphins. Previous studies suggested that kogiid whales can acquire *Anisakis simplex* (s.l.) by preying on infected fish and squid, but these species rarely develop into adults (Santoro et al., 2018, Cipriani et al., 2022). One possible explanation for the relatively high infection by *A. simplex* (s.s.) recorded in this pygmy sperm whale, is that the cetacean at the northern limit of its distribution range struggled to find its usual prey species, and opportunistically preyed on pelagic fish and squid species that commonly harbour high burdens of *A. simplex* (s.s.) in the area (Ross, 1979a; 1984; Klages et al., 1989; Plön, 2022; Taylor et al., 2012; Santos et al., 2006). *A. simplex* (s.s.) has been in fact reported at high infection rate in European flying squid (*Todarodees sagittatus*) off Scotland (Cipriani et al., 2021) and in blue whiting (*Micromesistius poutassou*) (Levsen et al., 2018; Sayyaf Dezfuli et al., 2021). Both this cephalopod and this pelagic fish species has been occasionally reported as prey of pygmy sperm whales in the NE Atlant (Desportes, 1985; Santos et al., 2006).

The results obtained remark a strong host-parasite relationship between kogiid whales and a group of anisakid species (i.e. *S. brevispiculata*, *S. paggiae*, and *P. ceticola*), passing through several intermediate/paratenic hosts, as results of a long co-evolutionary history, wherein the host-parasite species ecology has been shaped towards the distribution, diet, and ecology of both host and parasites, potentially adapting to the meso- and bathypelagic ecosystems. However, their preference for either of the two *Kogia* species, and their life cycles remain partially unknown. Poorly known are

also the pathologies associated with the infection by these nematodes towards their definitive hosts. Ulcers, often linked to clusters of *A. simplex* (s.l.) larvae, are frequently observed in extensive infections among delphinids and mysticetes, although occurrences in physeterids are comparatively rare (Smith, 1989; Abollo et al., 1998; Hrabar et al., 2017; Shamsi et al., 2019; Pons-Bordas et al., 2020; Gomes et al., 2021; Ryeng et al., 2021). There were no ulcers present in any section of the stomach of the stranded pigmy sperm whale here studied, despite the presence of several anisakid species.

The pigmy sperm whale is distributed in deep oceanic waters of the tropical and temperate Atlantic, Indian and Pacific Oceans, from approximately 50°S to 50-60°N (Kiszka & Braulik, 2020). This species has been rarely recorded in Scottish waters (seven stranding reports in the last 30 years from Scottish waters -SMASS report cit), and its occurrence in the area has been interpreted as a shift in the community composition, possibly associated to climate change (MacLeod et al. 2005; Santos et al., 2006). The observed occurrence of certain specific parasites in this whale species, such as *S. brevispiculata*, *S. paggiae*, and *P. ceticola*, previously recorded at the adult stage in tropical-temperate waters, and for the first time recorded at these latitudes, may have several explanations. Most likely this animal, before stranding at northern latitudes, was foraging in more southern regions, which are endemic areas for these anisakid species. Both the host and these parasites have been previously reported from the Atlantic Spanish waters (Abollo and Santiago, 2002). The lifespan of an adult anisakid in a definitive host has been estimated to be 1-2 months (Ugland et al., 2004), therefore, the animal would have had enough time to acquire the worms while feeding in southern waters and carry them until the stranding. Even though the presence of both pre-adult and adult stages of *S. brevispiculata* and *S. paggiae* could suggest that the animal acquired these nematodes at different times through its feeding activities during its last lifespan, some of them were possibly acquired recently before stranding, thus at northern latitudes. A second hypothesis, supporting the recent observations that kogiid whales might be spreading northward, most likely due to climate change (MacLeod et al., 2005; Santos et al., 2006), would suggest that the entire host community involving kogiid preys and their parasites are also shifting northwards, finding favourable abiotic and biotic conditions. According to Klimpel et al., (2011), *S. paggiae* is present in non-migratory mesopelagic fish species at northern latitudes, probably indicating that also early larval stages of this parasite species found abiotic and biotic conditions permitting to develop and infect intermediate and paratenic hosts.

These findings suggest that the stability of deep-water conditions is being affected by climate change events affecting upper water layers and influencing the complex interactions between hosts and parasites in the world's oceans, leading to shifts in their distribution and ecology. Further studies investigating these elusive species will shed light on their distribution range and the complex life cycles of their specific parasites.

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Conflicts of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

4. III - Integrative Taxonomy Reveals *Molicola uncinatus* and *Gymnorhynchus gigas* (Cestoda: Trypanorhyncha) Coinfection in the Atlantic Pomfret *Brama brama* from the Mediterranean Sea, With Notes on the Phylogenetic Position of *G. gigas* Within the Family Gymnorhynchidae. Published in: *Frontiers in Veterinary Science*, 9, Article 909163 (2022)

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Abstract -The cestode family Gymnorhynchidae Dollfus, 1935 (Trypanorhyncha) comprises three genera and six valid species that are typically intestinal parasites of large pelagic sharks. Members of Gymnorhynchidae show a wide geographic distribution and represent a global sanitary concern because as larvae they infect the edible flesh of several commercially important fishes with some species having allergenic potential. Larval Gymnorhynchidae collected from the muscles of the Atlantic pomfret *Brama brama* from various localities in the Mediterranean Sea were identified and characterized by combining traditional morphology, scanning electronic microscopy, and molecular analyses using newly generated nuclear 18S and 28S rRNA sequences. Overall, 98 larvae were collected from 20 (100%) Atlantic pomfrets (intensity of infection: 4.9; range: 1–12). High-quality sequences were obtained for 54 larvae. Of these, 11 and 43 larvae were identified as *Molicola uncinatus* and *Gymnorhynchus gigas*, respectively. The phylogenetic analysis showed the existence of three main clades within Gymnorhynchidae. The first included species of *G. gigas* and *M. uncinatus* from the Mediterranean and Atlantic; the second and third major clades included an unidentified species of *Molicola* from the Indian Ocean and specimens of *Gymnorhynchus isuri* from the Mediterranean and Atlantic, respectively. Finally, *Chimaerarhynchus rougetae* was the basal and most diverging taxon. The phylogenetic analysis suggested that *G. gigas* is more closely related to the members of *Molicola*. We demonstrated the coinfection of *M. uncinatus* and *G. gigas* from all localities studied and extended the intermediate hosts and geographical range of *M. uncinatus* by including the Atlantic pomfret and the Tyrrhenian and Ionian Seas, respectively. The present results supported the previous proposal that *G. gigas* and *Molicola* species should be included in the same genus. Our study demonstrated the usefulness of the integrative taxonomy for the unequivocal recognition of larval trypanorhynch species, resolving the current difficulties in the taxonomy, and elucidating the poorly known ecological and biological aspects of members of Gymnorhynchidae.

4.1. Introduction

The cestode family *Gymnorhynchidae* Dollfus, 1935 (order Trypanorhyncha) comprises three genera: *Gymnorhynchus* Rudolphi, 1819, *Molicola* Dollfus, 1935 and *Chimaerarhynchus* Beveridge and Campbell, 1989, with six valid species [i.e., *M. horridus* (Goodsir, 1841; Dollfus, 1935), *M. uncinatus* (Linton, 1924; Palm, 2004), *M. walteri* Palm, 2004, *G. gigas* (Cuvier, 1817; Rudolphi, 1819), *G. isuri* Robinson, 1959, and *C. rougetae* Beveridge and Campbell, 1989] (Palm, 2004; Robinson, 1959; Beveridge & Campbell, 1989). All species at the adult stage infect the intestine of large pelagic sharks (Palm, 2004; Beveridge & Campbell, 1989). The life cycle for members of this family has not yet been elucidated, but it has been proposed that copepods serve as first, pelagic euphausiids or schooling fish as second, and larger predatory fishes as third intermediate hosts (Palm, 2004). Species of *Gymnorhynchidae* show a wide geographic distribution and represent a global sanitary concern because as larvae they infect the edible flesh of several commercially important fishes (Palm, 2004).

According to the published records of *Gymnorhynchidae* in the Mediterranean Sea based on morphological identification of larvae, only *M. horridus* in the muscles of the swordfish *Xiphias gladius* (Caira et al., 1999; Dollfus, 1935), and in the liver of the ocean sunfish *Mola* spp. (Caira et al., 1999; Dollfus, 1935; Palm, 2004), and *G. gigas* in the muscle of the Atlantic pomfret *Brama brama* [syn. *Brama raji*; (Giarratana et al., 2018; Palm, 2004)] and of the silver scabbardfish *Lepidopus caudatus* (Giarratana et al., 2018; Panebianco et al., 2012) have been reported. Giarratana et al. (2018), describing the infection of larvae of *G. gigas* in the muscles of the Atlantic pomfret from Sicilia and Calabria waters of southern Italy, wrote: “Non si procedeva ad identificazione microscopica delle larve, considerata la nota, assoluta, prevalenza di *Gymnorhynchus* spp. in *B. raji* nell'ambito dei macroparassiti muscolari.” The same method for larvae identification was used when Panebianco et al. (2012) reported that larvae of *G. gigas* may interfere with values of total volatile basic nitrogen and trimethylamine nitrogen in the edible musculature of the silver scabbardfish. In both cases, Giarratana et al. (2018) and Panebianco et al. (2012) assumed that those larvae belonged to a single parasite species without even studying them. Recently, the application of molecular analyses for the identification of trypanorhynchs in Mediterranean fishes revealed that the larvae from the liver and muscles of the sunfish and the silver scabbardfish belonged to *G. isuri* (Palm et al., 2017) and *M. uncinatus* (Palm et al., 2015), respectively. This likely implies that the majority of the studies carried out so far from the Mediterranean Sea using questionable assumptions or morphological criteria alone for larvae identification reported misidentification of some *Gymnorhynchidae* species.

Nevertheless, misidentification of *Gymnorhynchidae* larvae using morphological criteria alone may occur because the isolation of their scoleces is difficult and often the partial or total invagination of their tentacles prevent the study of their most important taxonomic characteristics (Palm et al., 2015; Caira et al., 1999). When this occurs, the use of molecular analysis allows for species identification

of trypanorhynch larvae that are challenging or not possible (Palm et al., 2015; Caira et al., 1999; Giarratana et al., 2018). Correct identification of species is required to assess the biodiversity of an ecosystem. Misidentification of *Gymnorhynchidae* species can be confusing to our understanding of their ecological and biological aspects that include their poorly known life cycles, geographical distribution, and host specificity (Palm et al., 2017). Moreover, regarding the sanitary risks for consumers, the correct identification of *Gymnorhynchidae* larvae from the flesh of fishes used for human consumption is of pivotal importance because of the allergenic potential of some species, such as *M. horridus* and *G. gigas* (Buchmann & Mehrdana, 2016; Palm, 2004).

The aim of this study was to characterize, for the first time, by using an integrative taxonomic approach [scanning electronic microscopy (SEM) and molecular analysis], the larvae of *Gymnorhynchidae* that infect the edible musculature of the Atlantic pomfret in the Mediterranean and by generating new molecular data to discuss the phylogenetic relationships within the family.

4.2. Materials and Methods

4.2.1. General Data and Parasitological Analysis

In total, 20 individuals of Atlantic pomfret were studied for trypanorhynch larvae between May 2021 and March 2022. Fish were obtained from off the Tyrrhenian coasts of Campania (Sorrento, $n = 1$), Latium (Saline di Tarquinia, $n = 2$), Sicily (Gulf of Palermo, $n = 4$), and Tyrrhenian and Ionian coasts of Calabria (Vibo Valentia, $n = 3$; Bagnara Calabria, $n = 4$; Cariati, $n = 6$) regions of Italy (Figure 1). Fish were collected by professional fishermen at ≈ 500 m depth using a longline and purchased on landing. The fish were refrigerated ($+4^{\circ}\text{C}$) ($n = 6$) and examined within 24 h from capture or frozen (-20°C) ($n = 14$) for 2 weeks before thawing and dissection. They were measured (fork length—FL) to the nearest 0.1 cm and sex was determined by gonadal examination at dissection.

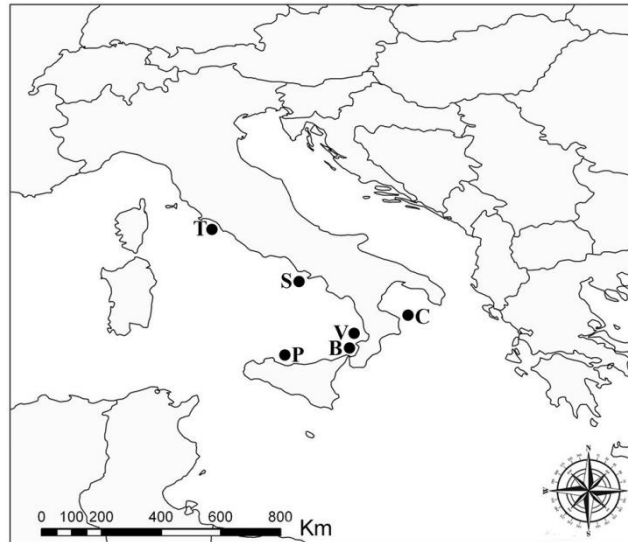


Figure 1. Sampling localities of the Atlantic pomfret in the Mediterranean Sea, 2021–2022. B, Bagnara Calabria; C, Cariati; P, Gulf of Palermo; S, Sorrento; T, Saline di Tarquinia; V, Vibo Valentia.

