New Parasite Records for the Sunfish *Mola mola* in the Mediterranean Sea and Their Potential Use as Biological Tags for Long-Distance Host Migration

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Studies describing the parasite fauna of sunfish species from the Mediterranean Sea are to date limited, despite information gained through parasitological examination may reveal unknown ecological and biological aspects of both hosts and parasites. Moreover, recent molecular studies on sunfish taxonomy revealed the presence of two species belonging to the genus *Mola* in the Mediterranean basin, namely *M. mola* and *M. alexandrini*. These two fish taxa have long been synonymized or confused among them, which implies that the majority of the studies carried out so far reported the parasites infecting both species under a single host species, generally referred to as *M. mola*. We hereby investigated the parasite fauna of a 43 cm long *M. mola* specimen from the Mediterranean Sea, whose identification was confirmed by molecular tool, and provided the first evidence of the occurrence of the nematode *Anisakis simplex* (s.s.) and of the cestode *Gymnorhynchus isuri* in *Mola* species anywhere. The use of helminth species as biological tags for the sunfish is also discussed.

Keywords: *Anisakis simplex* (s.s.), Molidae fishes, Tyrrhenian Sea, *Gymnorhynchus isuri*, metazoan parasites, Trypanorhyncha

INTRODUCTION

The sunfish genus *Mola* Koelreuter, 1766 has been considered, until recently, as composed of two species, namely *Mola mola* (Linnaeus, 1758), with a cosmopolitan distribution, and *Mola ramsayi* (Giglioli, 1883), restricted to the southern hemisphere (1). In the past few years, the use of molecular approaches revealed the presence of three valid species within this genus: *Mola alexandrini* (Ranzani, 1839) (syn. *M. ramsayi*) and *M. mola*, with a wide and sympatric distribution in the world’s oceans, including the Mediterranean Sea, and *Mola tecta* Nyegaard, Sawai, Gemmell, Gillum, Loneragan, Yamanoue and Stewart, 2017, mostly found from temperate waters of southern hemisphere (2–4).

*Mola* species are well-known to host a variegate parasite fauna, and in particular several papers focused in the past on the parasite fauna of “*M. mola*,” whose information was reviewed by de Figueiredo et al. (5). However, since the clarification of the worldwide taxonomy of the genus *Mola*,...
only Ahuir-Baraja et al. (6) investigated the endo-parasites of a *Mola* individual stranded on the Valencian coast (western Mediterranean Sea), identified by molecular tools as *M. alexandrini*. This likely implies that the majority of the studies carried out so far reported the parasites infecting the entire genus under the single species *M. mola* [see (5)].

Host specificity is often considered to be a result of various factors, including phylogenetic, physiological, and ecological aspects (7, 8). Since most marine parasites exhibit at least some degree of host-specificity or host-preference, it is plausible that a degree of host-specificity may exist for parasites infecting fishes of the genus *Mola*. Moreover, among the several methods applied to obtain biological and ecological data of fishes, the use of parasites as biological tags in marine environment has become a useful tool in producing data for the host stock identification and for studying their migration by using molecular tools to unequivocally identify both hosts and their parasites (9–12). In fact, parasites can be used as biological tags since their geographic ranges are definite, so that a host can only become infected when entering the parasite endemic range (9, 10).

We here investigated the whole parasite fauna of a *M. mola* specimen obtained from the Gulf of Naples (Tyrrhenian Sea) whose identification was confirmed through molecular analysis. We provide the first evidence in *Mola* species of the occurrence of larval forms of two helminth parasites typically known from the Atlantic Ocean, suggesting a potential long-distance migration of the examined host fish.

**MATERIALS AND METHODS**

**Sampling**

A juvenile male *Mola* specimen (4,190 kg in weight; 43 cm in total length) was fished at ~50 m depth on January 3, 2020 off Ischia Porto (~40.753766, 13.947723, Ischia Island, central Tyrrhenian Sea, Mediterranean) by an amateur fisherman, using a fishing line with the Mediterranean mussel (*Mytilus galloprovincialis* Lamarck, 1819) as bait. Once the fish was landed, it was first shown to the public and then discarded, being considered as bycatch. As one of the authors (FC) was there at that moment, and the specimen was already dead, it was collected by him and soon after frozen at −20°C for molecular and parasitological analyses.

**DNA Extraction and Molecular Analysis for Host Identification**

Total genomic DNA was extracted from a defrosted muscle sample, and a partial sequence of the cytochrome c oxidase subunit I (COX1) mitochondrial gene (mtDNA cox1) was amplified and sequenced following methods used in Osca et al. (13).

