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## Morphological and molecular characterization of two gastrointestinal parasites in *Mustelus mustelus* (Linnaeus, 1758) from Adriatic Sea

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### Abstract

Specimens of common smooth-hound, *Mustelus mustelus*, fished as bycatch in the Northwestern Adriatic Sea, were subjected to a complete parasitological examination. Parasites were processed for morphological and morphometric analyses aimed at identifying them to the species level. Parasite identity was confirmed through amplification and sequencing of 28S rDNA. The nematode *Acanthocheilus rotundatus* was recovered from the stomach and the proximal intestine, while the cestode *Calliobothrium verticillatum* was collected from the spiral valve. Our results confirm the occurrence of *C. verticillatum* in *M. mustelus* from the Mediterranean Sea and provide the first sequence data of *A. rotundatus* from the same area. Updated knowledge on the parasite fauna of *M. mustelus* from Adriatic Sea contributes to elucidating aspects of the ecology, biology and health status of elasmobranchs from the Mediterranean Sea, providing useful information for their proper management and conservation.

**Keywords:** *Helminths, parasites, Mustelus mustelus, sharks, Mediterranean Sea*

### Introduction

The correct management of elasmobranch populations for conservation purposes requires the acquisition of detailed information concerning their biology and ecology. Currently, in the Mediterranean Sea, important gaps of knowledge occur even for the most studied elasmobranch species.

Sharks are definitive hosts of a variety of trophically transmitted helminth parasites (Caira & Healy 2004). The analysis of the parasite fauna provides useful insights on diet and habitat preferences of their shark host (Klimpel et al. 2003). Moreover, the potential use of parasites as biological tags for the identification of different shark stocks has been explored successfully, highlighting a greater potential of intestinal cestodes (Yamaguchi et al. 2003).

Parasitological investigations focused on elasmobranchs from the Mediterranean Sea are scarce; the

compilation of accurate and updated checklists is further complicated by the occurrence of old records of infection and the doubtful identity of the shark host.

The common smooth-hound *Mustelus mustelus* (Linnaeus, 1758) occurs throughout the Eastern Atlantic and Mediterranean Sea, where it represents the target or bycatch of different fishing techniques (Marino et al. 2018). Parasitological data from Mediterranean *M. mustelus* are scarce and, in several instances, represented by old literature (Rudolphi 1819; Euzet 1959). In particular, the cestode fauna of this species is poorly known (Bernot et al. 2016). Recently, *M. mustelus* collected off Croatia were investigated with the aim of linking the parasite assemblage in the gastrointestinal tract with the trophic ecology of the species and providing preliminary insights on the role of *M. mustelus* in the life cycle of different groups of parasites (Gračan et al.

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2014). This species shows a non-specialised feeding behaviour, which varies in relation to members' geographic distribution and growth stage (Costantini et al. 2000); therefore, their parasite assemblage is expected to vary accordingly.

The conservation of *M. mustelus* is threatened by the high percentage of sexually immature individuals caught and landed; therefore, this species is currently classified as Endangered (EN) by the Italian Committee of the International Union for Conservation of Nature (IUCN), with the priority of setting an appropriate minimum size for commercial fisheries ([www.iucn.it/scheda.php?id=1856264254](http://www.iucn.it/scheda.php?id=1856264254)); similarly, the number of individuals captured for scientific research should be limited. In this context, the use of dead bycatch samples is a useful way to obtain biological and ecological data from those specimens that would otherwise have been discarded.

The present work provides a morphological and molecular characterisation of gastrointestinal parasites in *M. mustelus* collected off the Italian coasts of the Northern Adriatic Sea, updating information about the parasite fauna of this shark species; the integration of our data with previously published parasitological records highlights the possible occurrence of distinct parasite assemblages in smoothhounds from different areas of the Northern Adriatic Sea.

## Materials and methods

### *Study area and parasite collection*

Three specimens of *M. mustelus*, fished as bycatch in the Northwestern Adriatic Sea (FAO 37.2.1) from June to November 2019, were subjected to a complete parasitological examination: the skin, buccal and nasal cavities, gill chamber and visceral organs were inspected for the presence of metazoan parasites. All collected parasites were washed in saline and preserved in 70% ethanol for subsequent morphological and molecular analyses for species identification. Two taxa, one belonging to nematodes and one to cestode parasites, were macroscopically distinguished.

