UNIVERSITA'DEGLI STUDI DI MESSINA

Dipartimento di Scienze Veterinarie

DOTTORATO DI RICERCA IN SCIENZE VETERINARIE
XXXV CICLO
Settore Scientifico Disciplinare VET/06

Wild fish and parasites: Mediterranean surveys

Candidate:
Dott. Giovanni De Benedetto

Coordinator:
Ch.ma Prof.ssa Rosaria Laurà

Supervisor:
Ch.ma Prof.ssa Gabriella Gaglio

Anno Accademico 2021/2022

Preface

The following thesis is written in a 'thesis by publication' format and includes three published articles in peer-reviewed international journals.

This thesis, entitled "Parasitic fauna of wild teleost from Mediterranean Sea", is divided into three chapters:

Chapter 1. includes one research article published in the international journal 'Animals' focusing on the description of the parasite fauna of dusky grouper (*Epinephelus marginatus*) caught in the central Mediterranean Sea between the provinces of Messina and Syracuse.

Chapter 2. consists of an article published in 'Parasitology'. The chapter provides a morphological and molecular characterization of a new species of *Didymodiclinus* (Trematoda: Didymozoidae) infecting the dusky grouper, *Epinephelus marginatus* (Teleostei: Serranidae) from the Mediterranean Sea.

Chapter 3. presents a paper published in the journal 'Animals'. The chapter focuses on the description of granulomatous reaction due to *Anisakis pegreffii* larvae in *Sphiraena viridensis* compared to the same lesion in human.

The investigations presented in the form of research articles herein included were mainly carried out at the Department of Veterinary Sciences of the University of Messina during the candidate's PhD course.

A collaboration with the Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina, Italy was agreed upon for the execution of molecular analyses included in the research article in Chapters 2 and 3. In addition, a collaboration with the Department of Veterinary Medicine, University of Sassari, Italy was established for the Morphological description performed in the surveys reported in Chapter 2.

The thesis has been partially funded by European Project "PO-FEAMP 2014/2020, 2.56, FishPathNet" (cost of consumables and molecular analysis equipment).

Table of Contents

Abstract:…….page: 4

General introduction:……...page: 5

Chapter 1: Parasite Fauna of the Dusky Grouper (*Epinephelus marginatus*, Lowe 1834) from the Central Mediterranean Sea……..…….page: 11

Chapter 2: Morphological and molecular study of Didymodiclinus marginati n. sp. (Trematoda: Didymozoidae) gill parasite of Epinephelus marginatus from the central and western Mediterranean Sea……..page: 27

Chapter 3: *Anisakis pegreffii* Larvae in *Sphyraena viridensis* and Description of Granulomatous Lesions ……..……page: 47

General discussions and conclusions:…….page:66

Abstract

This doctoral thesis aims to provide original data on wild fish parasite fauna, to improve the current knowledge on teleost from the western and central Mediterranean Sea. The thesis is presented in the form of "thesis by publication" and is composed of three chapters.

Chapter 1 aimed to investigate the parasite fauna of dusky grouper (*E. marginatus*) from the central Mediterranean Sea, between the provinces of Messina and Syracuse. In this chapter, two main targets, the economic value and the current lack of studies of the study area, were considered. In the study, 70 dusky groupers caught between 2018 and 2020 were included. Forty-seven specimens (67.2%) were positive for endo and ectoparasites: nematodes, digenean and monogenean trematodes. The most retrieved species was *Prosorhynchus caudovatus* (42.9%), followed by *Podocotyle temensis* (28.6%), *Didymodiclinus* sp. (18.6%), *Philometra jordanoi* (5.7%), and *Anisakis* Type II larvae (5.7%). The different prevalence, found between warm and cold seasons, is probably related to *E. marginatus*, characterized by molluscs that are considered as intermediate hosts for the retrieved parasite species.

Chapter 2 provides a morphological and molecular characterization of a new species of *Didymodiclinus* (Trematoda: Didymozoidae) retrieved in *Epinephelus marginatus* (Teleostei: Serranidae) from Sicily and the island of Majorca. Between 1998 and 2020, 279 *E. marginatus* specimens were examined. The new species was morphologically different from the most similar congeneric species, retrieved in different hosts and locations within the host tissues, as described from other geographical areas. Moreover, this parasite represented the first didymozoid species reported in *E. marginatus* from the central and western Mediterranean Sea. For molecular evaluation, partial 28S, partial ITS-2 ribosomal RNA regions and mitochondrial COX1 gene were used. Comparison of *Didymodiclinus marginati* n.sp. sequences with other deposited sequences of 28S revealed that the new isolates cluster with several unidentified didymozoids. The morphological and genetic results obtained permitted to propose new identification keys for the genus *Didymodiclinus*.

Chapter 3 describes a gastric granulomatous reaction caused by *Anisakis pegreffii* in yellowmouth barracuda (*Sphyraena viridensis*). In this study, 68 *S.viridensis* specimens were sampled from different fish markets on the east coast of Sicily. Retrieved parasite specimens and pathological tissues were collected for histological and molecular analyses. Twelve specimens (p=17.6%, MA=0.9, MI=4.8) were positive for nematode larvae, identified as *Anisakis* sp. One large female *S. viridensis* specimen showed massive parasite infection associated with multiple modular lesions of the gastric wall. Histological evaluation showed that the parasite body was surrounded by a granulomatous reaction made up of macrophages, epithelioid cells, some lymphocytes, and an external connective layer. Molecular analysis performed with 18S rRNA and cox2 genes allowed to identify the retrieved parasite larvae as *A. pegreffii*. The lesions described in the present chapter, although macroscopically identical to the human eosinophilic granuloma, microscopically showed significant variations in the involved inflammatory cell population, as in the host immune reaction.

General introduction:

Understanding the distribution of high economic value teleost populations belonging to the same species is essential both to elaborate the dynamics and to manage sustainability, but also to identify possible fraud in fishing methods (Hilborn & Walters, 1992; Evans & Grainger, 2002). Stock identification is considered an important practice, closely related to the correct management of fish species, finalized to encourage acceptable exploitation of resources within variable ecosystems, improving sustainable fishing, limiting the risk of stock reduction, and preserving the genetic diversity of our seas (Begg & Waldman, 1999; Mattiucci et al., 2015; Waples, 1998; Begg et al., 1999), mainly regarding the less productive stocks (Begg GA & Waldman JR., 1999; Oliva and Sánchez, 2005; Catalano et al., 2014). Parasitism represents one of the most common lifestyles among eukaryotes (Poulin and Morand 2004). Since the 1930s, the use of parasites as biological tags of marine fish stocks has been described (Herrington et al., 1939). As reported by Williams et al. (1992), all taxonomic parasite groups, considering mainly the helminth parasites, have been used as tags for both marine and freshwater teleost and invertebrates. Among the most used techniques, molecular biology evaluation is still particularly used, even if the use of parasites as biological tags has gained wide acceptance since the 1990s (Williams et al. 1992; MacKenzie, 2002). Guidelines on the use of parasites as biological tags in fish population studies have been reported (Mackenzie, 2002; Lester and Mackenzie, 2009), indicating three selection criteria: different levels of infection between the sampling areas; presence of the target species for the entire duration of the study, and the inclusion of non-pathogen parasites for the host.

Parasites characterized by direct life cycles, such as monogenean trematodes and crustaceans, are considered the easiest species to include in these kinds of studies (Williams et al. 1992; MacKenzie, 2002). Those with complex life cycles, such as digenean trematodes, tapeworms, roundworms, and acanthocephalan, characterized by two or more intermediate different hosts, are more difficult to use as more information is needed on the biotic and abiotic factors that influence parasite transmission (Kabata, 1963; Sindermann, 1983; MacKenzie, 1987; Williams et al., 1992). As reported by Køie (1983), digenean trematodes are considered the best biological tags, due to the presence of mollusks as intermediate hosts; therefore, the endemic area of digenean trematodes is strictly related to the geographic distribution of its mollusk host. The most used parasites as tags are nematodes, mainly their larval stages, considering mainly their distribution in seawater and freshwater teleost (Sindermann, 1990 Abaunza et al., 1995; Garcia et al., 2011; Mattiucci et al., 2014, 2015).

One technique considers the entire parasitic load found from all different sampling areas, rather than evaluating a single species of parasite, to clearly describe the structure of the host

stocks (Mackenzie, 2002). Furthermore, the persistence of the parasite in the host must be known in order to enroll it as an effective indicator of the population (Lester & Mackenzie, 2009).

Genetic characteristics and population distribution of parasites can lead to the identification of their host population (Mulvey et al., 1991; Cross et al., 2007). Although parasite populations can change more rapidly than host populations (Mattiucci et al., 2015), parasites can help better locate hosts to their population of origin (Criscione et al., 2005). Therefore, the genetic evaluation of the parasite appears to be the best practice used for these procedures (Criscione et al., 2006; Mackenzie & Hemmingsen, 2015).

The use of parasites as tags is explained by the presence of fish that host a specific parasite species inside of geographic areas where this parasite is endemic. Specifically, by evaluating different parasite species, considered endemic in specific areas, more information can be obtained both on the area of origin and on the movements of the infected teleost (MacKenzie & Abaunza, 1998). In addition to being evaluated as useful biological tags during an ecological survey, parasites can provide very important information on host biology, such as feeding behavior, intraspecific dynamics, and the origin of single stock (Williams et al. 1992; Hemmingsen & MacKenzie 2001). Among endoparasite infections, the presence of helminths, characterized by complex life cycles, is particularly influenced by the feeding behavior of their hosts (Choisy et al. 2003; Parker et al. 2003). As previously reported, they can then be used as tags based on knowledge of their distribution areas, within which differences in host behavior and eating habits, as well as the abundance of intermediate hosts, can create levels of significantly different infections, capable of providing complete information on stock identification (MacKenzie et al. 2008). Among the other used techniques, the evaluation of food content in the gastrointestinal tract is considered an immediate but not exact analysis of the recent diet and eating habits of the hosts. On the other hand, parasitological analyses, following knowledge of biological cycles, can be considered in evaluating also previous interactions between predator and prey, which might also have occurred in distant environments and thus are not easy to observe in vivo (Klimpel & Palma, 2011). Molecular biology studies showed that zoonotic agents as *Anisakis* sp. larvae include a complex of sister species, characterized by different genetic profiles and geographic distributions (D'Amelio et al., 2013). Given the importance of these parasites in relation to public health, their taxonomy and distribution have been studied from different areas of the Mediterranean Sea (Mattiucci et al., 2008; Farjallah et al., 2008; Gutiérrez-Galindo et al., 2010; Angelucci et al., 2011; Meloni et al., 2011; Cavallero et al., 2012; Serracca et al., 2013). For these reasons, also the host-parasite interactions must be considered, to better understand the stock characteristics.

Surveys included in this thesis were carried out in Sicily (southern Italy) and Majorca (Spain) with the aim to provide original data on the description and identification of the parasite fauna of wild fish, to improve current knowledge on wild fish parasitology. In detail, Chapter 1 highlights the dusky grouper parasite fauna from the central Mediterranean Sea. Chapter 2 aims to provide a detailed morphological and molecular description of a new Didymozoid parasite species retrieved in *E. marginatus*, comparing two different stocks from the western and central Mediterranean Sea. Finally, Chapter 3 provides for the first time a detailed description of a granulomatous lesion, due to *Anisakis pegreffii*, in yellowmouth barracuda (*Sphyraena viridensis*) from the central Mediterranean Sea, comparing the retrieved lesions to those reported in humans.

References:

Abaunza, P., Villamor, B., Perez, J.R., 1995. Infestation by larvae of *Anisakis simplex* (Nematoda, Ascaridata) in horse mackerel, *Trachurus trachurus*, and Atlantic mackerel, *Scomber scombrus*, in ICES divisions VIIIb, VIIIc and IXa (N-NW of Spain). Sci. Mar. 59, 223e233.

Angelucci, G., Meloni, M., Merella, P., Sardu, F., Madeddu, S., Marrosu, R., Petza, F., Salati, F., 2011. Prevalence of *Anisakis* spp. and *Hysterothylacium* spp. larvae in teleosts and cephalopods sampled from waters off Sardinia. J. Food prot. 74, 1769–1775.

Begg GA, Waldman JR. An holistic approach to fish stock identification. Fish Res. 1999; 43: 35–44.

Begg GA, Friedland KD, Pearce JB. Stock identification and its role in stock assessment and fisheries management: an overview. Fish Res. 1999; 43: 1–8.

Catalano SR, Whittington ID, Donnellan SC, Gillanders BM. Parasites as biological tags to assess host population structure: guidelines, recent genetic advances and comments on a holistic approach. Int J Parasitol Parasites Wildl. 2014; 3: 220–226. doi: 10.1016/j.ijppaw.2013.11.001

Cavallero, S., Ligas, A., Bruschi, F., D'Amelio, S., 2012. Molecular identifica-tion of *Anisakis* spp. from fishes collected in the Tyrrhenian Sea (NWMediterranean). Vet. Parasitol. 187, 563–566.

Choisy M, Brown SP, Lafferty KD, Thomas F (2003) Evolution of trophic transmission in parasites: why add intermediate hosts? Am Nat 162:172–181

Criscione CD, Cooper B, Blouin MS. Parasite genotypes identify source populations of migratory fish more accurately than fish genotypes. Ecology. 2006; 87: 823–828.

Criscione CD, Poulin R, Blouin MS. Molecular ecology of parasites: elucidating ecological and microevolutionary processes. Mol Ecol. 2005; 14: 2247–2257.

Cross MA, Collins C, Campbell N, Watts PC, Chubb JC, Cunningham CO, et al. Levels of intra-host and temporal sequence variation in a large CO1 sub-units from *Anisakis simplex* sensu stricto (Rudolphi 1809) (Nematoda: Anisakidae): implications for fisheries management. Mar Biol. 2007; 151: 695– 702.

D'Amelio, S., Cavallero, S., Busi, M., Ingrosso, S., Giuffra, E., Farjallah, S.,Costa, G., 2013. *Anisakis*. In: Liu (Ed.), Molecular Detection of Human Parasitic Pathogens. CRC Press, Boca Raton, FL, pp. 487–499.

Evans, D. and Grainger, R. (2002). Gathering data for resource monitoring and fisheries management. In Handbook of Fish Biology and Fisheries, Vol. 2 (ed. Hart, P. J. B. and Reynolds, J. D.), pp. 84–102. Blackwell Publishing, Oxford.

Farjallah, S., Slimane, B., Busi, M., Paggi, L., Amor, N., Blel, H., Said, K., D'Amelio, S., 2008. Occurrence and molecular identification of *Anisakis* spp. from the North African coasts of Mediterranean Sea. Parasitol. Res.102, 371–379.

Garcia A, Santos MN, Damiano S, Nascetti G, Mattiucci S. 2008. The metazoan parasites of swordfish from Atlantic tropical-equatorial waters. *Journal of Fish Biology*, 73, 2274– 2287.

Gutiérrez-Galindo, J.F., Osanz-Mur, A.C., Mora-Ventura, M.T., 2010. Occurrence and infection dynamics of anisakid larvae in *Scomber scombrus*, *Trachurus trachurus*, *Sardina pilchardus*, and *Engraulis encrasicolus* from Tarragona (NE Spain). Food Control 21, 1550–1555.

Hemmingsen W, MacKenzie K (2001) The parasite fauna of the Atlantic cod, *Gadus morhua* L. Adv Mar Biol 40:2‑60

Herrington, W.C., Bearse, H.M., Firth, F.E., 1939. Observations on the life history, occurrence and distribution of the redfish parasite *Sphyrion lumpi*. United States Bureau of Fisheries Special Report No. 5, pp. 1e18.

Hilborn, R. and Walters, C. J. (1992). Quantitative Fisheries Stock Assessment: Choice, Dynamics, and Uncertainty. Chapman and Hall, New York.

Kabata, Z., 1963. Parasites as Biological Tags. ICNAF Special Publication No. 4, 31e37.
Klimpel S, Palm HW (2011) Anisakid nematode (Ascaridoidea) life cycles and distribution: increasing zoonotic potential in the time of climate change? In: Mehlhorn H (ed) Progress in parasitology, Parasitol Res Monographs 2. Springer, Heidelberg, pp 201‑222

Køie, M., 1983. Digenetic trematodes from *Limanda limanda* (L.) (Osteichthyes, Pleuronectidae) from Danish and adjacent waters, with special reference to their life histories. Ophelia 22, 201e208.

Lester, R. J. G., and Mackenzie, K. 2009. The use and abuse of parasites as stock markers for fish. Fisheries Research, 97: 1–2.

Mackenzie K, Hemmingsen W. Parasites as biological tags in marine fisheries research: European Atlantic waters. Parasitology. 2015; 142: 54–67. doi: 10.1017/S0031182014000341

MacKenzie K, Campbell N, Mattiucci S, Ramos P, Pinto AL, Abaunza P (2008) Parasites as biological tags for stock identification of Atlantic horse mackerel *Trachurus trachurus* L. Fish Res 89:136‑145

MacKenzie, K. (2002). Parasites as biological tags in population studies of marine organisms: an update. Parasitology 124, S153–S163.

MacKenzie, K. and Abaunza, P. (1998). Parasites as biological tags for stock discrimination of marine fish: a guide to procedures and methods. Fisheries Research 38, 45–56.

MacKenzie, K., 1987. Relationships between the herring, *Clupea harengus* L., and its parasites. Adv. Mar. Biol. 24, 263e319.

Mattiucci S, Cimmaruta R, Cipriani P, Abaunza P, Bellisario B, Nascetti G. Integrating *Anisakis* spp. Parasites data and host genetic structure in the frame of a holistic approach for stock identification of selected Mediterranean Sea fish species. Parasitology. 2015; 142: 90–108. doi: 10.1017/ S0031182014001103

Mattiucci S, Garcia A, Cipriani P, Santos MN, Nascetti G & Cimmaruta R. Metazoan parasite infection in the swordfish, *Xiphias gladius*, from the Mediterranean Sea and comparison with Atlantic populations: implications for its stock characterization. *Parasite*, 2014, 21, 35.

Mattiucci, S., Farina, V., Campbell, N., Mackenzie, K., Ramos, P., Pinto,A.L., Abaunza, P., Nascetti, G., 2008. *Anisakis* spp. Larvae (Nematoda: Anisakidae) from Atlantic horse mackerel: their genetic identificationand use as biological tags for host stock identification. Fish. Res. 89,146–151.

Meloni, M., Angelucci, G., Merella, P., Siddi, R., Deiana, C., Orru, G., Salati, F.,2011. Molecular characterization of *Anisakis* larvae from fish caught off Sardinia. J. Parasitol. 97, 908–914.

Mulvey M, Aho JM, Lydeard C, Leberg PL, Smith MH. Comparative population genetic structure of a parasite (*Fascioloides magna*) and its definitive host. Evolution. 1991; 45: 1628–1640.

Oliva ME, Sánchez MF. Metazoan parasites and commensals of the northern Chilean scallop *Argopecten purpuratus* (Lamarck, 1819) as tools for stock identification. Fish Res. 2005; 71: 71–77.

Parker GA, Chubb JC, Ball MA, Roberts GN (2003) Evolution of complex life cycle in helminth parasites. Nature 425:480‑484

Poulin R, Morand S (2004) Parasite Biodiversity. Smithsonian Institution Press, Washington, D.C.

Serracca, L., Cencetti, E., Battistini, R., Rossini, I., Prearo, M., Pavoletti, E., Fioravanti, M.L., Righetti, M., Di Donfrancesco, B., Ercolini, C., 2013.Survey on the presence of *Anisakis* and *Hysterothylacium* larvae in fishes and squids caught in Ligurian Sea. Vet. Parasitol. 196, 547–551.

Sindermann, C.J., 1983. Parasites as natural tags for marine fish: a review. NAFO Sci. Counc. Stud. 6, 63e71.

Waples RS. Separating the wheat from the chaff: patterns of genetic differentiation in high gene flow species. J Hered. 1998; 89: 438–450.

Williams, H. H., MacKenzie, K. and McCarthy, A. M. (1992). Parasites as biological indicators of the population biology, migrations, diet and phylogenetics of fish. Reviews in Fish Biology and Fisheries 2, 144–176.

Chapter 1

Parasite Fauna of the Dusky Grouper (*Epinephelus marginatus*, Lowe 1834) from the Central Mediterranean Sea

Adapted from:

Giovanni De Benedetto[1], Francesca Arfuso[1], Maria Catena Ferrara[2], Emanuele Brianti[1] and Gabriella Gaglio[1]

[1]Department of Veterinary Sciences, University of Messina, 98168 Messina, Italy,
[2]Zooprophylactic Institute of Sicily "A. Mirri", Territorial Area of Barcellona Pozzo di Gotto, 98051 Messina, Italy.

Animals 2021, 11, 2523

Simple Summary: The dusky grouper (*Epinephelus marginatus*) is one of the most expensive species present in the central Mediterranean Sea and the parasite fauna of this species has not been investigated, so far. The aim of the present survey was describing the dusky grouper parasites according to fish size and parasite charge. *E. marginatus* specimens in two groups (cold and warm months) were also divided to establish the relation between parasite fauna and fishing period. According to the results obtained, we can speculate that the infection differences between cold and warm periods could be related to the availability of different prey representing intermediate parasites host. None of the parasites found pose a threat to humans.