The sequence obtained was assembled using Sequencher v. 5.0.1 (GeneCodes Co.) and compared with reference sequences from the NCBI nucleotide (NT) database using BLASTn (14). Partial sequences of further *Mola* specimens were downloaded from GenBank and BOLD, together with those of *Masturus lanceolatus* (Liénard, 1840) to be used as outgroup (2). Nucleotide sequences were aligned with the Translator X server (15), using the MAFFT v7 (16) option with default settings. By subsequently deleting identical sequences, a total of 12 sequences of *Mola* specimens and of five of *M. lanceolatus* were used for the phylogenetic analyses (Supplementary Table 1).

Alignment format conversions were performed using the ALTER webserver (17). The best-fit model of substitution was determined using the Akaike information criterion (AIC) (18) implemented in PartitionFinder v.1.1.1 (19). Phylogenetic relationships were inferred performing maximum likelihood (ML) analyses in RAxML v.8.1.16 (20), using the rapid hill-climbing algorithm, and Bayesian inference (BI) analyses in MrBayes v.3.1.2 (21), running four simultaneous MCMC (Markov chain Monte Carlo) for 2 million generations, a sampling interval every 1,000 generations, and a burn-in of 25%. Two independent Bayesian inference runs were performed to increase the chance of adequate mixing of the Markov chains and of detecting failure to converge. Support for internal branches was evaluated by non-parametric bootstrapping (22) with 1,000 replicates (ML) and by posterior probabilities (BI). Finally, based on the results of the phylogenetic trees, diagnostic nucleotides were identified in our alignment as to further confirm the molecular identification of the specimen studied here.

**Parasitological Analysis**

During the fish necropsy, skin, musculature, gills, mouth cavity, digestive tract, liver, heart, testes, visceral cavity, and mesenteries of the sunfish were examined for metazoan parasites. Organs and tissues were removed and placed individually in plastic Petri dishes (200 mm in diameter); the organs were then dissected and the surfaces were examined visually. After the larger helminths were removed using tweezers, organs and tissues were washed through a 100 µm mesh screen. The remaining washed material from each organ was examined under a dissecting microscope (Leica M165 C), and parasites were collected, counted, washed in physiological saline, and preserved in 70% ethanol or frozen at −20°C (23). For identification, crustacean parasites and nematodes were clarified respectively in 20% potassium hydroxide and Amman's lactophenol, and then returned to 70% ethanol; digeneans and cestodes were stained with Mayer's acid carmine and mounted in Canada balsam. Parasites were studied with a compound microscope (Leica DM1000) and identified using published identification keys (24–27).

**DNA Extraction and Molecular Analysis for Selected Larval Parasites**

Total genomic DNA from a cestode and a nematode larva whose morphological identification at species level proved to be uncertain, was extracted using Quick-gDNA Miniprep Kit (ZYMO RESEARCH) following the standard manufacturer-recommended protocol. DNA was quantified by a NanoDrop® TC1-E20 spectrophotometer (BioTek Synergy HT).

A cestode larva morphologically considered as belonging to the family Gymnorhynchidae Dollfus, 1935 (Trypanorhynchida) was identified to species level by sequence analysis of the complete small subunit of the ribosomal RNA gene (ssrDNA) (28) and the partial large subunit ribosomal gene (lsrDNA) (29). Complete ssrDNA was amplified using the primers...
Molecular and Phylogenetic Analyses for Host Identification

A 606 bp partial sequence of the mtDNA cox1 gene was obtained for the fish specimen. The ML (\(-\text{lnL} = 1322.1\)) and BI (\(-\text{lnL} = 1356.15\) for run 1; \(-\text{lnL} = 1353.81\) for run 2) analyses arrived at similar tree topologies. Two major clades were defined with high support for both analyses (ML and BI), in which *M. tecta* and *M. alexandrini* were placed as sister groups. The other clade, with high support for ML and maximal support for BI, included sequences of *M. mola*. The latter clade also included the sequence of the specimen studied here (Supplementary Figure 1). The analysis of the positions of diagnostic nucleotides in our alignment revealed that *M. tecta* is diagnosed by 45 (G), 474 (A), 558 (G), and 576 (T), *M. alexandrini* is diagnosed by 168 (G), 240 (A), 291 (T), 516 (A), 597 (A), and 603 (G), and *M. mola* is diagnosed by 15 (A), 150 (T), 172 (G), 186 (C), 219 (C), 225 (A), 339 (T), 387 (C), 426 (G), 429 (C), 450 (G), 456 (A), 465 (G), 498 (A), 540 (G), 543 (T), 579 (T), and 606 (T). No differences with respect to present results were obtained when analyzing the unpublished cox1 sequence of *M. mola* obtained here. The sequence was deposited in GenBank under the accession number MT913440.