### *Morphological analyses*

After measuring the total length, nematode and cestode parasites were subjected to microscopical observation following clarification in Amman's lactophenol to study their morphology (Khalil et al. 1994; Gibbons 2010). Before clearing the worms, a little piece of the nematodes' body devoid of taxonomic features and one cestode proglottid were

excised with a sterile scalpel and stored at  $-20^{\circ}\text{C}$  for further molecular analysis.

Nematodes were additionally characterised by scanning electron microscopy; samples stored in 70% ethanol were dehydrated through a graded ethanol series, critical point dried, sputter-coated with gold, and observed using a JEOL JSM-5410 scanning electron microscope operating at 15 kV.

Measurements were taken with the imaging software NIS-Elements (Nikon, Campi Bisenzio [FI], Italy), and are given in micrometres ( $\mu\text{m}$ ; mean followed by the range in parentheses).

### *Molecular analysis*

For molecular analysis, genomic DNA was extracted from four cestodes and six nematodes using a PureLink® Genomic DNA Kit (Life Technologies, Carlsbad, California) following the manufacturer's instructions.

For cestodes, the amplification of the 28S rDNA region was performed with primers U178\_f (5'-GCACCCGCTGAAYTTAAG-3') and L1642\_r (5'-CCAGCGCCATCCATTTTCA-3') (Lockyer et al. 2003). For sequencing, the internal primers 900 F (5'-CCGTCTTGAAACACGGACCAAG-3') and EDC2 (5'-CCTTGGTCCGTGTTTC AAGACGGG-3') of Lockyer et al. (2003) were used. For nematodes, the amplification of the complete ITS rDNA region was performed with primers NC5\_f (50-GTAGGTGAACCTGCGGAAGGAT CATT-30) and NC2\_r (50-TTAGTTTCTT CCTCCGCT-30) (Zhu et al. 1998).

The polymerase chain reaction (PCR) products were electrophoresed on 1% agarose gel stained with SYBR® Safe DNA Gel Stain (Thermo Fisher Scientific, Carlsbad, California) in 0.5X TBE. Amplicons were purified with Nucleo-Spin Gel and PCR Cleanup (Mackerey-Nagel, Düren, Germany). All samples were sequenced with an ABI 3730 DNA analyser (StarSEQ, Mainz Germany). The trace files were assembled with ContigExpress (VectorNTI Advance 11 software, Invitrogen, Carlsbad, California), and the consensus sequences were compared with previously published data using BLAST tools (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). The sequences generated in this study were deposited in GenBank under the accession numbers MT967225–MT967230 and MT981401–MT981404.

## Results

The examined *M. mustelus* measured 44, 46 and 66 cm in total length. Parasitic helminths were



found in the digestive tract of a single specimen (total length 66 cm); in particular, larval and adult nematodes belonging to the species *Acanthocheilus rotundatus* (Rudolphi, 1819) (15 specimens) were recovered from the stomach and the proximal intestine, while adult cestodes belonging to the species *Calliobothrium verticillatum* (Rudolphi, 1819) (five specimens) were collected from the spiral valve.

Of these, morphometric data for eight *A. rotundatus* (one fourth-stage larva, five adult males and two adult females) and four *C. verticillatum* are reported below.

*Morphological analyses*

*Calliobothrium verticillatum* (Rudolphi, 1819)(Figure 1)

*Adult morphology:* Body 66,809 (62,612–75,629) long; scolex 453 (385–509) long × 326 (263–374) wide (Figure 1(a)), with four bothridia 430 (465–396) long × 121 (100–133) wide, each divided into three loculi by two transverse septa; anterior loculus 172 (136–189) long; middle loculus 71 (60–77) long; posterior loculus 73 (67–84) long; apical