Abstract: This study aimed to investigate parasite fauna of *E. marginatus* from the central Mediterranean Sea between Messina and Syracuse. In the present survey; parasite fauna of dusky grouper was investigated for two main reasons: the economic value of this species and the current lack of studies regarding the capture area. Seventy dusky groupers were caught from May 2018 to February 2020. Forty-seven out of the 70 specimens (67.2%) were infected with one or more parasite species. The most abundant species was *Prosorhynchus caudovatus* (42.9%), followed by *Podocotyle temensis* (28.6%), *Didymodiclinus* sp. (18.6%), *Philometra jordanoi* (5.7%), *Anisakis* Type II larvae (5.7%). Higher prevalence of infection of *P. jordanoi* and *Contracaecum* sp. was found in warm months (March to September), while *P. caudovatus* and *P. temensis* were mostly found during cold months. Weight and total length of *E. marginatus* were positively correlated with the parasitic load of *P. jordanoi* and *Didymodiclinus* sp. The different prevalence of parasite infection found between warm and cold months is probably related to the diet of the dusky grouper; which is characterized by mollusks that are intermediate hosts for parasite species found. None of the parasites found in the present survey is responsible for zoonosis.

Keywords: *Epinephelus marginatus*; nematodes; trematodes; Sicily; Italy; seasonality

1. Introduction

Among marine fish species distributed in warm water, the dusky grouper, *Epinephelus marginatus* (Lowe 1834) is one of the largest top predators in the western Mediterranean littoral ecosystems. Similar to other grouper species, *E. marginatus* shows an ontogenetic change in diet composition and an expansion of the trophic niche, with juveniles feeding primarily on Brachyura crustaceans and adults on cephalopods and fish [1,2]. In addition to its key role in coastal ecosystem food webs, the dusky grouper has a high economic value [3]. *Epinephelus marginatus* shows slow growth, fewer offspring, late maturation, large body size and long lifespan [4]. A decline in Mediterranean dusky grouper stocks has been observed over recent decades [5]. Both behavioral (e.g., site fidelity, inquisitive character) and biological (e.g., hermaphroditism, late sexual maturity, slow growth and longevity) features of this species together with artisanal fisheries and spear-fishing activities are the main factors increasing susceptibility to over-exploitation of *E. marginatus* and are accounted for as the foremost causes of species decline [4].

The occurrence of different parasites has been reported in dusky grouper populations [6–11]. In particular, natural outbreaks associated with gnathiid isopod larvae have been observed in wild and captive *E. marginatus*, in some cases associated to significant haematophagia [12,13]. Skin lesions and dermatitis, most likely associated with histozoic parasites, have been described in dusky groupers off the Libyan coast [14]. White capsules, tightly attached to the gills, pseudobranchs and orobranchial cavity with parasites identified as members of the didymozoid family (Trematoda: Didymozoidae) have been reported on wild dusky groupers in the Adriatic Sea [15] and the eastern Atlantic Ocean [16]. More recently, helminthological investigations on marine fish throughout the Mediterranean Sea reported adult philometrid nematodes in gonads of *E. marginatus* [17]. Polinas and co-authors [18] reported white and yellow didymozoid capsules and brown nodules on the gills and pseudobranchs of *E. marginatus* from the western Mediterranean Sea. Widespread occurrence of parasite infections in dusky grouper populations highlights the need for further research into the parasite fauna and infection mechanisms. Despite considerable progress in fish parasitology in recent decades, there are still major gaps in knowledge of parasitic infections affecting wild dusky groupers. Lack of knowledge in this field is related to the complexity of the marine environment. Due to extreme environmental variability, according to scientific literature, it is difficult to improve current knowledge on parasite control in wild fish. Therefore, the aim of the current survey was to characterize the gastrointestinal and gill parasite fauna of *E. marginatus* caught in the central Mediterranean Sea (Southern Italy). Moreover, as the

helminth fauna of the dusky grouper is unknown, parasitic infection based on *E. marginatus* development processes was investigated.

2. Materials and Methods

2.1. Fish Sampling

From May 2018 to February 2020, 70 internal organs (stomach, intestine, liver, spleen) and gills of dusky groupers were collected from different fish markets or seized (due to illegal fishing, in particular regarding size) during official checks by veterinarians on the east cost of Sicily (Southern Italy). All the fish were caught in the Central Mediterranean Sea (FAO area 37.2.2) (Figure 1), along the coast between Messina and Syracuse, an area characterized by the presence of cliffs and beaches, with a sandy and rocky seabed, sudden variations of sea depth and a mean water temperature of 20 ◦C during warm periods and 17 ◦C during cold periods. Fish were identified with a consecutive number, and the weight (PBA220, Mettler Toledo, accuracy of 1 g) and total length (with an accuracy of 0.1 cm) recorded during sampling. After collection, fish were immediately stored at +4 ◦C and transferred to the laboratory of Parasitology and Parasitic Diseases, University of Messina and examined within 12 h. The collected specimens were split into two groups according to the capture period, namely warm months (23 specimens), for those collected from March to September, and cold months (47 specimens), from October to April. At the same time, the specimens were divided into size classes according to the criteria defined by Reñones et al. [2] as follows: Class I (<30 cm TL); Class II (30–45 cm TL); Class III (45–60 cm TL); Class IV (>60 cm TL).

2.2. Anatomopathological and Parasitological Examination

A careful macroscopic examination was performed to highlight any lesions present. Examination of gills and internal organ surface was performed for each fish to investigate the presence of parasites with the aid of a stereomicroscope (SteREO Discovery.V12 Zeiss, Jena, Germany). All gastrointestinal organs were inspected for helminths with the total worm count technique (TWC). The parasites collected were stored in 70% ethanol until morphological identification. Parasites were stained, clarified in glycerin for 24 h, mounted and then identified with keys [2,19–24]. Trematods were stained with classic Semichon's carmine red technique [25], modified according to requirements. A biopsy of gills and all organs was performed for microparasite presence. All morphological analyses were formed under an optic microscope (Axioskop 2 plus Zeiss), and all pictures were taken with a digital camera (Axiocam Mrc Zeiss) and a digital system (Axiovision Zeiss).

2.3. Statistical Analysis

For each parasite species, the epidemiological indices of infection as prevalence (P, %), mean abundance (MA) and mean intensity (MI) were calculated according to Bush et al. [26], and Pearson's chi-square analysis applied to evaluate differences of most frequent parasite species in *E. marginatus* specimens between warm and cold months. Pearson's correlation coefficients

were computed to evaluate the relationship between the biometric data (weight and total length) of *E. marginatus* and the prevalence of infection of each parasite species. A linear regression model ($y = a + bx$) was applied to determine the degree of correlation between these parameters during the study period. Level of significance was set at p values < 0.05. Statistical analyses were performed using the software GraphPad Prism version 5.1 (GraphPad Software, San Diego, CA, USA).

3. Results

Examined *E. marginatus* specimens (3 males and 67 females) weighed from 280 g to 8 kg and had a total length (TL) ranging from 18 to 80 cm, divided as following: Class I (N = 21 fish); Class II (N = 26 fish); Class III (N = 11 fish); Class IV (N = 12 fish,). Parasites were isolated from several organs including the intestine, stomach, gills and gonads. Specifically, 47 out of the 70 *E. marginatus* specimens (67.2%) were positive for one or more parasite species or taxa (Table 1).

No microparasites were found by biopsy. *Prosorhynchus caudovatus* and *P. temensis* showed the highest rate of infection compared to the other parasite species found, also according to analysis of the size classes (Figure 2). According to species descriptions by Yamaguti [19] and Polinas et al. (2018) [18], three flukes identified as *Didymodiclinus* sp., *Pseudoempleurosoma* sp. and *Megalocotyle hexacantha* were found in gills (Figure 3a). Morphologic features of other parasites, observed after diaphanization, allowed the identification of the gastrointestinal flukes *Prosorhynchus caudovatus* (Figure 3b), *Podoctyle temensis*, *Hemipera* sp. (Figure 4a,b), and the nematode *Philometra jordanoi*, *Anisakis* sp. type II larvae, *Capillaria* sp. and *Contracaecum* sp.

Gross lesions in the coelomatic organs of *E. marginatus* specimens attributable to parasite infection were not observed, and macroscopic gonadal alterations resulting from the presence of *P. jordanoi* (one to five specimens per fish) or gill damage caused by *Didymodiclinus* sp. were not found. As reported in Table 1, statistical evaluation of the data showed a dynamic infection level in *E. marginatus* specimens caught throughout the study period. In particular, the most prevalent parasite species *P. caudovatus* ($\chi^2 = 7.55$, $p = 0.006$) and *P. temensis* ($\chi^2 = 7.61$, $p = 0.005$) were mostly found in cold months (October to March). The other parasite species showed no statistically significant difference in the prevalence of infection between warm and cold months. Biometric data, including weight and TL of *E. marginatus* investigated in the current survey, were positively correlated with the parasitic load of *P. jordanoi* and *Didymodiclinus* sp. (Table 2). The results of Pearson correlation were confirmed by the linear regression model (Figure 5).

4. Discussion

The present survey provides an overview of the parasite fauna of *E. marginatus* from a central Mediterranean Sea population that had not been investigated previously. *Prosorhynchus caudovatus* was the most prevalent species found in the current survey. Interestingly, this species has not been reported in *E. marginatus* of the Mediterranean area, so far. Only a morphological description of *P. caudovatus* isolated from one specimen of *E. marginatus* caught along the South African coast [27], and in two Epinephelus goreensis and in four *Lutjanus maltzani* caught along the Ghanaian coast, have been reported [28]. The species *P. temensis* found here with a prevalence of 28.6% represents the first report of this parasite in *E. marginatus* from the investigated area. Previously, this species was reported in a dusky grouper along Corsican coasts, where 22 specimens of *P. temensis* were found in the pyloric caeca of one *E. marginatus* [20]. The gonads of 4 out of the 70 studied fish were infected with one to five specimens of *P. jordanoi*, but no gross organ alterations were observed. This finding is different to that reported by Marino et al. [29] in *Pagellus erythrinus* massively infected by gravid *Philometra filiformis*; probably, the smaller size of subgravid females of *P. jordanoi* found in dusky grouper could account for this difference. Philometrid nematodes have been reported with a prevalence of 22% in *E. marginatus* gonads and, despite the difficulty of extraction and identification, according to Lòpez-Neyra [30] a male specimen was identified as *P. jordanoi*. Didymozoidae species found in the current survey was identified as *Didymodiclinus* sp. Although this is the first report of *Didymodiclinus* sp. from the central Mediterranean Sea, the presence of this species had previously been reported in an *E. marginatus* specimen caught off Majorca Island (Western Mediterranean Sea) [18]. The *Pseudempleurosoma* sp., *Contracaecum* sp., *M. hexacantha*, *Capillaria* sp., *Hemipera* sp. and larvae of *Anisakis* type II found in specimens, even though in low numbers, represent the first report in *E. marginatus* from the central Mediterranean Sea so far. Analysis of the data highlighted a very high prevalence for infections with *P. caudovatus* and *P. temensis*, whose life cycle depends on an intermediate host presence. Although current knowledge on the life cycle of these flukes is limited, the high prevalence of infection of *P. caudovatus* and *P. temensis* found here suggests an abundance and/or preference of intermediate hosts of these parasite species in the diet of the dusky grouper. The most frequently found parasitic species mostly found during warm months were *Philometra jordanoi* and *Contracaecum* sp., whereas *P. caudovatus* and *P. temensis* showed a higher prevalence of infection during cold months. Differences found in parasite infections between warm and cold months are probably related to the diet and availability of food source. According to Reñones et al. [2], juvenile *E. marginatus* specimens (<30 cm TL) caught in the Mediterranean Sea feed mainly on

Brachyura (46% of stomach contents), in medium size specimens, between 30 and 45cm, and from 45 to 60 cm (sub-adult stage) the diet is mainly represented by cephalopods (40% of stomach contents), in adult specimens, >60 cm, the diet is mainly represented by teleost. Some of these varied organisms (e.g., Crustaceans, small fish, cuttlefish and octopus) comprising the diet of *E. marginatus* could potentially act as intermediate hosts for identified endo- and/or ecto-parasite species in this study [1,2]. Due to the size of the most parasitized fish, it could be speculated that mollusks represented the main source of nourishment of the *E. marginatus* analyzed [2], making them possible intermediate hosts of the flukes and nematodes found. Moreover, the data collected in the present study showed that weight and total length of *E. marginatus* are strongly correlated with *Didymodiclinus* sp. and *P. jordanoi* infection suggesting that fish size and, therefore, age, are variables worthy of investigation for the parasitic fauna of the dusky grouper as well as of other fish species of commercial interest. A possible explanation for the positive correlation between fish size and presence of *Didymodiclinus* sp. and *P. jordanoi* once again falls on the diet of *E. marginatus* as, being young fish, they are unable to prey on intermediate hosts of these parasites, e.g., mollusks and other fish; this strong positive relationship between parasite abundance and fish size has previously been reported by Polinas et al. [18]. There are still many gaps in the life cycle of the parasite species infesting dusky grouper and, thus, justifications for the results of the present study are in part speculative. According to the findings obtained, the dusky grouper of the investigated sea area may be regarded as a fish species that is "poor" in parasites regarding both abundance and variety of parasite species. According to Adroher-Auroux and Benítez-Rodríguez [31], there are only two reports of *Anisakis* type II in mammals, both in laboratory rats during an experimental infection. For these reasons, it is impossible to suspect a possible zoonotic risk due to dusky grouper consumption. Nevertheless, the present survey allowed us to describe the presence of 10 parasite species in *E. marginatus* specimens caught in Sicilian seawaters which helps to better understand the parasite fauna of this fish species and the potential hazards posed, both to the host and to human consumers.

5. Conclusions

This investigation improves current knowledge on the parasitic fauna of *E. marginatus*, the most valued serranid of the fish population inhabiting Mediterranean coasts. Moreover, the current survey showed that the parasite species found and identified do not pose any public health concern.

Author Contributions: G.D.B. and G.G. conceived and designed the study. G.D.B., G.G. and M.C.F. performed the veterinary examinations and sampling. G.D.B. and G.G. carried out the laboratory work. G.D.B. drafted the first version of the manuscript. F.A., G.G. and E.B. critically reviewed the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Our study was planned on internal organs sampled from fish market. For this reason, according to national decree-law 26/2014 (2010-63-EU directive).

Data Availability Statement: No apply. The data presented in this study are available on request from the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest.

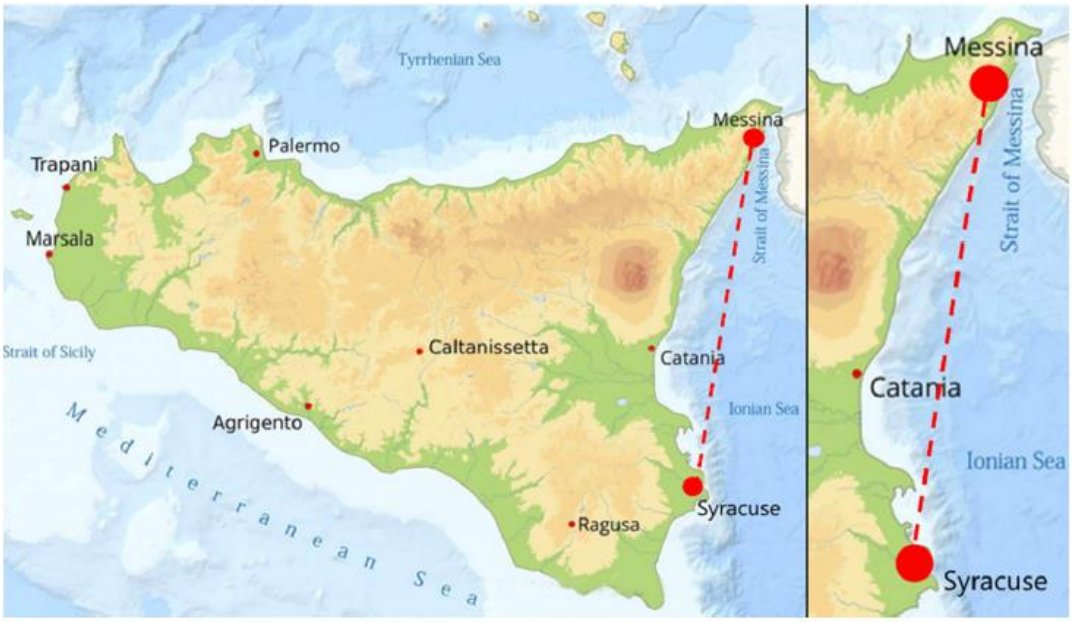

Figure 1. Study area between Messina and Syracuse

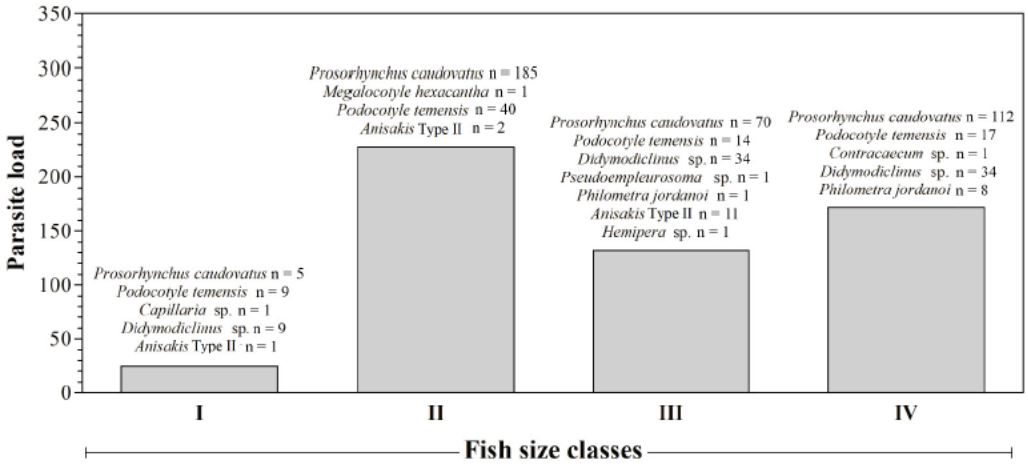

Figure 2. Number of parasites per species according to different size classes.

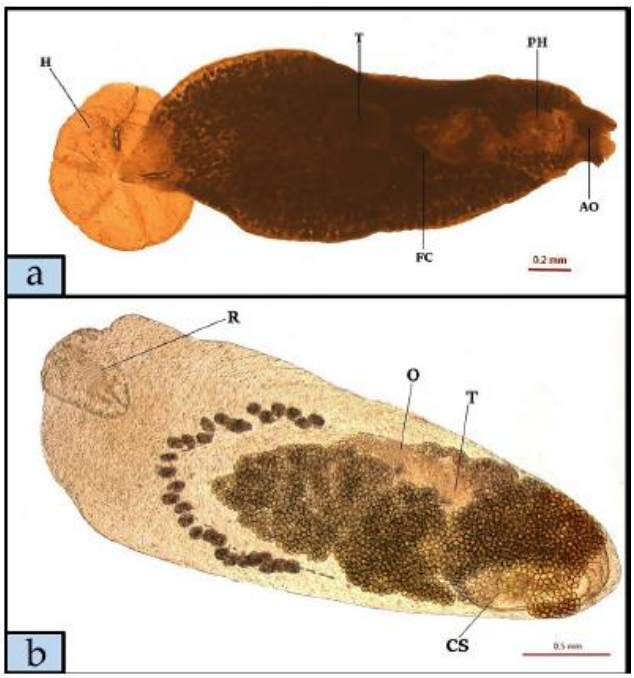

Figure 3. *Megalocotyle hexacantha* (**a**) specimen isolated from *Epinephelus marginatus* gills, glycerine diaphanization (H, Haptor; T, Testis; FC, Internal fertilization chamber; PH, Pharynx; AO, Anterior fixation organ) and *Prosorhynchus caudovatus* specimen (**b**) isolated from the *E. marginatus* stomach, glycerine diaphanization (R, Rhynchus; O, Ovary; T, Testis; CS, Cirrus sack).

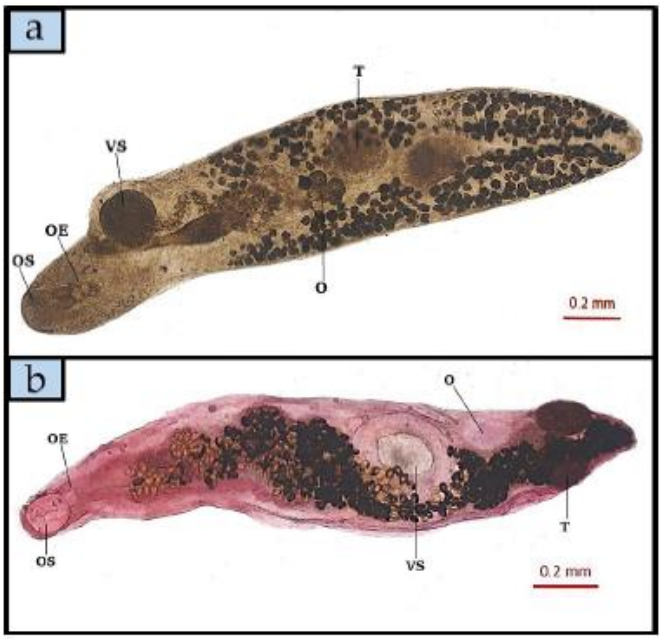

Figure 4. *Podocotyle temensis* specimen (**a**), glycerine diaphanization (O, Trilobal ovarian tissue; T, Testis; OE, Oesophagus; OS, Oral sucker; VS, Ventral Sucker) and *Hemipera* sp. specimen (**b**), after Semichon's carmine red technique staining (O, Ovary; T, Testis; OE, Oesophagus; OS, Oral sucker; VS, Ventral Sucker), both isolated from *Epinephelus marginatus* intestine.