Parasitological Analysis

A total of 46 metazoan parasites were found, belonging to 10 different species. These included eight helminth taxa (one monogenean, three digeneans, three cestodes, and one nematode) and two copepods (Table 1). All parasites were found at adult stage, except for the larval stages of the cestodes *H. trichiuri* and *Gymnorchynchus isuri* Robinson, 1959 (Figure 1) and the nematode *Anisakis simplex* (s.s.) (Rudolphi, 1809) (see below for the molecular identification of the selected larval parasites). Among the ectoparasites, the monogenean *Capsala martinieri* Bosc, 1811 and the copepods *Cecrops latreillii* Leach, 1816 and *Lepeophtheirus nordmanni* (Milne Edwards, 1836) were among those found on gills and skin.

Molecular Analysis for Selected Larval Parasites

According to the obtained sequences at the complete small subunit (ssrDNA) and the partial large subunit (1srDNA) ribosomal RNA gene, a larva was identified as *G. isuri*. The 1srDNA sequences (1,300 bp) and the ssrDNA sequences (1,968 bp) matched at 99–100% with the sequences of *G. isuri* from GenBank (25).

The BI obtained from the combined 1srDNA and the ssrDNA sequences dataset showed that the larva of *G. isuri* clustered together with the reference sequence of *G. isuri* from GenBank (25) (Figure 2). The obtained 1srDNA and ssrDNA sequences were deposited in GenBank under the accession numbers MT667258 and MT667257, respectively.

According to the sequences of 629 bp in length of the mtDNA cox2 gene locus, the *Anisakis* type 1 larva was assigned to the species *A. simplex* (s.s.). The obtained sequence matched 100% with the *A. simplex* (s.s.) sequences from GenBank. The obtained cox2 sequence was deposited in GenBank under the accession number MT667259.

DISCUSSION

*Mola mola* is usually considered a highly migratory fish, characterized by daily vertical movements following zooplankton preys and generally jellyfishes (1). Its horizontal movements are linked to seasonal variations of sea surface temperature and forage availability, with migrations shown to exceed 1,000 km in the northeast and northwest Atlantic (1, 35, 36). Recent studies suggest an ontogenetic shift in diet with age, with smaller sunfishes feeding on benthic invertebrates and larger individuals feeding on gelatinous zooplankton occurring in the water column (37–39). The present finding agrees with these statements, as our juvenile sunfish specimen (43 cm total length, corresponding to ca. 15-months of age according to captive growth rates for *M. mola*) [see (40)] was collected with a fishing line near the seabed, thus suggesting that the sunfish was foraging in the benthic zone.

The ecto-parasites found in the specimen from off Ischia Island were considered all *Mola* specialists with a direct life
TABLE 1 | Metazoan parasites found in a sunfish Mola mola from off Ischia Island (Tyrrhenian Sea, Mediterranean Sea), with number of individuals (N), developmental stage (DS), location in the host (L), life cycle type (LC), and known definitive host/s (DH).

| Parasites                  | N    | DS       | L       | LC        | DH                  |
|----------------------------|------|----------|---------|-----------|---------------------|
| **Copepoda**               |      |          |         |           |                     |
| Cecrops latreilli Leach, 1816 | 12   | Adult    | Gills   | Direct    | Molidae fishes      |
| Lepeophtheirus norhmanni (Milne Edwards, 1836) | 7    | Adult    | Skin    | Direct    | Molidae fishes      |
| **Monogenea**              |      |          |         |           |                     |
| Capsala martinieri Bosc, 1811 | 2    | Adult    | Skin    | Direct    | Molidae fishes      |
| **Digenea**                |      |          |         |           |                     |
| Accacladocoelium macracotyle (Diesing, 1858) Robinson, 1934 | 2    | Adult    | Intestine | Indirect | Molidae fishes      |
| Accacladocoelium nigroflavum (Rudolphi, 1819) Robinson, 1934 | 1    | Adult    | Intestine | Indirect | Mola spp.           |
| Accacoelium contortum (Rudolphi, 1819) Looss, 1899 | 6    | Adult    | Gills, rectum | Indirect | Molidae fishes      |
| **Cestoda**                |      |          |         |           |                     |
| Anchistrocephalus microcephalus (Rudolphi, 1819) | 5    | Adult    | Intestine | Indirect | Molidae fishes      |
| Hepatoxylon trichiuri (Holten, 1802) Bosc, 1811 | 1    | Plerocercoid larva | Body cavity | Indirect | Pelagic sharks      |
| Gymnorhynchus isuri Robinson, 1959 | 9    | Plerocercoid larva | Liver | Indirect | Pelagic sharks      |
| **Nematoda**               |      |          |         |           |                     |
| Anisakis simplex (s.s.) (Rudolphi, 1809) | 1    | Larva type I | Intestinal serosa | Indirect | Cetaceans           |