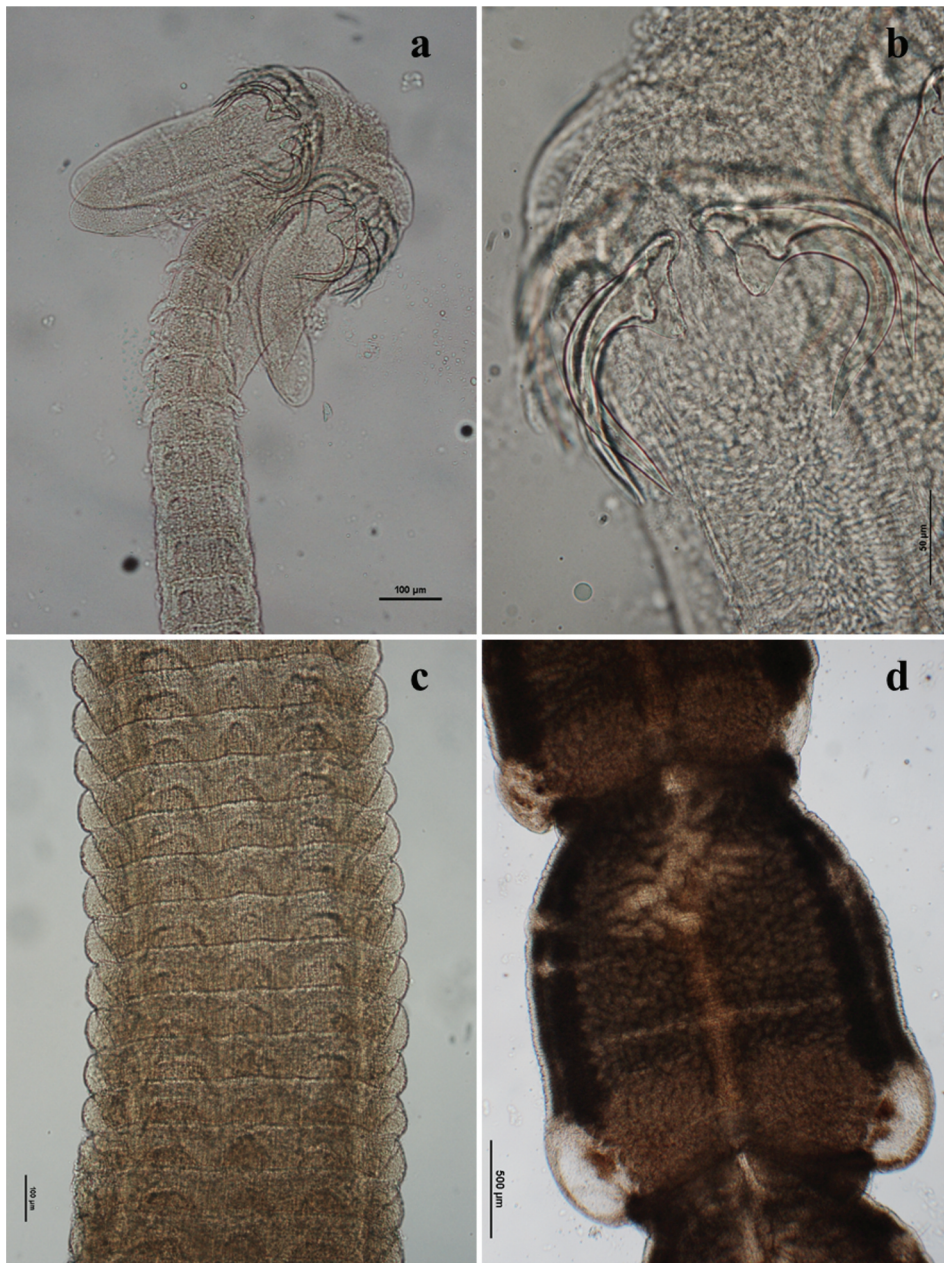


Figure 1. *Calliobothrium verticillatum* adult: (a) scolex; (b) detail of hooks; (c) immature proglottids; (d) mature proglottids.

region of each bothridium with anterior muscular pad 48 (40–52) long × 115 wide (93–129), bearing three apical suckers arranged in horizontal pattern; two pairs of articulated, unpronged hooks (Figure 1(b)): lateral axial hook 128 (117–140) long; medial axial hook 124 (112–143) long; lateral abaxial hook 141 (124–173) long; medial abaxial hook 128 (114–148) long. Strobila with 259 (168–316) segments, craspedote, lacinated, euapolytic. Pattern of proglottid lacination variable along length of strobila (Figure 1(c, d)); immature proglottids 155 (105–240) long × 565 (457–621) wide; mature proglottids 1497 (1137–2171) long × 1262 (888–1885) wide; genital pore lateral, irregularly alternating; testes numerous, in two lateral columns; postvaginal testes present on poral side; ovary at posterior end of proglottid; vagina anterior to cirrus sac; vitelline follicles lateral; uterus sacciform, medioventral; uterine pore medioventral.