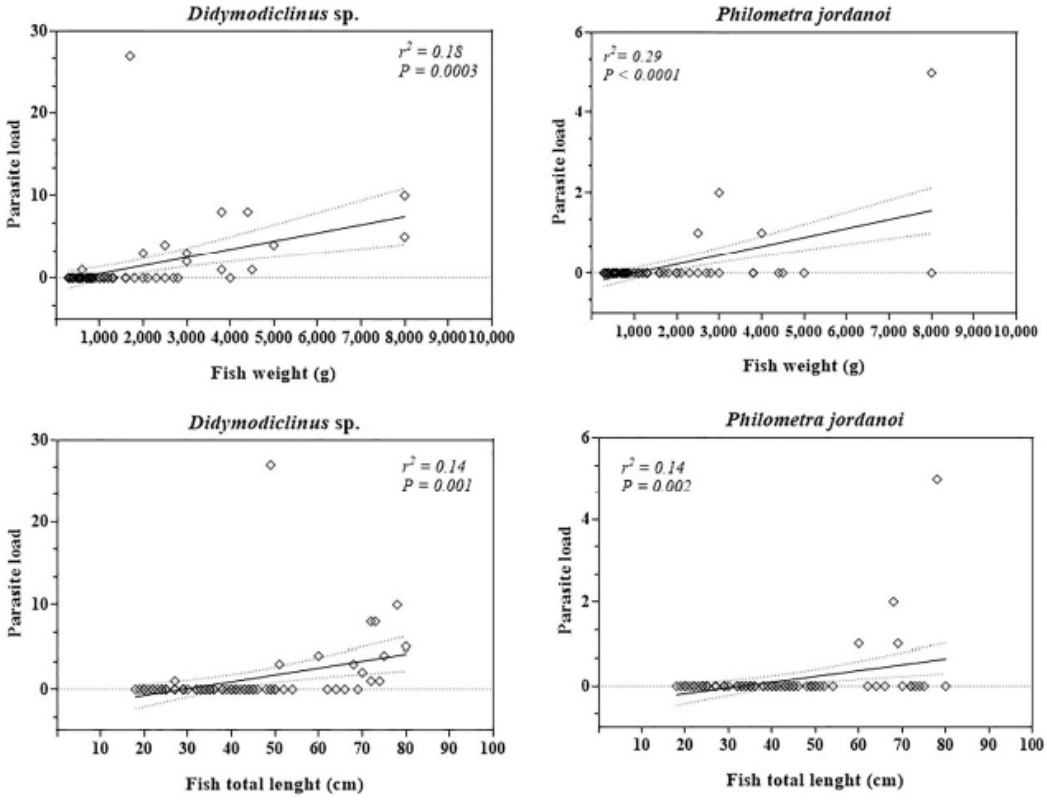

Figure 5. Linear regression values obtained between biometric data (weight and length) of *Epinephelus marginatus* and prevalence of infection of *Didymodiclinus* sp. and *Philometra jordanoi*.

Table 1. Prevalence (P, %), Mean abundance (MA) and mean intensity (MI) and infection site (Stomach = S, Intestine = I, Gills = G, Gonads = GO) of infection of parasite species retrieved in dusky grouper specimens.

Parasites with Indirect Life Cycle / Infection Site	P (%)			MA			MI		
	Total	Warm Months	Cold Months	Total	Warm Months	Cold Months	Total	Warm Months	Cold Months
Prosorhynchus caudovatus (S, I)	42.9	30.4 [A]	48.9	5.32	4.39	5.77	7.90	7.77	8.47
Podocotyle temensis (S, I)	28.6	17.4 [A]	34.0	1.14	0.43	1.49	1.70	0.77	2.19
Hemipera sp. (I)	1.4	0	2.1	0.01	0	0.02	0.02	0	0.03
Didymodiclinus sp. (G)	18.6	7.4	19.1	1.10	0.95	1.17	1.63	1.69	1.72
Philometra jordanoi (GO)	5.7	13.0	2.1	0.40	0.34	0.02	0.19	0.61	0.03
Anisakis sp. type II (S, I)	5.7	8.7	4.3	0.21	0.48	0.06	0.31	0.84	0.09
Capillaria sp. (S)	1.4	0	2.1	0.01	0	0.02	0.02	0	0.03
Contracaecum sp. (S)	1.4	4.3	0	0.10	0.04	0	0.02	0.08	0
Parasites with Direct Life Cycle / Infection Site	**P (%)**			**MA**			**MI**		
	Total	Warm Months	Cold Months	Total	Warm Months	Cold Months	Total	Warm Months	Cold Months
Pseudempleurosoma sp. (G)	1.4	4.3	0	0.01	0.04	0	0.02	0.08	0
Megalocotyle hexacantha (G)	1.4	4.3	0	0.01	0.04	0	0.02	0.07	0

[A] Significant difference found ($p < 0.05$).

Table 2. Correlation between biometric data of *Epinephelus marginatus* and parasites' prevalence.

	Biometric Data of *Epinephelus marginatus*	
Parasite Species	*Weight* (g)	*Total Length* (cm)
Pseudempleurosoma sp.	r = 0.07, $p = 0.56$	r = 0.13, $p = 0.27$
Megalocotyde hexacantha	r = −0.02, $p = 0.86$	r = 0.03, $p = 0.82$
Prosorhynchus caudovatus	r = 0.08, $p = 0.52$	r = 0.20, $p = 0.10$
Didymodiclinus sp.	**r = 0.42, $p = 0.0003$**	**r = 0.37, $p = 0.001$**
Philometra jordanoi	**r = 0.53, $p < 0.0001$**	**r = 0.14, $p = 0.001$**
Anisakis sp. type II	r = 0.06, $p = 0.65$	r = 0.12, $p = 0.32$
Podocotyle sp.	r = 0.02, $p = 0.88$	r = 0.12, $p = 0.32$
Hemipera sp.	r = 0.01, $p = 0.93$	r = 0.06, $p = 0.65$
Capillaria sp.	r = −0.07, $p = 0.54$	r = −0.10, $p = 0.40$
Contracaecum sp.	r = 0.11, $p = 0.37$	r = 0.19, $p = 0.12$

Significant correlations ($p < 0.05$) are indicated in bold letters.

References

1. Linde, M.; Grau, A.M.; Riera, F.; Massut´i-Pascual, E. Analysis of trophic ontogeny in *Epinephelus marginatus* (Serranidae). Cybium **2004**, 28, 27–35.
2. Reñones, O.; Polunin, N.V.C.; Goni, R. Size related shifts of *Epinephelus marginatus* in a western Mediterranean littoral ecosystem: An isotope and stomach content analysis. J. Fish. Biol. **2002**, 61, 122–137. [CrossRef]
3. Begossi, A.; Silvano, R.A.M. Ecology and ethnoecology of dusky grouper [garoupa, *Epinephelus marginatus* (Lowe, 1834)] along the coast of Brazil. J. Ethnob. Ethnom. **2008**, 4, 20. [CrossRef]
4. Tupper, M.; Sheriffn, N. Capture-based aquaculture of groupers. In Capture-Based Aquaculture; Global Overview; FAO Fisheries Technical Paper; Lovatelli, A., Holthus, P.F., Eds.; FAO: Rome, Italy, 2008; pp. 217–253.
5. IUCN. The IUCN Red List of Threatened Species. Version 2015.4. 2015. Available online: http://www.iucnredlist.org (accessed on 5 June 2018).
6. Mahmoud, N.E.; Alhindy, M.K.; Fahmy, M.M. Trypanorhynch cestodes infecting Mediterranean Sea fishes, Egypt: *Callitetrarhynchus gracilis* larvae (Pintner, 1931) as a bio-indicator of heavy metals pollution. J. Oceanogr. Mar. Sci. **2015**, 3, 133.
7. Merella, P.; Renones, O.; Garippa, G. Reinstatement of *Philometra jordanoi* (Lopez-Neyra, 1951) (Nematoda, Philometridae): A parasite of the Mediterranean dusky grouper *Epinephelus marginatus* (Lowe) (Osteichthyes, Serranidae). Syst. Parasitol. **2005**, 61, 203–206. [CrossRef] [PubMed]
8. Moravec, F.; Glamuzina, B.; Marino, G.; Merella, P.; Di Cave, D. Occurrence of *Philometra lateolabracis* (Nematoda: Philometridae) in the gonads of marine perciform fishes in the Mediterranean region. Dis. Aquat. Organ. **2003**, 53, 267–269. [CrossRef] [PubMed]
9. Roumbedakis, K.; Marchiori, N.C.; Paseto, A.; Gonçalves, E.; Luque, J.; Cepeda, P.B.; Sanches, E.; Martins, M. Parasite fauna of wild and cultured dusky-grouper *Epinephelus marginatus* (Lowe, 1834) from Ubatuba, Southeastern Brazil. Braz. J. Biol. **2013**, 73, 871–878. [CrossRef]
10. Roumbedakis, K.; Marchiori, N.C.; Garcia, P.; Pereira, J.; Castro, L.A.S.; Martins, M.L. *Helicometrina nimia* Linton, 1910 (Digenea: Opecoelidae) in dusky grouper *Epinephelus marginatus* (Lowe, 1834) (Teleostei: Serranidae) from southeastern Brazil. Braz. J. Biol. **2014**, 74, 472–479. [CrossRef]

11. Santos, C.P.; Buchmann, K.; Gibson, D.I. *Pseudorhabdosynochus* spp. (Monogenea: Diplectanidae) from the gills of *Epinephelus* spp. in Brazilian water. Syst. Par. **2000**, 45, 145–153. [CrossRef] [PubMed]
12. Genc, E. Infestation status of gnathiid isopod juveniles parasitic on dusky grouper (*Epinephelus marginatus*) from the northeast Mediterranean Sea. Parasitol. Res. **2007**, 101, 761. [CrossRef]
13. Marino, F.; Giannetto, S.; Paradiso, M.L.; Bottari, T.; De Vico, G.; Macrì, B. Tissue damage and haematophagia due to praniza larvae (Isopoda: Gnathiidae) in some aquarium seawater teleosts. Dis Aquat. Organ. **2004**, 59, 43–47. [CrossRef] [PubMed]
14. Rizgalla, J.; Bron, J.E.; Shinn, A.P.; Herath, T.K.; Paladini, G.; Ferguson, H.W. Ulcerative dermatitis in wild dusky grouper *Epinephelus marginatus* (Lowe) from Libyan waters. J. Fish. Dis. **2016**, 39, 1457–1466. [CrossRef] [PubMed]
15. Canestri-Trotti, G.; Fioravanti, M.L.; Patarnello, P.P.; Restani, R. Capsule branchiali da trematodi Didymozoidae in cernie (*Epinephelus guaza*) (Perciformes: Serranidae) dell'Adriatico meridionale. Parassitologia **1994**, 36, 26.
16. Gijón-Botella, H.; López-Romàn, R. Branquial parasites of *Epinephelus guaza* (L.) in the Canary Archipelago. Parasitol. Int. **1987**, 47, 620.
17. Moravec, F.; Chaabane, A.; Neifar, L.; Gey, D.; Justine, J.L. Descriptions of *Philometra aenei* n. sp. And *P. tunisiensis*. n sp. (Nematoda: Philometridae) from *Epinephelus* spp. off Tunisia confirm a high degree of host specificity of gonad-infecting species of *Philometra Costa*, 1845 in groupers (Serranidae). Syst. Parasitol. **2016**, 93, 115–128. [CrossRef]
18. Polinas, M.; Mele, S.; Padrós, F.; Merella, P.; Antuofermo, E.; Gouraguine, A.; Reñones, O. Ecological and histopathological aspects of *Didymodiclinus* sp. (Trematoda: Didymozoidae) parasite of the dusky grouper, *Epinephelus marginatus* (Osteichthyes: Serranidae), from the western Mediterranean Sea. J. Fish. Dis. **2018**, 41, 1385–1393. [CrossRef]
19. Yamaguti, S. Digenetic Trematodes of Hawaiian Fishes; Interscience Publishers: New York, NY, USA, 1970.
20. Bartoli, P.; Bray, R.A.; Gibson, D.I. Opecoelidae (Digenea) from western Mediterranean fishes: Three rare species. Syst. Parasitol. **2003**, 55, 81–95. [CrossRef]
21. Moravec, F.; Chaabane, A.; Justine, J.L.; Neifar, L. Two gonad-infecting species of *Philometra* (Nematoda: Philometridae) from groupers (Serranidae) off Tunisia, with a key to *Philometra* species infecting serranid gonads. Parasite **2016**, 23, 8. [CrossRef]

22. Anderson, R.C. Nematode Parasites of Vertebrates: Their Development and Transmission; CABI Publish: Wallingford, Oxon, UK, 2000.
23. Berland, B. Nematodes from some Norwegian marine fishes. Sarsia **1961**, 2, 1–50. [CrossRef]
24. Petter, A.J.; Maillard, C. Larves d'ascarides parasites de poissons en Méditerranée occidentale. Bull. Mus. Nam. Hist. Nat. Paris 4th Sér. Sect. A **1988**, 10, 347–369.
25. Cable, R.M. An. Illustrated Laboratory Manual of Parasitology; Burgess: Minniapolis, MN, USA, 1961.
26. Bush, A.O.; Lafferty, K.D.; Lotz, J.M.; Shostak, A.W. Parasitology Meets Ecology on Its Own Terms: Margolis et al. Revisited. J. Par. **1997**, 83, 575–583. [CrossRef]
27. Bray, R.A.; Justine, J.L. Bucephalidae (Digenea) from epinephelines (Serranidae: Perciformes) from the waters off New Caledonia, including *Neidhartia lochepintade* n. sp. Parasite **2013**, 20, 56. [CrossRef] [PubMed]
28. Fischthal, J.H.; Thomas, J.D. Digenetic trematodes of marine fishes from Ghana: Families Acanthocolpidae, Bucephalidae, Didymozoidae. Proc. Helminthol. Soc. Wash. **1968**, 35, 237–247.
29. Marino, F.; Busalacchi, B.; Bottari, T.; Rinelli, P.; Gaglio, G. Occurrence and prevalence of *Philometra filiformis* (Stossich, 1896) on *Pagellus erythrinus* (Linnaeus, 1758) in the southern Tyrrhenian Sea. J. Appl. Ichthyol. **2016**, 32, 687–692. [CrossRef]
30. Lòpez-Neyra, C.R. *Sanguinofilaria jordanoi* n. sp. (Nematoda: Filaroidea). Boletìn de la Universidad de Granada. Farmacia **1951**, 1, 291–293.
31. Adroher-Auroux, F.J.; Benítez-Rodríguez, R. Anisakiasis and *Anisakis*: An underdiagnosed emerging disease and its main etiological agents. Res. Vet. Sci. **2020**, 132, 535–545. [CrossRef]

Chapter 2

Morphological and molecular study of *Didymodiclinus marginati* n. sp. (Trematoda: Didymozoidae) gill parasite of *Epinephelus marginatus* from the central and western Mediterranean Sea

Adapted from:

Salvatore Mele[1]*, Giovanni De Benedetto[2]*, Alessia Giannetto[3], Kristian Riolo[3], Sabrina Oliva[3], Olga Reñones[4], Giovanni Garippa[1], Paolo Merella[1] and Gabriella Gaglio[2]

*Both authors contributed equally and share the first position.

[1]Department of Veterinary Medicine, University of Sassari, 07100 Sassari, Italy,
[2]Department of Veterinary Sciences, University of Messina, Italy,
[3]Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina, Italy,
[4]Instituto Español de Oceanografía (IEO, CSIC), Centro Oceanográfico de Baleares, Ecosystem Oceanography Group (GRECO), Palma 07015, Spain.

Parasitology, 2022, 1–10

Abstract:
The current study provides a morphological and molecular characterization of a new species of *Didymodiclinus* (Trematoda: Didymozoidae) infecting the dusky grouper, *Epinephelus marginatus* (Teleostei: Serranidae) from the Mediterranean Sea. A total of 279 dusky grouper specimens were examined for didymozoid gill parasites from the Mediterranean Sea between 1998 and 2020. New species differs from the most similar congeneric species by the rudiments of female reproductive organs in functional male specimens, and the seminal receptacle, Mehlis gland and accessory gland cells in functional female specimens, not observed in *Didymodiclinus branchialis* (Yamaguti, 1970), *Didymodiclinus epinepheli* (Abdul-Salam, Sreelatha and Farah, 1990) and *Didymodiclinus pacificus* (Yamaguti, 1938), respectively. These species are also characterized by their different hosts and location within the host tissues, being from other geographical localities. Moreover, this is the first species reported in *E. marginatus* from the central and western Mediterranean Sea. Genetic analyses were performed on partial 28S and partial internal transcribed spacer-2 ribosomal RNA regions and the mitochondrial cytochrome oxidase 1 (cox1) gene by polymerase chain reaction. Comparison of genetic sequences of *Didymodiclinus marginati* n. sp. with the available deposited sequences of 28S revealed that the new isolates cluster with several unidentified didymozoids and groups as a sister clade of the Nematobothrinae subfamily. Moreover, 28S and cox1 phylogenetic trees evidenced that Didymodiclinae is well separated from Didymozoinae and other gonochoric didymozoids. Following both morphological and genetic results, a key of identification for the genus *Didymodiclinus* is proposed.

Keywords: Didymoclinidae; didymozoid; dusky grouper; gill parasite; trematode

Introduction:

Didymozoids (Platyhelminthes, Trematoda) are poorly known parasites, often highly prevalent and abundant in several wild and reared marine fish (Munday et al., 2003; Mladineo and Tudor, 2004). Until 1985, Didymozoidae included 212 species placed in 81 genera, infecting mainly tropical and subtropical host species, with 23 species occurring in Mediterranean fish (Nikolaeva, 1985). However, in recent years the growing interest for fishery, protection and aquaculture of several fish from the Mediterranean Sea has increased the number of didymozoid species recorded in this region (40 taxa, Mele et al., 2016; Pérez-del-Olmo et al., 2016). Didymozoid parasites have been reported on the gills of the wild dusky groupers (*Epinephelus marginatus*, Lowe, 1834) (Osteichthyes, Serranidae), one of the largest top predators in the Atlantic and Mediterranean littoral ecosystems. In a first account, white capsules have been reported attached to the gills, pseudobranchs and orobranchial cavity of dusky grouper in the eastern Atlantic Ocean and ascribed them to *Didymodiclinus branchialis* (Yamaguti, 1970) (Gijón-Botella and López-Román, 1987). Also, Canestri-Trotti et al. (1994) reported similar findings on dusky grouper from the Adriatic Sea, with the uncertain attribution to the genera between *Gonapodasmius* (Ishii, 1935) and *Indoglomeritrema* (Madhavi and Hanumantha, 1983). Thus, the same parasitological lesions were observed on *E. marginatus* from several localities of the western Mediterranean Sea (Panebianco et al., 1995; Azzurro et al., 2002; Polinas et al., 2018). Recently, De Benedetto et al. (2021) found the infection also in fish from central Mediterranean Sea and ascribed it to *Didymodiclinus* sp. The present study provides the morphological and molecular description of a didymozoid gill parasite of *E. marginatus* from the Mediterranean Sea (Balearic Islands and Sicily), with the aim of clarifying the taxonomy of this organism. Moreover, a key of identification for the genus *Didymodiclinus* is proposed.

Materials and methods:

Fish sampling and parasitological analysis

A total of 279 dusky grouper specimens were examined for didymozoid gill parasites (Table 1). Two hundred and nine fish were caught off Majorca (western Mediterranean Sea) between 1998 and 2014; 70 were collected from different fish markets or seized during official controls by veterinarians in the east coast of Sicily (central Mediterranean Sea) between 2018 and 2020. Fish were measured (Total length) to the next millimetre and weighed (Total weight) to the next gram (Table 1) and then gills and opercula were examined for didymozoid capsules. Samples of infected gills and pseudobranchs were excised, placed in Petri dishes and dissected. Didymozoid capsules were opened to release the worms, which were recorded as alive, dead or degraded. Specimens were stored in 70% ethanol for morphological analysis or at −80°C for molecular analyses.

DNA isolation and polymerase chain reaction

Genomic DNA was extracted and purified using the NucleoSpin Plant II (Macherey-Nagel, Düren, North Rhine-Westphalia, Germany), according to the manufacturer's protocol. The obtained genomic DNA was used to evaluate 3 different markers, namely 2 ribosomal DNA markers, the 28S ribosomal RNA (28S) and partial internal transcribed spacer 2 (ITS-2) regions, and the mitochondrial cytochrome oxidase 1 (cox1) gene, by polymerase chain reaction (PCR). The loci of interest were amplified using the primer sets listed in Table 2 and the recombinant Taq DNA polymerase (Invitrogen, Carlsbad, California, United States) according to the manufacturer's instructions. PCR reactions (50 μL total volume) were performed in an Ep-Gradient Mastercycler (Eppendorf, Hamburg, Germany) using the following cycling parameters for 28S rDNA: 94°C for 30 s, 35 cycles of 94°C for 30 s, 58°C for 30 s and 72°C for 1 min, with a final step of 72°C for 10 min. For ITS-2 and Cox1 the following amplification profile was set: 94°C for 30 s, 35 cycles of 94°C for 30 s, 56°C for 90 s and 72°C for 90 s, with a final extension of 72°C for 10 min. PCR products were analysed by 1.5% agarose gel electrophoresis and samples that were successfully amplified were then purified using the E.Z.N.A. gel extraction kit (Omega Bio-tek, Norcross, Georgia, United States).