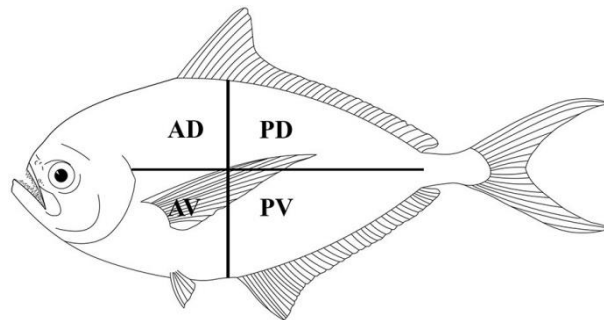


Figure 2. Schematic distribution of Gymnorhynchidae larvae in four muscle sections of the Atlantic pomfret. AD, anterior-dorsal; PD, posterior-dorsal; AV, anterior-ventral; PV, posterior-ventral.

Fish were manually eviscerated, and to assess the distribution of larvae in the skeletal muscles, the fillets of each fish were divided into four portions (i.e., anterior-dorsal, posterior-dorsal, anterior-ventral, and posterior-ventral; see Figure 2). Each portion of the fillets was first cut into thin slices and examined under a dissecting microscope for trypanorhynch larvae. Then, each slice was shredded in a Petri dish with purified seawater and all parasites were collected and counted (Palm et al., 1997). Live parasites were extracted from the muscles using scissors and tweezers and subsequently, were placed in Petri dishes with physiological saline or Ringer lactate solutions (Beveridge & Campbell, 1989; Palm, 2004). To free the scolex of larvae from the surrounding cyst, we used the methods described by Palm (2004), i.e., slicing lengthwise the part below the pars bulbosa of the larva, then the anterior part of the scolex was gripped and evaginated using forceps. The freed scoleces were placed overnight in saline or Ringer lactate solutions to allow for evagination of their tentacles (Beveridge & Campbell, 1989; Palm, 2004).

Table 1. Species, life-history (L, larva; A, adult), host species, locality of collection, and GenBank accession numbers of 28S and 18S sequences of taxa of the family Gymnorhynchidae included in the phylogenetic analysis shown in Figure 5.

Species	Life-history	Host species	Locality	28S	18S	References
<i>Chimaerahynchus rougetae</i>	A	<i>Squalus megalops</i>	New Caledonia	DQ642744	DQ642906	(36)
<i>Gymnorhynchus gigas</i>	L	<i>Brama brama</i>	Tyrrhenian Sea	ON197557-61	ON197572-76	This study
<i>Gymnorhynchus isuri</i>	A	<i>Isurus oxyrinchus</i>	USA	DQ642747	DQ642909	(36)
	L	<i>Mola mola</i>	Tyrrhenian Sea	MT667258	MT667257	(14)
<i>Molicola uncinatus</i>	L	<i>Thyrsites atun</i>	Australia	DQ642746	DQ642908	(36)
	L	<i>Lepidopus caudatus</i>	Malta coast	MT823197	MT823193	(15)
		<i>Mola mola</i>	Tyrrhenian Sea	ON197562-66	ON197567-70	This study
<i>Molicola</i> sp.	L	<i>Taractes rubescens</i>	Indonesia	FJ572949	FJ572913	(25)
	L	<i>Xiphias gladius</i>	SriLanka	KX712337-41	KX712332-36	(37)
<i>Pintneriella musculicola</i> (outgroup)	A	<i>Odontaspis ferox</i>	Indonesia	FJ572948	FJ572912	(25)

Before preserving the scoleces in 70% ethanol for morphological identification, a fragment from the posterior portion of each scolex was frozen (-20°C) for molecular analyses. For morphological identification, the scoleces were clarified in Amman's lactophenol, and the main characters of the scolex and their tentacular apparatus were used to identify the larvae at the lowest possible taxonomic level, according to the identification keys proposed by Palm (2004). The intensity of infection [$\pm$ standard deviation] followed Bush et al. (1997).

For SEM analyses, 10 scoleces were also fixed overnight in 2.5% glutaraldehyde, then transferred to 40% ethanol (10 min), rinsed in 0.1 M cacodylate buffer, postfixed in 1% OsO_4 for 2 h, and dehydrated in ethanol series, critical point dried, and sputter-coated with platinum. Observations were made using a JEOL JSM 6700F SEM operating at 5.0 kV (JEOL, Basiglio, Italy). Parasites were identified at the species level according to the descriptions of Palm (2004) and Casado et al. (1997, 1999).

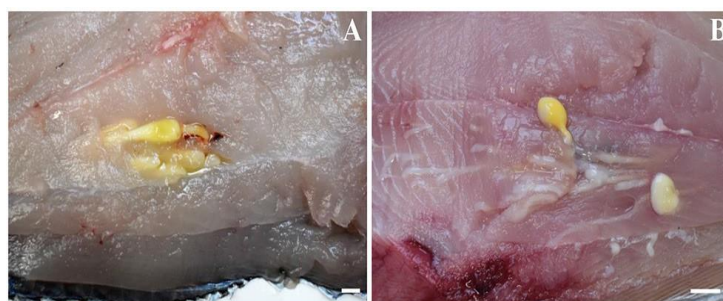


Figure 3. Larvae of *Molicola uncinatus* (A) and *Gymnorhynchus gigas* (B) sequenced in the present study from the muscles of the Atlantic pomfret.

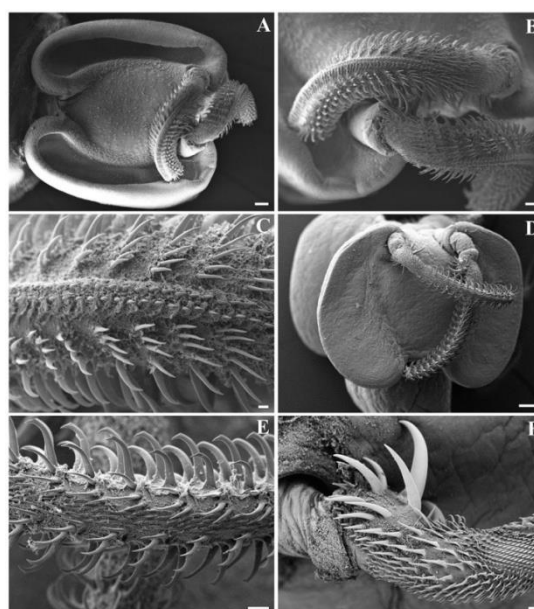


Figure 4. Scanning electron micrographs of *Gymnorhynchus gigas* (A–C) and *Molicola uncinatus* (D–F) sequenced in the present study. (A,D) Bothria and tentacular apparatus. (B, C, E, F) Particular of tentacles. Note the presence of winged chainette elements on the external surface of meta basal armature of *G. gigas* (B,C), and the beak like tips of principal hooks and the band of spiniform hooks on the external surface of metabasal armature of *M. uncinatus* (E). (F) Basal armature of tentacle of *M. uncinatus*.

4.2.2. Molecular and Phylogenetic Analyses

Total genomic DNA (gDNA) was extracted from 60 Gymnorhynchidae larvae, using the Quick-gDNA Miniprep Kit (Zymo Research), following the standard manufacturer-recommended protocol. The partial large subunit ribosomal DNA (lsrDNA, 28S) and the small subunit ribosomal DNA (ssrDNA, 18S) were amplified using the primers ZX-1 (5'-ACCCGCTGAATTTAAGCATAT-3'), 1500R (5'-GCTATCCTGAGGGAAACTTCG-3') (van der Auwera et al., 1994; Tkach et al., 2003), WormA (5'-GCGAATGGCTCATTAAATCAG-3'), and WormB (5'-CTTGTTACGACTTTTACTTCC-3') (Zhu et al., 2000), respectively.

Both polymerase chain reactions (PCRs) were carried out in a 25 µl volume containing 0.6 µl of each primer 10 µM, 2 µl of MgCl₂ 25 mM (Promega), 5 µl of 5 × buffer (Promega), 0.6 µl of deoxynucleoside triphosphates (dNTPs) 10 µM (Promega), 0.2 µl of Go-Taq Polymerase (5 U/µl) (Promega), and 2 µl of total DNA. PCR temperature conditions were the following: 94°C for 3 min (initial denaturation), followed by 35 cycles at 94°C for 30 s (denaturation), 53°C for 30 s (annealing), 72°C for 2 min (extension), followed by post-amplification at 72°C for 7 min. PCR amplicons were purified using the AMPure XP Kit (Beckman Coulter) following the standard manufacturer-recommended protocol and Sanger sequenced from both strands using the same primers through an Automated Capillary Electrophoresis Sequencer 3730 DNA Analyzer (Applied Biosystems), by the BigDye® Terminator v3.1 Cycle Sequencing Kit (Life Technologies). Contiguous sequences were assembled and edited using MEGA X v. 11 (Kumar et al., 2018). Sequence identity was checked using the Nucleotide Basic Local Alignment Search Tool (BLASTn) (Altschul et al., 1990). The 28S and 18S sequences obtained in the present study were aligned with the available sequences of Gymnorhynchidae using ClustalX v. 2.1 (Larkin et al., 2007). Regions of ambiguous alignment were defined in a NEXUS character exclusion set. The 28S and 18S sequences were combined, using SequenceMatrix (Vaidya et al., 2011), while the best partition schemes and best-fit models of substitution were identified using Partition Finder (Lanfear et al., 2012) with the Akaike information criterion (AIC; Akaike, 1974).

The phylogenetic trees were constructed using both the Bayesian inference (BI) and maximum likelihood (ML) method. BI analysis was performed by MrBayes, v. 3.2.7 (Ronquist et al., 2012), using the Markov Chain Monte Carlo (MCMC) algorithm, with four chains, 0.2 as the temperature of heated chains, nst = 6, rates = invgamma, ngammat = 4 models of evolution, 5,000,000 generations, with a subsampling frequency of 500, and a burn-in fraction of 0.25. Posterior probabilities (PP) were estimated and used to assess support for each node. Values with a 0.90 PP were considered well supported. ML analysis was performed using IQ-TREE (Nguyen et al., 2015) with 1,000 ultrafast bootstrap replicates (UFboot). Clades were considered to have high nodal support if the ML bootstrap resampling is ≥95%. The phylogenetic trees were rooted using *Pintneriella musculicola* Yamaguti, 1934 (Palm et al., 2004). For the phylogenetic tree construction, only the species with both 18S and 28S sequences were selected. The 28S and 18S sequences from

GenBank included in the phylogenetic tree are listed in Table 1. Genetic distances were computed using the Kimura 2-Parameters (K2P) model (Kimura, 1980) with 1,000 bootstrap resampling, by MEGA 7.0 (Kumar et al., 2016). Representative sequences obtained in the present study were deposited in GenBank under the accession numbers ON197557-66 for 28S and ON197567-76 for 18S.

4.3. Results

4.3.1. General Data and Parasitological Analysis

Out of the 20 Atlantic pomfret, nine were males and 11 were females ranging in FL from 26.5 to 47.5 cm. Overall 98 larvae were collected from 20 (100%) Atlantic pomfrets. The intensity of infection was 4.9 ± 3.2 ranging from 1 to 12. Most of the larvae were found in the posterior ventral portion (73.5%) of the fillets, the rest were from the posterior dorsal (14.3%) and anterior ventral portions (12.2%) of the fillets. Larvae from the skeletal muscles of the Atlantic pomfret were large and whitish or yellowish (Figure 3), with a sub-spherical blastocyst surrounding the invaginated scolex. Based on the morphological characters of the scolex, all larvae examined were conformed to earlier descriptions of genera of Gymnorhynchidae. We obtained only 18 scolices with at least a tentacle completely everted just from the refrigerated fish to be used for morphological study. These larvae based on the morphological characters were assigned to *M. uncinatus* ($n = 6$) and *G. gigas* ($n = 12$), respectively (Figure 4). In particular, the specimens of *M. uncinatus* showed a band of spiniform hooks on the external surface of the tentacle, uncinated hooks 1(1') of similar size to hooks 2(2'), hooks 4–6(4'–6') beaklike, and 12–15 macrohooks at the base of the tentacle (vs. 8–10 and 25–30 macrohooks in *M. horridus* and *M. walteri*, respectively). Conversely, the specimens of *G. gigas* showed a double chainette of elements on the middle of the external tentacle surface and more than 20 falciform macrohooks at the tentacle base (vs. 8–15 in *G. isuri*).

Table 2. K2P genetic distance $\pm$ standard error (SE) between species of family Gymnorhynchidae. 28S K2P values are reported below the diagonal and 18S K2P values are above the diagonal.