*Acanthocheilus rotundatus* (Rudolphi, 1819)  
(Figure 2)

*Fourth-stage larva morphology* (one specimen): body 15,426 long × 421 wide; anterior end (Figure 2(a)) with three small labia each provided with four small sclerified pieces with rounded tips; dorsal lip with two double papillae, latero-ventral lips each showing a double papilla and an amphid; oesophagus 1374 long becoming narrower at anterior end; ventriculus rounded, 137 long × 171 wide; nerve ring 466 from anterior end; excretory pore slightly posterior to nerve ring, 554 from anterior end; deirids 503 from anterior end; lateral alae extending slightly posteriorly from anterior end to mid-length of the tail; tail conical, 91 long with small terminal mucron (Figure 2(b)).

*Adult general morphology* (Figure 3): body large, becoming narrower towards the anterior extremity

(Figure 3(a)); lateral alae absent. Three small, semi-circular lips each provided on its internal face with two bifid teeth (Figures 3(c), 4(a)). Dorsal lip with two double papillae, latero-ventral lips each presenting a double papilla, a single latero-ventral papilla and an amphid; interlabia absent. Esophagus expanded at the anterior end. Nerve ring at around one-third to one-quarter of esophagus length. Deirids small, rounded, slightly posterior to the nerve ring; excretory pore slightly posterior to nerve ring (Figure 3(d)). Ventriculus small, rounded, wider than long (Figure 3(b)). Tail conical, with small terminal mucron.

*Adult male morphometry* (five specimens): body 31,922 (25,051–36,095) long × 802 (650–1060) wide; esophagus 2460 (2259–2912) long; ventriculus 268 (248–292) long × 363 (310–401) wide; nerve ring, excretory pore and deirids 821 (693–925), 848 (695–908) and 816 (731–905) from anterior end, respectively; spicules short, robust, and similar in length, pointed at distal end, 380 (263–523) long; gubernaculum absent; 41 (40–42) preloacal papillae (Figures 4b); tail 166 (126–204) long (Figure 3(f)).

*Adult female morphometry* (two specimens): body 39,709 (30,305–49,113) long × 1120 (947–1293) wide; esophagus 3062 (2723–3401) long; ventriculus 331 (318–344) long × 440 (411–467) wide; nerve ring, excretory pore and deirids 1053 (814–1293), 1171 (976–1365) and 1108 (907–1308), respectively, from anterior end; vulva 12,610 (12,045–13,174) from anterior end (Figure 3(e)); vagina muscular, directed posteriorly from vulva; two long uteri; tail 533 (520–546) long.

*Molecular analyses*

The 28S rDNA of four *C. verticillatum* were successfully sequenced and were identical to each other. The BLAST search gave 99.9% identity with *C. verticillatum* (KF685753) of Caira et al. (2014). The ITS

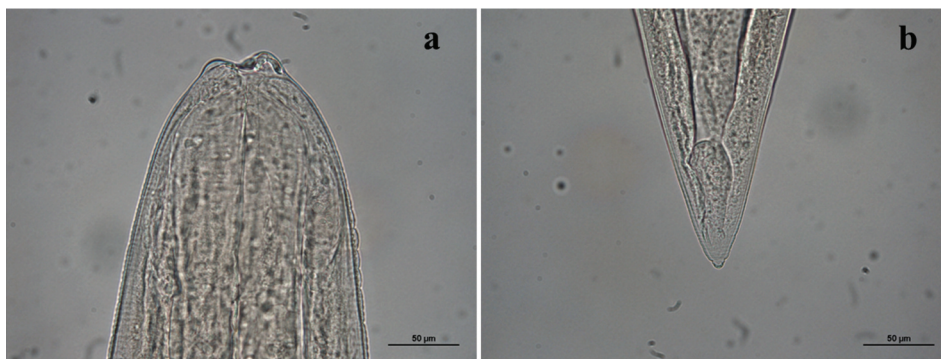


Figure 2. *Acanthocheilus rotundatus* fourth-stage larva: (a) anterior end; (b) caudal end.