DNA sequencing and alignment

Extracted samples were sequenced in both the forward and reverse directions on an Applied Biosystems 3730 DNA analyser (Thermo Fisher Scientific, Waltham, Massachusetts, United States) and the obtained DNA sequences were analysed by BLASTN similarity search against

the NCBI database (http:// blast.ncbi.nlm.nih.gov/Blast.cgi). The 28S, ITS-2 and cox1 sequences obtained from the isolates were aligned with the available nucleotide sequences of Didymozoidae (Table 3) using the MUSCLE algorithm and further used for phylogenetic analyses. Neighbour-joining (NJ) and maximum likelihood (ML) trees were constructed selecting the GTR + G + I nucleotide substitution model for all datasets with the bootstrap method (1000 replications) to evaluate the reliability of internal branches (Lefort et al., 2017). NJ and ML phylogenetic analyses were performed using MEGA X and PhyML 3.0 (Guindon et al., 2010; Kumar et al., 2018), respectively. The Bayesian inference (BI) analysis was carried out using MrBayes 3.2.6 (Ronquist and Huelsenbeck, 2003) with the GTR model (1 000 000 generations, sampling every 500 generations and burn-in fraction 0.25). The trees were rooted with *Prosogonotrema bilabiatum* (28S), Hemiuridae sp. (ITS-2) and *Genarchopsis goppo* (cox1) chosen as outgroups.

Results

Gills and pseudobranchs of 92 fish from Majorca and 13 from Sicily (prevalence 44.9 and 18.6%, respectively) were infected with didymozoids. The main morphological traits of these parasites did not correspond to those of any of the previously described species, indicating they belonging to a new one.

***Didymodiclinus marginati* n. sp.**

Type host: Epinephelus marginatus (Lowe, 1834) (Osteichthyes:Serranidae), dusky grouper.

Type locality: Majorca, Spain, western Mediterranean Sea.

Other localities: Sicily, Italy, Ionian Sea, Central Mediterranean Sea.

Type specimens: The holotype (NHM UK 2022.4.8.1), paratype (NHM UK 2022.4.8.2) and vouchers (NHM UK 2022.4.8.3-6) were deposited in the Invertebrates Collection at the Natural History Museum, London, UK.

Site in host: Gills and pseudobranchs.

Infection parameters: Prevalence: 38% of host, 45% in Majorcan fish and 19% of Sicilian fish; mean intensity: 10.0 worms per infected host, 9.8 in Majorcan fish and 11.8 in Sicilian fish.

Representative DNA sequences: GenBank accession numbers: MW489519 (ITS-2); MW489507 (28S rRNA gene) and MW540490 (COX1).

ZooBank registration: To comply with the regulations set out in Article 8.5 of the amended 2012 version of the *InternationalCode of Zoological Nomenclature* (ICZN, 2012), details of the new species have been submitted to ZooBank. The Life Science Identifier (LSID) for *D. marginati* n. sp. is urn:lsid:zoobank.org: act:3B39D377-55DC-46BE-BE9B-F5821E39A7C0.

Etymology: Specific name refers to the name of the host species, *E. marginatus*.

Description (Fig. 1)

[Based on 15 adult specimens, 6 functional females and 9 functional males from dusky groupers from Mallorca, western Mediterranean Sea. The values of the holotype NHM UK 2022.4.8.1 (functional female) and paratype NHM UK 2022.4.8.2 (functional male) are expressed in the text, whereas the range of the measurements is reported in Table 4.] Capsule containing a couple of parasites with convoluted posterior ends. Gonochoristic, with weak sexual dimorphism i.e. underdeveloped male and female organs in both functional female and male specimens, respectively. All measurements are given in micrometres. Functional male specimen filiform, not divided in longitudinal regions, smaller than functional female, with

anterior end dorsoventrally flattened and somewhat lanceolate, 53 861 long, 260 wide (Fig. 1A). Oral sucker terminal, spheroidal, 54 × 57. Pharynx spheroidal, 52 × 38: oesophagus bifurcating into 2 caeca, 437 wide. Ventral sucker 65 × 117, at 907 from the anterior end. Pharynx, oesophagus and proximal part of caeca covered with gland cells. Testes 2, parallel, 52 136−52 615 × 111–128, starting at 50 from the posterior end. Vasa deferentia sinuous and spermatic duct filled with spermatozoa. Vasa deferentia joint at 571 from the anterior end. Rudiments of ovary and vitellarium barely visible. Uterus with 1 loop first descending. Metraterm 60 wide, joined with spermatic duct in a very short hermaphroditic duct, 17 long, reaching genital pore ventrally to the oral sucker. In alive specimens the genital pore is located on an extensible temporary papilla. Descending uterus filled with few (2–100) apparently immature eggs, 15 × 10. Functional female filiform, not divided into regions, with anterior end dorso-ventrally flattened, 77 680 long, 226 wide. Oral sucker terminal, spheroidal, 62 × 47 (Fig. 1B). Pharynx ellipsoidal, 45 × 34. Oesophagus bifurcating into 2 caeca, 156 long, reaching the posterior end of body. Ventral sucker diameter 103, distant 796 from anterior end. Pharynx, oesophagus and proximal part of caeca covered with gland cells. Testes 2, parallel, 6304– 7307 × 44–54, starting at 55 805 from the posterior end (Fig. 1D). Vasa deferentia straight and spermatic duct filled with spermatozoa. Vasa deferentia joint at 442 from the anterior end. Ovary long, simple, tubular, sinuous, 33 wide, starting 852 from anterior end of body and reaching with a short oviduct the genital junction at 44 960 (3/5 of the body). Seminal receptacle cystic, 191 × 171, joining the oviduct. Vitellarium long, simple, tubular, posterior to genital junction, 36 wide, starting at 355 from posterior end and joining the oviduct (Fig. 1C). Mehlis gland present around the first part of the uterus. Uterus with 1 loop, first descending, filled with eggs. Metraterm well developed, 90 wide. Genital pore ventral to the oral sucker. In alive specimens the genital pore is located on an extensible temporary papilla. Mature eggs, 15–17 × 10–11 (Fig. 1E).

Remarks

This species morphologically agrees with the diagnosis of the genus *Didymodiclinus* (Pozdnyakov, 1993) by the number of characters: the encapsulation in gills and pseudobranchs, a not clearly marked sexual dimorphism, specimens of both sexes with long filiform not regionalized body, a simple short oesophagus, intestinal caeca reaching the posterior extremity, ventral sucker present. Moreover, glandular cells are present around oesophagus and proximal region of the intestinal caeca. Functional males are smaller than functional females, with 2 parallel testes reaching the posterior end, and similarly to *Didymodiclinus menpachi* (Yamaguti, 1970) with rudiments of the female reproductive

system. Functional females have long and narrow ovaries located in the anterior part of body and vitellarium with similar form, located in the posterior part. The central portion of the female reproductive system is situated approximately in the middle of the body. *Didymodiclinus marginati* n. sp. can be distinguished from *D. branchialis* because of the larger body length and shorter oesophagus, and also the presence of the seminal receptacle, not observed in the latter species (Table 4). The presence of Mehlis gland, larger pharynx and ventral sucker and shorter oesophagus distinguishes the new species from *Didymodiclinus epinepheli* (Abdul-Salam et al., 1990). *Didymodiclinus marginati* n. sp. has accessory gland cells around the oesophagus, a character not observed in *Didymodiclinus pacificus* (Yamaguti, 1938) and *Didymodiclinus pristipomatis* (Yamaguti, 1934). In addition, *D. marginati* n. sp. has smaller oral sucker and eggs and shorter oesophagus than these 2 species. Concerning the differences with the rest of the congeneric species, *D. marginati* n. sp. has larger body than *Didymodiclinus cypseluri* (Yamaguti, 1940) and *Didymodiclinus hainanensis* (Gu and Shen, 1983), but shorter than *D. menpachi* and *Didymodiclinus microovatus* (Reimer, 1980). It also has smaller eggs than *D. cypseluri, D. menpachi, Didymodiclinus reticulum* (Madhavi and Murugesh, 1994) and *Didymodiclinus spilonotopteri* (Yamaguti, 1970), but larger than *D. microovatus*. All previously described species are also characterized by their different hosts and location within the host tissues, being from other geographical localities. Among the most similar species: *D. branchialis* was recorded in the nostril of *Epinephelus quernus* Seale, 1901 from Hawaii; *D. epinepheli* on the gills of *Epinephelus tauvina* (Forsskål, 1775) from the Arabian Gulf and *Epinephelus coioides* (Hamilton, 1822) from the Philippines; *D. pacificus* on the gills of an epinephelid from the Pacific Ocean; *D. pristipomatis* in the mouth of *Epinephelus akaara* (Temminck and Schlegel, 1842) from the Japan Sea (Yamaguti, 1971; Abdul-Salam et al., 1990; Cruz-Lacierda et al., 2001).

Molecular results

All the isolates showed positive amplification for 28S, ITS-2 and cox1 genes. Partial sequences of 28S (746 nt, 15 replicates), ITS-2 (640 nt, 15 replicates) and cox1 (530 nt, 15 replicates) were obtained for *D. marginati* n. sp. The nucleotide sequences of the amplified products of each gene were identical among the isolates from specimens collected in Majorca and Sicily. The representative DNA sequences for *28S, ITS-2* and *cox1* were submitted to GenBank (accession numbers MW489507, MW489519 and MW540490, respectively). The representative sequences of 28S had 97.98 and 97.2% of similarity to that of Didymozoidae sp. (AY222193) and Didymozoidae sp. (AY222194) from GenBank, with 13 and 18 nt of differences, respectively. The ITS-2 sequences had 90.3% of similarity to that of

Didymozoidae (KP452505) from GenBank with 74 nt of difference. The obtained sequences of cox1 had 75.2% of similarity to *Didymosulcus katsuwonicola* (KF379726) from GenBank, with 71 nt of difference. The alignments of 28S sequences used 625 informative positions for NJ, ML and BI analyses. The dataset included sequences of all the species deposited in GenBank. The phylogenetic trees (Fig. 2) resulted in very similar topology with reasonably high posterior probabilities and bootstrap values in most of the nodes. *Didymodiclinus marginati* n. sp. (sub-family Didymodiclininae) grouped with unidentified didymozoids from *Epinephelus cyanopodus* (AY222193). The genetic distance between *D. marginati* n. sp. and the relatives within this clade ranged between 2 (12 nt, AY222193) and 5% (46 nt, AB725627 and AB725630). The alignments of ITS-2 and cox1 sequences used 151 and 244 informative positions, respectively. The results depicted a distribution of taxa similar to that of the 28S, however several basal nodes were not resolved (Figs 3 and 4). In fact, the sequences of *D. marginati* n. sp. clustered as the sister taxon of didymozoids from other coral reef fish (i.e. Didymozoidae sp. DC-280 and Didymozoidae sp. PC-298).

Discussion

Didymozoidae is one of the most taxonomically complex and rich in synonyms of digenean families; the main reliable morphological character used for the identification of the species of this family is the genital complex (Pozdnyakov and Gibson, 2008), which often represents a challenge due to the variety of body sizes and subjective/partial descriptions reported in literature (Pozdnyakov and Gibson, 2008; Mladineo et al., 2010). Pozdnyakov (1993) erected the family Didymodiclinidae to include gonochoristic didymozoids, arranging them into 3 subfamilies: Didymodiclininae, with filiform body without marked sexual dimorphism; and the other 2 with globular bodies, Nephrodidymotrematinae with strongly marked sexual dimorphism and body of partners fused, Koellikeriinae without these characteristics. The subfamily Didymodiclininae included only 2 genera separated by the presence (*Didymodiclinus*) or absence (*Paragonapodasmius*) of the ventral sucker. Pozdnyakov and Gibson (2008) rejected the family Didymodiclinidae, including the subfamilies Didymodiclininae within the Didymozoidae. Apart from the general description of *Didymodiclinus* (Pozdnyakov, 1993), the species herein described has some characters that can be useful to distinguish it from the other species representative of the genus: anterior end dorso-ventrally flattened between suckers; functional males with rudiments of the female reproductive system; functional females with Mehlis gland around the first part of descending uterus. Furthermore, the genital papilla was observed in live specimens, but it is a difficult discriminating character because it is visible only in alive specimens (hardly available). This structure seems to work as a muscular organ protruded for mating, but it is also evident in stressful situations (e.g. during the dissection of capsules in live specimens;

Salvatore Mele personal observation). Concerning molecular analysis, the comparisons of the results of 3 loci indicated that the sequences of the new species are related to those of unknown didymozoids of other serranid and haemulid hosts, especially to one found in *E. cyanopodus* from Australia (AY222193). However, the phylogenetic tree generated with the ITS-2 loci did not have enough significant power to resolve most of the nodes, because the region amplified was too short and included a large number of invariant sites (71% of pair of bases analysed). Conversely, the 28S alignment showed that *D. marginati* n. sp. clusters apart from the didymozoids of other coral reef fish from the Indian and Pacific Oceans and Yucatan (larval stage) and from the didymozoids of scombrid fish.

Key to identification of *Didymodiclinus* species

The following dichotomous keys are provided to facilitate the identification of the trematodes belonging to the genus *Didymodiclinus* based on the comparison between the species of the genus.

1.	Ventral sucker smaller than oral sucker	2
	Ventral sucker larger or equal than oral sucker	4
2.	Seminal receptacle absent	3
	Seminal receptacle present	*D. spilonotopteri*
3.	Eggs small, 15-18 x 9	*D. hainanensis*
	Eggs large, 20-21 x 14-15	*D. cypseluri*
4.	Ventral sucker equal or slightly larger than oral sucker	*D. epinepheli*
	Ventral sucker two times larger than oral sucker	5
5.	Oral sucker larger than 130, ventral sucker larger than 300, eggs small 10-13 x 9-10	*D. microovatus*
	Suckers smaller and eggs larger than above	6
6.	Gland cells around oesophagus and proximal part of caeca absent	7
	Gland cells around oesophagus and proximal part of caeca present	8
7.	Mehlis gland absent, ovary width less than 40, testes larger than 1500.	*D. branchialis*
	Mehlis gland present, ovary width more than 40, testes shorter than 1500	*D. pacificus*
8.	Uterus with three loops	*D. pristipomatis*
	Uterus with one loop	9
9.	Genital junction in the first third of body, eggs 18-20 x 10-15	10
	Genital junction in the middle of body, eggs 15-17 x 10-11	*D. marginati* n. sp.
10.	Seminal receptacle absent	*D. menpachi*
	Seminal receptacle present	*D. reticulum*

Data. The data presented in this study are available on request from the corresponding author.

Acknowledgements. The authors thank F. E. Montero for his critical comments and suggestions on an earlier draft of the manuscript.

Author contributions. S. M., G. D. B. and G. G. conceived and designed the study. S. M., G. D. B. and G. G. performed the veterinary examinations and sampling. A. G., S. O. and K. R. performed the molecular analysis. S. M., G. D. B. and A. G. wrote the article. G. G., P. M., O. R. and GI. G. critically reviewed the manuscript. All authors have read and agreed to the published version of the manuscript.

Financial support. This research received no specific grant from any funding agency, commercial or not-for-profit sectors.

Conflict of interest. The authors declare there are no conflicts of interest.

Ethical standards. Our study was planned on internal organs sampled from fish markets. For this reason, according to national decree-law 26/2014 (2010-63-EU directive), no institutional review board statement was required.

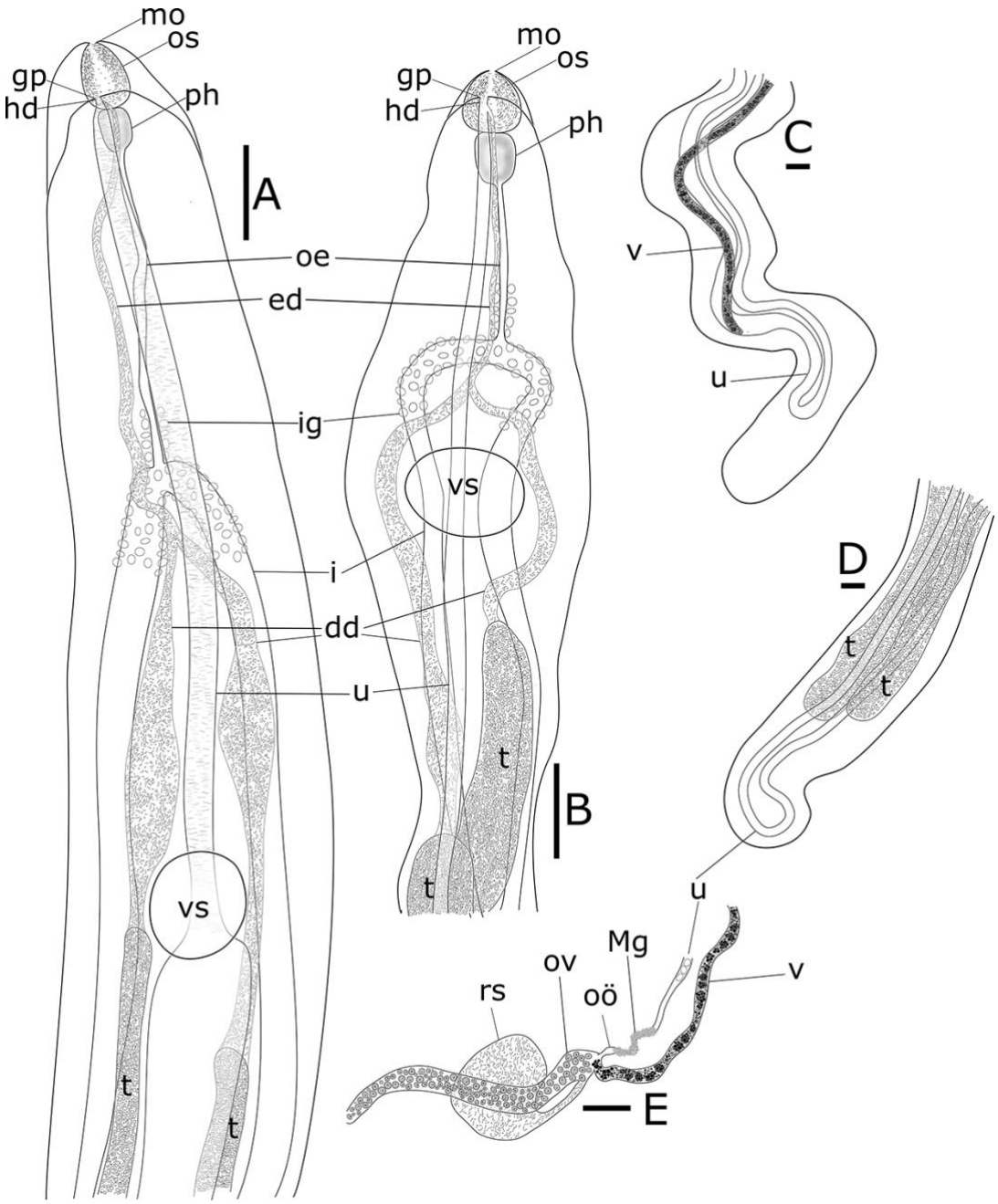

Fig. 1. *Didymodiclinus marginati* n. sp. ex the pseudobranch filaments of *Epinephelus marginatus*. (A) Functional female, holotype, anterior end of the body, ventral view. (B) Functional male, paratype, anterior end of the body, ventral view. (C) Functional female, holotype, posterior end of the body, ventral view. (D) Functional male, paratype, posterior end of the body, ventral view. (E) Functional female, holotype, genital junction. dd, deferent duct; ed, ejaculatory duct; gp, genital pore; hd, hermaphroditic duct; i, intestine; ig, intestinal glands; Mg, Mehlis gland; mo; mouth opening; oe, oesophagus; oö, oötype; os, oral sucker; ov, ovarium; ph, pharynx; rs, receptaculum seminis; vs, ventral sucker; t, testis; u, uterus; v, vitellarium. Scale bar: 100 μm.

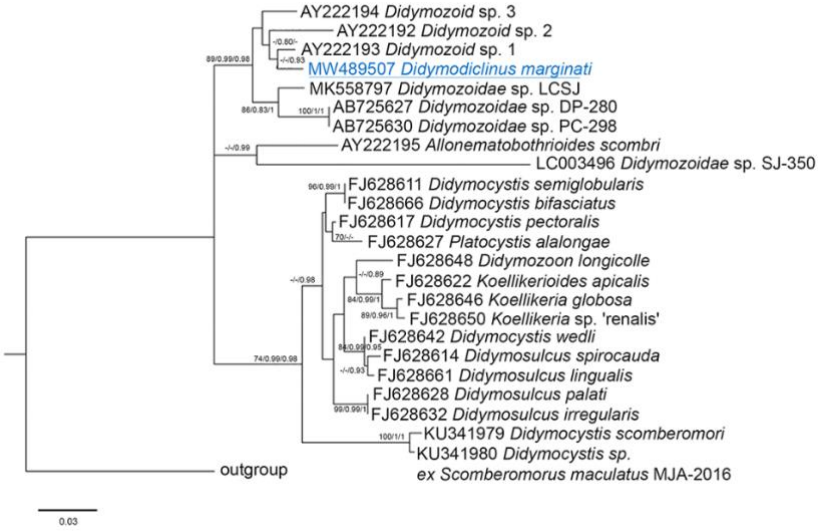

Fig. 2. Phylogenetic relationships between the isolates of the present study and other Didymozoidae as inferred from sequences of 28S rDNA analysed by NJ, ML and BI methods (ML tree is represented). Numbers at the nodes refer to NJ/ML/BI analysis; only bootstrap values above 70% (for NJ) or 0.7 (for ML and Bayesian posterior probabilities) are shown. GenBank accession numbers are indicated before species names. The species newly analysed in this study is underlined. Outgroup: *Prosogonotrema bilabiatum*.