	<i>M. uncinatus</i>	<i>Molicola sp.</i>	<i>G. gigas</i>	<i>G. isuri</i>	<i>C. rougetae</i>
<i>M. uncinatus</i>	-	0.0095 $\pm$ 0.0035	0.0039 $\pm$ 0.0023	0.0195 $\pm$ 0.0050	0.0522 $\pm$ 0.0082
<i>Molicola sp.</i>	0.0137 $\pm$ 0.0030	-	0.0055 $\pm$ 0.0027	0.0156 $\pm$ 0.0044	0.0497 $\pm$ 0.0081
<i>G. gigas</i>	0.0082 $\pm$ 0.0024	0.0156 $\pm$ 0.0033	-	0.0154 $\pm$ 0.0044	0.0495 $\pm$ 0.0080
<i>G. isuri</i>	0.0349 $\pm$ 0.0049	0.0279 $\pm$ 0.0046	0.0381 $\pm$ 0.0054	-	0.0559 $\pm$ 0.0089
<i>C. rougetae</i>	0.0744 $\pm$ 0.0078	0.0780 $\pm$ 0.0082	0.0771 $\pm$ 0.0080	0.0791 $\pm$ 0.0080	-

4.3.2. Molecular and Phylogenetic Analyses

High-quality sequences at both 28S (1,450 bp) and 18S (1,928 bp) gene loci were successfully obtained for 54 larvae. According to BLAST analysis, 11 larvae showed 100% similarity with the sequences (28S: MT823197-DQ642746 and 18S: MT823193-DQ642908) of *M. uncinatus*, available in GenBank. The remaining sequences of 43 larvae obtained at the 28S and 18S gene loci, of which 12 individuals had been morphologically identified as *G. gigas*, showed ≈ 98.90 and 99.40% similarities, respectively, with the sequences of *M. uncinatus* (28S: MT823197-DQ642746 and 18S: MT823193-DQ642908) available in GenBank. Coinfection by both parasite species was found in all localities studied. Genetic distance values between species of Gymnorhynchidae based on 28S and 18S sequences data sets are given in Table 2. While no intraspecific variation was found, a certain degree of genetic differentiation was observed at the interspecific level. In particular, the K2P value ranged between *M. uncinatus* and *G. gigas* from a minimum of 0.0082 ± 0.0024 and 0.0039 ± 0.0023 , to a maximum of 0.0791 ± 0.0080 and 0.0559 ± 0.0089 between *G. isuri* and *C. rougetae*, at the 28S and 18S gene loci, respectively. Phylogenetic tree was carried out using both separately (data not shown) and combined 18S and 28S gene loci data sets (Figure 5). Because BI and ML analyses yielded identical topologies, only ML is shown in Figure 5. Within the family Gymnorhynchidae, three clades were detected. Specimens of *M. uncinatus* and *G. gigas* were clustered in a first clade with high nodal support (PP = 1.0, UFboot = 99). In particular, the newly generated sequences of *M. uncinatus* clustered with high nodal support (PP = 1.0, UFboot = 100) with those of *M. uncinatus* previously deposited in GenBank. In the same clade, a novel sister clade of *M. uncinatus* was formed with high nodal support (PP = 1.0, UFboot = 100) by the newly generated sequences of *G. gigas*. The second major clade, with high nodal support (PP = 1.0, UFboot = 88), included an unidentified species of *Molicola*. The third clade was formed by specimens of *G. isuri*

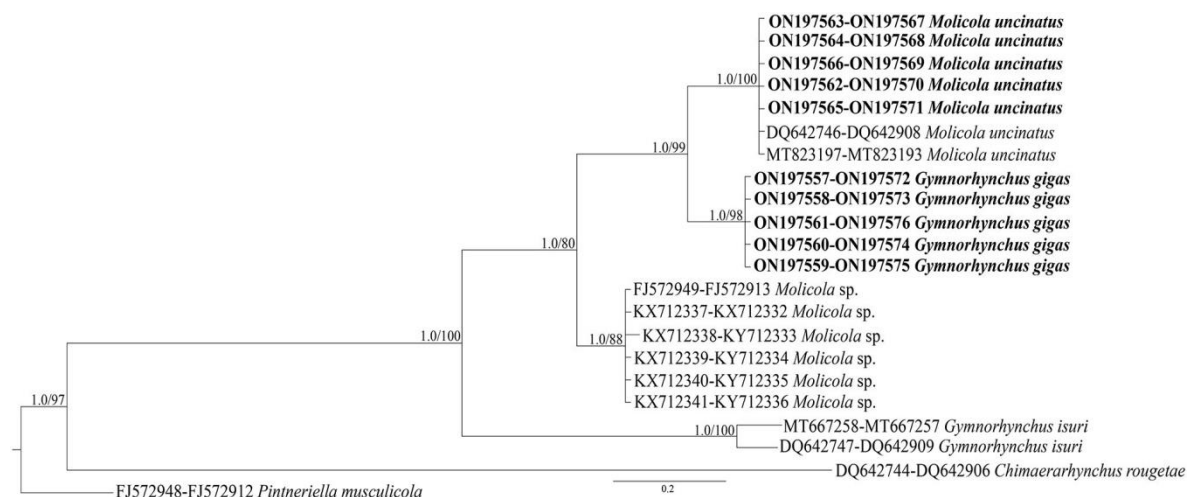


Figure 5. Phylogenetic concatenated tree from maximum likelihood (ML) based on 28S and 18S sequences obtained in the present study (inbold), with respect to the Gymnorhynchidae sequences at the same gene loci available in GenBank. Nodal support is indicated for BI (posterior probabilities) and ML (ultra fast bootstrap), respectively. *Pintneriella musculicola* was used as outgroup.

4.4. Discussion

Trypanorhynchs have a unique morphological feature, with both adults and larvae having the scolex equipped with a tentacular apparatus consisting of four retractile tentacles adorned with hooks (Palm, 2004; Marques et al., 2017). According to Palm (2004), the three genera of Gymnorhynchidae can be distinguished on the basis of the armature patterns of their tentacles (heterocanthous typical in *Molicola*; poeciloacanthous multitypical in *Gymnorhynchus*; and poeciloacanthous typical in *Chimaerarhynchus*) and the absence (in *Molicola*) or presence (in *Gymnorhynchus* and *Chimaerarhynchus*) of winged chainette elements on the surface of their tentacles. Morphological characters of the present specimens of *M. uncinatus* and *G. gigas* were in agreement with descriptions of Palm (2004) and Casado et al. (1994, 1996). Molecular analysis confirmed the morphological identification of *M. uncinatus*, with the sequences obtained being identical to those of *M. uncinatus* previously collected in *Thyrstites atun* and *L. caudatus* (Palm et al., 2004; Olson et al., 2010). The sequences of *G. gigas* obtained here, revealing closer similarity with those of *M. uncinatus*, represent the first molecular data available for *G. gigas*, which should be considered a starting point for comparative analyses and molecular identification of this species. In the present study, the identification of Gymnorhynchidae larvae found in the musculature of the Atlantic pomfret from various localities within the Mediterranean was obtained by combining traditional morphology, SEM, and molecular analyses because only a few live larvae everted their tentacles, which represent organs with essential diagnostic characters (Palm, 2004; Marques et al., 2017). Therefore, the use of integrative taxonomy allowed for accurate discrimination between *M. uncinatus* and *G. gigas*.

The supposed life-history strategy of Gymnorhynchidae might explain the coinfection with *M. uncinatus* and *G. gigas* in the Atlantic pomfret. According to the parasite-host list of Palm (2004) and subsequent literature (Palm et al., 2004; Olson et al., 2010), *M. uncinatus* infects *Alopias vulpinus* (Lamniformes) as definitive host and *Thyrstites atun*, *Taractes rubescens*, *Taractichthys steindachneri*, and *L. caudatus* (Scombriformes) as intermediate hosts. However, as a sporadic finding, it was also reported in the liver of *M. mola* (Tetraodontiformes), and in the musculature of *Allothunnus fallai* (Scombriformes) and *X. gladius* (Carangiformes) (Palm, 2004). Conversely, *G. gigas* infects *Carcharodon carcharias*, *Isurus oxyrinchus*, *Lamna nasus* (Lamniformes), and *Oxynotus centrina* (Squaliformes) as definitive hosts and *B. brama* as intermediate host (Palm, 2004). The present results confirm the importance of Scombriformes fishes in the life cycle of both trypanorhynch species. Moreover, all Scombriformes usually involved in their biological cycle are benthopelagic fishes (occurring along the upper slope down to 400–1,000 m), which prey on small fishes, cephalopods, amphipods, and euphausiids (Froese & Pauly, 2023). It has been suggested that different species of trypanorhynchs have evolved different life-history strategies occupying different ecological niches, also in terms of vertical distribution (Palm, 2004; Bush et al., 1997; Klimpel et al., 2001). Indeed, each parasite species has its depth preferences, following the most common feeding

ecology and depth range of its definitive host. Currently, there is no information on the feeding behavior of the Atlantic pomfret; however, studies on the feeding ecology of other Bramidae fishes and known intermediate and definitive hosts of *G. gigas* and *M. uncinatus* support that these fishes take consistent vertical movements in the water column for feeding (Froese & Pauly, 2023). The vertical migration could allow the infection with Gymnorhynchidae by feeding on infected prey, and at the same time, it could increase the possibility of parasite transmission, exposing the Atlantic pomfret to the attack of pelagic sharks. Our results suggest that the benthopelagic deep habitat may represent the preferred food-web system in which larvae of *M. uncinatus* and *G. gigas* are found in the Mediterranean.

The high migratory habits of the known intermediate and definitive hosts of both *M. uncinatus* and *G. gigas* confirm that their wide geographical dispersion may be associated with the movements of their hosts (Palm, 2004; Klimpel et al., 2001). Indeed, the geographical range so far reported for *M. uncinatus* and *G. gigas* seems to mirror the migratory behavior and distribution of their definitive hosts, with both species known from the north and southeast Pacific, northwest and south Atlantic, North Sea, and Mediterranean Sea (Palm, 2004; Olson et al., 2010). The Atlantic pomfret and the Tyrrhenian and Ionian Seas represent new host and geographical records for *M. uncinatus*, respectively. Indeed, in the Mediterranean, this parasite has only been recently found off the Malta coast (Olson et al., 2010).

Regarding the larvae distribution, the higher occurrence of both species in the ventral portions of fillets may be linked to the shortest distance of the larvae penetrating from the guts into the musculature, as proposed by Seyda (1976). Meanwhile, the higher occurrence in posterior portions could provide greater possibilities to enhance the transmission of parasites. This is in agreement with previous observations (Palm et al., 1997; Klimpel et al., 2001; Beveridge & Campbell, 2000) and may be explained by the hunting strategy followed by several sharks (definitive hosts), which use a tail-on approach, preying from behind.

The phylogeny of Gymnorhynchidae derived from the sequences available in GenBank, along with those generated in the present study, revealed the existence of three main clades. The first involved species of *G. gigas* and *M. uncinatus*, all from Mediterranean teleosts except for a specimen of *M. uncinatus* collected from the southeast Pacific (Australian waters) in *T. atun* (Palm et al., 2004). The second and third clades included an undetermined species of *Molicola* infecting the swordfish (*X. gladius*), the pomfret (*Taractes rubescens*), and *Taractichthys steindachneri* from the Indian Ocean, and *G. isuri* infecting *M. mola* and *I. oxyrinchus* from the Mediterranean and Atlantic, respectively. Finally, *C. rougetae* was the basal and most diverging taxon in all the elaborations from the two different datasets.

The phylogenetic analysis based on both genetic markers supported the hypothesis that *G. gigas* is very closely related to members of the genus *Molicola* rather than to the other species of the genus *Gymnorhynchus*. This finding suggests that the morphological character—the presence vs. the

absence of winged chainette elements on the external surface of their tentacles, as proposed by Palm (2004) to differentiate *Gymnorhynchus* and *Molicola*—has no taxonomic value for genus diagnosis. A similar situation was found in the phylogenetic inference of the anisakid species of the *Contracaecum osculatum* (s.l.) complex (Rudolphi, 1802) Baylis, 1920, plus *Phocascaris cystophorae* (Berland, 1963), which formed a well-supported monophyletic group (Mattiucci & Nascetti, 2006), despite the morphological character presence/absence of interlabia on which their distinctiveness at the genus level has been based. Unfortunately, the anatomical characters of adult *G. gigas*, the type species of the genus *Gymnorhynchus*, are unknown, as this species was described from larval material (Palm, 2004; Marques et al., 2017; Beveridge & Campbell, 2000; Dollfus, 1946). This prevents the identification of further distinctive morphological characters between the two genera until a redescription of adult *G. gigas* from the type locality is made.

The present phylogenetic results support Dollfus's (1946) proposal, which suggested that *G. gigas* and *Molicola* species be included in the same genus. Indeed, Dollfus (1946) placed *Gymnorhynchus* and *Molicola* in the same genus, suggesting the subgenus *Molicola* for species that had a band of hooks on the external surface of their tentacles instead of winged chainette elements. Later, based on tentacle characters, members of *Gymnorhynchus* were separated into the families Gymnorhynchidae and Molicolidae by Beveridge and Campbell (2000). A subsequent cladistic analysis based on shared morphological characters of 49 recognized genera of Trypanorhyncha supported the close relationship between Gymnorhynchidae and Molicolidae (Beveridge & Campbell, 2000). More recently, Palm (2004) included *Gymnorhynchus* and *Molicola* into the same family.

4.5. Conclusions

The present study demonstrated the usefulness of the integrative taxonomy for the unequivocal recognition of larval trypanorhynch species in fish when coinfection with similar species occurs. The present phylogenetic analysis also strongly supported the hypothesis that *G. gigas* is very closely related to species of *Molicola*. Finally, the coinfection within the musculature of the same fish species from various Mediterranean localities and the strict phylogenetic relationships between *G. gigas* and *M. uncinatus* could suggest a similar coevolutionary history that could have accompanied the speciation of these cestodes in their definitive hosts. Nevertheless, additional molecular and new co-phylogenetic data could clarify the patterns of host-parasite coevolutionary events between these parasites and their hosts.

Data Availability Statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article.

Ethics Statement

Ethical review and approval was not required for the animal study because the fish used in the present study were purchased dead on landing and no permit and/or ethical consideration were needed.