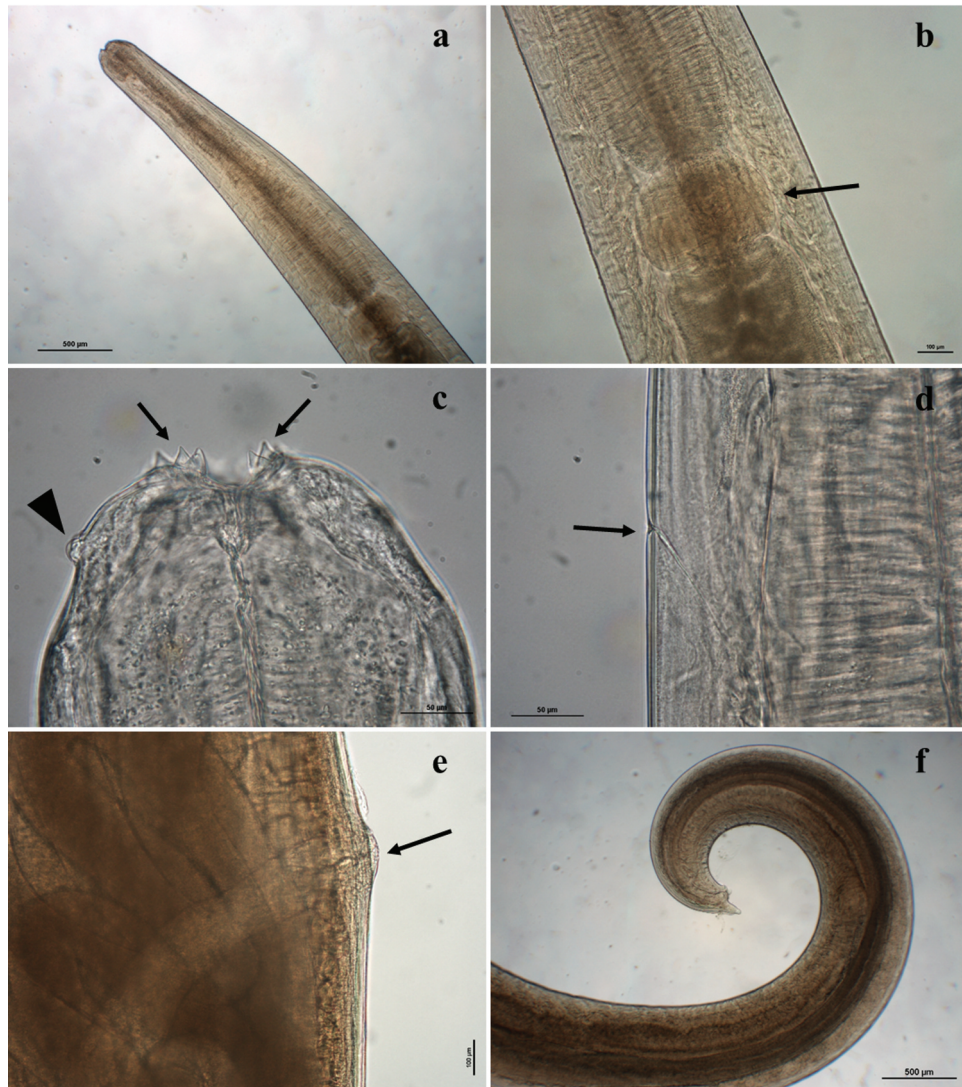


Figure 3. *Acanthocheilus rotundatus* adults: (a) male specimen, anterior end; (b) male specimen, detail of ventriculus (arrow); (c) male specimen, anterior end with detail of bifid teeth (arrows) and double papilla (arrowhead); (d) male specimen, detail of excretory pore (arrow); (e) female specimen, detail of vulva (arrow); male specimen, caudal end.

rDNA of six *A. rotundatus* were successfully sequenced and identical to each other. The BLAST analysis gave 98.6% identity with *Acanthocheilus rotundatus* (MF061679) of Li et al. (2018).

## Discussion

Parasitological examination of *M. mustelus* from the Northwestern Adriatic Sea allowed us to identify the cestode species *C. verticillatum* and the nematode species *A. rotundatus*.