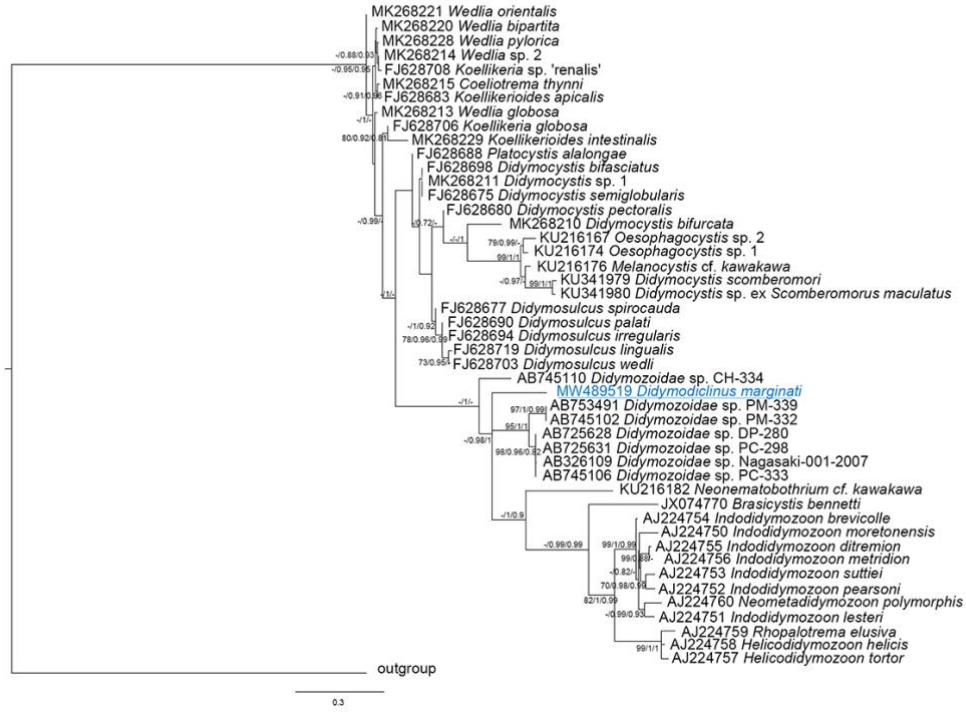

Fig. 3. Phylogenetic relationships between the isolates of the present study and other Didymozoidae as inferred from sequences of ITS-2 rDNA analysed by NJ, ML and BI methods (ML tree is represented). Numbers at the nodes refer to NJ/ML/BI analysis; only bootstrap values above 70% (for NJ) or 0.7 (for ML and Bayesian posterior probabilities) are shown. GenBank accession numbers are indicated before species names. The species newly analysed in this study is underlined. Outgroup: Hemiuridae sp.

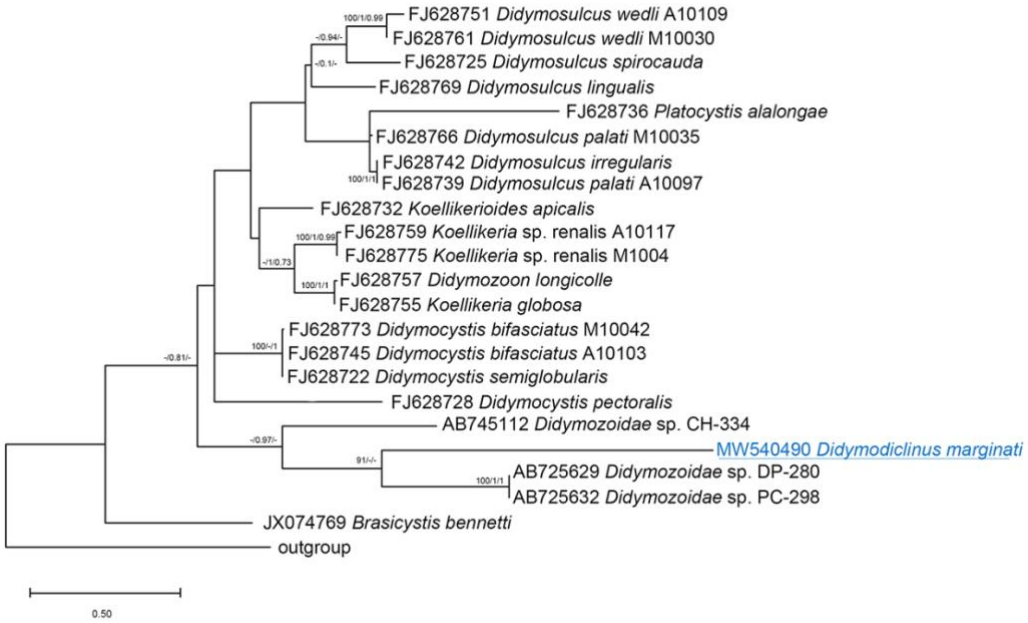

Fig. 4. Phylogenetic relationships between the isolates of the present study and other Didymozoidae as inferred from sequences of cox1 DNA analysed by NJ, ML and BI methods (ML tree is represented). Numbers at the nodes refer to NJ/ML/BI analysis; only bootstrap values above 70% (for NJ) or 0.7 (for ML and Bayesian posterior probabilities) are shown. GenBank accession numbers are indicated before species names. The species newly analysed in this study is underlined. Outgroup: *Genarchopsis goppo*.

Table 1. Data of *Epinephelus marginatus* from the Mediterranean examined.

Locality	Year	n	Total length (mm)	Total Weight (g)
Majorca	1998	30	116-802	18-9000
	1999	66	144-1035	58-18660
	2001	6	70-518	5-2000
	2002	83	68-930	84-16600
	2003	20	123-514	25-2368
	2014	4	317-1070	500-22000
Sicily	2018	16	180-700	280-3000
	2019	33	200-800	300-8000
	2020	21	270-750	600-5000

Table 2. List of targeted loci, sequence of the oligonucleotide forward (For) and reverse (Rev) primers used for PCR (Mladineo *et al.* 2015).

Locus	Sequence (5'- 3')
28S	For GTCCGATAGCGAACAAGTACCGT Rev AGCATAGTTCACCATCTTTCGGGTCTCAA
ITS-2	For GTCGTAACAAGGTAGCTGTA Rev TATGCTTAAGTTCAGCGGGT
cox1	For TTTTTTGGGCATCCTGAGGTTTAT Rev CAACAAATCATGATGCAAAAGG

Table 3. Nucleotide sequences of the 28S rRNA, ITS and cox1 markers used to evaluate the phylogenetic relations among our isolates and other Didymozoidae

Species	Isolate	Host	Locality	28S	ITS	COX1	Author
Allonematobothrioides scombri	na	*Scomber scombrus*	UK	AY222195			Olson *et al.* (2003)
Brasicystis bennetti	na	*Plagioscion squamosissimus* sciaenid fish	Brazil, Pará, Belem	JX074770	JX074770	JX074769	Melo *et al.* (2013)
Didymocystis bifasciatus	A1	*Thunnus thynnus*	Adriatic Sea		FJ628698	FJ628745	Mladineo *et al.* (2010)
Didymocystis bifasciatus	M1	*Thunnus orientalis*	California Bay	FJ628666	na	FJ628773	Mladineo *et al.* (2010)
Didymocystis linguatis	M1	*Thunnus orientalis*	California Bay	FJ628661	na	FJ628769	Mladineo *et al.* (2010)
Didymocystis linguatis	M3b/M1a	*Thunnus orientalis*	California Bay		FJ628719		Mladineo *et al.* (2010)
Didymocystis pectoralis	A1	*Thunnus thynnus*	Adriatic Sea	FJ628617	FJ628680	FJ628728	Mladineo *et al.* (2010)
Didymocystis scomberomori	na	*Scomberomorus maculatus*	USA: Gulf of Mexico	KU341979	KU341979		Schrandt *et al.* (2016)
Didymocystis semiglobulatis	A2	*Thunnus thynnus*	Adriatic Sea	FJ628611	FJ628675	FJ628722	Mladineo *et al.* (2010)
Didymocystis sp. ex *Scomberomorus maculatus* MJA-2016	MJA-2016	*Scomberomorus maculatus*	USA: Gulf of Mexico	KU341980	KU341980		Schrandt *et al.* (2016)
Didymodidinus marginati n. sp.	Em1-2020	*Epinephelus marginatus*		MW489507	MW489519	MW540490	This study
Didymosulcus irregularis	A1	*Thunnus thynnus*	Adriatic Sea	FJ628632	FJ628694	FJ628742	Mladineo *et al.* (2010)
Didymosulcus palati	A10019	*Thunnus thynnus*	Adriatic Sea	FJ628628	FJ628690	FJ628739	Mladineo *et al.* (2010)
Didymosulcus palati	M10035	*Thunnus orientalis*	California Bay			FJ628766	Mladineo *et al.* (2010)
Didymosulcus spirocauda	A10005	*Thunnus thynnus*	Adriatic Sea	FJ628614	FJ628677	FJ628725	Mladineo *et al.* (2010)
Didymosulcus wedli	A10033	*Thunnus thynnus*	Adriatic Sea	FJ628642	FJ628703	FJ628751	Mladineo *et al.* (2010)
Didymosulcus wedli	M10030	*Thunnus orientalis*	California Bay			FJ628761	Mladineo *et al.* (2010)
Didymozoid sp. 1-PO-2003	1-PO-2003	*Epinephelus cyanopodus*	Australia	AY222193			Olson *et al.* (2003)
Didymozoid sp. 2-PO-2003	2-PO-2003	*Taeniura lymma*	Australia	AY222192			Olson *et al.* (2003)
Didymozoid sp. 3-PO-2003	3-PO-2003	*Apogon cookii*	Australia	AY222194			Olson *et al.* (2003)
Didymozoidae sp. CH-334					AB745110	AB745112	Abe *et al.* (2014)
Didymozoidae sp. DP-280	DP-280	*Diagramma pictum*	Japan	AB725627	AB725628	AB725629	Abe *et al.* (2014)
Didymozoidae sp. PC-298	PC-298	*Plectorhinchus cinctus*	Japan	AB725630	AB725631	AB725632	Abe *et al.* (2014)
Didymozoidae sp. SJ-350	SJ-350	*Scomber japonicus*	na	LC003496			Abe and Okamoto (2015)
Didymozoidae sp. LCSJ-2019	LCSJ-2019	*Syacium papillosus*	Yucatan	MK558797			Vidal-Martínez *et al.* (2019)
Didymocystis bifurcata	na	*Thunnus obesus*	Solomon Islands	MK268210	MK268210		Moore *et al.* (2019)
Didymocystis sp. 1	na	*Thunnus obesus*	Ambon	MK268211	MK268211		Moore *et al.* (2019)
Wedlia globosa	na	*Thunnus obesus*	Solomon Islands	MK268213	MK268213		Moore *et al.* (2019)
Wedlia sp. 2	na	*Thunnus obesus*	Palabuhanratu	MK268214	MK268214		Moore *et al.* (2019)
Coeliotrema thynni	na	*Thunnus obesus*	Solomon Islands	MK268215	MK268215		Moore *et al.* (2019)
Koellikeria sp. 1	na	*Thunnus obesus*	Solomon Islands	MK268216	MK268216		Moore *et al.* (2019)
Wedlia bipartita	na	*Thunnus albacares*	Bali	MK268220	MK268220		Moore *et al.* (2019)
Wedlia orientalis	na	*Thunnus albacares*	Solomon Islands	MK268221	MK268221		Moore *et al.* (2019)
Wedlia pylorica	na	*Thunnus albacares*	Bali	MK268228	MK268228		Moore *et al.* (2019)
Koellikerioides intestinalis	na	*Thunnus albacares*	Solomon Islands	MK268229	MK268229		Moore *et al.* (2019)
Didymozoon longicolle	A1	*Thunnus thynnus*	Adriatic Sea	FJ628648		FJ628757	Mladineo *et al.* (2010)
Helicodidymozoon helicis	na	*Platycephalus fuscus*	Moreton Bay, Australia		AJ224758		Anderson and Barker (1998)
Helicodidymozoon tortor	na	*Platycephalus endrachtensis*	Moreton Bay, Australia		AJ224757		Anderson and Barker (1998)
Indodidymozoon brevicolle	na	*Platycephalus fuscus*	Moreton Bay, Australia		AJ224754		Anderson and Barker (1998)
Indodidymozoon ditremion	na	*Inegocia japonica*	Moreton Bay, Australia		AJ224755		Anderson and Barker (1998)
Indodidymozoon lesteri	na	*Platycephalus endrachtensis*	Moreton Bay, Australia		AJ224751		Anderson and Barker (1998)
Indodidymozoon metridion	na	*Suggrundus jugosus*	Moreton Bay, Australia		AJ224756		Anderson and Barker (1998)
Indodidymozoon moretonensis	na	*Platycephalus fuscus*	Moreton Bay, Australia		AJ224750		Anderson and Barker (1998)
Indodidymozoon pearsoni	na	*Platycephalus endrachtensis*	Moreton Bay, Australia		AJ224752		Anderson and Barker (1998)
Indodidymozoon suttiei	na	*Platycephalus fuscus*	Moreton Bay, Australia		AJ224753		Anderson and Barker (1998)
Koellikeria globosa	A1	*Thunnus thynnus*	Adriatic Sea	FJ628646	FJ628706	FJ628755	Mladineo *et al.* (2010)
Koellikeria sp. renalis	A1	*Thunnus thynnus*	Adriatic Sea	FJ628650	FJ628708	FJ628759	Mladineo *et al.* (2010)
Koellikeria sp. renalis	M1	*Thunnus orientalis*	California Bay			FJ628775	Mladineo *et al.* (2010)
Koellikerioides apicalis	A1	*Thunnus thynnus*	Adriatic Sea	FJ628621	FJ628683	FJ628732	Mladineo *et al.* (2010)
Melanocystis cf. *kawakawa*	MEL6	*Euthynnus alletteratus*	Spain, Mediterranean	KU290355	KU216176		Mele *et al.* (2016)
Neometadidymozoon polymorphis	na	*Priacanthus macracanthus*	Moreton Bay, Australia		AJ224760		Anderson and Barker (1998)
Neonematobothrium cf. *kawakawa*	N18	*Euthynnus alletteratus*	Spain, Mediterranean	KU290361	KU216182		Mele *et al.* (2016)
Oesophagocystis sp. 1	DZ14	*Euthynnus alletteratus*	Spain, Mediterranean	KU290353	KU216174		Mele *et al.* (2016)
Oesophagocystis sp. 2	DC5	*Euthynnus alletteratus*	Spain, Mediterranean	KU290346	KU216167		Mele *et al.* (2016)
Platocystis alalongae	A2	*Thunnus thynnus*	Adriatic Sea	FJ628627	FJ628688	FJ628736	Mladineo *et al.* (2010)
Rhopalotrema elusiva	na	*Platycephalus fuscus*	Moreton Bay, Australia		AJ224759		Anderson and Barker (1998)
Genarchopsis goppo (outgroup)	KT254045	*Rhinogobius kurodai*	Japan			AB703670	Urabe *et al.* (2012)
Prosaganotrema bilabiatum (outgroup)	1 DNA-2519	*Rhomboplites aurorubens*	USA	KU527431			
Hemiuridae sp. (outgroup)	TrM2	*Centropages hamatus*	Denmark		KM401880		Skovgaard and Mier-Jedrzejowicz (unpublished)

na, data not available.

Table 4. Morphometric data of species of the genus *Didymodiclinus*

	BL	BW	OSL	OSW	PL	PW	OL	VSL	VSW	DVS	OW	VW	TL	TW	EL	EW	DGJ
Didymodiclinus branchialis	13 000–102 000	220–500	37–86	46–78	25–58	23–50	100–500	70–130	70–130	350–1000	22	26			16–20	8–13	2300–8000
	4300–21 000	200–280	46–85	35–75	25–50	20–45	100–480	70–148	70–148	30–720			3160–14 600	80–120			
Didymodiclinus cypseluri	na	na	na	na	na	na	na	na	na	na	na	36			20–21	14–15	na
	17 000	130	60	57	23	23	175	27	27	600			13 300	15–45			
Didymodiclinus epinepheli	260 000–806 000	140–350	43–55	28–43	18–28	18–28	195–273	33–65	33–65	204–367	28–125	20–61			15–18	9–10	19 000–124 000
	4280–69 000	200–310	45–68	36–50	23–35	23–35	263–600	50–75	50–75	459–1020			2965–5297	20–93			
Didymodiclinus facialis	710–16 300	80–1400	40–80	30–52	18–24	18–24	76–137	no	no	no	40	27			18–20	11	3347
	1061	111	34	20	25	16	75	no	no	no			751	20			
Didymodiclinus hainanensis	67 714–271 900	568–668	84–100	84	100–117	117	636–685	48–50	50	1216	67	27			15–18	9	na
	52 112	668	84	67	45	45	735	42	42	1270			44 172	80			
Didymodiclinus kovaljovae	9980–20 100	630–1036	43–66	46–53	40–62	30–36	218–397	no	no		23–53	30–53			27–30	13–16	7631
	1400–3740	198–294	40–56	36–50	29–43	26–36		no	no	no			1176–2772	75–86			
D. marginati n. sp.	69 910–267 128	226–424	43	38	45	42	138	156	97	796	33	36			15–17	10–11	44 960
	30 356–53 861	160–335	41–54	38–57	38–52	30–42	125–437	65–117	43–117	563–895			40 515–52 615	33–128			
Didymodiclinus menpachi	498 000	800	63–75	50–60	40–45	40–55	250–400	120–180	120–180	540–900	80–140				18–20	12–15	65 000
	600 000	800	70–90	80–90	60	40–50	350–550	190–250	190–250	680–800			na	250–270			
Didymodiclinus microovatus	385 000	500–700	167	139	58	50	790	343	343	2008	65–80	45–80			10–13	9–10	21 670
	na	na	na	na	na	na	na	na	na	na			na	na			
Didymodiclinus pacificus	32 000–70 000	200–330	75	57–66	36–57	33–48	400–650	125–150	125–150	650–1100	50–70	21–54			18–20	12–13	650–1100
	31 700–42 500	200–330	88–93	60–67	38–48	36–40	45–55	150–135	150–135	800–1000			18 175–22 675	30–45			
Didymodiclinus pristipomatis	27 000–1 770 000	200–400	60	60	50	34	200	150	150	450	170	55			18–21	11–14	na
	57 000–71 000	180–440	na	na	na	na	na	na	na	na			na	220			
Didymodiclinus reticulum	67 000–90 000	440–490	90–104	64–66	50–56	36–40	320–464	112–140	92–104	na	15–16	na			18–20	10–12	na
	49 000–52 000	320–450	60–84	60–66	48–56	32–48	436–842	130–134	90–102	na			na	na			
Didymodiclinus spilonotopteri	56 000–141 000	280–380	93–112	58–93	28–46	28–46	250–530	33–54	33–54	450–1100					20–23	12–16	na
	6300–21 000	100–350	35–107	32–88	23–60	23–60	200–420	33–54	33–54	600–1200			6200–14 500	na			
Didymodiclinus tomex	na	na	na	na	na	na	na	na	na	na	na	na			na	na	na
	12 000	330	140	60–80	62	33	300	70	11	630			na	na	18	12	

BL, body length; BW, body width; OSL, sucker length; OSW, sucker width; PL, pharynx length; PW, pharynx width; OL, oesophagus length; VSL, ventral sucker length; VSW, ventral sucker width; DVS, distance between anterior tip of body and VS; OW, ovary width; VW, vitellarium width; TL, testis length; TW, testis width; EL, egg length; EW, egg width; DGJ, distance between the anterior tip and the genital junction; na, data not available.

References:

Abdul-Salam, J, Sreelatha, B and Farah, M (1990). *Gonapodasmius epinepheli* n. sp. (Didymozoidae) from the grouper *Epinephelus tauvina* from the Arabian Gulf. *Systematic Parasitology* **17**, 67–74.

Abe, N, Okamoto, M and Maehara, T (2014). Molecular characterization of muscle-parasitizing didymozoids in marine fishes. *Acta Parasitologica* **59**:354-8. doi: 10.2478/s11686-014-0234-2.

Abe, N and Okamoto, M (2015). Molecular characterization of muscle-parasitizing didymozoid from a chub mackerel, *Scomber japonicus*. *Acta Parasitologica* **60**:557-62. doi: 10.1515/ap-2015-0080.

Anderson, GR and Barker, SC (1998). Inference of phylogeny and taxonomy within the Didymozoidae (Digenea) from the second internal transcribed spacer (ITS2) of ribosomal DNA. *Systematic Parasitology* **41**: 87–94.

Azzurro, E, Berrilli, F, De Liberato, C, Di Cave, D and Orecchia, P (2002). Contributo alla conoscenza dei metazoi parassiti della cernia di scoglio (*Epinephelus marginatus* Lowe, 1834) del Mediterraneo. *Atti del IX Convegno Nazionale della Società Italiana di Patologia Ittica*: **14.**

Canestri-Trotti, G, Fioravanti, ML, Patarnello, PP and Restani, R (1994). Capsule branchiali da trematodi Didymozoidae in cernie (*Epinephelus guaza*) (Perciformes: Serranidae) dell'Adriatico meridionale. *Parassitologia* **36**, 26.