Author Contributions

MS, SM, and MP conceived and designed the study. MS, MP, and RA performed fish parasitological analysis, parasites collection, morphological and molecular analysis, and data elaboration. MS wrote the manuscript. All authors read, reviewed, and approved the final manuscript.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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5. IV - Distribution and genetic diversity of *Grillotiaadenoplusia* (Cestoda: Trypanorhyncha) in Small Deep Dwelling Sharks from Different Areas of the Mediterranean Sea

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Abstract -The order Trypanorhyncha (Cestoda) comprises a wide range of heteroxenous endoparasites, exhibiting a higher degree of host specificity in their adult stages compared to the larval forms (Palm & Caira, 2008). Among these, adults of *Grillotia adenoplusia* exclusively parasitize the bluntnose sixgill shark *Hexanchus griseus*. Various small deep-dwelling sharks act as intermediate/paratenic hosts, harboring the plerocercoid larval stages of the parasite. This study investigates the distribution, infection levels, and genetic diversity of *G. adenoplusia* from small deep-dwelling sharks from Mediterranean Sea waters, aiming to evaluate these parasites as indirect ecological indicators of deep-sea trophic-web stability, with *H. griseus* as the apex predator. A total of 29,031 *Grillotia* sp. plerocercoids were collected from 553 small deep-dwelling sharks (mainly *E. spinax* and *G. melastomus*), caught as by-catch during commercial trawling in five Mediterranean Sea regions: Costa Brava, Western Sardinian Sea, Port of Civitavecchia, Gulf of Naples, and Western Ionian Sea. Identification of *G. adenoplusia* (N = 344) was performed by sequence analyses of 18S and 28S rDNA gene loci; additionally, a novel mitochondrial gene locus (mtDNA *cox1*) was sequenced for the first time to explore the genetic variation within the taxon. The concatenated phylogenetic analysis of the sequences from 28S, 18S, and *cox1* in all N = 344 specimens showed that all clustered in a single clade, with a 100% probability value. Genetic analysis of 344 *G. adenoplusia* *cox1* sequences revealed 166 haplotypes, with significant genetic differentiation ($F_{st} = 0.3$) between two clusters, as evidenced by PCA and heatmap analyses. This suggests genetic sub-structuring of *G. adenoplusia* across the Mediterranean Sea, potentially indicating different parasite strains. This pattern may be linked to genetic sub-structuring in its definitive host, the deep-sea bluntnose sixgill shark, which has shown significant spatial genetic heterogeneity in recent studies. The high genetic polymorphism observed at the *cox1* locus likely correlate with the large population size of *G. adenoplusia* here reported in the Mediterranean. Infection levels increased with host size, with prevalence ranging from 56-87% in *E. spinax* to 100% in *G. melastomus*. These findings suggest that *G. adenoplusia* can be used as an indirect indicator of deep-sea trophic web stability, providing valuable insights into the ecological dynamics of Mediterranean deep-sea ecosystems, with *H. griseus* as an apex predator.

5.1 Introduction

Trypanorhynch cestodes represent a highly diverse group of marine parasites, with more than 270 species identified to date (Palm et al. 2009; Beveridge and Campbell 2013). The adult stage is commonly found in the gastrointestinal tracts of sharks and rays, whereas larvae infect a variety of invertebrates and bony fish (Campbell and Beveridge 1994; Palm 2004). These parasites are widely regarded as a valuable model for investigating the evolution and life strategies of helminths in fish (Palm 2004; Palm et al. 2007). A distinctive feature of trypanorhynchs is their tentacular apparatus, which consists of four retractable tentacles armed with hooks and connected to bulbs. This structure is key to taxonomic identification, although the retraction of tentacles can make identifying larval stages challenging without molecular tools (Campbell and Beveridge 1994; Palm 2004).

The genus *Grillotia* (Guiart, 1927) encompasses 18 recognized species, of which only seven (*G.yuniariae*, *G. rowei*, *G. adenoplusia*, *G. erinaceus*, *G.sasciae*, *G. pristiophori*, and *G. acanthoscolex*) were identified using molecular tools (Littewood et al., 1998; Palm et al., 2009; Olson et al., 2010; Oosthuizen et al., 2021; Lopez-Verdejo et al., 2024). Its life cycle typically begins with a copepod as the first intermediate host, followed by teleost fish or cephalopods as the second. In some cases, larger fish or elasmobranchs serve as paratenic hosts before the parasite reaches its definitive host, which is always an elasmobranch (Palm, 2004; Dallarés et al., 2016). *Grillotia* species have a cosmopolitan distribution, occurring in regions such as the Mediterranean Sea (Özer et al., 2014; Dallarés et al., 2017; Genç et al., 2018; Santoro et al., 2018, 2021) and the northeastern Atlantic Ocean (MacKenzie, 1990; Palm and Schröder, 2001; Alvarez et al., 2006). The larval stages, known as plerocerci, have been observed in different tissues, including muscles, of their second intermediate and paratenic hosts. Regarding the life cycle of *G. adenoplusia*, adults inhabit the digestive tract of the bluntnose sixgill shark (*Hexanchus griseus*) who act as definitive host (Lopez-Verdejo et al., 2024). Meanwhile larvae stages inhabit small to mid-sized benthonic sharks (i.e., *Etmopterus spinax*, *Galeus melastomus*, and *Scyliorhinus canicula*) in the Mediterranean Sea water (Santoro et al., 2021). These organisms are generally hermaphroditic, although cross-fertilization events -reciprocal fertilization between two hermaphroditic individuals- also appear possible (Olson et al., 2021). Such events promote genetic diversity; however, this theory is still under investigation due to limited data and challenges in studying these organisms and parasites in general.

Parasites are essential bioindicators that offer insights into various aspects of host biology, including behavior and population dynamics (Caira, 1990; Williams et al., 1992). They are particularly useful in studying deep-sea and rarely observed marine species (MacKenzie and Abaunza, 1998). By examining parasites, researchers can infer host population connectivity, migratory patterns, and localized movements, all of which are critical for understanding fish population structure and implementing effective fisheries management (Grutter, 1998; MacKenzie and Abaunza, 1998; Mattiucci et al., 2015). This information plays a vital role in conservation efforts by helping to identify demographically distinct fish populations (Gubili et al., 2016). Palomba et al.

(2023) recently emphasized the significance of using trophically transmitted helminth endoparasites (TTHs), such as nematodes (e.g., *Anisakis* spp.) and cestodes (e.g., order Trypanorhyncha), as indicators of anthropogenic impacts. These parasites, which rely on hosts from mid-to-upper trophic levels, are highly responsive to variations in host populations and environmental factors. Human-induced disturbances can influence their geographic distribution, prevalence, and genetic diversity.

The large ribosomal subunit gene (28S rDNA) demonstrates higher variability and divergence among phyla compared to the small subunit gene (18S rDNA) in *Grillotia* spp. (Hillis and Dixon, 1991). However, the concerted evolution of these ribosomal units results in high intragenomic similarity (Arnheim, 1983), where individual copies within a species are nearly identical, even as interspecies differences accumulate rapidly (Hillis and Dixon, 1991).

Despite its relatively low level of variation compared to other molecular markers, the variability observed in the 28S rDNA region is particularly useful for reconstructing recent evolutionary events. The rapid concerted evolution of ribosomal DNA, coupled with reduced or absent gene flow between geographic localities, tends to homogenize allelic variation within individuals and populations while accentuating variability between distinct populations. Palm et al. (2007) investigated the genetic diversity of the cestode *Tentacularia coryphaenae* by analyzing the 28S rRNA gene. Their results revealed a high degree of genetic homogeneity across populations, suggesting that the species exhibits a wide distribution and low host specificity. However, the limited intraspecific variation in the 28S rRNA gene restricted the ability to distinguish finer genetic structures within the species. To address this limitation and obtain more detailed insights into the genetic diversity and evolutionary history of parasitic cestodes, the authors emphasized the importance of incorporating additional molecular markers, such as the mitochondrial cytochrome c oxidase subunit I (*cox1*) gene. The *cox1* gene, with its higher resolution, offers a more effective tool for examining genetic differentiation and understanding the population structure of these parasites.

Isbert et al. (2023) investigated the genetic structure of *G. a* larvae found in the velvet belly lantern shark (*Etmopterus spinax*) in the Mediterranean Sea and Northeast Atlantic Ocean. To understand the genetic diversity and relationships of these parasites, the researchers used the 28S ribosomal DNA (rDNA) marker. Their analysis revealed that the 28S rDNA sequences from *Grillotia* larvae were almost identical to a species previously identified in the Mediterranean Sea. Two distinct genetic genotypes were found: one associated with the deep-sea shark *Dalatias licha*, and another shared by multiple host species, including *Etmopterus spinax*, *Galeus melastomus*, and *Scyliorhinus canicula*. This genetic variation suggests that these two genotypes may occupy different ecological niches or interact with distinct host species. The study highlights the effectiveness of using the 28S rDNA marker to explore the genetic structure and diversity of parasites, providing valuable insights into their ecology and the broader marine ecosystem.

The objectives of this study were: 1) to describe the infection levels with the cestodes larvae belonging to the genus *Grillotia* in four species of small-mided sized benthonic sharks that act as

intermediate/paratenic hosts (*Dalatias licha*, *Scyliorhinus canicula*, *Galeus melastomus* and *Etmopterus spinax*) in different geographical areas of the Mediterranean sea; 2) to develop and use highly polymorphic molecular markers to assess the population genetic structure of this cestode across the sampled regions; in the main scope to explore the potential use of *G. adenoplusia* as an indirect ecological indicator of the deep-sea food-web stability .

5.2. Materials and methods

5.2.1. Sampling and parasitological analysis

Between April 2023 and July 2024, a total of 545 specimens of mid-sized benthic shark's species (*G. melastomus*, *S. canicula*, *E. spinax*, and *D. licha*) were collected as bycatch during commercial trawling operations targeting red and pink shrimp. These individuals were captured at depths ranging from 50 to 800 meters across five Mediterranean locations: Palamos (Costa Brava), Port of Civitavecchia (Tyrrhenian Sea), Gulf of Naples (Tyrrhenian Sea), Western Sardinian Sea, and the western coast of the Ionian Sea. The precise number of specimens per species and sampling location is presented in Figure 1.

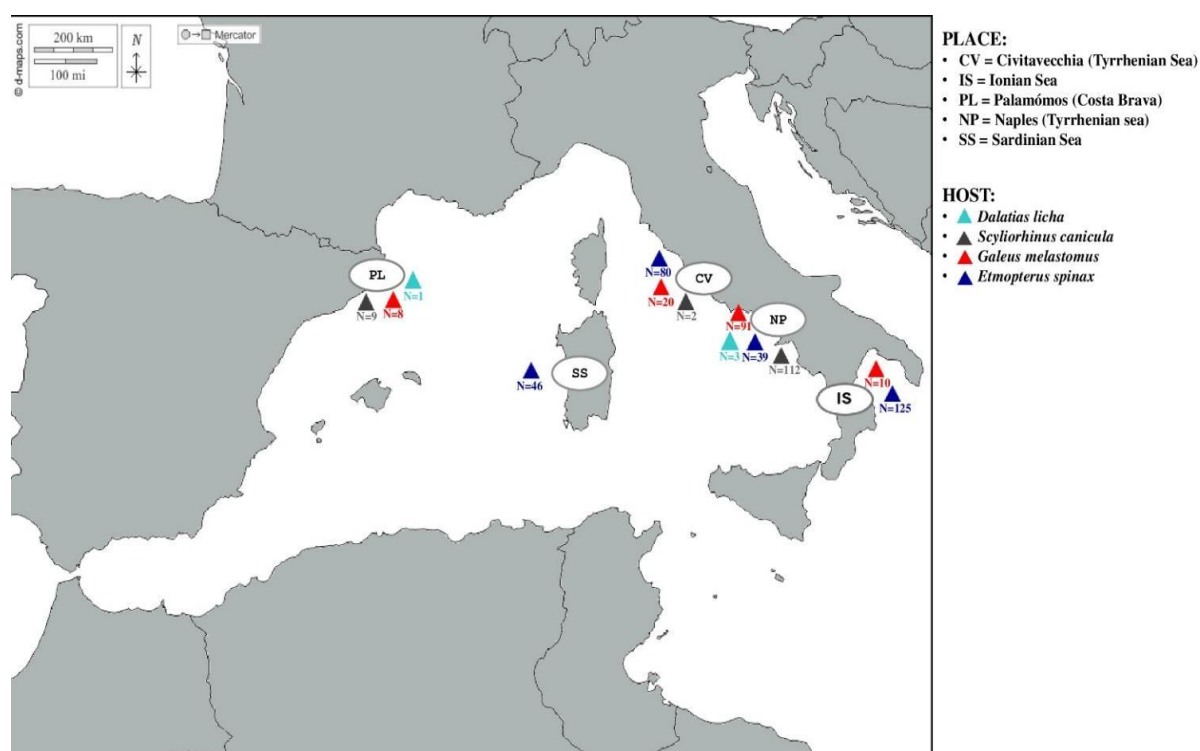


Figure 1: Sampling map (from d-maps.com), coloured and edited using Gimp 2.10.38. The Sampling locality of 546 specimens of mid-size benthic shark fished as bycatch in Mediterranean Sea localities. Each triangle in a different colour represents a shark species, with the number of sampled fish indicated below the triangle.

Specimens were immediately frozen after capture and shipped directly to the Experimental Ichthyogenic Centre of the Tarquinia Salt Pans (CISMAR) at Tuscia University. Morphometric data, including total length (TL), partial length (PL), total weight (TW), eviscerated weight (EW), and sex, were recorded. As previously described by Santoro et al. (2021), Trypanorhyncha plerocercoids

typically localize in the caudal musculature of their intermediate and paratenic hosts. The caudal regions of the specimens were sectioned into approximately 5 cm segments, extending from the base of the caudal fin to just below the dorsal fin (Figure 14). A total of 28,630 plerocercoids were examined using a stereomicroscope (Axio Zoom V16, Zeiss) and identified based on established morphological keys (Beveridge & Campbell, 2013; Caira et al., 2020, 2021; Gibson, 1976a, 1976b; Laubier et al., 1966; Palm, 2004; Williams, 1968). Identification was conducted using key morphological characteristics of the scolex and bothria, enabling species-level identification and genus assignment to *Grillotia* according to Palm's (2004) taxonomy. After identification, all plerocercoids were counted, washed in saline solution, and stored at -20°C for subsequent genetic analysis.



Figure 2: Macrophotographs of skeletal muscles of *G. melastomus*, from Civitavecchia, infected with plerocercoids of *G. adenoplusia*.