FIGURE 1 | Gymnorhynchus isuri infection in the liver of the sunfish (M. mola). Several white larvae are embedded in the liver (A); the arrow in (A) indicates the blastocyst of a G. isuri larva enlarged in (B) after disruption of the hepatic tissue. Bar scale: (A): 2 cm; (B): 100 μm.

parasites, except G. isuri and A. simplex (s.s.), were previously found in Mola species from the Mediterranean Sea (6, 41, 42).

In the present study, we have identified two parasite species [G. isuri and A. simplex (s.s.)] that potentially could be used as biological tags, having both some features able to producing data for fish stock identification and for a better understanding of the host migration routes [see (9–11)].

Nematodes of the genus Anisakis infect a wide range of marine hosts (with cetaceans as definitive hosts) including at least 12 species of dolphins, porpoises and whales (43). Small crustaceans (Euphausiidae), fishes, squids, and other invertebrates serve as first and second intermediate or paratenic hosts (43, 44). Anisakis larvae have been previously reported in Mola species from Spain, New Zealand, and Chile (5, 6, 42), although no molecular analyses were performed to identify those larvae to species level. Anisakis simplex (s.s.) is known from the Atlantic and Pacific Oceans, and has its southern limit of distribution in the North-Eastern Atlantic waters along the Spanish-Portuguese Atlantic coast, being never recorded in the Mediterranean Sea except from the Alboran Sea, a transitional zone between Atlantic and Mediterranean [see (43)], and in pelagic fishes like the Atlantic mackerel Scomber scombrus Linnaeus, 1758 and the Atlantic bluefin tuna Thunnus thynnus Linnaeus, 1758 in the Eastern Mediterranean (45, 46); however, it has been suggested that the latter findings were related to the large migratory routes of those intermediate/paratenic fish hosts (45, 46).

Trypanorhynchan cestodes use copepods as first and other invertebrates or small fishes as second intermediate hosts (47, 48). Species of the genera Gymnorhynchus occur as adults in pelagic sharks, with the larval stages infecting a wide range of teleosts and sharks (47, 48). The genus Gymnorhynchus includes two species, namely G. gigas and G. isuri (48). To the best of our knowledge, only G. gigas has to date been reported from the Mediterranean basin in the swordfish Xiphias gladius Linnaeus, 1758, the Ray’s bream Brama brama (Bonnaterre, 1788), and

cycle, and have been found through the whole geographical range of their hosts, while the endo-parasites were all trophically transmitted with a heteroxenous life cycle (6, 24–27, 41, 42).
the silver scabbardfish *Lepidopus caudatus* (Euphrasen, 1788) (49, 50). *Gymnorhynchus isuri* is known from the north, southwestern, and northeastern Atlantic and the Tasmanian Sea in the southwestern Pacific, infecting the shortfin mako *Isurus oxyrinchus* Rafinesque, 1810 and the blue shark *Prionace glauca* (Linnaeus, 1758) (30, 51, 52), and no record exists of *G. isuri* in *Mola* species anywhere.

A possible scenario seems to suggest that the sunfish specimen here analyzed had migrated from Atlantic Ocean waters into the Mediterranean Sea or at least from Alboran Sea waters. This hypothesis is based on the finding of parasite species typically known from Atlantic waters. No data exist on migration in juvenile *M. mola*, nor on Molidae in general within the Mediterranean Sea or from the Atlantic Ocean to the Mediterranean and *vice versa*, except for a study showing movements from the Atlantic Iberian coast to the Alboran Sea (36). Published information on the parasite fauna of the target fish species highlighted assemblages characterized by species with wide geographic range. This suggests the hypothesis that host migration route may be responsible for the parasite range expansion, as suggested for other marine vertebrates, that migrate from the Atlantic to the Mediterranean sea waters and *vice versa* (11, 45, 46, 53).