*Calliobothrium verticillatum*, the type species of the genus *Calliobothrium*, was originally described by Rudolphi (1819) from an undetermined shark host

referred to *Squali galei* collected off Italy. *Calliobothrium verticillatum* has been reported in *Mustelus vulgaris* (synonymised with *M. mustelus*) from the Northeastern Atlantic (van Beneden 1850) and subsequently reported by Euzet (1959) infecting Mediterranean *Mustelus*, namely *M. mustelus* and *Mustelus canis* (Mitchill, 1815); however, the latter species is now known not to occur in the Mediterranean area (Bernot et al. 2016). Our results would therefore confirm the occurrence of this host-parasite association in Mediterranean waters.

Euzet (1959) reported the presence of a highly diverse cestode fauna in *M. mustelus* from French coasts, including the phyllobothriideans *Phyllobothrium*

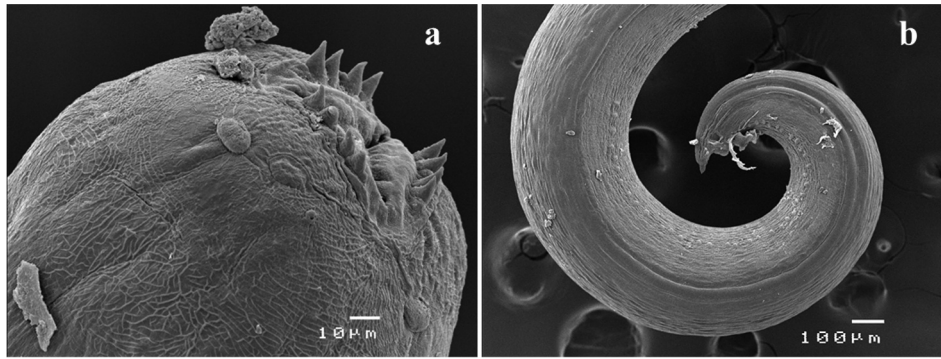


Figure 4. *Acanthocheilus rotundatus* adult male, scanning electron micrographs: (a) detail of anterior end; (b) caudal end.

*lactuca* Van Beneden, 1850, *Scyphophyllidium triacis* (Yamaguti, 1952) Caira, Jensen and Ruhnke in Caira, Jensen, Hayes and Ruhnke, 2020 and *Orygmatobothrium musteli* (Van Beneden, 1849) Diesing, 1863; the tetraphyllideans *C. verticillatum* (Rudolphi, 1819), *Symcallio eschrichti* (van Beneden, 1850) Bernot, Caira and Pickering, 2015, and *Symcallio lintoni* (Euzet, 1954) Bernot, Caira and Pickering, 2015; the onchoproteocephalidean *Acanthobothrium mathiasi* Euzet, 1959; the diphyllidean *Coronocestus musteli* (Pintner, 1889) Caira, Marques, Jensen, Kuchta and Ivanov, 2013; and the trypanorhynch *Eutetrarhynchus ruficollis* (Eysenhardt, 1829) Pintner, 1913 and *Lacistorhynchus tenuis* (Van Beneden, 1858) Pintner, 1913.

Regarding *C. verticillatum*, the total length of the specimens analysed in the present study is slightly shorter compared to the specimens analysed by Euzet (1959); however, the morphometry of the scolex features (hooks, bothridia, accessory suckers) and the number and size of segments in the strobila are in accordance with those reported by Euzet. Scolex morphology is also in accordance with the description provided in Caira and Ruhnke (1991).

Different species of *Mustelus* act as definitive hosts in the life cycle of *Calliobothrium* spp. Adults of *C. verticillatum* mature in the shark spiral intestine and produce hexacanth embryos that are released from gravid proglottids; hermit crabs act as intermediate hosts after ingesting hexacanth, and within their body the embryo develops firstly into a procercoid and subsequently into a plerocercoid larva; the life cycle is completed when infected hermit crabs are ingested by a shark (McDermott et al. 2010). Plerocercoids of *C. verticillatum* are found in the lumen of the anterior midgut caeca of hermit crab *Pagurus pollicaris* Say, 1817 [in Say, 1817–1818] from extra-Mediterranean areas (Caira & Ruhnke 1991), where remarkable prevalence values (over 95%; Cherry et al. 1991) have been reported.