5.2.2. Molecular analyses

5.2.2.1. Molecular identification of *Grillotia* by 28s and 18s gene loci (rDNA)

Genomic DNA was extracted from 396 plerocercoids using the Quick-gDNA Miniprep Kit (ZYMO RESEARCH), following the standard protocol recommended by the manufacturer. For molecular identification of the plerocercoids, by 28S (rDNA), to avoid non-specific amplifications, we designed new specific primers for this region. The primers used were 28GRIL-F (forward: 5'-GGAATAGCCCAGCACCGAA-3') and 28GRIL-R (reverse: 5'-ACAACCGACCAATGACCACA-3'). The 18s (rDNA) was amplified using primers WormA (5' - GCGAATGGCTCATTAATCAG - 3') and WormB (5' -CTTGTTACGACTTTTACTTCC - 3') (Littlewood & Olson, 2001). Both PCR

reactions were carried out in a 25 μ L. The master mix for 28s region volume containing 1 μ L of each primer (10 mM), 3 μ L of MgCl₂ (25 mM) (Promega), 5 μ L of 5x buffer (Promega), 0.6 μ L of dNTPs (10 mM) (Promega), 0.2 μ L of Go-Taq Polymerase (5 U/ μ L) (Promega), and 2 μ L of total DNA. The master mix for 18s region volume containing 0.6 μ L of each primer (10 mM), 2 μ L of MgCl₂ (25 mM) (Promega), 5 μ L of 5x buffer (Promega), 0.6 μ L of dNTPs (10 mM) (Promega), 0.2 μ L of Go-Taq Polymerase (5U/ μ L) (Promega) and 2 μ L of total DNA. The PCR cycling parameters for 28s region of *Grillotia plerocercoids* were: 94°C for 5 minutes, followed by 40 cycles at 94°C for 30 seconds, 54°C for 30 seconds, 72°C for 2 minutes, and a final extension at 72°C for 7 minutes. The PCR cycling parameters for 18s region were as follows: 94°C for 3 minutes, followed by 35 cycles at 94°C for 30 seconds, 53°C for 30 seconds, 72°C for 2 minutes, and a final extension at 72°C for 7 minutes. Purified PCR products were Sanger sequenced from both strands using an automated capillary electrophoresis sequencer 3730 DNA Analyzer (Applied Biosystems) and the BigDye Terminator v. 3.1 Cycle Sequencing Kit (Life Technologies). The sequence identity was verified using the Basic Local Alignment Search Tool (Blast, www.ncbi.nih.gov/BLAST/). Sequences were aligned and compared with reference sequences in the NCBI nucleotide (NT) database. The identity of the 28s and 18s (rDNA) sequences confirmed the species *G. adenoplusia*, showing 99%-100% similarity to published sequences (i.e., Lopez-Verdej et al., 2024; M. Santoro et al., 2021; W. Isbert et al., 2022).

5.2.2.2. Genetic characterisation of *G. adenoplusia* by mtDNA *cox1*

For the first time in this species, a mitochondrial gene was characterized. 396 genomic DNA plerocercoid samples previously extracted for genetic characterization through 28s and 18s (rDNA) were sequenced at mtDNA *cox1* gene locus. In this aim, new specific primers were designed for the Cytochrome oxidase 1 region (*cox1*). The primers used were GRCOX1BIS2-F (forward: 5' – TTTTAAACTGACTCCCGCTATTAC– 3') and GRCOX1-R (reverse: 5' – CCCTGAGGTGTATGTTTTGATTT– 3'). PCRs were performed in a 25 μ L volume containing 1.5 μ L of each primer (10 mM), 2 μ L of MgCl₂ (25 mM) (Promega), 5 μ L of 5x buffer (Promega), 0.8 μ L of dNTPs (10 mM) (Promega), 0.3 μ L of Go-Taq Polymerase (5 U/ μ L) (Promega), and 3 μ L of total DNA. The PCR cycling parameters were as follows: 94°C for 5 minutes, followed by 37 cycles at 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 1 minute, and a post-amplification at 72°C for 10 minutes. PCR product were sequenced by Sanger method from both strands using an Automated Capillary Electrophoresis Sequencer 3730 DNA Analyzer (Applied Biosystems) and the BigDye Terminator v. 3.1 Cycle Sequencing Kit (Life Technologies). The sequence identity was verified using the Basic Local Alignment Search Tool (Blast, www.ncbi.nih.gov/BLAST/). Sequences were aligned and compared with reference sequences in the NCBI database. Sequence similarities ranged from approximately 79.83% to 79.95% when compared to *cox1* sequences of cestodes deposited in GenBank (Jensen et al., 2026; Tang et al., 2017), which were insufficient for species identification.

5.2.3. Phylogenetic analysis and genetic diversity

The rDNA and mtDNA sequences were assembled and edited with ClustalX v. 2.1 (Larkin et al., 2007) and MEGAX v. 11 (Kumar et al., 2018), respectively. Bayesian inferences (BI) were constructed using MrBayes, v. 3.2.7 (Huelsenbeck & Ronquist, 2001). The Bayesian posterior probability analysis was performed using the MCMC algorithm, with four chains, 0.2 as the temperature of heated chains, 5,000,000 generations, with a subsampling frequency of 500 and a burn-in fraction of 0.25. Posterior probabilities were estimated and used to assess support for each node. Values with a 0.90 posterior probability were considered well supported. The species *Pintneriella musculicola* (a. n. FJ572948, FJ572912) was used as the outgroup for 28s and 18s; *Kotorella pronosoma* (a. n. EF103923) was used as the outgroup for the *cox1* gene locus. Concatenated phylogenetic trees were performed using SequenceMatrix, while the best partition schemes and best fit models of substitution were identified using Partition Finder with the Akaike information criterion. Genetic distances (shown as the percentage difference, i.e., n° of base substitutions per sites *100) were computed using the Kimura 2-Parameters (K2P) model (Kimura, 1980) with 1,000 bootstrap resampling, by MEGA Software. A principal component analysis (PCA) and an heatmap based on genetic distances (K2P) were performed with the R packages, i.e., Adegenet and ComplexHeatmap package. Genetic structure was assessed by calculating pairwise *Fst* values by using ARLEQUIN V3.5. The *Fst* value ranges from a maximum of 1, indicating complete differentiation among sequences of pre-defined populations to a value equal to a minimum of 0, which indicates no differentiation among the populations. Number of haplotypes (Nh), number of unique haplotypes (Nuh), nucleotide diversity (π), haplotype diversity (Hd), average number of differences (K), and number of polymorphic sites (S) were calculated using DnaSP V5.10.01 (<http://www.ub.edu/dnasp>).

5.2.4. Infection parameters

Infection parameters were estimated as prevalence (P%) of plerocercoids with 95% confidence intervals (CI, Clopper-Pearson) and mean abundance (mA) of plerocercoids with standard deviation ($\pm$ SD), following Bush et al. (1997). Prevalence represents the number of infected hosts relative to the total, calculated by species and for the whole area, while mean abundance indicates plerocercoids per host, also calculated by individual area. Calculations were performed using Quantitative Parasitology (QPweb) 3.0 software (Reiczigel et al., 2019; Reiczigel & Rozsa, 2005). This study examines P and mA of *G. adenoplusia* plerocercoids in the intermediate hosts *E. spinax*, *G. melastomus*, and *S. canicula*; it was not possible to calculate these parameters for *D. licha* due to limited samples.

5.3. Results

5.3.1. Epidemiological data on *Grillotia adenoplusia*

A total of 29,031 parasites were found within the muscle tissues of the caudal portion of the sampled mid-sized benthic sharks. All parasites were morphologically identified using a dichotomous key and attributed to the genus *Grillotia* (Palm, 2008). The key diagnostic features included the presence of a scolex with two bothria, from which a tentacular apparatus extends, consisting of four retractable tentacles equipped with hooks. All parasites were at the plerocercoid stage. Infection data were obtained for the most abundant benthic shark species, i.e., *Etmopterus spinax* (N= 244) and *Galeus melastomus* (N= 143), collected across the three sampled areas: Civitavecchia, Gulf of Naples, Western Ionian Sea, and Palamonos (Costa brava). The abundance of *G. adenoplusia* plerocercoids showed a linear increase with host size (Figure 3). Regression curves were generated, supported by statistically reliable number of samples from these areas.

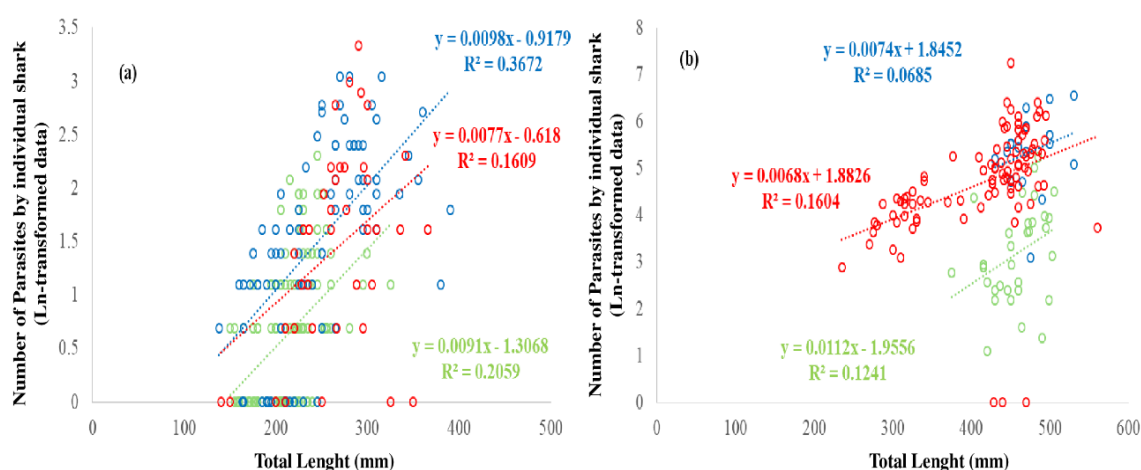


Figure 3: Linear relationships between the number of *G. adenoplusia* by individual shark and corresponding shark total length in samples of *Etmopterus spinax* (a) and *Galeus melastomus* (b) obtained from the Western Ionian Sea (green empty circles and related equation), Civitavecchia (blue empty circles and related equation), and Gulf of Naples (red empty circles and related equation). Equation parameters are provided inside the graphs. The mean abundance (mA) values of *E. spinax* in the sampling areas were 1.4 ± 0.00 (IS), 5.3 ± 0.01 (NP), 5.3 ± 0.01 (CV), and 5.8 ± 0.01 (SS) (Figure 20). Prevalence values ranged from 79.5% to 87% (SS) across the three areas (CV, SS, NP), decreasing to 56% in the Ionian Sea (IS). For *G. melastomus*, the mA values were 38.6 ± 0.08 (IS), 264.7 ± 0.40 (CV), 175.3 ± 0.20 (NP) and 251.5 ± 0.78 (CB), with consistently high values across all sampling areas.

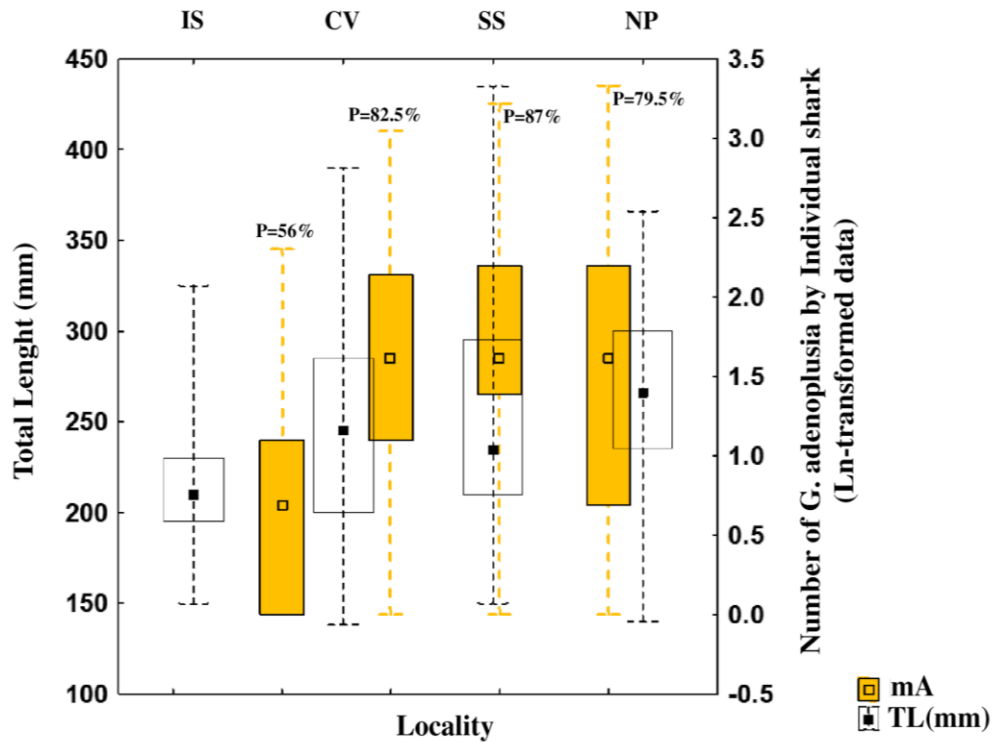


Figure 4: Box-plots representing the variation of the median, first and third quartiles of the number of parasites by individual shark (orange boxes, right y-axis) and total length (white boxes, left y-axis) of *E. spinax* obtained from the different areas: Western Ionian Sea (IS), Civitavecchia (CV), Western Sardinian Sea (SS) and Gulf of Naples (NP).