Cruz-Lacierda, ER, Lester, RJG, Eusebio, PS, Marcial, HS and Pedrajas, SAG (2001). Occurrence and histopathogenesis of a didymozoid trematode *Gonapodasmius epinepheli* in pond-reared orange-spotted grouper, *Epinephelus coioides*. *Aquaculture* **201**, 211–217.

De Benedetto, G, Arfuso, F, Ferrara MC, Brianti, E and Gaglio G (2021) Parasite Fauna of the Dusky Grouper (*Epinephelus marginatus*, Lowe 1834) from the Central Mediterranean Sea. *Animals* **11**:2523. https://doi.org/10.3390/ani11092523

Gijón-Botella, H and López-Román, R (1987). Branchial parasites of *Epinephelus guaza* (L.) in the Canary Archipelago. *Parasitology International* **47**, 620.

Gu, CD and Shen, JW (1983). Four new species of didymozoid trematodes from China. *Acta Zootaxonomica Sinica* **8**: 17-23

Guindon, S, Dufayard, JF, Lefort, V, Anisimova, M, Hordijk, W and Gascuel, O (2010). New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Systematic Biology* **59**, 307-321.

Kumar, S, Stecher, G, Li, M, Knyaz, C and Tamura, K (2018). MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution* **35**, 1547-1549. https://doi.org/10.1093/molbev/msy096.

Lefort V, Longueville J-E and Gascuel O (2017) SMS: Smart Model Selection in PhyML. *Molecular Biology and Evolution* **34**(9), 2422–2424.

Madhavi, R and Murugesh, M (1994). Gonapodasmid didymozoids (Digenea: Didymozoidae) from serranid fishes of the Visakhapatnam Coast, Bay of Bengal. *Rivista di Parassitologia* **55**, 47–55

Mele, S, Pennino, MG, Piras, MC, Macías, D, Gómez-Vives, MJ, Alemany, F, Montero, FE, Garippa, G and Merella, P (2016). Ecology of the Atlantic black skipjack *Euthynnus alletteratus* (Osteichthyes: Scombridae) in the western Mediterranean Sea inferred by parasitological analysis. *Parasitology* **143**, 1330-1339.

Melo, FT, Silva, JP, Gonçalves, EC, Furtado, AP, Giese, EG, Santos, CP and Santos, JN (2013). Taxonomic status and redescription of the genus *Brasicystis* Thatcher, 1979 (Digenea: Didymozoidae). *Parasitology International* **62**, 208-214.

Mladineo, I and Tudor, M (2004). Digenea of Adriatic cage-reared bluefin tuna *Thunnus thynnus*. *Bulletin of the European Association of Fish Pathologists* **24**, 114–153.

Mladineo I, Bott NJ, Nowak BF, Block BA (2010). Multilocus phylogenetic analyses reveal that habitat selection drives the speciation of Didymozoidae (Digenea) parasitizing Pacific and Atlantic bluefin tunas. *Parasitology* **137**, 1013–1025.

Mladineo I, Tomaš M and Stanić R (2015). Cross-fertilization as a reproductive strategy in a tissue fluke *Didymosulcus katsuwonicola* (Platyhelmintes: Didymozoidae) inferred by genetic analysis. *Parasitology* **142**:1422-9. doi: 10.1017/S0031182015000839.

Moore BR, Lestari P, Cutmore SC, Proctor C and Lester RJG (2019). Movement of juvenile tuna deduced from parasite data, *ICES Journal of Marine Science* **76**, 1678–1689, https://doi.org/10.1093/icesjms/fsz022

Munday, BL, Sawada, Y, Cribb, T, Hayward, CJ (2003). Diseases of tunas, *Thunnus* spp. *Journal of Fish Diseases* **26**, 187–206.

Nikolaeva, VM (1985). *Trematodes – Didymozoidae fauna, distribution and biology*. In *NOAA Technical Report NMFS Parasitology and Pathology of Marine Organisms of the World Ocean* (Ed. Hargis, WJ), Springfield, VA, USA, pp. 67–72.

Olson PD, Cribb TH, Tkach VV, Bray RA and Littlewood DTJ (2003). Phylogeny and classification of the Digenea (Platyhelminthes: Trematoda). *International Journal for Parasitology* **33**, 733–755.

Panebianco A, Giannetto S and Conte F (1995). Infestione da *Gonapodasmius epinepheli* in cernie del Mar Tirreno. Descrizione e considerazioni ispettive. *Atti Della Societa' Italiana Delle Scienze Veterinarie* **49**, 441-442.

Pérez-del-Olmo A, Kostadinova A and Gibson DI (2016) The Mediterranean: high discovery rates for a well-studied trematode fauna. *Systematic Parasitology* **93**(3), 249–256.

Polinas, M, Mele, S, Padrós, F, Merella, P, Antuofermo, E, Gouraguine, A, Reñones, O (2018). Ecological and histopathological aspects of *Didymodiclinus* sp. (Trematoda: Didymozoidae) parasite of the dusky grouper, *Epinephelus marginatus* (Osteichthyes: Serranidae), from the western Mediterranean Sea. *Journal of fish diseases* **41**, 1385–1393.

Pozdnyakov, SE (1993) The systematics of trematodes of the suborder Didymozoata. *Parazitologiya* **27**: 415-425

Pozdnyakov, SE and Gibson, DI (2008). Family Didymozoidae Monticelli, 1888. In Bray RA, Gibson DI and Jones A (Eds.), *Keys to the Trematoda*. Wallingford, UK: CABI Publishing and the Natural History Museum, pp. 631–734

Reimer, LW (1980). Beschreibung des Weibchens eines neuen Didymozoiden: *Gonapodasmius microovatus* spec. nov. *Angewandte Parasitologie* **21**: 26-31

Ronquist, F and Huelsenbeck JP (2003) "MrBayes 3: Bayesian phylogenetic inference under mixed models". *Bioinformatics* **19**: 1572-1574.

Schrandt MN, Andres MJ, Powers SP and Overstreet RM (2016). Novel Infection Site and Ecology of Cryptic *Didymocystis* sp. (Trematoda) in the Fish *Scomberomorus maculatus*. *Journal of Parasitology* **102**: 297–305. doi: https://doi.org/10.1645/15-772

Urabe M, Nishimura T and Shimazu T (2012) Taxonomic revision of three species of the genus *Genarchopsis* (Digenea: Hemiuroidea: Derogenidae) in Japan by molecular phylogenetic analyses. *Parasitology International* **61**(4), 554–560.

Vidal-Martínez VM, Velázquez-Abunader I, Centeno-Chalé OA, May-Tec AL, Soler-Jiménez LC, Pech D, Mariño-Tapia I, Enriquez C, Zapata- Pérez O, Herrera-Silveira J, Hernández-Mena DI, Herzka SZ, Ordoñez-López U and Leopoldina A-MM (2019) Metazoan parasite infracommunities of the dusky flounder (*Syacium papillosum*) as bioindicators of environmental conditions in the continental shelf of the Yucatan Peninsula, Mexico. *Parasites & Vectors* **12**(1), 381.

Yamaguti, S (1934). Studies on the helminth fauna of Japan. Part 2. Trematodes of fishes. I. *Japanese Journal of Zoology* **5**: 249-541

Yamaguti, S (1938). Studies on the helminth fauna of Japan. Part 24. Trematodes of fishes, V. *Japanese Journal of Zoology* **8**, 15–7

Yamaguti, S (1940). Studies on the helminth fauna of Japan Part 31. Trematodes of fishes, VII. *Japanese Journal of Zoology* **9**: 35-108

Yamaguti, S (1970). *Digenetic Trematodes of Hawaiian Fishes*. Keigaku Publishing Co. Tokyo, Japan.

Yamaguti, S (1971). *Synopsis of digenetic trematodes of vertebrates*. Keigaku Publishing Co. Tokyo, Japan. Vols I. and II: 247-277.

Chapter 3

Anisakis pegreffii Larvae in *Sphyraena viridensis* and Description of Granulomatous Lesions

Adapted from:

Giovanni De Benedetto[1], Alessia Giannetto[2], Kristian Riolo[2], Carmelo Iaria[2], Emanuele Brianti[1] and Gabriella Gaglio[1]

[1]Department of Veterinary Sciences, University of Messina, 98168 Messina, Italy,
[2]Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina, 98166 Messina, Italy

Animals ***2021****, 11, 3449*

Simple Summary:

Fish-borne zoonoses are caused by bacteria and parasites, while no viral fish-borne zoonoses have been reported, to date. Regarding zoonoses caused by parasites, *Anisakiasis* is one of the most important, with *Anisakis simplex* and *Anisakis pegreffii* agents in the central Mediterranean Sea. Humans can be infected by accidental ingestion of third-stage larvae in raw, undercooked or improperly processed fish or cephalopods. After ingestion, the larvae migrate from the gastrointestinal tract to gastrointestinal tissue, causing pain and, subsequently, inflammatory reaction leading to eosinophilic granuloma. This kind of reaction has not been described to date, in fish, and the aim of this study is to describe gastric wall lesions caused by *A. pegreffii* in *Sphyraena viridensis* and to compare them to those reported in humans, which appear macroscopically identical, albeit showing significant microscopic differences.

Abstract:

The aim of the present study was to describe gastric granuloma caused by *Anisakis pegreffii* in *Sphyraena viridensis* caught in the central Mediterranean Sea. Sixty-eight *S. viridensis* specimens were collected from different fish markets on the east coast of Sicily. Coelomic organs were observed both macroscopically and with the aid of stereomicroscope. Parasite specimens and lesioned tissues were collected for identification, histological and molecular analyses. Twelve specimens (P=17.6%) were positive for the presence of nematode larvae, morphologically identified as larvae of *Anisakis* sp., with values of mean abundance and mean intensity of 0.9 and 4.8, respectively. One large female specimen showed massive parasite infection associated with nodular lesions of the gastric wall. By histology, several nematode larvae encysted through the gastric wall were found. The parasite bodies were surrounded by a granulomatous reaction made up of macrophages, epithelioid cells, some lymphocytes, and an external connective sheet. Molecular analysis of *18S rRNA* and *cox2* genes from *Anisakis* sp. collected larvae, identified them as *A. pegreffii*. The lesions here described, though macroscopically superimposable on human eosinophilic granuloma, microscopically showed significant differences in the inflammatory cells involved and in the type of immune reaction.

Keywords: *Anisakis pegreffii*; Anisakidae; *Sphyraena viridensis*; Inflammatory reaction; gastric granuloma; humans

1. Introduction

Four species of Sphyraenidae have been reported in the Mediterranean Sea: *Sphyraena chrysotaenia* and *Sphyraena flavicauda* along the Red Sea coast, and *Sphyraena viridensis* and *Sphyraena sphyraena* in the central Mediterranean Sea [1,2]. Yellowmouth barracuda (*S. viridensis*, Cuvier, 1829) is a common coastal pelagic predator in the central Mediterranean Sea [3] where it can reach 90 cm in length, but generally ranged from 25 to 50 cm in length. It is a gregarious species, even if solitary specimens have been described [1]. According to the state of growth, *S. viridensis* feeds mainly on crustaceans and fish [1]. In the Mediterranean Sea it has been described from 50m depth to the water surface where *S. viridensis* moves to feed [1]. The mating season extends from September to April and the first stages of development are exclusively planktonic [1]. Though several studies have been carried out on the occurrence of this fish species in different areas, e.g., Azores Archipelago, Madeira, Cape Verde [4-8], limited and dated information on its biology and distribution in the central Mediterranean Sea are available [3].

Sphyrena viridensis, as a result of its diet based on benthonic fish and cephalopods, might be exposed to infection by *Anisakis* spp. [9]. Nematodes belonging to the Anisakidae are characterized by an heteroxenous life cycle, which involves various organisms of the marine ecosystem. Small crustaceans, belonging to the order Euphausiacea, represent the first intermediate hosts, fish and cephalopods are intermediate/paratenic hosts, where *Anisakis* larvae develop into the infesting third stage (L3). Adult parasites have been found in the stomach of marine mammals, such as cetaceans, that prey on intermediate hosts [10]. In fish, larvae are localized on the serosa of the coelomic organs and in some species also in the muscle [11].

The species *Anisakis simplex* and *Anisakis pegreffii* have been identified as the causative agents of human anisakidosis [12,13]. Humans act as accidental intermediate hosts, and become infected when eating raw or undercooked fish or cephalopods carrying L3 larvae [14]. In humans, L3 larvae move from the gastrointestinal tract to the gastrointestinal mucosa up to the submucosa, initially causing pain and subsequently a granulomatous reaction identified as eosinophilic granuloma [11]. The immune response during helminth infection is supported by T helper 2 (Th2) lymphocytes, with release of cytokines, as interleukin 3 (IL3), IL4, IL5, IL9 and IL13, eosinophilic granulocytes, mast cells and activated macrophages [15]. Since human is an accidental host, it is possible that the immune response during nematode infection is not properly modulated in this kind of atypical host [16]. Indeed, allergic-like reactions associated with *Anisakis* infections have been reported over the years [17,18]. The first case of human

infestation was reported in the Netherlands in 1960 [19]. About 20,000 cases referable to this zoonosis have been reported in humans worldwide, 90% in Japan [14]. In Italy, several cases of anisakiasis caused by *A. pegreffii* have been reported along the meridional coasts, where there is an increased consumption of raw or undercooked fish [20,21].

Granulomatous reactions have been reported, but not deeply described in European sea bass (*Dicentrarchus labrax*) and gilthead sea bream (*Sparus aurata*) after experimental infection by *Anisakis pegreffii* larvae [22,23] and in wild fish such as John dory (*Zeus faber*) sampled from the central Mediterranean Sea [24].

It is noteworthy that *A. pegreffii* has been found as the main nematode species infecting *S. viridensis* [9] in the western Mediterranean Sea; however, the typical lesions observed in human hosts including gastric eosinophilic granuloma have not been compared with granuloma caused in *S. viridensis* to date.

In this study, we describe gastric lesions and granuloma caused by *A. pegreffii* in *S. viridensis* and compare them with those reported in humans. This study represents the first report of a human-like gastric lesion caused by *A. pegreffii* infection in fish, and it provides clues for a better understanding of the pathogenic pathway power of this nematode infection in intermediate fish hosts.

2. Materials and Methods

2.1. Fish sampling

During this survey, 68 *S. viridensis* specimens, caught in the Mediterranean Basin (FAO area 37.2.2), were collected from different fish markets in the east coast of Sicily. All collected specimens were immediately stored at +4°C and transferred to the laboratory of Parasitology and Parasitic Diseases, University of Messina to perform necropsy and parasitological analysis. All fish were measured (total length, TL) and weighed (body weight, BW) (PBA220, Mettler Toledo, accuracy of 1 g), and measurements recorded.

2.2. Parasitological analysis

The fish were dissected and examined for *Anisakis* spp. larvae presence according to Arthur & Albert [25]. Briefly, coelomic organs were observed both macroscopically and with a stereomicroscope (Stereo Discovery.V12 Zeiss, Jena, Germany) as described by Piras et al. [9]. Parasites found were washed in physiological solution and fixed in 70° ethanol. Samples used for molecular analysis were stored at -80°C. Morphological larvae identification was performed using morphological keys suggested by Berland B. [26] and Sonko et al. [27], with the aid of an optical microscope (Axioskop 2 plus Zeiss) after glycerin diaphanization. Epidemiological indices of prevalence (P, %), mean abundance (MA) and mean intensity (MI) were calculated according to Bush et al. [28]. Pearson's correlation was calculated to evaluate the relationship between *S. viridensis* body weight (log transformed) and *A. pegreffii* load (number of larvae per subject). Statistical significance was set at p values < 0.05. Statistical analyses were executed using the software GraphPad Prism version 9.0 (GraphPad Software, San Diego, CA, USA).

2.3. Histological analysis

For histological evaluations, macroscopically visible lesions were excised and fixed in buffered 10% formalin solution for 48 hours, routinely processed and paraffin-embedded at 56°C. Four-micron thick sections were cut and routinely stained with hematoxylin and eosin (H&E) [29].

2.4. Molecular analysis

Total DNA was extracted from parasite specimens using the Nucleo Spin Plant II kit (Macherey-Nagel) according to the manufacturer's instructions. DNA quantity, purity, and integrity were verified by UV absorbance measurements at 260 and 280 nm (NanoDrop 2000, Thermo Scientific, Wilmington, MA). Polymerase chain reactions (PCR) were carried out for

the identification to species level using two specific primer sets (Table 1) and Taq DNA Polymerase Recombinant kit (Invitrogen).

The *18S rRNA* gene from *Anisakis* spp. was amplified using the primers Nem_18S_F and Nem_18S_R from Floyd et al. [30] and the following PCR conditions: after a first step of 94 °C for 5 min, DNA was subjected to 35 cycles of 94 °C for 30 s, 54 °C for 30 s, and 72 °C for 1 min, with a final extension of 72 °C for 10 min. The mitochondrial *cytochrome C oxidase subunit II* (*cox2*) gene was amplified using the primers 211 and 210 from Nadler and Hudspeth [31]. The PCR conditions were as follows: 94 °C for 3 min, 35 cycles of 94 °C for 30 s, 46 °C for 1 min and 72 °C for 90 s, followed by post amplification at 72 °C for 10 min [32].

The PCR products were resolved by 2.0% agarose gel electrophoresis and amplicons of the expected size were purified using the E.Z.N.A Gel Extraction Kit (OMEGA), according to the manufacturer's protocol. DNA sequencing of the purified fragments was performed on the Applied Biosystems 3730 DNA Analyzer (Thermo Fisher Scientific), using both forward and reverse primers for each gene analyzed. The sequences of *18S rRNA* (926 bp) and mtDNA *cox2* (629 bp) obtained from the *Anisakis* spp. specimens from *S. viridensis* were analyzed by BLASTN similarity search against the National Center for Biotechnology Information (NCBI) database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The *cox2* sequences were aligned with previously characterized sequences of other known *Anisakis* species and deposited in GenBank [33], by ClustalW, carried out using MEGA X software [34]. Phylogenetic analyses on the *cox2* sequence data sets were carried out by MEGA X using Maximum Parsimony (MP) and Neighbour-Joining (NJ), based on p-distance. Reliabilities of phylogenetic relationships were evaluated using nonparametric bootstrap analysis with 1,000 replicates for MP and NJ trees. Bootstrap values exceeding 70 were considered well supported [35].

3. Results

Host specimens sampled (48 males and 20 females) had a mean length of 68 ± 12.8 cm and a mean weight of 1,110 ± 638.7g. Twelve specimens (P=17.6%) scored positive for the presence of nematode larvae with values of MA and MI of 0.9 and 4.8, respectively. The total count of larvae ranged from 1 to 18 per specimen Morphologically, the nematode larvae were identified as *Anisakis* Type I larvae. No significative differences about parasite load were found between male and female specimens; Pearson's correlation test showed a positive correlation (R = 0.5 P <0.05) between the *Anisakis* larvae load and *S. viridensis* body weight considered as logarithm (Fig 1)

The larvae were found mainly attached to the serous walls of the celomic organs and within the stomach and intestine walls. In one case, a large female specimen of 4,064 g., a massive larvae infection was associated with nodular lesions on the gastric wall showing the presence of viable larvae inside (Figure 2 a, b).

By histology, several nematodes encysted through the gastric wall were found in both sub-serosa and sub-mucosa (Figure 3). Frequently, parasitic bodies were surrounded by a thick granulomatous reaction, made up of macrophages, epithelioid cells, some lymphocytes, and an external connective sheet, although singular parasites appeared to be simply encysted within a thin fibrous reactive capsule.

Up to five different stages of granuloma were detected (i.e., containing free larvae, encysted parasite, early, intermediate, mature (Figure 4 a, b, c, d); some granulomas without parasites inside were also found (Figure 5).

3.1. Molecular identification of Anisakis spp.

The genomic DNA of *Anisakis* larvae collected from *S. viridensis* was successfully amplified by both primer sets identifying *18S rRNA* and *cox2* molecular markers. The obtained nucleotide sequences were submitted to GenBank database under the accession numbers OK448176 and OK483324 for *18S rRNA* gene and *cox2* gene, respectively.

The amplification of the *18S rRNA* region produced a fragment of 926 bp; blast search showed that the *18S* DNA sequences identified in this study matched previously reported *18S rRNA* nucleotide sequences of the *Anisakis* species, *A. pegreffii* (MF072697.1 and EF180082.1), *A. simplex* (MF072711.1) and *Anisakis* sp. (U94365.1), with ~ 99% identity. Unfortunately, our isolates were not assignable to species level based on *18S rRNA* sequences since the amplified region shared the same sequence in both *Anisakis* species. A sequence of 629 pb coding for

the mtDNA *cox2* gene was obtained from *Anisakis* specimens isolated in this study. It is noteworthy that, the sequence analysis showed that the identified *cox2* gene matched exclusively with the *A. pegreffii cox2* sequences previously deposited in GenBank (139 hits found with E value of 0.0; 87 out of 139 showed >97% identity), therefore supporting the molecular identification of *A. pegreffii* larvae (Nematoda: Anisakidae) in *S. viridensis.* Phylogenetic analyses of the obtained *cox2* sequence with other *cox2* sequences from *Anisakis* species available in GenBank showed that our *cox2* sequence clustered together with known *cox2* sequences of *A pegreffii* previously deposited by Valentini et al. [32].