*A. rotundatus* was originally described by Rudolphi (1819) infecting *Galeorhinus galeus* (Linnaeus, 1758) from the Adriatic Sea and was subsequently re-described by Moravec and Nagasawa (2000) infecting *Mustelus griseus* Pietschmann, 1908 and *Mustelus manazo* Bleeker, 1855 from Japanese waters. *Acanthocheilus rotundatus* was reported infecting different shark species worldwide (Moravec & Nagasawa 2000, and references therein); in particular it was reported in *M. mustelus* from the Adriatic Sea, where it was identified with morphological methods (Petter et al. 1991). Our work provides the first sequence data of *A. rotundatus* from the Mediterranean Sea, since only sequences of specimens collected in extra-Mediterranean areas (Li et al. 2018) were previously available. Furthermore, the caudal end of male *A. rotundatus* is here characterised by scanning electron microscopy for the first time.

With respect to the morphometric data of *A. rotundatus* generated in this study, our measurements of male and female specimens are in accordance with morphometric data reported in previous studies (Diaz 1971; Petter et al. 1991; Moravec and Nagasawa 2000). In particular, the morphometric data of our adult females fall between those reported by these authors. Adult males examined are within the range of measurements reported by the same authors; however, we observed more numerous pairs of precloacal papillae (40–42 pairs) than those reported by Petter et al. (1991) (30–36 pairs) and by Moravec and Nagasawa (2000) (25–29 pairs); nevertheless, as pointed out by the latter authors, in ascaridoids the number of precloacal papillae is subject to a considerable degree of intraspecific variability. With respect to the fourth-stage larva, it was not possible to perform a full comparison due to the scarcity of morphometric data available in the literature for this larval stage. This work therefore represents the first detailed morphometric characterisation of *A. rotundatus* fourth-stage larva.

The life cycle of *Acanthocheilus quadridentatus* (syn. *A. rotundatus*) has been described by Diaz (1971): eggs are produced by gravid adult females in the intestine of the elasmobranchs and are released into the environment with the host's faeces; eggs hatch in seawater and release first-stage larvae, which undergo their first moult to second-stage larvae; the latter are found in the pyloric caeca of the hermit crab *Pagurus prideaux* Leach, 1815 [in Leach, 1815–1875], where they develop and moult into third-stage larvae, infective to the definitive elasmobranch host; in the stomach and proximal intestine of different elasmobranch species (i.e. *M. mustelus*, *Squalus acanthias*, *Raja asterias*), third-stage larvae moult into fourth-stage larvae, which undergo further growth and sexual maturation. Data on the distribution of *A. rotundatus* in elasmobranchs are scarce. In the intermediate host *P. prideaux*, remarkable prevalence (50%) and intensity (1–15) values of infection with *A. rotundatus* were reported (Diaz 1971); it is currently unknown whether other invertebrate or vertebrate hosts are involved in the transmission of the parasite to its definitive elasmobranch host.

*Mustelus mustelus* from the Northeastern Adriatic have been recently examined for gastrointestinal parasites by Gračan et al. (2014); these authors reported the presence of the trematode *Ptychogonimus megastomum* (Rudolphi, 1819) Lühe, 1900, the cestodes *Eutetrarhynchus* sp. and *Phyllobothrium* sp. and the nematode *Cucullanus micropapillatus* Tornquist, 1931 as the most prevalent and dominant species, and linked this information to the feeding habits of *M. mustelus* inferred from the analysis of gastrointestinal contents. Indeed, no prey items belonging to the genus *Pagurus* were reported in the gastrointestinal tract of the *M. mustelus* analysed in that study (Gračan et al. 2014).

Our findings, reporting the presence of different species of trophically transmitted parasites, would therefore suggest the possible occurrence of distinct parasite assemblages, arguably associated to different prey composition, in *M. mustelus* from different areas of the Northern Adriatic Sea.

Further screenings are needed to determine the parasite diversity of *M. mustelus* in different parts of the Mediterranean Sea. Moreover, additional information concerning the life cycle of the parasites identified in the present study would allow researchers to elucidate the full range of hosts potentially involved in parasite transmission to *M. mustelus* and provide valuable insights into its feeding ecology.

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## Disclosure statement

No potential conflict of interest was reported by the authors.

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