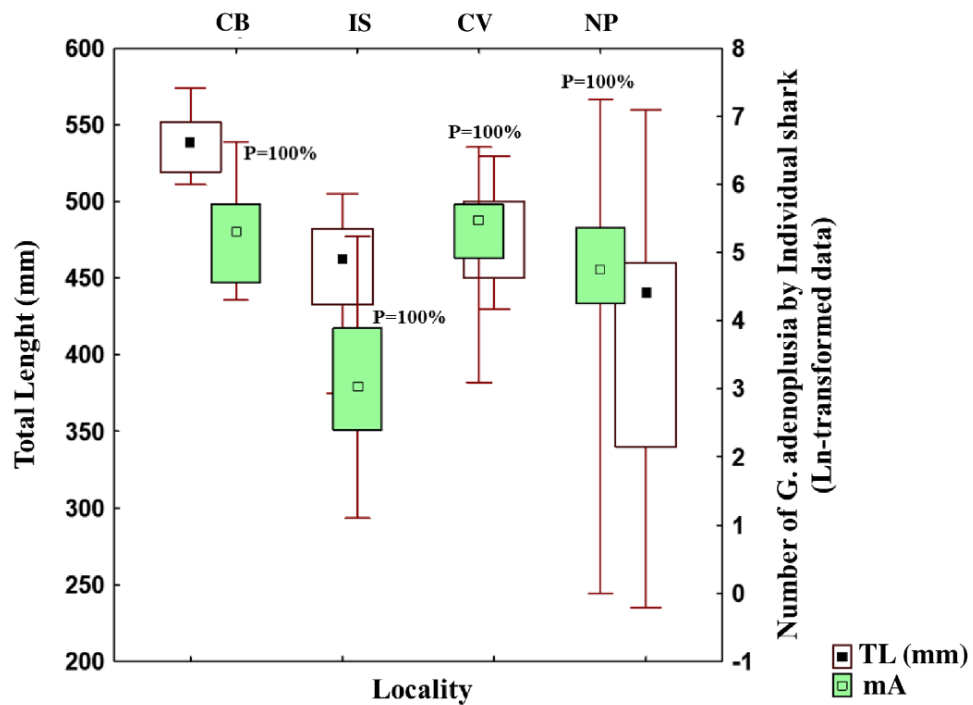


Figure 5: Box-plots representing the variation of the median, first and third quartiles of the number of parasites by individual shark (green boxes, right y-axis) and total length (white boxes, left y-axis) of *G. melastomus* obtained from different areas: Western Ionian Sea (IS), Civitavecchia (CV), Gulf of Naples (NP) and Costa Brava (CB).

5.3.2. Molecular identification of *Grillotia adenoplusia*

The species identity was confirmed by analyzing the 18s and 28s ribosomal DNA (rDNA) gene loci. A total of 396 individuals were sequenced at 28s gene locus (1082 bp), showing 100% identity with the sequences of *Grillotia adenoplusia* (a.n., PP153411 and PP153410) and with *Grillotia* sp. (a.n., LC730484 and MW838227), which had been previously deposited in GenBank and subsequently identified as *G. adenoplusia* (see Isbert et al., 2023). A subsample was also sequenced at the 18s gene locus, showing 98.02% identity with the sequences of *Paragrillotia similis* (a.n., KF685779). The *cox1* gene locus was amplified from the same specimens that were previously identified using the 28S and 18S rDNA markers. The sequence identity revealed approximately 80.04% of similarity with sequences of *cox1* from *Parabothriocephalus gracilis* (accession number KR780806) previously deposited in GenBank. However, this gene locus did not facilitate species confirmation, as no corresponding sequences for this locus are currently available in GenBank. However, it was used for subsequent population genetics studies. From the analysis of *cox1* gene locus sequences, the genetic diversity of *G. adenoplusia* across different geographical locations was observed. Genetic distance values among species within *Grillotia* genus, were calculated as inferred from each gene locus. Results are reported in Table 1 for the 28S rDNA gene locus, in Table 2 for the 18s gene locus and Table 3 for the *cox1* gene locus. The interspecific genetic distance values among species found at the 28s gene locus within the genus *Grillotia*, ranged from a minimum of 0.02 (K2P) between *G. acanthoscolex* and *G. rowei* to a maximum of 0.04 between *G. acanthoscolex* and *G. yuniariae*. The generated sequences of *G. adenoplusia* showed a minimum of 0.03 of genetic distance with respect to *G. acanthoscolex* and a maximum of 0.04 versus *G. pristiophori*.

Table 1: Interspecific genetic distances (i.e. interspecific variation) among species of *Grillotia* genus for 28s gene locus. Above the diagonal: *p*-values of genetic distance; Below the diagonal: genetic distances values.

Species	1	2	3	4	5
1. <i>G. rowei</i>	-	0.005	0.006	0.006	0.006
2. <i>G. adenoplusia</i>	0.034	-	0.006	0.006	0.005
3. <i>G. yuniariae</i>	0.042	0.037	-	0.006	0.004
4. <i>G. pristiophori</i>	0.039	0.038	0.035	-	0.005
5. <i>G. erinaceus</i>	0.035	0.033	0.029	0.029	-
6. <i>G. acanthoscolex</i>	0.020	0.028	0.038	0.034	0.031
7. <i>G. sasciae</i>	0.035	0.033	0.021	0.029	0.000

Interspecific genetic distance degrees among species found at the 18S rDNA gene locus within the genus *Grillotia*, ranged from a minimum of 0.012 (K2P) between *G. yuniariae* and *G. erinaceus* to a maximum of 0.022 between *G. erinaceus* and *G. rowei*. The generated sequences of *G.*

adenoplusia showed a minimum of 0.016 of genetic distance with respect to *G. pristiophori* and a maximum of 0.021 versus *G. erinaceus*. No intraspecific variation within species was observed.

Table 2: Interspecific genetic distances (i.e. interspecific variation) observed among *Grillotia* spp. as inferred from sequences of 18S rDNA gene locus. Above the diagonal: *p*-values of genetic distance; Below the diagonal: genetic distances values.

<i>Grillotia</i> spp.	1	2	3	4	5
1. <i>G. adenoplusia</i>	-	0.005	0.004	0.005	0.005
2. <i>G. rowei</i>	0.019	-	0.004	0.005	0.004
3. <i>G. pristiophori</i>	0.016	0.017	-	0.004	0.004
4. <i>G. erinaceus</i>	0.021	0.022	0.018	-	0.003
5. <i>G. yuniariae</i>	0.017	0.015	0.017	0.012	-

Interspecific genetic distance values as inferred from the mtDNA *cox1* among *Grillotia* spp., ranged from a minimum of 0.18 (K2P) between *G. adenoplusia* and *G. sasciae* to a maximum of 0.25 between *G. adenoplusia* and *G. pristiophori*. Values for *cox1* are higher; however, they do not allow intraspecific variation within the species to be observed.

Table 3: Interspecific genetic distances (i.e. interspecific variation) among species of *Grillotia* genus observed at the mtDNA *cox1* gene locus, excluding *Grillotia* species not identified at species level. Above the diagonal: *p*-values of genetic distance; Below the diagonal: genetic distances values.

<i>Grillotia</i> spp.	1	2	3
1. <i>G. adenoplusia</i>	-	0.025	0.021
2. <i>G. pristiophori</i>	0.250	-	0.025
3. <i>G. sasciae</i>	0.176	0.222	-

5.3.3 Phylogenetic analysis of genus *Grillotia*

The resulting concatenated phylogenetic tree from 28s and 18s gene locus revealed two main clades (Figure 6). The first clade, with high nodal support (PP = 1), was divided into two lineages. The first lineage included sequences of *G. acanthoscolex* and those of *G. rowei*, with robust nodal support (PP=1). The second lineage included sequences of *G. erinaceus*; *G. sasciae*; *G. yuniariae*; *G. pristiophori*, with a robust nodal support (PP=1). The second clade (PP=1) included only the obtained sequences of *Grillotia adenoplusia*. The phylogenetic tree derived from the *Cox1* gene locus of the genus *Grillotia* showed two main clades (Figure 7). As in the previous tree, *G. adenoplusia* formed a unique clade with high nodal support (PP=1). A phylogenetic tree for the genus *Grillotia* was also constructed by combining the sequences of the *Cox1*, 18s and 28s gene loci (Figure 8). The resulting tree was consistent with the topologies obtained from the previous analyses, confirming the robustness of the phylogenetic relationships within the genus.

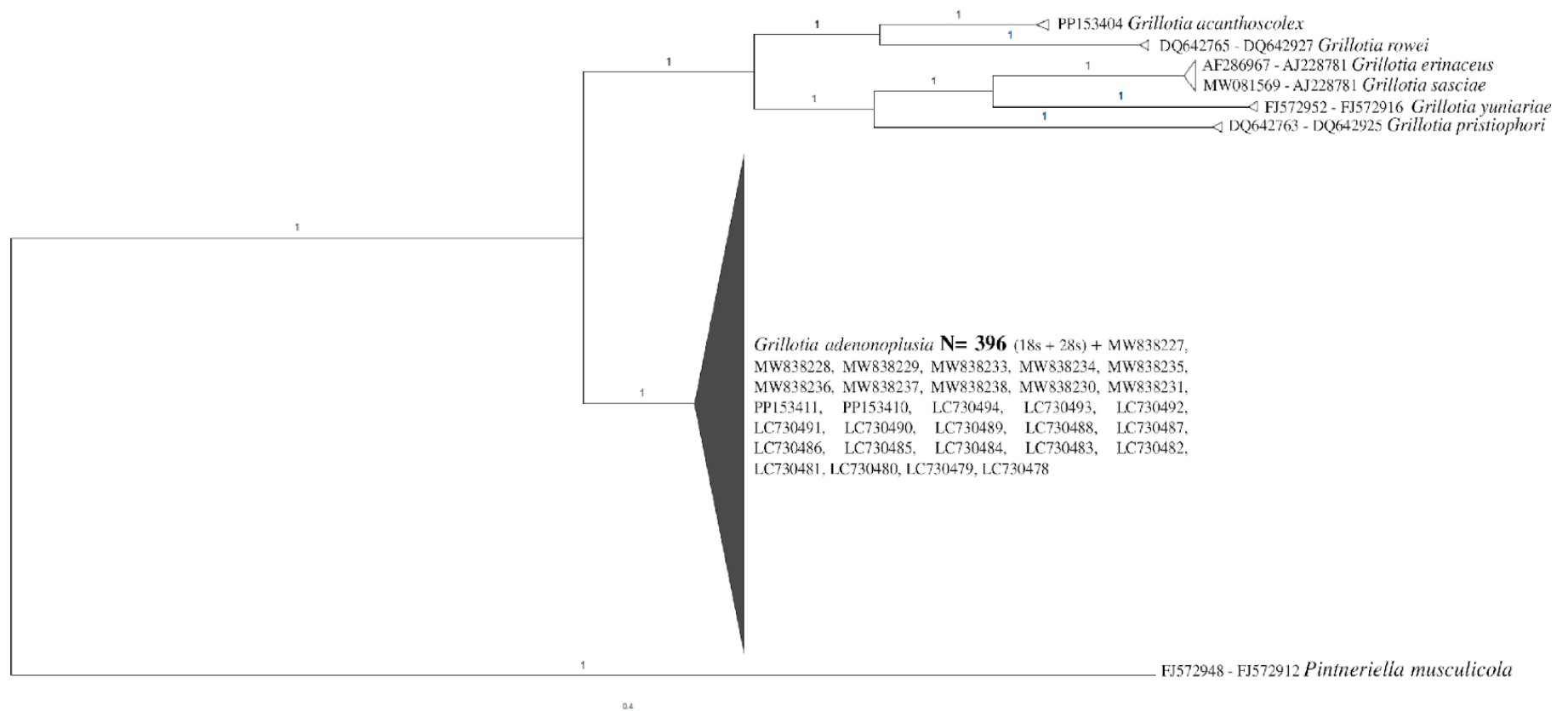


Figure 6: Concatenated phylogenetic tree of 28s and 18s (rDNA) gene locus of the genus *Grillotia* from Bayesian inference (BI) based on Markov Chain Monte Carlo (MCMC). The analysis was performed by MrBayes v. 3.2.7, using the TIM3 + I + G substitution model, as implemented in jModeltest 2.1.10.

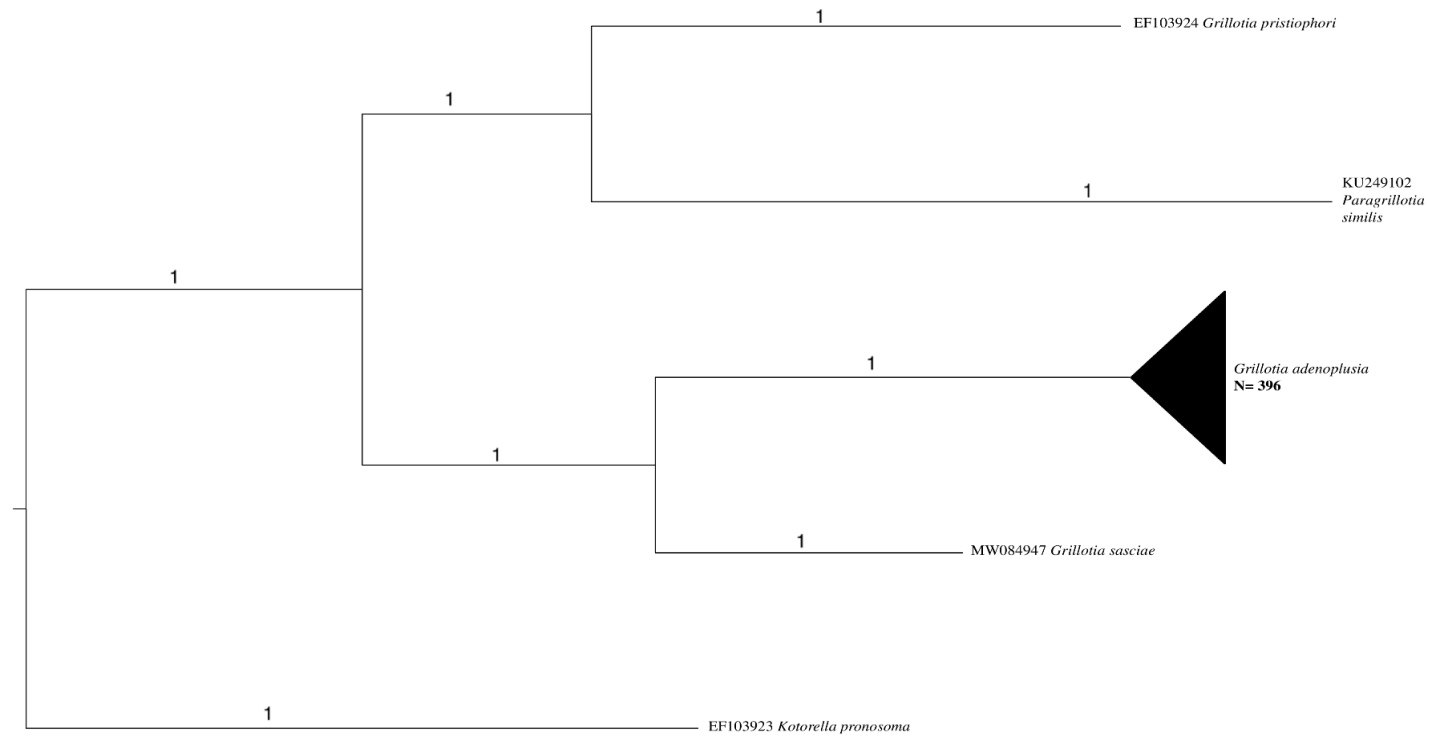


Figure 7: Phylogenetic tree of Cox1 (mtDNA) gene locus of the genus *Grillotia* constructed using Bayesian Inference (BI) based on Markov Chain Monte Carlo (MCMC). The analysis was performed by MrBayes v. 3.2.7, using the TIM3 + I + G substitution model, as implemented in jModeltest 2.1.1