Moreover, the phylogenetic trees from both NJ and MP analyses (Figure 5 and 1 Suppl.) showed that the mtDNA *cox2* gene from *Anisakis* specimens of this study clustered in the same, well-supported clade (100% bootstrap value) with the *cox2* sequences of *A pegreffii* previously deposited in GenBank. The trees derived from both MP and NJ analyses showed similar topologies, with *Anasakis* species clustering in two main clades: one including *A. paggiae*, *A. physeteris* and *A. brevispiculata* and the other one including all the anisakids analysed (*A. pegreffii, A. simplex* (*s. s.*), *A. simplex* C, *A. typica* and *A. ziphidarum*).

4. Discussion

The present study represents the first description of a gastric granuloma in *S. viridensis* by *A. pegreffii*. In fish, the migration of *Anisakis* larvae through the stomach wall mainly causes ulceration localized on the stomach mucosa surface, without negative effects on the physiological organ function [30]; in our case, this kind of lesion was not found. *Anisakis simplex* sensu lato is considered responsible for "Red belly syndrome" in Atlantic salmon (*Salmo salar*), characterized by hemorrhagic lesions in the area surrounding the vent, associated with the presence of non-encapsulated larvae [36], probably attributable to specific *A. simplex* tropism in *S. salar* and not superimposable on the lesion described in the present study.

In humans, ingested larvae may be expelled by digestive and peristaltic processes, but in some case, L3 larvae can penetrate the wall of the gastrointestinal tract causing local chronic granulomatous reaction [37]. This reaction is totally macroscopically superimposable on the granulomatous lesions herein described. The immune response and adaptation described in human during this kind of infection [15,16], did not clearly described in fish, so far, also because is not easy to perform this investigation in wild fish. From a histological point of view, human *A. pegreffii* lesions show a marked edema externally, localized in the submucosa layer often associated with abundant inflammatory infiltrate in the muscular layer, mainly composed of eosinophil granulocytes, followed by lymphocytes and plasma cells [38], whereas in *S. viridensis,* only sparse lymphocytes can be found in the muscle around granulomas. In this case, fish cannot be considered an appropriate animal model for the study of some human pathologies, as reported by Schmale et al. [39], also because the inflammatory response is different between teleosts and humans. A chronic granulomatous human lesion due to *A. pegreffii* could develop into abdominal peritonitis often associated to intestinal occlusion and abundant inflammatory reactions such as edema and fibrinous exudate [40,41]. In the present case, the gastric wall was involved, while inflammatory involvement of neighboring gastrointestinal tract, lumen occlusion and exudate reaction were not found.

The *Anisakis* larvae prevalence here observed reached much higher values (17.6 vs 5.7%) than those reported in a previous study from other areas of the Western Mediterranean Sea [9]. In agreement with the statistically significant correlation between fish body weight and parasite load found in the current study, larger fish are more likely to prey on a higher quantity and variability of intermediate or paratenic hosts. However, in some cases, as described by other authors [42] large or old fish may show a reduction of parasitic load compared to smaller specimens, probably because they are able to develop a better immune response against new

infections. The sequence and phylogenetic analyses of the mtDNA *cox2* gene supported the molecular identification of the zoonotic species *A. pegreffii* causing gastric granuloma in *S. viridensis* for the first time. This study corroborates the value of the mitochondrial *cox2* gene as an effective molecular marker for the identification of closely related species of the *Anisakis* genus. The phylogenetic trees obtained by NJ and MP analyses showed that anisakid nematode species separate in two major clades, with *A. paggiae*, *A. physeteris* and *A. brevispiculata* grouping in a separate clade, as reported by Mattiucci et al. [40]. This study highlights that the combined morphological and molecular approaches offer a valuable tool for the identification of *Anisakis* species.

5. Conclusions

In the present study, we described, granulomatous lesions caused by *A. pegreffii* in *S. viridensis* from the central Mediterranean Sea for the first time, focusing on the typology of lesions and comparing them with eosinophilic granulomas described in humans.

Although at gross anatomy the lesions here observed may be superimposable to the eosinophilic granulomas described in humans, some specific histological differences about the of inflammatory cells composition may be highlighted.

Author Contributions: G.D.B., E.B. and G.G. conceived and designed the study. G.D.B. and G.G. performed the veterinary examinations and sampling. G.D.B. and G.G. carried out the parasitological analysis. C.I. performed the histological examination. A.G. and K.R. performed the molecular analysis. G.G. and E.B. critically reviewed the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding

Institutional Review Board Statement: Our study was planned on internal organs sampled from fish markets. For this reason, according to national decree-law 26/2014 (2010-63-EU directive), no institutional review board statement was required.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest.

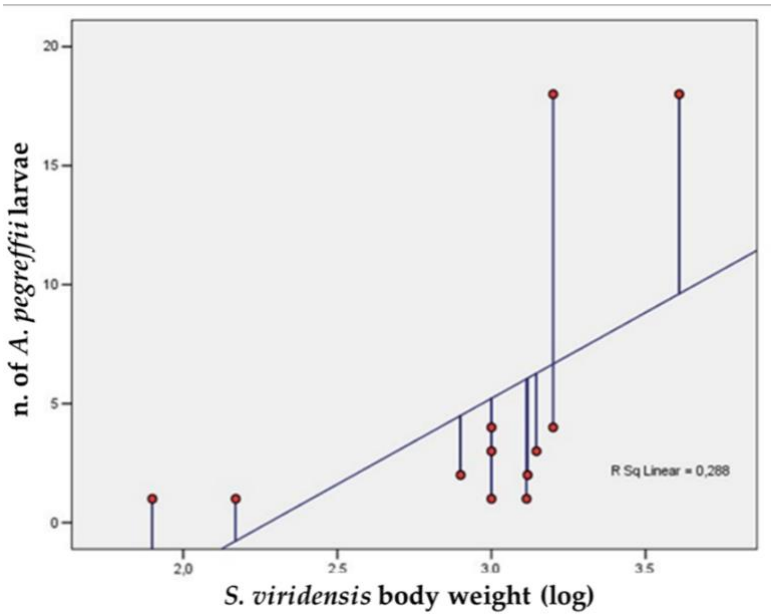

Figure 1. Correlation between *Anisakis pegreffii* larvae load and logarithm of *S. viridensis* body weight.

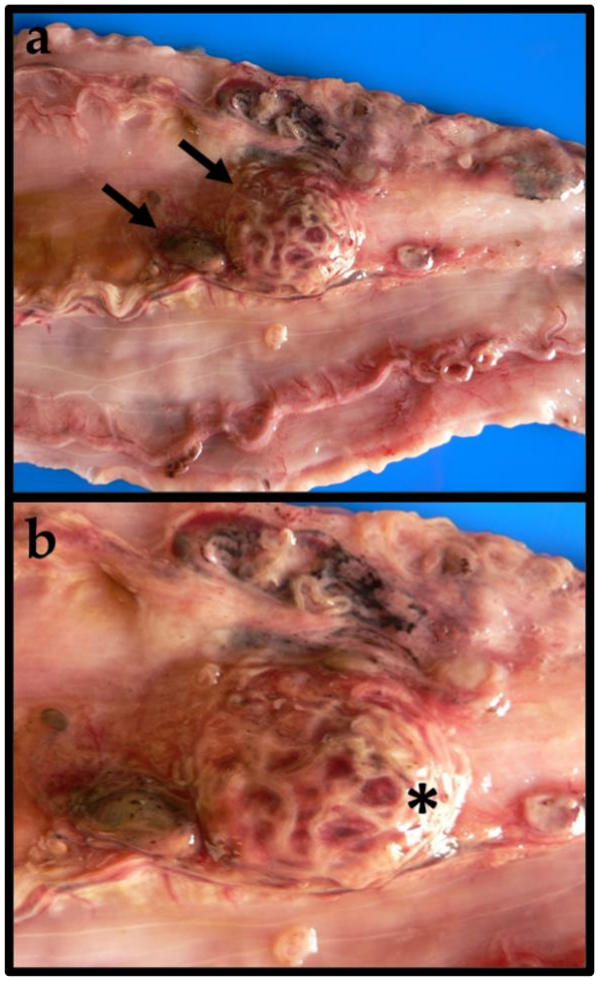

Figure 2. a) Gastric nodular lesions in *Sphyraena viridensis* caused by *Anisakis pegreffii (arrows)*. b) Note the presence of numerous *A. pegreffii* larvae inside the gastric nodule (asterisk).

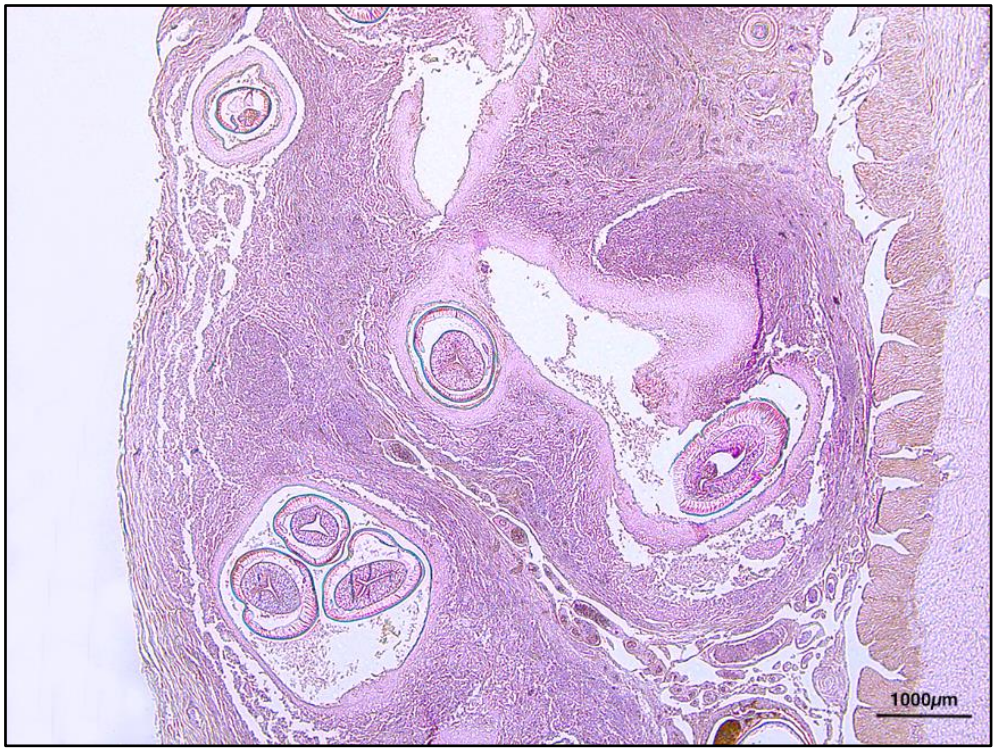

Figure 3. Mucosal granulomatous reaction with nematodes inside H&E 2.5x.

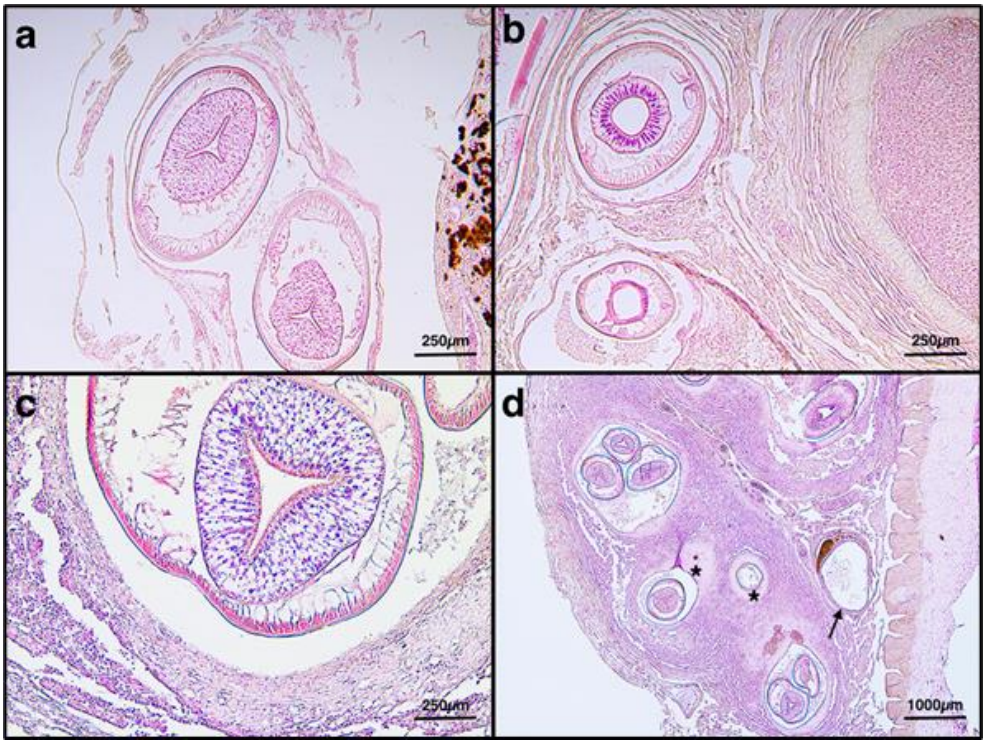

Figure 4. a) Nematode free larval stage found in the serosa H&E 10x. b) Encysted nematode stage H&E 10x. c) Early granuloma stage with nematodes inside a thin capsule of macrophages and epithelioid cells H&E 10x. d) intermediate (arrow) and mature (asterisks) granuloma stages with thick capsule surrounded by a fibrous reaction H&E 2.5x.

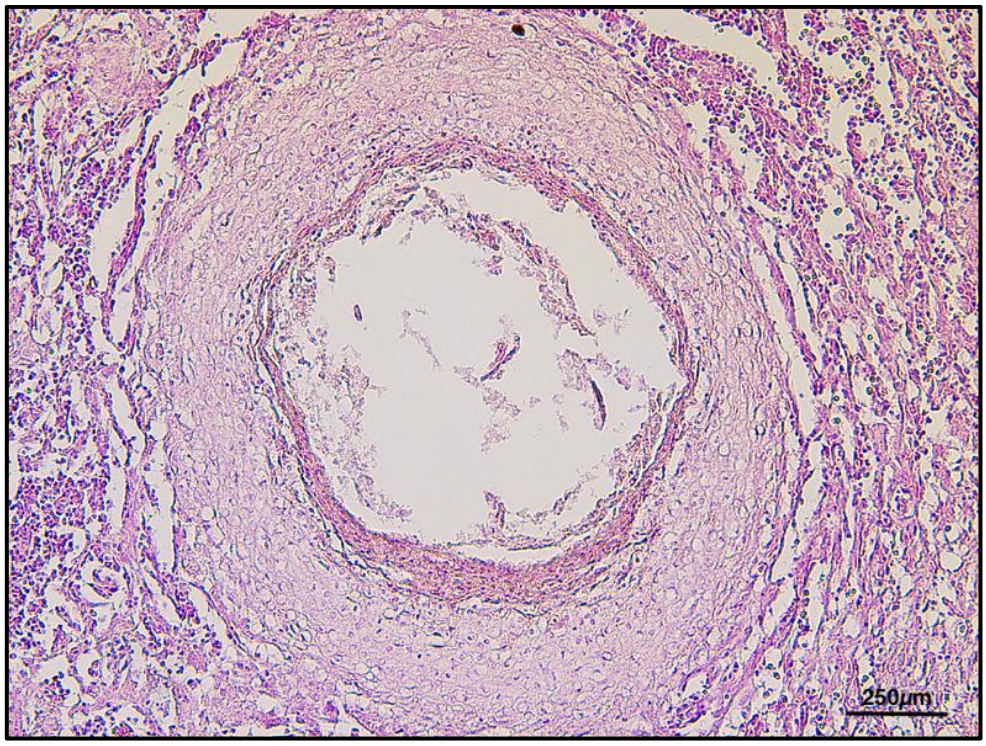

Figure 5. Thick granuloma reaction without parasite inside H&E10x.

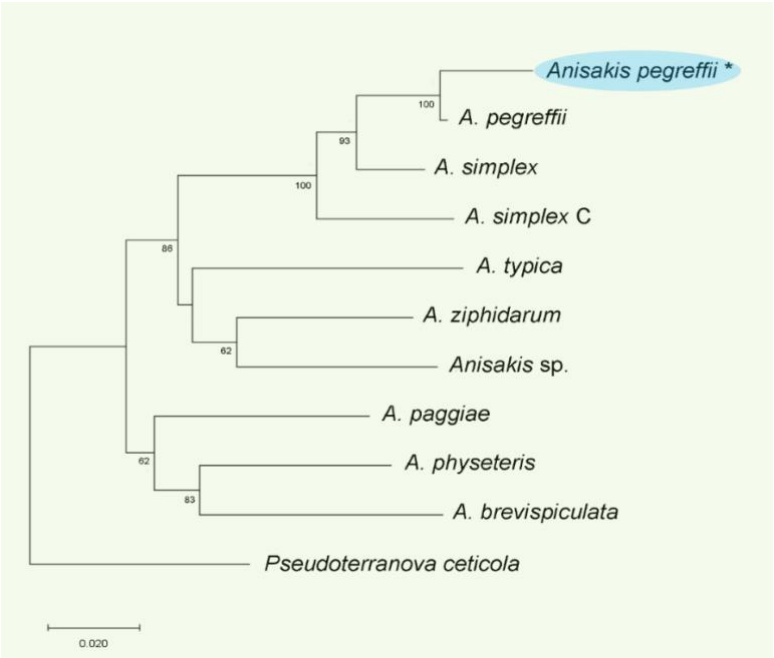

Figure 5. Phylogenetic relationships among *Anisakis* species as inferred by Neighbour-Joining (NJ) analysis of *cox2* gene. The accession numbers are as follows: *A. simplex* (*s. s*.) (DQ116426), *A. pegreffii* (DQ116428), *A. simplex* C (DQ116429), *A. typica* (DQ116427), *A. ziphidarum* (DQ116430), *A. physeteris* (DQ116432), *A. brevispiculata* (DQ116433), *A. paggiae* (DQ116434) and *Anisakis* sp. (DQ116431)]. *Pseudoterranova ceticola* (DQ116435.1) was used as the outgroup to root the tree. The bootstrap values (1, 000 replicates) are shown at the internal nodes (>70% only).

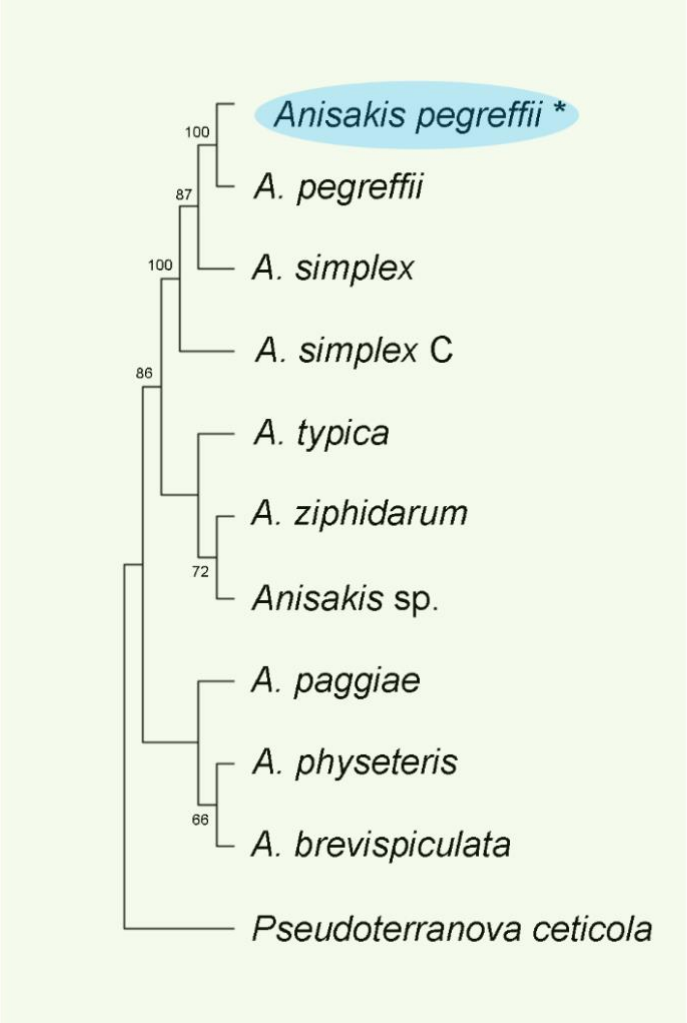

Figure 1. Suppl. Phylogenetic relationships among *Anisakis* species as inferred by maximum-parsimony (MP) analysis of *cox2* gene. The accession numbers are as follows: *A. simplex* (*s. s*.) (DQ116426), *A. pegreffii* (DQ116428), *A. simplex* C (DQ116429), *A. typica* (DQ116427), *A. ziphidarum* (DQ116430), *A. physeteris* (DQ116432), *A. brevispiculata* (DQ116433), *A. paggiae* (DQ116434) and *Anisakis* sp. (DQ116431)]. *Pseudoterranova ceticola* (DQ116435.1) was used as the outgroup to root the tree. The bootstrap values (1, 000 replicates) are shown at the internal nodes (>70% only).

Table 1. List of the primers used in this study.