Among trypanorhynchans, *Molicola horridus* (Goodsir, 1841) Dollfus, 1935 (Gymnorhynchidae) is often reported infecting the liver of *Mola* species (6, 41, 42). *Molicola* is closely related to *Gymnorhynchus* having a similar scolex morphology, surface ultrastructure, and tentacular armature, and members of both genera also cause similar pathological changes; however, whereas *M. horridus* is considered a specific parasite of *Mola* species, *G. isuri* is known from bradid fishes (48). As the plerocerci of these two genera are difficult to be differentiated by morphology only (encysted forms are often obtained damaged, dead and with tentacles retracted), there is the possibility that the two species were often mixed up or misidentified in the past literature. In fact, the most important diagnostic feature between the two genera is the arrangement of hooks on the external surface of tentacular armature, that is impossible to study in encysted dead larval stages (48). Indeed, in recent years, molecular tools for recognition of members of Gymnorhynchidae was successfully applied (30).

Regarding to the other helminths identified in the present study, the cestodes *H. trichiuri* (Sphyriocephalidae) and *A. microcephalus* (Triaenophoridae) parasitize as adults the intestine of pelagic sharks and Molidae fishes respectively (5, 6, 26, 41, 42, 47, 48). It is believed that copepods can serve as intermediate hosts for both cestode species (48, 54). Digeneans of the genera *Accacoelium*, and *Acca cladocoe lium* (Accacoeliidae) are specific parasites of Molidae fishes, that acquire the infection when feeding on nektomic organisms, and especially on cnidarians and ctenophores infected by accacoelid metacercariae (24, 55).

In conclusion, traditional parasitological studies implemented by molecular tools are still needed to understand if host specificity...
exists for most of the parasites infecting *Mola* species. However, despite we have here analyzed only a single *M. mola* specimen, we have showed that this sunfish species serves as an intermediate host for two cestodes (*H. trichiuri* and *G. isuri*) and as a definitive host for the remaining parasites except *A. simplex* (s.s.), considered here as an accidental finding. In fact, due to low prevalence and intensity of infection or absence of this nematode larvae, as also reported in other studies from Mediterranean (6, 41, 42), it is reasonable to conclude that this fish ecologically likely represents a dead end for the parasite (56). For the reasons listed above, the potential use of *A. simplex* (s.s.) as biological tag for sunfishes remains doubtful. In contrast, the cestode *G. isuri* has a useful potential to be used as biological indicator and could be used to study the sunfish movements at least in the Mediterranean and adjacent areas. However, despite the present study reveals for the first time the occurrence of *G. isuri* in the Mediterranean Sea and indicates *M. mola* as a new intermediate host record for this cestode species, a molecular screening of specimen morphologically identified in the past literature and in museum collections as *M. horridus* could confirm if the present finding of *G. isuri* in the Mediterranean is a new or casual event or the species is already widespread, but undetected and/or misidentified, in the basin.

**DATA AVAILABILITY STATEMENT**

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

**ETHICS STATEMENT**

Ethical review and approval was not required for the animal study because the sunfish was collected during amateur fishing with no additional experimental catches being performed. According to Italian law DL16/92 and European directive 2010/63/EU, this study did not require a specific permit. Procedures for this study were performed in accordance with the guide for the care and use of animals by the Italian Ministry of Health.

**AUTHOR CONTRIBUTIONS**

MS: performed fish necropsy, collection, morphological identification of parasites, and wrote the paper. SM and MP: performed molecular and phylogenetic analyses of selected larval stages of parasites. DO and FC: performed molecular and phylogenetic analyses of the sunfish. All authors contributed to manuscript revision, read, and approved the submitted version.

**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.579728/full#supplementary-material

**Supplementary Figure 1 | Phylogenetic relationships in the genus Mola based on mtDNA *cox*1 partial sequences downloaded from GenBank and BOLD (ID codes as in the Supplementary Table 1), and the specimen from off Ischia Island marked bold. Numbers above/below branches represent bootstrap values (ML) and posterior probabilities values (BI). Scale bar represents nucleotide substitution. Poor support values (<50%, 0.90) are not shown. **The putative misidentification (see Supplementary Table 1) is based on an incorrect entry in GenBank [see (2), where the same specimen is labeled as Mola sp. A = Mola alexandrini].

**Supplementary Table 1 | GenBank and Barcode of Life Data System mtDNA *cox*1 partial sequences used in the molecular systematic analyses and associated specimen data (locality obtained from web databases or relevant paper/s). 'The specimen from Ischia Island (central Tyrrhenian Sea, Mediterranean) analysed in the present study. **The putative misidentification is based on an incorrect entry in GenBank (where the same specimen is labelled *Mola* sp. A = *Mola alexandrini*).

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

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