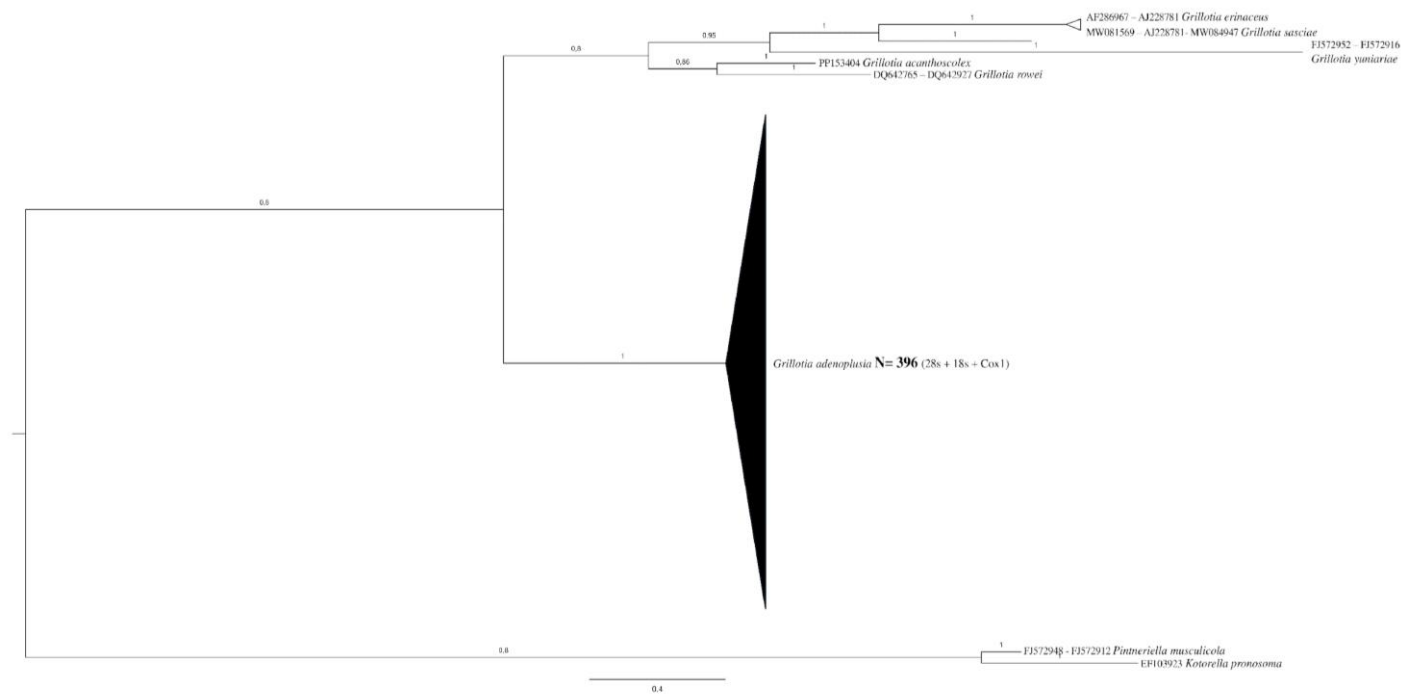


Figure 8: Concatenated phylogenetic tree (Cox1+18s+28s gene loci) of the genus *Grillotia* constructed using Bayesian Inference (BI) based on Markov Chain Monte Carlo (MCMC). The analysis was performed by MrBayes v. 3.2.7 and using the jModeltest 2.1.10

5.3.4. Genetic diversity in *Grillotia adenoplusia*

Analysis of *G. adenoplusia* *cox1* sequences (N = 344) revealed a total of 166 haplotypes, 151 private haplotypes, with a haplotype diversity (Hd) of 0.96 ± 0.007 . The average number of nucleotide differences (k) was 3.62, the number of polymorphic sites (S) 105, and the nucleotide diversity (π) was 0.0072 ± 0.0002 . PCA and heatmap analyses revealed a moderate genetic differentiation within the *G. adenoplusia* samples, with a significant F_{ST} value between the two depicted clusters of $0.330 (0.000 \pm 0.000)$ (Figure 9, a, b).

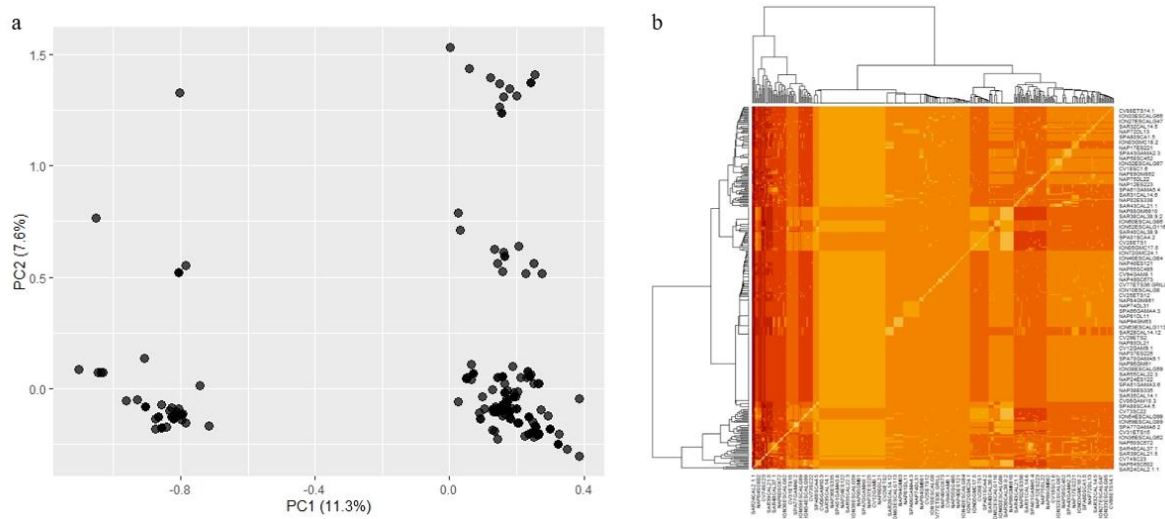


Figure 9:(a).PCA analysis obtained from the sequences of the mtDNA *cox1* gene locus in *G. adenoplusia*. (b). Cluster heat map reflecting the degree of mtDNA *cox1* genetic differentiation among *G. adenoplusia*.

However, the PCA analysis did not reveal a clear pattern of structuring with respect to either geographic area (Figure 10, a) or intermediate host species (Figure 10, b). Specifically, samples from different coastal regions showed a degree of overlap along PC1 and PC2, suggesting limited geographical differentiation in the studied populations. Similarly, no distinct clustering was observed among the host species, indicating a lack of intermediate host-driven genetic sub-structuring. This observation is further supported by non-significant F_{ST} values, which confirm the lack of substantial genetic differentiation among samples from species of small elasmobranchs as intermediate hosts.

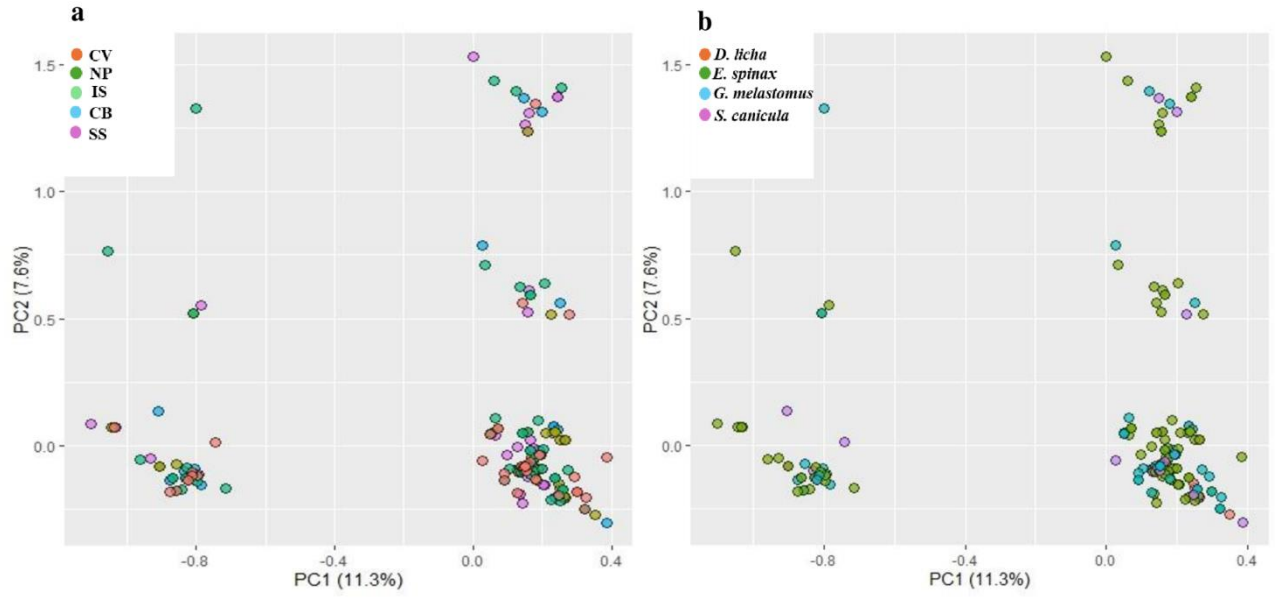


Figure 10: (a). PCA analysis obtained from the sequences of the mtDNA cox1 gene locus of *G. adenoplusia* among sampling areas. **(b).** PCA analysis assessed by the analysis of Cox1 gene locus of *G. adenoplusia* among intermediate/paratenic host species.

When dividing the samples by juvenile and adult intermediate/paratenic hosts, however, a moderate genetic structuring of the parasite was observed exclusively within juvenile hosts, particularly within the species *E. spinax* (Figure 12).

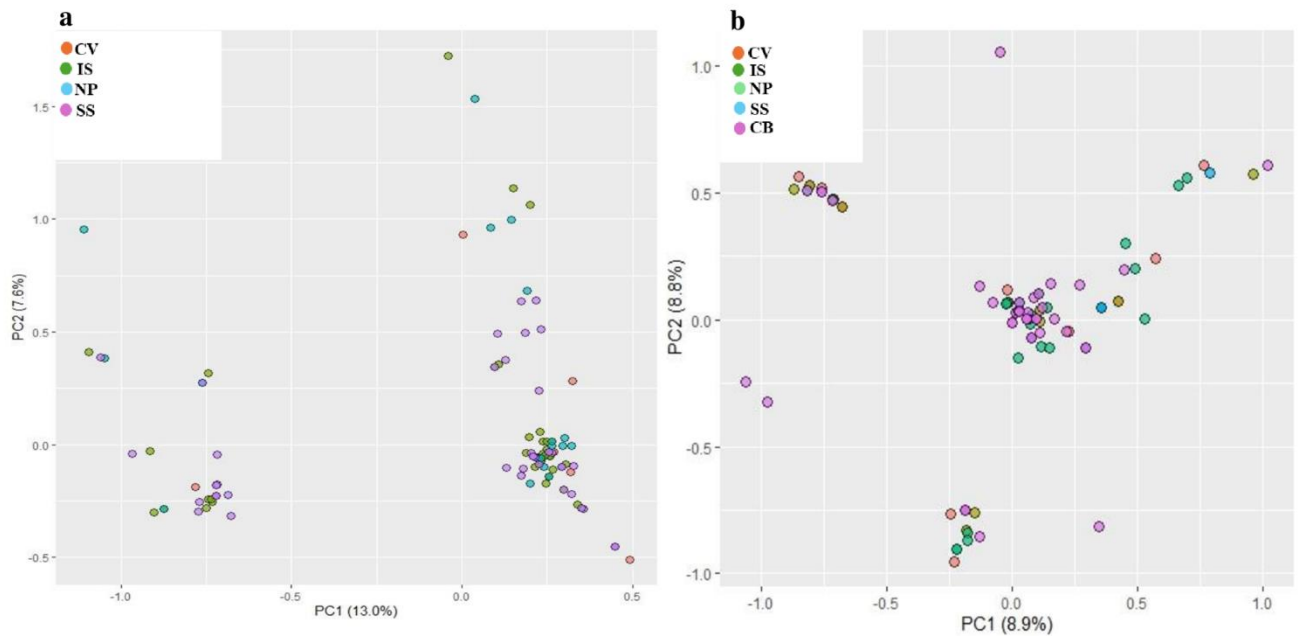


Figure 11: (a). PCA analysis assessed by the analysis of Cox1 gene locus of *G. adenoplusia* among areas of sampling for the juvenile. **(b).** PCA analysis obtained from the sequences of the mtDNA cox1 gene locus of *G. adenoplusia* among areas of sampling for the adult.