Gene	Forward primer sequence	Reverse primer sequence	Size (bp)	Reference
18S rRNA	CGCGAATRGCTCATTACAACAGC	GGGCGGTATCTGATCGCC	926	Floyd et al. [25]
cox2	TTTCTAGTTATATAGATTGRTTYAT	CACCAACTCTTAAAATTATC	629	Nadler and Hudspeth [26]

References:

1. Golani, D. B.; Öztöurk, B.; Basusta, N. The fishes of the eastern Mediterranean. *Turkish Marine Research Foundation* **2006**, Publication No. 24, Istanbul, Turkey.
2. Froese, R.; Pauly, D. - FishBase. World Wide Web electronic publication. www.fishbase.org, version (04/2014) (accessed on 23 June 2021).
3. Relini, M.; Orsi Relini, L. The two species of barracuda (Sphyraenidae) in the Western Mediterranean. Cybium **1997**, 21(2): 216-222.
4. Ben-Tuvia, A. (1986) - Sphyraenidae. In: Fishes of the North-east Atlantic and the Mediterranean (FNAM) Vol. III (Whitehead P.J.P., Bauchot M.-L., Hureau J.-C., Nielsen J. & E. Tortonese, eds), pp. 1194-1196. Paris: UNESCO.
5. Bizsel K.C.; Cihangir, B. A new fish record for the Turkish seas: Yellowmouth barracuda (Sphyraenidae: *Sphyraena viridensis* Cuvier, 1829). Turk. J. Zool. **1996**, 20: 357-359.
6. Wirtz, P. Twelve invertebrates and eight fish species new to the marine fauna of Madeira, and a discussion of the zoogeography of the area. *Helgol. Meeres* **1998**, 52: 197-207.
7. Vacchi, M.; Boyer, M.; Bussotti, S.; Guidetti, P.; Lamesa, G. Some interesting species in the coastal fish fauna of Ustica Island (Mediterranean Sea). *Cybium* **1999**, 23(4): 323-331.
8. Santos, R.S.; Porteiro, F.M.; Barreiros, J.P. Marine fishes of the Azores: An annotated checklist and bibliography. *Arquipélago Life Mar.Sci* **1997**, Supp. 1: 1-231.
9. Piras, M.C.; Tedde, T.; Garippa, G.; Virgilio, S.; Sanna, D.; Farjallah, S.; Merella, P. Molecular and epidemiological data on *Anisakis* spp. (Nematoda: Anisakidae) in commercial fish caught off northern Sardinia (western Mediterranean Sea). *Vet Parasitol*. **2014**, 16;203(1-2):237-40.
10. Mattiucci, S.; Nascetti, G. Advances and trends in the molecular systematics of anisakid nematodes, with implications for their evolutionary ecology and host-parasite co-evolutionary processes. *Adv Parasitol* **2008**, 66:47-148
11. Ishikura, H.; Namiki, M. Gastric anisakiasis in Japan: epidemiology, diagnosis, treatment. Springer-Verlag Tokyo **1989**,1989, 144.
12. D'Amelio, S.; Mathiopoulos, K.D.; Brandonisio O.; Lucarelli, G.; Doronzo, F.; Paggi, L. Diagnosis of a case of gastric anisakidosis by PCR-based restriction fragment length polymorphism analysis. *Parasitology* **1999**, 41:591-593.

13. Mattiucci, S.; Paoletti, M.; De Angelis, M.; Sereno, S.; Cancrini, G. Human anisakidosis in Italy: molecular and histological identification of two new cases. *Parassitologia* **2007**, 49:226.
14. Chai, J-Y.; Murrell, K.D.; Lymbery, A.J. Fish-borne parasitic zoonoses: status and issues. *Int. J. Parasitol*. **2005**, 35, 1233–1254.
15. Allen, J.E.; Maizels, R.M. Diversity and dialogue in immunity to helminths. *Nat Rev Immunol.* **2011**, 11:375–88. doi: 10.1038/nri2992
16. EFSA. Scientific opinion of the Panel on Biological Hazards on risk assessment of parasites in fishery products. *EFSA J.* **2010**, 8:10–43. doi: 10.2903/j.efsa.2010.1543.
17. Audicana, M.T.; Ansotegui, I.J.; de Corres, L.F.; Kennedy; M.W. *Anisakis simplex*: dangerous-dead and alive? *Trends Parasitol* **2002**, 18:20-25.
18. Audicana, M.; Kennedy, M.W. *Anisakis simplex*: from obscure infectious worm to inducer of immune hypersensitivity. *Clin Microbiol Rev* **2008**, 21:360-379
19. Van Thiel F.H.; Kuipers, F.C.; Roskam, R.T. A nematode parasitic to herring, causing acute abdominal syndromes in man. *Trop Geogr Med* **1960**, 2:97-113
20. Arthur, J.R.; Albert, E. A survey of the parasites of Greenland halibut (*Reinhardatius hippoglossoides*) caught off Atlantic Canada, with notes on their zoogeography in this fish. Canadian Journal of Zoology **1994**, 72: 765-778
21. Griglio, B.; Marro, S.; Marotta, V.; Testa, A.; Sattanino, G.; Civera, T.; Fazio, G.; Negro, M.; Cravero, M. Anisakidae: valutazione del rischio e indicazioni operative per i controlli ufficiali alla luce de quadro normativo. *AIVEMP Newslett* **2012**, 4, 7–20.
22. Macrì, F.; Lanteri, G.; Rapisarda, G.; Costa, A.; Marino, F. *Anisakis pegreffii* experimental challenge in *Dicentrarchus labrax*: an endoscopic study. *Aquaculture* **2012**, 338–341:297–299.
23. Marino, F.; Lanteri, G.; Passantino, A.; De Stefano, C.; Costa, A.; Gaglio, G.; Macrì F. Experimental susceptibility of gilthead sea bream, *Sparus aurata*, via challenge with *Anisakis pegreffii* larvae. *Biomed Res Int* **2013**, 2013:701828. doi: 10.1155/2013/701828.
24. Briguglio, G.; Di Caro, G.; Napoli, E.; Comignano, F.; Ferrantelli, V.; Gaglio, G.; Lanteri, G. Metazoan parasites of the John Dory *Zeus faber* Linnaeus, 1758 collected from the Mediterranean Sea. *Cah. Biol. Mar.* **2017**, 58. 83-89. 10.21411/CBM.A.4B08B2D9.

25. Arthur, J.R.; Albert, E. A survey of the parasites of Greenland halibut (Reinhardatius hippoglossoides) caught off Atlantic Canada, with notes on their zoogeography in this fish. Canadian Journal of Zoology 1994, 72: 765-778
26. Berland, B. Nematodes from some Norwegian marine fishes. *Sarsia* **1961**, 2:1–50
27. Sonko, P.; Chen, S. C.; Chou, C. M.; Huang, Y. C.; Hsu, S. L.; Barčák, D.; Oros, M.; Fan, C. K. Multidisciplinary approach in study of the zoonotic *Anisakis* larval infection in the blue mackerel (*Scomber australasicus*) and the largehead hairtail (*Trichiurus lepturus*) in Northern Taiwan. *J Microbiol Immunol Infect* **2019,** 53:1021–9.
28. Bush, A.O.; Lafferty, K.D.; Lotz, J.M.; Shostak, A.W. Parasitology Meets Ecology on Its Own Terms: Margolis et al. Revisited. J. Parasitol **1997**, 83; 575-583.
29. Feldman, A.T.; Wolfe, D. Tissue processing and hematoxylin and eosin staining. *Methods Mol Biol.* **2014,** 1180:31-43. doi: 10.1007/978-1-4939-1050-2_3. PMID: 25015141.
30. Floyd, R. M.; Rogers, A. D.; Lambshead, P. J. D.; Smith, C. R. Nematode-specific PCR primers for the 18S small subunit rRNA gene. *Mol. Eco. Notes* **2005**, 5(3), 611–612. doi:10.1111/j.1471-8286.2005.01009.
31. Nadler, S.A.; Hudspeth, D.S. Phylogeny of the Ascaridoidea (Nematoda: Ascaridida) based on three genes and morphology: hypotheses of structural and sequence evolution. *J. Parasitol*. **2000**, 86, 380-393.
32. Valentini, A.; Mattiucci, S.; Bondanelli, P.; Webb, S.C.; Mignucci-Giannone, A.A.; Colom-Llavina, M.M.; Nascetti, G. Genetic relationships among *Anisakis* species (Nematoda: Anisakidae) inferred from mitochondrial cox-2 sequences, and comparison with allozyme data. *J. Parasitol.* **2006**, 92, 156–166
33. Kumar, S.; Stecher, G.; Li, M.; Knyaz, C.; Tamura, K. MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. *Mol Bio and Evol* **2018**, 35:1547-1549.
34. Hills, D. M.; Bull, J. J. An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. *System Bio* **1993**, 42: 182–192.
35. Adroher-Auroux, F.J.; Benítez-Rodríguez, R. Anisakiasis and Anisakis: An underdiagnosed emerging disease and its main etiological agents. Res. Vet. Sci. **2020**, 132, 535–545.
36. Beck, M.; Evans, R.; Feist, S.W.; Stebbing, P.; Longshaw, M.; Harris, E. *Anisakis simplex* sensu lato associated with red vent syndrome in wild adult Atlantic salmon

Salmo salar in England and Wales. *Dis. Aquat. Org.* **2008**, 82, 61–65. https://doi.org/10. 3354/dao01979.

37. Mattiucci. S.; Paoletti, M.; Borrini, F.; Palumbo, M.; Palmieri, R. M.; Gomes, V.; Casati, A.; Nascetti, G. First molecular identification of the zoonotic parasite *Anisakis pegreffii* (Nematoda: Anisakidae) in a paraffin-embedded granuloma taken from a case of human intestinal anisakiasis in Italy. *BMC infect dis* **2011**, 11, 82. https://doi.org/10.1186/1471-2334-11-82
38. Mattiucci, S.; Paoletti, M.; Colantoni, A.; Carbone, A.; Gaeta, R.; Proietti, A.; Frattaroli, S.; Fazii, P.; Bruschi, F.; Nascetti, G. Invasive anisakiasis by the parasite *Anisakis pegreffii* (Nematoda: Anisakidae): diagnosis by real-time PCR hydrolysis probe system and immunoblotting assay. *BMC infect dis* **2017**, 17(1), 530. https://doi.org/10.1186/s12879-017-2633-0
39. Schmale, M.C.; Nairn, R.S.; Winn, R.N. Aquatic Animal Models of Human Disease. *Comp Biochem Physiol C Toxicol Pharmacol* **2007***,* 145(1): 1-4 223–226.
40. Valls Sánchez, A.; Daschner, Á.; De Frutos Moreno, C. Anisakiasis gastroalérgica. *JANO* **2009**, 1727, 19–23.
41. Dezfuli, B.S.; Bosi, G.; DePasquale, J.A.; Manera, M.; Giari, L. Fish innate immunity against intestinal helminths. *Fish Shellfish Immunol* **2016**, 50, 274–287. https://doi.org/ 10.1016/j.fsi.2016.02.002.
42. Serrat, A.; Lloret, J.; Frigola-Tepe, X.; Muñoz, M. Trade-offs between life-history traits in a Coldwater fish in the Mediterranean Sea: the case of blue whiting *Micromesistius poutassou. J. Fish Biol* **2019**, 95, 428–443. https://doi.org/10.1111/jfb. 13993.

General discussion and conclusions:

The data reported in the present thesis have provided additional information on the parasitic fauna of wild fish from the Mediterranean basin. As reported by some authors, parasites used as biological tags are considered an important resource for many reasons, such as the low impact on ecosystems and the possibility of providing clear data on host-parasite interactions.

Chapter 1 provides a description of dusky grouper (*E. marginatus*) parasitic fauna caught from the Ionian coast of the central Mediterranean Sea, an area previously unstudied. *Prosorhynchus caudovatus* and *Podocotyle temensis*, the most retrieved species in the present study, described for the first time in *E.marginatus*, was previously described in other Epinephelidae from different areas of the Mediterranean Sea (Bartoli et al., 2005; Bray & Justine, 2013; Fischthal & Thomas, 1968), characterized by different environmental characteristics. The presence of these species in the studied area better explains a parasite movement and adaptation between areas which is not exactly superimposable regarding environmental parameters. Moreover, the nematode *Philometra jordanoi* represents an important retrieved parasite in *E. marginatus*. While previously reported by Merella et al. (2005) in dusky grouper caught from different area of the central Mediterranean Sea (FAO 37.1.3), this finding represents the first report in the examined area (FAO 37.2.2).

The Didymozoidae extracts from *E. marginatus* gills are macroscopically identical to those described by Polinas et al (2018), but being present in all the seas worldwide, are not easily identifiable with accuracy, thus rendering them less useful as biological tags. For this reason, a more precise description is better reported in Chapter 2.

One of the other techniques used to identify fish stocks is the evaluation of their diet, albeit not as accurate as the study of the parasite fauna. In fact, in this study, possible differences between parasite fauna and fish diet have been reported, considering also the relation between the size of the host and their prey, confirming previously reported data (Reñones et al., 2002; Polinas et al., 2018).

In **Chapter 2**, a clear morphological and molecular identification of a new digenean trematode species was performed. Nowadays, to optimize the use of parasites as biological tags, given the global distribution of genus, it is necessary to proceed with an accurate description of the species, also proposing clear identification keys, useful in daily diagnostic activity.

The morphological differences reported in different parts of the world (Yamaguti, 1970; Pozdnyakov, 1993 Pozdnyakov & Gibson, 2008; Mladineo et al., 2010), considered characteristics of species, surely help to describe new parasite species, strictly related to a

specific host. Indeed, all the Didymozoid species previously described (Gu & Shen 1983; Madhavi & Murugesh, 1994; Yamaguti, 1971; Abdul-Salam et al., 1990; Cruz-Lacierda et al., 2001), were characterized by the specific host, sampled from a specific geographic area. This approach is certainly to be considered important in guest evaluations. Molecular evaluation, despite higher costs, is considered the most accurate technique for species differentiation; in association with morphological evaluation, it provides a clear, complete description of a new parasite species. For this reason, the performed molecular analysis allowed to clarify the genetic differences between didymozoid parasites previously described in the Indian and Pacific Oceans (Olson et al., 2003; Vidal-Martínez et al., 2019) and the new species described in the present chapter.

Chapter 3 better explains the importance of interactions between host and parasite. Some authors reported the importance of using zoonotic agent parasites as biological tags (Kotoulas et al., 1995; Marcogliese, 2005; Mattiucci et al., 2005; 2014; Garcia et al., 2011; Llarena-Reino et al., 2019). In this section, the host reaction against *Anisakis pegreffi* larvae was reported, focusing on a comparison with the same reaction in humans. As previously reported (Mattiucci et al., 2014) and explained in Chapter 2, the association between the morphological and molecular evaluation allowed to clearly identify the parasite species involved in the described case. Indeed, the molecular identification of *A. pegreffii* larvae in *Sphyrena viridensis* from the central Mediterranean Sea confirms the data previously reported by Piras et al. (2014). Another important data was the first description of a granulomatous reaction by *A. pegreffii* larvae in *S. viridensis*.

In conclusion, the data reported in this thesis improve the current knowledge on parasitic fauna in teleosts which are significantly appreciated by consumers. The usefulness of the association between morphological and molecular description allows to improve data and supports studies on the use of fish parasitic fauna used as biological tags.

References:

Abdul-Salam J, Sreelatha B and Farah M (1990) *Gonapodasmius epinepheli* n. sp. (Didymozoidae) from the grouper *Epinephelus tauvina* from the Arabian Gulf. Systematic Parasitology 17, 67–74.

Bartoli, P.; Bray, R.A.; Gibson, D.I. Opecoelidae (Digenea) from western Mediterranean fishes: Three rare species. Syst. Parasitol. 2003, 55, 81–95.

Bray, R.A.; Justine, J.L. Bucephalidae (Digenea) from epinephelines (Serranidae: Perciformes) from the waters off New Caledonia, including *Neidhartia lochepintade* n. sp. Parasite 2013, 20, 56.

Cruz-Lacierda ER, Lester RJG, Eusebio PS, Marcial HS and Pedrajas SAG (2001) Occurrence and histopathogenesis of a didymozoid trematode *Gonapodasmius epinepheli* in pond-rearedorange-spotted grouper, *Epinephelus coioides*. Aquaculture 201, 211–217.

Fischthal, J.H.; Thomas, J.D. Digenetic trematodes of marine fishes from Ghana: Families Acanthocolpidae, Bucephalidae, Didymozoidae. Proc. Helminthol. Soc. Wash. 1968, 35, 237–247.

De la Serna JM, Alot E, Godoy MD. 1992. Analysis preliminar de la madurez sexual del pez espada (*Xiphias gladius*) en el area Atlantica proxima al estrecho de Gibraltar. *ICCAT Collective Volume of Scientific Papers*, 39, 522–537.

Garcia A, Cecchetti S, Santos MN, Mattiucci S, Nascetti G, Cimmaruta R. 2011. Population structure of Atlantic Swordfish *(Xiphias gladius* L. 1758) (Teleostea, Xiphiidae) using mitochondrial DNA analysis: implications for fisheries management. *Animal Biodiversity and Conservation*, 34, 133–140.

Gu CD and Shen JW (1983) Four new species of didymozoid trematodes from China. Acta Zootaxonomica Sinica 8, 17–23.

Kotoulas G, Magoulas A, Tsimenides N, Zouros E. 1995. Marked mitochondrial DNA differences between Mediterranean and Atlantic populations of the swordfish, *Xiphias gladius*. *Molecular Ecology*, 4, 473–481.

Madhavi R and Murugesh M (1994) Gonapodasmid didymozoids (Digenea: Didymozoidae) from serranid fishes of the Visakhapatnam Coast, Bay of Bengal. Rivista di Parassitologia 55, 47–55.

Marcogliese, D.J. (2005). Parasites of the superorganism: are they indicators of ecosystem health? *International Journal for Parasitology* 35, n.7: 705-716.

Marino, F.; Busalacchi, B.; Bottari, T.; Rinelli, P.; Gaglio, G. Occurrence and prevalence of *Philometra filiformis* (Stossich, 1896) on *Pagellus erythrinus* (Linnaeus, 1758) in the southern Tyrrhenian Sea. J. Appl. Ichthyol. 2016, 32, 687–692.

Mattiucci, S., Farina, V., Garcia, A., Santos, M. N., Mariniello, L., Nascetti, G. 2005. Metazoan parasitic infections of swordfish (*Xiphias gladius* L., 1758) from the Mediterranean Sea and Atlantic Gibraltar waters: implications for stock assessment. *Col. Vol. Sci. Pap. ICCAT*, 58 (4), 1470–1482.

Mattiucci S, Garcia A, Cipriani P, Santos MN, Nascetti G & Cimmaruta R. Metazoan parasite infection in the swordfish, *Xiphias gladius*, from the Mediterranean Sea and comparison with Atlantic populations: implications for its stock characterization. *Parasite*, 2014, 21, 35.

Mladineo I, Bott NJ, Nowak BF and Block BA (2010) Multilocus phylogenetic analyses reveal that habitat selection drives the speciation of Didymozoidae (Digenea) parasitizing Pacific and Atlantic bluefin tunas. Parasitology 137, 1013–1025.

Olson PD, Cribb TH, Tkach VV, Bray RA and Littlewood DTJ (2003) Phylogeny and classification of the Digenea (Platyhelminthes: Trematoda). International Journal for Parasitology 33, 733–755.

Piras, M.C.; Tedde, T.; Garippa, G.; Virgilio, S.; Sanna, D.; Farjallah, S.; Merella, P. Molecular and epidemiological data on *Anisakis* spp. (Nematoda: Anisakidae) in commercial fish caught off northern Sardinia (western Mediterranean Sea). *Vet Parasitol*. 2014, 16;203(1-2):237-40.

Polinas, M.; Mele, S.; Padrós, F.; Merella, P.; Antuofermo, E.; Gouraguine, A.; Reñones, O. Ecological and histopathological aspects of *Didymodiclinus* sp. (Trematoda: Didymozoidae) parasite of the dusky grouper, *Epinephelus marginatus* (Osteichthyes: Serranidae), from the western Mediterranean Sea. J. Fish. Dis. 2018, 41, 1385–1393.

Pozdnyakov SE (1993) The systematics of trematodes of the suborder Didymozoata. Parazitologiya 27, 415–425.

Pozdnyakov SE and Gibson DI (2008) Family Didymozoidae Monticelli, 1888. In Bray RA, Gibson DI and Jones A (eds), Keys to the Trematoda. Wallingford, UK: CABI Publishing and the Natural History Museum, pp. 631–734.

Reñones, O.; Polunin, N.V.C.; Goni, R. Size related shifts of *Epinephelus marginatus* in a western Mediterranean littoral ecosystem: An isotope and stomach content analysis. J. Fish. Biol. 2002, 61, 122–137.

Vidal-Martínez VM, Velázquez-Abunader I, Centeno-Chalé OA, May-Tec AL, Soler-Jiménez LC, Pech D, Mariño-Tapia I, Enriquez C, Zapata-Pérez O, Herrera-Silveira J, Hernández-Mena DI, Herzka SZ, Ordoñez-López U and Leopoldina A-MM (2019) Metazoan parasite infracommunities of the dusky flounder (*Syacium papillosum*) as bioindicators of environmental conditions in the continental shelf of the Yucatan Peninsula, Mexico. Parasites & Vectors 12(1), 381.

Yamaguti S (1970) Digenetic Trematodes of Hawaiian Fishes. Tokyo, Japan: Keigaku Publishing Co.

Yamaguti S (1971) Synopsis of Digenetic Trematodes of Vertebrates, vols I and II. Tokyo, Japan: Keigaku Publishing Co., pp. 247–277