However, interestingly, when sub-dividing the juvenile samples by host species (i.e. *E. spinax*, *G. melastomus* and *S. canicula*), a certain genetic sub-structuring of the parasite was observed mainly within the *E. spinax* species (Figure 12), the fish species from which the largest number of the parasite samples was also obtained (N= 290).

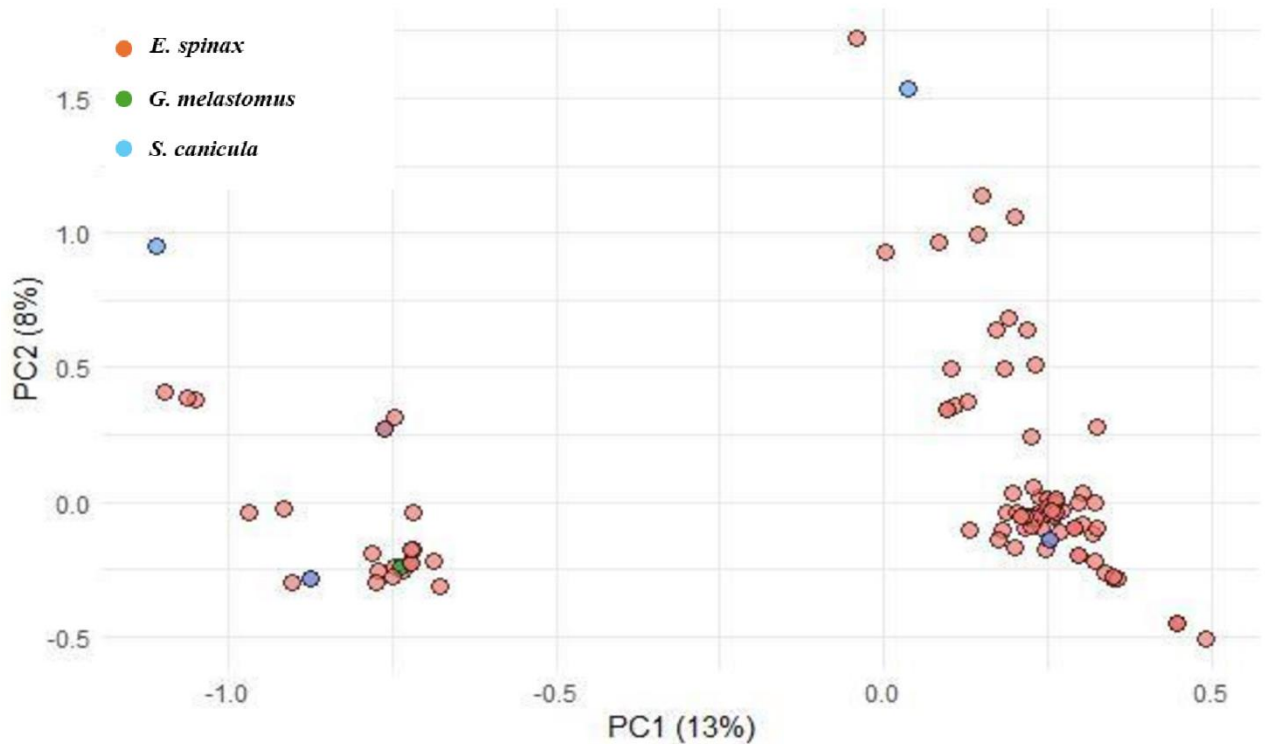


Figure 12: PCA analysis obtained from the mtDNA Cox1 sequences of *G. adenoplusia* among juvenile paratenic hosts.

5.4. Discussion

This study represents the first investigation into the genetic structure of *Grillotia adenoplusia* in the waters of the Mediterranean Sea. Based on the phylogenetic analysis performed using concatenated Bayesian Inference (BI) on the gene loci studied to date, the trypanorhynch larvae collected appear to belong to a single taxon. This taxon corresponds to *G. adenoplusia*, as confirmed by comparison with available 28S sequences in GenBank for congeneric species. However, the new mtDNA *cox1* locus sequenced in this study revealed a high level of polymorphism in the plerocercoids of *G. adenoplusia*. Our samples displayed significant genetic diversity, including a high number of haplotypes (N = 182), suggesting a large population size of this parasite in Mediterranean waters. This inference is supported by the high abundance and prevalence values observed in the present study, in Mediterranean *G. adenoplusia* populations. Prevalence values were measured by area and host species, never falling below 50%, supporting the host specificity of *G. adenoplusia* and classifying it as a "common" species both in

the sampled areas and host species. This indicates that the presence of *G. adenoplusia* in these hosts is not accidental. However, prevalence (P) and mean abundance (mA) values showed variability depending on the host species. In *Galeus melastomus*, both P and mA were significantly higher compared to other intermediate/paratenic hosts, as previously documented by Santoro et al. (2021), with a prevalence of 100% across all sampling areas and mA values ranging from 38.6 ± 0.08 (IS) to 264.7 ± 0.40 (CV). In *Etmopterus spinax*, mA values ranged from 1.4 ± 0.00 (IS) to 5.8 ± 0.01 (SS), while prevalence varied between 56% and 87%, generally lower than in other hosts, despite a much larger sample size for this species. These findings suggest a potential preference of the larval stage for specific intermediate/paratenic hosts, likely influenced by dietary differences that enhance direct or more frequent trophic interactions with the first intermediate host, which remains unknown. The high mA values for *G. melastomus* (N = 32) and *E. spinax* (N = 125) from the Western Ionian Sea (IS), being the highest observed overall, may reflect the specific ecological conditions of this basin, which could limit the passage of the definitive host and, consequently, egg release.

Despite the fact that according to the genetic data acquired, the parasite seems to belong to a single panmictic taxon, the significant genetic differentiation ($F_{st} = 0.3$) observed between the two clusters depicted by the Principal Component Analysis (PCA), suggests the existence of a certain genetic sub-structuring within *G. adenoplusia* in the Mediterranean Sea, likely supporting the existence of genetic "strains" of the parasite species. This sub-structuring seems to reflect similar genetic sub-structuring as that reported for its definitive host, the bluntnose sixgill shark (*Hexanchus griseus*) (Verdejo et al., 2023). Indeed, recent population genetic analysis of *H. griseus* has revealed significant spatial genetic heterogeneity at the intraspecific level (Vella and Vella, 2017). The authors' findings demonstrated that this shark species exhibits geographically distinct maternal lineages, indicating population structure across its geographic ranges. Population subdivisions of *H. griseus* exist not only between the Pacific and Atlantic Oceans, but also within these oceans and, on a smaller scale, within the Mediterranean Sea (Vella and Vella, 2017).

PCA and heat map analyses revealed that the haplotypes found in *G. adenoplusia* are not structured based on the intermediate hosts from which they were collected or the geographic areas where the fish hosts were sampled. Additionally, a low and non-significant F_{st} value was observed within each of the two clusters (Fig. 6.8), indicating no significant differentiation among *G. adenoplusia* individuals within the two groups. However, by dividing the total *G. adenoplusia* samples based on juvenile and adult stages of the fish species analyzed here—which may exhibit different migratory patterns in the Mediterranean Sea (Sion et al., 2004; Coelho and Erzini, 2010; D'Iglio et al., 2021; Coolen et al., 2014)—a structuring of *Grillotia* individuals was observed. Specifically, those sampled from juveniles (mainly *E. spinax*) were predominantly included in one cluster. Based on these findings, it can be hypothesized that juveniles, being more resident and feeding on infected intermediate hosts within their

residential areas, play a key role in maintaining this sub-structuring of the parasite population, which reflects that of the definitive host. Conversely, the greater vagility of adult intermediate/paratenic host species may contribute to broader dispersal of the parasite's haplotypes in Mediterranean waters, potentially influenced by the bioaccumulation effect. In this regard, it can be also considered that the parasite species' life cycle appears to be predominantly linked to a deep benthic environment; therefore, it likely involves a benthic first intermediate host (Palm, 2008) in the various geographic areas studied.

The current findings suggest that this parasite species may have followed the evolutionary history proposed for its definitive host (Vella and Vella, 2017). However, to corroborate this hypothesis, further studies are needed on additional *G. adenoplusia* populations collected from intermediate hosts in the eastern Mediterranean and northeastern Atlantic Ocean to explore potential co-phylogeographic patterns between this endoparasite and its definitive host. Furthermore, the use of additional genetic markers would be crucial to provide a more comprehensive understanding of these relationships and confirm the observed patterns.

5.5. Conclusions

The results obtained in this study provide baseline genetic data for this parasite for future research. The findings also highlight the potential to study co-phylogeographic patterns between *G. adenoplusia* and its definitive host, which could offer valuable insights into their evolutionary history.

Additionally, the high levels of polymorphism and parasitic load observed in this trophically transmitted parasite suggest that the population sizes of both its definitive and intermediate hosts are likely substantial. This indicates that a high stability in the trophic-chains sustaining the benthic parasite's life cycle. Finally, these results suggest that *G. adenoplusia* could serve as a useful ecological indicator for assessing the health and demographic status of its shark hosts, contributing to conservation strategies of this top predator in both Mediterranean and Atlantic waters.

6. Concluding Remarks

This thesis represents an investigation into certain target food-web transmitted endoparasite endoparasites (TTHs) in the European Sea waters, with a primary focus on two major parasitic groups: Anisakidae (Nematoda) and Trypanorhyncha (Cestoda). This work has contributed to enrich our understanding of these parasites biodiversity, genetic variation, ecological roles, and their application as indirect ecological bioindicators for assessing marine ecosystem health under anthropogenic pressures.

Objective 1: Biodiversity, distribution, and parasitic load of some food-web transmitted endoparasites

This research employed a multidisciplinary approach combining morphological and molecular techniques to document the biodiversity, distribution, and parasitic load of anisakid and trypanorhynch species from different host species as teleost fish (Intermediate/paratenic hosts), small-mid size sharks and cetaceas (Definitive hosts) in the European waters. These findings highlight the crucial role of host-parasite interactions in shaping parasite biodiversity and shed light on the ecological complexity of these marine systems.

For example, studies on *Anisakis* spp. in cetaceans demonstrated distinct host specificity patterns, with significant genetic differentiation observed among populations from the Norwegian Sea, North Sea, and Mediterranean waters. These results underscore the influence of geographic and ecological factors on the genetic structuring of parasite populations. Additionally, the study of *Anisakis physeteris* in the pygmy sperm whale (*Kogia breviceps*) at the edge of its distribution range provided novel insights into the dynamics of host-parasite interactions in understudied hosts. **Publication no. 1. and 2**

Publication no. 3. contributes significantly to expanding the knowledge of parasitic biodiversity by exploring the co-infection of *Molicola uncinatus* and *Gymnorhynchus gigas* in the Atlantic pomfret (*Brama brama*) from the Mediterranean Sea. Through integrative taxonomy and phylogenetic analysis, the study not only identifies these parasites but also sheds light on their ecological interactions and evolutionary relationships within the Gymnorhynchidae family. This work enriches our understanding of marine parasitology and highlights the importance of studying parasitic biodiversity to better comprehend marine ecosystems and their health. These larval cestodes, often found in high infection rates, highlighted the potential role of trypanorhynchs as bioindicators food-web structure in the Mediterranean ecosystem.

Moreover, the study provided a detailed assessment of *Grillotia adenoplusia*, focusing on its infection parameters and distribution patterns in various intermediate and definitive hosts. The results confirmed the suitability of trypanorhynchs, including *G. adenoplusia*, as ecological indicators for monitoring the stability and health of marine food webs. By analyzing their presence across different hosts and environments, this work underscores how parasitic load and host specificity reflect broader ecosystem conditions. This aspect of the work is particularly relevant in the context of ongoing anthropogenic pressures such as overfishing, pollution, climate change, and habitat degradation. **Paper no. 4.**

Objective 2: Genetic structure of some food-webs transmitted endoparasites

The second objective focused on the genetic variation of *Grillotia adenoplusia*, a trypanorhynch cestode frequently found in deep-dwelling sharks and teleost fish. By employing a polymorphic genetic marker, the study revealed significant genetic variation among populations of *G. adenoplusia* and demonstrated how host population dynamics may influence the genetic structure of this parasite.

The genetic data obtained also contribute to understanding the coevolutionary relationships between parasites and their hosts, offering a robust framework for future research on the impacts of environmental changes on host-parasite dynamics. **Publication no. 4.**

This thesis makes several substantial contributions to the field of marine parasitology and ecosystem monitoring.

Firstly, it has provided detailed biodiversity data on anisakid and trypanorhynch parasites, as integral components of marine food webs.

Secondly, it has demonstrated the utility of molecular markers in resolving taxonomic ambiguities and exploring genetic diversity, particularly in species with complex life cycles, such as *Anisakis spp.* and *Grillotia adenoplusia*.

Thirdly, it has highlighted the application of parasites as bioindicators for monitoring the health and stability of marine ecosystems under the influence of anthropogenic pressures.

The use of parasites as ecological indicators offers unique advantages. Their complex life cycles and trophic interactions make them sensitive to changes in host populations and environmental conditions. The findings of this thesis demonstrate how the parasitic load, and genetic diversity of these organisms can provide valuable indirect assessments of ecosystem health.

In conclusion, this thesis has provided advancements in the study of trophically transmitted helminths in European and Mediterranean Sea waters. The findings underscore the importance of integrating multidisciplinary approaches to enhance our understanding of parasite-host dynamics and their ecological implications.

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